



Regulatory and IgE⁺ B Cells in Allergic Asthma

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Abstract

Allergic asthma is triggered by inhalation of environmental allergens resulting in bronchial constriction and inflammation, which leads to clinical symptoms such as wheezing, coughing, and difficulty breathing. Asthmatic airway inflammation is initiated by inflammatory mediators released by granulocytic cells. However, the immunoglobulin E (IgE) antibody is necessary for the initiation of the allergic cascade, and IgE is produced and released exclusively by memory B cells and plasma cells. Acute allergen exposure has also been shown to increase IgE levels in the airways of patients diagnosed with allergic asthma; however, more studies are needed to understand local airway inflammation. Additionally, regulatory B cells (B_{regs}) have been shown to modulate IgE-mediated inflammatory processes in allergic asthma pathogenesis, particularly in mouse models of allergic airway disease. However, the levels and function of these IgE⁺ B cells and B_{regs} remain to be elucidated in human models of asthma. The overall objective for this chapter is to provide detailed methodological, and insightful technological advances to study the biology of B cells in allergic asthma pathogenesis. Specifically, we will describe how to investigate the frequency and function of IgE⁺ B cells and B_{regs} in allergic asthma, and the kinetics of these cells after allergen exposure in a human asthma model.

Key words Regulatory B cells, IgE⁺ B cells, Allergic asthma, Inhaled allergen challenge, Airway inflammation, Sputum, Human immunology

1 Introduction

1.1 Allergic Asthma

Asthma is a chronic inflammatory disorder of the lower airways, with a high degree of variability in its severity, natural progression, response to treatment, and degree of bronchial hyperresponsiveness and airflow obstruction likely secondary to a complex integration of inflammatory mechanisms [1]. Allergic asthma, the predominant form of asthma in childhood, is the result of hyperactive responses to repeated exposure to common environmental allergens. These exposures, in turn, lead to the development of allergen-specific memory T- and B-cell responses and the resulting production of inflammatory mediators [1] that aid in the recruitment of allergic immune effector cells including T and B cells [2]. Activated CD4⁺ T cells release type 2 cytokines which drive

many of the inflammatory processes in allergic asthma. Specifically, IL-4 and IL-13 play pivotal roles in the synthesis of IgE by B cells, while IL-3 and IL-5 lead to differentiation and proliferation of eosinophils, mast cells, and basophils, all key effector cells contributing to asthmatic bronchial hyperresponsiveness [3, 4].

Bronchoprovocation with inhaled allergen in subjects with asthma has proven to be an effective model to study the intricate pathways associated with the development of allergic asthma [5, 6]. It is postulated that the development of allergic disease is a result of the initial allergen exposure priming the immune system and leading to a shift toward type 2 inflammation mediated by IgE-dependent pathways [1]. Allergen presentation by antigen-presenting cells (APCs) to naïve CD4⁺ T cells is a pivotal event in the development of allergic responses [4]. Synthesis by B cells of antigen-specific IgE which binds to high-affinity IgE receptors (FcεRI) on the surface of dendritic cells, granulocytic cells, such as mast cells and basophils, and becomes cross-linked by allergen, initiates cell activation and release of their inflammatory mediators, particularly histamine, leukotrienes, and the type 2 cytokines IL-4, IL-5, and IL-13. This leads to the influx of CD4⁺ T cells and eosinophils into the airways [6], and thereby perpetuating type 2 inflammation. Thus, allergic asthmatics have been found to have higher levels of lymphocytes, eosinophils, basophils, and mast cells in their airways [6]. Eosinophilia is widely recognized as a hallmark of asthma, and fluctuation of these cells in blood and airways can be used to determine disease severity and treatment efficacy [6, 7]. Additionally, asthmatics also have higher levels of airway epithelial desquamation, goblet cell hyperplasia, and thicker basement membranes [4]. Ultimately, asthma has a broad presentation clinically with many different phenotypes, and cellularly with several different endotypes, and therefore, current management strategies must take these factors into consideration. Anti-IgE therapy has been used for several decades for treatment of asthmatic patients with high circulating IgE levels and has insofar proven to be clinically effective in the management of allergic asthma [8]. However, with the recognition of *in situ* IgE synthesis in asthma [9, 10], targeting local sources in the allergic tissue may be a more practical approach for treatment of allergic asthma. Thus, development of novel targets to regulate IgE synthesis for treatment of asthma and other allergic diseases is ongoing [11–13].

1.2 B Cells

Several diseases have B cells at the core of their pathogenesis, including, but not limited to, autoimmune disorders (multiple sclerosis [14, 15], systemic lupus erythematosus [16]), cancer (leukemia [17, 18]), and allergy (rhinitis [19], asthma [20–24]). B cells derive from the differentiation of hematopoietic stem cells. Most of their maturation occurs in the bone marrow, where they differentiate into functional effector or regulatory B cells [25–29].

Thereafter, they are released into peripheral compartments of the body (peripheral blood, secondary lymphoid organs, lymph nodes, and local tissues) [30]. B-cell surface markers allow for their characterization at different stages of development. These markers primarily include CD19, CD20, CD27, CD38, and CD138 [25–27, 31].

1.3 IgE⁺ B Cells

IgM, IgD, IgG, IgE, and IgA form the five isotypes of immunoglobulins produced by B cells, whose expression and function depend on the heavy chain constant region of the B-cell receptor (BCR) [26, 32]. At the initial phases of development, B cells express IgM. Once the B cell encounters an antigen and is co-stimulated with CD40L, it becomes activated. This activation triggers isotype switching, a process facilitated by the activation-induced cytidine deaminase (AID) enzyme through which heavy chain genes are rearranged [32]. The mechanism of class switching involves deamination of residues of gene segments upstream of the heavy chain genes, called switch regions. This deamination produces double-stranded breaks secondary to a break in two switch regions [32]. As a result, certain portions of the heavy chain genes are excised, leaving behind the gene encoding the heavy chain of interest to be expressed [33]. Mu (μ), delta (δ), gamma (γ), epsilon (ϵ), and alpha (α) encode the heavy chains for IgM, IgD, IgE, and IgA, respectively [26, 32].

To reiterate, the pathogenesis of allergic asthma is driven by binding of IgE to Fc ϵ RI and the subsequent activation and degranulation of mast cells and basophils as a result of cross-linking by a specific antigen [34]. In effect, Van de Pol et al. have demonstrated increases in the levels of total serum and allergen-specific IgE following a single inhalation of house dust mite extract by allergic asthmatics [35]. In the airways, Wilson et al. have shown that allergen-specific IgE levels in bronchoalveolar lavage (BAL) were significantly increased following allergen challenge relative to saline challenge. Similarly, Oliveria et al. have shown that sputum supernatant total IgE is higher in allergic asthmatics compared to controls, and further increased at 7 hours post-allergen challenge [9, 10, 36]. It can therefore be concluded that B cells, through their production of IgE, play a key role in the local airway inflammation characteristic to allergic asthma.

1.3.1 IgE⁺ B-Cell Kinetics and Compartmental Distribution

There are very few human studies evaluating IgE⁺ B cells; however, we have gained insight from studies of murine models. Memory B cells (MBCs) and plasma cells (PCs) were studied in a murine parasitic infection model by Nduati et al. [37]. Their results showed that although MBCs were detectable in the blood at all times, PCs were only detectable shortly after parasitic infections (primary and secondary) and were thus highly time-sensitive with respect to the challenge [37]. In this regard, it can be alluded that a short time

frame is needed to mobilize B cells into the blood following an inflammatory event. Similarly, Luger et al. found that mice who were given mucosal allergen challenge with ovalbumin (OVA) induced the production of PCs in the lung, spleen, and bone marrow but only for a short period following the challenge [38]. However, in both Luger et al. and Nduati et al.'s studies, a small population of those PCs were found to be long-lived in the spleen and bone marrow. These PCs were found to produce OVA-specific IgE, IgA, and IgG₁ [38]. Likewise, Chvatchko et al. demonstrated that, following inhalation challenge, murine B cells in the lungs were triggered toward increased antigen-driven differentiation, and, more specifically, toward OVA-specific IgE and IgG₁ production [39]. Despite these studies, the kinetics of B cells through various bodily compartments, such as lungs, blood, lymphatics, bone marrow, at different time points following challenges, remains to be elucidated, even in murine models. A further understanding of the expansion of B cells in asthma remains to be discovered. Although IgE⁺ B cells are very rare and difficult to enumerate, advances in flow cytometry have allowed for their measurement in humans [40]. Oliveria et al. have been able to detect IgE⁺ B cells in peripheral blood, bone marrow, tonsil, and airways by flow cytometry [10] and conducted studies to compare B-cell subsets across disease states (healthy controls, allergic non-asthmatics, and asthmatics), to examine the kinetics of these cells in peripheral blood (systemic compartment), bone marrow (hemopoietic compartment), tonsils (lymphatic compartment), and locally in sputum (airways compartment) after bronchoprovocation with inhaled allergen [9, 10]. They demonstrated higher levels of B cells and IgE⁺ B cells in the airways of allergic asthmatics compared to controls, but found no differences in blood levels of these cell types. Furthermore, sputum B cells were positively correlated with sputum eosinophils, and sputum supernatant IgE and BAFF [9]. In subjects with mild allergic asthma, levels of B cells and IgE⁺ B cells were noted to be elevated in the airways 7- and 24-hours post-bronchoprovocation challenge [10]; however no changes were observed in peripheral blood, bone marrow, or tonsils. Now that the levels of IgE⁺ B cells have been reported across disease states, and after allergic provocation of airways in a human in vivo model, there are questions about function, development, and memory of these rare B-cell subsets that need to be explored. Particularly, we still do not know much about the regulation of these IgE⁺ B-cell responses. There are B-cell-specific roles that modulate inflammation through cytokine or antibody-mediated processes, and these need to be explored within the context of allergy.

1.4 Regulatory B Cells (B_{regs})

In the early 1970s, the first postulated suppressive function of B cells was demonstrated through the transfer of B-cell-depleted splenocytes into OVA-sensitized guinea pigs which resulted in a decreased ability to dampen the inflammatory response [41, 42]. The exact mechanism by which the inflammatory response is dampened had not been further investigated until recently, wherein, in 1996, Wolf et al. demonstrated that B-cell-deficient mice could not recover from experimental autoimmune encephalomyelitis (EAE) [43, 44], suggesting a subset of B cells is required for immunomodulation. Similar observations were made regarding the B-cell-dependent immunomodulation in mouse models of inflammatory bowel disease [45], autoimmune diseases [46], and allergy [4, 47]. Interestingly, models of colitis [45], EAE [48], and arthritis [49] have found IL-10 to be a primary immunomodulatory cytokine, supporting the role of IL-10-producing B cells in these diseases. However, regardless of the disease, there is no universally accepted regulatory B-cell (B_{reg}) phenotype in the literature. Furthermore, the study of B_{reg} subsets and their functional mechanisms, specifically in allergic asthma, is still in its infancy and plenty of questions remain unanswered.

1.4.1 Development of B_{regs}

Significant attention was brought to the origin and function of B and T cells following the discovery of distinct lymphocyte subpopulations as unique aspects of the adaptive immune response over 40 years ago [50]. Cells expressing surface immunoglobulin receptors specific to antigenic epitopes are characterized as B (*bursal*) lymphocytes [50] and were demonstrated, in the early 1960s and 1970s, to be primarily responsible for the basic functions of antibody production and humoral immune responses [43]. However, several years later, as studies on B cells continued, complexities in their development, differentiation, maturation, and responses were observed. Of note was the role of B cells as antigen-presenting cells whereby they present peptides to T cells via MHC molecules on their surfaces [50]. Additionally, it was demonstrated that B cells have the ability to secrete a diverse array of cytokines that influence immune cell function and inflammatory processes [50]. B cells were found to have the ability to mount a robust secondary response with higher affinity to antigens through the process of somatic hypermutation within germinal centers [50]. The discovery of long-lived plasma cells and memory B cells provided crucial advancement in the understanding of the role of B cells in adaptive memory [51, 52]. B cells were found to have duality in their role in allergy and inflammation, wherein they possess the ability to class switch to IgE isotype in type 2 inflammatory environments and the further induction of the inflammatory cascade by IgE. Nevertheless, the recent discovery of regulatory B cells shed a different light [2]. Since the study of regulatory B cells is relatively novel in literature, the development and ontogeny of these cells remain to be elusive and future research is required.

