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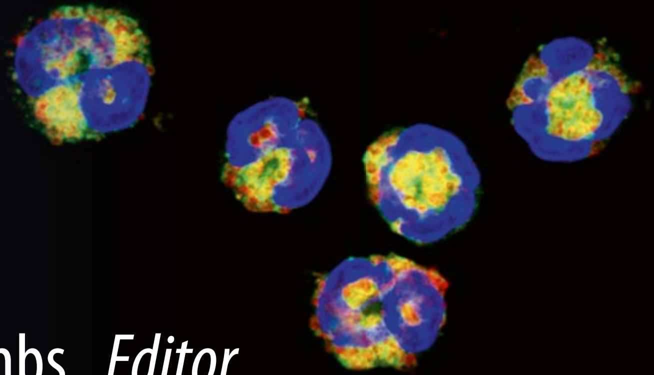

Kumi Nagamoto-Combs *Editor*

Animal Models of Allergic Disease

Methods and Protocols

METHODS IN MOLECULAR BIOLOGY

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Animal Models of Allergic Disease

Methods and Protocols

Edited by

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Preface

Over the last several decades, allergic diseases have become major health concerns worldwide. Common allergic conditions involve cardiorespiratory complications, gastrointestinal problems, and skin irritations. Although less acknowledged, allergic diseases have also been associated with altered brain homeostasis and neuroinflammation. While symptoms may vary significantly from person to person, it is well established that allergic diseases are manifestations of immune overreactions to otherwise innocuous stimuli and cause tremendous physical, psychological, and economic burdens to those who are afflicted.

With increasing prevalence in allergic diseases, research efforts have gathered momentum to elucidate disease etiology as well as to develop diagnostic and therapeutic approaches. Findings from clinical studies greatly facilitated our understanding of symptom variations and genetic tendency (atopy) in allergic diseases. However, it is difficult with human cohorts to control variables that might affect study outcomes, such as genetic backgrounds, diets, stress levels, commensal microbiota, and exposures to environmental agents. These factors have been suggested to influence the onset and extent of allergic diseases and therefore important variables to consider in a study. The use of animal models allows researchers to overcome the limitations in human studies and design experiments with well-controlled variables and extensive analyses of biological specimens.

Successful mouse models have been established for many types of allergic diseases, including food allergy, allergic asthma, atopic dermatitis, contact dermatitis, and allergic conjunctivitis. Experimental studies using these models have so far contributed to our knowledge in disease development, pathophysiologic features, and therapeutic strategies. The procedures used consistently across these models for achieving hypersensitivity involve a sensitization period with intermittent administrations of an allergen in the presence or absence of an adjuvant, followed by an allergen challenge. Nonetheless, selection of strains and sexes of mice, types of allergens and adjuvants, and the route of allergen exposure are critical determinants of developing appropriate disease models because they may affect sensitization efficacy and symptom presentations. Consequently, disease-specific protocols may differ greatly and therefore detailed instructions are particularly important in establishing mouse models of allergic diseases.

The purpose of this volume *Animal Models of Allergic Disease* in the Methods in Molecular Biology series is to provide explicit, step-by-step protocols for mouse models of allergic diseases along with general guidelines and considerations for choosing particular strains, allergen, adjuvant, and route of sensitization. This volume begins with an introductory review chapter, which provides a comprehensive overview of designing animal models for allergy research. Advantages as well as limitations with the use of animal models are also discussed. The following nine chapters describe how particular types of allergic disease are generated and assessed in rodents, demonstrating a variety of sensitization approaches to model after human conditions. Additional eight chapters specifically focus on detailed methods that are frequently employed to analyze the pathophysiology of allergic diseases at the molecular, cellular, and histological levels. The final three chapters cover manipulation of intestinal microbiota and desensitization of immune responses in animal models that are utilized for investigation of clinically relevant allergy development and therapeutic

strategies. Together, *Animal Models of Allergic Disease* offers a comprehensive collection of protocols and experience-derived know-hows to facilitate allergic disease research.

Lastly, I would like to send my deep appreciation to the contributors of *Animal Models of Allergic Disease* for sharing their valuable time, knowledge, and expertise. I would also like to thank the Series Editor, Dr. John Walker, and Anna Rakovsky of Springer Nature for this opportunity and their guidance.

Grand Forks, ND, USA ***Kumi Nagamoto-Combs***

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Chapter 1

Applications of Mouse Models to the Study of Food Allergy

Sara Benedé and M. Cecilia Berin

Abstract

Mouse models of allergic disease offer numerous advantages when compared to the models of other animals. However, selection of appropriate mouse models is critical to advance the field of food allergy by revealing mechanisms of allergy and for testing novel therapeutic approaches. All current mouse models for food allergy have weaknesses that may limit their applicability to human disease. Aspects such as the genetic predisposition to allergy or tolerance from the strain of mouse used, allergen dose, route of exposure (oral, intranasal, intraperitoneal, or epicutaneous), damage of the epithelial barrier, use of adjuvants, food matrix effects, or composition of the microbiota should be considered prior to the selection of a specific murine model and contemplated according to the intended purpose of the study. This chapter reviews our current knowledge on the application of mouse models to food allergy research and the variables that may influence the successful development of each type of model.

Key words Mouse model, Food allergy, Mouse strains, Adjuvants, Microbiota

1 Introduction

Guinea pigs [1], rats [2], dogs [3], or swine [4] have been used to study food allergy, but they are hard to handle and too expensive to use on a regular basis [5]. Mouse models of allergic disease offer numerous advantages when compared to the models of other animals such as their small size and short breeding cycle, the large reagent availability for studying this species, the detailed characterization of their immune system, and the similar sequence of immunological events involved in the development of sensitization and the elicitation of allergic reactions [6–8]. An ideal animal model should imitate human disease as closely as possible with respect to route of exposure, mechanisms underlying the disease, and associated clinical signs and, therefore, possess similar reactivity to proteins as humans [9].

Mouse models of food allergy have been used as a preclinical approach to study the efficacy of new therapeutic strategies, to predict allergenicity of new proteins, or to unravel mechanisms of action of the disease, among others [10]. However, all current

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mouse models have weaknesses that may limit their applicability to human disease. To date, a large diversity of protocols to induce food allergy in mice have been published [11], hindering the comparison of results. In experiments with mouse models of food allergy, it is important to take into account the several considerations such as the genetic predisposition to allergy or tolerance from the strain of mouse used, allergen dose, route of exposure (oral, intranasal, intraperitoneal, epicutaneous), damage of the epithelial barrier, use of adjuvants, food matrix effects, and composition of the microbiota, among others [12]. This chapter reviews our current understanding of the utility and limitations of mouse models for food allergy research and the critical factors influencing their outcomes.

2 Human Versus Mouse Immune System

An important question when assessing mouse models of food allergy is whether they have a significant relationship with the disease in humans. There exists a great degree of overlap in the immune system between mice and humans [13]. However, discrepancies between the mouse and human immune systems need to be considered [14].

In humans, most oral food-induced systemic anaphylaxis is mediated by crosslinking of IgE bound to high-affinity FcεRI receptors on mast cells and/or basophils leading to the release of an array of mediators, including histamine, platelet-activating factor (PAF), leukotrienes, and proteases, while the function of IgG is primarily limited to serving as potential competitors of IgE for binding of allergens [15]. In mice, in addition to the classic IgE-mediated pathway, an alternative pathway is activated by antigen–IgG complexes formed in blood before they bind FcγRIII on macrophages and leads to clinical signs triggered by PAF [16, 17]. There are some recent data that neutrophils might be involved in anaphylaxis and allergic reactions in mice and humans [18, 19]. IgG–antigen immune complexes can bind to Fcγ receptors, leading to neutrophil activation, degranulation, and platelet-activating factor release. This pathway is thought to play a significant role in drug allergy, but the possible role of this pathway in food allergy is not yet clear.

The relative importance of the two different pathways of systemic murine anaphylaxis depends on the balance between the quantity of antigen-specific IgG antibody produced and the quantity of antigen used to challenge mice. When antibody levels are low, only IgE-mediated anaphylaxis can occur. When antigen levels are low and antibody levels are high, IgG antibodies prevent IgE-mediated anaphylaxis by intercepting antigen, but IgG-mediated anaphylaxis does not occur because the quantity of

IgG–antigen complexes is too low. When antigen and antibody levels are both high and antibody levels are in excess to antigen levels, IgG antibodies block the binding of antigen to IgE, but IgG-mediated anaphylaxis occurs. When antigen and antibody levels are both high and antigen levels are in excess, both pathways can lead to anaphylaxis [17]. The type of pathway involved in the anaphylactic response might play an important role in the differences observed in parameters and manifestations of food allergy in different studies using mouse models [20].

Other differences between human and mouse allergic response also need to be considered. For example, hives, rashes, and vomiting are not reported in mouse models [21]. Moreover, in mice, the induction of disease is under the control of the investigator and up to 80% of total serum IgE is specific, while in patients only 0.1–15% of IgE is specific to a single food allergen [22]. Additionally, serum mast cell protease, MMCP-1, is used as a systemic readout for mouse mucosal mast cell activation in IgE-mediated food allergy [23], but no human equivalent exists in patients with food allergies [22].

There is some evidence in humans that the ability to elicit an IgE response to specific allergens may be associated with the expression of certain class subtypes of the human leukocyte antigen (HLA), which is homologous to the major histocompatibility complex (MHC) expressed in other animals [24]. In fact, identification of genetic variants contributing to peanut allergy responses has been associated with the HLA locus [25, 26]. However, most mouse strains are highly inbred, limiting the MHC class subtypes that they are capable of expressing [27]. This could require the use of multiple strains in order to accurately evaluate the allergenicity of novel proteins, restricting their usefulness as a general screening tool. Alternatively, the use of outbred strains may require a greater number of mice to identify allergenicity that is HLA allele dependent.

3 Mouse Strains

As for humans, several studies have documented that genetic predisposition in mice is important for measuring in vivo susceptibility to experimental food allergy [7, 28, 29], and therefore, selection of a mouse strain with a determined background may be crucial to study the capacity of a specific allergen to sensitize or to induce allergic inflammation after challenge [30].

Traditionally, in the field of food allergies, researchers have specially focused on certain strains of mice, such as C3H/HeOuJ and C3H/HeJ (TLR4 deficient) or BALB/c, which display Th2 responses more readily than other common murine strains [27] although differences in allergic manifestations exist between them

as discussed below [31]. C57BL/6 mice are known to have moderate responses to allergen sensitization compared to other strains [8, 20, 32], but due to the wide availability of genetically modified mice on this background strain, they can be a convenient tool for studying pathogenic mechanisms [33].

BALB/c mice generally do not show severe systemic anaphylactic reactions after oral challenge with allergen, but produce adequate levels of IgE and Th2 cytokines after sensitization with both milk and peanut allergens [20, 28, 34–36]. However, compared to C3H mice, BALB/c mice exhibit more pronounced gastrointestinal symptoms of food allergy after ingestion of the egg allergen ovalbumin as well as elevated allergen-responsive Th2 and Th1 cytokine production from splenocytes [23, 29, 37]. Susceptibility to gastrointestinal symptoms was shown to be associated with the presence of a population of agranular mucosal mast cells producing high levels of the cytokine interleukin (IL)-9 [38]. On the contrary, C3H mice show clinical anaphylactic responses upon allergen exposure as well as increased histamine levels and mast cell degranulation [31]. This strain also produces high levels of allergen-specific Th2 cytokines and immunoglobulins [20, 23, 28, 34, 36, 37].

C3H/HeJ mice, unlike the C3H/HeOuJ strain, carry a Toll-like receptor 4 (TLR4) mutation that makes them insensitive to lipopolysaccharide (LPS). However, these mouse strains are both widely used in studies of food allergy, raising a question regarding the role of TLR4 in susceptibility to food allergy. Some studies have demonstrated that the lack of TLR4 signaling results in augmentation of peanut sensitization and IgE-mediated anaphylactic responses in TLR4-deficient or mutant mice [39]. In contrast, we have shown that TLR4 deficiency is not sufficient to confer clinical reactions to peanut in BALB/c mice and that it attenuates anaphylaxis severity to a cow's milk allergen in C3H mice [35]. Furthermore, the TLR4-sufficient C3H/HeOuJ strain is widely used in food allergy research with observable responses [40, 41], indicating that mouse strains and allergens influence the role of TLR4 in food allergy and associated reactions. In addition to TLR4 mutation, C3H/HeJ mice also harbor a DOCK8 mutation [42], which is also a mutation leading to hyper-IgE and severe food allergy in humans [43]. Mice genetically deficient in DOCK8 specifically in the T-cell compartment generate hyper-IgE responses to sensitization [44], suggesting that DOCK8 contributes to the susceptibility of C3H mice to anaphylaxis.

In addition to DOCK8, the IL-4 receptor is an essential component of food allergy susceptibility in mice. Il4raF709 mice, with enhanced IL-4 signaling due to the disruption of the inhibitory signaling motif in the IL-4 receptor α-chain, demonstrate enhanced sensitization to oral antigens even in the absence of adjuvant [45]. These mice exhibit mast cell expansion and anaphylactic responses to food proteins and have an altered intestinal microbiota

that can transfer susceptibility to germ-free mice [46, 47]. Acute administration of IL-4 to mice (in the form of long-lived IL-4/IL-4-antibody complexes) has also been demonstrated to enhance susceptibility to anaphylaxis [48].

Another approach to studying genetic predisposition to food allergy comes from the collaborative cross, a large panel of new inbred mice that was developed to investigate the genetic basis of disease. Using this resource, a strain of mice, CC027/GeniUnc, was identified as susceptible to peanut allergy and prone to severe reactions after oral challenge [49]. The genetic basis of this susceptibility has not yet been identified.

Translating the findings in mice to humans is undoubtedly of utmost importance, and this is more easily achieved through humanized mice, which express human proteins that are vital to the sensitization and effector phase of allergy. One approach has been to use mice that express human Fc receptors, which addresses some of the important species differences in Fc receptor expression [50–52]. Other approaches have been to reconstitute immunodeficient mouse models transgenically expressing human growth factors with human hematopoietic stem cells to generate a "humanized mouse." While these models have been established [53, 54], they have not yet been widely used to study mechanisms or test therapeutics.

4 Sex and Age

In humans, both sexes are afflicted with food allergies. However, several studies have concluded that males are predominantly affected by food allergy in younger age while more females are affected as adults [55–57]. In mouse models, animal age seems to play a minor role [58]. Most mouse models have used young and adult female mice of 4–6 weeks. Males are difficult to house in groups as they have a tendency to fight, which is why female mice have been more commonly used for research studies requiring long-term housing. Some male mouse models have succeeded in providing novel insights into food allergy [59, 60], but sex differences have not been clearly established. A study using hazelnut allergy F1 hybrid mice model reported that female mice exhibited higher IgE responses compared to males [61]. In contrast, male C57BL/6 mice were more susceptible to whey protein sensitization than female mice [32] and showed significantly elevated plasma-specific IgE levels compared to female mice in a model of OVA-induced food allergy using C3H/HeJ mice [62]. Sex as a biological variable has been shown to influence many aspects of immunology related to food allergy, including antibody production and T-cell function related to B-cell help, but more research is needed in order to obtain conclusive data on the contribution of sex differences to food allergy.

5 Use of Adjuvants

As in healthy humans, administration of food antigens to genetically normal mice results in oral tolerance, with little or no immune response or disease [21]. To overcome physiological tolerance to ingested antigens in experimental studies, immune adjuvants are often coapplied with the food allergens during sensitization. These substances have the capacity to increase the immune response to an antigen and induce Th2 responses [63]. A variety of molecules such as glycans, proteases, chitin, mast cell/basophil-activating molecules, arachidonic acid metabolites, and other immunomodulatory lipids can act as adjuvants [63], although the most used in mouse models of food allergy are cholera toxin (CT), staphylococcal enterotoxin B (SEB), and aluminum hydroxide.

Cholera toxin, an endotoxin produced by *Vibrio cholera*, is one of the best-known mucosal adjuvants. The effect of CT depends on CD11b dendritic cells (DCs) and on intracellular cyclic adenosine monophosphate [64]. Oral administration of CT induces migration and activation of CD103+ DCs from the intestinal lamina propria to the mesenteric lymph nodes (MLNs) and promotes a Th2 polarizing DC phenotype by upregulation of the surface co-stimulatory molecules OX40L [65]. Chu et al. showed that administration of peanut allergen together with CT led to the production of IL-33 by intestinal epithelial cells, which was upstream of the change in DC phenotype [66]. Administration of CT to mice induces an upregulation of IL-1 locally within the intestinal tissue [67], upregulates the expression of the molecule TIM-4 on DCs [68, 69], and induces migration of DCs from the subepithelial dome to the T-cell area in the Peyer's patches [70], producing immune responses against co-administered antigens. Moreover, the imbalance of intestinal fluid balance has been suggested to contribute to CT actions as well [71]. CT has been widely used in mice to cause oral sensitization to egg white proteins, such as ovalbumin, lysozyme and ovomucoid [72], buckwheat [73], lupin proteins [74], shrimp tropomyosin [75], milk proteins [76], and peanut [77], among others [78, 79].

Staphylococcal enterotoxin B (SEB), an enterotoxin produced by the Gram-positive bacteria, Staphylococcus aureus, is another common adjuvant used together with food allergens to bypass the state of tolerance by oral administration [80]. It is commonly found at mucosal sites and, unlike *V. cholera*, has the potential to play a role in human allergic diseases, such as eczema, allergic rhinitis, asthma, and food allergy [81]. Similar to CT, SEB can change the phenotype of mucosal DCs. SEB induces the upregulation of TIM-4 on DCs and also increases the expression of the costimulatory molecules such as CD80 and CD86, skewing naïve T cells to a Th2 phenotype [82]. Moreover, dysfunction of Treg cells after SEB exposure has previously been shown in atopic dermatitis patients

[83], and intestinal expression of TGF-β and Foxp3 decreases after oral sensitization to ovalbumin and peanut extract in mice [80], indicating a suppression of local regulatory pathways. SEB has been mainly used in mice to induce oral sensitization to food proteins [84] although it is also an efficient and perhaps more physiologically relevant adjuvant for epicutaneous sensitization [85].

Systemic sensitization by the intraperitoneal route using aluminum hydroxide (alum) as an adjuvant is also common in food allergy studies with mice [70], mainly in combination with the chicken egg white allergen, ovalbumin. Its use is controversial primarily because of the nonphysiological route of exposure. Unlike CT and SEB, the clinical utility of alum in humans has been approved to enhance the immune response to vaccines [86]. Alum is an activator of the inflammasome [87], leading to DC activation and IL-1β production. Uric acid, which also activates the inflammasome, has been shown to be elevated in both peanut-sensitized mice and peanut-allergic children [88].

Other exogenous factors administered to induce oral sensitization include medium-chain triglycerides (MCTs), which enhance production of the Th2-inducing cytokines, IL-33, thymic stromal lymphopoietin (TSLP), and IL-25 in the intestine and induce sensitization to peanut in mice [59].

The use of adjuvant may not be useful when the intrinsic capacity of a protein to sensitize or host determinants of susceptibility are to be studied. Therefore, adjuvant-free animal models of food allergy have been developed. A single high-dose feed of peanut without adjuvant was reported to induce allergic sensitization in mice, resulting in anaphylaxis in response to systemic rechallenge [89]. Similarly, oral sensitization mouse models that do not use adjuvants have been described for soybean [90] and rice [91]. It is not clear in these model systems why oral exposure did not lead to the development of tolerance.

Adjuvant-free sensitization has been more widely described in response to skin exposure. Gangur and colleagues have described adjuvant-free sensitization by epicutaneous exposure of BALB/c mice to cashew nut, hazelnut, and milk whey protein [61, 92, 93]. Effective sensitization after topical exposure to peanut allergens or ovalbumin without adjuvants and anaphylaxis upon rechallenge have been demonstrated [85, 94].

6 Route of Exposure

The route of allergen sensitization may alter the resulting immune response, and as we described before, the most effective route could vary significantly between mouse strains due to genetic differences [28, 37]. Therefore, the sensitization route is an important and necessary consideration when using mouse models of food allergy.

Oral sensitization has traditionally been considered as the most physiological route for sensitization to food proteins, although epidemiologic evidence suggests that alternative routes may play a key role in sensitization to foods. Oral, cutaneous, intraperitoneal, and nasal administrations of allergens have been used for the establishment of food allergy in mouse models [84]. However, it remains largely unknown which route of sensitization is best to predict the severity of symptoms, therapeutic outcomes, or allergenic potency of food proteins.

Induction of oral sensitization to food proteins in mice depends on the dose, frequency of exposure, nature of the antigen [62, 95], and use of adjuvants [63] to overcome their strong tendency to develop oral tolerance by promoting Th2-polarized over Treg immune responses. Subsequent oral challenge with the allergen triggers systemic anaphylaxis, which can be evaluated by body temperature measurement or gastrointestinal symptom observation. Moreover, oral sensitization may be required to mimic the effect of digestion and the gut epithelium on sensitization to food proteins [7, 96].

Data from animal models show that allergic sensitization to food antigens, including egg, peanuts, and hazelnuts, can be readily induced by topical allergen exposure [97]. Similar to what is observed with the oral route, additional signals could be needed beyond allergen exposure to result in allergic sensitization. These can include co-administration of exogenous adjuvants or induction of damage signals by tape stripping to activate intraepithelial lymphocytes that participate in allergic sensitization [98, 99]. Tape stripping imitates excoriation of the skin induced by scratching in eczema, and the concept of damage or barrier defects contributing to allergic sensitization via the skin is supported by clinical observations that atopic dermatitis is a significant risk factor for the development of food allergy [81]. Models for ovalbumin, peanut, hazelnut, sesame, cow's milk, and cashew nut allergy have been described using this approach, inducing antigen-specific IgE, IgE-mediated anaphylaxis after oral challenge, and expansion of mast cells in the intestines [21, 23, 100]. These models have in common the local upregulation of IL-33 or TSLP in the skin that drive local sensitization, as well as distal effects that change the gastrointestinal milieu to promote allergic symptoms after oral challenge [85, 101, 102].

There is some evidence that transdermal exposure to food proteins in humans, particularly during infancy or childhood, might result in sensitization [103, 104]. In fact, cutaneous exposure in mice was more effective in triggering food sensitization than the intragastric, intranasal, or sublingual routes [98], and some allergens can induce sensitization through the skin in the absence of any exogenous adjuvant or damage. This is the case for peanut, which have intrinsic adjuvant activity, driving Th2 cell stimulation

by inducing IL-33 production from keratinocytes and modifying the phenotype of skin-draining DCs through the receptor ST2 [85]. Efficient cutaneous sensitization to milk proteins without skin abrasion has also been described [93, 95].

Exposure to an allergen via intranasal administration can also be used to induce food-derived allergies [84, 95, 98, 105, 106]. Although most of these studies have used an adjuvant to drive sensitization, Dolence et al. observed that peanut flour could induce the generation of T follicular helper cells and drive IgE production in the absence of adjuvant exposure [107].

Route of challenge is also a key factor in mouse models of food allergy. The physiological route of exposure on challenge is the oral route. Many investigators have found this to be the most difficult aspect of food allergy to model. While responses to oral challenge have been widely described in certain mouse models, these responses are often weak, difficult to measure objectively, and require extreme doses of antigen (100–200 mg per 20–30 g mouse, the equivalent of 60–200 g for a 20-kg child). Often, intraperitoneal challenge is needed to elicit reliable and robust anaphylaxis responses. This remains a key criticism of mouse models of food allergy.

7 Microbiota

There is increasing evidence that gut microbiota plays a critical role in allergic sensitization and tolerance induction in humans and rodents [108, 109], and therefore, composition of the gut microbiome may influence the outcome of food allergy mouse models. In fact, germ-free mice display a characteristic increased immune response to allergens with a remarkable Th2 bias compared to mice colonized with a diverse microbiota [110, 111]. In addition, antibiotic treatment of mice could increase the susceptibility to food allergy, and colonization of germ-free mice with microbiota from healthy infants or sensitized mice can suppress sensitization or transfer food allergy susceptibility, respectively [111, 112]. Moreover, commensal bacteria influence intestinal epithelial cell function and regulate its protective barrier properties [111], promoting immunoregulatory responses within mucosal tissue that protects against allergic sensitization [7, 97, 113]. Colonic Treg induction has been attributed mainly to the *Clostridium* species [111] although other microbial species are also implicated [114]. In contrast, altered microbial composition can also increase susceptibility to food allergy as it has been shown by several studies [45, 47, 115].

The gut microbiome of mice housed in different animal facilities can be influenced by the breeding environment [7], and it should be taken into account when studies carried out in different laboratories are compared. Moreover, it is known that some gut

microorganisms influence food sensitization, and therefore, gut microbiome should be taken into account when sensitizing capacity of proteins is studied in mouse models to avoid masking the results.

8 Food Extract Versus Purified Proteins

The use of whole foods in animal models has the advantage of presenting the proteins to the immune system in their natural context [7]. However, food matrix can potentiate or inhibit the sensitizing capacity of food proteins. Other proteins, fats, carbohydrates, micronutrients, and contaminants (e.g., LPS) may have various effects on the intrinsic allergenicity of proteins by changing their digestibility, bioaccessibility, and bioavailability, as well as on the host immune system by exerting immunomodulatory effects as adjuvants [3]. Proof of this is the fact that intranasal exposure to milk, but not to purified β-lactoglobulin, led to β-lactoglobulin-specific IgG1 production, suggesting an important role of the milk matrix in the presentation to the immune system [95]. In addition, the lipid fraction of peanut and Brazil nut has been demonstrated to be involved in their immunogenicity [116, 117]. Medium-chain triglycerides, common constituents in food-based oils, have been shown to stimulate allergic sensitization and anaphylaxis to peanut, likely by affecting intestinal absorption and availability of antigens and increasing Th2 cytokines in the spleen and intestines [59].

Another option is the use of protein extracts, although protein solubility may be influenced by the processing of the foods and the proportion of the extracted proteins may be altered in the final extract compared to the whole food, resulting in the testing of an incomplete panel of proteins [7]. If purified proteins are used, the purity and quality must be of a high standard because the predictive value of the animal model may be greatly influenced by contaminants, such as other proteins, endotoxin, or other contaminants [7].

9 Summary

Appropriate mouse models are critical to advance the field of food allergy by revealing mechanisms of allergy and for testing novel therapeutic approaches. Oral sensitization models with the use of an adjuvant to break tolerance or transdermal sensitization models are the most common models currently in use. Mouse models with defined genetics have highlighted the role of IL-4 and DOCK8 pathways in food allergy susceptibility, and the importance of genetics is exemplified by the strain dependence of the models. Mouse models have also advanced our understanding of the important role of the intestinal microbiota in regulation of susceptibility to food allergy. Despite major advances in the field of mouse

modeling of food allergy, including the development of humanized mouse models, a weakness of current model systems is the requirement for extreme doses by oral challenge or the use of intraperitoneal challenge to elicit objective symptoms.

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Chapter 2

Induction of Peanut Allergy Through Inhalation of Peanut in Mice

Joseph J. Dolence

Abstract

Peanut (PN) allergy is a common life-threatening disease; however, our knowledge on the immunological mechanisms remains limited. Here, we describe the first mouse model of inhalation-driven peanut allergy. We administered PN flour intranasally to naïve wild-type mice twice a week for 4 weeks, followed by intraperitoneal challenge with PN extract. Exposure of mice to PN flour sensitized them without addition of adjuvants, and mice developed PN-specific IgE, IgG1, and IgG2a. After challenge, mice displayed lower body temperature and other clinical signs of anaphylaxis. This inhalation model is an ideal system to allow for future examination of immunological mechanisms critical for the development of PN allergy.

Key words Peanut, Allergy, IgE, Anaphylaxis, Inhalation, Food allergy

1 Introduction

Peanut (PN) allergy is a growing public health concern [1]. Among children in the United States, the incidence of PN allergy increased fivefold from 0.4% in 1997 [2] to 2.0% in a national survey taken in 2010 [3]. While PN allergy remains increasingly problematic, especially for the youngest in our society, our understanding of how the disease initiates after the immune system encounters PN remains unclear. The majority of PN-allergic children experience their first allergic reaction to PN upon first ingestion of PN [4]. Recent clinical trials have provided strong evidence that eating PN early in life allows the development of an oral tolerance that protects children from developing allergic responses to PN [5, 6]. Since PN is readily detectable in household dust [7, 8], we examined whether mice could be sensitized to PN via inhalation. Here, we demonstrate mice exposed to PN via the airways developed clinical PN allergy [9]. Specifically, we demonstrate a 4-week-long, twice-weekly inhalation mouse model to establish PN allergy (Fig. 1). Using this model, we documented the development of PN-specific

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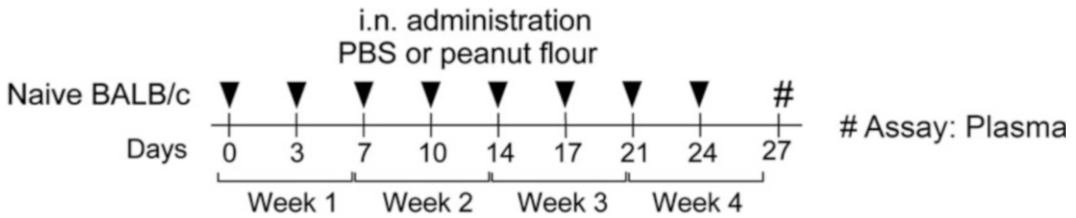

Fig. 1 Timeline of experimental model. Mice are exposed to either PBS or PN flour intranasally twice/week for 4 weeks. On Day 27, mice are bled to obtain plasma for PN-specific antibody ELISA analysis. On Day 28 (not pictured), mice are intraperitoneally challenged with PN to induce an anaphylactic reaction (Reproduced from ref. 9 with permission from Elsevier)

IgE, IgG1, and IgG2a responses (Fig. 2) and clinical symptoms resembling PN allergy in humans (Fig. 3) [9].

Due to the proposed link between eating PN and becoming sensitized to PN, many models of PN allergy are oral [10–12]. In addition, a majority of PN allergy mouse models require the use of mucosal adjuvants, such as cholera toxin and staphylococcal enterotoxin B [10–14]. To remove the adjuvant requirement from these models, mouse models that genetically inhibited Toll-like receptor 4 [15] and mice that expressed a disinhibited form of the IL-4 receptor were created [16]. These different manipulations, whether by adjuvant or genetics, make it difficult to elucidate the immunologic mechanisms involved in the initiation of peanut allergy. Moreover, data strongly support early oral exposure leads to tolerance, not sensitization, underscoring the importance of examining non-oral routes of sensitization [5, 6]. An adjuvant-free skin model of PN sensitization has been described, a finding consistent with the growing clinical evidence that demonstrates an association between atopic dermatitis and PN allergy in children [17]. Given environmental exposure to PN can occur three ways: eating (oral), touching (skin), or breathing (airway inhalation), we asked whether mice could become allergic to PN through the airways. Thus, we have described the first mouse model of inhalation-driven peanut allergy. This model is an ideal system for dissecting the immunological mechanisms that lead to the development of PN allergy and could be modified to test other food allergens in future studies.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C room temperature). Prepare and store all reagents at room temperature (unless otherwise specified). Follow all waste disposal regulations when disposing waste materials. We do not add sodium azide to any reagents.

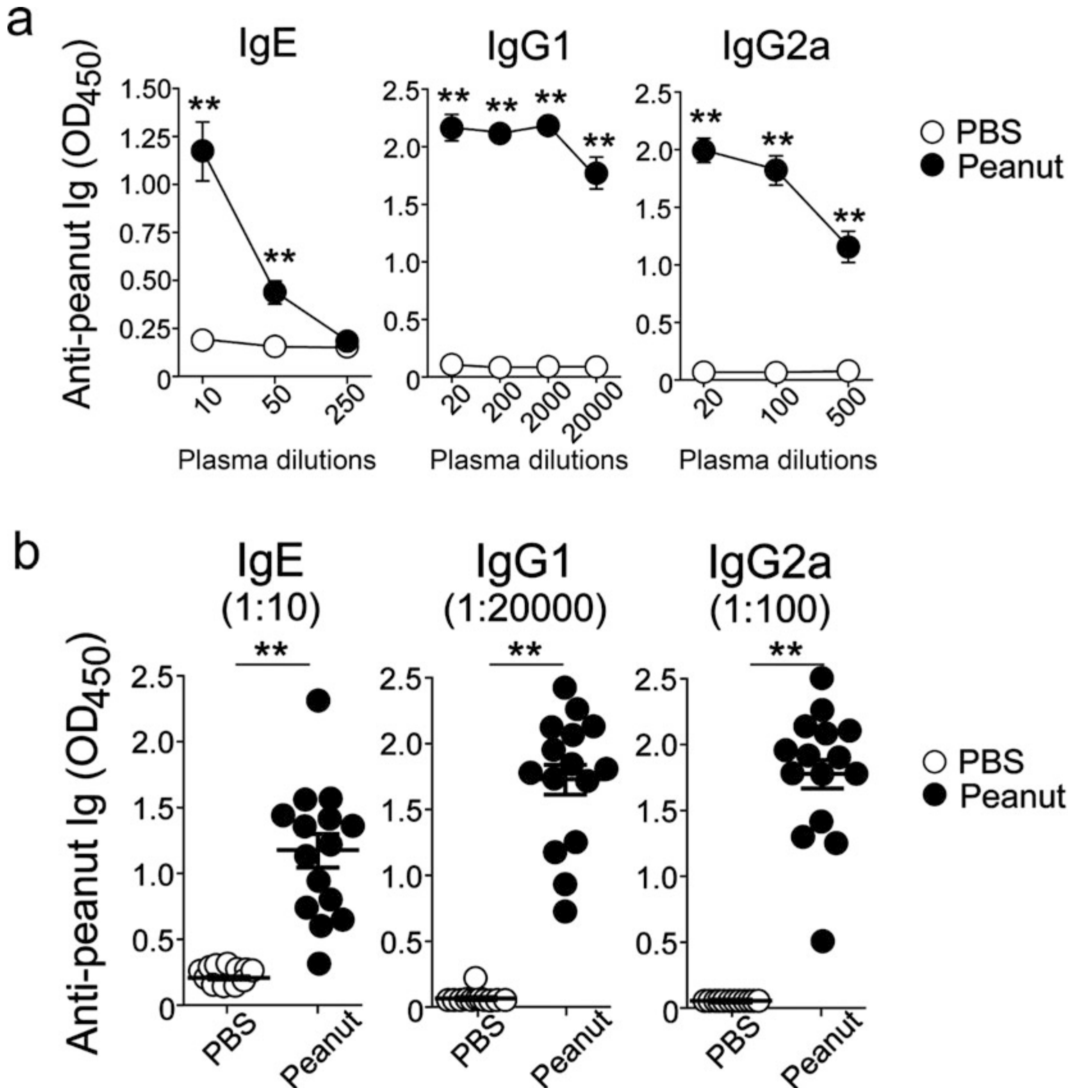

Fig. 2 Inhalation of PN stimulates PN-specific antibody responses. (**a**) Titers of anti-PN antibodies in plasma were determined on day 27 by ELISA. $^{**}P < 0.01$ compared to mice exposed to PBS. (**b**) Levels of anti-PN antibodies in each mouse are shown. Data are a pool from three experiments and are presented as mean $\pm$ SEM ($n = 12$–15 in each group) (Reproduced from ref. 9 with permission from Elsevier)

2.1 PN Flour Suspension

1. Phosphate-buffered saline (PBS): 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4. Combine 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, 8.0 g of NaCl, and 0.2 g of KCl in about 800 mL of water. After adjusting the pH to 7.4 with HCl, bring the final volume up to 1 L with water. PBS may be prepared as a 10× stock solution (14.4 g of Na_2HPO_4, 2.4 g of KH_2PO_4, 80.0 g of NaCl, and 2.0 g of KCl in 1 L of ultrapure water, pH 7.4). To prepare PBS using this 10× stock solution, dilute 5 mL of 10× PBS stock solution to a 50-mL conical tube and add water to a volume of 50 mL.

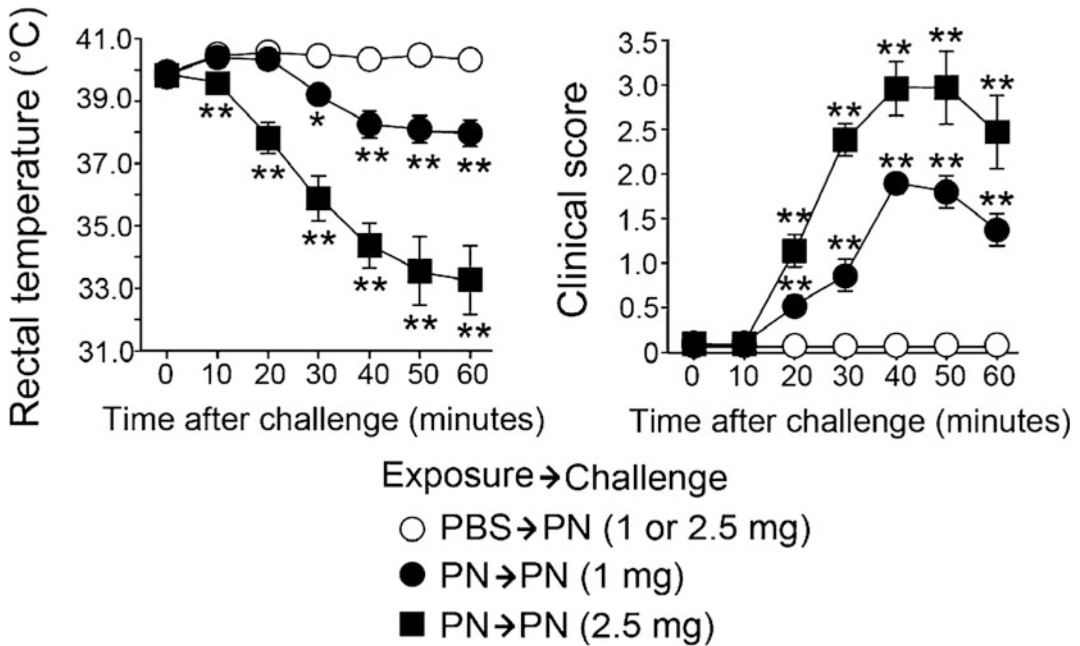

Fig. 3 Challenge with PN stimulates anaphylactic reaction in PN-sensitized, but not PBS-sensitized, mice. Changes in rectal temperature (left) and clinical scores (right) in mice challenged by intraperitoneal injection of CPE are shown. Data are presented as mean ± SEM ($n = 9$–15 in each group) and are a pool of three experiments. *$P < 0.05$ and **$P < 0.01$ compared with mice exposed to PBS (Reproduced from ref. [9] with permission from Elsevier)

Mix and store at room temperature. Each control mouse will be exposed to 50 μL PBS via inhalation.

2. 1.5-mL microcentrifuge tubes.
3. 15-mL conical tubes.
4. Pipettes and tips.
5. PN flour suspension stock solution: 5 mg of PN flour/mL of PBS. Place a 15-mL conical tube into a 100-mL beaker to balance the tube for weighing PN flour. Place onto an analytical balance that measures to the thousandths place (three decimal places). Weigh 25 mg of partially defatted (12% fat), light roast PN flour into the 15-mL conical tube. Add 5 mL of PBS to make 5 mg/mL of stock PN flour solution (*see* **Note 1**).
6. PN flour suspension final solution: 2 μg of PN flour/μL of PBS. Measure out 300 μL of PBS into a 1.5-mL microcentrifuge tube. Vortex the PN flour stock solution for 30 s and pipette 200 μL of the PN flour stock solution into a microcentrifuge tube containing 300 μL of PBS (*see* **Note 1**). Vortex to ensure even mixture of PN flour particles in the 2 μg/μL final PN flour solution (*see* **Note 2**). Each PN-exposed mouse will inhale 50 μL of the 2 μg/μL final PN flour solution, which will deliver 100 μg of PN into the airways.

2.2 Exposing Mice to PN via Inhalation

1. Adult mice: 6–12 weeks of age preferred, BALB/c or C57BL/6 backgrounds.
2. Isoflurane vaporizer (*see* **Note 3**).
3. 1.5-mL microcentrifuge tubes containing PBS or PN solution.
4. Pipettes and tips.

2.3 Crude PN Extract Suspension

1. PBS: pH 7.4. Prepared as described above (*see* Subheading 2.1).
2. Crude PN extract (CPE) stock solution: 50 mg of CPE/mL of PBS. Purchase CPE from Stallergenes Greer, Lenoir, NC, USA. Resuspend the CPE pellet with the requisite amount of PBS to obtain a 50 mg/mL solution, using the dry weight measurement found on the vial as a guide (*see* **Note 4**). Mix and store at 4 °C (*see* **Note 5**).
3. CPE Final Solution: 5 mg of CPE/mL of PBS. Conduct a 1:10 dilution of the CPE stock solution using PBS to generate the CPE final solution (*see* **Note 6**). Vortex to ensure even mixture of CPE in the final solution prior to use (*see* **Note 7**). During anaphylactic challenge on Day 28, each mouse will be injected intraperitoneally with 500 μL, which will deliver 2.5 mg of CPE.

2.4 Monitoring of Anaphylaxis in Mice

1. Electronic thermocouple thermometer with Type T input.
2. RET-3 rectal probe for mice.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

1. 0.5 M EDTA: pH 8.0. Place 186.1 g of disodium EDTA·$2H_2O$ and 800 mL of water in a 1-L beaker and stir on a magnetic stirrer. Adjust the pH to 8.0 with NaOH, and EDTA will dissolve completely. Bring the volume to 1 L with water.
2. High-binding ELISA 96-well microplates.
3. Coating buffer: 0.1 M carbonate–bicarbonate buffer solution, pH 9.5. Add about 800 mL of water to a glass beaker with a stir bar placed on a stir plate. Once water is added, start stirring. Weigh 8.40 g of $NaHCO_3$ (sodium bicarbonate) and slowly transfer to the beaker, ensuring that $NaHCO_3$ fully dissolves in the water. Weigh 3.56 g of Na_2CO_3 (sodium carbonate) and slowly transfer to the beaker, ensuring that Na_2CO_3 fully dissolves in the water (*see* **Note 8**). Adjust the pH to 9.5 if necessary. Add water to a volume of 1 L. Transfer to a bottle and store at 4 °C.
4. Coating antibody: Purified rat anti-mouse IgE heavy chain antibody, clone LO-ME-3. Store at 4 °C.
5. CPE: 2 μg of CPE/mL of the coating buffer (*see* **item 3** above). Conduct a 1:25,000 dilution of the CPE stock solution (same stock as described above) using the coating buffer to generate a

coating buffer containing CPE suitable for coating plates for IgG1 and IgG2a ELISA.

6. PBS: pH 7.4. Prepared as described above (*see* Subheading 2.1).
7. Wash buffer: PBS containing 0.05% Tween-20 (*see* **Note 9**). Store at room temperature.
8. Blocking buffer: PBS containing 1% (w/v) fraction V or molecular biology-grade bovine serum albumin (BSA). Store at 4 °C.
9. Assay diluent: PBS containing 1% (w/v) fraction V or molecular biology-grade BSA and 0.05% Tween-20 (*see* **Note 9**). Store at 4 °C.
10. Biotinylated CPE: BiotinTag™ Micro Biotinylation Kit or equivalent for labeling CPE with biotin (*see* **Notes 10** and **11**). Store at 4 °C.
11. Poly-HRP streptavidin reagent: Commercially available. Store at −20 °C.
12. Detection antibody for IgG1 ELISA: HRP-conjugated rat anti-mouse IgG1, Clone X56. Store at 4 °C.
13. Detection antibody for IgG2a ELISA: HRP-conjugated rat anti-mouse IgG2a, Clone R19–15. Store at 4 °C.
14. TMB (3,3′,5,5′-tetramethylbenzidine) substrate kit: Commercially available. Store at 4 °C.
15. Stop solution: 1 M HCl or 2 M H_2SO_4. Store at room temperature.
16. Microplate autoreader to measure absorbance at 450 nm.

3 Methods

3.1 Exposing Mice to PN via Inhalation: a 4-Week Model

1. Calculate the number of mice being exposed to either PBS alone (control) or PN flour suspension final solution. Based on this number, label two 1.5-mL microcentrifuge tubes as either PBS or PN. Measure out 500 μL of PBS into the PBS-labeled tube. Based on the instructions in Subheading 2.1 (**item 6**), make up 500 μL of PN final solution. Each mouse will be exposed via inhalation to 50 μL of either PBS or PN. Therefore, one tube of PBS can expose a cohort of up to ten control mice to PBS and one tube of PN final solution can expose up to ten additional mice to PN (*see* **Note 12**). If treating more mice, make additional tubes of PBS or PN final solution.
2. Place the first PBS mouse into the anesthesia chamber connected to an isoflurane vaporizer set up to deliver isoflurane. Establish the anesthesia conditions within the chamber by

turning on the connected oxygen to flow at 2 L/min and set the vaporizer to 3.5%. Monitor the mouse as it experiences the effects of the isoflurane. Once the mouse stops moving and is breathing deeply, open the chamber, take out the mouse, and shut the chamber to minimize loss of anesthesia conditions within the chamber.

3. Lay the mouse in the prone position. Grab it with one hand at the top of the neck/base of the head, lifting the mouse to hold it upright, tilting the hand backwards to ensure the nose is upright.
4. Pipette 50 μL of PBS slowly, but deliberately onto the tip of the nose in a dropwise fashion, allowing the mouse to breath in the liquid. To facilitate the airway aspiration of the solution, hold the mouse upright for 30 sec before placing the mouse into its cage on its back to recover from anesthesia (*see* **Note 13**).
5. Place the second and any subsequent PBS mice one-by-one into the anesthesia chamber, making sure to monitor, take out, and treat the mice one at a time as described in **steps 2–4**.
6. Once treatment of the PBS cohort of mice is complete, move onto treating the PN cohort. Place the first PN mouse into the anesthesia chamber. As the mouse is falling asleep, mix the tube with PN solution to put the PN into suspension (*see* **Note 14**).
7. Once the first PN mouse stops moving and is breathing deeply, take the mouse out and quickly shut the chamber to maintain the anesthesia conditions.
8. Lay and grab the mouse as described in **step 3**.
9. Pull up 50 μL of PN solution, pipetting up and down to ensure an even suspension of PN is captured (*see* **Note 15**).
10. Pipette 50 μL of PN solution slowly, but deliberately onto the tip of the nose using the same technique as described for PBS in **step 4**.
11. Place the second and any subsequent PN mice one-by-one into the anesthesia chamber, making sure to monitor, take out, and treat the mice one at a time as described in **steps 6–10**.
12. Once treatment of the PN cohort of mice is finished, Day 0 of treatment is complete. Repeat the PBS and PN treatments as previously described on Days 3, 7, 10, 14, 17, 21, and 24 (Fig. 1) (*see* **Note 16**).

3.2 Biotinylation of CPE

1. Carry out a 1:10 dilution of the CPE stock solution using PBS to generate the CPE solution useful for labeling.
2. Mix 12.2 μL of 1:10 diluted CPE and 87.8 μL of 0.1 M sodium phosphate buffer, pH 7.2 from BiotinTag™ Micro Biotinylation Kit for a final CPE mixture volume of 100 μL.

3. To make the biotinylation solution, dissolve the contents of one vial of BAC-SulfoNHS from the kit with 30 μL of DMSO, and add 970 μL of 0.1 M sodium phosphate buffer from the kit for a final volume of 1 mL.
4. Mix 100 μL of CPE mixture with 100 μL of the biotinylation solution for a final reaction volume of 200 μL. Once mixed, allow to incubate for 30 min at room temperature with gentle stirring (*see* **Note 10**).
5. During incubation, vortex one of the micro-spin Sephadex G-50 columns provided by the kit to resuspend the resin. Loosen the cap 1/4 turn and snap off the bottom closure. Place the column in a 1.5-mL microcentrifuge tube with its cap cut off. Spin for 1 min at 700 × *g*.
6. After the spin, add 200 μL of PBS to the column. Spin again for 1 min at 700 × *g*. Repeat this step by adding 200 μL of PBS to the column and spinning for 1 min at 700 × *g*.
7. Cut off the caps and label four 1.5-mL microcentrifuge tubes used to elute biotinylated CPE. After the 30-min incubation is complete, place the column into a first tube and apply the biotinylation reaction mixture to the column, being careful not to disturb the resin.
8. Spin for 2 min at 700 × *g* to collect the first elution of purified bio-CPE. Put a cap onto the first tube and set aside. Put the column into a second tube and apply 200 μL of PBS to the column.
9. Spin for 2 min at 700 × *g* to collect the second elution of purified bio-CPE. Put a cap onto the second tube and set aside. Repeat steps described to elute bio-CPE from the column into the second tube with a third and fourth tube. After the column has been eluted into four tubes, determine protein concentration for each tube and pool the contents of tubes containing similar concentrations of bio-CPE (*see* **Note 11**). Store at 4 °C.

3.3 ELISA for PN-Specific Antibodies

1. On Day 27 (Fig. 1), label the number of 1.5-mL microcentrifuge tubes necessary to collect plasma from each mouse.
2. Measure out 100 μL of 0.5 M EDTA into each of the labeled tubes.
3. Lightly anesthetize a first PBS mouse by isoflurane inhalation using technique described in Subheading 3.1, making sure to take out the mouse a few seconds after they stop moving (*see* **Note 17**).
4. Using a 100-μL glass capillary tube, retroorbitally bleed the first PBS mouse.

5. After collecting 100 μL of blood, dispense the content of the full glass capillary tube into tube labeled for the first PBS mouse. Gently mix the blood with EDTA to prevent clotting of the sample.
6. Repeat **steps 3–5** with each of the remaining mice.
7. Once blood collection is completed, centrifuge the tubes at 1500 × *g* for 5 min at room temperature.
8. Label new tubes for each sample, and once centrifugation is complete, collect supernatants from each tube. The supernatant is the plasma being analyzed by ELISA for PN-specific antibodies.
9. Freeze at −20 °C until Ig ELISA analysis of plasma samples is conducted (*see* **Note 18**).
10. Coat the wells of the ELISA microplate used to examine plasma levels of PN-specific IgE with 50 μL/well of the coating antibody diluted in the coating buffer at 5 μg/mL. Seal the plate with microplate seal.
11. Coat the wells of the ELISA microplates used to examine plasma levels of PN-specific IgG1 and PN-specific IgG2a with 50 μL/well of CPE diluted in the coating buffer at 2 μg/mL. Seal the plate.
12. Incubate the plates overnight at 4 °C.
13. Wash three times by filling the wells with the wash buffer, decanting, and tapping the plates on absorbent paper each time. The wash steps of this protocol can be carried out with a microplate washer or washing by hand with a squeeze bottle.
14. Block plates with 200 μL/well of the blocking buffer. Seal the plates and incubate for 1.5 h at room temperature.
15. During blocking incubation time, make the following dilution series for the PN-specific IgE ELISA by diluting plasma samples with the assay diluent in 1.5-mL microcentrifuge tubes: 1:10, 1:50, 1:250, 1:500 (*see* **Note 19**). Each plasma sample will have one set of these dilutions.
16. During blocking incubation time, make the following dilution series for the PN-specific IgG1 ELISA by diluting plasma samples with the assay diluent in 1.5-mL microcentrifuge tubes: 1:20, 1:200, 1:2000, and 1:20,000 (*see* **Note 20**). Each plasma sample will have one set of these dilutions.
17. During blocking incubation time, make the following dilution series for the PN-specific IgG2a ELISA by diluting plasma samples with the assay diluent in 1.5-mL microcentrifuge tubes: 1:20, 1:50, 1:100, and 1:500 (*see* **Note 21**). Each plasma sample will have one set of these dilutions.

18. After the blocking step is completed, wash as described in **step 13**.
19. For IgE ELISA, add 100 μL/well in duplicate for each plasma dilution (1:10, 1:50, 1:250, 1:500), using each column of the microplate/mouse (*see* **Note 22**). Running four dilutions in duplicate is eight samples down the microplate. After adding plasma dilutions, seal the plate and incubate for 2 h at room temperature.
20. For IgG1 ELISA, add 100 μL/well in duplicate for each plasma dilution (1:20, 1:200, 1:2000, 1:20,000), using each column of the microplate/mouse. Running four dilutions in duplicate is eight samples down the microplate. After adding plasma dilutions, seal the plate and incubate for 1 h at room temperature.
21. For IgG2a ELISA, add 100 μL/well in duplicate for each plasma dilution (1:20, 1:50, 1:100, 1:500), using each column of the microplate/mouse. Running four dilutions in duplicate is eight samples down the microplate. After adding plasma dilutions, seal the plate and incubate for 1 h at room temperature.
22. After plasma sample incubation is complete for both IgG1 and IgG2a ELISAs, wash as described in **step 13**.
23. Dilute IgG1 or IgG2a detection antibody at 1:1000 with the assay diluent. Add 100 μL/well of the diluted detection antibody. Seal each plate and incubate for 1 h at room temperature.
24. After plasma sample incubation is complete for IgE ELISA, wash as described in **step 13**.
25. Dilute biotinylated CPE to 1:2000 with the assay diluent for IgE ELISA. Add 100 μL/well of diluted biotinylated CPE. Seal the plate and incubate for 1 h at room temperature.
26. After the incubation with the detection antibody is complete for both IgG1 and IgG2a ELISAs (*see* **Note 23**), wash five times with the wash buffer.
27. Prepare the substrate solution from a TMB substrate kit following the kit instructions. Briefly, prepare a 1:1 mix of two solutions in the substrate kit.
28. Add 100 μL/well of the working substrate solution to IgG1 and IgG2a plates. Seal the plate and incubate at room temperature for 15 min in the dark. Turn on and set up the microplate autoreader.
29. After substrate incubation, add 100 μL/well of the stop solution. Read absorbance at 450 nm within 30 min of stopping reaction on a microplate autoreader (Fig. 2).

30. After incubation with biotinylated CPE is complete for IgE ELISA, wash five times with the wash buffer.
31. Dilute the poly-HRP streptavidin reagent 1:5000 with the assay diluent for IgE ELISA. Add 100 μL/well of the diluted poly-HRP streptavidin. Seal the plate and incubate for 30 min at room temperature.
32. After poly-HRP streptavidin incubation is finished, wash five times with the wash buffer.
33. Prepare the substrate solution as described in **step 27**.
34. Add 100 μL/well of working substrate solution to IgE plate. Seal the plate and incubate at room temperature for 30 min in the dark. Turn on and set up the microplate autoreader.
35. After substrate incubation, add 100 μL/well of the stop solution. Read absorbance at 450 nm within 30 min of stopping reaction on a microplate autoreader (Fig. 2).

3.4 Inducing and Monitoring Anaphylaxis to PN in Mice

1. On Day 28, prepare CPE final solution as directed above (*see* **Note 24**).
2. With a 1-mL syringe (*see* **Note** 7), pull up 500 μL of CPE final solution. Set the filled syringe aside.
3. Repeat **step 2**, filling the remaining syringes with CPE final solution.
4. Connect the RET-3 rectal probe to the electronic thermocouple thermometer. Turn on the thermometer. It should read the ambient temperature in the room.
5. Measure and record the rectal temperature for each mouse by inserting the probe into the anus of the mouse. Hold until temperature is stable. This is the zero (0)-min temperature reading. Normal rectal temperature for a mouse is between 37 and 40 °C [18]. When handling the mice, grab by the scruff of their neck, flip them over so their stomach faces upward, and curl the pinkie finger under a hind limb to stabilize the mouse.
6. Measure the clinical score for each mouse based on the following published criteria [19]: 0, no symptoms; 1, scratching of ear and mouth; 2, puffiness around eyes and mouth, pilar erection, labored breathing; 3, prolonged period of motionlessness; 4, severely reduced motility, tremors, severe respiratory distress; 5, death (*see* **Note 25**). Since this is the zero (0)-min time point, the clinical score is 0, no symptoms.
7. Using a clock with a second hand or digits as a guide, inject each mouse intraperitoneally with 500 μL of CPE final solution at the beginning of every minute (e.g., 9:00 AM, 9:01 AM, 9:02 AM, etc.) (*see* **Note 26**) until all mice have been injected. Record the time of each injection.

8. Ten minutes after the first injection (e.g., 9:10, 9:11, 9:12 AM, etc.), measure and record the rectal temperatures for each mouse beginning with the first mouse that was injected. Separated by 1 min, record the rectal temperature of each mouse in the experiment by order of injection. This is the 10-min temperature reading.
9. As the rectal temperature is being recorded, measure the clinical score for each mouse as described in **step 6**.
10. Repeat **steps 8** and **9** for each time point (20, 30, 40, 50, and 60 min), recording the rectal temperatures and clinical scores (*see* **Note 25**) of each mouse in the experiment by order of injection (each separated by 1 min) (*see* **Note 27**). Anaphylaxis is observed through a significant drop in rectal temperature and presence of clinical symptoms (Fig. 3).
11. After the 60-min time point has been recorded, sacrifice the mice. If desired, retroorbital blood can be taken at this time, prior to sacrificing the mice, to measure mediators of anaphylaxis (e.g., MCPT-1, histamine) in the plasma with ELISA [9].

4 Notes

1. PN flour does not fully go into suspension. Therefore, we make a fresh PN stock solution in order to generate fresh final PN solutions each day we expose mice to PN. In this way, we ensure that each final PN solution is fresh and poised to deliver 100 μg of PN into the airways per 50 μL. Since PN flour is an inexpensive reagent, we have always made these solutions fresh on the days of PN exposure. Therefore, we are uncertain if using stored stock PN flour solution would be different than the fresh version.
2. 500 μL of final PN flour solution will enable ten mice to be exposed to PN (50 μL/mouse). Scale as necessary to make more final PN flour solution to expose additional mice. To ensure we have enough of the final PN flour solution when we expose mice, we always make up twice as much as necessary.
3. Exposing mice to PN via inhalation is most optimally achieved by using an isoflurane vaporizer to put the mice under anesthesia. If the institution does not have one, a wide-mouth jar with an easily removable cover may be used as the chamber to anesthetize the mice, provided that the Institutional Animal Use and Care Committee of your institution approves this method. To put the mice initially under anesthesia in these conditions, place a cotton ball in the bottom of the jar and soak the cotton ball with 1 mL of isoflurane by pipetting the isoflurane directly onto the cotton. To maintain anesthesia

conditions (normally done after treating 2–3 mice, depending on the size of the jar), pipette 500 μL of additional isoflurane onto the cotton ball. Repeat maintenance isoflurane every 2–3 mice treated as necessary until all mice are treated.

4. One vial of CPE we previously used had a dry weight measurement of 535 mg/vial. We resuspended the CPE pellet with 10.7 mL of PBS to make the CPE stock solution (535 mg divided by 50 mg/mL equals 10.7 mL).
5. During storage, CPE falls out of suspension. When it is taken out for use, vortex to thoroughly mix CPE before using it to generate CPE final solution.
6. Each mouse is intraperitoneally exposed to 500 μL of CPE final solution, which delivers 2.5 mg of CPE. While we normally deliver 2.5 mg of CPE/mouse to induce anaphylaxis, we have been successful in stimulating anaphylactic reactions by delivering 1.0 mg of CPE/mouse (Fig. 3). In order to deliver 1.0 mg of CPE, carry out a 1:25 dilution using PBS to generate a 2.0 mg of CPE/mL CPE final solution. At this concentration (2.0 mg/mL), 500 μL of CPE delivers 1.0 mg of CPE.
7. We use 1-mL syringes (tuberculin syringes or an allergist tray with 27 G × 1/2-in needles) to deliver injections. We transfer CPE final solution from the 15-mL conical tube we use to generate the final solution to 1.5-mL microcentrifuge tubes, so we can pull up the CPE final solution into the syringes.
8. Alternatively, the carbonate–bicarbonate coating buffer solution can be made from mixing the contents of commercially available pre-made carbonate–bicarbonate buffer packs with water. We have used buffer packs that make 500 mL of 0.2 M carbonate–bicarbonate coating buffer, pH 9.4.
9. Tween-20 is viscous and difficult to pipette in small amounts. When making wash buffer and assay diluent, we cut off the last 1/4 in of a 1000-μL pipette tip. This simple step makes pipetting Tween-20 much easier.
10. Use a 1.5-mL microcentrifuge tube for biotinylation reaction. Also, use a small stir bar made for microcentrifuge tubes on the stir plate. To stabilize a microcentrifuge tube on a stir plate, place the reaction tube in a microcentrifuge tube foam floating rack to keep it upright and stable during the reaction time.
11. Eluted fractions from tubes 1–3 typically have similar protein concentrations (measured using BCA assay), so we pool these fractions to make up our final bio-CPE reagent, and discard the fourth tube. Using this kit, we have generated a stable bio-CPE reagent (0.24 μg/mL) usable to conduct PN-specific IgE ELISA for around 12–18 months.

12. While each prepared tube of either PBS or PN can expose ten mice, we make an additional tube of whatever reagent we need (PBS or PN) if exposing 8–10 mice to either PBS or PN to ensure we have enough reagent to treat all mice.
13. Depending on how deeply the mice are under anesthesia, they may wake up before the 30 s of holding them is completed. In this case, as they are about to be awaken, put back into the cage, even if it is earlier than 30 s. The critical part is the mouse inhaling the reagent, which takes place within seconds of it touching the tip of the nose.
14. The PN flour will fall out of suspension during the time it takes to treat the PBS mice, so before pipetting PN final solution, hold the microcentrifuge tube containing the PN solution between the index finger and thumb and vigorously shake it for 5–10 s. This will put the PN back into suspension and ready to be pipetted.
15. Quickly pipetting up and down about five times will ensure an even suspension of PN captured within the 50 μL of PN solution about to be delivered to the mouse.
16. The 4-week-long, twice weekly model (Fig. 1) takes some planning to set up the dates. We do the PBS and PN exposures (Days 0, 3, 7, 10, 14, 17, 21, and 24) on a 4-weekly rotation of Tuesdays and Fridays. Using this rotation, Day 27, the day mice are bled to collect their plasma, falls on a Monday and Day 28, the day mice are challenged with PN to induce anaphylaxis, is on a Tuesday. Alternatively, Day 0 could be on a Friday, and using a Friday–Monday rotation, where Day 27 is on a Thursday and Day 28 on a Friday. Choosing to start Day 0 (the first day) on a Wednesday, Thursday, Saturday, and Sunday places PBS- and PN-exposure days on weekends. Choosing a Monday for Day 0 start would mean that the Day 27 bleed would fall on a Sunday.
17. When retroorbitally bleeding mice, if the mice undergo the effects of anesthesia for as long as necessary to expose mice to PBS or PN via inhalation, bleeding becomes more difficult as their blood pressure drops. To ensure successful bleeding, we mentally count to five from the moment the mouse stops moving.
18. Subheading 3.3, **steps 10–12** should be done the day before collecting plasma (Day 26) if ELISA is to be done the same day as collection. Otherwise, plasma is incredibly stable, lasting for years when it is frozen at −20 °C.
19. To make up the dilution series for PN-specific IgE ELISA, we mix the following volumes for each plasma sample being analyzed: 1:10 is 30 μL of undiluted plasma + 270 μL of assay diluent; 1:50 is 60 μL of 1:10 dilution +240 μL of assay

diluent; 1:250 is 60 μL of 1:50 dilution +240 μL of assay diluent; and 1:500 is 25 μL of 1:50 dilution +225 μL of assay diluent.

20. To make up the dilution series for PN-specific IgG1 ELISA, we mix the following volumes for each plasma sample being analyzed: 1:20 is 15 μL of undiluted plasma + 285 μL of assay diluent; 1:200 is 30 μL of 1:20 dilution + 270 μL of assay diluent; 1:2000 is 30 μL of 1:200 dilution + 270 μL of assay diluent; and 1:20,000 is 30 μL of 1:2000 dilution + 270 μL of assay diluent.

21. To make up the dilution series for PN-specific IgG2a ELISA, we mix the following volumes for each plasma sample being analyzed: 1:20 is 15 μL of undiluted plasma + 285 μL of assay diluent; 1:50 is 5 μL of undiluted plasma + 245 μL of assay diluent; 1:100 is 60 μL of 1:20 dilution + 240 μL of assay diluent; and 1:500 is 60 μL of 1:100 dilution + 240 μL of assay diluent.

22. Using each column of the microplate/mouse would allow testing of a maximum of 12 mice per plate. This gives flexibility to examine plasma from wild-type (WT) PBS, WT PN, along with knockout mouse models of interest [9] in a side-by-side comparison on the same plate.

23. The order the plasma dilutions were added to the plate will dictate which ELISA plate finishes first. We pipette IgE plasma dilutions first, follow with IgG1 plasma dilutions, and then finish with IgG2a. We suggest this order (the steps listed are in this order) because the plasma incubation time for IgE is 2 h, rather than the 1 h for both IgG ELISAs. Based on this timing, IgG1 will finish first, then IgG2a, and finally, IgE.

24. Each mouse will be injected intraperitoneally with 500 μL. Make sure in the calculations to account for extra doses due to the dead volume of the 1-mL syringes being used to carry out the injections. We make up additional 2 mL (four doses) to ensure satisfactory amounts of CPE for injections. Once the CPE final solution is made, transfer to 1.5-mL microcentrifuge tubes (*see* **Note** 7).

25. Using this model, clinical symptoms usually show up around 20–30 min in the mice that were sensitized to PN for 4 weeks, but not the PBS-treated mice. Symptoms start with 1, scratching of ear and mouth. This manifests in mice scratching their ears vigorously, beyond normal grooming. Around 30 min, mice enter into the 2–3 range and exhibit hair standing on its end (pilar erection), labored breathing, and motionlessness. To test, we pick up the mice and place them down into the cages. If the mice move slowly to the side of the cage, we score as a 2; if the mice move even slower or barely move, we score it as a

3. If the mice fail to move, are shaking, and even worse breathing, we give that mouse a score of 4. Normally, scores of 4 show up around 40 min post-injection. We have never had a mouse die using this method of anaphylactic challenge, so we have never scored mice as a 5.

26. Due to recording rectal temperatures every 10 min for the 1-h post-CPE injection anaphylactic period, injecting a different mouse at the beginning of each minute will allow for a maximum of 9 mice in the 1-h period of the anaphylactic challenge. Separating the mice 1-min apart allows for much easier temperature measurements. During times we have more than nine mice, but not enough to justify a second 1-h round (e.g., 12 mice), we have separated the mice by 30 s.

27. It is normal for mice to begin to recover and show an increase in rectal temperature and decrease in clinical score by 50–60 min post-injection. It does not happen in every mouse, but it occurs.

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Chapter 3

Assessment of Immune Responses in an Animal Model of Wheat Food Allergy via Epicutaneous Sensitization

Norimasa Tamehiro, Reiko Adachi, and Kazunari Kondo

Abstract

Wheat allergy is a pathological event involving immunocompetent cells against ingested wheat allergen and is clearly associated with transdermal sensitization. However, the molecular mechanisms involved in the disease etiology are not completely understood. A complex cellular and tissue network linking to food allergy makes it difficult to understand the molecular mechanism of allergenicity. Animal models are valuable tools to deduce basic principles of human disease without invasive intervention trials. A mouse model of wheat allergy has provided insights into effects of skin exposure to wheat protein; it is a plausible route of human sensitization for wheat anaphylaxis. Further investigation of this model will capture the essential occurrence and flow of events, bringing useful clues to develop effective treatment and control strategies against wheat allergy. Here, we describe a method for analyzing the expression of cell surface molecules in single cells isolated from lymphoid tissue with flow cytometry. Sensitization by wheat extracts significantly increases antigen-specific T cells in the spleen. Collecting information regarding the contribution of immune cells to allergic sensitization in the development of wheat allergy would be useful in preventing and treating food allergies.

Key words Food allergy, Wheat allergy, Allergy mouse model, Transdermal sensitization, Immune cell responses, FACS

1 Introduction

Wheat allergy is one of the most common food allergies, resulting in unfavorable outcomes following ingestion of wheat protein [1, 2]. Symptoms occurring in patients with abnormal immune systems that recognize food antigens include itchiness, redness, hives, rashes, gastrointestinal discomfort, respiratory problems, and blood pressure drop. These immune reactions are primarily driven by IgE-mediated mast cell degranulation following antigen-specific IgE production by committed germinal center B cells in draining lymph nodes [3]. Therefore, antigen transport to local lymph nodes via antigen-presenting cells is critical for initiating allergic responses against food antigens. However, the contributions of various immune cells to allergic sensitization remain

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poorly understood. To elucidate the molecular and cellular mechanisms that underlie the onset of allergy to wheat, identification of immune cells involved in possible exacerbation pathways and characterization of both spatial and temporal sequence of events during food allergen sensitization are important.

In the past decade, progressive understanding in the field has been made to implicate that epicutaneous sensitization to antigens is a key route in the development and pathogenesis of food allergy [4]. There were many documented cases of wheat allergy caused by percutaneous sensitization via hydrolyzed wheat protein (HWP) present in facial soap in Japan [5]. This indicates the importance of skin sensitization for wheat allergy, as supported by circumstantial epidemiologic evidence. Therefore, we established a mouse model of HWP epicutaneous sensitization [6, 7]. The allergy response in this model closely resembles that in humans who are allergic to HWP-containing cosmetics and could be a promising and powerful tool for understanding the dynamics of immune cell maturation and phenotypic evolution in the development of wheat allergy.

Flow cytometry is a widely employed technology for analyzing the cell features by size and expression of lineage-specific cellular markers. A critical advantage of this approach is the ability to define different cell types in a heterogeneous cell population via simultaneous multiparameter analyses of single cells. Therefore, this technology allows for extensive and precise investigation into the immune responses involved in temporal changes in important contributors of allergic sensitization.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents.

2.1 Wheat Allergen Sensitization

1. Phosphate-buffered saline (PBS): 0.137 M NaCl, 2.7 mM KCl, 0.01 M Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4. Weigh 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 and make up to 1 L with water (*see* **Note 1**).
2. PBS-SDS: 0.5% SDS in PBS.
3. Antigen solution: Dissolve 100 mg of NIST® wheat flour (SRM® 1567b; *see* **Note 2**) in 500 μL PBS-SDS for extraction. Swirl or mix gently to prepare a homogenous suspension at 37 °C for 3 h. Store at 4 °C and use up within a month.
4. Age-matched female BALB/c mice: Ideally eight weeks old (*see* **Note 3**). At least five mice per group should be required for the determination of significance.

5. Sonicator.
6. Heat block.
7. Isoflurane inhalation apparatus: For anesthesia upon approval by the IACUC at your institution.
8. Pressure-sensitive tape.
9. Test patch: cotton disk, 9 mm in diameter, placed on hypoallergenic adhesive polyurethane film.
10. Nonwoven fabric jacket: A mouse-sized jacket may be made from nonwoven polyester fabric by placing two holes to accommodate the mouse's forelegs. Glue Velcro tape for closing.
11. Electric hair clipper.
12. Razor.
13. Antiseptic cotton wipes or swabs.

2.2 Allergen-Specific IgE ELISA

1. 96-Well ELISA plates.
2. Fluorescence microplate reader.
3. Microplate washer (optional).
4. 50 mM Carbonate buffer: 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.5.
5. Coating solution: 20 μg/mL of NIST® wheat flour in 50 mM carbonate buffer. Dissolve 100 μg of NIST® wheat flour (SRM® 1567b; *see* **Note 2**) in 5 mL of carbonate buffer.
6. PBS-T: 0.05% Tween-20 in PBS.
7. Blocking solution: PBS containing 0.1% casein.
8. Mg^{2+} blocking solution: 1.5 mM $MgCl_2{\cdot}6H_2O$ and β-mercaptoethanol in the blocking solution.
9. Phosphate buffer: 250 mM $NaH_2PO_4{\cdot}12H_2O$ and 62.5 mM $NaH_2PO_4{\cdot}2H_2O$, pH 7.4.
10. IgE standard solution: mouse anti-ovalbumin IgE.
11. Secondary antibody: 1:1000 dilution of rabbit anti-mouse RAM-IgE (Fc) in the blocking solution.
12. Detection antibody: 1:1000 dilution of β-galactosidase linked anti-rabbit IgG (H + L) in the Mg^{2+} blocking solution.
13. Substrate solution: 0.1 mM 4-methylumbelliferyl-β-galactopyranoside, 1.5 mM $MgCl_2$ $6H_2O$, 2 μM β-mercaptoethanol in phosphate buffer.
14. Stop buffer: 25 mM sodium carbonate.

2.3 Flow Cytometry

1. Scissors.
2. Forceps.
3. Pipettes and sterile tips.

4. PBS.
5. Glass slides: used to mash lymph node tissues.
6. Nylon mesh or cell strainer: 42 μm pore size, sterile.
7. 50-mL Conical centrifuge tubes.
8. ACK lysing buffer: 0.15 mM ammonium chloride, 10 μM potassium bicarbonate, and 10 μM ethylenediaminetetraacetic acid disodium salt 2-hydrate.
9. 0.32% Trypan blue solution: commercially available.
10. Hemocytometer.
11. Round-bottom 96-well microplates.
12. Multi-channel pipettes and tips.
13. Microplate centrifuge.
14. Staining buffer: 0.1% BSA and 0.05% sodium azide in PBS.
15. Antibodies: monoclonal antibodies specific for B220 (RA3-6B2), CD4 (GK1.5), CD8α (53-6.7), CD16/32 (2.4G), CD185 (L138D7), GL7 (GL7), IgD (11-26c.2a), IgE (RME-1), IgM (RMM-1), CD279(29F.1A12), TCRβ (H57–597), IL-4(11B11), CD95 (15A7), and IFN-γ (XMG1.2).
16. Culture medium: RPMI 1640 medium contain with 1× penicillin/streptomycin, 0.1 mM MEM nonessential amino acid solution, 10 mM HEPES buffer solution, 1 mM MEM sodium pyruvate solution, 14 mM β-mercaptoethanol, and 10% heat-inactivated fetal bovine serum (FBS).
17. Phorbol 12-myristate 13-acetate (PMA) stock solution: 20 μg/mL PMA in DMSO. Use at a final concentration of 20 ng/mL.
18. Ionomycin solution stock solution: 1 mg/mL ionomycin in DMSO. Use at a final concentration of 1 μg/mL.
19. (+)-Brefeldin A stock solution: 10 mg/mL brefeldin in DMSO. Use at a final concentration of 10 μg/mL.
20. Fixation buffer: 2% paraformaldehyde in PBS.
21. Permeabilization buffer: 0.1% saponin, 1% BSA, 2% FBS, and 0.05% sodium azide in PBS.
22. Flow cytometer with analysis software.
23. Sheath fluid: provided by the FACS machine manufacturer. Commercially available.

3 Methods

3.1 Animal Preparation Before Sensitization on Days 0, 15, and 22

1. Transfer mice to an appropriate procedure room for anesthesia.
2. Place mice in the anesthesia apparatus and administer 2–3% isoflurane for anesthetization until the pain response disappears as determined by toe pinch. Continue isoflurane administration at a constant flow during the entire procedure.
3. Remove back shoulder fur with an electric hair clipper and shave uncut fur with a razor so that back skin, approximately 2×2 cm^2, is exposed.
4. Return the mice to their holding cages until proceeding with the sensitization procedure the next day on Day 1 (*see* **Note 4**).

3.2 Allergen Sensitization on Days 1, 8, 15, and 22

1. Transfer mice to an appropriate procedure room for anesthesia.
2. Anesthetize mice with 2–3% isoflurane as described in Subheading 3.1, **step 2**.
3. Lightly press and successively remove ordinary adhesive tape from the previously depilated skin area ten times for removing the stratum corneum layers (*see* **Note 5**).
4. Wipe the stripped area with antiseptic cotton and keep it dry prior to sensitization.
5. Apply 50 μL of the antigen solution onto the cotton disk attached to the test patch. Vehicle (PBS-SDS)-only sensitization is required for comparison with antigen sensitization.
6. Apply the patch onto the stripped skin area.
7. Cover the patch with the fabric jacket (*see* **Note 6**) and put the mouse back in its cage (*see* **Note 7**).
8. On Days 4, 11, 18, and 24 (Fig. 1a), remove both the jacket and the test patch. Gently clean the test antigen residue on the skin with antiseptic cotton.
9. Conclude all sensitization procedures on Day 24 (*see* **Note 8**).
10. On Day 23 (i.e., 4 weeks), Anesthetize the mice with 2–3% isoflurane to collect blood for testing the development of sensitization to wheat antigen.
11. Draw 50–100 μL of peripheral blood from the superficial temporal vein using an animal lancet. Deposit blood drops directly into collection tubes (e.g., 1.5-mL microfuge tubes).
12. Compress the puncture wound to stop the bleeding and return the mice to their cages.
13. Allow the collected blood samples to clot for at least 20 min at room temperature.

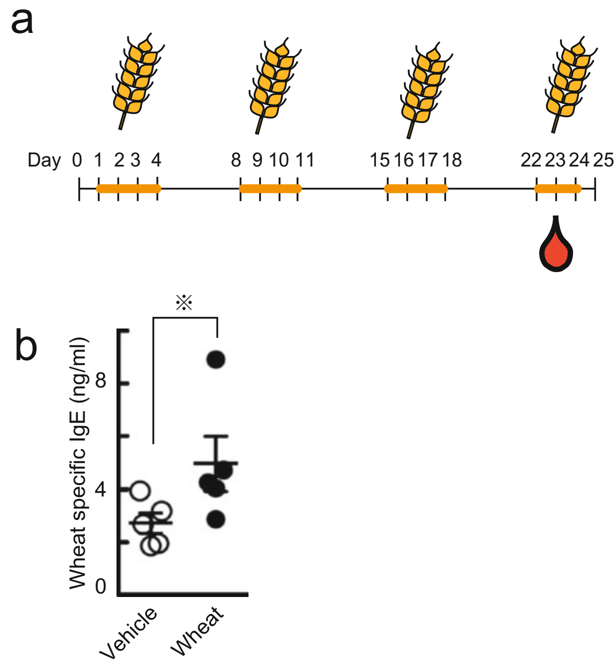

Fig. 1 Percutaneous sensitization of wheat extracts. (**a**) Schematic representation of sensitization protocol. The 3-day sensitization period for the first three rounds and a 2-day sensitization period for the last round are shown by the orange lines. The red droplet on Day 23 indicates the blood collection day as described in Subheading 3.2, **step 10**. (**b**) Wheat-specific IgE levels in sensitized mice. $*P < 0.05$. Data are represented as values (symbols) and means ± SD (bar)

14. Centrifuge the microfuge tubes at 900 × *g* for 15 min and transfer the sera into clean 1.5-mL microfuge tubes. Store frozen at −20 °C until used for ELISA.

3.3 ELISA for Antigen-Specific Immunoglobulin E (IgE) Quantification

1. Coat a 96-well ELISA plate with 50 μL/well of the coating solution containing NIST wheat flour. Seal the plate and incubate overnight at 4 °C.
2. Remove the coating solution and wash the plates three times with 300 μL/well of PBS-T or using a plate washer. Remove remaining liquid by patting the plate on a paper towel.
3. Block nonspecific protein binding with 200 μL/well of blocking buffer for 1 h at room temperature.
4. Aspirate blocking buffer and wash plates five times with PBS-T. Leave the wash solution in wells for 1 min between each wash step to efficiently remove unbound reagents.
5. Add 50 μL/well of diluted serum (*see* **Note 9**). Incubate the serum sample in the plate for at least 2 h at room temperature (*see* **Note 10**).

6. Remove the sera and wash the plate five times with PBS-T as described in **step 2**.
7. Add 50 μL of diluted secondary antibody solution to each well for 1 h at room temperature (*see* **Note 11**).
8. Remove the secondary antibody solution and wash the plate five times with PBS-T as described in **step 2**.
9. Add 50 μL/well of diluted detection antibody solution and incubate for 1 h at room temperature.
10. Remove the detection antibody solution and wash the plate five times with PBS-T as described in **step 2**.
11. Add 100 μL/well of substrate solution to each well and incubate for 1 h at room temperature.
12. Stop the reaction by adding 25 μL/well of stop buffer.
13. Read the fluorescence at 355 and 460 nm for excitation and emission, respectively. Data abstraction for each mouse is conducted in triplicate (*see* **Note 12**). The level of wheat antigen-specific IgE in sera are quantified by measurements from more than five mice (Fig. 1b).

3.4 Flow Cytometry

3.4.1 Preparation and Staining of Lymphoid Tissue Cells

1. On Day 25, anesthetize mice with 2–3% isoflurane using an inhalation anesthesia apparatus as described in Subheading 3.1, **steps 1** and **2**.
2. Remove the mouse only after complete anesthetization and perform cervical dislocation.
3. Apply ethanol on the carcass to avoid fur contamination during tissue separation.
4. Cut a small hole in the lower mid-abdominal region with scissors (Fig. 2) and expose the ventral region by pulling the cutaneous layers toward the head and lower limbs.
5. Remove axillary lymph nodes and the spleen with forceps (Fig. 2, *see* **Note 13**) and transfer to ice-cold PBS.
6. Mash the lymphoid tissues in PBS on glass slides and pass the resulting cell suspension through a 42-μm pore nylon mesh placed over a 50-mL conical centrifuge tube three times by pipetting to obtain single cell suspensions.
7. Centrifuge the strained cell suspensions at 900 × *g* for 5 min.
8. For lymphocytes, resuspend the cell pellets resulted from dissociating lymph nodes in ice-cold PBS.
9. For splenocytes, gently resuspend the cell pellets resulted from dissociating the spleens in 1 mL ACK lysing buffer. Incubate the cells at room temperature for 3 min.
10. Add 4 mL ice-cold PBS and centrifuge the splenocytes at 900 × *g* for 5 min. Resuspend cell pellet in ice-cold PBS.

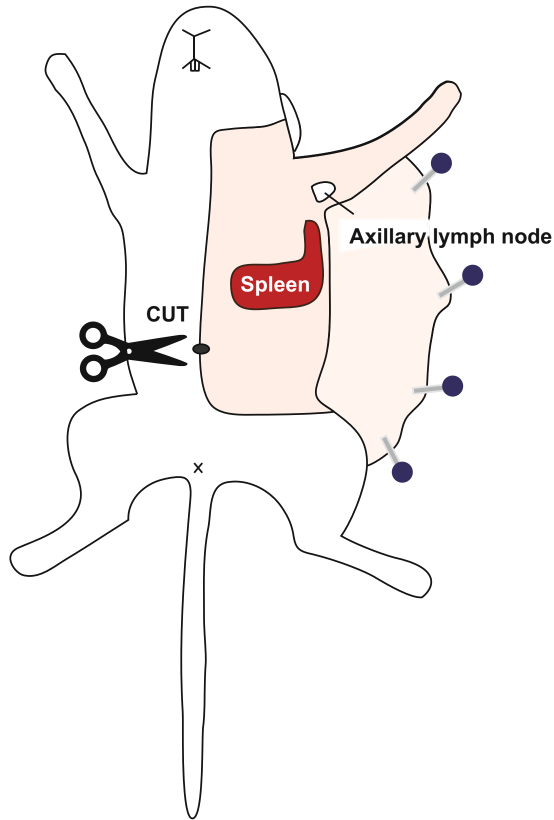

Fig. 2 Anatomy of the mouse

11. Take a small aliquot from each cell suspension and stain with trypan blue. Count the number of unstained live cells with a hemocytometer.
12. Dispense the splenocyte and lymphocyte suspensions into a 96-well round-bottom plate to seed 1×10^6 cells per well.
13. Spin the plate at $900 \times g$ for 2 min in a microplate centrifuge and remove the supernatant by turning it upside down.
14. Preincubate the cells with 10 μg/mL of CD16/32 for 5 min at 4 °C.
15. Add 10 μg/mL of fluorescence-labeled primary antibodies and stain cell surface proteins for 30 min at 4 °C, protected from light.
16. Centrifuge the plate at $900 \times g$ for 2 min and remove the supernatant.
17. Add 200 μL of ice-cold PBS, centrifuge at $900 \times g$ for 2 min, and remove the supernatant.
18. Repeat **step 17** two more times.

19. Resuspend the cell pellets in 100 μL of the staining buffer and filter the cell suspensions through 42-μm pore nylon mesh strainers.
20. Acquire fluorescence and scatter data of lymphoid cells using a flow cytometer and a compatible software. Post-acquisition analysis may be performed using the FlowJo software.

3.4.2 In Vitro Stimulation Assay

1. For an in vitro stimulation assay, seed splenocytes or lymphocytes into 96-well plates at 1×10^6 cells/well in triplicate with 100-μL cell culture medium containing 10 μg/mL of the antigen solution (dilute the stock antigen solution to 1: 2000). Culture the cells for 3 days at 37 °C under 5% CO_2 condition.
2. On Day 28, spin the cell culture plate at 900 × *g* for 5 min and carefully remove the supernatant.
3. Resuspend the cells in fresh culture medium containing 20 ng/ mL PMA, 1 μg/mL ionomycin, and 10 μg/mL of brefeldin A and incubate at 37 °C for 5 h (*see* **Note 14**).
4. Repeat **steps 13–18** in Subheading 3.4.1 to stain cell surface lineage makers.
5. Resuspend the cell pellets in 50 μL of the fixation buffer and fix the cells for 30 min on ice.
6. Centrifuge the cells at 900 × *g* for 5 min. Resuspend cell pellets with 50 μL permeabilization buffer and permeabilize for 30 min on ice.
7. To stain intracellular cytokines, incubate the cells with 10 μg/ mL of fluorescence-labeled primary antibodies diluted in permeabilization buffer for 30 min on ice, protected from light.
8. Centrifuge at 900 × *g* for 2 min and remove the supernatant.
9. Add 50 μL permeabilization buffer, centrifuge, and remove the supernatant.
10. Repeat **step 8** two more times.
11. Resuspend the final cell pellets in 100 μL of the staining buffer.
12. Resuspend the cell pellets in 100 μL of the staining buffer and filter the cell suspensions through 42-μm pore nylon mesh strainers.
13. Acquire fluorescence and scatter data of lymphoid cells using a flow cytometer and a compatible software. Post-acquisition analysis may be performed using the FlowJo software.
14. Cell population is compartmentalized with the expression of the lineage makers. Assess cytokine production from T cells by detecting intercellular staining of gated cell population (Fig. 3).

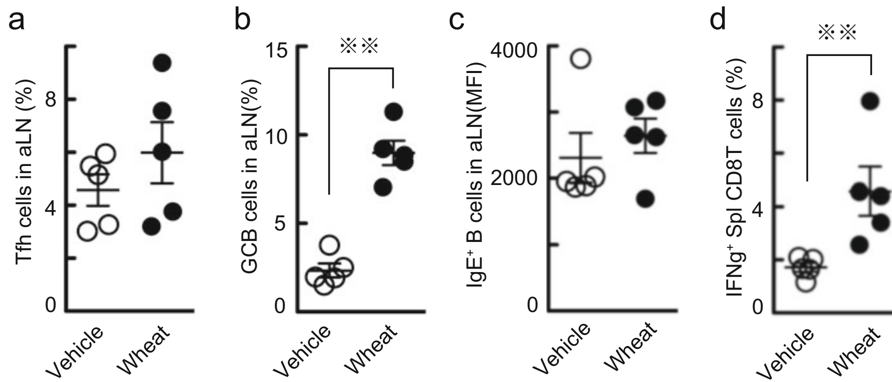

Fig. 3 Example results from flow cytometric assays of single-lymphoid cell suspensions. Frequency of (**a**) C185$^+$ PD-1$^+$ CD4$^+$ Tfh and (**b**) CD95$^+$ GL7$^+$ B220$^+$ germinal center B cells in axillary lymph node. (**c**) Mean fluorescence intensity of IgE in B220$^+$ B cells from axillary lymph node. (**d**) Frequency of IFN-γ expressing CD8$^+$ TCRβ^+ splenic CD8T cells

4 Notes

1. The pH should be approximately 7.4. Adjust pH before bringing the volume up to 1 L.
2. In our laboratory, we use NIST® SRM® 1567b wheat flour. NIST provides standard reference material and its quality is controlled for ISO 9000. Use of this standardized, quality-controlled material improves intra- and inter-laboratory reproducibility.
3. The temperature of experimental animal facility should be 20 ± 3 °C with relative humidity of 50 ± 10% under a 12-h light/dark cycle (or 14-h light/10-h dark cycle). Food and water should be provided *ad libitum*.
4. This 1-day rest allows for skin recovery from razor burns and scratches.
5. Pressure-sensitive tape should be applied and removed successively from the same skin area.
6. The jacket avoids slipping or peeling of the patch from the determined location. Ensure jacket wrapping is tight enough to prevent the mouse from rubbing it but not so tight that breathing is inhibited.
7. During the 3-day sensitization period, mice should be monitored to make sure that the dressing is maintained in place.
8. At the final round of sensitization, 2 days of immunization is often enough for induction of wheat allergy instead of 3 days of immunization. However, if the levels of wheat-antigen-specific IgE are not detected by ELISA, an extended sensitization

period is required. The duration of the sensitization period may be optimized by your laboratory, if necessary.

9. The concentration range of serum samples should be optimized to fit within the standard curve. In general, 50-fold dilution of mouse serum in blocking buffer is used for this IgE ELISA system.
10. At this step, the serum samples may be incubated at 4 °C overnight to maximize detection sensitivity.
11. An optimal secondary antibody concentration may be determined by serially diluting the antibody (10–10,000) to generate a capacity curve.
12. It is important to run each sample in triplicate to detect technical errors. It is likely that errors have occurred if the differences between the triplicates exceed more than 10%.
13. Extraneous tissues around the lymphoid tissue, such as adipose tissue, may be removed with sterile cotton gauze.
14. Restimulation with PMA/ionomycin induces cytokine production from T cells. Brefeldin A can block cytokine transport by accumulating protein at the Golgi complex/endoplasmic reticulum. These treatments are useful for intracellular staining of cytokine.

Acknowledgments

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Chapter 4

A Mouse Model of Oral Sensitization to Hen's Egg White

Sara Benedé, Daniel Lozano-Ojalvo, Rosina López-Fandiño, and Elena Molina

Abstract

Egg allergy is one of the most common food allergies in children, being the most important allergenic proteins found in the egg white (EW). Allergy to EW shows a complex phenotype that involves a multifaceted reaction that can only be assessed in vivo. Although other routes of sensitization have been described, oral exposure to food antigens is one of the most suitable in humans. In mice, oral administration of allergenic proteins results in the development of tolerance, and the use of adjuvants, such as cholera toxin (CT), is required to promote Th2-biased immune responses over tolerogenic responses. In this regard, among the mouse strains that readily display Th2 responses, Balb/c has been widely used. Here, we describe a frequently used protocol of oral EW sensitization by using CT as an adjuvant and we explain in detail the methods that we have developed to analyze the sensitizing and eliciting capacity of EW proteins including evaluation of signs, measurement of serum levels of specific immunoglobulins, mast cell degranulation, cytokine secretion profile of allergen-reactive T cells, phenotyping of mesenteric lymph node- and spleen-derived dendritic and T cells by flow cytometry, and quantification of intestinal gene expression.

Key words Food allergy, Egg white proteins, Intestinal gene expression, Th2 response, Balb/c model, T cells, Dendritic cells

1 Introduction

Hen's egg is the second most frequent source of allergic reactions in children [1]. The described overall prevalence of self-reported egg allergy reaches 2.5%, being higher in younger age groups (5.7% in children 2–5 years old) compared to older ones (2.0% people >18 years old) [1].

Egg allergy is an IgE antibody-mediated reaction defined as an adverse immune response triggered by the ingestion of egg proteins in sensitized individuals [2]. The main allergenic proteins of hen's egg are found in egg white (EW), ovomucoid and ovalbumin being the two most important proteins that can generate allergic reactions [2]. In most cases (25–50%), symptoms occur within 2 h after ingestion, including abdominal pain, bloating, vomiting, diarrhea (gastrointestinal symptoms), itching, urticaria, angioedema

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(cutaneous symptoms), asthma, and/or rhinitis (respiratory symptoms) [3].

Antigenicity of food proteins has been tested by in vitro methods including IgE antibody quantification and cell-based reactivity, mainly using mast cell or basophil activation assays [4, 5]. However, egg allergy shows a complex pattern that involves multifaceted reactions that can only be assessed in vivo. In this regard, murine models offer the opportunity to evaluate food-induced allergic responses, which would otherwise not be possible to estimate in human allergic patients.

Traditionally, in the field of food allergies, experimental models have been specially directed to certain mouse strains that readily display allergenic responses, Balb/c being one of the most widely used. As in humans, oral administration of food antigens to mice results in oral tolerance, with a little or no immune response or disease association [6]. To overcome the physiological tolerance to ingested antigens in experimental studies, immune adjuvants are often coapplied with food allergens in order to induce sensitization and to promote Th2-biased responses over Th1 [7]. The cholera toxin (CT), an endotoxin produced by *Vibrio cholera*, is one of the best-known mucosal adjuvants. Their nontoxic subunit B links to the allergen of interest, which acquires the capacity to bind to the ganglioside receptor GM-1 on intestinal epithelial cells [8]. In addition, CT induces migration and activation of dendritic cells (DCs) from the lamina propria to the mesenteric lymph nodes (MLNs) and promotes a Th2-associated DC phenotype, by the upregulation of the surface costimulatory molecule, OX40L [9].

The oral coadministration of CT together with egg proteins has been used by our group in order to evaluate the effect of food processing and matrix on their capacity to induce sensitization or elicit allergic responses [10], to compare the sensitizing capacity of different egg components (egg white and yolk) [11], and as a pre-clinical approach to study the efficacy of new preventive and therapeutic strategies [12].

In this experimental protocol, we explain how to establish EW-induced allergy in mice and how to evaluate their allergic response assessing their clinical symptoms, measuring their serum levels of antigen-specific immunoglobulins and mouse mast cell protease-1 (mMCP-1), and analyzing the phenotype of dendritic and T cells from MLNs and spleens and the intestinal gene expression. Moreover, we describe how to restimulate the T cells from MLNs and spleens with EW to study their capacity to secrete Th2 cytokines.

2 Materials

Prepare all solutions using deionized water and analytical grade reagents. Prepare and store all reagents at room temperature, unless indicated otherwise. Diligently follow all waste disposal regulations when placing waste materials.

2.1 Egg White Sensitization (See Note 1)

1. Phosphate-buffered saline (PBS): pH 7.4. Commercially available formulation without Ca^{2+} and Mg^{2+}. Suitable for cell culture.
2. Sensitizing solution for the experimental group: Dissolve 5 mg of EW lyophilized powder (*see* **Note 2**) and 10 μg of CT in 200 μL of PBS per mouse (*see* **Note 3**).
3. Sensitizing solution for the control group: Dissolve 10 μg of CT in 200 μL of PBS per mouse.
4. Five-week-old BALB/c female mice, ideally specified pathogen-free (*see* **Note 4**).
5. Freeze dryer.
6. Analytical balance.
7. 50-mL Centrifuge tubes.
8. Centrifuge.
9. 1-mL Syringes.
10. Feeding tubes: 30 mm.

2.2 Egg White Challenge

1. PBS: pH 7.4. Commercially available formulation without Ca^{2+} and Mg^{2+}. Suitable for cell culture.
2. Allergen challenge solution (without CT): Dissolve 50 mg of EW (*see* **Note 2**) in 200 μL of PBS per mouse (*see* **Note 3**).
3. 1-mL syringes.
4. Feeding tubes: 30 mm.
5. A rectal thermometer.

