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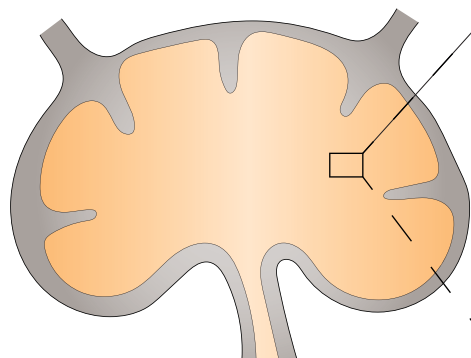
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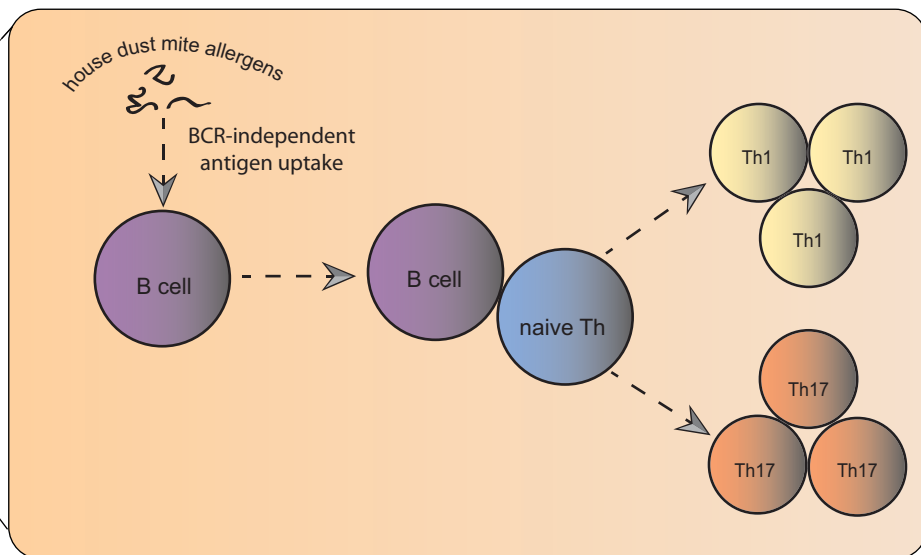
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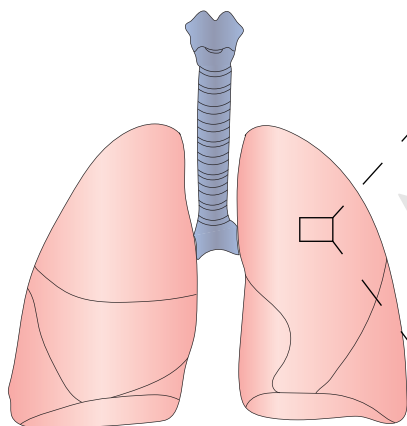
Primary response



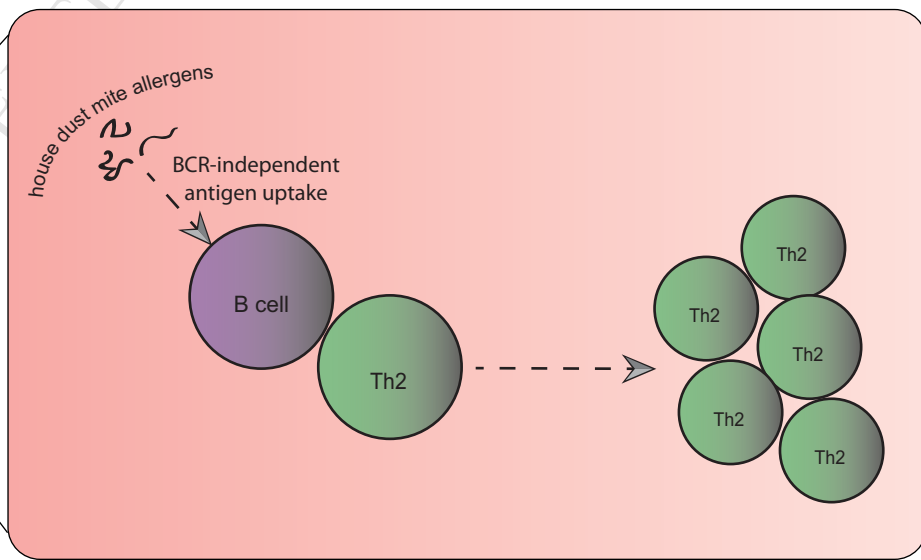
Mediastinal lymph node



Secondary response



Lung



Role of B cells in T helper cell responses in a mouse model of asthma

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Background: The importance of B lymphocytes to present antigens for antibody production is well documented. In contrast, very little is known about their capacity to influence CD4⁺ T cell activation during primary or secondary response to allergens.

Objective: Using mouse models of asthma, we investigated the role of B cells as antigen presenting cells in propagation and initiation of T helper cell responses.

Methods: Mice were immunized via intranasal route with house dust mite extract (HDM) derived from *Dermatophagoides pteronyssinus*. To investigate the importance of B cells in maintenance of allergic response, B cells were depleted in HDM-sensitized animals. To investigate the role of B cells in T cell priming, B cells were depleted before HDM sensitization; furthermore, HDM sensitization was performed in mice with MHC-II expression restricted to the B cell lineage.

Results: We found that B cells serve as potent antigen presenting cells *ex-vivo* and restimulate *in vivo*-primed HDM-specific T helper cells. HDM antigens were taken up by B cells independently of the B cell receptor specificity, indicating that HDM uptake and antigen presentation to CD4⁺ T cells is not restricted to rare B cells carrying HDM-specific B cell receptors. B cell depletion before HDM challenge in HDM-sensitized mice resulted in a dramatic reduction of allergic response, indicating the role of B cells in amplification of Th2 responses. In contrast, HDM sensitization of mice in which MHC-II expression was restricted to B cells revealed the inability of these cells to initiate Th2 responses, but highlighted their unexpected role in priming Th1 and Th17 responses.

Conclusion: Collectively, these data reveal new mechanisms leading to initiation and exacerbation of allergic response that may have implications for designing new therapeutic strategies to combat house dust mite allergy.

Key messages

- B cells acquire HDM antigens independently of their B cell receptor specificity and act as efficient APCs *ex-vivo*.
- B cells play an important role in amplification of Th2 responses *in vivo* and their depletion leads to reduction of allergic response.

- Although they are unable to initiate Th2 responses on their own, B cells efficiently prime naive CD4⁺ T cell precursors to differentiate into Th1 and Th17 subsets.

Capsule summary

B cells efficiently take up HDM antigens independently of their B cell receptor specificity and serve as potent antigen presenting cells, influencing both propagation and initiation of T helper cell response *in vivo*.

Keywords

Mouse model of asthma, house dust mite, B cells, antigen presentation, initiation of allergic response, maintenance of allergic response

Abbreviations

APC:	Antigen-presenting cell
BAL:	Bronchoalveolar lavage
BALF:	Bronchoalveolar lavage fluid
BCR:	B-cell receptor
CFSE:	Carboxyfluorescein succinimidyl ester
DC:	Dendritic cell
HDM:	House dust mite
HEL:	Hen egg lysozyme
IFN- γ :	Interferon gamma
Ig:	Immunoglobulin
ILC:	Innate lymphoid cell
LTC4:	Leukotriene C4 synthase
LTD4:	Leukotriene D4
LTE4:	Leukotriene E4
MLN:	Mediastinal lymph node
OVA:	Ovalbumin
PMA:	Phorbol-Myristate-Acetate
TLR4:	Toll-like receptor 4

TNF- α Tumor necrosis factor-alpha

Introduction

Allergy is a major problem in industrialized countries nowadays and its prevalence is on the rise. According to the World Health Organization, asthma is estimated to affect around 300 million people worldwide with 250,000 deaths per year due to the disease.¹ The hallmark of this disorder is a strong Th2 response with upregulated levels of IL-4, IL-5 and IL-13, which leads to enhanced IgE and IgG1 production and cell recruitment to the site of allergen entry. Antibodies of the IgE class, by binding to high affinity Fc ϵ RI on mast cells and basophils, cause immediate hypersensitivity reaction upon antigen re-exposure. This reaction is mediated by preformed mediators (e.g. histamine, serine proteases, carboxypeptidase A and proteoglycans), newly sensitized lipid mediators (e.g. LTC₄, LTD₄, LTE₄) and cytokines/chemokines,² that act in concert with responses by other cell types, and lead to exaggerated immune response and tissue damage.

The majority of mouse studies concerning allergic responses have employed ovalbumin (OVA) as a model antigen. The main advantages of these approaches came with development of TCR transgenic OT-I and OT-II mice which carried OVA-specific CD8⁺ and CD4⁺ T cells, respectively. These tools opened several new avenues to explore immune responses such as tracking of OVA-specific T cell migration or fate decisions. Also, using OVA narrowed immune responses to a single antigen and allowed easy isolation of high numbers of antigen-specific cells, creating a “neat” system to interpret experimental data. Early work using the OVA model provided evidence that B cells are not necessary for development of allergy³⁻⁵ and subsequent work showed that dendritic cells (DCs) play a non-redundant role in maintenance of allergic responses *in vivo*.⁶ However, although allergies to OVA may occur in real life, they are far rarer than allergies to other environmental antigens, such as birch pollen, cockroach or house dust mites. In contrast to these physiologically relevant allergens, OVA is a single protein, does not display enzymatic activity and does not carry microbial load. Therefore, B cell responses to OVA may not reflect those to complex, clinically relevant allergens. For these reasons, studies using the latter have recently started to emerge leading to important insights into the mechanisms

behind allergic inflammation. For example, using house dust mite extracts (HDM), central roles for epithelial cells, pulmonary DCs and ILC2 cells in orchestrating allergic sensitization have been unraveled.⁷ The role of B cells in HDM-induced responses has been also re-evaluated.⁸⁻¹⁰ However, while some studies indicate that B cells are necessary during HDM sensitization but dispensable during challenge,⁹ others indicate that B cells are dispensable for T cell priming but necessary for effector CD4⁺ T cell expansion.¹⁰

In this study, we evaluated the role of B cells as antigen presenting cells (APCs) in primary and recall responses using experimental models of HDM-induced allergy. We found that in contrast to the OVA model, B cells display unique capacity to take up HDM antigens *in vivo* independently of their B-cell receptor (BCR) specificity. Also, they acted as potent antigen presenting cells (APCs) *ex-vivo* and efficiently restimulated *in vivo*-primed HDM-specific effector/memory T helper cells. By depleting B cells before HDM challenge in HDM-sensitized mice, we observed a pronounced reduction in the allergic response, manifested by reduced numbers of effector/memory T helper cells, eosinophils and neutrophils in the lungs as well as impaired capacity of effector/memory T helper cells in lungs to secrete Th2-associated cytokines upon *ex-vivo* restimulation. By depleting B cells before HDM sensitization, we observed that they could induce Th1 rather than Th2 responses. Finally, with the use of a mouse strain in which MHC-II expression was restricted to the B cell lineage, we provide evidence that B cells are capable of initiating Th1 and Th17 but not Th2 responses against HDM *in vivo*. Overall, our data are consistent with previous reports underlining the importance of B cells in driving allergic inflammation. Additionally, we provide new evidence on the dual role of B cells depending on the stage of allergic reaction, which may help explain some of the controversies in the field.

