

Chapter 15

Regulatory B Cells in Allergic Airways Disease and Asthma

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Abstract

B cells have long been known to participate in both innate and adaptive immune responses by contributing to antigen presentation and by producing antigen-specific antibodies. Recent evidence shows that certain B-cell subsets can also inhibit T-cell immune responses. Like regulatory T cells (Treg), these regulatory B cells (Breg) appear to comprise several subpopulations. How Breg cells are generated and how they control immune responses in vivo are just beginning to be elucidated. Here, we provide detailed instructions for the identification, isolation, and functional characterization of Breg cells in a murine model of allergic airway disease.

Key words Allergy, Confocal microscopy, Suppressor B cells, Mouse, Tolerance

1 Introduction

B cells play a major role in the pathogenesis of asthma and are generally considered to be promoters of immune responses due to their ability to produce antigen-specific antibodies and to serve as antigen-presenting cells, thus facilitating optimal T-cell responses [1]. They are responsible for eliciting productive humoral immunity against a variety of bacterial, fungal, and viral infections. Human and murine B cells have traditionally been divided into two major subtypes (B1 and B2) based on their function and phenotypic differences [2]. In mice, B1 cells are characteristically B220^{lo}, IgM^{hi}, IgD⁺, CD43⁺, and CD23^{lo}. B1 cells are further classified based on CD5 expression as CD5⁺ B1a cells and CD5⁻ B1b cells [3]. B1 cells are an important source of natural antibodies and secrete higher amounts of IgM than IgG, and their immunoglobulin receptors show polyspecificity [4]. In comparison to B1 cells, B2 cells are conventional class switching B cells, which produce Ag-specific antibodies of different isotypes such as IgG, IgE, and IgA. In mice B2 cells are phenotypically B220^{hi}, IgM^{lo}, IgD⁺, CD43⁻, and CD23^{hi} [3]. B2 cells have much a more diverse B-cell receptor (BCR) repertoire than B1 cells [5]. B2 cells constitute

the majority of peripheral B cells found in lymphoid organs and in circulation [6].

Recent advances in B-cell biology have challenged the dogma that regarded antibody production as the only important function of B cells. The concept of B-cell-mediated immune suppression was first described in 1974, when the ability of B cells to suppress delayed-type hypersensitivity reactions was demonstrated [7, 8]. However, the concept that B cells can exert regulatory functions has not been fully appreciated until the last decade, when their modulatory effects have been demonstrated in a variety of murine models of chronic inflammation, including inflammatory bowel disease, rheumatoid arthritis, experimental autoimmune encephalomyelitis (EAE), and asthma [9, 10]. Similar to regulatory T (Treg) cells, Bregs elicit immune regulation by various mechanisms. Also, Bregs may exert their effects on T-cell responses indirectly, by inhibiting the innate immune system. Conditioning of dendritic cells (DCs) by CD5⁺ CD1d^{hi} B cells results in less T-cell proliferation compared to conditioning by non-Breg cells [11]. In addition, cocultivation with Breg cells inhibits DC maturation, antigen presentation and expression of the co-stimulatory molecules CD80 and CD86 via IL-10-dependent [12] and IL-10-independent mechanisms [13]. Antigen presentation by immature DCs induces T-cell tolerance rather than T-cell activation and pro-inflammatory cytokine production. Breg cells also can induce anergy in CD8⁺ T cells directly, by functioning as antigen-presenting cells with impaired IL-2 production [14].

Bregs modulate the immune response through production of anti-inflammatory cytokines and expression of inhibitory molecules. Perhaps the best-described Breg cell is the IL-10 secreting B10 cell [15]. B10 cells appear to have a B1a origin and are CD5⁺ and CD1d^{hi}. In mice, B10 cells have been shown to inhibit the progression of inflammation and/or hasten the recovery from inflammatory bowel disease [16, 17], experimental autoimmune encephalomyelitis (EAE) [18, 19], systemic lupus erythematosus [20, 21], and rheumatoid arthritis [22]. A comparable IL-10-competent B10 cell subset has been identified in human blood within CD24^{hi} CD27⁺ and/or CD24^{hi} CD38^{hi} B-cell subpopulations [23, 24]. In addition to these IL-10-producing B10 cells, a Breg subset that is capable of producing TGF- β after *in vitro* stimulation with LPS has been identified. Transfer of such TGF- β -producing B cells into B-cell-deficient nonobese diabetic (NOD) mice suppresses the development of diabetes in the recipient mice by inducing apoptosis of the effector T cells [25]. TGF- β -producing Breg cells may also participate in the induction of low-dose oral tolerance [26, 27] and in the inhibition of allergic airway disease [28, 29]. A human Breg cell characterized as CD25^{hi} CD27^{hi}

CD86^{hi} CD1d^{hi} IL-10^{hi} TGF- β ^{hi} is capable of enhancing Foxp3 and CTLA-4 expression in Treg cells via a mechanism dependent on direct contact between Breg and Treg cells and on TGF- β but not IL-10 [30]. In addition, both IL-10 producing CD19⁺ CD5⁺ B cells (B-10 cells) and TGF β -producing CD19⁺ CD5⁺ B cells have been shown to regulate cow's milk allergic responses in human subjects [31, 32].

