

Characterization of Group 2 Innate Lymphoid Cells in Allergic Airway Inflammation Models in the Mouse

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Abstract

Allergic asthma is a chronic inflammatory lung disease mediated by type 2 cytokines produced by T helper 2 (Th2) cells as well as the recently discovered group 2 innate lymphoid cells (ILC2). Due to a lack of unique markers, the accurate phenotypic characterization and quantification of ILC2 requires a comprehensive panel of fluorescently labeled antibodies. The markers that are currently used to characterize ILC2 have not been standardized and often vary between research groups, which poses significant challenges when comparing data. Intranasal administration of the pro-inflammatory cytokine IL-33 in mice is associated with strong, Th2 cell-independent ILC2 activation. ILC2 are also activated in mouse models of allergic asthma based on the physiologically relevant house dust mite (HDM) allergen, which parallel eosinophilic airway inflammation observed in asthma patients. Here, we describe the analysis of ILC2 by flow cytometry in these two commonly used allergic airway inflammation models in the mouse.

Key words Allergy and asthma, Group 2 innate lymphoid cells (ILC2), House dust mite, Interleukin-33, Murine airway inflammation model

1 Introduction

Asthma is a disease of the airways involving chronic inflammation and remodeling and is characterized by episodes of coughing, wheezing, and shortness of breath. Patients can be clustered into several endotypes based on symptoms, disease mechanisms and immunological profile, the most common being allergic asthma [1]. The T helper 2 (Th2) cell is classically placed in the center of the pathophysiology of allergic asthma. The Th2 signature cytokines IL-4, IL-5, and IL-13 are key orchestrators of the hallmarks of asthma. These include persistent inflammation, smooth muscle cell hyperplasia, mucous cell metaplasia, and airway hyperresponsiveness and remodeling [2]. Recently, innate counterparts of Th2 cells referred to as group 2 innate lymphoid

cells (ILC2) have been identified as an innate source of type 2 cytokines and have been hypothesized to contribute to the pathogenesis of allergic asthma [3, 4].

Observations that a non-B/non-T cell population was capable of producing IL-5 and IL-13 in response to IL-25 were first made by Fort et al. in 2001 [5]. It was reported some years later that this IL-25-dependent population is an important source of type 2 cytokines and that these cells are critically involved in helminth expulsion [6]. In 2010, several independent research groups characterized this novel non-B/non-T lymphocyte population in fat associated lymphoid clusters, mesenteric lymph nodes, spleen, and liver and found them to be highly responsive to both IL-25 and IL-33, although different names were assigned to the cells at the time [7–9]. Cells with a similar phenotype and cytokine profile were also discovered in the lung in the context of influenza infection [10, 11]. A universal nomenclature was proposed and it is now generally accepted that in the mouse ILC2 are negative for classic hematopoietic lineage markers, express Sca-1, CD117 (c-kit), CD25 (IL-2R α), CD127 (IL-7R α), and T1/ST2 (IL-33R) on their cell surface, and are dependent on the transcription factor GATA3 [7–9, 12–15].

IL-25/IL-33-responsive ILC2 have also been found in human lungs and are enriched in the nasal polyps of patients suffering from chronic rhinosinusitis, a typical type 2 inflammatory disease [16]. These human ILC2 are defined by the expression of the seven-transmembrane prostaglandin D2 receptor CRTH2/CD294 and the natural killer marker CD161 and also require GATA3 [16, 17]. More recently, studies in asthmatic patients revealed increased numbers of ILC2 in peripheral blood and sputum, compared with healthy controls [18–20]. Moreover, this population appeared to be steroid insensitive, which is in agreement with in vitro studies showing that TSLP is a major determining factor in steroid sensitivity of ILC2 [20].

To explore the role of ILC2 in the complex inflammatory processes of allergic asthma, a number of mouse models have been developed that mimic allergy and asthmatic responses to physiological allergens. Intranasal administration of the protease allergen papain leads to activation of ILC2 and induces eosinophilic airway inflammation in wild-type as well as T and B cell-deficient *Rag1*^{−/−} mice. However, *Rag2*^{−/−}*Il2rg*^{−/−} mice that additionally lack ILC2 do not mount an inflammatory response to papain and adoptive transfer of wild-type ILC2 into these mice restored inflammation [21]. In addition, ILC2-derived IL-13 promotes migration of activated lung dendritic cells towards the lymph nodes where subsequent T cell priming occurs, further suggesting a central role for ILC2 as early initiators of inflammation [22]. Experimental asthma models using the fungal allergen