2.3 Fecal and Organ Sample Collection

1. CO_2 for euthanasia.
2. Dissection instruments: scissors and forceps.
3. Sample tubes: 50 and 1.5 mL.
4. Collection buffer for MLNs: 25 mM Hepes; 238.3 g/mol) and 0.1% glucose in PBS. Weigh 3 g of Hepes and 0.5 g of glucose. Dissolve solutes in 500 mL of PBS. Mix and filter through a pore size membrane of 0.2 μm. Store at 4 °C.
5. Collection buffer for spleens and intestines: 0.1% glucose in PBS. Weigh 0.5 g of glucose and dissolve in 500 mL of PBS. Mix and filter through a pore size membrane of 0.2 μm. Store at 4 °C.

6. RNA preservation buffer (lysis buffer for lysing cells and tissues recommended by commercial RNA isolation kits).
7. Tissue homogenizer: Ultra-Turrax T8 or equivalent.

2.4 Splenocyte and MLN Cell Isolation

1. 70-μm Cell strainers.
2. PBS: *See* Subheading 2.2.
3. Completed RPMI: Roswell Park Memorial Institute (RPMI) 1640 culture medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin.
4. FACS buffer: 1 mM ethylenediaminetetraacetic acid (EDTA) and 2% bovine serum albumin (BSA) in PBS. Weigh 0.29 g of EDTA and 20 g of BSA. Dissolve solutes in 1 L of PBS. Store at 4 °C.
5. Red blood cell (RBC) lysis buffer: Commercially available.

2.5 Splenocyte and MLN Cell Culture

1. Sterile 48-well culture plates.
2. Negative control stimulating solution: completed RPMI (*see* Subheading 2.4).
3. Positive control stimulating solution: 25 μg/mL Concanavalin A (ConA) dissolved in completed RPMI (*see* Subheading 2.4).
4. Egg white stimulating solution: 2 mg/mL EW lyophilized powder (*see* **Note 2**) dissolved in completed RPMI (*see* Subheading 2.4).

2.6 Measurement of EW-Specific Immunoglobulins, mMCP-1, and Cytokines by ELISA

1. Clear flat-bottom polystyrene high-bind 96-well plates.
2. Egg white lyophilized powder (*see* **Note 2**).
3. 10× PBS stock solution: 80.9 mM $Na_2HPO_4 \cdot 12H_2O$, 14.7 mM KH_2PO_4, 1370 mM NaCl, and 26.8 mM KCl. Weigh 29 g of $Na_2HPO_4 \cdot 12H_2O$, 2 g of KH_2PO_4, 80 g of NaCl, and 2 g of KCl. Dissolve solutes in 800 mL of distilled water. Adjust the pH to 7.4 and make up to 1 L with distilled water.
4. PBS: Mix 100 mL of PBS 10× with 900 mL of distilled water.
5. PBS–BSA: 1% BSA in PBS (w/v). Weigh 1 g of BSA and dissolve solute in 100 mL of PBS. Store at 4 °C.
6. 0.1 M Sodium bicarbonate ($NaHCO_3$) buffer: pH 9.6. Weigh 0.84 g of $NaHCO_3$ and dissolve solute in 90 mL of PBS. Adjust pH to 9.6 and make up to 100 mL with distilled water. Store at 4 °C.
7. PBS-Tween (PBS-T): 2.5% Tween 20 in PBS (v/v). Dissolve 5 mL of Tween 20 in 200 mL of PBS. Store at 4 °C.

Table 1
Overview of immunoglobulin ELISA

ELISA	Layer	Component	Dilution (dil.) or concentration	Diluent
sIgE	Capture	EW[a]; rat anti-mouse IgE	5 μg/mL; 2 μg/mL	PBS
	Block	Tween	2.5%	PBS
	Samples	Serum samples; mouse-IgE	1:20 dil.; 7–500 ng/mL[b]	PBS-T
	Detection	Biotinylated rat anti-mouse IgE	2 μg/mL	PBS-T
	Developing	Avidin–horseradish peroxidase	1:1000 dil.	PBS-T
sIgG1	Capture	EW; rat anti-mouse IgG1	2 μg/mL; 2 μg/mL	PBS
	Block	BSA	1%	PBS
	Samples	Serum samples; mouse-IgG1	1:1000 dil.; 2–150 ng/mL[b]	PBS–BSA
	Detection	Biotinylated rat anti-mouse IgG1	0.1 μg/mL	PBS–BSA
	Developing	Avidin–horseradish peroxidase	1:4000 dil.	PBS–BSA
sIgG2a	Capture	EW; rat anti-mouse IgG2a	2 μg/mL; 2 μg/mL	PBS
	Block	BSA	1%	PBS
	Samples	Serum samples; mouse IgG2a	1:100 dil.; 4–1000 ng/mL[b]	PBS–BSA
	Detection	Biotinylated rat anti-mouse IgG2a	0.1 μg/mL	PBS–BSA
	Developing	Avidin–horseradish peroxidase	1:4000 dil.	PBS–BSA
sIgA	Capture	EW; rat anti-mouse IgA	1 μg/mL; 2 μg/mL	0.1 M $NaHCO_3$
	Block	Tween	2.5%	PBS
	Samples	lyophilized feces; mouse IgA	1:10 dil.; 3–50 ng/mL[b]	PBS
	Detection	Biotinylated rat anti-mouse IgA	1 μg/mL	PBS–BSA
	Developing	Avidin–horseradish peroxidase	1:2000	PBS–BSA

[a]Separated from whole hen's egg and lyophilized (*see* Subheading 2.5)
[b]Indicates the range of mouse immunoglobulin concentrations used for the standard curve

8. Wash buffer: 0.05% (v/v) Tween 20 in PBS. Dissolve 500 μL of Tween 20 in 200 mL of PBS. Store at 4 °C.
9. Specific antibodies against IgE, IgG1, IgG2a, and IgA and mouse IgE, IgG1, IgG2a, and IgA. For an overview of all antibodies and reagents, *see* Table 1.
10. ELISA kits for mMCP-1 and cytokines of interest, such as IL-4, IL-5, IL-10, IL-13, and IFNγ.
11. 3,3′,5,5′-Tetramethylbenzidine (TMB).
12. Stop Solution: 0.5 M H_2SO_4. Add 2.8 mL of concentrated H_2SO_4 (*see* **Note 5**) to 50 mL of distilled water and make up to 100 mL with distilled water.
13. Plate shaker.
14. ELISA plate washer (optional).
15. Absorbance microplate reader.

2.7 Phenotypic Analysis of Dendritic and T Cells From MLNs and Spleens

1. Clear V-bottom polystyrene high-bind 96-well plates.
2. PBS: *See* Subheading 2.2.
3. FACS buffer: *See* Subheading 2.3.
4. Specific antibodies against DC and T-cell markers. For an overview of all antibodies and reagents, *see* Table 2.
5. Viability staining solution: Dilute 0.1 μL of LIVE/DEAD™ Fixable Cell Stain in 200 μL of PBS per sample (*see* **Note 3**).
6. Fc-blocking solution: Dilute 0.25 μL of anti-CD16/CD32 in 25 μL of FACS buffer per sample (*see* **Note 3**).
7. Antibody cocktail for detection of MLNs (*see* **Note 6**): Dilute 0.3 μL of Alexa Fluor 700-anti-CD4, 0.4 μL of APC-anti-CXCR3, 0.4 μL of PerCP Cy5.5-anti-CD69, 0.7 μL of PE-anti-ST2, 2.5 μL of PE Cy7-anti-CD39, and 2 μL of FITC-anti-CD25 in 45.5 μL of FACS buffer per sample (*see* **Note 3**).
8. Antibody cocktail for detection of DCs (*see* **Note 6**): Dilute 1 μL of PE Cy7-anti-CD11c, 0.2 μL of Alexa Fluor 700-anti-CD11b, 2 μL of PE-anti-CD103, 1.5 μL of APC-anti-CD80, 1.5 μL FITC-anti-CD86, and 1 μL PerCP Cy5.5-anti-CD8α in 42.8 μL of FACS buffer per sample (*see* **Note 3**).
9. Flow cytometer.
10. 5-mL Flow cytometry tubes.
11. Flow cytometry data analysis software.

Table 2
Antibodies used for FACS analysis of DCs and T cells

Specificity	Clone	Isotype	Target	Supplier
Anti-CD16/CD32	2.4G2	Rat IgG2b, κ	DCs, T cells	BD Biosciences
PE Cy7-anti-CD11c	N418	Armenian Hamster IgG	DCs	BioLegend
Alexa Fluor 700-anti-CD11b	M1/70	Rat IgG2b, κ	DCs	BioLegend
PerCP Cy5.5-anti-CD8α	53-6.7	Rat IgG2a, κ	DCs	eBiosciences
PE-anti-CD103	2E7	Armenian Hamster IgG	DCs	BioLegend
APC-anti-CD80	16-10A1	Armenian Hamster IgG	DCs	eBiosciences
FITC-anti-CD86	GL1	Rat IgG2a, κ	DCs	eBiosciences
Alexa Fluor 700-anti-CD4	GK1.5	Rat IgG2b, κ	T cells	eBiosciences
PerCP Cy5.5-anti-CD69	H1.2F3	Armenian Hamster IgG	T cells	eBiosciences
FITC-anti-CD25	3C7	Rat IgG2b, κ	T cells	BioLegend
PE-anti-ST2	RMST2-2	Rat IgG2a, κ	T cells	eBiosciences
APC-anti-CXCR3	CXCR3-173	Armenian Hamster IgG	T cells	eBiosciences
PE Cy7-anti-CD39	Duha59	Rat IgG2a, κ	T cells	BioLegend

2.8 Intestinal Gene Expression

1. Tissue homogenizer.
2. NucleoSpin RNA Isolation Kit or equivalent (*see* **Note 7**).
3. Agilent 2100 Bioanalyzer (*see* **Note 8**).
4. PCR tubes or plates.
5. cDNA synthesis kit: PrimeScript RT Reagent Kit or equivalent.
6. Real-time PCR system: SYBR Premix Ex Taq II (Tli RNase H Plus) or equivalent.
7. Primer pairs: The primer sequences used in the analyses of intestinal gene expression by reverse transcriptase quantitative PCR (RT-qPCR) are shown in Table 3.
8. Real-time PCR thermocycler.

3 Methods

3.1 Egg White Sensitization and Blood Sample Collection

1. Administer 200 μL per mouse of the sensitizing solutions by oral gavage during three consecutive days on the first week and once per week during the following 5 weeks (*see* **Note 9** and Fig. 1).
2. Extract blood samples by cheek puncture 3 days after the last sensitization dose to measure EW-specific immunoglobulin levels. Keep them on ice until continuing with the serum isolation step described below.
   (a) Centrifuge blood samples at 2000 × *g* for 15 min at 4 °C.
   (b) Take sera from supernatants and transfer to a set of new tubes.
   (c) Freeze serum samples and store at −20 °C until analysis.

3.2 Egg White Challenge and Anaphylactic Response Evaluation

1. One week after the last sensitization dose (*see* Fig. 1), administer 200 μL of the allergen challenge solution by oral gavage.
2. Thirty minutes after oral challenge, evaluate the severity of anaphylaxis by measuring the body temperature with a rectal thermometer. Score the severity of the clinical signs according to the graded score scale from Table 4.
3. Extract blood samples by cheek puncture 30 min after oral challenge to analyze mMCP-1 serum levels. Keep them on ice until continuing with the isolation step described in Subheading 3.1, **step 2**.

3.3 Fecal Sample Collection

1. Collect feces from the cage in a 5-mL tube once a week (*see* **Note 10**).
2. Lyophilize and weigh the fecal samples. Dissolve one part of each fecal sample in six parts of PBS (w/v).

Table 3
Primer sequences used for RT-qPCR analyses of intestinal relative gene expression

Gene	Primer pair	Extension temperatures
Actb[a]	*fw* 5′ AGCTGCGTTTTACACCCTTT 3′ *rv* 5′ AAGCCATGCCAATGTTGTCT 3′	60 s at 60 °C
Aldh1 a1	*fw* 5′ CTCTGTTCCCCAGGTGTTGT 3′ *rv* 5′ TCATGCAAGGGTGCCTTTAT 3′	
Foxp3	*fw* 5′ ACAACCTGAGCCTGCACAAGT 3′ *rv* 5′ GCCCACCTTTTCTTGGTTTTG 3′	
Gata3	*fw* 5′ CCTTAAAACTCTTGGCGTCC 3′ *rv* 5′ AGACACATGTCATCCCTGAG 3′	
Il12p40	*fw* 5′ *AGGTGCGTTCCTCGTAGAGA* 3′ *rv* 5′ *AAAGCCAACCAAGCAGAAGA* 3′	
Il17a	*fw* 5′ TGCCTGTGGCACTGAAGTAG 3′ *rv* 5′ TTCATGGCTGCAGTGAAAAG 3′	
Il22ra2	*fw* 5′ TCAGCAGCAAAGACAGAAGAAAC 3′ *rv* 5′ GTGTCTCCAGCCCAACTCTCA 3′	
IL-25	*fw* 5′ ACAGGGACTTGAATCGGGTC 3′ *rv* 5′ TGGTAAAGTGGGACGGAGTTG 3′	
IL-33	*fw* 5′ ATTTCCCCGGCAAAGTTCAG 3′ *rv* 5′ AACGGAGTCTCATGCAGTAGA 3′	
Irf4	*fw* 5′ TCCTCGTCCCTTGCTGAAAC 3′ *rv* 5′ GGGCTTTGGGGCTTCTAGTT 3′	
Irf8	*fw* 5′ ACCGGCGGCAGGATGT 3′ *rv* 5′ ACAGCGTAACCTCGTCTTCC 3′	
Jag2	*fw* 5′ GGCAAAGAATGCAAAGAAGC 3′ *rv* 5′ GCTCAGCATTGATGCAGGTA 3′	
Muc2	*fw* 5′ GCTGACGAGTGGTTGGTGAATG 3′ *rv* 5′ ATGAGGTGGCAGACAGGAGAC 3′	
Rorc	*fw* 5′ TCACCTGTGAGGGGTGCAAG 3′ *rv* 5′ GTTCGGTCAATGGGGCAGTT 3′	
T-bet	*fw* 5′ GTATCCTGTTCCCAGCCGTTTC 3′ *rv* 5′ ACTGTGTTCCCGAGGTGTCC 3′	
Tslp	*fw* 5′ *AGGCTACCCTGAAACTGAGA* 3′ *rv* 5′ *GGAGATTGCATGAAGGAATAC* 3′	

(continued)

Table 3 (continued)

Gene	Primer pair	Extension temperatures
Actb[a]	*fw* 5′ AGCTGCGTTTTACACCCTTT 3′ *rv* 5′ AAGCCATGCCAATGTTGTCT 3′	45 s at 58 °C 15 s at 60 °C
Aldh1 a2	*fw* 5′ ACCGTGTTCTCCAACGTCACTGAT 3′ *rv* 5′ TGCATTGCGGAGGATACCATGAGA 3′	
Cldn2	*fw* 5′ GTCATCGCCCATCAGAAGAT 3′ *rv* 5′ ACTGTTG GACAGGGAACCAG 3′	
Il6	*fw* 5′ TTCCATCCAGTTGCCTTCTTG 3′ *rv* 5′ GGGAGTGGTATCCTCTGTGAAGTC 3′	
Il10	*fw* 5′ GCCTTATCGGAAATGATCCA 3′ *rv* 5′ AGGGGAGAAATCGATGACAG 3′	
Il22	*fw* 5′ CATGCAGGAGGTGGTACCTT 3′ *rv* 5′ CAGACGCAAGCATTTCTCAG 3′	
Il27	*fw* 5′ CTCTGCTTCCTCGCTACCAC 3′ *rv* 5′ GGGGCAGCTTCTTTTCTTCT 3′	
Tfgb1	*fw* 5′ TTGCTTCAGCTCCACAGAGA 3′ *rv* 5′ TACTGTGTGTCCAGGCTCCA 3′	
Tjp1	*fw* 5′ TACCTCTTGAGCCTTGAACTT 3′ *rv* 5′ ACAGAAATCGTGCTGATGTGC 3′	

fw forward primer, *rv* reverse primer
[a]*Actb* may be used as a reference gene

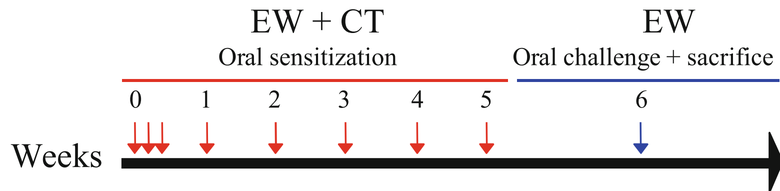

Fig. 1 Experimental protocol for oral sensitization of Balb/c mice with egg white (EW) plus cholera toxin (CT)

3. Homogenize the samples in PBS using an electric tissue homogenizer.
4. Centrifuge the samples at 2000 × *g* for 15 min at 4 °C.
5. Collect the supernatants, transfer to new tubes, and store them at −80 °C until use.

3.4 Organ Collection

1. Thirty minutes after challenge, sacrifice the mice by CO_2 inhalation and individually collect the whole spleen and the MLNs in 1 mL of cold, tissue-specific collection buffer. Keep samples on ice until cell isolation.

Table 4
Clinical severity score

Score	Clinical signs
0	No signs
1	Scratching nose and mouth less than ten times in 15 min
2	Puffiness around eyes and mouth, scratching nose and mouth more than ten times in 15 min
3	Wheezing and labored respiration, cyanosis around the mouth and tail, diarrhea and difficulty in walking normally
4	No activity after prodding
5	Death

2. Collect the whole gut in 5 mL of cold collection buffer specific for intestines. Flush the intestinal contents with the collection buffer, three times using a 5-mL syringe.
3. Place a 2-cm segment portion from each of the cleaned duodenum, jejunum, ileum, caecum, or colon in 350 μL of RNA preservation buffer. Store at −80 °C until its use.

3.5 Splenocyte and MLN Cell Isolation

1. Transfer the whole spleen or the MLNs to a 70-μm cell strainer held on a 50-mL conical tube and mechanically disrupt the organ using the plunger of a 1-mL syringe.
2. Rinse the filter five times using 5 mL of PBS.
3. Centrifuge the tubes at 450 × *g* for 10 min at 4 °C, and discard the supernatant.
4. Add 2.5 mL of RBC lysis buffer to the tube. Homogenize the cell suspension by pipetting and incubate for 3 min at room temperature.
5. Add 25 mL of PBS and divide the cell suspension into two tubes.
6. Centrifuge the tubes at 450 × *g* for 10 min at 4 °C, discard the supernatants, and resuspend the cells in the appropriate volume of completed RPMI (for cell culture) or FACS buffer (for flow cytometry staining) to obtain a final density of 4×10^6 cell/mL.

3.6 Splenocyte and MLN Cell Culture

1. Seed 1 mL of the splenocytes and MLN cells resuspended in completed RPMI in a 48-well plate.
2. Add 100 μL of stimulating solution to appropriate wells:
   (a) Negative control: completed RPMI.
   (b) Positive control: 2.5 μg/mL ConA.
   (c) Egg white stimulation: 200 μg/mL EW.

3. Incubate the cell culture for 72 h in a 5% CO_2 incubator at 37 °C.
4. Centrifuge the plate at 450 × *g* for 10 min at room temperature.
5. Collect the supernatants and store them at −80 °C for cytokine secretion analysis. Collect the remaining cells in RNA preservation buffer (such as RNAlater™ or TRIzol™) and store them at −80 °C until gene expression analysis.

3.7 Quantification of EW-Specific Murine IgE, IgG1 e IgG2a in Sera and IgA in Feces

1. Make coating solutions for EW-specific immunoglobulin ELISAs with the lyophilized EW. Determine the protein concentration of lyophilized EW powder by the Kjeldahl method (*see* **Note 2**) and prepare the following EW solutions with the specified concentrations in PBS.
   (a) For coating IgE ELISA plate: 5 μg/mL EW.
   (b) For coating IgG1 or IgG2a ELISA plate: 2 μg/mL EW.
   (c) For coating IgA ELISA plate: 1 μg/mL EW.
2. Coat 96-well plates with 100 μL/well of appropriate EW solutions from **step 1**.

   For the reference curves, coat the designated wells with 100 μL of rat anti-mouse IgE, IgG1, and IgG2a in PBS or rat anti-mouse IgA in 0.1 M $NaHCO_3$, pH 9.6, each at 2 μg/mL (Table 1). Incubate the plates overnight at 4 °C.
3. Aspirate the wells and wash four times with 250 μL/well of the wash buffer (*see* **Note 11**).
4. Block the wells with 250 μL/well of PBS-T. Incubate at room temperature for 2 h.
5. Prepare a 1:20 dilution of serum samples in PBS-T for IgE ELISA, a 1:1000 dilution in PBS–BSA for IgG1 ELISA, and a 1:100 dilution in PBS–BSA for IgG2a ELISA. For IgA ELISA, prepare a 1:10 dilution of fecal samples in PBS. For the reference curves of mouse IgE, IgG1, IgG2a, and IgA, prepare serial dilutions using the ranges indicated in Table 1.
6. Repeat **step 3** and add 100 μL/well of diluted samples. Seal the plate and incubate at room temperature for 2 h (*see* **Note 12**).
7. Repeat **step 3** and add 100 μL/well of biotinylated rat anti-mouse IgE, IgG1, IgG2a, and IgA (2 μg/mL for IgE, 0.1 μg/ml for IgG1, and IgG2a, and 1 μg/mL for IgA ELISA). Buffers used for biotinylated antibody dilutions are PBS-T for IgE and PBS–BSA for IgG1, IgG2a, and IgA ELISA. Seal the plate and incubate at room temperature for 1 h.
8. Repeat **step 3** and add 100 μL/well of avidin–horseradish peroxidase (1:1000 dilution for IgE, 1:4000 dilution for IgG1 and IgG2a, and 1:2000 for IgA ELISA). Buffers used for biotinylated antibody dilutions are PBS-T for IgE and PBS–

BSA for IgG1, IgG2a, and IgA ELISA. Seal the plate and incubate at room temperature for 30 min.

9. Repeat **step 2** and add 100 μL/well of TMB. Incubate at room temperature for 15 min in the dark.
10. Add 50 μL/well of the stop solution.
11. Read plate at 450 nm on an absorbance microplate reader.

3.8 Quantification of mMCP-1 in Sera and Cytokines Levels in Supernatants of Cultured Splenocytes and MLNs

1. Determine the levels of mMCP-1 in serum samples by using a commercial ELISA kit, according to the manufacturer's protocol.
2. Determine cytokines released during cell culture (IL-4, IL-5, IL-10, IL-13, IFN-γ, and TNF-α) by using commercial ELISA kits or Luminex immunoassay kits, according to the manufacturer's protocol.
3. Collect Luminex data in a Luminex Instrument 100 IS (Luminex Corporation, Texas, US).
4. Analyze data using ProcartaPlex Analyst 1.0 software (Fig. 2).

3.9 Phenotypic Analysis of Dendritic Cells and T Cells From MLNs and Spleens

3.9.1 Staining for Flow-Cytometric Analysis

1. Distribute the cells isolated from the MLNs and spleens (*see* Subheading 3.3) in two 96-well plates each (1×10^5 cells per well) (*see* **Note 13**). Prepare a pool of cells with leftover cell suspensions of MLNs and spleens for unstained, viability, Fluorescence Minus One (FMO), and single staining controls, and distribute 1×10^5 cells per well (one per control) on each plate. Centrifuge cells at $500 \times g$ for 5 min at 4 °C.
2. Resuspend the cell pellets in 200 μL of LIVE/DEAD™ viability staining solution and incubate for 30 min at 4 °C on ice, protected from light (*see* **Note 14**).
3. Add 100 μL of PBS and centrifuge for 5 min at $500 \times g$, 4 °C.
4. Add 100 μL of FACS buffer and centrifuge for 5 min at $500 \times g$, 4 °C.
5. Resuspend the cell pellets in 25 μL of Fc-blocking solution and incubate for 15 min at 4 °C on ice, protected from light (*see* **Note 15**).

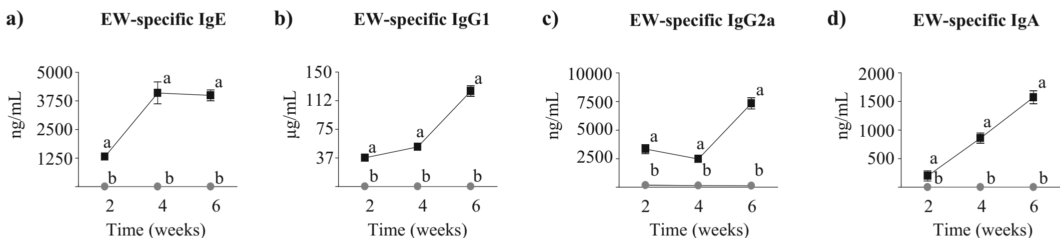

Fig. 2 Egg white (EW)-specific IgE (**a**), IgG1 (**b**), IgG2a (**c**), and IgA (**d**) antibodies from mice after administration of EW plus cholera toxin (CT) or CT alone (Adapted with permission from Lozano-Ojalvo et al. [13])

6. Add 150 μL of FACS buffer and centrifuge at 500 × *g* for 5 min at 4 °C.
7. Resuspend the cell pellets in 200 μL of FACS buffer and centrifuge for 5 min at 500 × *g* at 4 °C.
8. Resuspend the cell pellets in 50 μL of appropriate antibody solutions and incubate for 30 min at 4 °C on ice, protected from light (*see* **Note 16**).
9. Add 150 μL of FACS buffer and centrifuge for 5 min at 500 × *g* at 4 °C.
10. Add 200 μL of FACS buffer and centrifuge for 5 min at 500 × *g* at 4 °C.
11. Resuspend the cell pellets in 200 μL of FACS buffer and centrifuge for 5 min at 500 × *g* at 4 °C.
12. Resuspend the cell pellets in 200 μL of FACS buffer and keep at 4 °C until data acquisition on the flow cytometer (*see* **Note 17**).

3.9.2 Flow-Cytometric Gating Strategy for DCs

1. Gate cells on the basis of forward scatter-integral (FS-I), live cells, and CD11c population (*see* Fig. 3).
2. Further gate on CD11b and CD103, which are markers used for identifying different DC populations. CD103+CD11b+ and CD103+CD11b− populations correspond to lamina-

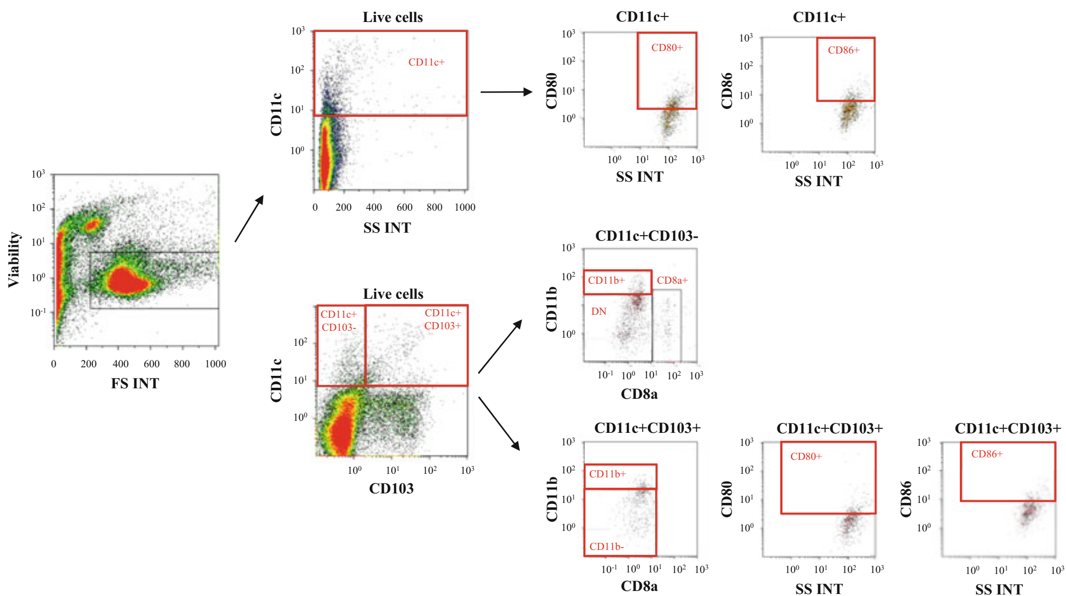

Fig. 3 Gating strategy for identification of dendritic cell (DC) subsets in the mesenteric lymph nodes (MLNs) of mice sensitized to egg white (EW). Cells are gated based on the viability and CD11c staining and subsequently analyzed based on CD80, CD86, CD11b, CD8α, or CD103 expression. Pictures show representative dot plots of DC analysis in MLNs (Adapted with permission from Pablos-Tanarro et al. [11])

propria-derived DCs that bear the integrin α-chain CD103 [13] with the capacity to present antigens to naïve T cells and induce a Th2-skewed response in the MLNs. CD103− DCs (either CD11b+CD8α−, CD11b− CD8α+ or CD11b−CD8α−) correspond to resident MLN-DCs [14].

3. Further gate on CD80 and CD86 to identify the upregulation of these costimulatory molecules.

3.9.3 Flow-Cytometric Gating Strategy for T-Cells Subsets

1. Gate cells on the basis of FS-I and live cells (*see* Fig. 4).
2. Further gate CD4+ cells.
3. To detect different subsets of activated T cells, gate on CD69 +CXCR3+ for Th1 cells, CD69+ T1/ST2+ for Th2 cells, and CD25+CD39+ for regulatory T cells (Treg).

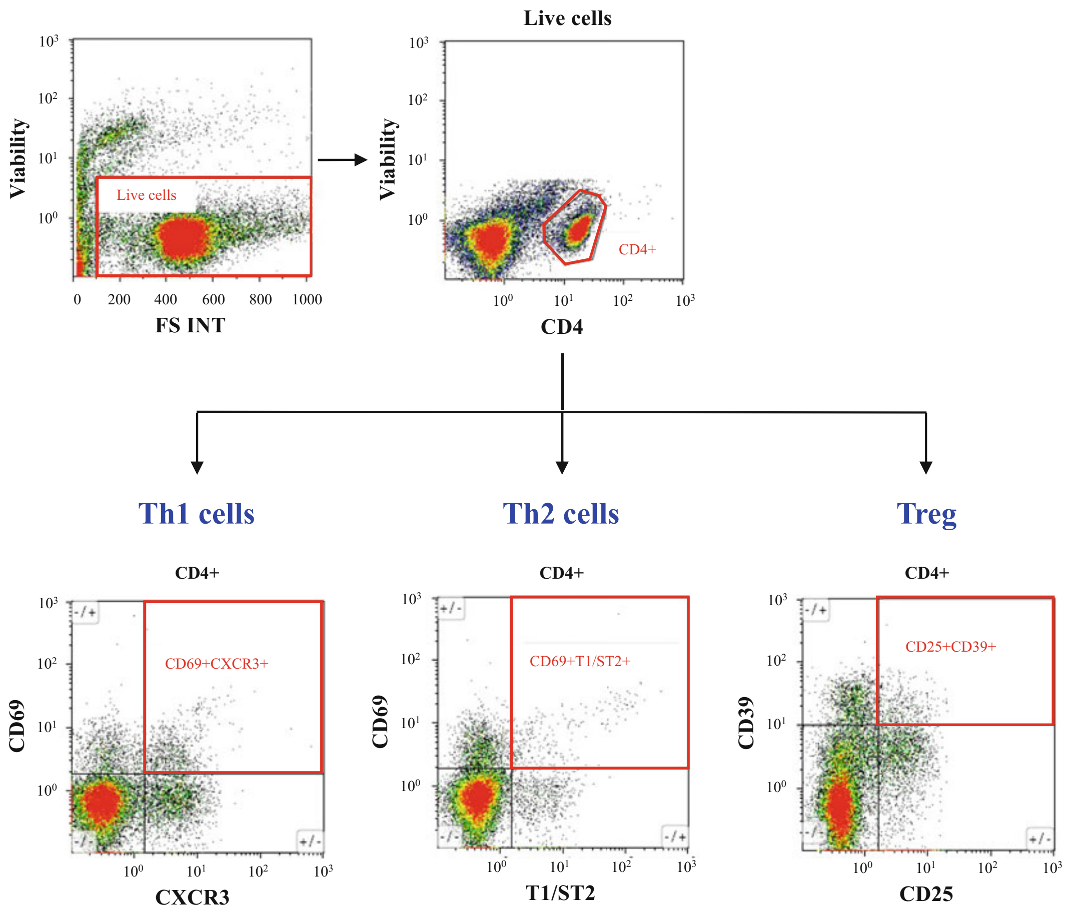

Fig. 4 Gating strategy for identification of T-cell subsets in the mesenteric lymph nodes (MLNs) and spleens of mice sensitized to egg white (EW). Cells are gated based on the viability and CD4 staining, and subsequently analyzed based on their CD69, CXCR1, T1/ST2, CD25, and CD39 expression. Pictures show representative dot plots of T-cell analysis in the spleen

3.10 Intestinal Gene Expression

1. Disaggregate and homogenize the thawed intestinal samples using an electronic tissue homogenizer.
2. Perform RNA isolation following the specific instructions given by the RNA isolation kit manufacturer. Elute the RNA with 60 μL of RNase-free water to obtain approximately 1500 ng/mL of RNA.
3. Determine the integrity, purity, and concentration of the isolated RNA using an Agilent 2100 Bioanalyzer (*see* **Note 8**).
4. Use 2 μg of the RNA samples to synthesize cDNA following the specific instructions given by a selected cDNA synthesis reagent kit.
5. Prepare a master mix following the instructions provided by a selected real-time PCR reagent system. Use less than 100 ng of the synthesized cDNA as the template for qPCR reactions.
6. Reconstitute lyophilized primers (store at 10 μM and −80 °C) in RNase-free water and use them at a final concentration of 100 nM in the reaction (*see* **Note 18**).
7. Perform the qPCR in a real-time thermocycler. The thermal cycling conditions include an initial denaturation step of 30 s at 95 °C, followed by 40 cycles consisting of a denaturation step of 15 s at 95 °C and a polymerase extension step. For the polymerase extension step appropriate for each primer set, follow the thermal conditions indicated in the Table 3. After qPCR cycling, perform a melting curve analysis to confirm the specific product formation.
8. For the analysis of the results, determine the relative gene expression by normalizing the data to the expression of a reference gene (e.g., *Actb*, *see* Table 3), as described by Livak and Schmittgen [15].

4 Notes

1. All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee and must follow officially approved procedures for the care and use of laboratory animals.
2. Separate the egg white from whole fresh hen's eggs and lyophilize it to concentrate EW. Its endotoxin content may be quantified using a commercial endotoxin detection kit, and protein composition (based on total nitrogen content) may be determined by the Kjeldahl's method (The procedure and performance characteristics of the method for determination of total nitrogen content of egg are described in both AOAC Method 991.20 [16]). Egg white solutions in sterile PBS can be made beforehand and stored in the −20 °C freezer for 2 months.

3. Prepare an excess amount of each solution enough for three additional numbers of mice (or samples).
4. Five to six mice per group are usually enough to obtain statistically significant results.
5. Handle in a fume hood, with gloves, lab coat, and safety glasses.
6. Several panel designs targeting the same cell surface markers are possible. If another panel is designed, or if antibodies are bought from different suppliers, we recommend choosing the same antibody clones. New antibodies should first be validated, their optimal dilution determined, and the staining conditions decided before using them in the complete staining mixture.
7. This isolation kit is used as an example in this chapter, but any RNA isolation kit commercially available from different brands can be chosen depending on the experimental design.
8. The integrity of the isolated RNA can also be determined by agarose gel electrophoresis, and its purity and concentration can be determined by a NanoDrop spectrophotometer. A 260/280 ratio of ~2.0 is generally accepted for pure RNA and 260/230 values are commonly in the range of 2.0–2.2.
9. Pay attention and do not put the feeding tubes into the trachea.
10. Animals are individually isolated in an empty box for 5–10 min until 2–3 fecal pellets are obtained per animal.
11. Blot the plate on absorbent paper to remove any residual buffer.
12. For maximal sensitivity, incubate overnight at 4 °C.
13. One plate will be used for T-cell staining and a different one for DC staining.
14. Add 200 μL of PBS to tubes containing the unstained and single staining controls instead of the LIVE/DEAD™ viability staining solution.
15. Resuspend the cells for unstained and viability controls with 25 μL of FACS buffer instead of Fc-blocking solution.
16. For FMO controls, add all fluorophore-conjugated antibodies in the antibody mixture, *except* one. Use FACS buffer in the place of the excluded antibody. For single staining controls, resuspend the cell pellets in 50 μL of FACS buffer containing only one fluorophore-conjugated antibody.
17. Acquire cells immediately or keep them in a fridge, covered in parafilm and foil for a maximum of 24 h. For longer storage periods (no more than 3 days), fix the cells after staining with 4% paraformaldehyde at room temperature for 5–10 min.
18. To determine the appropriate template amount, make serial dilutions of the synthesized cDNA. The optimal quantity varies depending on the number of target copies present in the template solution.

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Chapter 5

Induction of Hypersensitivity with Purified Beta-Lactoglobulin as a Mouse Model of Cow's Milk Allergy

Nicholas A. Smith and Kumi Nagamoto-Combs

Abstract

Cow's milk allergy is one of the most prevalent food allergies in both children and adults. As dairy products are common dietary ingredients and the prevalence of chronic conditions is on the rise, milk allergy is a growing public health concern. To elucidate underlying mechanisms and develop therapeutic strategies, reliable animal models are essential research tools. Sensitization to a milk protein is the principal procedure for establishing animal models of cow's milk allergy. However, the methods of sensitization vary from laboratory to laboratory, using different milk proteins with different amounts, routes, and durations of allergen exposure during sensitization of varying sex and strains of mice, likely resulting in diverse immunological and physical responses. Furthermore, the sources and potential impurities of milk protein may also produce variable responses. Thus, standardization of sensitization protocol is important, particularly when results are compared across studies. Here, we describe a method to generate a mouse model of cow's milk allergy using purified β-lactoglobulin as the milk allergen with cholera toxin as an adjuvant in a 5-week oral sensitization protocol.

Key words Allergy, Bovine whey, β-Lactoglobulin, Cholera toxin, Cow's milk, Gavage, Hypersensitivity, C57BL/6J, Mouse

1 Introduction

Food allergy afflicts approximately 8% of children and 11% of adults in the United States [1, 2]. Upon exposure to an offending allergen, sensitized individuals often experience a spectrum of symptoms, including gastrointestinal discomfort, hives, respiratory distress, and systemic anaphylaxis. Including the costs of medical treatments, special food, and lost productivity, total economic burdens to allergic patients and caregivers are estimated to be $24.8 billion annually in the United States alone [3, 4]. The prevalence, symptom severity, and economic burdens of food allergy make this chronic disease an important area of research, in which animal models are an essential tool to investigate underlying etiology and potential therapeutic strategies.

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The prevalence and symptoms of food allergies are well documented to be variable depending on sex [1, 5], age [1, 6], and race [1, 5]. With these inherent variables, it is crucial to maintain a consistent sensitization protocol when establishing an animal model of food allergy. To establish food allergy in a mouse model, an allergen is typically introduced to mice with an adjuvant to provoke innate immune responses. Existing models of food allergy, however, employ diverse methods to establish sensitivity. For example, the amount of an allergen used in sensitization often varies between 0.5 and 20 mg [7–13] though evidence suggests higher amounts of allergen during sensitization reduces efficacy [10]. The types of adjuvants used also vary from alum [14–16] to cholera toxin [7–9, 12, 17] to staphylococcus enterotoxin [18], and in other cases, no adjuvant at all [19, 20]. In addition, the routes of exposure may be intragastric [8, 9, 12], intraperitoneal [14, 16, 20], epicutaneous [21], or subcutaneous [22]. Furthermore, age, genetic background, and sex of animals have been shown to affect the development of hypersensitivity responses and symptom presentations [9, 11, 15, 23]. These differences in sensitization methodology and experimental animals introduce additional variables and must be considered when comparing results across studies or establishing a model in your laboratory.

It is important to carefully select a consistent sensitization protocol and animals that produce immunological and physical responses appropriate for the purpose of your study. For example, the goal of our study is to produce subclinical sensitization to a milk protein via the most likely route of exposure for investigating the effect of mild food allergy on brain function and behavior. Since severe anaphylaxis obscures observation of more subtle behavioral changes, we utilize C57BL/6J mouse strains that exhibit milder physical responses with allergen re-exposure than other mouse strains [9, 12]. Mice are then orally administered with an adjuvant, cholera toxin (CT), without or with purified bovine β-lactoglobulin (BLG: Bos d 5) as the cow's milk allergen to produce a mouse model of cow's milk allergy. Since the property of CT as a mucosal adjuvant has been demonstrated [17], this intragastric sensitization method has been used with various forms of bovine milk allergens to investigate diverse aspects of milk allergy [8, 17, 24–26].

In this chapter, we describe a detailed protocol to establish hypersensitivity to BLG via intragastric gavage. Using this protocol, we have previously demonstrated successful sensitization of C57BL/6J mice and BLG-specific immunologic responses and documented sex-dependent anxiety-like behavioral changes without producing anaphylactic reactions [12].

2 Materials

2.1 Oral Sensitization

1. Mice: appropriate number, strain, age, and sex(es) should be determined by the investigator (*see* **Note 1**).
2. Whey-free rodent diet (*see* **Note 2**).
3. Vehicle: 0.2 M sodium bicarbonate ($NaHCO_3$), pH 9.0. Dissolve 8.4 mg of $NaHCO_3$ in about 400 mL of purified water. Adjust pH to 9.0 with sodium hydroxide and bring the volume to 500 mL with purified water. Sterilize using a 0.2-μm filter unit and store at 4 °C.
4. Sterile plastic tubes: for making and storing sensitization solutions. Select 5-, 10-, or 50-mL tubes to fit the volume of solutions required for the number of mice to be sensitized.
5. 0.2-μm Sterile syringe filters: use to filter sensitization solutions.
6. Cholera toxin from *Vibrio cholerae*: 2 μg/μL in sterile purified water, azide-free. Prepare a stock solution by adding 500 μL of sterile water to 1 mg cholera toxin. Store at 4 °C in a tightly sealed container.
7. β-Lactoglobulin (BLG): purified from bovine milk, endotoxin-free. Store at 4 °C.
8. Allergen solution: Prepare 200 μL/mouse of the vehicle containing 1 mg BLG and 10 μg cholera toxin. Combine 10 mg of BLG and 50 μL of 2 μg/μL cholera toxin stock solution in 2 mL of bicarbonate buffer, and sterile filter the solution. Prepare immediately before use (*see* **Note 3**).
9. Sham solution: Prepare 200 μL/mouse of the vehicle containing 10 μg of cholera toxin only. Add 50 μL of 2 μg/μL cholera toxin stock solution in 2 mL of sterile-filtered sodium bicarbonate buffer. Prepare immediately before use (*see* **Note 3**).
10. 1-mL Sterile disposable syringes.
11. Gavage needles: 22-G, 25-mm-long soft-tip plastic feeding tubes (*see* **Note 4**).
12. Empty cage with a metal-rack top: used to securely grasp mice.
13. Small waste container: Use to collect biohazardous waste, such as gavage needles, syringes, and plastic tubes containing cholera toxin solutions.
14. 5-mL Conical microfuge tubes.

2.2 BLG Challenge and Assessment of Allergic Reactions

1. BLG challenge solution: Prepare 200 μL/mouse of the vehicle containing 50 mg BLG. Dissolve 1.0 g of BLG in 4 mL of bicarbonate buffer, and sterile filter the solution. Prepare immediately before use.

2. 5-mL Conical microfuge tubes.
3. Mouse restrainer.
4. Rectal thermometer with a RET3 probe.

3 Methods

All procedures must be approved by your Institutional Animal Care and Use Committee. Be sure to have sufficient amounts of required solutions by preparing enough for 2–3 extra mice.

3.1 Oral Sensitization

In this protocol, mice are orally administered with either the sham solution (10 μg CT in vehicle) or allergen solution (1 mg BLG + 10 μg CT in vehicle) weekly over a 5-week period. Before and after the oral administration, mice are fasted for 2 h to avoid potential sensitization to other dietary proteins. The timeline of sensitization procedure is depicted in Fig. 1.

3.1.1 Preparation of Mice

1. Divide mice randomly into sham and BLG groups. Place all mice on a whey-free diet at least for 1 week prior to initiating the sensitization procedure (*see* **Notes 1** and **2**).
2. Record baseline body weight and temperature 1 day before sensitization (*see* **Note 5**).
3. On the day of sensitization, remove food from the feeding rack of the cages to fast mice for 2 h prior to gavage. Allow mice an ad libitum access to water (*see* **Note 6**).

3.1.2 Oral Sensitization via Intragastric Gavage

1. Following the 2-h fasting period, prepare a workspace to accommodate 1–2 mouse cages, the sham and BLG solutions, syringes, gavage needles, a small waste container, and an empty cage with a metal-rack top. Place each material within a comfortable reach (Fig. 2).
2. Place a gavage needle onto a 1-mL syringe and draw 200 μL of the sham solution. Set the syringe aside to hold a mouse (*see* **Note 7**).

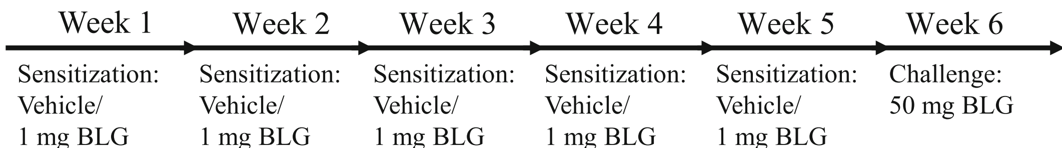

Fig. 1 The 5-week sensitization schedule followed by BLG challenge. Starting at 4 weeks of age, mice are subjected to weekly oral administrations of either sham or BLG solution for 5 weeks as described in Subheading 3.1. On the sixth week, all mice, now 9 weeks old, are challenged with the vehicle containing 50 mg of BLG as described in Subheading 3.2

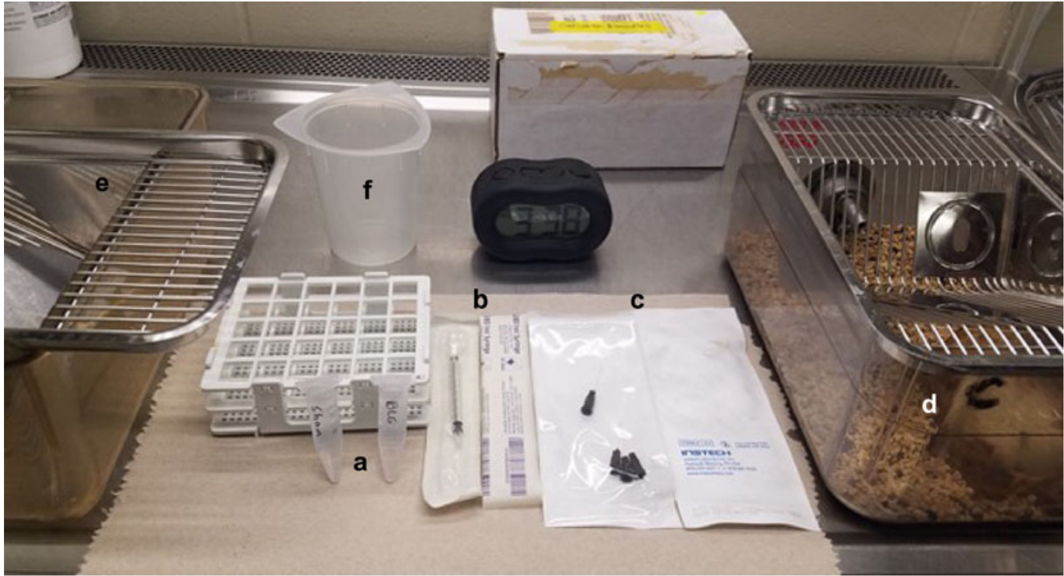

Fig. 2 An example setup for sensitization workstation. The allergen and sham solutions (**a**), 1-mL syringes (**b**), and soft-tip gavage needles (**c**) are conveniently placed within arm's length. To minimize stress, mice are kept in home cage till immediately prior to gavage (**d**). A mouse is placed on metal cage rack (**e**) and gently pulled by the tail by an investigator so that the mouse grips the metal bar. This position allows the investigator to securely grasp the mouse by the nape for gavage. After the administration of sensitization solution, the mouse is then placed in a separate cage temporarily to minimize the risk of repeated treatment. Once all the mice in a cage have been treated, they are returned to their home cage. Used syringes and needles are collected in a container (**f**) and disposed of in an appropriate biohazardous waste container

3. Grasp the nape of the mouse with your thumb and index finger of one hand. Use the other fingers to securely hold the mouse (Fig. 3a). Be sure that the forelimbs of the mouse are pulled back so that they cannot reach the mouth (*see* **Note 8**).
4. Place the gavage needle against the body of the mouse and approximate the length of the needle from mouth to the sternum (Fig. 3b). This step is important, especially for a smaller mouse, to prevent the needle from injuring the stomach.
5. Entering the mouth from the side to avoid the incisors, gently insert the gavage needle down the esophagus (*see* **Note 4**). In order to avoid inserting the needle into the trachea, follow the roof of the mouth with the needle once inserted into the mouth (Fig. 3c).
6. Hold the mouse and the syringe vertically, making a straight line from the mouth to the stomach. Slowly and steadily, dispense the solution in the syringe (*see* **Note 9**).
7. Gently remove the needle and return the mouse to its cage. Repeat **steps 3**–**7** for each mouse, using a new gavage needle, if necessary (*see* **Note 10**).

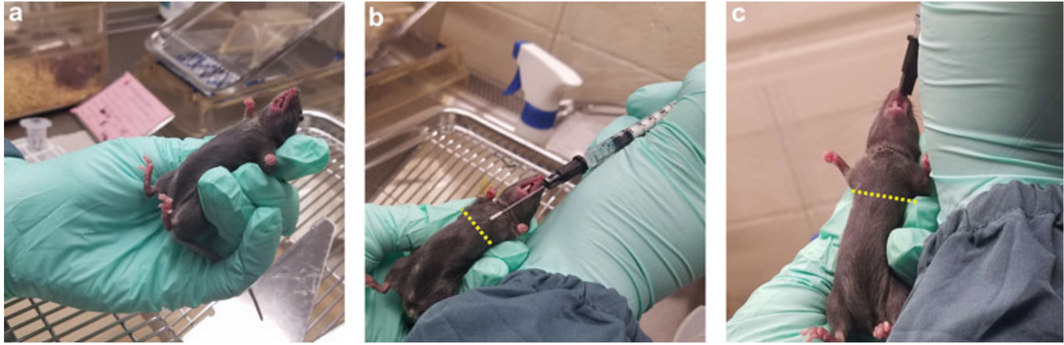

Fig. 3 Intragastric gavage technique. (**a**) A properly held mouse. Note that the nape of the neck is held securely to restrain the head, the fur on the back is held to pull the forelimbs away from the mouth, and the tail is restrained to limit the hind limb movement. (**b**) Estimating the length of the gavage needle to reach the stomach. The approximate level of the sternum is marked by the yellow dotted line, which is approximately one-third of the total length of the mouse's body. These guidelines can be used to estimate the proper distance into the distal esophagus and the stomach. (**c**) The gavage needle that has been properly inserted into the mouth. Note that the entry point of the needle is adjacent to the incisors to prevent the mouse from chewing the needle

8. Once all mice in the sham group have received the sham solution, discard the tube and syringe used for the sham solution. Using a new syringe and gavage needle, repeat **steps 3**–**7** with the BLG solution for the BLG group.
9. Return all mice to their home cages and continue to fast for additional 2 h. Monitor mice for their well-being during this time.
10. Dispose of used syringes, gavage needles, and any remaining solutions in proper biohazardous waste containers.
11. Repeat this procedure once a week for 5 weeks on the same day of each week to develop hypersensitivity in BLG-sensitized mice (*see* **Note 11)**.
12. Be sure to record the weight of each mouse at least once a week to confirm healthy growth (*see* **Note 5**).

3.2 BLG Challenge and Assessment of Allergic Reactions

1. As with the weekly sensitization protocol, fast mice for 2 h prior to allergen challenge. During the fasting period, prepare the BLG challenge solution.
2. Following the gavage technique described in Subheading 3.1.2 **steps 3**–**7**, gavage each mouse with 200 μL of the BLG challenge solution (50 mg BLG/mouse) and note the time of administration (*see* **Note 12**).
3. At 20–30 min post-challenge, observe the mouse's physical status to assess allergic reactions. Based on the anaphylactic symptom scoring table (Table 1), score the mouse's reactions to the BLG challenge (*see* **Note 13**).

Table 1
Scoring of post-challenge anaphylactic symptoms

Score	Symptoms
0	No reaction/clinical symptoms
1	Scratching and rubbing around the nose and head
2	Puffy eyes and mouth, pilar erecti, reduced activity
3	Dyspnea and/or wheezing; cyanosis around the mouth and tail
4	No activity after prodding; exhibit tremor and/or convulsion
5	Death

Thirty minutes after BLG challenge, observe the physical status of mice and record appropriate scores based on their symptom presentations. Symptoms are categorically ranked based on severity from 0 to 5. The scoring criteria were adopted from Li et al. [25]

4. At 30 min post-challenge, place each mouse in a mouse restrainer that allows an access to the rear. Lift the tail, gently insert the probe of a rectal thermometer, and record body temperature (*see* **Note 14**).
5. Return the mouse to its home cage or an individual cage if an extended observation is required to monitor its reactions.
6. If your experiment requires the mice to recover from the challenge, wait for 2 h and return the whey-free diet to the cage. Keep monitoring the mice for their well-being by observing their physical appearance, mobility, food consumption, and body weight.
7. Alternatively, challenged mice may be sacrificed immediately after the recording of anaphylaxis score and body temperature. Collect biological samples, such as blood, intestines, gastrointestinal-associated lymphoid tissues, and the spleen to assess immunologic responses. Methods for these assessments are described elsewhere and therefore not covered in this chapter. Sensitization of C57BL/6J mice using this protocol resulted in increased BLG-specific serum IgE and IgG1 (Fig. 4) (*see* **Note 15**).

4 Notes

1. We typically obtain 3-week-old mice and place them on a whey-free diet while being acclimated to the facility for a week. We initiate the sensitization protocol when the mice are 4 weeks old.
2. It is important to remove BLG from their normal diet to prevent uncontrolled exposures to the allergen. Many

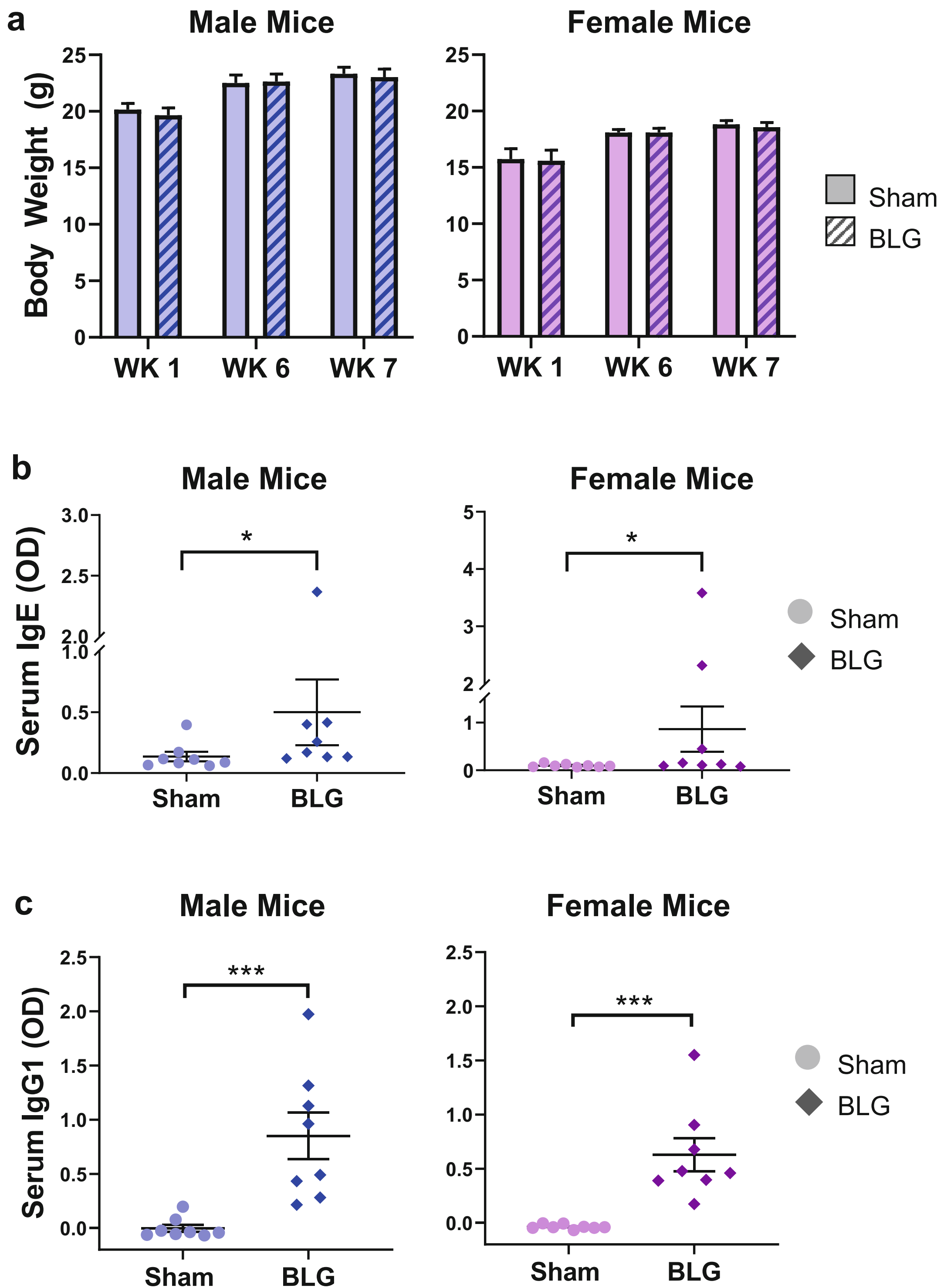

Fig. 4 Physical growth and adaptive immunity development during BLG sensitization. (**a**) Weights of mice were recorded during weeks 1, 6, and 7 of sensitization to assess potential impact of the sensitization regime on overall health and growth. (**b**, **c**) Serum isolated from the terminal blood was used to quantify the levels of BLG-specific IgE (**b**) or IgG1 (**c**) using ELISA. Values shown in the graphs indicate the group average $\pm$ SEM, *$p < 0.05$; ***$p < 0.001$. (Mann–Whitney test), male sham: $n = 8$; male BLG: $n = 8$; female sham: $n = 8$; female BLG: $n = 8$ (Reproduced from Smith et al. Front Cell Neurosci 2019 Jul 16;13:320 [12])

commercial mouse chows contain whey proteins. We use Envigo Teklad 2018 Rodent Diet, which contains no animal-derived proteins.

3. The provided volume (2 mL) is sufficient to administer 7–8 mice by oral gavage (200 μL/mouse). To compensate for volume losses during filtration and administration, prepare extra amounts of solutions.
4. The use of soft-tip plastic gavage needles may be safer for mice and less likely to cause gastroesophageal injury during gavage. However, these tips are not resistant to chewing and bending, and mice may bite them off the syringes. Alternatively, semi-malleable round-tip metal gavage needles may also be used.
5. Bodyweight and temperature are recorded prior to sensitization in order to obtain baseline physical status. Bodyweight should be monitored regularly during the sensitization period to monitor the well-being of mice. Impeded growth or weight loss may indicate deteriorated health, and appropriate measures should be promptly taken according to your IACUC-approved protocol.
6. The 2-h fasting period is a good time to prepare the sham and allergen solutions required for that day. Maintaining the time of sensitization consistent avoids introducing potential variability.
7. To prevent potential confusion, administer the sham solution to all sham mice first before proceeding to administer the allergen solution to the BLG group mice.
8. We recommend holding the mouse in the investigator's non-dominant hand so that the investigator has a better control of the syringe with their dominant hand.
9. Mice lack the capacity to vomit so that the dispensed solution will remain in the stomach if the gavage needle is inserted properly. If the solution is dispensed in the upper esophagus, mice may spit out and receive a smaller amount of the solution than intended [27].
10. If sharing a gavage needle does not pose problems for your experiment, the same needle may be used for the same group as long as the needle is intact. If a needle is damaged from biting or cross-contamination is a concern, a new sterile gavage needle should be used for each mouse. We change needles between sham and BLG groups and males and females, regardless of needle conditions.
11. Determined from the amount of BLG-specific serum IgE, we have found no difference in the development of hypersensitivity between two groups of mice that were sensitized for either 5 or 8 weeks (unpublished observation).

12. For consistent assessment of post-challenge reactions, be sure to record the time of BLG challenge. Prior to a challenge day, we generate a table with a specific challenge time for each mouse, allowing about 3–5 min between mice for recording body temperature. For example, Mouse #1, 2, and 3 are, respectively, challenged at 9:00, 9:05, and 9:10 a.m. and returned to their home cage. Then, the body temperature of Mouse #1 is taken at 9:30 a.m. Clean the mouse restrainer and the rectal probe, and proceed to record the temperature from Mouse #2 at 9:35 a.m. Repeat the cleaning procedure and record the temperature from Mouse #3 at 9:40 a.m.
13. Highly sensitized mice may exhibit severe anaphylactic reactions, including respiratory distress, immobility, and death. If using a mouse strain known to display severe reactions (e.g., C3H background [13]), an appropriate termination protocol should be promptly implemented. In our mouse model of mild cow's milk allergy, BLG-sensitized C57BL/6J mice show no to moderate signs of anaphylaxis upon BLG challenge [12].
14. Hypothermia is one of the common anaphylactic reactions demonstrated by highly sensitized mice [28, 29].
15. Some variability in the magnitude of allergen-specific immunoglobulin induction is often observed within a treatment group. However, we routinely detect significantly elevated levels of BLG-specific IgE and IgG1 when the difference between sham and BLG group averages are statistically analyzed (Fig. 4).

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Chapter 6

Diet-Induced Mouse Model of Atopic Dermatitis

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Abstract

Atopic dermatitis (AD) is a common skin disease characterized by chronic inflammation and itchiness. Although skin barrier dysfunction and immune abnormalities are thought to contribute to the development of AD, the precise pathogenic mechanism remains to be elucidated. We have developed a unique, diet-induced AD mouse model based on the findings that deficiencies of certain polyunsaturated fatty acids and starches cause AD-like symptoms in hairless mice. Here, we present a protocol and tips for establishing an AD mouse model using a custom diet modified from a widely used standard diet (AIN-76A Rodent Diet). We also describe methods for evaluating skin barrier dysfunction and analyzing itch-related scratching behavior. This model can be used not only to investigate the complex pathogenic mechanism of human AD but also to study the puzzling relationship between nutrition and AD development.

Key words Atopic dermatitis, Animal model, Diet, Itch, Skin barrier dysfunction, Hairless mice

1 Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease. Itching (or pruritus) is the most bothersome symptom of AD because the resultant scratching exacerbates the skin lesions and elicits further itching, thereby establishing a vicious itch-scratch cycle [1]. Multiple genetic and environmental factors are thought to affect AD development [2, 3]. Aberrant Th2 immune responses in the skin have long been recognized as contributing factors of AD [4]. However, recent accumulating evidence has implicated that skin barrier dysfunction plays a primary role in AD [5, 6]. Although various cells and molecules have been shown to be involved in AD pathogenesis [7, 8], the precise mechanism of AD remains to be elucidated.

Animal models are useful in the study of disease pathogenesis and the development of new therapies. Existing mouse models of

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human AD can be generally classified into three types [9]: (1) several strains of mice that spontaneously develop AD-like skin lesions; (2) models induced by the epicutaneous application of sensitizers; and (3) genetically modified mice that either overexpress or lack AD-associated molecules. In most of these models, immunological triggers primarily cause the AD phenotypes.

We have reported a unique, diet-induced, alternative AD mouse model [10]. HR-1 hairless mice fed a commercial special diet (named HR-AD) develop pruritic dermatitis resembling human AD. In HR-AD-fed mice, skin barrier dysfunction precedes the development of skin inflammation and systemic immune changes. Furthermore, we have recently shown that HR-AD-fed mice have skin gene expression profiles that more closely resemble human AD than other common AD mouse models (Fujii M et al., unpublished observation). Thus, HR-AD-fed mice can be used as an AD model that is based predominantly on skin barrier dysfunction.

The method using the special HR-AD diet is simple and highly reproducible; however, there are several limitations in some cases. Specifically, there is no appropriate control diet for HR-AD; consequently, the nutrient compositions of the HR-AD diet and the control diet could be very different. Another limitation is that HR-AD is not easily available in countries outside of Japan. However, we have succeeded in replicating a similar AD phenotype in hairless mice using a custom diet modified from a widely used standard diet (AIN-76A Rodent Diet) based on the findings that deficiencies of certain polyunsaturated fatty acids and starches are primarily responsible for the diet-induced AD in mice [11, 12].

Here, we present a protocol and tips for establishing an AD mouse model using a special diet deficient in unsaturated fatty acids and starch. We also describe a method for evaluating skin barrier dysfunction as the primary symptom of diet-induced AD mice. Skin barrier function has been evaluated by noninvasively measuring transepidermal water loss (TEWL). Moreover, we introduce a protocol to analyze pruritus in this model. Diet-induced AD mice exhibit characteristic scratching responses; for example, (1) long-duration scratching bouts are spontaneously observed [10] (*see* Videos S1 and S2) and (2) oral administration of ethanol markedly increases scratching via a central nervous system action [13]. Although AD-related inflammatory and immune changes such as epidermal thickening, infiltration of inflammatory cells (e.g., $CD4^+$ cells, eosinophils, and mast cells), and increased expression of proallergic cytokines (e.g., thymic stromal lymphopoietin) are detected in the skin of diet-induced AD mice [10, 12], describing the methods for analyzing these changes is beyond the scope of this chapter and will not be covered here.

2 Materials

1. Female HOS:HR-1 hairless mice: 4-week-old, weighing 11–14 g (Hoshino Laboratory Animals, Ibaraki, Japan) (*see* **Note 1**).
2. Control diet: AIN-76A Rodent Diet; product # D10001 (Research Diets, New Brunswick, NJ).
3. Special diet: Product # D03052309 (Research Diets), diet compositions are shown in Table 1 (*see* **Note 2**).
4. Tewameter® TM210 or equivalent device to measure TEWL.
5. Polyvinyl observation chamber with 12 partitions: A single unit size: H: 15 cm × W: 12 cm × L: 12 cm (*see* **Notes 3** and **4**).
6. Digital video camera.
7. Originally developed counter (*see* **Note 5**).
8. 30% Ethanol in purified water.
9. Flexible feeding tube: Length, 38 mm; ball diameter, 2 mm (*see* **Note 6**).

Table 1
Compositions of the control and special diets

	Control diet	Special diet
General ingredient (g%)		
Protein	20	20
Carbohydrate	66	66
Fat	5	5
Composition of diet (g/kg diet)		
Casein, 80 mesh	200	200
DL-Methionine	3	3
Corn starch	150	0
Sucrose	500	650
Cellulose, BW200	50	50
Corn oil	50	0
Coconut oil, hydrogenated	0	50
Mineral mix S10001	35	35
Vitamin mix V10001	10	10
Choline bitartrate	2	2
Total	1000	1000

The special diet lacks both unsaturated fatty acids and starch by replacing (1) corn oil with hydrogenated coconut oil and (2) corn starch with sucrose

3 Methods

AD-like symptoms will occur 8–12 weeks after the start of special diet feeding. Figure 1 shows typical results obtained from mice fed the control diet or the special diet at the twelfth week of feeding. During AD development in the special diet-fed mice, various physiological and behavioral parameters could be measured. In addition, at the end of the experiments, skin and immunological samples could be collected and examined. The experiments should be completed within several weeks after the development of AD because long-term (>15 weeks) feeding of the special diet often causes debilitating effects. All experimental procedures using live animals must be approved by your local animal ethics committee.

3.1 Animal Housing and Feeding

1. Soon after the mice arrive at the animal facility, feed the control diet or the special diet according to the experimental design (*see* **Note 7**). Maintain the mice on the same diet throughout the study.
2. Change bedding once a week. However, after AD development is observed in special diet-fed mice, change the bedding more frequently because AD mice have increased TEWL, and the cage will likely become humid.
3. Check mice regularly during the experiments. We also usually measure mouse body weight. A typical result is shown in Table 2.

3.2 Skin Barrier Function Measurement

1. Launch the Tewameter® and wait for 15 min for the probe to automatically warm up to skin temperature (*see* **Note 8**).
2. Hold mouse securely with one hand, taking care to keep the head and forelimbs immobilized (Fig. 2a).
3. Place the probe lightly on the upper back skin of the mouse (Fig. 2b) (*see* **Note 9**).
4. Press the start button and wait until the TEWL value becomes stable (it usually takes 30–60 s).

3.3 Analysis of Spontaneous Scratching

1. Place mice in the observation chamber and allow them to become acclimatized for at least 10 min.
2. Videotape the mice for an appropriate period. We typically record the mice for 60 min (*see* **Note 10**).
3. To measure hindlimb scratching, watch the recording and document the cumulative duration and the frequency of scratching bouts by the original counter (*see* **Notes 5** and **11**).
4. Calculate the duration of one scratching bout by dividing the cumulative duration by the frequency.

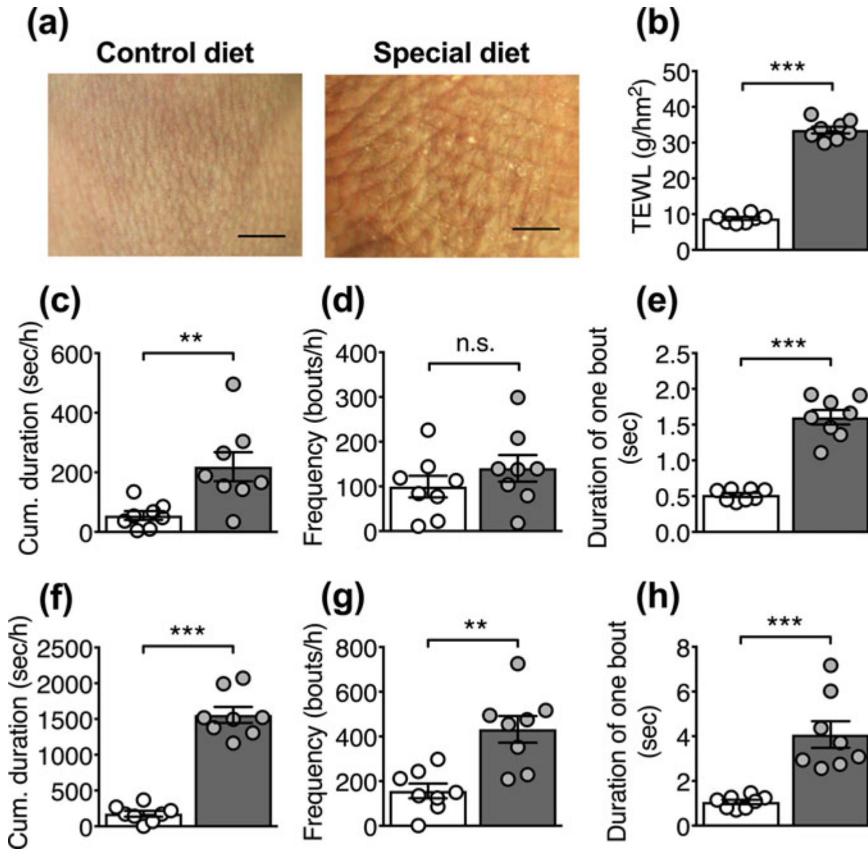

Fig. 1 AD-like symptoms induced by feeding a special diet to hairless mice for 12 weeks. (**a**) Appearance of the back skin. Special diet-fed mice exhibit scaly and wrinkled skin. Bar represents 3 mm. (**b**) TEWL. (**c**–**e**) Spontaneous scratching. (**f**–**h**) Ethanol-induced scratching. Data represent the mean ± S.E. of eight animals. ** and ***: $P < 0.01$ and 0.001, respectively, unpaired *t*-test. *Cum* cumulative; *n.s.* not significant. Reproduced from ref. 12 with permission

3.4 Analysis of Ethanol-Induced Scratching

In AD patients, pruritus is often exacerbated by alcohol intake [14]. Interestingly, in diet-induced AD mice, scratching behavior is similarly increased by the oral administration of ethanol [13] (Fig. 1f–h).

1. Measure mouse body weight.
2. Acclimatize mice to the observation chamber as described in Subheading 3.3, **step 1**.
3. Remove the mice from the chamber.
4. Grasp the nape of the neck using the thumb and index finger and hold the mouse in an upright position (*see* **Note 12**).
5. Insert the feeding tube carefully into the mouth.
6. Administer a 30% ethanol solution at a volume of 10 mL/kg.

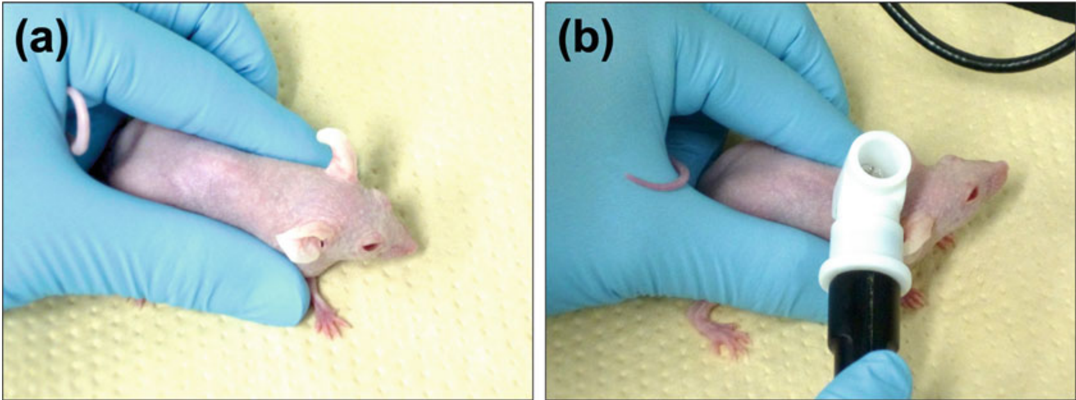

Fig. 2 Manner of holding mouse (**a**) and placing the probe on the mouse skin (**b**) for TEWL measurement

Table 2
Body weight changes and diet composition in hairless mice fed the control and special diets

	Body weight (g)					Diet consumption (g/animal/day)
Week after feeding	0	2	4	8	12	9–10
Control diet	12.5 ± 0.5	21.9 ± 0.3	22.5 ± 0.3	23.2 ± 0.2	25.8 ± 0.5	3.59 ± 0.06
Special diet	12.6 ± 0.4	21.0 ± 0.3	21.6 ± 0.3	22.0 ± 0.4	22.8 ± 0.6***	3.64 ± 0.04

Body weight represents the mean ± S.E. of eight animals. Diet consumption was measured 9–10 weeks after the start of feeding and represents the mean ± S.E. of five measurements. ***: $P < 0.001$, two-way ANOVA with Bonferroni's multiple comparison test

7. Immediately after administration, return the mouse to the observation chamber and record them as described in Subheading 3.3, **step 2**.
8. Analyze scratching behavior as described in Subheading 3.3, **steps 3** and **4**.

4 Notes

1. Weaning-age mice should be prepared. Using adult mice (even at 5 weeks old) delays the onset of diet-induced AD. Male HR-1 hairless mice can be used; however, we typically use female mice because male mice fight among themselves. Although other mouse strains such as BALB/c and C57BL/6 are available, they develop diet-induced AD differently. As reported previously [15], the onset is earlier, and the severity is greater in HR-1 hairless mice than in other mice. This may be

due to a hypomorphic mutation in the hairless (*Hr*) gene carried on the HR-1 strain. Although we obtain HR-1 hairless mice from a Japanese supplier (Hoshino Laboratory Animals), other hairless strains carrying the same mutation, such as SKH1, are commercially available worldwide [16].

2. The food of the custom diet can be colored with food dyes to distinguish between diets.
3. It is important that mice do not observe other mice. The scratching behavior of a mouse (observer) could be affected by the observation of another mouse scratching (demonstrator) [17, 18].
4. During videotaping, mice sometimes jump out of the observation chamber. An acrylic transparent cover with vent holes prevents the mouse from jumping out.
5. This counter was made with four digit up/down counters (KIT129E; EK Japan, Fukuoka, Japan), a programmable crystal oscillator (SPG8651A; Seiko Epson, Nagano, Japan), and a snap switch (D2VW-5L2A-1M, Omron, Kyoto, Japan). This allows for the analysis of scratching behavior in two ways: (1) the number of times an observer touches the switch indicates the number of scratching bouts and (2) the length of time that an observer touches the switch documents the cumulative duration of the scratching behavior (*see* Video S3). Note that the precision of this counter is 0.1 s.
6. Stainless steel feeding needles should not be used for gavage administration in mice. A flexible tube with a soft tip decreases the risk of perforations of the esophagus and stomach.
7. Mice should be equally distributed in cages with a 12-h light/dark cycle and free access to water and food. Although diet-induced AD symptoms occur under either conventional or specific pathogen-free conditions, some inflammatory and immune parameters might be variable between different facilities.
8. Measurement of TEWL should be conducted at a temperature of 23 ± 1 °C and 50% ± 10% humidity.
9. The sensor in the probe is fragile. Care should be taken to avoid any direct contact with the sensor.
10. Complete the videotaping of the mice (especially special diet-fed AD mice) within 2 h. Long-term videotaping without access to water may cause dehydration.
11. Alternatively, the researcher can visually determine only the number of scratching bouts; however, there is no reproducible difference in the frequency of spontaneous scratching between normal and AD mice (Fig. 1d).
12. Ensure that the mouse is comfortable by monitoring its breathing.

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Chapter 7

Animal Models of Contact Dermatitis: 2,4-Dinitrofluorobenzene-Induced Contact Hypersensitivity

Mario C. Manresa

Abstract

Allergic contact dermatitis (ACD) is a common skin disease with high prevalence in work environments. Human allergic contact dermatitis is triggered by the exposure to haptens that leads to an initial phase known as sensitization. During this phase, hapten–protein complexes presented by antigen-presenting cells activate a T-cell-mediated response, leading to the generation of memory cells against the hapten. Upon re-exposure to the same hapten, the elicitation phase is initiated. This phase is characterized by a quicker acute inflammatory response involving activation and/or infiltration of a variety of immune cell populations. Human ACD can be studied through the use of animal models of contact hypersensitivity (CHS). The 2,4-dinitrofluorobenzene (DNFB)-induced CHS model is a commonly used mouse model that has been helpful in the study of the mechanisms as well as potential therapeutic interventions of ACD. In this chapter I will provide a detailed protocol to develop acute DNFB-induced CHS in mice in a period of 7 days. In addition, I will discuss several key considerations for experimental design including best controls, potential expected outcomes, and sample collection.

Key words Allergic contact dermatitis (ACD), Contact hypersensitivity (CHS), Hapten, 2,4-Dinitrofluorobenzene (DNFB), Skin inflammation

1 Introduction

Allergic contact dermatitis (ACD) is one of the most common skin diseases. It is estimated that around 20% of health complaints in work environments are due to ACD, making it a highly prevalent occupational disease [1, 2]. As a result, ACD has a high impact from health and economic perspectives. Human ACD can be accurately mimicked with the use of murine models of contact hypersensitivity (CHS). These mouse models of skin allergy are powerful tools to study the causes, mechanisms, and potential therapeutic interventions of ACD. As in the case of human ACD, CHS models are characterized by the existence of two phases. The initial phase, known as sensitization, involves exposure of mice to an hapten that activates the innate immune response. Haptens combine with

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proteins to form hapten-protein complexes and are subsequently presented by antigen-presenting cells, ultimately leading to the priming of effector T cells [1–4]. This is followed by a later challenge phase in which mice are re-exposed to a lower dose of the same hapten in an area of the skin remote from the site of sensitization [5, 6]. The challenge phase leads to a rapid multistep inflammatory response that is led by T cells and characterized by the infiltration and/or activation of different immune cell populations, including neutrophils, eosinophils, and mast cells [7–9].

Various chemical agents can be used as haptens to evoke CHS in mice. Of the currently available chemical hapten-mediated models, 2,4-dinitrofluorobenzene (DNFB)-induced CHS is a commonly used model that has been useful in investigating many features of allergic skin disease. For example, key roles of neutrophils in the elicitation phase of CHS have been described using this model. Some of these studies revealed that the expression of FasL and perforin by neutrophils is required for infiltration of T cells to challenge sites, as well as an essential role of the neutrophil chemoattractant CXCL1 [7, 10, 11]. In keeping with this, we recently described a reduction in DNFB-induced inflammation at challenge sites when neutrophils were depleted via pharmacologic inhibition of oxygen-sensing hypoxia-inducible factor hydroxylases [12]. The DNFB model has also helped to clarify the role of mast cells in CHS. Genetic depletion of mast cells in mice resulted in reduced DNFB-induced CHS [8]. In addition, cross-talk between mast cells and dendritic cells has been found to play a key role in the recruitment of T cells to inflamed skin in DNFB-induced CHS [13]. Moreover, the model has also helped in understanding the roles of different molecular signaling pathways such as p38 and STAT5 in CHS [9, 14]. Therefore, the DNFB model is a powerful tool for the study of CHS that shows reproducibility in different mouse strains and does not pose a high threat to the general health condition of the mice, making it highly attractive as a laboratory model of allergic skin disease.

In this chapter I will provide a detailed protocol to safely perform the DNFB model in mice. The protocol outlined herein describes an example experiment on female C57BL/6 mice, starting at the time the mice reach 10 weeks of age. Mice are divided into two groups of five mice each, for negative and positive controls. The experiment is performed over a period of 8 days and allows the analysis of acute inflammatory responses at the challenge site. In this model, ear thickness increases with time after challenge, providing a direct measurement of inflammation (or edema). This read-out can be used to monitor the correct development of the model and to assess variables such as the effects of treatments or genetic deletions on the elicitation of CHS. Ear skin samples are easily obtained for histological and molecular analyses.

2 Materials

2.1 Sensitization of Animals

1. Mice: 9-week-old female C57BL/6 mice (*see* **Note 1**). Randomly group into sensitization or control group (*see* **Note 2**). Acclimatize for 1 week before sensitization, which should start once the mice reach 10 weeks of age.
2. Anesthesia station (*see* **Note 3**).
   (a) Isoflurane.
   (b) Isoflurane vaporizer with pressure-controlled oxygen supply.
   (c) Induction box.
   (d) Waste gas scavenging system.
3. Trimmer: for shaving the abdominal region of mice.
4. Pipettor: P100 (100 μL).
5. Sterilized pipette tips: 100 μL.
6. Acetone/olive oil 3:1 (v/v) mixture: Combine three parts of molecular grade acetone and one part of low acidity olive oil and vortex gently until homogenized (*see* **Note 4**).
7. Sensitizing agent (hapten): 0.5% DNFB (2,4-dinitrofluorobenzene) in acetone:olive oil 3:1 mixture (*see* **Note 4**).

2.2 Allergen Challenge

1. Anesthesia station: *See* Subheading 2.1, **item 2**. Connect a second vaporizing channel to a vaporizing mask for maintenance of anesthesia.
2. Vaporizing mask (breathing circuit): placed on top of the heating pad to deliver vaporized isoflurane. Secure the position on the pad using tape.
3. Heating pad: set to 36–38 °C.
4. Adhesive tape: used to fix the mouse and vaporizing mask to the heating pad to avoid movement during anesthesia.
5. Vehicle: acetone/olive oil 3:1 mixture to be used as a negative control (*see* **Note 5**).
6. Challenge agent: 0.3% DNFB in acetone/olive oil 3:1 to be used as a positive control (*see* **Note 5**).
7. Pipettor: P10 or P20 (10–20 μL).
8. Sterilized pipette tips: 10–20 μL.
9. A digital thickness gauge: sensitive to the micrometer range.

2.3 Sample Collection

1. Euthanasia materials (*see* **Note 6**).
2. Surgical scissors.
3. Liquid nitrogen: for snap freezing of mouse ear skin samples.

4. Formalin: for fixation of mouse ear skin samples to be used for histological analysis.
5. RNA later or equivalent for tissue sample storage and RNA preservation.
6. Cryotubes.

2.4 Preparation of Histological Samples

1. 70% Ethanol.
2. Paraffin or optimal cutting temperature (OCT) compound.
3. Tissue processor.
4. Tissue embedding station.
5. Plastic or metallic histology molds.
6. Tissue embedding cassettes.
7. Tissue embedding sponges.
8. Microtome for paraffin block sectioning or cryostat for frozen sectioning.
9. Glass slides.
10. Water bath: set at 37 °C (only for paraffin sections).

2.5 Preparation of Protein Samples

1. Radioimmunoprecipitation assay (RIPA) buffer.
2. Tissue homogenizer.
3. 5-mm Stainless steel beads.
4. 1.5-mL Microcentrifuge tubes.
5. Benchtop microcentrifuge with a maximum speed reaching up to 18,000 Relative Centrifugal Force (RCF).

2.6 Preparation of RNA Samples

1. RNA stabilizing reagent: RNAlater™ or equivalent.
2. RNA isolation reagent: TRIzol™ or equivalent.
3. Chloroform.
4. Tissue homogenizer.
5. 5-mm Stainless steel beads.
6. Nuclease-free microcentrifuge tubes.
7. Benchtop microcentrifuge with a maximum speed reaching up to 18,000 RCF.

3 Method

The following methodology describes an example experiment with ten female C57BL/6 mice, in which five mice are sensitized with 0.5% DNFB and challenged with vehicle (negative control), while the other five mice are sensitized with 0.5% DNFB and challenged with 0.3% DNFB (positive control) (*see* **Note 2**). Increments in the

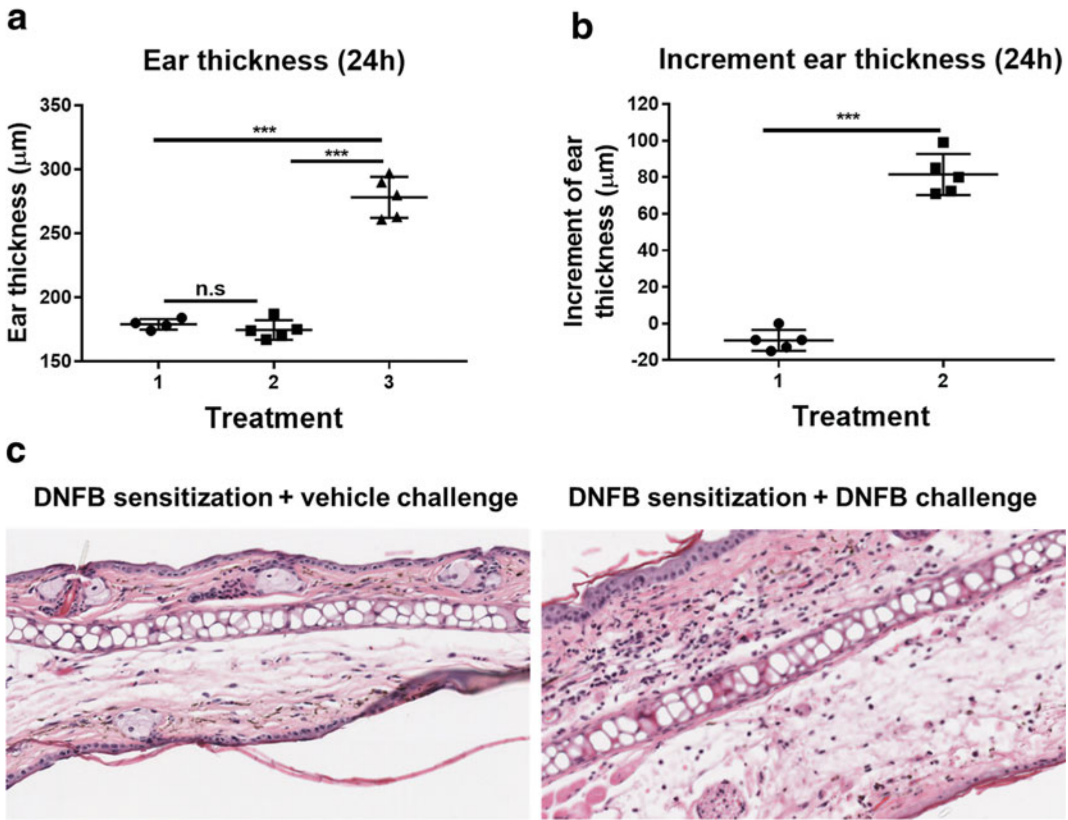

Fig. 1 (**a**) Comparison of ear thickness between mice sensitized with 0.5% DNFB on 2 consecutive days and challenged with vehicle (1) or animals not sensitized with DNFB but challenged with 0.2% DNFB (2) or animals sensitized with 0.5% DNFB on 2 consecutive days and challenged with 0.2% DNFB (3), at 24 h postchallenge ($n = 4$–5 mice/group). (**b**) Increment of ear thickness comparing mice sensitized with 0.5% DNFB on 2 consecutive days and challenged with vehicle (1) or animals sensitized with 0.5% DNFB on 2 consecutive days and challenged with 0.3% DNFB (2), between Time 0 and 24 h postchallenge ($n = 5$ mice/group). (**c**) Representative H&E staining of mouse ear specimens from mice treated as in (**b**). Data adapted from Manresa et al., *Allergy*, 2018 [12]

ear thickness at 24 h postchallenge are used as read-outs of inflammation (Fig. 1). The thickness of the mouse ear under basal conditions should be around 150 μm (Fig. 1a). Most common use of DNFB as a challenge agent is application of 20–40 μL of 0.2–0.3% DNFB to the mouse ear, although we found no significant differences in the effects of the two doses on the increment of ear thickness at 24 h postchallenge in mice presensitized to DNFB as in the experiment detailed here (Fig. 2) [15, 16]. Therefore, both doses should lead to a comparable and effective induction of skin inflammation within 24 h.

3.1 Sensitization

This protocol consists of two sensitizations. Therefore, the exact procedures detailed in **steps 1–6** are repeated on Day 2 (Fig. 3). Mice are then allowed to rest from Day 3 to Day 6 (4 days) before challenged (Fig. 3).

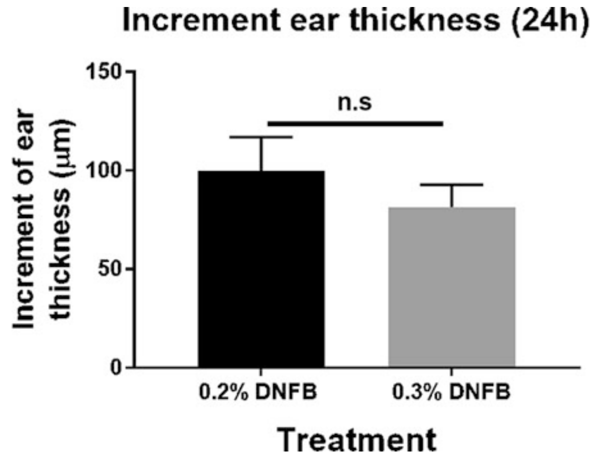

Fig. 2 Comparison of the increments of ear thickness between Time 0 and 24 h postchallenge in mice sensitized with 0.5% DNFB on 2 consecutive days and challenged with 0.2% or 0.3% DNFB. Data adapted from Manresa et al., *Allergy*, 2018 [12]

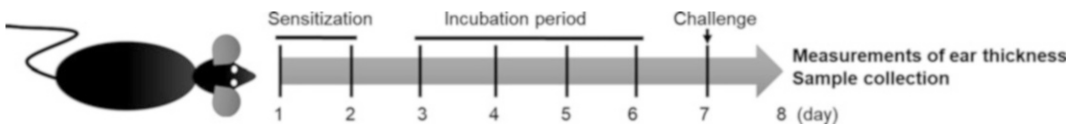

Fig. 3 Timeline of the proposed DNFB experiment

1. On Day 1, anesthetize mice on the anesthesia station. Adjust the oxygen supply to 1 LPM and the vaporizer to position 5 to deliver 5% isoflurane (*see* **Note 3**). Start the vaporizer 5 min before introducing the mouse to precondition the induction box.
2. Place a mouse in the induction box and close the box. The mouse should be fully anesthetized in 2–3 min. To validate the depth of anesthesia, use a classic method such as toe pinching. Pinch between toes and monitor mouse reactions. Fully anesthetized mice should not react to pinch.
3. Grasp the mouse by pulling the fur from the back and back of the neck. Flip the mouse to expose the abdomen. Trim the fur of the abdominal area, from the lower part of the chest down to approximately 2 cm above the genital area.
4. With a 100-μL pipette, apply 100 μL of 0.5% DNFB in acetone/olive oil 3:1 to the shaved area. Spread the sensitizing agent evenly by moving the pipette tip along the abdomen on a zigzag path.

5. Return the mouse to the induction box with the anesthetic flow reduced to 2% on the vaporizer (low dose used for maintenance of anesthesia). Allow the sensitizing agent to be absorbed into the abdominal skin for an additional 5 min with constant application of maintenance anesthesia.
6. Return the mouse to its cage and monitor recovery from anesthesia. Repeat the procedure for each mouse.
7. For Day 2, repeat **steps 1–6** above.
8. Following the administration of the sensitizing agent on Days 1 and 2, allow mice to rest for 4 days. The 4-day resting period allows a priming of the immune response with the generation of memory T cells. Minor irritation or skin lesions may be observed in the area of application of the sensitizing agent during the first days, which should resolve spontaneously.
9. Monitor the mice during this period by checking the shaved abdominal area once per day.

3.2 Challenge and Inflammation Monitoring

1. Anesthetize mice as described in Subheading 3.1, **steps 1** and **2**.
2. Once fully anesthetized, place the mouse on the heating pad with its back upward and its mouth and nose inside the vaporizing mask (*see* **Note** 7). Reduce the anesthetic flow to 2% on the vaporizer for maintenance of anesthesia.
3. Fix the vaporizing mask and the mouse to the heating pad using tape to facilitate measurement of ear thickness and administration of the DNFB challenge. Make sure to use the tape on hairless areas on the paws to avoid damage to the hair and underlying skin during the procedure.
4. Turn on the digital thickness gauge and ensure that it reads 0 under baseline conditions. Lift the spindle of the gauge, place the center of the ear to be challenged between the edges of the spindle, and release the spindle (Fig. 4a). Record the measurement value that appears on the screen within the first 3 seconds (*s*) (*see* **Note 8**). This measurement serves as a reference of initial ear thickness at Time 0 (*see* **Note 9**).
5. Slowly apply 10 μL of the vehicle or challenge agent to each side of the ear of the mouse for a total volume of 20 μL. If both ears are to be challenged, repeat the procedure on the second ear. Make sure that the liquid spreads around the surface of the ear (*see* **Note 10**).
6. Keep the mouse on the heating pad for 10–15 min to allow complete absorption of the challenge agent or vehicle (*see* **Note** 7).
7. Return the mouse to its cage and monitor the recovery from anesthesia.

