

Journal Pre-proof

Deletion of IL-4R α signalling on B cells limits hyperresponsiveness depending on antigen-load.

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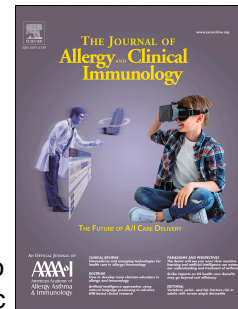
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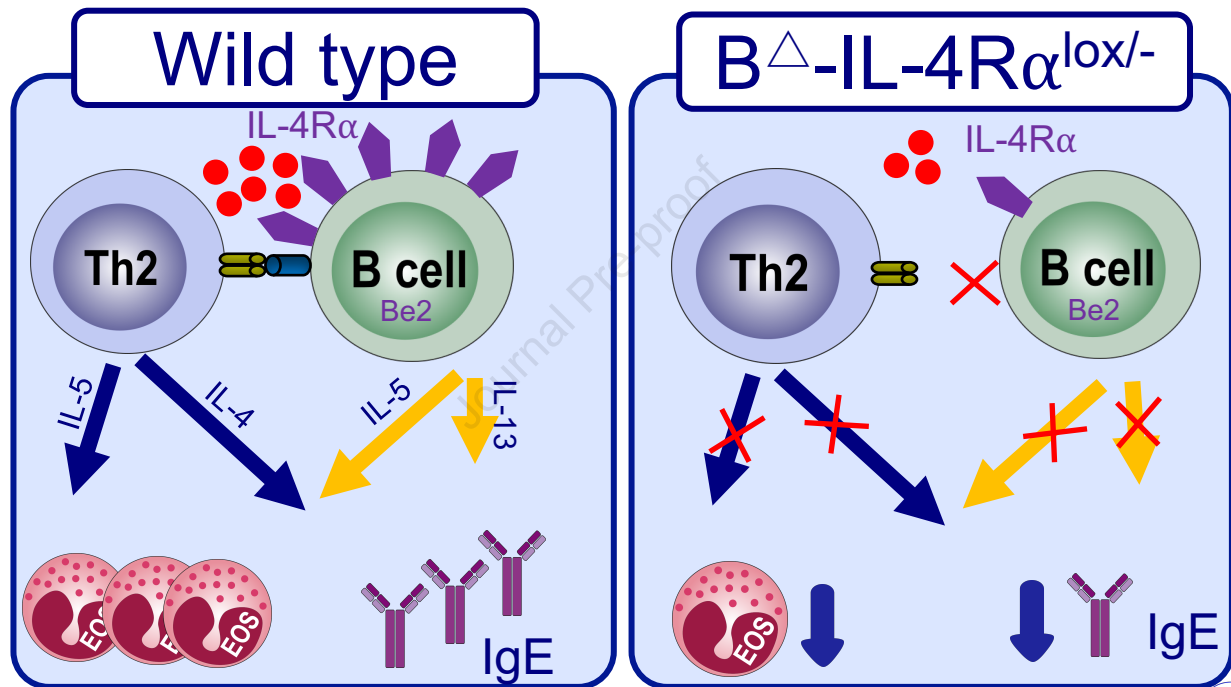
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IL-4R α on B cells is required for optimal CD4 and B cell interaction in GC



IL-4R α (Interleukin 4 receptor alpha); Be2 (B effector 2); GC (Germinal Centre)

Figure E1: Characterisation of IL-4R α expression on B cells.

A, Representative histogram plots of IL-4R α expression on B cells in mediastinal lymph nodes.

B, Quantification of IL-4R α expression on B cells in mediastinal lymph nodes and lungs represented as median fluorescent intensity.

C, Frequencies of eosinophils (Live⁺CD11c^{low} CD11b^{high} Ly6G^{low} SiglecF^{hi}) and neutrophil (Live⁺CD11c^{low}CD11b^{high}Ly6G^{high}) analysed by Flow cytometry (Part of Figure 1).

D, Total number of lung CD4 T cells and CD4 T cells producing IFN- γ after 5 hr stimulation with PMA/ionomycin in the presence of monensin in mb1^{cre}IL-4R α ^{-/lox} and littermate control IL-4R α ^{-/lox} mice.

Shown is mean $\pm$ SDs from one representative experiment of 3 or more (n=6-7). Significant differences between groups were performed by student t-test (Mann-Whitney) and are described as: **p< 0.01.

Figure E2: IL-4R α -responsive B cells are essential in TH2 and IL-21 production.

Representative flow cytometry plots showing lung CD4 T cells producing IL-4, IL-5, IL-13 and IL-21. Frequencies are CD4+cytokine+ cells are quantified on the right. This is part of Figure 2 and Figure 3D.

Shown is mean $\pm$ SDs from one representative experiment of 3 (n=4-9). Significant differences

between groups were performed by student t-test (Mann-Whitney) and are described as:

*p<0.05, **p< 0.01.

23

24 **Figure E3: IL-4R α -responsive B cells are essential in TH2 cytokine release after anti-CD3**
 25 **stimulation.**

26 Mediastinal lymph nodes were stimulated with anti-CD3 (10 μ g/mL) for 5 days and
 27 supernatants were used to measure levels of IL-4, IL-5 and IL-13. Cytokines were not
 28 detected in unstimulated or HDM (30 μ g) stimulated mLN. Shown is mean $\pm$ SDs from one
 29 experiment (n=6-7). Significant differences between groups were performed by student t-
 30 test (Mann-Whitney) and are described as: *p<0.01.

31

32 **Figure E4: B cells produce other TH2 type cytokines.**

33 **A,** Frequencies of B cells (Live⁺B220⁺CD19⁺MHCII⁺) in mediastinal lymph nodes of mb1^{cre}IL-
 34 4R α ^{-/lox}, littermate control IL-4R α ^{-/lox} and IL-4R α ^{-/-} mice.

35 **B,** Frequencies of follicular (FO) B cells (Live⁺B220⁺CD19⁺MHCII⁺CD23⁺CD21/CD35^{low}) and
 36 marginal zone B cells (Live⁺B220⁺CD19⁺MHCII⁺CD23^{low}CD21/CD35⁺) in mediastinal lymph
 37 nodes of mb1^{cre}IL-4R α ^{-/lox}, littermate control IL-4R α ^{-/lox} and IL-4R α ^{-/-} mice.

38 **C,** Frequencies of IL-5 producing B cells (Live⁺B220⁺CD19⁺MHCII⁺IL-5⁺) in the lungs of
 39 mb1^{cre}IL-4R α ^{-/lox}, littermate control IL-4R α ^{-/lox} and IL-4R α ^{-/-} mice.

40 **D,** Total numbers of IL-13 producing B cells (Live⁺B220⁺CD19⁺MHCII⁺IL-13⁺) in the lungs of
 41 mb1^{cre}IL-4R α ^{-/lox}, littermate control IL-4R α ^{-/lox} and IL-4R α ^{-/-} mice.

42 Shown is mean $\pm$ SDs from one representative experiment of 3 (n=6-7). Significant
 43 differences

between groups were performed by student t-test (Mann-Whitney) and are described as:

****p**< 0.01.

Figure E5: Antigen uptake and processing is intact in IL-4R α -deficient B cells.

A, Quantification of CD80 expression on B cells (live⁺B220⁺CD19⁺) in mediastinal lymph nodes

represented as median fluorescent intensity.

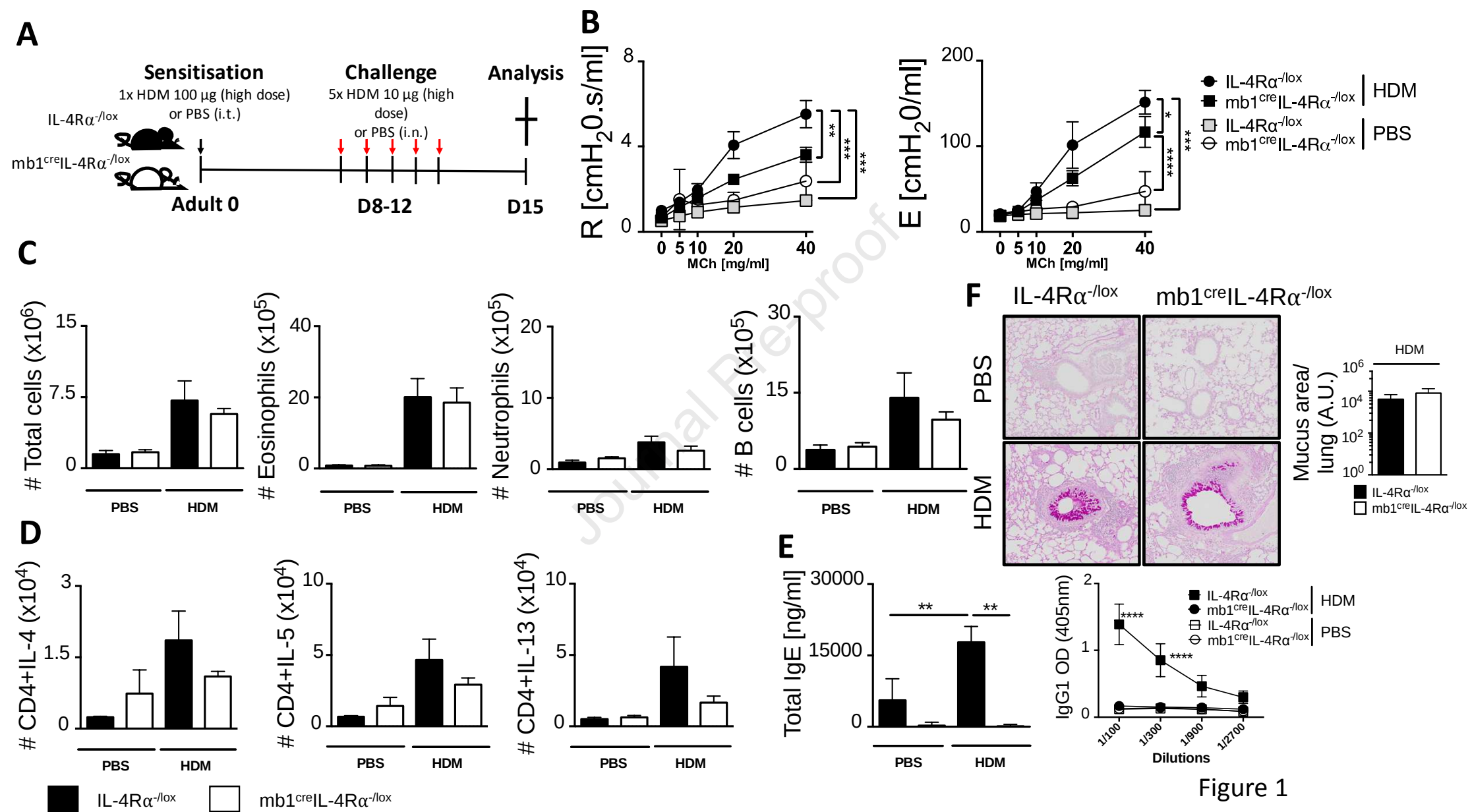
B, Quantification of MHCII expression on B cells (live⁺B220⁺CD19⁺) in mediastinal lymph nodes

and lungs represented as median fluorescent intensity.

