

Murine Models of Allergic Asthma

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Abstract

Allergic asthma is a heterogeneous inflammatory lung disease affecting millions of people worldwide and with a steadily increasing incidence. Mouse models have been of utmost importance in uncovering key inflammatory cell types, cytokines, and pathways in the development and maintenance of allergic asthma. Historically, the mainstay in experimental asthma research was sensitizing rodents to the model protein antigen ovalbumin (OVA) with the pro-Th2 adjuvant aluminum hydroxide, followed by repetitive OVA exposures to the airways to initiate a Th2-skewed adaptive immune response leading to eosinophilic airway inflammation and airway hyperreactivity (AHR). In the last 5 years, OVA is often replaced by naturally occurring allergens such as house dust mite (HDM) or cockroach extracts, but the principle of first sensitizing and then repetitively challenging mice with the same antigen is unchanged. Here, we describe an often used and relevant HDM-based protocol to establish acute allergic asthma, and the methods we have developed to rapidly analyze inflammatory cell infiltration in the bronchoalveolar lavage fluid by flow cytometry. Moreover, we explain the methods to restimulate T cells from lung-draining mediastinal lymph nodes with HDM to allow the measurement of cytokine secretion profiles of allergen reactive T cells.

Key words Acute allergic asthma, Bronchoalveolar lavage, Eosinophilia, Flow cytometry, House dust mite (HDM), Mediastinal lymph nodes, Murine model, Ovalbumin (OVA)

1 Introduction

Worldwide, more than 300 million people suffer from asthma and its incidence is continuously increasing. Asthma is a chronic airway disorder characterized by lung inflammation, airway hyperreactivity (AHR) to nonspecific stimuli and recurring episodes of airway obstruction, sometimes leading to permanent airway remodeling and impaired airway function. Allergic asthma, which accounts for about 80 % of asthma cases, is often described as a Th2-driven disease characterized by IL-5 dependent lung eosinophilia, IL-4 dependent elevated IgE serum levels and IL-13 dependent AHR and goblet cell metaplasia (GCM) [1, 2]. Not surprisingly, high levels of Th2 cytokines can be detected in lung tissue and bronchoalveolar lavage (BAL) fluid. Current treatments for asthma

mainly consist of inhaled corticosteroids and long acting bronchodilators, and act in reducing the symptoms without curing the disease. Although these treatments are effective in most patients, they can be unsuccessful in some patients and may have side effects [3]. Thus, there is a continuous search for better targeted treatment strategies that are based on mechanistic insights into disease development and progression.

Mouse models have been of invaluable importance in elucidating key cytokines, cellular networks and pathways in both the development and effector phases of allergic asthma. Recently several new drugs that were discovered in mouse models (such as neutralizing IL-5 antibodies in eosinophil-rich asthma) have been approved by the Food and Drug Administration. Until a decade ago, the model antigen ovalbumin (OVA) was the mainstay in murine asthma models. In these protocols, mice were first immunized systemically once or twice with OVA via intraperitoneal (i.p.), intradermal or subcutaneous injection, typically together with the adjuvant aluminium hydroxide (alum) to skew the resulting immune response towards a Th2-driven response. This was followed by repetitive intratracheal (i.t.), intranasal (i.n.), or aerosolized OVA-challenges, resulting in an acute and robust influx of eosinophils in the lungs and the establishment of AHR [4]. OVA-models are rather cheap and are easy to use considering the tools available. For example, the TCR transgenic OT-I and OT-II mice, that possess OVA-specific CD8+ and CD4+ T cells, respectively, are commercially available. Moreover, OVA-based models have proven extremely valuable in uncovering critical steps in the induction and maintenance of Th2-mediated asthma. For example, it has been shown that the observed eosinophilia and AHR are independent of IgE or B cells, but require the activation of CD4+ T lymphocytes via IL-4 mediated activation of the STAT6 pathway [5, 6]. The resulting Th2 cells secrete amongst others IL-5 and IL-13, which play a prominent role in eosinophil activation, and airway remodeling and mucus hyperplasia, respectively [7, 8]. The critical role of dendritic cells (DCs) in the OVA asthma model has been studied in models where DCs were depleted during either sensitization or challenge to OVA and in studies in which the i.p. sensitization of OVA/alum was shown to be completely dependent on monocyte derived DCs [9–11].