Breg cells also express a number of signaling molecules that may play key roles in their suppressive actions. Breg expression of glucocorticoid-induced TNF receptor ligand (GITRL) may play an essential role in homeostasis by maintaining natural Treg cell (nTreg) numbers above a threshold required for the prevention of autoimmunity. In the murine EAE model, Breg expression of GITRL, but not IL-10, is required for proliferation of Treg cell and EAE recovery [33]. Another mechanism of suppression by Bregs is killing CD4⁺ T cells by Fas/FasL-dependent mechanisms. In NOD mice, the number of FasL-expressing splenic B cells inversely correlates with the severity of diabetes [24]. FasL-expressing B cells mediate immune tolerance in a skin graft model [34] and in cockroach antigen-induced murine asthma [35]. This FasL-dependent apoptotic function has been attributed to CD5⁺ B-cell subsets [10]. Between 50 and 70 % of mouse peritoneal CD5⁺ B cells also express the programmed death-1 ligand PL-L2 [36]. PL-L2 has been implicated in mucosal homeostasis and the modulation of oral tolerance and asthma [37], but the potential regulatory role of these PL-L2-expressing CD5⁺ B cells remains to be determined. Both IL-10-expressing [38, 39] and TGF- β -expressing Breg cells [28] also demonstrate high expression of CD1d. CD1d is required for the presentation of phospholipid and glycolipid antigens to NKT cells, and CD1d^{hi} B cells have been shown to promote immune tolerance through activation of NKT cells in different mouse models [40, 41], suggesting a potential regulatory mechanism involving Breg and iNKT cell interaction.

Thus, as with Tregs, it is likely that several populations of Bregs exist and that different subsets may be preferentially generated based on the types of stimuli, local cytokine environment, and anatomical sites of antigen exposure. The origins of these Bregs and how they develop are just beginning to be elucidated. More work toward understanding the mechanism of regulation and phenotypic characteristics of Bregs in different settings is highly important. In the following sections, we present detailed instructions for the *in vivo* generation, identification, isolation, and functional characterization of Breg cell populations that can be used in multiple model systems.

2 Materials

2.1 Animal Sensitization and Aerosol Challenge

2.1.1 Sensitization

1. Stock ovalbumin (OVA) solution: 5 mg/ml (chicken egg albumin, Sigma #A-5503) in sterile 0.9 % saline (*see Note 1*).
2. Alum (aluminum hydroxide).
3. 0.45 μm syringe filters.

2.1.2 Aerosol Challenge

1. Ovalbumin: 1 % OVA in 0.9 % saline.
2. BANG nebulizer and inhalation chamber (CH Technologies) (*see Fig. 1*).
3. Compressor at 125 PSI (Craftsman).
4. Mercer cascade impactor (In-Tox Products).



Fig. 1 Aerosol exposure chamber and mouse holding tubes. Mice are inserted into individual holding tubes that expose their noses to the aerosol stream. The aerosol is delivered by a compressed-air-driven nebulizer, which is connected to a reservoir containing OVA. The exposures are performed in a fume hood to minimize exposure to technicians

2.2 Mouse Sacrifice: Blood, Tissue, and Bronchoalveolar Lavage Collection

1. Anesthetic: add 0.3 ml xylazine (AnaSed, 20 mg/ml, Akron Inc) to 10 ml Ketaset (Ketamine HCl 100 mg/ml, 10 ml per bottle, Fort Dodge Animal Health) for a final volume of 10.3 ml.
2. 1.5-ml Eppendorf tubes and Eppendorf centrifuge.
3. Tracheal cannula made from a dulled 21-gauge, 1" needle and ~1 in. of 0.032" tubing, with end cut at ~45° angle (*see Note 2*).

2.3 Tissue Preparation

1. Hanks' buffered salt solution (HBSS) pH 7.2.
2. Collagenase/RPMI digestion solution: 5 % fetal calf serum (FCS), 1 mM MgCl₂, 1 mM CaCl₂, collagenase 150 U/ml (Gibco #17018-37) in RPMI.
3. 10× Tris-buffered ammonium chloride (TAC, lysis solution for red blood cells): 0.13 M Tris-Cl pH 7.2, 0.15 M ammonium chloride in distilled H₂O.
4. FACS buffer: 0.2 % bovine serum albumin (BSA), 0.1 % NaN₃ in DPBS.
5. Cell strainer caps (BD Falcon 40 µm, for spleen, lung).
6. Hemocytometer slides.
7. Methanol fixative (Fisher Scientific #A 454-4).
8. Differential stain: May-Grunwald stain (Sigma #MG-1L); Giemsa stain (Sigma #GS-1L), dilute 1:20 in distilled water.
9. May-Grunwald/Giemsa buffer: 6.63 g potassium phosphate, monobasic (Baker 5-3246), 2.56 g sodium phosphate, dibasic (Fisher S373-500), bring to 1 l volume with water, pH 7.2.
10. Nigrosin: 0.2 % in 0.9 % saline.
11. Buffered formalin for pathology specimens.
12. Cytospin 4 (Thermo Shandon).