Shown is mean $\pm$ SDs from one representative experiment of 3 (n=6-7). Significant differences

between groups were performed by student t-test (Mann-Whitney) and are described as:

****p**< 0.01.



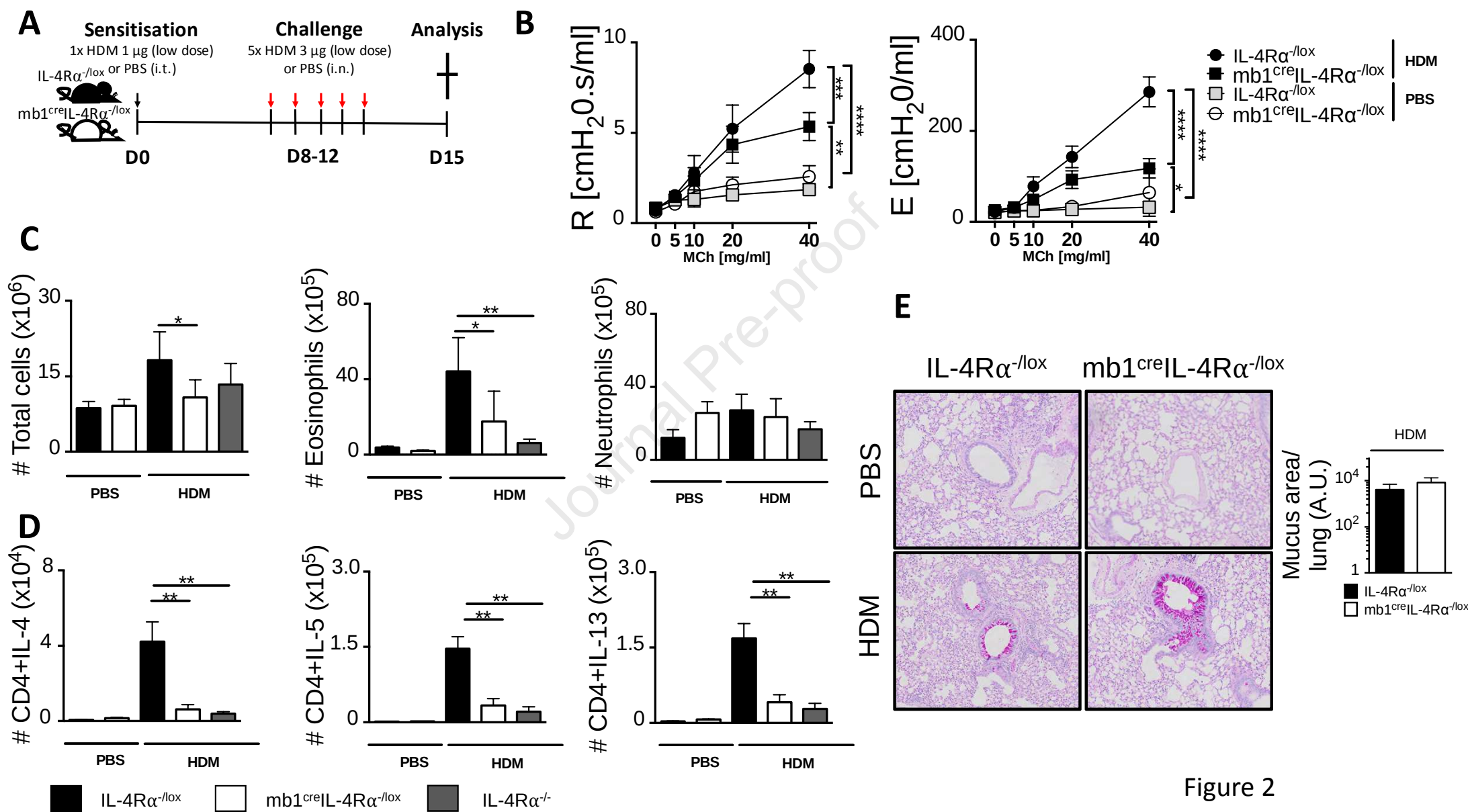


Figure 2

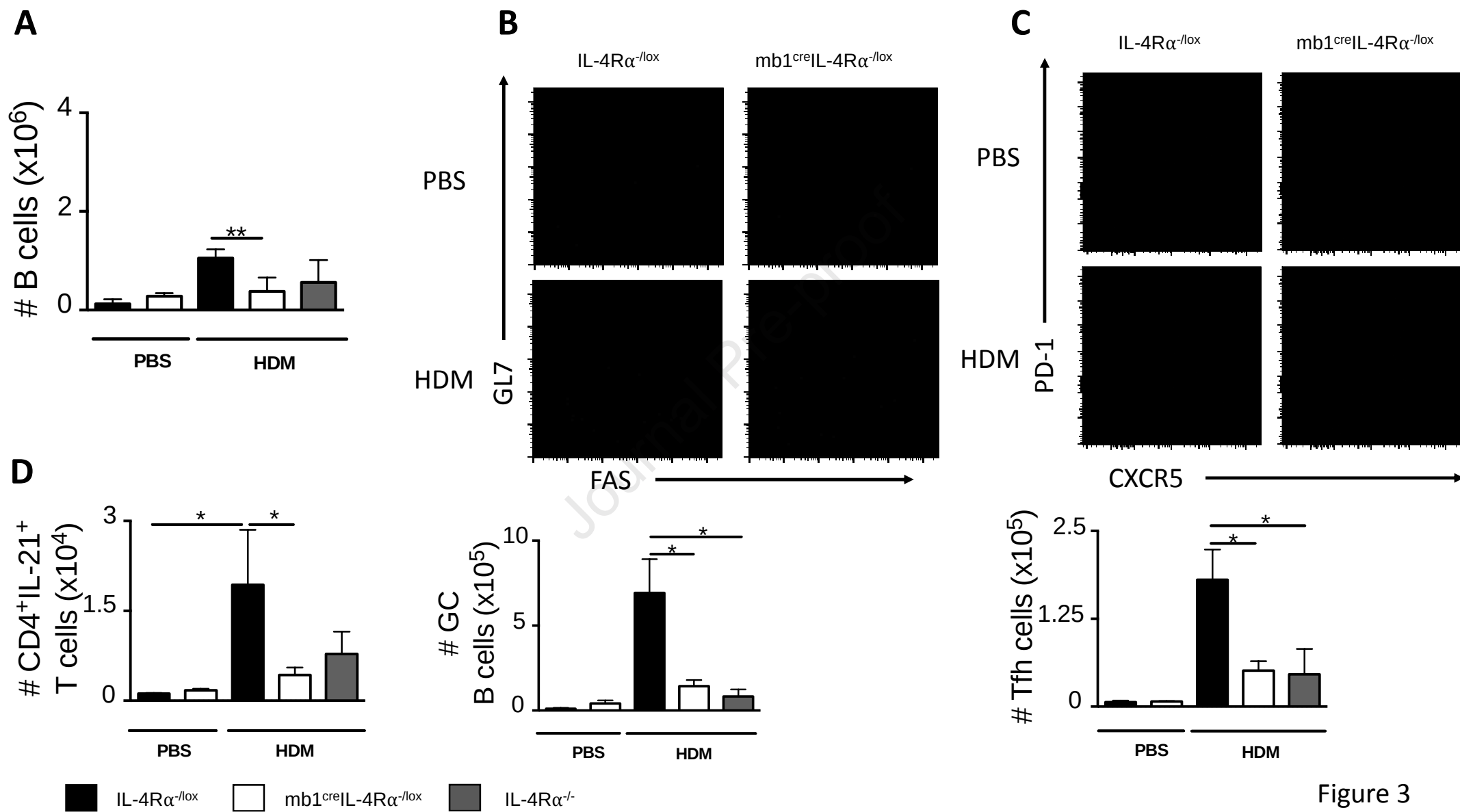


Figure 3

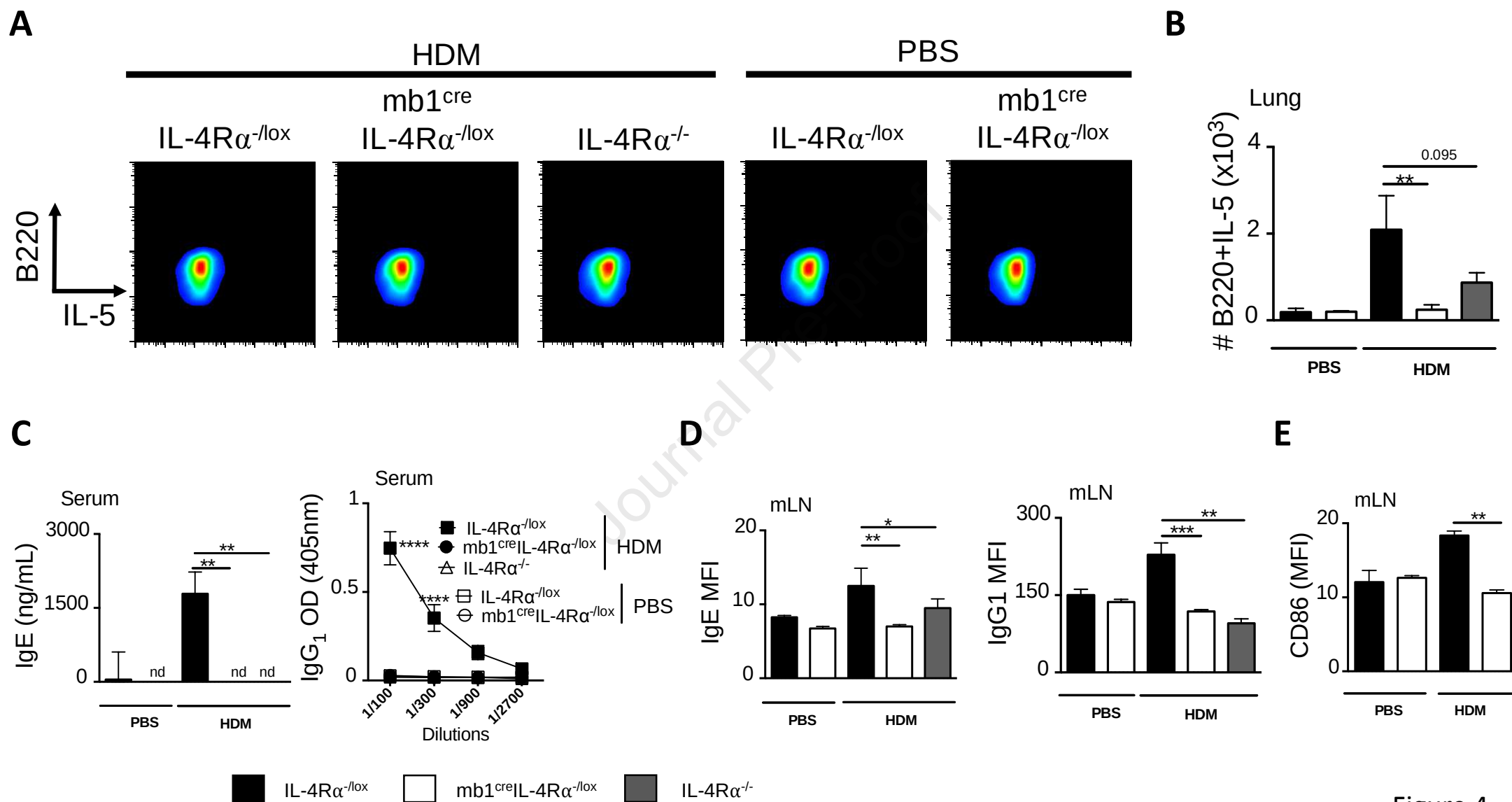


Figure 4

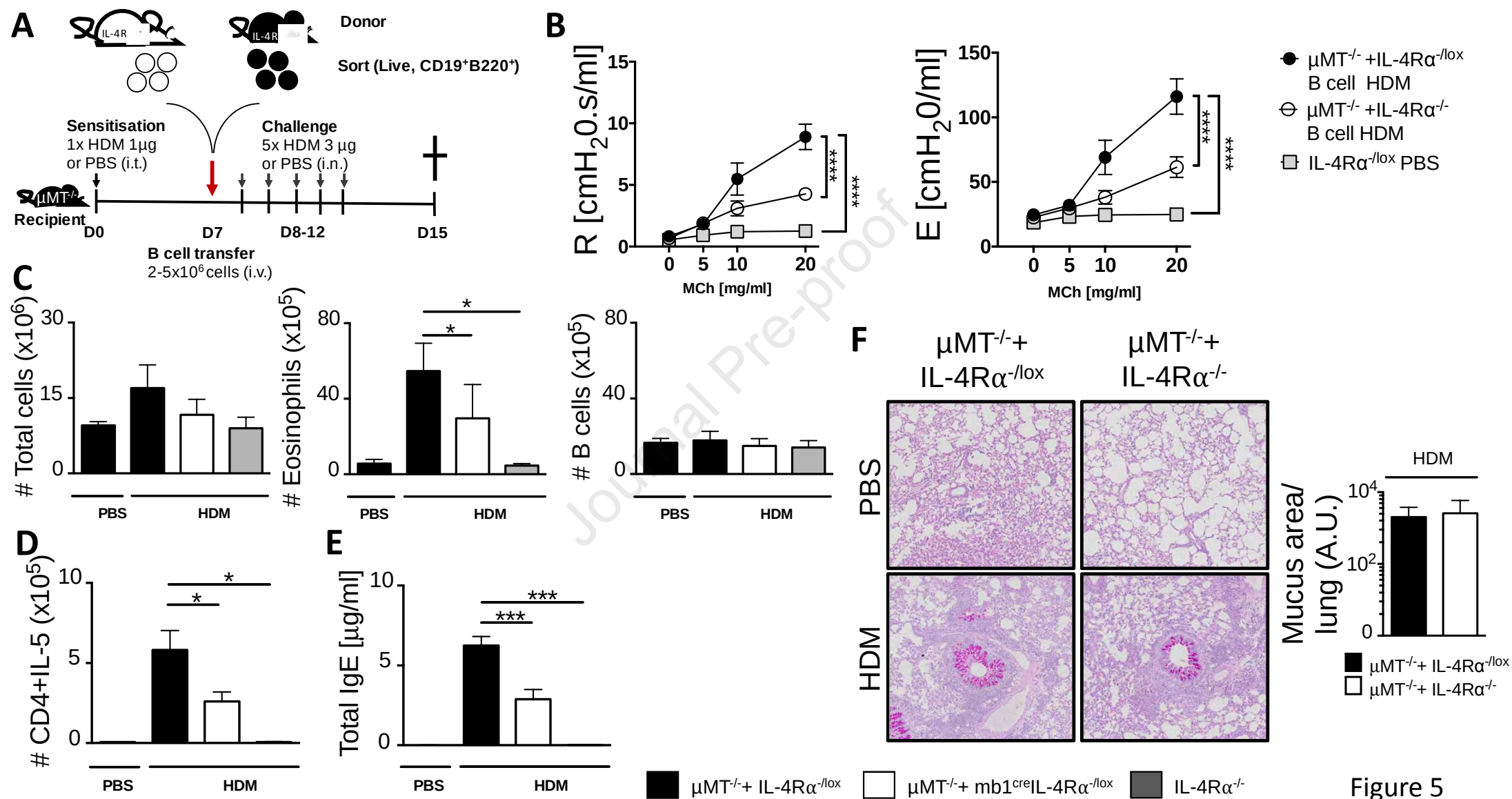


Figure 5

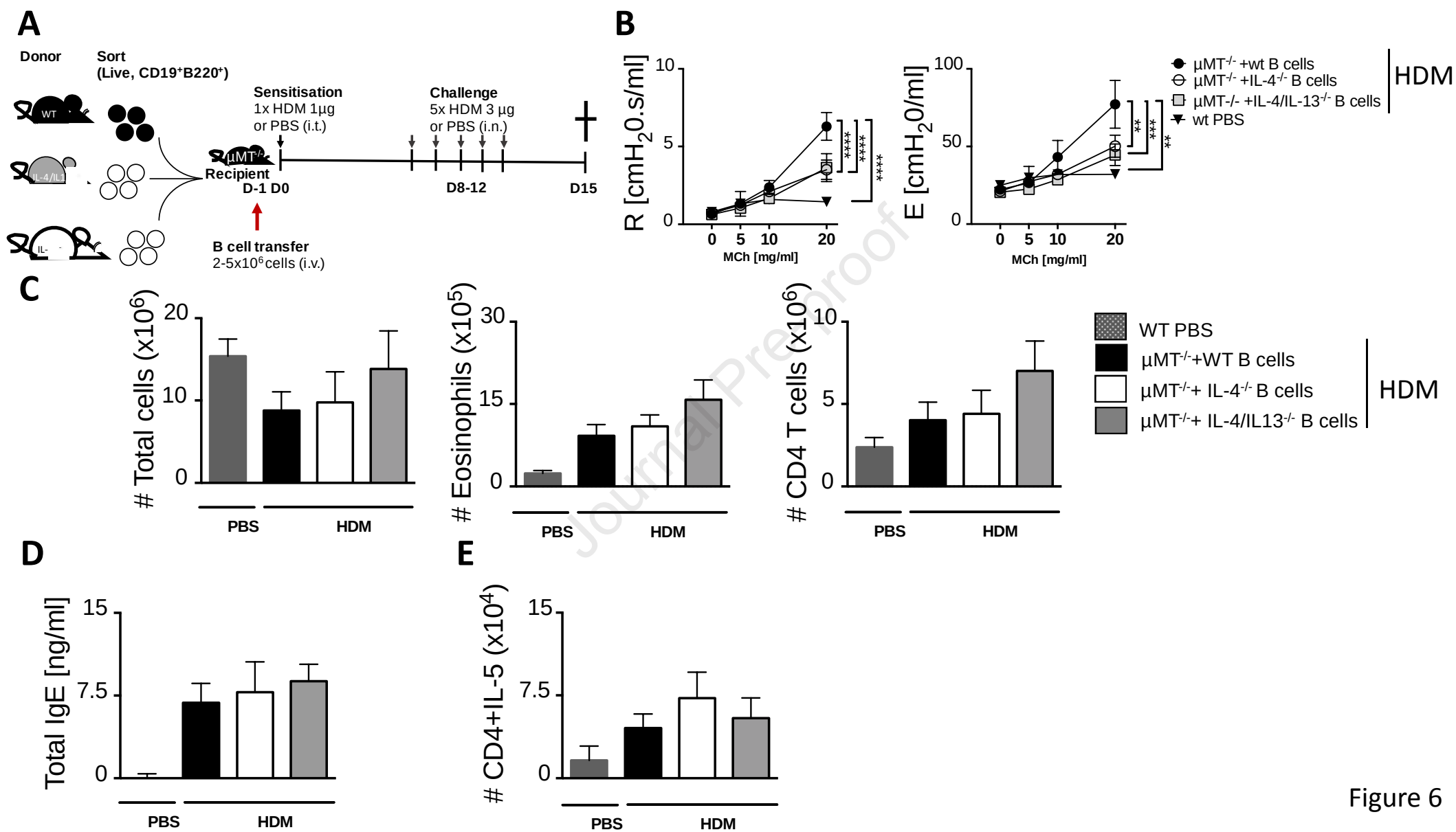


Figure 6