However, newly developed drugs targeting the identified key players and showing efficacy in the murine OVA-model often did not prove successful in clinical trials [12–14]. Differences in the underlying mechanisms of disease between OVA-mice and human patients may account for the failures in clinical translation. Repetitive OVA sensitization via the lung only leads to a very weak response after OVA-challenges. In the absence of TLR agonists, the outcome of OVA inhalation can even be immunological tolerance. Robust sensitization via the pulmonary route requires the concomitant administration of the TLR4 agonist LPS [15]. In this scenario of

pulmonary sensitization, TLR activation by LPS and/or other immunostimulating pathogen associated molecular patterns (PAMPs) is necessary for the maturation and activation of antigen presenting cells (APCs), like DCs. Moreover, the LPS dose can influence the resulting immune response; whereas OVA administered together with high LPS doses induces a Th1-like response, low doses of concomitant LPS lead to a Th2-like immune response. The use of alum bypasses the requirement for PAMPs, but might therefore also bypass innate signals and pathways that naturally occur in human sensitization and can modify the resulting immediate and effector immune responses to allergens.

Recently, new acute models of allergic asthma have been validated that make use of naturally occurring allergens, such as extracts of house dust mite, cockroach or *Alternaria alternata*. In these models, sensitization is carried out via the pulmonary route, without any need for adjuvant, and triggers a vast immediate innate immune response next to the initiation of adaptive immunity [16]. These models mimic human disease more closely and aid in unraveling important aspects of allergic sensitization that were not significant in the OVA-induced model, such as the necessity of epithelial expressed TLR4 for efficient sensitization to HDM [17].

It should be taken into account that also these models show caveats worth mentioning. Whereas human asthma is a chronic condition, inflammation and AHR typically resolve in acute murine models when challenges are stopped [18]. Next, it is now appreciated that asthma is a very heterogeneous disease with multiple phenotypes and endotypes. For example, one subset of patients has very high levels of neutrophils and Th17-associated cytokines in BAL, which is not reflected in the standard HDM-induced asthma model [19]. Depending on the research question, chronic model or models of neutrophilic asthma can be used to bypass these issues. Still, to resolve questions about the eosinophil-rich, steroid-sensitive endotype of human asthma, which is present in about half of the patients, the HDM-induced asthma model proved to be a valid model.

In this protocol, we explain how to establish HDM-induced or OVA-induced acute allergic asthma in mice and how to analyze the influx of inflammatory cells in BAL fluid by flow cytometry. Moreover, we describe how to restimulate the T cells from mediastinal lymph nodes (MLNs) with HDM to study their capacity to secrete Th2 and other cytokines. These are two main read-outs informative for the strength and the nature of the adaptive immune response.

2 Materials

2.1 Establishment of a Mouse Model of HDM-Induced Acute Allergic Asthma

1. 6–8 week old C57Bl/6 mice, ideally specified-pathogen free (*see* Notes 1 and 2).

2. Small animal anesthesia device compatible with isoflurane and a connected induction chamber.
3. Vertical restraining device for intratracheal installation (*see* Fig. 1).
4. Non-sharp forceps.
5. Isoflurane.
6. House dust mite (HDM) extract from Greer Laboratories, diluted in sterile phosphate buffered saline (PBS) to a concentration of 5 mg protein content/mL (*see* Notes 3 and 4).

2.2 Establishment of a Mouse Model of OVA-Induced Acute Allergic Asthma

1. Standard laboratory equipment including a shaker, a balance, ethanol 70% (v/v), syringes, and 26 gauge needles.
2. Small animal aerosol inhalation chamber with nebulizer.
3. OVA grade V and grade III from Sigma Aldrich.
4. Sterile aluminum hydroxide ($\text{Al}(\text{OH}_3)$).
5. Sterile PBS.

2.3 Analysis of the Infiltration of Inflammatory Cells in BAL Fluid

1. Standard laboratory equipment including syringes, needles (23 and 26 gauge), a centrifuge, an aspirator, a round bottom 96-well plate, and fluorescence-activated cell sorting (FACS) tubes.
2. Dissection material including forceps and scissors.
3. A three laser flow cytometer.
4. A light microscope, Bürker chamber, and trypan blue for manual cell counting.
5. Cannulas, made of 23 Gauge needles with a small piece of polyethylene tubing around the sharp needle points.
6. Small strings.