2.4 Confocal Microscopy

2.4.1 Frozen Cryostat Sectioning

1. Optimal cutting temperature (O.C.T.) compound—embedding medium.
2. Cryomold (Tissue-Tek).
3. Pre-charged superfrost slides (Fisher Scientific).
4. Acetone.
5. Ice-cold PBS.
6. Image analysis software like ImageJ (public domain, Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997–2012) or Imaris Suite® (Bitplane AG, Zurich, Switzerland).

2.4.2 T- and B-Cell Staining

1. Staining buffer: 2 % FCS, 2 % normal goat serum in sterile PBS.
2. PAP pen for immunostaining (Sigma-Aldrich, Z377821).

3. Primary and secondary antibodies (listed in Table 1).
4. Fc receptor block: anti CD16/CD32 (eBioscience).
5. Foxp3 staining buffer set (eBioscience).
6. Immu-mount, mounting medium (Thermo Scientific).

2.5 Functional Breg Assays

1. B-cell and T-cell isolation kits (all from Miltenyi Biotec): CD19 beads; CD4 CD25 kit; B-cell isolation non-B-cell depletion kit; CD5 (Ly-1) microbeads.
2. LD, LS, and MS columns (Miltenyi Biotec).
3. MACS buffer: 0.5 % BSA, 2 mM ethylenediaminetetraacetic acid (EDTA) in 1× PBS. May be prepared 1 day in advance.
4. Cell culture medium (may be prepared 1 day in advance): 10 % FCS, 4 mM L-glutamine, 200 U/ml penicillin, 200 µg/ml streptomycin, 50 µM 2-mercaptoethanol in RPMI-1640.
5. CFSE (Molecular Probes, #C34554): immediately before use, reconstitute the contents of one vial of CFSE to a stock solution of 5 mM by adding 18 µl dimethyl sulfoxide (DMSO, supplied). Dilute 1:1,000 for the final working concentration (5 µM).
6. 24-well sterile plates.
7. Nitex mesh for screening out debris and cell clumps.
8. Anti-CD19 and anti-CD5-FITC mAbs for cell sorting (for details, *see* Table 1).
9. Irradiator (Gammacell® 1000, Best Theratronics).

Table 1
Representative monoclonal antibodies used in these studies

Confocal antibody	Clone	Vendor	Fluorochrome	Dilution
αB220	RA3-6B2	eBioscience	AF488	1:100
αCD4	GK1.5	RA3-6B2	AF647	1:100
αFoxp3	FJR-16s	eBioscience	PE	1:100

Functional Breg antibody				
αCD3	145-2C11	eBioscience	PerCPCy5.5	1:200
αCD19	1D3	BD Biosciences	PE	1:200
αB220	RA3-6B2	eBioscience	APC-EF780	1:200
αCD4	GK1.5	BD Biosciences	PE-Cy7	1:200
αCD5	53-7.3	BD Biosciences	FITC	1:100
αCD25	PC61.5	eBioscience	AF700	1:200
αFoxp3	FJR-16s	eBioscience	APC	1:200

3 Methods

3.1 Animal Sensitization and Aerosol Challenge

Over the past decade, we have extensively characterized a biphasic response of Ag-sensitized mice to inhaled antigen challenge (*see* Fig. 2). After initial intraperitoneal sensitization to OVA, daily aerosol challenge with OVA for 3–10 days results in allergic airway disease, characterized by antigen-specific IgE production, airway eosinophilia, airway Th2 cytokine elaboration, increased pulmonary B and T lymphocytes, and airway hyperresponsiveness to methacholine. In contrast, during 6 weeks of continuous daily aerosol challenge with OVA, mice are able to reestablish airway homeostasis, with resolution of the above inflammatory changes despite continued systemic IgE production and cutaneous responses [42, 43]. We have termed this chronic phase local inhalational tolerance, to emphasize the focal nature of the anti-inflammatory process. How mice are able to downregulate the allergic airways disease (AAD) response is a complex process, involving CD25⁺Foxp3⁺CD4⁺ Treg cells [44], NKG2A⁺CD8⁺ T cells [45], and CD5⁺ TGFβ-producing Breg cells [28, 29]. Further understanding of the generation and regulatory roles of these T and B cells may lead to immunization strategies designed to induce local inhalation tolerance (LIT) in humans, thereby attenuating or curing their asthma.

3.1.1 Intraperitoneal (i.p.) Sensitization

1. Right before injecting animals, add to a 50-ml centrifuge tube: 120 mg alum, 0.3 ml of 5 mg/ml ovalbumin stock, 29.7-ml sterile saline for a final volume of 30 ml. Shake well before filling 1-ml syringe.
2. Each mouse is injected with 0.5 ml i.p.