1.4.2 Functions of B_{regs}

A paradigm shift was created upon the discovery of a tolerogenic role for B cells which consequently also shifted our understanding of B-cell function and role in diseases. Stimulation by TLR ligands, such as CpG and LPS, CD40:CD40L axis, and BCR agonists, activate B cells toward a regulatory phenotype [43]. The B_{regs} ability to produce IL-10, either constitutively or in response to stimulation, is often the definition of “activated” in the literature, though this definition can vary. The immunomodulatory function of B_{regs} has primarily been studied, in both mice [53] and humans [54], through its ability to modulate T-cell function in favor of a regulatory phenotype. Additionally, B_{regs} have also been shown to play an indirect immunomodulatory role in suppressing inflammatory processes through their ability to induce regulatory phenotypes in a variety of other immune cell subsets (e.g., DCs, T cells, and mast cells) [55]. Through their ability to produce anti-inflammatory cytokines (e.g., IL-10, TGF- β , and IL-35), B_{regs} indirectly aid in the suppression of Th1 and Th17 differentiation of CD4⁺ T cells [55]. B_{regs} also play a crucial role in the maintenance of T_{reg} populations [24, 56]. When analyzing both the T_{reg} and B_{reg} regulatory functions, they have many distinct similarities and differences. Similar to the thymus-derived T_{regs} , the expression of genes responsible for the suppressive phenotype of B_{regs} , a distinct lineage of B cells, is influenced by specific factors [43]. However, the development and ontogeny of these cells remain unclear [43]. Like T_{regs} , B_{regs} possess regulatory capabilities through their ability to secrete anti-inflammatory cytokines that provide direct exhaustion and deletion of effector cells [57–60]. However, it is still unclear by which mechanism regulatory B cells elicit this function, and further research is required. Nevertheless, through their IgG₄ isotype, a type of blocking antibody that competes with the binding of IgE to allergen, B_{regs} have the unique ability to suppress the humoral immune response [61]. Despite the vast and continually growing knowledge of the role of B cells in immunity that we have gained in the literature, more remains to be elucidated and the persistence of scientific endeavors is crucial to providing clarity into the suppressive nature of B_{regs} .

1.4.3 IL-10 Production by B_{regs}

IL-10, produced primarily by clonally expanded Tr1 (regulatory T cells) and professional antigen-presenting cells, and at times also by mast cells, monocytes, and NK cells, is an important anti-inflammatory cytokine [62, 63]. IL-10 possesses anti-inflammatory properties through its ability to suppress naïve T-cell activation by blocking co-stimulatory signaling through CD2, CD28, and inducible T-cell co-stimulation (ICOS) in a rapid signal transduction cascade [62, 64, 65]. Specifically, CD28 tyrosine phosphorylation is inhibited which prevents the binding of phosphatidylinositol 3-kinase p85 [65, 66]. As a result, the entire

CD28 signaling pathway is inhibited, and thus, T-cell co-stimulatory pathways [65, 66]. Furthermore, IL-10 hinders the type 1 immune response by inducing expression of the suppressor of the cytokine signaling 3 (SOCS3) gene and the resulting inhibition of IFN- γ -induced phosphorylation of STAT1 [67]. IL-10 has the capacity to suppress total and allergen-specific IgE antibodies, and skew the humoral response from the IgE isotype to the non-inflammatory isotypes, IgG₄ and IgA, and hence plays a critical role in allergy and immune tolerance [68–70].

In summary, the ability to suppress activation of effector cells, production of inflammatory cytokines, and hence inflammation in general, characterizes IL-10 as an inflammatory cytokine [71]. In fact, evidences in allergic asthma demonstrating the capacity of regulatory T cells to suppress inflammation through secretion of IL-10 have been reported [72, 73]. The term regulatory B cell was coined in the early 2000s following the discovery of a subset of B cells with the capacity to produce IL-10 [74]. Currently, the terms regulatory B cells or B_{regs} and B10 cells are acceptable nomenclatures for IL-10-producing B cells. However, the phenotype of B_{regs} creates controversies in its studies due to its wide variability [75, 76]. Nevertheless, the commonly accepted attribute of B_{regs} is their ability to produce IL-10. In Iwata et al.'s study, B_{regs}, identified with the CD19⁺CD24⁺CD27⁺ phenotype, were studied in both mice and humans and were found to be competent in the production of cytoplasmic IL-10 [76]. However, there is no clear knowledge of the pathways leading to the manifestation of these B cells. Additionally, B_{regs} were found to produce antigen-specific antibodies, specifically IgG₄, as was demonstrated in the study of Maseda et al., thus indicating that B_{regs} have the dual role of producing IL-10 as well as antigen-specific antibodies [61, 77, 78].

James et al. and van de Veen et al. demonstrated that IgG₄, produced by B cells following the induction of IL-10, can act as a blocking antibody for IgE-specific antigens. By doing so, the downstream IgE-mediated allergic cascade is inhibited. In fact, in James et al.'s study, grass pollen allergens were neutralized by IgG₄ antibodies, which subsequently resulted in reduced IgE-mediated events [77]. Similarly, in van de Veen et al.'s study, inflammatory immune responses induced by bee venom were dampened through IL-10-producing B_{regs} and their production of IgG₄ antibodies [61].

In summary, IL-10 induces B_{regs} to produce IgG₄ and together, these anti-inflammatory mediators suppress CD4⁺ T-cell proliferation, IgE, and the allergic cascade [61, 77]. In a study of cow's milk allergy, Noh et al. found that B_{regs} produced IL-10 when stimulated with allergen. However, this same stimulation also increased apoptosis in the regulatory B-cell subpopulation [79–81]. Although numerous and phenotypically diverse

regulatory B-cell subpopulations exist, they are all defined by their ability to produce IL-10 and their resulting significant roles in many autoimmune diseases, allergies, cancers, and infectious diseases. The phenotype and function of B_{regs} involved in such pathways, particularly with regard to allergic asthma, remain to be discovered.

1.4.4 Role of B_{regs} in Allergic Disease

Allergic diseases have complex and vastly heterogeneous pathogenesis. Type 2 inflammatory responses to a specific allergen mediated by ways of sensitization and development of B- and T-cell responses are the means by which allergic diseases are established [2]. Hence, the focus of novel therapies are to shift the type 2 inflammatory response toward a healthy tolerogenic state [47, 82–84], which can be accomplished through various immunomodulatory approaches [2]. The in vivo induction of tolerance to the regimented and controlled administration of increasing allergen concentrations, called allergen immunotherapy (AIT), is a potentially curative therapy for allergic disease and represents a clinical model for tolerance induction [2].

Two mechanisms describe the role of B_{regs} in the allergic response: the cell-mediated and the humoral suppressive responses. The cell-mediated suppressive response is carried through the release of anti-inflammatory mediators (e.g., IL-10 and TGF- β). The humoral suppressive response is classified by the B cells' ability to isotype switch and produce IgG4 blocking antibodies.

The earliest discoveries of the role of B_{regs} in allergies occurred in guinea pigs through ovalbumin (OVA)-induced skin hypersensitivity reactions [41]. Similarly, a study in 1998, examined the frequency of IL-10⁺ B cells in patients with bee venom allergy receiving immunotherapy and is the human study to first note evidence for B_{regs} [68]. Compared to IL-10⁻ B cells, transcription analysis identified IL-10⁺ B cells as being CD19⁺CD73⁻CD25⁺CD71⁺ [61].

These IL-10⁺ B cells were appropriately named B_{regs} given that they are similar to Tr1 cells with regard to their ability to suppress antigen-specific T-cell proliferation via the IL-10-mediated response [61]. Those with bee venom allergy undergoing immunotherapy in the aforementioned study were found to have a two- to fivefold increase in the Br1 CD19⁺CD73⁻CD25⁺CD71⁺IL-10⁺ B-cell subset, specific for the major bee venom phospholipase A2 (PLA) approximately 4 months post-therapy [61]. In conclusion, following high-dose allergen exposure, we assist to an expansion of B_{reg} populations and thus to the maintenance of a tolerogenic response to specific allergens [61]. Moreover, B_{regs} have the ability to increase IgG₄ production relative to serum IgE levels, thus demonstrating humoral effects in allergic disease. B_{regs} are central to the humoral effects in allergic disease [61]. In fact, in the

aforementioned study, IL-10 played an integral role in the B cells' ability to differentiate into the IgG₄-producing plasma cells and resulted in a 100-fold decrease in the IgE/IgG₄ ratio [61]. Hence, there is sufficient evidence to support the notion that B_{regs} play an integral part of the modulation of allergic disease, both through their ability to suppress effector T cells and skew the humoral response through the IgG₄ isotype. This has expanded our understanding of allergic disease and shows promise in the development of future therapies.

Through limited studies in allergic airway inflammation, we have gained tremendous insight into the mechanistic role played by B_{regs} [85]. Murine models of house dust mite (HDM) airway hyperresponsiveness have demonstrated that CD9⁺ B cells were crucial to the production of suppressive mediators [86]. B1 cells, plasma cells, and marginal zone B cells are shown to express the adhesion molecule CD9 [86]. The adoptive transfer of CD9⁺ B cells results in the suppression of Th2- and Th17-mediated inflammatory responses through IL-10-mediated mechanisms [86]. Additionally, FasL expression was upregulated on CD5⁺ B1 cells following stimulation with cockroach allergen and mediated the apoptosis of effector CD4⁺ T cells and thus controlled allergen-induced airway inflammation [23]. Furthermore, human trials have shown that the suppression of AHR-mediated inflammation is highly regulated by IL-10⁺ B_{regs}. Specifically, IL-10 secretion resulted in the reversal of allergic airway inflammation through CD19⁺CD1d^{hi}CD5⁺CD21^{hi}CD23⁺IgD⁺IgM^{hi} B_{regs} and positive expression of CD1d [87]. B_{regs} also recruit T_{regs} to the lungs, further suppressing airway inflammation [24, 85].

Further emphasizing the importance of B_{regs}, Amu et al., Sing et al., and Lundy et al. found that B_{reg}-deficient mice had higher levels of serum IgE, type 2 cytokines (IL-4 and IL-5), and eosinophilia [23, 24, 85]. They also found that adoptive transfer of B_{regs} into mice with allergic airway disease reversed the inflammatory phenotype [23, 24, 85].

In summary, B_{regs} produce IL-10 resulting in the production of IgG₄ and the recruitment of T_{regs} into the lungs, thus suppressing the allergic cascade and airway inflammation [23, 24, 85]. However, research regarding the role of B_{regs} in the pathogenesis and pathobiology of allergic asthma is limited and remains to be further elucidated.

1.4.5 Phenotypes of B_{regs}

Despite our partial understanding of the role of B_{regs} in various disease states, we still do not have a defined characterization of the B_{reg} phenotype. Moreover, the exact mechanism of their inception within specific disease contexts has not yet been elucidated. Currently two hypotheses have been considered: one states that B_{regs} are specific to a distinct B-cell lineage, whereas the other states that

B_{regs} are derived from a certain immunological microenvironment inducing B cells to take on this specific phenotype and function. In this light, a primary aim of future research in this field will be characterizing these phenotypes and highlighting those with the most suppressive functions to further understand their role in diseases. In a B_{reg} phenotype study by Oliveria et al., these phenotypes were evaluated, particularly $\text{FoxP3}^+ B_{\text{regs}}$ were found to be lower in level in allergic asthmatics compared to controls. Furthermore, $\text{FoxP3}^+ B_{\text{regs}}$ were evaluated in bone marrow, blood, and sputum, and it was demonstrated that at 7 hours post-challenge, $\text{FoxP3}^+ B_{\text{regs}}$ increased in the blood, and at 24 hours post-challenge they decreased in the bone marrow and increased in the airways [88]. All together, these findings suggest that B_{regs} are trafficking to the airways after allergic stimulation. Oliveria et al. also conducted in vitro studies evaluating the role of IL-4 stimulation of B cells and found that IL-4 stimulation increases the levels of $\text{FoxP3}^+ \text{IL-10}^+$ B cells after 72 hours of culture [88]. Thus, the allergic cascade has a role in the propagation of regulatory B cells and may be transiently increasing their functions to mitigate inflammatory mechanisms, but more comprehensive studies need to be done to confirm these conclusions. Particularly, for allergic diseases, we would benefit from gaining knowledge regarding B_{reg} trafficking and compartmentalization in the early and late phase of the allergic response. Among the investigated phenotypes, $\text{CD1d}^+ \text{CD5}^+ B_{\text{regs}}$, $\text{CD5}^+ \text{FoxP3}^+ B_{\text{regs}}$, and $\text{CD24}^+ \text{CD38}^+ B_{\text{regs}}$ have shown promise in reversing allergic airway inflammation, particularly in human and murine models.