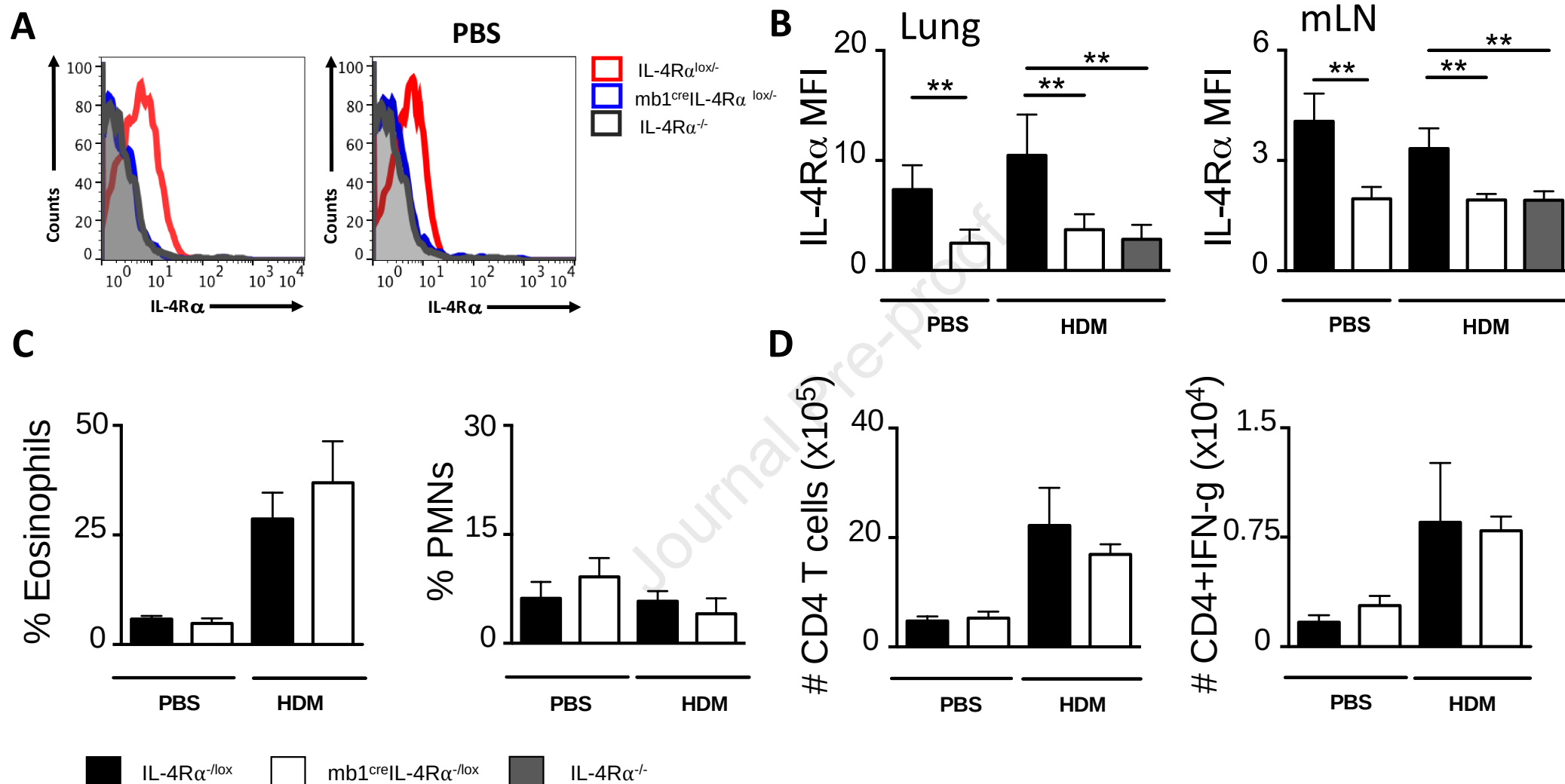


Figure E1

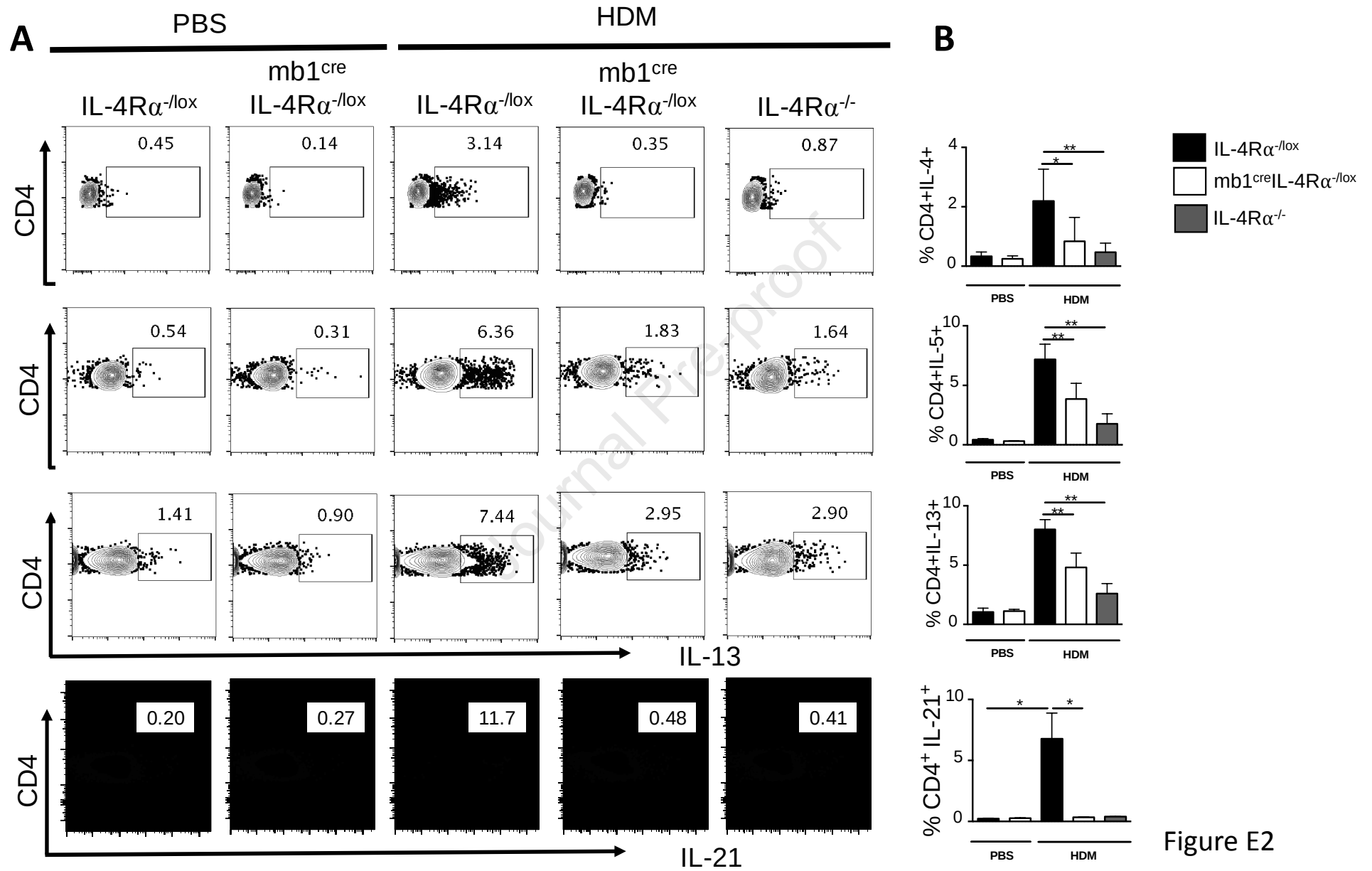


Figure E2

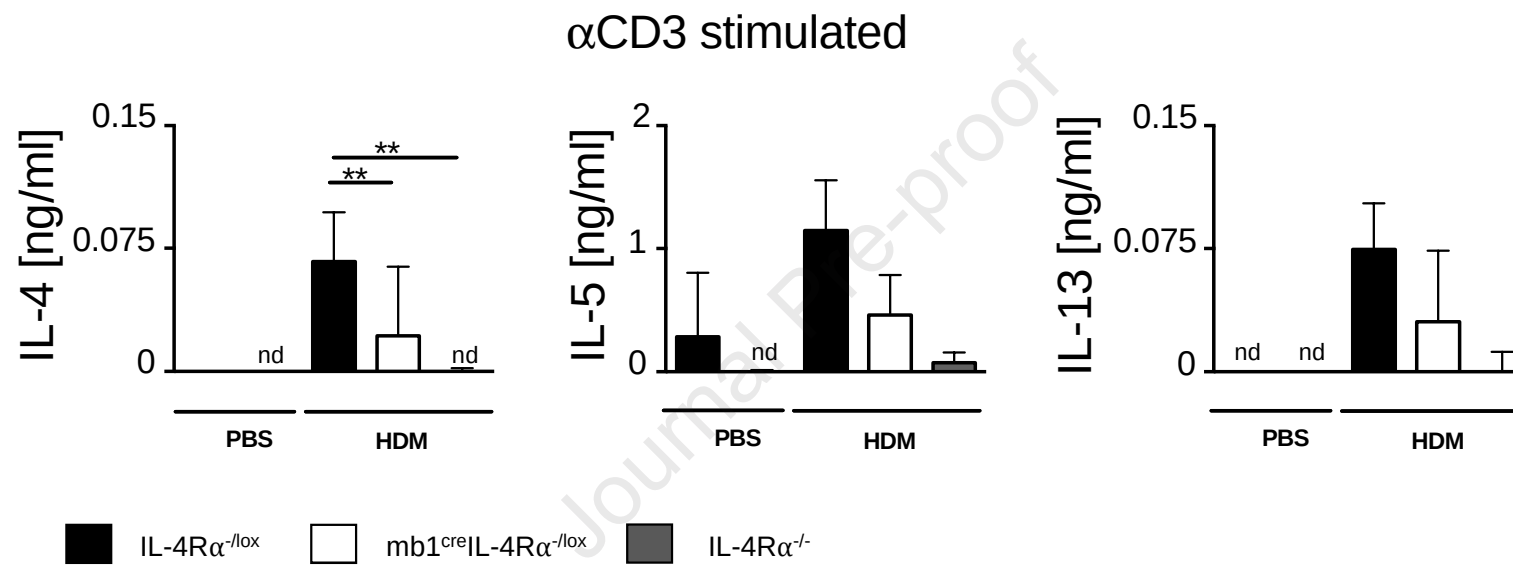


Figure E3

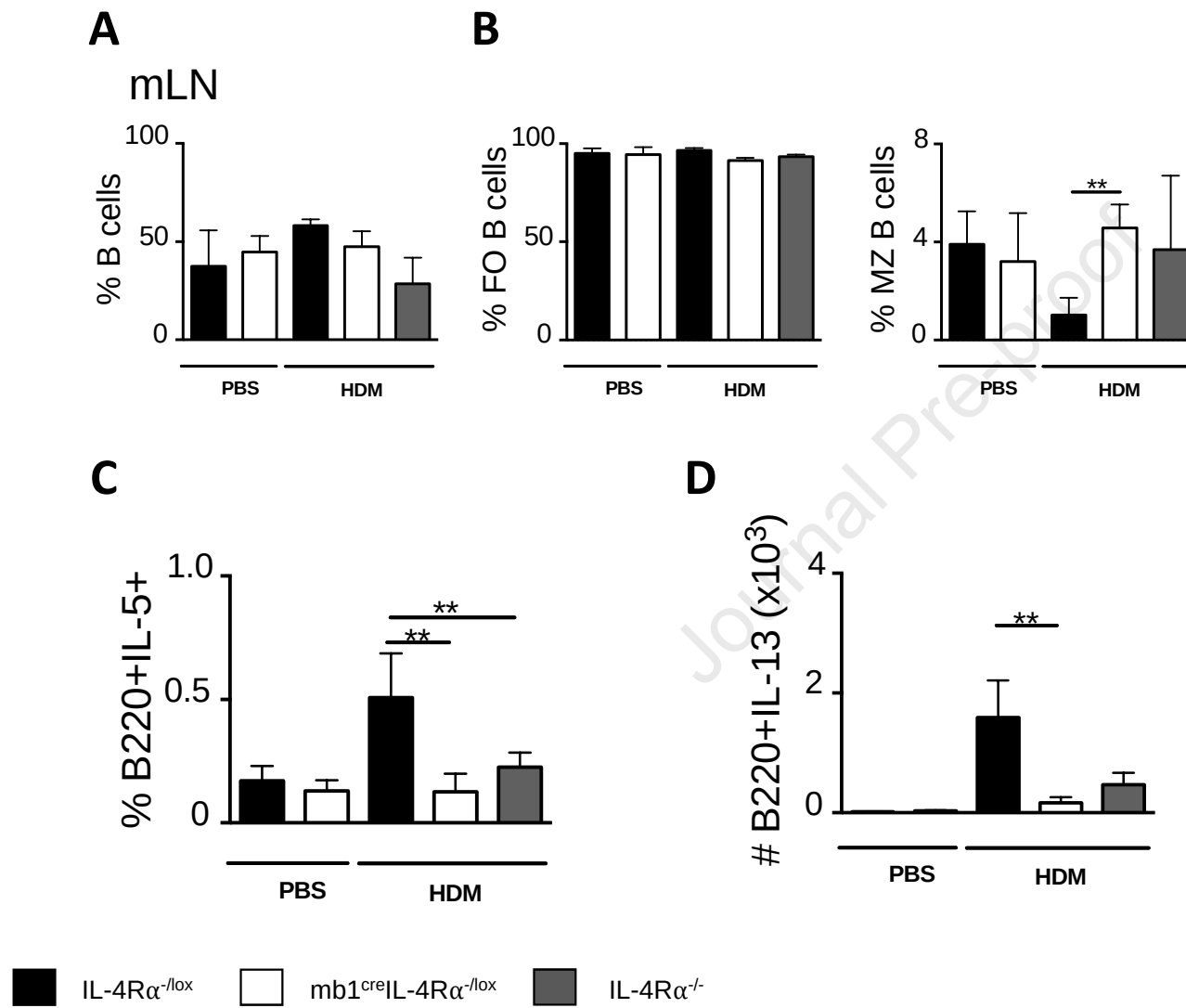


Figure E4

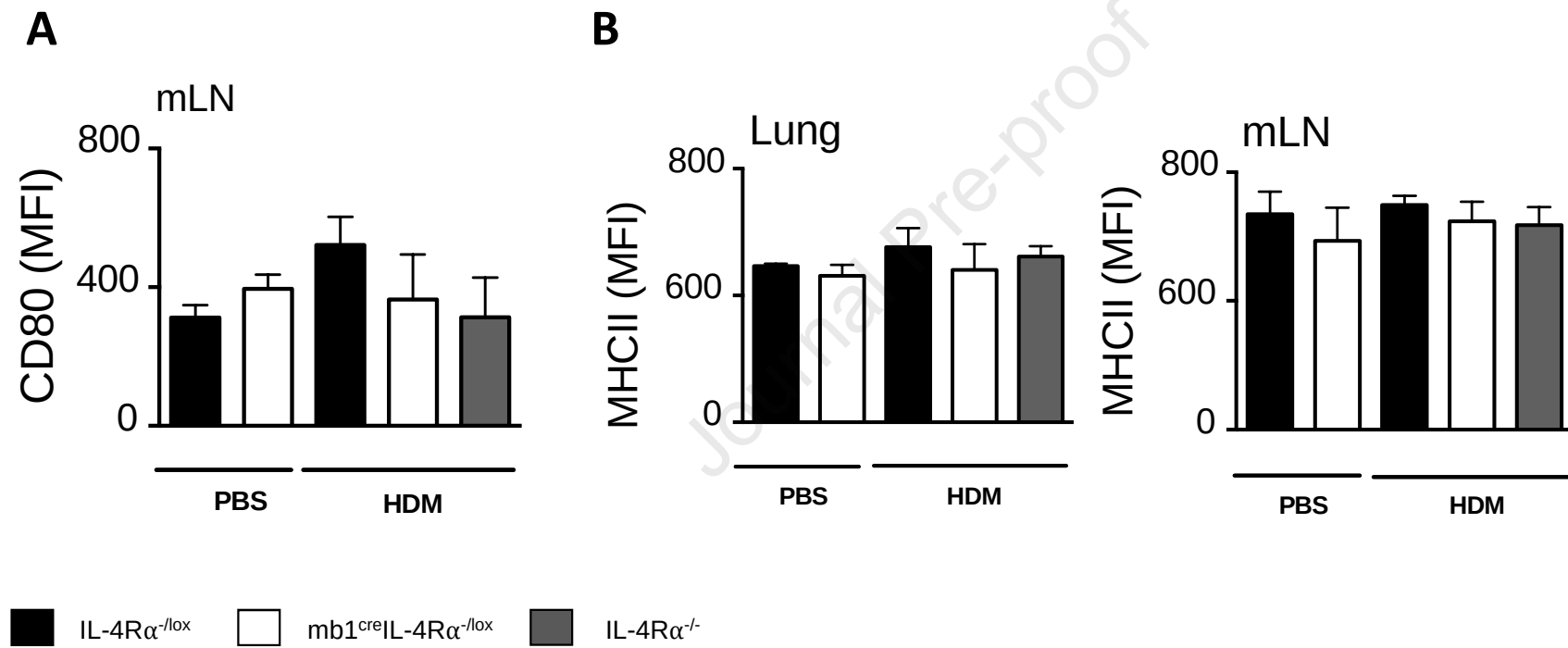


Figure E5

Full title: Deletion of IL-4R α signalling on B cells limits hyperresponsiveness depending on antigen-load.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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ABSTRACT

Background: B cells play an important role in allergies through secretion of IgE. Interleukin 4 receptor α (IL-4R α) is key in allergic asthma and regulates type 2 cytokine production, IgE secretion and airway hyperresponsiveness (AHR). IL-4 activation of B cells is essential for class-switching and contributes to the induction of B effector 2 (Be2) cells. The role of Be2 cells and signalling via IL-4R α in B cells is not clearly defined.

