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House dust mite driven asthma and allergen specific T cells depend on B cells when the amount of inhaled allergen is limiting

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Abstract (max 250 words: 243)**Background**

Allergic asthma is a CD4 Th2-lymphocyte driven disease characterized by airway hyper-responsiveness and eosinophilia. B cells can present antigens to CD4 T cells and produce IgE immunoglobulins which arms effector cells, however mouse models are inconclusive whether B cells are necessary for asthma development.

Objectives

To address the role of B cells in a house dust mite (HDM) driven Th2-high asthma mouse model.

Methods

WT and B cell deficient muMT Mice were sensitized and challenged via the airways with HDM extracts. Antigen presenting capacities of B cells were studied using new TCR transgenic 1-DER mice specific for *Der p 1* allergen.

Results

In vitro, activated B cells from HDM-exposed mice presented antigen to 1-DER T cells and induced a Th2 phenotype. *In vivo*, B cells were dispensable for activation of naive 1-DER T cells, but necessary for full expansion of primed 1-DER T cells. At high HDM challenge doses, B cells were not required for development of pulmonary asthmatic features, yet contributed to Th2 expansion in the MLN but not in the lungs. When the amount of challenge allergen was lowered, muMT mice had reduced asthma features. Under these limiting conditions, B cells contributed also to expansion of Th2 effector cells in the lungs and central memory T cells in the MLN

Conclusion

B cells are a major part of the adaptive immune response to inhaled HDM allergen, particularly when the amount of inhaled allergen is low, by expanding allergen specific T cells.

Key words: allergic asthma, B cells, house dust mite, T helper 2 cells, antigen presentation

Key messages:

- B cells are essential for optimal expansion and activation of Th2 cells in the MLN upon airway HDM exposure.
- Only when the allergen dose is limiting, does the absence of B cells lead to reduced lung Th2 immunity and asthmatic features to HDM.

Capsule summary:

B cells are essential for optimal expansion and activation of Th2 cells in the MLNs upon airway HDM exposure. Only when the allergen dose is limiting, does the absence of B cells also lead to reduced lung Th2 immunity and asthmatic features to HDM. The degree to which HDM asthma features are affected in B cell deficient animals is equally dependent on the dose of allergen used as on the tissue site where Th2 cytokine production is probed.

Abbreviations: HDM: house dust mite; AAI: allergic airway inflammation; Th2: T helper 2 cell; MLN: mediastinal lymph node; BAL: broncho-alveolar lavage; DCs: dendritic cells, Ig: Immunoglobulin; APC: Antigen Presenting Cells, ILCs: innate lymphoid cells.

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Introduction

Allergic asthma is a chronic inflammatory disease of the conducting airways affecting 5-10% of the western population. It is characterized by reversible airway obstruction, airway hyper-responsiveness, eosinophilia and a CD4 Th2 lymphocyte dominated immune response associated with elevated serum IgE against innocuous environmental allergens^{1,2,3}. Upon allergen exposure, airway epithelial cells produce chemokines and innate cytokines, such as IL-1, thymic stromal lymphopoietin, granulocyte-macrophage colony stimulating factor and IL-33, which instruct dendritic cells (DCs) and activate ILC2s³. In the lymph nodes, DCs induce the differentiation of Th2 cells and IL-21 and IL-4 producing T follicular helper cells (T_{FH}) cells, helped in this process by basophils⁴. Th2-derived IL-4, in turn, activates B lymphocytes and promotes immunoglobulin (Ig) class switching to IgE^{1,5}.

The most documented effector function of B cells is production of antigen-specific immunoglobulins (Ig) of which IgG, IgM and IgA are far more abundant compared with IgE. Circulating IgE can bind to high affinity FcεRI receptor and cross-linking of IgE-FcεRI complexes on mast cells and basophils leads to degranulation and immediate release of inflammatory mediators causing allergic symptoms⁶, while allergen focusing via IgE armed FcεRI on DCs can lower the threshold for allergen recognition and further promote Th2 responses⁷. Whereas allergen-specific IgE is mostly considered pathogenic, allergen-specific IgG (particularly IgG4) and IgA are believed to play a protective role by capturing and neutralizing allergens at the mucosal surface⁸⁻¹⁰. Besides Ig production, activated B cells also play a role as accessory antigen presenting cells, particularly when T cells are first primed by DCs when antigens are captured via the surface immunoglobulin receptor¹¹⁻¹³. They may not be as potent as DCs in priming naive T cells, still their abundant presence in T cell inductive sites, the production of cytokines that influence the activation and positioning of DCs and naive T cells, and their expression of surface molecules such as CD86, ICOS-L, CD40L and OX40-L endow B cells with relevant antigen presenting and/or accessory functions to

promote and sustain type 2 adaptive immune responses¹⁴⁻¹⁹. The antigen presenting and Th2 promoting effects of B cells have been demonstrated in models of parasitic infection^{20,21,17,22,23} and in Th2 driven asthma models^{24,25,26,27} mainly using the model allergen ovalbumin, which requires injection of peritoneal alum adjuvant to boost Th2 immunity.

Even though a causal relationship between IgE and allergic asthma has long been recognized²⁸ and animal models of ovalbumin-driven asthma have revealed the contribution of different classes of immunoglobulins in mice^{29,30}, the role of B cells in the allergic process to natural allergens like house dust mite (HDM; *Dermatophagoides pteronyssinus*, *Der p*) remains elusive. The aim of this study was to establish the exact role of B cells in the course of HDM induced allergic airway inflammation (AAI). House dust mite is the most prevalent cause of perennial allergic sensitization in asthmatics (50-85%) and derives its allergenic nature from mite-derived proteins that can be found in the excreta of mites. Mite fecal pellets, inhaled as small particles that contain fecal TLR ligands and crude extracts of entire *Dermatophagoides* organisms, have the capacity to trigger epithelial cells and DCs of the lung, leading to Th2 and Th17 priming in the absence of adjuvant addition^{31,32,33}. A major focus of our study was to understand how B cells contribute to antigen presentation to CD4 T cells taking advantage of a newly developed TCR transgenic mouse strain, the 1-DER mice, in which all CD4 T cells are naive and react to a peptide found in the cysteine proteinase *Der p 1* protein, a major allergen of HDM^{4,33,34}.

We found that B cells are massively recruited to HDM allergen challenged lungs and mediastinal nodes, produce Igs reactive to HDM and presented allergens to naive 1-DER T cells *in vitro*. *In vivo* however, the contribution of B cells to the induction of cellular type 2 inflammation and asthmatic features was dependent on allergen dose and on the tissue site where Th2 cytokine production is probed. Only when the amount of allergen challenge dose was low, were B cells necessary for optimal expansion of HDM-reactive Th2 and Tcm cells

126 and for development of full blown asthma features like eosinophilic airway inflammation and
127 bronchial hyperreactivity.

Methods

Mice

C57BL/6 mice were purchased from Harlan. B cell deficient muMT mice (C57BL/6 background, Jackson Laboratories) were bred at the animal facility of the Ghent university Hospital. Wild type or heterozygous littermates were used as controls. 1-DER mice express an MHCII-restricted TCR specific for Der p1 on their CD4 T cells and were generated as described^{4,33}, and maintained on the *Rag2*^{-/-} background so that CD4 T cells had not seen any bystander antigens. Experiments were approved by the animal ethical committee of Ghent University.

HDM asthma model

To induce allergic airway inflammation, mice were sensitized intra-tracheally (i.t.) with 1 µg crude HDM extract (Greer Laboratories) on day 1 and subsequently challenged with 10 µg HDM extract intranasally (i.n.) on days 7-11. In some experiments the challenge dose was lowered to 1 µg or 3 µg daily. Control mice received sham sensitization and challenge with PBS. Four days after the last challenge, mice were sacrificed and organs were collected for analysis. Broncho-alveolar lavage (BAL) was performed with 3 x 1 ml of PBS containing EDTA. Lungs were collected and frozen at -80°C for Periodic acid-Schiff (PAS) staining to evaluate lung inflammation and mucus cell metaplasia. Pictures were obtained with Analysis getIT. To confirm mucus cell metaplasia, *Muc5ac* mRNA expression was measured in lung tissues. RNA was obtained using the TriPure isolation reagent (Roche) and isolated according to manufacturer's instructions. RNA was reverse transcribed with a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) and samples were analyzed by SYBR green-based RT-PCR with a LightCycler 480 system (Roche). PCR primers for *Muc5ac* and reference genes *Rpl13a*, *Hprt* and *Sdha* were as follows:

Muc5ac: FW: GAGGCTCCCACATGTCCA; Rev: TGAAGGCATTACTGTCACAGGG

Rpl13a: FW CCTGCTGCTCTCAAGGTTGTT; Rev TGGTTGTCACTGCCTGGTACTT

Hprt: FW TCCTCCTCAGACCGCTTT; Rev: CCTGGTTCATCATCGCTAATC

Sdha: FW: TTTCAGAGACGGCCATGATCT; Rev: TGGGAATCCCACCCATGTT

Single cell suspensions were obtained for further analysis; lymph nodes and lungs were digested using Liberase TM and DNase (both Roche). For the measurement of airway hyperresponsiveness, mice were anesthetized with urethane, paralyzed using d-tubocurarine, and tracheotomized, followed by mechanical ventilation on a Flexivent machine. Increasing concentrations of methacholine (Mch) were administered via a jugular vein catheter (0–200 µg/ml) or nebulized (0–400 mg/ml). Dynamic resistance and compliance were recorded after a standardized inhalation maneuver given every 10 s for 2 min. Baseline resistance was restored before administering the subsequent doses of Mch.

Th2 cytokine assessment

To analyze Th2 cytokine secretion, 2×10^5 cells/well Mediastinal Lymph Node (MLN) cells were restimulated for 3 days with 30 µg/mL HDM extract. Cytokine production was measured in the supernatants by cytokine-specific ELISAs (eBioscience Ready-Set-Go kits). In some experiments, splenic or lymph node sorted CD19⁺ B cells (2×10^5 , >95% pure) or GM-CSF bone marrow-derived dendritic cells (BMDC, 2×10^4) cultured as previously described³⁵ were added to HDM restimulated MLN cells.

To analyze cytokine production by intracellular cytokine staining, 50 ng/ml PMA and 1 mM ionomycin (both from Sigma) were added to cultures of total MLN or lung cells for 3 hours in the presence of BD GolgiStop. The BD Fixation/Permeabilization buffer set was used for intracellular staining of cells.

B and T cell cocultures

1-DER T cells were isolated from spleens and lymph nodes of *Rag*^{-/-} 1-DER mice (CD45.1) using CD4⁺ T Cell Isolation Kit (Miltenyi) and were labeled with Cell Trace Violet (CTV, Invitrogen). 1-DER T cells were isolated either from naive animals (referred to as naive T cells since they were >93% CD44⁻) or from animals having received 1ug HDM 1 week prior (referred to as primed T cells).

Three days post HDM challenge, B cells (CD19⁺MHCII⁺CD3⁻) were sorted from MLNs and cocultured with 1*10⁵ naive or primed 1-DER T cells in the presence of 3μM der p1 protein. After 3 days of culture, cells were analyzed using flow cytometry for proliferation, IL33R (T1ST2) expression and Tfh differentiation (% PD1⁺CXCR5⁺ cells). IL-5, IL-13 and IFNγ were measured in the supernatants by ELISA (eBioscience ready-set-go).

Proliferation indices were computed as the sum of the cells in all generations, including the parental, divided by the computed number of original parent cells theoretically present at the start of the experiment (# parent cells in nth division: (# cells nth division)/(2ⁿ)).

T cell adoptive transfer

Naive or primed 1-DER T cells (CD45.1) were isolated as above, labeled with Cell Trace Violet (CTV, Invitrogen) and injected into CD45.2 WT or muMT recipients via the tail vein. Following transfer, mice were inoculated with HDM as described.