$\text{CD1d}^+ \text{CD5}^+ B_{\text{regs}}$

$\text{CD1d}^+ \text{CD5}^+ B_{\text{regs}}$ represent a splenic B-cell subset which exerts immunomodulatory effects through the production of IL-10 and plays a crucial role in the suppression of the acute phase reaction in contact hypersensitivity [46, 89, 90]. Moreover, it has been demonstrated that, in mice, $\text{CD19}^+ \text{IL-10}^+ \text{CD1d}^{\text{hi}} \text{CD5}^+ \text{CD21}^+ \text{CD23}^+ B_{\text{regs}}$ suppress allergic airway inflammation through the secretion of IL-10 and expression of CD1d [87]. Furthermore, murine and human models of autoimmune disease have demonstrated the involvement of B_{regs} in their pathogenesis, particularly systemic lupus erythematosus (SLE) and rheumatoid arthritis. In fact, defective CD1d expression resulted in altered B_{reg} stimulation and as a result, impaired invariant natural killer T cell (iNKT) number and function [91]. Normally, natural killer T (NKT) cells are activated through activation of their surface-membrane T-cell receptors (TCRs) secondary to CD1d expression. Overall, the expression of CD1d is a specific marker of B_{reg} that is implicated in both allergic and non-allergic diseases secondary to its unique suppressive pathways [92–94].

CD5⁺FoxP3⁺ B_{regs}

The expression of FoxP3, an important transcription factor in T_{reg} development, was first identified in CD19⁺CD5⁺ B cells by Noh et al. in 2010 [75]. In human PBMCs, CD5⁺ B-expressing FoxP3 made up $8.5 \pm 3.5\%$ of the CD19⁺ B-cell pool and showed a significantly elevated apoptotic frequency of 63.44% [75]. These cells have been extensively studied in food allergy. In one study, subjects allergic to cow's milk protein (e.g., casein) presented decreased CD5⁺FoxP3⁺ B_{regs} upon allergen stimulation compared to milk-tolerant controls who actually showed an increase in this cell population [81]. Conversely, both allergic and tolerant individuals demonstrated an increase in CD4⁺FoxP3⁺ T_{reg}, suggesting that B_{regs} play a much broader role in food allergy beyond merely T_{reg}-mediated suppression [81]. More remains to be discovered regarding the role of CD5⁺FoxP3⁺ B_{regs} in the pathogenesis of allergic asthma.

CD24⁺CD38⁺ B_{regs}

CD24⁺CD38⁺ regulatory B cells constitute a subset of transitional B cells localized primarily in the peripheral tissues [46, 55, 76, 95]. Following stimulation with CD40, these cells produce the highest levels of IL-10 among all other B_{regs} in peripheral blood (CD24⁺CD27⁺ B_{regs}, CD1d⁺ B_{regs}) [46]. Additionally, they are the only subset currently demonstrated to directly suppress Th1 differentiation of CD4⁺ cells which is speculated to be partially dependent on cellular contact between CD80–86 on B cells and CD28 on T cells, and they have the capacity to induce the differentiation of CD4⁺ T cells into T_{regs} or Tr1 cells [95]. According to studies by Blair et al., CD24⁺CD38⁺ B_{regs} from SLE patients were defective in number and showed impaired IL-10 production following CD40 stimulation [95]. These patients were also unable to suppress Th1 responses secondary to defective STAT3 phosphorylation [95]. Although SLE could be the result of genetic predispositions, the above findings lead to the hypothesis that the observed B_{reg} dysfunction is the result of overstimulation secondary to the chronic inflammatory environment leading to B_{reg} exhaustion and loss of function [74]. CD24⁺CD38⁺ B_{regs} also contribute to immune dysfunction in HIV infection where they hinder viral clearance [96]. This subtype of B_{reg} plays an integral role in the establishment of graft tolerance through suppression of effector T cells and thus prevents the development of chronic graft versus host disease [97, 98]. To conclude, B_{regs} have a vast role in various disease processes, and our understanding of their function will potentially give rise to novel therapies and the possibility of creating transplantation tolerance. Nevertheless, their role in allergic disease is still not well understood.

CD24⁺CD27⁺ B_{regs}

CD24⁺CD27⁺ B_{regs} comprise a subset of memory B cells and represent the majority of IL-10-producing B10 cells [46, 55]. The production of TNF- α by circulating monocytes is regulated by these CD24⁺CD27⁺ B_{regs} [76]. It was found that individuals with allergic asthma have a significantly reduced number of CD24⁺CD27⁺ B_{regs} in addition to their defective production of IL-10 upon LPS stimulation relative to healthy patients [29]. As a result, the induction of T_{regs} in response to the Der p1 allergen was weaker, and this could potentially be responsible for the propagation of allergic asthma and hence warrants further research in CD24⁺CD27⁺ B_{regs} [29].

Other B_{regs} Phenotypes

There is no standard definition of the phenotypic characterization of B_{regs}, and as a result, certain established B_{reg} subtypes do not conform to the definition of IL-10-mediated suppression. A subset of plasmablasts identified as CD27^{hi}CD38^{hi} is known as being IL-10-rich and is postulated to have a role in the anti-inflammatory humoral response [99]. Another unique B_{reg} subset, characterized by the CD25^{hi}CD71^{hi}CD73^{lo} phenotype, is postulated to have a role in the induction of allergen tolerance through the production of IgG4 antibodies and the suppression of antigen-specific CD4⁺ T cells [61]. In addition to IL-10-enriched B_{regs}, granzyme B (GrB)-expressing B cells, characterized by the CD19⁺CD38⁺CD1d⁺IgM⁺CD147⁺ phenotype, have been described to exert a suppressive function [100, 101]. Another subtype of B_{regs} characterized by the CD39⁺CD73⁺ phenotype was shown to drive the microenvironment away from an AT-mediated pro-inflammatory environment, through interaction with adenosine [102]. Additionally, the CD35⁺TSP-1⁺ B_{regs} subset has been shown to suppress allergic inflammation in the intestine through their ability to downregulate dendritic cell co-stimulatory molecules via TGF- β production, thus resulting in T_{reg} differentiation [103].

1.5 Summary

To summarize, B-cell subsets play important roles in the promotion and regulation of allergic disease. Future studies are required to further characterize and define subsets of IgE and B_{reg} phenotypes, and in particular, their individual contributions to allergic disease. Given that these cells have been shown to modulate the inflammatory processes in allergic asthma, much remains to be understood in this field. We provide a comprehensive overview of IgE⁺ B cells and regulatory B cells in the studies outlined in this chapter, utilizing a human model of allergic airway inflammation, rare samples spanning the airway, hematopoietic, lymphatic and peripheral compartments, and relevant experimental strategies in the field of immunology (*see* Fig. 1) [9, 10, 88]. Further, the advent of novel technologies being able to study these cells more in depth will allow us to better understand their undiscovered roles in disease.

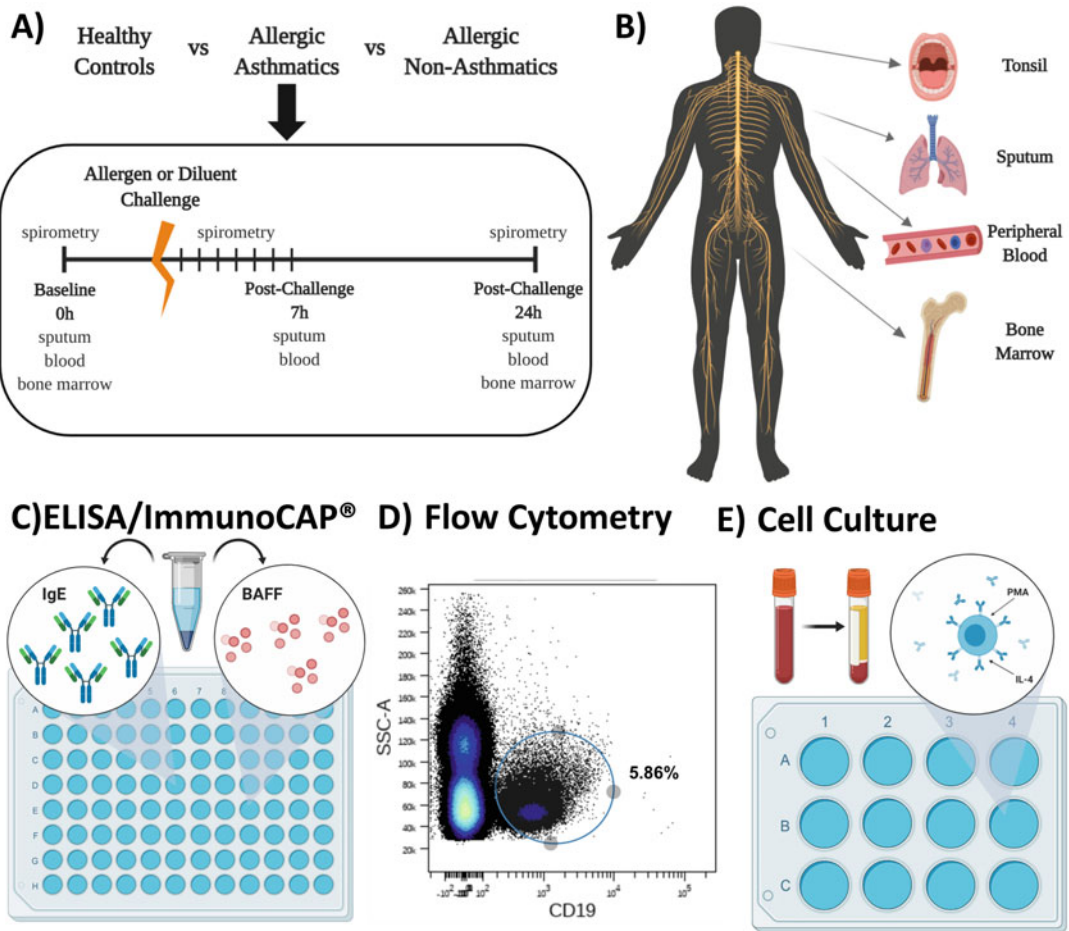


Fig. 1 Experimental workflow evaluating IgE⁺ B cells and regulatory B cells in allergic asthmatics. (a) Groups of individuals evaluated and experimental timeline for the human allergen inhalation challenge. (b) Specimens collected from human volunteers and evaluated for IgE⁺ B cells and regulatory B cells analysis. (c) ELISA and ImmunoCAP measurements for inflammatory mediators including BAFF, total IgE, and specific IgE. (d) Flow cytometry analysis of various sample cell suspensions at baseline between disease groups and pre- and post-allergen challenge. (e) Cell culture techniques employed to stimulate B cells and evaluate their regulatory capacity

2 Materials

2.1 Selection of Participants with Mild Allergic Asthma

2.1.1 Skin Prick Test

1. Histamine (positive control).
2. Diluting solution: 0.9% saline (negative control).
3. Allergen extracts resuspended in diluting solution.
4. Lancet to perform a small abrasion on the skin with the allergen extracts.
5. Pen and ruler to measure the wheal diameters.
6. Alcohol swabs to wipe allergen extract from skin.
7. Anti-histamines as needed by the participants.

2.1.2 Spirometry and Methacholine Challenge Test

1. Methacholine.
2. Wright nebulizer.
3. Electronic spirometer meeting American Thoracic Society/European Respiratory Society requirements.
4. Nose clips to prevent air leakage.
5. Bronchodilator as needed by the participants, to return them to baseline levels.

2.1.3 Allergen Inhalation Challenge

1. Aqueous allergen extracts.
2. Wright nebulizer.
3. 0.9% saline for diluent challenges.
4. Electronic spirometer meeting American Thoracic Society/European Respiratory Society requirements.
5. Nose clips to prevent air leakage.
6. Bronchodilator to ensure individuals are returned to baseline levels.