Alternaria alternata have led to similar results, showing an IL-33-mediated inflammatory response orchestrated by activated ILC2 rather than Th2 cells [23, 24]. Together with data showing that ILC2 are capable of enhancing Th2 cell differentiation and function, these findings support a model in which ILC2 can become rapidly activated to provide an early source of type 2 cytokines and bridges the gap between innate and adaptive immunity [25, 26]. In contrast, *Rag1*^{-/-} mice exposed to *Aspergillus fumigatus*, another fungal allergen, fail to develop airway inflammation and hyperresponsiveness. Moreover, ovalbumin-driven airway inflammation is attenuated upon depletion of CD4⁺ T cells [27, 28]. In house dust mite (HDM)-induced allergic airway inflammation, blockade of CD28 signaling strongly diminishes airway hyperresponsiveness and pulmonary infiltrates of inflammatory cells in the lung [29]. Interestingly, chronic exposure to a cocktail of *Alternaria*, *Aspergillus*, and HDM synergistically induces airway inflammation, hyperresponsiveness, and remodeling that is dependent on adaptive immunity [30]. Taken together, these models instead indicate a critical role for T cells in mediating and maintaining allergic airway inflammation. Therefore, the contribution of ILC2 in the induction of allergic inflammation appears highly dependent on the allergen model.

Here, we describe in detail a T cell-independent and a T cell-dependent murine model for airway inflammation via intranasal administration of IL-33 or HDM, respectively. The alarmin cytokine IL-33 is constitutively expressed in the nucleus of airway epithelial cells and is released upon cellular stress or injury that occurs during exposure to a number of different environmental allergens [31]. IL-33 is a potent stimulator of ILC2 proliferation and cytokine production and intranasal administration of IL-33 leads to eosinophilic airway inflammation reminiscent of allergic asthma, which provides a unique tool to specifically investigate ILC2 function during inflammation [32–34]. A more physiologically relevant mouse model for allergic asthma employs HDM extract. Intranasal sensitization followed by provocation challenge with HDM produces eosinophilic infiltrates in the lung and airway hyperresponsiveness that closely resembles clinical observations in patients with allergic asthma [2]. Although there is abundant literature on mouse ILC2 in a variety of inflammatory diseases, the markers used to characterize ILC2 have not been standardized, which poses difficulties when comparing data from various publications. In this report, we provide a detailed insight into the induction of airway inflammation in mouse models based on exposure to IL-33 and HDM, focusing on the characterization of the ILC2 phenotype in bronchoalveolar lavage (BAL) fluid, lungs, and mediastinal lymph nodes (MLN) using flow cytometry.

2 Materials

Standard laboratory equipment including plates, tubes, syringes, pipettes, and centrifuges is not listed.

2.1 Induction of IL-33 and HDM-Induced Airway Inflammation

1. 8–16-week-old C57BL/6 mice housed in a specific-pathogen-free facility, fed ad libitum, and age- and gender-matched (*see Note 1*).
2. Recombinant mouse IL-33 (BioLegend, USA; 0.5 µg/40 µL; *see Note 2*).
3. HDM extract from *Dermatophagoides pteronyssinus* (Greer, USA; 1 µg/40 µL for sensitization and 10 µg/40 µL for challenge; *see Note 3*).
4. PBS.
5. Isoflurane anesthesia device.

2.2 Collection of BAL Fluid, Lungs, and MLN

1. Anesthetic and tools suitable for sacrificing mice (*see Note 4*).
2. Cannula: 23-gauge × 1¼ in. needle with the tip sheathed by ~10 mm of polyethylene tubing (inner diameter 0.58 mm/0.023 in.) to prevent puncturing of the airways (Fig. 1a).
3. Suture thread.
4. PBS containing 0.5 mM ethylenediaminetetraacetic acid (EDTA).
5. Hank's balanced salt solution (HBSS).

2.3 Preparation of Single-Cell Suspension

1. PBS containing 0.5 mM EDTA supplemented with 0.5 % bovine serum albumin (BSA).
2. Osmotic lysis buffer: 8.3 % NH₄Cl, 1 % KHCO₃, and 0.04 % Na₂EDTA in Milli-Q.
3. 100 µm nylon cell strainers.
4. RPMI 1640 Cell culture medium containing 10 % fetal bovine serum (FBS).