Methods

Mice. C57BL/6 and BALB/c inbred mice (WT, Wild type) were purchased from Harlan Italy Srl and Envigo RMS Srl, respectively. B-MHC-II and Flox mice were provided by Dr. Gregory Wu from Washington University, St. Louis, USA and have previously been described.²⁰ MD4 (002595) mice were obtained from The Jackson Laboratory. All mice were bred and maintained under specific pathogen-free conditions. Mice of female gender at the age of 6-8 weeks were

used for experiments. They were treated in accordance with guidelines of the Swiss Federal Veterinary Office and the experiments were approved by the Dipartimento della sanità e della socialità of Canton Ticino.

Antigens and immunizations. House dust mite (HDM) extract from *Dermatophagoides pteronyssinus* (Greer Laboratories) and endotoxin-free ovalbumin (OVA) protein (Hyglos) were resuspended in phosphate-buffered saline (Sigma-Aldrich). Low molecular weight compounds, such as peptides were excluded from the HDM extract with the use of PD-10 desalting columns (GE Healthcare). Before intranasal administration, mice were anesthetized with isoflurane (4% in air) for 5 minutes and treated with 20 µg of HDM resuspended in 20 µl PBS. As a negative control, 20 µl of PBS was administered. Solutions were applied on day 0, 7, 8, 9, 10, 11 and mice sacrificed on day 14. Alternatively, mice were immunized on days 0, 11, 12, 13 and sacrificed on day 14. To study priming of T cell responses, 100 µg of HDM in 33 µl of PBS was used. As a negative control, 33 µl of PBS was applied. Mice were immunized on days 0 and 1 and sacrificed on day 7.

In some experiments, monoclonal antibody (clone 18B12) against murine CD20 was introduced (250 µg intravenously + 130 µg intranasally) two days before or nine days after HDM sensitization in order to deplete B lymphocytes. As a control, isotype matched control antibody against human CD20 (clone 2B8) was administered in the same manner. In some experiments, HDM or OVA proteins were labeled with the use of AF647 labeling kit (Invitrogen), eluted with PBS and administered intranasally at the dose of 20 micrograms.

CD4⁺ T cell transfer. Spleens and mesenteric lymph nodes were collected from naive WT C57BL/6 mice and smashed through the 40 µm nylon cell strainer (Falcon) to obtain a homogenous suspension. CD4⁺ T cells were isolated with the use of CD4⁺ T cell isolation kit (Miltenyi Biotec) according to manufacturer's instructions. Purity of cells was confirmed by flow cytometry and was always >97%. 10⁷ cells were injected intravenously into Flox and B-MHC-II mice 15 days prior to HDM immunization in order to reconstitute CD4⁺ T cell compartment.

Bronchoalveolar lavage fluid, lungs and mediastinal lymph nodes collection.

Bronchoalveolar lavage (BAL) for cytokine measurement was performed using 1 ml of PBS. BAL fluid was spun down, supernatant collected and stored at -20 °C until further processing. Lungs were perfused with 10 ml of PBS before collection, finely cut with scissors and digested for 1 hour at 37 °C in a solution of Collagenase D (Sigma Aldrich) at the concentration of 2 mg/ml and DNase I (Sigma Aldrich) at the concentration of 0.1 mg/ml in PBS. This was followed by smashing of lung pieces through 40 µm nylon cell strainer. Cell suspensions were washed twice with MACS buffer before downstream applications. Mediastinal lymph nodes were collected, smashed through 40 µm nylon cell strainer, washed once with MACS buffer and used for downstream applications.

Cell sorting. For sorting of lung CD4⁺ T cells, B cells and dendritic cells, lung cell suspensions were incubated with anti-CD4 microbeads (clone: L3T4, Miltenyi Biotec), anti-CD19 beads (Miltenyi Biotec) and anti-CD11c microbeads (Miltenyi Biotec) and isolated using LS columns (Miltenyi Biotec) according to manufacturer's guidelines. This was followed by sorting on a FACSaria III cell sorter. Activated CD4⁺ T cells were sorted as CD4⁺ CD44⁺ CD11c⁻ SiglecF⁻. Lung B cells were sorted as CD19⁺ B220^{high} CD11c⁻ CD4⁻. Dendritic cells were sorted as CD11c⁺ SiglecF⁻ CD4⁻. For sorting cells from mediastinal lymph nodes (MLNs), cell suspensions were directly stained with antibody cocktail and sorted on a FACSaria III cell sorter. Total CD4⁺ T cells were sorted as CD4⁺ B220⁻ CD8⁻ CD11c⁻ while DCs as CD11c⁺ B220⁻ CD8⁻ CD4⁻.

Flow cytometry. The following antibodies or streptavidin, coupled to biotin, PerCP-Cy5.5, FITC, Alexa Fluor (AF) 488, APC, AF647, Pacific Blue, Pacific Orange, APCCy7, PE, PECy7 were purchased from Biolegend, eBioscience, BD Biosciences or Invitrogen: CD19 (clone: 6D5), B220 (RA3-6B2), CD3 (145-2C11), CD4 (RM4-5), CD11c (N418), CD8α (53-6.7), CD45.1 (A20), CD45.2 (104), CD44 (IM7), CXCR5 (2G8), MHC-II (M5/114.15.2), SiglecF (E50-2440), CD11b (M1/70), CCR3 (TG14/CCR3), Gr-1 (RB6-8C5), CD80 (16-10A1), CD86 (GL1), CD40 (3/23), OX40L (RM134L), CD43 (S7), CD5 (53-7.3), IgM (R6-60.2), IgD (11-26c). Dead cells were excluded with 7-AAD (eBioscience). Flow cytometry was performed either on a FACSCanto II or BD Fortessa (BD Biosciences) and data analyzed using FlowJo software (Tree Star).

Ex-vivo experiments. For evaluation of B cell capacity to restimulate *in vivo* primed T cells, 20 000 lung CD4⁺ CD44⁺ T cells were co-cultured with 20 000 antigen presenting cells in the presence or absence of HDM at the concentration of 40 µg/ml or in the presence of titrated doses of HDM (40 µg/ml, 13.3 µg/ml, 4.44 µg/ml, 1.48 µg/ml). In experiments evaluating capacity of B cells to restimulate *in vivo* primed T cells in absence of exogenous antigens, 100 000 lung B cells from HDM-immunized mice or 100 000 lung B cells from naive mice were co-cultured with 100 000 lung CD4⁺ CD44⁺ T cells from HDM-immunized mice. In experiments employing B cell depletion before challenge, 500 000 total lung cells were plated in the presence of HDM (40 µg/ml) for 4 days. In the same experiments, 10 000 lung CD4⁺ CD44⁺ T cells were co-cultured with 10 000 lung DCs in the presence of HDM (40 µg/ml). In experiments addressing priming of CD4⁺ T cells, 10 000 CD4⁺ T cells from mediastinal lymph nodes or 10 000 lung CD4⁺ T cells were co-cultured with 3000 MLN DCs or lung DCs, respectively. In some experiments, T cells were labeled with CFSE before plating. CFSE (Thermo Fisher) labeling was performed at the concentration of 2µM in 1% fetal bovine serum (Gibco®, Thermo Fisher) for 7 minutes at 37°C. Cells were subsequently washed twice and plated. In each *ex vivo* experiment, cells were washed and cultured in RPMI medium supplemented with 10% fetal bovine serum (Gibco®, Thermo Fisher), 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin, 2 mM glutamine (all from Life Technologies) and 0.1% 2-mercaptoethanol (LuBio Science). All cultures were incubated in a 96-well round bottom plates (Costar) at 37°C in 5% CO₂ for 4 days and the supernatant was collected for analysis. In some experiments, proliferation of T cells was assessed on a FACSCanto II or BD Fortessa based on CFSE dilution.

Cytokine and immunoglobulin measurement. Cytokine measurements were performed with the use of Mouse Th1/Th2/Th17/Th22 13plex Multiplex (eBioscience). Alternatively, ready-set-go kits for IL-4, IL-5, IL-10, IL-13, IL-17 and IFN-γ (eBioscience) were used. In all cases, protocols were according to manufacturer's guidelines. HDM-specific immunoglobulin levels were measured by ELISA. 96-well half-area plates (Costar) were coated with 10 µg/ml HDM in PBS overnight at 4 °C. Plates were blocked for 2 hours at RT in 1% BSA (Sigma Aldrich) in PBS and serial dilutions of sera incubated for 2 hours at RT. Alkaline-phosphate-labeled

anti-mouse IgE (Southern Biotech) were incubated for 1 hour at RT followed by addition of pNPP substrate (Sigma Aldrich). Absorbance was measured at 405 nm wavelength with the use of microplate reader (Molecular Devices).

Ovalbumin (OVA) model of asthma. Protocol of OVA-induced asthma was adapted from Swedin et al., 2010.¹⁵ Briefly, mice were sensitized on days 0 and 7 via intraperitoneal injection of 10 µg OVA protein in 1 mg of ImjectAlum (Thermo Fisher) and challenged with 20 µg OVA protein in PBS on days 14, 15 and 16. Mice were sacrificed for experiment on day 17.