Flow cytometry and antibodies

All stainings were performed at 4°C for 20-30 minutes. Prior to antibody staining, cells were incubated with 2.4G2 Fc receptor antibody (L. Boon) to reduce non-specific binding, together with fixable live/dead marker (eBioscience) in PBS. To determine the cellular composition of BAL fluid, cells were stained with MHCII (M5/114.15.2), CD11c (N418), Gr1 (RB6-8C5), CD19 (1D3), CD3 (145-2C11) (from eBioscience) and Siglec F (E50-2440, BD Biosciences).

For phenotyping of B cells, T cells, DC and other cell types in cell suspensions of lungs and MLN, the following antibodies were used: CD3 (145-2C11), CD11c (N418), CD19 (1D3), CD38 (90), CD103 (2E7), B220 (RA3-6B2), FcεRI (MAR-1), Ly6G (1A8), IL-13 (eBio13A), IFNγ (XMG1.2) (all eBioscience), CD4 (RPA-T4), CD8 (RPA-T8), CD11b (M1/70), CD64 (X54-5/7.1), CD138 (281-2), Fas (JO-2), GL7, IgM (II/41), IgD (11-26c,2a), IgE (R35-72), Ly6C (AL21), Siglec F (E50-2440), CD45.1 (A20), IL-4 (BVD4-1D11), IL-5 (TRFK5) (all BD Biosciences), MHCII (M5/114; Biolegend), IL-33R/T1/ST2 (DJ8, MDBiosciences). Surface IgE was measured by standard extracellular staining with anti-human IgE, which quantifies FcεRI-bound IgE. FMO controls were taken along to allow for proper gating.

Acquisition was performed on a LSR Fortessa cytometer. Cell sorting was performed on a FacsARIA II cytometer and after cell sorting purity was checked (always >95%).

Statistical analysis

All data are represented as mean $\pm$ SEM. Statistical significance between groups was calculated with ANOVA multiple comparisons analysis and/or Mann-Whitney U testing using Graphpad Prism software. P values are shown as follows: *p=0.01-0.05 ; **p=0.001-0.01; ***p<0.001.

Lung function transfer resistance (Rtr) values were log-transformed and analyzed as repeated measurements using the residual maximum likelihood (REML) as implemented in Genstat v18 (Payne et al. 2013). A linear mixed model was fitted to the data, with time points set as equally spaced. The antedependence correlation structure of order 2 was selected as best model fit based on the Aikake Information Coefficient (AIC). Significance of main (genotype, treatment, time) and all possible interaction effects was assessed by an F-test. The experiment was set up as a randomized block design.

RESULTS

B cell accumulation in HDM sensitized and challenged mice

House dust mite (HDM) is an important human allergen and when instilled in airways of previously sensitized mice (Figure 1A), it induces allergic airway inflammation with features resembling human Th2-high allergic asthma such as eosinophilia in broncho-alveolar lavage (BAL) fluid (Figure E1A), goblet cell metaplasia (Figure E1B), increased production of Th2 cytokines in mediastinal lymph node (MLN) cultures (Figure E1C) and a rise in serum IgG1 and IgE (Figure 1B), not seen in control animals that were sham PBS sensitized and PBS challenged. The levels of IgE induced were sufficient to arm FcεRI-expressing effector cells like mast cells, basophils and inflammatory DCs with surface IgE (Figure 1C, E2C).

To assess the contribution of B cells to HDM allergic airway inflammation, we studied their distribution in MLNs and lungs in steady state (PBS) and after HDM challenge. In the control situation, naive B cells made up the majority of B cells in the lymph nodes and lung. HDM treatment induced a strong increase in total B cell numbers both in MLNs and lungs (resp. 10- and 18-fold increase) (Figure 1D-G). Besides massive influx of naive B cells, we found on average 12.2% GL7⁺ Fas⁺ germinal center B cells, 11.9% IgD⁻IgM⁻ switched memory B cells, ±5.9% IgM⁺ B cells and 2.7% CD138⁺ plasma cells in the MLN (Figure E2A, Figure 1E,G). Germinal center B cells were less abundant in the lungs, but still accounted for ±2% of total lung B cells (Figure E2A, Figure 1E,G) in HDM-treated mice, whereas this was less than 0.3% in PBS mice.

Lung immunofluorescence staining revealed B220⁺ B cell rich lymphocyte accumulations around blood vessels, which contained naive (IgD⁺) B cells and IgM⁺, IgG1⁺ and some IgE⁺ antibody secreting B cells (Figure 1H). Although these structures are reminiscent of inducible bronchus-associated lymphoid tissue (iBALT), organized collections of GL7⁺ cells in germinal centers were not detected with the microscope. Together, these results demonstrate that B cells contribute actively to the immune response to HDM.

B cells present Der p 1 allergen to naive and primed 1-DER T cells *in vitro*

To evaluate whether B cells play any role in T cell activation, we first analyzed their antigen presenting and Th2 inducing capacity *in vitro*, by coculturing B cells sorted from HDM-treated mice with naive or primed Der p1-specific *Rag*^{-/-} 1-DER T cells in the presence of Der p 1 protein. Primed 1-DER T cells were isolated *ex vivo* from mice that received 1µg HDM i.t. 1 week earlier. Primed as well as naive T cells cultured in the presence of B cells and Der p 1 protein underwent up to four divisions in 3 days. Up to 40% of 1-DER T cells differentiated into Th2 effector cells expressing IL-33R, secreted IL-5 and IL-13 and to a lesser extent differentiated into T follicular helper cells (Tfh, CXCR5⁺PD1⁺)(Figure 2A,B). Strikingly, there was also induction of IFNγ producing Th1 effector cells. In the presence of B cells, naive T cells divided and differentiated into Th2 and Th1 cells as efficiently as previously primed T cells, although they were less prone to develop into Tfh. The Th2 response increased relative to the number of B cells added to the culture, whereas IFNγ secretion was inversely influenced, particularly when primed T cells were stimulated by B cells. When comparing the antigen presenting capacity of antigen experienced B cells (HDM-B cells) to B cells that never encountered antigen (PBS-B cells, close to 95% naive B cells), the latter were also adept to present Der p 1 protein to 1-DER T cells, particularly when the T cells were already primed previously by antigen exposure *in vivo* (Figure 2C). We further observed that *ex-vivo* sorted B cells from HDM allergic mice did not stimulate 1-DER T cell division in the absence of exogenous Der p 1 protein, suggesting that the amount of antigen captured and/or processed *in vivo* was very low and below the detection limit of the assay. In support, we found that fluorescently labeled HDM extract could not be recovered in MLN B cells after intra-tracheal injection, whereas it could be readily detected in MLN DCs (Figure 2D). In conclusion, B cells isolated from the airways of HDM sensitized and challenged mice

are capable antigen presenting cells and can induce 1-DER Th2, Th1 and Tfh differentiation *in vitro*, but only when exogenous antigen is supplied.

B cells are not required for initial expansion of allergen reactive T cells *in vivo*

The fact that B cells sorted *ex vivo* 3 days after allergen encounter did not present *in vivo* captured antigen to 1-DER T cells could be due to rapid down regulation of MHC-peptide complexes, as previously described to occur within 24 h in B cells presenting Ag to cognate CD4 T cells³⁶. We therefore also performed an *in vivo* Ag presentation experiment in B cell deficient muMT mice. These mice lack mature B cells due to absence of the IgM membrane domain which is necessary for positive selection of immature B cells³⁷. Naive CD45.1 *Rag*^{-/-} 1-DER T cells were adoptively transferred into naive CD45.2 muMT mice receiving HDM extract to study sensitization (Figure 2E). In this setting, 1-DER T cell division in muMT mice measured at three days mirrored WT mice (Figure 2F), with comparable proliferation indexes (Figure 2G) and CD44 upregulation (Figure 2H) observed in MLNs and lungs. These data demonstrate that B cells are not necessary for mounting primary T cell responses to HDM, at least in part because they fail to take up antigen *in vivo*.

B cells are not necessary for development of asthma features to standard HDM allergen

Although, antigen presentation to naive allergen-reactive T cells was not affected by lack of B cells in muMT mice, it was still important to study the impact of B cell deficiency on features of asthma, as Igs are crucial for arming asthma effector cells like basophils and mast cells, with IgE. It has, however, been reported that muMT mice can still develop B cells through an IgM-independent pathway, and can even secrete IgE antibodies in conditions of inflammation³⁸⁻⁴⁰, we first tested whether B cells were absent from the lungs of muMT mice subjected to a protocol of HDM-induced asthma, induced by challenge of the lungs with 10µg of HDM extract. Both in lungs and MLNs of muMT mice we found only trace amounts

of B cells (Figure 3A&B, Figure E3A&B) demonstrating the absence of any IgM-independent B cell populations. Compared to wild type (WT) littermates, serum and BAL fluid IgE, IgG1 and IgA were extremely low to absent in muMT mice and did not increase upon HDM treatment (Figure 3C, D, E3C). HDM-specific IgG1 was also not detectable in muMT sera when it was in WT sera (Figure E3C). To verify absence of IgE, we checked the occupancy of FcεRI on basophils in blood and lungs by IgE surface staining. Surface IgE staining was significantly down on basophils of muMT mice compared with WT (Figure 3E, F, Figure E4) confirming near-complete absence of IgE in the muMT mice. We further confirmed that muMT mice had a block in Tfh development in the MLNs in response to HDM (Figure 3G), as expected in the absence of B cells in muMT mice.

Unexpectedly, HDM-treated muMT mice did develop similar levels of eosinophilia in BAL fluid (Figure 3H) and goblet cell metaplasia comparable to their WT littermates (Figure 3I). Lung function was measured by assessing bronchial hyperreactivity (BHR) to metacholine. HDM-treated muMT mice exhibited a (non-significant) trend towards lower BHR at intermediate metacholine doses, but at the highest dose tested, they reacted comparable to WT mice (Figure 3J). Altogether, these results establish that even though muMT mice lack B cells and Igs, they develop HDM-induced airway inflammation comparable to WT mice, demonstrating the redundancy of B cells in this standard dose model of HDM asthma.

B cells are necessary for stimulating cytokine production in mediastinal lymph nodes

Although the induction of pulmonary eosinophilia is a reflection of adequate adaptive Th2 cell immunity to HDM in B cell deficient mice, we still measured Th2 and Th1 cytokine production after *in vitro* HDM restimulation of MLN lymphocytes. The Th2 cytokines IL-4, IL-5, and IL-13, as well as IL-10 and IFNγ were all drastically reduced in muMT MLN cultures, compared with WT (Figure 4A). To address whether these effects in muMT mice were indeed solely due to lack of B cells, the ideal experiment would be to reconstitute

muMT mice with B cells *in vivo*. However, previous work^{41,42} as well as our own experience has shown that adoptively transferred B cells are rejected in muMT mice a few days after injection, precluding such *in vivo* reconstitution experiment. We therefore took an *ex vivo* approach and complemented restimulated muMT lymphocyte cultures with either naive MLN B cells from unmanipulated WT mice or B cells isolated from MLNs of HDM-treated WT mice. Results in Figure 4B show that addition of naive B cells only marginally increased production of Th2 cytokines, while antigen primed B cells were able to partially restore Th2 cytokine production. The addition of DCs was also able to restore Th2 cytokine production in muMT cultures, as previously reported by others as well⁴¹. Naive B cells were as capable as BM-DC to restore IL-10 and boosted IFN γ production of muMT lymphocytes above WT levels (Figure E5A). Supplementation of WT HDM MLN lymphocyte cultures with exogenous BM-DC, also boosted their Th2 secretion (Figure E5B), whereas no Th2 cytokines were detected when MLN cultures from PBS treated WT mice were supplemented with APC (Figure E5C). The need for exogenous APC for *in vitro* Th2 cytokine production, could result from a scarcity of DC in the MLNs of muMT mice, as has been described by others⁴³. However, the frequency and numbers of CD11c⁺MHCII^{hi} DCs in the MLNs of muMT mice was comparable to WT mice, both after sensitization and after induction of HDM-driven asthma (Figure E6).