2.4 Flow Cytometry

1. FACS-buffer containing 0.25 % BSA, 0.5 mM EDTA, and 0.05 % NaN₃ in PBS with a final pH of 7.2.
2. For intracellular transcription factor detection: eBioscience FoxP3/transcription factor staining kit containing (1) fixation and permeabilization concentrate and (2) diluent and (3) 10× wash buffer to be diluted in Milli-Q.
3. For intracellular cytokine detection: phorbol myristate acetate (PMA), ionomycin, and GolgiStop (BD Biosciences, USA). 4 %



Fig. 1 Stepwise procedure for the collection of BAL fluid, lungs, and MLN. (a) A cannula used for the collection of BAL fluid. The polyethylene sheath prevents puncturing of the airways and is colored red to indicate its placement. (b) Preparation of the trachea for cannula insertion. The connective tissue is removed and a small incision is made between the tracheal cartilage along the *dotted blue line*. (c) Inserted cannula secured by suture thread. (d) The thoracic cavity can be opened by removing the diaphragm and cutting the ribs on both sides along the *dotted blue line*. (e) MLN can be exposed by lifting the right lung lobes and is situated below a blood vessel

paraformaldehyde (PFA) in PBS as stock solution, diluted 1:2 in PBS to a 2% working solution. Permeabilization and wash buffer of 0.5% saponin (Sigma-Aldrich, USA) in FACS-buffer.

4. Purified and unlabeled CD16/CD32 antibody (Fc-block, clone 2.4G2).
5. Fixable live/dead cell stain (Fixable Viability Dye eFluor® 506, eBioscience, USA).
6. Antibodies used for flow cytometry, listed in Table 1.
7. Data acquisition is performed using an LSR II flow cytometer (Beckton Dickinson, USA) equipped with three lasers and FACSDiva™ software (Beckton Dickinson, USA) and analyzed by FlowJo v10 (Tree Star Inc., USA) software.

Table 1
Antibodies used for flow cytometric analysis of ILC2, eosinophils, and neutrophils

Antibody	Conjugate	Clone	Company
ILC2 lineage mix			
B220	PE	RA3-6B2	eBioscience
CD3e	PE	145-2c11	eBioscience
CD4	PE	GK1.5	eBioscience
CD5	PE	53-7.3	eBioscience
CD11b	PE	M1/70	eBioscience
CD11c	PE	N418	eBioscience
CD19	PE	1D3	BD Biosciences
CD8a	PE	53-6.7	eBioscience
FcεRIα	PE	MAR-1	eBioscience
Gr-1	PE	RB6-8C5	BD Biosciences
NK1.1	PE	PK136	eBiosciences
TER-119	PE	TER-119	eBioscience
ILC2 surface markers			
CD45	PE-CF594	13/2.3	Abcam
ICOS	APC	C398.4A	eBioscience
KLRG1	Biotin	2F1/KLRG1	BioLegend
MHCII	Brilliant Violet 650	M5/114.15.2	BD Biosciences
Sca-1	Brilliant Violet 786	D7	BD Biosciences
T1/ST2	Biotin	DJ8	MD Bioproducts
T1/ST2	FITC	DJ8	MD Bioproducts
Streptavidin	APC-eFluor 780		eBioscience
CD25	PerCP-Cy5.5	PC61.5	eBioscience
CD127	PE-Cy7	A7R34	eBioscience
CD127	eFluor450	A7R34	eBioscience
ILC2 transcription factors			
GATA3	eFluor 660	TWAJ-14	eBioscience
ILC2 cytokines			
Amphiregulin	Biotin	Polyclonal	R&D Systems
IL-4	Brilliant Violet 711	11B11	BD Biosciences
IL-5	APC	TRFK-5	BD Biosciences
IL-13	eFluor 450	eBio13A	eBioscience
Eosinophil and neutrophil surface markers			
Gr-1	APC-eFluor 780	RB6-8C5	eBioscience
Siglec-F	PE	E50-2440	BD Biosciences

3 Methods

3.1 Intranasal Injection

1. Mice are placed in a small gas chamber and are exposed to 2.5 % isoflurane anesthesia with an oxygen airflow of 1 L/min (VerEquip).
2. When the breathing rate has dropped to approximately two breaths per second, hold the mouse by the scruff of the neck with the abdomen facing you and tilt the body to a 45° angle.
3. Carefully place a drop of liquid on the tip of the nose using a pipette and wait for the mouse to inhale (*see* **Note 5**).
4. Once the droplet has been successfully inhaled and the breathing rate of the mouse starts to increase, place it on its back in the cage.