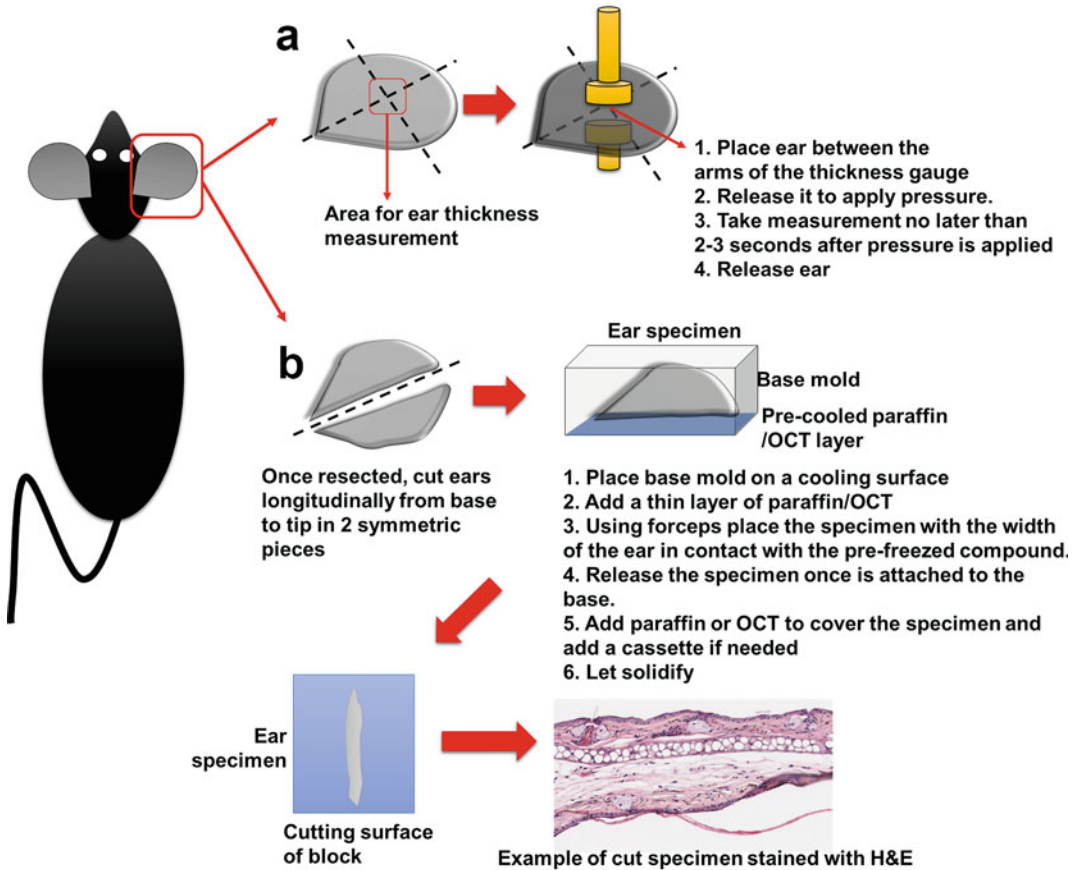

Fig. 4 (**a**) Schematic instructions depicting the best area and method to measure ear thickness using a thickness gauge. (**b**) Schematic instructions depicting the method of mouse ear resection and the ideal orientation and method to obtain high-quality mouse ear skin specimens for histology

8. Repeat the ear thickness measurements as described above at different time points to obtain a read-out of tissue edema and to monitor the establishment of inflammation (*see* **Note 9**).

3.3 Sample Collection

1. Sacrifice mice using the appropriate method in your Institutional Animal Care and Use Comitte (IACUC)-approved protocol. Some commonly used methods of sacrifice might exert effects on inflammation (*see* **Note 11**).
2. Resect both ears using surgical scissors. For histological analysis, bisect each ear longitudinally from the base to the tip (Fig. 4b). Appropriately oriented half ears will be enough for histological analysis. *See* Fig. 4b for the best orientation for histological analysis of ear specimens in paraffin or OCT blocks.
3. The remaining half of each ear can be used for the extraction of proteins (*see* **Note 12**). To preserve ear samples for later protein

extraction, place the samples in cryotubes and snap-freeze in liquid nitrogen. Store at −80 °C until use.

4. If RNA is also to be extracted from the ears for downstream assays, do not cut both ears in halves. Use half of an ear for histology and the remaining half ear for protein extraction. Use the other full ear for RNA (*see* **Note 13**).
5. To obtain paraffin-embedded samples for histological analysis, immerse half ear samples in formalin for 16–24 h at room temperature. If both ears are to be analyzed by histology, one-half of each ear should be used.
6. After fixation, transfer the samples to new microcentrifuge tubes containing 1 mL of 70% ethanol. These samples can be stored at 4 °C for longer storage or can be processed immediately for embedding in paraffin blocks.
7. To obtain frozen specimens for histological analysis, immediately freeze half ears in OCT compound as described below in Subheading 3.4, **step 4** (Fig. 4b).
8. For RNA isolation, immerse full-ear specimens in RNA later reagent and store at 4 °C for up to 12 h. These samples can then be stored at −20 °C for longer storage.

3.4 Preparation of Histological Samples

1. Place ear samples in the indicated orientation and embed in paraffin or OCT compound as shown in Fig. 4b.
2. Cut paraffin blocks on a microtome or frozen specimens on a cryostat to produce 4-μm-thick sections.
3. When cutting paraffin sections, preheat the sections in a water bath at 37 °C before mounting them to a glass slide. Stretch the tissue on the water and let it adhere to the slide (*see* **Note 14**).
4. Once the sections are collected on a slide, place the slide on a rack and allow it to dry at room temperature. The slides can then be stored or stained. For staining, deparaffinize and rehydrate the tissue slides prior to staining. Examples of H&E-stained samples using this procedure are included in Figs. 1c and 4b.
5. When cutting frozen sections on a cryostat, mount the sections directly onto a glass slide at room temperature (*see* **Note 15**).

3.5 Preparation of Protein Samples

1. Thaw the frozen-stored half-ear samples and immediately place them in 300 μL of RIPA buffer or other appropriate lysis buffer.
2. Homogenize the tissue with presterilized 5-mm stainless beads in appropriate tubes for 10 min at an oscillation frequency of 25–30 Hz using Qiagen Tissue Lyser II or equivalent (*see* **Note 16**).

3. Centrifuge the homogenate at 18,000 × *g* for 10 min at 4 °C to separate protein supernatants from tissue pellets. The obtained supernatant will be cloudy and may have abundant debris.
4. Recover the homogenate supernatant avoiding the pellet debris and place it in a new sterile microcentrifuge tube.
5. Repeat centrifugation 2–3 more times at 18,000 × *g* at 4 °C for 10 min to clear the supernatant.
6. Collect the final supernatants and aliquot into clean tubes. Use the resulting protein samples immediately or store them at −80 °C until use (*see* **Note 12**).

3.6 Preparation of RNA Samples

1. Thaw the samples collected in RNA later if stored at −20 °C. Place each sample in an appropriate tube with 750 μL of TRIzol and a 5-mm stainless steel bead (*see* **Note 17**).
2. Homogenize the samples using a Qiagen Tissue Lyser II as described for protein extraction in Subheading 3.5, **step 2**.
3. Centrifuge the TRIzol homogenates at 18,000 × *g* for 10 min at 4 °C to separate the tissue debris. Collect the supernatants into clean nuclease-free microcentrifuge tubes.
4. If using a classic chloroform-based extraction method, add 200 μL of chloroform to the obtained TRIzol supernatants, mix vigorously, and proceed to extraction. If a commercial column-based method is chosen, proceed to RNA extraction according to the manufacturer's instructions.

4 Notes

1. Standard housing conditions should be applied. Mice should be kept at a controlled temperature between 18 and 23 °C with humidity between 40% and 60%. Mice should be exposed to a 14-h light 10-h dark cycle and allowed free access to food and water throughout the experiment.
2. Another possible control could be the use of mice that have not been sensitized but are challenged with DNFB. We performed a study comparing the ear thickness at 24 h postchallenge of mice sensitized with DNFB and challenged with vehicle to mice not sensitized but challenged with DNFB or mice sensitized with DNFB and challenged with DNFB. We found that the ear thickness of mice challenged with vehicle after sensitization with DNFB was not different from that of mice not sensitized (vehicle) but challenged with DNFB (Fig. 1a). A comparison of the increments in ear thickness from Time 0 to 24 h between mice sensitized with DNFB and challenged with vehicle and mice sensitized and challenged with DNFB clearly showed a significant increment in ear thickness in those

sensitized and challenged with DNFB (Fig. 1b, c). Thus, a group of mice sensitized to DNFB but challenged with vehicle serves as a good negative control for local inflammation in the ear skin during the challenge phase. However, for studies aiming to investigate the immune events developed during the sensitization phase, DNFB-sensitized mice should be compared to vehicle-sensitized mice instead.

3. For successful and safe general anesthesia, a vaporizer, anesthetic agent, induction box, oxygen supply, and scavenging system are required. The oxygen supply should be connected to the vaporizer. The oxygen will enter the vaporizer and, when in contact with the liquid isoflurane, induce vaporization. The resulting anesthetic gas is conveyed to the induction box by the vaporizer. The scavenging canister should be connected to the induction box. This will allow clearance of residual isoflurane. For additional safety, the induction box can be placed inside a laminar flow hood.

   Isoflurane should be added to the vaporizer using an appropriate adaptor/application system. This is designed to avoid leakage that would result in direct exposure to the agent, as isoflurane is highly volatile at room temperature and atmospheric pressure. The applicator should be inserted into the appropriate receptor on the vaporizer and the bottle inclined to 45° to fill the vaporizer.

   If anesthesia is required more than once per day, this should be specified in your IACUC protocol.

4. The number of mice included in the experiment should be considered when preparing the required volume of acetone/olive oil 3:1 (v/v). For example, for sensitization of ten mice, 1.1 mL of acetone/olive oil 3:1 should be prepared by mixing 825 μL of acetone and 275 μL olive oil to allow 0.1 mL excess volume to account for any minor volume losses. For sensitization of ten mice using 100 μL of 0.5% DNFB per mouse, add 5.5 μL of DNFB in 1.1 mL of homogenized acetone/olive oil 3:1 mixture. Prepare freshly on the day of sensitization. Do not store for later use.

5. In an example experiment consisting of five negative-control mice and five positive-control mice, five of them are challenged with vehicle (acetone:olive oil, 3:1) and five with 0.3% DNFB. Prepare 1.2 mL of the vehicle by mixing 900 μL of acetone and 300 μL of olive oil and vortex to ensure homogeneity. Separate 200 μL of the vehicle into a sterile microcentrifuge tube, which is sufficient for the negative-control (vehicle-challenged) group of five mice. To the remaining 1 mL of vehicle, add 3 μL of DNFB and vortex to ensure a proper mixture. This will be your challenge agent. For each mouse, only 20 μL of the vehicle or challenge agent per ear (0.3% DNFB) will be needed. However,

an excess volume of challenge agent should be prepared, since it is important to keep the volume of DNFB high enough to ensure accurate pipetting and mixing.

6. Materials needed for euthanasia will depend on the selected and preapproved method.

7. During the challenge phase, mice should be placed on a heating pad to ensure maintenance of body temperature. The measurement of ear thickness and the absorption of the DNFB challenge to the ear skin can together take 10–15 min, and the heating pad will help maintain the mouse's body temperature.

8. As the spindle continues to apply pressure, the thickness reading will constantly decay. To avoid misleading results, record the measurement that appears on the screen within the first 3 s (Fig. 4a). Take the measurements in the middle area of the ear as shown in Fig. 4a. If the measurements are taken around the tip area of the ear, increments in ear thickness during the course of the experiment might not be detected. The tip part is thinner, and the pressure applied by the apparatus might mask any increases.

   In my experience, both ears can be used in either DNFB challenged or control (vehicle-challenged) mice. This increases the number of samples obtained per mouse. If this method is followed and both ears are challenged, the ear thickness should be measured in both ears and the inflammation observed should be similar. Calculate the average ear thickness between both ears at Time 0 and at later time points. To obtain the increment in ear thickness, deduct the ear thickness at Time 0 from the ear thickness at later time points and calculate the average between these values.

9. When selecting time points, one must take into account that the measurement of ear thickness will require anesthesia, so the frequency of measurements will also depend on ethical considerations. Typically, significant increases in ear thickness can already be observed 8 h after the challenge, and reach a maximum between 24 and 48 h [8, 12, 15].

   Shortly after the challenge agent is applied, scratching behavior may be observed. This can be recorded as a read-out of CHS.

10. Apply the solution starting around the tip area of the ear and move downward to ensure a homogeneous distribution. When pipetting on the internal side, it is important to prevent the liquid from entering the auditory canal by pipetting slowly from the upper part of the ear.

11. Some studies have analyzed the effects of different euthanasia methods on parameters of inflammation, such as cytokine levels and lymphocyte proliferation [17, 18]. Although effects

of the method of euthanasia on DNFB-induced CHS have not been investigated, it would be appropriate to consider these prior studies when choosing a method of euthanasia for the CHS model described here.

12. To obtain sufficient amounts of proteins, a half to a full-ear sample will be needed. The abundance of proteins may be higher in inflamed samples, and therefore, protein concentrations in all samples should be measured and adjusted before used for analysis (such as by enzime-linked immunosorbent assay or western blot).

13. A full ear may be required to obtain high enough RNA yields. In case the ear tissues from one mouse are to be divided for histology, RNA, and protein samples, the ideal approach would be to use a half of one ear for histology, the remaining half of the ear for protein, and the other full ear for RNA. Based on the data obtained for ear thickness changes, the inflammation should be very consistent between both ears. This should be confirmed in data obtained by histology and RNA and/or protein analysis.

14. When trimming a block on a microtome to reach the embedded section, you may set the section thickness at 10 μm until the tissue becomes visible on the surface of the block. Once the tissue is evident, reduce the thickness setting to 4 μm, which is an ideal thickness for various types of staining.

15. The OCT compound will immediately melt at room temperature. To avoid tissue degradation, fix the tissue slides in 1% paraformaldehyde for 20 min and wash three times with phosphate-buffered saline, 5 min each. Use these slides immediately for staining.

16. Different homogenization methods may be used for this step. I have successfully used a Qiagen Tissue Lyser II station with 5-mm stainless steel beads in appropriate tubes. If this or another similar system is available, homogenize the tissues using the system appropriate adapters/holders in the homogenizer as described in Subheading 3.5. The ear skin is difficult to homogenize, which is why it requires high speed and long homogenization times. The resulting homogenates should be cloudy but free of obvious macroscopic ear structures.

    If a different homogenization method is used, optimization of the protocol may be necessary to ensure clean protein extracts with high yields.

17. If a method other than TRIzol or other phenol–chloroform-based extraction is used, RNA from the ear samples should be extracted according to the manufacturer's instruction to ensure proper preservation during extraction.

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Chapter 8

Induction of Airway Hypersensitivity to Ovalbumin and Dust Mite Allergens as Mouse Models of Allergic Asthma

Mei-Chi Chen, Jesse W. Tai, and Cheng-Jang Wu

Abstract

Mouse models of allergic asthma have been utilized to establish the role of T helper type 2 (Th2) cells in driving lung inflammation, airway hyperresponsiveness, and obstruction. Here, we present the allergic asthma models, in which mice are hypersensitized to ovalbumin (OVA) and house dust mite (HDM). These models mimic the major characteristics of human asthma including the eosinophilic inflammation and hyperactivity of the airway, overproduction of Th2 cytokines in the lung, and elevated total and allergen-specific immunoglobulin E (IgE) in serum.

Key words Airway hypersensitivity, Allergic asthma, Pulmonary eosinophilia, House dust mite, Immunoglobulin E, Mouse model, Ovalbumin, T helper type 2 cytokines

1 Introduction

Allergic asthma is a chronic inflammatory disease of the airways associated with enhanced T helper type 2 (Th2) responses to inhaled environmental allergens, such as cat dander, pollen, and house dust mite proteins [1–3]. Such Th2 responses lead to bronchial eosinophil infiltration, airway hypersensitivity, mucus hypersecretion, and elevated serum immunoglobulin E (IgE) levels [4]. Some Th2 cytokines, such as interleukin (IL)-5, promote the differentiation and activation of eosinophils, while IL-4 and IL-13 enhance IgE production and further enhance the severity of asthma [5, 6]. Mouse models have been developed to mimic the features of human allergic asthma and are essential to better understand the pathophysiologic mechanisms in asthma development [7, 8]. Here, we describe the mouse models of allergic asthma induced by the experimental allergen chicken egg ovalbumin (OVA) and naturally occurring allergen house dust mite (HDM) [9, 10]. Moreover, the detailed information to determine the severity of asthma responses will be also included, such as pulmonary function test, eosinophil count in bronchoalveolar lavage (BAL) fluid, lung histology, lung

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Th2 cytokine measurement by quantitative reverse transcription-polymerase chain reaction (RT-PCR) or flow cytometry, and serum IgE measurement.

2 Materials

2.1 Allergic Asthma Mouse Models

1. 10× Phosphate-buffered saline (PBS) stock solution: pH 7.4. Dissolve 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4 in 800 mL of distilled water. Adjust pH to 7.4 and make up to 1 L with distilled water. Sterilize by autoclave. Dilute 10 mL of 10× PBS stock solution in 90 mL of distilled water to make PBS (1×).
2. Imject™ Alum adjuvant: 40 mg/mL aluminum hydroxide and 40 mg/mL magnesium hydroxide with inactive stabilizers.
3. OVA solution: 1 mg/mL ovalbumin (OVA) in sterile PBS. Weigh 100 mg of OVA (*see* **Note 1**) in 100 mL of PBS, filter through a 0.22-μm filter for sterilization. Aliquot 1 mL in sterile 1.5-mL microcentrifuge tubes and store at −80 °C.
4. HDM extract solution: Dissolve 2 mg/mL HDM extract (*Dermatophagoides pteronyssinus*) in PBS. Aliquot 1 mL in sterile 1.5-mL microcentrifuge tubes and store at −80 °C.
5. Rotator: used to mix OVA and adjuvant.
6. 1-mL syringe with a 25-gauge needle.
7. Isoflurane and isoflurane vaporizer connected with oxygen gas.
8. Pipettors and tips.

2.2 Pulmonary Function Test

1. Equipment for pulmonary function test with ventilator: Scireq flexiVent or equivalent.
2. Anesthetic agents: Freshly prepare xylazine (10 mg/kg body weight) and ketamine (100 mg/kg body weight) by diluting in sterile PBS.
3. 70% Ethanol: Dilute 700 mL of absolute ethanol (100%) with 300 mL of distilled water.
4. Methacholine solutions: Dissolve methacholine in PBS at different concentrations (0, 3, 24, and 48 mg/mL) for pulmonary function test.

2.3 Examination of Bronchoalveolar Lavage (BAL) Fluid Cells

1. Lavage solution: 0.1 mM ethylenediaminetetraacetic acid (EDTA) prepared in PBS.
2. Enzyme-linked immunosorbent assay (ELISA) kits for Th2 cytokines: commercially available.
3. Trypan blue solution: Prepare a 0.4% solution in PBS to stain bronchoalveolar lavage (BAL) cells.

4. Hemocytometer for counting cell number.
5. Shandon Cytospin® for depositing monolayer cells on slides.
6. Precleaned microscope slides.
7. Wright–Giemsa staining kit: commercially available.
8. Mounting medium.
9. Coverslips.
10. Microscope.

2.4 Lung Histology

1. 10% Formalin solution: prepare in PBS.
2. Ethanol solutions for tissue dehydration/rehydration steps: prepare 70%, 80%, 95%, and 100% solutions.
3. Xylene for tissue dehydration.
4. Paraffin wax: Heat to 58 °C for tissue embedding.
5. Paraffin embedding molds.
6. Microtome.
7. Hematoxylin and eosin (H&E) stain.
8. Perform periodic acid-Schiff (PAS) stain.

2.5 Quantitative RT-PCR of Lung Tissue

1. TRIzol RNA isolation reagent or equivalent.
2. Homogenizer or 1-mL Luer Lock Syringe with a 20-gauge needle.
3. NanoDrop or spectrophotometer to determine RNA concentration.
4. Complementary DNA (cDNA) synthesis kit.
5. PCR tubes and caps.
6. Pipettors and tips: PCR grade.
7. SYBR™ Green real-time PCR reagents master mixes.
8. Primer pairs for IL-4, IL-5, IL-13, IFN-γ, and glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (*see* Table 1).
9. Real-time thermal cycler.

Table 1
Forward and reverse primers for the detection of Th2 cytokine and GAPDH reference gene expression using quantitative RT-PCR

Gene	Forward primer	Reverse primer
IL-4	5′-TTGAACGAGGTCACAGGAGA-3′	5′-AAATATGCGAAGCACCTTGG-3′
IL-5	5′-GCAATGAGACGATGAGGCTT-3′	5′-CATTTCCACAGTACCCCCAC-3′
IL-13	5′-TGCCAAGATCTGTGTCTCTCC-3′	5′-CCAGGTCCACACTCCATACC-3′
IFN-γ	5′-GCGTCATTGAATCACACCTG-3′	5′-GAGCTCATTGAATGCTTGGC-3′
GAPDH	5′-CGTCCCGTAGACAAAATGGT-3′	5′-TCAATGAAGGGGTCGTTGAT-3′

2.6 Lung Phenotyping by Flow Cytometry

1. 1.5-mL microcentrifuge tubes.
2. Surgical scissors.
3. Conical tubes: 15-mL and 50-mL tubes.
4. Liberase™ TL: 25 mg/mL. Add 200 μL of distilled water into 1 vial of 5 mg Liberase.
5. DNAse I: 10 mg/mL. Dissolve 100 mg DNAse I in 10 mL of distilled water. Aliquot in 1.5-mL microcentrifuge tubes and store at −20 °C.
6. Digestion medium: RPMI 1640 medium supplemented with 1× penicillin/streptomycin and 20 mM hydroxyethyl piperazineethanesulfonic acid (HEPES).
7. Orbital shaker.
8. Complete RPMI 1640 medium: Supplement with 5% fetal bovine serum (FBS), 1× penicillin/streptomycin, 2 mM L-Glutamine, 1× nonessential amino acid (NEAA), 1 mM sodium pyruvate, 55 μM β-mercaptoethanol, and 10 mM HEPES.
9. Sterile 40-μm cell strainers.
10. 100-mm Petri dishes.
11. Sterile plungers from 1-mL syringes.
12. 47% Percoll gradient solution: Mix 47 mL of 100% Percoll solution with 53 mL of complete RPMI 1640 medium.
13. Refrigerated centrifuge.
14. Microplate centrifuge.
15. 96-well V-bottom plate.
16. Lymphocyte stimulating reagents: 200 μg/mL phorbol 12-myristate 13-acetate (PMA), 250 μg/mL ionomycin, 1 mg/mL Brefeldin A, all prepared in dimethyl sulfoxide (DMSO). Store at −20 °C.
17. CO_2 incubator: set to 37 °C.
18. Fluorescence-conjugated anti-mouse antibodies: anti-CD4, anti-Foxp3, anti-IL-4, anti-IL-5, anti-IL-13, anti-IFN-γ, and cell viability dye.
19. Fixation and permeabilization buffer set for flow cytometry: commercially available.
20. 2% paraformaldehyde.
21. Fluorescence-activated cell sorting (FACS) buffer: 0.5% bovine serum albumin (BSA) prepared in PBS.
22. Multicolor flow cytometer.

2.7 Total and OVA-Specific IgE Measurement

1. 1-mL Syringe with a 25-gauge needle.
2. 1.5-mL Microcentrifuge tubes.
3. Refrigerated centrifuge.
4. ELISA kit for total IgE and OVA-specific IgE: commercially available.

3 Methods

3.1 Allergic Asthma Mouse Model

Mouse models of allergic asthma induced by experimental allergen OVA or clinically relevant allergen HDM are described here (*see* **Note 2**). In the OVA-induced asthma protocol as shown in Fig. 1, 8-week-old mice, housed under specific-pathogen-free conditions, are sensitized intraperitoneally with 50 μg OVA emulsified with adjuvant aluminum hydroxide on Days 0 and 12 and then challenged intranasally with 20 μg OVA on Days 24, 26, and 28 [9]. In the HDM-induced asthma protocol (Fig. 1), mice are exposed intranasally to 100 μg HDM extract once a week for total four doses of challenge [9]. Prepare all reagents and perform all procedures using sterile technique.

3.1.1 Sensitization and Challenge Protocol for OVA-Induced Asthma Model

1. Before sensitization, freshly prepare OVA emulsified with Imject™ Alum adjuvant. Thaw an aliquot of 1 mg/mL OVA solution prepared in PBS. Mix 250 μL of the OVA solution, 100 μL of Imject, and 650 μL of PBS in a 1.5-mL microcentrifuge tube, and emulsify the mixture on a rotator for 1 h at 4 °C.

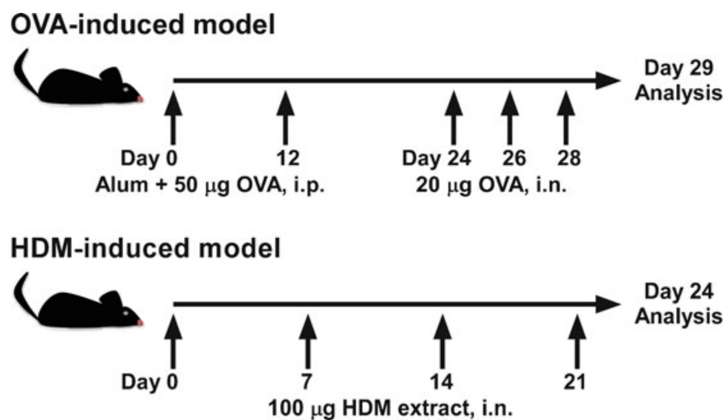

Fig. 1 Schematic diagram depicting the protocols for OVA- and HDM-induced allergic asthma models as described in Subheading 3. *i.p.* intraperitoneal, *i.n.* intranasal

2. Inject each mouse on Days 0 and 12 intraperitoneally with 200 μL of the OVA/Imject mixture using a 1-mL syringe with a 25-gauge needle. Each mouse will receive 50 μg OVA and 0.8 mg of aluminum hydroxide per dose. Inject control mice with 200 μL of PBS/Imject solution (*see* **Note 3**).
3. Challenge mice on Days 24, 26, and 28. Before challenge, thaw 1 tube of 1 mg/mL OVA solution and dilute with PBS to make 400 μg/mL OVA solution.
4. Transfer a previously sensitized mouse to the induction chamber for inhalational anesthesia by vaporizing isoflurane and oxygen gas mixture. Remove the mouse from the induction chamber once the respiratory rate slows down (*see* **Note 4**).
5. Holding the mouse with one hand, pipette 50 μL of 400 μg/mL OVA intranasally (*see* **Note 5**). Total of 20 μg OVA is given per challenge per mouse. Inject control mice intranasally with 50 μL of PBS.
6. Sacrifice the mouse for further assessment of asthma phenotype on Day 29, 1 day after the last challenge.

3.1.2 Challenge Protocol for HDM-Induced Asthma Model

1. Before challenge, thaw an aliquot of 2 mg/mL HDM extract solution.
2. Transfer a mouse to the anesthetic chamber with vaporizing isoflurane and oxygen gas mixture. Monitor respiratory rate for anesthetic depth. Proceed to the next step once respiration slows.
3. Pipette 50 μL of 2 mg/mL HDM extract solution (100 μg per dose per mouse) to the mouse intranasally as described in the OVA-induced protocol in Subheading 3.1.1. Inject control mice intranasally with 50 μL of PBS. Challenge mice on Days 0, 7, 14, and 21.
4. Sacrifice mice for further evaluation on Day 24, 3 days after the last challenge (*see* **Note 6**).

3.2 Pulmonary Function Test

Airway hypersensitivity is one of the characteristic features of asthma [11]. One day after the last challenge for the OVA-induced model, or 3 days after the last challenge for the HDM-induced model, the airway resistance is measured in intubated and ventilated mice by stimulating with increasing concentrations of methacholine (0, 3, 24, and 48 mg/mL in PBS) using a computer-controlled ventilator.

1. Anesthetize the mouse with the anesthetic reagent (xylazine and ketamine mixture) intraperitoneally.
2. Verify that the mouse shows no reaction to a toe pinch and reaches a surgical level of anesthesia.

3. Disinfect the throat area with 70% ethanol. Cut the skin and gently remove the submaxillary gland to expose the trachea. Then, perform a tracheotomy to insert a 20-gauge metal cannula inside the trachea and use a surgical stitch to fix the cannula in place.
4. Connect the mouse to the ventilator (*see* **Note 7**). Expose the mouse to nebulized PBS for baseline measurement, and subsequently to increasing concentrations (3, 24, and 48 mg/mL) of nebulized methacholine in PBS. Record the resistance index (RI) at each dosage for 3 min. The dynamic airway resistance can be determined by using Scireq software or equivalent.
5. Euthanize the intubated mouse by gradually filling the induction chamber with carbon dioxide (*see* **Note 8**). Mice euthanized after the pulmonary function test are used to collect BAL fluid as described in Subheading 3.3.

3.3 Examination of BAL Fluid Cells

Eosinophils are the key inflammatory cells involved in asthma pathophysiology [12]. In asthma mouse models, allergic airway inflammation can be assessed by counting the total number of cells and eosinophils in BAL fluid.

1. Mice euthanized after the pulmonary function test in Subheading 3.2 are used to collect BAL fluid.
2. Lavage the lung with 0.7 mL of PBS containing 0.1 mM EDTA by connecting a 1-mL syringe to the metal cannula intubated in Subheading 3.2. Then, transfer the BAL fluid to a 1.5-mL microcentrifuge tube on ice.
3. Centrifuge the cells in BAL fluid for 5 min at 800 × *g* at 4 °C. Collect the supernatants of BAL fluid in a new 1.5-mL microcentrifuge tube, and store at −80 °C for airway cytokine measurements. Determine Th2 cytokine levels in the BAL fluid by using commercial ELISA kits according to the manufacturer's instructions.
4. Resuspend BAL cells in 1 mL of PBS and count the absolute number of BAL cells using trypan blue dye exclusion under a microscope.
5. Centrifuge the BAL cells onto slides at 1000 rpm for 3 min in a Shandon Cytospin (*see* **Note 9**). Air-dry the slides before proceeding with staining.
6. Stain the BAL cells with Wright–Giemsa stain in a staining jar for 3 min, and then rinse with distilled water three times and air-dry (*see* **Note 10**).
7. Mount the slide with mounting medium and coverslip. Characterize and count the cell types, that is, eosinophils, neutrophils, lymphocytes, and macrophages based on staining morphology profiles (Fig. 2a) under a microscope at 1000× magnification for a total of 200 cells per slide.

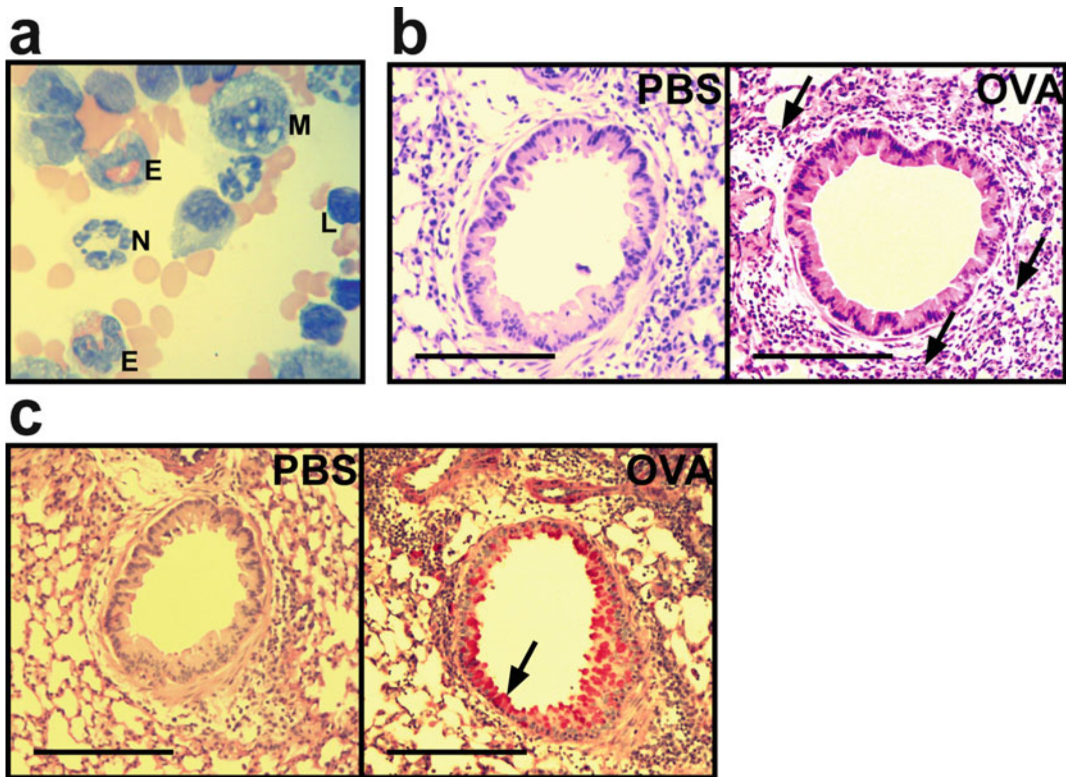

Fig. 2 (**a**) Wright–Giemsa staining of the BAL from sensitized mouse airway. *M* macrophages, *L* lymphocytes, *N* neutrophils, *E* eosinophils. Magnification, ×1000. (**b**) H&E-stained lung sections from unsensitized (PBS) and sensitized (OVA) mice are shown. Cells are largely infiltrated in the perivascular and peribronchial spaces in sensitized lung tissue. Scale bar, 200 μm. (**c**) PAS staining of lung sections from unsensitized (PBS) and sensitized (OVA) mice is indicated. The arrow indicates a PAS^{+} mucus-secreting cell in a bronchiole. Scale bar, 200 μm

3.4 Lung Histology

The lung inflammation can also be assessed by lung histology. Hematoxylin and eosin (H&E) staining is used to stain cell and tissue structures [13]. Periodic acid-Schiff (PAS) stain is performed to detect mucus hypersecretion by goblet cells [14].

1. Harvest the lung tissue, and immediately fix it in 10% formalin solution.
2. After fixation, dehydrate the lung tissues with the following steps to embed the lung tissue in paraffin wax for section:
   (a) 70% Ethanol: 2 × 1 h each.
   (b) 80% Ethanol: 1 × 1 h.
   (c) 95% Ethanol: 1 × 1 h.
   (d) 100% Ethanol: 3 × 1 h each.
   (e) Xylene: 3 × 1 h each.
   (f) Paraffin wax at 58 °C: 2 × 2 h each.
3. Embed tissues in paraffin blocks.

4. Cut the paraffin-embedded lung tissues at 10 μm by a microtome and transfer the sections onto slides.
5. Deparaffinize and rehydrate the lung sections with the following wash sequence:
   (a) Xylene: 3 × 3 min each.
   (b) 100% Ethanol: 3 × 3 min each.
   (c) 95% Ethanol: 1 × 3 min.
   (d) 70% Ethanol: 1 × 3 min.
   (e) Wash slides in distilled water.
6. Stain lung paraffin sections with hematoxylin and eosin stain according to the manufacturer's instructions for examining lung pathology due to airway allergic reaction. Eosinophilic inflammation can be detected in the perivascular and peribronchial spaces in sensitized lung tissue (Fig. 2b).
7. Perform periodic acid-Schiff (PAS) stain following the manufacturer's instructions to detect goblet cell hyperplasia (Fig. 2c). Quantitate the level of goblet cell hyperplasia in the airway by counting PAS^+ epithelial cells in individual bronchioles. Assess at least 10 bronchioles.

3.5 Th2 Cytokine Determination in the Sensitized Lung

Th2 cells produce cytokines IL-4, IL-5, and IL-13, which have been shown to drive disease pathology in patients with asthma [15]. For detecting cytokine levels in sensitized lungs, Th2 cytokines can be detected in BAL fluid from sensitized airway by commercial ELISA kits (*see* Subheading 3.3, **steps 2** and **3** for collection of BAL fluid). Alternatively, the gene expression level of Th2 cytokines can be determined by quantitative RT-PCR of lung tissue or by analyzing cytokine-producing T cells via flow cytometry. Both approaches are described here.

3.5.1 RNA Isolation and Quantitative RT-PCR of Lung Tissue

1. Harvest one lobe of lung (upper right) and store in 1 mL of TRIzol reagent at −80 °C freezer before RNA extraction.
2. Thaw the lung tissue sample at room temperature and homogenize the tissue in TRIzol reagent using a homogenizer (*see* **Note 11**).
3. Extract total lung RNA by using TRIzol reagent following the manufacturer's instructions. Determine the RNA concentration by a NanoDrop or spectrophotometer.
4. Generate cDNAs according to the instruction provided by the kit's manufacturer.
5. Perform quantitative real-time PCR using SYBR Green PCR kits. The thermal cycling conditions used for all genes are 95 °C for 10 min, and 40 repeats at 95 °C for 15 s and 60 °C for 1 min. Calculate the relative expression of each cytokine gene by normalizing with housekeeping gene GAPDH expression.

3.5.2 Ex Vivo Lung Phenotyping by Flow Cytometry

1. Dissect remaining lobes of the lung, wash in plain RPMI 1640, and transfer to 1.5-mL microcentrifuge tubes (*see* **Note 12**).
2. Chop the lung tissues into small pieces by small surgical scissors.
3. Enzymatically digest the lung tissue in 10 mL of the digestion medium containing Liberase™ (0.16 U/mL, Liberase TL) and 0.05% DNAse I in a 50-mL conical tube for 30 min at 37 °C on an orbital shaker at 190 rpm (*see* **Note 13**).
4. Inactivate the enzyme reaction by adding 10 mL of cold complete RPMI 1640 medium.
5. Pass the digested tissue mixture through a 40-μm strainer on a 100-mm Petri dish. Mash up the tissue on the strainer with the plunger flange of a 1-mL syringe. Collect the filtered cell suspension in a 50-mL conical tube.
6. Add additional 10 mL of cold complete RPMI 1640 medium to the 40-μm strainer and repeat tissue mashing.
7. Collect the filtered cell suspension into the same collection tube.

   Repeat **steps 6** and 7 one more time for a total volume of 40 mL.
8. Centrifuge the cell suspension for 5 min at 400 × *g* at 4 °C and discard the supernatant.
9. Resuspend the cell pellet with 10 mL of 47% Percoll gradient solution, transfer to a 15-mL conical tube, and centrifuge for 10 min at 400 × *g* at 4 °C to enrich lymphocytes.
10. Wash cell pellet with 10 mL of complete RPMI 1640 medium and count the cell number.
11. Centrifuge the cell suspension, discard the supernatant, and adjust the cell concentration to 1×10^6 cells/mL with complete RPMI 1640 medium.
12. To detect cytokine production, place 200 μL of the lung suspension from **step 12** into a 96-well V-bottom plate (2×10^5 cells per well).
13. Centrifuge the plate, discard the supernatant, and resuspend the cells with 200 μL of complete RPMI 1640 medium containing a working concentration of each of the lymphocyte stimulating reagents (50 ng/mL PMA, 0.5 μg/mL ionomycin, and 1 μg/mL Brefeldin A) to stimulate cytokine production. Incubate for 4 h at 37 °C in a CO_2 incubator.
14. Centrifuge the plate, discard the supernatant, and then wash the cells with 200 μL of FACS buffer twice.
15. Resuspend the cells with 50 μL FACS buffer containing Fixable Viability Dye and a fluorescence-conjugated anti-mouse CD4 antibody for CD4 T-cell staining. Incubate the cells at 4 °C for 30 min in the dark.

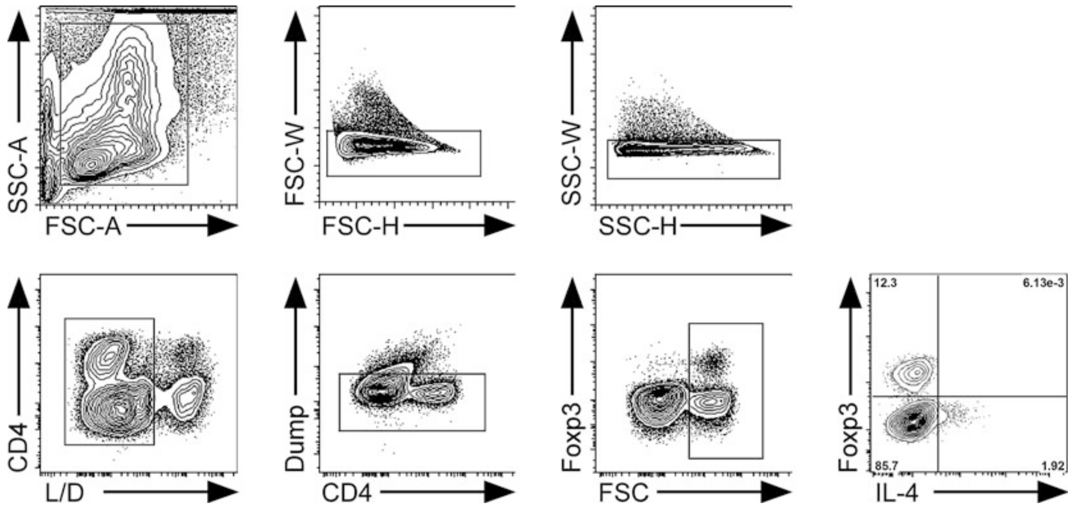

Fig. 3 Flow cytometry analysis gating strategy to identify cytokine-secreting T effector cells. Gating sequence: upper panel, from left to right; and then bottom panel, from left to right

16. To the existing FACS buffer, add 150 μL of FACS buffer to each well, centrifuge the plate, and discard the supernatant. Wash the cells with 200 μL of FACS buffer.
17. Fix the cells with 200 μL of fixation and permeabilization buffer and incubate at 4 °C for 30 min or overnight, according to the manufacturer's instructions.
18. Add 150 μL of the permeabilization buffer to each well, centrifuge the plate, and discard the supernatant. Wash the cells with 200 μL permeabilization buffer.
19. Resuspend the cells with 50-μL permeabilization buffer containing anti-mouse Foxp3, IL-4, IL-5, IL-13, and IFN-γ antibodies for intracellular staining for 45 min at room temperature in the dark.
20. Add 150-μL permeabilization buffer to each well, centrifuge the plate and discard the supernatant. Resuspend the cells with 200 μL of 2% paraformaldehyde.
21. Run the stained samples on a flow cytometer, acquiring 2×10^5 cell events to analyze data. Figure 3 shows the gating strategy to identify cytokine-positive T effector cells. Determine the relative frequencies for cytokine-positive T effector cells.

3.6 Total and OVA-Specific IgE Measurement

IgE plays a central role in the pathogenesis of allergic asthma. Elevated serum IgE level is one of the main features of human asthma [16]. Total and OVA-specific IgE levels can be determined by enzyme-linked immunosorbent assay (ELISA).

1. Withdraw cardiac blood with a 1-mL syringe with a 25-gauge needle and transfer the blood to 1.5-mL microcentrifuge tubes (*see* **Note 8**). Allow the blood to coagulate at room temperature for 30 min.
2. Centrifuge the peripheral blood samples for 5 min at 800 × *g* at 4 °C.
3. Transfer the serum samples to new 1.5-mL microcentrifuge tubes. Approximately 200 μL serum can be collected from one mouse. Serum samples can be immediately used in the next step or can be kept in a −80 °C freezer for long-term storage. If frozen, ensure that serum samples are thawed on ice prior to use.
4. Make a two- to fivefold dilution of serum samples using sample dilution buffer provided by the selected ELISA kit. Determine total and antigen-specific IgE in serum by commercial ELISA kits according to the manufacturer's instructions.

4 Notes

1. Many commercially available recombinant OVAs have some levels of endotoxin (lipopolysaccharide, LPS) contamination that will largely affect the readouts of asthma responses. We, therefore, recommend that OVA used in asthma models will be carefully screened for endotoxin contamination by an endotoxin quantitation kit.
2. Protocols described here are for the C57BL/6J mouse strain. Protocols used in other mouse strains need to be adjusted accordingly. We have adjusted the asthma protocol in the BALB/c strain by sensitizing mice on Days 1, 2, 3, 14 and challenging the mice on Days 14, 17, 20, 23, and 26 [17]. All mice should be maintained and handled in accordance with the Institutional Animal Care and Use Guidelines and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.
3. Gently invert the tube with OVA/Imject solution between injections to make sure that OVA and the alum adjuvant are evenly distributed in the solution and that all mice are sensitized with the equal amount of OVA/Imject solution.
4. Carefully monitor the anesthetic depth either by a toe pinch or by observing the respiratory rate via movement of the chest wall. Do not wait until the mouse starts hiccoughing, as this will cause the solution to flow out during intranasal administration.

5. Inject the antigen immediately once the respiratory rate slows down. Administer approximately half of the amount in each nostril. Hold the mouse still for a few seconds to allow the mouse to inhale the antigen solution into the lung.
6. To mimic natural asthma induction, mice are challenged directly with HDM extract without sensitization in the HDM-induced asthma model. We observe the highest pulmonary inflammation at 3 days following the last challenge.
7. Align the animal to the ventilator at the same level to avoid a possible tracheal twist. Conscious active breathing by the mouse will largely distort the lung function measurement by the intubated ventilation method. Thus, double check that the respiratory system of the mouse remains passive under deep anesthesia to obtain valid resistance value during measurements.
8. If you want to collect blood to save serum samples, collect the blood right after mouse is euthanized by carbon dioxide to avoid blood clotting. Pull back the syringe plunger slowly to prevent collapsing, which can stop blood flow into the syringe.
9. Alternatively, BAL cell slides can be prepared without using a Cytospin. Concentrate the BAL cell suspension by centrifugation at 800 × *g* at 4 °C for 5 min, resuspend the BAL cell pellet with 0.1 mL of PBS, then spread the concentrated cell suspension onto a slide evenly by using another clean slide. Allow the slide to air-dry before proceeding with the staining protocol.
10. Wright–Giemsa stain can be reused within 1 month. Adjust the staining duration accordingly, for example, prolong staining time for 10 more seconds when Wright–Giemsa stain has been reused after more than 1 week.
11. If a homogenizer is not available, tissue homogenization can also be performed by passing the tissue in TRIzol reagent through 1-mL Luer Lock syringe with a 20-gauge needle up to 10 times.
12. To obtain actual lung tissue-infiltrating lymphocytes, perform whole-body perfusion with PBS before harvesting the lung tissues. Take 10 mL of PBS by a 10-mL syringe with a 25-gauge needle, and then slowly infuse all of the PBS into the right ventricle of the heart, and the lungs should turn white in color.
13. Collagenase is cheaper and can be used as an alternative to digest lung tissue, but Liberase digestion will have better cell viability and recovery rate.

Acknowledgments

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Chapter 9

Generation and Characterization of Inducible Lung and Skin-Specific IL-22 Transgenic Mice

Li Zhou, Tao Zheng, and Zhou Zhu

Abstract

IL-22 is an IL-10 family cytokine that is increased in asthma and atopic dermatitis (AD). However, the specific role of IL-22 in the pathogenesis of allergic lung inflammation and AD in vivo has yet to be elucidated. We aimed to develop mouse models of allergic diseases in the lung and skin with inducible and tissue-specific expression of IL-22, using a tetracycline (Tet)-controlled system. In this chapter, we describe a series of protocols we have developed to generate a construct that contains the TRE-Tight promoter and mouse IL-22 cDNA based on this system. Furthermore, we describe how to generate TRE-Tight-IL-22 mice through pronuclear microinjection. In our approach, two Tet-on (CC10-rtTA or SPC-rtTA) and a Tet-off (K5-tTA) transgenic mouse lines are selected to crossbreed with TRE-Tight-IL-22 mice to generate inducible tissue-specific transgenic lines. The transgenic strains, CC10-rtTA/TRE-Tight-IL-22 (CC10-rtTA-IL-22) or SPC-rtTA/TRE-Tight-IL-22 (SPC-rtTA-IL-22) mice, do not produce detectable levels of IL-22 in their bronchoalveolar lavage (BAL) samples in the absence of doxycycline (Dox). However, oral Dox treatment of these mice induces IL-22 expression in the BAL, and the airway and lung epithelial cells. For K5-tTA/TRE-Tight-IL-22 (K5-tTA–IL-22) mice, to avoid potential IL-22 toxicity to mouse embryos, Dox is given starting at the time of breeding to suppress tTA and to keep the IL-22 transgene off until the K5-tTA–IL-22 mice are 6 weeks old. Experiments are then initiated by withdrawing Dox from the drinking water. In all cases, IL-22 protein can be detected by immunohistochemistry in the skin of Tg(+) animals, but not in the skin of Tg(−) animals. Utilizing transgenic technology based on the Tetracycline-controlled system, we have established inducible transgenic mouse models in which cytokine IL-22 can be expressed specifically in the lung or skin. These models are valuable for studies in vivo in a broad range of diseases involving IL-22 and will provide a new platform for research and for seeking novel therapeutics in the fields of inflammation, asthma, and allergic dermatitis.

Key words IL-22, Allergic disease, Asthma, Atopic dermatitis, Tetracycline-inducible system, Tissue-specific

1 Introduction

IL-22, a member of the IL-10 family cytokines, plays an important role in innate and adaptive immunity. In the gastrointestinal tract, innate lymphoid cells (ILCs) are a dominant source of IL-22 [1–4]. Other cells, including CD4+ Th1, Th17, Th22 cells, CD8+ Tc17, Tc22 cells, and γδ T cells and NK cells can also produce IL-22 [5–9].

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In clinical studies, IL-22 expression has been found to be highly elevated in the allergic dermatitis (AD) skin [10] and in the blood of asthmatic patients, which correlates with disease severity [11]. Accumulating evidence indicates that IL-22 may have immune-modulatory effects on the development of allergen-induced pulmonary inflammation [12–14]. Also, robust progressive activation of Th2 and Th22 characterizes the nature of dysregulated immunity in both acute and chronic AD [3, 10, 15–20], and alterations in Th2 and Th22 cytokines correlate positively with AD disease severity [21]. As the roles of IL-22 in the development and maintenance of AD and allergic asthma have not been well explored, it is therefore desirable to develop inducible tissue-specific IL-22 overexpression animal models for detailed studies of this cytokine.

The Tetracycline-controlled (Tet) system has several inducible expression vectors for the expression of gene of interest in mammalian cells and has been developed and applied to a number of transgenic rodents [22]. In this system, tissue-specific expression of a Tet-activator (tTA) or reverse-Tet-activator (rtTA) fusion protein controlled by a specific promoter [23–25], in combination with the administration of Dox, which represses or activates gene expression, respectively, downstream of a tet operator (TetO) CMV promoter element [26]. Withdrawal of doxycycline (Dox) reverses the effects. To date, it is the most widely used inducible system, with over 500 Tet rodent lines created (*see*: https://www.tetsystems.com/support/transgenic-rodent-lines).

In this chapter, we describe how to use this Tet system and generate three new mouse strains that are engineered to express IL-22 selectively in mice under the control of the CC10 and SPC promoter (rtTA) in airway and lung epithelial cells, and K5 promoter (tTA) in the skin. We also describe the methods to confirm IL-22 expression in the Tg(+) mice using immunohistochemistry and show examples of characterization of IL-22 expression at mRNA and protein levels.

2 Materials

2.1 Plasmids Preparation

1. Transfection-grade plasmid DNA isolation kit (e.g., QIAGEN EndoFree Plasmid Mini Kit or Qiagen HiSpeed Plasmid Midi Kit).
2. LB medium with 50 μg/mL ampicillin: Make 1 L of LB medium according to supplier's instruction and sterilized by autoclaving. Allow to cool and add 0.5 mL of 100 mg/mL ampicillin before use.
3. Competent cells: *E. coli* Top 10.

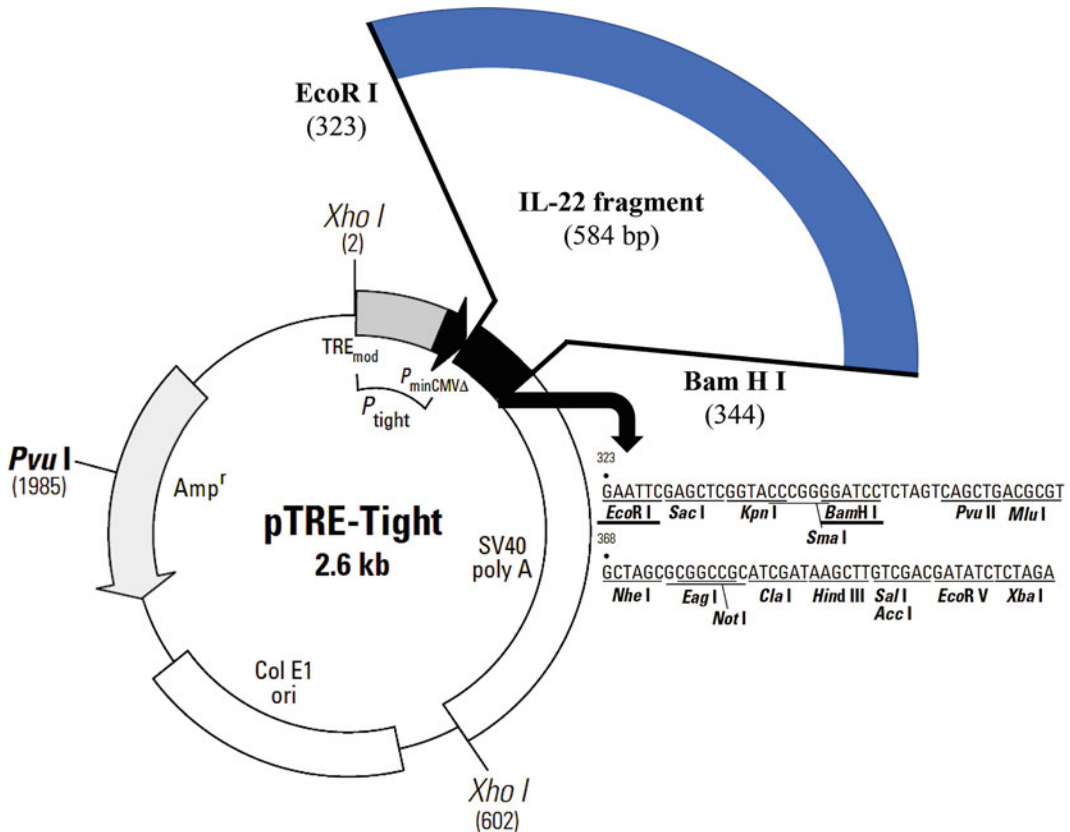

Fig. 1 Schematic DNA construct of the TRE-Tight-IL-22 transgene. IL-22 DNA is inserted into the multiple cloning site (MCS) of pTRE-Tight vector (PT3720-5, Clontech) using restriction enzymes for microinjecting fertilized eggs as described in Subheading 3.4.2 [29]

4. Tris-EDTA (TE) buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Dissolve 15.8 g of Tris-HCl in 800 mL of ultrapure water. Add 2.9 g of EDTA and adjust the pH to 8.0. Bring the volume up to 1 L and store at room temperature. Also, commercially available.
5. pTRE-Tight plasmid vector (*see* **Note 1** and Fig. 1): Dissolve purified plasmid DNA in TE buffer.
6. pTet-on plasmid vector (*see* **Note 2**): Dissolve purified plasmid DNA in TE buffer.
7. Phenol: saturated with TE buffer.
8. Ethanol.
9. Isopropanol.
10. 3 M Sodium acetate (NaOAc) buffer: pH 5.2.
11. Shaking incubator.
12. Spectrophotometer.
13. Refrigerated centrifuge.

2.2 Generation of a TRE-Tight-IL-22 Construct

1. 1.5-mL Microfuge tubes.
2. Pipettors and tips.
3. PCR tubes.
4. Donor vector pENT_DTOPO_mIL-22 plasmid: The vector contains mIL-22 cDNA sequence, which is used as the PCR template.
5. Taq DNA polymerase system: Platinum™ Taq DNA Polymerase Kit (Invitrogen) or equivalent.
6. 25 mM dNTPs: commercially available.
7. Primers: *See* Table 1 for the list of required primers.
8. Thermal cycler.
9. pTRE-Tight plasmid vector (*see* **Note 1** and Fig. 1).
10. Restriction enzymes: *Eco*RI, *BamH*I, and *Xho*I.
11. Agarose.
12. Tris-acetate-EDTA (TAE) buffer: 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.5. Dissolve 4.84 g Tris-base, 1.14 mL acetic acid, and 0.15 g EDTA in 800 mL of water. Adjust the pH to 8.5 and bring the volume up to 1 L. Also, 50× TAE buffer is commercially available.
13. Low-melting agarose gel.
14. Electrophoresis apparatus.
15. 10 mg/mL Ethidium bromide solution.
16. Ultraviolet transilluminator.
17. QIAquick Gel Extraction Kit (Qiagen) or equivalent.
18. T4 ligase.
19. Competent cells: *E. coli* Top 10.
20. Transfection-grade plasmid DNA isolation kit (e.g., QIAGEN EndoFree Plasmid Mini Kit or Qiagen HiSpeed Plasmid Midi Kit).
21. Ethanol.
22. Isopropanol.
23. Spectrophotometer.
24. Refrigerated centrifuge.
25. Shaking incubator: set at 37 °C.
26. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. *See* Subheading 2.1, **item 4**.
27. Agar plates with LB medium with 50 μg/mL ampicillin: Dissolve 15 g of Bacto agar in 1 L of LB medium and sterilize by autoclaving. Cool to 50 °C in a temperature-controlled water bath. Add 0.5 mL of 100 mg/mL ampicillin. Pour into plates.

Table 1
Primers

Primer	Orientation	Sequences
mIL-22	Sense	5′-GCGAATTCCCCCTTCACCGC-3′
	Antisense	5′-CGCGGATCC TTCCAGTTTAAT-3′
TRE-Tight-IL-22	Sense	5′-TCCCTATCAGTGATAGAGAACGA-3′
	Antisense	5′-CGCGGATCCTTCCAGTTTAAT-3′
SPC-rtTA	Sense	5′-GAC ACATATAAGACCCTGGTC A-3′
	Antisense	5′-AAAATCTTGCCAGCTTTCCCC-3′
CC10-rtTA	Sense	5′-TGCCCAAACACCCCACAAGT-3′
	Antisense	5′-CCTATCTAACATCTCAATGGCTAAGGC-3′
K5-tTA	Sense	5′-AGGGCATCGGTAAACATCTG-3′
	Antisense	5′-GATGTCGTCATAGAGGCTGTTGG-3′

2.3 Induction of IL-22 Expression In Vitro

1. A549 cells: human lung carcinoma cell line. Commercially available.
2. Culture medium: Dulbecco's Modified Eagle's Medium, 10% heat-inactivated fetal bovine serum (v/v), Opti-MEM® I Medium, and 1× antibiotics penicillin/streptomycin.
3. 0.25% Trypsin solution: commercially available.
4. Cell culture dishes: 6-well plates and T25 flasks.
5. Lipofectamine 2000.
6. Water bath: set at 37 °C.
7. Centrifuge: with a swinging bucket rotor.
8. CO_2 incubator: humidified with 5% CO_2, 37 °C.
9. Doxycycline (Dox): Prepare 0, 0.01, 0.1, or 1 mg/L solutions in the culture medium.
10. ELISA kit for mouse IL-22: commercially available ELISA kit and reagents.

2.4 Generation of TRE-Tight-IL-22 Mice

1. 1.5-mL Microfuge tubes.
2. Pipettors and tips.
3. PCR tubes.
4. Linearized and purified TRE-Tight-IL-22 DNA fragment (*see* Subheading 3.2).
5. Microinjection buffer: 8 mM Tris-HCl, 0.15 mM EDTA, pH 7.5. Commercially available.
6. Inverted microscope combined with the microinjector system.

7. Wild-type, 8-week-old, female mice, C57BL/6 background: used either for obtaining superovulated zygotes or for pseudo-pregnant mice (*see* **Note 3**).
8. Ear punch tool.
9. Equine chorionic gonadotropin (eCG).
10. Human chorionic gonadotropin (hCG).
11. M2 medium.
12. Hyaluronidase.
13. Human Tubal Fluid (HTF) medium.
14. Liquid paraffin.
15. Petri dishes.
16. Micromanipulator system.
17. CC10-rtTA (Stock No: 006232) or SPC-rtTA transgenic mice (stock no: 006235) are maintained on the C57BL/6 background and available from Jackson Laboratory. These mice are used to cross with TRE-Tight-IL-22 transgenic mice.
18. Keratin 5 (K5)–tetracycline transcriptional activator (tTA) mouse line: provided by Dr. Adam Glick [27]. These mice are maintained on C57BL/6 background and used to cross with TRE-Tight-IL-22 transgenic mice.
19. Dox-containing drinking water: 0.5 mg/mL Dox, 4% (w/v) sucrose in ultrapure drinking water.
20. Allele-In-One Mouse Tail Direct Lysis Buffer: commercially available.
21. Heating block: set at 37 °C.
22. Primers: *See* Table 1 for the list of required primers.
23. Taq DNA polymerase system: commercially available.
24. 25 mM dNTPs. commercially available.
25. Agarose.
26. TAE buffer: *See* Subheading 2.2, **item 12**.
27. 10 mg/mL Ethidium bromide solution.
28. Thermal cycler.
29. Refrigerated centrifuge.

2.5 Histology and Immunohistochemistry

1. Euthanasia chamber with CO_2.
2. Dissection tools.
3. 10% Neutral buffered formalin.
4. Paraffin wax.
5. Ethanol solutions: 100%, 95%, 70%, and 50%.
6. Xylene.

7. Glass slides.
8. Microtome.
9. Hematoxylin and eosin stain kit: commercially available.
10. Alcian blue staining kit: commercially available.
11. 1% Hydrogen peroxide in methanol.
12. Microwave oven.
13. Citrate buffer: 10 mM sodium citrate, 0.05% Tween 20, pH 6.0.
14. Phosphate buffered saline (PBS): pH 7.4. Mix 8 g NaCl, 1.44 g Na_2HPO_4, 0.2 g KCl, 0.24 g KH_2PO_4 in 800 mL of water. Adjust the pH to 7.4 and bring the volume to 1 L.
15. Blocking solution: 2% goat serum in PBS.
16. Rat anti-mouse major basic protein (MBP) monoclonal antibody: for staining eosinophils (*see* **Note 4**).
17. Anti-mouse IL-22 antibody (*see* **Note 5**).
18. Avidin–biotin complex (ABC) staining systems: commercially available as kits that include a choice of secondary antibody and diaminobenzidine (DAB) as a chromogen.
19. Mounting medium.
20. Coverslips.

3 Methods

3.1 Plasmid Preparation

In this protocol, *E. coli* Top 10 competent cells are first transformed with the pTet-on or pTRE-Tight plasmids and amplified. The plasmids are then and purified using the Qiagen HiSpeed Plasmid Midi Kit according to the manufacturer's protocol with some modifications.

1. Transform the pTet-on plasmid or pTRE-Tight plasmid to *E. coli* Top 10 competent cells according to the supplier's instruction.
2. Spread the transformed cells onto an LB plate with 50 μg/mL ampicillin and culture at 37 °C overnight.
3. Select and grow *E. coli* Top 10 colonies containing the pTet-on or pTRE-Tight plasmid overnight in 100 mL of LB broth with 50 μg/mL ampicillin at 37 °C in a shaking incubator.
4. Centrifuge the broth for 15 min at 3500 × *g*. Discard the cell culture supernatant.
5. Resuspend the pellet in 6 mL of ice-cold Buffer P1 from the Qiagen HiSpeed Plasmid Midi Kit containing 50 μg/mL RNase A and vortex.

6. Add 6 mL of Buffer P2 and mix by inverting 5 times. Incubate for 5 min at room temperature.
7. Add 6 mL of ice-cold Buffer P3 and mix it by inverting 5 times.
8. Pour the lysate into the QIAfilter Cartridge and incubator for 10 min at room temperature. Filter the lysate onto the pre-equilibrated HiSpeed Tip.
9. Wash the HiSpeed Tip with 20 mL of Buffer QC followed by elution of the DNA with 5 mL of Buffer QF.
10. Add 3.5 mL of isopropanol, mix, and incubate for 5 min at room temperature.
11. Filter the mixture through the QIAprecipitator and wash the DNA by filtering with 2 mL of ethanol. Dry the membrane of the QIAprecipitator by passing air through it.
12. Elute the DNA into the collection tube by adding TE buffer.
13. Add an equal amount of TE-saturated phenol, vortex, and centrifuge at $12{,}000 \times g$ for 5 min.
14. Transfer the aqueous phase into a new tube and add 0.1 volume of 3 M NaOAc (pH 5.2) and 2.5 volume of ethanol.
15. Precipitate the DNA by centrifugation at $12{,}000 \times g$ for 10 min at room temperature and wash the pellet once with 70% ethanol.
16. Air-dry the pellet for 10 min and resuspend it in 50–100 μL of modified TE. Quantify the plasmids' concentration using a spectrophotometer.
17. pTet-on and pTRE-Tight plasmids are stored at −20 °C until use.

3.2 Construction of a TRE-Tight-IL-22 Construct

1. Amplify mouse IL-22 cDNA fragment via PCR from a pENT_DTOPO_mIL-22 plasmid containing mouse IL-22 using primers: 5′-GC<u>GAATTC</u> CCCCTTCACCGC-3′ and 5′-CGC<u>GGATCC</u> TTCCAGTTTAAT-3′ with *Eco*RI and *Bam*HI sites, respectively (Table 1).
2. Digest the amplified mouse IL-22 cDNA fragment and pTRE-Tight vector at the *Eco*RI and *Bam*HI restriction sites (Fig. 1).
3. Separate the restriction fragments by electrophoresis through a low-melting agarose gel.
4. Using an ultraviolet transilluminator, identify and isolate the proper bands containing the restriction fragments (*see* **Note 6**).
5. Extract the restriction fragments according to the manufacturer's protocol for the QIAquick Gel Extraction Kit.
6. Elute the DNA from the QIAquick column using TE buffer and measure the DNA concentration by spectrophotometer. Store at −20 °C until use.

7. Mix the IL-22 DNA fragment and pTRE-Tight fragment mentioned above and ligate using T4 ligase at 16 °C overnight.
8. Transform the ligation mixture into *E. coli* Top 10 competent cells according to the supplier's instruction.
9. Spread onto an LB plate containing 50 μg/mL ampicillin and culture at 37 °C overnight.
10. Pick colonies and extract plasmids using the Qiagen HiSpeed Plasmid Midi Kit according to the manufacturers' protocol with some modifications as described in Subheading 3.1.
11. Confirm that the plasmids contain the correct construct by performing PCR, restriction digestion analysis, and sequencing.
12. Excise the DNA fragment containing the TRE-Tight promoter, IL-22 cDNA, and the SV40 polyadenylation signal sequence (TRE-Tight-IL-22 fragment) with *Xho*I.
13. Identify the 1152 bp length of TRE-Tight-IL-22 fragment on a low-melting agarose gel electrophoresis and extract the DNA fragment as described in Subheading 3.2, **step 3**–**6**.
14. Elute the DNA with microinjection buffer and determine the DNA concentration using a spectrophotometer and adjust to a working concentration of 0.1 g/L (*see* **Note 7**).
15. Dispense the TRE-Tight-IL-22 fragment to new tubes and store at −80 °C until use. This linearized fragment is used to generate transgenic TRE-Tight-IL-22 mice in Subheading 3.4.

3.3 Induction of IL-22 Expression In Vitro

1. For transfection, seed 4 × 10^5 A549 cells in 2 mL of DMEM with 10% FBS in a 6-well plate. Culture in a CO_2 incubator at 37 °C to achieve 80–90% confluence on the day of transfection.
2. At the time of transfection, mix pTet-on vector and TRE-Tight-IL-22 fragment DNA, 0.5 μg each in 0.15 mL Opti-MEM® I medium, with 10 μL Lipofectamine 2000 in 0.15 mL Opti-MEM® I medium. Diluted DNA to diluted Lipofectamine 2000 should be at 1:1 ratio and mix to form DNA–lipid complex. Incubate for 5 min at room temperature.
3. Add the DNA–lipid complex to A549 cells.
4. Change the medium at 6 h post-transfection with fresh 10% FBS DMEM containing Dox at the concentrations, 0, 0.01, 0.1, or 1 mg/L.
5. At 24 h post-transfection, collect the cell culture supernatant and store at −80 °C until used for IL-22 protein detection by ELISA according to the manufacturer's instruction (Fig. 2).

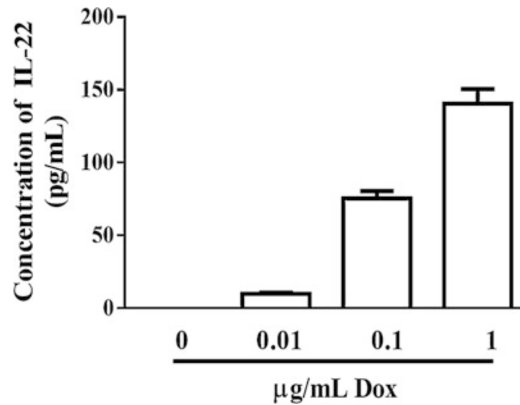

Fig. 2 Expression of IL-22 induced by Dox in vitro. A549 cells were transfected with pTet-on vector and TRE-Tight-IL-22 fragment DNA and cultured with DMEM containing doxycycline (Dox) at indicated concentration (0, 0.01, 0.1, 1 μg/mL with 4% sucrose). The IL-22 transgene was activated by Dox, and the expression of IL-22 in the cell culture supernatant was determined by an IL-22 ELISA kit. Without Dox treatment, no IL-22 was detected in the cell culture supernatant. Reproduced and modified with permission [32]

3.4 Generation of Transgenic Mice

In this section, transgenic mice on C57BL/6 genetic background carrying the transgene TRE-Tight-IL-22 are first generated. The transgenic TRE-Tight-IL-22 mice are identified by genotyping and crossed with SPC-rtTA or CC10-rtTA transgenic mice and keratin 5 (K5)–tetracycline transcriptional activator (tTA) transgenic mice to generate double transgenic mice, SPC-rtTA-IL-22 or CC10-rtTA-IL-22 mice and K5-tTA–IL-22 mice, respectively.

3.4.1 Preparation of Mice and Zygotes

1. Intraperitoneally inject 5 IU of eCG into C57BL/6 wild-type (WT) female mice.
2. Intraperitoneally inject 5 IU of hCG about 48 h later and mate these females with C57BL/6 WT male seed mice.
3. Identify the females with vaginal plugs on the following morning.
4. Sacrifice the plugged females. Remove the oviducts into Petri dishes containing M2 medium.
5. Transfer the oviduct to M2 medium containing 300 μg/mL hyaluronidase and introduce the zygotes–cumulus cell complex into the medium by teasing the ampulla of the oviduct with a 26-gauge needle. Separate the oocytes from cumulus cells by pipetting.
6. Wash the zygotes twice with M2 medium.
7. Incubate the zygotes in M2 medium in a 5% CO_2 incubator until use (*see* **Note 8**).

3.4.2 Generation of TRE-Tight-IL-22 Mice

Pronuclear injection is typically performed in an institutional transgenic mouse core facility.

1. Dilute the TRE-Tight-IL-22 DNA fragments from Subheading 3.2 to a final concentration of 0.1 g/L in a microinjection buffer.
2. Microinject the linearized TRE-Tight-IL-22 DNA fragments into the pronuclei of zygotes by the micromanipulator system.
3. Two-cell embryos developing 1 day after microinjection are transferred to oviducts of pseudopregnant females that have been mated with vasectomized males. Allow these females to deliver their pups.
4. Offspring born from the injected zygotes are termed "founders" and are usually screened for the presence of the transgene.
5. Collect tail biopsies from offspring to new microtubes. Tail tips <0.5 cm may be biopsied from pups ≤21 days old without the use of an analgesic.
6. Add 200 μL of Allele-In-One Mouse Tail Direct Lysis Buffer to these microtubes and incubate them in a 55 °C heating block overnight. Each sample can be directly subjected to PCR as the template (1–2 μL per reaction).
7. Perform PCR genotyping to identify TRE-Tight-IL-22 transgenic animals using the following primer pairs: 5′-TCCCTAT CAGTGATAGAGAACGA-3′ and 5′-CGCGGATCCTTCCA GTTTAAT-3′. Use the following conditions for all PCR reactions: 10 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 5 min at 72 °C.
8. Run the PCR reaction on an agarose gel for electrophoresis. PCR products of 857 bp indicate transgenic mice.

3.4.3 Generation of Lung-Specific Inducible IL-22 Transgenic Mice

1. To obtain mice that can express IL-22 specifically and inducibly in the lung, cross TRE-Tight-IL-22 mice with SPC-rtTA or CC10-rtTA transgenic mice to produce double transgenic SPC-rtTA-IL-22 or CC10-rtTA-IL-22 mice (Fig. 3).
2. Determine the genotypes of the mice by PCR using specific primers for TRE-Tight-IL-22 as described in Subheading 3.4.2. Use 5′-GACACATATAAGACCCTGGTC A-3′ and 5′-AAAATCTTGCCAGCTTTCCCC-3′ for SPC-rtTA, or 5′-T GCCCAAACACCCCACA AGT-3′ and 5′-CCTATCTAA CATCTCAATGGCTAAGGC-3′ for CC10-rtTA (Table 1).
3. The breeding also produces single transgenic mice (CC10-rtTA, SPC-rtTA, or TRE-Tight-IL-22). Use these mice for further breeding. Use transgenic negative [Tg(−)] littermates in the experiments as negative controls.

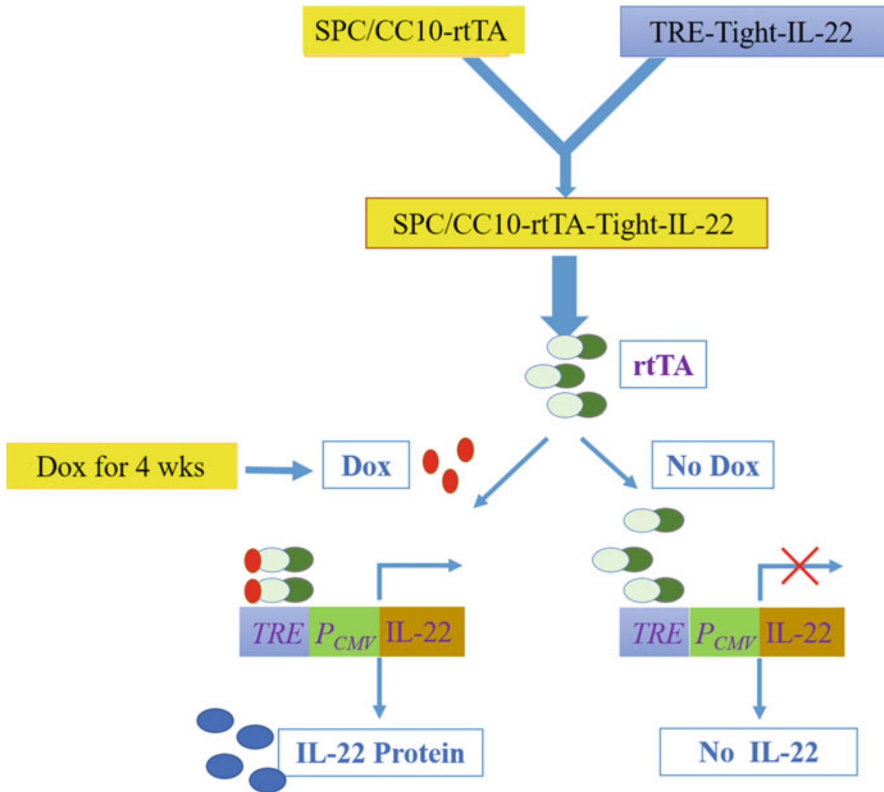

Fig. 3 Generation of SPC- or CC10-rtTA-TRE-Tight-IL-22 (SPC- or CC10-IL-22) mice. SPC-rtTA or CC10-rtTA mice are crossbred with TRE-Tight-IL-22 mice to obtain SPC- or CC10-IL-22 double-positive mice. The IL-22 transgene is activated by doxycycline (Dox) in the drinking water for 4 weeks. ELISA and IHC may be performed to identify the expression of IL-22 in the BAL fluid and lung tissues, respectively. Without Dox, no IL-22 should be detected in the BAL or the lung. Reproduced from Fang et al. PLoS One 2014; 9 (9):e107454 [30]

4. Activate the IL-22 transgene when the mice are 4 weeks old by giving mice the Dox-containing drinking water [28]. For all experiments, randomly assign Tg(+) and WT littermates to receive normal or Dox water for 4 weeks (Fig. 3) [29].

3.4.4 Generation of Skin-Specific Inducible IL-22 Transgenic Mice

1. To express IL-22 specifically and inducibly in the skin, cross the TRE-Tight–IL-22 mouse line with the keratin 5 (K5)–tetracycline transcriptional activator (tTA) mouse line to produce double-transgenic K5-tTA–IL-22 mice (Fig. 4).
2. Determine the genotypes of the mice by PCR using specific primers, 5′-AG GGCATCGGTAAACATCTG-3′ and 5′-GAT GTCGTCATAGAGGCTGTTGG-3′ for K5-tTA. For TRE--Tight–IL-22, use the primer set from Subheading 3.4.2 [30, 31].

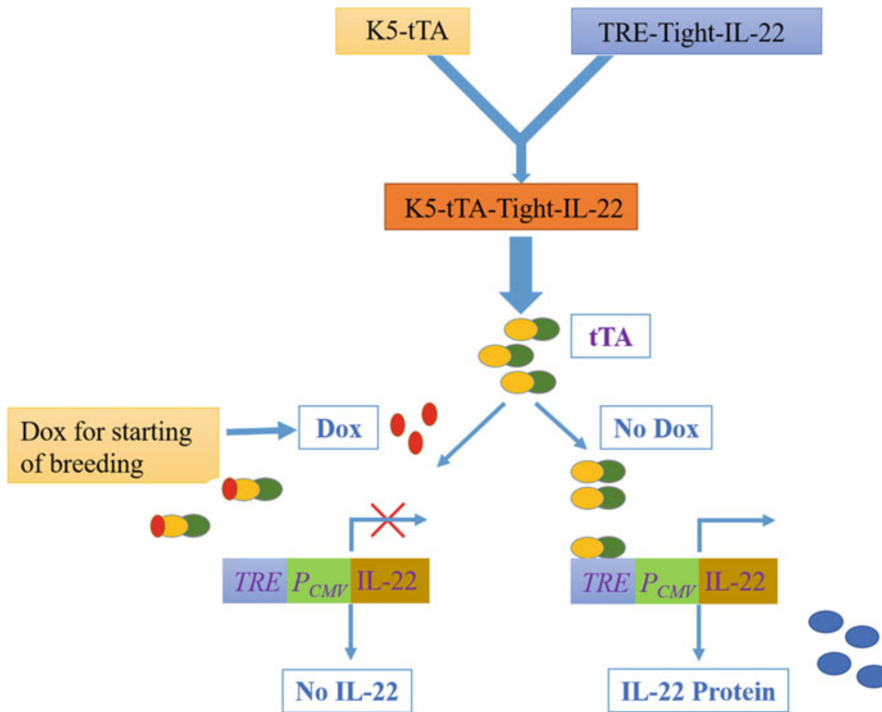

Fig. 4 Generation of inducible skin-specific IL-22 transgenic (K5-tTA-IL-22) mice. K5-tTA mice are crossbred with TRE-Tight-IL-22 mice to obtain K5-tTA-IL-22 double-positive mice. The IL-22 transgene is suppressed by Dox in the drinking water starting at the time of breeding. When mice are 6-weeks old, Dox is withdrawn from the drinking water to activate the IL-22 transgene. IHC may be performed to identify the expression of IL-22 in the skin. Without Dox, IL-22 is detected in the skin

3. The breeding also produces single transgenic mice (K5-tTA or TRE-Tight-IL-22). Use these mice for further breeding. Use Tg(−) littermates in the experiments as negative controls.
4. Starting at breeding, supply the Dox-containing drinking water to suppress tTA and to keep the IL-22 transgene off until the K5-tTA-IL-22 mice are 6 weeks old.
5. Initiate experiments by withdrawing Dox from the drinking water. In all experiments, give Tg(−) littermate controls the same amount of Dox or no Dox for the same length of time [30] (Fig. 4).

3.5 Histology and Immunohistochemistry (IHC)

1. Euthanize mice with an approved method of euthanasia, such as CO_2 gas inhalation (*see* the American Veterinary Medical Association's 2013 Guidelines for the Euthanasia of Animals https://www.avma.org/KB/Policies/Documents/euthanasia.pdf).
2. Excise the lung or skin and fix them in neutral buffered formalin at 4 °C overnight.

3. Dehydrate and clear the tissue through increasing concentrations of ethanol solutions and in xylene.
4. Embed the tissue in paraffin and section at 5 μm to mount on to glass slides.
5. Stain the sections with hematoxylin and eosin (H&E) and Alcian blue (AB) stains for histological analysis according to the supplier's protocol.
6. For immunohistochemistry experiments, rehydrate the tissue sections and quench endogenous peroxidase with 1% hydrogen peroxide diluted in methanol for 7 min at room temperature.
7. Rinse the tissue slides well with PBS and pre-block the tissue with the blocking solution for 30 min at room temperature.
8. To stain eosinophils, incubate the tissue in rat anti-mouse MBP antibody at 1:500 dilution overnight at 4 °C.
9. To stain IL-22 positive cells, incubate the tissue in goat anti-mouse IL-22 at 1:180 dilution overnight at 4 °C.
10. Use the ABC staining systems with the appropriate secondary antibodies to visualize the target proteins in the tissues.
11. Dehydrate the slide and coverslip using a mounting medium. Positive IHC staining in the SPC-rtTA-IL-22 and CC10-rtTA-IL-22 mice, or the K5-tTA–IL-22 mice indicate successful IL-22 transgene expression in the airway epithelial cells or skin cells, respectively, in an inducible faction (Figs. 5 and 6).

4 Notes

1. The pTRE-Tight vector consists of multiple cloning sites (MCS). We have cloned the mouse IL-22 cDNA fragment into MCS of this vector (Fig. 1).
2. The pTet-on vector expresses the reverse tet-responsive transcriptional activator (rtTA) from the strong immediate early promoter of cytomegalovirus (Pcmv). The rtTA binds to TRE, thus activating transcription of TRE downstream gene in the presence of doxycycline.
3. The most popular hybrid mouse strains such as B6D2F1 (C57BL/6 × DBA/2) or B6C3F1 (C57BL/6 × C3H/He) can be used as donors. Inbred strains such as C57BL/6 or C3H/He can be used, but they have a lower embryonic development rate than hybrid strains.
4. We have used a kind gift of rat anti-mouse MBP monoclonal antibody from Drs. Nancy and James J. Lee, Mayo Clinic, Scottsdale, AZ, at 1:500 dilution. The optimal dilution of a selected antibody should be determined by each laboratory.

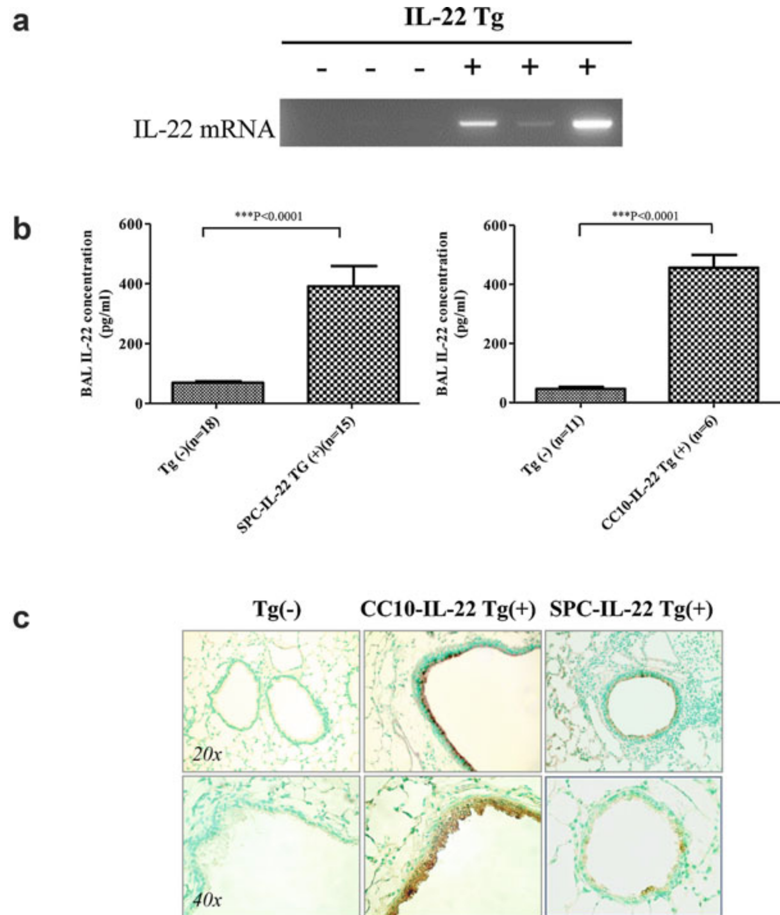

Fig. 5 Targeted IL-22 expression in the airway epithelial cells in the lung of IL-22 Tg(+) mice. After Dox induction, BAL fluid and lung tissues were harvested for analysis of IL-22 expression as described [29]. (**a**) RT-PCR analysis of IL-22 mRNA expression in the lung tissue. (**b**) ELISA measurement of IL-22 protein in the BAL fluid samples from Tg(−) littermate controls, SPC-IL-22 Tg(+), and CC10-IL-22 Tg(+) mice. (**c**) Immunohistochemical staining of lung tissue sections. Reproduced from Fang et al. PLoS One 2014; 9(9):e107454 [29]

5. We have successfully used a goat polyclonal anti-mouse IL-22 antibody from R&D Systems at 1:180 dilution.
6. Be sure to use protective gear when isolating bands on an ultraviolet transilluminator.
7. Determination of precise vector concentrations is important because the viability of embryos and PITT efficiency are sensitive to the concentrations of injected DNA. We sometimes quantify the DNA using several methods (e.g., NanoDrop Spectrophotometer).
8. Egg transfer is performed by transferring ten eggs per oviduct. Therefore, a total of 30 embryos are transferred to a recipient female mouse.

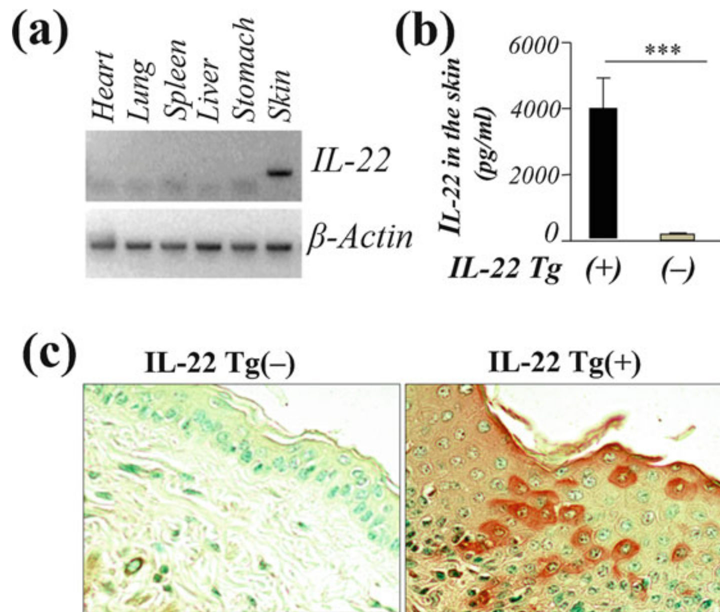

Fig. 6 Targeted IL-22 expression in the epidermis of the skin of IL-22 Tg(+) mice. After Dox withdrawal to activate the IL-22 transgene in the skin, tissue samples were harvested and analyzed for IL-22 expression. (**a**) RT-PCR analysis of IL-22 mRNA in skin tissues; (**b**) ELISA measurement of IL-22 in skin tissues; (**c**) IHC of IL-22 in the skin tissue sections from IL-22 transgenic mice and littermate controls. Reproduced from Lou et al. J Immunol 2017 198(7):2543–2555 [31]

Acknowledgments

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Chapter 10

Experimental Mouse Models of Ragweed- and Papain-Induced Allergic Conjunctivitis

Akira Matsuda, Toshiaki Hirakata, Yosuke Asada, and Susumu Nakae

Abstract

Mouse models of allergic conjunctivitis mimic various aspects of human allergic conjunctivitis. They are useful as acute models of allergic conjunctivitis to study immunological aspects of this condition. In this chapter, we will describe ragweed-pollen-induced experimental allergic conjunctivitis (mostly driven by adaptive immunity), and papain-soaked contact lens-induced experimental allergic conjunctivitis (mostly driven by innate immunity). Giemsa staining of histological sections is used for quantification of the number of infiltrating eosinophils, which is useful to evaluate the severity of the allergic inflammation. Immunohistochemical staining and quantitative PCR are used to clarify spatiotemporal expression of proinflammatory molecules in the conjunctival tissue. Flow cytometric analysis of conjunctival tissue is used for the detection of innate lymphoid cell type 2 (ILC2) in the ocular surface tissues.

Key words Mouse conjunctivitis model, Allergy, Ragweed pollen, Papain, Immunohistochemistry, Quantitative PCR, IgE, Innate lymphoid cell type 2 (ILC2)

1 Introduction

Allergic conjunctivitis is a common disorder in developed countries. There are four subtypes of allergic conjunctivitis: seasonal/perennial allergic conjunctivitis (SAC/PAC), vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), and giant papillary keratoconjunctivitis (GPC). In this chapter, we describe methods for the induction and analysis of a ragweed-induced experimental allergic conjunctivitis model (RW-EAC) and a papain-soaked contact lens-induced allergic conjunctivitis (papain-CL conjunctivitis) model, which mimic human SAC [1] and GPC [2], respectively. The RW-EAC model is IgE-dependent, whereas the papain-CL conjunctivitis model is IgE-independent [2, 3]. Mouse models are superior to other allergic conjunctivitis models using guinea pigs or rats because of the availability of a wide range of antibodies for functional analysis, genetic information, and genetically modified animals.

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2 Materials

2.1 RW EAC Mouse Model

1. Ragweed pollen (RW): 19–20-μm sized ragweed pollen collected from *Ambrosia artemisiifolia*.
2. Phosphate-buffered saline (PBS): 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4. Dissolve 1.44 g of Na_2HPO_4, 8.0 g of NaCl, 0.24 g of KH_2PO_4, and 0.2 g of KCl in distilled water and adjust the pH to 7.4. Bring the final volume to 1 L with distilled water.
3. Imject™ Alum Adjuvant: a commercially available formulation containing 40 mg/mL aluminum hydroxide and 40 mg/mL magnesium hydroxide.
4. Female BALB/c mice: 8–12 weeks old (*see* **Note 1**).
5. 1-mL Syringe.
6. 1.5-mL Microfuge tubes for eye and blood collection.
7. Micropipettes and tips.

2.2 Papain-CL Conjunctivitis Mouse Model

1. Soft contact lens: 2 mm in diameter, negatively charged, disposable. Trim commercially available contact lenses using a 2 mm diameter biopsy punch.
2. Papain solution: 25 mg/mL in PBS, pH 7.4. Prepare a fresh solution just before use (no need to be sterilized). Heat-inactivated papain solution is made by heating the papain solution at 95 °C for 30 min.
3. 96-Well cell culture plates: Used to soak contact lenses in the papain solution.
4. 8-0 nylon surgical suture.
5. Female C57BL/6 mice: 8–12 weeks old (*see* **Note 1**).

2.3 Tissue Collection

1. 1-mL Syringes.
2. Pentobarbital: 6.5 mg/mL in saline solution for general anesthesia.
3. 0.4% (w/v) Oxybuprocaine hydrochloride: for topical anesthesia of the eye.
4. Microscissors.
5. Dissection microscope.

2.4 Quantitative PCR (qPCR) of the Mouse Conjunctival Tissue

1. RNA stabilization solution: RNAlater™ or equivalent.
2. Nuclease-free 1.5-mL microfuge tubes.
3. RNA isolation kit: NucleoSpin II or equivalent.
4. Reverse transcriptase kit for the preparation of cDNA.
5. RNase inhibitor.

Table 1
Primers used for quantitative PCR

Gene	Forward primer	Reverse primer
il4	5′-TCCAAGGTGCTTCGCATATTTT-3′	5′-CAGCTTATCGATGAATCCAGGC-3′
il5	5′-TCCAAGGTGCTTCGCATATTTT-3′	5′-TCCAATGCATAGCTGGTGATT-3′
il13	5′-GGCAGCAGCTTGAGCACATT-3′	5′-GGCATAGGCAGCAAACCATG-3′
ccl5	5′-ACTATGGCTCGGACACCA-3′	5′-ACACACTTGGCGGTTCCT-3′
ccl11	5′-AGATGCACCCTGAAAG-3′	5′-GCATCCTGGACCCACT-3′
il33	5′-TCCAACTCCAAGATTTCCCCG-3′	5′-CATGCAGTAGACATGGCAGAA-3′
gapdh	5′-AAGGGCTCATGACCACAGTC-3′	5′-GGATGACCTTGCCCACAG-3′

6. NanoDrop spectrophotometer or equivalent for quantitation of RNA.
7. PCR tubes with lids.
8. Forward and reverse primers: *See* Table 1.
9. Universal quantitative PCR reaction master mix: KAPA SYBR® FAST or equivalent.
10. Real-time qPCR thermal cycler.

2.5 Histological Analysis of Mouse Conjunctival Tissue

1. 10× Phosphate-buffered saline stock solution (10× PBS): 100 mM Na_2HPO_4, 18 mM KH_2PO_4, 1.37 M NaCl, 27 mM KCl, pH 7.4. Dissolve 14.4 g of Na_2HPO_4, 80 g of NaCl, 2.4 g of KH_2PO_4, and 2 g of KCl in distilled water and adjust the pH to 7.4. Bring the final volume to 1 L with distilled water.
2. 12% (w/v) Paraformaldehyde (PFA) stock solution: Dissolve 120 g PFA in 800 mL of distilled water on a hot plate (50–50 °C) with a magnetic stirrer. Add a few drops of 1 M NaOH until the solution becomes translucent. Adjust the final volume to 1 L with distilled water. Store at 4 °C.
3. 4% (w/v) PFA in PBS: Just before use, prepare 100 mL of 4% PFA solution by mixing 25 mL of 12% PFA stock solution, 10 mL of 10× PBS, and 65 mL of distilled water.
4. Ethanol solutions for dehydration and rehydration: 100% and 70% ethanol solutions for rehydration; 50%, 70%, 80%, 90%, 95%, and 100% ethanol solutions for dehydration.
5. Xylene.
6. Paraffin wax: histological grade.
7. Plastic molds: for embedding tissue in paraffin blocks.
8. Microtome for paraffin block sectioning.

9. Glass slides: histological grade, adhesive coated.
10. Giemsa staining solution: commercially available.
11. Mounting medium.
12. Coverslips.
13. 30% (w/v) Sucrose in PBS: Dissolve 300 g of sucrose in 1 L of PBS. Prepare just before use.
14. Plastic tissue-embedding mold: 15 mm × 15 mm × 5 mm.
15. Tissue-embedding compound: O.C.T. compound or equivalent.
16. Cryostat.
17. Blocking buffer: 10% normal donkey serum and 1% bovine serum albumin in PBS.
18. Hydrophobic pen.
19. Primary antibodies for immunohistochemical staining:
    (a) Goat anti-mouse IL-33 polyclonal antibody.
    (b) Rat anti-mouse major basic protein (MBP) antibody.
20. Secondary antibodies for immunohistochemical staining:
    (a) Donkey Alexa 488-conjugated anti-rat IgG antibody.
    (b) Donkey Alexa 594-conjugated anti-goat IgG antibody.
21. Anti-fade and nuclear staining mounting medium: 0.3 μg/mL DAPI, 3% DABCO, and 50% glycerol in distilled water.
22. Confocal laser scanning microscope.