Finally, we evaluated *in vivo* Th2 responses by performing intracellular staining for Th2 cytokines and the Th2 lineage transcription factor GATA-3 on BAL fluid and MLN lymphocytes. We found that BAL fluid of HDM-treated muMT mice contained normal numbers of Th2 cytokine-secreting and GATA-3⁺ CD4⁺ effector T cells as compared to WT littermates (Figure 4C). In the MLN however, MuMT mice presented significantly less IL-4-expressing and GATA-3⁺ CD4⁺ T cells (Figure 4D), most likely representing Tfh cells. Altogether, these data demonstrate that B cells are necessary for mounting full blown cytokine responses in the MLN during inhaled allergen HDM challenges.

B cells are necessary for full expansion of primed 1-DER T cells during secondary allergen challenge

To study how lack of B cells might reduce the cytokine response to inhaled HDM allergen challenge in the lymph nodes, primed CD45.1 *Rag*^{-/-} 1-DER T cells were adoptively transferred into already sensitized CD45.2 muMT mice, immediately prior to receiving 5 daily HDM doses (Figure 5A). In this setting, primed 1-DER T cells divided significantly less in MLNs, lungs and spleens of muMT mice compared to WT mice (Figure 5B). Although the proportion of divided cells was only marginally lower in muMT mice (on average 89,5% in muMT vs. 97,6% in WT MLNs), the number of divisions that T cells underwent was significantly reduced, as illustrated by the substantially reduced proliferation index (on average 11,4 vs. 4,5 in WT and muMT MLNs resp., Figure 5C). The proliferation index of 1-DER T cells in mLN of muMT mice resembled the one of 1-DER T cells in non-draining nodes of WT mice. Furthermore, 1-DER T cells from muMT mice were less activated as evidenced by reduced expression of the CD44 T cell activation marker (Figure 5B, D). In conclusion, expansion and activation of previously primed allergen-specific T cells occurred much less efficiently in the MLNs of B-cell deficient muMT mice.

Role for B cells in HDM driven asthma is revealed by limiting the allergen challenge dose

We next reasoned that the reduced expansion of allergen-specific T cells observed in muMT MLNs was not translating into reduced airway inflammation because the dose of challenge allergen used in the experimental setup was sufficient either for local antigen presentation to primed resident Th2 cells in the lung effector site^{44,45}, or that the few MLN expanded effector T cells that still made it to the lung (Fig 4C) were sufficient to mediate airway inflammation when challenged with standard doses of allergen. To address if eosinophilia in B cell

deficient mice was influenced by allergen dose, we titrated the dose of challenge allergen given over a period of 5 days. Whereas muMT mice challenged with 10 μ g of HDM extract had identical eosinophil influx in the lungs compared with WT mice, those challenged with 3 μ g doses had reduced eosinophil influx compared with WT mice (Figure 6A). This was -like for the standard dose challenge- supported by a strong decrease in the production of Th2 and Th1 cytokines by MLN cells upon *in vitro* HDM restimulation with low dose allergen challenge (Figure 6B). We verified that serum IgG1 and IgE in muMT mice treated with 3 μ g HDM were still low compared to WT littermates (Figure 6C). In line with reduced BAL eosinophilia, BHR to metacholine challenge was reduced in low dose HDM challenged muMT mice compared to WT ($p < 0.05$; Figure 6D). Unanticipated finding was that low dose HDM muMT mice exhibited identical levels of goblet cell metaplasia (GCM, measured by *Muc5ac* mRNA induction) compared to WT mice (Figure 6E), despite clearly reduced levels of the GCM-inducing cytokine IL-13. GCM thus seems to be B cell-independent. In line with decreased BAL eosinophilia and severely reduced *in vitro* MLN Th2 cytokine production, Th2 cytokine producing T cells were significantly reduced both in BAL and MLNs from muMT mice receiving low dose HDM compared to WT littermates (Figure 6F). As in mice receiving standard HDM challenge doses, proliferation and activation of adoptively transferred primed CD45.1 *Rag*^{-/-} 1-DER T cells was lower in muMT mice compared to WT mice receiving low dose HDM (Figure 6G). In conclusion, we demonstrated that the role for B cells in HDM-induced asthma features is to be revealed only when the dose of challenge allergen is low, as a consequence of a drop in the number of Th2 effector cells in the lung effector site of muMT mice.

It has been shown that during secondary immune responses to allergen, there is continued need for new waves of effector cells generated in the draining MLN from recirculating T central memory cells (T_{cm}), fueling the ongoing response with new effector cells^{44,46}. In addition, pathogenic Th2 cells were found to be lung resident memory cells (T_{rm}) that

depend on IL-2 signaling and are increased in the absence of B cells⁴⁵. Hence, as a final investigation, we explored if lack of B cells altered the differentiation of 1-DER T cells to become either Tcm or Trm. Therefore, naive CD45.1+ *Rag*^{-/-} 1-DER T cells were adoptively transferred in WT or muMT hosts, which subsequently underwent the full HDM protocol with low dose antigen and were left untreated for 3 weeks (Figure 7A). We utilized a brief intravenous labeling with an antibody directed against the pan-leukocyte marker CD45 to label all cells that were still in the bloodstream, and could therefore not be considered “resident” cells of the lungs⁴⁷. First, 1-DER T cells persisted at similar numbers in muMT and WT MLNs and lungs (Figure 7B), suggesting that B cells do not influence the overall survival of 1-DER T cells following adoptive transfer. Additionally, lung resident CD45^{i.v.-negative} CD4⁺CD44^{hi} 1-DER T cells were present at normal frequencies in muMT compared to WT mice (Figure 7C, E7A) and exhibited comparable expression of the Trm marker CD69 (39,2% of WT and 40,4% of muMT 1-DER Trm cells). More strikingly however, there was a significant reduction in the number of CCR7+CD44⁺ Tcm 1-DER T cells in the MLN of muMT mice 3 weeks after the last challenge, and 1-DER T cells expressed less CD69 and CD44 compared to WT (Figure 7D, E7B). These data demonstrate that in a low dose HDM challenge model, lack of B cells mainly affects the generation of Tcm cells, while leaving Trm formation intact.

Discussion

Here, we set out to dissect the role of B cells in a mouse model of HDM-induced allergic asthma, with a focus on antigen presentation. The use of HDM-specific 1-DER T cells *in vivo* allowed us to directly and for the first time address the antigen presenting capacities of B cells to both naive and primed allergen specific T cells, and to follow their fate in mice lacking B cells. We ventured to do this because the available literature on the role of B cells in asthma mouse models is discordant, with some groups reporting that B cells are essential for BHR only⁴⁸, whereas others demonstrate that B cells are redundant for the development of eosinophilic airway inflammation and/or bronchial hyperreactivity^{24-27,39,49,50}. At the same time, some of those groups reported normal Th2 cytokine production^{27,49} whereas others reported reduced Th2 cytokines in B cell deficient mice^{21,26,48,50}. These discrepant findings were largely explained as the result of intrinsic differences in model setup (number and timing of allergen doses), mouse strain (muMT or JH^{-/-} on BALB/C or B6 background) and antigen (OVA, HDM, *Aspergillus*, Cockroach, or a mixture). The controversy in this field has recently been fired up by two high profile papers addressing the role of B cells in HDM asthma^{45,51}. Ballesteros-Tato *et al.* found muMT mice to have impaired pulmonary Th2 effector responses, due to a defect in formation of Tfh cells, proposed by this group to be the immediate progenitors of effector Th2 cells⁵¹. Hondowicz *et al.* to the contrary⁴⁵, observed just like us, that B cells are dispensable for pulmonary effector responses and BHR. The mechanistic explanation offered by this group was that defective Tfh differentiation caused by lack of B cells favors differentiation of CD4 T cells to an alternative fate of cytokine producing Trm cells that can mediate asthma features upon allergen encounter. Our findings in an inhaled HDM model of asthma in C57Bl/6 mice resolves an important part of this recent controversy by showing that the degree to which airway eosinophilia and Th2 cytokine production is affected in B cell deficient animals is dose dependent, and conclusions and

mechanistic explanations offered will also heavily depend on the precise location where Th2 cytokine production is measured.

Mechanistically, we show that lack of B cells mainly affects the proliferation of primed allergen-specific CD4 T cells in the mediastinal nodes. As the amount of Trm cells developing from a pool of naive Der p 1-specific T cells was normal in mice lacking B cells (despite a dramatic reduction in Tfh differentiation), we believe that lack of expansion of primed T cells is the main explanation for the drop in lung Th2-cytokine producing cells and consequent asthma development when the amount of recall allergen is limiting. Rescue of *ex vivo* cytokine production of MLN cells by exogenous APC, together with normal frequencies of Th2 producing cells in muMT MLNs, demonstrates that muMT T cells indeed harbor the potential to produce Th2 cytokines, but that there is an intrinsic defect in stimulation, most likely caused by defective antigen presentation. We have previously shown that in the sensitization setting, DCs are sufficient for Th2 priming^{34,52} and our current data demonstrate that this holds true in the absence of B cells. In the challenge setting in the absence of B cells, on the other hand, we assume that DCs become limiting to present the available allergen to primed 1-DER T cells especially in the MLN, where allergen reactive T cells massively expand after a few days of proliferation. In that situation, bystander B cells, which were also activated upon HDM exposure (cfr Fig1: GC reaction; upregulation of MHC-II and costimulatory molecule CD86, MDullaers unpublished data) could further amplify 1-DER T cell responses, allegedly by providing additional MHCII molecules⁴¹. This concept is supported by our and others' ⁴¹ *in vitro* experiments showing that either B cells or DCs could rescue Th2 function of muMT MLN T cells. Impaired Th2 responses in B cell deficient mice were also reported in various other model systems^{21,26,48,50,41,53}. A lack of proper Th2 expansion emerged as a mechanistic explanation in a Th2 model of keyhole limpet hemocyanin (KLH)/alum vaccination, where *in vivo* reconstitution of muMT mice with either activated splenic B cells or DC could restore short term (<5 days) CD4 T cell expansion and

Th2 differentiation⁴¹. Likewise, chimeric mice lacking MHC-II only on B cells showed defects in T cell expansion and differentiation towards Th2^{54,51}.

The fact that HDM-specific B cells, but not naive B cells, partially restored Th2 cytokine secretion in our muMT MLN restimulations, could be related to the activation status of the B cells or their enhanced antigen uptake capacity via a HDM-specific BCR. In support, we found that B cells taken from HDM exposed mice had higher levels of the costimulatory molecule CD86. Unfortunately, we could not perform *in vivo* reconstitution experiments with B cells in muMT mice, as B cells are rapidly rejected in these mice and do not survive well beyond 5 days^{41,42}. In our experiments, we found that B cells sorted from MLNs of HDM exposed mice contained only minute amounts of HDM allergen, generating a conceptual problem, as antigen presentation is virtually always preceded by antigen uptake. As we performed this experiment on a pool of polyclonal B cells, we cannot fully exclude that HDM-specific B cells would be more adept to take up HDM antigen and stimulate HDM-specific T cells. Further, B cells were shown to take up papain (a Th2 inducing protease) after footpad injection and subsequently amplified Th2 responses in the draining LNs in an ICOS-L dependent manner¹⁸. This non-BCR mediated phenomenon seems to be specific for papain, since OVA administered through the same route, was not taken up by B cells. Our group has preliminary evidence that proteolytic activity of allergens is decisive as to whether they leak through the airway epithelium or not. It is very likely that the enzyme papain gains access to B cells in the lymph nodes after footpad injection by leaking into the lymphatics, whereas in our model, most HDM reaches the LN in a CCR7 dependent manner, requiring DCs³⁴.