3.2 IL-33-Induced Airway Inflammation

1. Mice are administered 0.5 µg recombinant IL-33 in 40 µL PBS via intranasal injection under isoflurane anesthesia, as described above, three times with 1 day of rest between each injection.
2. Organs of interest are harvested 1 day after the final injection.
3. Control mice are treated with PBS.

3.3 HDM-Induced Airway Inflammation

1. Mice are sensitized with 1 µg HDM extract dissolved in 40 µL PBS via intranasal injection under isoflurane anesthesia as described above.
2. After a resting period of 7 days, mice are challenged daily to 10 µg HDM extract dissolved in 40 µL PBS by intranasal injection for 5 consecutive days.
3. Organs of interest are harvested 1 day after the final challenge.
4. Control mice are sensitized with PBS and are challenged with HDM.

3.4 Collection of BAL Fluid, Lungs, and MLN

1. Mice are sacrificed, using 75 mg/kg ketamine combined with 1 mg/kg medetomidine intraperitoneally (i.p.).
2. A midline incision is made to retract the skin and the connective tissue surrounding the trachea is carefully removed.
3. A suture thread is placed underneath the trachea by carefully lifting it with tweezers. This will be used to secure the cannula in the next step.
4. A small incision between the tracheal cartilage is made to insert a cannula, be careful not to sever the entire trachea (Fig. 1b). The cannula is secured in place using the suture thread in **step 3** by tying a knot around the portion of the trachea housing the cannula (Fig. 1c).

5. A syringe containing 1 mL PBS supplemented with 0.5 mM EDTA is attached to the cannula. The lungs are subsequently washed by gently flushing. Afterwards, the syringe is uncoupled and the aspirated BAL fluid is collected in a tube.
6. **Step 5** is repeated two more times for a total of ~3 mL BAL fluid.
7. The thoracic cavity is then opened by removing the diaphragm and cutting the ribs on both sides along the dorsal plane. The connective tissue between the thymus and the ribcage is removed and a transversal cut is made above the heart to completely expose the lungs (Fig. 1d).
8. The MLN is situated underneath the right lung lobes below a blood vessel and can be carefully extracted using tweezers and placed in HBSS for further processing (Fig. 1e).
9. Lungs lobes are separated from the bronchi and placed in HBSS for further processing.

3.5 Preparation of Single-Cell Suspensions

Lung and MLN tissue are mechanically disrupted on a 100 μ m cell strainer using the plunger of a syringe. The strainers are flushed with 5 mL PBS containing 0.5 mM EDTA and 0.5 % BSA to extract an optimal amount of cells.

1. BAL fluid, lung, and MLN cell suspensions are centrifuged at 4 °C 400 $\times g$ for 7 min and the supernatant from the BAL fluid may be collected for the detection of cytokine levels by ELISA (not discussed here). Lung and MLN supernatants are discarded.
2. BAL and MLN cell pellets are resuspended in an appropriate volume of cell culture medium for cell counting and are ready for flow cytometric applications.
3. The cell pellet from the lung is resuspended in 1 mL osmotic lysis buffer and incubated at room temperature for 2 min. Lysis is stopped by adding 10 mL cold PBS and the suspension is centrifuged at 4 °C 400 $\times g$ for 7 min and the supernatant is removed.
4. Cells are resuspended in cold cell culture medium and after a sample is taken to determine absolute cell numbers, they are ready for flow cytometric applications.

3.6 Flow Cytometry

3.6.1 Intracellular Flow Cytometry Staining Protocol for ILC2 Detection

1. To detect cytokine production, one million cells per well in a 96-well plate are stimulated with PMA (50 ng/mL; diluted from a 1000 $\times$ stock solution) and ionomycin (300 ng/mL diluted from a 1000 $\times$ stock solution), and GolgiStop (1:1500) diluted in cell culture medium for 4 h at 37 °C prior to antibody staining.
2. For surface marker staining, one million cells are placed in each well of a 96-well plate and are incubated with 40 μ L primary antibody mix in FACS buffer containing Fc-block for 30 min at 4 °C.