2.6 Mouse Serum IgE Quantification

1. 96-Well ELISA plates and sealers.
2. Multichannel pipettors and tips.
3. ELISA kit for mouse IgE: commercially available.
4. Coating buffer: 100 mM $NaHCO_3$, 33.6 mM Na_2CO_3, pH 9.5. Dissolve 8.4 g $NaHCO_3$ and 3.56 g Na_2CO_3 in 800 mL of distilled water. Adjust the pH and bring the volume to 1 L.
5. Wash buffer: 0.05% (v/v) Tween 20 in PBS, pH 7.4.
6. Assay diluent: 1% bovine serum albumin in PBS.
7. Orbital shaker.
8. TMB substrate reagents: commercially available ELSIA reagent set with 0.005% (w/v) 3,3,5,5-Tetramethylbenzidine solution and hydrogen peroxide.
9. Stop solution: 2 N H_2SO_4.
10. Spectrophotometric microplate reader.

2.7 Flow Cytometric Analysis of Mouse Conjunctival Tissue for Innate Lymphoid Cell Type 2 (ILC2)

1. Dissection tools.
2. Plungers from 10-mL syringes.
3. Hank's balanced salt solution (HBSS): Dissolve 400 mg KCl, 60 mg KH_2PO_4, 350 mg $NaHCO_3$, 8 g NaCl, 48 mg Na_2HPO_4, and 1 g D-glucose, in 800 mL of distilled water. Adjust the final volume to 1 L with distilled water.
4. FACS buffer: 2% fetal calf serum (FCS) in HBSS. Add 2 mL of FCS in 98 mL HBSS.
5. Cell strainers: 70 μm, sterile.
6. 50-mL Conical tubes.
7. Digestion buffer: HBSS containing 1 mg/mL collagenase type IV and 0.5 mg/mL DNase I.
8. 3-mL Syringes.
9. Red blood cell (RBC) lysis buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 8.0. Mix 8.29 g NH_4Cl, 1 g $KHCO_3$, and 0.2 mL of 0.5 M EDTA in 1 L of distilled water.
10. Fc receptor blocking reagent: rat anti-mouse CD16/CD32 antibody, clone FCR4G8.
11. Fluorochrome-conjugated monoclonal antibody cocktails: Mix 0.5 μg each of the following monoclonal antibodies per 1×10^6 cells in FACS buffer.
    (a) Brilliant Violet 421-anti-mouse-CD45 (30-F11).
    (b) PE-conjugated anti-mouse lineage marker cocktail: CD3 (145-2C11), CD19. (6D5), NK1.1 (PK136), CD11b (M1/70), Gr1 (RB-8C5), and Ter119 (Ter-119).
    (c) FITC-conjugated anti-mouse ST2L (DJ8).
    (d) Cy7-conjugated anti-mouse CD127 (A7R34).
    (e) APC-conjugated anti-mouse CD25 (PC61).
    (f) APC-Cy7-conjugated anti-mouse CD90.1 (30-H12).
12. FACS tubes with 70-μm nylon mesh tops.
13. 7-AAD (7-amino-actinomycin D) nuclear staining solution: commercially prepared 50 μg/mL 7-ADD solution in PBS with 0.09% sodium azide, pH 7.2. Store in the dark at 2–8 °C. Use at the final concentration of 2 μg/mL in FACS buffer.
14. MAQS Quant® flow cytometer or equivalent.

3 Methods

3.1 RW EAC Mouse Model

3.1.1 Sensitization

1. Mix 1 mg of RW with 1 mL of Imject™ Alum and stir overnight at room temperature to make RW emulsion (*see* **Note 2**).
2. For the first sensitization, inject 50 μL of the emulsified RW into the left hind foot and root of the tail subcutaneously on Day 0.
3. Collect the serum from the tail vein blood for quantitation of presensitization IgE levels.
4. For the second sensitization, inject the emulsified RW into the right footpad subcutaneously on Day 14.

3.1.2 Allergen Challenge with RW Eye Drop Solution

1. Make an eye drop solution by mixing RW with PBS (2 mg in 10 μL/eye) and vortex (*see* **Note 2**).
2. Using a micropipette, instill 10 μL of the RW eye drop solution per eye from Days 26 to 29, once per day for 4 days (*see* **Note 3**).
3. Twenty minutes after the last eye drop challenge, Evaluate EAC using the following 12-point scoring system based on the criteria described by Magone et al. [4]. (Fig. 1; *see* **Note 4**).
   (a) Chemosis: 0 = absent; 1 = mild; 2 = obvious; 3 = severe.
   (b) Redness: 0 = absent; 1 = mild; 2 = moderate; 3 = severe.
   (c) Lid edema: 0: absent; 1 = mild; 2 = moderate; 3 = severe, unable to open eye.
   (d) Tearing and discharge: 0 = absent; 1 = mild; 2 = obvious; 3 = severe.

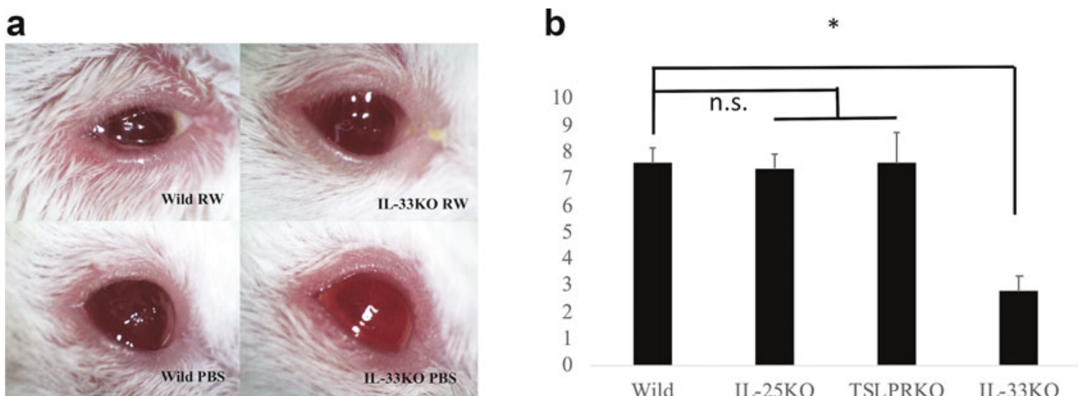


Fig. 1 Representative photographs of RW-EAC models, using wild-type mice and IL-33 knockout (KO) mice challenged either with RW-PBS (upper row) or with PBS alone (lower row) taken 20 min after the last eye drop challenge (**a**). The clinical scores of the RW-challenged EAC models are shown (**b**). *$P < 0.05$, Mann–Whitney U-test. *Wild* wild-type, *KO* IL-33 KO mice. Reproduced from Invest Ophthalmol Vis Sci. 56: 5194–5202, 2015 [1]

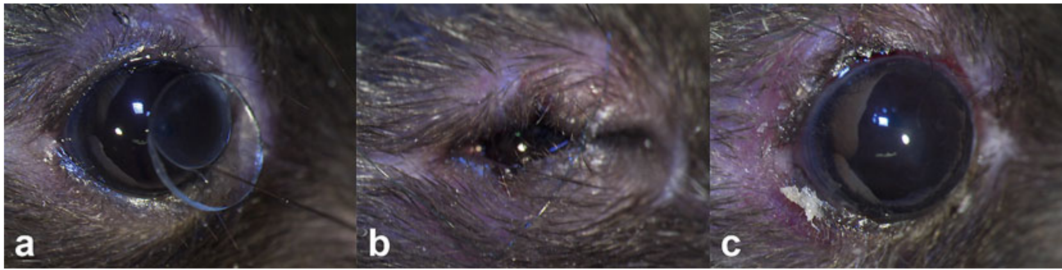


Fig. 2 Papain-contact lens (papain-CL)-induced inflammation in the mouse eye. On Day 0, a papain-CL is inserted into the conjunctival-sac of the right eye (**a**), and the eyelid is sutured with 8-0 nylon (**b**). The papain-CL is exchanged once on Day 2. The second papain-CL is removed, and the eye is sampled for further analysis on Day 5 (**c**). Reproduced from Immun Inflamm Dis. 5: 515–525, 2017 [2]

4. At 24 h after the last eye drop challenge, euthanize the mice by a method approved by your institution. Collect the eyeballs (with lids and conjunctival tissue) for histological and gene expression analysis, and blood for postantigen challenge IgE levels in the serum.

3.2 Papain-CL Conjunctivitis Mouse Model

1. Soak negatively charged soft contact lenses (CLs) in 25-mg/mL papain solution for 24 h to prepare papain-CLs (*see* **Note 5**). For this purpose, dispense 200 μL of the papain solution to 96-well cell culture plates and immerse a 2-mm diameter soft contact lens in each well.
2. Anesthetize the animals by administering an intraperitoneal injection of pentobarbital at 50 mg/kg body weight. Instill oxybuprocaine hydrochloride topical anesthesia eye drops into the eyes (*see* **Note 6**).
3. After general anesthesia, place a papain-CL in the conjunctival sac of the right eye (Day 0). Suture the eyelid with an 8-0 nylon suture (Fig. 2). Use either heat-inactivated papain-CLs or PBS-soaked CL as a negative control.
4. Two days after the initial surgery (Day 2), repeat **steps 1–3** above to replace the first papain-CL with a newly prepared papain-CL in the same conjunctival sac. Resuture the eyelids.
5. Three days after the second surgery (Day 5), euthanize the mice and remove the second papain-CL. Collect the eyelid and conjunctival tissue for gene expression analysis, histological analysis, and/or fluorescence-activated cell sorting (FACS) analysis.
6. For gene expression analysis, immerse the enucleated eyes in RNAlater™ solution. Collect conjunctival tissue under a dissecting microscope using microscissors and forceps (Fig. 3). Store the tissue RNAlater™ solution at −20 °C until analysis.

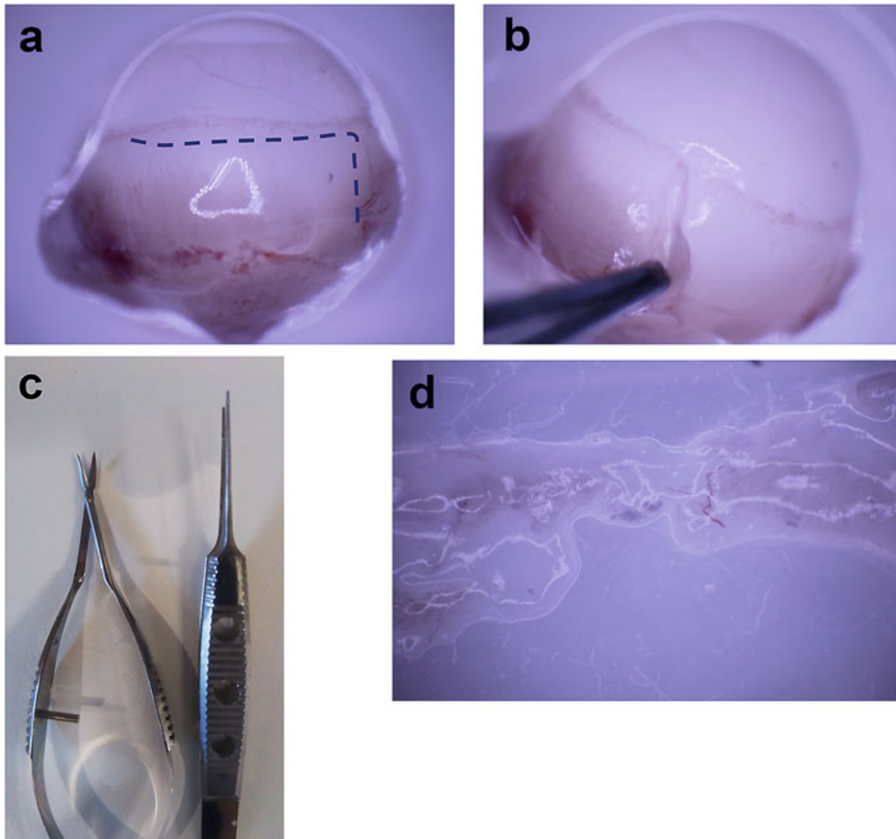


Fig. 3 Conjunctival tissue separation from an eyeball. Cut (**a**: as dotted line) and peel off (**b**) conjunctival tissue from the eyeball using microscissors and forceps (**c**). Isolated conjunctival tissue is shown as a sheet (**d**)

7. For histological analysis, enucleate the eye with the eyelid using microscissors and fix the tissue immediately with 4% PFA.
8. For FACS analysis, keep the enucleated eyes and microdissected conjunctival tissue in FACS buffer.

3.3 Quantitative PCR (qPCR) for Gene Expression Analysis of Th2 Cytokines

1. Isolate conjunctival tissues from mouse eyes using microscissors and forceps under a dissecting microscope (Fig. 3). Immerse the isolated tissues immediately in RNAlater solution and keep at −20 °C until RNA extraction.
2. Extract total RNA from the conjunctival tissues using a NucleoSpin II RNA Isolation Kit or equivalent according to the manufacturer's instruction (*see* **Note 7**). Quantify the amount of RNA in each sample using a NanoDrop spectrophotometer.
3. Prepare cDNA from the conjunctival RNA extracts using a reverse transcriptase kit with random primers and RNase inhibitors according to the manufacturer's protocol.

4. Quantify the relative expression of the target genes by qPCR using a real-time thermal cycler using the primers listed in Table 1 (*see* **Note 8**). The conditions of qPCR depend on the machine and reagents used for qPCR analysis. If KAPA SYBR® FAST qPCR master mix is used with a Light Cycler (Roche Diagnostics), use the following condition: 3-min preincubation at 95 °C; 40 cycles of 3-step amplifications (95 °C for 10 s, 60 °C for 20 s, 72 °C for 1 s).

3.4 Histological Analysis of the Mouse Conjunctiva

3.4.1 Giemsa Staining for Eosinophil Counting

1. Dissect mouse eyes with conjunctival tissues and the eyelids intact and fix in 4% PFA for 3 h at 4 °C.
2. Dehydrate the tissue through ethanol gradients by incubating in 50%, 70%, 80%, 90%, 95%, and 100% ethanol solutions. Transfer the tissue in xylene to clear.
3. Incubate the tissue in melted paraffin at 58–60 °C. Place the tissue in a plastic mold and embed it in a paraffin block.
4. Make vertical 2-μm-thick sections on a microtome and mount them on glass slides (*see* **Note 9**).
5. Deparaffinize, rehydrate, and stain the sections with Giemsa solution according to the supplier's instructions.
6. Dehydrate the sections as described in **step 2**. Coverslip to observe under the microscope (Fig. 4).
7. Count the infiltrating eosinophils in the lamina propria of the tarsal and bulbar conjunctiva throughout each section in the central portion of the eye.

3.4.2 Immunohistochemical Staining of the Conjunctival Tissue

1. Trim the eye and eyelids under a dissecting microscope after 30 min fixation in 4% PFA.
2. Remove the lens and make toroidal tissue including the eyelid, cornea, conjunctiva, iris, and retina (Fig. 5).
3. After dissection, fix the eye tissue again in 4% PFA for another 30 min.
4. Immerse the tissue in 30% sucrose-PBS for cryoprotection for 30 min.
5. Take the tissue out of the sucrose-PBS solution and remove the excess solution using filter paper (*see* **Note 10**).
6. Transfer the tissue in a plastic mold to embed in O.C.T. compound and freeze on a liquid nitrogen-cooled metal plate. Do not freeze the tissue directly in liquid nitrogen to prevent cracking of the tissue.
7. Make 5-μm-thick frozen sections using a cryostat and air-dry the tissue sections on glass slides.
8. Rinse the slides in PBS and incubate in the blocking buffer for 30 min at room temperature.

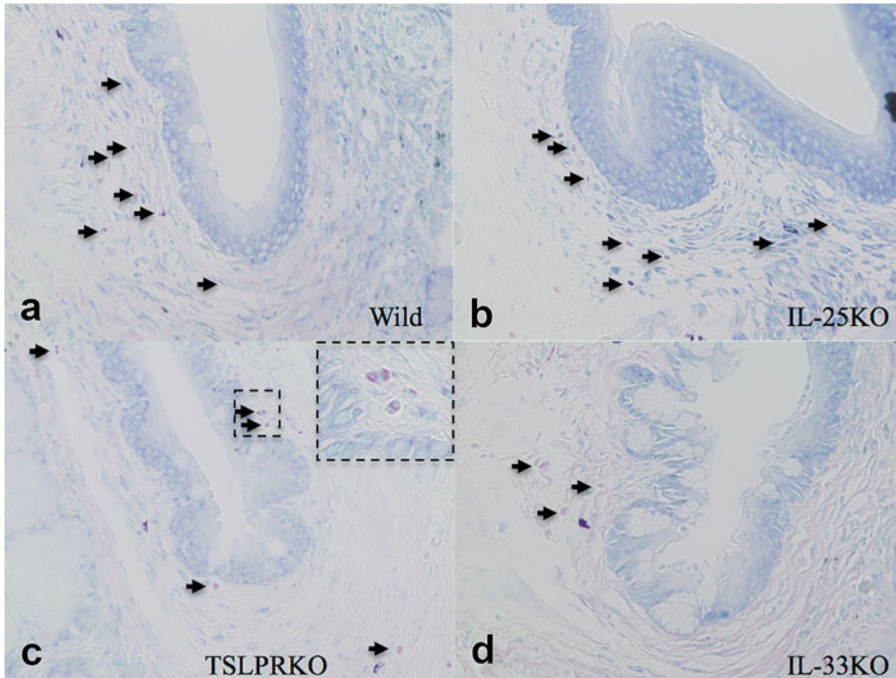


Fig. 4 Infiltrating eosinophils are identified by Giemsa staining. Compared to wild-type (**a**) and IL-25 KO (**b**) mice, significant reduction in eosinophil numbers is observed in the models using TSLP receptor (**c**: TSLPRKO) and IL-33(**d**: IL-33KO) knockout mice. Infiltrating eosinophils are shown at a higher magnification in the inset (dotted box in **c**). *Wild* wild-type, *KO* knockout mice. Reproduced from Immun Inflamm Dis. 5: 515–525, 2017 [2]

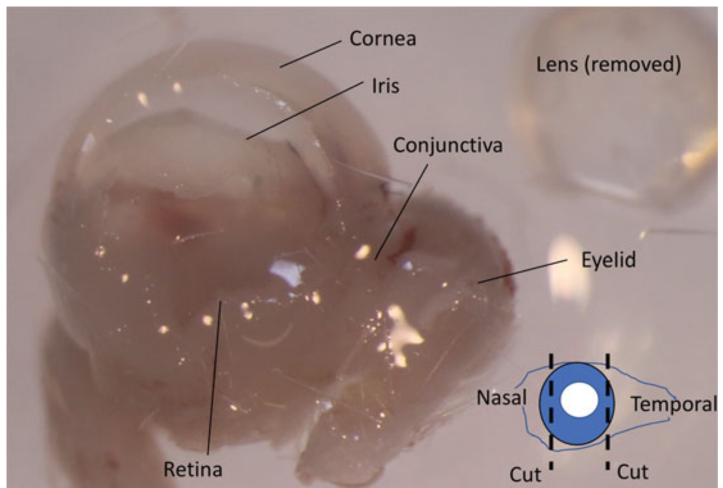


Fig. 5 Enucleated eye and eyelids are dissected using microscissors. Parts of corneal and scleral tissues are removed from both the nasal and temporal sides. The lens is also removed. The toroidal-shaped eye tissue can be embedded in O.C.T. compounds using a plastic mold

9. Use a hydrophobic pen to draw circles around the tissue sections on the slides to minimize the amount of antibody solution.
10. To perform double immunostaining, apply anti-IL-33 (1:100 dilution using donkey blocking buffer) and anti-MBP antibody solutions (1:500 dilution with the blocking buffer) within the circles on the slides, covering the eye sections completely. As negative controls, incubate additional specimens to be incubated with control goat IgG or rat IgG at the same concentrations as the IL-33 and MBP primary antibodies, respectively.
11. Incubate the slides in the antibodies for 2 h at room temperature or overnight at 4 °C.
12. Remove the antibody solution and wash the tissue sections in PBS for 5 min, 3 times.
13. Apply two secondary antibodies (Alexa-488-anti-rat IgG and Alexa-594-anti-goat IgG antibodies, dilution 1:1000 each) to the slides for 30 min at room temperature.
14. Remove the antibody solution and wash the tissue sections in PBS for 5 min, 3 times.
15. Coverslip using an anti-fade mounting medium. Use a confocal laser scanning microscope to observe MBP-positive cytoplasmic staining in green (a marker for eosinophils) and IL-33 positive nuclear staining in red (Fig. 6).

3.5 Mouse Serum IgE Quantification

1. Collect blood samples from mice at presensitization (Day 0) and postchallenge (Day 30) to determine the total serum IgE levels.
2. Leave the collected blood samples in 1.5-mL microfuge tubes for 20–30 min at room temperature to coagulate the blood.
3. After coagulation, centrifuge the tubes for 5 min at 20,000 × *g* and transfer the supernatant (serum) in new microfuge tubes. Store at −80 °C until analysis.
4. Select a commercial ELISA kit for total IgE detection. One day before ELISA analysis, coat 96-well microwell ELISA plates with mouse IgE capture antibody diluted in the coating buffer. Seal the plate and incubate overnight at 4 °C.
5. Remove the captured antibody and rinse the wells with the wash buffer. Block the wells with the assay diluent for 2 h at room temperature.
6. If frozen serum samples are used, thaw the sample completely and keep in the ice until dilution. Make 1:10 dilution of Day 0 (preimmune) sera and 1:100–1:1000 dilution of Day 30 (postchallenge) sera using the assay diluent of the selected ELISA system (*see* **Note 11**).

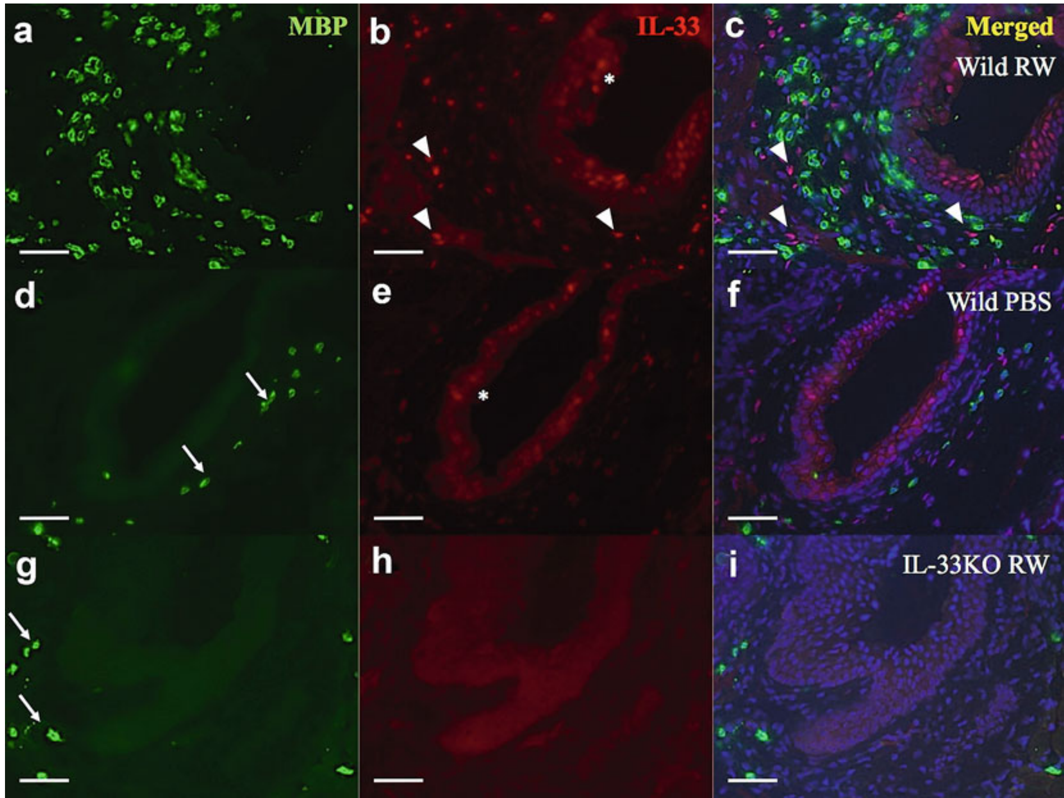


Fig. 6 MBP (**a**, **d**, **g**) and IL-33 (**b**, **e**, **h**) immunostaining of the conjunctivae obtained from RW-EAC models is shown. Massive infiltration of MBP-positive eosinophils (green) is observed in the substantia propria of RW-challenged EAC in wild-type mice (**a**). On the other hand, sparse infiltration of eosinophils is observed in PBS-challenged wild-type mice (**d**) and RW-challenged IL-33 KO mice (**g**). IL-33 protein expression (asterisks) can be observed in the conjunctival epithelial cells of the wild-type mice (**b**, **c**, **e**, **f**). The cells infiltrating the substantia propria in the vicinity of MBP-positive eosinophils (arrowheads) are also immunopositive for IL-33 (**b**, **c**). No IL-33 positive cells are observed in the conjunctiva of IL-33 KO mice (**h**, **i**). *Wild* wild-type, *KO* knockout mice. Scale bar = 50 μm. Reproduced from Invest Ophthalmol Vis Sci. 56: 5194–5202, 2015 [1]

7. After blocking the plate, remove the assay diluent and add 100 μL of the IgE standard and serum samples to the wells and incubate for 2 h with gentle shaking.
8. Wash the plate with the wash buffer 4 times. Add 100 μL of the detection antibody and incubate for 1 h at room temperature.
9. Remove the detection antibody and wash it again 4 times with the wash buffer.
10. Add 100 μL of TMB substrate solution and incubate in the dark for 20 min.
11. Add 100 μL of stop solution to terminate the color development and read the absorbance at 450 nm using a microplate reader.

3.6 FACS Analysis of Mouse Conjunctival Tissue

3.6.1 Cell Preparation

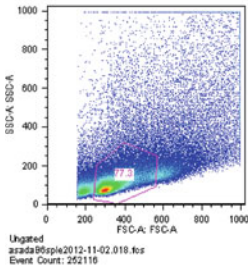
1. For spleen cells, harvest the spleen from each mouse and use one half of the spleen to prepare spleen cell suspension. Triturate the spleen tissue with the flat end of the plunger of a 10-mL disposable syringe.
2. For conjunctival cells, collect the conjunctival tissue around the sensitized eye from each mouse (*see* **Note 12**). Pool conjunctival tissues from 10 mice (=10 eyes) and mince the tissues using scissors. Incubate the tissue fragments in the digestion buffer for 15 min at 37 °C, and then triturate the tissue fragments using the end of a 10-mL syringe plunger.
3. Set a 70-μm cell strainer on top of a 50-mL centrifuge tube and pass the cell suspension prepared above through the cell strainer. Fill the tube with 10–15 mL of HBSS for washing.
4. Centrifuge the samples at 400 × *g* for 5 min at 4 °C and discard the supernatant.
5. Resuspend the cells with 1 mL of RBC lysis buffer and incubate for 5 min at room temperature.
6. Fill the tube with 10–15 mL of HBSS for washing.
7. If cell debris is seen, repeat **step 3** and strain the debris.
8. Centrifuge the samples at 400 × *g* or 5 min at 4 °C and discard the supernatant.
9. Resuspend the cells in 500 μL of FACS buffer and transfer to 1.5-mL microfuge tubes. Usually, 1–5 × 10^6 cells are obtained from conjunctival tissues pooled from 10 mice, and 1–5 × 10^8 cells are obtained from one-half of spleen tissue.

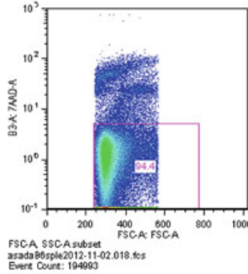
3.6.2 Cell Staining

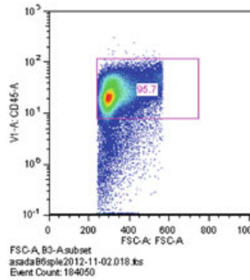
1. To the cell suspensions from Subheading 3.6.1, **step 9** above (2–5 × 10^6 conjunctival cells and 1 × 10^8 spleen cells), add 1 μg of Fc receptor blocking reagent. Incubate for 15 min at 4 °C.
2. Add 100 μL of the fluorochrome-conjugated primary antibody cocktail or fluorochrome-conjugated isotype-matched control antibodies in FACS buffer. Mix gently by pipetting and incubate for 30 min at 4 °C in the dark.
3. Wash the cells 3 times with 1 mL of FACS buffer and centrifuging at 800 × *g* for 1 min at 4 °C. Discard the supernatants and resuspend the cells with 200 μL of FACS buffer.
4. Pass the cell suspensions through a 70-μm nylon mesh into FACS tubes.
5. Add 200 μL of 7-AAD nuclear staining solution and incubate for 5 min at room temperature.
6. Analyze the cell proportion in the cell suspension by flow cytometry as below.

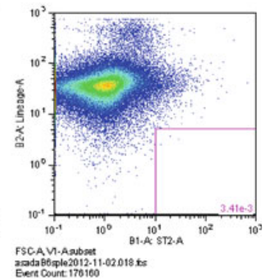
Spleen cell

Select lymphoid cell | Remove dead cell | Select CD45⁺ cell | Select Lin⁻ST2⁺ cell



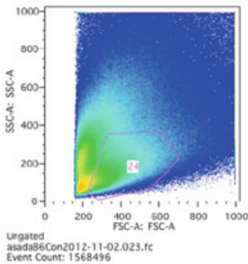


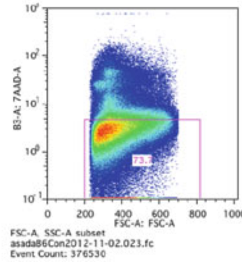


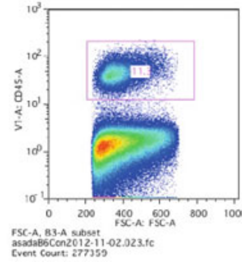


Conjunctival cell

Select lymphoid cell | Remove dead cell | Select CD45⁺ cell | Select Lin⁻ST2⁺ cell







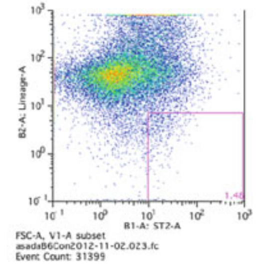


Fig. 7 Gating strategy for ILC2 detection. Spleen cells are used as controls. Select lymphoid-shaped cells using SSC/FSC gating. Next, exclude 7AAD$^+$ dead cells, and select CD45$^+$ cells. Finally, select Lin$^-$ST2$^+$ cells for further analysis

3.6.3 Flow Cytometric Analysis

1. Select the population of lymphocytes by gating using the scatter plot of FSC and SSC (Fig. 7: Select lymphoid cells).
2. Select the population of live cells by gating the 7-AAD-negative region (Fig. 7: Remove dead cells).
3. Select the population of cells by gating the CD45-positive region in live cells (Fig. 7: Select CD45+ cells). Most of the spleen cells are CD45-positive lymphoid cells.
4. Select the population of cells by gating the lineage marker-negative and ST2-positive region (Fig. 7: Select Lin-ST2+ cells).
5. Further confirm the identity of the cells as ILC2 [5] by assessing the cell surface expression of CD25, CD127, and CD90.1 (Fig. 8). Most of the Lin$^-$/CD45$^+$/ST2$^+$ cells are ILC2.

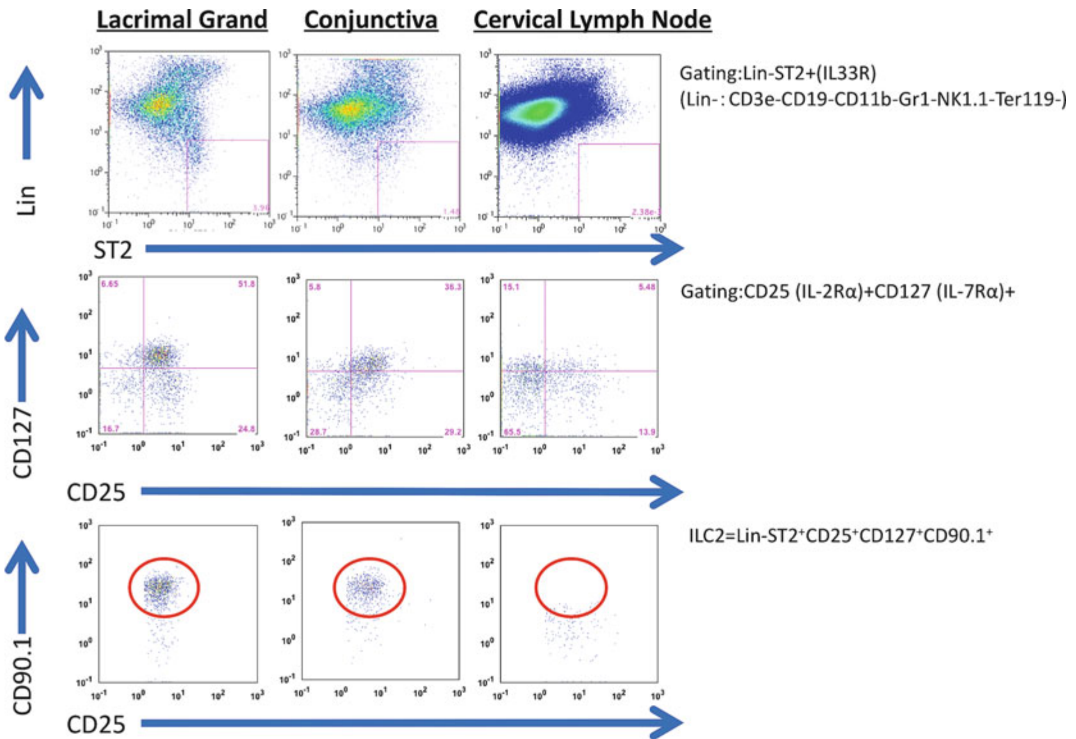


Fig. 8 Detection of ILC2 in the mouse conjunctival tissue by flow cytometry. ILC2s (Lin$^-$ST2$^+$CD25$^+$CD127$^+$CD90.1$^+$) are identified in the conjunctival tissue (bottom left) and lacrimal gland tissue (bottom center) from the papain-CL model. On the other hand, only a few ILC2s are detected in the cervical lymph node (bottom right). Reproduced from Immun Inflamm Dis. 5: 515–525, 2017 [2]

4 Notes

1. The BALB/c strain is preferred for the RW-EAC model because the mouse has a Th2-prone nature. We use the C57BL/6 strain for the papain-CL conjunctivitis model because of the availability of genetically modified mice.
2. It is essential to stir overnight to make the alum-RW emulsion. Mix RW-PBS eye drops well before instillation into the eye. We usually prepare 300 μL of solution for 10 mice.
3. After the instillation of the RW eye drops, the mouse should be kept on a rough mesh on top of the mouse cage by gently holding the tail to keep the mouse from escaping. Allow the mouse to blink and scratch for 3 min. This procedure facilitates RW particle retention within the conjunctival sac and eyelids.
4. For clinical grading, we recommend video recording of the mouse eye under a dissecting microscope using a constant magnification. Clinical evaluation can be carried out by

A

B

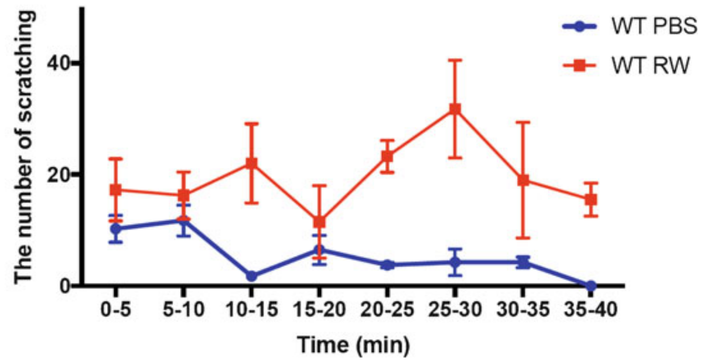


Fig. 9 A mouse challenged with RW eye drop is showing scratching behavior (**a**). The number of scratching behaviors is counted (**b**). Time (min) indicates the time after RW eye drop instillation. *WT PBS* wild-type mouse challenged with PBS eye drops. *WT RW* wild-type mouse challenged with RW eye drops. Typically, the number of scratching behaviors peaks at 20–30 min after RW eye drop instillation

examiners familiar with ophthalmological findings. Counting the number of scratching behaviors is one of the indicators of the itching sensation [6] (Fig. 9).

5. It is essential to use negatively charged contact lenses to retain papain molecules on the surface of the contact lens because papain is positively charged. Negatively charged disposable soft contact lenses can be obtained from commercial sources; we use 1-Day Acuvue Moist and trepan the lenses using a 2-mm diameter biopsy punch.

6. General anesthesia of mice may be performed in various ways. We also carry out general anesthesia using sevoflurane with a vaporization machine. Methods of anesthesia should adhere to the regulations of the animal research committee at your institution.

7. Typically, conjunctival tissue from one eye yields 500–1000 ng of RNA. The RNA concentration and quality should be estimated using the NanoDrop spectrophotometer (Thermo Fisher) or equivalent. RNA yield and quality may be affected by the method of conjunctival tissue disruption. We perform tissue disruption in the lysis buffer of the RNA isolation kit using microscissors. Tissue Ruptor (Qiagen) is also useful.

8. Primer pairs for SYBR Green-based qPCR analysis can be designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) software. The relative gene expression is quantified by comparative Ct methods using mouse *gapdh* expression in the same cDNA as the controls.

9. Care should be taken to make vertical (not oblique) sections and not to include the nictitating membrane. It is important to fix the eye with eyelid tissue in the wide-open position. If the eyelids are closed, it is difficult to maintain the vertical plane of the eyeball at the time of paraffin embedding.
10. This step prevents curling of the tissue during the cryosection procedure.
11. The optimal dilutions of serum samples need to be determined by individual laboratories. The total serum IgE concentration is affected by the environment of the experimental mouse. For example, total IgE tends to be lower in the specific-pathogen-free condition compared to the conventional condition.
12. For the papain conjunctivitis model, only one eye may be sensitized to establish conjunctivitis in a mouse. Therefore, conjunctival tissues from one eye are harvested from each mouse.

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Chapter 11

Intravital Imaging of Vascular Permeability by Two-Photon Microscopy

Gyohei Egawa, Sachiko Ono, and Kenji Kabashima

Abstract

The regulation of vascular permeability is critical in inflammation. It controls the distribution of water and plasma contents such as immunoglobulins in peripheral tissues. To regulate allergic diseases, it is important to study vascular biology especially in inflammation. Since the vascular permeability changes in minutes upon the exposure to proinflammatory mediators, intravital imaging system is a powerful technique to capture such dynamic responses. We here describe how to evaluate vascular permeability in vivo using multiphoton microscopy. We use various sizes of fluorescence-labeled dextran to visualize how leaky the blood vessels are in the steady state and in inflammation. Using this assay system, we can illustrate the dynamic kinetics of vascular permeability in vivo in real-time. This assay system provides a novel convenient way to study vascular biology that is beneficial in the assessment of various animal models of allergic disease.

Key words Vascular permeability, Dextran, Histamine, Multiphoton microscopy, Intravital imaging

1 Introduction

The permeability of blood vessels is tightly regulated to maintain the homeostasis of peripheral tissues [1]. A variety of plasma proteins, such as hormones, cytokines, amyloids, lipoproteins, carrier proteins, and immunoglobulins circulate in the blood, and the distribution of these proteins depends on the permeability of blood vessels. The permeability changes within minutes upon the exposure to proinflammatory mediators including histamine, bradykinins, vascular endothelial growth factor (VEGF), and platelet-activating factor (PAF). To capture such a dynamic change of vascular permeability, intravital imaging system is regarded as a powerful technique.

Multiphoton (MP) microscopy, also referred to as two-photon excitation microscopy, was first developed by Denk et al. [2] and has been used especially in the field of immunology. In comparison to a conventional single-photon excitation microscope, an MP microscope allows deeper tissue penetration of laser with less

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photodamage, achieving high spatiotemporal resolution in vivo [3]. Various cutaneous immune responses have been visualized by intravital imaging [4].

By employing the intravital imaging techniques, we previously reported an intravital evaluation system for vascular permeability [5]. The major advantages of this method are as follows: (1) the dynamic kinetics of vascular permeability is determined in real-time, (2) the permeability can be evaluated without introducing inflammation, (3) the types of blood vessels, such as arterioles, venules, and capillaries, are readily distinguished. Thus, this method should provide a novel convenient way to study vascular biology in vivo. In this chapter, we provide our experimental protocol in detail.

2 Materials

2.1 Preparation of Mice

1. Six- to eight-week-old mice (*see* **Note 1**).
2. Sterile 1-mL syringe.
3. Pentobarbital sodium: 10% (v/v) solution in sterile phosphate-buffered saline (PBS): 1.37 M NaCl, 0.027 M KCl, 0.081 M Na_2HPO_4, 0.0147 M KH_2PO_4. Use at 10 μL/g body weight (*see* **Note 2**).
4. Hair removal cream (*see* **Note 3**).

2.2 MP Microscopy

1. Isoflurane inhalation anesthesia apparatus for small animals.
2. Cover glasses: 2 sizes, 24 mm × 24 mm and 24 mm × 50 mm.
3. Silicone grease (e.g., Beckman Coulter silicone vacuum grease).
4. Two-photon microscope with a tunable (690–1040 nm) Ti/sapphire laser (e.g., Mai Tai DeepSee, Spectra-Physics) (*see* **Note 4**).
5. Immersion oil.

2.3 Tail Vein Cannula and Dye Injection

1. 30-Gauge insulin syringes.
2. 30-Gauge syringe needles.
3. Polyethylene tubing: 0.28 mm inner diameter, 0.61 mm outer diameter (e.g., Beckton Dickinson PE 10).
4. Heparin solution: 100 U/mL heparin lithium salt in sterile PBS.
5. Warm water (40–50 °C) for immersing mouse tails to dilate veins.
6. Superglue.
7. Fluorescence-conjugated dextran solution: 2 mg/mL in sterile PBS (*see* **Notes 5** and **6**).

2.4 Induction of Vascular Hyperpermeability

1. Fluorescence-conjugated dextran solution: 2 mg/mL in sterile PBS.
2. Histamine solution: 2 mg/mL histamine dihydrochloride in sterile PBS (*see* **Note 7**).

3 Methods

3.1 Preparation of Mice

1. Anesthetize mice with 10 μL/g body weight of 10% pentobarbital sodium solution via intraperitoneal injection.
2. Apply the hair removal cream to the ventral and dorsal side of the left ear skin. Three minutes later, remove the cream from the ear using wet cotton swab or running water (*see* **Note 8**).
3. Allow the mice to rest at least 24 h before proceeding with imaging (*see* **Note 9**).

3.2 Placement of a Mouse on a MP Microscope

1. Anesthetize the mice again as described in Subheading 3.1, **step 1**. Keep the mouse on a heating pad at 37 °C throughout the imaging procedure (*see* **Note 10**).
2. Apply the grease to the dorsal side of the ear using a cotton swab (*see* **Note 11**) and attach a piece of 24-mm × 24-mm cover glass to the dorsal side of the ear. The mouse is ready to be placed on a MP microscope for imaging.
3. Place a single drop of immersion oil on the objective lens when using oil immersion lens.
4. Cover the central hole of the microscope stage with a piece of 24-mm × 50-mm cover glass and fix it on the stage with tape (*see* **Note 12**).
5. Place a drop of immersion oil onto the cover glass just above the objective lens.
6. Place a mouse onto the stage. Sandwich the ear between two cover glasses (*see* **Note 13**).
7. Connect the mouse to the anesthesia apparatus and stabilize the mouse using plastic tape (Fig. 1). Flow 1% isoflurane at a rate of 1 L/min (*see* **Note 14**).
8. Set the appropriate excitation laser wavelength. The ideal excitation wavelength is 780 nm for FITC and 800–840 nm for TRITC [3]. Collagen fibers in the dermis can be visualized by second-harmonic generation (*see* **Note 15**).

3.3 Visualization of Vascular Permeability at a Homeostatic Condition

1. Prepare a tail vein cannula by cutting a 20-cm segment from the polyethylene tubing (*see* **Note 16**) and attaching it to a needle that has been pulled out of a 30-gauge insulin syringe (Fig. 2).

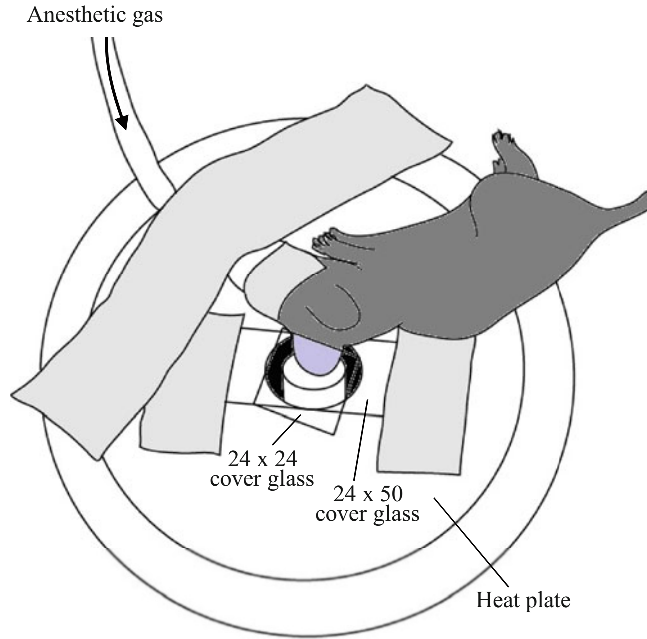

Fig. 1 How to fix a mouse on the stage. The ear lobe is sandwiched between two different sizes of cover slips

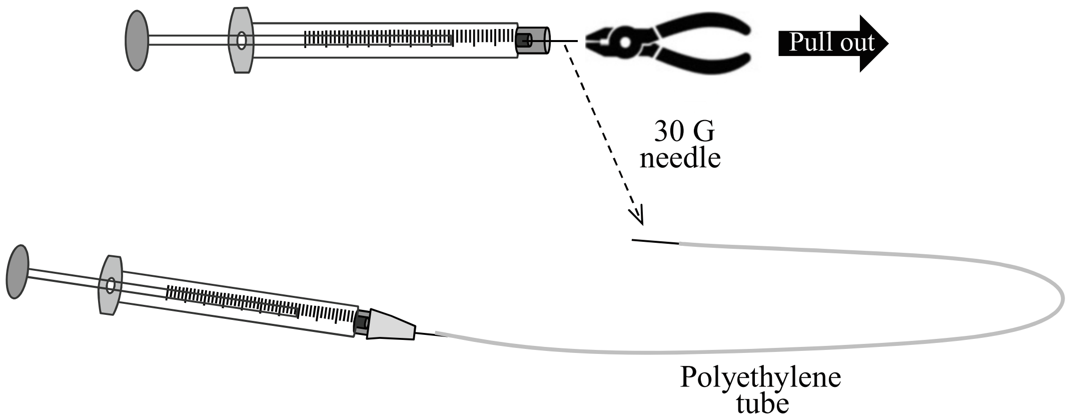

Fig. 2 How to make a cannula for the tail vein

2. Connect a 30-gause syringe with a needle to the other end of the polyethylene tubing and prefill the tube with the heparin solution.
3. Immerse the tail of the anesthetized mouse in the warm water for 1–2 min to dilate the tail vein.
4. Insert the needle of the prepared cannula to the tail vein and fix it in place with superglue.

5. Connect a syringe filled with 400–500 μL fluorescence-conjugated dextran solution to the open end of the tube attached to the cannula and slowly inject the dextran solution. Blood vessels should be visible immediately after the dextran injection.
6. Commence imaging soon after the injection of fluorescence-conjugated dextran. We typically take stacks of 10–15 images, spaced 2–4 μm apart, every minute for 1 h.

3.4 Induction of Vascular Hyperpermeability by Histamine Injection

Here, we describe a method to induce vascular hyperpermeability with histamine injection. If using mice with pre-induced inflammatory conditions, such as contact hypersensitivity response, increased vascular leakage should be observed without histamine administration.

1. Prepare a canula as described in Subheading 3.3, **steps 1** and **2**.
2. Insert the cannula into the tail vein and fix it on the tail as described in Subheading 3.3, **steps 3–5**.
3. Inject 400–500 μL fluorescence-conjugated dextran solution as described in Subheading 3.3, **step 6** (*see* **Note 17**). Commence imaging soon after the injection as described in Subheading 3.3, **step 7**.
4. After 10 min of imaging the vasculatures at a steady state, slowly inject 400 μL of the histamine solution.
5. Continue imaging for 30 min (*see* **Note 18**). Example images for steady state and post-histamine injection are shown in Fig. 3.

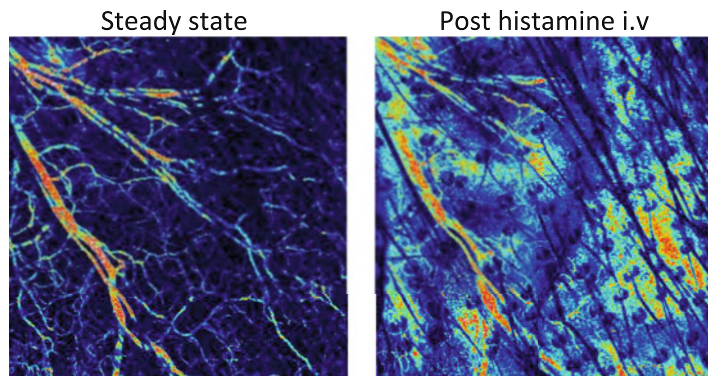

Fig. 3 Sample images. 150-kDa FITC-conjugated dextran was injected via tail vein. Images were acquired at a steady state (left) and 10 min after histamine injection (right)

4 Notes

1. If possible, use albino strains such as BALB/c mice or *Tyr*-deficient mice. Melanin granules cause nonspecific signals and may also produce heat by two-photon laser emission that damages surrounding tissues.
2. The anesthesia is maintained for 1–1.5 h with intraperitoneal administration of pentobarbital sodium. Anesthetic gases such as isoflurane are suitable for longer anesthesia.
3. Hair removal cream for humans is sufficient.
4. An inverted microscope is better suited for the observation of skin than an upright microscope because it is easier to fix the ear on the stage.
5. Fluorescence-conjugated dextran of various sizes (4–2000 kDa) is commercially available. Choose a size of dextran appropriate for your experiment. The retention time of dextran in blood depends on the size of dextran. Dextran larger than 70 kDa will be retained for a longer time (more than several hours) in blood, while dextran smaller than 70 kDa will leak from the blood within 1 h [5]. *See* Table 1 for the molecular sizes of plasma proteins.
6. Fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) are the most widely used fluorescent dye conjugated to dextran.
7. Histamine solution can be kept in 4 °C for a week.
8. Ensure that the hair removal cream does not stay on the ear for more than 5 min. The cream is an irritant and may modify skin inflammation. Even without hair removal, we can observe the vasculature in the ear skin with two-photon microscopy, although the field of view will become limited.
9. If imaging needs to be performed immediately, do not use hair removal cream. Vascular permeability is very easily affected by subtle skin inflammation, which may be caused by the cream.
10. The body temperature of the mouse will drop, particularly after the anesthesia. Keep the mouse on a heating pad and warm it sufficiently.
11. Handle the ear gently to prevent injuries arising from friction.
12. Avoid the formation of bubbles when immersion oil is placed between the objective lens and the cover glass.
13. Do not apply strong pressure to the ear as it may disturb blood and lymph circulation, which are important for intravital imaging studies.
14. The flow rate of isoflurane must be adjusted depending on the body weight of the mouse.

Table 1
The molecular sizes of commercially available FITC-conjugated dextran and some proteins in plasma

Commercially available FITC-dextran	Plasma proteins
4 kDa	
10 kDa	
20 kDa	Cytokines (10–20 kDa)
40 kDa	
70 kDa	Albumin (66 kDa)
150 kDa	Immunoglobulin G (150 kDa)
250 kDa	
500 kDa	
	Immunoglobulin M (990 kDa)
2000 kDa	

15. Second harmonic generation is a kind of autofluorescence phenomenon caused by two-photon laser, in which photons interacting with nonlinear materials, such as collagen fibers, are combined to form new photon with a half wavelength.
16. The longer the length of the polyethylene tube is, the bigger the dead space will be. The tube must be prefilled by heparin solution or PBS to avoid injecting air into the vein.
17. For this purpose, use fluorescence-conjugated dextran larger than 70 kDa. Dextran smaller than 70 kDa will rapidly leak from the blood even without inflammation.
18. In inflammatory states, most of the intravenously injected fluorescent dextran will leak from the blood within 30 min.

Acknowledgments

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Chapter 12

Isotype-Specific Detection of Serum Immunoglobulins Against Allergens

Danielle L. Germundson and Kumi Nagamoto-Combs

Abstract

Type-I hypersensitivity is commonly characterized by increased levels of antigen-specific immunoglobulin (Ig) E. Therefore, it is important for clinical and research investigators to reliably measure serum levels of IgE in allergic patients and animal models. While current ELISA-based methods are simple and commonly performed for the detection of allergen-specific IgE using serum or plasma, they may produce misleading results. This is in part due to decreased sensitivity for IgE in the presence of other Ig isotypes in the same sample, such as IgG, that are typically more abundant than IgE. When assessment of multiple Ig isotypes is necessary, performing optimized assays for individual isotypes requires high sample volumes. Here, we describe an approach to increase the sensitivity for IgE detection while conserving the sample volume needed. This method not only improves the accuracy of serum IgE measurements but also allows simultaneous analysis of other allergen-specific immunoglobulins.

Key words Adsorption, Allergy, Enzyme-linked immunosorbent assay (ELISA), Immunoglobulins, Protein-G

1 Introduction

Allergic sensitization results from inappropriate recognition and subsequent presentation of normally benign substances as harmful antigens. This process is mediated by the major histocompatibility complex II (MHC-II), a receptor complex expressed by professional antigen presenting cells such as dendritic cells and macrophages. MHC-II forms a complex with a processed antigen peptide and presents them to CD4+ T cells, which in turn induce isotype class switching in B cells for antigen-specific immunoglobulin (Ig) E production [1]. The produced IgE bind to the Fcε receptors on mast cells and basophils for rapid release of histamine, cytokines, and other inflammatory factors upon re-exposure to the allergen, causing swelling and redness commonly associated with type-I immediate hypersensitivity reactions [2]. Because serum IgE concentrations are normally very low, testing allergic individuals for

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elevated levels of IgE for particular antigens is useful in identifying offending allergens. This test is commonly practiced in clinics for diagnosis of allergy in addition to skin prick tests [1].

However, detection of other Ig isotypes, especially IgG and IgA, can also provide important information for understanding the development and progression of allergy. While IgG is classically associated with delayed type hypersensitivity reactions, IgG1, IgG2a, and IgG2b subclasses have been shown to potentially contribute to or independently cause anaphylaxis in experimental studies and possibly allergic individuals [3]. In contrast, an increase of serum antigen-specific IgG4 has been implicated in allergy resolution [4]. Additionally, individuals with decreased IgA are thought to be more susceptible to development to various allergies [5]. While the precise roles of these other Igs in allergy development are still elusive, IgE levels alone may not provide a complete measure of humoral responses in allergic individuals.

The enzyme-linked immunosorbent assay (ELISA) has become a widely used method to detect a variety of Ig isotypes over the past decades. However, the assay may produce biased and unreliable outcomes if the presence of allergen-specific IgG is predominant and competes for the same antigen against a relatively small amount of allergen-specific IgE in the same serum sample [6] (Fig. 1). Additionally, when multiple Ig isotype levels are to be measured in smaller animals such as mice, it is often not feasible to dedicate a large volume of serum to carry out the assays for multiple Ig isotypes individually. Therefore, the current ELISA approach may not be optimal in some cases, especially when the Ig isotype of interest is present in low abundance.

Here, we outline a method to separate Ig isotypes from a single serum sample by adsorbing total IgG using magnetic protein G beads to provide a more accurate measure of IgE in the sample by ELISA (Fig. 2). Since each serum sample is highly diluted prior to the separation process, only 6 μL of serum is required to individually assay multiple Igs. Moreover, the adsorbed IgG can be subsequently eluted and used for detection of total or allergen-specific IgG using a standard ELISA protocol with minor modifications.

2 Materials

Use ultrapure water (18.2 Ω) and analytical grade reagents. Prepare and store all reagents at 4 °C unless indicated otherwise. Do not add sodium azide to buffers.

2.1 Preparation of Serum Samples

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4. Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 800 mL water. Adjust pH to 7.4 and add water to a final volume of 1 L. Store at room temperature.

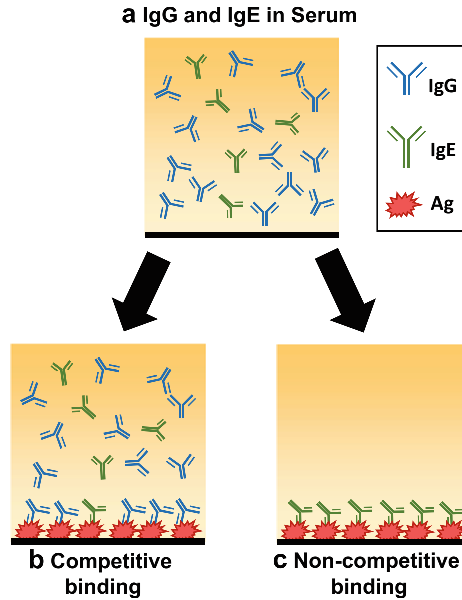

Fig. 1 Potential masking of IgE by IgG during ELISA. IgE is present in a relatively low amount in serum samples compared to IgG (**a**). When untreated serum is used for ELISA, antigen-specific IgG competes for the antigen binding sites (**b**). Pretreatment of serum samples with protein G adsorption removes the IgG, allowing antigen-specific IgE to bind to the antigen (**c**)

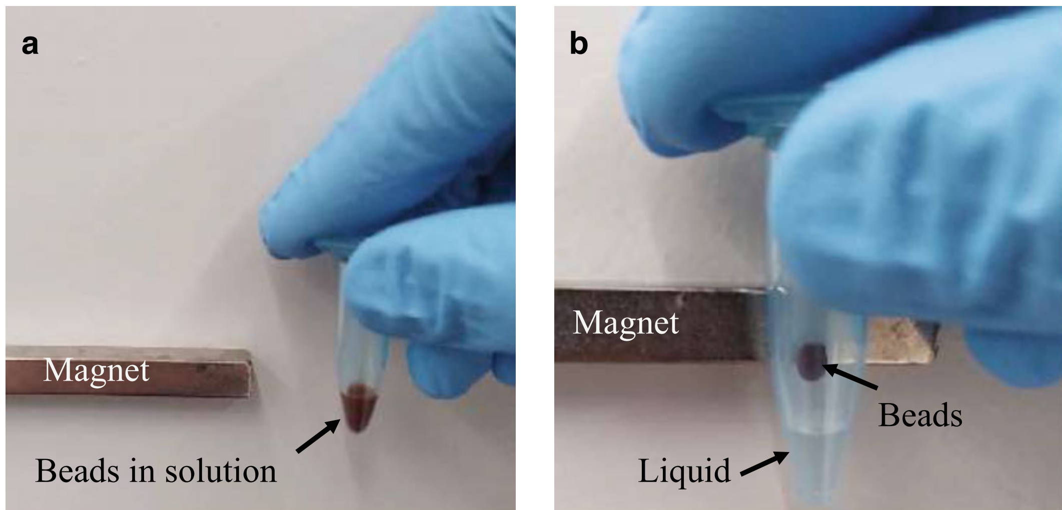

Fig. 2 Separation of protein G beads with a magnet. After vortexing, protein G beads are well suspended in the solution (**a**). With a magnet applied to the side of the tube, the beads become easily separated from the solution (**b**). Remove the solution with a pipette and prepare the beads as described in Subheading 3.1.1

2. Assay buffer: 0.05% (v/v) Tween-20 and 0.5% (w/v) bovine serum albumin (BSA) in PBS, pH 7.4. Add 500 μL of Tween-20 and 5 g of BSA in 1 L of PBS.
3. 0.5-mL Microfuge tubes.
4. Magnetic protein G beads (*see* **Note 1**).
5. Magnet.
6. Vortexer.
7. Elution buffer: 50 mM Glycine-HCl, pH 2.8. Dissolve 0.38 g of glycine in 80 mL of H_2O. Adjust pH and add water to a final volume of 100 mL.
8. Tris buffer: 1 M Tris-HCl, pH 7.5. Dissolve 12.1 g Tris in 80 mL of H_2O. Adjust pH and add water to a final volume 100 mL.

2.2 Antigen-Specific IgE/IgG ELISA

1. High-binding 96-well ELISA plate or strips: clear, flat-bottom.
2. Coating buffer: 44.0 mM $NaHCO_3$, 6.04 mM Na_2CO_3, pH 9.6. Add 3.7 g $NaHCO_3$ and 0.64 g Na_2CO_3 to 1 L of water.
3. 100× Target antigen stock solution: 2 mg/mL in the coating buffer for coating ELISA plate (*see* **Note 2**). Dissolve 10 mg target antigen in 5 mL the coating buffer (*see* **Note 3**).
4. 10-mL Syringe.
5. 0.2-μm Syringe filter.
6. PBS: *See* Subheading 2.1, **item 1**.
7. Wash buffer: 0.05% Tween-20. Add 500 μL of Tween-20 to 1 L of PBS.
8. Assay buffer: *See* Subheading 2.1, **item 2**.
9. Species-specific biotinylated secondary antibody.
10. Horseradish peroxidase-conjugated avidin (HRP-avidin).
11. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution: Commercially available stabilized TMB solution.
12. Stop solution: 2 N H_2SO_4.
13. Micropipettors: multichannel pipettors optional.
14. Pipette tips.
15. Plate sealers.
16. Plate rocker or orbital shaker.
17. Microplate reader capable of reading at 450 nm.

3 Methods

3.1 Preparation of Serum Samples

This section describes a method to separate IgG from serum samples to be used for antigen-specific IgE detection by ELISA. To avoid freezing and thawing of the samples, this brief procedure should be performed when an ELISA plate has been coated and ready to be used, or when a pre-coated ELISA plate is used.

3.1.1 Adsorption of IgG from Diluted Samples

1. If frozen aliquots of serum samples are being used, thaw quickly and keep on ice until diluted in the assay buffer.
2. Dilute 6 μL of each serum sample with 234 μL of the assay buffer to a total volume of 240 μL and set aside (*see* **Note 4**).
3. Vortex magnetic protein G beads in the original container for 1 min and then immediately transfer 50 μL of the beads into a 0.5-mL microfuge tubes. Place a magnet on the side of the tube and wait briefly for beads to aggregate on the tube wall (Fig. 2). Discard the liquid, leaving only the beads in the tube.
4. Add the diluted serum from **step 2** to the beads from **step 3** and close the lid tightly. Vortex briefly for 3 s and place the tube on a rocker to keep beads agitated in suspension for 10 min at room temperature.
5. Place the magnet on the side of the tube and collect the supernatant (Fig. 3a), which is now devoid of IgG (Fig. 3b). Transfer the supernatant in a clean tube and save for allergen-specific IgE ELISA described in Subheading 3.2 (*see* **Note 5**). If performing IgG detection, immediately proceed to Subheading 3.1.2 to elute IgG adsorbed by the beads (*see* **Note 6**).

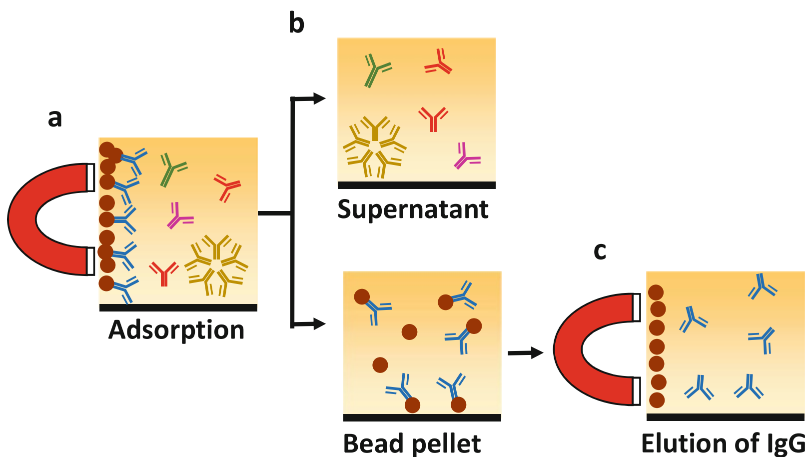

Fig. 3 Adsorption of total immunoglobulin G. A diluted serum sample is added to magnetic protein-G beads. The beads bind to IgG and are subsequently pelleted with a magnet (**a**). The remaining supernatant, which contains IgE but not IgG (**b**), is collected and used for total or antigen-specific IgE ELISA (**b**). Bead-bound IgG is immediately eluted with a low-pH buffer, neutralized, and used for total or antigen-specific IgG ELISA (**c**)

3.1.2 Elution of IgG from Magnetic Beads

1. Immediately add 200 μL of wash buffer to the beads from Subheading 3.1.1, **step 5**. Gently mix by pipetting to avoid foaming.
2. Place the magnet to the side of the tube and discard the wash buffer. Immediately add 25 μL of elution buffer to the beads and gently mix by pipetting. Incubate for 2 min at room temperature.
3. Neutralize the elution buffer with 25 μL of Tris buffer and mix well (*see* **Note 7**). Adjust the volume to 240 μL with the assay buffer and collect the IgG-containing supernatant in a clean microfuge tube (Fig. 3c). Keep on ice until proceeding to antigen-specific IgG ELISA described in Subheading 3.2.

3.2 Detection of Antigen-Specific IgE or IgG with ELISA

3.2.1 Preparation of ELISA Plate with an Antigen

1. If a nonsterile 96-well ELISA plate or 8-well ELISA strips are used, sterilize under ultraviolet light in a biological safety cabinet (*see* **Note 8**).
2. Dilute the 100× antigen stock solution with coating buffer to make 1× antigen-coating solution. For enough 1× antigen-coating solution for one 96-well plate, dilute 100 μL of the 100× antigen stock solution in 10 mL of the coating buffer. Sterile filter the 1× antigen-coating solution using a 0.2-μm filter attached to a syringe.
3. Pipette 100 μL of the 1× antigen-coating solution to all wells. Seal the plate and incubate on a rocker with gentle agitation overnight at 4 °C.
4. Remove the antigen-coating solution and rinse wells with 300 μL of the wash buffer three times, 1 min each. Blot dry between the rinses by tapping the inverted plate on an absorbent surface, such as a stack of paper towels (*see* **Note 9**).
5. Place 200 μL assay buffer in each well to block nonspecific binding. Seal the plate and incubate on a rocker for 2 h at room temperature.
6. Remove the assay buffer from the wells and place 100 μL of prepared samples from Subheading 3.1.1 (for IgE) or Subheading 3.1.2 (for IgG) in their designated wells (*see* **Note 10**). Seal plate and incubate overnight at 4 °C.

3.2.2 Colorimetric Reaction and Plate Reading

1. Aspirate serum samples and wash wells with 300 μL of wash buffer three times, 1 min each. Blot dry between the rinses as described in Subheading 3.2.1, **step 4**. Place 100 μL of a biotinylated secondary antibody at a working concentration in each well (*see* **Note 11**). Seal the plate and incubate on a rocker with gentle agitation for 2 h at room temperature.

2. Remove the secondary antibody solution and rinse wells with wash buffer four times, 1 min each. Blot dry between the rinses. Add 100 μL of HRP-avidin at a working concentration to each well (*see* **Note 11**). Seal the plate, protect from light, and incubate on a rocker with gentle agitation for 1 h at room temperature.
3. Remove HRP-avidin and rinse the wells with the wash buffer three times, 1 min each.
4. Place 100 μL of TMB substrate solution (*see* **Note 11**) to each well and incubate on a rocker for 20–30 min, protected from light (*see* **Note 12**).
5. Stop the color reaction with 100 μL of the stop solution and immediately read the absorbance at 450 nm on a microplate reader. If possible, additionally read with a reference wavelength at 540–570 nm (*see* **Note 13**). Example of results are shown in Fig. 4.

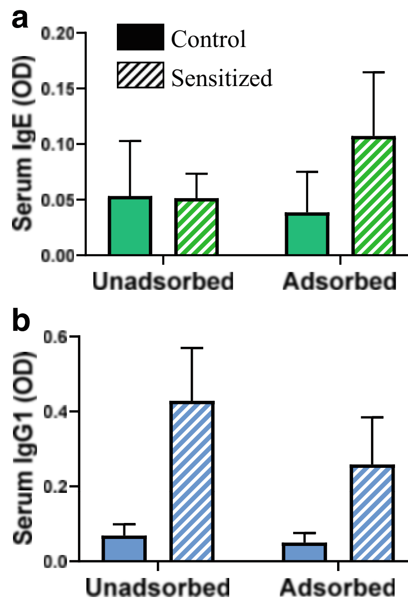

Fig. 4 Comparison of allergen-specific IgE and IgG1 ELISAs using adsorbed and unadsorbed serum samples. Male C57BL6/J mice were orally sensitized to a cow's milk protein, β-lactoglobulin. Allergen-specific IgE and IgG1 ELISAs comparing the sera from sham control and sensitized mice were performed without or with the IgG adsorption pretreatment. The difference in the IgE levels between the control and sensitized mice was more evident with the pretreatment (**a**) while the relative differences observed for IgG1 with the eluted sera remained unchanged (**b**). Mean ± SEM, $n = 5$. *OD* optical density

4 Notes

1. This protocol describes the use of magnetic protein G beads, which bind to all IgG isotypes with high affinity. However, other protein bead options, such as protein A beads, that show variable affinities to other antibody isotypes are also available [7].
2. An important factor in coating the plate adequately with a given antigen is the pH of the buffer used. The pH of the buffer facilitates the attachment of the antigen to the microplate and thus has a significant effect on the strength of the color reaction. The most common buffer used is a bicarbonate buffer (pH 9.6), although PBS (pH 7.4) is occasionally used. If the color reaction is weak, adjusting the pH of the coating buffer may facilitate better binding of some antigens. Optimal coating conditions should ultimately be determined for specific antigens used by individual laboratories.
3. The antigen concentration of 2 μg/mL (using the 1× stock solution) has been optimized for the detection of IgE against a purified bovine milk whey protein, β-lactoglobulin (Bos d 5) [8]. Optimal coating concentrations of other antigens may vary and therefore need to be determined by individual laboratories. For example, we have used 20 μg/mL to coat plates with bovine whey proteins [9], while others have used up to 50 μg/mL of antigens to coat plates with shrimp or peanut extract [6].
4. While 6 μL of serum is a sufficient volume for the detection of IgE against β-lactoglobulin in our orally sensitized mouse model of milk allergy, a greater amount of sera may be necessary depending on the mouse model being investigated. The optimal serum concentration for ELISA should ultimately be determined by each laboratory.
5. These aliquots may be kept on ice until the IgG elution procedure is completed. Avoid repetitive freezing and thawing of the samples.
6. If adsorbed IgG needs to be eluted from the beads, immediately proceed to Subheading 3.1.2 and add wash buffer to the remaining beads. Do not leave the beads to dry.
7. With a short incubation time, a repeating pipettor is a convenient tool to add neutralization buffer quickly.
8. We perform ELISA plate coating under a sterile condition to avoid potential microbial growth during the coating and sample incubation processes. Microbial growth can alter Ig binding and the later developement of the color reaction.

9. Care should be taken to remove as much of the wash buffer as possible without completely drying out the wells. Firmly tapping the plate 3–4 times on a stack of paper towels laid on a laboratory bench should be sufficient.
10. The supernatants from the protein G adsorption should yield enough (approximately 200 μL) to run each sample in duplicates. While the postadsorption supernatant can also be used to detect serum IgA, it should not be used for IgM ELISA since this isotype is partially absorbed by protein G.
11. Use working concentrations of secondary antibodies and HRP-avidin as suggested by the suppliers. However, optimal concentrations for these reagents to detect each Ig should ultimately be determined by individual laboratories.
12. For an initial reaction, periodically inspect the development of the yellow reaction product to avoid color saturation.
13. The reference wavelength can help to correct for optical imperfections in the wells, and the reading can be subtracted from the measurements at 450 nm.

Acknowledgments

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Chapter 13

An Overview of Flow Cytometry: Its Principles and Applications in Allergic Disease Research

Taylor Schmit, Mitchell Klomp, and M. Nadeem Khan

Abstract

Flow cytometry is a popular technique used for both clinical and research purposes. It involves laser-based technology to characterize cells based on size, shape, and complexity. Additionally, flow cytometers are equipped with the ability to take fluorescence measurements at multiple wavelengths. This capability makes the flow cytometer a practical resource in the utilization of fluorescently conjugated antibodies, fluorescent proteins, DNA binding dyes, viability dyes, and ion indicator dyes. As the technology advances, the number of parameters a flow cytometer can measure has increased tremendously, and now some has the capacity to analyze 30–50 or more parameters on a single cell. Here, we describe the basic principles involved in the mechanics and procedures of flow cytometry along with an insight into applications of flow cytometry techniques for biomedical and allergic disease research.

Key words Fluorescence, Compensation, Light scatter, Immunophenotyping, FACS

1 Introduction

Flow cytometry, or sometimes also referred to as fluorescence-activated cell sorting (FACS), is a cutting-edge methodology used to characterize cellular and molecular phenotypes of diverse cell populations in a single-cell suspension. FACS is a flow cytometer additionally equipped with the capacity to physically sort cells based on their specific cellular and molecular phenotypes. First developed in the 1950s, flow cytometry has now advanced to allow simultaneous analysis of more than 30 parameters in a sample, making the method one of the most powerful tools in biomedical research. The multiparametric analysis of single cells by flow cytometry is enabled by labeling of cells with fluorophore-conjugated monoclonal antibodies against specific molecules expressed by the cells of interest. Initially used primarily for the detection of surface antigens, the use of the flow cytometer has expanded to determine various aspects of cell functions, including, but not limited to, intracellular proteins, cellular viability, proliferation, and enzymatic activity in addition to

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their shapes, sizes, and granularity. In this chapter, we will present an overview of flow cytometry and its application for immune cell phenotyping in allergic disease research. A more detailed, step-by-step protocol is provided in our subsequent chapter.

2 Principles of Flow Cytometry

Flow cytometers contain three main components: fluidics, optics, and electronics systems. The fluidics system allows transportation of cells in a suspension from the sample tube to the flow cell. When a sample tube, often referred to as FACS tube, is placed in a flow cytometer, the sample becomes mixed with saline solution, called sheath fluid, and the cells are taken up through the nozzle into the flow cell. The sample and sheath fluid each creates a stream running side by side in the same direction. As the sample cells move through the flow cell, they become aligned into a single file via a process called hydrodynamic focusing, one of the most important features of flow cytometers, creating what is called laminar flow. Sheath fluid, flowing at a higher velocity and in the same direction as a slower moving sample stream, focuses the cells as they move from the fluidics system through the optics system of the flow cytometer one at a time (Fig. 1) [1]. It is by this process that we can obtain the phenotype of a single cell in a heterogenous solution.

The components of the optical system include excitation light sources, lenses, and filters that collect the emitted light en route to various detectors. Once through the flow cell, individual cells pass through laser beams, each of which can detect a certain number of

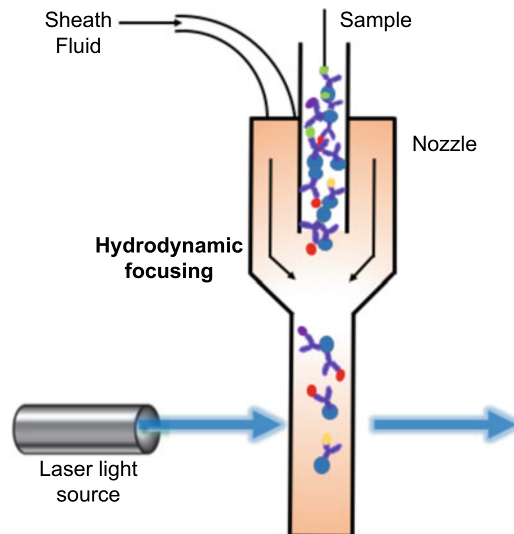

Fig. 1 Fluidics system of a flow cytometer. The fluidics system focuses cells into a single-file fashion by the process of hydrodynamic focusing

fluorescence wavelengths. The light emitted by each cell is then captured by detectors and converted into information regarding size, internal complexity, and antigen presence as measurable parameters [2]. Forward scattering of light (forward scatter or FSC) after hitting a cell is sensed by a detector in front of the light source and measured as a parameter to determine the size of the cell. Side scattering of the light (side scatter or SSC) by a cell is sensed by a detector placed perpendicular to the forward scatter detector. SSC is proportional to the granularity and complexity of cells and serves as another parameter for cell phenotyping [3]. When the cells are labeled with fluorescent molecules, such as fluorophore-conjugated antibodies, the emitted light from the excited molecules is detected through fluorescence filters for specified wavelengths and recorded as another parameter for antigen presence. The capacity of a flow cytometer to analyze multiple parameters therefore depends on the number of lasers and filters the machine is equipped with.

With the electronics system of a flow cytometer, photonic measurements by the detectors are converted into voltages, which reflect the intensities of the lights emitted. Each of the detectors sends photocurrents that are digitized and processed into numeric values and recorded by the electronic system. The time delays between detectors ensure that the signals from multiple lasers are attributed to the correct events [4]. Typically, data from at least 10,000–100,000 events are collected per sample and stored by the software that supports the operation of the flow cytometer. The data are saved and subsequently analyzed using a specialized analysis software tool.

3 Designing an Antibody Panel for Flow Cytometry

As previously mentioned, newer flow cytometers have the capability to measure 30 or more fluorophores on one cell at a time in a single read. However, this multiparametric assessment of cell phenotyping relies on the availability of fluorophore-conjugated antibodies to the cell markers as much as the number and type of lasers and fluorescence filters equipped in the flow cytometer. To take advantage of a flow cytometer for detection of multiple fluorophores in a single-cell suspension, cells in a sample are first incubated or "stained" with a mixture of fluorophore-conjugated antibodies specific to antigens or epitopes present on the sample cells. This mixture of antibodies, also referred to as a "panel," must be carefully designed to encompass all of the markers required for cell type differentiation in your experiment while choosing fluorophores that complement each other and minimize spectral overlap of fluorescence signals. The following sections describe some of the important factors that should be considered when designing antibody panels for multiparametric analysis.

3.1 Machine Laser Configuration

It is important to be familiar with which lasers are equipped in your flow cytometer and how they are aligned. Most manufacturers of flow cytometers offer layouts of the lasers with excitation and emission wavelengths for each fluorophore. This information helps to determine where your selected fluorophores emit lights and what fluorophores your flow cytometer is compatible with. For instance, if a flow cytometer has 4 lasers, the ideal 4-color panel would include one color from each laser, as it allows minimal spectral overlap. The inclusion of more colors in the panel will require choosing more than one color from each laser. Using an online resource, such as the BioLegend Fluorescence Spectra Analyzer, can help in assessing the potential spectral overlap of your selected fluorophores.

3.2 Expression Level of Antigens

The abundance of target antigens in your samples can vary due to cell activation and functional differences. When choosing fluorophore-conjugated antibodies to target a mixture of antigens with low, medium, and high expression levels, you must consider these differences to balance the fluorescence signals of the antibodies. For example, if an antigen of interest is expressed more abundantly than other antigens in your samples (e.g., CD4 and CD8 on T cells), a dimmer fluorophore, such as Pacific Blue or Alexa Flour 700, should be selected for the antibody that targets the antigen. In contrast, brighter fluorophores, such as PE, Brilliant Violet, and APC, should be reserved for the antibodies that detect low expressing molecules such as cytokines [5].

3.3 Cell Viability Marker

It is important to include a cell viability dye in the panel to exclude dead cells from the data. This is important for many reasons, including that dead cells within a sample can aggregate, absorb dyes non-specifically, and autofluoresce, skewing sample results. If cells are to be analyzed on a flow cytometer immediately after preparation without requiring fixation, exclusion dyes such as Propidium Iodide or DAPI can be used [6]. If fixation is required, or subsequent intracellular cytokine staining is to be performed, there are a host of fixable viability dyes or amine-reactive dyes available, such as ViViD, Aqua Blue, Zombie dyes™ (BioLegend), Ghost Dyes™ (TONBO Biosciences), or eFluor® Fixable Viability Dyes (eBiosciences) [7]. Upon inclusion of a viability dye in your samples, you must therefore take the wavelength of the dye into account when designing your antibody panel.

4 Antibody Titration

Once an antibody panel has been designed and the fluorophores have been selected, the antibodies in the antibody panel must be titrated to determine the optimal concentrations before using them

for your experimental samples. An optimal antibody titration creates maximal separation between stained and unstained populations, minimizing false-positive and false-negative data. If an excess amount of a particular antibody is present in the antibody panel, the fluorescence signal from the antibody can bleed into other channels and cause false positives. On the other hand, if the amount of an antibody is not sufficient in the panel, the target antigen is underrepresented by the antibody and leads to a false-negative result. Additionally, titrating antibodies help in determining the brightness of the fluorophore by calculation of the Signal-to-Noise ratio or the Stain Index. The Signal-to-Noise ratio allows accurate detection of the differences between stained and unstained cell populations by assessing the mean fluorescence intensity (MFI) of the fluorescence positive cells and dividing this number by the MFI of the fluorescence negative cells. The Stain Index is much like calculating the Signal-to-Noise ratio, but also takes into account the intensity distribution of the unstained (fluorescence negative) cell population [8]. Because the width of the unstained cell population affects the separation of the fluorescence negative and positive cell populations, the Stain Index is the recommended statistic for assessing fluorophore brightness and population separation [9]. Once an antibody is titrated, it does not require re-titration for the next experiment as long as the product from the same lot or batch is used.

For titration, the antibodies in the panel are serially diluted and used to stain cell samples or beads. Ideally, antibodies are titrated on the tissue or sample that is to be used in your experiment. The cells or beads stained with different concentrations of each antibody are subsequently run on a cytometer at photomultiplier voltage (PMT) values. Because the sample is stained with one fluorophore, compensation does not need to be performed. The Stain Index is then determined for each antibody dilution using the following formula:

$$\text{Stain Index} = \frac{\text{MFI}_{\text{pos}} - \text{MFI}_{\text{neg}}}{2 \times \text{SD}_{\text{neg}}}$$

The top row of the formula, $\text{MFI}_{\text{pos}} - \text{MFI}_{\text{neg}}$, represents the difference (D) in the median fluorescence intensity of the positive and negative (stained and unstained) cell populations [10]. Because the Stain Index takes into account the width (W) of the peak of the negative cell population, the difference between the two medians is divided by two times the standard deviation of the negative population, also known as the robust standard deviation or rSD (Fig. 2). The dilution with the highest stain index indicates maximum spread between positive and negative cell populations.

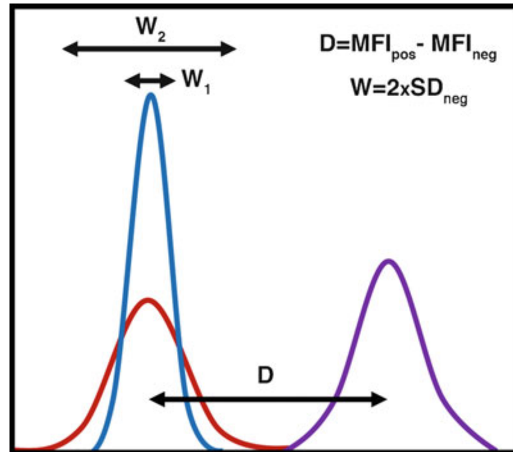

Fig. 2 Schematic representation of a positive and negative cell populations. A positive cell population (purple line) and two negative cell populations (blue and red line) have equal Signal-to-Noise ratios but different Stain Indexes

5 Compensation

If more than one dye is used simultaneously in flow cytometry, the potential problem of fluorescence spillover arises. This is when the signal for one fluorescent dye bleeds into the channel of another dye. The spillover, or spectral overlap, occurs because the fluorescence emission spectrum of each fluorophore has a wider wavelength range than what the optical filters in flow cytometers measure, causing one fluorophore to be detected in multiple detectors (Fig. 3) [4, 11]. This can lead to false-positive populations, messy graphs, and inconsistent or unreliable data. This spillover is corrected by a process called "compensation," which is the mathematical correction of fluorescence spillover by removing a percentage of the total signal from each detector. The need for compensation increases as the number of fluorophores in a panel increases. There are a variety of programs available for use to calculate the compensation settings of an antibody panel, including FLOWJO™. Compensation uses single-stain controls of each fluorophore in your experiment, and the signal of each fluorophore is calculated in its own detector and in all other detectors that will be used for the antibody panel (e.g., FITC in PE). An example of compensation between FITC and PE is shown in Fig. 4. In the left panel ("Uncompensated"), a group of cells detected by both FITC and PE detectors is present (P3). After compensation, however, the fluorescence signal of FITC detected by the PE detector is subtracted from the total fluorescence detected by the PE detector, resulting in a distinct P3 (Fig. 4). Accurate compensation of a flow cytometry panel is therefore crucial and depends on the quality of

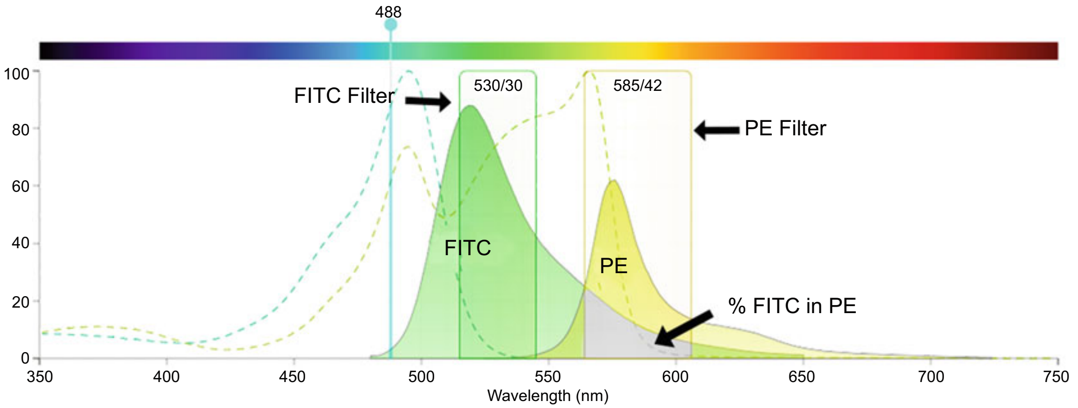

Fig. 3 Spectral overlap. Excitation (dashed line) and emission (solid line) spectrum of FITC (green) and PE (yellow). The gray color represents the amount of FITC signal that overlaps into the PE detector. This signal is removed or "subtracted" from the total signal in the PE detector by calculating compensation

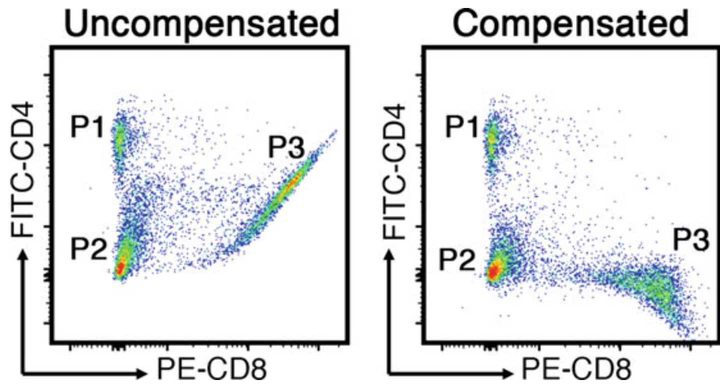

Fig. 4 Compensation example. Prior to compensation, the presence of FITC into the PE filter is noticeable by observing the smear that is population P3. After compensation, the presence of FITC in the PE detector is subtracted and the result is 3 distinct cell populations

the controls used while compensating. A few of the most important controls to consider are discussed in Section 8.

6 Sample Preparation

Sample preparation is the final step following the validation of the antibody panel. Because the specimen must pass through the flow cell in a single-file fashion, the cells in the sample suspension must be well dispersed for flow cytometry. While this is not an issue for cells isolated from blood or bone marrow samples, preparation of

single-cell suspensions from composite (solid) animal tissues, such as lungs, spleens, and liver, must be performed thoroughly. Tissues that are dense in fibrous proteins, like collagen, begin with a mincing step using a scalpel, blade, or scissors. These samples are subsequently incubated with digestive enzymes, for example, collagenase, dispase, or hyaluronidase, to breakdown the extracellular matrix [12]. Tissues that are not dense in fibrous proteins, such as the spleen, do not require this enzymatic digestion step. An additional important enzyme is DNase I that degrades DNA and, therefore, prevents aggregation of cells. Interestingly, some enzymes, including DNase and collagenase, have been found to reduce the expression level of certain epitopes. Notably, CD4, CD44, and CD8 on T lymphocytes were reduced while other important markers of T lymphocytes (e.g., TCR, CD3) were unaffected [13]. This observation stresses the importance of enzyme purity and enzyme concentration optimization by each laboratory. Following enzymatic digestion, these tissues are mechanically dissociated, passed through a 70-μm filter, and treated with a red blood cell lysis buffer for the removal of red blood cells. Finally, the number of viable cells is counted using a dye exclusion method such as trypan blue. The goal of sample preparation is to obtain a single-cell suspension with high viability while preventing the loss or alteration of cell-surface markers. Once a viable single-cell suspension is prepared from an organ or tissue of interest, the sample is ready for surface staining, intracellular cytokine staining, or a cell-based assay that involves stimulating the cells with an antigen or protein and staining the cells for flow cytometry.

7 Gating Strategies

The term "gating" in flow cytometry refers to identifying a group of cells that exhibit common parameters and defining them as a distinct population of cells. These parameters to characterize particular types of cells include cell size (FSC), complexity or granularity (SSC), viability, singularity, and antigen presence [14, 15]. One of the most common manual gating strategies used in flow cytometry is FSC vs. SSC. This gating strategy allows the removal of dead cells (Fig. 5a) and the visualization of different cell populations within a sample (Fig. 5b). Single-parameter or double-parameter gating can be performed from within each distinct population (Fig. 5c) or from the entire sample (Fig. 5d). Additionally, a strategy referred to as "backgating" can be used to visualize the presence of an antigen or protein by gating the parameter of interest within the entire sample and plotting it on FSCA vs. SSCA (Fig. 5d). Alternative to manual gating, there has been an increased interest in the automatization of the gating process in flow cytometry using computer gating software. This

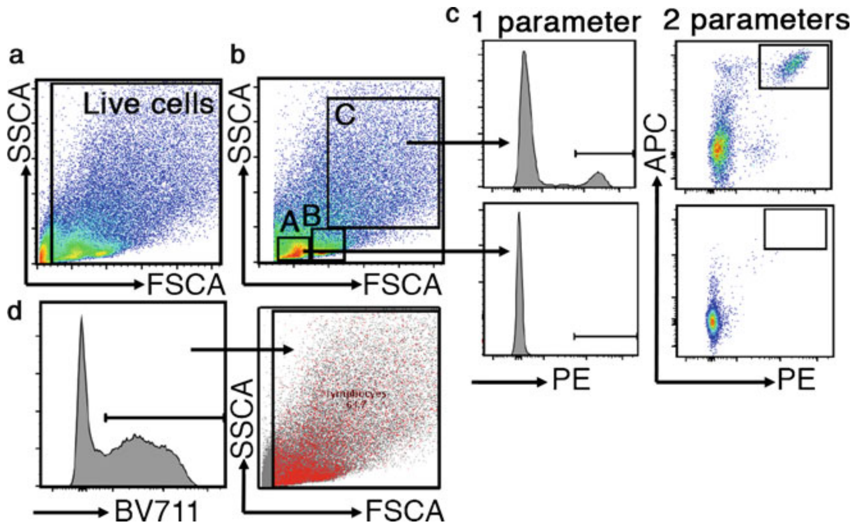

Fig. 5 Manual gating strategies for flow cytometry. (**a**) Dead cells/debris are removed by gating all cells except the bottom left corner of the dot plot. (**b**) Different cell populations can be visualized utilizing a FSCA vs. SSCA dot plot that includes lymphocytes (A) monocytes (B) and granulocytes (C). (**c**) Single-parameter histogram of PE from within populations A and C shows no presence in population A and a presence in population C. Double-parameter gating of PE and APC within populations A and C show a clear double-positive population in population C that is not present in population A. (**d**) Single-parameter and double-parameter gating of all cells in a sample can be backgated to show from what cell populations they arise

software, often open access, uses single-stain and fluorescence-minus-one controls to make gates on all types and variations of cell populations included in an antibody panel. However, these gates often need to be cross-checked and fine-tuned for gating accuracy [15].

8 Controls

When analyzing samples on a flow cytometer, appropriate controls must be included to identify false-positive/negative signals. Some commonly used controls and their use are described below.

8.1 Unstained Control

An unstained control is useful for determining the autofluorescence or background fluorescence present within the sample. This is especially true if sample cells are treated in vitro or if the sample contains a large number of granular cell types (e.g., macrophages, dendritic cells) [16]. The best way to accommodate for autofluorescence is to be sure that the unstained population used during compensation is the same cell type as the stained population (cell type to cell type, cells to cells, beads to beads). A recommended method is to use compensation beads that are equipped with an equal amount of autofluorescence [17].

8.2 Single-Stain Control

Single-stain controls are used in determining the spectral overlap or spillover of one fluorophore into the detector of another fluorophore. Hence, they are important tools for compensation (*see* Subheading 5). Importantly, not all fluorophores are the same and, when compensating, the same antibody that is used in your experiment must be used for single-stain controls. For example, FITC and GFP will both fluoresce at the same wavelength but will not have the same background or spillover into other channels. Single-stain controls must also be as bright as, if not brighter than, the experimental samples. If using cells instead of compensation beads, it is a good idea to use cells that are treated or stimulated to increase the amount of target antigen (i.e., using cells from the experimental group as compared to the control group) [18].

8.3 Isotype Control

An isotype control is used when determining background caused by non-specific binding of an antibody. An isotype control should be an immunoglobulin of the same class as the primary antibody used in the antibody panel. It should also have the same fluorophore and does not target an antigen present in the sample. It is important to note that isotype controls should not be used for applying gates on a parameter or distinguishing between positive and negative populations. A better control for applying gates is a fluorescence-minus-one control [19].

8.4 Fluorescence-Minus-One (FMO)

An FMO refers to a sample that was prepared and stained under the same conditions as the experimental samples but lacks one fluorophore in its antibody panel. Unlike background induced by non-specific binding (isotype control) or autofluorescence (unstained control), an FMO accounts for fluorescence background caused by spillover from other fluorophores in the panel and is an essential control in the use of multi-parametric flow cytometry [20]. Because an FMO is the only control that accounts for fluorescence spillover, it is encouraged to use this control when applying gates during analysis.