2.2 Sample Collection and Tissue Preparation

2.2.1 Peripheral Blood Collection and Processing

1. Two 10 mL heparin-coated, green top, vacutainer tubes of whole blood.
2. Cryovials for plasma storage.
3. Frosted, glass slides for smear.
4. 50 mL conical tubes.
5. Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS).
6. Density gradient medium for centrifugation-based isolation of peripheral blood mononuclear cells.
7. Ammonium chloride-based red cell lysing reagent.
8. Sterile disposable 10 mL and 20 mL pipettes.
9. Centrifuge.
10. CD19⁺ isolation kit to isolate and purify B cells (*see Note 1*).

2.2.2 Bone Marrow Aspiration and Processing

1. Bone marrow aspiration needle.
2. Local anesthetic such as lidocaine.
3. 10 mL syringe.
4. 1 mL liquid heparin in syringe.
5. Cryovials for plasma storage.
6. Frosted, glass slides for smear.
7. 50 mL conical tubes.
8. RPMI 1640 medium supplemented with 10% FBS.

9. Density gradient medium for centrifugation-based isolation of peripheral blood mononuclear cells.
10. Ammonium chloride-based red cell lysing reagent.
11. Sterile disposable 10 mL and 20 mL pipettes.
12. Centrifuge.
13. Kit, device (magnet, column, etc.) and solutions for immunomagnetic separation of human CD138⁺ cells (*see Note 2*).
14. 100 μ m sterile filter.

2.2.3 Tonsil biopsy Processing and Cell Isolation

1. Sterile, through-cut forceps.
2. Local anesthetic such as lidocaine.
3. 10 mL conical tubes.
4. Phosphate buffered saline (PBS): 8.00 g sodium chloride, 0.20 g potassium chloride, 1.44 g sodium phosphate, 0.24 g potassium phosphate, 1000 mL distilled water, pH adjusted to 7.4.
5. Petri dish.
6. RPMI 1640 medium supplemented with 10% FBS.
7. Disposable scalpel.
8. Sterile squeeze bulb pipettes.
9. 0.1% collagenase solution: 1 mL of 1% collagenase type I in 9 mL PBS.
10. 37 °C incubator with shaker.
11. 100 μ m sterile filter.
12. Centrifuge.

2.2.4 Sputum Induction and Processing

1. 3%, 4%, and 5% saline.
2. Nebulizer (jet or ultrasonic) with output 0.2–1.0 mL/min.
3. Sterile cups with screw-on caps for sputum expectorant.
4. Petri dish.
5. Sterile forceps.
6. 10 mL and 50 mL conical tubes.
7. PBS.
8. 0.2% sputolysin reagent: a concentrate of dithiothreitol (DTT) in phosphate buffer.
9. Cryovials for sputum supernatant storage.
10. Trypan blue live/dead cell stainer.
11. Hemocytometer.
12. 50 μ m nylon mesh cloth.

13. Frosted, glass slides for sputum cytopins.
14. Cytopsin centrifuge.
15. Diff-Quik™ stain with hematoxylin and eosin (Microptic).

2.3 Circulating Protein Measurements

1. Squeeze bottle for wash buffer.
2. Paper towels and kim wipes.
3. Multi-channel pipettes.
4. Ice box and ice.
5. Human BAFF ELISA kit. Many different suppliers provide ELISA kits of sufficient quality to be used for BAFF detection. We used the BAFF ELISA kit from R&D Systems.
6. Total human IgE ELISA kit. Many different suppliers provide ELISA kits of sufficient quality to be used for BAFF detection. We used the total IgE ELISA kit from Abcam.
7. ELISA plate reader.
8. Phadia® 250 platform by ImmunoCAP® (ThermoFisher Scientific).

2.4 Flow Cytometry Staining

1. Trypan blue live/dead cell stainer.
2. Hemocytometer.
3. Round-bottom, 5 mL, polystyrene tubes with filter caps.
4. Cell staining medium (CSM): 0.5% bovine serum albumin (BSA), 0.1% sodium azide, 5 mM ethylenediaminetetraacetic acid (EDTA) in PBS.
5. Human FcR block reagent. (*see Note 3*).
6. Fluorochrome-conjugated antibodies for flow cytometry staining of surface (*see Table 1*) and intracellular (*see Table 2*) molecules of IgE⁺ B cells and B_{regs}.
7. Homemade or commercially distributed fixation and permeabilization buffers.
8. Centrifuge.
9. Table top vortex.
10. Compensation beads.
11. Flow cytometer and flow cytometry data analysis software.

2.5 B-Cell Isolation, Culture, and Stimulation

2.5.1 B-Cell Isolation

1. Four 10 mL heparin-coated, green top, vacutainer tubes of whole blood.
2. CD19⁺ isolation kit to isolate and purify B cells (*see Note 4*).
3. 50 mL conical tubes.
4. PBS.
5. Magnetic separation system.

Table 1
Surface antibodies used to characterize IgE⁺ B cells and B_{regs} subsets

Target	Clone	Fluorophore
CD4	RPA-T4	APC-H7
CD8	RPA-T8	FITC
CD5	UCHT2	Alexa Fluor 700
CD19	SJ25-C1	APC-Cy7
CD19	MOPC-21	PE-Cy7
CD1d	CD1d42	APC
CD24	ML5	PE-CF594
CD27	M-T271	PE-Cy7
CD27	M-T271	V450
CD38	HIT2	PE
CD38	HB7	V450
CD45	2D1	AmCyan
CD45	2D1	APC-H7
CD45	HI30	V450
CD138	MI15	FITC
CD138	MI15	PerCP-Cy5.5

Table 2
Intracellular antibodies used to characterize IgE⁺ B cells and B_{regs} subsets

Target	Clone	Fluorophore
FoxP3	PCH101	Alexa Fluor 488
IgE	MB10-5C4	APC
IgE	MB10-5C4	PE
IgG	IS11-3B2.2.3	APC
IgG	IS11-3B2.2.3	FITC
IL-10	JES3-9D7	PE

6. Sterile squeeze bulb pipette.
7. B-cell culture medium: RPMI medium supplemented with 10% FBS, 2.5% 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (HEPES), 0.5% Penicillin/Streptomycin, and 1% sodium pyruvate.

2.5.2 B-Cell Culture and Stimulation

1. Round-bottom, 96-well, individually packaged, sterile culture plates.
2. Trypan blue live/dead cell stainer.
3. Hemocytometer.
4. Purified human IL-4.
5. Phorbol 12-myristate 13-acetate (PMA).
6. Ionomycin.
7. 5% CO₂, 37 °C cell culture incubator.
8. Protein transport inhibitor cocktail made of monensin and brefeldin A.
9. Fluorochrome-conjugated antibodies for flow cytometry staining of surface (*see* Table 1) and intracellular (*see* Table 2) molecules of IgE⁺ B cells and B_{regs}.

3 Methods

3.1 Selection of Participants with Mild Allergic Asthma

When recruited, study participants complete all allergic asthmatic testing aimed to confirm the allergic status (skin prick test) and airway hyperresponsiveness (methacholine challenge) (*see* Table 3). The results of the methacholine challenge and skin prick test confirm the eligibility for subjects with mild asthma to enter the study, while the results of the skin prick test titration are used to determine the allergen dose for inhalation, as previously described [104–110].

3.1.1 Determination of Allergic Status Using Skin Prick Testing

1. Prick the skin with a lancet containing a small amount of common aeroallergens. Additionally, histamine and saline are used as positive and negative controls, respectively.
2. Measure wheal size using a pen and ruler 10 min after skin prick. A wheal diameter > 2 mm is considered to be positive [104–106].

Table 3
Subject characterization using methacholine challenge and skin prick test results

	Methacholine challenge result	Skin prick test result
Allergic asthmatics	Positive	Positive
Allergic non-asthmatics	Negative	Positive
Healthy controls	Negative	Negative

3.1.2 Measurement of lung Function Using Spirometry

1. Subjects must perform spirometric maneuvers with full flow volume loop.
2. Determine the percent predicted forced expiratory volume in one second (FEV₁) using the third National Health and Nutrition Examination Survey (NHANES III) reference values. A FEV₁ $\geq$ 70% of predicted [107] is considered to be normal lung function.
3. Subjects that develop asthmatic exacerbations or respiratory tract infections within 4 weeks of the study start date or are historically diagnosed by a physician with other underlying diseases other than asthma must be excluded. Spirometry must be performed following American Thoracic Society guidelines [108].

3.1.3 Airway Hyperresponsiveness Testing Using the Methacholine Challenge Test

1. The methacholine challenge test that determines airway responsiveness [109] should be conducted by a trained, experienced technician using the tidal breathing method from a Wright Nebulizer.
2. The methacholine provocative concentration causing at least a 20% fall in FEV₁ (PC₂₀) is calculated by linear interpolation.
3. The methacholine PC₂₀ is required to be $\leq$ 16 mg/mL [107] for a subject to have airway hyperresponsiveness (*see Note 5*).

3.1.4 Inducing Symptomatic Allergic asthma Using the Inhaled Allergen Challenge

1. As detailed in [111], allergen challenge should be conducted by a trained, experienced technician with a physician at arms-length in the event of complications.
2. The allergen challenge is conducted with increasing concentrations of allergen inhaled by tidal breathing for 2 min each. FEV₁ is measured 10 min post-inhalation.
3. Allergen inhalation is stopped when the FEV₁ falls by at least 20%, then FEV₁ is measured regularly until 7 h post-challenge.
4. The early asthmatic response is the lowest fall in FEV₁ between 0 and 2 h, while the late asthmatic response is the lowest fall in FEV₁ between 3 and 7 h.
5. The diluent control challenge (negative control) consisted of three doses of the diluting solution instead of diluted allergen extract (*see Fig. 1a*).

3.2 Sample Collection and Tissue Preparation

All samples must be collected by trained physicians or technicians, ensuring excellent quality sampling, and stored in controlled conditions (*see Fig. 1b*). Our group is uniquely positioned where we have extensive experience employing the allergen inhalation challenge model, in addition to being able to obtain a wide array of human biological specimens (e.g., bone marrow, peripheral blood, sputum, and tonsil biopsies).

3.2.1 Collection of Venous Blood through Venipuncture

1. Peripheral blood must be collected into vacutainers containing heparin by an experienced, trained phlebotomist.
2. Perform total cell count using a hemocytometer. Prepare smears and stain with Diff-Quik™ for cell differentials.
3. Centrifuge 2 mL of the blood at $1000 \times g$ for 10 min to collect plasma for measurement of total IgE levels.
4. Dilute the remaining blood in RPMI medium supplemented with 10% FBS at 1:1 and then layer on density gradient medium. Centrifuge at $800 \times g$ for 20 min without break.
5. Collect the PBMC layer and store at 4 °C for flow cytometry staining (*see Note 6*).

3.2.2 Aspiration of Bone Marrow Sample from the Iliac Crest

1. Bone marrow must be aspirated from the iliac crest following local administration of lidocaine by a trained, experienced hematologist.
2. Collect 9 mL of bone marrow into a bone marrow aspiration syringe containing 1 mL of heparin.
3. The 10 mL of bone marrow is further diluted 1:1.5 with RPMI medium supplemented with 10% FBS and layered on top of 15 mL density gradient medium at room temperature. Centrifuge at $800 \times g$ for 20 min without break (*see Note 7*).
4. The bone marrow mononuclear cell (BMMC) layer was collected and stored at 4 °C for flow cytometry staining of regulatory B cells (*see Note 8*).

3.2.3 Homogenization of Tonsil biopsy and Cell Isolation

1. Tonsil tissue must be collected from patients using a through-cut forceps technique following local administration of lidocaine by a trained, experience otolaryngologist (*see Note 9*).
2. Disrupt the biopsy mechanically with a disposable scalpel in a petri dish and then incubate the processed tissue in 0.1% collagenase for 2 h at 37 °C to further breakdown tissue collagen (*see Note 10*).
3. Wash cells with PBS, centrifuge at $500 \times g$ for 10 min, and filter through a 100- μ m filter. Cells can then be stained for flow cytometry analysis.

3.2.4 Induction of Sputum Samples

1. Induce sputum by inhalation of 3%, 4%, and 5% saline and select and process mucous plugs as previously described [112, 113] (*see Notes 11 and 12*).
2. Treat sputum with sputolysin reagent and filter through a nylon mesh. Pellet the cells by centrifugation and perform a total cell count.

3. Prepare two cytopspins and stain with Diff-Quik for cell differentials. Store the remaining cells at 4 °C for flow cytometry staining.
4. Froze the fluid phase at –80 °C for measurements of total IgE, BAFF, and other inflammatory mediators.