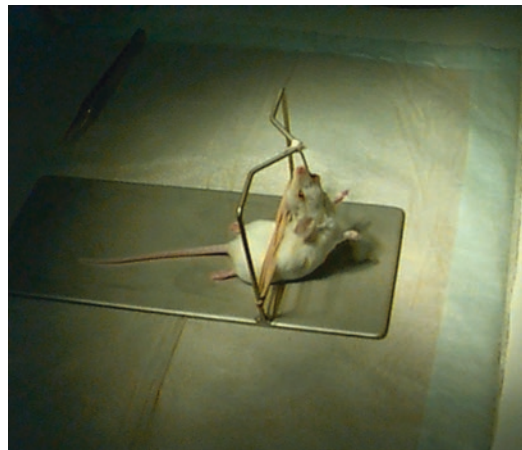


Fig. 1 Vertical mouse restraining device for intratracheal instillation

7. Pentobarbital or another anesthetic.
8. PBS containing 0.5 mM EDTA for taking BAL fluid.
9. FACS buffer consisting of PBS containing 0.25 % BSA, 0.5 mM EDTA, and 0.05 % NaN₃ (*see Note 5*).
10. (Optional) osmotic lysis buffer: ddH₂O containing 0.83 % NH₄Cl, 0.1 % KHCO₃, and 0.1 M EDTA at a pH between 7.1 and 7.4 (adjusted with HEPES).
11. Staining mix comprised of antibodies in Table 1, diluted in FACS buffer (*see Notes 5 and 6*).

2.4 Restimulation of MLN Cells with HDM

1. Standard laboratory equipment including a 24-well plate, 100 or 70 µm meshes, small petri dishes, and syringes (all sterile).
2. Dissection material including forceps and scissors.
3. A CO₂ incubator.
4. A sterile 96-well culture plate.
5. A light microscope, Bürker chamber, and trypan blue for manual cell counting.
6. House dust mite (HDM) extract (Greer Laboratories), diluted in sterile PBS to a concentration of 5 mg protein content/mL.
7. Tissue culture medium (TCM), consisting of RPMI- 1640 medium with GlutaMAX-1 (diluted according to manufactur-

Table 1

Antibody composition of the flow cytometry staining mix for analysis of BAL fluid (*see also Notes 5 and 6*)

Specificity	Clone	Fluorochrome	Excitation laser	Species	Isotype	Supplier	Dilution
Siglec-F	E50-2440	PE	488 or 561 nm	Rat	IgG2a	BD Pharmingen	1/1000
CD3e	145-2C11	PE-Cy5	488 or 561 nm	Armenian Hamster	IgG	Tonbo Biosciences	1/200
CD19	eBio1D3	PE-Cy5	488 or 561 nm	Rat	IgG2a	eBioscience	1/400
CD11c	N418	PE-Cy7	488 or 561 nm	Armenian Hamster	IgG	eBioscience	1/800
CD11b	M1/70	BD Horizon V450	405 nm	Rat	IgG2b	BD Pharmingen	1/800
Ly-6G	1A8	AF700	633 nm	Rat	IgG2a	BD Pharmingen	1/500
MHC-II	M5/114.15.2	APC- eFluor780	633 nm	Rat	IgG2b	eBioscience	1/1000
CD16/CD32 (Fc-block)		Unlabeled					1/400

er's instructions), 5% fetal calf serum, gentamycin (diluted according to manufacturer's instructions) and 5×10^{-5} M β -mercaptoethanol.

3 Methods

3.1 Establishment of a Mouse Model of HDM-Induced Acute Allergic Asthma

1. Dilute the HDM stock (5 mg/mL) to 12.5 μ g/mL in sterile PBS (1 μ g in 80 μ L/mouse) under sterile conditions (*see* **Note 7**).
2. Intratracheal instillation: on day 0, anesthetize mice with 2–2.5% isoflurane in air or O₂, then place them on the restraining device in a vertical head-up position with the hind legs on the bench, and by fixing the two front teeth in a loop of string (*see* Fig. 1). Gently pull the tongue out with non-sharp forceps to prevent any swallowing. Pipet 80 μ L of diluted HDM extract, or 80 μ L PBS as a control, into the mouth and keep mice in this position until all the fluid is inhaled (*see* **Notes 8 and 9**).
3. Dilute the HDM stock (5 mg/mL) to 250 μ g/mL in sterile PBS (10 μ g in 40 μ L/mouse) under sterile conditions (*see* **Note 7**).
4. From days 7 to 11, challenge the mice daily intranasally by pipetting 40 μ L of diluted HDM extract directly into the nostrils of isoflurane (2%)-sedated mice (*see* **Note 10**).

3.2 Establishment of a Mouse Model of OVA-Induced Acute Allergic Asthma

1. Prepare an OVA/alum solution of 500 μ L sterile PBS per mouse containing 20 μ g/mL OVA grade V and 2 mg/mL Alum. Just before use, rotate for 30 min at room temperature.
2. Intraperitoneal (i.p.) injection: on days 0 and 7, disinfect and clean the belly of the mice by spraying 70% ethanol (v/v) and inject 500 μ L OVA/alum solution, or 500 μ L PBS as a control in the left lower quadrant just beneath the nipple with a 26 G needle. Hold the mice with their head downwards to make sure organs are not pricked (*see* **Note 11**).
3. From days 14 to 20, daily challenge mice in an inhalation chamber by aerosolizing about 10 mL of 1% OVA grade III in sterile PBS with a nebulizer (*see* **Note 12**).