Statistics. Data were plotted and analyzed with Prism 6 (Graphpad software). When comparing two groups, unpaired two-tailed Student t-test (for data displaying Gaussian distribution) or Mann-Whitney test (non-Gaussian distribution) was used. When comparing more than two groups, One-Way ANOVA (Gaussian distribution) or Kruskal-Wallis test (non-Gaussian distribution) with correction for multiple comparisons (Tukey test) was employed. Data distribution was assessed by D'Agostino-Pearson omnibus normality test. Graphs show the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Results

B cells are present in the lungs at the steady state and after HDM immunization and outnumber dendritic cells

In this study we used a mouse model of allergic sensitization whereby BALB/c mice are repeatedly exposed to allergens administered intranasally. To analyze abundance of B lymphocyte population in the lungs at steady state and upon allergen sensitization, mice were treated with PBS or HDM on days 0, 7, 8, 9, 10 and 11 and sacrificed on day 14 (referred hereafter as HDM-sensitized mice and PBS-treated mice). Lungs were collected and processed for flow cytometric analysis. B cells constituted 20-25% of CD45⁺ cells in the lungs of both groups of mice (Fig. 1A,B). Upon HDM sensitization, the total number of cells in the lungs increased and so did the number of B cells (Fig. 1B), consistent with other models of allergic inflammation.¹⁰⁻¹² Importantly, both in PBS-treated and in HDM-sensitized mice, B cells

outnumbered DCs. The majority of B cells present in the lungs were CD19⁺CD43⁻ B-2 cells, while CD19^{high} CD43⁺ B-1 cells¹³ constituted around 8% of total B cells (Fig. 1C,D). Interestingly, most B-2 cells were of naive phenotype (IgD⁺ IgM^{lo}), both in PBS-treated mice and in mice sensitized with HDM (Fig. 1E,F). Consistent with their localization in non-lymphoid tissue, lung B cells expressed reduced levels of CXCR5 compared to circulating, splenic or mediastinal lymph node B cells (Fig. 1G,H).

Lung B cells efficiently capture HDM antigens *in vivo* independently of their BCR specificity

To assess whether B cells residing in the lungs could efficiently capture HDM antigens, we sensitized mice with HDM on days 0, 11, 12 and challenged them on day 13 with fluorescently labeled HDM (HDM-AF647). Twenty-four hours after challenge, 3.4-5.2% of lung B cells stained positive for HDM-AF647 (Fig. 2A,B). In the same samples, a large proportion of lung DCs (7.4-21.1%) but only few lung CD4⁺ T cells (0.3-1.5%) stained positive. Given the high abundance of B cells in the lungs, the total numbers of HDM-AF647⁺ B cells and HDM-AF647⁺ DCs were comparable. Uptake of HDM was observed also in B cells from MD4 mice which carries rearranged heavy and light chain transgenes encoding for a HEL-specific monoclonal antibody¹⁴ (Fig. 2C,D), indicating that this process did not rely on the BCR specificity. However, lung B cells from BALB/c mice were less efficient in taking up fluorescently-labeled OVA (OVA-AF647) compared to lung DCs, even when co-injected with unlabeled HDM (Fig. 2E-G), indicating that efficient antigen internalization by B cells is a property characteristic of the antigenic features of HDM extract but not of OVA.

B cells serve as potent antigen presenting cells *ex vivo*

Given the efficiency of B cells in HDM uptake, we investigated if they could serve as antigen presenting cells for effector/memory T cells. Mice were sensitized with HDM on days 0, 7, 8, 9, 10, 11 and lungs were collected on day 14. Lung CD4⁺ CD44⁺ T cells were isolated and cultured with lung B cells (HDM B cells) or lung DCs (HDM DC) from HDM-sensitized mice, or splenic B cells from PBS-treated mice (CTRL B cells), in absence or presence of HDM extract. In these experiments we excluded the potentially confounding presence of peptides in the HDM extract via size exclusion chromatography. Interestingly, B cells from different sources were equally

efficient in presenting HDM antigens and in inducing cytokine production by CD4⁺ CD44⁺ T cells, and their efficacy was similar (in the case of IL-4) or even higher (in the case of IL-5 and IL-13) than that of lung DCs (Fig. 3A). However, in contrast to DCs, B cells were unable to restimulate effector CD4⁺ T cells to produce IL-17 (Fig. 3A).

To better evaluate the efficiency of B cells in HDM antigen presentation, we set up co-cultures using different concentrations of HDM. Similarly as in figure 3A, B cells from lungs of HDM-sensitized mice and lungs or spleen of PBS-treated mice were equally efficient in presenting HDM antigens to CD4⁺ CD44⁺ T cells, and their efficacy was similar (in the case of IL-4) or higher (in the case of IL-5 and IL-13) than that of splenic DCs. However, they were unable to restimulate T cells to produce IL-17 and less efficient to restimulate T cells to produce IFN- γ (Fig. 3B).

We then asked whether lung B cells from HDM-sensitized mice capture enough antigen *in vivo* for presentation to CD4⁺ CD44⁺ T cells. Mice were sensitized with HDM on days 0, 11, 12, 13, total lung cells were isolated on day 14 and co-cultured with HDM-primed CFSE-labeled CD4⁺ CD44⁺ T cells in absence of exogenous antigen. As shown in figure 3C,D, lung B cells from HDM-immunized mice were able to induce proliferation and cytokine production by CD4⁺ CD44⁺ T cells, while B cells from PBS-treated mice were not. These data indicate that lung B cells captured sufficient amounts of HDM antigens *in vivo* to be able to restimulate *in vivo*-primed CD4⁺ CD44⁺ T cells.

Finally, to ask whether these potent antigen presenting capacities are restricted to HDM, we employed a well-established OVA model of allergic asthma.¹⁵ Mice were sensitized with OVA/Alum via intraperitoneal injection on days 0 and 7, challenged intranasally with OVA protein on days 14, 15 and 16 and analyzed on day 17. As depicted in figure 3E, both lung and splenic B cells were far less efficient than DCs in restimulating *in vivo* primed CD4⁺ CD44⁺ T cells in the OVA model. This data indicates that efficacy of B cells in restimulating *in vivo* primed CD4⁺ CD44⁺ T cells is a property characteristic of HDM but not OVA model of allergic asthma.

Lung B cells upregulate MHC-II and CD86 upon HDM immunization

Because B cells displayed efficient antigen presenting capacity *ex vivo*, we wondered if they upregulate MHC-II and co-stimulatory molecules expression upon HDM treatment *in vivo*. Upon

HDM sensitization (days 0, 11, 12, 13), around one third of total lung B cells upregulated their MHC-II expression (Fig. 4A,B). Furthermore, these MHC-II^{hi} B cells had significantly higher levels of CD86, but not of CD80, OX40L or CD40, compared to MHC-II^{int} B cells from PBS or HDM-treated mice (Fig. 4B). Intriguingly, HDM treatment reduced the surface levels of ICOSL on lung B cells (Fig. 4B).

Since HDM sensitization results in potent Th2 response, we wondered if lung B cells can directly contribute to Th2 cytokine milieu in our model. B cells have been shown to produce cytokines in various experimental models. Based on their cytokine secretion profile, they are classified into three main subsets: regulatory B cells (secreting IL-10 and TGF- β), Be1 cells (secreting IFN- γ , IL-12 and TNF- α) and Be2 cells (secreting IL-4, IL-6, IL-13 and TNF- α).¹⁶ To assess cytokine production by B cells from HDM-sensitized mice, we restimulated total lung cells with PMA/Ionomycin or HDM extract for 6 hours, and analyzed cytokine production by intracellular cytokine staining on CD19⁺ B220⁺ B cells by flow cytometry. As summarized in figure 4C, lung B cells were unable to produce IL-4, IL-6, IL-13 or IL-10. To study the cytokine production after longer restimulation period, CD19⁺ B220⁺ B cells were sorted from lungs of PBS-treated or HDM-sensitized mice and restimulated with HDM, PMA/Ionomycin or α -IgM/CpG for 3 days. Consistent with the intracellular cytokine staining, lung B cells from HDM-immunized mice were unable to secrete IL-4, IL-6, IL-13 or IL-10. Collectively, these results demonstrate that lung B cells do not differentiate into Be2 subset upon HDM sensitization and do not directly contribute to Th2 cytokine milieu in the lung tissue. Also, they did not secrete non Th2-related cytokines in our model, such as GM-CSF as has been shown for the innate response activator (IRA) B cells¹⁷ (data not shown).

We next assessed if B cells contribute to HDM inflammation by secreting elevated levels of HDM-specific IgE or IgG. We therefore treated mice intranasally with PBS or HDM on days 0, 11, 12 and 13 and isolated plasma on day 14. We found no upregulation of HDM-specific IgE at this time point (Fig. 4D), which is consistent with data obtained by others^{18,19} and most likely reflects relatively short period of immunization. Similarly to IgE, we found no upregulation of HDM-specific IgG1 or IgG in our model.

Taken together, we conclude that in the model used lung B cells upregulate MHC-II and CD86 upon HDM treatment but their production of either cytokines or HDM-specific IgE is below the detection limit.

Depletion of B cells before challenge reduces the allergic response

To investigate the contribution of B cells to allergic response in the challenge phase of HDM immunization, we depleted B cells in mice previously sensitized to HDM using α -CD20 antibody. In these experiments, all mice were treated with HDM intranasally on day 0 and either treated with α -CD20 antibody on day 9, and challenged intranasally with HDM on days 11, 12 and 13 (α -CD20-HDM group), or treated with isotype control antibody (Ig Ctrl) on day 9, followed by challenges with HDM (Ig Ctrl-HDM group) or PBS (Ig Ctrl-PBS group) (Fig. 5A). α -CD20 treatment dramatically reduced numbers of B cells in the lungs (Fig. 5B). Enumeration of the lung immune infiltrate showed that mice in the α -CD20-HDM group had fewer CD4⁺ CD44⁺ T cells, eosinophils and neutrophils compared to Ig Ctrl-HDM group, which reflected reduced allergic inflammation in the tissue (Fig. 5B). We then sorted CD4⁺ CD44⁺ T cells and plated them in a co-culture with HDM-pulsed DCs. CD4⁺ CD44⁺ T cells from the α -CD20 group secreted reduced levels of Th2 cytokines compared to their Ig Ctrl counterparts. Importantly, their response was not skewed into a different subset, since these cells secreted lower amounts of IL-10 and IFN- γ , while IL-17 production was not significantly altered (Fig. 5C). Finally, α -CD20 treatment resulted in reduced concentrations of cytokines, particularly IL-4 and IL-13 in the bronchoalveolar lavage fluid (BALF) (Fig. 5D).

The strain of mice used in these experiments (BALB/c) is prone to develop strong Th2 response unlike another common laboratory strain, C57BL/6. In order to investigate if perpetuation of allergic inflammation by B cells is a feature restricted to allergy prone strains, we repeated the experiments employing C57BL/6 mice. Interestingly, we obtained similar results as in the case of BALB/c mice. Numbers of effector/memory CD4⁺ T cells, eosinophils and neutrophils were reduced upon α -CD20 treatment (Fig. 6A). Also, CD4⁺ CD44⁺ T cell response after α -CD20 treatment was impaired in a similar manner as in BALB/c mice. We observed reduced production of Th2 cytokines (IL-4, IL-5 and IL-13) after 4 days of co-culture with DCs in the presence of HDM. Also, there were lower levels of IL-10 and IFN- γ , while the levels of IL-17 were not changed (Fig. 6B).