3.3 Circulating IgE and BAFF Measurements

1. Use commercially available, sandwich, enzyme-linked immunosorbent assay (ELISA) kits to quantify total and allergen-specific IgE and BAFF in peripheral blood plasma and sputum fluid phase (*see* Fig. 1c and **Note 13**).
2. The lower limit of detection (LLOD) is 0.5 IU/mL for total IgE based on the Abcam kit, 0.1 IU/mL for allergen-specific IgE based on the ImmunoCAP® platform, and 2.68 pg/mL for BAFF based on the R&D Systems kit.
3. Quantify the absorbance using an appropriate plate reader (*see* **Note 14**).

3.4 Flow Cytometry Experiments

The ones presented here below are the first protocols to be developed to evaluate IgE⁺ B cells and regulatory B cells (B_{regs}) in the sputum, as well as in other peripheral compartments, of allergic asthmatics using the allergen inhalation challenge model [9, 10, 88].

Classically, IgE⁺ B cells have been difficult to detect in the blood due to cell rarity. However, methodological advances in flow cytometry with more lasers have allowed for B-cell subsets to be measured using greater than 10 fluorochrome-conjugated antibody panels. Recent papers have reported the measurement of IgE⁺ B cells in the blood [40], nasal biopsies, and nasal lavage of patients with allergic rhinitis [114, 115]. However, with our methods we were able for the first time to consistently detect IgE⁺ B cells in the blood, bone marrow, tonsil, and sputum of subjects.

We also evaluated different phenotypes of B_{regs} implicated in allergic disease [29, 81, 116] using flow cytometry. We observed a consistent decrease in the frequency of FoxP3-expressing B_{regs} in the bone marrow and an increase in the blood at 24 h post-allergen inhalation challenge. Although we were not able to measure different phenotypes of B_{regs} in the airways due to limited sample availability, we were the first to measure the numbers of CD19⁺FoxP3⁺ B cells in the sputum.

3.4.1 General Cell Staining Protocol for Flow Cytometry

1. Aliquot the isolated cells in cell staining tubes (*see* **Note 15**) and wash twice with CSM.
2. Incubate cells for 10 min with human FcR block reagent diluted in accordance to manufacturer's instructions to a final volume of 50 µl.

3. After 10 min, add the surface antibodies without washing. Depending on the specific sample (sputum, PBMC, BMMC, tonsil) being analyzed, incubate cells for 30 min at 4 °C with a different combination of antibodies directed against specific surface markers at a final volume of 200 μ l (*see* Table 1 and Subheadings 3.4.2 and 3.4.3).
4. Following incubation, wash cells by 5 min centrifugation at $500 \times g$ at 4 °C (*see* Note 16).
5. Proceed with the fixation/permeabilization step according to the manufacturer's instructions of the employed commercial kit (*see* Note 17).
6. Incubate cells with mouse and rat serum for 10 min (*see* Note 18).
7. After 10 min, add the intracellular antibodies without washing. Incubate cells with the antibodies directed against specific intracellular markers at a final volume of 200 μ l for 30–60 min at room temperature (*see* Table 2 and Subheadings 3.4.2 and 3.4.3). Appropriate isotypes and fluorescence minus one (FMO) must be used as negative controls for fluorescent staining background and antibody specificity, respectively. Use compensation beads to adjust settings.
8. Following incubation, wash cells and acquire the samples using a flow cytometer capable of detecting up to 15 fluorochromes simultaneously.
9. Export the .fcs files from the flow cytometer and analyze them with a dedicated software (*see* Fig. 1d).

3.4.2 Gating Strategy to Identify the Rare Population of IgE⁺ B Cells

1. To identify IgE⁺ B cells among total sputum cells, samples should be surface stained with antibodies to CD45 (APC-H7), CD19 (PE-Cy7), and intracellularly stained with antibodies to IgE (PE). As shown in Fig. 2a, place a gate around all the cells, excluding debris and doublets, in the forward scatter (FSC) and side scatter (SSC) plots. Plot SSC-A and CD45 and gate on the SSC^{low}CD45⁺ lymphocyte population (*see* Fig. 2b). Then draw a gate around the CD19⁺ B-cell population on the CD45 and CD19 plot (*see* Note 19). Plot CD19⁺ B cells with IgE and analyze IgE⁺ B cells in the airways (*see* Note 20 and Fig. 2b). Figure 2 also shows a representative analysis of how the frequency of the CD19 population changes among the participants of the allergic asthma study (*see* Fig. 2c) and in the course of allergen challenge (*see* Fig. 2d).
2. To identify IgE⁺ B cells among memory B cells (MBCs, CD27⁺CD38⁻), plasmablasts or pre-plasma cells (PPCs, CD27⁺CD38⁺) and plasma cells (PCs, CD38⁺CD138⁺) from peripheral blood and tonsil, samples should be surface stained

CD19⁺ B Cells and IgE⁺ B Cells in Sputum

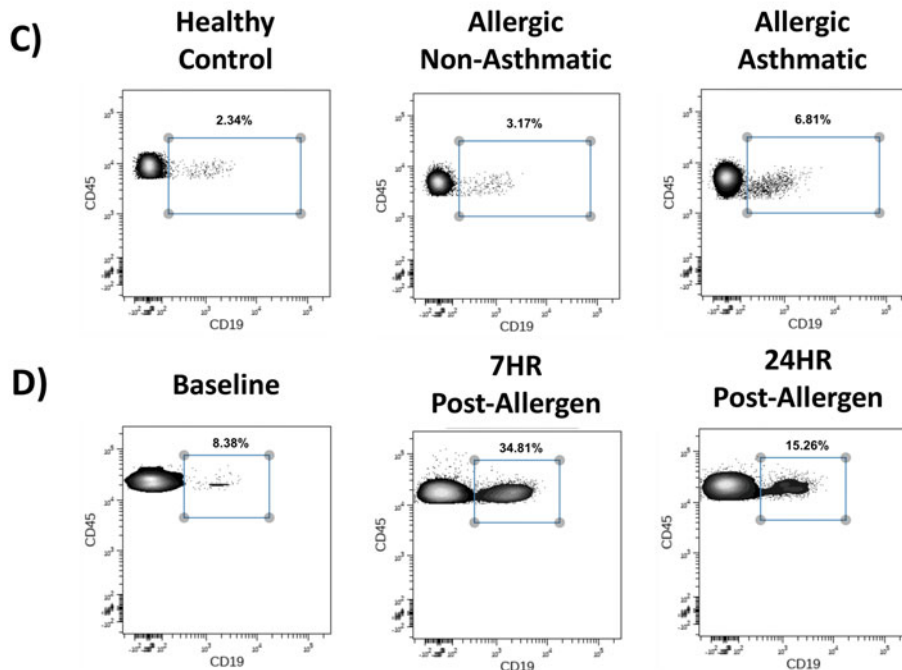
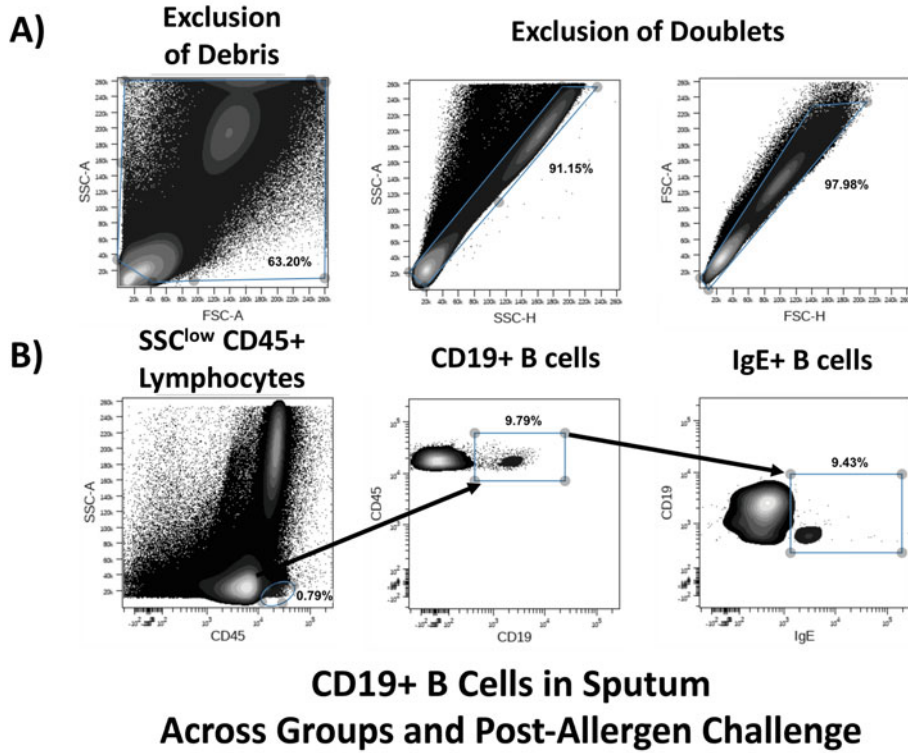


Fig. 2 Representative gating strategy for CD19⁺ and IgE⁺ B cells in sputum. **(a)** Exclusion of debris and isolation of single cells. **(b)** Identification of total lymphocytes, CD19⁺ B cells, and IgE⁺ B cells. **(c)** CD19⁺ B-cell proportions as a percent of CD45⁺ lymphocytes across groups (controls and allergic asthmatics). **(d)** CD19⁺ B cell proportions as a percent of CD45⁺ lymphocytes in allergic asthmatics pre- and post-allergen inhalation challenge

with antibodies to CD45 (APC-H7), CD19 (PE-Cy7), CD27 (V450), CD138 (FITC), CD38 (PE), and intracellularly stained with antibodies to IgE (APC) and IgG (APC). As shown in Fig. 3a, place a gate around all the cells, excluding debris and doublets, in the forward scatter (FSC) and side scatter (SSC) plots. Plot SSC-A and CD45 and gate on the lymphocyte population ($\text{SSC}^{\text{low}}\text{CD45}^+$). Then draw a gate around the CD19^+ B-cell population on the CD45 and CD19 plot (*see Note 21*). To identify memory B cells, draw a gate around the CD27^+ population on the CD19 and CD27 plot (*see Fig. 3b*). To identify plasmablasts or pre-plasma cells, draw a gate around the $\text{CD27}^+\text{CD38}^+$ population on the CD38 and CD27 plot (*see Fig. 3c*). To identify plasma cells, draw a gate around the $\text{CD38}^+\text{CD138}^+$ population on the CD38 and CD138 plot (*see Fig. 3d*). Further evaluation of IgE^+ and IgG^+ MBCs, PPCs, and PCs can be conducted (*see Note 22, Fig. 3b-d*).

3. To identify IgE^+ B cells among plasmablasts or pre-plasma cells (PPCs, $\text{CD38}^+\text{CD138}^-$) and plasma cells (PCs, $\text{CD38}^+\text{CD138}^+$) from bone marrow, samples should be surface stained with antibodies to CD45 (PerCP-Cy5.5), CD3 (PE-Cy7), CD19 (APC-Cy7), CD38 (FITC), CD138 (PE), and intracellularly with antibodies to IgE (APC) and IgG (APC). As shown in Fig. 4a, place a gate around all the cells, excluding debris and doublets, in the forward scatter (FSC) and side scatter (SSC) plots. Plot SSC-A and CD45 and gate on the CD45^+ lymphocytes population. Then draw a gate around the CD3^- non-T cells population on the CD19 and CD3 plot (*see Note 23*). To identify plasmablasts and plasma cells, draw a gate around the $\text{CD38}^+\text{CD138}^-$ population for PPCs and $\text{CD38}^+\text{CD138}^+$ population for PCs on the CD38 and CD138 plot (*see Fig. 4a*). Further evaluation of IgE^+ and IgG^+ PPCs and PCs can be conducted (*see Fig. 4b-c*).