3.3 Collection and Analysis of Bronchoalveolar Lavage (BAL) Fluid

1. Three or four days after the last challenge, euthanize mice by giving them an overdose of pentobarbital (100 μ L i.p. with 26 G needle).
2. Cut the chest wall open up to the chin and remove fat, salivary glands and the surrounding tegument to expose the trachea. Make a small incision in the trachea between the first and second cartilage ring, bring in a cannula and retain it by knotting

a string around the cannula and trachea. Collect BAL fluid in a 15 mL tube by flushing the lungs three times with 1 mL of PBS containing 0.5 mM EDTA via a 1 mL syringe inserted in the cannula (*see* **Notes 13** and **14**). Repeat this step two times to reach a volume of 3 mL.

3. Centrifuge the BAL fluid for 7 min at $400\times g$ and 4°C . Discard the supernatant, resuspend the pellet containing the cells in 200 μL FACS buffer, and transfer to a 96-well round bottom plate. Centrifuge the plate for 3 min at $400\times g$ and 4°C , remove supernatant and again resuspend in 200 μL FACS buffer (*see* **Notes 15** and **16**).
4. Take a small sample (10 or 20 μL) and manually count cells.
5. Take some cells from every sample, pool and redistribute in seven wells for single stains and an unstained control. For one single stain, 50,000 cells are sufficient.
6. Centrifuge the plate for 3 min at $400\times g$ and 4°C , discard supernatant and stain a maximum of 5×10^6 cells in 100 μL staining mix, consisting of Fc-block, SiglecF-PE, CD3-PE-Cy5, CD19-PE-Cy5, CD11c-PE-Cy7, CD11b-V450, Ly6G-AF700, and MHC-II-APC-eFluor780 in FACS buffer. Incubate for 30 min at 4°C in the dark (*see* **Notes 5** and **6**).
7. Add 100 μL FACS buffer and centrifuge the plate ($400\times g$, 4°C , 3 min). Remove supernatant, resuspend in 200 μL FACS buffer and transfer to FACS tubes for acquisition with a flow cytometer.

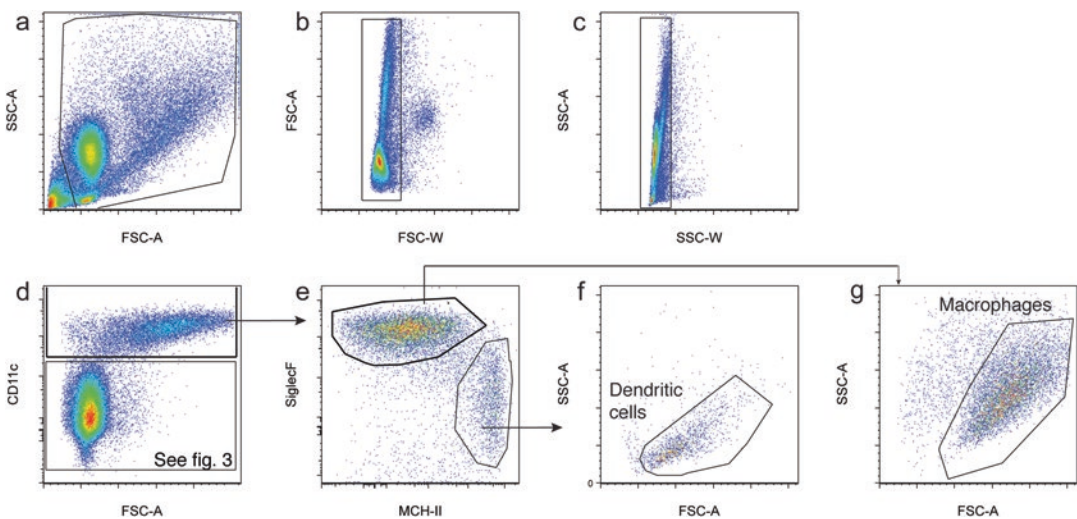


Fig. 2 Flow cytometry gating strategy for inflammatory cell populations in BAL fluid. Hierarchical gating of non-debris, single cells, CD11c^{hi} cells, and DCs and macrophages. See text for details