Depletion of B cells before priming results in reduced capacity of CD4⁺ T cells to produce IFN- γ but not IL-4 or IL-13

In order to evaluate the importance of B cells in shaping CD4⁺ T cell responses at the time of priming, we set up a protocol to deplete B cells before sensitization with HDM. For this, mice received α -CD20 or Ig Ctrl antibody two days before HDM administration. On day 7 post-immunization, we collected their lungs and evaluated the numbers of eosinophils, neutrophils and effector T helper cells (experimental set-up shown in figure 7A). As depicted in figure 7B, anti-CD20 treatment resulted in decreased numbers of eosinophils and neutrophils but not of effector T helper cells in the lung tissue. Additionally, we collected mediastinal lymph nodes and sorted CD4⁺ T cells and DCs. CD4⁺ T cells were labeled with CFSE and co-cultured with DCs in the presence of HDM (Fig. 7A); on day 4 proliferation was assessed by CFSE dilution. As shown in figure 7C, B cell depletion before priming did not impair the ability of CD4⁺ T cells to proliferate upon *ex-vivo* restimulation. Also, secretion of Th2 cytokines (IL-4 and IL-13) was not changed (Fig. 7D). However, B cell depletion before priming resulted in reduced ability of CD4⁺ T cells to produce IFN- γ . Collectively, these results demonstrate a dual feature of B cells in this model. On one hand, they can induce granulocyte recruitment into the lung tissue and priming of Th1 responses but on the other, they are dispensable for priming of Th2 responses and recruitment/proliferation of effector T helper cells.

B cells are able to initiate CD4⁺ T cell responses upon HDM priming

Next, we asked whether B cells are sufficient to prime naive T cells and to induce Th1 differentiation or other responses, such as Th17 or Treg. To address this question, we took advantage of a mouse strain in which MHC-II expression is restricted to the B cell lineage (B-MHC-II).²⁰ The parental strain of these mice carries a floxed polyadenylation stop sequence in the IA β locus, making this strain MHC-II deficient. However, by crossing it to CD19-cre mice, it is possible to restore MHC-II expression in B cells and obtain B-MHC-II strain after the second generation. Because of lack of MHC class II expression in thymic epithelial cells, both flox mice and B-MHC-II mice have a severe reduction in peripheral CD4⁺ T cells. In order to correct for this, we first restored the CD4⁺ T cell compartment in flox mice and B-MHC-II mice by injecting 10⁷ polyclonal CD4⁺ T cells from naive WT C57BL/6 mice, as previously described²¹ (Fig. 8A). Flox and B-MHC-II mice were sensitized by intranasal administration of HDM on day 0 and 1. CD4⁺ T cells were isolated from mediastinal lymph nodes (MLNs) and lungs on day 7, labeled with CFSE and co-cultured with WT DCs from PBS-treated mice in the presence of

HDM for four days. As depicted in figure 8B, MLN and lung CD4⁺ T cells from B-MHC-II mice proliferated extensively in response to HDM restimulation *ex-vivo*. Despite this, they were unable to secrete Th2 associated cytokines (IL-4, IL-5 and IL-13). However, they produced high levels of IL-17 and IFN- γ (Fig. 8C).

Collectively, these data indicate that B cells are able to prime HDM-specific naïve CD4⁺ T cells *in vivo*. However, they are unable to induce Th2 differentiation but instead, prime naïve T cells to become IL-17 and IFN- γ producing cells.

Discussion

The field of antigen presentation in initiation and propagation of allergic responses has been dominated by DCs. It is now generally believed that this cell type plays a non-redundant role in this process.²² However, this conclusion is largely based on the ovalbumin models of allergy,³⁻⁶ which may not reflect responses to complex, physiologically relevant allergens. Using the latter, recent studies have pointed towards the role of B cells as APCs in allergic responses.^{9,10} Also, the cross-talk between B cells and DCs in helminth infections has recently been highlighted.⁸

In this study, using HDM model of asthma, we have shown that in contrast to the OVA model, B cells efficiently take up antigens *in vivo* and serve as potent antigen presenting cells *ex-vivo*. This is in line with a recent report showing that B cells can stimulate Der p 1-specific T helper cells to produce IL-5 and IL-13 *in vitro*.¹⁰

Interestingly, the ability of B cells to acquire HDM antigens did not rely on the specificity of the B cell receptor since B cells with irrelevant surface Ig were equally efficient. Similar results have been recently obtained using papain.²³ This is somewhat surprising because only antigen-specific B cells are traditionally considered potent at antigen internalization. The mechanisms behind the efficient HDM uptake by B cells remain to be determined but it is possible that internalization requires binding of one or more HDM antigens to a surface receptor.

The most striking observation of our study was that depletion of B cells before challenge had pronounced effects on reduction of allergic response. These included reduced numbers of effector/memory T helper cells, eosinophils and neutrophils as well as impaired capacity of CD4⁺ CD44⁺ T cells to secrete cytokines upon *ex-vivo* restimulation. Importantly, the effects of

B cell depletion were observed in two different laboratory mouse strains: BALB/c and C57BL/6. This is in line with a conclusion reached in a recent paper by Dullaers and colleagues where they pointed to a crucial role for B cells in optimal proliferation and activation of Th2 cells in the mediastinal lymph nodes.¹⁰ Given the fact that B cells produce neither cytokines nor HDM-specific IgE in our model but act as efficient APCs, it is most likely that it is abrogation of sufficient T helper cell stimulation by antigen presenting B cells which confers the observed effects. Nevertheless, existence of other mechanisms cannot be excluded. For example, the cross-talk between B cells and DCs in the induction of Th2 responses upon nematode infection has previously been reported.²⁴ In this model, secretion of lymphotoxin by B cells drove CXCL13 expression in the mediastinal lymph nodes and controlled co-localization of B cells and CXCR5⁺ DCs. Importantly, its disruption led to a reduced development of IL-4-producing T cells. Whether similar cross-talk between B cells and DCs or B cells and other cell types in the lungs could play a role in our model of asthma remains to be investigated.

Depletion of B cells employed in the above experiments is reminiscent of the B cell depletion therapy used in human patients suffering from various cancers and autoimmune disorders. The rationale behind the use of α -CD20 therapy in the treatment of autoimmune diseases has been the role B cells play in the production of autoantibodies. However, in some cases clinical improvement did not correlate with decreased autoantibody levels, pointing to the existence of additional factors playing a role in the therapy.²⁵⁻²⁸ For example, a study was conducted on atopic eczema patients who had elevated levels of immunoglobulin E specific to various allergens, including birch and grass pollens, cat dander and house dust mites. All of the patients receiving α -CD20 therapy showed a remarkable improvement by 4 to 8 weeks post treatment, which among others, included reduced expression levels of IL-5 and IL-13 in CD4⁺ T cells. Importantly however, the levels of allergen-specific IgE were not changed.²⁸ This example highlights that the mechanism unraveled in our work may also be relevant for human patients suffering from house dust mite allergies.

The conclusion that B cell depletion strongly reduces ongoing allergic inflammation may be somewhat surprising since it has been proposed that DCs are necessary and sufficient to promote ongoing allergic inflammation.⁶ In this study, authors employed diphtheria toxin-based depletion of CD11c⁺ cells specifically before the challenge phase of immunization. However, in order to study allergic inflammation, they used ovalbumin model of asthma which, as underlined

throughout our study, greatly differs from the HDM model. This drawback of above work has been corrected by Plantinga and colleagues who employed HDM model of asthma to study the role of DCs in initiation and maintenance of Th2 immunity.²⁹ In this study, authors sensitized and challenged *Flt3l*-deficient mice, which show a strong reduction of cDC numbers. Interestingly, they observed lower numbers of eosinophils, lymphocytes and neutrophils in the BALF upon HDM sensitization and 5 consecutive challenges. However, this was only the case in a low dose model of HDM immunization (sensitization with 1µg HDM, challenges with 10µg HDM) but not in the high dose model (sensitization with 100 µg HDM, challenges with 100 µg HDM), indicating that the cDC requirement may depend on the dose of antigen used. Unfortunately, the capacity of total lung cells or CD4⁺ T cells to produce cytokines was not reported. Therefore, the direct evidence for the importance of DCs for T helper cell responses is missing.

The main caveat of the above study is usage of gene knockout mice to investigate responses during the challenge phase of immunization. Based on this approach, it is impossible to conclude whether the priming or the challenge phase of the response had been affected. It may very well be that it is differentiation of Th2 cells from naive precursors which had been impaired and this is the reason why influx of effector cells was impaired after the challenge. Hence, the conclusion about the role of DCs specifically in the secondary response cannot be reached. This issue should definitely be clarified.

Our data showing reduced allergic response upon B cell depletion before HDM challenge but not before sensitization are in contrast with findings from Ballesteros-Tato and colleagues, who showed that B cells are necessary for the development of allergic inflammation during the sensitization phase, but are dispensable during the challenge phase of the response.⁹ The reasons for this discrepancy is not clear, although it may be due to differences in the experimental approach. B cell-deficient mice (µMT) may differ compared to B cell-depleted WT mice in terms of organization and cellular composition of lymphoid organs and/or lungs and in T cell function and distribution³⁰⁻³². Furthermore, antibody deficiency in µMT mice might have shaped composition of intestinal and lung microbiota in a different way than in WT animals, which might result in major physiological differences.³³ Alternative explanations include different immunization protocols used and/or composition of house dust mite extract.