9 Applications

Flow cytometry is a powerful tool in research as well as in clinical diagnostics and pharmacology. While it is considered a viable experimental method in nearly all branches of biology, the technology is particularly useful in the area of immunology, including allergy research. One of the primary applications of flow cytometry is immunophenotyping. Immunophenotyping is a process in which distinct populations of cells are identified by fluorophore-conjugated antibodies against cell-surface or intracellular immunological markers and their numbers are quantified [21]. For example, helper T cells can be distinguished from cytotoxic T cells by their

expression of $CD4^+$ or $CD8^+$ on their cell surfaces to differentiate between T cell functions. In addition, specific subsets of T helper cells (T_h1, T_h2, T_h17, T_{fh}, T_{reg}, etc.) can be further classified and quantified from single-cell populations. Moreover, if cells are permeabilized with a mild detergent prior to staining, immunophenotyping can also be applied to intracellular proteins [22]. Quantification of transcription factors (e.g., phosphorylated STAT), intracellular cytokines, heat shock proteins, or other intracellular proteins can be determined with minor modifications to staining protocols to assess differences in cellular functions between control and diseased subjects [23, 24].

In allergy research, a fluorescence-activated cell sorting, or FACS, is also valuable. For FACS, individual cells are treated in the same manner as conventional flow cytometry, except that each individual cell is given an electronic charge as it passes through the flow cell. This charge is dependent on the fluorescence inside of the liquid droplet encompassing the cell. Once the droplet leaves the nozzle of the flow cytometer, the droplet moves between deflection plates that either attract or repel electronic charges [25]. Depending on the property of the electronic charge, the droplet is repelled or attracted in one direction and subsequently discarded or collected as a cell type of interest and used in cell culture experiments, adoptive transfer experiments, or other plethora of applications. FACS procedure, however, must be performed under aseptic conditions to prevent contamination of cells for downstream use.

Flow cytometry is also useful for assessing cell proliferation and cell cycle analysis. Cell proliferation in response to inflammatory stimuli, such as allergens, and the extent by which the stimulated cells proliferate are important parameters in allergy research. Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent dye that is readily taken up by cells and binds to intracellular lysine residues on proteins inside of cells. As cells undergo mitosis, the relative fluorescence intensity of CSFE is halved for each daughter cell that is produced. The fluorescence of CSFE stain can be measured by a flow cytometer and, when plotted as a histogram, each new mitotic cycle produces a distinct peak as the fluorescence intensity lessens. Over generations of daughter cells, the relative intensity of CSFE continues to dwindle and the respectively produced peaks represent how readily a population of cells has proliferated (Fig. 6). Similarly, intracellular dyes that stain for each of the four distinct cell cycles can be used to determine the current state of cell division.

Cell death, such as apoptosis, necroptosis, or pyroptosis, can also be determined, analyzed, and quantified via flow cytometry. Phosphatidylserine, a phospholipid that resides in the inner leaflet of cell membranes, begins to flip to the outer membrane of apoptotic cells. Annexin V binds to phosphatidylserine and, thus, the relative intensity of Annexin V in a given sample correlates with

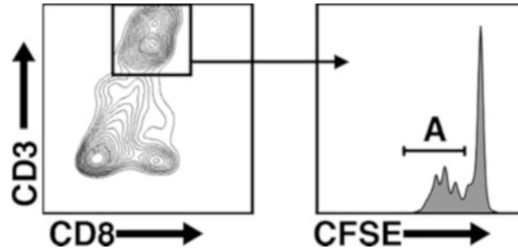

Fig. 6 Changes in CFSE signals with cell proliferation. CFSE-labeled CD3 + CD8+ lymphocytes incubated for 72 h show 3 peaks with decreased levels of CFSE expression representing 3 generations of cell proliferation

apoptotic cells. Propidium iodide (PI) can be costained with Annexin V to distinguish apoptotic cells from necrotic cells as PI can enter necrotic cells but is excluded from apoptotic cells [26]. 7-Aminoactinomycin D (7-AAD) intercalates with guanine–cytosine-rich regions of DNA. 7-AAD does not readily pass through intact membranes, but it can pass through the weak or "leaky" membranes of apoptotic or necrotic cells and bind to their DNA. Cell death can be characterized by quantifying the relative intensity of 7-AAD signals increased in dead or dying cells.

Flow cytometry can also be utilized for in vitro studies, in which plasmids, expressing fluorescent markers, are transfected into cultured cells. The efficiency of transfection as well as the expression of reporter genes can be assessed by quantifying fluorescence proteins expressed by successfully transfected cells. Commonly used fluorescent markers include green fluorescent protein (GFP), enhanced green fluorescent protein, cyan fluorescent protein, yellow fluorescent protein, red fluorescent protein, and mCHERRY. Ex vivo phagocytosis can be conducted by coating the test particle (i.e., bacteria, allergen) with a dye that is minimally fluorescent at neutral or high pH and is extremely fluorescent at low or acidic pH such as in the phagosome. Using this method, the fluorescence, caused by phagocytosis of the fluorescently labeled particles, can be observed via a flow cytometer [27]. In the case of in vivo studies, CFSE-labeled bacteria or allergens can be used to assess dissemination by analyzing the fluorescence in different organs. Phagocytosis assays are important supporting evidence when studying host response or antibody function and there are now ways to utilize flow cytometry to conduct in vivo phagocytosis assays of allergens or pathogens. 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFDA/SE) is the diacetate form of CFDA that can pass through cell membranes and label viable bacteria. After entering the cell, the molecule is converted to CFSE. This method has been described to successfully observe phagocytized bacteria by analyzing fluorescence using the FITC filter on a flow cytometer [27, 28]. Additionally, GFP-tagged

mouse reporter strains are readily available and can be used to observe cell recruitment, protein expression on specific cell types, transcription factor activation, etc.

Multiplexed Bead Arrays are increasing in popularity as they allow the simultaneous measurement of 30–50+ parameters using just two lasers. These arrays use beads that are pre-coated with antibodies to specific proteins or nucleic acids. Beads are differentiated based on their differing sizes and levels of fluorescence, allowing the detection of multiple analytes [29]. For allergy studies, these arrays can be useful for the detection of multiple cytokines in bronchiolar or peritoneal lavage supernatants, serum, and tissue homogenate supernatants. Additionally, arrays that can quantitate the presence of various class and sub-class types of antibodies exist, and they are especially useful in studying mouse models of allergic disease.

As discussed here, flow cytometry is a versatile tool and its applications continue to expand as the equipment technology advances and diverse fluorophores and antibodies are developed. It is one of the most employed methodologies in immunological research and immune cell phenotyping in animal models of allergic disease.

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Chapter 14

The Application of Flow Cytometry for Simultaneous and Multi-parametric Analysis of Heterogenous Cell Populations in Basic and Clinical Research

Taylor Schmit, Mitchell Klomp, and M. Nadeem Khan

Abstract

The use of flow cytometry allows simultaneous measurement and multiparametric analysis of single cells in a heterogenous solution. The purpose of flow cytometry can vary depending on the use of antibodies and dyes targeted for specific cell molecules. The method of immune-phenotyping with fluorescently conjugated antibodies to label cell proteins or DNA works in tandem with fluidic, optic, and electrical systems present in the flow cytometer. Some flow cytometers can detect numerous fluorescent molecules on a single cell, allowing the measurement of more than 30 parameters. This ability to detect, measure, and quantitate multiple fluorescent markers on a single cell makes the flow cytometer a useful tool for analyzing various aspects of cell phenotype and function. Here we describe a standardized protocol for surface and intracellular immune-phenotyping of murine lungs, beginning with the building of an optimal antibody panel and ending with data analysis and representation, including sample gating strategies for innate and adaptive immune responses.

Key words Flow cytometry, Flow cytometer, Cell-staining, Cell antibody staining, Cell surface staining, FACS

1 Introduction

Flow cytometry is a useful research tool, which enables phenotyping of various cell types in a single heterogeneous cell suspension based on the expression of cell-surface or intracellular molecules. Flow-cytometric analysis of single cells is preceded by the labeling of cells with fluorophore-conjugated monoclonal antibodies against specific molecules expressed by the cells of interest. Initially used primarily for the detection of surface antigens, the use of flow cytometry has expanded to determine various cellular phenotypes, including but not limited to the expression of intracellular proteins, cellular viability, proliferation, and enzymatic activity [1]. All of these applications make flow cytometry an immensely powerful tool in science and medicine.

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Successful use of flow cytometry for cell phenotyping can provide an extensive amount of data when performed properly with a detailed and well-planned protocol. Flow cytometry begins with designing an optimal antibody panel that contains fluorophores that can be detected with your flow cytometer, minimizes spectral overlap, and encompasses as many antigens or cell-specific epitopes as possible. Once an optimal panel is designed, a single-cell suspension is prepared from a sample and stained with the antibodies. All newly acquired antibodies are titrated to determine their dilutions that allow maximum distance between stained and unstained populations. After optimal antibody dilutions are determined, a process called "compensation" is performed on the flow cytometer to minimize fluorescent or spectral overlap. After sample staining, events can be acquired and analyzed with proper software for detailed multiparametric phenotyping of cells in a heterogenous mixture.

Here we describe in detail our laboratory's complete flow cytometric procedure, beginning with designing optimal antibody panels and concluding with gating and analysis of acquired events. Although this protocol can be extrapolated to phenotype a variety of cells from clinical or experimental tissue or blood samples, it has been modified for this chapter to provide a specific methodology for lung immune cell surface phenotyping in animal models. Additional modifications are provided in Subheading 4.

2 Materials

Use distilled or ultrapure water and sterile reagents. Prepare and store all reagents on ice during use or at 4 °C, unless indicated otherwise. Perform all steps in a sterile hood when applicable.

2.1 Antibody Panel Design

1. Layout of laser configuration/fluorophore capability of your flow cytometer.
2. Fluorochrome reference chart: a reference for available fluorochromes with wavelength, lasers, filters, detectors, brightness (e.g., BD Biosciences Fluorochrome/Laser Reference Poster).
3. List of desired epitopes and antigen expression levels on cells.

2.2 Preparation of Mouse Lungs

1. Lung tissue from mice.
2. Sterile phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS).
3. Cell strainers 70-μm.
4. A 5-mL syringe.
5. Petri dish.
6. Conical tubes of 15-mL.

7. ACK (ammonium-chloride-potassium) lysing buffer: 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4, or commercially available [2].
8. Digestion buffer: sterile PBS containing 10% FBS, 10 mM HEPES, 1 mg/mL collagenase type IV and 0.5 mg/mL DNase I (enzymes added on the day of sample preparation).
9. Surgical scissors and forceps.
10. Trypan blue solution 0.4%.
11. Cell counter: electronic counter or hemocytometer.
12. Centrifuge.

2.3 Antibody Titration

1. Anti-mouse antibodies against target epitopes: Conjugated to fluorophores compatible with your flow cytometer.
2. PBS containing 2% FBS.
3. FACS tubes or 6-well v-bottom plate: 5.0-mL round-bottom polystyrene or polypropylene tubes.
4. Data analysis software: FlowJo (FlowJo, LLC) or equivalent.

2.4 Cell Staining

1. PBS.
2. Sterile 2% FBS in PBS.
3. FACS buffer: 2% FBS, 0.5 mM EDTA in 1× PBS.
4. Fluorophore-conjugated anti-mouse antibodies against target epitopes.
5. Centrifuge.
6. Viability stain: Ghost Dye™, LIVE/DEAD™ or alternative.
7. A 96-well v-bottom plate or FACS tubes.
8. If performing intracellular cytokine staining, cell permeabilization buffer: Cytofix/Cytoperm™ and Perm/Wash™ buffer or equivalent.

2.5 Acquisition and Compensation

1. Compensation beads: commercially available bead kit with positive and negative beads or a single-vial bead system, such as UltraComp eBeads™ Compensation Beads (ThermoFisher) or equivalent.
2. Fluorophore-conjugated antibodies against the target markers of interest.
3. FACS tubes.
4. Cytometer Setup and Tracking (CS&T) beads: used for quality control of a flow cytometer.
5. Sheath fluid: filtered PBS.

3 Methods

3.1 Antibody Panel Design

1. Use the laser configuration of your flow cytometer to identify what lasers are available.
2. Use a reference guide, such as Fluorochrome/Laser reference Poster (BD Biosciences) to identify compatible fluorophores based on available lasers.
3. Choose anti-mouse antibodies of target epitopes that are conjugated to fluorophores that are compatible with your flow cytometer. Use an online spectral analyzer to minimize fluorescent overlap (*see* **Notes 1** and **2**).
4. Be sure to include a Ghost Dye™ of a compatible fluorophore into your panel (*see* **Note 3**).

3.2 Sample Preparation of Mouse Lungs

The general workflow of flow cytometry from sample preparation to analysis is highlighted in Fig. 1. The preparation of single-cell suspensions detailed below is optimized for murine lungs or other collagen dense tissues. If preparing single-cell suspensions from solid tissues that are not high in collagen, skip to **step 6**. If preparing single-cell suspensions of liquid tissues, such as bone marrow, skip to **step 8**.

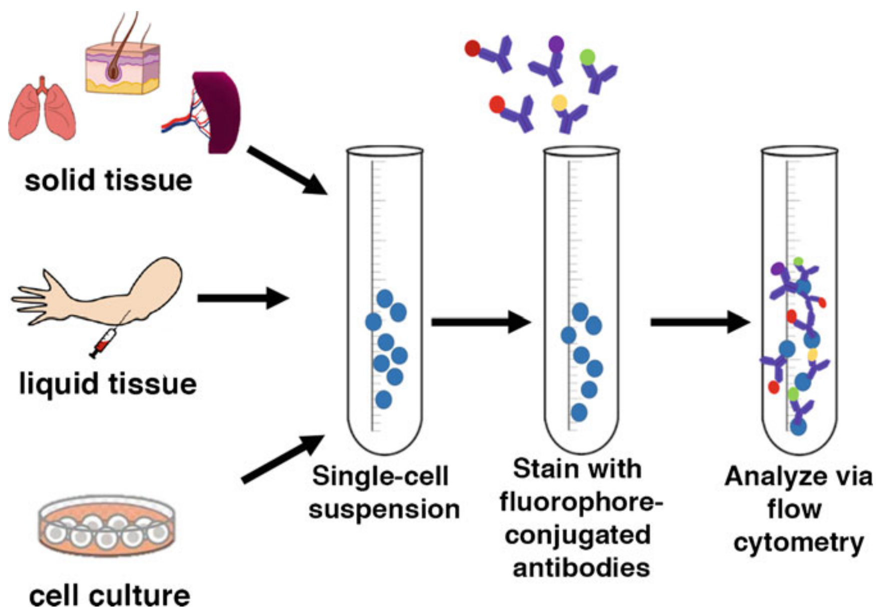

Fig. 1 General experimental procedure for flow cytometric analysis. The first step is to create a single cell suspension of the sample. Second, the suspended cells are incubated with fluorescently conjugated antibodies for use with a flow cytometer. Lastly, a flow cytometer is used to measure the fluorescence intensity of each fluorophore

1. At the end of your experiment, euthanize mice by a method approved by the IACUC at your institution. Using sterile surgical instruments, make a vertical incision along the thoracic cavity to expose the lungs. Use forceps and surgical scissors to remove the lungs.
2. Place the lung tissues in 2% FBS in PBS (*see* **Note 4**).
3. Use surgical scissors to mince or cut the tissue into small pieces in a Petri dish.
4. Suspend the tissue in 5 mL of the digestion buffer [3] (*see* **Note 5**).
5. Incubate at 4 °C for 35 min, inverting or vortexing tubes intermittently to prevent settling.
6. Place the tissue in a 70-μm strainer set over a 50-mL conical tube or Petri dish and gently mash the tissue with the flat end of a 5-mL sterile syringe plunger to allow cells to pass through the filter.
7. Rinse the end of the plunger and the filter 2–3 times with 5–10 mL of cold 2% FBS in PBS and collect the cells in a 15-mL conical tube.
8. Centrifuge the cells at 250 × *g* for 7 min.
9. Decant the plate and gently resuspend the pellet in 1 mL of ACK lysis buffer.
10. Incubate for 3 min at room temperature.
11. Neutralize ACK lysing buffer by adding 5–10 mL of cold 2% FBS in PBS and inverting the tube.
12. Centrifuge at 250 × *g* for 7 min and wash 2 additional times with 5 mL of cold 2% FBS in PBS. Resuspend the final cell pallet in a desired volume of cold 2% FBS in PBS. If you have only a portion of the lung, resuspend in 1 mL. If using an entire mouse lung, resuspend in 2 mL of cold 2% FBS in PBS.
13. To obtain cell count and viability, mix 10 μL of the cell suspension from **step 12** and 90 μL of trypan blue. Place 10 μL of the mixture onto electronic cell counter cartridge or hemocytometer. If counting manually, count the number of unstained live cells in all four quadrants of the hemocytometer (*see* **Note 6**). *See* Fig. 2 for counting cells using a hemocytometer.
14. If cell viability is 70% or lower, *see* **Note 7** for troubleshooting strategies.
15. Adjust the cell density to 4×10^7 cells/mL in 2% FBS in PBS by increasing the final volume or centrifuging sample and resuspending in the appropriate volume of 2% FBS in PBS.

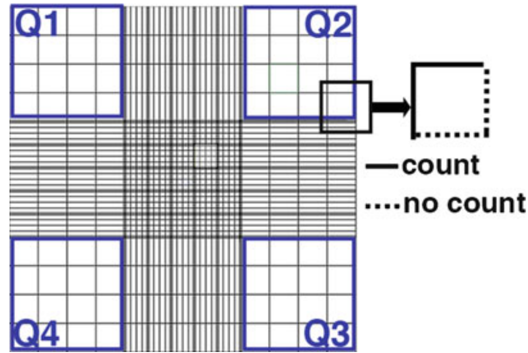

Fig. 2 Counting cells using a hemocytometer. A hemocytometer contains 4 quadrants as outlined by the blue lines. There are 16 squares in each quadrant. When using a hemocytometer, cells are counted in all of the 16 squares. Subsequently, the number of cells in the 4 quadrants is averaged. To assure cells are not double counted, cells on the line represented by the solid black lines are counted while cells on the lines represented by the dotted lines are not

3.3 Antibody Titration

An optimal antibody titration creates maximal separation between stained and unstained cell populations [4]. It also minimizes false positive and false negative populations within the data. Because of this, it is important to titrate antibodies before their use in the experiment. Since the abundance of epitopes may vary depending on the sample tissue, it is recommended to titrate using samples from your experiment. Antibodies of the same lot or batch do not need retitration, but it is recommended to retitrate the same antibodies from different lots.

1. Assign one column of a 96-well v-bottom plate to dilute each of newly acquired antibodies (e.g., Column 1 for Antibody A, Column 2 for Antibody B, etc.). Make a 1:25 dilution of each antibody with 2% FBS in PBS in a total volume of 50 μL and place it in Row A of the corresponding column. Add 25 μL of 2% FBS in PBS to Rows B–H for each of the columns assigned to an antibody.
2. Use 25 μL from Row A to perform 1:2 serial dilutions of each antibody 6–8 times down each column so that the following antibody dilutions are made: 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200. Remove 25 μL from each well in Row H after the dilution.
3. Place 25 μL of the cell suspension containing 1×10^6 cells into each well containing 25 μL of an antibody dilution. Each well now contains 50 μL of antibody/cell mixture. The highest antibody concentration should be 1:50 in the mixture of 25 μL of cell suspension and 25 μL of 1:25 antibody dilution (Row A).

4. Gently mix the contents of the wells using a multichannel pipette, cover the plate with tin foil and incubate for 30 min at room temperature in the dark.
5. Add 150 μL of 2% FBS in PBS.
6. Spin the plate at 250 × *g* for 7 min.
7. Decant the plate and resuspend in 200 μL of FACS buffer.
8. Acquire on a flow cytometer on optimal CS&T values (*see* Subheading 3.6) using a high-throughput sequencer. Alternatively, transfer to FACS tubes and volume to 400–500 μL using FACS buffer.
9. Export FCS files and load into flow cytometry analysis software (FACSDiva™, FlowJo, or equivalent).
10. For each antibody, place the *x*-axis as SSC-A and *y*-axis as the appropriate fluorescence signal for the antibody. Create 1 gate that encompasses the negative cell population and another that encompasses the positive cell population. Apply the gates to all dilutions as shown in Fig. 3.
11. Using statistical analysis functions available in your software, display the median fluorescence intensity of the positive (MFI_{pos}) and negative (MFI_{neg}) cell populations, as well as the standard deviation of the negative population (σ_{neg}). This can be done using the table editor in FlowJo and modifying the statistic and parameter.

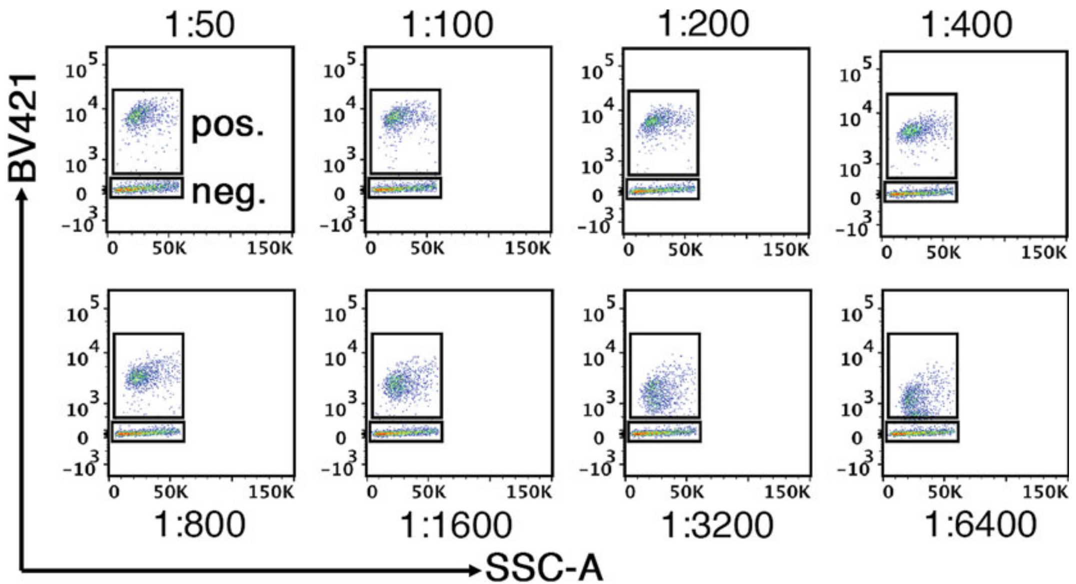

Fig. 3 Gating positive and negative cell populations for antibody titrations. Dead cells and debris have been gated out using FSC-A vs. SSC-A. Next, the positive and negative population gates are created as shown for each antibody concentration. These gates are used to calculate the MFI of the positive and negative population and the standard deviation of the negative population to calculate the Stain Index of each antibody dilution

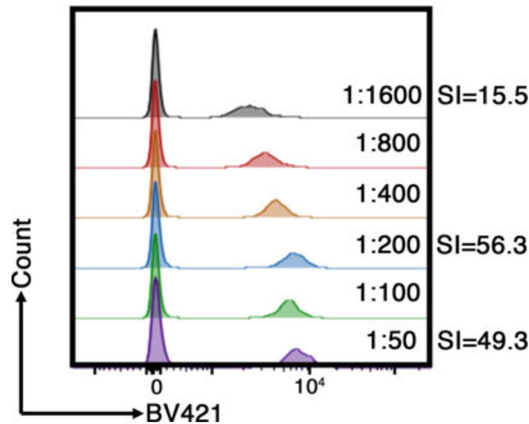

Fig. 4 Offset histograms of antibody titration. The optimal antibody titration is the lowest amount of antibody with the largest amount of separation (highest Stain Index, SI). As shown, the ideal antibody dilution is 1:200

12. Determine the Stain Index of each antibody dilution by using the formula [5]:

$$\text{Stain Index} = \frac{\text{MFIpos} - \text{MFIneg}}{2\sigma\text{neg}}$$

13. The dilution with the highest Stain Index is the optimal dilution for that antibody (Fig. 4).

3.4 Cell Staining

With the advancements of flow cytometric analysis, there are many different types of staining that can provide information for data analysis. This section is subdivided to describe the procedures addressing surface marker staining and intracellular marker staining. For staining controls to consider, *see* **Notes 8** and **9**.

3.4.1 Staining of Cell Surface Marker Proteins

1. In a 96-well v-bottom plate, place 1×10^6 cells per sample into a well and centrifuge the plate at $250 \times g$ for 7 min (*see* **Note 10**).
2. While centrifuging, prepare 50 μL per sample of a viability stain cocktail. If using Ghost Dye™, dilute 1 μL of the dye in 1000 μL of PBS (1:1000 ratio). If using an alternative viability stain, prepare according to the manufacturer's instructions.
3. After centrifugation, decant the plate by quickly inverting plate over a sink.
4. Resuspend samples in 50 μL/well of the viability stain cocktail prepared at **step 2**.
5. Cover the plate in tinfoil and incubate for 20 min at 4 °C.
6. Add 150 μL of 2% FBS in PBS to each sample and spin plate at $250 \times g$ for 7 min.

7. Prepare 50 μL per sample of a surface marker staining cocktail in 2% FBS in PBS. Determine the buffer amount necessary for the number of samples plus 2–3 additional samples to compensate for potential volume loss during pipetting.
8. Add the required dilution of each antibody as determined via antibody titration in Subheading 3.3. Keep the antibody cocktail on ice, shielded from light.
9. Decant the plate after centrifuging and resuspend the pellet in 50 μL of the staining cocktail. If performing intracellular marker staining, bring permeabilization reagents to room temperature at this time.
10. Wrap the plate in tinfoil and incubate for 30–60 min at room temperature in the dark. If performing intracellular staining, limit surface stain to 30 min.
11. Remove the tinfoil from the plate and add 150 μL 2% FBS in PBS to the wells. Centrifuge the plate at 250 × *g* for 7 min to wash the cells.
12. If performing intracellular marker staining, skip the next step and proceed to Subheading 3.4.2. If phenotyping the cells only by the surface markers, continue to **step 13**.
13. Decant the plate to remove the supernatant and resuspend the cells in 200 μL of FACS buffer.
14. Run your samples on a high throughput sequencer-compatible flow cytometer. Alternatively, transfer your samples to FACS tubes and fill them to a final volume of 400–500 μL with FACS buffer. If samples are not being run immediately, *see* **Note 11**.

3.4.2 Intracellular Marker Staining

As an example, we will describe cell permeabilization for intracellular cytokine staining using the BD Cytofix/Cytoperm™ kit (*see* **Note 12**).

1. After washing, gently resuspend cells in 60 μL of BD Cytofix/Cytoperm™. Cover plate in tinfoil and incubate in the dark at room temperature for 30 min.
2. Add 150 μL of 1× BD Perm/Wash™ and centrifuge at 250 × *g* for 7 min to wash cells.
3. Decant the plate by quickly inverting over a sink and perform an additional wash using 200 μL of 1× BD Perm/Wash™.
4. Using 1× BD Perm/Wash™ for the intracellular staining buffer diluent, calculate 50 μL of diluent for each sample plus an additional 2–3 samples for volume loss while pipetting.
5. Add the intracellular antibodies to the intracellular staining buffer in the titrated ratio from Subheading 3.3. Keep the antibody cocktail shielded from light.

6. After washing the cells, decant and resuspend in 50 μL of intracellular antibody cocktail. Cover plate in tinfoil and incubate in dark at room temperature for 30 min.
7. After incubation, wash cells by adding 150 μL of 1× BD Perm/Wash™ and spinning at 250 × *g* for 7 min. Perform an additional washing step using 200 μL of 1× BD Perm/Wash™ before finally resuspending in 200 μL of FACS buffer.
8. Run your samples on a high throughput sequencer-compatible flow cytometer. Alternatively, transfer your samples to FACS tubes and fill them to a final volume of 400–500 μL with FACS buffer.

3.5 Compensation

Once the antibody panel has been designed and the fluorophores have been selected, the spectral overlap needs to be minimized through a process called "compensation." Compensation minimizes the amount of bleeding of one fluorophore into the detector of another fluorophore [6]. Do not substitute one fluorophore for another of similar spectrum (do not use FITC instead of GFP, for example). Because the optimal voltages may change over time, run compensation immediately prior to running samples. The protocol below describes compensation using UltraComp eBeads (ThermoFisher), which include both positive and negative compensation beads (*see* **Note 13**).

1. Obtain compensation beads compatible with the antibodies in your panel and flow cytometer lasers. Before use, shake the beads well and place 100 μL (1 drop) of compensation beads into each FACS tube. You will need one tube for each individual fluorophore on the panel plus one tube of unstained beads.
2. Add 1 μL of each antibody to one tube of beads, vortex, and incubate in the dark at room temperature for 10 min.
3. Add 500 μL of PBS to the tube and cover to hide from light. The single stain controls are now ready for compensation.

3.6 Acquisition

1. Before using a flow cytometer, adequately clean and prime the machine while allowing the lasers time to warm up (approximately 10–30 min depending on the flow cytometer).
2. Run a quality control (QC) check on the flow cytometer's optic, fluidic and electronic systems (*see* **Note 14**). To perform QC using FACSDiva™, go to "cytometer" > "CST" at the top bar.
3. Prepare CS&T beads by adding 1 drop of CS&T beads and 300 μL of filtered 1× PBS to a FACS tube.
4. In the pop-up window, verify that the lot matches the lot number on the bottle of CS&T beads. Click "run" on the setup control window.

5. Press "run" on the flow cytometer and adjust the flow rate to "low." After verifying that the sheath fluid is full and the waste container is empty, press "ok."
6. Verify that the machine has "passed" its quality control. Unload the CS&T tube and close the CS&T window. If it does not pass, consult with the administrative operator who is responsible for the maintenance of the flow cytometer.
7. Upon entering back into FACSDiva™, a prompt will appear. Click "Use CST Settings." The cytometer is now ready for acquisition.
8. Apply CS&T values or other optimal photomultiplier tube (PMT) voltage values generated during QC.
9. Use the single stain controls from Subheading 3.5 to compensate your antibody panel. Select the parameters for your experiment by selecting only your fluorophores and eliminating the colors or parameters not in your panel.
10. Edit the compensation settings of your antibody panel. Adjust the PMT voltages for each fluorophore in the panel until the desired separation is achieved with minimal bleeding into other fluorophores. Do this by obtaining a minimum half-log separation between the selected fluorophore and other fluorophores in the panel (Fig. 5).
11. Once you have screened all of your single stains to make certain there is not less than a half-log of separation between fluorophores, record a minimum of 1000–5000 events for each stain.
12. Calculate the compensation for your panel and apply those compensation settings to your experiment. If your antibody panel is used repeatedly, such as in the event of time-course experiments, consider standardizing your assay by using Rainbow Beads (*see* **Note 15**).
13. In your experiment, create a tube and run a sample with histograms open of all colors in the panel to make sure that the stained samples are on scale. If cell populations exist in one experimental group that do not exist in others, use a sample from each experimental group to make certain all fluorophores are on scale. If a population is off scale, adjust PMT voltages or redo compensation.
14. Adjust the forward and side scatter of the sample to make sure that all of the cell populations are within range as shown in Fig. 6. Set the flow cytometer to record a minimum of 100,000 events, run, and save (*see* **Note 16**).

3.7 Data Analysis

Followed by acquisition is the analysis of flow cytometry data [7]. Export the FCS files from your experiment and load into flow cytometry analysis software (i.e., FlowJo).

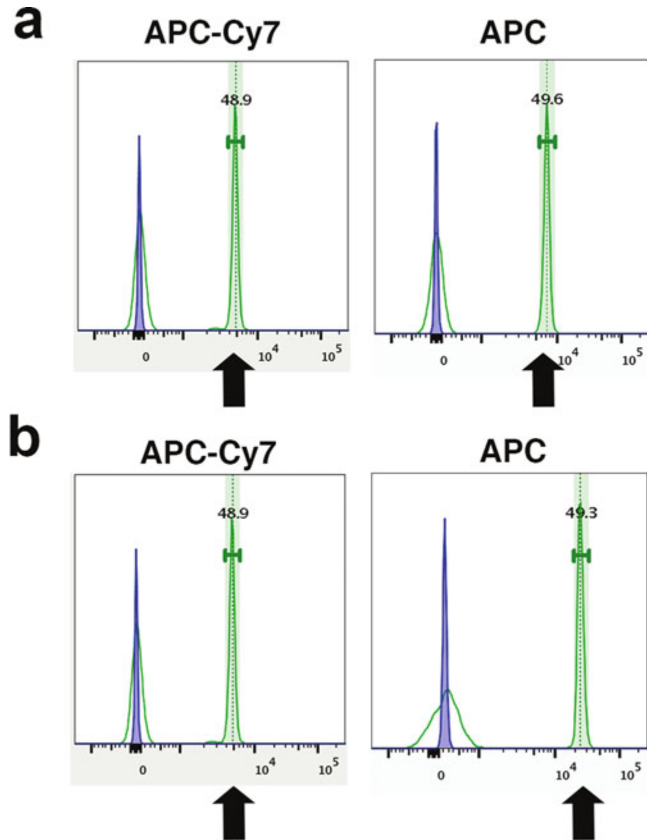

Fig. 5 Calculation of compensation using compensation beads. The peak fluorescence of APC and APC-Cy7 (shown in green) are close together indicating a risk for significant spectral overlap (**a**). To minimize the overlap, the PMT voltages of APC are increased to gain at least one-half log separation between the peak fluorescence of each color (**b**)

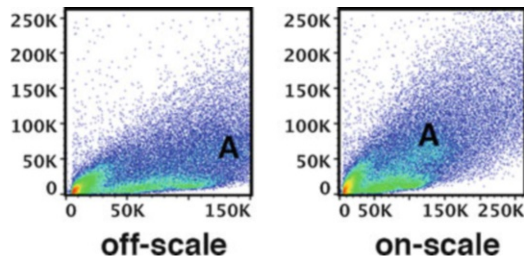

Fig. 6 Examples of on-scale and off-scale event acquisition. When forward scatter and side scatter are off-scale (left), the cell population “A” is not entirely shown within the plot. This can be corrected by modifying the voltages of the forward scatter and side scatter as shown in the on-scale (right) plot

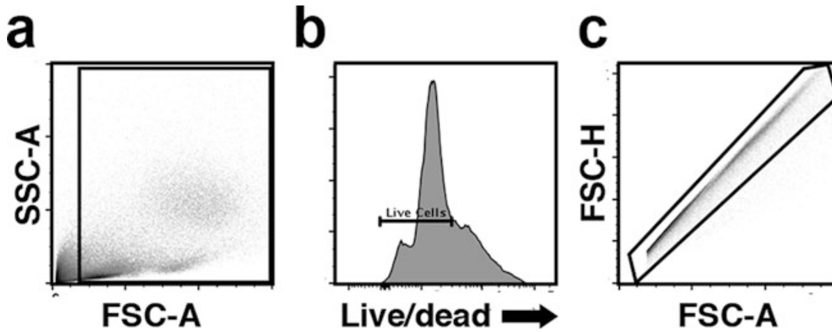

Fig. 7 Initial gating steps. Gating strategies to eliminate debris (**a**), dead cells (**b**) and doublets (**c**) are shown

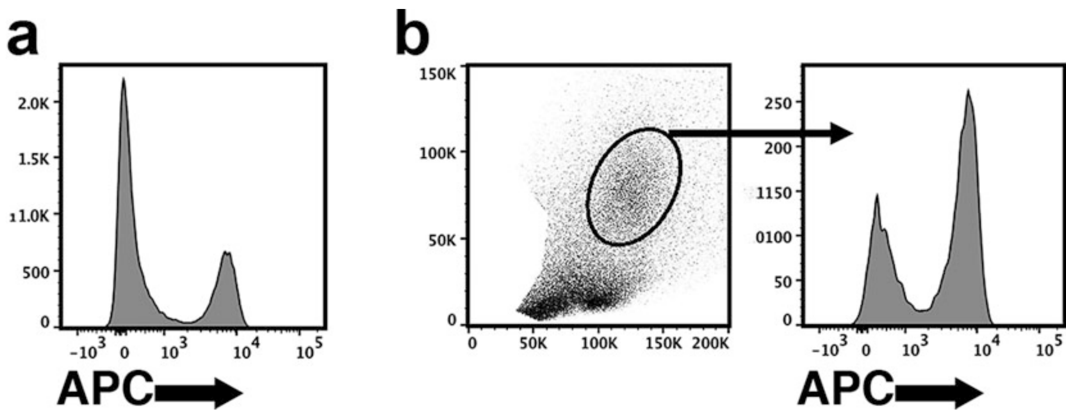

Fig. 8 Histograms showing APC signals in two populations of cells. Fluorescence signals can be measured from total cell population (**a**) or after selecting a distinct cell population (**b**). In this example, APC signals within granulocyte population is shown (**b**)

1. To eliminate doublets and dead cells from your samples, follow the gating strategies shown in Fig. 7.
2. Look for fluorescence signal within the sample (Fig. 8a) or within distinct cell populations (Fig. 8b).
3. Use the fluorescence-minus-one (FMO) controls, which include all antibodies in your panel except one fluorophore (*see* **Note 8**), to create a gate that lacks the signal from the fluorophore. Apply this gate to all other samples, as shown in Fig. 9.
4. For a sample gating strategy of innate immune cell populations from the murine lung, *see* Fig. 10. For a sample gating strategy of adaptive immune cells, *see* Fig. 11.
5. After gating your populations, apply gates to all samples. If using a flow cytometry analysis software, such as FlowJo™, drag the frequencies of each population, listed in the "statistic" column of the program, into the table editor. Using the table editor, modify the statistical output of each population of

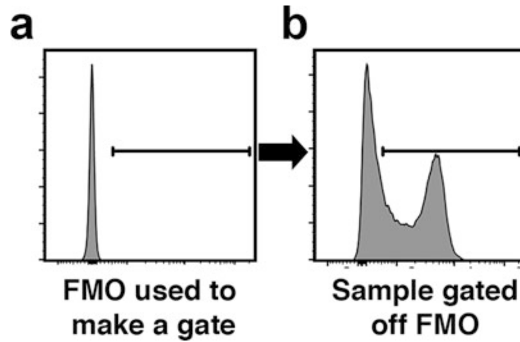

Fig. 9 Gating using FMO. Histogram gate of FMO of AF-700 (**a**) is used to create a gate of AF-700 positive populations (**b**)

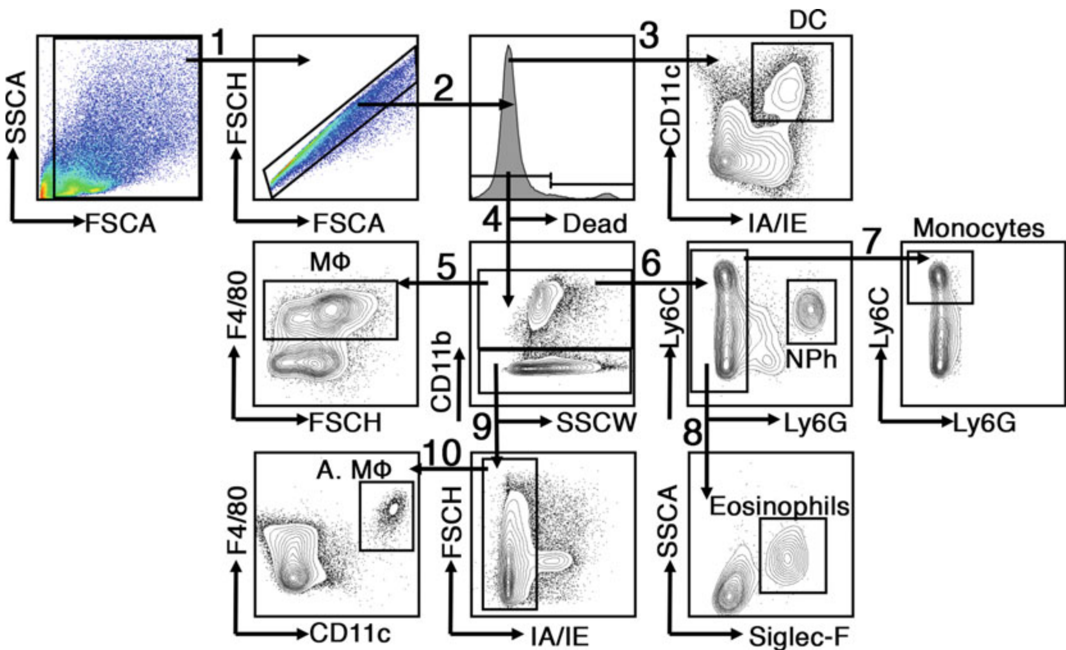

Fig. 10 Sample gating strategy of innate immune cells in the murine lung. Cells are first gated to remove debris and dead cells by excluding the bottom left corner of the dot plot. (1) Doublets are removed from the cell population. (2) Dead cells are removed using a viability stain and gating the negative cell population. From the population of "live" cells, cells are gated for (3) dendritic cells (DC, $CD11c^{+}IA/IE^{+}$) or (4) $CD11b^{+}$ cells. Gating on $CD11b^{+}$ cells, (5) macrophages (MΦ, $CD11b^{+}F4/80^{+}$), (6) neutrophils (NPh, $CD11b^{+}Ly6C^{+}Ly6G^{+}$), (7) monocytes ($CD11b^{+}Ly6C^{+}Ly6G^{-}$), and (8) eosinophils ($CD11b^{+}Ly6G^{-}SiglecF^{+}$) can be gated. (9, 10) Alveolar macrophages (A.MΦ, $CD11b^{-}IA/IE^{-}F4/80^{+}CD11c^{+}$) can be gated from the $CD11b^{-}$ population

interest to display alternative information such as the frequency of total events, the median fluorescence intensity, or the standard deviation of a population. These numbers can be compared between experimental groups to observe changes in immune responses under different conditions.

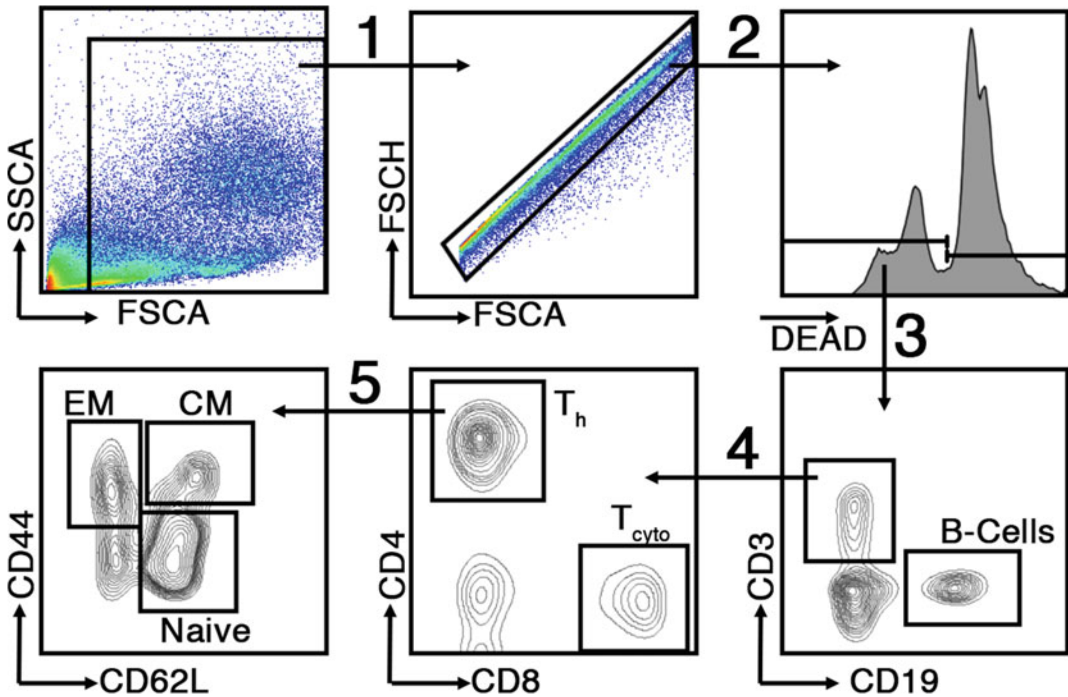

Fig. 11 Sample gating strategy of adaptive immune cells in the murine lung. Cells are first gated to removed debris and dead cells by excluding the bottom left corner of the dot plot. (1) Doublets are removed. (2) Dead cells are removed by using a viability stain and gating the negative population. (3) Cells are then gated for B-cells ($CD3^{-}CD19^{+}$), (4) T-helper cells (T_h, $CD3^{+}CD4^{+}CD8^{-}$), and cytotoxic T-cells (T_{cyto}, $CD3^{+}CD4^{-}CD8^{+}$). Within all T-helper cell populations (5), cells are gated for effector memory T-cells (EM, $CD44^{+}CD62L^{-}$), central memory T-cells (CM, $CD44^{+}CD62L^{+}$), and naïve T-cells (Naïve, $CD44^{-}CD62L^{+}$)

6. If you do not see noticeable shifts in the population gates or changes in frequencies, consider plotting the MFI of that parameter in a bar graph and comparing it between experimental groups. This is also an acceptable comparison between groups.
7. Lastly, choose diagrams from your flow data that are representative of your statistics. These can be displayed as dot plots, density plots, or contour plots.

4 Notes

1. When choosing fluorophores for desired epitopes, select fluorophores that minimize spectral overlap to avoid the detection of one fluorophore in the detector of another fluorophore. Do this by choosing the minimum number of fluorophores that are detectable on each laser. Useful online tools, such as the BioLegend Spectra Analyzer (https://www.biolegend.com/spectraanalyzer), to visualize the fluorescent overlap of the colors in your panel.

2. For best results, assign bright fluorophores to low expressing antigens and dim fluorophores to abundantly expressed antigens. For instance, a dim Pacific Blue would be reserved for an abundantly expressed CD3 epitope and a bright PE would be reserved for a low expressed cell surface receptor or intracellular cytokine.
3. Alternatively, Annexin V and PI or alternative cell viability dyes can be used according to the manufacturer's protocol. However, the associated fluorophore must be accommodated into your panel. Additionally, if performing intracellular cytokine staining, it must be a fixable cell viability dye. If using Annexin V, do not include EDTA into your FACS buffer.
4. Any nutrient-rich medium to aid preserving cell viability prior to sample processing is acceptable. If cell viability becomes a problem (<70%), consider adjusting the medium or decreasing the processing time.
5. Sterile PBS containing 10% FBS and 10 mM HEPES can be prepared ahead of time and stored at 4 °C for an extended period of time. Collagenase type IV and DNase I should be preserved appropriately either in frozen aliquots or in lyophilized forms. These enzymes should be mixed with the buffer immediately prior to lung tissue digestion.
6. Average the total number of cells in all four quadrants and multiply by the dilution factor (10) and by 10^4 for the total number of cells per milliliter for each sample. If there are too many or not enough cells to count, adjust the dilution of cells and trypan blue and recount.
7. If cell viability is low, consider decreasing processing times, collagenase, and DNase I concentrations, the amount of ACK lysis buffer, and/or the time incubated with ACK. The quality of each buffer is also an important consideration. Be sure to use a viability dye in your flow panel if cell viability is low.
8. To make a gate on a specific protein or marker of interest, an important control is a fluorescence-minus-one (FMO) staining [8]. To create an FMO, stain 1×10^6 cells with all fluorophore-conjugated antibodies in the panel, minus one antibody. Prepare FMO for each of the antibodies on the panel and use it to stain your experimental samples. Use an antibody isotype control for proteins that are less abundant or if the high background is an issue.
9. If you are staining samples with large populations of monocytes, macrophages, B-cells, and/or dendritic cells, consider adding an F_c block in your live/dead stain. F_c block is also referred to as anti-mouse CD16/CD32 antibody, and it prevents nonspecific binding of antibodies to F_c receptors on these cell types.

10. Alternatively, samples can be stained directly into FACS tubes. Samples can be decanted by inverting it upside down over the waste container and resuspended via pipette, lightly flicking the tube or by gently sliding the tube along the 5-mL test tube rack. Stain volumes can remain the same but wash volumes should be increased to 0.5–1.0 mL. Gently tap tube on Kim-wipe or use a pipette to remove residual supernatant.
11. If samples are not being immediately analyzed on a flow cytometer, resuspend the cells in 1–4% formaldehyde in PBS after the final wash. Wrap samples in tin foil and keep in the dark at 4 °C for up to 3 days. If samples sit too long or are exposed to light, tandem dyes may begin breaking and the integrity of the data could be compromised [9].
12. If performing intracellular cytokine staining, proceed with the intracellular cytokine staining protocol by permeabilizing cell membranes at this time. The permeabilization kit recommended and used in this protocol is BD Cytofix/Cytoperm™. However, this kit does not work for nuclear staining. For nuclear staining (such as Foxp3 or other transcription factors), consider Foxp3/ Transcription Factor Staining Buffer Kit by Tonbo Bioscience. When performing these protocols, we have found that cell permeabilization and intracellular cytokine staining work best at room temperature. Additionally, intracellular cytokines should be stained with bright fluorophores (PE, BV421, etc.) and may need to be stained at a ratio of about 1:25 to 1:50.
13. To perform compensation using cells, stain the cells in conditions consistent with your experimental samples, that is, staining 1×10^6 cells with the antibody dilution determined by titration. Because the expression of epitopes may be low at the basal level, it is best to compensate with cells using experimental samples. If proteins are intracellular or antigens are dim, it is recommended to compensate using beads. Whatever the choice, be sure to keep the method (beads or cells) consistent across all single stains.
14. Quality control (QC) of your flow cytometer should be performed to identify any variation in the performance and its baseline defined during instrument set-up. This is important for minimizing the background and making sure that the lasers are running optimally and consistently between experiments. For some flow cytometers, QC is carried out by running Cytometer Setup and Tracking (CS&T) beads. This will adjust laser delays, area scaling factors, and photomultiplier tube (PMT) voltages, if necessary.

15. If your antibody panel is to be used repeatedly, such as in the event of time-course experiments, consider standardizing your flow assay using Rainbow Beads (Spherotech, Cat/RFP-30-5A Rainbow Fluorescent Particles Mid-Range) for improved reproducibility and reducing set-up variation. After determining optimal voltages for your panel, add 1 drop of rainbow beads to 300 μL FACS buffer and acquire. Create a gate around the bead population and generate a histogram of each fluorophore. Draw a tight gate the width of the peak. Alternatively, create a gate the width of the histogram and display the MFI. Record and save this template. In future experiments, open this template and adjust the PMTs using rainbow beads to fit within the gates drawn or match the MFI. After voltage adjustments, record compensation and experimental samples [10].
16. If your sample clogs the machine during acquisition, consider filtering your sample through an additional 70-μm filter to remove any clumped cells or debris. If recording a minimum of 100,000 cells/sample is not an option, be sure to collect a consistent number of cells for each sample.

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Chapter 15

Cellular and Biochemical Analysis of Bronchoalveolar Lavage Fluid from Murine Lungs

Rama Satyanarayana Raju Kalidhindi, Nilesh Sudhakar Ambhore, and Venkatachalem Sathish

Abstract

Bronchoalveolar lavage (BAL) is a technique used to collect the contents of the airways. The fluid recovered, called BAL fluid (BALF), serves as a dynamic tool to identify various disease pathologies ranging from asthma to infectious diseases to cancer in the lungs. A wide array of tests can be performed with BALF, including total and differential leukocyte counts (DLC), enzyme-linked immunosorbent assays (ELISA) or flow-cytometric quantitation of inflammatory mediators, such as cytokines, chemokines and adhesion molecules, and assessment of nitrate and nitrite content for estimation of nitric oxide synthase (NOS) activity. Here, we describe a detailed procedure for the collection of BALF for a variety of downstream usages, including DLC by cytological and flow-cytometry-based methods, multiplex cytokine analysis by flow cytometry, and NOS activity analysis by determining nitrate and nitrite levels.

Key words Differential leukocyte count, Cytokines, Nitric oxide synthase, Flow cytometry, Tracheostomy

1 Introduction

The lung is considered the most exposed organ in the body for its continuous interactions with external airborne antigens and other toxins [1]. Such constant exposures to exogenous substances at the airway mucosal surface can trigger immunological reactions in the lungs, resulting in disorders such as asthma and chronic obstructive pulmonary disease (COPD) [2–8]. When interstitial lung disorders are suspected, biopsy is not the first choice for identifying pathological and biochemical changes due to its invasive nature. Bronchoalveolar lavage (BAL) is often performed instead to examine the immune cells present in the lungs and to determine cytokine, chemokine, and adhesion molecule profiles.

BAL is a saline-based wash of the airways first established in 1970 [9–11]. In humans, it is a minimally invasive procedure to investigate lung pathophysiologies [2, 3, 12–17] and often used to

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diagnose patients suffering from interstitial lung disorders [2]. The fluid recovered by BAL is known as BAL fluid (BALF) and contains a multitude of airway constituents, including cells, lipids, proteins, and other chemical or biological substances from the mucosal surface of the bronchial tree [18]. Most proteins present in BALF include albumin, immunoglobulin, α1-anti trypsin, transferrin, fibronectin, collagen, and collagenase. In addition, prostaglandins and a few metabolites that are either locally synthesized or reach the lungs via active transport by immunoglobulins or via passive transport by albumin are also found in BALF [4]. The cellular contents of BALF are predominantly leukocytes, with a few exceptions like erythrocytes and platelets [19, 20]. Identifying the pattern and population of differential leukocyte counts (DLC) plays a crucial role in characterizing various lung diseases [10]. In laboratory research using in vivo animal models for various lung disorders [21–23], BAL serves as an important and most commonly used technique to study inflammatory cell infiltration, biochemical, and molecular changes [2, 24].

Conventionally, DLC is performed by cytological staining of air-dried BALF smears with Wright-Giemsa, May-Grunwald-Giemsa, or Differential-Quik stain [25, 26]. Following staining, 200–500 cells are counted under a microscope and manually classified into neutrophils, eosinophils, macrophages, basophils, etc. However, DLC by this conventional method is prone to human errors and its diagnostic validity may be challenged for yielding false results. In this context, with the advancements of microfluidics technology and abundance of cell-specific antibodies, flow-cytometry-based DLC has become a more rapid and trustworthy tool.

In addition to DLC, BALF has been used for the detection of cytokines, chemokines, and adhesion molecules. However, independent ELISA kits were used to quantify the proteins, which is both time and cost consuming. In contrast, flow-cytometry-based multiplex assays provide a solution to minimize these challenges by facilitating the simultaneous detection of multiple proteins in a single sample. Furthermore, markers used for detecting oxidative stress in the lungs can also be measured in BALF. Oxidative stress plays a crucial role in the pathophysiology of lung disease by generating reactive oxygen species (ROS), which when combined with nitric oxide, forms potent peroxy nitryl radicals, resulting in the nitrosylation of proteins leading to lipid peroxidation [27–33].

In this chapter, we describe detailed procedures to perform the collection of BALF from mice followed by performing DLC using both conventional cytological and flow-cytometric methods. In addition, we also describe a procedure to assay multiple cytokines in BALF with flow-cytometry using a commercially available multiplex kit. Finally, we describe a method to quantify nitrate and nitrite levels in BALF as an indicator of NOS activity, which suggests the extent of ROS generation in the airways.

2 Materials

2.1 BALF Collection

1. Mice: 8–12 weeks old (*see* **Note 1**).
2. Anesthetics: ketamine (100 mg/kg) and xylazine (10 mg/kg) cocktail (9:1 ratio).
3. Sodium pentobarbital: 100 mg/kg body weight for euthanasia.
4. 18-Gauge cannula: used for tracheostomy.
5. Surgical thread.
6. Phosphate-buffered saline (PBS) with protease and phosphatase inhibitor: 2.66 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCl, 8.06 mM Na_2HPO_4-$7H_2O$, pH ~7.4. Add protease and phosphatase inhibitor cocktail (1×).
7. 2-mL Syringe.
8. Surgical dissection tools: pointed forceps, serrated forceps, pointed bent scissors, scalpel, and conventional small scissors.
9. Microfuge tubes: 1.5-mL and 2-mL sizes for collection and storage of BALF.
10. Ice in an ice bucket.

2.2 DLC by Cytology

1. 0.4% (w/v) Trypan blue solution: weigh 0.4 g of trypan blue and dissolve in 100 mL of PBS.
2. Automated or manual cell counter: Countess™ Cell Counting Chamber Slides with Countess II FL Automated Cell Counter or a hemocytometer using a microscope.
3. Cytospin (*see* **Note 2**).
4. Glass slides: 25 × 75 × 1.0 mm.
5. Coverslips: 22 × 22 mm.
6. Romanowsky-Giemsa (modified Giemsa) staining kit: Diff-Quik Stain Kit or commercially available equivalent with a fixative (methanol), eosinophilic xanthene dye (eosin Y), and basophilic thiazine dye (methylene blue).
7. Absolute ethanol: histological grade. Used for dehydration of cells.
8. Xylene.
9. Mounting medium for placing cover slip.
10. Microscope.
11. Cell counter.

Table 1
Antibodies (clones) and their working concentrations for identifying different leukocytes in murine BALF

Antibody target	Clone name	Conjugation	Cell type	Working concentration
CD36	SMΦ	Alexa Fluor® 488	Monocytes/ macrophages	0.4 μg/mL final concentration
CD2	3B6	PE	T lymphocytes	0.4 μg/mL final concentration
CD19	B-1	Alexa Fluor® 594	B lymphocytes	0.4 μg/mL final concentration
CD45	2D-1	Alexa Fluor® 680	Lymphocytes	0.4 μg/mL final concentration
CD294 (CRTH2)	No3m1scz	Alexa Fluor® 647	Granulocytes	0.4 μg/mL final concentration

2.3 DLC by Flow Cytometry

1. Flow cytometer: equipped with two lasers capable of distinguishing 575–585 nm and 660 nm.
2. Flow cytometry data analysis software.
3. 3% (w/v) Bovine serum albumin (BSA): Dissolve 3 g of BSA in 100 mL of PBS.
4. Monoclonal antibody cocktail: Combine antibodies against CD36, CD2, CD19, CD45, and CD294 in PBS at their recommended concentrations (*see* Table 1 for more details).
5. DAPI nuclear stain solution: 100 μg/mL DAPI in PBS. Make the working concentration of DAPI by diluting a 1 mg/mL DAPI stock solution to 1:10 in PBS.

2.4 Multiplex Cytokine Assay by Flow Cytometry

1. Flow cytometer: equipped with two lasers capable of distinguishing 575–585 nm and 660 nm.
2. Flow cytometry data analysis software.
3. Multichannel pipettors: 5–200 μL.
4. Reagent reservoirs for multichannel pipettors.
5. Mouse cytokine multiplex detection kit: BioLegend 13-plex L EGENDplex™ Inflammation Panel or equivalent (*see* **Note 3**).
6. Microplate vacuum manifold or centrifuge: used for washing the filter or V-bottom 96-well plate provided in the multiplex kit.
7. Wash buffer: Thaw the entire container of 20× wash buffer from the multiplex kit and bring to room temperature. Add 475 mL of deionized water to make 1× wash buffer. This solution can be stored at 2–8 °C for upto 1 month.

8. 1.5-mL Polypropylene microfuge tubes.
9. Refrigerated centrifuge.
10. Vortex mixer.
11. Sonicator bath.
12. Aluminum foil.
13. Paper towels.
14. Plate shaker.

2.5 Nitric Oxide Synthase (NOS) Assay

1. Reaction buffer: 50 mM HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.4, 5 μM FAD (flavin adenine dinucleotide), 0.1 mM NADPH (nicotinamide adenine dinucleotide phosphate hydrogen), 0.2 U/mL nitrate reductase in 290 μL of distilled water.
2. 1 mM Potassium ferricyanide prepared in Millipore water. Prepare this solution freshly on the day of experiment.
3. Greiss reagent: Dissolve 0.2% (w/v) N-(1-Naphthyl) ethylenediamine (NED), 2% (w/v) sulphanilamide, and 5% (v/v) 95% phosphoric acid in double distilled water and stir it using a magnetic stirrer until the solution appears free of any particles.
4. UV-visible microplate spectrophotometer.
5. Clear 96-well plates.

3 Methods

3.1 BALF Collection

1. Euthanize mice with an overdose of sodium pentobarbital (100 mg/kg body weight) injected intraperitoneally.
2. Place the mice in supine position and make an incision at the cervical region using surgical scissors (or scalpel) and removing the skin (Fig. 1).
3. Carefully separate the tissues near the thyroid region to either side. Make a vertical incision on the external connective tissue (adventitia) to expose the trachea. Take precautions not to disturb any blood vessels to avoid bleeding (*see* **Note 4**).
4. Using a sharp scalpel, make a small horizontal incision on the trachea without severing (*see* **Note 5**). Slowly insert an 18-gauge cannula into the trachea, with the needlepoint facing toward the lungs. Secure the cannula in place by tying a surgical thread around the trachea (Fig. 1a, b).
5. Using a 5-mL syringe, slowly inject 1 mL of PBS with protease and phosphatase inhibitor into the cannula. Collect the BALF by slowly drawing the injected PBS back into the syringe (*see* **Note 6**). Place the collected BALF in a 2-mL microfuge tube and place it on ice until BALF samples from all mice are collected.

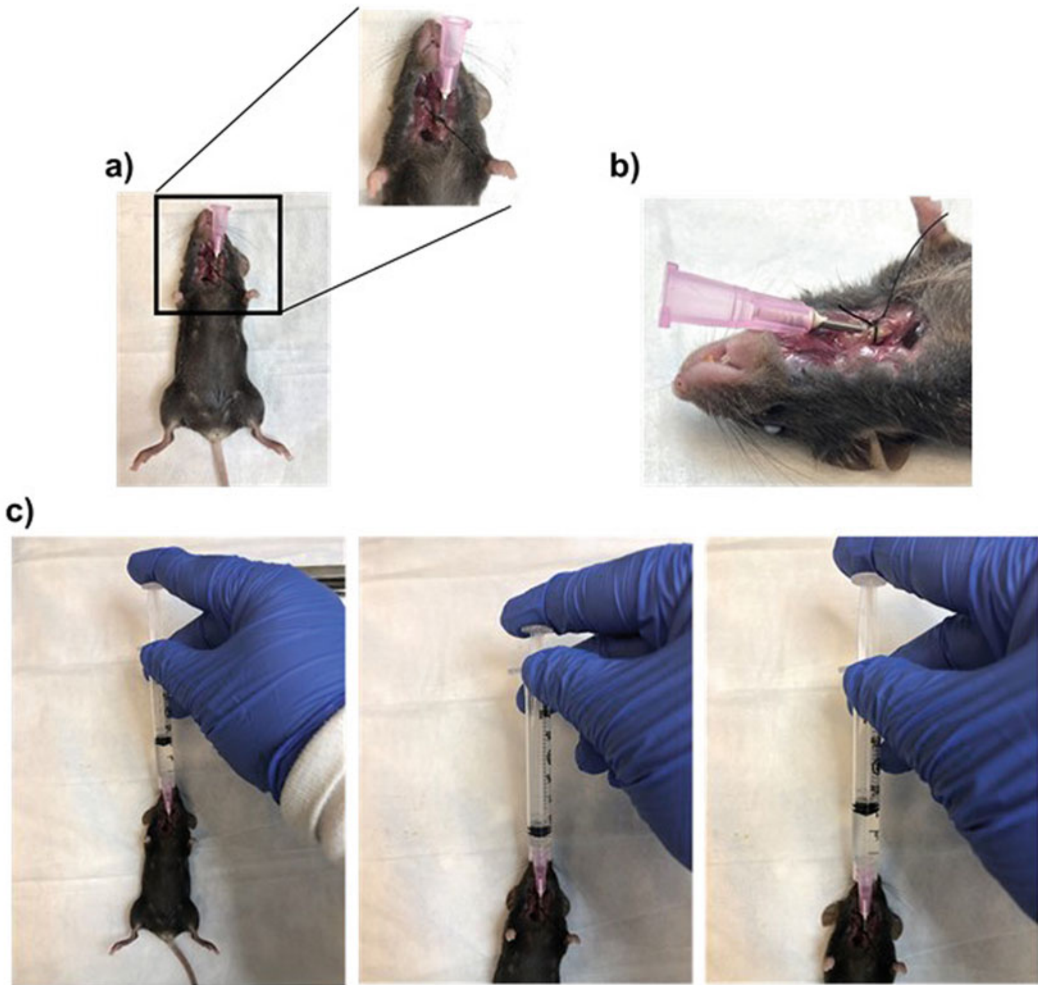

Fig. 1 Collection of bronchoalveolar lavage (BAL). (**a**) Image showing tracheostomized mice in supine position. Inset shows magnified area of trachostomy. (**b**) Lateral view of tracheostomized mice. (**c**) From left to right: images showing the collection of BAL from tracheostomized mice using syringe connected to the canula

6. After collecting the BALF from all the experimental mice, centrifuge the BALF at 2000 × *g* for 5 min at 4 °C.
7. Transfer the supernatants into new microfuge tubes (*see* **Note 7**). Store them in aliquots of 100 μL at −80 °C until use (*see* **Note 8**).
8. Resuspend the cell pellets in 200 μL of PBS and keep at 4 °C until use (*see* **Note 9**).

3.2 Total and Differential Leukocyte Counts

3.2.1 DLC by Cytological Staining

1. Transfer 20 μL of the cell suspension from Subheading 3.1 **step 8** into a microfuge tube and add an equal volume of trypan blue. Mix gently.
2. Place the solution on a Countess™ Cell Counting Chamber Slide and count the total number of cells using Countess II FL Automated Cell Counter. Alternatively, count the cells manually under a microscope using a hemocytometer and a cell counter.
3. Place 100 μL of the cell suspension from Subheading 3.1 **step 8** and onto a glass slide. Use a Cytospin to disperse the cells uniformly onto the slide (*see* **Notes 2** and **10**).
4. Leave the slide at room temperature for 30 min (*see* **Note 11**). Fix the air-dried cells for 30 s in the methanol fixative solution provided in the Diff-Quik Stain Kit.
5. Stain the slide with Diff-Quick Solution II for 30 s followed by counterstaining with Solution I for 30 s. Drain well between the stains.
6. Rinse the stained slide in tap water to remove excess stain and dehydrate in absolute ethanol. Place coverslip using two drops of mounting medium.
7. Perform the differential cell count using a digital light microscope at 100× magnification by oil immersion technique. Using a cell counter, count at least 200 cells per slide along a zigzag path, left to right and right to left as shown in Fig. 3.
8. Identify individual cell types based on the color and appearance as described in Table 2 and shown in Fig. 2.

3.2.2 DLC by Flow Cytometry

1. To 100 μL of the BALF cell suspension from Subheading 3.1, **step 8**, add 100 μL of 3% BSA in PBS and incubate for 1 h at room temperature as a blocking step. Prepare an additional cell sample as a no-stain (no antibodies) control to be used for flow cytometry (*see* **Note 12**).
2. Centrifuge the samples at 600 × *g* for 5 min and discard the supernatant.
3. Resuspend the cell pellet in 80 μL of PBS, add 20 μL of the antibody cocktail (Table 1), and incubate at room temperature for 1 h. Protect the cells from light (*see* **Note 13**).
4. Centrifuge the samples at 600 × *g* for 5 min and discard the supernatant (*see* **Note 14**).
5. Resuspend the pellets in PBS and centrifuge at 600 × *g* for 5 min and discard the supernatant. Repeat this step for one more time.
6. Add 100 μL of the diluted DAPI solution to the cell pellet and incubate for 10 min at room temperature.

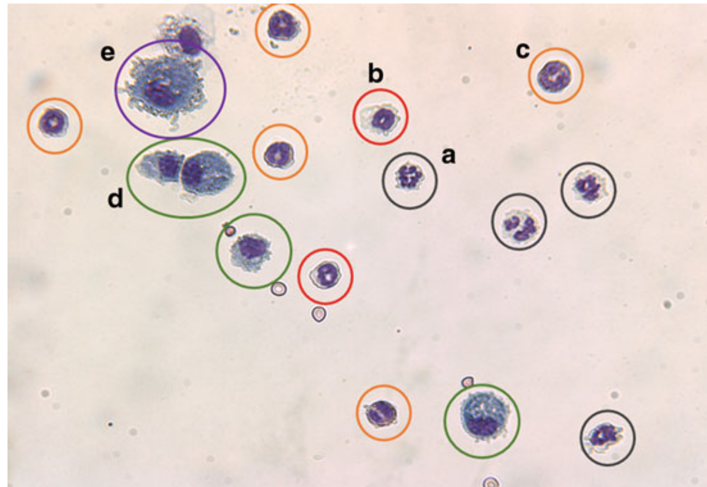

Fig. 2 Figure showing major leukocytes found in BAL fluid. (**a**) Neutrophil (black circles); (**b**) eosinophil (red circles); (**c**) basophil (orange circles); (**d**) monocyte (green circles), and (**e**) macrophage (purple circles)

Table 2
Morphological description of different leukocytes in BALF stained with Diffquik

Cell type	Color and appearance
Neutrophils	Have a dark blue multilobed nucleus and pale pink cytoplasm with purple granules.
Eosinophils	Have a blue bilobed nucleus and cytoplasmic granules varying from red to reddish orange.
Basophils	Have a purple to dark blue nucleus and black or dark purple granules.
Monocytes	Have a purple nucleus with sky blue cytoplasm.
Macrophages	Have a purple nucleus with sky blue cytoplasm as monocytes but larger than other leukocytes.

7. Wash the cells with PBS and centrifuge the samples at 600 × *g* for 5 min and discard the supernatant to remove excess DAPI stain.
8. Resuspend the cell pellet in 100 μL of PBS and immediately perform flow cytometry.
9. Set the flow cytometer to capture at least 40,000 nucleated events.
10. Using the no-stain control sample from **step 1** on an FSC vs. SSC plot, apply gating to eliminate unstained cells.
11. On an SSC vs. CD45 plot, apply gating to isolate total lymphocyte count.
12. Identify T-lymphocytes on a CD45 vs. CD2, where $CD2^+$ cells are T-lymphocytes and $CD2^-$ cells are B-lymphocytes.

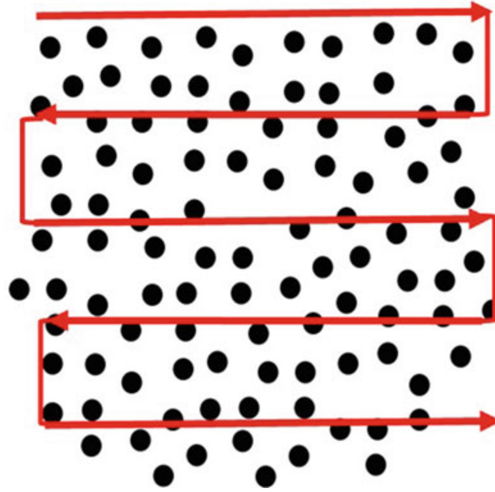

Fig. 3 Representative image showing the pattern of counting cells on a stained BAL smear

Alternatively, on a CD45 vs. CD19 plot, identify $CD19^+$ as B-lymphocytes and $CD19^-$ cells as T-lymphocytes.

13. To identify eosinophils and neutrophils, plot SSC on the *x*-axis and CD294 on the *y*-axis. Here, eosinophils appear toward the *y*-axis and away from the *x*-axis while neutrophils appear away from the *y*-axis and toward the *x*-axis (*see* **Note 15**).
14. Determine the number of each cell type in a BALF sample using a flow cytometry data analysis software.

3.3 Multiplex Cytokine Assay by Flow Cytometry

1. Completely thaw the BALF supernatant from Subheading 3.1, **step** 7, and keep on ice prior to performing the assay.
2. Create a template for loading the standards and samples (*see* Table 3).
3. Sonicate the bottle of pre-mixed beads from the multiplex cytokine assay kit for 1 min (*see* **Note 16**).
4. Reconstitute the mouse inflammation panel standard cocktail using 250 μL of the assay buffer, keep it at room temperature for 10 min, and label it as C7.
5. Prepare 1:4 dilutions serially in the following sequence: C6, C5, C4, C3, C2, and C1. Use the assay buffer alone for the 0 pg/mL standard.
6. Prior to initiating the assay, wet the wells of the 96-well filter plate with 100 μL of the wash buffer and let it sit at room temperature for 1 min.
7. Remove the wash buffer by placing the filter plate on a vacuum manifold (*see* **Note 17**).

Table 3
Sample template for flow cytometric analysis of cytokines using BioLegend 13-plex LEGENDplex™ inflammation panel. C0–C7 are standards and S1–S40 are samples

	A	B	C	D	E	F	G	H	I	J	K	L
1	C0	C4	S1	S5	S9	S13	S17	S21	S25	S29	S33	S37
2	C0	C4	S1	S5	S9	S13	S17	S21	S25	S29	S33	S37
3	C1	C5	S2	S6	S10	S14	S18	S22	S26	S30	S34	S38
4	C1	C5	S2	S6	S10	S14	S18	S22	S26	S30	S34	S38
5	C2	C6	S3	S7	S11	S15	S19	S23	S27	S31	S35	S39
6	C2	C6	S3	S7	S11	S15	S19	S23	S27	S31	S35	S39
7	C3	C7	S4	S8	S12	S16	S20	S24	S28	S32	S36	S40
8	C3	C7	S4	S8	S12	S16	S20	S24	S28	S32	S36	S40

8. With the filter plate on an inverted plate lid, first add 25 μL of assay buffer to all the wells, and then add 25 μL of standards prepared at **steps 3** and **4**, or BALF supernatants to respective standard or sample wells (*see* **Note 18**).
9. Briefly vortex the bead mixture for 30 s and add 25 μL to each of the standard and sample wells with the filter plate on the inverted plate lid.
10. Seal the plate with a plate sealer, wrap the plate with aluminum foil, and incubate for 2 h at room temperature on a plate shaker at 500 rpm.
11. Remove the solution as described in **step 6** and add 200 μL of the wash buffer to each well on the inverted plate lid (*see* **Note 19**).
12. Remove the wash buffer by applying vacuum to the filter plate on the manifold and blot any residual wash buffer using a paper towel.
13. Repeat **steps 11** and **12** one more time.
14. Add 25 μL of the detection antibody solution from the kit to each well. Seal the plate with a new plate sealer, wrap it with aluminum foil, and incubate for 1 h at room temperature on a plate shaker at 500 rpm.
15. After the 1-h incubation with the detection antibody, add 25 μL of SA-PE from the kit directly to each well. Seal the plate with a new plate sealer, wrap the plate in an aluminum foil and incubate for 30 min at room temperature on a shaker at 500 rpm.
16. Wash the plate twice by repeating **steps 11** and **12**. Add 150 μL of the wash buffer to each well on the inverted plate

lid. Using a plate shaker, shake the plate briefly to resuspend the beads. The beads are ready for flow cytometry and should be analyzed on the same day.

17. Vortex the plate for 5 s and place it on an autosampler.
18. Set the flow rate to low and set the number of beads to be acquired at 300 per sample.
19. Analyze the data using the LEGENDplex™ data analysis software provided with the kit [34] (*see* **Note 20**).

3.4 Nitric Oxide Synthase Activity Assay

1. Completely thaw the BALF supernatants from Subheading 3.1, **step 7** and keep on ice prior to performing the assay. Prepare all the reagents freshly on the day of the assay (*see* **Note 21**).
2. Prepare another identical set of tubes, omitting nitrate reductase. This sample set is used for determining nitrite content alone.
3. Incubate 100 μL of the BALF supernatant samples with 400 μL of the reaction buffer at 37 °C for 30 min to convert nitrate to nitrite.
4. Add 500 μL of 2 mM potassium ferricyanide to the sample tubes to make the final concentration to 1 mM. Incubate at 25 °C for 10 min to oxidize any unreacted NADPH in the reaction buffer.
5. Add 1 mL of Griess reagent and incubate at 25 °C for 10 min. Read the absorbance at 543 nm (*see* **Note 22**). The linear limit of detection for the assay is 1 mM [35].

4 Notes

1. Mice should be housed under constant temperature and a 12-h light/dark cycle with food and water provided *ad libitum*. All procedures must be conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approval by the Institutional Animal Care and use Committee at your institution. Always use personal protective equipment while handling mice or biological samples.
2. If a cytospin is not available, BALF cells may be spread onto the slide by smearing a droplet of cell suspension across the slide with another glass slide held at a 30–45° angle.
3. Some multiplex kits offer a choice of either a filter plate or V-bottom plate for the 96-well sample plate. Here we describe a procedure using a filter plate.

4. To avoid damaging blood vessels, carefully remove the skin layer and use two blunt forceps to pull apart the thyroid tissue to expose the trachea.
5. While performing tracheostomy, be extremely cautious not to disturb any blood vessels near the tracheal incision as it will contaminate the BAL samples and the cellular analysis will be compromised.
6. It is almost impossible to extract the whole amount of BAL (1 mL) as there will be a 20% loss, which is expected. Massaging the thorax region may facilitate maximum recovery of the injected PBS. BALF appears as a slightly cloudy solution with clearly visible particulate matter.
7. After centrifuging the BALF sample at $600 \times g$ for 5 min, take precaution while separating the supernatant from the cells. Leave the last 50 μL of the supernatant to prevent collecting any cells.
8. The supernatant from the BALF samples should be stored at −80 °C until analysis. Avoid repeated freeze–thaw cycles to prevent any degradation of cytokines. It is highly recommended to store the sample in equally divided aliquots of 100 μL each.
9. Keep the resuspended cells from BALF samples on ice and process immediately on the same day for DLC to avoid cell loss due to lysis.
10. During the cytospin procedure, proper assembling of the slide, filter paper, and the solution-holding accessory is vital. Make sure the openings of all three components align to prevent loss of cells during the centrifuging step. Carefully dismantle the cytospin components to prevent smudging of the smear.
11. Do not let the smear dry for more than 30 min.
12. The cell suspension for flow analysis should always be placed in a dark container and the tubes need to be wrapped in aluminum foil to prevent bleaching of fluorescent molecules.
13. When DLC is performed using flow cytometry, wash the cells thoroughly after the incubation with primary antibodies to avoid any artifacts, which compromise the integrity of data.
14. Thereafter, the bottle of beads should be vortexed for 30 s just prior to adding to samples.
15. Eosinophils are less granular and hence appear toward the *y*-axis, whereas neutrophils appear away due to dense granules. In terms of CD-294 staining, eosinophils stain positive for CD294 and appear away from the *x*-axis, whereas neutrophils stain negative for CD294 and hence appear closer to the *x*-axis. If the plot is separated into 4 quadrants, eosinophils appear on

the top left quadrant and neutrophils appear on the bottom right quadrant.

16. The vacuum pressure should always be set to 10 mmHg to prevent any damage to the filter located at the bottom of the plate.
17. Centrifuge the samples and perform the assay with the supernatants to prevent any particulate matters from clogging the bottom of the plate during vacuum application. Perform a protein assay to quantify the protein concentrations of the supernatants and use the same amount of proteins for all samples when performing the cytokine or NOS assays.
18. Do not touch the bottom of the well with the pipette tip as you can damage the filter; instead, introduce the samples or any solutions along the sides of the wells.
19. If the filter becomes clogged at the bottom, use a pipette to pipette up and down the contents of the well. Clear the bottom of the clogged well with a clean wipe and apply vacuum.
20. If the data obtained is not within the standard range, adjust the dilution of the samples and repeat the assay.
21. For consistency of the reaction condition, assay all the samples to be compared at the same time.
22. If the sample values are too diluted, use a concentrating centrifuge tube like Vivaspin 6 Centrifugal Concentrator and repeat the assay.

Acknowledgments

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Chapter 16

Procedures to Evaluate Inflammatory and Pathological Changes During Allergic Airway Inflammation

Savita P. Rao, Stephanie Rastle-Simpson, Mythili Dileepan, and P. Sriramarao

Abstract

Cellular inflammation, with elevated levels of Th1/Th2 cytokines, airway mucus hypersecretion, and thickening of the airway smooth muscle, are characteristic features of the allergic lung. Assessment of pathophysiological changes in allergic lungs serves as an important tool to determine disease progression and understand the underlying mechanisms of pathogenesis. This can be achieved by evaluating the lung tissue for inflammation and airway structural changes along with the measurement of important pro-inflammatory mediators such as Th1/Th2 cytokines and eotaxins. This chapter describes procedures to histologically evaluate inflammatory and pathological changes observed during allergic airway inflammation using lung tissue from mice.

Key words Allergic inflammation, Lung tissue, Tissue lysis, Th1, Th2, Cytokines, Histology, Airway mucus secretion, Airway smooth muscle hypertrophy, Fibrosis

1 Introduction

Allergic airway inflammation (AAI), including allergic asthma, is associated with increased pulmonary recruitment of inflammatory cells such as eosinophils, mast cells, and activated $CD4^+$ T cells, along with elevated levels of Th2 cytokines and chemokines, which together orchestrate various aspects of allergic inflammation [1, 2]. Additionally, prolonged allergen exposure leads to airway remodeling due to structural changes in the airways caused by mucus hypersecretion by airway goblet cells, increased airway smooth muscle mass from hyperplasia or hypertrophy of smooth muscle cells, and excessive collagen deposition leading to airway fibrosis, which in turn contribute to airflow obstruction and airway hyperresponsiveness [3]. Mouse models of allergic asthma are widely used to gain a better understanding of the mechanisms underlying the pathophysiology of this disease and to evaluate newly developed therapeutic strategies for safety and efficacy

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[4, 5]. Allergen-challenged mice replicate many features of human asthma and together with the availability of novel reagents, genetically manipulated strains, and innovative technology offer a unique opportunity to interrogate the role of specific inflammatory mediators in promoting aspects of allergic asthma such as airway inflammation and remodeling.

We have extensively investigated the role of pro-inflammatory mediators [6–11] and inhibitors targeting signaling molecules [12] or specific enzymes [13] on the outcome of allergic asthma, specifically airway inflammation, Th1/Th2 cytokines, eotaxins, and lung pathophysiology in mouse models. Here, we have compiled methods routinely used in these studies to evaluate the pathophysiology of the allergic lung in mice.

2 Materials

2.1 Collection and Processing of Lungs for Pathophysiological Evaluation

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4.
2. Syringes: 1 mL.
3. Microcentrifuge tubes: graduated, 2 mL.
4. Cannula/needles: blunt, 21-gauge.
5. Forceps: curved, blunt end.
6. Craft thread: for tying off airways.
7. Dissection scissors.
8. 16% Paraformaldehyde stock solution: 16% aqueous paraformaldehyde, electron microscopy grade, is commercially available. It is typically sold in 10 mL ampules. It is stored at room temperature and diluted with PBS to prepare 4% paraformaldehyde as needed.
9. 4% Paraformaldehyde fixative: Prepare freshly from the paraformaldehyde stock solution by making 1:4 dilution in PBS.
10. Centrifuge tubes: 15 mL, for paraformaldehyde fixation.
11. Liquid nitrogen: for snap-freezing lung tissue.
12. 70% Ethanol solution: Prepare 70% ethanol solution in distilled water using absolute ethanol. Make 1–2 L at a time or as needed and store in airtight plastic gallon-size jugs at room temperature.
13. Biopsy embedding cassettes with lids: 1.5 in × 1 in.
14. Plastic container with lid: for biopsy embedding cassettes.
15. Microtome: for sectioning of paraffin-embedded tissue.
16. Microscope slides: Superfrost™ Plus or equivalent.

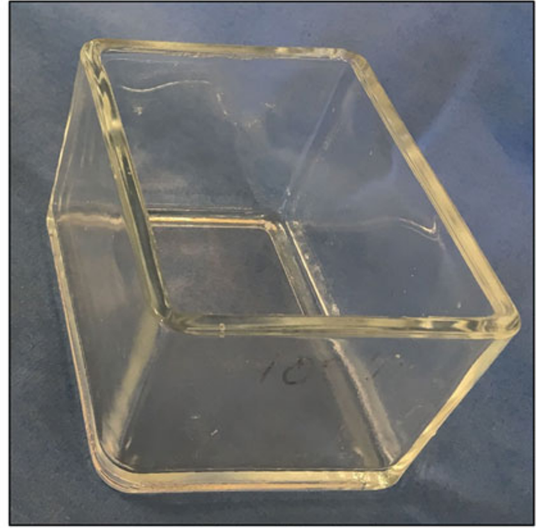
2.2 Preparation of Lung Lysates

1. Protease inhibitor cocktail: 100×. Store at −20 °C.
2. Phosphatase inhibitor cocktail: 100×. Store at −20 °C.
3. 200 mM Phenylmethylsulfonyl fluoride (PMSF): Prepare 10 mL of 200 mM PMSF in isopropyl alcohol and store as 200 μL aliquots at −20 °C.
4. Lysis buffer: 0.5% Triton X-100 in PBS. Store at 4 °C. Just before use, add 1/100 volume of 100× protease inhibitor cocktail, 1/100 volume of 100× phosphatase inhibitor cocktail, and 1/100 volume of the 200 mM stock PMSF to final concentration 2 mM. Chill on ice.
5. Microcentrifuge tubes: graduated, 2 mL.
6. Tissue homogenizer.
7. Ultrasonic cell disrupter.
8. Microcentrifuge.
9. BCA protein assay system.
10. BD™ Cytometric Bead Array (CBA) Mouse Th1/Th2 Cytokine Kit and Mouse IL-13 Flex Set (*see* **Note 1**). Store at 4 °C.
11. Enzyme-linked immunosorbent assay (ELISA) plates.
12. Eotaxin-1 and -2 ELISA kits (*see* **Note 2**).
13. Flow cytometer.
14. ELISA plate reader.

2.3 Lung Histology

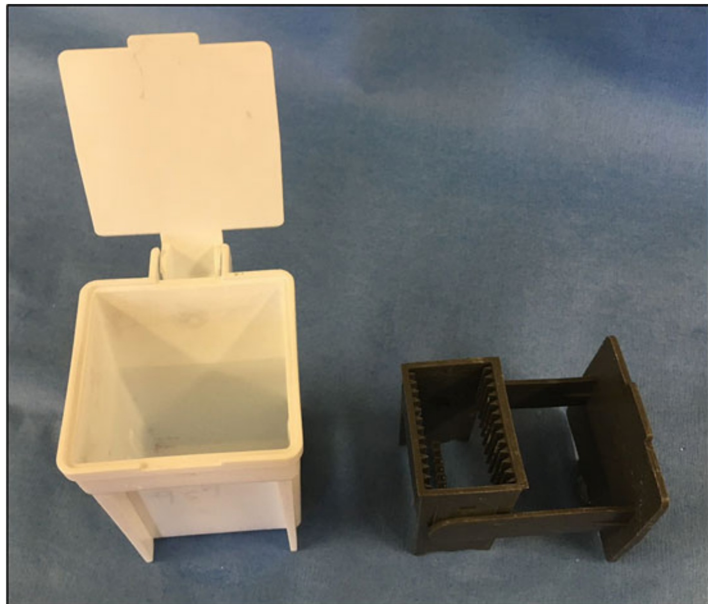
1. Glass staining slide rack (slotted) and staining dish (Fig. 1).
2. Slide staining jar and slide staining rack (Fig. 2).
3. Microscope coverslips: glass, for mounting.
4. Tray: firm plastic, large enough for holding all sample slides.
5. CitriSolv™ Hybrid solvent and clearing agent: ready to use.
6. 95% and 70% Ethanol solutions: Prepare 95% and 70% ethanol solutions in distilled water using absolute ethanol. Make 1–2 L at a time or as needed and store in airtight plastic gallon-size jugs at room temperature.
7. Hematoxylin and alcoholic eosin Y solutions: Obtain ready-to-use solutions.
8. 1% Acid alcohol solution: Add 2 mL of 37% hydrochloric acid to 200 mL of 70% ethanol and mix well. Prepare just before use.
9. 0.2% Ammonia water solution: Add 2 mL of 28–30% ammonium hydroxide solution to 1 L of distilled water and mix well. Prepare just before use.
10. Mounting medium: Low-viscosity Cytoseal™ 60 or equivalent.

Slotted slide rack (glass)



Staining dish (glass)

Fig. 1 Glass-slotted slide rack and staining dish for deparaffinization and rehydration of tissue sections



EasyDip™ slide staining jar and slide staining rack (plastic)

Fig. 2 EasyDip™ slide staining rack and slide staining jar for staining of tissue sections

11. Periodic acid-Schiff staining kit: commercially available kit containing 0.5% periodic acid, Schiff's reagent, and Gill's hematoxylin solution, No. 3, all ready to use.
12. 1% Acetic acid: Dilute 8.8 mL of 1 N acetic acid with 41.2 mL of distilled water.

13. Bouin's solution: 1% picric acid, 9% formaldehyde, and 5% acetic acid solutions. Bouin's solution is a fixative and commercially available as a ready-to-use solution. Store at room temperature.
14. Weigert's iron hematoxylin staining kit: commercially available kit containing Weigert's iron hematoxylin solution A (1% certified hematoxylin in ethanol) and Weigert's iron hematoxylin solution B (1.2% (w/v) ferric chloride and 1% (v/v) hydrochloric acid).
15. Masson's trichrome staining kit: commercially available kit containing Biebrich scarlet-acid fuchsin solution (0.9% Biebrich scarlet, 0.1%, acid fuchsin in 1.0% acetic acid), 10% phosphotungstic acid solution, 10% phosphomolybdic acid solution, and aniline blue solution (2.4% aniline blue in 2% acetic acid).
16. Hydrophobic barrier PAP pen.
17. 0.12% Trypsin solution: Trypsin Digest-All 2 kit or equivalent. This kit contains 0.5% trypsin and liquid diluent. Prepare a 0.12% trypsin solution with the diluent provided.
18. Tris-buffered saline (TBS): 150 mM NaCl, 20 mM Tris, pH 7.6.
19. TBS containing 0.01% Tween-20 (TBST): Add 10 μL Tween-20 per 100 mL of TBS.
20. 0.3% Hydrogen peroxide (H_2O_2) solution: Dilute 30% H_2O_2 solution at 1:100 in distilled water.
21. Mouse monoclonal antibodies against α-smooth muscle actin.
22. Avidin–biotin staining kit with anti-mouse IgG secondary antibody: VECTASTAIN® Elite® ABC HRP kit (Vector Laboratories) or equivalent.
23. AEC (3-amino-9-ethylcarbazole) peroxidase substrate kit.
24. Normal mouse IgG.
25. Glycerol gelatin, aqueous slide mounting medium: Store refrigerated (*see* **Note 3**).

3 Methods

3.1 Collection of Lungs from Mice

Detailed procedures describing mouse dissection to collect lungs can be found elsewhere [14, 15] and are not described herein. Fixation and embedding of harvested tissue are necessary before sectioning to maintain cell structure and tissue morphology. The two most commonly used materials for embedding mouse tissue are paraffin wax and optimal cutting temperature (O.C.T) compound, which contains a mixture of soluble glycols and resins for tissue sectioning at low temperatures.

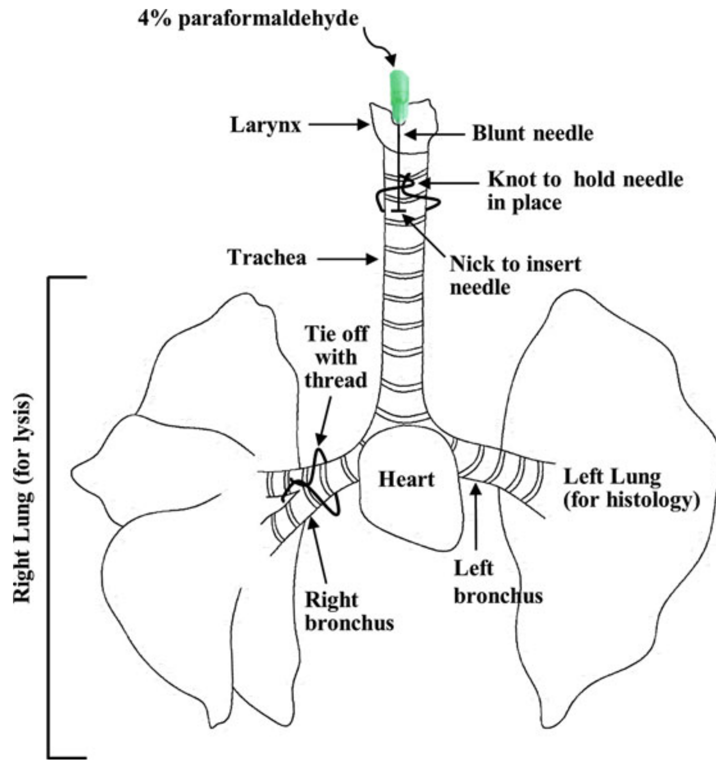


Fig. 3 Schematic of mouse lung collection for analysis. First, the right bronchus is tied off with craft thread, and individual lobes of the right lung are collected in separate tubes. Next, the left lung is infused with 4% paraformaldehyde and then fixed in paraformaldehyde for immunohistochemical staining

1. Once the trachea and lungs of the allergen-challenged mouse are exposed and the trachea is freed from surrounding tissue, tie off the main bronchus leading into the right lung with craft thread (Fig. 3).
2. Remove the lobes of the right lung gently without disturbing the knot and place individual lobes in labeled microcentrifuge tubes. Snap-freeze in liquid nitrogen and store at −80 °C for later analysis.
3. Slightly raise the trachea with a pair of curved forceps to exert mild tension on the trachea.
4. Make a small "V"-shaped nick on the top of exposed trachea using dissection scissors. Insert a piece of craft thread, approximately 2.5 in. long, under the trachea.
5. Insert a 21-gauge blunt needle into the "V"-shaped nick in the trachea.
6. Once the needle has been inserted into the trachea, tie a knot around the trachea with the craft thread inserted in **step 4** to hold the needle in place.

7. Connect a 1-mL syringe containing 4% paraformaldehyde to the inserted needle and slowly infuse the lung with 0.5 mL of 4% paraformaldehyde until the lung inflates (*see* **Note 4**). Employ a flat angle approach such that the paraformaldehyde is directed toward the lung.
8. After inflating the lung, remove the needle by a gentle twisting motion and quickly clamp trachea by tightening the knot placed around the trachea (in **step 6**) to prevent the paraformaldehyde from leaking out of the hole in the trachea left from the removal of the needle.
9. Using the free ends of the thread, lift trachea carefully, and dissect the infused lung along with the heart out of the thoracic cavity by cutting the trachea above the knot.
10. Transfer the lungs to a labeled 15-mL tube containing 4% paraformaldehyde (≈5 mL/tube), ensuring that the tissue is well submerged in paraformaldehyde. Fix lungs in 4% paraformaldehyde at 4 °C for up to 48 h (*see* **Note 5**).
11. After fixation, trim other tissue (i.e., heart and other connective tissue) off the lung, dissect lobes, and transfer into labeled 15-mL tubes containing 70% ethanol (*see* **Note 6**).
12. After 12–24 h in 70% ethanol, transfer the lobes to a biopsy embedding cassette (*see* **Note 7**).
13. Place cassettes in a plastic container containing 70% ethanol (to prevent tissue from drying) and embed in paraffin. Paraffin-embedding of tissue is most often automated and is therefore not described here.
14. Section the paraffin-embedded tissue, 4 μm thick, using a microtome (*see* **Note 8**).