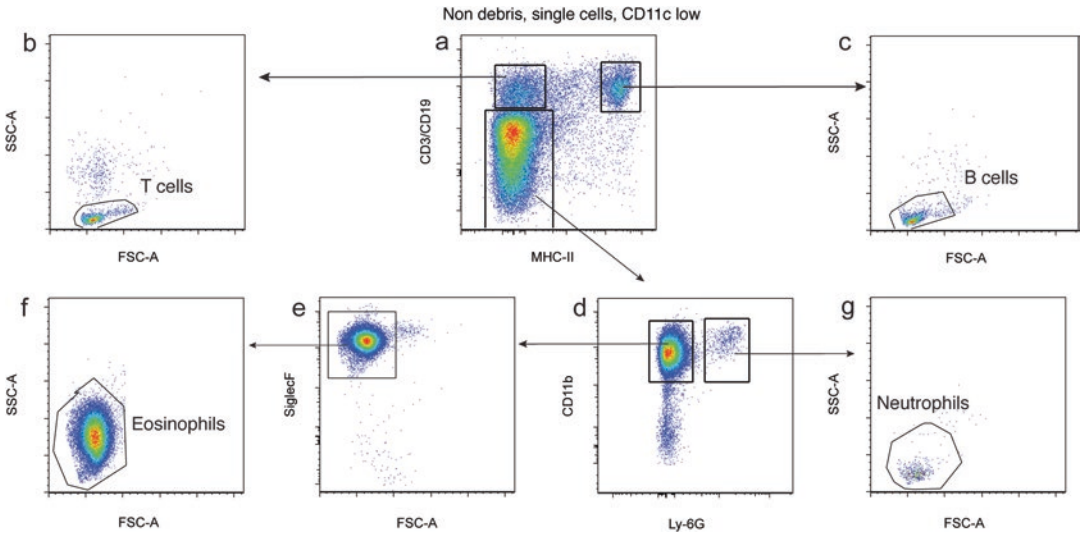


Fig. 3 Flow cytometry gating strategy for inflammatory cell populations in BAL fluid. Hierarchical subdivision of CD11c^{low} cells (see Fig. 1) in T cells, B cells, neutrophils, and eosinophils. See text for details

8. Several gating strategies are possible, but we propose a straightforward and validated strategy depicted in Figs. 2 and 3 that is consistently used in our lab (see Note 17).
 - (a) Plot forward scatter-area (FSC-A) against side scatter-A (SSC-A) and exclude the debris that can be recognized as events with a very low signal for one or both parameters, in the lower left corner and at the edges of this plot (Fig. 2a). FSC-A and SSC-A are dependent on the size and the complexity of the cell, respectively. Events with very low FSC-A or SSC-A are too small to be cells and should therefore not be analyzed further.
 - (b) Discriminate the doublets from the analysis. When a cell doublet passes the laser, the width (W), which is the time necessary for the doublet to pass the laser beam, will be higher than the W of a singlet. In contrast, the height (H), which is the maximum signal of the event and is dependent on the pulse voltage, is similar between singlets and doublets. Therefore, doublets show a disproportion when comparing the parameters W and H and can be excluded by plotting FSC-W against FSC-H, followed by plotting SSC-W against SSC-H. Similarly, one can also plot FSC-W against FSC-A ($A = W \times H$) followed by plotting SSC-W against SSC-A (Fig. 2b, c).
 - (c) (Optional) If desirable, exclude dead cells from the analysis (see Note 5). Plot the fixable Live/Dead (L/D) marker against FSC-A. Gate out the dead cells that are L/D^{high} and small (low FSC-A). Cells with an intermediate to high

L/D signal and a high FSC-A tend to be big autofluorescent cells such as macrophages and are considered alive.

- (d) Separate the remaining alive single cells in two populations based on CD11c expression (Fig. 2d). CD11c^{high} (hi) cells comprise both dendritic cells (DCs) and macrophages, whereas all other cell populations are CD11c^{low}.
 - (e) In the CD11c^{hi} gate, macrophages and DCs can be distinguished from each other based on SiglecF and MHC-II signal (Fig. 2e). Alveolar macrophages express high levels of SiglecF whereas DCs are negative for SiglecF. Both cell populations express MHC-II, but macrophages at intermediate (int) levels and DCs at high levels. Check whether the SiglecF^{hi}MHC-II^{int} cells delineated as macrophages have high FSC-A and SSC-A values, which is expected for the big and complex alveolar macrophages of the lungs (Fig. 2g). SiglecF⁻MHC-II^{hi} cells that range from FSC-A^{low} SSC-A^{low} to FSC-A^{int} SSC-A^{int} are defined as DCs (Fig. 2f).
 - (f) In the CD11c^{low} gate, lymphocytes can be distinguished as cells expressing either CD3 (T cells) or CD19 (B cells). In this gating strategy, antibodies to both CD3 and CD19 are coupled to the same fluorochrome (PE-Cy5), therefore T and B cells are to be discriminated from each other based on the MHC-II expression on B cells that is not present on T cells (Fig. 3a). Both CD11c^{low}CD3/CD19⁺MHC-II⁻ T cells and CD11c^{low}CD3/CD19⁺MHC-II⁺ B cells are small and not complex cells with a low FSC-A and SSC-A (Fig. 3b, c).
 - (g) Cells that are both low for CD11c, CD3 and CD19 and high for CD11b are either eosinophils or neutrophils (Fig. 3d). Neutrophils are CD11b^{hi}Ly-6G⁺ cells that have a similar FSC-A as lymphocytes but a slightly higher SSC-A (Fig. 3g). CD11b^{hi}Ly-6G⁻ cells, that moreover highly express SiglecF, are denoted as eosinophils (Fig. 3e). The small but very complex eosinophils occupy a notable area in the FSC-A-SSC-A plot, consisting of the FSC-A^{low} SSC-A^{hi} events (Fig. 3f).
9. For every cell population, absolute numbers can be calculated by multiplying percentages obtained from the FACS software with total cell counts from manual counting, and dividing the result by 100. A representative result is shown in Fig. 4 (see Note 18).
 1. After taking the BAL from mice, cut the rib cage open, move the lungs upwards or sideways and collect mediastinal lymph nodes (MLNs) in a 24-well plate in a sterile, rich buffer (HBSS or RPMI) on ice. MLNs are located underneath the right lung