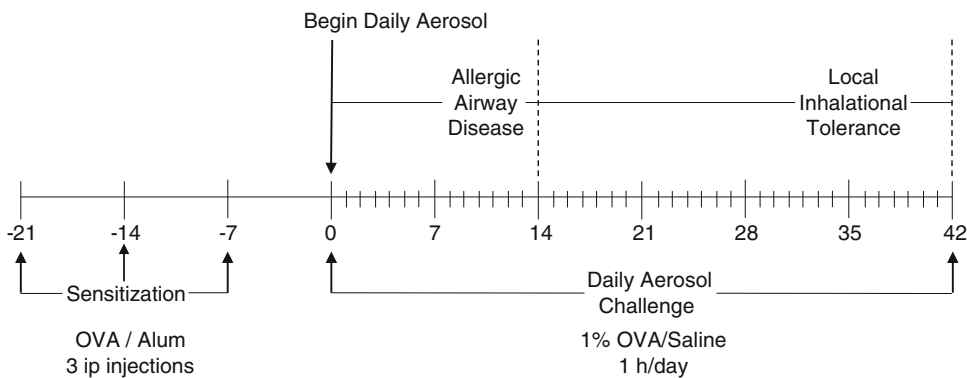


Fig. 2 Protocol for generation of murine allergic airway disease and local inhalational tolerance. Mice are sensitized to OVA-alum (i.p.) weekly for three consecutive weeks. Beginning on day 0, mice are challenged with OVA aerosol, 1 h/day for consecutive days. Animals are sacrificed 24 h after 7–14 days of OVA aerosols for assessment of allergic airway disease and after 6 weeks of daily OVA aerosols for established local inhalational tolerance

3.1.2 *Aerosol Challenge*

1. Vortex/shake 1 % OVA tube to ensure it has completely dissolved, then pour into basin. Pull up into a 20-ml syringe and place a 0.45- μm syringe filter on tip. Push OVA solution through filter into nebulizer cup. Make up OVA solution fresh for each run.
2. Attach nebulizer cup to nebulizer.
3. Place mice into restrainers into the inhalation chamber (*see Note 3*).
4. Adjust aerosol chamber airflow (at a rate of ~ 6 l/min) to generate OVA particles of mass median aerodynamic diameter and geometric standard deviation of approximately 1.4 μm and 1.6 μm , respectively (as determined by a cascade impactor).
5. Also periodically, adjust OVA-aerosol exposure time to provide an estimated daily inhaled OVA dose of 40–80 $\mu\text{g}/\text{mouse}$ (*see Note 4*).

3.2 *Mouse Sacrifice and Blood and Bronchoalveolar Lavage Collection*

3.2.1 *Animal Sacrifice*

1. 24 h after the last OVA aerosol, mice are sacrificed by anesthetic overdose (0.14 ml 3:100 xylazine/ketamine) and exsanguination.
2. When deeply anesthetized (*see Note 5*), open chest through diaphragm and puncture heart with a 1-ml syringe. Collect as much blood as possible, usually ~ 0.5 ml.
3. Slowly expel blood into a 1.5-ml Eppendorf tube (remove needle to avoid hemolysis).
4. Let it stand at room temperature (RT) for 60 min to allow blood to clot. Break up clot with wooden stick, then centrifuge for 10 min at $1,800\times g$ in Eppendorf 5415C.
5. Carefully pipette off clear serum, aliquot, and freeze at -80°C .

3.2.2 *Bronchoalveolar Lavage (BAL)*

1. Open rest of chest and throat to expose trachea.
2. With mouse nose toward you, pull a 6" strand of silk under trachea with curved small forceps. With small sharp scissors, make an incision in trachea posterior to larynx.
3. Insert cannula into incision and advance toward lungs until it meets resistance (bifurcation); pull back 1–2 mm and secure with two silk square knots.
4. Attach Luer from cannula into three-way stopcock attached to two 5-ml syringes, one of which is filled with 5-ml physiologic saline (or PBS). Gently push 0.8–1 ml saline into lungs and observe them inflate (*see Note 6*). Switch stopcock to empty syringe which acts as a reservoir and withdraw as much saline as possible. Repeat inflation and deflation until saline is gone.
5. Record volume of lavage collected in syringe and expel into 15-ml centrifuge tube on ice.

6. BAL fluid is spun down for 10 min at $600\times g$ at 4 °C.
7. BAL supernatant is transferred to another tube to be concentrated for protein or cytokine profile. BAL cells are resuspended in 200 μ l saline for total counts and further processing for differentials and FACS (*see Note 7*).
8. For total cell count, suspend cells from mouse BAL (*see Note 8*) in 200 μ l saline. Mix 15 μ l cell suspension with 15 μ l 0.2 % nigrosin in a 96-well plate and count cells with a hemocytometer (*see Note 9*).
9. To determine BAL leukocyte differentials, after total BAL cells are counted, 1×10^5 BAL cells are spun onto labeled $1\times 3''$ frosted slides in a Cytospin 4 centrifuge ($100\times g$, 5 min) and then air-dried. Dried slides are stained in Coplin jars as follows: fix 5 min in methanol (can stop at this point and resume later); stain 5 min in May-Grunwald stain; incubate 1–5 min in May-Grunwald buffer, pH 7.2; and stain 15–20 min in 1:20 Giemsa stain. Rinse briefly in distilled water; air-dry. At least 100 cells are then counted and sorted according to their morphology (*see Note 10*).