Together, our data reveal that the dose of allergen during secondary challenge of already primed mice is decisive whether or not lack of B cells and concomitant reduction in the expansion of Th2 cells in the MLN also leads to reduced airway inflammation. We believe

that with standard doses of allergen challenge, B cells are redundant because local antigen presentation by inflammatory and conventional DCs to tissue resident memory Th2 cells³⁴ (whose formation is fully intact in B-cell deficient mice) is sufficient to cause full blown airway eosinophilia. In support of this hypothesis, with standard dose HDM, we found normal numbers of CD4⁺ T cells and Th2 producing T cells in the lung effector site, probed in the BAL compartment, in the face of a profound decrease in CD4⁺ T cell and Th2 cell numbers in MLNs of muMT mice. It has been shown that during secondary immune responses to allergen, there is continued need for new waves of effector cells generated in the draining MLN from recirculating Tcm or lymph node resident T effector memory cells, fueling the ongoing tissue response with new effector cells that migrate to the lung^{44,46}. Our current work reveals that expansion of such primed Th2 effector cells as well as Tcm cells in the MLN requires B cells, in addition to migratory DCs^{46,55}. By lowering the amount of allergen during challenge, we indeed observed that effector responses became more dependent on these “new recruit” effectors. Consequently, in the absence of B cells, insufficient Th expansion in the MLN leads to lack of new effectors and a decline in airway eosinophilia, BHR and Th2 effector cells in the lungs of muMT mice.

Further work is needed to fully understand the impact of B cells in asthma. Lack of B cells and secreted immunoglobulins in muMT mice subjected to asthma also came with lack of IgE on effector cells such as basophils and mast cells. A causal relationship between B cell-derived IgE and allergic asthma in humans has long been recognized²⁸ and anti-IgE therapeutics are being used successfully for treatment of severe allergic asthma, suggesting an important role for B cell-derived IgE in human asthma⁵⁶. It is therefore also possible that upon reduction of the allergen dose in muMT mice, the enhancing effect of IgE “arming” of these effector cells became relatively more important. As there are many parallel paths to causing tissue eosinophilia, B cell deficient mice would see reductions in tissue inflammation because of a lack of adequate effector function of basophils and mast cells only when the

amount of allergen is lowered. Alternatively, DCs might also use the high affinity IgE receptor to capture allergens more efficiently, and this effect might see its major consequence only if the amount of allergen is limiting⁷. Studies are underway in our laboratory to dissect these roles of FcεRI on various cell types in the HDM model. Since the heterogeneity of human asthma is as wide-ranging as the mouse models mentioned above, we assume that B cells will contribute to asthmatic features in some patient groups but probably not in all. This remains to be investigated further, but is certainly supported by the observation that the clinical effectiveness of anti-IgE therapies in human asthmatics is also only seen in 30-40% of cases. We predict based on our findings that asthma therapies aimed at B cells (including anti-IgE strategies) will work best when combined with allergen avoidance measures.

Taken together, we show here, using a novel TCR transgenic model to read out HDM-specific T cell responses, that B cells are dispensable for primary CD4⁺ T cell responses, yet crucial for optimal expansion of primed Th2 cells and generation of Tcm cells in the MLNs. Only when the amount of inhaled allergen is limiting, does the enhancement of T cell effector responses by B cells (or their derived products) become crucial for optimal induction of asthma features.

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- 719

Figure Legends**Figure 1: B cells contribute actively to the HDM-induced immune response in MLNs**

and lungs. A. Mouse model of HDM-induced asthma. C57Bl/6 mice received a sensitization dose of 1µg HDM intra-tracheally (i.t.) followed by 5 daily intranasal (i.n.) challenges of 10µg HDM. Control animals received PBS at all administrations. Three days after the last challenge, mice were sacrificed for analysis. B. Total IgG1 and IgE serum concentration as determined by ELISA. C. IgE surface expression on effector cells as measured by flow cytometry. Mast cells were gated as CD45⁺CD3⁻CD19⁻MHCII⁻CD11c⁻ckit⁺FcεRI⁺, basophils as CD45^{int}CD3⁻CD19⁻MHCII⁻CD11c⁻ckit⁻FcεRI⁺ and inflammatory DC as CD45⁺CD3⁻CD19⁻MHCII⁺CD11c⁺ckit⁻FcεRI⁺. D, E, F, G. Lymphocyte and B cell subsets in MLNs and lungs were analyzed with flow cytometry. Number of cells, as determined by trypan blue counting, are plotted for CD4⁺ and CD8⁺ T cells, B cells and B cell subsets in mice receiving either PBS or HDM. E,G. The area of the pie charts represents the cell numbers. H. Confocal imaging of consecutive frozen lung sections immuno-stained as indicated with B220, GL7, IgM, IgD, IgE and IgG1. Sections were counterstained with DAPI in blue. Results shown are mean +/- SEM of 6 mice/group. Representative of 3-6 similar experiments.

Figure 2: B cells present Der p1 allergen to naive and primed 1-DER T cells *in vitro* but

are not required for initial expansion of 1-DER T cells *in vivo*. A, B. C57Bl/6 mice received a sensitization dose of 1µg HDM i.t. followed by 5 daily i.n. challenges of 10 µg HDM. Three days after the last challenge, B cells were sorted from MLNs and cocultured at the indicated numbers with either 1*10⁵ naive or primed 1-DER T cells in the presence of 3µM der p1 protein. After 3 days of culture, cells were flow cytometrically analyzed for proliferation (Cell Trace Violet=CTV), IL33R (T1ST2) expression and Tfh differentiation (%)

PD1⁺CXCR5⁺ cells) and IFN γ , IL-5 and IL-13 were measured in the supernatants by ELISA. Repeated measures one-way ANOVA tested significant for all parameters tested ($p < 0.05$).

C. Comparison of 1-DER T cell divisions induced by B cells sorted either from PBS treated or HDM treated mice. D. Naive C57Bl/6 mice received 50 μ g AlexaFluor647-labeled HDM i.t. Eighteen hours later B cells and DCs from MLNs were analyzed for their AF647-HDM content by flow cytometry. B cells were gated as CD3⁻CD19⁺MHCII⁺CD11c⁻, DCs as CD3⁻CD19⁻MHC⁺CD11c⁺.

E. 3*10⁶ CTV labeled naive CD45.1 Rag^{-/-} 1-DER T cells were adoptively transferred in naive CD45.2 WT and muMT C57/Bl6 mice. F. Representative proliferation plots of 1-DER T cells (CD3⁺CD4⁺CD45.1⁺CD45.2⁻) from MLNs of WT and muMT mice analyzed after 3 days. G,H. Proliferation index and CD44 expression of 1-DER T cells in MLNs and lungs of WT and muMT mice 3 days after HDM. Data shown are mean $\pm$ SEM from 1 out of 2 independent experiments, 5-6 mice per group.

Figure 3: B cells are not necessary for development of HDM-induced allergic airway inflammation. WT and muMT mice received the full HDM asthma model as shown in figure 1A. All analyses were performed on day 15. Data shown are from 1 out of 2-4 similar experiments, 4-6 mice per group. A,B. T cells (CD3⁺MHC-II⁺) and B cells (CD19⁺MHCII⁺) were enumerated in MLNs and lungs by flow cytometry and trypan blue counting. C,D. Total IgG1, IgE and IgA in serum and BAL fluid as determined by ELISA. E,F. Surface IgE (shown in mean fluorescence intensity=MFI) was determined by flow cytometry on blood and lung basophils (CD45^{int}SSC^{int} Fc ϵ RI⁺MHCII⁺). G. Follicular T helper cells (Tfh, CXCR5⁺PD1⁺) as frequency of CD4⁺ T cells. H. Numbers of eosinophils (SiglecF⁺CD11c⁻) as assessed by flow cytometry. I. Periodic acid – Schiff (PAS) staining for mucus cell metaplasia on frozen lung sections, showing an intermediate airway in the lower left corner with associated blood vessel. J. Lung function was measured by assessing bronchial

hyperreactivity to metacholine (Mch). Shown is the airway resistance (Rrs) at different concentrations of i.v. Mch. Data shown are mean $\pm$ SEM from 1 out of 2-4 independent experiments, 5-6 mice per group.

Figure 4: B cells are necessary for mounting full blown Th2 responses in mediastinal lymph nodes (10 μ g HDM).

A. WT and muMT mice received the full HDM asthma model as shown in figure 1A. On day 15, MLNs (2×10^5 /well) were *in vitro* restimulated with 15 μ g/mL HDM extract and 3 days later cytokines were measured in the supernatants by ELISA. B. muMT MLNs were complemented with naive MLN B cells or B cells from mice receiving 5 doses of 10 μ g HDM (1×10^5 /well) or bone marrow derived dendritic cells (DCs, 2×10^4 /well) during HDM restimulation *in vitro*. After 3 days, cytokines were measured in the supernatants by ELISA. Data shown are mean $\pm$ SEM from 1 out of 2-4 independent experiments, 5-6 mice per group. P values shown in graphs were calculated using Dunn's multiple comparisons test following ordinary ANOVA comparing WT against the different muMT conditions. C. WT and muMT mice received the full HDM asthma model as shown in figure 1A. On day 15, CD4⁺ T cells from BAL fluid and MLN were stimulated with PMA and Ionomycin for 3 hours before performing intracellular staining for IL-4, IL-5, IL-13 and GATA-3. Numbers were calculated based on trypan blue viability counts.

Figure 5: B cells are necessary for full expansion of primed 1-DER T cells during secondary allergen challenge

A. 1×10^6 CTV-labeled, HDM primed CD45.1 Rag^{-/-} 1-DER T cells were adoptively transferred in sensitized WT and muMT C57Bl6 mice that subsequently received 5 i.n. HDM challenges. Mice were analyzed on day 15. B. Representative flow cytometry plots from MLNs of WT and muMT mice showing CTV proliferation and CD44 upregulation. C. Proliferation indices of 1-DER T cells (CD3⁺CD4⁺CD45.1⁺CD45.2⁻) in MLNs, lungs, non

draining lymph nodes (ndLN= brachial LN) and spleen of WT and muMT mice. D. Frequency of CD44⁺CTV⁻ 1-DER T cells. Data shown are mean +/- SEM from 1 out of 2 independent experiments, 5-6 mice per group.

Figure 6: Role for B cells in HDM driven asthma is revealed by limiting the allergen challenge dose (3µg HDM).

A. WT and muMT mice received the HDM asthma model as shown in figure 1A with either 1, 3 or 10µg HDM as challenge dose. Numbers of eosinophils (SiglecF⁺CD11c⁻) were assessed by flow cytometry. B. MLNs (2*10⁵/well) of mice receiving 3µg HDM challenge dose were *in vitro* restimulated with 15µg/mL HDM extract and 3 days later cytokines were measured in the supernatants by ELISA. C. Total serum IgG1 and IgE of mice receiving 3µg HDM challenge dose as determined by ELISA. D. Lung function of mice receiving 3µg HDM challenge dose was measured by assessing bronchial hyperreactivity to metacholine (Mch). Shown is transfer resistance (Rtr) at different concentrations of nebulized Mch. Using a linear mixed model, the response in resistance to the HDM treatment differs significantly between muMT and WT (p<0.05). E. Lung *Muc5ac* expression as measured with qPCR. F. CD4⁺ T cells from BAL fluid and MLNs of mice treated with 3µg HDM were stimulated with PMA and Ionomycin for 3 hours before performing intracellular staining for IL-4, IL-5 and IL-13. G. 1*10⁶CTV-labeled, HDM primed CD45.1 Rag^{-/-} 1-DER T cells were adoptively transferred in sensitized WT and muMT mice that subsequently received 3µg HDM challenges. Mice were analyzed on day 15. Shown are proliferation indices and frequency of CD44⁺CTV⁻ of 1-DER T cells in MLNs, lungs, and spleen of WT and muMT mice. Data shown are mean +/- SEM from 1 out of 2-4 independent experiments, 5-6 mice per group.

Figure 7. B cells are needed for Tcm expansion in MLNs but not for lung Trm development.

A. 1×10^5 CTV-labeled, HDM naive CD45.1 Rag^{-/-} 1-DER T cells (CD45.1) were adoptively transferred in WT and muMT C57Bl6 mice that subsequently received the low dose HDM protocol. Mice were analyzed on day 33, 3 weeks after the last challenge. B: 1-DER T cell numbers were calculated based on trypan blue viability counts. C, D: Frequencies of lung Trm (CD69⁺ CD44⁺CD45iv⁻) and MLN Tcm (CD44⁺CCR7⁺) in the 1-DER T cell populations are shown as well as CD69 and CD44 expression (expressed as mean fluorescence intensity).