3.4 Restimulation of Mediastinal Lymph Node (MLN) Cells with HDM

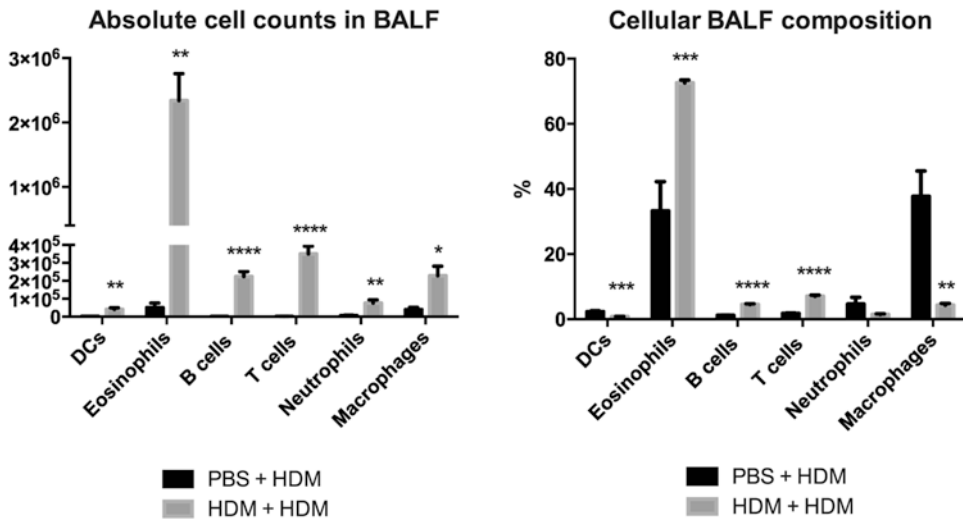


Fig. 4 Representative inflammatory cell infiltration in the BAL fluid (BALF) for a standard HDM-induced asthma protocol. Mice are sensitized intratracheally with 1 μ g HDM extract or mock-sensitized with PBS, followed by five consecutive HDM-challenges (10 μ g intranasally) 1 week later. Four days after the last challenge, eosinophil counts in BAL fluid are expected to be around two million. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

- lobes next to the right lateral border of the trachea and just below the thymus (see [Note 19](#)).
2. Homogenize the MLNs under sterile conditions with the tamping bar of a syringe, through a 70 or 100 μ m mesh, in a small petri dish in 2 $\times$ 500 μ L TCM. Pipet the cell suspension from the petri dish into a 15 mL tube.
 3. Manually count cells and bring samples to a concentration of two million cells/mL by adding TCM, or by centrifuging and resuspending in the appropriate volume of TCM.
 4. For every sample, seed 6 wells of a round bottom 96-well culture plate with 200,000 cells in 100 μ L TCM per well.
 5. Dilute the HDM stock (5 mg/mL) to 30 μ g/mL in TCM (6 $\times$ 100 μ L per MLN) and add 100 μ L of diluted HDM extract to every well.
 6. Incubate for 3–4 days in a CO₂ incubator at 37 $^{\circ}$ C and 5 % CO₂.
 7. Spin cells down (400 $\times g$, 3 min, 4 $^{\circ}$ C).
 8. For every sample, pool supernatant of the 6 wells into 1 well of a 24-well plate and freeze at –20 $^{\circ}$ C for subsequent analysis of cytokines by ELISA. A representative result is shown in [Fig. 5](#).