Finally, our study revealed an unexpected capacity of B cells to initiate CD4⁺ T cell responses from naive precursors. The experimental approach relied on a mouse strain in which MHC-II expression is restricted to the B cell lineage. This approach offers some advantages compared to others. It avoided the use of BCR-transgenic mice or antigen specific B cell transfer into naive hosts,^{34,35} avoiding a non-physiological excess of Ag-specific B cells at the time of priming. It also allowed us to directly assess whether B cells can prime CD4⁺ T cell responses from naive precursors or only expand DC-primed effector CD4⁺ T cells still residing in the lymph node. Finally, it enabled us to dissect the capacity of B cells to prime T helper cell responses from their necessity to do so, since redundancy in antigen presentation was not a relevant factor in this system. These two last issues are in contrast to studies employing B cell deficient mice or mice with MHC-II expression abrogated specifically in the B cell lineage.^{3-5, 36-39}

Our conclusion that B cells may initiate priming of CD4⁺ T cell responses in a system where clonality of B cells is not artificially skewed most likely derives from the fact that B cells do not have to rely on rare antigen-specific BCRs to capture HDM. In experimental approaches relying on model antigens, such as ovalbumin, such notion would be less expected. Indeed, using B-MHC II strain of mice, Barnett and colleagues failed to observe initiation of T helper cell differentiation upon OVA immunization.²¹

Unexpectedly, we observed that B cells can initiate CD4⁺ T cell differentiation into IFN- γ and IL-17 producing cells. Classically, Th1/Th17 differentiation is perceived as a process requiring strong stimulation, including MHC-II-peptide-TCR interaction (signal 1), co-stimulation (signal 2) and cytokine signaling (signal 3). B cells, in contrast to DCs, do not secrete polarizing cytokines in response to HDM and express lower levels of co-stimulatory molecules. Therefore, their ability to prime Th2 or Treg responses, if any, would be rather suspect. However, this was not the case: B cells were unable to induce polarization of IL-4/13 or IL-10-secreting Th cells but efficiently primed IL-17 and IFN- γ responses. It is most likely that there are other cell types, such as DCs activated by HDM, which provide cytokines necessary for the development of these subsets. Though MHC-II deficient, they may capture antigens in the tissue, mature and migrate into lymph nodes where they secrete cytokines. Subsequently, by diffusing across the lymphoid tissue these cytokines *in trans* may influence CD4⁺ T cell priming by B cells. This scenario requires further investigation.

The observation that sensitization to HDM induces only Th1/Th17 response in B-MHC-II mice while a Th2/Th1 phenotype predominates in WT animals exemplifies the heterogeneity of T helper cell fates and the complexity behind their regulation. Previously, such a phenomenon has been demonstrated in our lab where T helper cells specific to *Candida albicans* antigens bearing the same T cell receptors but belonging to different subsets were detected.⁴⁰ Evaluation of TCR specificity in Th2 subset of WT and Th1/17 subsets of B-MHC-II mice overlaps would be an important step to further support this concept and underline plasticity of the T helper cell fate. Going one step further and defining molecular cues leading to this heterogeneity is the next challenge to undertake.

In conclusion, this study highlighted an important role of B cells in propagation and possibly initiation of T helper cell responses to house dust mite, a complex, physiologically relevant allergen, and may pave the way for further discoveries in this field as well as for development of new strategies to combat house dust mite allergy.

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Author contributions

T.P.W. conceived the project, designed and performed experiments, analyzed the data and wrote the manuscript. R.M. performed experiments and analyzed data. G.W. provided strains of mice and edited the manuscript. A.L. edited the manuscript. F.S. supervised the work and wrote the manuscript.

Conflicts of interest

The authors declare no conflict of interest

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Figure legends

Figure 1. B cells are present in the lungs and outnumber dendritic cells. A,B. BALB/c mice were sensitized intranasally with HDM or treated with PBS as control on days 0, 7, 8, 9, 10, 11 and analyzed on day 14. Representative FACS plots of B cells among total lung CD45⁺ cells (A) and total numbers of B cells (CD45⁺ CD19⁺ B220⁺) and DCs (CD45⁺ CD11c⁺ SiglecF⁺) in lungs (mean $\pm$ SEM, n=4 mice/group). **C,D.** Representative FACS plots of B-1 cells (CD19^{hi} CD43⁺) among total B cells (CD45⁺ CD19⁺ B220⁺) in blood, spleen and lungs of a naïve mouse (C) and cumulative data of percentage (mean $\pm$ SEM) of B-1 and B-2 cells from 8 mice (D). **E,F.** Representative FACS plots of IgM/IgD staining of lung B cells from PBS- or HDM-treated mice (E) and cumulative data (mean $\pm$ SEM) from 7 PBS-treated and 13 HDM-treated animals (F). IgM⁻ IgD⁻ B cells consisted mainly of IgG⁺ cells (around 73% in the PBS group and 78% in the HDM group). Additionally, around 19% of IgM⁻ IgD⁻ B cells in both groups were IgA⁺ B cells. We found virtually no IgE⁺ B cells at steady state or upon HDM immunization while IgG1⁺ B cells were present at steady state and increased in frequency upon allergic inflammation (9% and 22%, respectively). **G,H.** Surface CXCR5 expression on B cells isolated from the indicated organs of naive mice. Representative FACS plot (G) and cumulative data (mean $\pm$ SEM) from 8 mice (H). Data are representative of at least three independent experiments. * p<0.05, **** p<0.0001.

Figure 2. Lung B cells efficiently capture HDM antigens independently of their BCR specificity. A. BALB/c mice were sensitized intranasally with HDM on days 0, 11 and 12 and received AF647-labeled HDM on day 13. Percentage of HDM-AF647⁺ B cells, DCs and CD4⁺ T cells in lungs was measured on day 14. Representative FACS plots (A) and cumulative percentage and total cell number (mean $\pm$ SEM) from 5 mice (B). **C,D.** C57BL/6 or BCR-transgenic MD4 mice (on C57BL/6 background) were treated intranasally with HDM or HDM-AF647 on day 0 and were analyzed one day later. Representative FACS plots (C) and percentage and geometric mean fluorescence intensity (GMFI, mean $\pm$ SEM) of lung HDM-AF647⁺ B cells from 4 mice/group (D). **E,F.** BALB/c mice were treated intranasally with OVA-AF647 on day 0 and analyzed one day later. Representative FACS plots (E) and percentage and total number of OVA-AF647⁺ cells (mean $\pm$ SEM) in lungs from 4 animals (F). **G.** BALB/c mice were treated

intranasally with HDM-AF488 or with OVA-AF647 plus unlabelled HDM and analysed one day later. Shown is the percentage of fluorescent-positive cells (mean $\pm$ SEM) in lungs from 3 (HDM) and 4 (OVA+HDM) animals. Data are representative of at least two independent experiments. ** $p < 0.01$, *** $p < 0.001$.

Figure 3. B cells efficiently present HDM antigens to *in vivo*-primed CD4⁺ CD44⁺ T cells. A. BALB/c mice were sensitized intranasally with HDM or treated with PBS as control on days 0, 7, 8, 9, 10, 11 and analyzed on day 14. Lung B cells and lung DCs from HDM-treated mice (HDM B cells and HDM DCs, respectively) and splenic B cells from PBS-treated mice (CTRL B cells) were isolated and co-cultured with lung CD4⁺ CD44⁺ T cells from HDM-treated mice in absence or presence of HDM (40 μ g/ml). Graphs show cytokine concentrations in the day 4-culture supernatants measured by ELISA. Data are mean $\pm$ SEM. **B.** Splenic B cells or DCs from PBS-treated mice (splenic CTRL B cells and splenic CTRL DC, respectively) or lung B cells from PBS- or HDM-treated mice (lung HDM-B cells and lung CTRL B cells, respectively) were co-cultured with lung CD4⁺ CD44⁺ T cells from HDM-treated mice in the presence of different HDM concentrations. Graphs show cytokine concentrations in the day 4-culture supernatants measured by ELISA. IL-21 was below the limit of detection. **C,D.** To assess whether lung B cells exposed to HDM *in vivo* carry HDM peptide-MHC complexes on the surface for presentation to T cells, BALB/c mice were treated with HDM or PBS on days 0,11,12,13 and lung B cells were isolated 24 hours later (day 14). B cells were directly co-cultured with CFSE-labeled lung CD4⁺ CD44⁺ T cells. Representative FACS plots showing CFSE profile of proliferating T cells (C) and concentration of IL-5 and IL-13 measured by ELISA (IL-4, IL-17, IFN- γ were below the limit of detection). (D). **E.** BALB/c mice were sensitized intraperitoneally with OVA/Alum on days 0 and 7, challenged with OVA protein intranasally on days 14, 15, and 16. On day 17, lung CD4⁺ CD44⁺ T cells were isolated and co-cultured with lung DCs or lung B cells from OVA-treated mice, or splenic B cells from PBS-treated mice in the presence of different concentrations of OVA protein. Supernatants were collected 4 days later and cytokine concentrations measured by ELISA. The levels of IFN- γ and IL-21 were below the limit of detection. Data are representative of at least three (A-D) or two (E) independent experiments. * $p < 0.05$.

Figure 4. Lung B cells upregulate MHC-II and CD86 upon HDM sensitization. **A,B.** BALB/c mice were sensitized with HDM or treated with PBS as control on days 0, 11, 12, 13 and analyzed on day 14. Representative FACS plots of MHC-II expression by total B cells (left) and CD86 expression (right) by total B cells (in PBS-treated mice) or MHC-II^{int} and MHC-II^{hi} B cells (in HDM-treated mice) (A). Geometric mean fluorescent intensities of MHC-II and different co-stimulatory molecules (GMFI, mean $\pm$ SEM) from 5 mice per group (B). **C.** BALB/c mice were treated as in A. Lung B cells were restimulated with HDM or PMA/Ionomycin in the presence of Brefeldin A for 6 hours and analyzed by intracellular cytokine staining. Graphs illustrate percentage of cytokine producing B cells (mean $\pm$ SEM) from 5 PBS-treated and 5 HDM-treated mice. **D.** Plasma was collected from HDM- and PBS-treated mice and HDM-specific IgE, total IgG and IgG1 was measured by ELISA. Graph shows optical densities (mean $\pm$ SEM, n=5). Data are representative of four (A) or two (B-D) independent experiments. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Figure 5. Depletion of B cells before challenge reduces the allergic response. **A.** Schematic representation of the experimental set-up. BALB/c mice were sensitized with HDM on day 0 and injected with α -CD20 or isotype-matched antibody on day 9 (α -CD20 and Ig Ctrl). Mice were challenged on days 11, 12 and 13 with PBS or HDM and analyzed on day 14. **B.** Absolute numbers of T cells (CD45⁺ CD3⁺ CD4⁺ CD44⁺), eosinophils (CD45⁺ CD11c^{lo/-} SiglecF⁺ CCR3⁺ CD11b^{int} Gr-1^{int}) and neutrophils (CD45⁺ CD11b^{hi} Gr-1^{hi} CD11c⁻ SiglecF⁻) in lungs were quantified by flow cytometry. **C.** Lung CD4⁺ CD44⁺ T cells were restimulated with HDM in co-cultures with lung DCs for 4 days and cytokine production was measured by ELISA. IL-21 was below the limit of detection. **D.** Bronchoalveolar lavage fluid (BALF) was collected on day 14 and cytokine production was measured by ELISA. IL-21 was below the limit of detection. Data are pooled from four independent experiments. Graphs show the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 6. Depletion of B cells before challenge reduces the allergic response in C57BL/6. **A.** C57BL/6 mice were treated as in figure 5A and the absolute numbers of CD4⁺ CD44⁺ T cells, eosinophils and neutrophils in lungs were quantified as in figure 5B. **B.** Lung CD4⁺ CD44⁺ T cells were restimulated with HDM in co-cultures with lung DCs for 4 days. Supernatants were