Objective: Here, we asked whether IL-4R α -responsive B cells or Be2 function were essential in experimental allergic asthma.

Methods: Mice lacking IL-4R α on B cells (mb1^{cre}IL-4R α ^{-/lox}) or littermate controls (IL-4R α ^{-/lox}) and mice lacking IL-4 or IL-4/IL-13 on B cells were sensitised and challenged with high dose HDM (>10 μ g) or with low dose HDM (<3 μ g). We also adoptively transferred naïve IL-4R α ^{-/lox} or IL-4R α ^{-/-} B cells into μ MT^{-/-} mice a day before sensitisation or a day before challenge. We analysed lung inflammation, cellular infiltrate and AHR.

Results: We found that IL-4R α signalling on B cells was important for optimal TH2 allergic immune responses mainly when the load of antigen is limited. IL-4R α signalling on B cells was essential for germinal centres (GC) and in the effector phase of allergic responses. Be2 cells were essential in AHR, but not in other parameters.

Conclusion: IL-4R α signalling on B cells is deleterious in allergic asthma as it is required for optimal TH2 responses, Be2 function, GC formation and T follicular helper cells, especially when the load of the antigen is limiting.

Keywords: IL-4R α ; TH2 cells; B cells; germinal centre; T follicular helper cell; B effector 2 cells

73 Abbreviations used:

74 IL-4R α : interleukin 4 receptor alpha

75 BAL: Bronchoalveolar lavage

76 GC: Germinal Centre

77 HDM: House dust mite

78 i.n.: Intranasal

79 i.t.: intratracheal

80 medLN: Mediastinal lymph node

81 TFH: T follicular helper

82 AHR: airway hyperresponsiveness

83 Be2: B effector 2 cells

84

85 Key Messages

- 86 • IL-4R α -responsive B cells play a critical role in HDM induced allergic asthma when
87 the load of HDM is limited.
- 88 • IL-4R α -signalling on B cells is required at both sensitisation and effector stages of
89 allergic disease.
- 90 • IL-4R α -responsive B cells are required for B effector 2 function of B cells and help
91 maintain optimal TH2 during allergic asthma.

92 **CAPSULE SUMMARY**

93 B cells expressing high level IL-4R α are important in class switching to IgE in GC. Targeting IL-
94 4R α signalling on B cells has clinical benefit in allergic asthma even in high IgE setting.

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INTRODUCTION

Asthma is chronic debilitating disease affecting over 300 million people worldwide with at least 250000 people dying from complications associated with the disease ¹. The immune response to the disease is characterised by T helper 2 (TH2) immune cells such as eosinophils and type 2 cytokines IL-4, IL-5 and IL-13 and B cells secreting IgE ^{2,3}. Secreted IgE binds to high affinity receptors FcεR on the surfaces of mast cells and basophils, resulting in activation and degranulation of these cells and release of histamines, proteases and membrane phospholipids such as leukotrienes and prostaglandins ^{4,5}. Priming of long-lived type 2 memory T cells is attributed to dendritic cells (DCs) ^{6,7}, with earlier studies demonstrating a minimal contribution from B cells, despite their ability to present antigens to T cells ^{8,9}.

The role of B cells in experimental allergic asthma is contradictory, earlier studies using ovalbumin (OVA) as a model antigen showed a redundant role for B cells in allergic asthma ¹⁰⁻¹². Mice deficient of B cells ($\mu\text{MT}^{-/-}$) developed similar airway hyperreactivity (AHR), eosinophilia and TH2 airway responses when sensitised and challenged with OVA ¹⁰⁻¹². More recent evidence using a clinically relevant allergen, house dust mite (HDM), suggests an essential function of B cells in allergic asthma ¹³⁻¹⁵. However, despite empirical evidence demonstrating a key role played by B cells in allergic asthma, caveats and contradictions in literature still exist and require further clarification. Some studies have suggested that B cells are either not important at all ⁹ or are only essential in priming of CD4 T cells and induction of T follicular T helper (T_{FH}) cells during the sensitisation stage, but play no part during challenge stages of HDM-induced asthma ¹³. Other studies have shown that B cells are essential in both priming of CD4 T cells ¹⁵ and effector stages of HDM-induced allergic

asthma¹⁴. Furthermore, the load of antigen seems to be critical in the involvement of B cells in HDM-induced allergic asthma¹⁴. At high doses of HDM, B cells play minimal role in antigen uptake, processing and presentation to CD4 T cells, whereas at low dose of HDM antigen, B cells have more access to antigen and can uptake, process and present antigen to T cells, playing an essential part in the development of T_{FH}¹⁴. Interestingly, the inability of B cells to present antigen at the sensitisation stage leads to TH1 and TH17 airway responses and not TH2 responses¹⁵.

Interleukin 4 receptor alpha (IL-4R α) is central in TH2 allergic airway asthma^{16–18} and other type 2 diseases^{19,20}. In allergic asthma, we and others have shown temporal²¹ and cell-specific requirement of IL-4R α in DCs²², T cells²³ and epithelial cells²⁴ and also redundant role of this IL-4/IL-13 signalling receptor in macrophages²⁵ and airway smooth muscle cells^{26,27}.

We have recently shown that early IL-4 production by B cells influences type 2 CD4 T cells differentiation in lymph nodes which leads to protective type 2 responses against certain parasitic infection^{28–30}. Given the complexity around the importance of B cells in allergic asthma, we set to investigate the role of IL-4R α signalling specifically in B cells during HDM-induced allergic asthma.

We challenged mice with high dose or low dose HDM to assess whether antigen load matters in the requirement of IL-4R α responsive B cells in allergic asthma. We found that although mice lacking IL-4R α on B cells (mb1^{cre}IL-4R α ^{-/lox}) had reduced airway hyperresponsiveness at high dose HDM, in other parameters, they were comparable to littermate control mice. This was contrary to what we observed at low dose HDM, where mb1^{cre}IL-4R α ^{-/lox} mice had reduced AHR, type 2 responses, eosinophilia, T_{FH} and ability to

produce TH2 cytokines. By adoptively transferring naïve IL-4R α -deficient B cells into μ MT^{-/-} mice sensitised to low dose HDM, we demonstrated the importance of IL-4R α signalling on B cells at both sensitisation and effector stages. Interestingly, lack of IL-4 or IL-4/IL-13 production by B cells resulted in reduced AHR which suggested a key contribution in this parameter, but less so in airway inflammation or antibody production. Here, we show an essential role for IL-4R α responsive B cells in optimal type 2 allergic airway inflammation especially when the load of HDM is limited.

METHODS

Mice

To generate mice deficient of IL-4R α only on B cells (mb1^{cre}IL-4R α ^{-/lox}), we intercrossed homozygous mb1^{cre} mice³¹ with IL-4R α ^{-/-} 32 on Balb/c background. We then further mated mb1^{cre} IL-4R α ^{-/-} mice with homozygous IL-4R α ^{lox/lox} mice¹⁹ to generate hemizygous mb1^{cre}IL-4R α ^{-/lox} 33 which were backcrossed up to 10 generations in Balb/c background. Hemizygous littermates (IL-4R α ^{-/lox}) expressing single functional IL-4R α allele was used as a wild-type control in all experiments. Mice were housed in independently ventilated cages under specific pathogen-free conditions at the University of Cape Town Animal Facility. All mice were used at eight to 10 weeks of age and animal procedures were performed according to strict recommendation by the South African Veterinary Council and were approved by the University of Cape Town Animal Ethics Committee (Reference number 018/013).

House Dust-mite induced allergic airway disease

A high dose and a low dose treatment schedule were used to induce symptoms of allergic asthma in mice¹⁴. Mice were anaesthetised with ketamine (Anaket-V; Centaur Labs,

Johannesburg, South Africa) and xylazine (Rompun; Bayer, Isando, South Africa). For the high dose schedule, mice were sensitised intratracheally (i.t.) on day 0 with 100 µg of HDM (Stellergens Greer Laboratories, Lenoir, U.S.A.) and intranasally challenged with 10 µg HDM on days 8, 9, 10, 11 and 12. For low dose treatment, mice were sensitised with 3 µg of HDM and challenged with 1 µg HDM on days 8, 9, 10, 11 and 12. AHR was measured on day 15. After the procedure, mice were euthanised and tissue samples were collected for analysis.

Adoptive transfer

Spleens were collected from naïve IL-4Rα^{-/lox}, mb1^{cre}IL-4Rα^{-/lox}, IL-4^{-/-} or IL-4/IL-13^{-/-} mice and passed through 40 µm strainer to obtain single cell suspensions. Cells were stained with FITC-B220 and APC-CD19 for 30 min at 4°C. A dead cell exclusion dye (7AAD) was added before sorting on BD FACS Aria I to at least 96% purity. 2-5 × 10⁶ cells were adoptively transferred intravenously (i.v.) into μMT^{-/-} recipient mice a day before HDM sensitisation. In other experiments, sorted B cells were adoptively transferred i.v. into low dose HDM sensitised mice a day before challenge with low dose HDM.

Airway Hyperresponsiveness

Airway resistance and elastance of the whole respiratory system (airways, lung chest wall) after intranasal challenge was determined by forced oscillation measurements as described previously²⁵ with the Flexivent system (SCIREQ, Montreal, Canada) by using the single compartment (“snapshot”) perturbation. Measurements were carried out on mice with increasing doses (0, 5, 10, 20 and 40 mg/mL) of acetyl-β-methylcholine (methacholine, Sigma-Aldrich, Aston Manor, South Africa) treatment. Differences in the dose-response curves were analysed by repeated-measures Two-way ANOVA with the Bonferroni post-

test. Only mice with acceptable measurements for all doses (coefficient of determination >0.90) were included in the analysis.