3. The samples are then washed once with FACS buffer and once with PBS.
4. Following this, the cells are incubated with fixable live/dead stain with 40 μ L secondary antibody mix in PBS and incubated for 15 min at 4 °C.
5. For intracellular transcription factor detection: cells are washed in PBS, fixed and permeabilized using eBioscience fixation and permeabilization buffer for 30 min at 4 °C, followed by two washing steps using eBioscience wash buffer. Next, the pellet is incubated with 40 μ L tertiary antibody mix in wash buffer for 60 min at 4 °C. Finally, the samples are washed, once with wash buffer and once with FACS buffer, before being resuspended in FACS buffer and measured by a flow cytometer within 1 day (*see Note 6*).
6. For intracellular cytokine detection: cells are washed in PBS and fixed using 2 % PFA for 15 min at 4 °C followed by two washes with PBS. After fixation, the cells are permeabilized with permeabilization and wash buffer containing 0.5 % saponin for 15 min at 4 °C. Next, the cell pellet is incubated with 40 μ L tertiary antibody mix in perm/wash buffer for 60 min at 4 °C. Finally, the samples are washed once with perm/wash buffer and once with FACS buffer before being resuspended in FACS buffer and acquired within 1 day (*see Note 6*).

3.6.2 Analysis of Allergic Airway Inflammation by Flow Cytometry

The number of eosinophils present in the BAL fluid can be used as an indicator for the severity of allergic inflammation. Successful induction of airway inflammation yields large numbers of granulocytes, characterized by high side scatter (SSC) and relative low forward scatter (FSC) values, of which 90 % or more consist of Siglec-F⁺ eosinophils (Fig. 2a). These percentages generally translate to between ~0.5 and ~1.0 million eosinophils in the BAL fluid. Cellular infiltrates can also be seen in the lungs and typically accumulate around the bronchioles and blood vessels. In addition, thickened alveolar septa are also observed (Fig. 2b).

3.6.3 Analysis of ILC2 by Flow Cytometry

To quantify ILC2 in the BAL fluid, living cells that are negative for the live/dead stain are selected. However, it should be kept in mind that macrophages are autofluorescent and therefore positive in the live/dead staining, irrespective of their viability, and are therefore also excluded. Furthermore, it is possible to remove any debris in this step that may interfere with subsequent gating. ILC2 are relatively small cells and fall within the general lymphocyte gate in the FSC/SSC plot. CD45 expression is used to exclude all non-leukocyte populations and the lineage markers are compiled in a “dump” channel to readily exclude major hematopoietic cell populations. ILC2 do not express lineage markers and express high levels of surface Sca-1. Furthermore, they also express the IL-33R