3.3 Tissue Collection and Cell Preparation

3.3.1 Lymph Node Preparation

1. Hilar (mediastinal) lymph nodes (HLN) are identified below and slightly posterior to the carina (*see Note 11*).
2. Place nodes in HBSS solution on ice.
3. Using two frosted slides, crush node into one well of 6-well plate or 100-mm culture dish containing 4 ml HBSS. Pipette thru 5-ml tube with cell strainer cap, count, ready for FACS.

3.3.2 Lung Preparation

1. Pre-warm water bath to 37 °C.
2. After perfusion of mouse heart with 5 ml PBS and heparin, lungs are removed and placed in 30×10 mm dish with 4 °C $1\times$ HBSS containing 1.3 mM EDTA.
3. Use forceps and scissors to mince the lung into very fine pieces.
4. Pipette lung fragments into pre-labeled 50-ml tube and fill to 20 ml with HBSS containing 1.3 mM EDTA.
5. Centrifuge for 5 min at $450\times g$.
6. Pipette off supernatant to ensure that lung pieces stay for digest.
7. Digest lung in collagenase/RPMI digestion solution in a 50-ml tube at 37 °C with shaking for 60 min.
8. Pour into a new 50-ml tube through cell strainer. Mash cells with masher and use pipette to rinse with HBSS, filling to 20 ml.
9. Centrifuge for 5 min at $450\times g$; discard supernatant.

10. Resuspend pellet in 1 ml TAC solution per lung tissue for 1 min and add 10 ml HBSS.
11. Centrifuge for 5 min at $450\times g$. Resuspend pellet on 4 ml FACS buffer and divide as needed for flow cytometry analysis. Otherwise, resuspend cells in RPMI for culture.
12. Count dead and live cells with nigrosin. $\sim 1\text{--}2\times 10^6$ cells are needed for flow cytometry analysis.

3.3.3 Spleen Lymphocyte Preparation

1. Remove spleen before perfusion and place in HBSS on ice.
2. Place cell strainer over 50-ml tube and mash spleen through with rubber tip from 5-ml syringe, rinse with 10 ml HBSS.
3. Centrifuge for 5 min at $450\times g$; decant off supernatant.
4. Add 1 ml TAC solution per spleen and gently swirl the tube. Wait for 1–2 min and fill tube with 25 ml HBSS.
5. Centrifuge for 5 min at $450\times g$; decant off supernatant.
6. Add 5–10 ml HBSS thru screen again. Cells at this point are stable on ice for several hours.
7. Count cells with nigrosin, both dead and viable. Ready for FACS staining.

3.4 Confocal Microscopy

Confocal microscopy is a valuable tool that allows the user to visualize cells in deeper tissue sections and create images in three dimensions. The major advantage of a confocal microscope over a conventional optical microscope is the presence of confocal pinholes. These allow light only from the plane of focus to reach the detector producing images of much greater resolution and clarity. The confocal microscope also enables users to collect optically sliced images at varying depth for creating a 3D representation of the sample (*see* Fig. 3).

3.4.1 Tissue Preparation for Cryostat Sectioning

1. Pour a thin layer of O.C.T. compound in the Cryomold without filling the entire volume of the mold. This ensures that the tissue does not directly come in contact with the Cryomold.
2. Isolate tissues of interest (e.g., HLN), making sure to carefully remove surrounding fat tissue and not breaking the tissue architecture.
3. Place tissue directly on the mold in its desired orientation, and fill rest of the mold with O.C.T.
4. Freeze the tissues in -80°C . Frozen tissues in O.C.T. can be preserved indefinitely.
5. Collect sections of $20\text{ }\mu\text{m}$ thickness directly onto the charged slides. For consistency, middle sections from each tissue could be used for staining.
6. Fix the sections in acetone for 5–10 min, air-dry the slides, and preserve them at -20°C .