3.2 Preparation of Lung Lysates

1. Take the tubes with frozen lung tissue (from Subheading 3.1, **step 2**) out of −80 °C storage and transfer onto the ice. Immediately add ≈300 μL of chilled lysis buffer to the tube (*see* **Note 9**).
2. Homogenize tissue with a tissue homogenizer (3–4 cycles, 30 s each cycle), keeping the tube on the ice during the entire process.
3. Next, sonicate the tissue homogenate (3 cycles, 15 s each with a 10–15 s break between cycles) on ice using an ultrasonic cell disruptor with the power setting at 3 W (*see* **Note 10**).
4. Centrifuge the samples in a refrigerated microcentrifuge at 14,000 × *g* for 10 min to pellet debris.
5. Collect supernatants in new, sterile, labeled tubes and discard the debris.

6. Measure the protein concentration of the supernatants by BCA protein assay as per the manufacturer's instructions [16] (*see* **Note 11**).
7. Store the supernatants as aliquots in labeled tubes at −80 °C until measurement of cytokines and chemokines.

3.3 Analysis of Th1/Th2 Cytokines and Eotaxins

1. Thaw the lung lysate supernatants from Subheading 3.2 on ice (*see* **Note 12**).
2. Measure Th1/Th2 cytokines in the supernatants by flow cytometry using CBA Mouse Th1/Th2 Cytokine Kit and Mouse IL-13 Flex Set and analyze results as per the manufacturer's instructions [8] (*see* **Note 1**).
3. Measure eotaxin-1 and -2 in the lung supernatants using ELISA kits and analyze results using a plate reader as per the manufacturer's instructions [11] (*see* **Note 2**).
4. Cytokine and chemokine data generated with commercially available kits are usually expressed as pg/mL of sample; however, for lung tissue levels, cytokine and chemokine levels can be expressed as pg cytokine (or chemokine)/mg protein. To obtain cytokine or chemokine levels as pg/mg protein, divide cytokine or chemokine pg/mL (derived from assay) by mg protein/mL in lung lysate supernatant obtained from BCA assay in Subheading 3.2, **step 6**.

3.4 Deparaffinization and Rehydration of Tissue Sections for Histological Analyses

The procedures described in this section are applicable to formalin-fixed, paraffin-embedded mouse lung tissue.

1. To deparaffinize, place slides with lung sections in a slotted glass slide staining rack (Fig. 1) and immerse into a glass staining dish containing CitriSolv for 3 min. Repeat two more times, using fresh CitriSolv each time (*see* **Note 13**).
2. To rehydrate sections, transfer the rack of slides to a staining dish containing absolute ethanol for 3 min. Repeat this step two more times, using fresh absolute ethanol each time.
3. Immerse the slide rack in 95% ethanol for 2 min followed by immersion in 70% ethanol for another 2 min.
4. Transfer the slide rack to a fresh staining dish containing tap water and rinse the slides by gently running tap water through the dish for 3 min to complete the rehydration process.
5. Rinse the slides once in distilled water and gently shake the slides to remove excess water. Remove residual water by holding the slides vertical on a paper towel with the long edge of the slides making contact with the paper towel (*see* **Note 13**).
6. For histological analysis of lung cellular inflammation, airway mucus secretion or lung fibrosis, proceed to Subheading 3.5,

Subheading 3.6 or Subheading 3.7, respectively. For antibody-based immunohistochemical staining of α-smooth muscle actin, proceed to Subheading 3.8.

3.5 Hematoxylin and Eosin (H&E) Staining for Detection of Lung Cellular Inflammation

1. Place deparaffinized slides in a slide staining rack (Fig. 2). Immerse the rack in a slide staining jar containing the hematoxylin solution for 10–30 s at room temperature (*see* **Note 14**).
2. Transfer the rack to a staining dish and rinse the slides by gently running tap water through the dish for 3 min.
3. Transfer the rack to a staining jar containing 1% acid alcohol for 5 s to differentiate the staining.
4. Rinse the slides as described above in **step 2** for 1 min.
5. Transfer the rack to a staining jar containing 0.2% ammonia water for 1 min (*see* **Note 15**).
6. Rinse the slides as described in **step 2**.
7. Dip the slide rack 10 times in a staining jar containing 95% ethanol.
8. Transfer the slide rack to a staining jar containing the alcoholic eosin Y solution for 30 s to counterstain the sections (*see* **Note 16**).
9. Dehydrate the sections by transferring the slide rack to a jar containing 95% ethanol for 1 min followed by two changes in absolute ethanol, 10 dips each time.
10. Allow the slides to air-dry.
11. Place the slides on a horizontal surface and apply two small drops of mounting medium to cover the tissue sections.
12. Carefully place a coverslip on top of the mounting medium (*see* **Note 17**).
13. Allow the slides to dry completely before evaluation.
14. Observe the slides under a light microscope. Hematoxylin stains negatively charged nucleic acids bluish purple. Eosin stains proteins pink. In a tissue section, nuclei stain dark purple while the cytoplasm and extracellular matrix show varying shades of pink staining. H&E-stained lung sections from an allergen-challenged mouse and a control (nonallergen challenged) mouse are shown in Fig. 4.

3.6 Periodic Acid–Schiff (PAS) Staining for Detection of Airway Mucus

1. Place deparaffinized slides in a slide staining rack. Immerse the rack in a slide staining jar containing the periodic acid solution for 5 min at room temperature (*see* **Note 18**).
2. Transfer the slide rack to a clean staining jar containing distilled water and rinse the slides with at least five changes of distilled water.

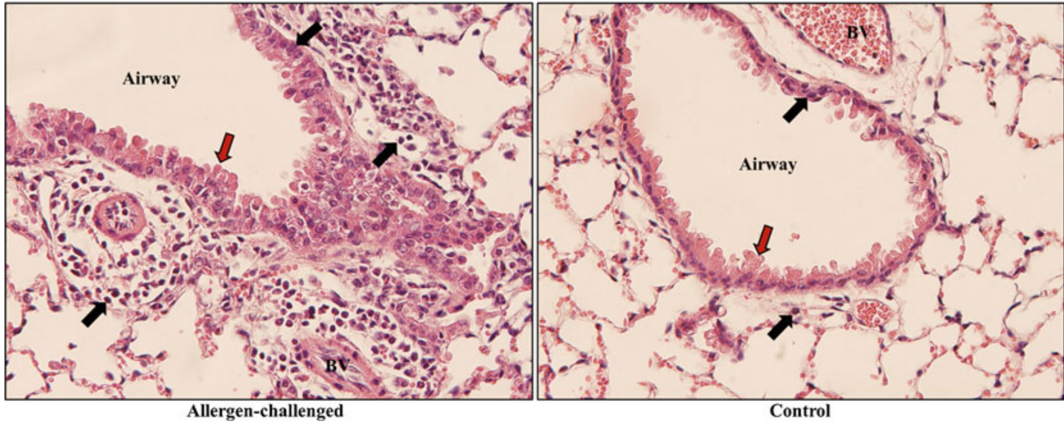


Fig. 4 H&E-stained mouse lung tissue. Representative images of lung sections from allergen-challenged (left) and control (nonallergen challenged, right) mice are shown. BV, blood vessel. Black arrows indicate nuclei stained dark purple and red arrows indicate cytoplasm stained pink. Magnification $\times$200. Note the increased presence of cells (dark purple nuclei) around the airways and BV in the allergen-challenged lung section

3. Gently shake the slides to remove excess water. Remove residual water by holding the slides vertical on a paper towel with the long edge of the slides making contact with the paper towel.
4. Return the slides to the slide rack and immerse the rack in a staining jar containing Schiff's reagent for 15 min at room temperature.
5. Transfer the slide rack to a staining dish containing tap water and rinse the slides by gently running tap water through the dish for 5 min. Remove excess water as described above in **step 3**.
6. Airway mucus secretion/production can be quantified [11] using an image analysis program such as ImageJ [17]. If quantification is required, omit the following counterstain step and proceed to **step 9**.
7. Counterstain the slides by transferring the slide rack to a staining jar containing the hematoxylin solution, Gill No. 3 for up to 30 s (*see* **Note 19**).
8. Rinse the slides in gently running tap water as described in **step 5**.
9. Allow the slides to air-dry before mounting as described in Subheading 3.5, **steps 11–13**.
10. Observe the slides under a light microscope. In slides counterstained with hematoxylin solution, Gill No. 3, PAS-positive areas, such as mucus, stain pink while nuclei stain bluish purple. An example of PAS-stained airways from an allergen-challenged mouse and a control (nonallergen challenged) mouse after counterstaining is shown in Fig. 5.

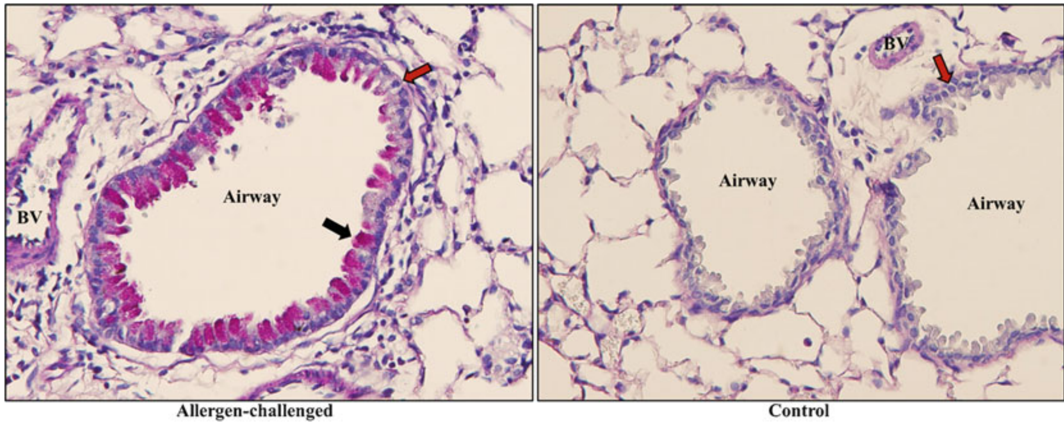


Fig. 5 PAS-stained mouse lung tissue counterstained with hematoxylin. Representative images of lung sections from allergen-challenged (left) and control (right) mice are shown. BV, blood vessel. Black arrows indicate airway mucus stained pink and red arrows indicate nuclei stained blueish purple. Magnification ×200. Note the increased staining for mucus in airway epithelial goblet cells (pink) in the allergen-challenged lung section

3.7 Trichrome Staining for Detection of Airway Fibrosis

1. Place deparaffinized slides in a slide staining rack and immerse the rack in a slide staining jar containing Bouin's solution.
2. Incubate overnight at room temperature.
3. Transfer the rack with slides to a staining dish containing tap water and rinse by gently running tap water through the dish for 2–3 min to remove the yellow color from the sections.
4. Prepare a working solution of Weigert's iron hematoxylin by mixing equal parts of solution A and B from the Weigert's iron hematoxylin staining kit.
5. Place the slide rack in a staining jar containing the working solution of Weigert's iron hematoxylin for 5 min.
6. Wash the slides in tap water as described above in **step 3** for 5 min.
7. Transfer the slide rack to a staining dish containing distilled water and rinse quickly.
8. Place the slide rack in a staining jar containing Biebrich scarlet-acid fuchsin solution for 5 min.
9. Transfer the slide rack to a staining dish containing distilled water and rinse for 5 min with 2–3 changes of distilled water.
10. Prepare a working solution of phosphotungstic/phosphomolybdic acid by mixing one volume *each* of the phosphotungstic acid solution and the phosphomolybdic acid solution provided with the Masson's trichrome staining kit with two volumes of deionized water.

11. Place the slide rack in a staining jar containing the working solution of phosphotungstic/phosphomolybdic acid for 5 min.
12. Transfer the slide rack to a staining jar containing the aniline blue solution from the Masson's trichrome staining kit for 5 min.
13. Place the slide rack in a staining jar containing 1% acetic acid for 2 min.
14. Transfer the slide rack to a staining dish containing tap water and rinse the slides using three changes of tap water.
15. Dehydrate the sections through ethanol as described in Subheading 3.5, **step 9**.
16. Clear the sections by placing the slide rack in a jar containing CitriSolv for 1 min. Repeat this step two more times, using fresh CitriSolv each time.
17. Allow the slides to air-dry and mount as described in Subheading 3.5, **steps 11–13**.
18. Observe the slides under a light microscope. Masson's trichrome staining kit stains collagen blue and cytoplasm different shades of red. An example of lung sections from an allergen-challenged mouse and a control mouse after Masson's trichrome staining is shown in Fig. 6.

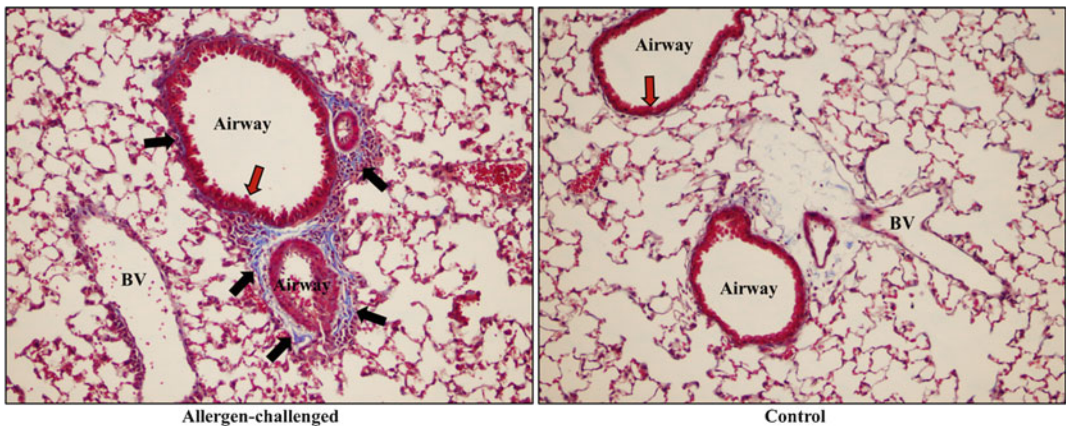


Fig. 6 Trichrome-stained mouse lung tissue. Representative images of lung sections from allergen-challenged (left) and control (right) mice are shown. BV, blood vessel. Black arrows indicate collagen stained blue and red arrows indicate cytoplasm stained red. Magnification ×100. Note the increased collagen deposition (blue) beneath the airway epithelium in the allergen-challenged lung section

3.8 α-Smooth Muscle Actin Immunohistochemical Staining for Detection of Smooth Muscle Mass

α-Smooth muscle actin is expressed by smooth muscle cells and is commonly used as a molecular marker for the detection of smooth muscle mass [7, 9, 18].

3.8.1 Antigen Retrieval

Antigen retrieval methods to unmask cross-linking of protein antigens caused by tissue fixation are antibody-specific. The antigen retrieval method for α-smooth muscle actin antibody is described below:

1. After deparaffinization and rehydration of tissue sections as described in Subheading 3.4, draw a large circle around the section(s) on the slide with a hydrophobic barrier PAP pen (Fig. 7a, b) (*see* **Note 20**).
2. Place the slides on a firm tray, cover sections uniformly with 50–100 μL of trypsin solution, and incubate at 37 °C for 20 min (*see* **Note 21**).
3. Discard the trypsin solution by orienting the slides vertical along their long edge (Fig. 7b).
4. Transfer the slides to a slide rack and place the rack in a staining dish containing TBST. Rinse slides by gently rocking the dish two times. Let slides sit in TBST for 5 min. Repeat this step two more times, replacing the TBST after each rinse.

3.8.2 Quenching of Endogenous Peroxidase Activity

1. Place the slide rack with slides in a staining jar containing 0.3% hydrogen peroxide solution at room temperature for 30 min.
2. Wash the slides with TBST as described in Subheading 3.8.1, **step 4**.

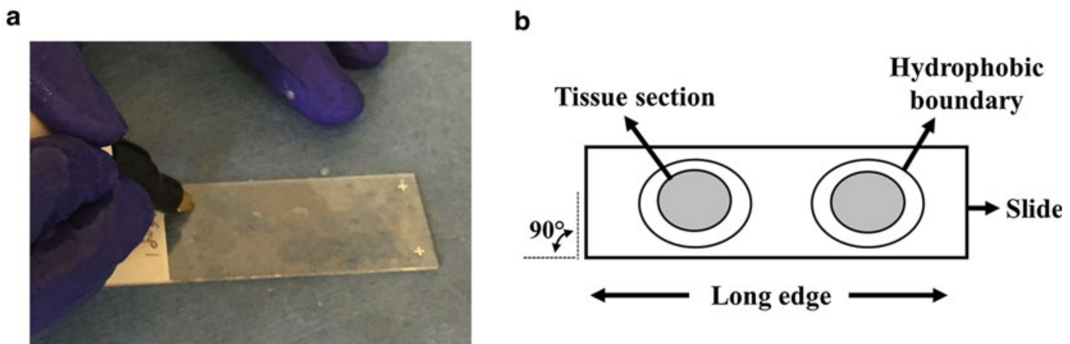


Fig. 7 Illustration of a hydrophobic barrier around a specimen on a slide. (**a**) Application of a boundary around a specimen with a PAP pen. (**b**) Cartoon showing a hydrophobic boundary around specimen on a slide vertically oriented along its long edge

3.8.3 Antibody Incubation

The primary antibody used for the detection of α-smooth muscle actin is a mouse monoclonal antibody. The next few steps are carried out using components from the Vectastain® Elite® ABC HRP Kit for the detection of mouse IgG.

1. Prepare blocking buffer by adding 15 μL of horse serum (provided as part of the Vectastain® Elite® ABC HRP Kit for mouse IgG) per 1 mL of TBST, i.e., 1.5% (*see* **Notes 22** and **23**).
2. After washing with TBST as described in Subheading 3.8.1, **step 4**, place the slides on a firm tray and cover each section uniformly with 50–100 μL of the blocking buffer. Transfer the tray with slides to a humidified chamber and incubate for 50 min at room temperature (*see* **Note 24**).
3. During incubation with the blocking buffer, prepare a primary antibody solution by diluting the α-smooth muscle actin antibody to a final concentration of 2 μg/mL in the blocking buffer (*see* **Note 25**). Also, prepare a solution of normal mouse IgG of the same concentration to be used as a negative control (*see* **Note 23**).
4. At the end of incubation with the blocking buffer, discard the buffer by orienting the slides vertical along their long edge and remove residual blocking buffer by holding the slides vertically on a paper towel with the long edge of the slide making contact with the paper towel; ensure that the sections are not disturbed. It is not necessary to wash slides after this step.
5. Return the slides to the tray and add 50–100 μL/section of the prepared primary antibody or normal mouse IgG to the appropriate sections.
6. Place the tray with slides in a humidified chamber and incubate overnight at 4 °C.
7. At the end of incubation with the primary antibody, allow the slides to equilibrate to room temperature for 15 min.
8. During this equilibration, prepare the secondary antibody solution by adding 5–6 μL of biotinylated horse anti-mouse IgG (provided as part of the Vectastain® Elite®ABC HRP Kit) per 1 mL of the blocking buffer (*see* **Note 23**).
9. Remove the primary antibody solution from the sections as described earlier in **step 4**.
10. Transfer the slides to a slide rack and rinse three times with TBST as described in Subheading 3.8.1, **step 4**. Gently shake off excess buffer and remark sections with hydrophobic barrier PAP pen if needed.
11. Place the slides on a tray and add 50–100 μL/section of the prepared secondary antibody to the sections. Incubate in a humidified chamber for 1 h at room temperature.

12. Halfway through incubation with the secondary antibody, prepare avidin-biotin complex (ABC) reagent (provided as part of the Vectastain® Elite® ABC HRP Kit for mouse IgG) as follows: Add 20 μL of avidin DH (Reagent A of Vectastain® Elite® ABC HRP Kit) per 1 mL of TBST in a tube; then add 20 μL of biotinylated-HRP (Reagent B of Vectastain® Elite® ABC HRP Kit) and mix immediately and let stand (*see* **Note 23**). This reagent should be prepared 30 min before use.
13. At the end of incubation, remove the secondary antibody solution as described in **step 4**.
14. Transfer the slides to a slide staining rack and rinse three times with TBST as described in Subheading 3.8.1, **step 4**. Gently shake off excess buffer and return slides to the tray.
15. Add 50–100 μL/section of the prepared ABC reagent to the sections and incubate in a humidified chamber for 30 min at room temperature. This timing can be increased depending on the desired intensity.
16. Remove ABC reagent from the sections as described in **step 4** and rinse three times with TBST as described in Subheading 3.8.1, **step 4**. Gently shake off excess buffer and place slides on the tray.
17. Just before use, prepare peroxidase substrate solution using components from the AEC (3-amino-9-ethylcarbazole) substrate kit as follows (*see* **Note 26**): Add two drops (approximately 72 μL) of buffer stock solution, three drops (approximately 90 μL) of AEC stock solution and two drops (approximately 80 μL) of hydrogen peroxide solution to 5 mL of distilled water and mix well (*see* **Note 23**).
18. Add 50–100 μL/section of the prepared peroxidase substrate solution to the sections and incubate at room temperature. The incubation time can vary from 2 to 30 min and should be standardized by individual users.
19. At the end of incubation, remove the peroxidase substrate solution as described in **step 4**.
20. Transfer slides to a staining rack and immerse in a staining dish containing tap water; rinse with several changes of tap water.
21. Remove slides from staining rack, gently shake off excess water and remove residual water on a paper towel as described in **step 4**. Return slides to the tray.
22. Airway smooth muscle mass can be quantified [11] using an image analysis program such as ImageJ [17]. If quantification is required, omit the following counterstain step and proceed to **step 27**.

23. Add 50–100 μL/section of hematoxylin and incubate for 10–30 s at room temperature to counterstain the tissue sections.
24. At the end of incubation, remove hematoxylin as described in **step 4** and transfer slides to a slide rack.
25. Place slide rack in a staining jar containing tap water and rinse in gently running tap water for 5 min to promote nuclear bluing.
26. Remove slides from staining rack and gently shake off excess water. Remove residual water on a paper towel as described in **step 4** and return to the tray.
27. Apply one small drop of aqueous glycerol gelatin mounting medium to each section (*see* **Note 3**).
28. Carefully place a coverslip on top of the mounting medium (*see* **Note 17**) and let the medium spread evenly. Press the coverslip to remove remaining air bubbles and allow to air-dry.
29. Observe under a light microscope. In counter-stained slides, smooth muscle actin-positive areas stain dark brown while nuclei stain blue. An example of lung sections from an allergen-challenged mouse and a control mouse after immunohistochemical staining with α-smooth muscle actin monoclonal antibodies is shown in Fig. 8.

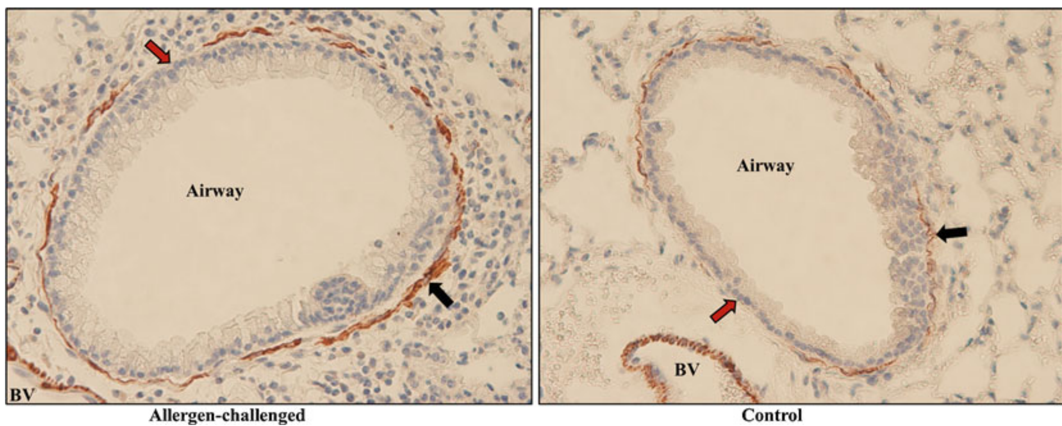


Fig. 8 SMA-stained mouse lung tissue counterstained with hematoxylin. Representative images of lung sections from allergen-challenged (left) and control (right) mice are shown. BV, blood vessel. Black arrows indicate smooth muscle actin-positive areas stained dark brown and red arrows indicate nuclei stained blue. Magnification ×200. Note the increased thickness of the smooth muscle layer (dark brown) beneath the airway epithelium in the allergen-challenged lung section

4 Notes

1. Mouse Th1/Th2 Cytokine Kit contains cytokine capture beads, phycoerythrin (PE)-conjugated cytokine detection reagent, and standards for quantitative analysis of IL-2, IL-4, IL-5, IFN-γ, and TNF-α. Mouse IL-13 Flex Set contains IL-13 capture beads, PE-conjugated IL-13 detection reagent, and Mouse IL-13 standard for quantitative analysis of IL-13.
2. Each solid-phase sandwich ELISA kit contains capture and detection antibody, recombinant standard and streptavidin-conjugated horseradish peroxidase (HRP) for quantitative analysis of eotaxin-1 and eotaxin-2.
3. Glycerol gelatin is highly viscous and should be warmed to approximately 50–60 °C in an incubator or hot water bath before use. The mounting medium cools rapidly, limiting the number of slides that can be cover-slipped at one time. Ensure that the bottle containing the mounting medium remains warm by placing it in a beaker containing hot water between slides.
4. It is important to use 0.5 mL of paraformaldehyde for the infusion of one lung. Infusing more than 0.5 mL of paraformaldehyde can lead to over-inflation and "blow up" lung alveoli, while using volumes under 0.5 mL can cause the lung to collapse and result in poor sectioning.
5. The recommended duration of fixation for mouse tissue that is no larger than 1.5 cm thick is 24–48 h. Paraformaldehyde solution should be examined every 24 h; if the solution is cloudy, it should be replaced with fresh 4% paraformaldehyde.
6. Transferring tissue to 70% ethanol will halt formalin fixation and acclimatize tissue to the first stage of the dehydrating process.
7. Lobes can be stored in 15-mL tubes containing 70% ethanol at 4 °C and paraffin embedded later. Before embedding, lung tissue samples should be adequately trimmed for optimal processing and embedding. Maintain a maximum height of 3 mm and leave tissue loosely in the embedding cassette for ample penetration of paraffin.
8. The directions for using a microtome vary according to the make and model of the equipment and are therefore not described here.
9. The volume of lysis buffer used to lyse lung tissue should be determined by the size of the lung tissue. The recommended volume of lysis buffer is 5–6 μL/mg of lung tissue.

10. The power setting for the cell disruptor can vary depending on the make and model of the cell disruptor and should be standardized by the user.
11. BCA protein assay is most often performed using a commercially available kit as per the manufacturer's instructions.
12. It is important to determine the amount of sample needed for Th1/Th2 cytokine analysis by flow cytometry using CBA Mouse Th1/Th2 Cytokine Kit and Mouse IL-13 Flex Set and/or measurement of eotaxins by ELISA in advance and thaw only the necessary number of frozen aliquots of lung lysate supernatants. Freeze–thaw cycles can lead to sample degradation.
13. It is important to not let tissue sections become dry between steps.
14. The incubation time indicated is for staining with hematoxylin from Leica. This timing will need to be standardized by the end user and may vary depending on the source of the hematoxylin solution used. Hematoxylin solution can be reused at least three times with reproducible staining.
15. This step is called bluing and the initial soluble red color of the hematoxylin in the nucleus is converted to an insoluble bluish purple color by the alkaline pH (pH 10.0) of ammonia water.
16. The incubation time indicated is for staining with alcoholic eosin Y from Leica. This timing will need to be standardized by end user and may vary depending on the source of the eosin Y solution used.
17. To prevent air bubbles from being trapped under the coverslip, use forceps to angle the coverslip over the section with one edge of the coverslip touching the slide and gently lower the coverslip into place to cover the section.
18. All reagents in the periodic acid–Schiff staining kit should be brought to room temperature before use.
19. The timing of incubation with hematoxylin solution, Gill No. 3, can vary and should be standardized by the end user.
20. Drawing a hydrophobic boundary around tissue sections enables the use of smaller antibody volumes and evaluation of multiple sections on the same slide stained with different antibodies, e.g., isotype control IgG and antigen-specific IgG. Remarking with PAP hydrophobic barrier pen may be needed after washes.
21. Optimal incubation time with the trypsin solution may vary depending on the degree of fixation and should be standardized by the end user.

22. Sections should be blocked with a blocking solution to prevent nonspecific binding of the primary antibody to the tissue. The species of the serum used in blocking solutions should be the same as the source of the secondary antibody.
23. The total volume to be prepared will depend on the number of slides to be stained and the number of sections per slide with a requirement of 50–100 μL/section.
24. A plastic box with a lid containing moist paper towels at the bottom can be used as a humidified chamber.
25. The concentration of the primary antibody will need to be standardized by the end user based on the vendor and lot number and of the antibody being used.
26. All reagents required for preparing the peroxidase substrate solution are included in the AEC substrate kit.

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Chapter 17

Assessment of Lung Eosinophils In Situ Using Immunohistological Staining

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Abstract

Eosinophils are rare white blood cells that are recruited from circulation to accumulate in the lung in mouse models of allergic respiratory inflammation. In hematoxylin–eosin (HE) stained lungs, eosinophils may be difficult to detect despite their bright eosin staining in the secondary granules. For this reason, antibody-mediated detection of eosinophils is preferable for specific and clearer identification of these cells. Moreover, eosinophils may degranulate, releasing their granule proteins into surrounding tissue, and remnants of cytolysed cells cannot be detected by HE staining. The methods here demonstrate the use of eosinophil-specific anti-mouse antibodies to detect eosinophil granule proteins in formalin-fixed cells both in situ in paraffin-embedded lungs, as well as in cytospin preparations from the lung. These antibody staining techniques enable either colorimetric or fluorescence imaging of eosinophils or their granule proteins with the potential for additional antibodies to be added for detection of multiple molecules.

Key words Eosinophils, Immunohistochemistry, Lung, Eosinophil peroxidase, Major basic protein, Staining, Formalin-fixed, Granule proteins, Fluorescence

1 Introduction

Eosinophils are considered the hallmark cell that mediates destructive [1–3] and immune regulating [4–8] activities in asthma pathologies [9–11]. To analyze eosinophils in situ, lung sections are often used as a measure of their numbers and states of activation in allergic respiratory pathology. Allergen models of pulmonary inflammation induce many characteristics of human pathology including increased mucus secretion, smooth muscle thickening, airway inflammation, and eosinophilic infiltration [12, 13]. Although evidence of degranulation is controversial in most acute allergen models [14, 15], some chronic models develop significant release of granule proteins in the lungs [16], a feature found in human asthmatic lung biopsies and in lung injury [17]. These pathologic changes are often viewed with use of

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standard dyes that characterize inflammation (HE), mucus production and goblet cell metaplasia, periodic acid–Schiff (PAS) or collagen deposition as with Masson's trichrome or picrosirius red.

In order to identify eosinophils in situ, the lungs of euthanized mice are often formalin-fixed, embedded in paraffin, and thinly sliced (5 μm) onto glass slides. These slides are then deparaffinized and stained with dyes meant to highlight eosinophils based on the unique nature of their granule proteins. The most common dyes are acidic and chosen due to their tendency to stain cationic eosinophil granule proteins. In short, eosinophils are eosin-*philic* (i.e., eosin-loving), due to the acidic eosin dye accumulating on the highly positively charged and acidophilic granule proteins as discovered by Dr. Paul Ehrlich over a century ago [18]. Additional dyes that are used for identifying eosinophils include Congo red and Luna. With these dyes, however, distinction between neutrophils and eosinophils is challenging, and nonspecific staining is present. For these reasons, they tend to produce less specific staining than immunohistochemistry (IHC) or immunocytochemistry (ICC), which in contrast utilizes antibodies that recognize eosinophil-specific antigens [19]. Although eosinophils can be identified by dyes, they must be differentiated manually with a trained eye. These dyes are commercially available, have easily accessible instructions on their use, and will not be discussed in this chapter.

Immunostaining assays such as IHC, ICC, and immunofluorescence (IF) use antibodies that recognize specific proteins of interest [20]. Monoclonal antibodies are superior to polyclonal antibodies due to their specificity for unique epitopes (for reviews [21–23]). Secondary granule proteins in mouse eosinophils include eosinophil peroxidase (EPX), major basic protein (MBP-1), and the divergent homologs mouse ribonucleases (mEARs) that are related to human eosinophil-derived neurotoxin (hEDN) and eosinophil cationic protein (hECP) [24]. mEARS have been found in macrophages, and MBP-1 is a low-abundant protein in basophils. In humans, ECP and EDN are also found in neutrophils [25, 26]. An additional eosinophil-associated molecule that may be targeted with antibodies is Siglec-F [27], although this is found on alveolar macrophages and eosinophils [28]. Out of all the granule proteins identified so far in eosinophils, EPX is considered the most specific to this cell type based on mouse knockout studies, and antibodies targeting EPX can be used together with those that recognize MBP-1 for highly sensitive and specific detection of eosinophils in tissues [16, 29, 30]. For this reason, our laboratory has developed monoclonal antibodies that recognize mouse EPX [31] and MBP-1 [32, 33] to specifically target eosinophils for immunostaining of lung tissue and cytospins of bronchoalveolar lavage (BAL).

First, we describe how lungs are isolated and prepared for different immunostaining techniques. Lungs must be carefully

inflated and removed to maintain resting lung architecture for proper analysis. Next, we describe five staining protocols: MBP IHC, EPX IHC, EPX fluorescent IHC, EPX indirect IF on lung slices, and dual EPX/MBP indirect IF on cytospin-prepped eosinophils. The techniques employed here have advantages depending on the desired end result and equipment available [34, 35]. Colorimetric IHC is highly stable and may be viewed/imaged repeatedly using brightfield microscopy. IF methods allow co-localization/multiplex imaging of antigens at once, yet photobleaching is problematic for repeated viewing/imaging. Conventional IHC utilizes an indirect approach where an enzyme-conjugated secondary antibody recognizes the primary antibody, and a signal is developed by chromogen deposition. Using secondary antibodies and enzyme development leads to high amplification of signal. This method is employed in MBP IHC, EPX IHC, and EPX fluorescent IHC. In brief, the most common enzyme protocols are peroxidase-based horseradish peroxidase (HRP)-conjugated secondary antibodies or phosphatase-based alkaline phosphatase (AP)-conjugated secondary antibodies that react with colorimetric dyes to form precipitate at the location of the antibody (i.e., in situ). The most common dye used is 3,3′-diaminobenzidine (DAB) that produces a brown/black color, although many alternatives are available that produce a range of blue, red, and purple colorimetric stains. Alternatively, fluorescent IHC or ICC can be performed by using tyramide signal amplification (TSA), which highly amplifies the fluorescence signal through HRP activation of the fluorophore-conjugated tyramide molecule [36]. This method allows very high spatial resolution in situ compared to colorimetric IHC. Fluorescent IHC/ICC or fluorophore-conjugated secondary antibody techniques permit fluorescence imaging up to three antigens/markers with most fluorescence microscopes [37]. The fluorescence method chosen may depend on availability of antibodies, as well as the auto-fluorescence intensity of the formalin-fixed tissue or cell. Additional methods are available for multiplex imaging of >30 antigens in situ in formalin-fixed tissues that often require cyclical staining, multispectral microscopes, and sophisticated imaging software [38–40]. Fluorescence imaging is superior to conventional chromogenic staining in that it is quantifiable and allows many more stains. This chapter will encompass standard IHC and up to 3-color IF imaging for eosinophils using standard laboratory equipment, microscopes, and imaging software.

2 Materials

All reagents should be prepared, stored, and used at room temperature unless otherwise indicated. Follow local waste disposal guidelines. Scale working solution volumes up or down dependent on your experiment.

2.1 Lung Collection for Fixation and Embedding

1. 10-mL Luer-lock syringe.
2. Support stand with rod and clamp.
3. 3-way stopcock.
4. Catheters: 20G, 30-mm length.
5. Butterfly needle infusion set: 21GA × 3/4 in. with a 12-in. tubing.
6. 70% Ethanol: To make 200 mL, add 140 mL ethanol to 60 mL distilled water.
7. Surgical scissors.
8. Two surgical forceps.
9. 10% Formalin.
10. 50-mL Conical tubes or containers.
11. Paper towels.
12. 4-0 Non-absorbable silk sutures: Cut into 5-in. lengths per mouse.
13. Euthanasia: pentobarbital or ketamine-xylazine.

2.2 Deparaffinization/ Rehydration of Slides

1. Tissue-Tek® Slide Holder (*see* **Note 1**) (Fig. 1).
2. Tissue-Tek® Staining Dish (*see* **Note 2**).
3. Incubator (55 °C).
4. Xylene (*see* **Note 3**).
5. 50:50 Xylene/ethanol solution: To make 200 mL, add 100 mL of xylene to 100 mL of ethanol.
6. 100% Ethanol: 200 proof (*see* **Note 4**).
7. 95% Ethanol: To make 200 mL, add 190 mL of ethanol to 10 mL of distilled water.
8. 75% Ethanol: To make 200 mL, add 150 mL of ethanol to 50 mL of distilled water.

2.3 MBP IHC with a Red Alkaline Phosphatase Substrate as a Chromogen

1. Tissue-Tek® Slide Holder (*see* **Note 1**) (Fig. 1).
2. Tissue-Tek® Staining Dish (*see* **Note 2**).
3. Shandon™ Sequenza™ Staining Rack and coverplates (*see* **Note 5**) (Fig. 2).
4. Wash buffer: 0.05 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.6. Alternatively, a 10× concentrate may be prepared and diluted to a 1:10 ratio before use by adding 10 mL of the concentrate to 90 mL of ultrapure water. Working solution can be stored at room temperature for 1 week.
5. Digest-All™ 3: ready-to-use pepsin solution. Store at 4 °C (*see* **Note 6**).

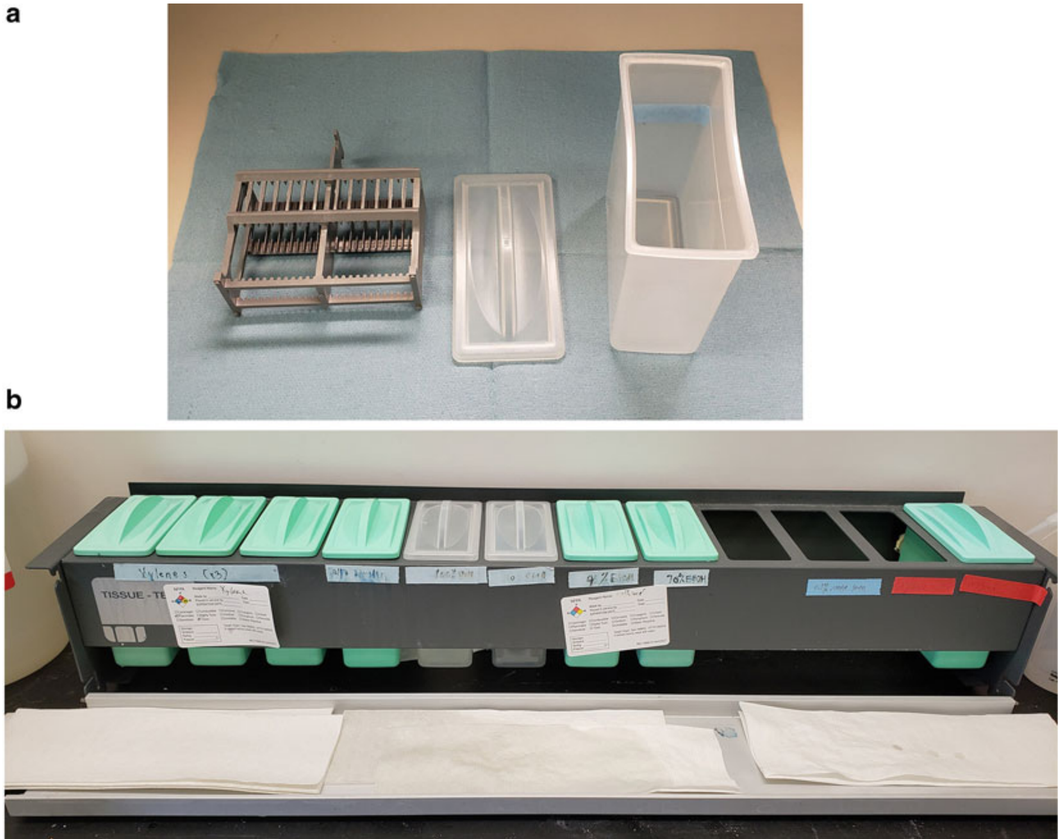

Fig. 1 Tissue-Tek® Slide Holder and Rack. (**a**) Tissue-Tek® Slide Holder that can hold up to 24 slides (grey) and staining lid and dish. The grey rack fits inside the holder. Slides are fully immersed in liquid when 200 mL of fluid is in the container. (**b**) Tissue-Tek® Rack is a convenient way to organize multiple staining dishes used for deparaffinization and rehydration of tissue sections

6. Antibody Diluent, Background Reducing (Agilent Dako): ready-to-use. Store at 4 °C.
7. Dual Endogenous Enzyme Blocker (Agilent Dako): ready-to-use. Store at 4 °C (*see* **Note 7**).
8. Blocking buffer: 5% normal goat serum in the wash buffer. Dilute to a 1:20 ratio by adding 10 μL of serum to 190 μL of the wash buffer (*see* **Note 8**).
9. Rat anti-MBP primary antibody: 1 mg/mL, Clone MT2-14.7.3 (Mayo Clinic, Arizona). Dilute to a 1:1000 ratio before use by adding 1 μL of the antibody to 999 μL of the antibody diluent to make a final concentration of 1 μg/mL. Use diluted antibody same day (*see* **Notes 9** and **10**).
10. Secondary antibody (ImmPRESS®-AP anti-rat polymer, Vector Labs): ready to use. Store at 4 °C (*see* **Note 11**).

Fig. 2 The use of Shandon™ Sequenza™ Staining Rack and coverplates allows for controlled flow of 200 μL of fluid over slides and for several slides to be processed at the same time. (**a**) Shandon™ Sequenza™ Staining Rack, lid, and coverplate. There is room for ten slides per rack. (**b**) Slide preparation rack filled with distilled water. (**c**) Instructions of how to load slides onto coverplate: (1) A container is filled with water; (2) Coverplate is submerged under water; (3) A slide is lowered onto the coverplate face-down, creating a small water filled void between the slide and coverplate; (4) Hold in place and then slide into the rack firmly

11. Chromogen (ImmPACT® Vector® Red AP Substrate, Vector Labs): To prepare 2.5 mL of Vector Red working solution, add 1 drop of Reagent 1 and 1 drop of Reagent 2 to 2.5 mL of the diluent and mix well before use. Use immediately after preparation (*see* **Note 12**).
12. 0.1% Methyl green: Add 200 mg of methyl green to 200 mL of ultrapure water.
13. Nonaqueous permanent mounting medium.
14. #1.5 Glass coverslip.

2.4 EPX IHC with DAB as a Chromogen

1. **Items 1–6** from Subheading 2.3.
2. Rodent Decloaker Antigen Retrieval, 10× (Biocare Medical): Dilute the concentrate to a 1:10 ratio with ultrapure water before use (*see* **Note 13**).
3. Decloaking chamber™ (*see* **Note 14**) (Fig. 3).
4. Mouse anti-EPX primary antibody: 1 mg/mL, Clone MM25.82.2.1 (Mayo Clinic, AZ). Dilute to a 1:500 ratio before use by adding 1 μL of the antibody to 499 μL of the antibody diluent to a final concentration of 2 μg/mL. Use diluted antibody same day (*see* **Notes 9** and **10**).
5. Rodent Block M (Biocare Medical): ready-to-use. Store at 4 °C (*see* **Note 15**).
6. Goat anti-mouse IgG (H + L), HRP-conjugated secondary antibody: 0.4 mg/mL. Dilute to a 1:250 ratio before use by adding 1 μL of the antibody to 249 μL of the antibody diluent. Store the antibody at −20 °C. Use diluted antibody same day.
7. DAB chromogen: SignalStain® DAB Kit (Cell Signaling Technologies) or equivalent. To prepare the DAB working solution, add 1 drop (30 μL) SignalStain® DAB chromogen concentrate to 1 mL of SignalStain® DAB diluent and mix well before use. Working solutions are stable for up to 14 days when stored at 4 °C or up to 5 days when stored at room temperature (*see* **Note 16**).

Fig. 3 Decloaking chamber (BioCare) with a plastic coplin jar. Water (500 mL) is placed inside the decloaker to distribute the heat around the coplin jar

8. Hematoxylin: Ready-to-use solution is commercially available.
9. Acid rinse solution: To prepare 200 mL, add 4 mL of glacial acetic acid to 196 mL of ultrapure water (*see* **Note 17**).
10. Bluing solution: To prepare 200 mL, add 3 mL of 30% ammonium hydroxide to 197 mL of 70% ethanol (*see* **Note 18**).
11. Nonaqueous permanent mounting medium.
12. #1.5 Glass coverslip.

2.5 EPX Fluorescent IHC with Tyramide Signal Amplification (TSA)

1. **Items 1–6** from Subheading 2.3.
2. Rodent Decloaker Antigen Retrieval, 10× (Biocare Medical): Dilute the concentrate to a 1:10 ratio with ultrapure water before use (*see* **Note 13**).
3. Decloaking Chamber™ (*see* **Note 14**) (Fig. 3).
4. Mouse anti-EPX primary antibody: 1 mg/mL, Clone MM25.82.2.1 (Mayo Clinic, AZ). Dilute to a 1:500 ratio before use by adding 1 μL of the antibody to 499 μL of the antibody diluent to a final concentration of 2 μg/mL (*see* **Notes 9** and **10**).
5. Rodent Block M (Biocare Medical): ready to use. Store at 4 °C (*see* **Note 15**).
6. Goat anti-mouse IgG (H + L) secondary antibody: 0.4 mg/mL, HRP-conjugated. Dilute to a 1:250 ratio before use by adding 1 μL of the antibody to 249 μL of the antibody diluent. Store the stock at −20 °C. Use diluted antibody same day.
7. TSA cyanine 3 (Cy3) kit: TSA™ Plus Cyanine 3 Kit or equivalent. Reconstitute TSA Plus stock with DMSO (HPLC-grade) according to manufacture recommendations. Dilute the stock solution to a 1:800 ratio before use by adding 1 μL of TSA dye to 799 μL of 1× Amplification Diluent to make TSA Plus working solution (*see* **Note 19**).
8. Phosphate-buffered saline (PBS): 1.5 mM KH_2PO_4, 155 mM NaCl, 2.7 mM Na_2HPO_4-$7H_2O$, pH 7.4. To prepare 1 L, add 210 mg KH_2PO_4, 9 g NaCl, and 726 mg Na_2HPO_4-$7H_2O$ to 900 mL distilled water. Adjust pH and raise volume to 1 L with distilled water.
9. 4′,6-Diamidino-2-phenylindole, dilactate (DAPI): 10.9 mM DAPI. Prepare a stock solution by dissolving 5 mg of DAPI in 1 mL ultrapure water. Aliquot and store the stock at −20 °C. To prepare working solution, dilute the stock to 1:5000 in PBS to 1 μg/mL. Store the working solution at 4 °C (*see* **Note 20**).
10. Mounting medium: ProLong™ Diamond Antifade Mountant (Invitrogen) or equivalent. Ready to use. Store at −20 °C (*see* **Note 21**).
11. #1.5 Glass coverslip.

2.6 EPX Indirect IF

1. **Items 1–6** from Subheading 2.3.
2. Rodent Decloaker Antigen Retrieval, 10× (Biocare Medical): Dilute the concentrate to a 1:10 ratio with ultrapure water before use (*see* **Note 13**).
3. Decloaking Chamber™ (*see* **Note 14**) (Fig. 3).
4. Mouse anti-EPX primary antibody: 1 mg/mL, Clone MM25.82.2.1 (Mayo Clinic, AZ). Dilute to a 1:100 ratio before use by adding 2 μL of the antibody to 198 μL of the antibody diluent to a final concentration of 10 μg/mL. Use diluted antibody same day (*see* **Notes 9** and **10**).
5. Rodent Block M (Biocare Medical): ready-to-use. Store at 4 °C (*see* **Note 15**).
6. Anti-mouse IgG secondary antibody: Alexa 594-conjugated. Dilute to a 1:500 ratio by adding 1 μL of the antibody to 499 μL the antibody diluent. Store stock at 4 °C. Use diluted antibody same day (*see* **Note 22**).
7. Phosphate-buffered saline (PBS): 1.5 mM KH_2PO_4, 155 mM NaCl, 2.7 mM Na_2HPO_4-$7H_2O$, pH 7.4. To prepare 1 L, add 210 mg KH_2PO_4, 9 g NaCl, and 726 mg Na_2HPO_4-$7H_2O$ to 900 mL distilled water. Adjust pH and raise volume to 1 L with distilled water.
8. 4′,6-Diamidino-2-phenylindole, dilactate (DAPI): 10.9 mM DAPI. Prepare a stock solution by dissolving 5 mg of DAPI in 1 mL of ultrapure water. Aliquot and store the stock at −20 °C. To prepare working solution, dilute stock 1:5000 in PBS to 1 μg/mL. Store working solution at 4 °C (*see* **Note 20**).
9. Mounting medium: ProLong™ Diamond Antifade Mountant (Invitrogen) or equivalent. Ready to use. Store at −20 °C (*see* **Note 21**).
10. #1.5 Glass coverslip.

2.7 MBP and EPX Dual Fluorescent Immunocytochemistry (ICC)

1. Cells from peripheral blood or bronchoalveolar lavage in 5% BSA in PBS, stored at 4 °C.
2. ThermoScientific Cytospin™ 3 or 4 Cytocentrifuge and components, funnel filter paper, and clip.
3. Phosphate-buffered saline (PBS): 1.5 mM KH_2PO_4, 155 mM NaCl, 2.7 mM Na_2HPO_4-$7H_2O$, pH 7.4. To prepare 1 L, add 210 mg KH_2PO_4, 9 g NaCl, and 726 mg Na_2HPO_4-$7H_2O$ to 900 mL distilled water. Adjust pH and raise volume to 1 L with distilled water.
4. 5% (w/v) BSA: To make 100 mL, add 5 g of BSA to 100 mL of PBS. Store at 4 °C and use within 1 day (*see* **Note 23**).
5. Permeabilization buffer (PBT): PBS containing 0.2% (v/v) Triton™ X-100. First prepare a stock solution of 10% (v/v) Triton™ X-100 by adding 200 μL of Triton™ X-100 to

9.8 mL of PBS. To make working solution, dilute 10% stock to a 1:50 ratio by adding 10 μL of the stock to 490 μL of PBS (*see* **Note 24**).

6. Wash buffer (PBST): PBS containing 0.1% TWEEN® 20. First prepare a stock solution of 10% TWEEN® 20 by adding 100 μL of TWEEN® 20 to 9.9 mL of PBS. To make working solution, dilute 10% stock to 1:100 by adding 5 μL of the stock to 495 μL of PBS (*see* **Note 24**).
7. Antibody diluent: 1% BSA in PBST. To prepare 5 mL, add 1 mL of 5% BSA to 4 mL of PBST. Store at 4 °C.
8. Blocking buffer: 5% normal donkey serum in antibody diluent. To prepare 1 mL, add 50 μL of normal donkey serum to 950 μL of antibody diluent. Use on the same day (*see* **Note 8**).
9. Primary antibody mix: mouse anti-EPX [1 mg/mL] (Clone MM25.82.2.2, Mayo Clinic AZ) and rat anti-MBP [1 mg/mL] (Clone MT2-14.7.3, Mayo Clinic AZ). To prepare 1 mL, dilute the antibodies to 1:200 by adding 5 μL of anti-EPX and 5 μL of anti-MBP to 990 μL of antibody diluent (*see* **Notes 9** and **10**).
10. Secondary antibody mix: donkey anti-mouse Alexa 594 and donkey anti-rat Alexa 488. To prepare 1 mL, dilute the antibodies to 1:500 by adding 2 μL of anti-mouse Alexa 594 and 2 μL of anti-rat Alexa 488 to 996 μL of the antibody diluent (*see* **Note 19**).
11. 4′,6-Diamidino-2-phenylindole, dilactate (DAPI): 10.9 mM DAPI. Prepare a stock solution by dissolving 5 mg DAPI in 1 mL ultrapure water. Aliquot and store stock at −20 °C. To prepare working solution, dilute stock 1:5000 in PBS to 1 μg/mL. Store working solution at 4 °C (*see* **Note 20**).
12. ProLong™ Diamond Antifade Mountant (Invitrogen): ready to use. Store at −20 °C (*see* **Note 21**).
13. #1.5 Glass coverslip.

3 Methods

3.1 Lung Collection for Fixation and Embedding

This protocol is optimized for BALB/c or C57BL/6 mice that are >6 weeks of age or 18–40 g in weight. Procedures must be approved by IACUC committee and under the assurances of the Office for Laboratory Animal Welfare. All incubations should be performed at room temperature unless noted otherwise. The mice used in these procedures have undergone a house dust mite allergen sensitization and challenge protocol [41].

1. Set up a syringe with a stopcock, a butterfly needle, and a catheter on the support stand (*see* **Note 25**) (Fig. 4).

Fig. 4 Syringe and catheter setup to prepare formalin-inflated lungs. A 10-mL syringe is held in place by a clamp such that the 10-mL mark on the syringe is 20 cm above the benchtop. The blue stopcock controls the flow of formalin. The catheter is placed into the trachea during instillation of formalin

2. Ensure the stopcock is off (perpendicular to syringe) and fill the syringe with the formalin solution (*see* **Note 26**). With the tip of the catheter placed into a disposable container, open the stopcock to let the formalin fill the length of the catheter and tubing. Make sure there are no air bubbles in the tubing line. Stop the flow by turning the stopcock to the off position.
3. Euthanize a mouse with a lethal dose of sodium pentobarbital or ketamine-xylazine (*see* **Note 27**), and lay the mouse on its back on top of paper towels to absorb excess fluids. Wet the fur around the throat and torso with 70% ethanol.
4. To remove the skin over the chest area, grab the skin under the jaw with forceps, creating a tent, and cut the skin with scissors from the length of the jaw to the bottom of the rib cage.
5. Lift up on the rib cage by grabbing bottom part of the sternum (the xiphoid process) with forceps and make an incision along the edge (beneath) of the rib cage from right to left to expose the diaphragm.

6. Cut the diaphragm away from the ribs (cutting left to right). Be careful not to poke or cut the lung. Any tears will lead to formalin leakage and lung deflation, altering the architecture.
7. While lifting the xiphoid process with forceps up away from the body, use the scissors to cut the rib cage on both sides about 2/3 distance to top of rib cage, approximately right below the clavicles. A final cut is made across the top of the ribcage to remove it and expose the heart and lungs.
8. The clavicles must be cut in order to remove the lungs from the mouse. Cut the clavicle on each side such that the section of bone remaining over the thymus and heart can be carefully removed from the mouse. This allows for total exposure of the trachea, heart, thymus, and lungs.
9. Expose the ventral side of the trachea by moving away the thyroid gland (pull apart, splitting the middle). Carefully cut the muscle layer over the trachea so as to expose the cartilage of the trachea.
10. Carefully loop the 5″ suture material underneath the trachea using forceps and then loosely form a knot immediately below the thyroid cartilage/voice box. Do not tighten.
11. Cut the trachea horizontally just enough to allow a 20G catheter insertion at the thyroid cartilage/voice box as this provides a solid and wide location to support this type of cut and provides a reference point (*see* **Note 28**).
12. Put the catheter into the trachea such that it is inserted only a few millimeters, past the loose knot, yet avoid going so far that there is resistance. Holding the catheter in place, tighten the knot until snug.
13. Open the stopcock and allow the lungs to fill. Turn off the stopcock once the lungs are fully inflated.
14. When the lung is fully inflated, at the same time, remove the catheter and tighten the knot completely, so no liquid escapes.
15. While holding trachea with forceps at the knot, cut above the forceps to sever trachea and cut any connective tissue holding the lungs in place.
16. Place the whole lung into a 50-mL conical filled with 30 mL formalin and store for 24 h (*see* **Note 29**).
17. Prepare for embedding and sectioning. This is beyond the scope of this chapter but is described elsewhere [42]. Sections stained in this protocol are 5-μm thick coronal slices of formalin-fixed and paraffin-embedded (FFPE) tissue.

3.2 Deparaffinization/Rehydration FFPE Slides

1. Place slides in Tissue-Tek® Slide Holder and Tissue-Tek® Staining Dish (Fig. 1) and incubate the slides at 55 °C for 30 min with lid on the dish (*see* **Note 30**).
2. In a fume hood, set up the indicated number of Tissue-Tek dishes with 200 mL of each solution, and place the slide holder into the staining dishes for the indicated times:
   (a) Three dishes of xylene, 5 min each (*see* **Note 31**).
   (b) One dish of 50:50 xylene/ethanol, 2 min.
   (c) Two dishes of 100% ethanol, 2 min each.
   (d) One dish of 95% ethanol, 2 min.
   (e) One dish of 75% ethanol, 2 min.
3. Rinse the slides in running distilled water for 30 s. Store slides in water until next steps to keep hydrated.

3.3 MBP IHC with a Red AP Substrate as a Chromogen

1. After deparaffinization/rehydration of slides, load the slides into Shandon™ Sequenza™ Staining Rack with coverplates (*see* **Note 32**) (Fig. 2).
2. Add 200 μL of Digest-All™ 3 pepsin to the slides and incubate for 10 min.
3. Wash three times in wash buffer for 2 min each.
4. Add 200 μL of Dual Endogenous Enzyme Block to the slides and incubate for 10 min.
5. Wash three times in wash buffer for 2 min each.
6. Add 200 μL of the blocking buffer to the slides and incubate for 30 min (*see* **Note 33**).
7. Add 200 μL of the diluted anti-MBP antibody (1 μg/mL) to the slides and incubate overnight at 4 °C. For negative control slides, add diluent without the antibody (*see* **Note 34**).
8. Wash three times in wash buffer for 5 min each.
9. Add 200 μL of ImmPRESS Anti-Rat AP polymer to the slides and incubate for 30 min.
10. Wash three times in wash buffer for 5 min each.
11. Add 200 μL Vector Red chromogen to slides and incubate for 5 min (*see* **Note 35**).
12. Wash once with distilled water for 2 min, then transfer slides to a dish filled with distilled water to keep tissue hydrated.
13. To counterstain with methyl green, place slides in methyl green for 15 s (*see* **Note 35**), and wash slides in running distilled water until water is clear (about 10 s).
14. Dehydrate the slides (*see* **Note 36**) by placing them once in 95% ethanol for 1 min and twice in 100% ethanol for 1 min. Air-dry the slides.
15. Dip slides in xylene and coverslip with nonaqueous permanent mounting medium (Fig. 5).

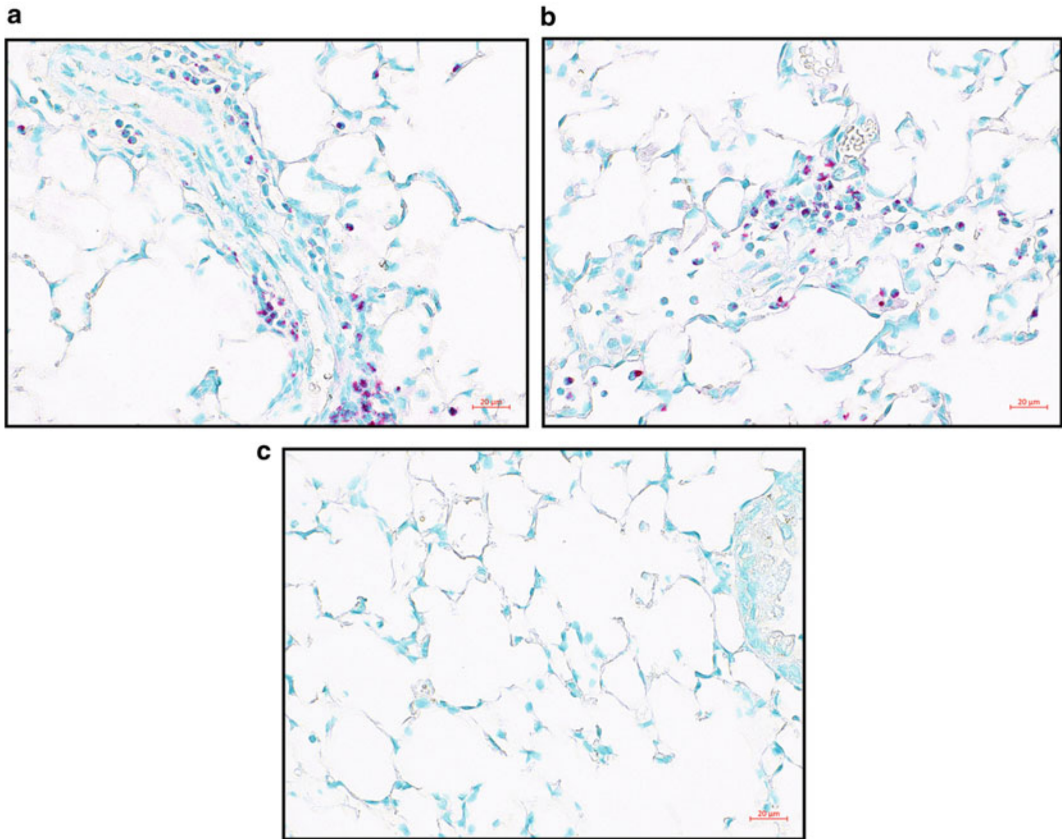

Fig. 5 Allergen-challenged FFPE lung sections with MBP IHC with the red chromogen. (**a**, **b**) Two examples of MBP IHC in allergen-challenged lung FFPE slices. MBP is stained red showing the location of eosinophils, and methyl green counterstains nuclei green. (**c**) Negative control staining. Images were taken on Zeiss Imager.M2 with a ×40 objective

3.4 EPX IHC with DAB as a Chromogen

3.4.1 Antigen Retrieval

1. Add 500 mL distilled water to the Decloaker or equivalent.
2. Submerge deparaffinized and dehydrated slides into a staining jar with diluted antigen retrieval solution (*see* **Note 37**) and place them in the Decloaker.
3. Incubate the slides in Decloaker at 95 °C for 40 min, then 85 °C for 10 min. Remove the staining jar from the Decloaker, keeping the slides in the retrieval buffer, and allow to cool on benchtop for 20 min.
4. Rinse the slides in running distilled water until all the antigen retrieval solution is removed (*see* **Note 38**).

3.4.2 Antibody Incubation and Color Development

1. Load slides into Shandon™ Sequenza™ Staining Rack (*see* **Note 32**) (Fig. 2).
2. Add 200 μL of Digest-All™ 3 pepsin to the slides and incubate for 10 min.

3. Wash three times in wash buffer for 2 min each.
4. Add 200 μL Rodent M Block to slides and incubate for 30 min.
5. Wash three times in wash buffer for 2 min each.
6. Add 200 μL anti-EPX antibody (2 μg/mL) to the slides and incubate overnight at 4 °C. For negative control slides, add diluent without the antibody (*see* **Note 34**).
7. Wash three times in wash buffer for 5 min each.
8. Add 200 μL of anti-mouse HRP secondary to slides and incubate for 30 min.
9. Wash three times in wash buffer for 5 min each.
10. Add 200 μL of DAB chromogen to slides and incubate for 10 min (*see* **Note 35**).
11. Wash once with distilled water for 2 min, then transfer slides to a slide holder submerged in distilled water to keep tissue hydrated.

3.4.3 Hematoxylin Counterstaining

1. Incubate slides in hematoxylin for 5 min.
2. Wash slides in running distilled water until water is clear.
3. Immerse slides ten times into acid rinse solution.
4. Immerse slides ten times into distilled water.
5. Incubate slides for 1 min in bluing solution.
6. Immerse slides ten times into distilled water.

3.4.4 Dehydration and Coverslipping

1. Incubate slides in 75% ethanol for 1 min.
2. Incubate slides in one wash of 95% ethanol for 1 min each.
3. Incubate slides in two washes of 100% ethanol for 1 min each.
4. Air-dry slides.
5. Dip slides in xylene and coverslip with nonaqueous permanent mounting medium (Fig. 6).

3.5 EPX Fluorescent IHC with TSA

1. Perform antigen retrieval as described in Subheading 3.4.1.
2. Pretreat and block the slides as described by Subheading 3.4.2, **steps 1–5**.
3. Add 200 μL of anti-EPX antibody [2 μg/mL] to slides and incubate overnight at 4 °C. For negative control slides, add diluent without the antibody (*see* **Note 34**).
4. Wash three times in wash buffer for 5 min each.
5. Add 200 μL of anti-mouse HRP secondary antibody to the slides and incubate for 1 h.
6. Wash three times in wash buffer for 5 min each.

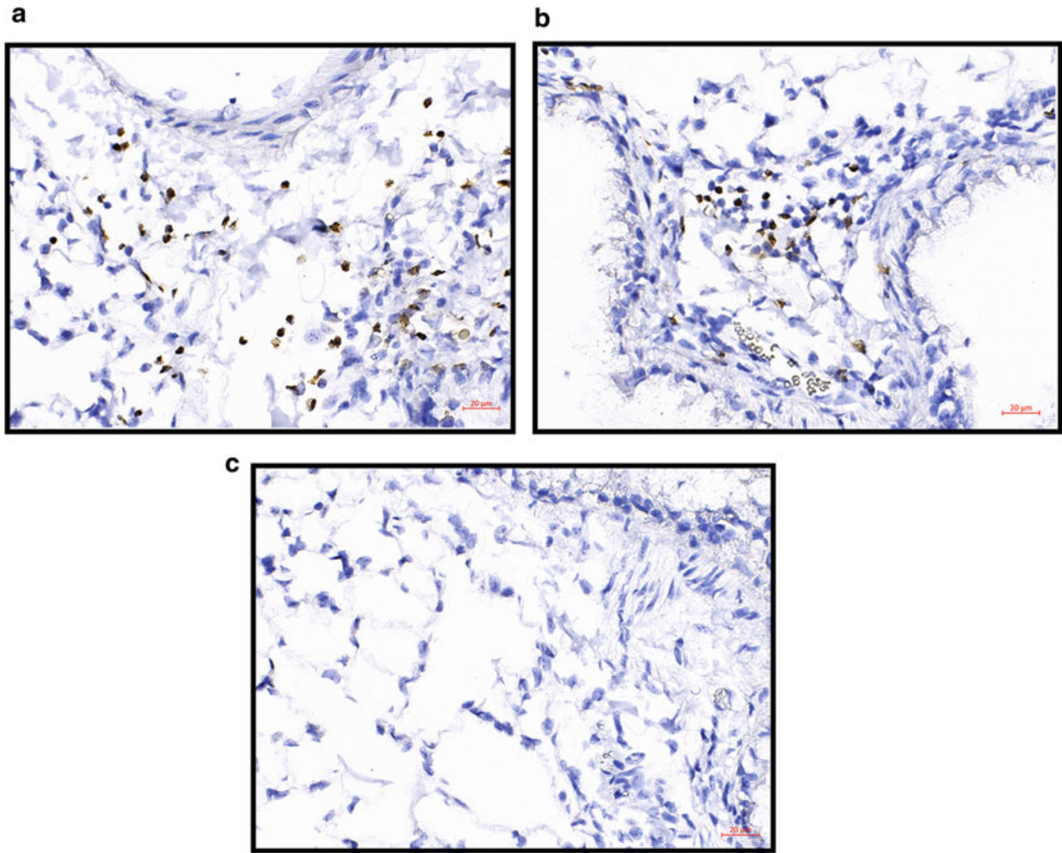

Fig. 6 Allergen-challenged FFPE lung sections with EPX IHC with DAB as the chromogen. (**a**, **b**) Two examples of EPX IHC in allergen-challenged lung FFPE slices. EPX is stained brown showing the location of eosinophils, and hematoxylin counterstains nuclei blue/purple. (**c**) Negative control staining. Images were taken on Zeiss Imager.M2 with a $\times 40$ objective

7. Add 200 μL of TSA Cy3 dye solution to slides and incubate for 10 min protected from light. All following steps should be protected from light to reduce photobleaching.
8. Wash three times in wash buffer for 5 min each and rinse with PBS.
9. Counterstain nuclei by adding 200 μL of DAPI to the slides and incubate for 7 min.
10. Wash three times in PBS for 2 min each.
11. Remove one slide at a time from the rack and coverslip using ProLong™ Diamond Antifade mountant (*see* **Note 39**).
12. Lay slides flat and allow to dry overnight protected from light before imaging (*see* **Note 40**) (Fig. 7).

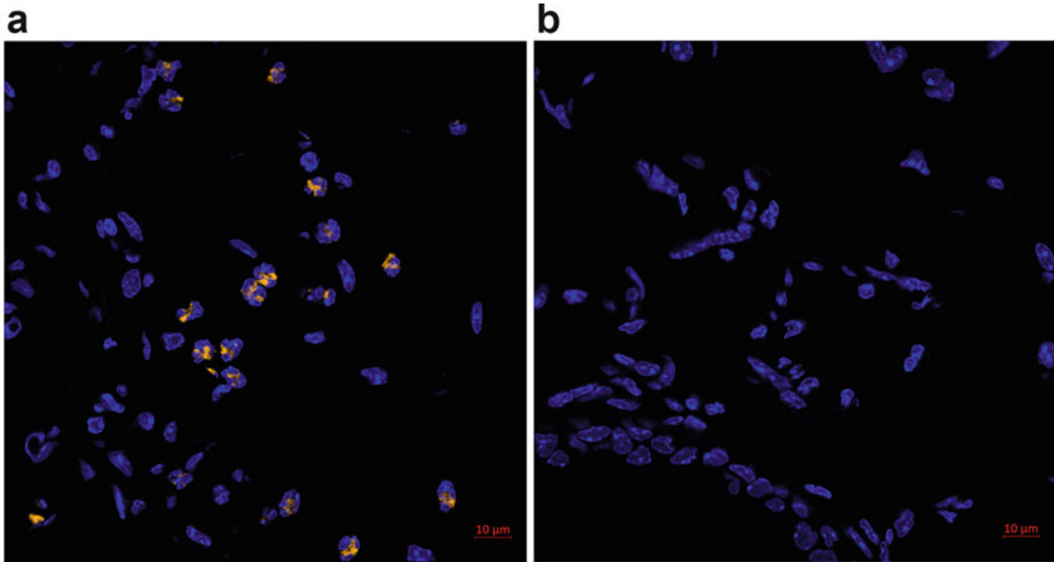

Fig. 7 Allergen-challenged FFPE lung sections with EPX fluorescent IHC with TSA. (**a**) Eosinophils are stained for EPX with Cy3-conjugated tyramide substrate (orange). Nuclei are counterstained with DAPI (blue). (**b**) Negative control without the primary antibody. Image was acquired with a Plan-Apochromat ×63 objective on a Zeiss LSM 800 microscope

3.6 EPX Indirect IF

This FFPE lung staining method may be adapted for dual IF by adding an additional primary antibody such as a rat or rabbit antibody, combined with an appropriate fluorophore-conjugated secondary antibody (such as goat anti-rat or goat anti-rabbit Alexa 647) (*see* **Note 41**). Optimization of antigen retrieval and blocking agents will be required for additional primary antibodies.

1. Perform antigen retrieval as described in Subheading 3.4.1.
2. Pretreat and block the slides as described by Subheading 3.4.2, **steps 1–5**.
3. Add 200 μL of anti-EPX antibody [10 μg/mL] to the slides and incubate overnight at 4 °C. For negative controls, add diluent without the antibody (*see* **Note 34**).
4. Wash three times in wash buffer for 5 min each.
5. Add 200 μL of anti-mouse Alexa594 secondary antibody to the slides and incubate for 1 h protected from light. All following steps should be protected from light to prevent photobleaching.
6. Rinse, stain with DAPI, and coverslip the slides as described in Subheading 3.5, **steps 8–12** (Fig. 8).

3.7 MBP and EPX Dual Fluorescent ICC.

1. Resuspend cells from peripheral blood or bronchoalveolar lavage at 1 × 10^6 cells/mL in cold 5% BSA/PBS (*see* **Note 42**).

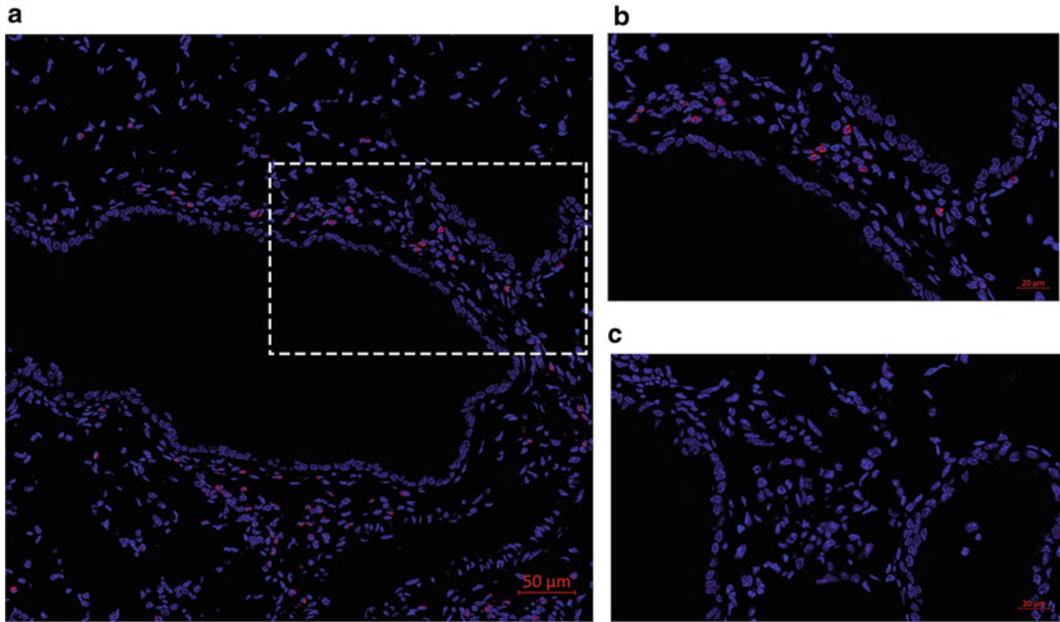

Fig. 8 Allergen-challenged FFPE lung sections with EPX indirect IF. Eosinophils are stained for EPX (red) and nuclei are counterstained with DAPI (blue). (**a**) Tile (5 × 5) image was acquired with a Plan-Apochromat ×63 objective on a Zeiss LSM 800 microscope. (**b**) Zoomed in image of (**a**). (**c**) Negative control without the primary antibody

2. Set up cytospin cages with microscope slides and funnels. Load into a cytocentrifuge (Fig. 9).
3. Pre-wet the slides by adding 50 μL of 5% BSA to the funnels, bringing the cytocentrifuge up to 500 RPM (~28 × *g*) and stopping (*see* **Note 43**).
4. Add 50 μL of the cells to each funnel, then add 50 μL of 5% BSA.
5. Spin at 500 rpm, slow acceleration, for 5 min.
6. Remove the slides and immediately immerse in 4% formaldehyde for 15 min.
7. Wash three times in PBS for 5 min each.
8. Load the slides into Shandon™ Sequenza™ Staining Rack (*see* **Note 32**) (Fig. 2).
9. Rinse slides with PBS.
10. Add 200 μL of PBT to slides and incubate for 10 min.
11. Wash two times in wash buffer for 2 min each.
12. Add 200 μL of blocking buffer and incubate for 30 min at room temperature (*see* **Note 33**).

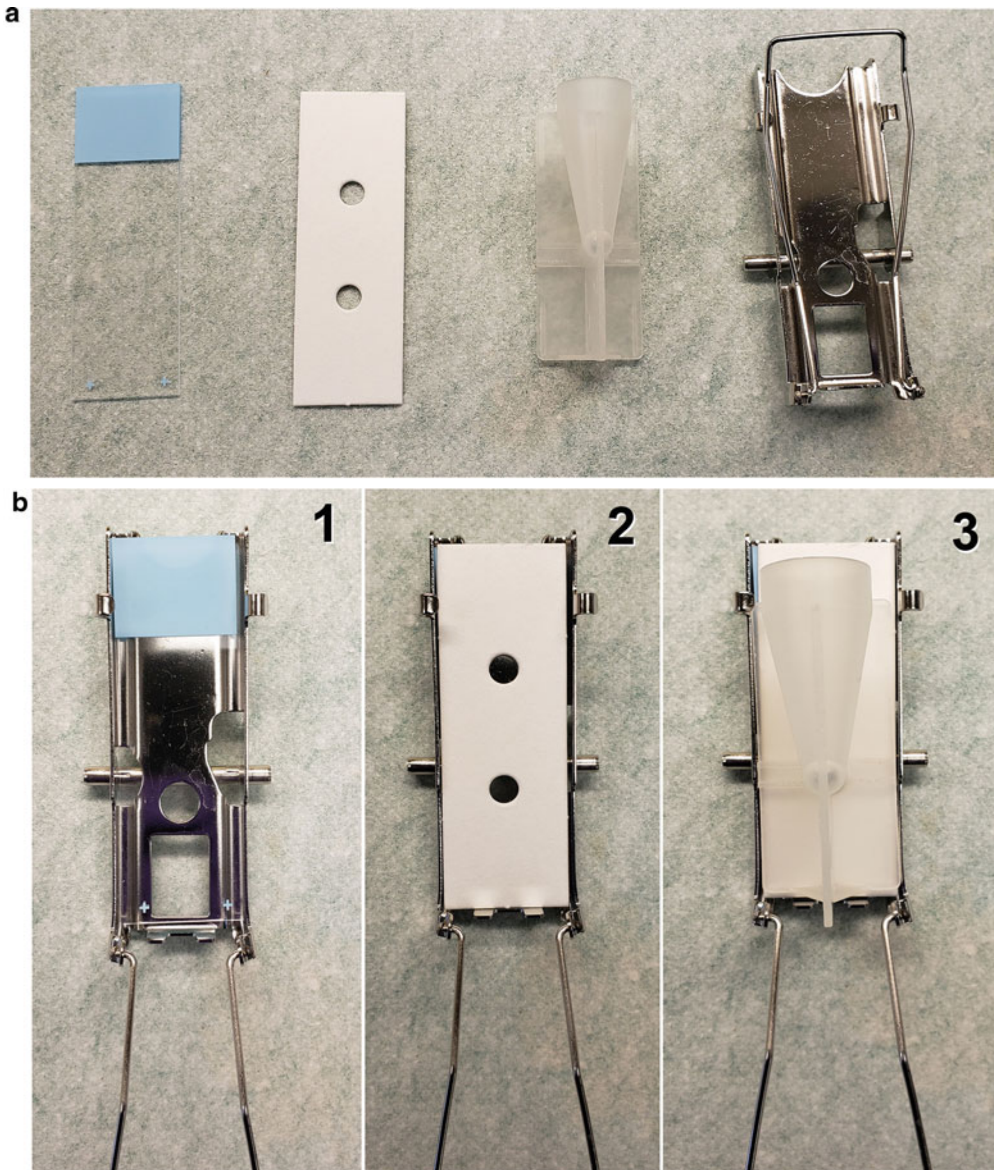

Fig. 9 Cytospin materials and slide preparation. (**a**) Materials include, from left to right, a labeled new clean slide, a filter card, a funnel, and a cage. (**b**) Setup sequence: (1) Place the slide in the cage; (2) Cover the slide with the filter paper, making sure to align its bottom edge flush with the bottom of the cage; (3) Place the funnel over the filter paper and slide such that the bottom of the funnel is directed toward the hole in the filter paper. Clamp shut and place in cytocentrifuge. The cell suspension is placed into the funnel, and the cells will be distributed onto the slide upon centrifugation

13. Add 200 μL of the primary antibody mixture and incubate overnight at 4 °C. For negative control slides, add the diluent without the antibodies (*see* **Note 34**).
14. Wash three times in wash buffer for 5 min each.

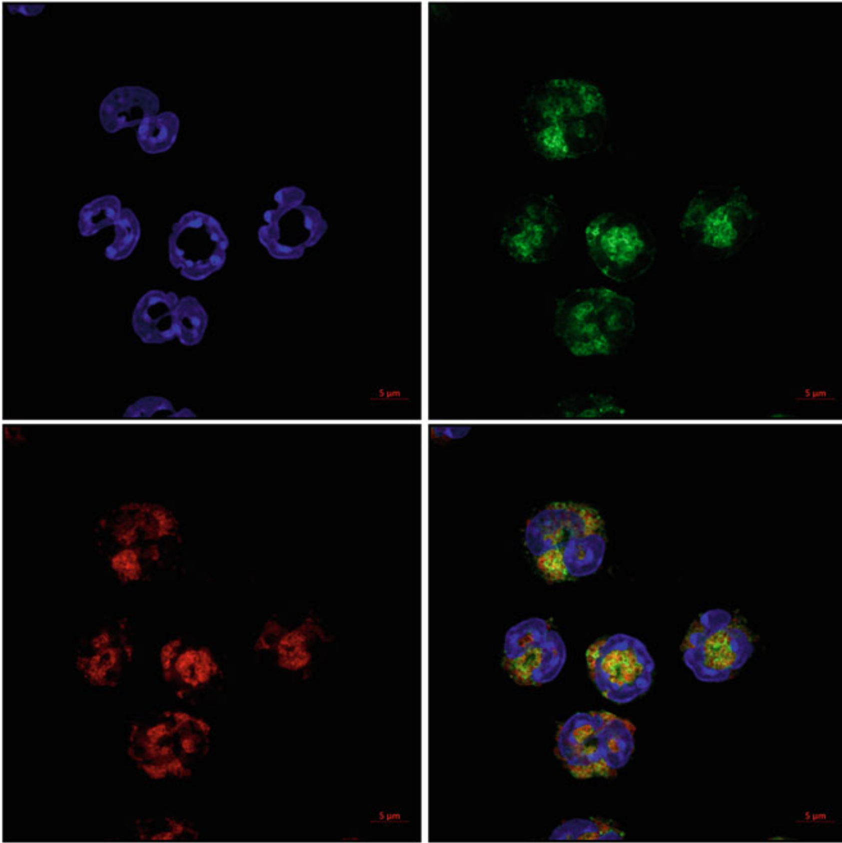

Fig. 10 MBP and EPX dual fluorescent ICC. Cells were prepared by cytocentrifugation and then stained for both EPX and MBP. Eosinophils are stained for EPX (red) and MBP (green). Nuclei are counterstained with DAPI (blue). Image was acquired with a Plan-Apochromat ×63 objective using Airyscan on a Zeiss LSM 800 microscope

15. Add 200 μL of the secondary antibody mixture and incubate for 1 h protected from light. All following steps should be protected from light to reduce photobleaching.
16. Rinse, stain with DAPI for 2 min, and coverslip the slides as described in Subheading 3.5, **steps 8–12** (Fig. 10).

4 Notes

1. Slide holders are not mandatory, but they are convenient for the deparaffinization/rehydration steps when working with multiple slides at once. These come in different sizes to meet your needs. Slide mailers are a cheap alternative that can be used to submerge slides into solutions.
2. Staining dishes are plastic, solvent resistant, and can tolerate the high temperature of a pressure cooker. They can handle rapid temperature changes and have a lid to reduce evaporation of

solvents. Coplin jars or any solvent resistant container can be used as an alternative. These hold up to 24 slides, and we use a volume of 200 mL to submerge slides.

3. Xylene is highly flammable and should be kept under a fume hood in a closed container to avoid evaporation of fumes.
4. Ethanol is flammable and should be stored in a closed container to avoid evaporation.
5. The Shandon™ Sequenza™ Staining Rack requires a minimum 200 μL of solution per slide. The staining rack is convenient as all staining and washing steps are performed in a portable rack. Once the slides are loaded, there is no need to move them until the very end of the protocol. The coverplate/rack system also keeps the slides uniformly hydrated, preventing issues associated with cell or tissue dehydration. Alternatively, staining can be performed using traditional methods (hydrophobic pen/incubation in humidified chamber). However, it is important to keep the tissue wet throughout staining. Traditional methods require lower reagent volumes to be applied to each slide, which is a benefit over the rack method.
6. This protocol has been optimized using commercially available Digest-All™ 3 pepsin solution. Other pepsin solutions would require further optimization.
7. Dual Endogenous Enzyme Blocker (Agilent Dako) reagent helps to block endogenous peroxidases and phosphatases that may react with the chromogen and develop nonspecific background staining [43–45]. It is compatible with both HRP-based and AP-based detection protocols.
8. This step helps to block nonspecific binding of the primary antibody, as well as the secondary. The species of the serum may match the species in which the secondary antibody was raised, although goat serum is a common serum used for many protocols and sufficient with monoclonal primary rat and mouse antibodies. Normal sera can be stored short term at 4 °C, while long-term storage can be done at −20 °C. Centrifuge stock serum at 13,000 × g for 5 min before use to remove precipitates.
9. Rat anti-MBP (clone: MT2-14.7.3) [33] and mouse anti-EPX (clone: MM25-82.2.1) [31] are only available through Mayo Clinic at this time and can be obtained by contacting the senior author of this chapter and as described here [32]. These antibodies are highly purified by IgG column purification and prepared without sodium azide for storage. Stocks are validated in-house before shipment. Antibodies are aliquoted and shipped as 50 μg lyophilized samples that are stable for many years at −80 °C. Lyophilized antibodies are reconstituted with

molecular grade water to generate 1 mg/mL antibody solution. Reconstituted antibodies are stable for greater than 6 months at 4 °C.

10. Antibody dilutions may require adjustment per tissue stained or fixation methods.
11. We have had great success using this specific secondary antibody, but this may be substituted for another AP-polymer secondary antibody system. Alternatively, as the dual enzyme block is used in this protocol, the AP detection system can be swapped for an HRP-based system with an appropriate chromogen (i.e., DAB). Various substrates with different colors and properties are available for both enzymes, so one might choose one enzyme over the other based on the substrate of interest [46, 47].
12. This chromogen is also fluorescent and can be viewed using Texas red filter (600–650 λ).
13. This specific retrieval buffer is important in blocking endogenous mouse IgG, which can cross-react with the secondary antibody, and be a source of background staining. This buffer also inactivates endogenous peroxidases, serving as an enzyme block and reducing background staining in HRP-based detection systems.
14. During fixation, epitopes are masked and heat-induced antigen retrieval helps to unmask these epitopes, so the primary antibody can bind the antigen of interest [20, 48, 49]. We prefer to use the Decloaker (Biocare) because of its precise control of temperature and time. This protocol does not call for high temperature/pressure, so any incubator that can reach 95 °C may be used.
15. This commercial blocking reagent helps to block endogenous mouse IgG and reduce nonspecific background staining in mouse tissues. When performing a mouse-on-mouse protocol, the secondary antibody cannot distinguish between the primary antibody and any endogenous IgG found within the tissue. If this endogenous IgG is not blocked sufficiently, it becomes a cause for high background staining.
16. Alternative DAB kits or HRP substrates can be used in place of this kit, but incubation times may require adjustment. Endogenous phosphatases might not be effectively blocked, so we do not recommend using an AP detection system with this protocol.
17. The acid rinse helps to remove nonspecific hematoxylin staining.
18. Hematoxylin will stain nuclei a reddish-purple, and this reagent changes it to a bluish-purple.

19. Cyanine 3 dye in the TSA kit is light-sensitive and requires protection from light. Working solution can be stored at 4 °C for up to 1 month. The concentration of the dye can be adjusted to increase the staining intensity, but EPX is a very abundant protein, and we have found that 1:800 gives a good signal-to-noise ratio. Too high concentrations of the dye can lead to increased background and signal developing outside the cell. Not only does TSA highly amplify the fluorescence signal, it is compatible with highly multiplexed techniques (reviewed here [36]) because the dye is covalently attached to the tissue.
20. DAPI is light-sensitive, so protect all solutions from light. DAPI is also a suspected carcinogen, so handle with proper personal protection equipment. We have found that DAPI containing mounting media causes background and prefer to do a separate staining step prior to mounting. Stock solution is stable for at least 6 months. The dilactate formulation is more water soluble than the dihydrochloride.
21. ProLong™ Diamond is a hardening reagent whose refractive index is highest once fully cured. Slides can be imaged immediately after coverslipping, but for optimal imaging allow reagent to cure. There is no need to seal the slide edges.
22. Protect fluorophore-conjugated antibodies from light. Centrifuge the antibody solution briefly to pellet aggregates—only use the supernatant. The fluorochrome(s) can be changed depending on the experiment and microscope setup. Alexa-based fluorophores are more stable than original fluorophores, such as FITC or rhodamine, when exposed to ambient light [50]. Autofluorescence in formalin-fixed samples can be seen at all visible wavelengths, but the intensity is the highest around the blue-green region (475–525 λ), so we prefer to use red-shifted colors (>525 λ) [34]. Various immunostaining methods to reduce FFPE autofluorescence in lung tissues are described elsewhere [51].
23. BSA takes a while to dissolve, and it is best to prepare ahead of time. After adding BSA to PBS, incubate at room temperature until fully dissolved (about 45 min for 5 g). To remove BSA stuck to the side of the container, gently swirl the solution but be careful not to over agitate, which will cause it to foam. For long-term storage at 4 °C, filter solution through 0.2 μm flask filter and maintain aseptic techniques.
24. Stock detergent solutions are very viscous. Aspirate and dispense slowly. We have found that swirling the pipette while dispensing into PBS helps to get the detergent into solution faster. 10% solution is not as viscous and is easier to pipette.
25. The syringe holding formalin needs to be 20–25 cm above the table to ensure proper pressure to inflate lungs to 25 cm H_2O. This height results in approximately 70% of the air lung

capacity being, providing optimal structural integrity for imaging, rather than complete lung collapse. By the time of embedding and slide preparation, though, the volume of the lung after dehydration and processing is not equivalent to a live viable lung [52, 53].

26. The most commonly used fixative is 10% neutral-buffered formalin (pH 7.0). Depending on the epitope and antibody parameters, many fixatives, such as zinc-formalin or glutaraldehyde-formalin (http://www.ihcworld.com/_protocols/histology/fixatives.htm), provide unique advantages but should be optimized before use as these fixatives may alter the antigenicity of the epitope of interest. Cryofixation and sectioning avoid the covalent crosslinking of these fixatives, as well as processing-induced removal of lipid-based compounds from tissues. However, these methods are beyond the scope of this chapter. The eosinophil antibody protocols listed here all use phosphate-free neutral-buffered formalin (ThermoFisher), which is the equivalent of a 4% (v/v) formaldehyde solution.
27. Although carbon dioxide (CO_2) exposure is a common method of euthanasia, we recommend either ketamine-xylazine or sodium pentobarbital-based euthanasia method as CO_2 may result in hemorrhaging of the lung [54, 55]. Depending on the physiological kinetics of the molecules being studied, other considerations may be taken into account when selecting euthanasia methods [56]. Please review AVMA (American Veterinary Medical Association) Guidelines for the Euthanasia of Animals (https://www.avma.org/kb/policies/documents/euthanasia.pdf) or appropriate guidelines for animal use at your institution.
28. Before the lung is filled with formalin, the lungs may be manipulated for additional usages. For example, one may perform a bronchoalveolar lavage (BAL) by inserting an 18G catheter with a syringe filled with 1 mL of PBS at a tracheotomy site [57]. However, this may lead to some structural changes in lung architecture due to the pressure changes to obtain BAL. If perfusion is needed to clear the circulatory system of blood, this may be performed once the heart is exposed soon after euthanasia to avoid clotting. If only one lobe of the lungs is needed for IHC, suture material may be used to tie off the right or left lobe and cut off the main bronchus of that lobe distal to the knot and trachea. The separated lobe may be used for flow cytometry or other measures. The knot creates a closure so that the lobe left behind is still filled with formalin without leakage and may be used for histology.

29. Fixation time and temperature can alter the extent of covalent bonds and therefore the epitope availability for IHC [49, 58, 59]. For long-term storage, formalin-fixed samples may be dehydrated and stored in 100% ethanol (200 proof).
30. This step softens paraffin prior to deparaffinization. Slide should be kept upright and incubated for a minimum of 15 min and up to 1 h. We have found 30 min to be optimal. If problematic, the incubation can be done immersed in xylene so long as ventilation is good, and lid remains sealed on container.
31. Xylene is used to dissolve paraffin wax. During all washes, agitate slides once every minute by lifting them up and down.
32. To avoid trapping air bubbles, load slides onto coverplates submerged in distilled water (Fig. 2). After loading onto the rack, add water to slides to ensure the flow is slow and consistent. Rapid draining is indicative of an incorrect setup. In this case, try to reload the coverplate and slide and repeat the drain test. Make sure all incubations are done with the lid of the rack on to maintain humidity. This reduces evaporation of reagents on slides. If not using a rack, make sure slides are kept wet in a humid enclosure.
33. Allow at least 30-min incubation to efficiently block the tissue at room temperature. Incubations can be extended without any detrimental effects. Overnight incubations at 4 °C are often acceptable as well. Do not wash off blocking buffer before adding primary antibodies. The staining rack will drain excess blocking buffer when antibodies are added. If not using a staining rack, remove blocking buffer by tapping side of slide on a paper towel before adding antibodies.
34. This is to control for nonspecific binding of the secondary antibody. IgG isotype antibody can also be used to control for nonspecific binding of the primary antibody. Always run a negative control slide (not containing primary antibody) with experiments and, if possible, have the negative control be a serial section of the sample or at minimum the same tissue origin and conditions.
35. Increase or decrease incubation time to optimize staining intensity.
36. While the slides can be air-dried overnight, the dehydration allows the slides to be coverslipped within 10 min.
37. Depending on the number of slides being stained, we use a plastic coplin jar (<5 slides) or staining dish (6–24 slides) to hold our slides during antigen retrieval.

38. Gently run water into the container until all foam/bubbles are gone. Ensure the water stream is not directly on the sections to avoid damaging tissues.
39. Try to remove as much buffer as possible without letting specimen dry by gently tapping the slide on a paper towel. ProLong™ is a viscous reagent. If using a micropipette to dispense the reagent, ensure to pipette slowly to prevent bubbles. We usually load a pipette tip with the mountant before removing slides from the staining rack to prevent excessive drying of the tissue. If bubbles form on specimens, use a 10-μL micropipette tip to pop or aspirate bubbles. If bubbles form in the stock reagent, transfer to a microcentrifuge tube and centrifuge at 10,000 × *g* for 2 min. Protect ProLong™ from light for it is light sensitive.
40. ProLong™ Diamond is a hardening reagent whose refractive index is highest once fully cured. Slides can be imaged immediately after coverslipping, but for the best images, wait for the reagent to cure. There is no need to seal the slide. Caution must be taken when handling/imaging slides that have not been cured as the coverslip can slide around.
41. The lung FFPE EPX IF and TSA protocols can be adapted for multiplex staining by the addition of other primary antibodies and their corresponding secondary antibodies. This will require optimization of antigen retrieval, blocking steps, and antibody dilutions similar to as described above and in literature [60, 61].
42. Techniques for peripheral blood isolation or brochoalveolar isolation are described elsewhere [57, 62]. Make sure the cells stay cold on ice to maintain viability. Cell numbers can be modified to fit experimental needs, but this density of cell suspension yields a nice uncrowded distribution of cells.
43. Pre-wetting the slides with BSA helps the cells stick to the slides. Set up as many cytospin cages as needed for your experiment. Make sure to always have an even number of cages to counterbalance the centrifuge. The majority of cytospin centrifuges have their speed setting in RPM, which is equivalent of approximately 28 × *g*.