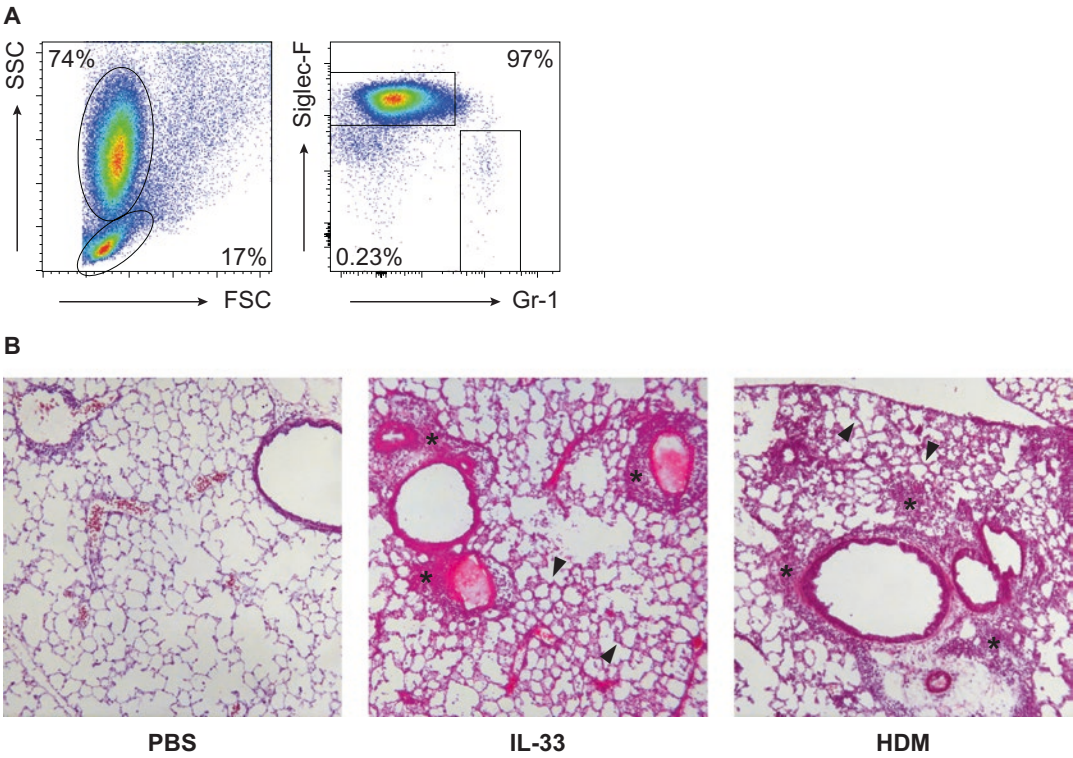


Fig. 2 Extent of eosinophilic inflammation in BAL fluid and lungs after IL-33 or HDM treatment. **(a)** Flow cytometric identification of eosinophils and neutrophils in the BAL fluid of IL-33-treated mice. Granulocytes are gated on the basis of high SSC and low FSC values and discriminated as eosinophils or neutrophils using Siglec-F and Gr-1, respectively. **(b)** Hematoxylin and eosin (H&E) staining of lung sections from PBS, IL-33, and HDM-treated mice, showing perivascular infiltrates (indicated by *asterisks*) and thickening of alveolar walls (indicated by *arrow heads*)

component T1/ST2 and intracellular GATA3 (Fig. 3a). Because GATA3, T1/ST2 and Sca-1 are also expressed by Th2 cells, it is critically important to optimize the lineage mix and to conservatively gate for lineage-negative cells to ensure no T cells are included within the ILC2 gate. The level of GATA3 expression within the ILC2 population is variable between different tissues (Fig. 3b). This likely reflects the activation state, as we observed that GATA3 levels increase when ILC2 are activated by IL-33 stimulation.

Although GATA3 is a consistent marker for ILC2, it is a nuclear protein that requires fixation and permeabilization steps for its detection. For applications where this is not desirable, such as live cell sorting, surface CD127 may be used as a replacement in conjunction with Sca-1 and T1/ST2. Although CD25 is also frequently associated with ILC2, its expression is variable (Fig. 3c). The observed differential expression on ILC2, depending on the type of stimulus applied, precludes the usage of CD25 as a surface