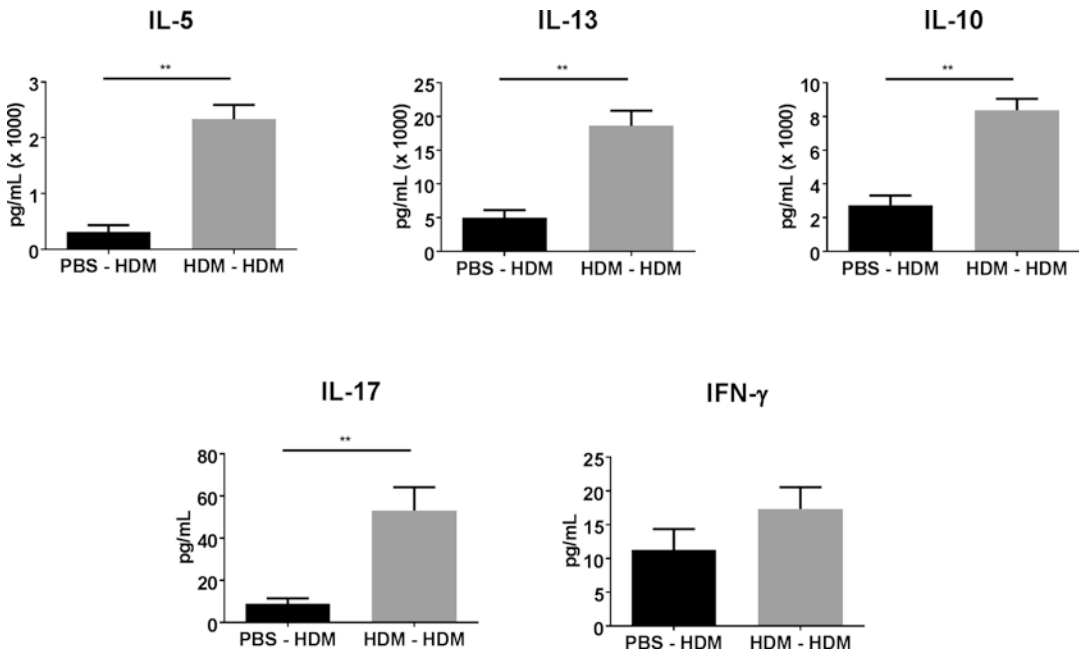


Fig. 5 Representative cytokine secretion levels in supernatants of HDM-restimulated MLN cell cultures for a standard HDM-induced asthma protocol. Mice are sensitized intratracheally with 1 μ g HDM extract or mock-sensitized with PBS, followed by five consecutive HDM-challenges (10 μ g intranasally) 1 week later. Four days after the last challenge, MLNs are collected, homogenized to single cell suspensions and restimulated in vitro with HDM for 3 days. MLN cells from HDM-sensitized and -challenged mice secrete significantly more Th2, Th17 and Treg cytokines in their culture supernatants. ** $p < 0.01$

4 Notes

1. 6–8 mice per group are usually enough to obtain statistically significant results. For control groups, in which variation tends to be lower, three or four animals may suffice.
2. The strength and nature of responses to OVA, HDM, Der p 1 (a major HDM allergen), and other aeroallergens differ between mouse strains. Generally, in models of acute asthma, pulmonary eosinophilia and Th2 cytokine levels in BAL are significantly higher in C57Bl/6 mice than in BALB/c mice, whereas they exhibit a less pronounced AHR [20–22]. Mouse strains might also differ in the underlying pathophysiological mechanisms leading to the hallmarks of allergic airway disease. For example, eosinophils are necessary for AHR induction in C57Bl/6 mice, a requirement not observed in BALB/c mice [6, 23, 24]. Furthermore, A/J mice exhibit intrinsic AHR without any allergen exposure and that is independent of T and B cells and IL-4 signaling [25]. For this reason, carefully select the mouse strain best fit for your research question.