Figure 1

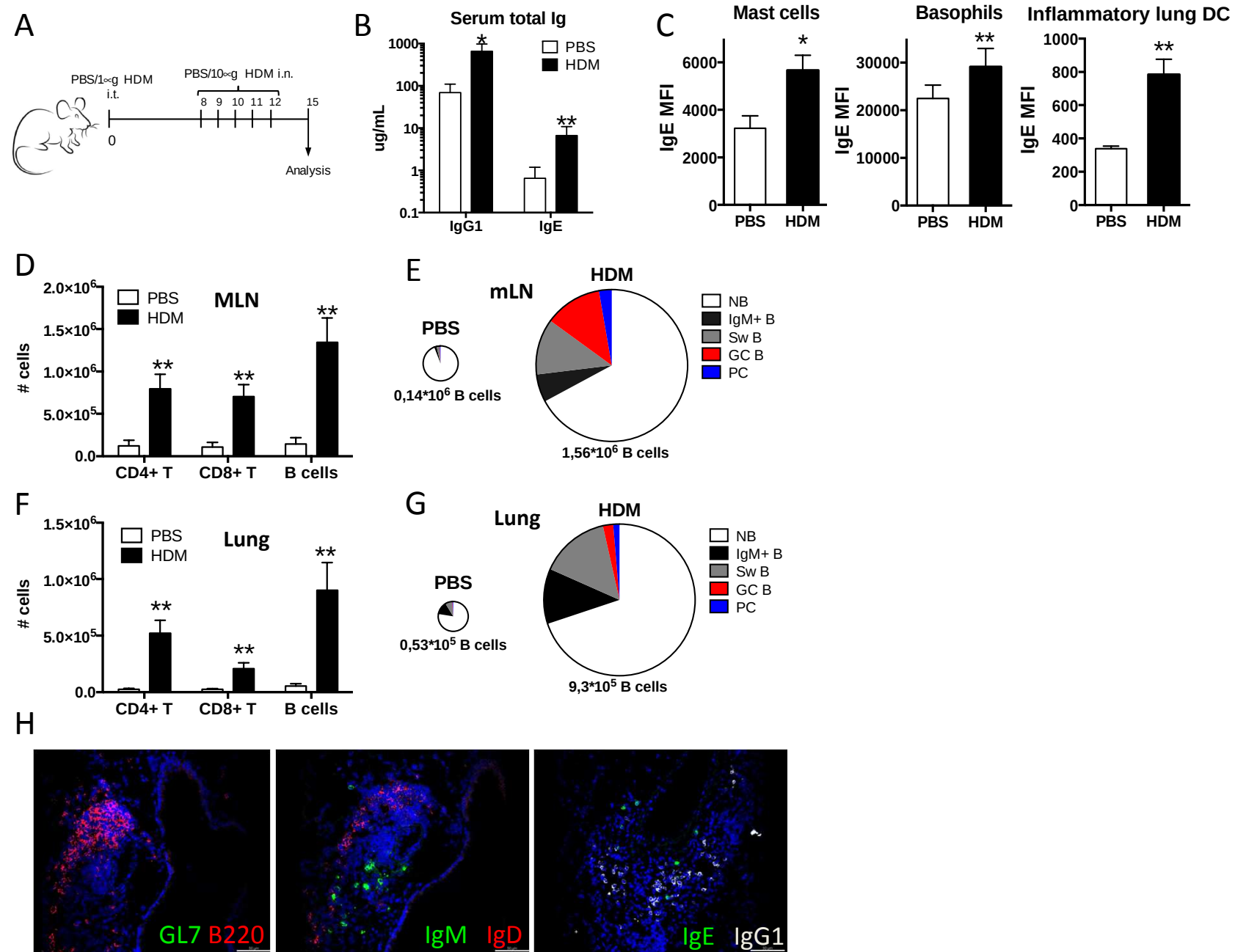


Figure 2

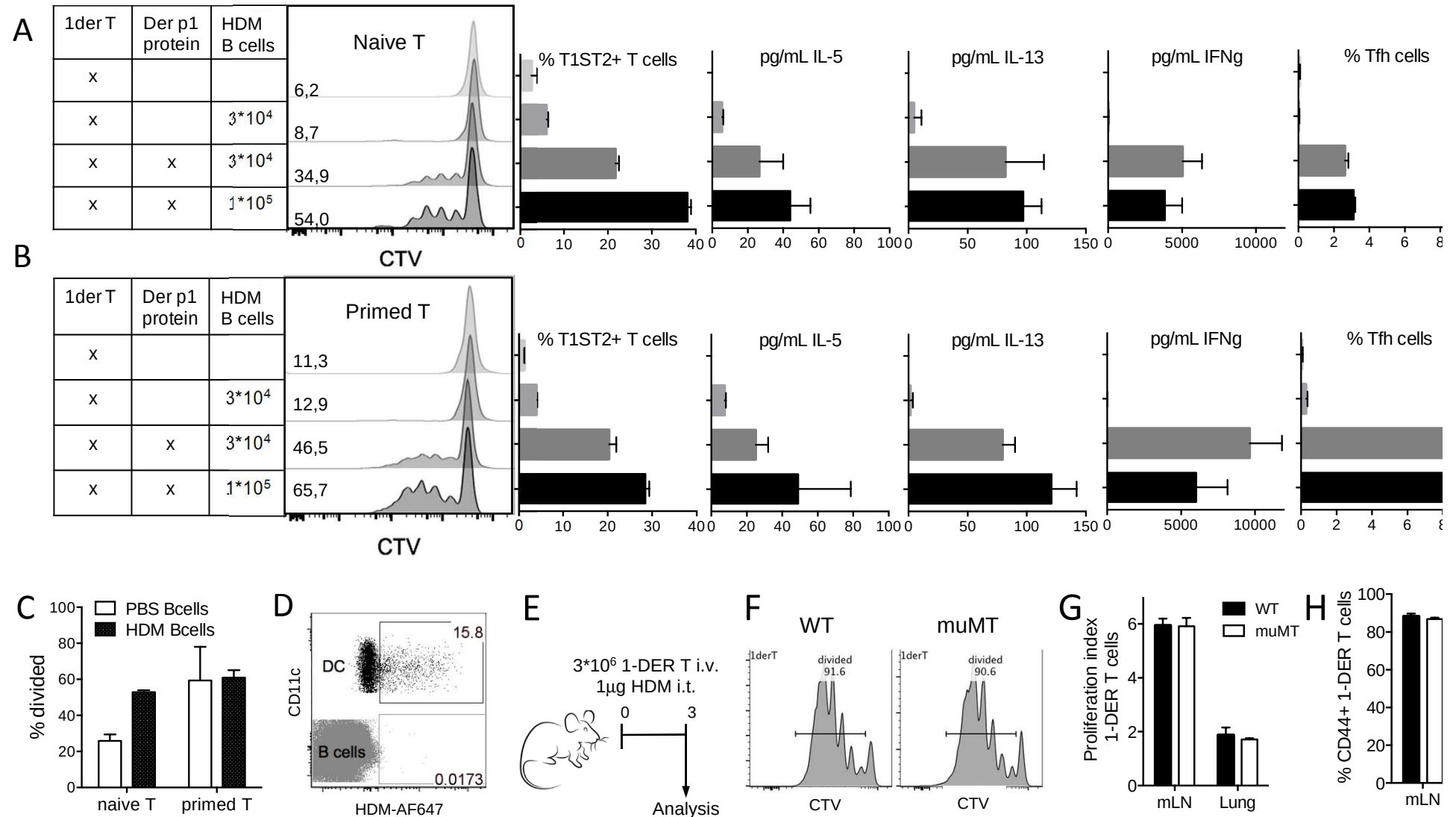


Figure 3

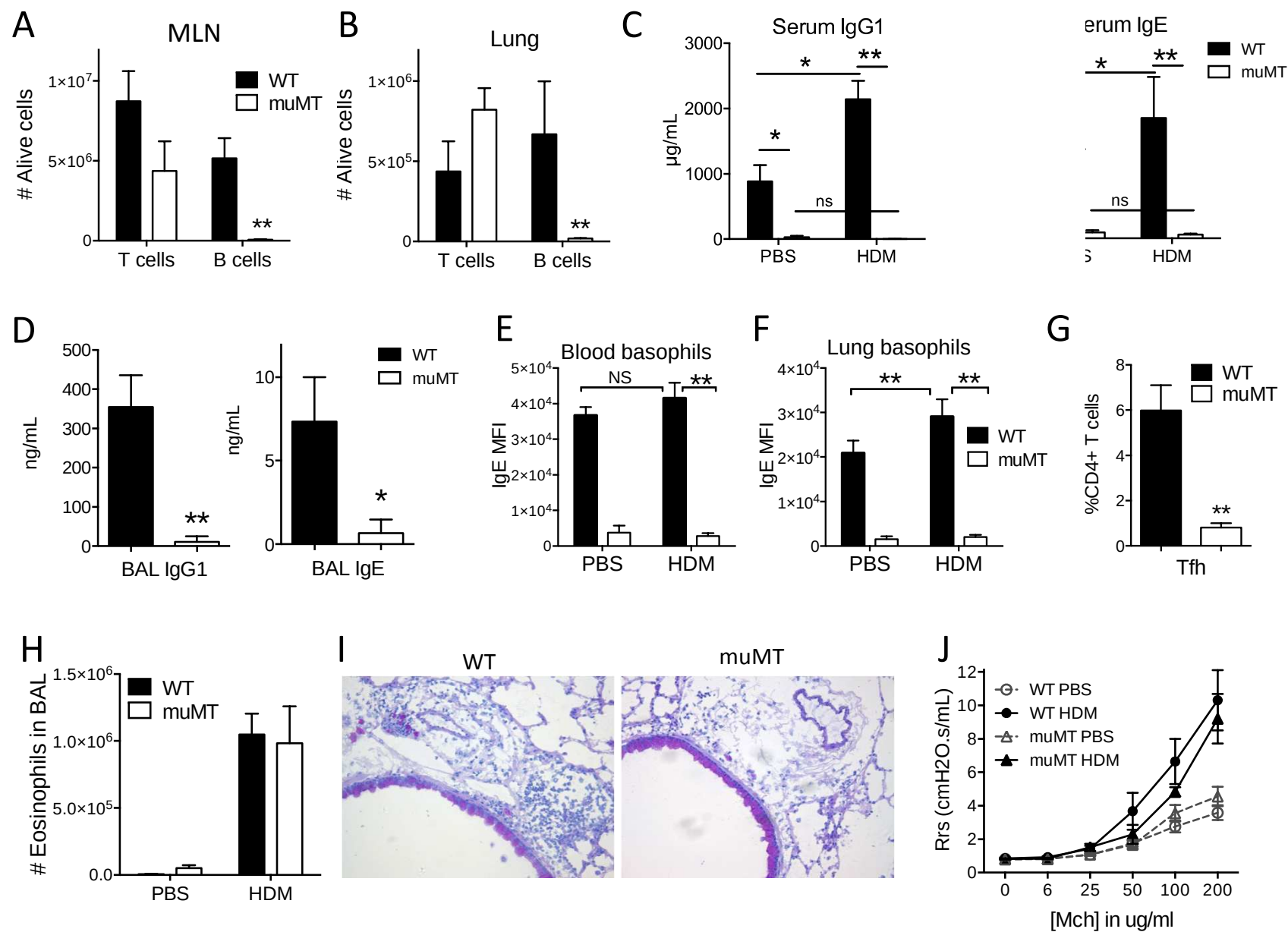


Figure 4