collected and cytokine production was measured by ELISA. IL-21 was below the detection limit. Data are pooled from three independent experiments. Graphs show the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Figure 7. Depletion of B cells before priming with HDM results in reduced numbers of eosinophils and neutrophils in the lungs but does not influence the capacity of mediastinal lymph node Th cells to secrete IL-4 or IL-13. **A.** Experimental setup of the experiment. C57BL/6 mice were treated with α -CD20 or isotype control antibody (Ig Ctrl) on day -2, treated with HDM or PBS intranasally on days 0 and 1 and analyzed on day 7. Lungs were collected and numbers of eosinophils, neutrophils and effector T helper cells evaluated. Additionally, CD4⁺ T cells were isolated from mediastinal lymph nodes (MLNs), labeled with CFSE and co-cultured with MLN DCs in the presence of HDM for 4 days. **B.** Enumeration of eosinophils, neutrophils and effector T helper cells in the lung tissue. **C.** Proliferation, as assessed by CFSE dilution, was measured by flow cytometry. Representative FACS plots and cumulative data from 4 mice per group. **D.** Supernatants from the co-cultures were collected on day 4 and cytokines were measured by ELISA. IL-5, IL-17 and IL-21 were below the limit of detection. Data are representative (B,C) or pooled (C) from 2 (B) or 4 (C,D) independent experiments. Graphs show the mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$.

Figure 8. B cells are able to prime CD4⁺ T cells in response to HDM. **A.** Experimental set-up. WT C57BL/6 mice were sensitized with HDM or treated with PBS on days 0 and 1 and analyzed on day 7 (left). B-MHC-II and Flox mice (both on C57BL/6 background) received intravenous injection of 10^7 polyclonal CD4⁺ T cells from WT C57BL/6 mice prior to HDM sensitization in order to reconstitute the CD4⁺ T cell compartment. Mice were subsequently sensitized with HDM on day 0 and 1 and analyzed on day 7 (right). **B.** Mediastinal lymph nodes (MLNs) or lung CD4⁺ T cells were labeled with CFSE and co-cultured with MLN DCs in the presence of HDM for 4 days. Proliferation was assessed by CFSE dilution. **C.** Cytokine concentrations in the day-4 culture supernatants were measured by ELISA. IL-21 in both data sets and IL-5 in the mediastinal lymph node data set were below the limit of detection. Data are representative (B) or pooled (C) from 3 independent experiments. Graphs show the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 1