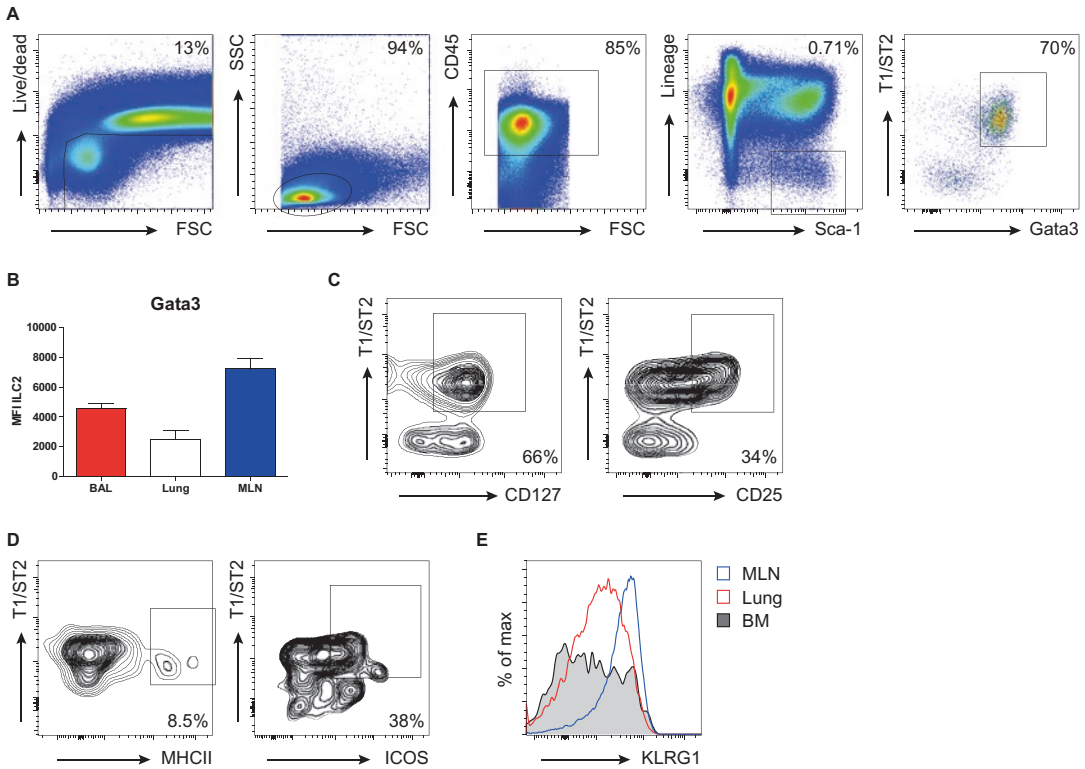


Fig. 3 Gating strategy for the identification of ILC2 in BAL fluid of HDM-treated mice. **(a)** After exclusion of dead cells and debris and gating for CD45⁺ lymphocytes, ILC2 are lineage-negative and express high levels of Sca-1, T1/ST2, and intracellular GATA3. **(b)** GATA3 expression level by ILC2 is variable between tissues, as shown by mean fluorescence intensity (MFI) values. **(c)** Alternative gating strategy for ILC2 from Lineage[−] Sca-1⁺ cells without intracellular staining, using CD127 as an additional marker. CD25 expression is variable within this population. **(d)** Analysis of MHCII and ICOS expression in ILC2. **(e)** Comparative analysis of KLRG1 expression in ILC2 derived from bone marrow (BM), lung, and MLN. Data are shown as mean $\pm$ SEM of $n=5$ mice per group. All flow cytometry panels are pooled data from >4 mice

marker for accurate quantification of ILC2 numbers. A fraction of ILC2 express MHCII and ICOS (Fig. 3d) and have sparked investigations into the interaction of ILC2 with other immune cells [25, 35]. Expression of the co-inhibitory killer-cell lectin like receptor G1 (KLRG1) has been proposed as a marker acquired by mature ILC2 under inflammatory conditions [15, 36] and is differentially expressed between tissues (Fig. 3e).

ILC2 produce IL-5 and IL-13 in steady state and rapidly expand and upregulate their cytokine production upon stimulation. Amphiregulin production is low in resting ILC2 and is significantly upregulated when exposed to IL-33 or HDM (Fig. 4a). CD25 is expressed by $\sim 50\%$ of ILC2 in the lung in steady state. Administration of IL-33 results in a homogeneous ILC2 population that consistently expresses high levels of CD25. However, HDM stimulation leads to the expansion of CD25^{lo/-} ILC2 that

still possess the same cytokine profile as ‘classical’ CD25⁺ ILC2 (Fig. 4b, c). Additionally, these CD25^{lo/-} ILC2 are present in higher numbers in this model and significantly contribute to the total population of type 2 cytokine producing cells (Fig. 4d). It is conceivable that the CD25^{lo/-} ILC2 population is also prominent in other allergen-based mouse models of lung inflammation. We therefore suggest the use of this marker as a method to discriminate between subpopulations of ILC2 rather than a characteristic to quantify ILC2 numbers.