3.4.3 Gating Strategy to Identify Different Regulatory B-Cell Subsets

1. To identify FoxP3^+ B cells among total sputum cells, samples should be surface stained with antibodies to CD45 (V450), CD19 (PE-Cy7), and intracellularly with antibodies to FoxP3 (Alexa Fluor 488). As shown in Fig. 5a, place a gate around all the cells, excluding debris and doublets, in the forward scatter (FSC) and side scatter (SSC) plots. Plot SSC-A and CD45 and gate on the lymphocyte population ($\text{SSC}^{\text{low}}\text{CD45}^+$). Then draw a gate around the CD19^+ B-cell population on the CD45 and CD19 plot, plot CD19^+ B cells with FoxP3, and analyze FoxP3^+ B cells in the airways (*see Fig. 5b*).
2. To identify regulatory B cells among total B cells from peripheral blood and bone marrow, samples should be surface stained with antibodies to CD19 (PE-Cy7), CD1d (APC), CD5 (Alexa

IgE⁺ B Cells in Blood and Tonsil

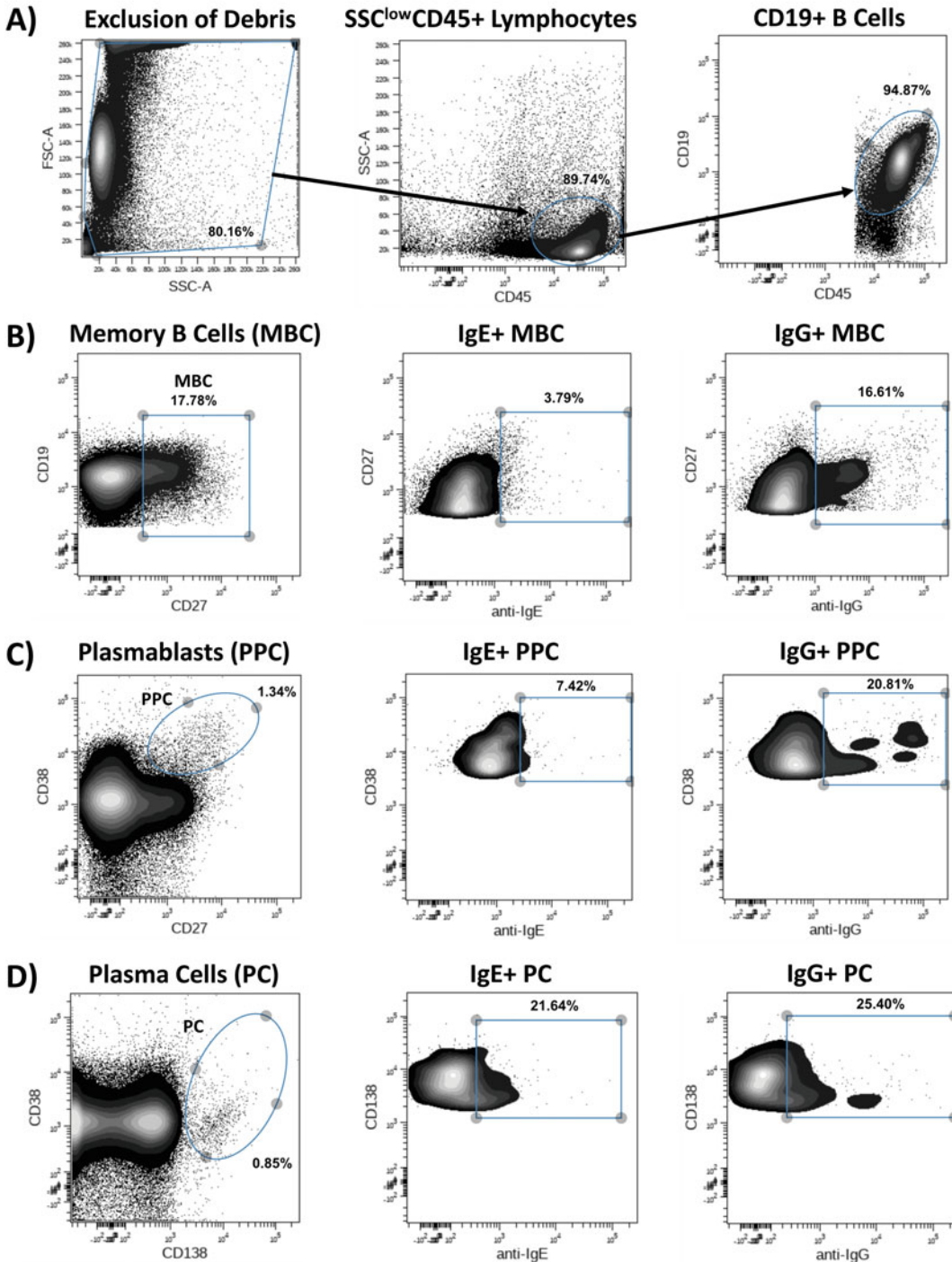


Fig. 3 Representative gating strategy for IgE⁺ B cells in peripheral blood and tonsil. (a) Exclusion of debris and isolation of single cells and identification of CD19⁺ B cells. (b) Identification of memory B cells and IgE⁺ and IgG⁺ memory B cells. (c) Identification of plasmablasts and IgE⁺ and IgG⁺ plasmablasts. (d) Identification of plasma cells and IgE⁺ and IgG⁺ plasma cells

IgE+ B Cells in Bone Marrow

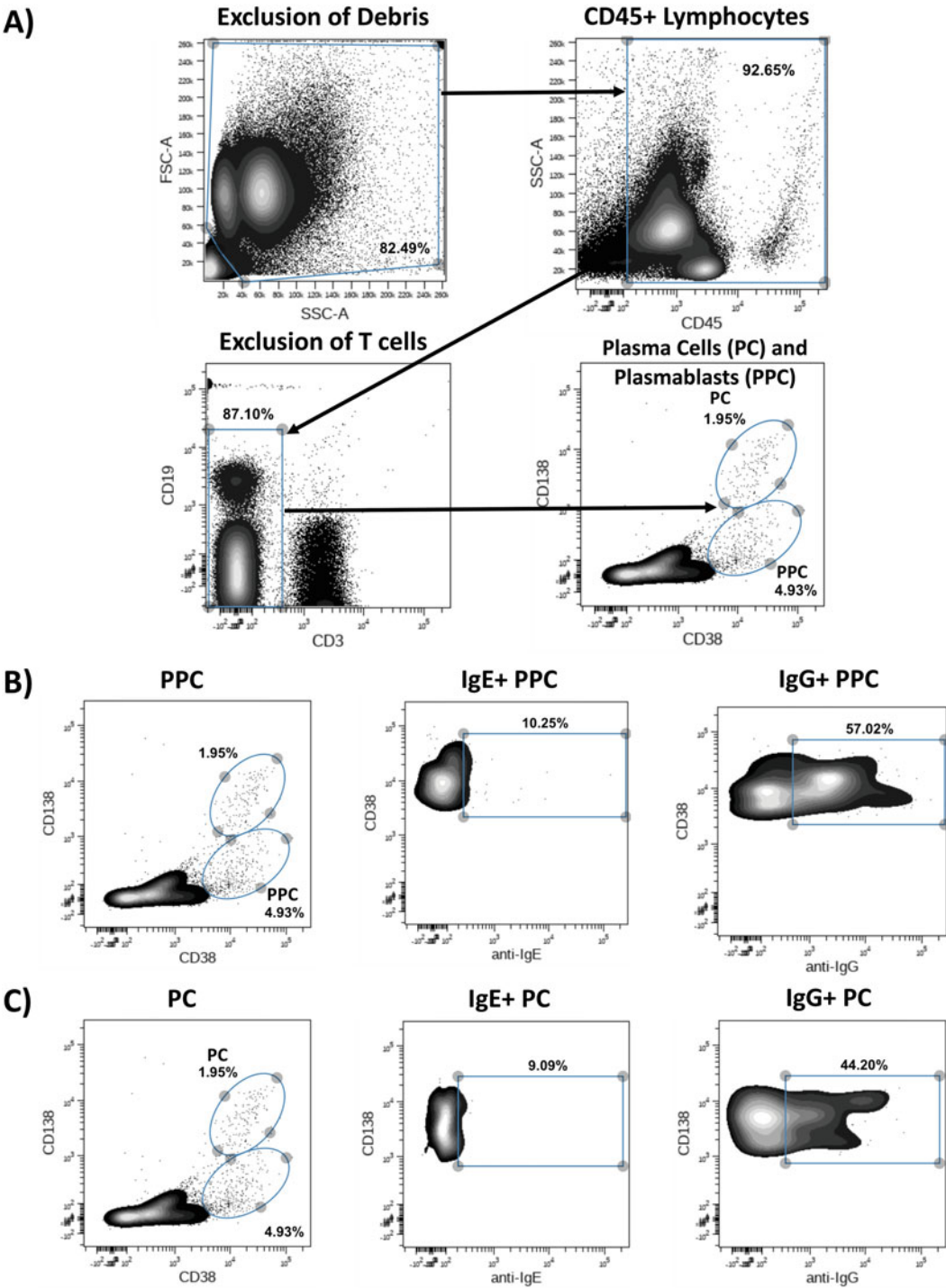


Fig. 4 Representative gating strategy for IgE⁺ B cells in the bone marrow. (a) Exclusion of debris and isolation of single cells, identification of CD3⁻ cells, and identification of plasmablasts and plasma cells. (b) Identification of plasmablasts and IgE⁺ and IgG⁺ plasmablasts. (c) Identification of plasma cells and IgE⁺ and IgG⁺ plasma cells

FoxP3⁺ B Cells Sputum

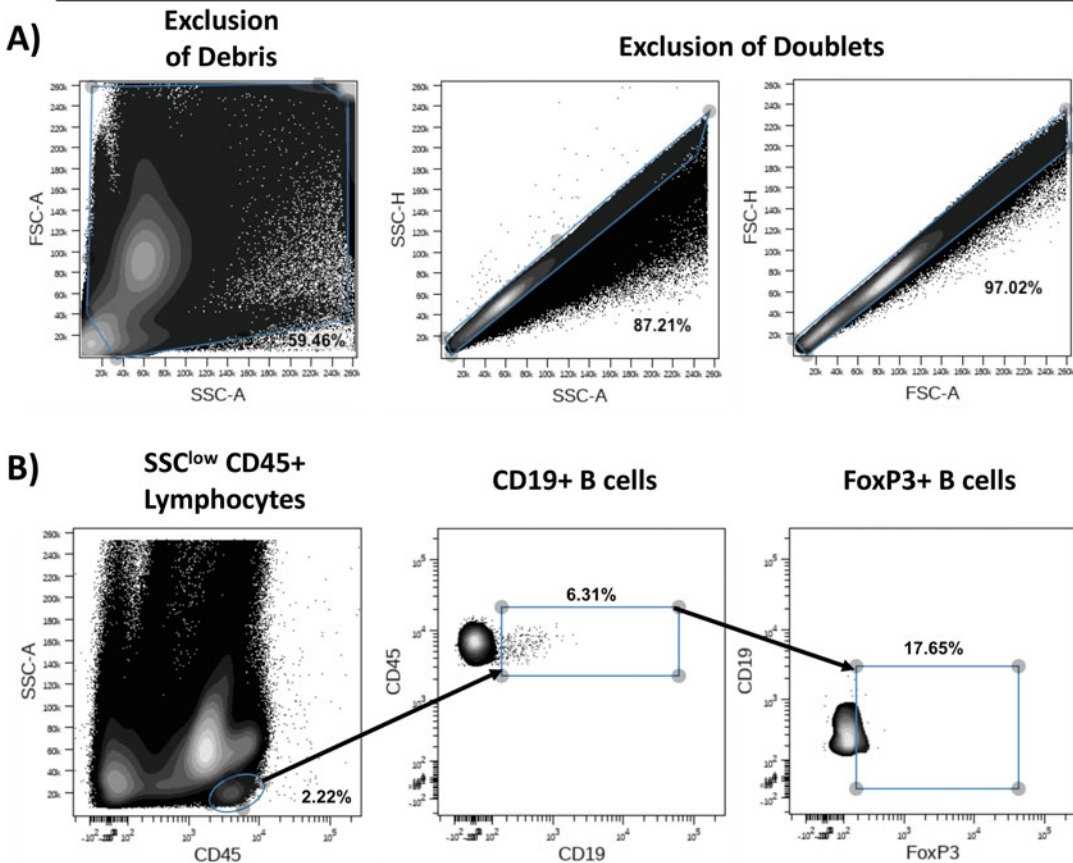


Fig. 5 Representative gating strategy for FoxP3⁺ B cells in sputum. **(a)** Exclusion of debris and isolation of single cells. **(b)** Identification of total lymphocytes, CD19⁺ B cells and FoxP3⁺ B cells

Fluor 700), CD24 (PE-CF594), CD27 (V450), CD38 (PE), and intracellularly with antibodies to IgE (APC), IL-10 (PE), and FoxP3 (Alexa Fluor 488). As shown in Fig. 6a, place a gate around all the cells, excluding debris and doublets, in the forward scatter (FSC) and side scatter (SSC) plots. Plot SSC-A and CD19 and gate on the B-cell population (SSC^{low}CD19⁺). To identify the different regulatory B-cell subsets in peripheral blood and bone marrow, a combination of plots are used as seen in Fig. 6b-c. Briefly, plot CD24 and CD27 and gate on the CD24⁺CD27⁺ B_{reg} population. Second, plot CD24 and CD38 and gate on the CD24⁺CD38⁺ B_{reg} population. Third, plot CD1d and CD5 and gate on the CD1d⁺CD5⁺ B_{reg} population. Last, plot CD5 and FoxP3 and gate on the CD5⁺FoxP3⁺ B_{reg} population.