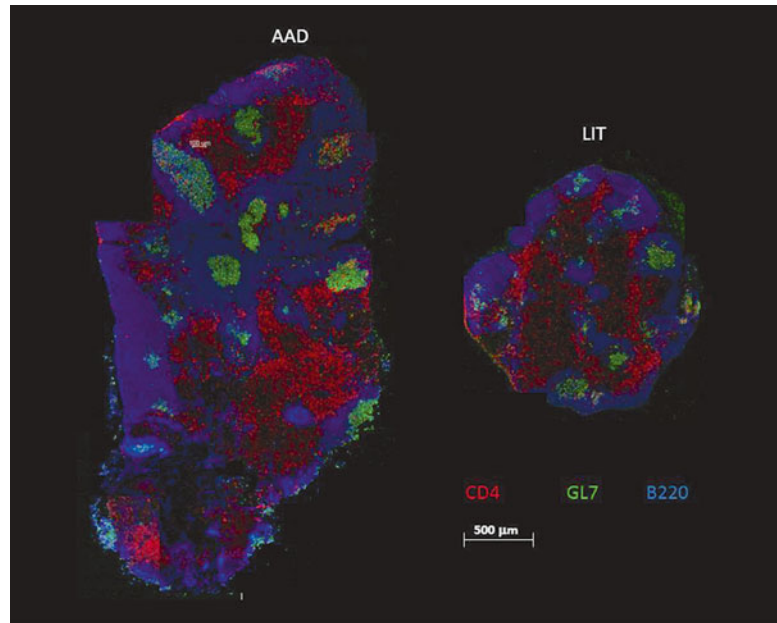


Fig. 3 Representative confocal images of HLNs from mice at allergic airway disease and at local inhalational tolerance. Confocal microscopy was performed to determine the presence of germinal centers at these stages. CD4 in red (CD4⁺ T cells), B220 in blue (B cells), GL7 in green (germinal center marker). Samples are 20 μ m thick midsections from the nodes; image magnification is 20 $\times$

3.4.2 Staining Procedure for Evaluation of Surface Marker Expression

1. Circle the sections with PAP pen making a ridge to contain the Ab solution and prevent it from spreading over the slide.
2. Soak slides in ice-cold PBS to melt off O.C.T. from around tissue sections.
3. Dab PBS from back of slides and excess from around tissue carefully but keep tissues damp.
4. Prepare Ab solutions with primary Abs (*see* Table 1) in the staining buffer at a desired concentration.
5. Add 300 μ l primary Ab solution per slide.
6. Incubate for 1 h at RT (or overnight at 4 $^{\circ}$ C).
7. Tap off Ab onto a paper towel.
8. Wash slides by soaking them in ice-cold PBS for 5 min; aspirate off PBS; repeat procedure two additional times.
9. Wipe off excess PBS as above and add secondary Ab solution as above.
10. Incubate for 1 h at RT and wash three times with PBS (as in **step 8** above).
11. Hold slide at a slanting position and add one drop of Immumount on the higher part of the section and allow it to flow down the slide.

12. Place a coverslip at an angle on the higher side of the section and slowly lower to cover the entire section to avoid air bubbles.
13. Clean off excess Immu-mount around the edge of the coverslip; store slides at 4 °C.
14. Let the slides set at 4 °C overnight.
15. Capture images using a confocal microscope and analyze images using ImageJ or Imaris Suite®.

3.4.3 Staining Procedure for Evaluation of Intracellularly Expressed Molecules

For the identification of intracellularly expressed molecules (e.g., Foxp3), surface-stained cells must first be permeabilized using fixation/permeabilization buffer, according to the manufacturer's protocol, before staining with Abs (e.g., anti-Foxp3 Ab).

1. After surface staining the sections, permeabilize the sections using the Foxp3 staining buffer set.
2. Add 300 µl cytofix/cytoperm solution and incubate at 4 °C for 1 h.
3. Wash the slides twice with 1× permeabilization buffer and wipe off excess buffer.
4. Add 300 µl Ab solution in permeabilization buffer and incubate an hour and a half at RT or overnight at 4 °C.
5. Wash the slides with permeabilization buffer and mount the slides using mounting medium.

3.5 Functional Breg Assays

The identification of Breg cells may be based on cell surface phenotyping and cytoplasmic IL-10 staining, as extensively described [15]. However, they may also be characterized functionally, based on their suppressive functions in vitro and in vivo. In our murine model, we have shown that Breg cells isolated from HLNs of animals with local inhalational tolerance can inhibit T-cell proliferation in coculture assays and induce CD4⁺ T-cell Foxp3 expression via a TGFβ-dependent pathway. In addition, these Breg cells attenuate the development of AAD when adoptively transferred into sensitized and subsequently OVA-aerosol challenged mice [28, 29]. Details of these functional assays are presented below.

3.5.1 T-Cell Proliferation Assay

To assess the ability of Breg cells to inhibit T-cell proliferation, a T-cell proliferation assay must be performed after coculture of CD19⁺ Breg cells (or CD19⁺CD5⁺ Breg cells) with CD4⁺CD25⁻ T cells. The suppression of T-cell proliferation is achieved by assessing CFSE dilution on flow cytometry.

CD19⁺ Breg Cells Isolation from HLNs of Mice

CD19⁺ Breg cells are isolated using positive selection with CD19⁺ beads, according to the manufacturer's directions. All steps are done under sterile conditions.