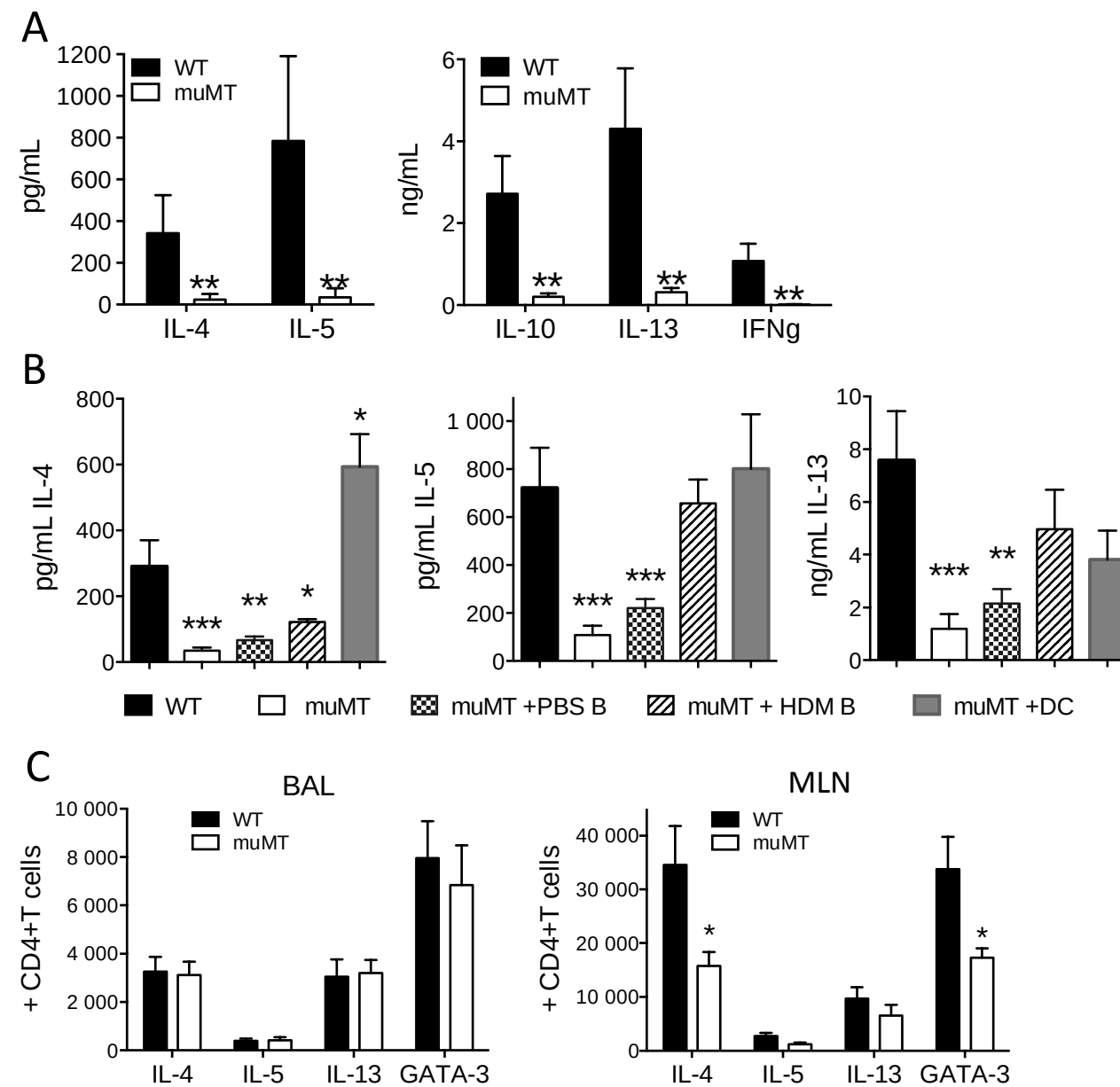


Figure 5

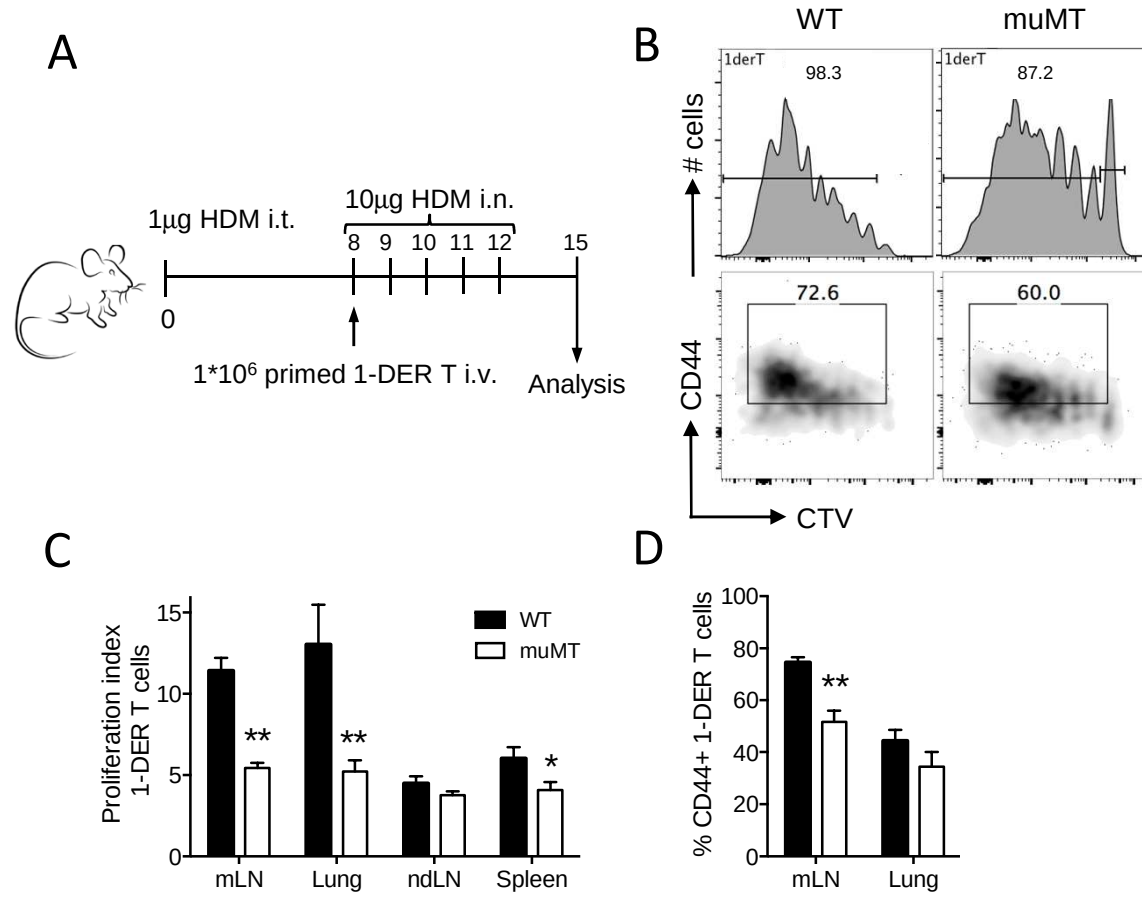


Figure 6

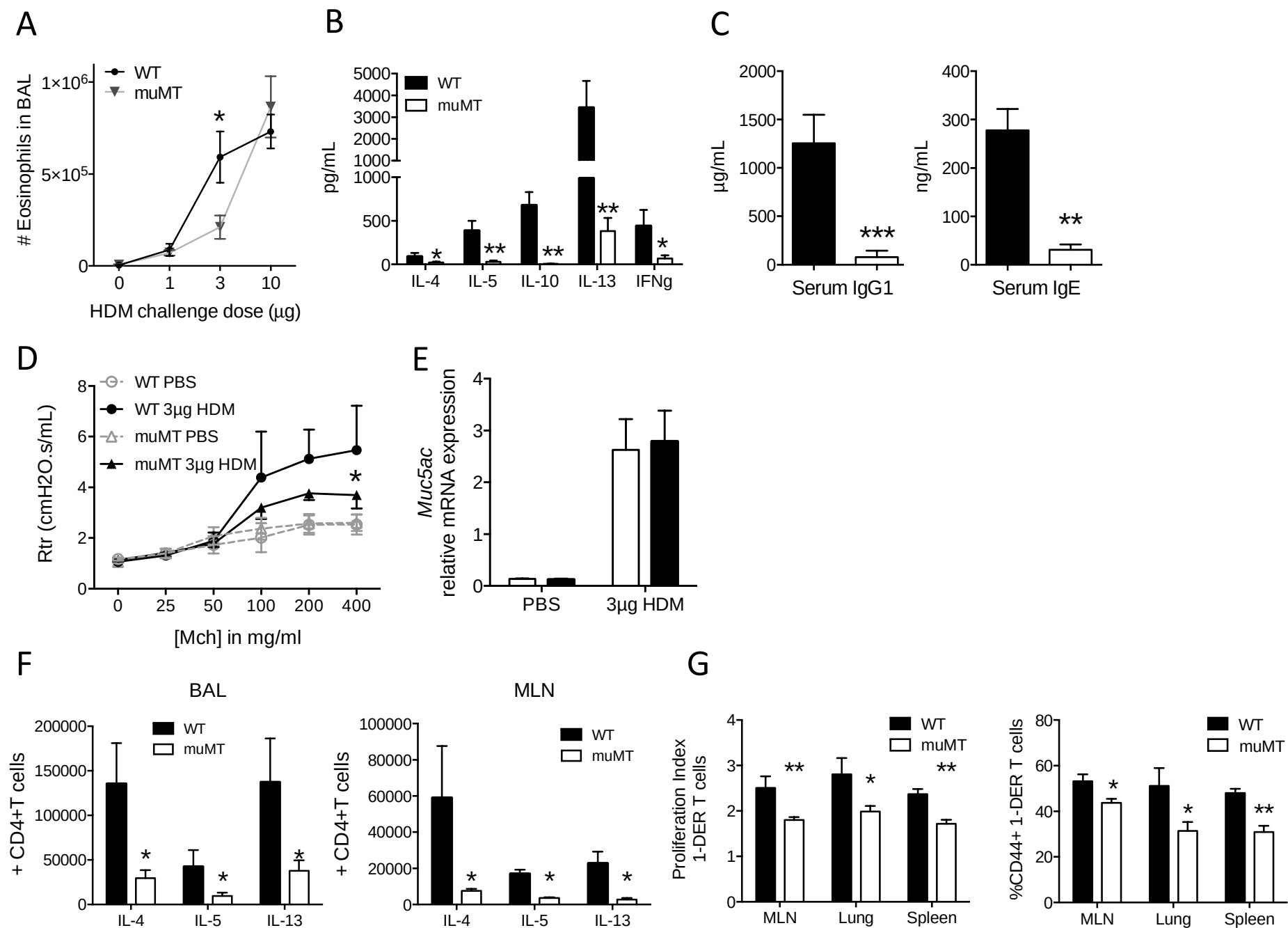
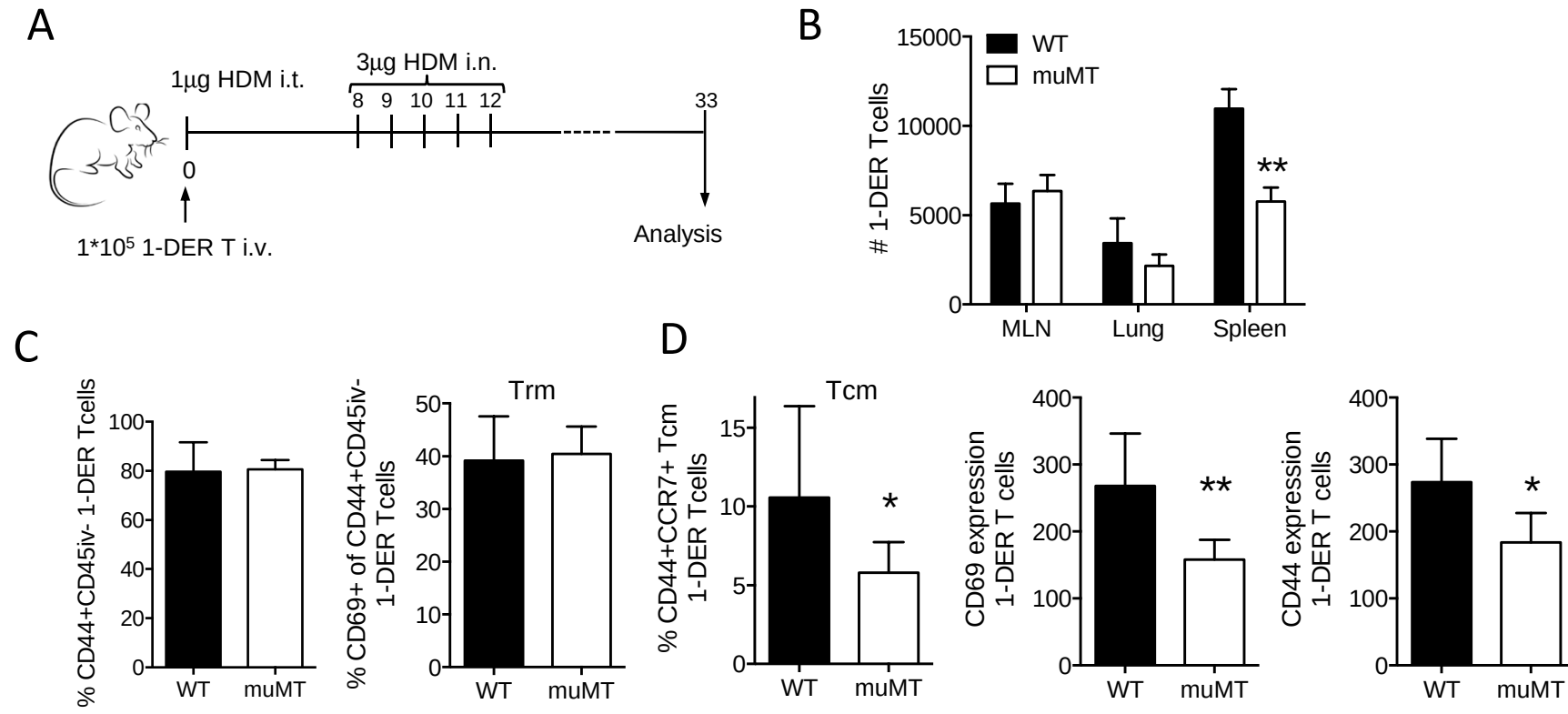