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Chapter 18

Preservation and Processing of Intestinal Tissue for the Assessment of Histopathology

Juliane Rieger, Lisa-Marie Pelckmann, and Barbara Drewes

Abstract

The intestine is often examined histologically in connection with allergies and in search for pathological changes. To be able to examine the intestine histologically with a microscope, it must be sampled and processed correctly. For microscopic analysis, the samples have to be cut into thin sections, stained, and mounted on slides. Since it is not possible to cut fresh samples without damaging them, they must first be fixed. The most common method, which is described herein, is the fixation in formalin with subsequent embedding in paraffin and staining of the slides with hematoxylin and eosin (H&E). Hematoxylin solutions (in this case Mayer's hemalum solution) stain the acidic components of the cell, i.e., cell nuclei, blue. The staining with eosin gives a pink staining of cytoplasm. This chapter describes the method of processing intestinal tissue for paraffin-embedding, sectioning, and staining with H&E. Tissue processing can be done in tissue processing machines or manually. We describe the manual processing that is often used for smaller batches of samples.

Key words Histology, Pathology, Fixation, Formalin, Hematoxylin and eosin, Intestinal tissue, Histological techniques

1 Introduction

Many allergic diseases are related to the intestines. Especially the composition of the diet and microbiota have a great influence here [1]. For this reason, the intestine is often examined in connection with allergies and, in particular, pathological changes are brought into focus. To be able to examine the intestine histologically, it must be sampled and processed swiftly to stop the immediate decay of the tissue. Intestinal samples may be obtained via biopsy, surgery, or necropsy. After sampling, there are several processing options available. The most common method is the fixation in formalin with subsequent embedding in paraffin and staining of the slides with hematoxylin and eosin (H&E) (Fig. 1). Formaldehyde solution (formalin) is used as a common fixative in histology. It is a protein cross-linking additive fixative. It stops the autolysis of tissue

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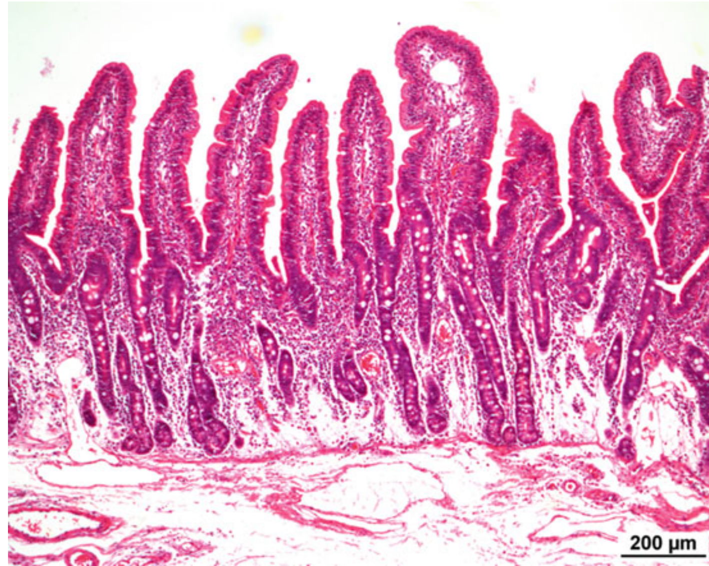

Fig. 1 The picture shows an intestinal sample (jejunum) of a pig. The sample was fixed in 10% formalin, embedded in paraffin, and stained with H&E. The mucosa and submucosa can be seen. The nuclei are stained in blue to violet with hematoxylin, and the cytoplasm and connective tissue are stained in pink with eosin

samples, making them durable [2]. H&E staining is one of the major tissue stains used in histology and is often the gold standard in pathology. Hematoxylin solutions stain the acidic components of the cell, i.e., cell nuclei, but also rough endoplasmic reticulum (recognizable in terms of metabolically active cells) and acidic mucus in blue. The staining with eosin gives a pink to reddish-yellowish staining of cytoplasm and connective tissue [3]. H&E staining does not always contrast all the desired structures, and in these cases, more specific stains and methods are available.

The staining intensity and the shade of the histological preparations can vary greatly. Clarke and Treanor have written an excellent article on this topic with a focus on digital image processing [4].

2 Materials

Use demineralized or ultra-purified water for all preparations. All steps are carried out at room temperature unless described otherwise.

2.1 Tissue Sampling

1. Modified Ringer solution: 154 mM NaCl, 5.6 mM KCl, 2.2 mM calcium chloride dihydrate, pH 7.4. Dissolve 9.0 g NaCl, 0.42 g KCl, and 0.33 g calcium chloride dihydrate in 1 L water. Store at 4 °C.

2. Neutral buffered formalin (NBF): 10% formalin in phosphate-buffered saline (PBS), pH 7.2 (*see* **Note 1**). For 1 L of 10% NBF, mix 108 mL 37% formalin (*see* **Note 2**) with 500 mL of 2× PBS (*see* **Note 3**). Bring the volume up to 950 mL with water. Check the pH and, if necessary, adjust to 7.2 with 1 M NaOH or 1 M HCl. Fill with water to 1 L. Store over night at room temperature or at 4 °C. The solution is usable as long as the pH remains constant.
3. Beakers: appropriately sized for rinsing samples.
4. Syringes: used for rinsing samples.
5. Cutting board.
6. Dissection tools: scalpels and forceps.
7. Embedding cassettes: 41 mm × 27.5 mm × 12 mm.
8. Sample containers: appropriately sized container with a snap-on lid or screw cap, such as 300-mL wide mouth jars, for holding samples in embedding cassettes during fixation.
9. Ice and ice bucket: used for cooling samples during transport and handling.

2.2 Tissue Processing

1. Ethanol solutions for tissue dehydration: Prepare 70%, 80%, 96%, and 100% solutions using denatured absolute ethanol (*see* **Note 4**).
2. Xylene or equivalent histological reagent: used for dehydrating tissues (*see* **Note 5**).
3. Long tweezers.
4. Magnetic stirrer and stirring bar.
5. Sample containers: specimen jars or other types of sample containers with lids.
6. Paraffin wax pellets: melting point of 56 °C.
7. Embedding molds: stainless, 37 mm × 24 mm × 5 mm or 40 mm × 25 mm × 10 mm.
8. Microtome for paraffin sectioning.
9. Cold and warm water baths: for floating paraffin sections. Set at room temperature and at 40 °C, respectively.
10. Glass slides for histology.
11. Hot plate or slide warmer: set to 50–60 °C.
12. Incubator or oven: set to 40 °C.

2.3 H&E Staining

1. Solutions for the dewaxing and rehydration station: Prepare the indicated number of slide staining dishes containing xylene (or substitute) × 2, 100% ethanol × 2, 96% ethanol × 1, 80% ethanol × 1, 70% ethanol × 1; and demineralized water × 2.

2. Mayer's hemalum solution: Dissolve 1 g hematoxylin in 1000 mL water on a magnetic stirrer. Add 200 g sodium iodate ($NaIO_3$) and 50 g of potassium alum (potassium aluminum sulfate dodecahydrate) and dissolve. The color of the solution is blue–violet. Add 50 g chloral hydrate and 1 g of citric acid and dissolve. The color changes to red–violet (*see* **Note 6**).
3. Eosin solution: 0.1% eosin dissolved in water (*see* **Note 7**).
4. Solutions for the staining and dehydrating station: Prepare the indicated number of slide staining dishes containing Mayer's hemalum solution × 1, demineralized water × 1, tap water × 1, demineralized water × 1, eosin solution × 1, demineralized water × 1, 70% ethanol × 1, 80% ethanol × 1, 96% ethanol × 1, 100% ethanol × 3, xylene (or substitute) × 2.
5. Mounting medium.

3 Methods

The sampling of intestinal specimens has to be well planned, as a delay between taking the samples and fixation will greatly influence histological quality (*see* **Note 8**).

3.1 Tissue Sampling

1. Label embedding cassettes in pencil with sample identifiers beforehand.
2. Euthanize your experimental animals according to an approved protocol.
3. Dissect the intestine and other organs of interest from the animal as soon as possible after death. Cut the samples to a desired form and size (*see* **Note 9**). Intestinal samples, skin samples, or similar may be cut into 1 × 2 cm rectangles. Organ samples such as liver, kidney, lung or musculature should be cut into a maximum size of 1 cm^3 (*see* **Note 10**).
4. Rinse specimens by swirling briefly in cold-modified Ringer's solution or PBS. If compatible with the study objective, rinse off blood and other debris (*see* **Note 11**).
5. Place the trimmed samples in the prepared embedding cassettes. Close the cassettes tightly and place in the container filled with the NBF fixative. The volume of the fixative should be at least 20 times or preferably 40 times the volume of the tissue. Place each sample in its own container or embedding cassette (*see* **Note 12**).
6. Fix the samples for 24 h up to 4–5 days at room temperature or 4 °C (*see* **Note 13**).

3.2 Tissue Processing

Here, manual tissue processing up to embedding in paraffin is described. An embedding machine may also be used, if available.

3.2.1 Dehydration

1. Decant the NBF fixative from the sample container into an appropriate disposal container. Stack the samples, still in embedding cassettes, in a sample container and place a stir bar in the container.
2. On a magnetic stirrer, rinse the samples for 24 h with at least five batches of 70% ethanol. Do not use water (*see* **Note 14**).
3. Continue rinsing the samples for another 24 h in at least five batches of 80% ethanol.
4. On the third day, dehydrate the samples in two batches of 96% ethanol and three batches of 100% ethanol for 1 h each.
5. Place the samples in a 1:1 mixture of ethanol and xylene for 40 min, and in two batches of pure xylene, 30 min each.

3.2.2 Paraffin Embedding

1. After the incubation with xylene in Subheading 3.2.1, **step 5**, incubate the samples in paraffin overnight at 58–59 °C (*see* **Note 15**).
2. On the fourth day, incubate the samples in two more batches of paraffin, 2 h each.
3. Take out the tissues from embedding cassettes and place them into embedding molds to embed in fresh paraffin (Fig. 2). If necessary, align the sample in liquid paraffin with warm tweezers, working quickly and avoiding the introduction of any air bubbles into the paraffin.
4. Label each of the paraffin molds with an appropriate sample identifier by placing a piece of paper with the sample identifier in the liquid paraffin or by putting the embedding cassette on top of it.
5. Place the molds on a cooling plate until the paraffin is hardened.
6. Take the hardened paraffin blocks out of the molds and store them for later use.

3.2.3 Slide Preparation

1. Before a sample block is cut on a microtome, precool the block in a refrigerator or an ice bath.
2. Cut the block into 3–5 μm sections (*see* **Note 16**). Guide the sections with a brush or tweezers from the microtome blade and float them in the "cold" (room temperature) water bath, carefully smoothing out any wrinkles. To ensure the same orientation of all sections, place all with the matte side up on the water. Subsequently, transfer the sections with a slide to float them in the warm water bath for further stretching (*see* **Note 17**).

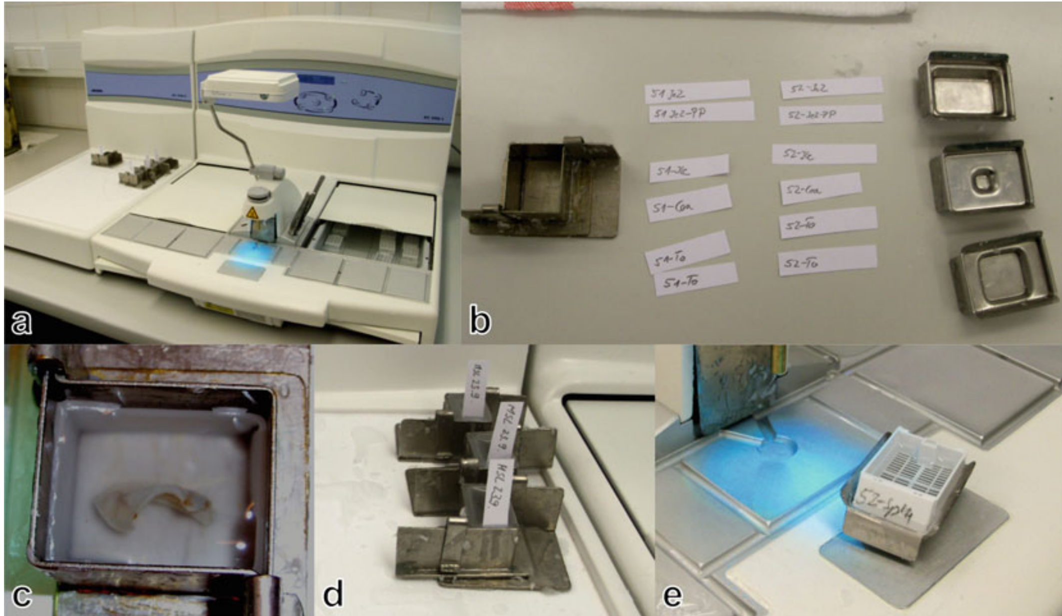

Fig. 2 (**a**) A paraffin embedding station with sample storage containers with liquid paraffin (right) and cooling plate (left). (**b**) Some metal molds for different sample sizes and prepared sample labels are shown. (**c**) A piece of porcine intestine upright in liquid paraffin. The side from which you want to cut the sample later must face down. (**d**) Finished molded blocks rest on the cooling plate. They are provided with paper signs for identification. (**e**) An embedding cassette is placed in the liquid paraffin for identification. This allows the sample to be clamped well into the microtome later

3. To mount each of the smoothed-out sections on a glass slide (*see* **Note 18**), gently submerge the slide into the water. Place the slide under the section in the water and position the section in the center of the slide. Then drag the slide toward the section so that the surface of the slide comes in contact with an edge of the section. Carefully pull out the slide with the section obliquely out of the water (*see* **Note 19**).
4. Dry the slide on a hotplate at 50–60 °C for about 1 h and in an incubator or oven at about 40 °C overnight (*see* **Note 20**).
5. Store the slides in slide boxes at room temperature until used.

3.3 H&E Staining

1. Place the tissue slides in a staining rack and prepare the dewaxing and rehydration station and the staining and dehydration station (*see* **Note 21**).
2. Dip the staining rack through the dewaxing and rehydration station in the following order, gently moving the rack up and down to rinse off the remainder of the solution from the previous bath in the new solution in the next dish (*see* **Note 22**):
   (a) In xylene, 15 min × 2.
   (b) In 100% ethanol, 10 min × 2.

(c) In 96%, 80% and 70% ethanol solutions in series, 2 min each.

(d) In demineralized water, 2 min each × 2.

3. Transfer the staining rack from the second batch of water to the dish with hemalum solution and gently move it up and down a few times to completely wet the sections. Leave the slides in the staining solution for 3–7 min (*see* **Note 23**).
4. Rinse the staining rack for a few seconds in the dish with demineralized water to remove excess staining solution and residues from the slides.
5. Place the staining rack under running tap water for 10 min to develop blue color. Alternatively, rinse the rack in a dish by changing water every minute.
6. Rinse the staining rack in demineralized water for 1–2 min.
7. Transfer the staining rack from the demineralized water into the dish with eosin solution and leave in the staining solution for 5–10 min (*see* **Note 24**).
8. Rinse the staining rack in demineralized water for a maximum of 5 s by dipping the rack three times.
9. Differentiate the staining and dehydrate the tissue by rinsing the staining rack through the dehydration station in the following order (*see* **Note 25**):

(a) In 70% ethanol, 30 s × 1.

(b) In 80% ethanol, 30 s × 1.

(c) In 96% ethanol, 1 min × 1.

(d) In 100% ethanol, 3–5 min × 3.

(e) In xylene, 5 min × 2.

10. Coverslip the slides with a suitable mounting medium, avoiding the formation of air bubbles. Keep slides lying flat overnight under the hood to evaporate the solvent from the mounting medium. Refrain from handling at least until the edges of the coverslips are dry and fixed on the slide. It takes several days for the mounting medium to completely harden.

4 Notes

1. The fixative should be prepared as freshly as possible. Concerning the concentration designation, 4% or 10%, there is a long debate in the literature. Here, we follow the argumentation chain that "formol or formalin" designates the aqueous solution of formaldehyde in water. Formaldehyde is soluble in water up to a concentration of about 40%, so that 10% formol, here in buffered form as NBF, corresponds to a concentration

of 4% formaldehyde in water. Prolonged storage in the refrigerator causes the formaldehyde in the solution to polymerize back to paraformaldehyde, making the solution unusable. The solution must be clear after shaking. After prolonged storage in the refrigerator (several weeks or months), check the pH before use. Always ensure that sufficient quantities of the fixative are available. The volume of the fixative should be at least 10 times and preferably 40 times the tissue volume. The fixative can be replaced with a fresh solution after a few hours or the next morning, especially for samples containing a lot of blood.

For most uses, including for immunohistochemistry, it is not necessary to depolymerize formalin from paraformaldehyde. Commercially available histological-grade formalin stabilized with methanol or calcium carbonate is sufficient. The only important thing is that it is buffered with PBS (TRIS buffer is not possible because tromethamol (Tris) decomposes on contact with formalin). For some antigens, however, fixation with, for example, methacarn (methanol-Carnoy) gives better results. Even for histological special stains or the presentation of certain components, such as intestinal mucus, tissue fixation with 10% NBF is not always the best choice. It is therefore very important to plan before tissue sampling which options one would like to keep open in addition to your routine histological staining. If necessary, fix additional tissue samples in another fixative or freeze them unfixed in liquid nitrogen.

2. Use a commercially available 37% formalin solution. Alternatively, 35% formalin may be used. In this case, use 114 mL of 35% formalin to make 1 L of 10% NBF. Formalin is a hazardous substance. It is not only toxic and causes severe burns to the skin and mucous membranes (eyes), but also has an allergic effect and classified as a suspected carcinogen that cause genetic damage. Handle only with personal protective equipment (lab coat, gloves, possibly goggles) and under the hood.
3. To make 2× PBS (274 mM NaCl, 5.4 mM KCl, 20 mM Na_2HPO_4, 3.6 mM KH_2PO_4, pH 7.2), dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 450 mL of ultrapure water. Adjust pH with 1 M NaOH or 1 M HCl and bring the volume up to 500 mL. Store at 4 °C. 2× PBS may also be prepared from commercially available powder or from 10× concentrated PBS. For 500 mL 2× PBS, dissolve 9.55 g of powder in 500 mL of water.
4. Analytically pure ethanol is not necessary. Ethanol is flammable; therefore, keep away from ignition sources. Avoid eye contact.

5. A xylene substitute may be used. Xylene is a hazardous substance. Irritation of skin and mucous membranes (eyes, respiratory system), risk of damage to the brain, liver, and kidney during prolonged or frequently repeated inhalation, therefore only handle under the hood.
6. Hematoxylin itself is colorless to yellowish and must be oxidized to hematin in order to be used as a dye together with polyvalent metal ions, in this case, aluminum. In the present recipe, the oxidation is achieved by the addition of sodium iodate. The dyeing solution is therefore usable immediately. Other hematoxylin solutions cause oxidation by atmospheric oxygen and must "ripen" for a few days to weeks before use. The solution can be kept in a tightly closed container at room temperature and is stable for about 1 year or as long as it is still reddish. If it turns blue, it is no longer usable. A ready-to-use staining solution is also commercially available.
7. If staining is only moderate or not successful, the eosin solution can be acidified with 1 drop of glacial acetic acid per 100 mL staining solution. However, do not use more glacial acetic acid than necessary because hematoxylin may be released from the tissue due to reduction of pH. If more reddish (carmine) staining is desired, substitute erythrosine for eosin. Prepare and use erythrosine in the same way as eosin. The staining solution is stable for several weeks at 4 °C, although it should be discarded if flakes appear (fungi).
8. The sampling must be completed within minutes after euthanasia because the intestine decomposes very quickly. If necessary, samples taken can be temporarily stored in ice-cold Ringer's solution. Figure 3 shows an example of a gut sample taken too late after death of the animal.
9. If there is not enough time during the sampling to accurately cut the samples, this can also happen after or during fixation. First and foremost, it is important to fix the samples quickly. Depending on the study question, it will be necessary to observe the intestinal samples longitudinally or transversally. The tissue orientation should therefore be clear. If intestinal rings are cut, orientation is no problem. If the intestine is cut open and rectangles shall be embedded, then having a short and a long cutting edge may be helpful. It should be borne in mind that in some sections of the intestine, especially in the ileum, the side of the intestinal wall facing the mesentery differs in fine construction from the antimesenteric side (lymphatic tissue).
10. Do not squeeze the tissue during trimming. The inner surface of the intestine is very sensitive. Even light touches put artifacts; therefore, discard any points in the final cutting of the

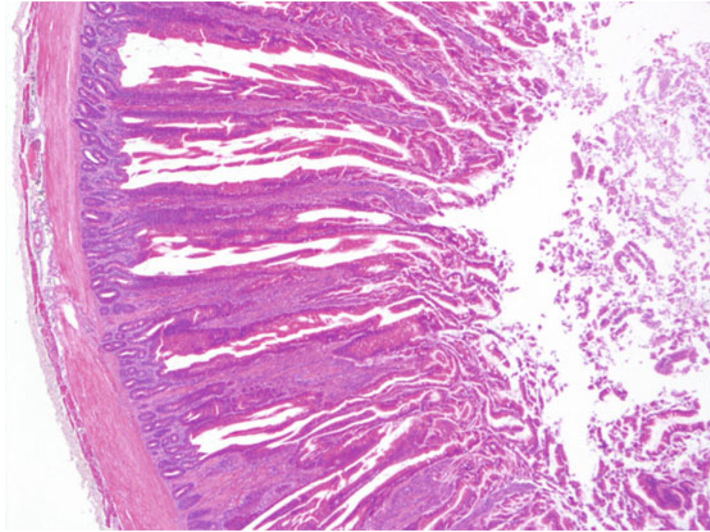

Fig. 3 A formalin-fixed, paraffin-embedded, and H&E-stained jejunoileum of the chicken. A delay in fixation caused the tissue damage present at the tips of the villi, as well as separation of the epithelium from the lamina propria. The sample was either left outside for too long or the animal was dead for a while before the tissue was harvested

samples that have previously come in contact with tweezers. If the intestinal tube is to be cut open, always carefully insert the blunt leg of the scissors into the lumen and later remove the cut edges with a scalpel. Cut with the scalpel "pulling," not with pressure. During preparation, the surface should be kept moist by gentle wetting with Ringer's solution.

11. It should be considered in the planning whether the intestinal content should be preserved, or the samples may be rinsed. Washed samples are easier to work with, as no hard feed ingredients will scratch the tissue when sectioning. Especially large intestine samples from large experimental animals, such as the pig, are very difficult to remove and process without intestinal contents removed. If it is necessary, cryopreservation should be considered. If the contents of the intestine are to be rinsed out, fill a 20 mL disposable syringe with cold Ringer's solution, carefully insert the nozzle of the syringe into the opening of the intestinal tube and push in the piston with moderate pressure.
12. With intestinal samples of large animals, it is often necessary to open them and fix only in parts. In order to prevent the sliced intestinal samples from rolling up, it is possible to pin them, mucosal side upwards, on cork plates. Do not pull too tight, because the samples can shrink during fixation. Then place the cork plates in the NBF fixative with the sample facing down.

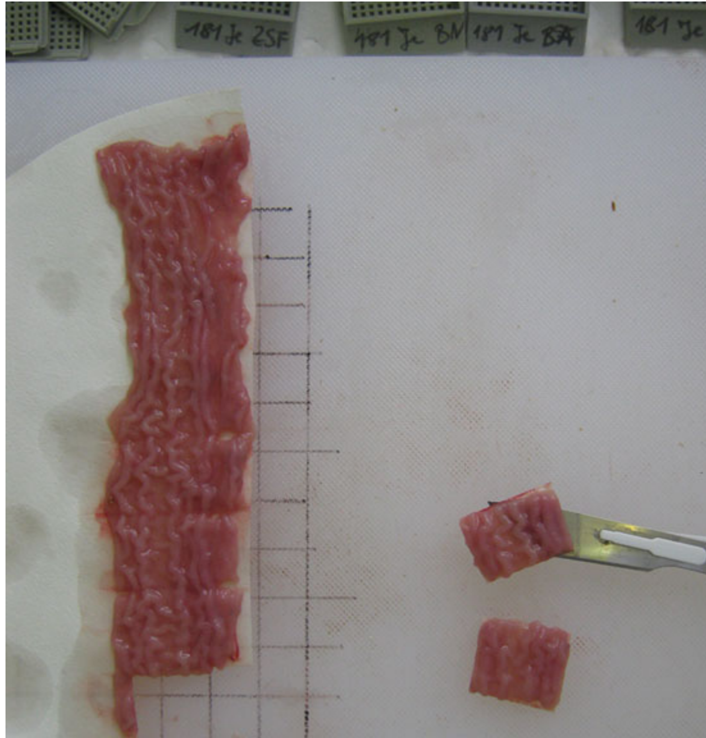

Fig. 4 The small intestine of a pig placed directly on the filter paper, opened, and then cut to size. Placing the specimens with the filter paper in the embedding cassette largely prevents the intestinal tissue from rolling up during fixation

After fixation, remove the pins and retrim the samples. Alternatively, you can adhere the samples to thick filter paper (Fig. 4). Therefore, place the rinsed intestinal sample on the paper and cut them open with a scissor. Now cut the sample to the desired form together with the filter paper. Place with the paper underlay in the embedding cassette. The serosa adheres to the filter paper and curling can usually be prevented. Remove the filter paper after fixation or during dehydration.

13. When choosing the fixation temperature, the following should be considered: At room temperature, the fixative penetrates more quickly into the tissue while at 4 °C, the activities of degrading enzymes are significantly reduced. We therefore usually fix at 4 °C. Although polymerization of formaldehyde to paraformaldehyde takes place more quickly at a lower temperature, this is not an issue since the fixative is present in excess.
14. It is important to wash the unbound formalin well out of the tissue. While water is acceptable for this purpose, alcohol is preferred, especially if the fixation time is short, in case the tissue is under-fixed. Rinsing in alcohol is particularly

important for delicate tissues such as the intestine, pancreas, and so on. As with the subsequent dehydration steps, cover the samples completely with the alcohol wash solutions during rinsing. The most effective means for removing formalin and water from the tissue is increasing the number of alcohol rinses rather than the volume of the alcohol rinses. The incubation time in 70% and 80% ethanol solutions can be exceeded without adversely affecting the tissues and staining quality, and the samples may be stored in these alcohol solutions over a weekend if required. From the point of rinsing in 96% ethanol, however, the indicated incubation times are optimized for intestine samples with an edge length of 1×2 cm, and therefore should be followed. If over-incubated in absolute ethanol and xylene, the tissue hardens too much, and sectioning becomes difficult. On the other hand, under-incubation should also be avoided because it would prevent complete penetration of xylene and paraffin into the tissue and lead to "friability" of the tissue when the blocks are cut. If required, methyl benzoate can be added as an intermediate between ethanol and xylene, which can still absorb residual amounts of water and does not lead to further hardening of the tissue. It is also possible to store the samples in methyl benzoate overnight or over a weekend.

15. The temperature of the paraffin baths should not exceed 60 °C if immunohistochemical staining is to be performed with the tissues in the blocks in addition to histological staining. Therefore, the melting temperature should be taken into consideration when selecting paraffin.
16. If you experience difficulty during cutting, a paraffin block may be trimmed to make the cut surface smaller. You can also vary the speed of cutting, breathe on the cut surface, or slide another paraffin block over the cut surface. When cutting thinner sections of 2–3 μm, noticeable variability in section thickness can occur.
17. If tissue sections are already smooth, you can transfer the cut tissue sections straight to the warm water bath. In most cases, however, it is easier to gently straighten out wrinkles with two brushes in the room-temperature water bath first. Be sure to have the correct temperature setting of the warm water bath. If it is not warm enough, the sections do not stretch well, and if it is too hot, the paraffin melts around the tissues.
18. Typically, standard slides are sufficient for use for H&E staining. If the sections are to be used for immunohistochemistry, silanized or adhesion slides may be used, especially if the sections need to be pretreated as part of heat-induced epitope retrieval. For tissues that generally do not adhere very well to

slides, there are various adhesion methods to improve the bonding of the tissues to the slides. It is important to label slides before mounting sections on them so that you can identify the sections later. Make sure that the label and the section are always on the same side of the slide to avoid tissue losses during handling of the slides.

19. Drain the water by briefly shaking the slide. It is important that there are no water bubbles under the tissue. These bubbles deform the tissue when it dries and the water escapes from them poorly. The tissue will either fail to stick at this point or tissue expansion remains visible after staining. After mounting the stretched section, you can leave the slide on a slide holder to drain before you put it on the hot plate.
20. If you are in a hurry to dye the tissue, you can also dry the sections for 1 h at 80 °C on the hotplate, and then deparaffinize to dye. For immunohistochemistry, do not heat tissue above 60 °C, except during the heat-induced antigen retrieval.
21. Always filter the dye solutions to remove precipitated dye before use. Precipitated dye particles are difficult or impossible to remove from the slides later.
22. In this step, deparaffinize the sections for clear and streak-free specimens. Before going to the next dish, keep the slide rack slightly slanted over the current dish to allow the solution to drip off. This minimizes carrying the solution from one dish over to the next. You may shorten the time for dewaxing by stirring the solution in the dish on a magnetic stirrer. Longer dewaxing time does not affect staining. Dewaxing overnight is sometimes recommended for immunohistochemistry.
23. The staining with hematoxylin is progressive, and the dye is difficult to remove once bound to the tissue. Optimal staining should be obtained by starting with a shorter staining time and repeating the process as needed. Staining results also depend on the age of the staining solution and the number of slides stained using the solution. Thus, the staining time indicated here is approximate. Fresh or still usable hematoxylin solution should be reddish; discard if it turns blue.
24. The staining with eosin (or erythrosin) is regressive. The sections will be over-stained and during the subsequent rinsing in demineralized water and dehydration steps in alcohol, dye diffuses out of the section. If the color is too weak after rinsing in absolute ethanol, the sections can be placed back into water, and the staining process repeated with a longer staining time. If the section is over-stained, simply prolong the rinse time in demineralized water and changing the rinsing water, if necessary.
25. Move the staining rack up and down several times. Especially in 70% and 80% ethanol, the dye will dissolve from the sections.

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Chapter 19

Antibiotic Treatment in an Animal Model of Inflammatory Lung Disease

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Abstract

Allergic disease is on the rise and yet the underlying cause and risk factors are not fully understood. While lifesaving in many circumstances, the use of antibiotics and the subsequent disruption of the microbiome are positively correlated with the development of allergies. Here, we describe the use of the antibiotic vancomycin in combination with the papain-induced mouse model of allergic disease that allows for the assessment of microbiome perturbations and the impact on allergy development.

Key words Antibiotics, Vancomycin, Microbiome, Allergic disease, Papain

1 Introduction

One in three Canadians will be diagnosed with an allergic disease during their lifetime [1]. Although effective treatments are available, there are currently no cures for allergic diseases. Children have the greatest burden of disease and experience deaths due to anaphylaxis and asthma exacerbations, as well as hospital admissions due to unabated airway inflammation. In 1989, Strachan proposed the *hygiene hypothesis*, suggesting that decreased pre/perinatal exposure to infectious disease results in the increased susceptibility to allergic disease [2]. Various epidemiological studies support this hypothesis and demonstrate a rising incidence of asthma and allergic disease concomitant with industrialization, improvements in sanitation and hygiene, increased use of antibiotics, and decreased rural/farm living [3]. Since Strachan's hypothesis, we have learned that a variety of genetic and environmental factors interact to contribute to susceptibility, and severity of allergic disease, most notably, the composition and perturbation of the early life microbiome [3, 4].

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The role of the microbiome in atopic disease has been studied using microbiome-depletion in mice. The most extreme microbiome studies employ the use of germ-free (GF) animals, which are devoid of all microorganisms [5, 6]. Antibiotics can be used as a tool to create controlled disturbances of the microbiota [3, 4, 7]. Vancomycin is a glycopeptide antibiotic that sterically hinders peptidoglycan polymerase to prevent the formation of the backbone glycan chains and interferes with cell wall synthesis in many Gram-positive bacteria [8]. Due to low oral bioavailability, orally administered vancomycin is poorly absorbed into the bloodstream and therefore acts mainly on gut bacterial communities [9]. Typically reserved as an antibiotic of last resort, the rates of vancomycin administration are on the rise as resistance to other front-line antibiotics becomes more commonplace [10]. Mice treated with low-dose vancomycin display dramatic changes in their bacterial microbiota [11]. Oral administration of vancomycin depletes microbes producing short-chain fatty acids including the epigenetic modifier butyrate and supplementation of antibiotic-treated mice with exogenous butyrate reverses many of the symptoms linked to allergy through modification of T-cell and dendritic cell responses [12].

In mice, it has been demonstrated that a lack of microbial colonization in early life increases sensitivity to atopic models [7], including mouse models of asthma [13]. One model of asthma is the papain model, which uses the cysteine-protease allergen from papaya and mimics the phenotype of occupational asthma [14]. The allergenic activity of papain is protease dependent. The protease activity of the enzyme causes damage to the mucosa resulting in the release of alarmins from the epithelium that induces eosinophilic inflammation and robust levels of IgE in the periphery [15, 16].

The combined use of antibiotic treatment and a model of allergic disease is important to decipher some of the underlying causes of allergic disease and subsequently may allow one to address some of the fundamental issues that include subclinical allergen-sensitization, overlapping wheezing phenotypes (potential clinical precursors to asthma), absence of truly predictive biomarkers, and a lack of diagnostic tests. Currently, these issues lead to chronic under-diagnosis and under-treatment.

2 Materials

2.1 Antibiotic Treatment

1. Vancomycin hydrochloride from *Streptomyces orientalis:* Prepare a 100-mg/mL stock solution in autoclaved water. Store as 1-mL aliquots at −20 °C for up to 1 year.
2. C57BL/6J mice: used as breeding pairs (*see* **Note 1**).

2.2 Papain-Induced Asthma Model

1. Sterile phosphate-buffered saline (PBS): (137 nM NaCl, 2.7 nM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4).
2. Papain: ≥16 units/mg protein. Prepare a solution at a concentration of 0.25 μg/μL in sterile PBS. This can be pre-made and frozen as a 10× stock for up to 1 year. Thawed aliquots should not be refrozen.
3. Anesthesia vaporizer system with isoflurane.
4. C57BL/6J mice: at least 6 weeks old, reared from vancomycin-treated and control breeding pairs (*see* **Note 2**).
5. P200 pipette with tips.

2.3 Collection of Bronchiolar Lavage (BAL) Fluid, Lungs, and Blood Samples

1. 2,2,2-Tribromoethanol (Avertin).
   (a) Make a stock of 100% avertin by mixing 10 g of 2,2,2-tribromoethyl alcohol with 10 mL of tert-amyl alcohol. Make sure the solution is completely dissolved by using a stir bar and heating the solution to approximately 50 °C. Store 100% Avertin at 4 °C. Avertin is light sensitive. Store in the dark.
   (b) Make a working Avertin solution by diluting 100% Avertin stock 1:40 with PBS. Add Avertin stock dropwise to warm PBS on a stir plate, heat the solution, but do not boil. Avertin may take a long time to dissolve completely. Add the drops very slowly.
2. Dissection board with pins.
3. Surgical scissors and forceps.
4. Lavage buffer: 2% fetal bovine serum (FBS) and 2 mM EDTA in PBS.
5. 18G–22G Catheters.
6. 1-mL Tuberculin syringes: Three syringes per mice are required for BAL fluid collection.
7. 22G Syringe needles.
8. 15-mL conical tubes.
9. Formalin, commercially available as 10% formalin solution, buffered to a neutral pH.
10. TRIzol™ reagent or equivalent for RNA extraction.
11. 2-mL Microcentrifuge tubes.

2.4 Histology

1. Lung tissues fixed in 10% neutral-buffered formalin (*see* Subheading 2.3).
2. Ethanol gradients for de/rehydration: 100%, 95%, and 70% ethanol solutions.
3. Xylene.

4. Microtome.
5. Silane-coated glass slides for histology.
6. Slide staining jars.
7. Mayer's hematoxylin: commercially available as 1 g/L.
8. Saturated lithium carbonate solution.
9. Slide drying oven: Set at 37 °C.
10. Permount mounting medium or equivalent.
11. Coverslips: 1.5 mm thickness.

2.5 Reverse-Transcription Quantitative PCR (qPCR)

1. Lung tissues collected in TRIzol reagent (*see* Subheading 3.3, **step 10**).
2. Stainless steel beads: 5 mm diameter.
3. Refrigerated centrifuge.
4. RNase-free water: commercially available.
5. Chloroform.
6. Isopropanol.
7. 75% Ethanol: Make fresh with RNase-free water.
8. Nanodrop spectrophotometer or equivalent.
9. Filtered pipet tips: For P1000, P200, and P20 pipettes. Nuclease-free.
10. Reverse-transcriptase cDNA synthesis kit: High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) or equivalent.
11. Universal SYBR green qPCR kit: KAPA SYBR® FAST qPCR kit (KAPA Biosystems) or equivalent.
12. Primers: IL4, IL5, IL13, and GAPDH.
    (a) IL4: forward 5′-TCGGCATTTTGAACGAGGTC-3′ and reverse 5′-CAAGCATGGAGTTTTCCCATG-3′.
    (b) IL5: forward 5′-GATGAGGCTTCCTGTCCCTACTC-3′ and reverse 5′-TCGCCACACTTCTCTTTTTGG-3′.
    (c) IL13: forward 5′-CCTGGCTCTTGCTTGCCTT-3′ and reverse 5′-GGTCTTGTGTGATGTTGCTCA-3′.
    (d) GAPDH: forward 5′-CATCAAGAAGGTGGTGAAGC-3′ and reverse 5′-CCTGTTGCTGTAGCCGTATT-3′.
13. 96-Well qPCR plates.
14. qPCR plate sealers.
15. 0.2-mL PCR tubes
16. Quantitative PCR thermal cycler.

2.6 Assessment of IgE Levels in Serum Samples

1. Serum samples from experimental mice.
2. Refrigerated centrifuge.
3. 1.5-mL Microfuge tubes.
4. Single-channel pipettes and tips.
5. Multi-channel pipette and tips.
6. IgE ELISA Set: commercially available kit with capture and detection antibodies, enzyme reagent, and standards (e.g., BD OptEIA™).
7. 96-well flat-bottom ELISA plates.
8. Plate sealers.
9. Coating buffer: 0.1 M sodium carbonate, pH 9.5. To make 1 L, dissolve 7.13 g $NaHCO_3$, and 1.59 g Na_2CO_3 in ultrapure water and adjust pH with NaOH. Store at 4 °C.
10. Assay diluent: 10% fetal bovine serum in PBS. Store at 4 °C.
11. Wash buffer: PBS containing 0.05% Tween-20.
12. Tetramethylbenzidine (TMB) substrate solution set for ELISA: A kit containing TMB and hydrogen peroxide solutions are commercially available.
13. Stop solution: 1 M H_3PO_4 or 2 N H_2SO_4.
14. Automated plate washer or squirt bottle.
15. Microplate reader: for measuring absorbance at 450 nm.

2.7 Flow Cytometry

1. ACK (ammonium-chloride-potassium) lysis buffer: commercially available red blood cell lysis buffer.
2. PBS.
3. Flow cytometry (FACS) buffer: 2% FBS, 2 mM EDTA in PBS, pH 7.4.
4. Blocking buffer: FACS buffer with 10% normal rat serum and 5 μg/mL purified anti-CD16/32 (clone 2.4G2).
5. Refrigerated centrifuge.
6. 96-Well V-bottom plates.
7. Carboxylate modified latex beads: 4% (w/v), 10 μm. Approximately 1×10^8 beads/mL. Commercially available. Store at 4 °C.
8. 7-AAD viability stain.
9. Fluorochrome-conjugated antibodies (name, clone, fluorochrome, dilution):
   (a) Anti-neutrophil, 7/4, FITC,1/200.
   (b) Siglec-F, E50–2440, PE, 1/400.
   (c) CD3e, 145-2C11, PE-Cy7, 1/200.
   (d) CD11c, N418, Alexa Fluor 647, 1/200.

(e) CD45, I3/2, Pacific Blue, 1/200.
(f) B220/CD45R, RA3-6B2, APC, 1/200.

10. FACS tubes.
11. Flow cytometer: BD LSRII analyzer or equivalent.

3 Methods

3.1 Antibiotic Treatment

1. Place one breeding pair per cage. Randomly assign each breeding pair to receive either control or vancomycin treatment.
2. Prepare drinking water for the vancomycin group by making a 1:500 dilution of the vancomycin stock solution in autoclaved water. The final concentration of vancomycin in the drinking water is 200 mg/L.
3. Administer the vancomycin water during breeding and nursing. Control mice should receive equivalent autoclaved water without antibiotic addition.
4. Once pups are born from these breeding pairs, maintain them on respective water for the duration of the experiment. Offspring should be separated from the breeders once they reach weaning age (*see* **Note 3**).

3.2 Papain-Induced Asthma Model

In this model, a papain solution prepared in PBS is administered on Days 0, 1, 14, and 20. Control mice receive PBS only.

1. Anesthetize mice with 3.5–4% isoflurane using oxygen (2.0 L/min) until breathing rate reduces to approximately 1 breath/s.
2. Using a P200 pipette, administer 10 μg of papain in a 40-μL volume intranasally dropwise through the nares.
3. Monitor mice until they recover from anesthesia, then return to the cage.
4. Repeat **steps 1–3** on the next day and again on days 14 and 20.

3.3 Collection of Bronchiolar Lavage (BAL) Fluid, Lungs, and Blood

1. Anesthetize mice with avertin injected intraperitoneally (20 μL/g of body weight). Transfer the mice to a dissection board once deep anesthesia is confirmed by the loss of pedal reflex.
2. Pin the mouse to the dissection board, ventral side up.
3. Dissect the fur, skin, and muscle on the neck carefully using surgical scissors and forceps to expose the parotid and submaxillary glands. Bluntly dissect the parotid and submaxillary glands away from the trachea, taking care not to rupture the jugular vein.
4. Expose the trachea by bluntly dissecting away the muscle layer around it. Make a small incision into the trachea inferior to the larynx. Be careful not to cut all the way through the trachea.

5. Insert the 18G catheter into the small incision in the trachea.
6. Fill three 1-mL syringes with 1 mL of lavage buffer. Lavage the lungs once with each syringe and complete three lavages total (*see* **Note 4**). Pool the BAL fluid from the three syringes into one 15-mL conical tube and store on ice.
7. Collect blood via cardiac puncture by inserting a 22G needle and a 1-mL syringe into the closed thoracic cavity. Store blood on ice.
8. Open the thoracic cavity to expose the lungs. Collect the left lung for histology and rinse once with PBS (*see* **Note 5**).
9. Place the left lung in a 15-mL conical tube containing 5 mL of 10% neutral buffered formalin on ice.
10. Collect the right lung lobe for RNA analysis. Store the lung in 1 mL of TRIzol™ reagent in a 2-mL microcentrifuge tube. Store at −80 °C for long-term storage.

3.4 Histology

1. Keep the lung tissue collected, as described in Subheading 3.3, **step 8**, in 10% neutral buffered formalin overnight at room temperature.
2. The next day, transfer tissues to 70% ethanol for an additional 16–24 h.
3. Fixed lungs were sent to an external service for paraffin embedding.
4. Paraffin embed lungs, cut 3–6 μm sections.
5. Dry slides at 37 °C overnight.
6. Deparaffinize and hydrate the slides by incubating in xylene, ethanol solutions, and water in the following order for the indicated time:
   (a) Xylenes: 3 × 5 min.
   (b) 100% Ethanol: 2 × 3 min.
   (c) 95% Ethanol: 1 × 3 min.
   (d) 70% Ethanol: 1 × 3 min.
   (e) dH_2O: 3 min.
7. Stain the slides in Mayer's hematoxylin solution for 5 min.
8. Wash in running tap water for 5–10 min.
9. Bluing in sodium lithium carbonate solution for 1 min.
10. Wash in running tap water for 5–10 min.
11. Rinse in 95% ethanol by dipping 10 times.
12. Counterstain in eosin–phloxine solution 30–60 s.
13. Dehydrate the slides by incubating in ethanol solutions and xylene in the following order for the indicated time:
    (a) 70% Ethanol: 2 min.
    (b) 95% Ethanol: 2 min.

(c) 100% Ethanol: 3 × 2 min.

(d) Xylene/100% ethanol (1:1): 2 min.

14. Place slides in xylene for 2 min.
15. Mount slides using Permount and coverslips.
16. Score H&E-stained sections blindly on a scale of 0–12 as previously described [17]. Briefly, each lung section will get a score from 0 to 4 for infiltration of immune cells into the perivascular space, the peribronchiolar space, and the parenchyma.

3.5 Reverse-Transcription qPCR

For the following steps, nuclease-free, sterile filter tips should be used to protect RNA from degradation.

3.5.1 RNA Isolation from Lung Tissue

1. Thaw lungs placed in TRIzol™ on ice. Add one clean steel bead per 2-mL tube.
2. Seal tubes with parafilm and homogenize lung tissue using a mechanical homogenizer such as the Qiagen TissueLyser at room temperature for 10 min at 19 rpm.
3. While tissue is homogenizing, precool a microcentrifuge to 4 °C.
4. In a fume hood, add 200 μL of chloroform to each tube and invert tubes for 15 s by hand. Spin tubes at 12,000 × *g* for 15 min at 4 °C.
5. Transfer 500 μL of the upper phase to new sterile RNase-free 1.5-mL microcentrifuge tubes with care to avoid the interphase.
6. Add 500 μL of isopropanol to each tube and invert to mix. Spin tubes in a microcentrifuge at 12,000 × *g* for 15 min at 4 °C. After centrifugation, a small white pellet of RNA should be visible at the bottom of the tube.
7. Decant isopropanol and dab the top of the microcentrifuge tube on a paper towel to wick away excess isopropanol. Take precautions to perform this step gently, as the pellet can be easily dislodged.
8. Add 1 mL of RNase-free 75% ethanol by swirling around the edges of the tube. Take precautions to perform this step gently, as the pellet can be easily dislodged.
9. Decant ethanol and dab the top of the microcentrifuge tube on a paper towel to wick away excess ethanol.
10. Add 50–100 μL of RNase-free water to each tube. Leave tubes at room temperature for 10 min until the pellet is completely dissolved.
11. Store RNA at −20 °C for short-term storage or −80 °C for long-term storage. Avoid multiple freeze-thaws.

3.5.2 Reverse-Transcription

1. Determine RNA concentrations using a Nanodrop spectrophotometer. Add 2000 ng of RNA to a 0.2-mL PCR tube. Add RNase-free water to a total volume of 10 μL.
2. Make a master mix for each sample according to the instructions provided by the manufacturer of selected reverse-transcriptase cDNA synthesis kit (*see* **Note 6**).
3. Add 10 μL of the master mix from **step 2** to each RNA sample from **step 1**.
4. Place tubes in a PCR thermal cycler with the following program: 25 °C for 10 min, 50 °C for 50 min, 85 °C, 5 min, hold at 4 °C.

3.5.3 Real-Time Quantitative PCR

1. Add 80 μL of RNase-free water to each cDNA sample resulted from Subheading 3.5.2. Mix well.
2. Add 2 μL of each cDNA sample from **step 1** and one blank (water only, no template control) to a qPCR plate in duplicate or triplicate.
3. Make qPCR SYBR master mix for each primer according to the manufacturer's instruction. For the KAPA SYBR® FAST, the master mix components per sample are 5 μL SYBR reagent, 2 μL RNase-free water, and 1 μL of a 50/50 mix of forward and reverse primers each at a final concentration of 2 nM.
4. Add 8 μL of the master mix to 2 μL of each cDNA sample for a total volume of 10 μL per well.
5. Seal the qPCR plate well by sticking down all edges of the plate sealer.
6. Centrifuge the plate for a few seconds to make sure all components are well mixed in the conical bottom of the plate.
7. Place the plate in a real-time PCR thermal cycler with the following program:
   (a) Stage 1 (polymerase activation): 95 °C for 0:20.
   (b) Stage 2 (denaturing; extension/annealing): 95 °C for 1:00; 60 °C for 0:20, collect data here. Repeat the cycle 40 times.
   (c) Dissociation stage (melting curve analysis): 95 °C for 0:15, 60 °C for 0:15, collecting data between these two stages, 95 °C for 0:15.
8. To analyze qPCR results, normalize the mean CT value for each sample to the mean CT of the housekeeping gene (e.g., GAPDH) for that same sample. Calculate ΔCT using the following equation:

$$\Delta CT = 2^{(\text{CT housekeeping gene})} / 2^{(\text{CT gene of interest})}.$$

3.6 Assessment of IgE Levels in Serum Samples

1. Incubate whole blood for 1 h at room temperature or overnight at 4 °C to allow clotting.
2. Separate serum from whole blood by centrifugation at 600 × *g* for 10 min. Transfer serum to a new 1.5-mL microcentrifuge tube. Store at −20 °C for up to 1 year.
3. Perform ELISA for total serum IgE according to the manufacturer's instructions. If BD OptEIA™ IgE ELISA Set is used, no modifications are required. A serum dilution test experiment is recommended. For allergic mice, typically a 1:10–1:20 dilution is necessary, but this should be validated as allergic models and their IgE levels can vary depending on mouse unit or mouse strain.
4. Coat an ELISA plate with the capture antibody, seal the plate, and incubate overnight.
5. Wash the ELISA plate with wash buffer according to instructions and add the assay diluent. Seal the plate and incubate for 1 h at room temperature.
6. Prepare IgE standard dilutions and sample dilutions in a 96-well plate during the 1 h incubation. IgE standard dilutions must be included with each plate. Follow the manual for dilution instructions.
7. Wash the ELISA plate and add prepared standards and samples. Seal the plate and incubate for 2 h at room temperature.
8. Wash the ELISA plate. More washes are required at this step. Add the detection antibody and enzyme mixture. Seal the plate and incubate for 1 h at room temperature.
9. Wash the ELISA plate more extensively. Add the substrate solution, protect the plate from light exposure, and incubate for a maximum of 30 min (*see* **Note 7**). Samples containing IgE will show a blue color change.
10. Stop the reaction with the stop solution. Blue wells should turn yellow. Read absorbance at a wavelength of 450 nm within 15 min of adding the stop solution.
11. Calculate standard curve and sample IgE concentrations according to manual instructions.

3.7 Flow Cytometry

1. Record the volume of the BAL fluid collected in Subheading 3.3.
2. Centrifuge the BAL samples at 500 × *g* for 5 min. Remove the supernatant.
3. Wash the resulting cell pellet twice with PBS to remove EDTA.
4. Resuspend the cell pellet in 3 mL of ACK lysis buffer, incubate for 5 min at room temperature, then dilute the cell suspension with 3 mL of FACS buffer. Centrifuge at 500 × *g* for 5 min.

5. For papain-induced animals, resuspend the cell pellet in 800 μL of FACS buffer. Aliquot 200 μL (1/4 of total volume) into a well of a 96-well V-bottom plate. For naïve or PBS-treated animals, resuspend the cell pellet in 200 μL of FACS buffer and aliquot the total volume into a well of a 96-well V-bottom plate. Pool remaining cells from all samples and aliquot 100 μL for each FACS controls. FACS controls should include a negative (unstained) control, single color controls for each of the antibodies, and fluorescence minus one (FMO) controls for each antibody used.
6. Centrifuge the plate at 500 × *g* for 3 min. Flick the plate to remove supernatant (the pellet is securely attached to the bottom following centrifugation). Resuspend cell pellets in 50 μL of the blocking buffer. Incubate cells at 4 °C for 20 min.
7. During the incubation, prepare the sample antibody cocktail, including all antibodies to be tested in the blocking buffer. Additionally, make up single-color controls for machine setup, and fluorescence minus one (FMO) controls for data analysis. For the single color controls, only one antibody is added. For the FMO controls, all but one antibodies are added.
8. Centrifuge the cells at 500 × *g* for 3 min to remove the blocking buffer from **step 6**. Flick the plate to remove the supernatant.
9. Resuspend the cells in 100 μL/well of the antibody cocktail or control antibodies prepared in **step 7**. Incubate the samples for 20 min at 4 °C in the dark.
10. Centrifuge plate at 500 × *g* for 3 min. Flick the plate to remove supernatant.
11. Wash the samples twice with FACS buffer.
12. Resuspend the samples in 100 μL of FACS buffer containing diluted beads (following manufacturer's recommendation) and 0.5 μg/mL 7-AAD viability dye.
13. Analyze the samples using a flow cytometer the BD LSRII analyzer, or equivalent. The gating strategy is specified in Table 1. Between 500,000 and 1,000,000 events should be acquired.
14. Determine the total number of cells using the beads as a reference:

    (Number of cells counted) × (number of beads loaded ÷ number of beads counted).
15. Determine the total number of cells per mL of lavage: (Total number of cells) × (Dilution factor [% of total BAL plated for flow cytometry])/(mL of lavage).
16. Determine the BAL differential using fluorescent markers (Table 1).

Table 1
Flow cytometry gating strategy for lung immune cell subset characterization

Cell type	Surface markers
Alveolar macrophages	Live, CD45+, CD11c+, Siglec F+, auto-fluorescence+
Dendritic cells	Live, CD45+, CD11c+, Siglec F−
Eosinophils	Live, CD45+, CD11c− Siglec F+
Neutrophils	Live, CD45+, 7/4 +
T cells	Live, CD45+, CD3+, B220−
B cells	Live, CD45+, CD3e− B220+

4 Notes

1. Breeder mice should be at least 6 weeks old at the time of breeding pair setup. All animal experiments must be conducted according to your institution's animal care guidelines.
2. Mice should be age- and sex-matched. All animal experiments must be conducted according to your institution's animal care guidelines.
3. To avoid cross-contamination of microbes from control mice to antibiotic-treated mice, be sure to use fresh gloves when handling the mice, food, and/or bedding.
4. In order to recover a maximum volume of BAL fluid, move the syringe plunger slowly.
5. When dissecting the lung for histology, take care not to damage the lung with forceps to preserve the histology.
6. Master mix components per sample from High-Capacity cDNA Reverse Transcription Kit should contain 2 μL of random primers, 0.8 μL of DNTPs, 2 μL of reaction buffer, 1 μL of enzyme, and 4.2 μL RNase-free water.
7. The incubation time can be shorter depending on concentration of samples. It is recommended to sporadically check the intensity of color change. Stop the reaction if samples appear to reach outside of the standard range.

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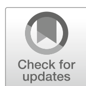

Chapter 20

Methods for Experimental Allergen Immunotherapy: Subcutaneous and Sublingual Desensitization in Mouse Models of Allergic Asthma

Laura Hesse, Arjen H. Petersen, and Martijn C. Nawijn

Abstract

Allergic asthma is characterized by airway hyperresponsiveness, remodeling, and reversible airway obstruction. This is associated with an eosinophilic inflammation of the airways, caused by inhaled allergens such as house dust mite or grass pollen. The inhaled allergens trigger a type-2 inflammatory response with the involvement of innate lymphoid cells (ILC2) and Th2 cells, resulting in high immunoglobulin E (IgE) antibody production by B cells and mucus production by airway epithelial cells. As a consequence of the IgE production, subsequent allergen reexposure results in a classic allergic response with distinct early and late phases, both resulting in bronchoconstriction and shortness of breath. Allergen-specific immunotherapy (AIT) is the only treatment that is capable of modifying the immunological process underlying allergic responses including allergic asthma. Both subcutaneous AIT (SCIT) as well as sublingual AIT (SLIT) have shown clinical efficacy in long-term suppression of the allergic response. Although AIT treatments are very successful for rhinitis, application in asthma is hampered by variable efficacy, long duration of treatment, and risk of severe side effects. A more profound understanding of the mechanisms by which AIT induces tolerance to allergens in sensitized individuals is needed to be able to improve its efficacy. Mouse models have been very valuable in preclinical research for characterizing the mechanisms of desensitization in AIT and evaluating novel approaches to improve its efficacy. Here, we present a rapid and reproducible mouse model for allergen-specific immunotherapy. In this model, mice are sensitized with two injections of allergen adsorbed to aluminum hydroxide, followed by subcutaneous injections (SCIT) or sublingual administrations (SLIT) of allergen extracts as an immunotherapy treatment. Finally, mice are challenged by intranasal allergen administrations. We will also describe the protocols as well as the most important readout parameters for the measurements of invasive lung function, serum immunoglobulin levels, isolation of bronchoalveolar lavage fluid (BALF), and preparation of cytospin slides. Moreover, we describe how to perform ex vivo restimulation of lung single-cell suspensions with allergens, flow cytometry for identification of relevant immune cell populations, and ELISAs and Luminex assays for assessment of the cytokine concentrations in BALF and lung tissue.

Key words Allergic asthma, Subcutaneous immunotherapy (SCIT), Sublingual immunotherapy (SLIT), BALB/cByJ and C57BL/6 mouse models, Grass pollen (GP), House dust mite (HDM), FlexiVent, Flow cytometry, Bronchoalveolar lavage, Eosinophilia

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1 Introduction

Asthma is the result of a complex interaction between genetic susceptibility and environmental factors. The most common asthma phenotype is allergic asthma, which is caused by inhaled allergens such as grass pollen (GP), ragweed, cat and dog allergens, and house dust mites (HDM) [1, 2]. Patients suffering from allergic asthma have reversible airway obstruction associated with eosinophilic inflammation as well as airway hyperresponsiveness (AHR) and remodeling. The worldwide prevalence of allergic asthma has dramatically increased over the last 25 years, currently affecting over 300 million people [3].

The inflammatory responses in allergic asthma are characterized by the presence of high levels of cytokines such as IL-4, IL-5, and IL-13, produced by both innate lymphoid cells (ILCs) and T helper 2 (Th2) cells [4]. These cytokines contribute to the pathological changes of the airways observed in allergic asthma, including influx of eosinophils, mucus hypersecretion, airway hyperresponsiveness, and airway wall remodeling. In addition, the Th2-dominated adaptive immune response to inhaled allergens results in the presence of allergen-specific IgE. Allergen-induced crosslinking of IgE that is bound to the cell surface of mast cells and basophils through the high affinity IgE receptor triggers degranulation of these cells resulting in acute allergic responses, leading to bronchoconstriction and vasodilation. The subsequent influx of inflammatory cells, including Th2 cells, into the tissue will result in activation of these cells and late-phase responses. Upon recurrent exposures to the allergen, chronic and poorly resolving inflammation around the small airways is induced, resulting in permanent structural changes to the airway wall.

Currently available asthma therapies are focused on controlling the chronic inflammatory process, mainly using inhaled corticosteroids in combination with long-acting beta agonists or leukotriene receptor antagonists [5]. Notwithstanding the clinical success in achieving asthma control, current asthma treatment regimens fail to cure the disease. This lack of a cure is evidenced by ongoing airway wall remodeling even in well-controlled asthma patients [6, 7]. Moreover, a subset of patients with severe asthma does not respond to steroid treatment [8, 9]. These shortcomings of current mainstream asthma therapy indicate that this therapeutic approach fails to address the underlying, causative immune mechanisms, achieving merely a transient suppression of symptoms of asthma in most patients.

The only treatment known to date that is capable of modifying the immunological process underlying allergic asthma is allergen-specific immunotherapy (AIT) [10]. AIT provides long-term protection against asthma attacks, which is even maintained upon

cessation of therapy and reduces medication use in allergic asthma. AIT involves the administration of gradually increasing amounts of allergen for a period of 3–5 years, aiming to achieve a state of immunological tolerance and a subsequent reduction of clinical manifestations of the disease [11]. Although the immune mechanisms behind successful immunotherapy remain unknown, the beneficial effects of AIT associated with a shift from Th2 activity toward a T-regulatory (Treg) profile that suppresses allergen-specific responses are known [12]. Successful AIT is characterized by increased levels of neutralizing antibodies, production of IL-10 and TGF-β, and increased $CD4^{+}FoxP3^{+}$ Treg numbers [13–16].

While subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) have been widely accepted as effective therapeutic alternatives for allergic rhinoconjunctivitis, application in asthma is hampered by the long duration of treatment, the variable efficacy in allergic asthma, and concerns regarding the safety of treatment. SCIT injections of allergen extracts have a risk of inducing anaphylactic reactions, with an incidence of severe anaphylactic responses at around one in a million injections [13]. Therefore, outpatient clinic visits are required for the administration and monitoring of the therapy [14]. In developing more convenient alternatives, SLIT has been developed as a less invasive alternative with proven clinical effects for patients suffering from allergic rhinitis [15]. Herein, uptake of the allergen involves the oral mucosa, where mucosal Langerhans cells in humans and oral macrophage-like cells in mice have been implicated to be important [16]. Although the exact mechanism of action remains to be elucidated, advantages of SLIT include the ease of use (droplets or fast-dissolving tablets), the application in a home setting, and relative costs. A better understanding of the mechanisms by which AIT suppresses allergen-induced asthma phenotypes is needed to improve the efficacy and safety of AIT, in particular in asthmatic patients.

Previously, animal models have proven to be valuable as a preclinical model to improve AIT by unraveling the immune mechanisms of allergen desensitization. The development of a predictive and reproducible AIT protocol was based on the classic OVA-driven mouse model of allergic asthma [17]. In this study, mice were sensitized to OVA in seven intraperitoneal injections. Two weeks later, SCIT treatment was performed using three injections of OVA (1 mg), followed by allergen challenges after another 2 weeks by OVA (2 mg/mL) inhalation once a day (5 min) for 8 consecutive days. In these initial studies, no adverse events of SCIT treatment were recorded in the BALB/c strain of mice, while the experimental SCIT treatment effectively suppressed airway inflammation and AHR and induced serum levels of antigen-specific immunoglobulin (spIg)G1 and spIgG2a. In addition, spIgE levels were increased, which matches the initial rise of

spIgE in human subjects treated with SCIT. Importantly, SCIT treatment in the OVA mouse model prevented the increase of spIgE levels after allergen challenges, which readily occurs in control-treated mice [18]. After several improvements, the protocol for sensitization was reduced to two intraperitoneal injections of OVA using a sensitizing adjuvant, Alum (mixture of aluminum hydroxide and magnesium hydroxide), and three intranasal challenges containing a high dose of aerosolized OVA. Importantly, OVA SCIT treatment was shown to be also effective in sensitized mice that were challenged by OVA inhalation prior to SCIT treatment, indicating the ability of SCIT treatment to suppress an established allergic airway inflammatory response [19]. Throughout the years, this mouse model for SCIT has been used to characterize the mechanisms of desensitization [17, 20–22], including the relevance of the neutralizing antibody responses [19], the role of IL-10 in the induction of tolerance, and the contribution of $CD4^{+}FoxP3^{+}$ T-regulatory cells (Treg). In addition, the role of dendritic cells (DCs) and their phenotypic modulation has been investigated extensively. DCs play a key role in the generation of adaptive T-cell subsets and can respond in either immunogenic or in a tolerogenic fashion [23]. Tolerogenic DCs have a semimature or immature phenotype, characterized by high expression of major histocompatibility complex class II (MHC-II) and B7-2, low expression of CD40, and lack of proinflammatory cytokine expression (IL-6 and TNFα). Studies have shown that incubation of immature DCs with $CD4^{+}$ T cells induces antigen-specific Tregs [24, 25], indicating that immature DCs can play a critical role in Treg cell generation and peripheral tolerance. Based on these findings, it has been studied whether AIT can be improved when allergen administration is accompanied by inhibition of DC maturation or prevention of DC-dependent costimulation. One approach is the use of $1,25(OH)_2$Vitamin D_3 ($VitD_3$), the active metabolite of vitamin D, which suppresses DC differentiation and maturation. Indeed, using the OVA-SCIT mouse model, administration of $1,25(OH)_2VitD_3$ has been shown to potentiate AIT [20]. In addition, CTLA4-Ig (Abatacept) was found to enhance the efficacy of SCIT in the OVA model, most likely by affecting the DC function [26].

Although using this mouse model of immunotherapy has provided insight into the immunological mechanisms of AIT, its value as an experimental preclinical model is limited by the use of a purified protein (OVA) that lacks the properties of natural allergens and induces tolerance when inhaled by naïve mice [27]. Therefore, the classical model allergen OVA was more recently replaced with a natural allergen extract that is also used in human SCIT, such as GP and HDM. The GP and HDM SCIT protocols for allergic asthma have been optimized first with regard to the allergen dosage needed to achieve suppression of phenotypes of allergic asthma [28–

30]. Second, other administrative routes were optimized, based on a SLIT mouse model of allergic rhinitis [28, 31]. SCIT and SLIT have been validated and standardized allowing a head-to-head comparison [28]. This model therefore allows in-depth characterization of the mechanisms of SCIT and SLIT treatment for allergic asthma as well as their optimization using novel approaches including peptide SCIT treatment or the use of alternative formulations and adjuvantia. Herein, we found that, while SLIT suppresses mainly AHR, GP SCIT suppresses Th2 profile and induces neutralizing antibodies. Furthermore, we showed that using purified allergens derived from crude extracts of HDM, like *Dermatophagoides pteronyssinus (Der p)* Der p1 and Der p2, allows suppression of AHR and inflammation, but also has superior activity toward suppression of type 2 cytokines [29].

The use of crude allergen extracts with IgE-crosslinking capacity has safety concerns, and although occurring in very low frequency, there is a risk of anaphylaxis [32, 33]. When studying mechanisms of allergen-induced tolerance induction in murine models, the same risk should be taken into consideration [34–36]. BALB/c mice have traditionally been considered an appropriate strain for developing allergy mouse models [35, 37]. The allergic phenotype of these mice has led this strain to be widely used for characterizing classic (IgE-FcεRI-mast cell-histamine) and alternative dependent pathways (IgG-FcγRIII-macrophage-platelet-activating factor) and for establishing the immunoregulatory mechanism underlying tolerance, which suppresses both Th 1 and Th 2 responses. Smit et al. demonstrate that in three different mouse strains (BALB/c, C3H/HeOuJ, and C57BL/6), components of the classic and alternative anaphylactic cascade are differently expressed, leading to different outcomes in parameters of allergic disease and food-induced systemic anaphylaxis. To overcome strain-dependent differences in optimizing allergen immunotherapy for allergic asthma, we performed our GP SCIT protocol in C57BL/6J mice and found that these mice are more prone toward anaphylaxis than BALB/cByJ mice (*unpublished data*).

In the protocol provided here, we explain how subcutaneous and sublingual routes of allergen-specific immunotherapy can be applied in both BALB/cByJ and C57BL/6 mouse models of allergic asthma using natural allergen extracts. We provide detailed methods to obtain the most important outcome parameters for translational studies, including invasive lung function measurements for AHR, specific IgE and IgG levels in serum, ear swelling tests for the early phase response, and inflammation of lung tissue and airways. Moreover, we describe how to restimulate lung cells with allergen extracts, perform flow cytometric measurements to identify populations of relevant immune cells, and perform ELISAs and Luminex assays to measure the cytokine concentrations in bronchoalveolar lavage fluid (BALF) and lung tissue. In C57BL/6 mice, we

included an adapted SCIT treatment protocol, including monitoring of immediate responses such as severity of shock and body temperature after the first injections, to avoid anaphylaxis in this mouse strain.

2 Materials

2.1 Subcutaneous and Sublingual Immunotherapy in a Mouse Model of Allergic Asthma

1. BALB/cByJ mice or C57BL/6 mice: 7- to 9-week-old females housed in individually ventilated cages (IVC).
2. 1-mL Syringes and 25G needles.
3. P20 pipette and tips.
4. 15-mL tubes.
5. Sterile phosphate buffered saline (PBS): 1 mM potassium phosphate monobasic (KH_2PO_4), 154 mM sodium chloride (NaCl), 3 mM sodium phosphate dibasic (Na_2HPO_4-$7H_2O$), pH 7.4. Dissolve 144 mg of KH_2PO_4, 9.0 g of NaCl, and 795 mg of Na_2HPO_4-$7H_2O$ in approximately 800 mL of ultrapure water. Adjust the pH and make the final volume up to 1 L. Use a 0.25-μm filter or autoclave to sterilize.
6. Rough extract of grass pollen (GP, *Phleum pratense*; *Phl p*): Dissolve 204 mg of dry matter of *Phleum pratense*, 225 (MP225PHLpra, 1006674 or 1031225) in 2.125 mL of sterile PBS to obtain a solution containing 60 kSQ/μL (~96 μg/μL). Aliquot this stock in 100-μL portions and store at −20 °C.
7. Crude extract of house dust mite (HDM, *Dermatophagoides pteronyssinus*; *Der p*): Dissolve 25.0 mg of DP extract FD 12C27 in 500 μL of sterile PBS to get a solution containing 50 μg/μL HDM and aliquot this stock in 25-μL portions and store at −20 °C.
8. Imject® Alum or equivalent aluminum hydroxide adjuvant: 20% $Al(OH)_3$.
9. Isoflurane: used for anesthesia at 4.5% with 1 mL/min O_2.
10. Heating mats with temperature control.

2.2 Blood Withdrawal via Orbital Puncture

1. Small animal anesthesia device compatible with isoflurane and a connected induction chamber.
2. Isoflurane: 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane. Used for inhalation anesthesia at 4.5% with 1 mL/min O_2.
3. Sterile phosphate buffered saline (PBS): *See* Subheading 2.1, **item 5**.
4. 30G Insulin syringes: 0.3 mL, needle 0.3 mm × 8 mm (e.g., BD Micro-Fine™ 0.3 mL).

5. 1-mL MiniCollect® serum tubes (Greiner Bio-One) or equivalent.
6. Glass microcapillary tubes: micro hematocrit tubes (Na-Heparinized 80 IU/mL).
7. 1.5-mL Microfuge tubes.
8. Centrifuge.

2.3 Ear Swelling Test

1. Positive allergen test solutions: Prepare either 1 μg of *Phl p* or 0.5 μg of *Der p* in 10 μL of sterile PBS per mouse as positive allergen test solutions for GP or HDM sensitivity, respectively.
2. Negative allergen test solution: sterile PBS, pH 7.4.
3. Mitutoyo Digimatic Micrometer or equivalent: 0.5 ± 0.15 N. Used for measuring ear thickness.
4. Regular hand tissues, hand gloves, and sterile tissues.

2.4 Lung Function Measurement

1. SCIREQ® FlexiVent (SCIREQ Scientific Respiratory Equipment Inc.).
2. A computer installed with FlexiWare software (SCIREQ Scientific Respiratory Equipment Inc.).
3. Silicon tubing: 0.28 mm and OD. 0.61 mm.
4. Syringes: 1 mL and 5 mL.
5. Anesthesia: Combine 100 mg/mL ketamine and 1 mg/kg Domitor as shown in Table 1.
6. Manometer with syringe and closing valves.
7. Weighing scale (precision >0.1 g).
8. Surgical microscope (40×).
9. Dissection instrument set.
10. Ligatures: 6/0 and 3/0.
11. 25G needles.
12. 20G intravenous cannula: for tracheal cannulation and calibration (pink, 20GA 1.16IN 1.1 × 30 mm BD Insyte-W™ or equivalent).
13. Bulldog clamp.
14. Sterile PBS.
15. Rocuronium bromide: Prepare a working solution of 0.125 mg/mL from a 10-μg/mL stock in sterile PBS.
16. Sterile methacholine solutions: For concentrations and dosage, *see* Tables 2 and 3.
17. Micro pulse-oximeter for small animals.
18. Heating mats for small animals.

Table 1
Dilution scheme for anesthesia

	Solution	Administration	Dose
Ketamine	100 mg/mL	75 mg/mL	0.75 μL/g mouse
Domitor	0.5 mg/mL	1 mg/mL	1 μL/g mouse
Dilution scheme (100 mg/mL)			
	1 mL	**5 mL**	**10 mL**
Domitor	200 μL	1000 μL	2000 μL
Ketamine	75 μL	375 μL	750 μL
Saline	725 μL	3625 μL	7250 μL

Table 2
The dosages of methacholine and the concentrations of methacholine solutions used for intravenous injections during the lung function test

Dose (μg/kg)	Dose mg/mL
0	0
50	0.01562
100	0.03125
200	0.0625
400	0.125
800	0.25

19. Supplemented PBS: 3% (w/v) bovine serum albumin (BSA, heat shock fraction, protease free, low endotoxin, suitable for cell culture, ≥98%) with protease inhibitor prepared in PBS, pH 7.4. Dissolve 0.3 g of BSA and 1 tablet of commercially available protease inhibitor cocktail tablet in 10 mL of sterile PBS.
20. Sterile RPMI 1640: supplemented with 10% fetal calf serum (FCS), L-glutamine (200 mM in 0.85% NaCl stock solution), 100 U/mL penicillin, 100 μg/mL streptomycin. To make 500 mL, add 50 mL of FCS (complement inactivated) and 0.5 mL of 2 mM L-glutamine in 0.85% NaCl working solution (freshly diluted from stock), and 5 mL of PenStrep (10,000 U/mL penicillin and 10,000 μg/mL streptomycin). (*see* **Note 1**).
21. 1.5-mL Microfuge tubes.
22. 15-mL Tubes.
23. 24-Well cell culture plates.

Table 3
Weights of mice and corresponding volumes of the methacholine solutions to be injected

Weight (g)	Injection volume (μL)
20	64
21	67
22	70
23	74
24	77
25	80
26	83
27	86
28	90
29	93
30	96
31	99
32	102
33	106
34	109
35	112
36	115
37	118
38	122
39	129
40	128

24. 1-mL MiniCollect® serum gel tubes (Greiner Bio-One) or equivalent.
25. 2-mL cryogenic vials.

2.5 Analysis of the Infiltration of Inflammatory Cells in BALF

1. Microscope slides: 76 × 26 mm.
2. Shandon filter cards.
3. Cytospin cuvette.
4. Cytospin metal slide holder and its driver.
5. Cytocentrifuge (e.g., Shandon Cytospin 3).
6. Temperature-controlled centrifuge.
7. Aspirator.

8. 70% Ethanol: To make 100 mL, mix 70 mL of absolute ethanol with 30 mL of ultrapure water.
9. Micropipettes with associated pipette tips.
10. Automated cell counter or hemocytometer (e.g., Coulter Counter Z1, single-threshold model, Beckman Coulter).
11. Red blood cell (RBC) lysis buffer: commercially available (e.g., Lyzerglobin, Avantor B.V. Deventer).
12. Sterile PBS.
13. 5% bovine serum albumin (BSA): Dissolve in PBS, pH 7. Use heat shock fraction, protease free, low endotoxin, suitable for cell culture, $\geq$98%.
14. Sterile lysis buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA.
15. Diff-Quick staining set: commercially available.
16. Light microscope with 20×, 40×, and 100× objective lenses.
17. Immersion oil.
18. Differential cell counter.

2.6 Preparation of Single-Cell Suspensions of Lung Tissue, Spleen, and Draining Lymph Nodes (DLNs)

1. Ice in a bucket.
2. 24-Well culture plates.
3. Petri dishes.
4. Sterile scalpels and scalpel blades.
5. Micropipettes with associated pipette tips.
6. 50-mL Conical tubes.
7. 70-μm Nylon cell strainers.
8. 5-mL Syringe.
9. Biosafety cabinet: down-flow cabinet with a closed suction system.
10. Temperature-controlled centrifuge.
11. Sterile RPMI 1640: supplemented with 10% fetal calf serum (FCS), L-glutamine (200 mM in 0.85% NaCl stock solution), 100 U/mL penicillin, 100 μg/mL streptomycin. To make 500 mL, add 50 mL of FCS (complement inactivated) and 0.5 mL of 2 mM L-glutamine in 0.85% NaCl working solution (freshly diluted from stock), and 5 mL of PenStrep (10,000 U/mL penicillin and 10,000 μg/mL streptomycin). *see* **Note 1**.
12. Collagenase A: 4 mg/mL.
13. DNase I: 0.1 mg/mL.
14. Cell counter.
15. 10-mL Coulter counter cups.

16. Flow cytometry diluent reagent: ISOTON® II Diluent (Beckman Coulter) or equivalent.
17. Red blood cell (RBC) lysis buffer: commercially available (e.g., Lyzerglobin, Avantor B.V. Deventer).
18. Cryogenic vials.
19. Benchtop cooler or ice bucket.
20. −80 °C Freezer.
21. Liquid nitrogen storage.
22. Hanks' Balanced Salt solution (HBSS): 140 mM NaCl, 5.0 mM KCl, 1.0 mM $CaCl_2$, 0.4 mM $MgSO_4{\cdot}7H_2O$, 0.5 mM $MgCl_2{\cdot}6H_2O$, 0.3 mM $Na_2HPO_4{\cdot}2H_2O$, 0.4 mM KH_2PO_4, 6.0 mM D-glucose, and 4.0 mM $NaHCO_3$, pH 7.4. To make 1000 mL, dissolve 8.0 g of NaCl, 0.4 g of KCl, 140 mg of $CaCl_2$, 100 mg of $MgSO_4{\cdot}7H_2O$, 100 mg of $MgCl_2{\cdot}6H_2O$, 60 mg of $Na_2HPO_4{\cdot}2H_2O$, 60 mg of KH_2PO_4, 1 g of D-glucose, and 350 mg of $NaHCO_3$ in about 800 mL of ultrapure water. Adjust pH to 7.4 and bring the volume up to 1000 mL. Sterilize with a 0.25-μm filter. Store at 4 °C.
23. Storage medium for cells in liquid nitrogen: 40% FCS and 10% DMSO in HBSS.

2.7 Restimulation of Lung Cells and Draining Lymph Node (DLN) Cells

1. Biosafety cabinet: down-flow cabinet with a closed suction system.
2. Single-cell suspension resulted from Subheading 3.10.
3. Temperature-controlled centrifuge.
4. CO_2 incubator.
5. Petri dishes.
6. Sterile scalpels and scalpel blades.
7. Micropipettes with associated pipette tips.
8. Supplemented RPMI 1640: *See* Subheading 2.6, **item 11**.
9. Sterile U-bottom 96-well cell culture plate.
10. GP rough extract or HDM crude extract: *See* Subheading 2.1, **items 6** (GP) and 7 (HDM).

2.8 Quantification of Lung Single-Cell Suspensions Using Flow Cytometry

1. FACS tubes (polystyrene) for samples and singles during staining.
2. 30-μm Filter top FACS tubes.
3. Temperature-controlled centrifuge.
4. Three-laser flow cytometer (e.g., FacsVerse).
5. FACS buffer: 1% BSA in PBS.

6. Block buffer for extracellular blocking: 2% normal rat serum (NRS) and 5% FcBlock (purified and unlabeled CD16/32 antibody) in FACS buffer.
7. Block buffer for intracellular blocking: 2% NRS and 5% FcBlock in PERM buffer (*see* **item 10** in this section).
8. Extracellular staining antibodies: diluted in FACS buffer according to suppliers' recommendations (*see* Tables 2 and 3). In some cases, the dilution of every antibody can be adjusted depending on the intensity of the fluorescence signal.
9. Fixable LIVE/DEAD™ (L/D) V450 cell stain: Predilute to 1:1000 in PBS prior to use.
10. Foxp3/transcription factor staining buffer set: a commercial kit containing the following components:
    (a) Fixation/permeabilization concentrate.
    (b) Fixation/permeabilization diluent (FIX).
    (c) Permeabilization buffer (PERM buffer).

2.9 Homogenization of Lung Tissue for Total Protein and Cytokine Analysis

1. Homogenizer (e.g. IKA Werke T10 basic Ultra-Turrax, Germany).
2. 1.5-mL Microfuge tubes.
3. 96-Well flat-bottom ELISA plates.
4. Cryogenic vials.
5. ELISA plate reader.
6. BCA protein assay kit: commercially available.
7. Demineralized water.
8. Tween 20.
9. 70% Ethanol.
10. Luminex buffer: 50 mM Tris-HCl, 150 mM NaCl, 0.002% Tween 20, pH 7.5. To make 100 mL, add 0.6 g of Tris-HCl, 0.9 g of NaCl, and 2.0 μL of Tween 20. Optionally, add 1 tablet of complete protease inhibitor cocktail (e.g., cOmplete Mini) per 100 mL of buffer and 1 tablet of PhosSTOP phosphatase inhibitor cocktail per100 mL of buffer. Luminex buffer can be aliquoted and stored at −20 °C.

2.10 Biotinylation of Allergens for spIgE ELISA

We use a "home-made" biotinylated allergen for the detection of allergen-specific IgE using ELISA. Biotinylation of GP and HDM is performed using a commercially available biotinylation reagent.

1. Biotinylation reagent: EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) or equivalent.
2. PBS: pH 7.4. Used as reaction buffer.
3. Desalting columns or dialysis units: Slide-A-Lyzer Dialysis Cassettes 0.1–0.5 mL or equivalent with a molecular-weight cutoff of 3500 kDa. Use for buffer exchange.

2.11 Analysis of Immunoglobulin Levels in Serum with ELISA

For total IgE, IgA, IgG1, IgG2a, and spIgE, unconjugated rat monoclonal antibodies against respective mouse immunoglobulins are used as the capture antibodies. For spIgG1 and spIgG2a ELISA, the allergen extracts are used to coat ELISA plates. For spIgE ELISA, biotinylated antigens are used for detection (*see* Subheadings 2.10 and 3.13, **step 1**). *See* Table 6 for an overview of all antibodies, reagents, and sample types used for these ELISAs.

1. NUNC Maxisorp 96-well flat-bottom ELISA plates or equivalent.
2. Multichannel pipettes and associated tips.
3. ELISA plate washer (optional).
4. Plate shaker.
5. Spectrophotometric microplate reader.
6. ELISA buffer: 50 mM Tris-HCl, 136.9 mM NaCl, 0.05% Tween 20, 2 mM EDTA, 1% BSA, pH 7.2. To make 1000 mL, dissolve 6.06 g of Tris, 8 g of NaCl, 0.744 g of EDTA, and 10 g of BSA in ultrapure water. Adjust pH.
7. Wash buffer: 0.05% Tween 20 in PBS.
8. Capture antibody: rat antimouse IgE (R35-72), rat anti-mouse IgG1 (A85-3), rat antimouse IgG2a (R11-89), or rat anti-mouse IgA antibody (RMA-1).
9. Coating antigen: GP rough extract or HDM crude extract. *See* **items 6** and 7 in Subheading 2.1.
10. Immunoglobulin standards: mouse IgE κ isotype control (C38-2), mouse IgG1 κ isotype control (MOPC-31C), mouse IgG2a κ isotype control (G155-178), mouse IgA, or pooled positive reference serum.
11. Samples: mouse sera collected at different time points (*see* Fig. 1).
12. Detection antibody/antigen: biotinylated rat antimouse IgE, (R35-118), biotinylated rat antimouse IgG1 (A85-1), biotinylated rat antimouse IgG2a (R19-15), biotinylated rat anti-mouse IgA (C10-1), biotinylated GP rough extract, or HDM crude extract (*see* Subheadings 2.10 and 3.13, **step 1**).
13. Avidin horseradish peroxidase (Avidin-HRP).
14. Peroxidase substrate: *o*-phenylenediamine dihydrochloride (OPD).
15. Stop solution: 4 M H_2SO_4.

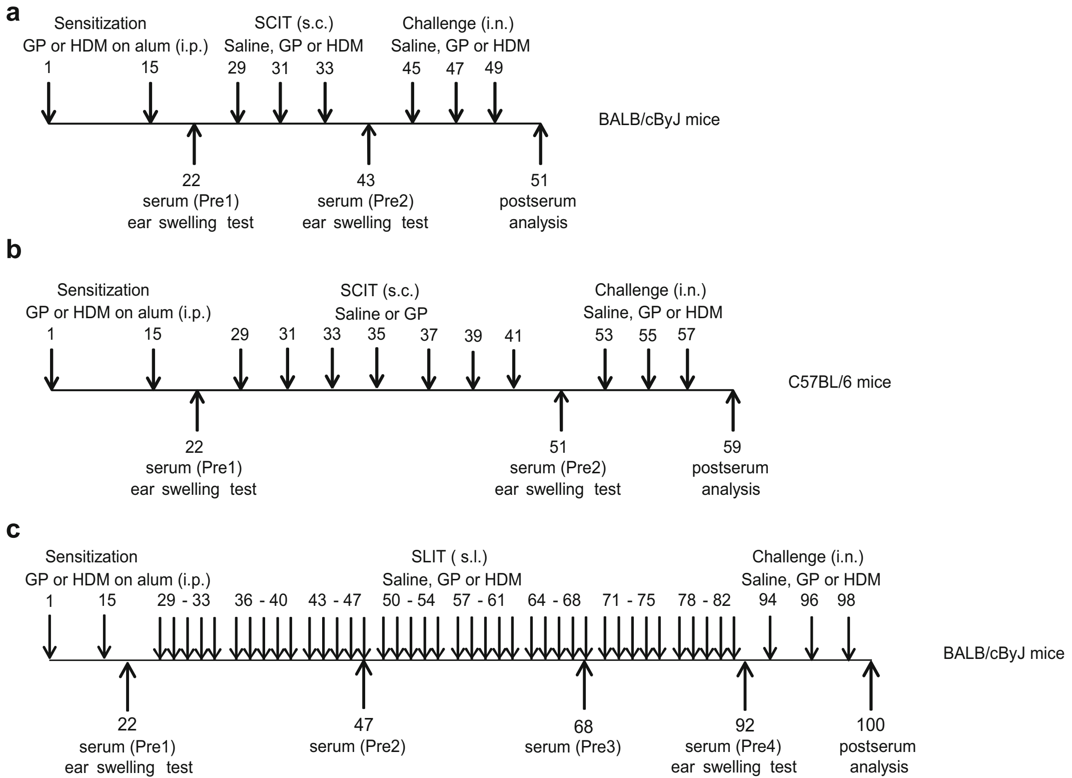

Fig. 1 Overview of AIT-treatment protocols in BALB/cByJ and C57BL/6 mice. (**a**, **b**) Outline of the SCIT protocols in both mouse strains. (**c**) Outline of the SLIT protocol. Serum is taken before SCIT (Pre1), before challenge (Pre2 in case of SCIT and Pre4 in case of SLIT), and after challenges (Post). In case of SLIT, we include two extra serum time points (Pre2 and Pre3) during SLIT. Ear swelling tests (ESTs) are performed before AIT (to confirm sensitization) as well as after AIT (to confirm the effect of AIT).

2.12 Analysis of Cytokine Levels in BALF, Supernatant of Restimulated Single-Cell Suspensions and Lung Tissue Homogenates

1. Commercially available ELISA kits for cytokines of interest, such as IL-4, IL-5, IL-10, IL-13, and IFNγ.
2. Mouse cytokine multiplex assay: Mouse Magnetic Luminex Screening Assay (R&D Systems) or equivalent multiplex assay. Choose your cytokines of interest and combine them in a single assay system.
3. Multichannel pipettes and associated tips.
4. ELISA plate washer (optional).
5. Plate shaker.
6. Spectrophotometric microplate reader.

3 Methods

3.1 Sensitization

For allergen sensitization, both BALB/cByJ and C57BL/6 mice are given either GP or HDM allergen by intraperitoneal injections on Days 1 and 14, using alum-adsorbed allergen extracts.

1. Before preparing the sensitization solution, thaw the aliquoted stocks of grass pollen (GP, *Phleum pratense*; *Phl p*) or house dust mite (HDM, *Dermatophagoides pteronyssinus*; *Der p*) and mix Imject Alum (or an equivalent aluminum hydroxide adjuvant) well by shaking or vortexing until thoroughly emulsified.
2. Under a sterile condition, add 20 μL of the alum adjuvant to 80 μL of each allergen stock. The final concentration of allergens will be 8 μg of *Phl p5a* for GP sensitization and 5 μg of *Der p* for HDM sensitization, in a total of 100 μL of allergen-alum solution per mouse. Prepare fresh.
3. Randomly assign mice to GP or HDM sensitization groups.
4. Using 1-mL syringes with 25G needles, intraperitoneally inject 100 μL of the appropriate sensitization solution to each mouse assigned for the allergen (*see* **Note 2**).
5. Repeat **steps 1–4** again on Day 14.

3.2 SCIT Treatments

3.2.1 SCIT Treatment of BALB/cByJ Mice

For SCIT treatment of BALB/cByJ mice, subcutaneous allergen injections are given on Days 29, 31, and 33. *See* Fig. 1a for the timeline of the treatment schedule.

1. Randomly assign the allergen-sensitized mice to SCIT or control groups to start the treatments on Day 29.
2. On Day 29, thaw the aliquoted stocks of grass pollen (GP) or house dust mite (HDM) to prepare allergen solutions for SCIT injections. Dilute each allergen stock in sterile PBS to achieve the final concentrations of SCIT solutions below (100 μL/mouse).
   (a) For GP: Dilute 5.2 μL of the *Phl p* stock (96 μg/μL) in 94.8 μL of sterile PBS to the final concentration of 500 μg *Phl p* in 100 μL of PBS.
   (b) For HDM: Dilute 5 μL of the *Der p* stock (50 μg/μL) in 95 μL of sterile PBS to the final concentration of 250 μg of *Der p* in 100 μL of PBS.
3. Subcutaneously inject 100 μL of the appropriate SCIT solution to each mouse using 1-mL syringes with 25G needles (*see* **Note 3**). Use sterile PBS to inject SCIT control mice.
4. Repeat **steps 1–3** again on Days 31 and 33.

3.2.2 SCIT Treatment of C57BL/6 Mice

For SCIT treatment of C57BL/6 mice, subcutaneous allergen injections are given on Days 29, 31, 33, 35, 37, 39, and 41. *See* Fig. 1b for the timeline of the treatment schedule. Given the sensitivity of C57BL/6 for anaphylactic responses, we use an incremental updosing scheme for SCIT treatments (*see* **step 2** below). In addition, the mice should be monitored for potential adverse responses during the first injections. For this reason, we routinely

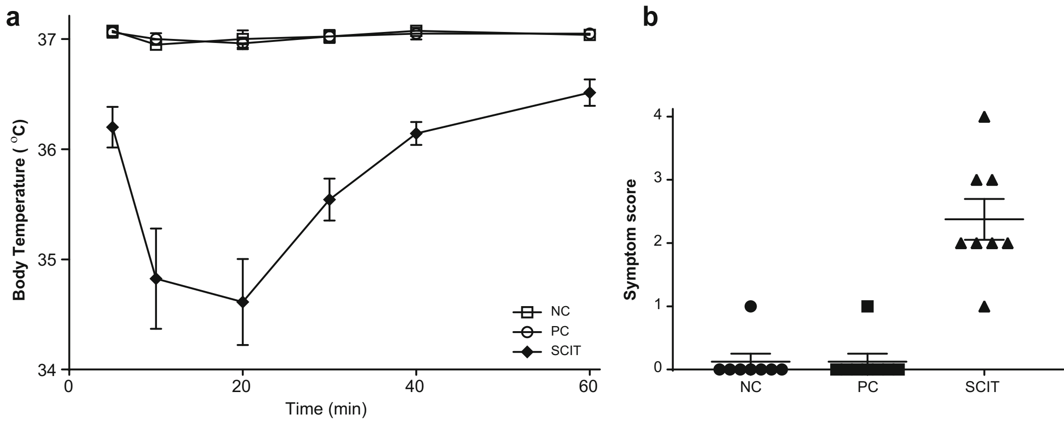

Fig. 2 Monitoring symptoms of anaphylaxis in sensitized mice following the first subcutaneous GP injections. (**a**) Changes in rectal temperature following SCIT at the indicated time points. (**b**) Peak anaphylactic symptom score of each individual mouse within 40 min after the first SCIT. Absolute values are expressed as mean ± SEM ($n = 8$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$ compared to the positive control. *NC* negative control, PBS challenged, *PC* positive control, *GP* challenged; SCIT-treated mice (50 μg of GP), *GP* challenged

measure body temperature and score shock symptoms after each injection (*see* **Note 4**, Fig. 2).

1. Randomly assign the allergen-sensitized mice to SCIT or control groups to start the treatments on Day 29.
2. Thaw the aliquoted stocks of GP or HDM to prepare allergen solutions for SCIT injections (*see* **Note 4**). Dilute each allergen stock in sterile PBS to achieve the final concentrations of SCIT solutions below (100 μL/mouse).
   (a) For GP: Dilute 0.5 μL of the GP stock (96 μg/μL) in 99.5 μL of sterile PBS to the final concentration of 50 μg of *Phl p* in 100 μL of PBS.
   (b) For HDM: Dilute 5.0 μL of the HDM stock (50 μg/μL) in 95.0 μL of sterile PBS to the final concentration of 250 μg of *Der p* in 100 μL of PBS.
3. Subcutaneously inject 100 μL of the appropriate SCIT solution to each mouse using 1-mL syringes with 25G needles (*see* **Note 3**). Use sterile PBS to inject SCIT control mice.
4. Measure body temperature at 20, 40, and 60 min after the SCIT injection using a rectal thermometer (*see* Fig. 2). Observe mice and record shock symptom scores according to the scoring criteria listed in **Note 4**.

5. Repeat **steps 1–4** again on Days 31, 33, 35, 37, 39, and 41, *except* change the concentrations of GP SCIT solution by appropriately diluting the GP stock as follows (100 μL/mouse):
   (a) Day 31: Dilute 1.0 μL of the GP stock in 99.0 μL of sterile PBS to the final concentration of 100 μg of *Phl p* in 100 μL of PBS.
   (b) Day 33: Dilute 2.1 μL of the GP stock in 97.9 μL of sterile PBS to the final concentration of 200 μg of *Phl p* in 100 μL of PBS.
   (c) Day 35: Dilute 4.2 μL of the GP stock in 95.8 μL of sterile PBS to the final concentration of 400 μg of *Phl p* in 100 μL of PBS.
   (d) Days 37, 39, and 41: Dilute 5.2 μL of the GP stock in 94.8 μL of sterile PBS to the final concentration of 500 μg of *Phl p* in 100 μL of PBS.

   For HDM SCIT in C57BL/6 mice, simply recalculate a similar updosing scheme as described above.

3.3 SLIT Treatments

SLIT treatment is applied 5 days a week for a total of 8 consecutive weeks from day 29 through day 82 by sublingual administration (*see* **Note 5**) of the allergen in PBS. *See* Fig. 1c for the timeline of the treatment schedule.

1. Randomly assign the allergen-sensitized mice to SLIT or control groups to start the treatments on Day 29.
2. Thaw the aliquoted stocks GP) or HDM to prepare allergen solutions for SLIT applications. Dilute each allergen stock in sterile PBS to achieve the final concentrations of SLIT solutions below 5 μL/mouse.
   (a) For GP: Dilute 5.2 μL of the GP stock in 94.8 μL of sterile PBS to the final concentration of 500 μg of *Phl p* in 100 μL of PBS.
   (b) For HDM: Dilute 5 μL of the HDM stock in 95 μL of sterile PBS to the final concentration of 250 μg of *Der p* in 100 μL of PBS.
3. Sublingually apply 5 μL of the appropriate SLIT solution to each mouse using a P20 pipette (*see* **Note 5**). Use sterile PBS for SLIT control mice.
4. Repeat **steps 1–3** again 5 days a week for 8 consecutive weeks until day 82 (*see* Fig. 1c).

3.4 Allergen Challenges

Allergen challenges are performed by intranasal administration (*see* **Note 6**; Fig. 1) 2 weeks after the cessation of SCIT or SLIT treatments. For SCIT-treated BALB/cByJ mice, the challenge

days are days 45, 47, and 49, while SCIT-treated C57BL/6 mice are challenged on days 53, 55, and 57. For SLIT treatment, BALB/cByJ mice are challenged on days 94, 96, and 98.