1. Sterilely remove HLNs under hood into 5 ml ice-cold MACS buffer. Then crush the HLNs between two slides (frosted ends).
2. Centrifuge the crushed cell suspension for 10 min at $300 \times g$ at 4 °C.
3. Aspirate supernatant and resuspend pellet in 3 ml MACS solution.
4. Count cells with hemocytometer and suspend in MACS buffer at a concentration of 90 μ l MACS buffer per 10^7 cells.
5. Add 10 μ l CD19⁺ MicroBeads per 10^7 cells. Incubate in refrigerator for 15 min.
6. Prepare an LS column by placing into magnet stand, then washing with 3 ml MACS buffer.
7. Wash cells after the incubation with 15 ml MACS buffer; resuspend in 0.5 ml MACS buffer and apply to column through Nitex mesh.
8. Pass cell suspension over prepared LS column. Wash with 3×3 ml MACS buffer. Collect flow through for non-CD19⁺ cells if desired.
9. Remove column from magnet and place on a 15-ml tube. Add 5-ml MACS buffer on top of the column and push the positively selected cells using the plunger provided with the column.
10. Count CD19⁺ cells with hemocytometer (*see* **Note 12**).

Isolation of CD19⁺CD5⁺
B Cells

To isolate CD19⁺CD5⁺ B cells from HLNs or lung single cell suspensions, perform B-cell selection using non-B-cell depletion kit followed by CD5⁺ microbead selection, according to manufacturer's protocol.

Isolation
of CD4⁺CD25⁻ T Cells

CD4⁺CD25⁻ T cells are isolated from spleens of naïve mice, using positive selection with CD4⁺ beads, according to the manufacturer's directions.

1. Sterilely remove spleens in the hood into 5 ml ice-cold MACS buffer. Then crush spleens between two slides (frosted ends).
2. Centrifuge crushed cell suspension for 10 min at $300 \times g$ at 4 °C.
3. Aspirate supernatant and resuspend pellet in 1 ml sterile per spleen TAC solution for 1 min at RT. Add 45 ml MACS solution and spin cells for 10 min at $300 \times g$.
4. Count cells with hemocytometer and suspend at a concentration of 1×10^7 cells in 40 μ l MACS buffer.
5. Add 10 μ l of Biotin-Antibody Cocktail per 10^7 total cells. Mix well and refrigerate for 10 min (4–8 °C).
6. Add 30 μ l of buffer, 20 μ l of Anti-Biotin MicroBeads and 10 μ l of CD25-PE antibody per 10^7 total cells. Mix well and refrigerate for an additional 15 min in the dark (4–8 °C).

7. Prepare an LD column by placing into magnet stand, then washing with 2 ml MACS buffer.
8. Wash cells by adding 1–2 ml of buffer per 10^7 total cells and centrifuge at 10 min at $300\times g$. Aspirate supernatant completely. Resuspend 10^8 cells in 500 μ l buffer. Pass over prepared LS column through Nitex.
9. Wash column twice with 1 ml MACS buffer. Collect flow through for isolated CD4⁺ T cells.
10. Centrifuge isolated CD4⁺ T cells at $300\times g$ for 10 min. Aspirate supernatant completely. Resuspend cell pellet in 90 μ l of buffer per 10^8 cells.
11. Add 10 μ l of Anti-PE MicroBeads per 10^8 cells. Mix well and refrigerate for 15 min in the dark (4–8 °C).
12. Place MS Column in the magnetic field of a suitable MACS Separator. Prepare column by rinsing with 500 μ l of buffer.
13. Wash cells by adding 1–2 ml of buffer and centrifuge at $300\times g$ for 10 min. Aspirate supernatant completely. Resuspend up to 10^8 cells in 500 μ l of buffer.
14. Apply cell suspension onto the column through Nitex. Let cells pass through and wash column with 500 μ l of buffer. Perform the washing step three times by adding buffer times only once the column reservoir is empty. Collect the flow through for CD4⁺CD25⁻ T cells.

CFSE Labeling
of CD4⁺CD25⁻ T Cells

1. Prepare T cells at 10^7 /ml in PBS (*see Note 13*).
2. Treat cells with an equivalent volume of 5 μ M CFSE stock solution (final CFSE concentration 2.5 μ M CFSE) for 4 min at RT, rocking gently every minute.
3. After 4 min, add to tube an equivalent volume of newborn or fetal bovine serum. Invert the tube and leave for a minute.
4. Spin cells and wash twice in cell culture medium.
5. Coculture naïve spleen CD4⁺CD25⁻ T cells (1×10^6) for 60 h with viable irradiated HLN CD19⁺ Breg cells (1×10^6 , or 2×10^5 HLN CD19⁺CD5⁺ Breg cells) and soluble anti-CD3 and anti-CD28 (both at 1 μ g/ml) in RPMI 1640 supplemented with 10 % FCS and 50 μ M 2-mercaptoethanol.
6. After 60 h of coculture, harvest cells from the plate, stain with CD4, and observe for proliferation via CFSE dilution on flow cytometry.

3.5.2 Treg
Induction Assay

All subsequent steps are performed under sterile conditions.

1. Isolate HLN CD19⁺ Breg cells and spleen CD4⁺CD25⁻ T cells as described above.
2. Irradiate HLN CD19⁺ Breg cells with 2,600 rads.