Figure 7



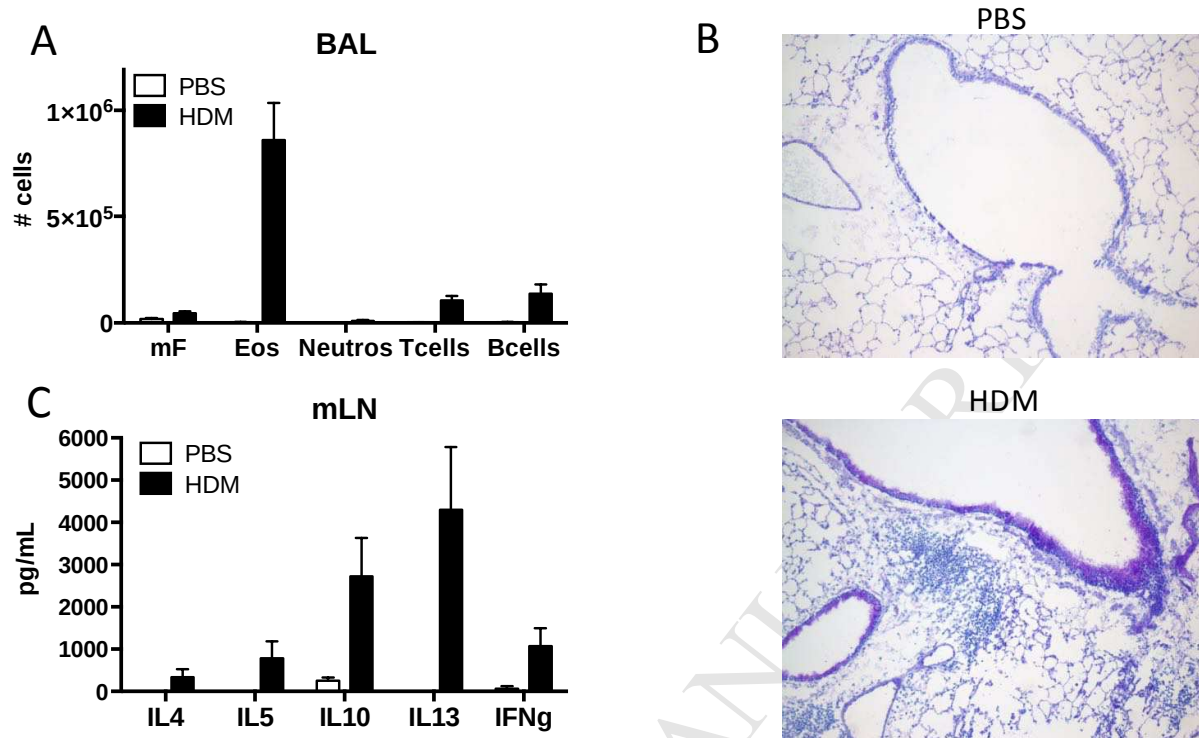


Figure E1: Characteristic features of the HDM-asthma model. C57/Bl6 mice received a sensitization dose of 1 μ g HDM intratracheally (i.t.) followed by 5 daily intranasal (i.n.) challenges of 10 μ g HDM as shown in figure 1A. Control animals received PBS at all administrations. A. Cellular composition of BAL fluid showing a remarkable increase in eosinophils upon HDM treatment. B. Periodic acid – Schiff (PAS) staining for mucus cell metaplasia on frozen lung sections. C. On day 15, mLNs (2 $\times$ 10⁵/well) were *in vitro* restimulated with 15 μ g/mL HDM extract and 3 days later cytokines were measured in the supernatants by ELISA.

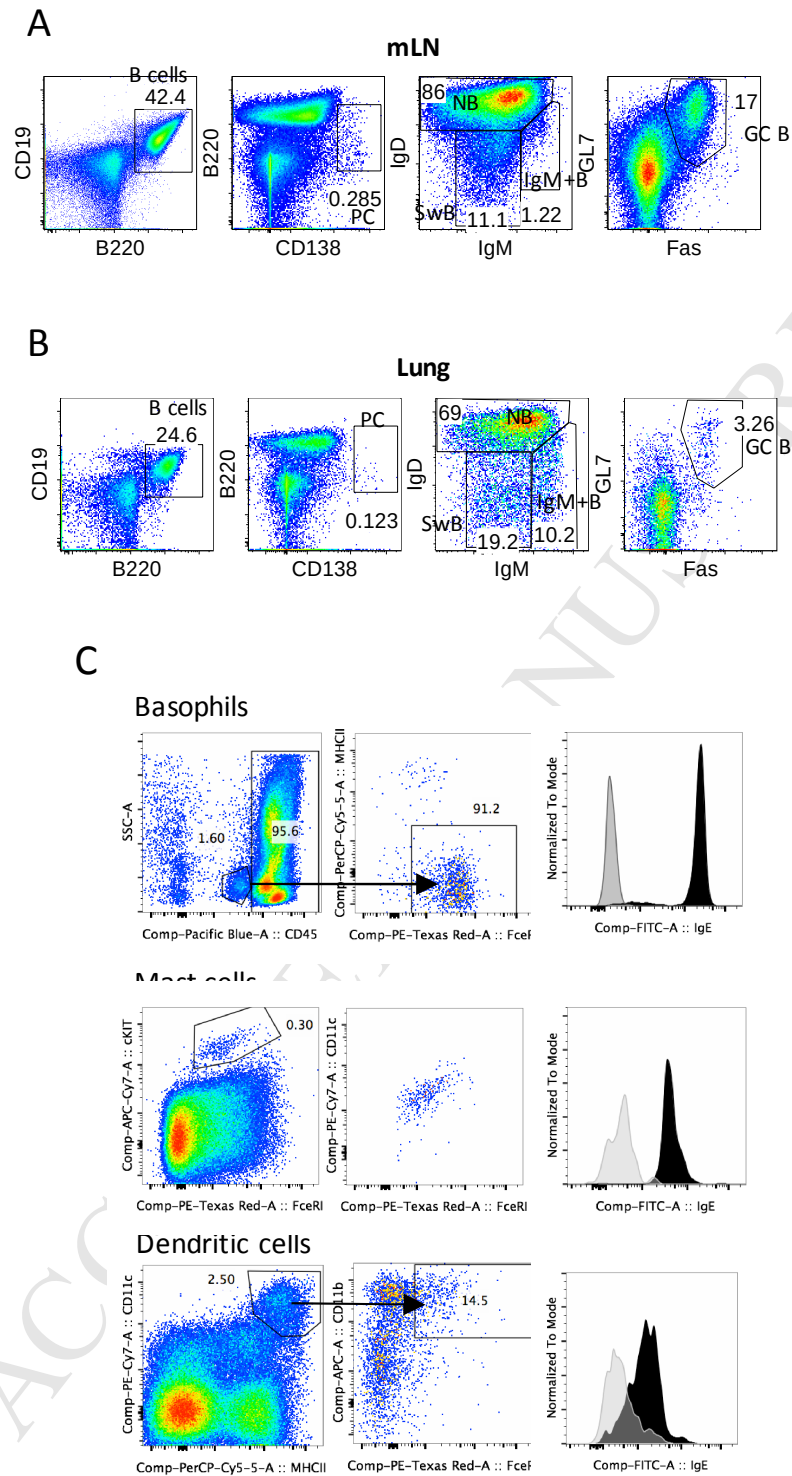


Figure E2: A+B: Flow cytometric gating strategy of B cells in mLN and lungs. C: Flow cytometric gating and IgE surface staining of lung basophils, mast cells and dendritic cells. Surface IgE was measured by extracellular staining with anti-human IgE, which quantifies FcεRI-bound IgE. Shown in overlays are FMO controls (grey) and IgE stain (black) of HDM treated mice.

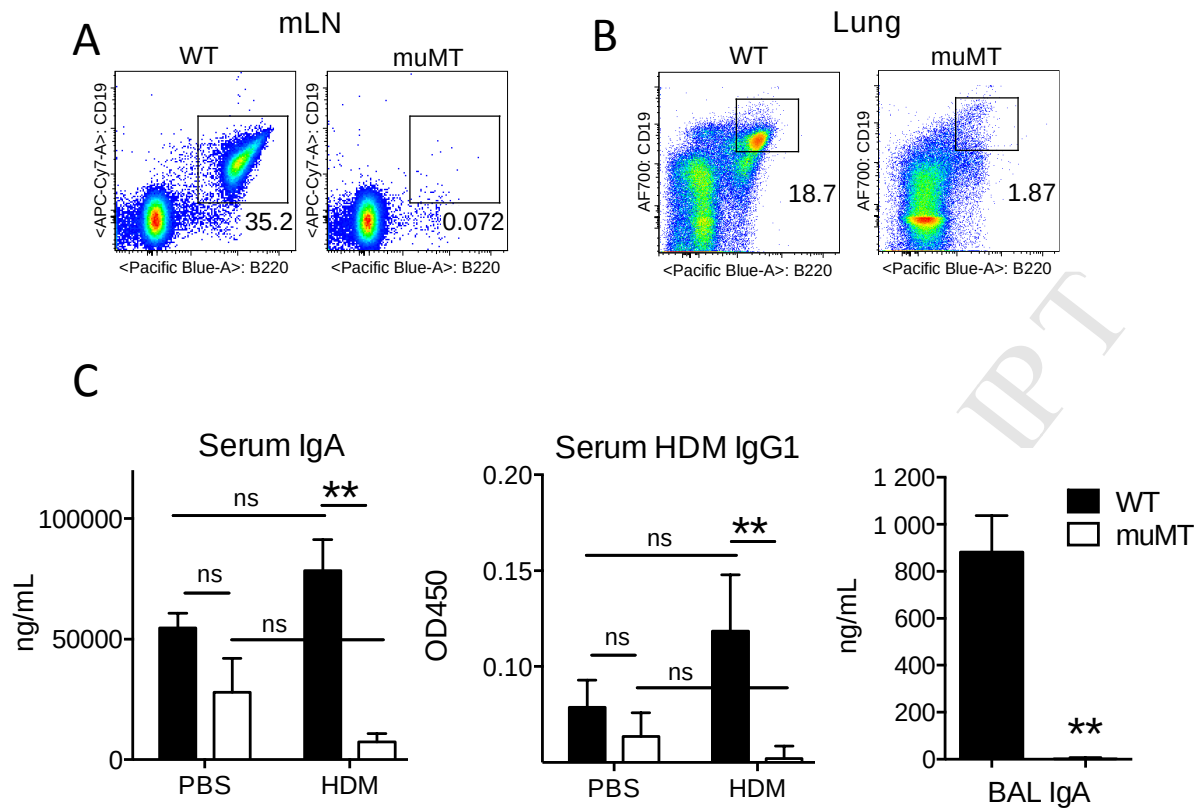


Figure E3: Characterization of the B cell compartment in muMT mice after the full HDM model. A, B. Flow cytometric analysis of B cells in mLN and lungs of WT and muMT mice. C. Total IgA and HDM-specific IgG1 as measured by ELISA in the serum and total IgA from BAL fluid.