Flow cytometry

Bronchoalveolar lavage (BAL) fluid cells were obtained as previously described²⁶. Single-cell suspensions were prepared from lymph nodes in Roswell Park Memorial Institute (RPMI) media (Gibco, Paisley, United Kingdom) by passing them through 100µm strainer. To obtain single cell suspensions from lung tissues, a left lobe was digested for 1 hour at 37°C in RPMI containing 13 mg/mL DNase I (Roche, Randburg, South Africa) and 50 U/mL collagenase IV (Gibco, Waltham, Massachusetts) and passed through 70µm strainer. Antibodies used in these experiments included, phycoerythrobilin (PE)- conjugated anti-Siglec-F (clone, E50-2440), anti-CD124 (IL-4Rα, clone, M-1), anti-IL-5 (clone, TRFK5), anti-CD44 (clone, KM114), FITC- conjugated anti-Gr-1 (clone, RB6-8C5), CD45 (clone, 30-F11), IL-4 (clone, 11B11), PerCP Cy5.5- conjugated anti-Ly6C (clone, AL-21), -CD45.1 (clone, A20), anti-IL-17 (clone, TC11-18H10), Allophycocyanin (APC)- conjugated anti-CD11c (clone, HL3), anti-FoxP3 (clone, MF23), V450 conjugated anti-CD11b (clone, M1/70), anti-CD62L (clone, MEL-14), anti-IgG1 (clone, A110-1), AlexaFluor 700- conjugated anti-CD3ε (clone, 145-2C11) -anti IFN-γ, V500- anti-CD4 (clone, RM4-5) and anti- B220 (clone, RA3-6B2), APC-Cy7-conjugated anti-CD19 (clone, 1D3) and anti-CD8 (clone, 53-6.7), BV786 conjugated anti-IgE (clone, R35-72) and anti-IL-33R (ST2) (clone, U29-93), biotin-CD25 (clone, 7D4) were purchased from BD Pharmingen (San Diego, CA). PE-Cyanine7 anti-F4/80 (clone, BM8), anti-IL-13 (clone, eBio13A), AlexaFluor 700- conjugated anti-MHC II (clone, M5/114), APC- conjugated anti-IL-21 (clone, FFA21) and Live/dead Fixable Yellow stain (Qdot605 dead cell exclusion dye) were purchased from eBiosciences. Biotin-labelled antibodies were detected by Texas Red

conjugated PE (BD Biosciences). For staining, cells (1×10^6) were stained and washed in PBS, 3% FCS FACS buffer. For intracellular cytokine staining, cells were restimulated with phorbol myristate acetate (Sigma-Aldrich) (50 ng/mL), ionomycin (Sigma-Aldrich) (250ng/mL), and monensin (Sigma-Aldrich) (200mM in IMDM/10% FCS) for 5h at 37°C then fixed in 2% PFA, permeabilised with Foxp3 transcriptional factor staining buffer kit (eBioscience) before intracellular staining with appropriate cytokine antibodies and acquisition through LSR Fortessa machine (BD Immunocytometry system, San Jose, CA, USA) and data was analysed using Flowjo software (Treestar, Ashland, OR, USA).

Histology

Left upper lung lobes was fixed in 4% formaldehyde/PBS and embedded in paraffin. Tissue sections were stained with periodic acid-Schiff for mucus secretion, and haematoxylin and eosin (H&E) stain for inflammation. Slides were scanned at 20x magnification on the virtual slide VS120 microscope (Olympus, Japan). Downstream processing of images was done through Image J (FIJI) for image extraction at series 15 and Ilastik software was used for mucus area quantification on whole lung sections. Data shown is from 1 experiment from at least 3 independent experiments (n = 5-7 mice per experiment).

Antibody and cytokine ELISAs

Antibody ELISAs were carried out as previously described²⁶ using 5 µg/ml HDM to coat for specific IgGs. Total IgE in serum was measured using anti-mouse IgE (BD Biosciences, 553413) to coat, mouse IgE (κ, anti-TNP, BD Biosciences, 557079) as standard and biotin anti-mouse IgE (BD Biosciences, 553419) as secondary antibody.

For *in vitro* cytokine production analysis, single cell suspensions were prepared from mediastinal lymph nodes of HDM-treated and littermate control mice. Cells (2×10^5 cells, in 200 μ L) were incubated for 5 days in IMDM/10% FCS (Delta Bioproducts, Kempton Park, South Africa) in 96-well plates. Cells were either stimulated with HDM (30 μ g/mL) or anti-CD3 (10 μ g/mL) and supernatants were collected after a 5-day incubation period. Concentrations of IL-4, IL-5 (BD Biosciences) and IL-13 (R&D Systems, Minneapolis, Minn), were measured using ELISA assays according to the manufacturer's protocol.

Statistical analysis

P-values were calculated in GraphPad Prism 6 (GraphPad Software, Inc) by using nonparametric Mann-Whitney Student's *t*-test or Two-way ANOVA with Bonferroni's post-test for multiple comparisons, and results are presented as standard error of the mean (SEM) or mean of standard deviation (SD). Differences were considered significant if *P* was <0.05.

RESULTS

IL-4R α -responsive B cells are not essential in high dose HDM induced allergic asthma.

The role of B cells in asthma is controversial^{9,14} and recent evidence suggested that the load of antigen is crucial in influencing the role of B cells¹⁴. We used a standard high dose of 100 μ g HDM to sensitise mice at day 0 and challenged with a reduced dose of 10 μ g in days 8 to 12^{34,35} (Figure 1, A). Firstly, we showed that at both steady state and during HDM challenge, there was reduced IL-4R α expression in both lung and mediastinal lymph nodes (mLNs) in mice lacking IL-4R α on B cells (mb1^{cre}IL-4R α ^{-/lox}) when compared to littermate (IL-4R α ^{-/lox}) control mice or IL-4R α -deficient mice (Suppl. Figure 1, A-B). We found that mice lacking IL-4R α on B cells had a moderately reduced airway resistance and elastance when compared to littermate mice sensitised and challenged with high dose HDM (Figure 1, B).

We then measured cellular infiltrates within the lung tissue after HDM challenge and observed a comparable increase in total cellular infiltration, which was mainly represented by eosinophils in both mb1^{cre}IL-4R α ^{-/lox} and littermate mice challenged with HDM (Figure 1, C and Suppl. Figure 1C). We then measured type 2 cytokines produced by CD4 T cells in the lung after stimulation with phorbol myristate acetate (PMA)/ionomycin for 5 hours. We observed increased but comparable levels of CD4 T cells producing IL-4, IL-5 and IL-13 in both mb1^{cre}IL-4R α ^{-/lox} and littermate mice challenged with high dose HDM (Figure 1, D). Levels of CD4 T cells producing IL-4, IL-5 and IL-13 were low in mb1^{cre}IL-4R α ^{-/lox} and littermate control IL-4R α ^{-/lox} mice challenged with phosphate buffered saline (PBS) when compared to high dose HDM challenged mice of the same genotype (Figure 1, D). We also observed no differences in CD4 T cell numbers or IFN- γ -producing CD4 T cells in all mutants challenged with high dose HDM (Suppl. Figure 1, D). We observed significantly higher total

IgE production and HDM-specific IgG1 titres in IL-4R α ^{-/-lox} mice when compared to mb1^{cre}IL-4R α ^{-/-lox} challenged with high dose HDM (Figure 1, E). We observed no differences in mucus production and inflammation between mb1^{cre}IL-4R α ^{-/-lox} and IL-4R α ^{-/-lox} mice (Figure 1, F). Overall, these results demonstrated that IL-4R α on B cells plays a minimal role in the development of allergic asthma after a challenge with high dose HDM.

IL-4R α -responsive B cells play an essential role in low dose HDM induced allergic asthma.

B cell-deficient mice (μ MT^{-/-}) showed increased eosinophilic airway inflammation when challenged with high dose HDM, comparable to that observed in wild type mice, even under chronic challenges^{14,36}. Titration of HDM below 3 μ g reduced influx of eosinophils, proliferation of Derp-1-specific T cells and type 2 cytokine production when compared to wild type mice¹⁴. We then used this low dose HDM sensitisation and challenge protocol to assess whether type 2 airway inflammation depended on the dose of inhaled HDM (Figure 2, A). We found robust differences in airway resistance and elastance in mb1^{cre}IL-4R α ^{-/-lox} sensitised and challenged with low dose HDM when compared to littermate IL-4R α ^{-/-lox} mice (Figure 2, B). Mb1^{cre}IL-4R α ^{-/-lox} and IL-4R α ^{-/-lox} mice challenged with saline had similarly low levels of resistance and elastance compared to HDM exposed mice (Figure 2, B). We then analysed total lung infiltrate and found significant increase in total cells and eosinophils in IL-4R α ^{-/-lox} littermates compared to mb1^{cre}IL-4R α ^{-/-lox} or global IL-4R α -deficient mice challenged with low dose HDM (Figure 2, C). We did not observe any changes in neutrophil numbers when comparing low dose HDM challenged mice and control mice that were challenged with saline (Figure 2, C). We then analysed type 2 cytokine production by CD4 T cells in the lung and found significant increase in percentages and number of CD4 T cells producing IL-4, IL-5 and IL-13 in IL-4R α ^{-/-lox} littermate mice when compared to mb1^{cre}IL-4R α ^{-/-lox}

$^{-/lox}$ and IL-4R $\alpha^{-/-}$ mice sensitised and challenged with low dose HDM (Figure 2, D and Suppl. Figure 2, A and B). We also found similar trends of reduced TH2 cytokines levels in IL-4R α B cell-deficient mLN stimulated for 5 days with anti-CD3 (Suppl. Figure 3). There were low number of cytokine producing CD4 T cells in both mb1^{cre}IL-4R $\alpha^{-/lox}$ and littermate control mice sensitised and challenged with saline (Figure 2, D). We analysed lung tissue for signs of inflammation and stained for mucus producing cells (Figure 2, E). We found similar levels mucus area in both mb1^{cre}IL-4R $\alpha^{-/lox}$ and IL-4R $\alpha^{-/lox}$ littermate control mice and there were no detectable mucus producing cells in control mice challenged with saline (Figure 2, E). Overall these results demonstrate that at low dose HDM exposure, IL-4R α on B cells contributes significantly to the development of allergic asthma and Th2-type lung inflammation.

IL-4R α -responsive B cells are important for accumulation of germinal centre B cells and T follicular helper cells in secondary lymphoid tissue at low antigen load.

B cells have been shown to be important in the development of T follicular cells (T_{FH}) and these T_{FH} acted as precursors for IL-4/IL-13 committed CD4 T cells that migrated to the lung to recruit eosinophils and caused disease¹³. Firstly, we measured percentages and number of B cells in the mLN and found frequencies to be intact (Suppl. Figure 4, A). However, total numbers were significantly reduced when comparing mb1^{cre}IL-4R $\alpha^{-/lox}$ to IL-4R $\alpha^{-/lox}$ littermate mice and global IL-4R α -deficient mice (Figure 3, A). We then compared germinal centre (GC) B cells in mLN and found comparably low levels between mb1^{cre}IL-4R $\alpha^{-/lox}$ and IL-4R $\alpha^{-/lox}$ littermate control mice (Figure 3, B). We observed significantly higher percentages (represented by high expression of GL7 and FAS) and numbers of GC B cells in IL-4R $\alpha^{-/lox}$ littermate mice when compared to mb1^{cre}IL-4R $\alpha^{-/lox}$ mice challenged with low dose HDM

(Figure 3, B). We did not observe major changes in frequencies of follicular B cells (Suppl. Figure 4, B), but observed significantly increased frequencies of marginal zone B cells (Suppl. Figure 4, B) in $mb1^{cre}IL-4R\alpha^{-/lox}$ when compared to $IL-4R\alpha^{-/lox}$ littermate mice challenged with low dose HDM, probable as a compensatory mechanism to increase non-GCs antibody production. We then looked for T_{FH} of which we know B cells play a crucial role in their development particularly at low dose HDM. We found significantly reduced frequencies and numbers of T_{FH} (represented by high expression of PD-1 and CXCR5) in mLN of $mb1^{cre}IL-4R\alpha^{-/lox}$ and $IL-4R\alpha^{-/-}$ when compared to $IL-4R\alpha^{-/lox}$ littermate controls (Figure 3, C). To understand whether these T_{FHs} could be contributing to effector TH2 cells in the lung, we analysed IL-21 intracellular levels produced by CD4 T cells in the lung³⁷. We found significantly reduced frequencies and numbers of IL-21 producing T cells in $mb1^{cre}IL-4R\alpha^{-/lox}$ and $IL-4R\alpha^{-/-}$ when compared to $IL-4R\alpha^{-/lox}$ littermate controls (Figure 3, D and Suppl. Figure 2, A-B). Our data suggested that IL-4R α -responsive B cells in secondary lymphoid tissues are important for the accumulation of GC B cells and development of T_{FH} cells, which might be contributing to overall TH2 cells.