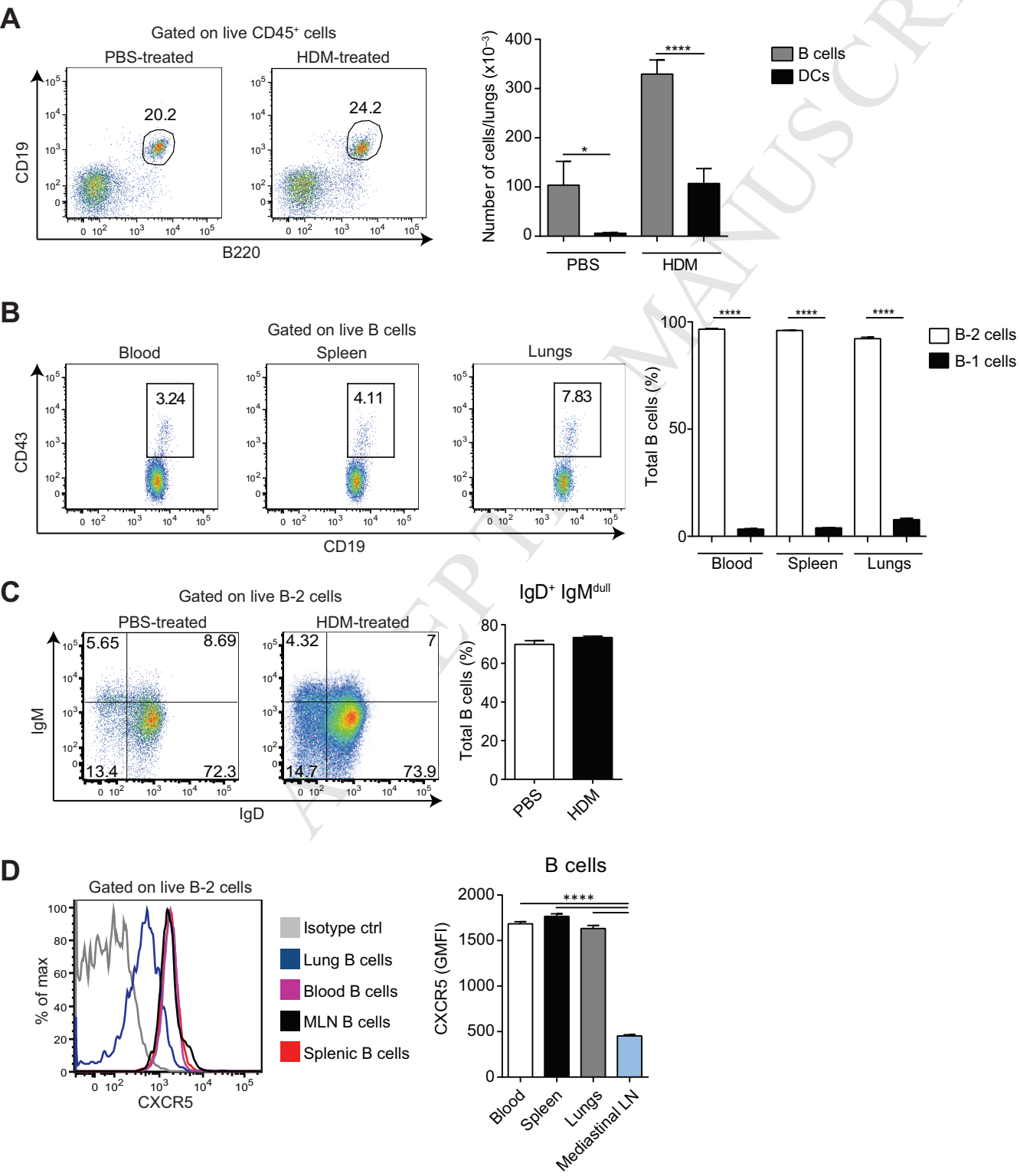


Figure 2

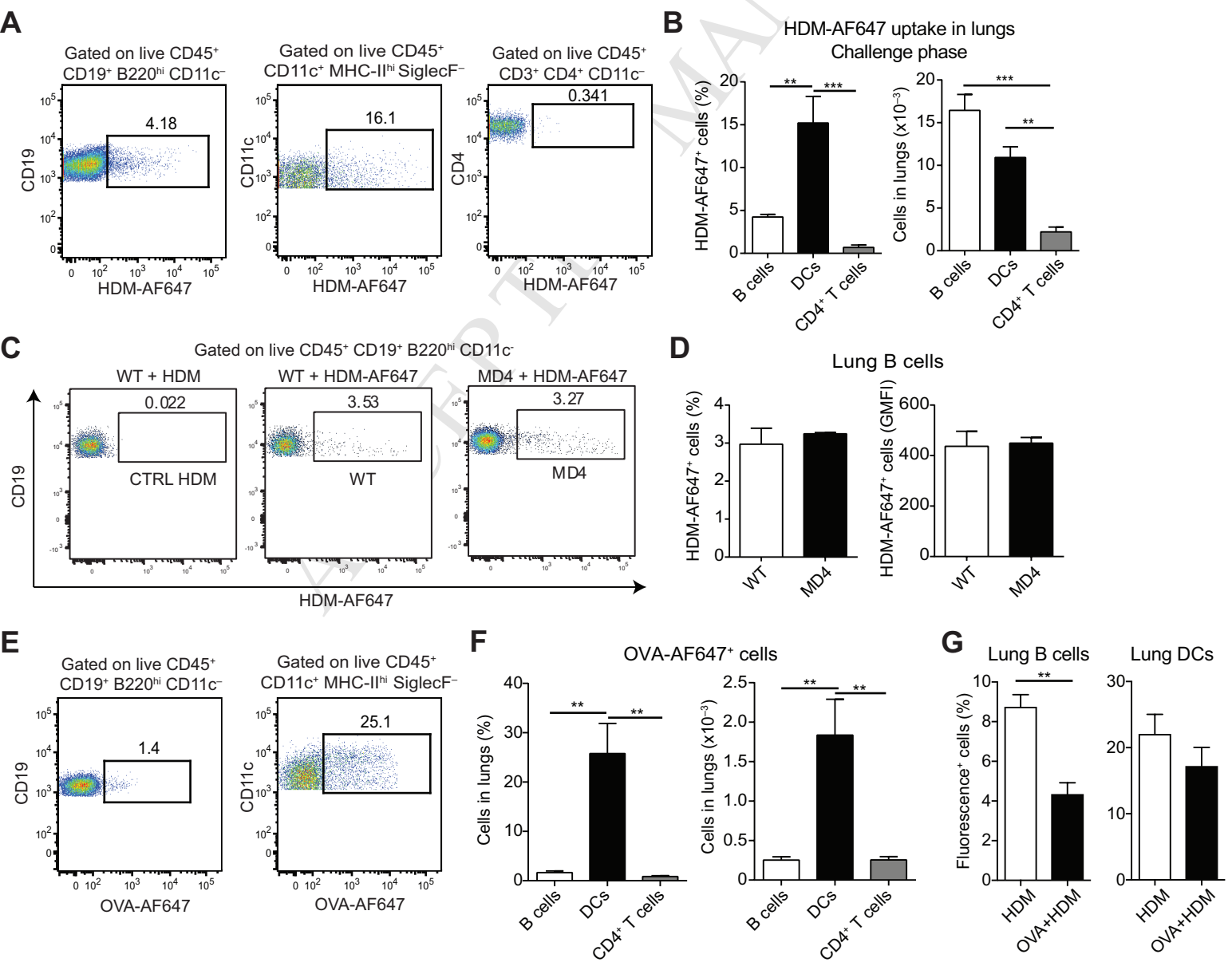
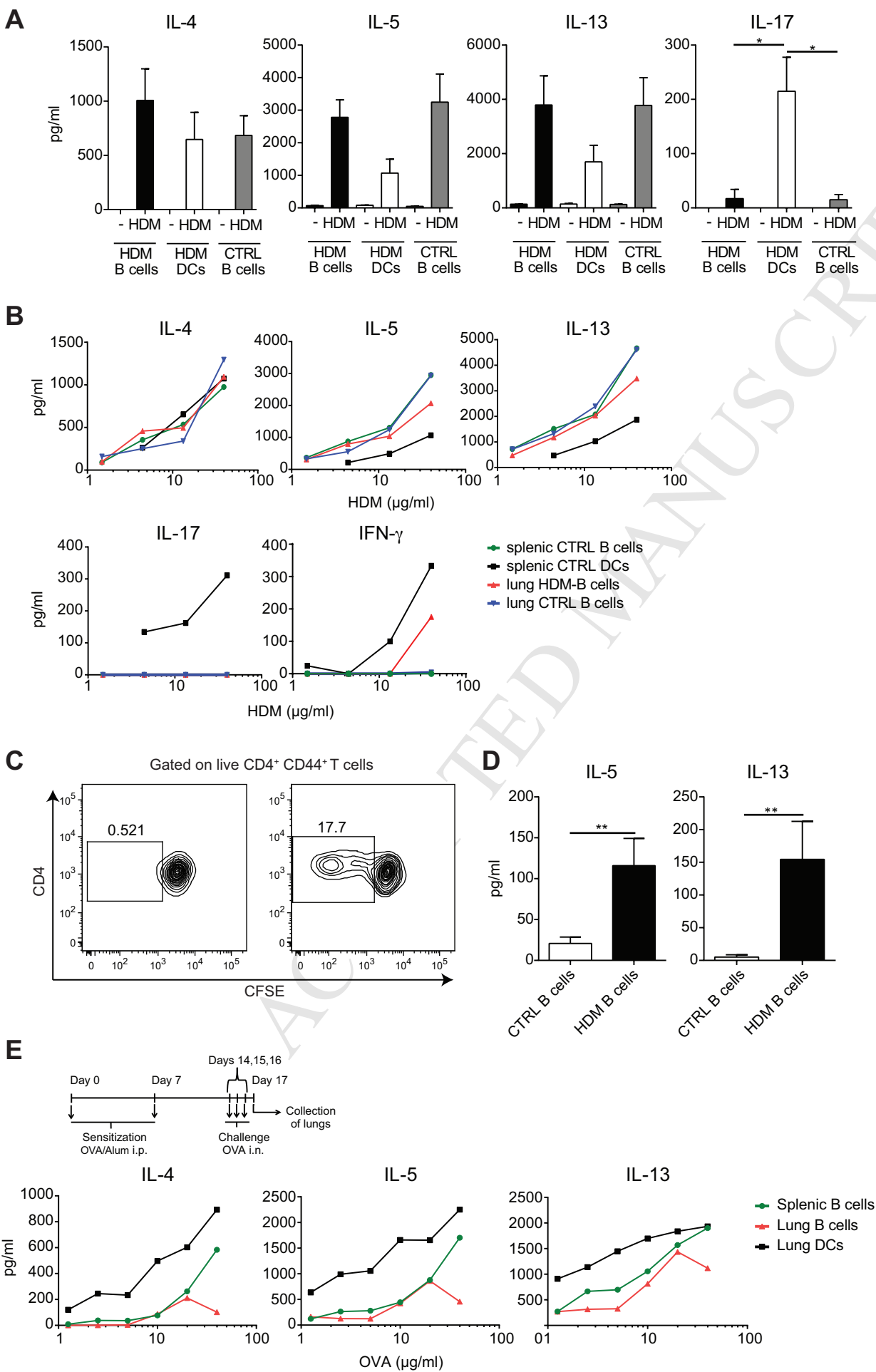
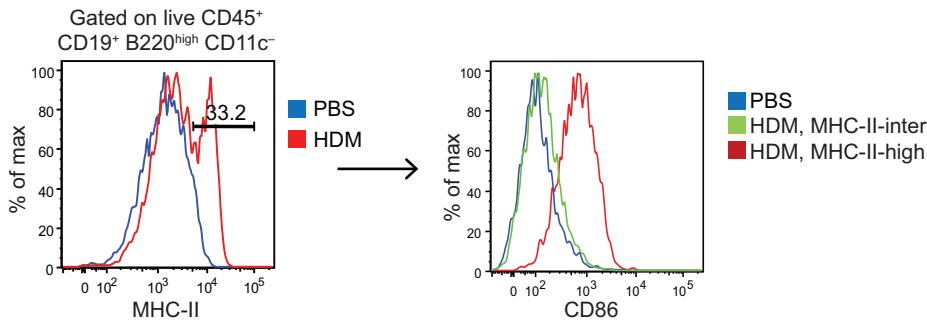


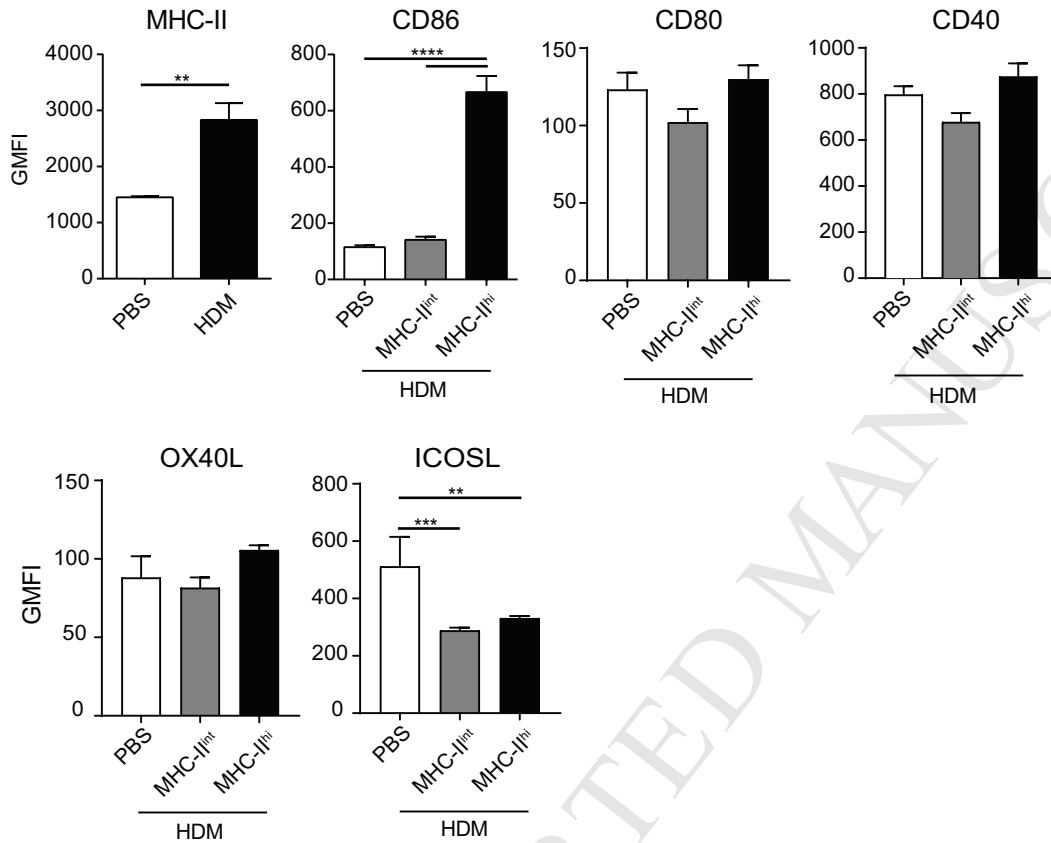
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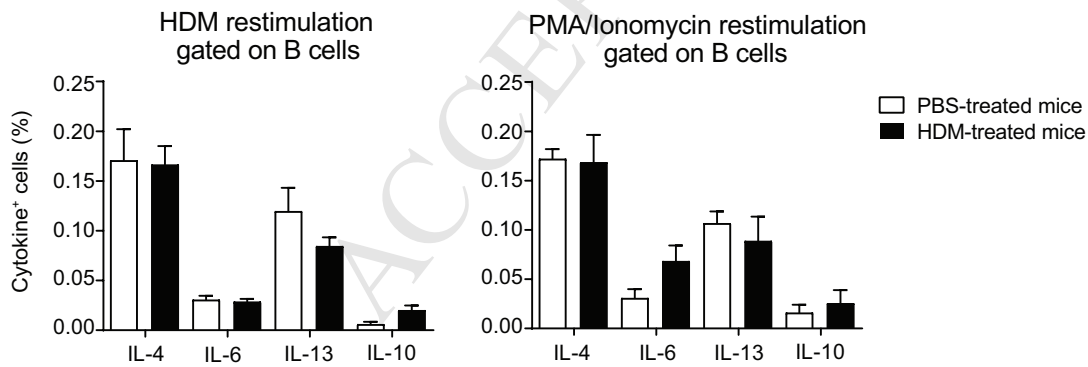
A