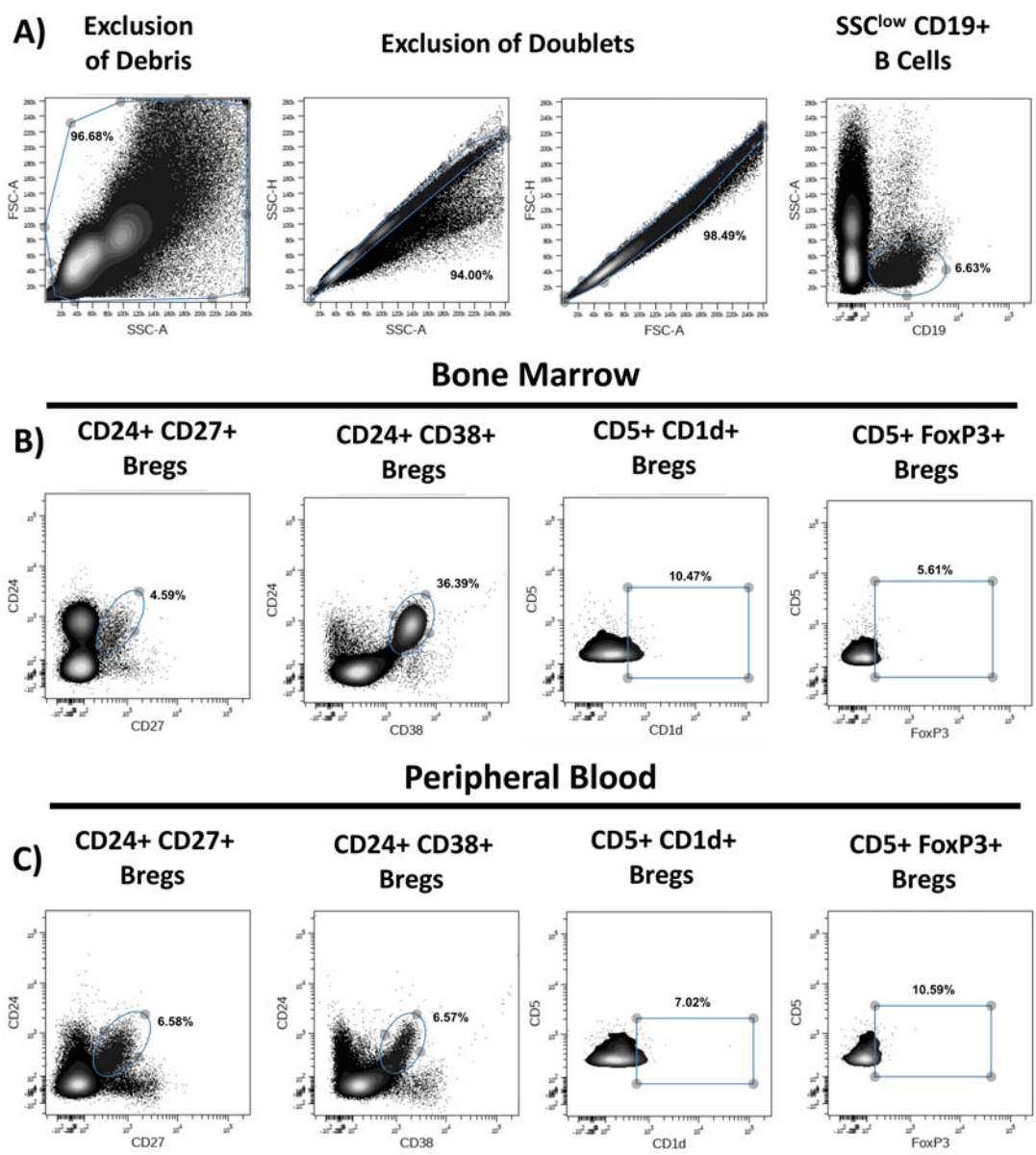


Fig. 6 Representative gating strategy for regulatory B cells in the bone marrow and peripheral blood. (a) Exclusion of debris and isolation of single cells. (b) B_{reg} populations in the bone marrow. (c) B_{reg} populations in peripheral blood

3.5 B_{regs}
Functional Assay

The initiation of type 2 inflammation in allergic asthmatics includes the production of the IL-4 and IL-13 cytokines which are known to promote maturation of IgE-producing B cells [9, 10]. In a growing body of literature, B_{regs} have been proposed to suppress IgE-mediated allergic inflammation through the secretion of IL-10 [24, 61, 81, 117]. In murine models, a deficiency in B_{regs} has been associated with increased serum IgE, increased type-2-cytokines, and increased eosinophilia [24, 85]. In light of these

considerations, when studying B_{regs} in the context of allergic asthma, it is very important to investigate the functional capacity of B_{regs} to express IL-10 in vitro. In this section we provide the protocols to assess how molecules produced during inflammation in allergic asthmatics, such as IL-4, affect the B-cell capacity to express FoxP3 and IL-10, which are both important markers for B_{regs} functions.

3.5.1 Isolation of Peripheral Blood CD19⁺ B Cells by Magnetic-Based Cell Separation

1. Collect the PBMC layer after density gradient centrifugation of peripheral blood (*see* Subheading 3.2.1).
2. Isolate human CD19⁺ B cells from whole blood by immuno-magnetic separation (*see* **Note 4**). Follow the manufacturer's instructions of the employed kit.
3. At the end of the isolation process, wash and count purified cells. Before culturing, check the purity of isolated B cells by assessing the expression of the CD45, CD19, and CD20 markers by flow cytometry (*see* **Note 24**).
4. Suspend isolated cells at a concentration of 1×10^6 B cells/mL in B-cell culture medium (*see* **Note 25**).

3.5.2 B-Cell Stimulation with IL-4 to Determine Functional Expression of IL-10 and FoxP3

1. Plate isolated B cells in a round-bottom 96-well plate at a starting density of 2×10^5 cells in a final culture volume of 200 μ l (*see* Fig. 1e).
2. Culture B cells in B-cell culture medium alone (negative control) or supplemented with either 15 ng/mL PMA and 0.5 μ g/mL ionomycin (positive control) or with increasing concentrations (5 ng/mL, 25 ng/mL, and 50 ng/mL) of IL-4.
3. Incubate B cells in a humidified 37 °C, 5% CO₂ incubator for 72 h. Every 12 h, gently agitate the culture plate for approximately 3 sec (*see* **Note 26**).
4. Six hours before the end of culture, add to the culture suspension a protein transport inhibitor cocktail of 1 μ mol/l brefeldin A and 1 μ mol/l monensin to block cytokine secretion (*see* **Note 27**).
5. Collect cells and transfer to staining tubes. Proceed with cell surface (CD19, CD5, CD24, CD27) and intracellular (IL-10, FoxP3) staining as described in Subheading 3.4.1.
6. Analyze by flow cytometry the frequency of different B_{reg} subpopulations (*see* Subheading 3.4.3).

3.6 Next Generation Methods

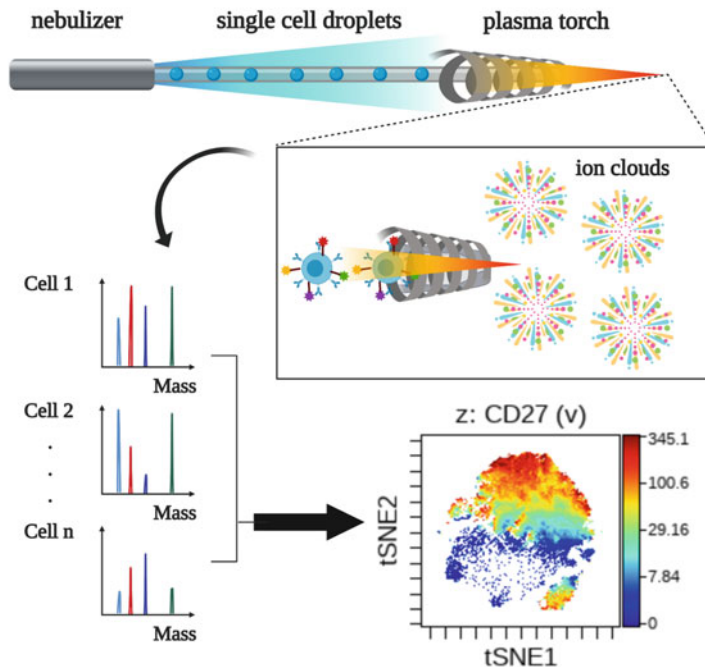
The ones reported here below are not methodological protocols but rather the descriptions of two cutting edge techniques that allow to simultaneously study IgE⁺ B cells and regulatory B cells. Mass cytometry (CyTOF) and multiplexed imaging (IMC and MIBIScope) are next generation technologies still in their infancy

of utility that can permit to quantify and characterize IgE⁺ B cells and B_{regs} with much more resolution than what is already available in literature.

3.6.1 Mass Cytometry (CyTOF)

A big limitation when studying IgE⁺ B cells and B_{regs} is the technological limitation in being able to quantify these cells. With the advancements in flow cytometry techniques and the availability of many new fluorochromes, it has been possible to adequately study IgE⁺ B cell and B_{regs} subsets in separate flow cytometry experiments. However, the ability to simultaneously study IgE⁺ B cells and B_{regs} within one experiment would be ideal since it would give us the ability to accurately compare the ratio of these cell types and heterogenous cell states; better examining the balance in cellular quantities between inflammation and regulation within the context of allergic asthma pathogenesis. Specifically, utilizing mass cytometry or cytometry by time-of-flight mass spectrometry (CyTOF), and specifically quantifying and characterizing IgE⁺ B cells and B_{regs} would be beneficial to the better understanding of the proportions of these cell subpopulations [118] (*see* Fig. 7a). Mass cytometry is still a fairly underused, versatile technology used for the analysis of cells on a single-cell level [119–126]. Unlike flow cytometry, mass cytometry allows for simultaneous measurement of more intracellular and extracellular biomarkers, with greater precision [120, 122]. In flow cytometry, cells of interest are labeled with fluorochrome-conjugated antibodies, and the interactions of the cell with a laser lead to fluorescent emission of light at variable wavelengths, allowing for the detection and quantification of up to 20 cellular markers [122]. Mass cytometry involves the labeling of cells with metal-tagged antibodies for the detection and quantification of up to 50 cellular markers, which is almost double compared to flow cytometry [122]. The ability to simultaneously explore more cellular parameters allows for better understanding of complex cellular systems and signaling pathways that contribute to our current understanding of B-cell biology [127]. Glass et al. prepared the first B-cell atlas, evaluating the expression of 351 surface molecules on B cells using mass cytometry, as well as interrogate transcription, translation, metabolism, and signaling in B cells [127, 128]. They identified four specific markers (CD45RB, CD11c, CD73, CD95) that distinguished phenotypically and isotypically distinct B-cell subsets [127]. The next step would be to explore the intracellular environment of B cells, evaluating transcription factors, cytokine expression, and other inflammatory mediators, particularly within the context of regulatory B cells. One of the most practical approach to deeply learn B-cell biology is to continue employing next generation technologies like mass cytometry. Quantifying cells based on mass and charge allows us to circumvent fluorochrome light detection overlap and autofluorescence, which are large limitations of flow cytometry [122]. Lastly,

A) Mass Cytometry (CyTOF)



B) Multiplexed Ion Beam Imaging (MIBIScope)