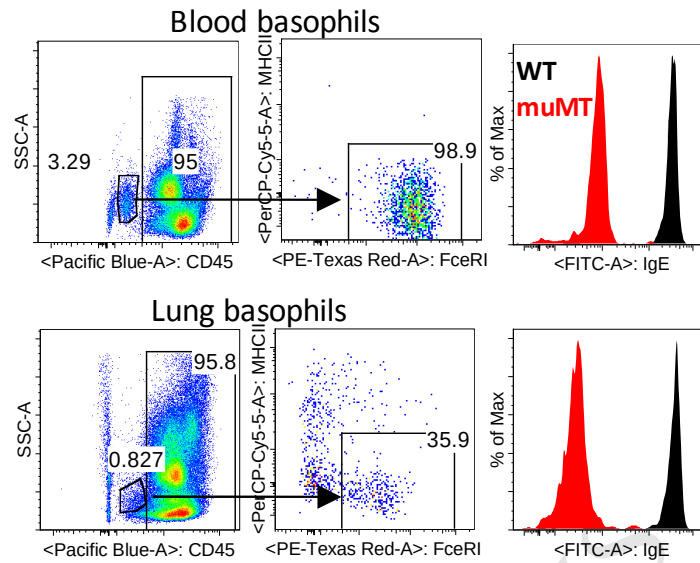


Figure E4: Gating strategy for basophils from blood and lungs and their surface IgE expression. Surface IgE was measured by extracellular staining with anti-human IgE, which quantifies FcεRI-bound IgE. Shown in overlays are muMT (red) and WT (black) surface IgA values.

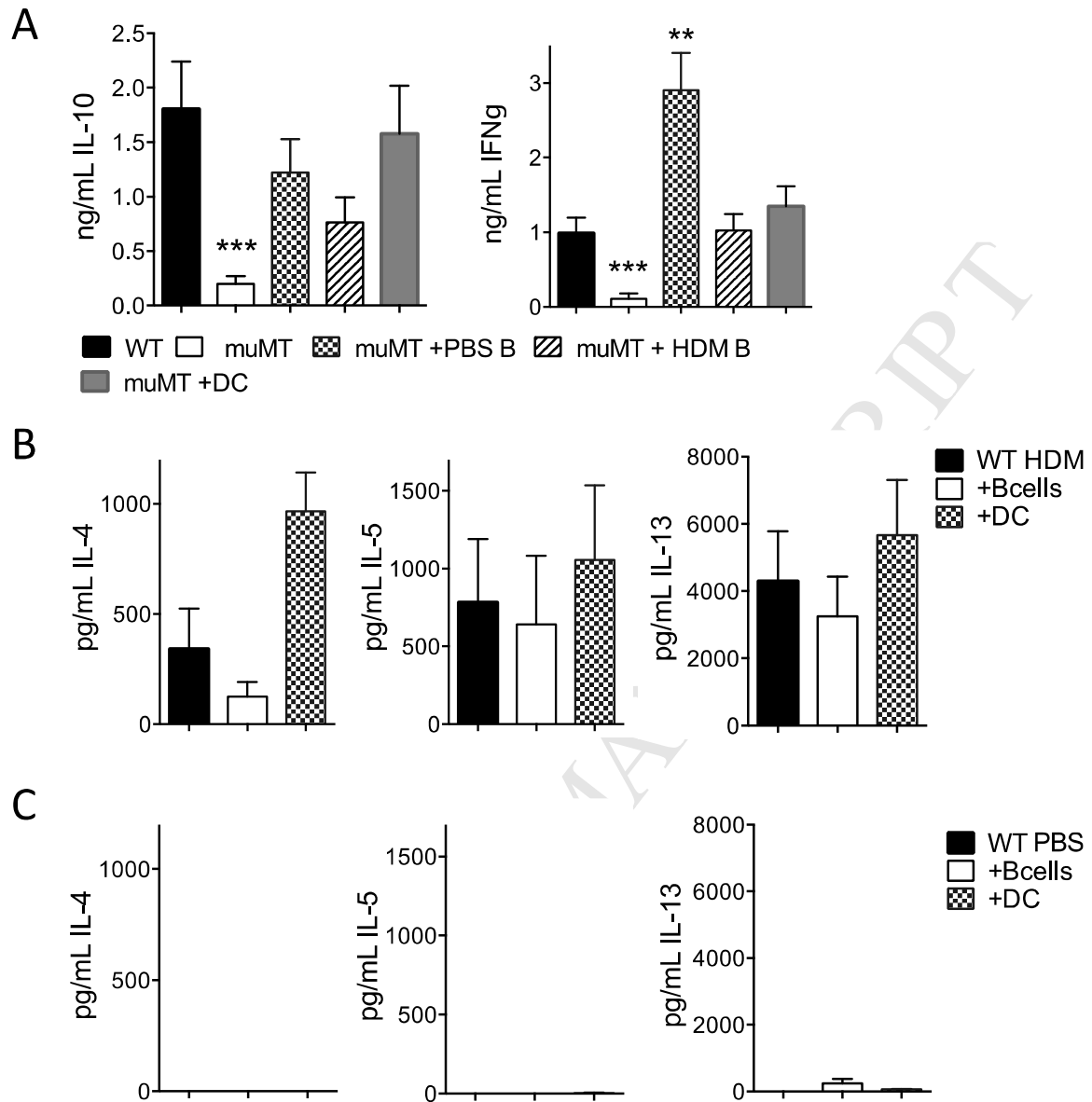


Figure E5: Supplementary results on the *in vitro* complementation of mLN cultures for Th2 cytokine production. WT and muMT mice received the full HDM asthma model as shown in figure 1A. On day 15, mLNs (2×10^5 /well) were *in vitro* restimulated with 15μg/mL HDM extract in the presence or absence of exogenous B cells or BM-DC. A. IL-10 and IFNγ produced by muMT MLNs supplemented with exogenous B cells or DC as indicated. B. Th2 cytokines produced by WT HDM treated MLNs (splenic naïve B cells). C. Th2 cytokines produced by WT PBS treated MLNs.

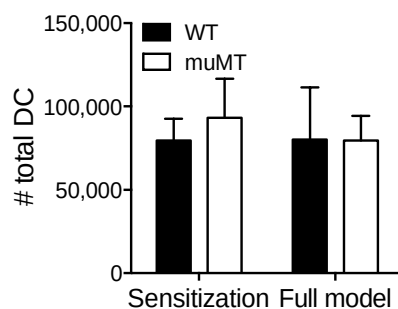


Figure E6: Total DC numbers in mLN after sensitization or the full model with 10 μ g HDM challenges. Numbers were obtained using manual Trypan Blue viability cell counting.

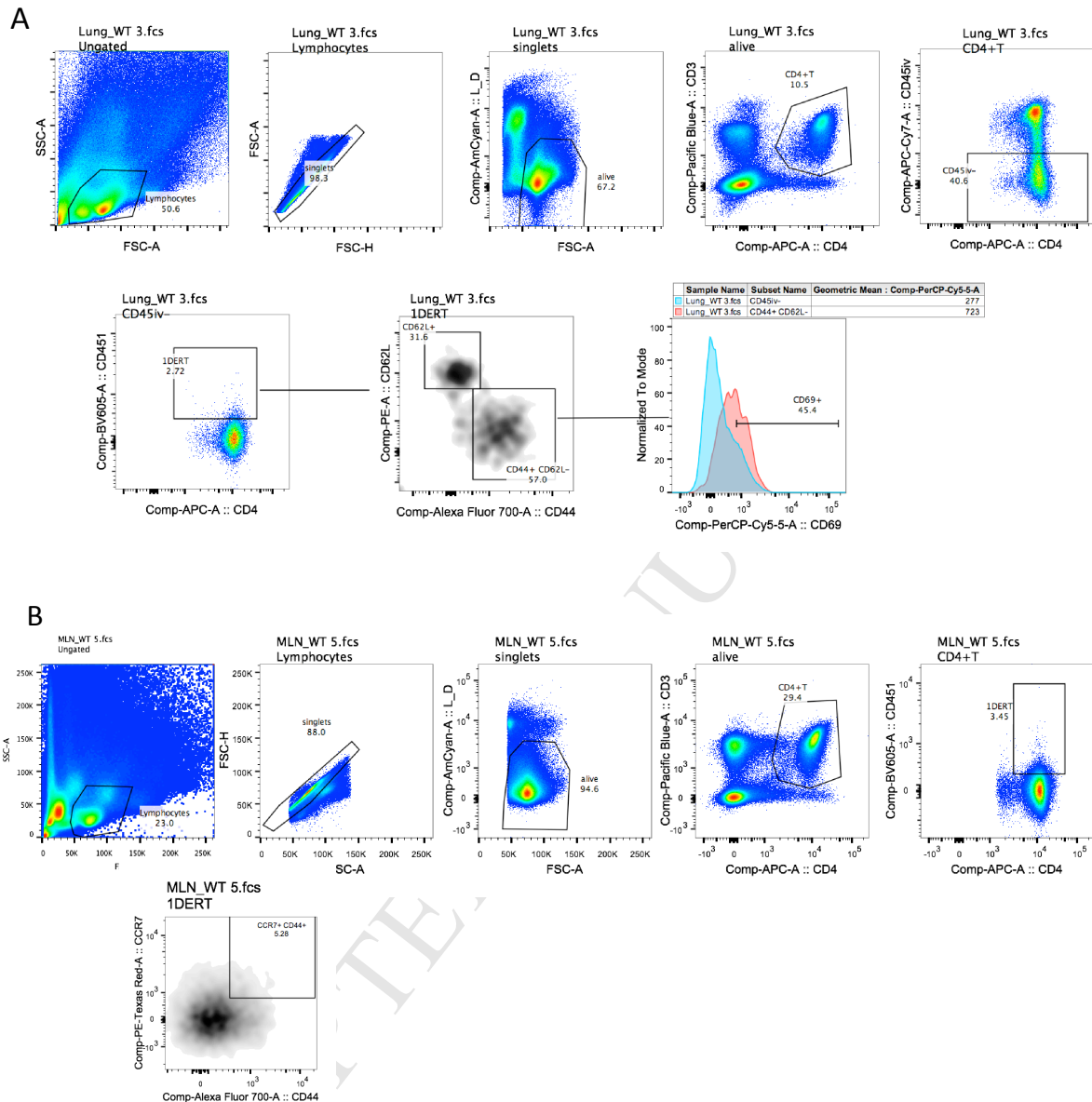


Figure E7: Gating strategy for 1-DER T cell memory subsets. Mice received anti-CD45 Ab iv 10 minutes prior to sacrifice. Organ single cell suspensions were stained for 1DER (CD45.1 congenic mark) and memory T cells markers. After exclusion of cellular debris, doublets and dead cells, 1DER T cells were gated as CD45.1+ CD3+CD4+.

A. Gating for lung Trm cells: Lung resident cells were gated by excluding CD45iv- cells. Within the CD44high population, CD69 expression was determined. CD69+ CD44high cells were defined as Trm.

B. Gating for MLN Tcm cell: within the 1-DER T cell gate, CCR7+CD44+ cells were defined as Tcm cells.

The endogenous CD4+ T cell population was used to position gates for the memory markers.