1. Thaw the aliquoted stocks of GP or HDM to prepare allergen solutions for intranasal challenge. Dilute each allergen stock in sterile PBS to achieve the final concentrations of allergen solutions below 25 μL/mouse.
   (a) For GP: Dilute 1 μL of the GP stock in 59 μL of sterile PBS to the final concentration of 40 μg of *Phl p* in 25 μL of PBS.
   (b) For HDM: Dilute 1 μL of the HDM stock in 50 μL of sterile PBS to the final concentration of 25 μg of *Der p* in 25 μL of PBS.
2. Anesthetize a mouse using 4.5% isoflurane in combination with 1 mL/min oxygen until its breathing starts to slow down. Remove the mouse from anesthesia.
3. Restrain a mouse by gently gripping the nape with one hand and anchoring the tail between the small finger and the palm. Hold the mouse in a supine position with the head elevated.
4. Right before the mouse wakes up (approximately within 1 min), position the end of a micropipette at or in the external nares. Administer 25 μL of the appropriate allergen solution to each mouse, by placing it as a droplet on the nose. Watch as the mouse strongly inhales the droplet, usually splitting into both nasal cavities (*see* **Note 6**).
5. Repeat **steps 1–4** until all the mice are challenged.

3.5 Blood Withdrawal via Retro-Orbital Puncture

To monitor the response to SLIT or SCIT treatments, blood is collected by retro-orbital puncture after allergen challenge. The timing of blood collections for each experimental paradigm is indicated as “Pre1 and Pre 2” for SCIT and “Pre1–4” in the “SLIT protocol” in Fig. 1a–c.

1. Anesthetize a mouse using 4.5% isoflurane in combination with 1 mL/min oxygen. Confirm deep anesthesia with the absence of a pedal reflex.
2. Place the anesthetized mouse on a flat surface. Gently press the body to force its blood from the thorax to the head.
3. Using the forefinger of the same hand holding the mouse down, pull the dorsal eyelid back to produce slight exophthalmos (bulging of the eye).
4. Penetrate the orbital conjunctiva at the medial or lateral canthus of the eye with a glass microcapillary tube. As soon as blood accumulates in the capillary, lift up the mouse and hold it above the MiniCollect tube to collect 10 drops of blood (*see* **Note 7**).

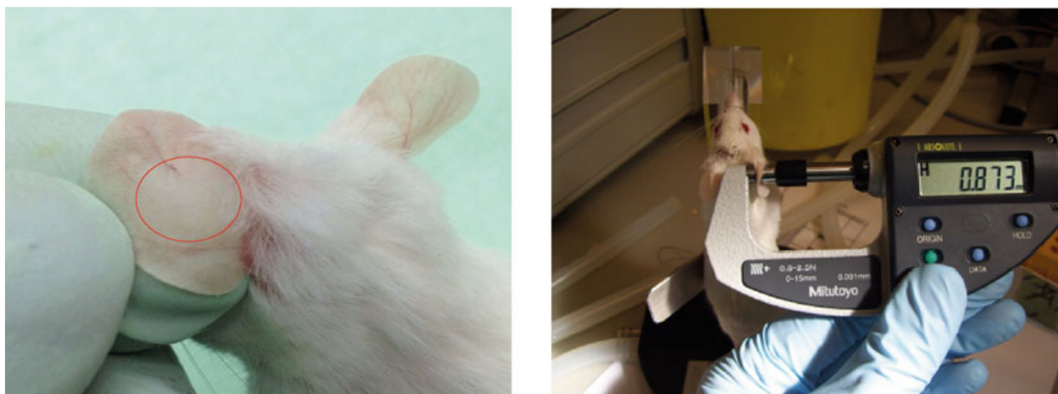

Fig. 3 The ear swelling test. *Left*: Anesthetized mice are intradermally injected with 10 μL of PBS in the left ear as a control using a small insulin syringe. A small swelling will be visible just below the skin. *Right*: after 2 h, ear thickness of both ears is measured using a micrometer. It is important to keep the micrometer in a horizontal position

5. Process further to collect serum. Ensure that the MiniCollect® Cross-Cut Cap is properly placed back on the tubes and the tubes are centrifuged at 3000 × *g* for 10 min at room temperature. Gel separation tubes should be centrifuged no later than 2 h after collection.

3.6 Ear Swelling Test

To monitor modulation of the early phase response to allergen provocation by SCIT or SLIT, the ear swelling test (EST) is performed before and after the treatments. To minimize discomfort to the experimental animals due to repeated anesthesia, the EST is carried out at the same time as the blood draws (Fig. 1).

1. Prepare test solutions to inject in the ear to determine local responses. For a positive control, use either 1 μg of *Phl p* or 0.5 μg of *Der p* in 10 μL of sterile PBS. For a negative control, use sterile PBS only.
2. Anesthetize a mouse using 4.5% isoflurane/min O_2 as described in Subheading 3.5, **step 1**.
3. Inject the mouse with the selected allergen test solution intradermally in the right ear (Fig. 3).
4. Inject sterile PBS intradermally in the mouse's left ear as a negative control reference.
5. After 1–2 h, anesthetize the mice again using 4.5% isoflurane/min O_2 and measure the ear thicknesses in micrometers (*see* **Note 8**).
6. Calculate the allergen-induced net increase in ear thickness (Δ, in μm) by subtracting the left ear thickness from that of the right ear (Fig. 4a).

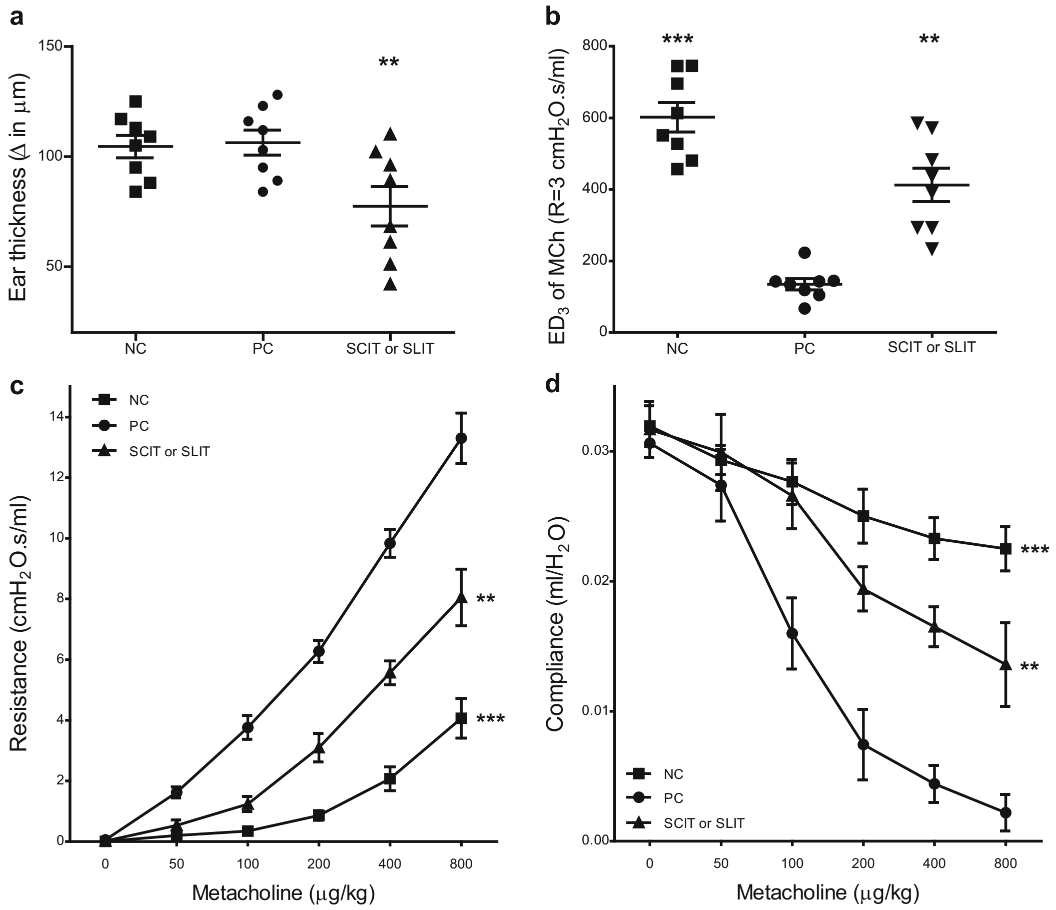

Fig. 4 Clinical manifestations after AIT. (**a**) IgE-dependent allergic response performed after AIT. Ear thickness (μm) in the right ear was measured 2 h after GP injection (1 kSQ) and differences in the thickness were compared to the left ear, which received PBS as a control. Placebo-treated mice (NC and PC) showed a similar swelling since both had not been challenged yet. (**b**) Effective Dose (ED) of methacholine, when the airway resistance reaches 3 $cmH_2O.s/mL$. Airway hyperactivity (AHR) was measured by FlexiVent and plotted as (**c**) airway resistance (R in cmH2O.s/mL) and as (**d**) airway compliance (**c** in mL/cmH2O). Absolute values are expressed as mean ± SEM ($n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$ compared to positive control. *NC* negative control, PBS challenged, *PC* positive control, *GP* challenged, SCIT- or SLIT-treated mice (300 kSQ), *GP* challenged

3.7 Lung Function Measurement

Lung functions of experimental mice are tested 2 days after the final allergen challenge. Here, we describe the method to assess lung functions using the FlexiVent version 5.3. In this setup, we routinely use intravenous methacholine administration in combination with the use of flexible cannulas for tracheal intubation, although the use of inhaled methacholine and rigid tracheal intubation has also been described by others [38]. In our experience, invasive measurement of airway resistance affects immunohistochemical analyses of lung tissue, including airway wall remodeling and

inflammation. Therefore, we recommend that histological analyses be performed in a separate group of mice to make sure that lung tissue architecture is not disrupted by the prior FlexiVent analysis.

3.7.1 Preparation

1. Prior to the measurement, calibrate the computer-controlled small-animal ventilator (e.g., FlexiVent) according to the manufacturer's instructions (*see* **Note 9**). Perform weight-adjusted calibration of both airway and cylinder pressure for each animal using a 1-mL syringe, a manometer, and a closed and an open cannula (*see* **Note 9**).
2. Two days after the final allergen challenge, weigh and anesthetize mice with 75 mg/kg ketamine (100 mg/mL) and 1 mg/kg domitor (0.5 mg/mL) in sterile PBS by intraperitoneal injection (10 μL of mix per gram bodyweight). *See* the dilution scheme shown in Table 1.
3. Upon confirmation of deep anesthesia with the absence of a pedal reflex, place the mouse in a supine position on an operating table for tracheal and jugular cannulations (*see* **Note 10**).
4. For tracheal cannulation, first, make an incision in the middle of the neck and carefully remove the underlying tissue with two sharp pairs of forceps to reveal the muscle bundles that cover the trachea. Do not touch the glands around the trachea, as this will trigger enhanced mucus production.
5. Move the muscle bundles aside to reveal the trachea, which is then freed from the underlying tissue.
6. Place two ligatures under the trachea, one more proximal to the oral cavity but still leaving room for making the incision to insert the cannula, and the other more distally, close to the bifurcation leading to the primary bronchi.
7. Open the trachea by making a small cut in between the tracheal cartilage rings, leaving the two ligatures below the incision site. Place the cannula in the trachea and fix the tracheal canula in an airtight fashion by carefully closing both ligatures to prevent the cannula from sliding up or down within the trachea.
8. Attach the tracheal cannula to the FlexiVent and ventilate using the standard breathing program with the script running on basic breathing (*see* **Note 11**).

3.7.2 Methacholine Challenge

1. Prepare methacholine solutions with different concentrations for intravenous injections according to Tables 2 and 3.
2. Methacholine will be introduced via the jugular vein. To position the jugular vein, draw an imaginary line from the right ear and its left armpit, and between the chin and its right armpit. The crossing point of these two lines identifies the location of

incision (Suppl. Fig 1). Make a 1.5-cm vertical incision downward.

3. Carefully remove the underlying tissue with two sharp pairs of forceps until the jugular vein is revealed, clearing any fat or surrounding connective tissue (*see* **Note 12**).
4. Place a ligature (6/0) around the upper part of the jugular vein and close it lightly (Suppl. Fig 1). Secure the ligature to the operating table with a small piece of tape, in order to put some tension on it.
5. After the tension is sufficient to stretch the jugular vein slightly, place a second ligature (6/0) approximately 0.5 cm below the first and close it lightly but not completely, still allowing sufficient space for the cannula to be placed into the jugular vein.
6. Place a bulldog clamp on the lower ligature to increase the tension on the jugular vein and make a small cut in the vein between the two ligatures. Carefully place the cannula into the jugular vein through this opening, and tightly close the bottom ligature (*see* **Note 13**).
7. Once the proper placement of the cannula is confirmed, fix it in position by tightly closing both upper and lower ligatures. Now, the jugular vein cannula can be used for intravenous methacholine delivery when prompted by the FlexiVent's protocol (*see* **Note 11**).
8. Prior to the FlexiVent measurements, administer an intraperitoneal injection of rocuronium bromide (1 μL/g mouse body weight).
9. Set the Positive end-expiratory pressure (PEEP) at 20 mmH_2O.
10. Administer the appropriate volume of saline (0 μg/kg methacholine) intravenously through the jugular cannula as a blank for the subsequent methacholine injections. *See* Table 3 for injection volumes.
11. Measure airway responsiveness by obtaining airway resistance (R in $cmH_2O.s/mL$), the Newtonian resistance (the resistance of the central or conducting airways, Rn in $cmH_2O.s/mL$), and lung compliance (C in mL/H_2O).
12. Inject the next dose of methacholine (50 μg/kg body weight) listed in Table 3. After the injection of methacholine solution, immediately flush the tubing of the jugular vein cannula with 30 μL of saline to make sure that all the methacholine enters the body in a single bolus (*see* **Note 14**). Measure airway responsiveness as described in **step 11**.

Table 4
Antibodies used for FACS analysis of innate lymphocytes

Specificity	Clone	Isotype	Staining
Brilliant Violet 605™ anti-mouse Ly-6A/E (Sca-1) antibody	D7	Rat IgG2a, κ	Extracellular
PerCP/Cy5.5 anti-mouse/human KLRG1 (MAFA) antibody	2F1/ KLRG1	Syrian hamster IgG	Extracellular
Anti-Mouse CD3e PE	145-2C11	Armenian Hamster IgG	Extracellular
Anti-Mouse CD5 PE	53-7.3	Rat IgG2a, κ	Extracellular
Anti-Mouse CD19 PE	eBio1D3 (1D3)	Rat IgG2a, κ	Extracellular
Anti-Mouse NK1.1 PE	PK136	Mouse IgG2a, κ	Extracellular
Anti-Mouse Fc epsilon Receptor I alpha (FceR1) PE	MAR-1	Armenian Hamster IgG	Extracellular
Anti-Mouse CD11b PE	M1/70	Rat IgG2b, κ	Extracellular
Anti-Mouse CD11c PE	N418	Armenian Hamster IgG	Extracellular
Anti-Mouse Ly-6G (Gr-1) PE	RB6-8C5	Rat IgG2b, κ	Extracellular
Anti-Mouse TER-119 PE	TER-199	Rat IgG2b, κ	Extracellular
T1/ST2 (IL-33R) Monoclonal Antibody, FITC	DJ8	IgG1	Extra- and Intracellular
Anti-Mouse CD45 APC	30-F11	Rat IgG2b, κ	Extracellular
Anti-Mouse CD127 APC-eFluor® 780	A7R34	Rat IgG2a, κ	Extracellular
Anti-Human/Mouse Gata-3 PE-Cyanine7	TWAJ	Rat IgG2b, κ	Intracellular

13. Continue with 100, 200, 400, and 800 μg/kg methacholine doses (*see* Tables 3 and 4), with an additional administration of rocuronium bromide after the methacholine dosage of 100 μg/kg. Repeat the measurements as described in **step 11** after each methacholine administration.
14. For the analysis of the lung function test, export all FlexiVent data as a comma separated value (CSV) format and store the data files in an appropriate location such as a backed-up network drive for analysis (*see* **Note 15**).

3.8 Collection of Blood, Bronchoalveolar Lavage, and Lung Tissue

1. Immediately after the completion of FlexiVent measurements, sacrifice the mouse by collecting a large volume of blood through the vena cava (post-serum). Under continued anesthesia, open the abdomen and reposition the bowels to reveal the vena cava. After removing fat, puncture the vena cava using

a 25G needle on a 1-mL syringe. Up to 1 mL of blood should be collected.

2. For the collection of the bronchoalveolar lavage fluid (BAL), open the diaphragm to allow the lungs to collapse. It is important to make sure not to damage (puncture) the lungs when opening up the thoracic cavity.
3. Using the tracheal cannula and a 1-mL syringe, lavage the lungs with 1 mL of the supplemented PBS at room temperature. During the first drawback of this BALF, a small volume may remain behind in the lungs. Store this first 1 mL of BALF separately in a 1.5 mL tube and keep on ice until the cells and fluid are separated by centrifugation in **step 5** below.
4. Repeat the lung lavage four more times using regular nonsupplemented PBS at room temperature. Poole the BALF from the four lavages in a 15-mL tube and keep on ice.
5. Spin down the initial BALF collected in **step 3** at 590 × *g* at 4 °C for 5 min. Transfer the supernatant in clean microcentrifuge tubes in 100 μL aliquots and store at −80 °C until used for cytokine ELISA in Subheading 3.14.
6. Resuspend the resulting cell pellets from **step 5** in 1 mL of PBS and combine with the rest of the BALF sample collected in a 15-mL tube in **step 4**. Keep this BAL cell suspension on ice until used for cytospin slide preparation or flow cytometry in Subheading 3.9.1 or Subheading 3.12, respectively.
7. Finally, collect individual lung lobes and any other necessary organs in 1 mL of ice-cold RPMI 1640 medium for preparation of a single-cell suspension in Subheading 3.10.
8. Alternatively, collect individual lung lobes in cryogenic vials, snap-freeze immediately by submerging the tubes in liquid nitrogen, and store at −80 °C until processed for ELISA in Subheadings 3.13 and 3.14 (*see* **Note 16**).

3.9 Analysis of the Infiltration of Inflammatory Cells in BALF

Cell compositions of BALF or lung tissue can be analyzed either by cytospin preparations or by flow cytometry. The cytospins require minimal time investment on the section day of the experiment but need to be differentially counted thereafter, which requires expertise and additional time. In contrast, flow cytometric measurements have a greater capacity to phenotype cells based on their granularity and allow immediate analysis.

3.9.1 Cytological Analysis with Cytospin Preparations

1. Label cytospin slides for the identification of the samples.
2. To coat the slides with BSA, assemble each cytospin holder by inserting a labeled slide and a Shandon filter card and attaching a cuvette in the holder above the filter card.

3. Place the assembled holder into the cytocentrifuge. Add 20 μL of PBS containing 1% BSA in the cuvette and spin at 550 × *g* for 1 min.
4. Centrifuge the BALF cells from **step 6** in Subheading 3.8 at 590 × *g* for 5 min at 4 °C. Discard the supernatant.
5. Resuspend the cell pellets in 500 μL of RBC lysis buffer and incubate for 1 min at room temperature.
6. Centrifuge the cells 590 × *g* for 5 min at 4 °C, discard the supernatant, and resuspend the pellet in 200 μL of PBS containing 1% BSA.
7. Count the cell number in the BALF using a cell counter or hemocytometer for total BALF cell count (Fig. 5a). Adjust the

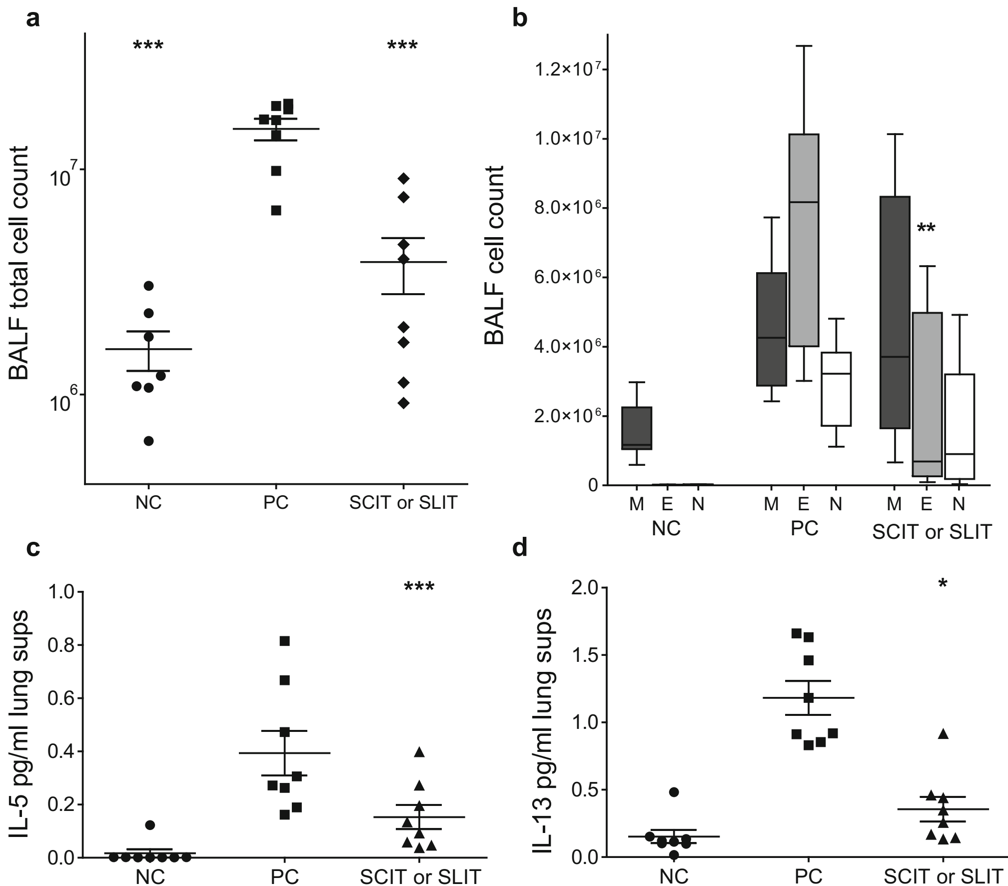

Fig. 5 Eosinophilic and proinflammatory cytokine responses after AIT. (**a**) Total cell counts in bronchoalveolar lavage fluid (BALF). (**b**) Differential cytospin cell counts in BALF. *M* mononuclear cells, *E* eosinophils, *N* neutrophils. Absolute numbers are plotted as median and 10–90 interquartile. (**c**, **d**) Concentrations of IL-5 and IL-13 measured in restimulated single-cell suspensions of lung cells. Concentrations were calculated as the concentration after 5 day restimulation minus unstimulated control (PBS) and expressed as mean ± SEM ($n = 8$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$ compared to positive control

volume of the cell suspension with PBS with 1% BSA to achieve a BALF cell density of 1×10^6 cells/mL.

8. Prepare cytospins by spinning down 100 μL of the cell suspension (1×10^5 cells) onto the coated slides at 550 × *g* for 5 min at room temperature. Carefully release the slides from the holder and let the slides air-dry at room temperature for 10 min. Clean the cuvettes using demineralized water and 70% ethanol solution.
9. Stain the cytospin slides using a Diff-Quick staining set according to the manufacturer's protocol. Dry thoroughly and coverslip the slides.
10. Perform differential counts of 300 cells per cytospin by identifying mononuclear cells (M), neutrophils (N), and eosinophils (E) by standard morphology using a light microscope at 100× magnification (*see* **Note 17**). The results may be graphed as shown in Fig. 5b.
11. Alternatively, the BAL cell suspension from **step 7** may be used for flow cytometric analysis. *See* Subheading 3.11 to proceed with flow-cytometry-based BALF cell analyses.

3.10 Preparation of Single-Cell Suspensions of Lung Tissue, DLNs, and the Spleen

3.10.1 Single-Cell Suspensions from Lung Tissue and DLNs

1. After dissecting the lung tissue from the mouse in **step 7**, Subheading 3.8, transfer the largest left lung lobe to a Petri dish in a biosafety cabinet (*see* **Note 18**).
2. Using a scalpel, cut the lobe into a homogenous paste and resuspended in 2 mL of RPMI 1640 medium with 4 mg/mL Collagenase A, 0.1 mg/mL DNase I, and 1% BSA. Incubated the cells for 1.5 h at 37 °C.
3. To remove tissue fragments, run the lung cell suspension through a 70-μm cell strainer into a 50-mL tube. Wash the cell strainer with 2–5 mL of RPMI 1640 at room temperature in order to flush out the remaining cells.
4. Centrifuge the cell suspension at 350 × *g* for 5 min at 4 °C and discard the supernatant. Resuspend the cell pellet in 1 mL of RBC lysis buffer and incubated for 3 min at room temperature.
5. Centrifuge the cell suspension again at 350 × *g* for 5 min at 4 °C and discard the supernatant. Count the cells using an automated cell counter or hemocytometer.

3.10.2 Single-Cell Suspensions from the Spleen

Single-cell suspensions of spleen cells are obtained in a similar fashion as from the lung tissue, although enzymatic digestion is only required for analysis of DC subsets.

1. Remove the spleen from the mouse in **step 7**, Subheading 3.8. Mince the tissue with a scalpel and strain through a 70-μm cell strainer in a biosafety cabinet as described in **steps 2** and **3** of

Subheading 3.10.1. Rinse the strainer with 5 mL of RPMI 1640.

2. Centrifuge the spleen cell suspension at 550 × *g* for 5 min at 4 °C and discard the supernatant. Resuspend the cell pellet in 1 mL of RBC lysis buffer and incubate for 10 min at room temperature.
3. Centrifuge again at 550 × *g* for 5 min at 4 °C and resuspend in 1 mL of RMPI 1640.
4. Repeat **step 3** one more time, and the cells are ready for further use (*see* **Note 19**).

3.11 Restimulation of Lung Cells and DLN Cells

To evaluate the T-cell responses to allergen recall, lung and DLN cells prepared in Subheading 3.10 are restimulated with allergens in culture.

1. In U-bottom 96-well plates, seed 200,000 cells/well in 250 μL of RPMI 1640 supplemented with 10% FCS, pen/strep; 50 μM beta-mercaptoethanol in the presence of either 0 μg or 30 μg of GP or HDM extract in triplo (*see* **Note 20**).
2. Culture the cells for 5 days at 37 °C and 5% CO_2.
3. Collect the culture media from all wells and store at −80 °C for analysis of cytokines (*see* Subheading 3.14).

3.12 Quantification of DCs, T-Cell Populations, and Innate Lymphoid Cells in Lung Single-Cell Suspensions Using Flow Cytometry

3.12.1 Staining of Extracellular and Intracellular Targets

1. Divide all lung single-cell suspensions in FACS tubes, using one tube per mouse for each staining. In addition, pipette approximately 5 μL out of every mouse sample and prepare a pooled sample containing a small number of cells from each mouse for the single stains.
2. Wash the cells using 300 μL of the FACS buffer and centrifuge the cells at 590 × *g* for 5 min at 4 °C to remove the supernatant.
3. Resuspend the cells in 100 μL of the FACS buffer. Add 100 μL of the block buffer (to a total of 200 μL) for extracellular blocking and incubate for 10 min at room temperature.
4. Centrifuge at 590 × *g* for 5 min at 4 °C to remove the supernatant. Resuspend the cells in 100 μL of FACS buffer.
5. Add 100 μL of an antibody cocktail containing appropriately diluted primary antibodies for extracellular staining to a total of 200 μL (*see* Tables 4 and 5). Incubate for 30 min at room temperature in the dark.
6. Centrifuge at 590 × *g* for 5 min at 4 °C and wash in 1 mL of FACS buffer. Repeat the centrifugation and wash again in 1 mL of PBS. Centrifuge again to remove the supernatant.

Table 5
Antibodies used for FACS analysis of T cells and DCs

Specificity	Clone	Isotype	Staining
Brilliant Violet 605™ anti-mouse CD4 Antibody	GK1.5	Rat IgG2b, κ	Extracellular
Anti-Mouse CD103 (Integrin alpha E) FITC	2E7	Armenian Hamster IgG	Extracellular
Anti-Mouse CD11b APC	M1/70	Rat IgG2b, κ	Extracellular
Anti-Mouse CD11c APC-eFluor® 780	N418	Armenian Hamster IgG	Extracellular
Anti-Mouse F4/80 Antigen PE-Cyanine7	BM8	Rat IgG2a, κ	Extracellular
Anti-Human/Mouse Gata-3 PerCP-eFluor® 710	TWAJ	Rat IgG2b, κ	Intracellular
Anti-Mouse/Rat Foxp3 PE	FJK-16 s	Rat IgG2a, κ	Intracellular

7. Resuspend the cells in 100 μL of PBS and add 75 μL of fixable L/D V450 cell stain (1:1000 prediluted in PBS). Incubate for 15 min at room temperature in the dark.
8. Wash once in 1 mL of PBS as described in **step 6** and resuspend the cells in 1 mL of FIX. Incubate for 30 min at room temperature in the dark.
9. Without washing, add 1 mL of PERM buffer. After one minute, centrifuge at 590 × *g* at 4 °C and remove the supernatant. Resuspend the cells in 100 μL of intracellular block buffer and incubate for 10 min at room temperature.
10. Centrifuge at 590 × *g* at 4 °C, remove the supernatant, and resuspend in 100 μL of PERM buffer.
11. Add 100 μL of the antibody cocktail containing primary antibodies appropriately diluted in PERM buffer for intracellular staining (*see* Tables 4 and 5). Incubate for 30 min at room temperature in the dark.
12. Without washing, add 1 mL of PERM buffer, centrifuge 590 × *g* at 4 °C, and remove the supernatant. Wash once in 1 mL of FACS buffer and remove the supernatant.
13. Resuspend the cell pellet in 200 μL of FACS buffer and transfer the cells to a FACS tube with a 35-μm cell strainer cap to remove any clumps. The samples are now ready for flow cytometry.

3.12.2 Flow Cytometry

Use different gating strategies depending on the cell types of interest. Here, the gating strategy for ILC2s is described as an example.

1. Set compensation using single stain controls for the antibody panel used.

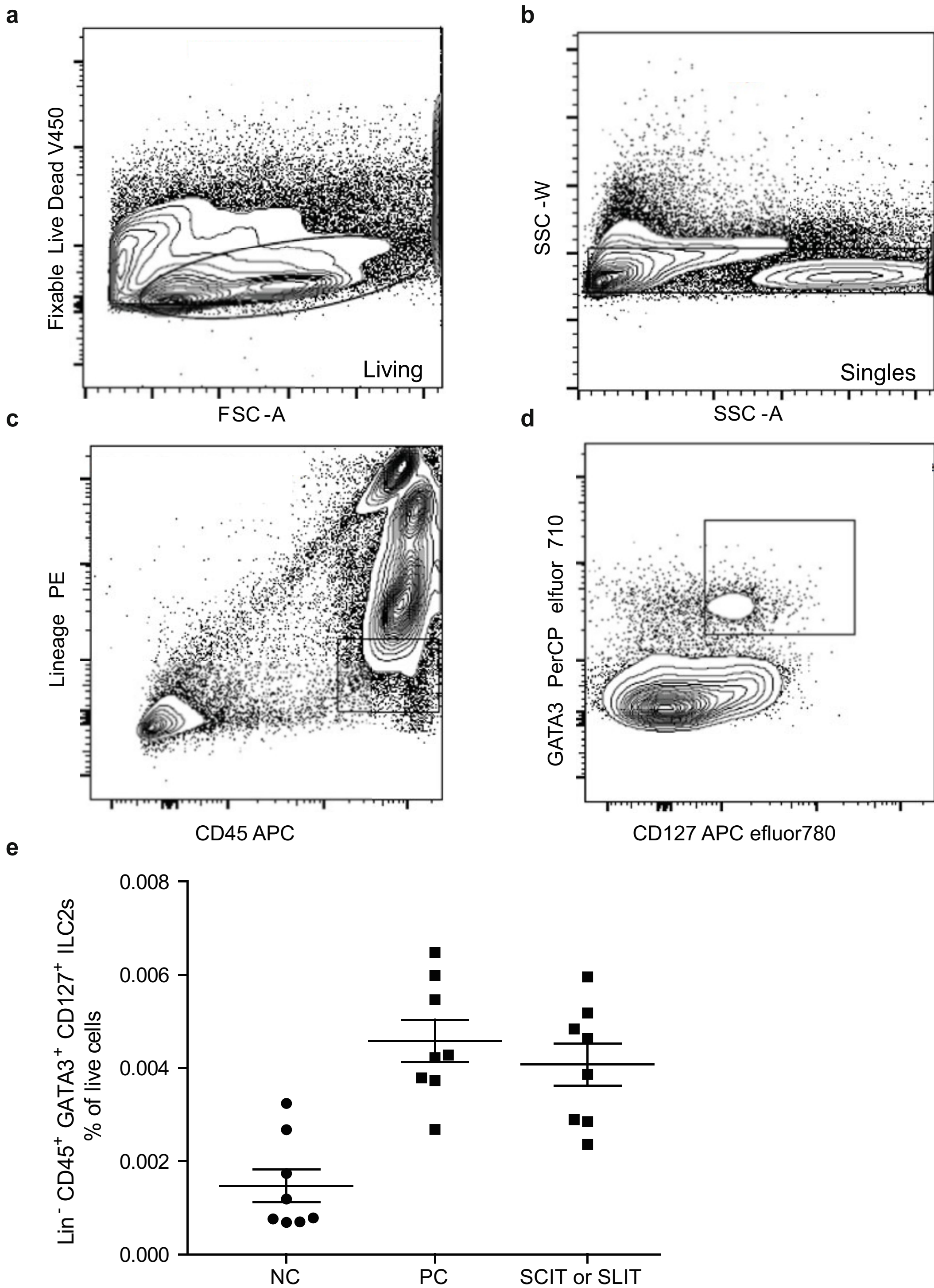

Fig. 6 Gating strategy for the identification of innate lymphoid cells type 2 (ILC2s) in the lung. (**a**) Gating of live cells identified by L/D V450 stain. (**b**) Doublet cell exclusion. (**c**) Gating out the lineage negative cells and including the CD45 positive cells. (**d**) Gating for only the GATA3 and CD127 double-positive cells. (**e**) Lineage^{-}CD45^{+}GATA3^{+} CD127^{+} ILC2s plotted as % of live cells

2. Exclude all dead cells by plotting the area of the forward scatter (FSC-A) against the fixable L/D marker (Fig. 6a).
3. Exclude all doublets using a small selection gate in the plot of the area of the side scatter (SSC-A) against the width of the side scatter (SSC-W, Fig. 6b).
4. ILC2s may be plotted as Lineage negative cells, CD45-positive cells (*see* Table 4, Fig. 6c), followed by gating on the GATA3- and CD127-positive cells, markers that are both expressed by ILC2s (Fig. 6d).
5. For the identification of other cell types, use CD4 and GATA3 for Th2 cells, CD4 and FoxP3 for Treg cells, and CD11b and CD103 for subpopulations of conventional DCs (*see* Table 5).

3.13 Homogenization of Lung Tissue for Total Protein and Cytokine Analysis

The levels of specific cytokines, chemokines, or other mediators can be measured from either freshly dissected lung lobes or from snap-frozen lung lobes stored at −80 °C. Use the identical lung lobe for all mice in the experimental and control groups. Keep the lung tissue on ice at all times.

1. Weigh the cryogenic vials containing the lung lobe and correct for the empty cryogenic vial weight to obtain the net lung lobe weight (milligram of lung tissue).
2. Add Luminex buffer at a ratio of 1:5 (weight: volume). For example, to 1 mg of lung tissue, add 4 μL of the buffer.
3. Homogenize the lung with a homogenizer on ice for at least 1 min, until no large pieces are visible anymore. Clean the homogenizer with tap water and ethanol between samples.
4. Centrifuge the lung homogenate samples at 12,000 × *g* for 20 min at 4 °C.
5. Collect the supernatants in clean microcentrifuge tubes in 100-μL aliquots (*see* **Note 21**). Store the samples at −80 °C until used for ELISA in Subheading 3.14. Discard the pellets.

3.14 Analysis of Immune Responses by ELISA

Immunoglobulin levels in serum and cytokine levels in BALF, culture media of restimulated single-cell suspensions, and lung tissue homogenates can be determined using ELISA (*see* **Note 22**).

3.14.1 Biotinylation of Allergens for spIgE ELISA

Biotinylation of *Phl p* and *Der p* is performed using a commercial biotinylation reagent such as Thermo Scientific EZ-Link Sulfo-NHS-LC-Biotin. The biotinylated allergens are specifically used to detect spIgE by ELISA (*see* Subheading 3.12.2, **step 7**). According to the manufacturers' protocol, we can adjust the molar ratio of Sulfo-NHS-LC-Biotin to a protein to obtain the level of incorporation desired (~ 4–6 biotin groups per allergen particle). Since the rough extracts contain a mixture of proteins with various molecular weights, we make an estimate based on SDS-PAGE results. For the rough extract of GP, we use an average molecular weight of 10,000 Dalton.

1. Calculate millimoles of biotin reagent to add to the reaction for a 20-fold molar excess: *mmol biotin* = mL protein × (mg protein/mL protein) × (mmol protein/mg protein) × (20 mmol biotin/mmol protein).

   An example: 1000 μL GP × (3.9 mg of GP/1 mL GP) × (1 mmol GP/10,000 mg of GP) × (5 mmol biotin/1 mmol GP) = 1.95×10^{-3} mmol biotin.
2. Calculate microliters of 10 mM biotin reagent solution (stock) to add to the reaction: *μL biotin* = mmol biotin × (1,000,000 μL/L) × (l/10 mmol).

   An example: 1.95×10^{-3} mmol biotin × (1×10^{6} μL/L) × (l/10 mmol) = 195 μL of biotin.
3. For the biotinylation reaction, take the following steps: add 180 μL of ultrapure water to the 1 mg microtube to prepare a 10 mM solution of the biotin reagent (stock) and take out the calculated volume of biotin to add to your protein solution.
4. Incubate for 2 h on ice or for 30 min at room temperature.
5. Hydrate the membrane of a Slide-A-Lyzer™ G2 Dialysis Cassette in PBS for 2 min in order to purify the labeled proteins for optimal performance.
6. Using a 1-mL syringe with an 18G needle, slowly fill the cassette with your biotin–allergen mixture and withdraw any remaining air.
7. Dialyze in 2 L of PBS for 2 h at room temperature.
8. Refresh the 2 L PBS and dialyze for another 2 h at room temperature.
9. Change the buffer again and dialyze overnight at 4 °C.
10. The next day, collect the purified sample using a 1-mL syringe with an 18G needle. Store in aliquots at −20 °C.

3.14.2 Detection of Analytes in Serum, BALF, Culture Media, and Tissue Homogenates by ELISA

For the detection of immunoglobulins, ELISA is carried out using antibodies and reagents listed in Table 6. For cytokine measurements in BALF, the supernatant of restimulated single-cell suspensions and lung tissue homogenates, ELISA and multiplex are carried out using kits available from a wide range of commercial suppliers according to the manufacturers' instructions.

1. Coat a high-affinity binding 96-well flat-bottom ELISA plate with 100 μL/well of an appropriate capture antibody diluted in PBS overnight at 4 °C (*see* Table 6).
2. Wash the plate five times using 300 μL of the wash buffer either using an ELISA plate washer or by hand.
3. Block the ELISA plate using 300 μL of ELISA buffer with 1% BSA at room temperature for 1 h.

Table 6
Overview of immunoglobulin ELISA antibodies

ELISA	Layer	Antibody	Stock	Dilution	Supplier
Total IgE	Capture	Purified rat anti-mouse IgE (R35-72)	0.5 mg/mL	1:500	BD Bioscience
	Block	ELISA buffer containing 1% BSA	Pure	300 μL	Lab EXPIRE
	Sample	Mouse serum samples (pre- and post-sera)	Pure	Pre 1:30, post 1:60	Animal Centre
	Standard	Purified mouse IgE κ isotype control (C38-2)	0.5 mg/mL	Start 2500 ng/mL, twofold dilution steps	BD Bioscience
	Detection	Biotin rat anti-mouse IgE (R35-118)	0.5 mg/mL	1:500	BD Bioscience
Total IgG1	Capture	Purified rat anti-mouse IgG1 (A85-3)	0.5 mg/mL	1:200	BD Bioscience
	Block	ELISA buffer containing 1% BSA	Pure	300 μL	Lab EXPIRE
	Sample	Mouse serum samples (pre- and post-sera)	Pure	1:300,000	Animal Centre
	Standard	Purified mouse IgG1, κ isotype control (MOPC-31C)	0.5 mg/mL	Start 750 ng/mL, threefold dilution steps	BD Bioscience
	Detection	Biotin rat anti-mouse IgG1 (A85-1)	0.5 mg/mL	1:500	BD Bioscience
Total IgG2a	Capture	Purified rat anti-mouse IgG2a (R11–89)	0.5 mg/mL	1:200	BD Bioscience
	Block	ELISA buffer containing 1% BSA	Pure	300 μL	Lab EXPIRE
	Sample	Mouse serum samples (pre- and post-sera)	Pure	1:50	Animal Centre
	Standard	Purified mouse IgG2a κ isotype control (G155-178)	0.5 mg/mL	Start 500 ng/ml, twofold dilution steps	BD Bioscience
	Detection	Biotin rat anti-mouse IgG2a (R19-15)	0.5 mg/mL	1:500	BD Bioscience
Total IgA	Capture	Purified rat anti-mouse IgA Antibody (RMA-1)	0.5 mg/mL	1:100	BioLegend
	Block	ELISA buffer containing 1% BSA	Pure	300 μL	Lab EXPIRE
	Sample	Mouse serum samples (post-sera)	Pure	Post 1:50	Animal Centre
	Standard	Purified mouse IgA	1000 ng	Start 1000 ng/mL, threefold dilution steps	Bethyl Laboratories
	Detection	Biotin rat anti-mouse IgA (C10-1)	0.5 mg/mL	1:250	BD Bioscience

(continued)

Table 6 (continued)

ELISA	Layer	Antibody	Stock	Dilution	Supplier
spIgE	Capture	Purified rat anti-mouse IgE (R35-72)	0.5 mg/mL	1:500	BD Bioscience
	Block	ELISA buffer containing 3% powdered milk (ELK)	Pure	300 μL	Campina
	Sample	Mouse serum samples (pre- and post-sera)	Pure	Pre 1:30, Post 1:60	Animal Centre
	Standard	Pooled positive reference serum	Pure	Start 1:2, twofold dilution steps	Animal Centre
	Detection	Biotinylated allergen	3.9 mg/mL	1:150	Lab EXPIRE
spIgG1	Capture	Crude or rough extract allergen	1 mg/mL	1:100	ALK Abello/Citeq Biologics
	Block	ELISA buffer containing 1% BSA	Pure	300 μL	Lab EXPIRE
	Sample	Mouse serum samples (pre- and post-sera)	Pure	1:300,000	Animal Centre
	Standard	Pooled positive reference serum (*see* **Note 14**)	Pure	Start 1:50,000, twofold dilution steps	Animal Centre
	Detection	Biotin rat anti-mouse IgG1 (A85-1)	0.5 mg/mL	1:500	BD Bioscience
spIgG2a	Capture	Crude or rough extract allergen	1 mg/mL	1:100	ALK Abello/Citeq Biologics
	Block	ELISA buffer containing 1% BSA	Pure	300 μL	Lab EXPIRE
	Sample	Mouse serum samples (pre- and post-sera)	Pure	1:50	Animal Centre
	Standard	Pooled positive reference serum (*see* **Note 14**)	Pure	Start 1:25, twofold dilution steps	Animal Centre
	Detection	Biotin rat anti-mouse IgG2a (R19-15)	0.5 mg/mL	1:500	BD Bioscience

4. Empty the plate by flicking and wash once with the wash buffer, making sure that the wells are empty.
5. Add the samples and standards, both in duplo, appropriately diluted in the ELISA buffer in a total volume of 100 μL (*see* **Note 22** and Table 6 for appropriate dilutions for serum samples and standards). Incubate for 2 h at room temperature on a bench-top plate shaker at 300 rpm.
6. Wash the plate five times with 300 μL of the wash buffer each time. In each well, add 100 μL of a respective detection antibody, or biotinylated allergen for spIgE (*see* Subheading 3.13, **step 1**), appropriately diluted in ELISA buffer (*see* **Note 22** and

Table 6) and incubate for 1.5 h at room temperature on the shaker at 300 rpm.

7. Wash the plate again five times with 300 μL of wash buffer each time. Add 100 μL of Avidin-HRP diluted 1:200 in ELISA buffer and incubate for 1 h at room temperature on the shaker at 300 rpm.
8. Wash the plate again three times with 300 μL of the wash buffer each time. Add 100 μL of the OPD peroxidase substrate to each well and incubate for 10 min in the dark until the coloring reaction is complete, visible by the color development in the standard wells.
9. Stop the reaction by adding 75 μL of 4 M H_2SO_4 to each well. Read the optical density at 490 nm on an ELISA plate reader.
10. Calculate the concentrations of analytes in the serum samples based on the standard curve, using a four-parameter model. The fit of the standard curve should be at least $r^2 = 0.95$. When performing with multiple 96-well plates, each plate should contain a standard curve as well as a few reference samples that are included on all plates for plate–plate comparisons.

4 Notes

1. The medium may be optionally supplemented with 3% BSA (w/v, cell culture grade) and 5 mL of 100× MEM Vitamin Solution, which is commercially available as a growth supplement.
2. Sensitization with intraperitoneal injections is performed on nonanesthetized animals. Restrain a mouse by gently gripping the nape with one hand and anchoring the tail between the small finger and the palm to secure the lower body. Tilt the mouse head down at a 35–40° angle so that the intestines fall away from the injection site. The needle is inserted into the lower right quadrant of the abdomen slightly off the midline anterior to the bladder. Slight negative pressure is applied to the syringe. To avoid the likelihood of puncturing or lacerating abdominal organs, a 1/2 in., 27G needle or insulin syringe is recommended. This route of administration allows for a volume of up to 1 mL to be given safely to a mouse.
3. For subcutaneous injections, a nonanesthetized mouse is held by the nape using one hand. A syringe with a 25G needle is inserted with the other hand at the base of a skin "tent" created by the thumb and forefinger. A slight negative pressure is applied to the syringe to ensure that the needle placement is subcutaneous and did not accidentally damage a local vein. A total of 100 μL is injected.

4. During the first SCIT injections in C57BL/6 mice, it is important to monitor immediate responses like severity of shock and drop in body temperature. The severity of shock is scored as follows: (1) mild shock (itching, ruffling of fur, dyspnea, self-isolation, and decrease in spontaneous activity); (2) moderate shock (prostration, sluggish gait, no response to whisker stimuli, puffiness around eyes and or mouth, and slight activity after prodding); (3) severe shock (complete paresis and no activity following prodding with or without convulsions); (4) death within 30 min. At the same time, body core temperature must be registered every 20 min using a rectal thermometer after the first SCIT injection.
5. For sublingual administration, restrain a nonanesthetized mouse by gently gripping the nape with one hand and tuck its tail between the fingers to secure the lower body. Hold the mouse up at a fully vertical position. Tighten the grip of the fur between the thumb and index finger until the mouse sticks out its tongue slightly. Using a P20 pipette, place 5 μL of the allergen solution under the tongue, while ensuring that it stays there for 30 s minimum. It is of paramount importance that the mouse does not swallow the allergen during the administration. After the administration, maintain the restrained animal for 30 s vertically to prevent swallowing before returning the mouse to the individual ventilated cage (IVC).
6. The intranasal challenges are performed with the mice that have been lightly anesthetized. This method facilitates the inhalation of the allergen into the lungs as the mouse wakes up. Check visually whether the allergen solution has been inhaled by the mouse.
7. Retro-orbital puncture targets the venous sinus located behind the eye. When correctly performed on these long-anesthetized mice using 4.5% isoflurane in combination with 1 mL/min O_2, the eyes and health of the animal remain unaffected. The rate of blood flow through the tube may vary significantly from mouse to mouse. When punctured properly, act quickly. The maximum amount of blood that should be collected from a mouse is 8 mL/kg body weight in 14 days.
8. When measuring ear thickness using a micrometer, it is important to keep the device perfectly horizontal. Therefore, all mice should be maintained in the same horizontal position during the measurement. Mice receiving a light anesthesia using 4.5% isoflurane in combination with 1 mL/min O_2 can be placed in a stand, similar to that for intratracheal administrations, to make sure the ears are in the same horizontal position every time (*see* Fig. 3 right panel).

9. For airway pressure, the first measurement is set to zero, while the second point is the pressure measurement after administration of 1 mL of air. For the cylinder pressure, a dynamic tubing calibration is required, using the open and closed cannula as per the manufacturer's instructions.
10. Maintain the anesthesia by repeating the injections every 20 min with 25% of the initial dose. Keep the anesthetized mice warm and record the heart rate, blood pressure, and O_2 saturation during the entire procedure.
11. A standard script is available within the FlexiWare software, which can be adjusted to the following format:

```
FlexiVent script
================
Script: MCh IV DRC
Author: LH
Date: 15-02-2012
Changes: LH: 8-6-2016
Adapted to new software and doses
Adapted to new doses and comments of Scireq crew
1. Added snapshot after TLC (for quality control)
2. Quick prime 3 instead of quick prime 2 (+ reduction of
number of perturbations)
3. In template adaptation: addition of constant phase model for
the quick prime perturbations
(c) SCIREQ Inc. 2001-04
// Start of command section
// ------------------------
Start Script, Format = 3.0;
Title = MCh DRC with TLC;
//Start DEFAULT ventilation (Depending on Template)
0:00 Ventilation DEFAULT;
// Perform TLC maneuver immediately after attaching the animal
0:02 Prompt MESSAGE=Attach the animal, then click OK to perform
two TLC maneuvers.;
0:00 Marker TEXT=Performing two TLC maneuvers at the start of
the experiment.;
0:00 Ventilation MODE=CFlow; F=10 br/min; Vt=40 mL/kg; IER=1;
Pmax=30 cmH2O;
0:05 Ventilation DEFAULT;
0:15 Ventilation MODE=CFlow; F=10 br/min; Vt=40 mL/kg; IER=1;
Pmax=30 cmH2O;
0:05 Ventilation DEFAULT;
//Do a snapshot for quality control (check for leakage)
0:05 Perturbation NAME=Snapshot-240;
// Log the substance
```

```
0:05 Marker TEXT=Start of MCh Dose–response measurement.;
// Start loop for Baseline
0:02 Loop Begin
// Log next dose
0:00 Marker TEXT=Next dose: Baseline ;
// Prepare to inject the mice
0:00 Prompt BEEP ;
0:00 Prompt MESSAGE=Press OK to start baseline measurement.
0:02 Marker TEXT=Mouse is being injected: Baseline;
// Take 6 measurements directly after each other
0:00 Loop Begin
0:00 Perturbation NAME=QPrime3;
0:00 Loop Return MAXCOUNT=6;
// Take 4 measurements for 4 times
0:00 Loop Begin
0:00 Perturbation NAME=QPrime3;
0:40 Loop Return MAXCOUNT=4
// Prompt user for next dose
0:20 Query MESSAGE=Continue with next dose? (No to restart
previous dose);
0:00 Loop Return REPLY=NO ;
// Start loop for 0 µg/kg
0:02 Loop Begin
// Log next dose
0:00 Marker TEXT=Next dose: 0 ;
// Prepare to inject the mice
0:00 Prompt BEEP ;
0:00 Prompt MESSAGE=Press OK when ready to inject mouse (0 µg/
kg), and inject immediately thereafter. ;
0:02 Marker TEXT=Mouse is being injected:0 µg/kg;
// Take 6 measurements directly after each other
0:00 Loop Begin
0:00 Perturbation NAME=QPrime3;
0:00 Loop Return MAXCOUNT=6;
// Take 4 measurement for 4 times
0:00 Loop Begin
0:00 Perturbation NAME=QPrime3;
0:40 Loop Return MAXCOUNT=4;
// Prompt user for next dose
0:20 Query MESSAGE=Continue with next dose? (No to restart
previous dose);
0:00 Loop Return REPLY=NO;
// Start loop for 50 µg/kg → repeat for the dosages 100, 200,
400, and 800 µg/kg
// End of script
```

12. Any fat or surrounding connective tissue needs to be cleared from the jugular vein, allowing sufficient space for a curved surgical tweezer to maneuver underneath the jugular vein.
13. To test whether the cannula is correctly in place, slightly pull the plunger of the syringe connected to the cannula. A properly cannulated jugular vein will allow blood to flow into the cannula upon the application of negative pressure.
14. Before putting a new syringe on the IV-line, make sure there is no air remaining in the syringe and cannula (use a 5-mL syringe with saline to fill the 1-mL syringe).
15. Analysis of resistance and compliance data can be performed with the exported files using any spreadsheet or statistical program and plotted against the methacholine doses (Fig. 4c, d). In short, highlight the values of interest (resistance and compliance), the COD, and the PEEP. Every dose of methacholine gives a peak value in resistance ($cmH_2O.s/mL$) and a minimum value in compliance (mL/H_2O). Select these values for every dose of methacholine and for every mouse and plot them.

    To ensure high-quality data, use the coefficient of determination (COD)-value with a cutoff of 8.0. This is a quality control parameter measuring the quality of the single-compartment model fit. Next, check whether the PEEP was registered as ~20 mmH_2O or ~2 cmH_2O. Alternatively, all values that are measured with sufficient technical quality (use the COD value as a cutoff) can be plotted to calculate an area under the curve for each individual methacholine dose. Another relevant variable is the effective dose (ED) of methacholine (MCh) necessary to increase AHR to an R of 3 $cmH_2O.s/mL$ (ED3) (*see* Fig. 4b). As the data are not normally distributed, but represent linked measurements in a dose-range of methacholine challenges, an appropriate statistical evaluation is ANOVA, or in case of missing values, a GEE analysis [39].
16. For quantification of net weight of lung tissue collected, it is imperative to weigh the empty cryogenic vials prior to dissection and again after collecting the lung lobes.
17. Differential counting of BALF cells should be performed by an observer who is blinded to the experimental groups.
18. Cells originating from the lung DLN can be processed in an identical fashion as the lung tissue cells.
19. For long-term storage of the cells in liquid nitrogen, resuspend the cell pellet in 50% HBSS, 40% FCS, and dropwise add 10% DMSO, then divide the cell suspension over cryogenic vials, and place the vials in a Stratagene® box at −80 °C

overnight. The day thereafter, transfer the vials to liquid nitrogen.

20. Since DLNs do not usually yield as many cells as the lung, DLN cell cultures may only be performed in duplo.
21. Protein concentrations retrieved from homogenization of lung lobes vary considerably between individual mice. Therefore, a BCA protein assay should be performed to quantify the protein content of the lung tissue homogenates. The protein concentration for each sample is used to normalize cytokine levels detected by ELISA when evaluating cytokine responses between experimental groups.
22. The serum samples taken at the three (in SCIT) or five (in SLIT) different time points (pre1–4 and post-serum; *see* Fig. 1a–c) will be used to measure the total as well as allergen-specific IgG1, IgG2a, and IgE. Usually, pre-sera samples will be diluted 1:30 and post-sera samples 1:60 in ELISA buffer, although several dilutions can be tested to determine the optimal dilution. For accurate quantification of immunoglobulin or cytokine levels by ELISA, samples should be stored at −80 °C until used. Samples should be processed within 3 months from isolation.

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Chapter 21

T-Cell Epitope Immunotherapy in Mouse Models of Food Allergy

Christine Y. Y. Wai, Nicki Y. H. Leung, Ka Hou Chu, and Patrick S. C. Leung

Abstract

Food allergy has been rising in prevalence over the last two decades, affecting more than 10% of the world population. Current management of IgE-mediated food allergy relies on avoidance and rescue medications; research into treatments that are safer and providing guaranteed and durable curative effects is, therefore, essential. T-cell epitope-based immunotherapy holds the potential for modulating food allergic responses without IgE cross-linking. In this chapter, we describe the methods in evaluating the therapeutic capacities of immunodominant T-cell epitopes in animal models of food allergy. Moreover, we explain in detail the methods to measure the allergen-specific antibody levels, prepare single-cell suspension from spleen, and prepare small intestine for immunohistochemical analysis of eosinophils and Foxp3+ cells.

Key words Shellfish allergy, Egg allergy, Cow's milk allergy, Oral immunotherapy (OIT), Mouse model, ELISA, Immunohistochemical staining (IHC), Swiss roll, Eosinophils, Regulatory T cell (Treg)

1 Introduction

Food allergy is an immune-mediated hypersensitivity resulted from the failure to develop or the abrogation of oral tolerance against food proteins that are harmless to most of the population. Most cases of food allergy are IgE-mediated, by which type 2 immune response activated by the culprit allergen triggers the release of allergen-specific IgE that cross-link the allergenic proteins on mast cells or basophils, leading to the release of inflammatory mediators such as histamine, prostaglandins, and leukotrienes. These events result in immediate hypersensitivity symptoms involving a single or multiple organs, and they vary from mild reactions at skin, respiratory, and gastrointestinal tracts to life-threatening anaphylaxis such as hypovolemic shock and deterioration in respiratory functions.

Food allergy poses substantial burden on global health affecting approximately 8–10% of children and adults, respectively, based

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on self-reports and approximately 3% adults and children when diagnosed by oral food challenges [1, 2]. The prevalence of food allergy has doubled over the past two decades and has loomed as a "second wave" of allergy epidemic after the rise in prevalence of asthma and allergic rhinitis [3]. Although the prevalent allergens vary with cultural and geographical factors, shellfish, peanut, cow's milk, and egg often top the lists worldwide [4, 5].

Despite the high impact, treatment for food allergy has remained unchanged for decades; strict avoidance and rescue medication with antihistamines and epinephrine autoinjector are the mainstay "treatments." Allergen-specific immunotherapy (AIT) deploying standardized food extracts or unmodified allergens is by far the only disease-modifying option. AIT is highly effective against aeroallergens, but frequent adverse events, low patient adherence, and lack of evidence in developing durable immunological and clinical tolerance remain the major obstacles in food allergy AIT [6]. In this regard, immunotherapy using allergen-specific peptides has been shown to be safe and effective. These peptides are essentially small in size without secondary or tertiary structure, making them incapable of cross-linking IgE on the effector cells. Thus far, immunodominant T-cell epitopes of major food allergens such as peanut Ara h 1 and 2, ovalbumin (OVA) and ovomucoid (Ovm), β-lactoglobulin (BLG), and shrimp tropomyosin have been identified [7–14]. Administration of the T-cell epitopes has been shown to suppress anaphylactic and/or diarrhea scores, decrease allergen-specific IgE levels, increase the inhibitory IgG_{2a} antibodies, reduce Th2 cytokine responses, and increase Th1 and regulatory T-cell responses such as the expression of intestinal *FOXP3* and *TGF-β* in animal models of food allergy [13, 15, 16]. Whereas the therapeutic efficacy of oral peptide immunotherapy was superior to subcutaneous treatment [17], peptide-based treatment for food allergy still warrants further optimization and in-depth characterization of the mechanisms of desensitization involved.

In this chapter, we detail how the therapeutic efficacies of T-cell epitope immunotherapy can be evaluated in mouse models of different food allergies, including shellfish, cow's milk, and egg allergies. Based on our laboratory experiences, we also provide protocols of the most important efficacy assessments for translational studies. These include methods of immediate allergic responses assessment, blood sampling, serum allergen-specific IgE and IgG analyses, and preparation of mouse splenocytes for flow cytometry and cytokine level measurements, as well as detailed methodologies in preparing the small intestine for immunohistochemical (IHC) staining of eosinophils and Foxp3+ T cells.

2 Materials

2.1 Allergen Sensitization and T-Cell Epitope Immunotherapy

1. Female BALB/c mice: 3–5 weeks old.
2. Culprit allergen: recombinant shrimp tropomyosin (purified rMet e 1), OVA, Ovm, or BLG.
3. Cholera toxin: from *Vibrio cholera*, azide-free.
4. Aluminum hydroxide: $Al(OH)_3$, Imject® Alum. An aqueous solution of 40 mg/mL aluminum hydroxide and 40 mg/mL magnesium hydroxide plus inactive stabilizers.
5. T-cell epitope peptides: 15–22 mer, >95% purity. Purified by high-performance liquid chromatography (HPLC) and validated by mass spectrometry. Reconstitute individual peptides in ultrapure water at 1 μg/μL concentration, aliquot, and store at −20 °C until use. Avoid repeated freeze-thaw cycles (*see* **Note 1**).
6. Sterile phosphate-buffered saline (PBS): 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4. Dissolve 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 8.0 g NaCl, and 0.2 g KCl in 800–900 mL of ultrapure water. Adjust the pH to 7.4 with HCl and add water to make the final volume of 1 L. Sterilize by using a 0.25-μm filter unit or autoclaving.
7. 1-mL Syringe with a 22-gauge stainless gavage needle.
8. 1.5-mL Microfuge tubes.
9. 1-mL Syringe with a 23-gauge needle.

2.2 Allergen Challenge

1. 1-mL Syringe without needle.
2. 22-Gauge stainless steel gavage needle.
3. Microprobe thermometer with a RET-3 mouse-specific rectal probe.
4. Restrainer.
5. Warming chamber.
6. 23-Gauge syringe needle.
7. Capillary tube.
8. 1.5-mL Microfuge tubes.

2.3 ELISA for Allergen-Specific Antibodies

1. 96-Well flat-bottom enzyme-linked immunosorbent assay (ELISA) plates.
2. Coating buffer: 50 mM Na_2CO_3, 130 mM $NaHCO_3$, 3 mM NaH_3. Dissolve 5.3 g Na_2CO_3, 10.9 g $NaHCO_3$, and 1.953 g NaH_3 in 1 L of ultrapure water. Store at 4 °C.
3. PBS-T: 0.05% (v/v) Tween-20 in PBS. Add 0.5 mL Tween-20 in 1 L PBS, pH 7.4.

4. Blocking buffer: 5% fetal bovine serum (FBS) in PBS. Add 2.5 mL of FBS in 47.5 mL of PBS.
5. Mouse serum: Dilute 1:10 in the blocking buffer.
6. Secondary antibody: biotin-labeled goat anti-mouse IgE, IgG_1, and IgG_{2a} antibodies. Dilute 1:1000 in the blocking buffer.
7. Horseradish peroxidase-labeled avidin D (Av-HRP): Dilute 1:1000 in the blocking buffer.
8. TMB substrate solution set: 3,3′,5,5′-Tetramethylbenzidine (TMB). Commercially available.
9. Stopping solution: 2 N H_2SO_4. Dissolve 55.6 mL of 18 M H_2SO_4 in 944.4 mL of ultrapure water.
10. Plate reader: for colorimetric quantitation.

2.4 Mouse Splenocytes Preparation

1. 70% Ethanol: use for sterilizing the tools and mouse fur during dissection.
2. Dulbecco's Modified Eagle Medium (DMEM), high glucose.
3. Complete culture medium: 10% FBS in DMEM. Add 50 mL of FBS and 500 μL of penicillin–streptomycin (10,000 U/mL) in 500 mL of DMEM, high glucose.
4. Red blood cell depletion reagent: 0.85% NH_4Cl. Dissolve 0.85 g NH_4Cl in 100 mL of ultrapure water.
5. 0.4% (w/v) Trypan blue solution: commercially available as a ready-to-use solution.
6. Dissection board.
7. Sterile forceps and scissors: autoclaved. Keep in a sterile beaker with 70% ethanol.
8. 5-mL Syringe plunger.
9. 40-μm Nylon cell strainer.
10. 60-mm Tissue culture dishes.
11. 24-Well tissue culture plates.
12. 50-mL Centrifuge tubes.
13. 1.5-mL Microfuge tubes.

2.5 Intestinal Preparation for Histological Analysis

1. 70% Ethanol.
2. PBS: pH 7.4, ice-cold.
3. Fixative: 4% paraformaldehyde (PFA). Dissolve 4 g of paraformaldehyde in 100 mL of PBS, pH 7.4. Heat at 56 °C to dissolve completely. Sterile filter through 0.4 μm polyethersulfone (PES) membrane (*see* **Note 2**).
4. Dissection board.

5. Sterile forceps and scissors: autoclaved. Keep in a sterile beaker with 70% ethanol.
6. Petri dish.
7. 10-mL Syringe.
8. 20-Gauge stainless steel gavage needle.
9. 15-mL Centrifuge tubes.
10. Cardboard.
11. Toothpicks.
12. Embedding cassettes.
13. Adhesive microscope slides: positively charged.

2.6 Immunohistochemical Staining for Eosinophils and Foxp3+ Cells

1. Xylene: Prepare two baths of xylene for deparaffinization of tissue sections.
2. Ethanol: Prepare 100%, 95%, 70%, 50%, and 30% ethanol solutions for rehydration of tissue sections.
3. Tris-EDTA antigen retrieval buffer: 5% Tris, 1.5% 2-butoxyethanol, and 5% sodium ethylenediaminetetraacetic acid (EDTA), pH 9.0.
4. 1-L Glass beaker.
5. Microwave oven and/or hotplate.
6. Hydrophobic barrier pen for histology.
7. Hydrogen peroxide solution for eosinophil staining: 3% hydrogen peroxide in PBS.
8. Hydrogen peroxide solution for Foxp3+ cell staining: 0.3% hydrogen peroxide in absolute methanol.
9. Pepsin solution: 0.25% pepsin with 0.1% sodium azide as preservative in Tris buffer, pH 2.0. Commercially available.
10. PBS: pH 7.4.
11. Blocking buffer: 10% (v/v) goat serum in PBS, pH 7.4. Add 1 mL of goat serum in 9 mL of PBS. Store at 4 °C.
12. 0.1% (v/v) Triton-X 100: Prepare in PBS, pH 7.4. Dissolve 100 μL of Triton-X 100 in 100 mL of PBS. Store at room temperature.
13. Rat anti-mouse major basic protein (MBP) antibody. Dilute 1:500 to 1:15,000 in the blocking buffer for eosinophil staining.
14. Rat anti-mouse Foxp3 antibody. Dilute 1:100 in PBS with 0.1% Triton-X 100.
15. HRP-conjugated anti-rat IgG antibody.
16. Diaminobenzidine (DAB) chromogen substrate kit: commercially available.

17. Hematoxylin Gill #3: commercially available.
18. Mounting medium: Permount or equivalent.
19. Coplin jars.
20. Moisture chamber/slide tray: used for preventing evaporation of antibody solutions during incubation.
21. Glass coverslips.

3 Methods

3.1 Allergen Sensitization

1. Prepare the sensitization allergen and adjuvant. For intragastric sensitization, mix 100 μg (recombinant shrimp tropomyosin) or 1 mg (OVA and Ovm) allergen with 10 μg cholera toxin in 400 μL of sterile PBS per mouse (*see* **Note 3**).
2. For intraperitoneal sensitization, prepare 50 μg (BLG) or 100 μg (recombinant shrimp tropomyosin) allergen per mouse adsorbed in 1–2 mg of $Al(OH)_3$ by adding thoroughly vortexed adjuvant solution into the allergen solution drop by drop. The final total volume should be 200–300 μL per mouse (*see* **Note 3**).
3. Randomly house the mice in cages (*see* **Note 4**). Using a 1-mL syringe and 22-gauge stainless steel gavage needle, sensitize the animals by orally administering the allergen solution prepared in **step 1** above at weekly intervals for 4 weeks as illustrated in Fig. 1.
4. Alternatively, use a 1-mL syringe with a 23-gauge needle to sensitize the animals by intraperitoneally administering the allergen solution prepared in **step 2** above at weekly intervals for 3 weeks as illustrated in Fig. 1.

3.2 T-Cell Epitope Treatment

1. Randomly assign the animals sensitized in Subheading 3.1 into the positive control and treatment groups with 6–8 animals per experimental group. Validating allergen-specific IgE levels prior to immunotherapy is recommended (*see* **Note 5**).
2. For single peptide immunotherapy, administer 1 mg of single peptide per mouse. For cocktail peptide immunotherapy, first prepare a mixture of peptides (3–6 peptides, each at 0.2–1 mg) before administering to mice.
3. Treat mice with the peptide(s) 2–3 times a week for 3–4 weeks intragastrically or subcutaneously as described below (also *see* Fig. 1).
   (a) For shrimp allergy: administer 1.2 mg cocktail peptides via intragastric gavage.
   (b) For egg white allergy (Ovm): administer 1 mg single peptide or 3 mg cocktail peptides via intragastric gavage.

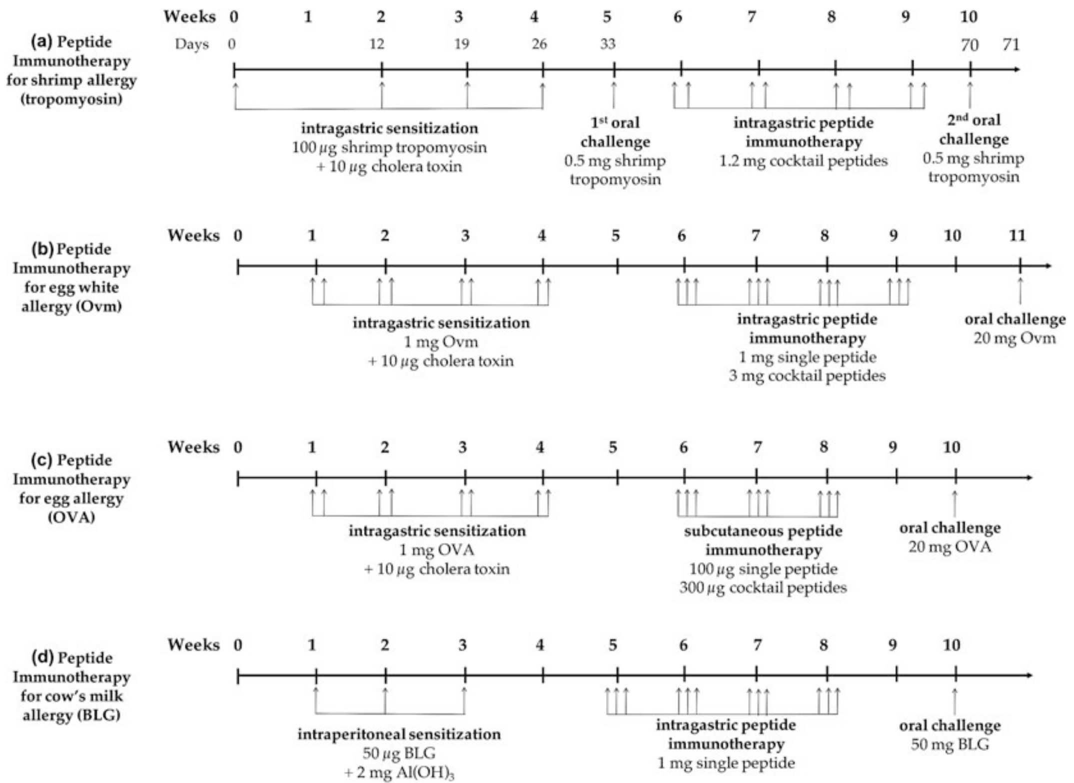

Fig. 1 Regimen designs for T-cell peptide immunotherapy of food allergy. (**a**) Peptide therapy for shrimp tropomyosin allergy [13]. BALB/c mice are sensitized with 100 μg recombinant shrimp tropomyosin with 10 μg cholera toxin (CT) on Days 0, 12, 19, and 26 followed by an oral challenge with 0.5 mg tropomyosin on Day 33. Mice are then intragastrically treated with a mix of six T-cell peptides, each at 0.2 mg (total = 1.2 mg), twice a week for 4 weeks. A second 0.5 mg tropomyosin oral challenge is given on Day 70 (1 week after last treatment). (**b**) Peptide treatment for egg white allergy [16]. BALB/c mice are sensitized with 1 mg ovomucoid (Ovm) and 10 μg CT twice a week for 4 weeks. One week after the last sensitization, mice are intragastrically treated with either 1 mg single peptide or 3 mg cocktail peptides three times a week for 4 weeks. An oral challenge with 20 mg Ovm is given 2 weeks after the last treatment. (**c**) Peptide treatment for egg allergy [15]. BALB/c mice are sensitized with 1 mg ovalbumin (OVA) and 10 μg CT twice a week for 4 weeks. One week after the last sensitization, mice are subcutaneously treated with 100 μg single peptide or 300 μg cocktail peptides three times a week for 3 weeks. An oral challenge with 20 mg OVA is given 2 weeks after the last treatment. (**d**) Peptide treatment for cow's milk allergy [17]. BALB/c mice are intraperitoneally sensitized with 3 weekly doses of 50 μg β-lactoglobulin (BLG) and 2 mg $Al(OH)_3$. Single peptide at 1 mg is then given intragastrically three times a week for 4 weeks. A 50 mg BLG oral challenge is finally given 2 weeks after the last treatment

(c) For egg white allergy (OVA): administer 100 μg single peptide or 300 μg cocktail peptides via subcutaneous injection.

(d) For cow's milk allergy (BLG): administer 1 mg single peptide via intragastric gavage.

3.3 Allergen Challenges

Allergen challenge can be introduced to sensitized mice 1 week after the last sensitization and 1–2 weeks after the last peptide treatment (*see* **Note 6**). General protocols only involve challenging the animals after the peptide treatment and comparing the allergic parameters among different experimental groups to assess the therapeutic effects of the T-cell epitopes (*see* **Note 7**).

3.3.1 Preparation and Administration of Culprit Allergens

1. Fast the animals overnight for 16–18 h before allergen challenge. Water is allowed.
2. Prepare the challenge solution by reconstituting the appropriate culprit allergen in PBS. For each mouse, use 500 μg recombinant shrimp tropomyosin, 20 mg OVA, 20 mg Ovm, or 50 mg BLG in sterile PBS. The total volume should not exceed 600 μL per mouse (*see* **Note 3**).
3. On the day of challenge, record the rectal temperature using a microprobe thermometer before allergen challenge, and then intragastrically deliver the culprit allergen with a 1-mL syringe and a 22-gauge stainless steel gavage needle.
4. Return the mice to their home cage for observation and proceed to Subheading 3.3.2.

3.3.2 Assessment of Immediate Allergic Responses

1. Record the rectal temperature at 15, 30, and 45 min postchallenge (*see* **Note 8**).
2. Observe and record the immediate hypersensitivity symptoms within 40 min postchallenge using a scoring system listed in Table 1 [18]. Scoring should be performed by an independent investigator in a blinded manner (*see* **Note 9**).
3. Observe and record the condition of feces for signs of diarrhea using a scoring system listed in Table 2 [19]. Scoring should be performed by an independent investigator in a blinded manner (*see* **Note 9**).

3.3.3 Tail Vein Blood Sampling

Blood sampling is recommended at 24 h postchallenge for maximum allergen-specific IgE titer.

1. Keep the mouse inside a restrainer and apply local anesthetic cream on the tail surface 30 min before blood sampling.
2. Warm the animal in a warming chamber until the tail vein becomes visible.
3. Insert a 23-gauge needle into the blood vessel and use a capillary tube to collect blood into a 1.5-mL microfuge tube.
4. Put gentle pressure on the bleeding spot upon completion of blood collection.
5. Centrifuge the whole blood samples at 10,000 × g for 20 min at room temperature to separate the serum portion. Store serum samples at −20 °C until analyses.

Table 1
Scoring system of systemic allergic responses

Score	Symptoms
0	No symptom
1	Scratching and rubbing around the nose, head, and tail
2	Puffiness around eyes and/or mouth; reduced activity
3	Labor respiration
4	No activity after prodding; tremor; convulsion
5	Death

Table 2
Scoring system of fecal condition

Score	Fecal condition
0	Hard feces
1	Soft feces
2	Liquid feces
3	White, mucus-like feces

3.4 ELISA for Allergen-Specific Antibodies

1. Prepare a culprit allergen in the coating buffer at 5 μg/mL concentration.
2. Coat a 96-well plate with 100 μL/well of the diluted allergen overnight at 4 °C (*see* **Note 10**).
3. Wash plate with PBS-T twice (*see* **Note 11**).
4. Block the plate with 200 μL/well of the blocking buffer for 2 h at room temperature.
5. Wash plate with PBS-T once.
6. Prepare serum samples in duplicate and add 100 μL of diluted serum samples to each well. For measuring the levels of IgE, IgG_1, and IgG_{2a} antibodies, prepare dilutions at 1:10, 1:10,000, and 1:200 with the blocking buffer, respectively (*see* **Note 12**). Incubate the plate overnight at 4 °C.
7. Wash plate with PBS-T for three times (*see* **Note 11**).
8. Incubate the plate with 100 μL/well of the diluted secondary antibody for 1 h at room temperature (*see* **Note 12**).
9. Wash plate with PBS-T for four times (*see* **Note 11**).
10. Incubate the plate with 100 μL/well of the diluted Av-HRP for 30 min at room temperature in the dark.

11. Wash plate with PBS-T for five times and PBS once (*see* **Note 11**).
12. Allow TMB substrate to reach room temperature before use and add 100 μL/well of TMB substrate for signal development; protect the plate from light.
13. After sufficient color development, stop the signal reaction by adding 50 μL/well of 2 N H_2SO_4 (*see* **Note 13**).
14. Measure the absorbance of each well (Optical Density at 450 nm) with a plate reader (*see* **Note 14**).

3.5 Mouse Splenocytes Preparation

1. At the end of the experiment, euthanize mice according to a method approved by the Institutional Animal Care and Use Committee of your institution.
2. Place a sacrificed mouse on a clean dissection board and rinse the mouse with 70% ethanol to sanitize the fur (*see* **Note 15**). Dissect and remove spleen (*see* **Note 16**). Place the spleen in a 60-mm tissue culture dish with 3 mL of the complete culture medium.
3. Place a 40-μm nylon cell strainer in a new 60-mm tissue culture dish with 5 mL of DMEM. Place the spleen into the cell strainer and press the spleen gently using the seal of a sterile 5-mL syringe plunger until only the connective tissues are left behind (*see* **Note 17**).
4. Transfer the spleen cells with all medium to a 50-mL centrifuge tube.
5. Add 10 mL of DMEM (without any supplements) to the same culture dish. Pipette up and down to remove any residual spleen cells (*see* **Note 18**). Transfer the medium to the same 50-mL centrifuge tube.
6. Centrifuge the spleen cells at 250 $\times$ g for 5 min at room temperature. Discard supernatant using pipettes.
7. Add 5 mL of the red blood cell depletion reagent to the spleen cells. Pipette up and down to resuspend the cells (*see* **Note 18**). Slowly add the resuspended spleen cells to 1 mL of FBS.
8. Centrifuge at 250 $\times$ g for 5 min at room temperature. Discard supernatant using pipettes.
9. Wash spleen cells by resuspending in 10 mL of unsupplemented DMEM (*see* **Note 18**). Centrifuge at 250 $\times$ g for 5 min at room temperature. Discard supernatant using pipettes. Repeat this step twice.
10. Resuspend the spleen cells in 5 mL of the complete culture medium. Take 200 μL of cells into a new microfuge tube and add 300 μL DMEM and 500 μL 0.4% trypan blue solution (dilution factor = 5). Apply the mixture to a hemocytometer for counting the number of viable cells (*see* **Note 19**).

11. For flow cytometric analysis, resuspend the cells at a density of 1×10^6 to 1×10^8 cells/mL in an appropriate staining buffer and proceed to cell staining immediately.
12. For cytokine analysis, resuspend the cells at a density of 5×10^6 cells/mL in the complete culture medium. Place 1 mL of the cell suspension in each well and culture the cells in a 24-well tissue culture plate with 50–100 μg of the culprit allergen.
13. After culturing for 72 h at 37 °C in a 5% CO_2 incubator, harvest the cells in microfuge tubes. Centrifuge at $250 \times g$ for 5 min at room temperature. Collect the supernatant in microfuge tubes and store at −20 °C until analysis.
14. Quantify the levels of target cytokine(s) using commercial mouse cytokine ELISA kits using the harvested cell supernatants.

3.6 Intestinal Preparation for Histological Analysis

1. Place the sacrificed mouse on a clean dissection board and rinse the mouse with 70% ethanol. Dissect and identify the stomach to trace its junction with the small intestine.
2. Carefully make an incision at the junction and gently isolate the small intestine from the mesenteric membrane. Make another incision at junction with the cecum and transfer the entire small intestine from the abdomen to a clean Petri dish containing ice-cold PBS.
3. Divide the small intestine into three segments by locating the duodenum, jejunum, and ileum.
4. Fill a 30-mL syringe with ice-cold PBS and attach a gavage needle to the syringe. Flush the contents of each intestinal segment with ice-cold PBS.
5. Fill a new 10-mL syringe with ice-cold 4% PFA and attach a gavage needle to it. Gently fill each intestinal segment for immediate fixation (*see* **Note 20**).
6. Transfer each segment into a 15-mL centrifuge tube. Fill each tube full of 4% PFA. Allow fixation for 4 h at 4 °C (*see* **Note 21**).
7. Transfer the intestinal tissue on a cardboard and orient each intestinal segment such that the proximal (stomach) end is closest to the operator. Then, use a pair of forceps to hold one end of the small intestine with gentle tension, and with another, use scissors to cut open the intestinal segment longitudinally in a straight line along the mesenteric line (*see* **Note 22**).
8. Keep the luminal side of the intestinal segment facing upward and the segment flat open on the cardboard.

9. Hold the distal (cecum) end with a pair of forceps and wrap the edge of the distal end around a toothpick held with another hand. Gently roll the toothpick with fingers to wrap the intestinal segment around the toothpick to form a "Swiss roll" (*see* **Note 23**). Be sure to overlay each successive rolled layer so that the edges are flush. Avoid rolling the intestinal tissues too tightly as this will lead to compression of the villi and degrade the morphological appearance.
10. Upon rolling up the entire intestinal segment, slide the Swiss roll off the toothpick gently with a pair of forceps (*see* **Note 24**). Place the Swiss roll in the original 15-mL centrifuge tube filled with 4% PFA overnight at 4 °C for fixation (*see* **Note 25**).
11. On the next day, transfer the fixed tissue to a clean Petri dish and rinse the tissue with ice-cold PBS until the fixative is completely removed. Place the cleaned tissue in an embedding cassette.
12. Proceed with processing the PFA-fixed tissues within 2 weeks to avoid potential tissue damage. Embed the processed tissue in paraffin with the tissue oriented on its side such that the entire length of each intestinal segment is exposed.
13. Cut 4-μm-thick sections with a microtome and collect the sections on positively charged adhesive slides (*see* **Note 26**). Bake the slides in a 55 °C oven for 2 h. Either let cool to room temperature or keep in 4 °C until using them for staining.

3.7 Immunohistochemical Staining for Eosinophils and Foxp3+ Cells

1. Deparaffinize tissue sections of slides by immersing the slides sequentially in xylene and ethanol solutions in the following order: two baths of xylene for 5 min each; one bath each of 100%, 95%, 70%, 50%, and 30% ethanol for 1 min each. Place the slides in a water bath and rinse gently in running water for 1 min (*see* **Note 27**).
2. For Foxp3 staining only, perform heat-induced antigen retrieval. Boil 500 mL Tris-EDTA buffer in a 1-L glass beaker with a microwave. Place the beaker with the boiled buffer on a hotplate with a high-heat setting. Transfer the deparaffinized slides from tap water to the boiled buffer and heat the slides for 10 min.
3. Remove the beaker from the hotplate with the slides still in it and allow the slides and Tris-EDTA buffer to cool to room temperature.
4. Circumscribe tissue sections on the slides with a hydrophobic barrier pen.
5. Block endogenous peroxidases with a hydrogen peroxide solution at the following concentrations in PBS:

(a) For eosinophil staining: immerse slides in Coplin jar filled with 3% hydrogen peroxide in PBS for 8 min at room temperature.

(b) For Foxp3+ cell staining: immerse slides in Coplin jar filled with 0.3% hydrogen peroxide in absolute methanol for 30 min at 4 °C.

6. Wash slides once with PBS for 5 min on a rocker.
7. For eosinophil staining only, incubate each tissue section with 200 μL of the pepsin solution for 5 min at room temperature. Wash slides thrice with PBS, each for 5 min on a rocker.
8. Block each tissue section with 200 μL of the blocking buffer for 1 h at room temperature. Wash slides once with PBS for 5 min on a rocker.
9. Add 100 μL of diluted primary antibody (*see* **Note 28**) to tissue section slides that have been appropriately pretreated for the particular antibody (*see* **steps 2**, **5**, and 7 above).

   (a) For eosinophil staining: dilute rat anti-mouse MBP antibody at 1:500–1:15,000 in the blocking buffer.

   (b) For Foxp3+ cell staining: dilute rat anti-mouse Foxp3 antibody at 1:100 in 0.1% Triton-X 100 in PBS.

   Incubate the slides in a moisture chamber/slide tray overnight at 4 °C to prevent drying of the slides that may result in inconsistent staining (*see* **Note 29**).

10. Rinse slides with PBS once quickly, then wash the slides with PBS thrice, each for 5 min on a rocker. Apply 100 μL of HRP-conjugated anti-rat IgG antibody to each tissue section for 30 min at room temperature.

    (a) For eosinophil staining: 1:250 dilution in the blocking buffer.

    (b) For Foxp3+ cell staining: 1:100 dilution in 0.1% Triton-X 100 in PBS.

11. Rinse slides with PBS once quickly, then wash the slides with PBS thrice, each for 5 min on a rocker. Bring the DAB substrate solution to room temperature before use and incubate each tissue section slide with 100 μL of DAB color development substrate until a brownish color is developed.
12. Discard the DAB substrate, and then rinse slides with PBS once followed by washing the slides in distilled water thrice, each for 5 min on a rocker (*see* **Note 30**).
13. Subject slides to hematoxylin counter stain, and then rinse the slides under running tap water for 5 min. Dehydrate the slides by sequentially immersing the slides as follows: rinse in 70% and 95% ethanol; two baths of 100% ethanol for 2 min each;

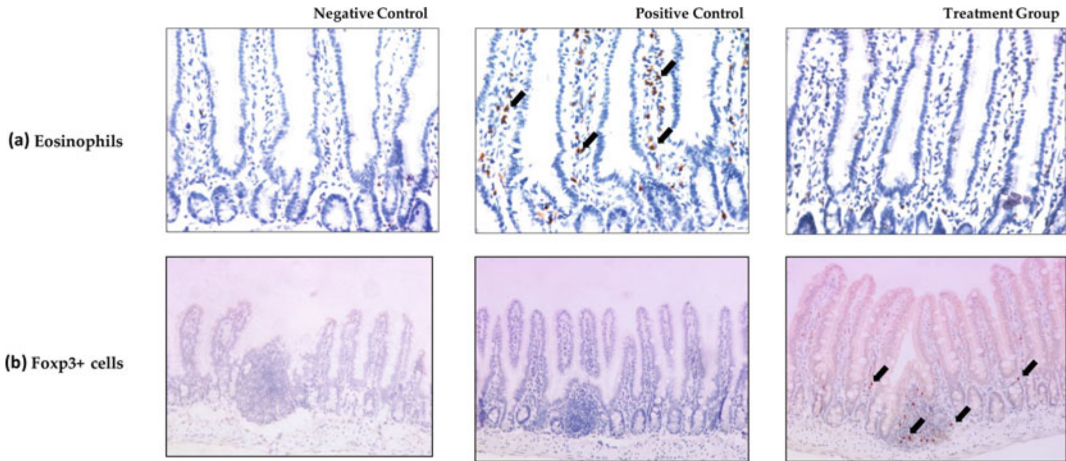

Fig. 2 Immunohistochemical staining of eosinophils and Foxp3+ cells. Representative photomicrographs of (**a**) eosinophils (×400 magnification) and (**b**) Foxp3+ cells (×200 magnification). Black arrows indicate eosinophils and Foxp3+ cells stained brown in IHC

one bath of 50% xylene/absolute ethanol for 2 min; and two baths of xylene for 2 min each.

14. Allow the slides to air-dry. Mount glass coverslips onto the slides with Permount.
15. Select random fields on the slide and capture images at 200× (Foxp3) and 400× (eosinophils) magnifications by an investigator unfamiliar with the key codes on the slides.
16. Count the number of cells stained brown (Fig. 2) using the ImageJ software, and express the cell count as number of cells/mm^2 of villi or crypt area.

4 Notes

1. All peptides should first be dissolved in ultrapure water. If the peptide cannot be completely dissolved, add 10–30% acetic acid solution or trifluoroacetic acid (TFA; <50 μL) to solubilize if the overall charge of the peptide is positive. For peptide that has a negative overall charge, add NH_4OH (<50 μL). Add organic solvents like acetonitrile, methanol, or isopropanol to solubilize peptide that have an overall charge of zero. Add a small amount of dimethylsulfoxide (DMSO) for insoluble and highly hydrophobic peptides. 6 M guanidine–HCl or 8 M urea can be added for peptides that tend to aggregate.
2. Use autoclaved PBS to prepare the 4% PFA fresh and use within 2 days. Paraformaldehyde is recommended for fixation; the use of standard fixatives such as 10% buffered formalin yields intestinal tissue that is stiff and increases difficulty in bundling the

intestinal tissue and subsequent immunohistochemical staining.

3. The minimum recommended volume of allergens and peptides for sensitization, treatment, and challenge is 350 μL. While the maximum volume should not exceed 600 μL, dead volume within the syringe and gavage needle need to be considered when calculating the total amount of sensitization solution.
4. A 1-week acclimatization period is recommended before commencing the sensitization procedures. Mice should be provided with a diet free of the allergen used for sensitization.
5. In our general experience, sensitization success rate is approximately 70–90% with the intragastric route and depending on the source of cholera toxin used. Blood sampling 1 week after the last sensitization or 24 h after the first allergen challenge for measuring allergen-specific IgE levels with ELISA (*see* Subheading 3.2) is recommended prior to choosing candidates for the immunotherapy stage. Mice with allergen-specific IgE ELISA reading of equal to or greater than 0.3 OD (optical density) at 450 nm should be selected.
6. Introduction of allergen challenge 1–2 weeks posttreatment only evaluates the short-term effects of the peptide immunotherapy. An allergen challenge at 1–3 months off-treatment is recommended to examine the long-term effects of the peptide immunotherapy.
7. We also recommend introducing an allergen challenge after sensitization but before peptide treatment to compare the changes in antibody levels and immediate allergic symptoms within the same animal.
8. The changes in rectal temperature in challenged mice showing hypersensitivity reaction can be up to −8 °C.
9. Most common allergic symptoms in challenged mice include continuous scratching and rubbing around the nose, head, and tail for over 15 s, puffiness around mouth, and reduced activity. Convulsion is also common in mice sensitized through the intraperitoneal route using $Al(OH)_3$ as adjuvant. Postchallenge death in mice is not common except for peanut allergy.
10. Positive and negative controls should always be included along with the samples to ensure proper interpretation of results. Include "blank controls" by coating dedicated microwells with the culprit allergen and blocking with the blocking buffer. No serum samples or detector antibodies are added to the blank control wells, but only blocking buffer is added in place. "Nonspecific binding controls" should also be added to assess the contribution of the labeled detector antibody. The nonspecific binding control microwells are coated with

the culprit allergen and blocked with the blocking buffer. No serum sample is added, but the addition of detector antibodies is included as usual.

11. Improper plate-washing can lead to inconsistent results, low signals, or high background across replicate samples and control wells. For all washing steps, use approximately 400 μL/well of PBS-T or PBS with thorough aspiration of microwell contents. Allow the wash buffer to sit in the wells for 15–30 s before aspiration. Blot-dry on tissue papers between each wash. To minimize cross-well contamination, avoid over-flooding of microwells during the wash steps. If alkaline phosphatase-conjugated antibodies are used for colorimetric reactions, use Tris-buffered saline instead of PBS.
12. Suboptimal dilutions of serum and detection antibodies can also lead to inconsistent results. Optimization of serum and detection antibody dilutions can be accomplished by serial dilution of the serum and/or detection antibody across the plate [20].
13. Incubate plate at room temperature until the desired color develops or stop the reaction before any well displays a green product. Color development usually takes 15–30 min at room temperature.
14. The expected OD signal for blank control wells is as low as zero. High ODs in blank control wells may indicate a plate-washing problem. The desired OD signal for nonspecific binding control wells is slightly over the blank control wells but not over the sample wells. High ODs in nonspecific binding control wells may indicate improper blocking procedures and/or concentrations of the detection antibodies. Increasing the concentration of FBS up to 8% or replacing FBS by Bovine Serum Albumin (BSA) may reduce nonspecific binding signals. Optimize dilutions of detection antibody as described in **Note 11**.
15. All procedures should be performed under sterile conditions in a biological safety cabinet to prevent contamination.
16. Immediate processing of the spleen into single-cell suspensions is recommended. If immediate processing is not possible, keep the spleen in 5 mL of the complete culture medium in a clean 15-mL centrifuge tube at 4 °C to maintain cell viability. However, avoid temperature shock from ice and warming up to room temperature.
17. Lift the cell strainer off the bottom of the Petri dish regularly during the straining process to allow the cells to pass through.
18. Gentle pipetting is recommended to avoid damaging the cells when resuspending the cells during each washing step.

19. The anticipated recovery of live lymphocytes from the spleen per mouse is 5–15 $\times$ 10^7 cells.
20. Delay in fixation would lead to irreparable damage to the small intestine tissue. Immediately place the intestinal segments into iced-cold PBS and instill 4% PFA into the gut lumen within 10 min to reduce potential tissue damage.
21. Before cutting intact intestinal segments open, fixation in 4% PFA for 3–4 h is recommended to minimize distortion of the villi and to prevent unrolling of the intestinal tissues. Avoid overnight fixation of intact intestinal segments in 4% PFA as overnight fixation yields stiff intestinal tissues that are too hard to roll. Flexibility of the tissue is critical in the Swiss roll technique.
22. When cutting open the intestines, uneven edges make it more difficult to keep the intestine flat and result in poorer bundling of the tissue. Keep a steady but gentle tension on the intestine with forceps and cut smoothly and steadily to give a straight edge.
23. Bundling all intestinal segments from distal (cecum) to proximal (stomach) ends is recommended to minimize compression of the villi and for estimating the anatomical location in histological analysis.
24. Unrolling of the bundled intestinal tissues represents a common problem; however, pinning of the intestinal roll is not recommended, as this would degrade the fixation quality.
25. Avoid overfixation as this leads to masking of epitopes and failure of immunohistochemical staining. A maximum of 24-h fixation in 4% PFA is recommended.
26. Adhesive slides are recommended over uncoated slides to reduce the tendency of tissue detachment especially when heat-induced epitope retrieval is required prior to immunohistochemical staining.
27. Use fresh xylene in deparaffinization to prevent high background staining. Warm the slides in a 55 °C oven for 10–15 min to soften paraffin if the slides were stored at 4 °C prior to use.
28. Optimization of the dilution of the primary antibody for IHC is required. The recommended dilution for rat anti-mouse MBP antibody for eosinophil staining is between 1:500 and 1:15,000, whereas that for anti-mouse Foxp3 antibody is at 1:100. The dilution adopted in our experiment for eosinophil staining is 1:15,000 with overnight incubation for maximum signal.
29. An isotype control antibody should be included as a control to rule out nonspecific antibody staining.

30. Optimization of the incubation time with DAB substrate is recommended to maximize signal while minimizing background noises. Recommended incubation time for eosinophil staining is 1 min 30 s and 4 min for Foxp3+ cell staining. Handle DAB with great caution for it is potentially carcinogenic. Discard used DAB substrate solution into a designated container and dispose of it according to the guidelines of your institution.

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