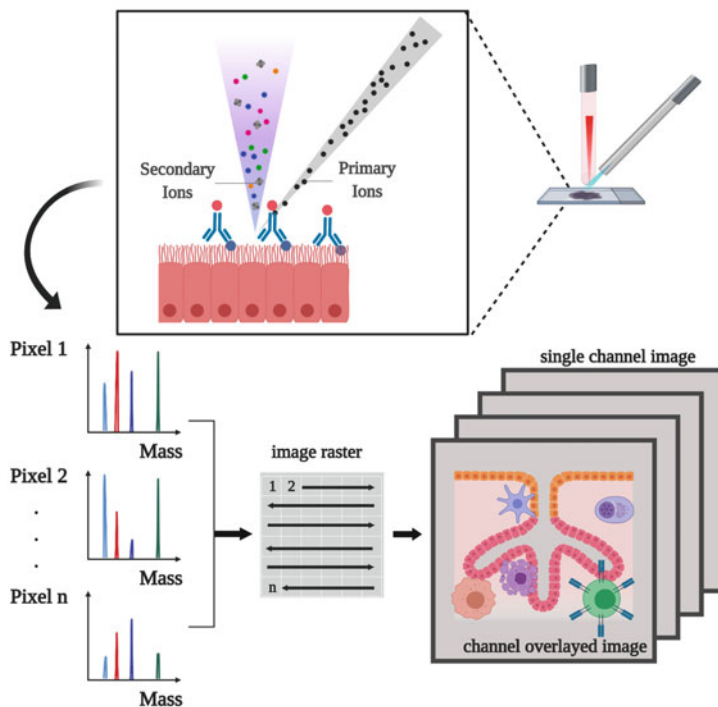


Fig. 7 Next generation technologies to evaluate IgE⁺ B cells and regulatory B cells. (a) Multiparameter cytometry using mass cytometry (CyTOF) technology with the ability to simultaneously measure more than 40 antigens simultaneously, on the cell surface or intracellularly. (b) Multidimensional imaging using multiplexed ion beam imaging (MIBIScope) technology with the ability to simultaneously measure more than 40 targets in tissues, simultaneously revealing spatial localization of structural, cellular, and subcellular features

mass cytometry allows for cellular barcoding, which improves the efficiency and sensitivity of single-cell analysis, allowing researchers to better understand shifts and functional potential of specific cellular subsets and sub-phenotypes [119, 122, 123, 129–132]. Capitalizing on the utility of cellular barcoding to increase the efficiency in the acquisition of cells from multiple patients and/or cells from different compartments, such as spleen, blood, and bone marrow, significantly increases the throughput of precious biological samples [119, 123]. Furthermore, Hartmann et al. developed and quality controlled a method using CD298 platinum-conjugated antibodies to barcode live cells, which broadens the downstream capabilities of single-cell monitoring using mass cytometry [132].

Taken together, since there is currently no consensus on the surface phenotype of B_{regs} in the literature, the use of mass cytometry to better understand the distribution and quantities of different B_{regs} phenotypes will aid in understanding the roles that these cells play in allergic asthma pathogenesis. Additionally, since we do not know the distribution of B_{regs} in the body after allergic stimulation, we can take advantage of barcoding and mass cytometry to simultaneously analyze multiple compartments (bone marrow, blood, airways) after allergen inhalation challenge. Lastly, mass cytometry can be further used to better understand the single-cell proteomic profile of cells by intracellular staining of different cytokines being produced by the cells (e.g., type 2 cytokines; IL-4 and IL-13). By utilizing our allergen inhalation challenge model and mass cytometry, we can answer questions regarding B_{regs} development, transiency, distribution, and function.

3.6.2 Multiplexed Ion Beam Imaging Time of Flight (MIBI-TOF)

Although multiparametric mass cytometry can be a useful tool in revealing novel states of B cells giving a more granular look on different B cell states (developmental progression, functional state, memory status, isotype class), much more work is needed to understand these cells in health and disease. Particularly, as seen in Oliveria et al.'s work [9, 10, 88], it is important to study these different B-cell subsets in the diseased organ (e.g., the lungs such as biopsies or surrogate samples like sputum or bronchoalveolar lavage in allergic asthmatic individuals). With the advent of more advanced imaging technologies (imaging mass cytometry by Fluidigm Sciences and MIBIScope by IonPath), the spatial localization of total B cells and of their different subsets, as well as the cell–cell interactions between B cells and other immune and structural cells, can be evaluated and specifically quantified (*see* Fig. 7b). Multiplexed imaging is still in its infancy and a lot more work is needed to: (i) learn more interesting biological mechanisms [133, 134], (ii) work with the high-dimensional data and develop more robust and innovative analytical pipelines [134, 135], and (iii) develop

concurrent methodological approaches leveraging the imaging technology platforms to perform multi-omic-type experimental studies. Similar to how mass cytometry is the next generation technology for flow cytometry, imaging mass cytometry (IMC) and MIBIScope is the next generation technology for immunohistochemistry [129]. Multiplexed imaging yields rich spatial information that cannot be achieved by mass or flow cytometry [129, 134]. Imaging has always been able to provide spatial information, such as localization of cell surface or intracellular markers, points of cell contact and interaction, spatial heterogeneity of various cell populations. However, with multiplexed imaging and through the using of the full potential of greater than 40 antigens measured simultaneously, novel cell subsets, different cellular clusters/niches can be revealed, and spatial arrangement of immune cells can be uncovered [134, 136–138]. In the work by Zhao et al., authors determined that different B-cell subsets occupy different niches in lymphoid tissues, where marginal zone B cells and memory B cells are spatially located in different anatomical spaces within the gut lymphoid tissues [136]. This data shows that there is B-cell diversity and heterogeneity that is still widely unknown, even within healthy individuals, let alone various disease states. Much more work using high-dimensional imaging platforms to study B cells is needed, particularly in allergic diseases, since IgE produced by B cells plays an important role in disease pathogenesis [138].

4 Notes

1. To isolate CD19⁺ B cells from whole blood, we use the RosetteSep™ CD19⁺ B-cell enrichment kit (STEMCELL Technologies). This negative selection kit enriches the B-cell population through a two-step protocol: first non-B cells are bound to a tetramer-antibody cocktail, while the following centrifugation with the RosetteSep™ density gradient medium will enrich B cells in the visible layer, and pellet non-B cells and red blood cells.
2. We used the StraightFrom® Whole Blood CD138 MicroBeads (Miltenyi Biotec) since it allows the isolation of CD138⁺ cells directly from blood or blood products without the need for density gradient centrifugation. The AutoMACS running buffer and AutoMACS rinsing solution were used accordingly.
3. The FcR block reagent is used to prevent unwanted binding of antibodies to human Fc receptor-expressing cells. In alternative to the FcR block reagent, both mouse and rat serum can be used during staining for flow cytometric analysis to inhibit non-specific binding of mouse and rat monoclonal antibodies, respectively. Although many different suppliers provide the FcR block reagent, we used the one from Miltenyi Biotec.

4. EasySep™ CD19⁺ B-cell isolation kit (STEMCELL Technologies) is used to isolate and purify CD19⁺ B cells by positive selection. This kit isolates CD19⁺ B cells directly from whole blood and the isolation process is quick and efficient, yielding high purity of functional B cells. Compared to the RosetteSep CD19⁺ B-cell enrichment kit, the EasySep CD19⁺ B-cell isolation kit provides a final product with a much greater purity, and this is fundamental when cells must be put in culture for downstream experiments. Indeed, although good at depleting other cells, B-cell isolation through negative selection still has other cellular contaminations, which can be easily removed *in silico* (e.g., gating desired cells and/or gating out undesired cells) but are not suitable with cell culture.
5. To be considered to have airway hyperresponsiveness, the methacholine PC₂₀ must be below 16 mg/mL. Methacholine is a smooth muscle agonist, and by inhaling concentrations of methacholine higher than 16 mg/mL, even healthy subjects with healthy airways can experience bronchoconstriction.
6. For the peripheral blood studies evaluating the kinetics of IgE⁺ B cells [10], peripheral blood can be collected into heparin tubes and enriched for B cells using the RosetteSep™ isolation kit. B cells can then be stained for flow cytometry.
7. The suggested dilution is based on experience evidences that show that this dilution generates the optimal viscosity to perform an undisrupted layer on the density gradient.
8. Since the bone marrow is a reservoir for CD138⁺ plasma cells, a CD138⁺ isolation kit can be used to isolate and enrich plasma cells in the bone marrow studies evaluating the kinetics of IgE⁺ B cells [10]. Incubate the mononuclear cell layer with anti-CD138⁺ microbeads before proceeding with immunomagnetic separation. If using the magnetic-activated cell sorter (MACS) system from Miltenyi, then you can increase CD138⁺ cell purity by passing the labeled cells through a LS column twice, and by slowly plunging the LS column each time. Isolated plasma cells can be stained for flow cytometry.
9. The physician should consistently collect 0.5 cm³ pieces of tonsil tissue from willing donors. This will allow to yield enough cells in order to stain 2×10^6 cells per sample tube for flow cytometry analysis.
10. After mechanical disruption of the tissue, the petri dish must be thoroughly washed with PBS three times to ensure that all cells were collected before further chemical digestion. Place the petri dish above a black colored piece of paper to visualize any remnants of cells. Moreover, filter three times the digested tissue to ensure there is no clumping of cells or remnants of collagen milieu.

11. The collection of mucous plugs must be done by trained and experienced technicians. Indeed, this staff is trained to collect “milky white” mucous plugs rich in cells, and to make sure to not include excess saliva and other debris, which could contaminate the sample with buccal epithelial cells. Sputum mucous plug collection must be done above a black colored piece of paper to visualize no remnants of cells. The increasing salt concentrations gradually increase ciliary transport of sputum from the trachea and allow for the mucous plugs to be coughed up by the sputum donor.
12. Encouragement of the participant during expectoration of sputum and live demonstration by the technician was shown to result in optimal samples with adequate cell numbers for flow cytometry staining. Visualization of the expectoration process yields optimal samples.
13. All samples should be pre-aliquoted after collection and processing, and stored at -80°C . This ensures no freeze-thaw of samples which helps to avoid any inaccurate results from unmeasurable protein degradation.
14. It is important to note whether the samples for protein measurements have been diluted or run neat. For example, our sputum supernatant values were multiplied by the sputum processing dilution factor of 8 in order to get the correct detected levels.
15. To consistently obtain reliable bone marrow and peripheral blood B-cell measurements, the quantity of cells stained per sample tube should be 2×10^6 cells. This needs to be consistent throughout the duration of the study. If there are not enough cells (a very rare situation), use 1×10^6 cells per sample tube. To consistently obtain reliable sputum B-cell measurements, 5×10^5 cells per sample tube is required.
16. Perform centrifugation at a speed of $500 \times g$ before fixation and permeabilization, and drop down in speed of $300 \times g$ after fixation and permeabilization to ensure cells remain intact.
17. The addition of fixation and permeabilization solution should be done at a slow, dropwise fashion while the cell suspension is maintained under slow vortexing to avoid any clumping of cells.
18. The use of mouse and rat serum is useful to decrease the background signal of intracellular antibodies in flow cytometry.
19. In addition, selected CD19⁺ B cells can be analyzed for CD27 and CD138 expression to isolate memory B cells (MBCs) and plasma cells (PCs). IgE⁺ MBCs and PCs can also be enumerated in the airways.

20. IgG⁺ B cells, MBCs, and PCs can also be enumerated in the airways.
21. By using the RosetteSep™ CD19⁺ B-cell enrichment kit, the CD19⁺ B-cell population was above 90% of the total CD45 parent gate.
22. This gating strategy can be used for cells isolated from tissue (e.g., tonsil) and peripheral blood.
23. Since a CD138⁺ isolation kit was used to isolate CD138⁺ cells, a CD3 in silico depletion was needed to remove any T cells from the analysis, as a subset of T cells also express CD138.
24. By using the EasySep™ CD19⁺ B-cell isolation kit, the purity was consistently above 98.5%.
25. Resuspend the isolated B cells in B-cell culture medium to ensure they remain “happy” after magnetic isolation and let them rest for 2 h at 37 °C before beginning culture protocols.
26. Since it is known that B cells suffer during culture, a trypan blue viability count was performed before and 72 h after the start of culture to count viable and death cells. B-cell viabilities were above 99.3% and 82.1%, respectively.
27. Every 2 h during the 6 h protein transport inhibition period, gently agitate the culture plate for approximately 3 sec.

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