B



C



D

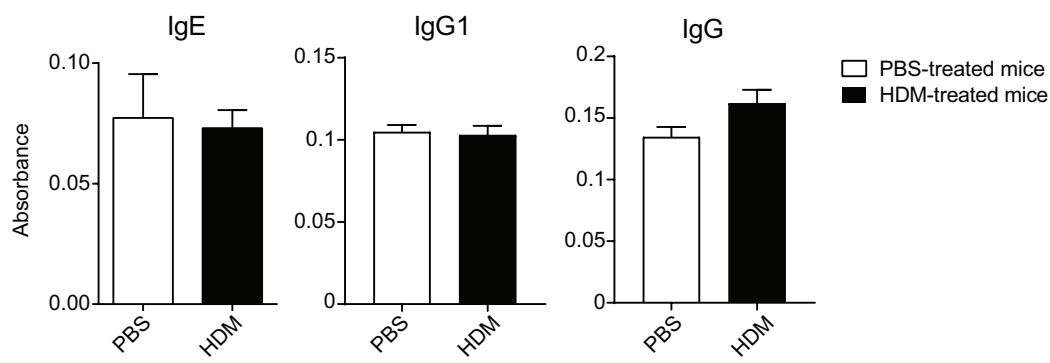
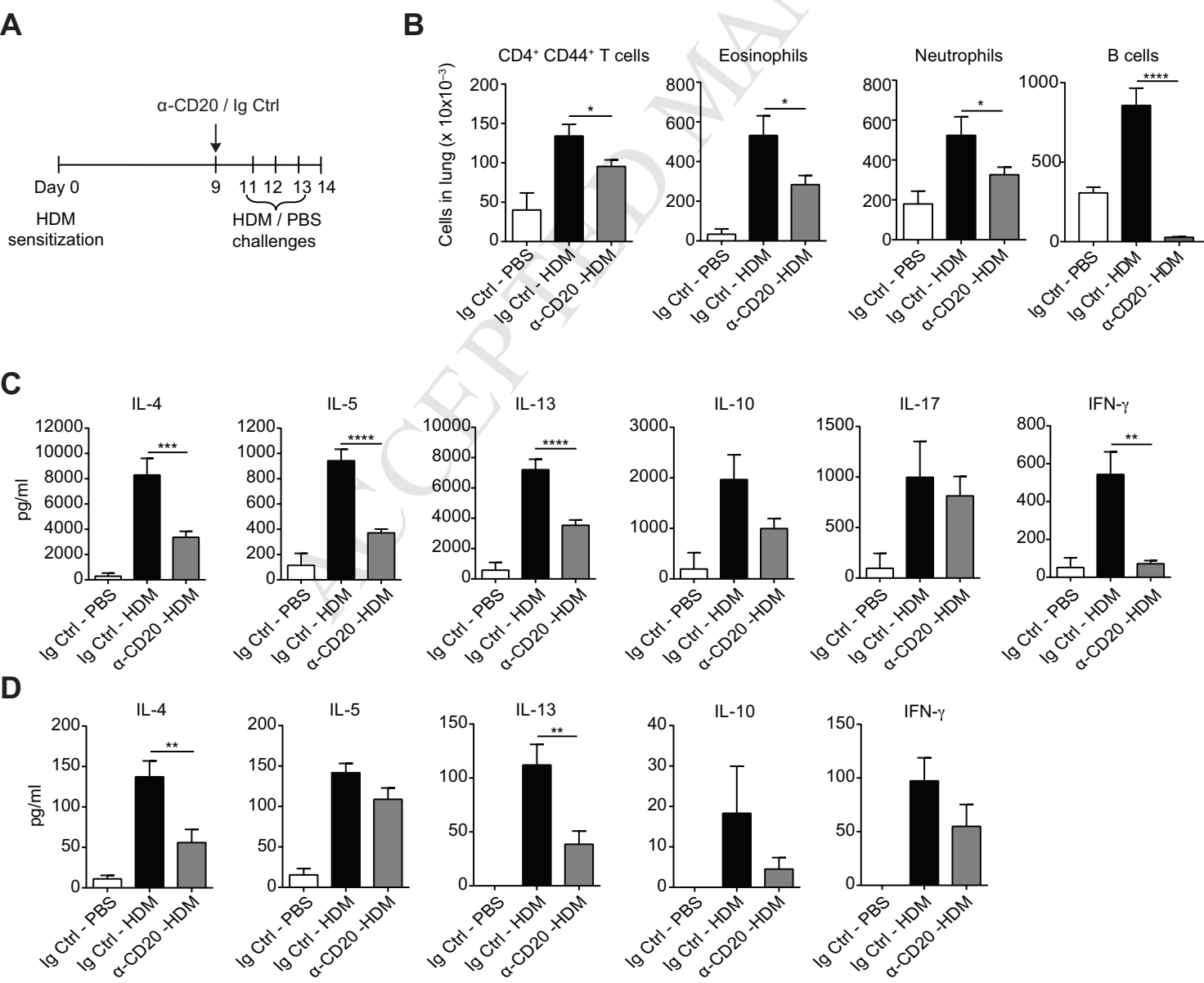


Figure 5



A

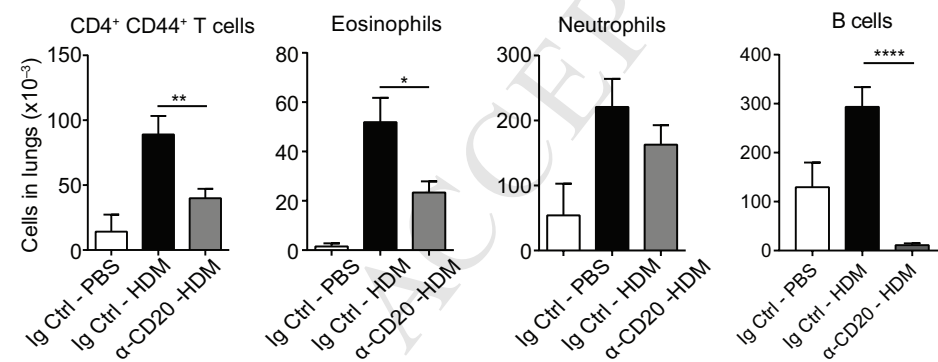


Figure 6

B

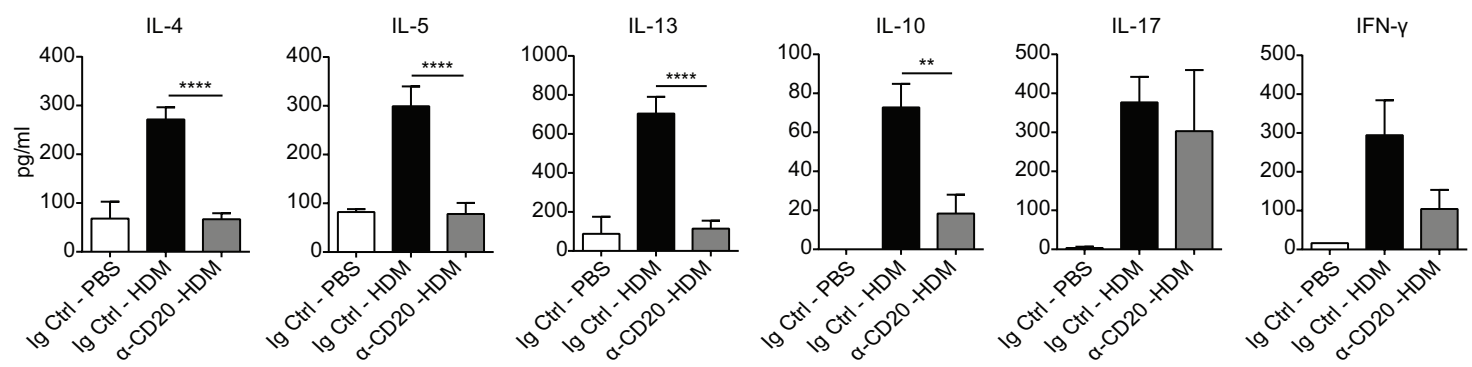


Figure 7

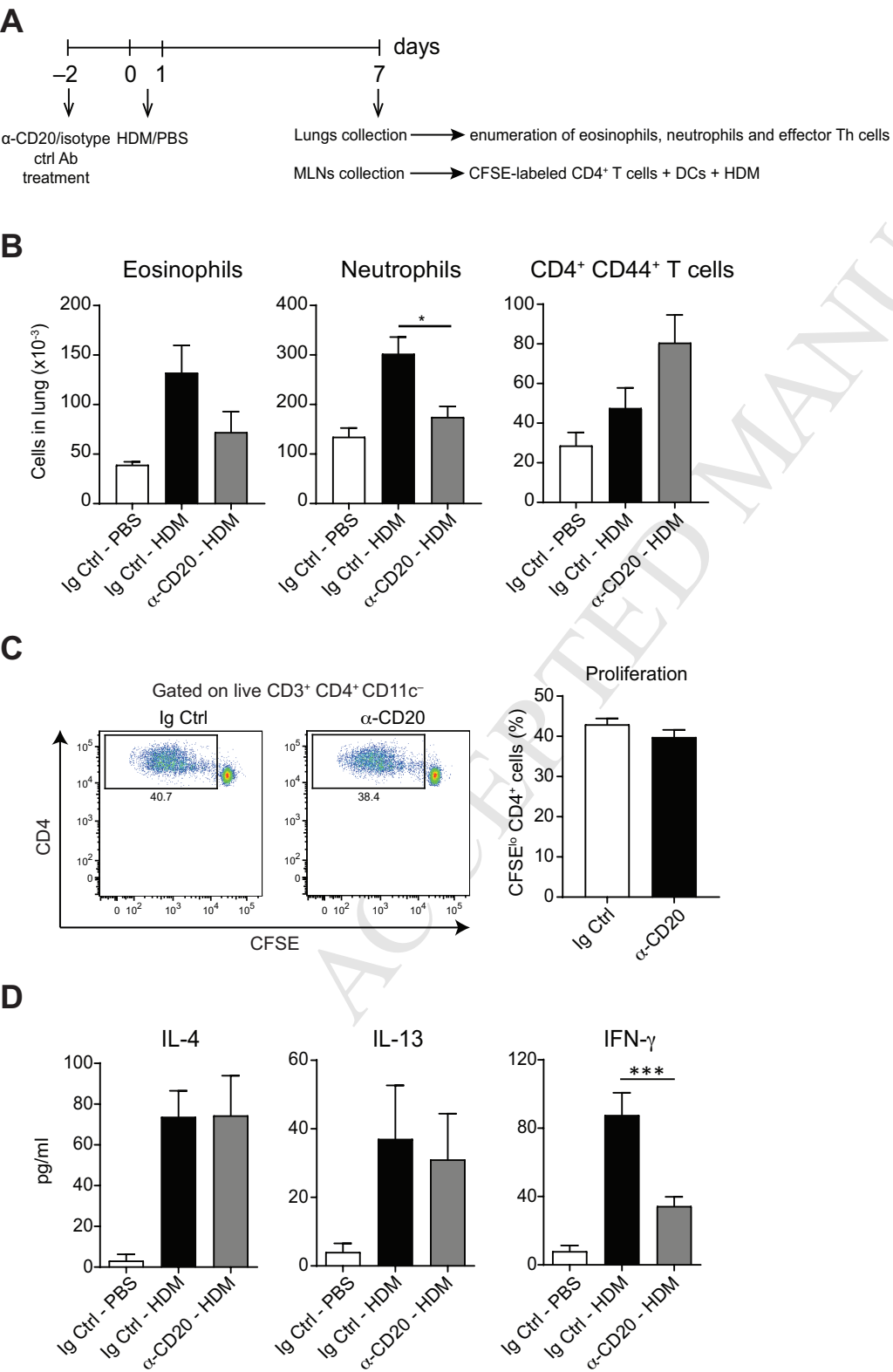


Figure 8