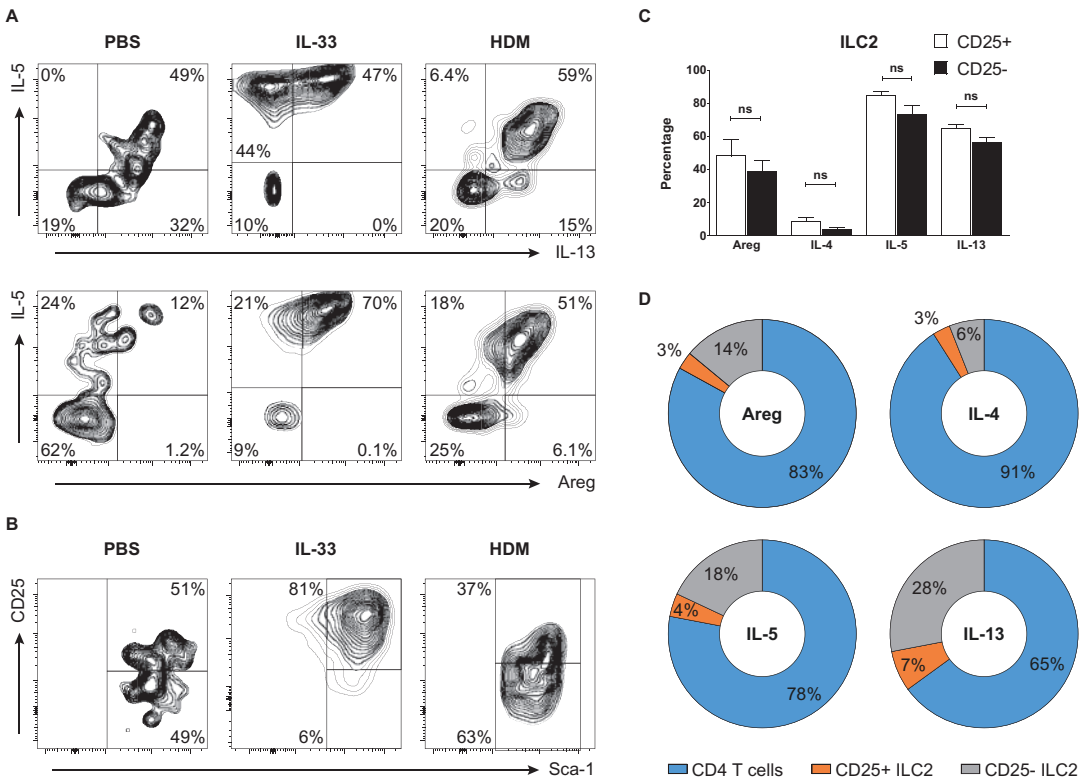


Fig. 4 CD25⁻ and CD25⁺ ILC2 secrete similar amounts of cytokines in HDM-treated mice. **(a)** Flow cytometric analysis of intracellular cytokine production of BAL fluid ILC2 in PBS, IL-33, or HDM-treated mice after stimulation of single-cell suspensions with PMA/ionomycin and GolgiStop for 4 h. **(b)** Expression of CD25 in BAL fluid derived ILC2 is dependent on the type of stimulation used. **(c)** Analysis of cytokine profiles of CD25⁻ and CD25⁺ ILC2 from BAL fluid of HDM-treated mice. **(d)** Relative contribution of various cell populations to amphiregulin (Areg), IL-4, IL-5, and IL-13 producing cells in BAL fluid of HDM-treated mice. Single-cell suspensions were stimulated with PMA/ionomycin in the presence of GolgiStop for 4 h and intracellular cytokine expression was quantified. Proportions of cytokine producing cells (expressed as percentages of the total number of cytokine-producing cells) were determined by flow cytometry in ILC2 and CD4 T cells. Data are shown as mean $\pm$ SEM of $n=4-6$ mice per group. All flow cytometry panels are pooled data from >4 four mice. A Mann-Whitney U test was used to determine statistical significance between CD25⁻ and CD25⁺ ILC2 in HDM-treated mice

4 Notes

1. Our observation is that female C57BL/6 mice tend to develop more severe eosinophilic lung inflammation than male mice and therefore it is crucial to gender match between experimental groups. Difference in the severity of inflammation also exists between various mouse strains.
2. The potency of recombinant mouse IL-33 diminishes over time and has an effective shelf life of 3 months if stored at -80°C and 1 week if stored at 4°C . Avoid repeated freeze–thaw cycles.
3. HDM extract is a natural product and has a variable composition between batches. It is important to note the endotoxin content of each batch and to compare the immune response generated between new and old batches in order to ensure similar levels of inflammation. If necessary, perform titrations of different amounts of HDM during challenge.
4. Cervical dislocation is not a viable option due to a high chance of tearing the trachea, thus preventing the placement of a cannula for BAL fluid extraction. Euthanasia by inhalation of CO_2 gas potentially damages the immune cells in the lungs, and thus we recommend methods employing intraperitoneal overdose of anesthetic agents like ketamine/medetomidine.
5. If the mice are placed in the isoflurane chamber for extended periods of time (>1 min), they may become too deeply anesthetized and have trouble adequately inhaling the droplet. This is characterized by an extremely slow breathing rate of less than once per second. To avoid this, do not anesthetize more than four mice simultaneously to ensure optimal anesthesia. Additionally, it is normal for some mouse strains such as the C57BL/6 mice to hold their breath for a short amount of time during intranasal administration. We recommend not to exceed a volume of $80\text{ }\mu\text{L}$ for intranasal injections.
6. It is possible to leave the cells in the fixation step overnight at 4°C and continue the FACS stain the next day if time is an issue.

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