3. On a bottle of HDM extract, two concentrations are depicted: use the protein content and NOT the dry weight (wt) to make your dilution.
4. HDM batches, even when bought from the same company, are not standardized and may therefore vary in composition (e.g., LPS, β -glucan and chitin content, protease activity). Recently it has been shown in an HDM-based model of allergic asthma that adding LPS and β -glucans to the HDM leads to a strong pulmonary infiltration of neutrophils on top of eosinophils [26]. Also other experiments showed that LPS can vastly influence the asthmatic response, and that the precise effect is dependent on the dose and the timing of administration [17, 27]. Thus, HDM batches varying in these components can profoundly affect Th-skewing properties. When using a new HDM batch for the first time, it is very useful to first characterize the response induced by your particular batch by doing some standard read-outs of interest. Great care should be taken when you switch to a new batch within the same project, and results from the two batches should carefully be compared.
5. BAL fluid contains very little dead cells, however, some scientists still prefer to add a Fixable Live/Dead stain to the antibody mix, for example L/D-eFluor506. In this case, use PBS instead of FACS buffer for all steps.
6. Several panel designs targeting the same cell surface markers are possible. If another panel is designed, or if antibodies are bought from different suppliers, it is most safe to choose for the same antibody clones. New antibodies should first be validated and checked for their optimal dilution and staining conditions before using them in the complete staining mix.
7. In our lab, a standard sensitization dose of 1 μ g HDM extract is used, followed by a standard challenge dose of 10 μ g for five times (the “1/10” protocol). This can be adapted to lower doses if there is a suspicion that mild effects are masked by the robust response after sensitization and challenge, or to higher doses when the read-out(s) of interest are hard to detect. Always keep in mind that changing the doses of sensitization or challenges might change the mechanisms behind the observed responses. For example, *Flt3l*^{-/-} mice, lacking CD103⁺ cDCs and CD11b⁺ cDCs, show a significantly reduced influx of eosinophils when using the standard “1/10” protocol. This suggests that monocyte-derived DCs (moDCs), the only remaining lung DC population, are not capable of inducing asthma in this setting. However, when sensitization and challenge doses are increased to 100 μ g, asthma is induced in *Flt3l*^{-/-} mice, demonstrating that moDCs do sensitize mice for HDM when the sensitization dose is high [28]. Similar dose dependent effects have also been described

in a cockroach model of asthma [29] and in OVA-protocols, for example in AHR induction, which is mast cell-dependent in low challenge frequency/dose protocols, but mast cell-independent in protocols with higher frequency and dose of challenges [30].

8. It may take some practice to identify when the mice are sedated enough (to have adequate time to carry out the i.t. instillation) but not too much to avoid them breathing in little shocks. This depends on the device but typically, we wait until the respiratory rate is slow and restful, then wait another 10–20 s before removing the mice from the isoflurane device for instillation.
9. C57Bl/6 and BALB/c mice show a very different behavior when being exposed to isoflurane. When using BALB/c mice, the timeframe during which the mice are optimally sedated for i.t. and i.n. instillation (enough but not too much) is shorter.
10. The exact timing when an i.n. challenge is given is critical and it may take practice to recognize when the mice should be removed from the isoflurane device. Typically, the percentage of administered isoflurane is slightly lower than for i.t. instillation (2 versus 2.5 %) and the mice can be challenged from the moment the breathing is slow and restful without additional waiting. When carried out correctly, it takes less than a minute for the mice to wake up.
11. Always gently mix the OVA/alum solution by inverting the tube (or syringe) a few times to make sure that OVA and alum are homogeneously distributed in the tube (or syringe) and that all the mice are injected with the same amounts of OVA/alum.
12. 1 % OVA dilutions in sterile PBS can be made beforehand and stored in the –20 °C freezer for a couple of months.
13. Pay attention not to put the cannula too deep into the trachea; it should not reach the bifurcation of the bronchi.
14. Sometimes, it can be hard to recover the BAL fluid from the lungs because a vacuum is created; in this case, the vacuum can be disrupted by carefully moving the cannula a bit less deep in the trachea. Watch out not to pull it out completely.
15. We use this two-step redilution scheme because after aspiration of the supernatant from the 15 mL tube, a little bit of fluid is always left at the edges of the tube and consequently cells may not be resuspended in exactly the same volume after the addition of FACS buffer. This is important since the absolute cell counts is one of the main read-outs of this protocol.
16. Usually, the cell suspensions contain very little red blood cells (RBCs). However, if some cell pellets stain red, you may facilitate the manual cell counting by lysing the RBCs. After the

first centrifugation round, discard the supernatant and add 300 μ L osmotic lysis buffer directly to the 15 mL tubes at room temperature. After 3 min, neutralize with an excess of PBS (or FACS buffer), centrifuge and resuspend the samples in 200 μ L FACS buffer for transfer to the 96-well plate.

17. Eosinophils and neutrophils are the most significant inflammatory cell populations in the BALF. By making use of a hierarchical gating in which both cell types are only identified in the end, possible contaminations of these gates with other cell types are minimized.
18. As a rule of thumb, eosinophil percentages above 50 % indicate successful sensitization and challenging.
19. In PBS controls, MLNs can be extremely small and hard to see. It helps to carefully push with a forceps on the surrounding fat and tissue to move the MLNs upwards and out of their surroundings. If MLNs are really big, pay attention not to break them. It may be useful to first detach the MLNs from their surroundings by gently pulling at the strings keeping them in place. Do not confuse the MLNs with the thymus just above them, which is larger and stiffer.

Acknowledgments

Eline Haspeslagh is supported by the Research Foundation Flanders (FWO).

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