IL-4R α -responsive B cells are required for optimal T helper 2 airway responses and antibody production.

Effector B cells producing type 2 cytokines (Be2) have been shown to be important during parasitic infections and early expression of IL-4 by these B cells promotes differentiation of type 2 CD4 T cells^{28,30,38}. We measured cytokine production by effector B cells and found increased frequencies and numbers of mediastinal lymph node B cells producing IL-5 in $IL-4R\alpha^{-/lox}$ littermate mice when compared to $mb1^{cre}IL-4R\alpha^{-/lox}$ and $IL-4R\alpha^{-/-}$ mice challenged with low dose HDM (Figure 4, A-B and Suppl. Figure 4, C). We also observed similar

reduction in number of IL-13 producing B cells in $mb1^{cre}IL-4R\alpha^{-/lox}$ and $IL-4R\alpha^{-/-}$ when compared to $IL-4R\alpha^{-/lox}$ littermate mice challenged with low dose HDM (Suppl. Figure 4, D). We then measured total serum IgE and HDM-specific IgG1 by ELISA and found significantly reduced titres in $mb1^{cre}IL-4R\alpha^{-/lox}$ and $IL-4R\alpha^{-/-}$ when compared to $IL-4R\alpha^{-/lox}$ littermate mice challenged with low dose HDM (Figure 4, C). We analysed IgE and IgG1 surface expression by B cells using flow cytometry. We found significantly reduced levels of IgE and IgG1 expression in B cells when comparing $mb1^{cre}IL-4R\alpha^{-/lox}$ to $IL-4R\alpha^{-/lox}$ littermate mice (Figure 4, D). B and T cell engagement through CD86 and CD28 in T cell zones is essential for T_{FH} generation and class switching to IgE³⁹. We measured CD86 and other co-stimulatory molecule on the surface of B cells and found reduced expression of CD86, but not CD80 and MHCII (Figure 4, E, Suppl. Figure 5A-B), which may suggest an incomplete T cell engagement via CD28 and explain lack of class switching. Thus far, our data suggested that IL-4R α signalling on B cells is essential for Be2 function and class switching to IgE and this contributes to overall TH2 responses.

IL-4R α -responsive B cells are essential in type 2 airway inflammation during effector phase.

Previous studies had suggested that B cells were important in T_{FH} development at sensitisation stage, but not at effector stage and played minimal role in disease if adoptively transferred after sensitisation¹³. We asked whether IL-4R α -responsive B cells were only important at sensitisation stage. We sensitised B cell deficient $\mu MT^{-/-}$ mice with low dose HDM and a day before challenge, we transferred naïve B cells either sufficient or lacking IL-4R α (Figure 5, A). We found that $\mu MT^{-/-}$ with B cells from $mb1^{cre}IL-4R\alpha^{-/lox}$ had significantly reduced resistance and elastance when compared to $\mu MT^{-/-}$ receiving B cells from $IL-4R\alpha^{-/lox}$

littermate mice (Figure 5, B). This reduced AHR in $mb1^{cre}IL-4R\alpha^{-/lox}$ mice was accompanied by reduced eosinophil recruitment in the lung, but not total cells or B cell numbers (Figure 5, C). We then analysed CD4 T cell numbers that were producing type 2 cytokines and found significantly reduced IL-5 producing CD4 T cells in the lung of $\mu MT^{-/-}$ mice that received $mb1^{cre}IL-4R\alpha^{-/lox}$ B cells when compared to $\mu MT^{-/-}$ mice receiving $IL-4R\alpha^{-/lox}$ B cells (Figure 5, D). We then measured total IgE by ELISA and found significantly increased IgE in $\mu MT^{-/-}$ mice that received $IL-4R\alpha^{-/lox}$ B cells compared to $\mu MT^{-/-}$ mice that received $mb1^{cre}IL-4R\alpha^{-/lox}$ B cells (Figure 5, E). We observed comparable mucus area between $\mu MT^{-/-}$ mice receiving $IL-4R\alpha^{-/lox}$ B cells or $mb1^{cre}IL-4R\alpha^{-/lox}$ B cells (Figure 5, F). No mucus producing cells were detected in control mice challenged with saline (Figure 5, F). Our findings suggested that IL-4R α signalling on B cells was also essential at effector phase for optimal TH2 airway responses.

IL-4/IL-13 producing B cells contribute to airway hyperresponsiveness but not inflammation.

We then investigated whether the production of type 2 cytokines by these B cells is essential for allergic airway inflammation. We adoptively transferred naïve B cells either sufficient or deficient of IL-4 or double deficient of IL-4/IL-13 into $\mu MT^{-/-}$ mice, sensitised and challenged with low dose HDM (Figure 6, A). We measured lung function and found both resistance and elastance to be significantly reduced in $\mu MT^{-/-}$ mice that received IL-4-deficient or IL-4/IL-13-double deficient B cells when compared to $\mu MT^{-/-}$ mice that received WT B cells (Figure 6, B). We then measured cellular infiltrate in the lung and found no major changes in total lung infiltrate, eosinophils and CD4 T cells in all recipient mice (Figure 6, C).

The number of B cells were lower in $\mu\text{MT}^{-/-}$ mice that were adoptively transferred with B cells from various strains compared to control mice, however, no differences were observed between mice that were challenged with a low dose of HDM (Figure 6, C). Total IgE measured by ELISA was not changed between groups of $\mu\text{MT}^{-/-}$ mice adoptively transferred with B cells, sensitised and challenged with low dose HDM (Figure 6, D). We also did not observe any changes in type 2 cytokines produced by CD4 T cells in the absence of type 2 cytokines produced by B cells when comparing $\mu\text{MT}^{-/-}$ mice that received WT B cells to those that received IL-4-deficient or IL-4/IL-13-double deficient B cells (Figure 6, E). Taken together, these results suggested that although B cells producing type 2 cytokines are essential in airway hyperresponsiveness, they play minimal role in airway inflammation.

DISCUSSION

B cells secrete IgE are important in activating mast cells and basophils degranulation, which initiates a cascade of inflammatory signals. However, contradictory findings on the requirement of B cells exists in studies using mice lacking B cells. More recent evidence suggested that antigen load determines the importance of B cells, particularly in interactions with T helper cells and generation of T follicular helper cells. IL-4 is critical in class switching of B cells to generate IgE⁴⁰, however, whether signalling through the IL-4R α on B cells is required for generation of T_{FH} cells and IgE have not been investigated in the context of allergic asthma. Here, we showed that IL-4R α responsive B cells are important mainly when the load of HDM antigen is limiting. We further showed that IL-4R α responsive B cells regulate AHR, IgE secretion, T_{FH} cells generation and that B cells derived type 2 cytokines are required for optimal TH2 responses.

We first challenged mice with high dose HDM and found IL-4R α responsive B cells to be important in AHR and IgE production, but not for eosinophil recruitment or type 2 cytokine production. This is consistent with previous studies where B cell-deficient mice showed similar levels of eosinophilia and type 2 cytokines when sensitised and challenged with high dose HDM¹⁴. B cell-deficient mice have significantly increased AHR at high dose HDM, a phenotype different to what we observe in IL-4R α -deficient B cells, which suggested that IL-4R α responsive B cells may be involved in driving AHR during allergic asthma. Differences in diseases susceptibility is also observed between μ MT^{-/-} mice and mb1^{cre}IL-4R α ^{-/lox} mice during chronic *Schistosoma mansoni* infection, which is attributed to differences in immunomodulatory IL-10 production seen in μ MT^{-/-} mice, but not in mb1^{cre}IL-4R α ^{-/lox} mice^{28,29}.

Similarly to what was reported by Dullaers et al.¹⁴, $mb1^{cre}IL-4R\alpha^{-/lox}$ mice that were sensitised with 1 μ g HDM and challenged with 3 μ g HDM displayed a great reduction in AHR, eosinophil recruitment and type 2 cytokine production compared to littermate $IL-4R\alpha^{-/lox}$ mice. We also observed significant reduction in GC B cells, T_{FH} cells, but no changes in follicular and slight increase in marginal zones B cells, probable as a compensatory mechanism to increase non-GCs antibody production. The ability of B cells to produce type 2 cytokines was also significantly reduced, suggesting that $IL-4R\alpha$ B cells help contribute to the overall type 2 immune response output. This is consistent with previous studies, where $IL-4R\alpha$ responsiveness by B cells is crucial in early IL-4 production in mLN, defining a dichotomy in subsequent CD4 T helper cells differentiation^{28–30,38}. B cells receive IL-4 signal from CD4 T cells in GC to initiate class switching to produce IgE, which is likely to be dependent on $IL-4R\alpha$ signalling on B cells^{33,41}. Although, IgE can directly be generated from IgM particularly with low antigen load⁴², we think that in our model, lack of $IL-4R\alpha$ signalling on B cells lead to reduced sequential class switching from IgM to IgG1 and to IgE. Sequential class switching occurs in the GCs and results in high affinity plasma cell IgE^{41,43,44}. These high affinity IgE plasma cells are direct precursors of IgG1 plasma cells as they share similar CDR3 repertoire in the context of helminth infections, skin cancers and alum/OVA induced asthma^{44,45}. In human B cells from the tonsil, $IL-4R\alpha$ signalling is required for GC maintenance and generation of high affinity IgE BCRs that select for plasma cell compartments^{46,47}.

There has been contrasting evidence regarding whether B cells are important during the sensitisation phase, effector phase or in both phases of allergic response^{13,15}. B cells were found to be critical in shaping IL-4 committed T_{FH} cells during HDM sensitisation stage which contributed to effector TH2 pool during challenge stages. However, blocking of T_{FH} cells with BLC6 inhibitor after sensitisation stage did not reduce TH2 allergic airway inflammation,

which was attributed to redundant role of B cells at this stage. This is in contrast with recent findings where blocking B cells with anti-CD20 before HDM challenge, significantly reduced TH2 airway responses¹⁵. To consolidate these findings, we transferred IL-4R α -deficient B cells after HDM sensitisation and before challenge. Our data showed that IL-4R α -responsive B cells are required at the challenge stage, as we observed reduced AHR, TH2 cytokines and total IgE when we transferred B cells lacking IL-4R α into recipient mice. Both previous studies had used a similar dose of 20 μ g of HDM to sensitise and challenge and we used 3 μ g of HDM to challenge. It is likely that the choice of method used to target B cells or their function might be a major contributing factor between the two studies and not necessarily the load of HDM antigen. In our studies, we transferred naïve B cells a day before challenge, whereas Ballestos-Tato et al.¹³ had blocked T_{FH} cells using BLC6 inhibitor and Wypych et al.¹⁵ targeted B cells using anti-CD20 monoclonal antibody. All in all, our data demonstrated that IL-4R α on B cells is important in TH2 allergic asthma at both sensitisation and challenge stages and contributes to overall TH2 responses when the antigen load is limited.

Previous studies have shown a controversial role of IL-21 in T_{FH}s that eventually developed into committed TH2 cells. Ballesteros-Tato et al.¹³ suggested that lung TH2 cells were direct descendants of IL-21⁺Bcl6⁺ T_{FH} cells and developed 6 days after multiple sensitisation with 25 μ g of HDM exposure. In contrast, Coquet et al.³⁷ found that IL-21⁺ T_{FH} cells did not differentiate efficiently into ST2⁺ TH2 cells and migrated into the lung without all key features of T_{FH} cells such as CXCR5 expression. This idea was recently supported by Tibbitt et al.⁴⁸, where a trajectory single cell analysis of differentiating TH2 cells up until day 10, suggested that naïve CD4 T cells acquired many features of T_{FH} cells but did not express Bcl6 or CXCR5 which suggested that TH2 cells did not descend directly from GC T_{FH} cell

precursors. In our study, the absence of IL-4R α signalling on B cells resulted in reduced IL-21 production in the lung, which might explain reduced GCs and TH2 cells. Appropriate experiments to answer this complex function of IL-21 in TFH cells that commit to TH2 cells are needed and should employ a double (Bcl6 and IL-21) or triple (Bcl6, IL-21 and IL-4) reporter transgenic mouse or a fate reporter transgenic mouse that can trace naïve CD4 T cells as they differentiate into intermediate and committed TH2 cells in multiple tissues.