3. Coculture naïve spleen CD4⁺CD25⁻ T cells (1×10^6) for 60 h with 1×10^6 viable irradiated HLN CD19⁺ Breg cells (or 2×10^5 HLN CD19⁺CD5⁺ Breg cells) in the presence of soluble anti-CD3 and anti-CD28 (both at 1 µg/ml) in RPMI 1640 supplemented with 10 % FCS and 50 µM 2-mercaptoethanol.
4. After 5 days in culture, harvest cells from the wells and stain for intracellular Foxp3. Foxp3 expression is measured in gated CD4⁺ T cells by flow cytometry.

3.5.3 Adoptive Transfer Studies

In order to evaluate the ability of Breg cells to attenuate the development of AAD, CD19⁺ (or CD5⁺CD19⁺) Breg cells are sorted from LIT HLNs and adoptively transferred into sensitized, and subsequently OVA-aerosol-challenged, mice.

1. Isolate CD19⁺ B cells from LIT HLNs as described in Subheadings 3.3.1 and “Isolation of CD19⁺CD5⁺ B cells”.
2. Spin down the cells and discard supernatant. Dislodge the cell pellet (should be around 200 µl of volume left behind).
3. Stain cells for 20 min with CD19-PE (and CD5-FITC if desired) monoclonal antibodies: CD19-PE at 1:100 and CD5-FITC at 1:50. Wash cells in MACS buffer (*see* **Note 14**). Resuspend in about 1–2 ml of MACS buffer for the sort.
4. Sort for CD19⁺ (or CD5⁺CD19⁺) Breg cells by flow cytometry.
5. Resuspend cells in 200 µl sterile saline and transfer 1×10^6 CD19⁺ Breg cells (or 2×10^5 CD5⁺CD19⁺) Breg cells into sensitized recipients via tail vein injections, 2 days prior to starting aerosol exposures.
6. Sacrifice after 7 days of OVA aerosol to assess changes to AAD by transferred cells (*see* **Note 15**).

4 Notes

1. OVA stock solution (5 mg/ml) may be frozen for future use. Divide into 0.3 ml aliquots and freeze at -20°C in 12×75 -mm plastic tubes.
2. Blacken the end of cannula with a sharpie to better visualize its tip.
3. Mice should be snugly in place, with their noses inserted into the chamber so that they are unable to turn around in the restrainers.
4. The airflow and exposure time adjustment as discussed in the aerosol challenge section are checked periodically.
5. Use toe pinch to make sure mouse is anesthetized, look for agonal breathing. Blood return is enhanced if the heart is still beating during the collection.

6. When performing bronchoalveolar lavage, instill slowly and steadily, watching lungs inflate. Stop instillation when lungs appear fully inflated and when pressure increases, so that the lungs do not rupture.
7. Remove any other organ at this time, e.g., lungs, trachea, and lymph nodes. Lungs for histology are put into 25 ml buffered formalin in a 50-ml tube and held under the surface of the liquid with a piece of gauze. Lungs to be FACS stained are digested for 1 h at 37 °C with collagenase (*see* Subheading 3.3.2).
8. This method is not only used to count cells from mouse BAL but also to count cells obtained from tissue processing.
9. Apply cell suspension to hemocytometer with pipette tip. Examine hemocytometer under a microscope with an objective power of 40× and count viable leukocytes within the 25 squares. The number of cells in the 25 squares multiplied by 2 (for the dilution in nigrosin) gives the number of cells $\times 10^4$ in 1 ml of cell suspension. By multiplying for the volume of suspension, one obtains the total number of BAL (or tissue) cells.

Do not count red blood cells (bumpy, rough outer coat, no nuclei; look like popcorn) or cells stained blue with nigrosin (dead cells).

10. Cells are identified according to their specific staining characteristics, as follows:
 - Macrophages are large pleomorphic cells with lightly stained nuclei and a large amount of foamy, light blue cytoplasm. These cells normally constitute 97–98 % of BAL cells in naïve mice.
 - Eosinophils have dark blue to purple bilobed or donut-shaped nuclei and red to orange-red granules within light blue cytoplasm.
 - Polymorphonuclear neutrophils have dark blue to purple multilobed nuclei with few reddish purple granules in pale pink cytoplasm.
 - Lymphocytes/monocytes are small round cells with densely stained nuclei and very little sky-blue cytoplasm.
11. Hilar lymph nodes are tiny and barely identifiable (≤ 1 mm diameter) in naïve and sensitized mice. They enlarge during AAD to 2–4 mm in diameter and shrink slightly during LIT (1–2 mm diameter).
12. In our experience, we isolate five to ten million B cells from pooled 10–15 LIT HLNs.
13. Fewer than 10^7 /ml cells results in killing of cells by CFSE. For cleaner staining, wash cells once in cold PBS.

14. Never use FACS buffer in these studies. It will fix the cells during staining because of its azide content, and they will not remain functional.
15. AAD can be quantified by several methods, including degree of airway eosinophilia, airway Th2 cytokine levels or lung tissue Th2 cytokine production, histologic lung inflammation, and/or increased responsiveness or sensitivity to methacholine.

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