B effector 2 (Be2) cells producing IL-4 or IL-13 have been shown to be important in worm expulsion or in *Leshmania major* diseases susceptibility^{28-30,38,49}. These Be2 cells are dependent on IL-4 and IL-4R α and require presence of intact TH2 cells^{28,38,49}. Since we had observed that IL-4R α signalling on B cells was essential for optimal TH2 allergic airway immune responses, we then investigated whether production of cytokines by these Be2 cells was essential for optimal TH2 immune responses. We transferred B cells from IL-4 or IL-4/IL-13-deficient mice into μ MT^{-/-} before sensitisation. Interestingly, Be2 cells were essential for AHR, but played no role in lung eosinophil recruitment, total IgE production or type 2 cytokine production by CD4 T cells. This suggested that although the presence of IL-4/IL-13 cytokines production by Be2 cells was required for AHR, it was redundant in other parameters. It is likely that TH2 cells can compensate for the lack of IL-4/IL-13 production by B cells, however, on how TH2 cells fail to compensate for AHR is currently unclear and requires further investigation. B cells in lymph nodes secrete early IL-4 production which may be important for CD4 T cell differentiation^{28,29}. It is likely that B cells produce early IL-4 in mediastinal lymph nodes, which act in an autocrine fashion to upregulate IL-4R α , but on whether this IL-4 plays major role in CD4 TH2 differentiation, we can only speculate.

Blocking B cells with anti-CD20 before sensitisation did not affect TH2 cytokine production *ex-vivo*, but resulted in reduction in eosinophils and IFN- γ secretion¹⁵. Unfortunately, AHR was not investigated in this setting making it difficult to draw parallel conclusions regarding the function of B cells in AHR. We can speculate that other intrinsic Be2 cell mechanisms are at play in regulation AHR. B cells are known to take up HDM and present it to naïve T cells priming them to become TH2 cells both *in vitro* and *in vivo* and lack of MHCII in B cells results in reduced TH2 priming^{14,15}. IL-4R α -deficient B cells have been shown to have reduced MHC II expression and antigen uptake which contributed in reduced TH2 priming upon secondary exposure to *N. brasiliensis*, leading to increased worm burdens³⁰. We did not observe any changes in CD80 or MHCII expression on IL-4R α signalling deficient B cells when compared to IL-4R α sufficient mice (Suppl. Figure 5, A-B). However, we did observe a reduction in CD86 co-stimulatory molecule (Figure 4, E), which may suggest an incomplete T cell engagement via CD28 and reduced IgE potentiation³⁹. Our findings do not suggest antigen uptake and processing as a potential mechanism for reduced TH2 priming, but a lack of complete co-stimulatory engagement in the absence of IL-4R α signalling on B cells.

In conclusion, we showed that IL-4R α -responsive B cells play a non-redundant role in allergic asthma in an antigen load dependent manner. We further showed that IL-4R α signalling on B cells is crucial at both sensitisation and challenge stages and produce cytokines that help in optimal TH2 allergic airway responses. We further showed that Be2 cells function is only important for AHR, but redundant in eosinophilia. Our study highlighted a previously unappreciated function of IL-4R α signalling on B cells and brings evidence for targeting of this signalling axis in allergic asthma.

AUTHOR CONTRIBUTIONS

Conceived and supervised study: SH FK FB. Performed the experiments: SH SM AN MS JK FK.
Performed revision experiments: HN NM. Analysed the data: SH. Wrote the paper: SH. All
authors discussed the results and commented on the manuscript.

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FIGURE LEGENDS

Figure 1: IL-4R α -responsive B cells regulate AHR and IgE production during high dose HDM exposure, but have little impact on airway inflammation and TH2 responses

A, Schematic diagram showing sensitisation and challenge protocol where mice (mb1^{cre}IL-4R α ^{-/-lox}) and wild type littermate control (IL-4R α ^{-/-lox}) were sensitised with HDM 100 μ g intra-tracheally on days 0 and challenged with HDM 10 μ g on days 8-12. Analysis was done on day 15.

B, Airway resistance and elastance were measured with increasing doses of acetyl methacholine (0 -40 mg/mL).

C, Total lung cell numbers, eosinophil numbers neutrophil numbers and B cell numbers were stained and analysed by Flow cytometry and enumerated from % of live cells.

D, Number of lung CD4 T cells producing IL-4, IL-5 and IL-13 after 5 hr stimulation with PMA/ionomycin in the presence of monensin.

E, Total serum IgE and HDM-specific IgG1 titres measured by ELISA.

F, Histology analyses of lung sections (magnification x20), stained with Periodic Acid Schiff.

Shown is mean $\pm$ SDs from one representative experiment of 2 (n= 4-6). Significant differences between groups were performed by student t-test (Mann-Whitney) (C, D, F) or by Two way ANOVA with Benforroni post-test (B, E) and are described as: *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.

Figure 2: IL-4R α -responsive B cells are essential in optimal TH2 immune responses during low dose HDM exposure.

A, Schematic diagram showing sensitisation and challenge protocol where mice ($mb1^{cre}IL-4R\alpha^{-/lox}$) and wild type littermate control ($IL-4R\alpha^{-/lox}$) were sensitised with HDM 1 μ g intra-tracheally on days 0 and challenged with HDM 3 μ g on days 8-12. Analysis was done on day 15.

B, Airway resistance and elastance were measured with increasing doses of acetyl methacholine (0 -40 mg/mL).

C, Total lung cell numbers, eosinophil numbers and neutrophil numbers were stained and analysed by Flow cytometry and enumerated from % of live cells.

D, Number of lung CD4 T cells producing IL-4, IL-5 and IL-13 after 5 hr stimulation with PMA/ionomycin in the presence of monensin. Representative FACS plots are in shown in Figure E2.

E, Histology analyses of lung sections (magnification x20), stained with Periodic Acid Schiff.

Shown is mean $\pm$ SDs from one representative experiment of 3 (n= 6-7). Significant differences between groups were performed by student t-test (Mann-Whitney) (C, D, E) or by Two way ANOVA with Benforroni post-test (B) and are described as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3: IL-4R α signalling on B cells is essential for germinal centre formation and T_{FH} cells during low dose HDM exposure.

A, Total number of B cells in the mediastinal lymph nodes in $mb1^{cre}IL-4R\alpha^{-/lox}$, littermate control ($IL-4R\alpha^{-/lox}$) and $IL-4R\alpha^{-/-}$ mice sensitised and challenged as in Figure 2.

B, Representative flow cytometry plots of germinal centres (GCs) and numbers of GC (Live⁺B220⁺CD19⁺MHCII⁺GL7⁺FAS⁺) in the mediastinal lymph nodes in mb1^{cre}IL-4Rα^{-lox} and littermate control IL-4Rα^{-lox} mice.

C, Representative flow cytometry plots of T follicular helper cells (Live⁺CD3⁺CD4⁺CD44⁺PD-1⁺CXCR5⁺) and numbers of T_{FH} in the mediastinal lymph nodes.

D, Number of lung CD4 T cells producing IL-21 after 5 hr stimulation with PMA/ionomycin in the presence of monensin. Representative FACS plots are shown in Suppl. Figure 2.

Shown is mean ±SDs from one representative experiment of 3 (n=4-6). Significant differences between groups were performed by student t-test (Mann-Whitney) and are described as: *p< 0.05.

Figure 4: IL-4Rα signalling on B cells is essential for B effector 2 function and class switching.

A, Representative flow cytometry plots of IL-5 producing B cells (Live⁺B220⁺CD19⁺MHCII⁺IL-5⁺) in the lung of mb1^{cre}IL-4Rα^{-lox}, littermate control IL-4Rα^{-lox} and IL-4Rα^{-/-} mice.

B, Quantification of total number of IL-5 producing B cells in the lung.

C, Total serum IgE and HDM-specific IgG1 titres in mb1^{cre}IL-4Rα^{-lox}, littermate control IL-4Rα^{-lox} and IL-4Rα^{-/-} mice measured by enzyme linked immunosorbent assay.

D, Surface expression of IgE (Live⁺B220⁺CD19⁺MHCII⁺IgE⁺) and IgG1 (Live⁺B220⁺CD19⁺MHCII⁺IgG1⁺) on mediastinal lymph node B cells, represented as median fluorescent intensity.

E, CD86 surface expression on mediastinal lymph node B cells (Live⁺B220⁺CD19⁺MHCII⁺CD86⁺), represented as median fluorescent intensity.

Shown is mean $\pm$ SDs from one representative experiment of 3 (n=4-6). Significant differences between groups was performed by student t-test (Mann-Whitney) (B, D, E) or by Two way ANOVA with Benforroni post-test (E) and are described as: ***p<0.001, ****p<0.0001.

Figure 5: IL-4R α signalling on B cells is essential at effector phase of allergic asthma through regulation of AHR and TH2 airway responses

A, Schematic diagram showing sensitisation and challenge protocol where μ MT^{-/-} mice were sensitised with HDM 1 μ g intra-tracheally on days 0 and naïve B cells (live⁺B220⁺CD19⁺) from mb1^{cre}IL-4R α ^{-/lox} or IL-4R α ^{-/lox} mice (2-5 x10⁶ cells) were adoptively transferred intravenously a day before challenge with HDM 3 μ g on days 8-12. Analysis was done on day 15.

B, Airway resistance and elastance were measured with increasing doses of acetyl methacholine (0 -20 mg/mL).

C, Total lung cell numbers, eosinophil numbers and B cell numbers were stained and analysed by Flow cytometry and enumerated from % of live cells.

D, Number of lung CD4 T cells producing IL-5 after 5 hr stimulation with PMA/ionomycin in the presence of monensin.

E, Total serum IgE production from 2 independent experiments pooled together.

F, Histology analyses of lung sections (magnification x20), stained with Periodic Acid Schiff.

Shown is mean $\pm$ SEM from 2 independent experiments pooled (n=10-14). Significant differences between groups were performed by student t-test (Mann-Whitney) (C-F) by Two way ANOVA with Benforroni post-test (B) and are described as: *p< 0.05, ***p< 0.001, ****p< 0.0001

Figure 6: TH2 cytokine production by B cells is only important in regulation of AHR but not eosinophilia or TH2 airway responses.

A, Schematic diagram showing sensitisation and challenge protocol where naïve B cells (live⁺B220⁺CD19⁺) from IL-4^{-/-} or IL-4^{-/-}IL-13^{-/-} mice (2-5 x10⁶ cells) were adoptively transferred intravenously into μ MT^{-/-} mice a day before sensitisation with HDM 1 μ g intra-tracheally on days 0 and challenged with HDM 3 μ g on days 8-12. Analysis was done on day 15.

B, Airway resistance and elastance were measured with increasing doses of acetyl methacholine (0 -20 mg/mL).

C, Total lung cell numbers, eosinophil numbers and CD4 T cell numbers were stained and analysed by Flow cytometry and enumerated from % of live cells.

D, Total IgE production from 2 independent experiments pooled together.

E, Number of lung CD4 T cells producing IL-5 after 5 hr stimulation with PMA/ionomycin in the presence of monensin.

F, Histology analyses of lung sections (magnification x20), stained with Periodic Acid Schiff.

Shown is mean $\pm$ SEM from 2 independent experiments pooled (n=10-14). Significant differences between groups were performed by student t-test (Mann-Whitney) (C-F) by Two

833 way ANOVA with Benforroni post-test (B) and are described as: * $p < 0.05$, *** $p < 0.001$,

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