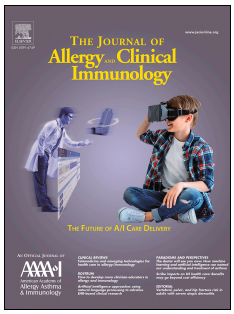

Deletion of IL-4Rα signalling on B cells limits hyperresponsiveness depending on antigen-load.

Sabelo Hadebe, PhD, Jermaine Khumalo, PhD, Sandisiwe Mangali, MSc, Nontobeko Mthembu, MSc, Hlumani Ndlovu, PhD, Martyna Scibiorek, MSc, Amkele Ngomti, BSc (Hons), Frank Kirstein, PhD, Frank Brombacher, PhD

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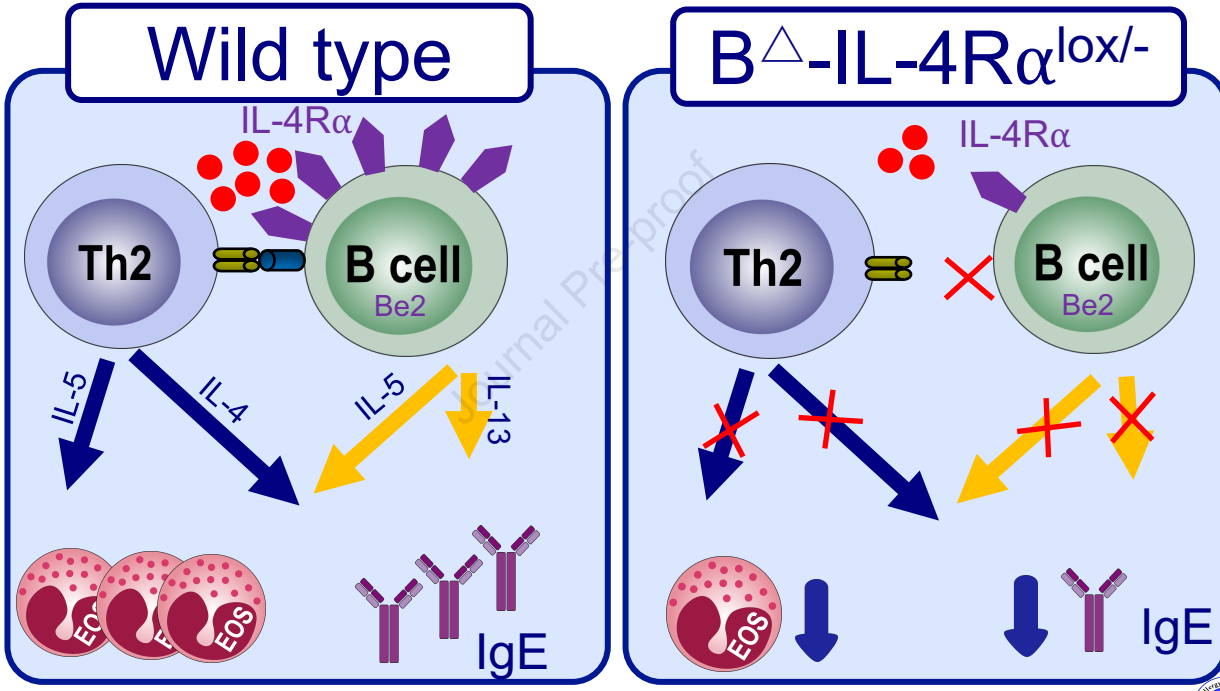
IL-4Rα on B cells is required for optimal CD4 and B cell interaction in GC



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

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

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

B, Quantification of IL-4Rα 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 $mb1^{cre}IL\text{-}4R\alpha^{-/lox}$ and littermate control $IL\text{-}4R\alpha^{-/lox}$ mice.

Shown is mean ±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-4Rα-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 ±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.

Figure E3: IL-4Rα-responsive B cells are essential in TH2 cytokine release after anti-CD3 stimulation.

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

Figure E4: B cells produce other TH2 type cytokines.

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

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

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

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

Shown is mean ±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 E5: Antigen uptake and processing is intact in IL-4Rα-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 ±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.

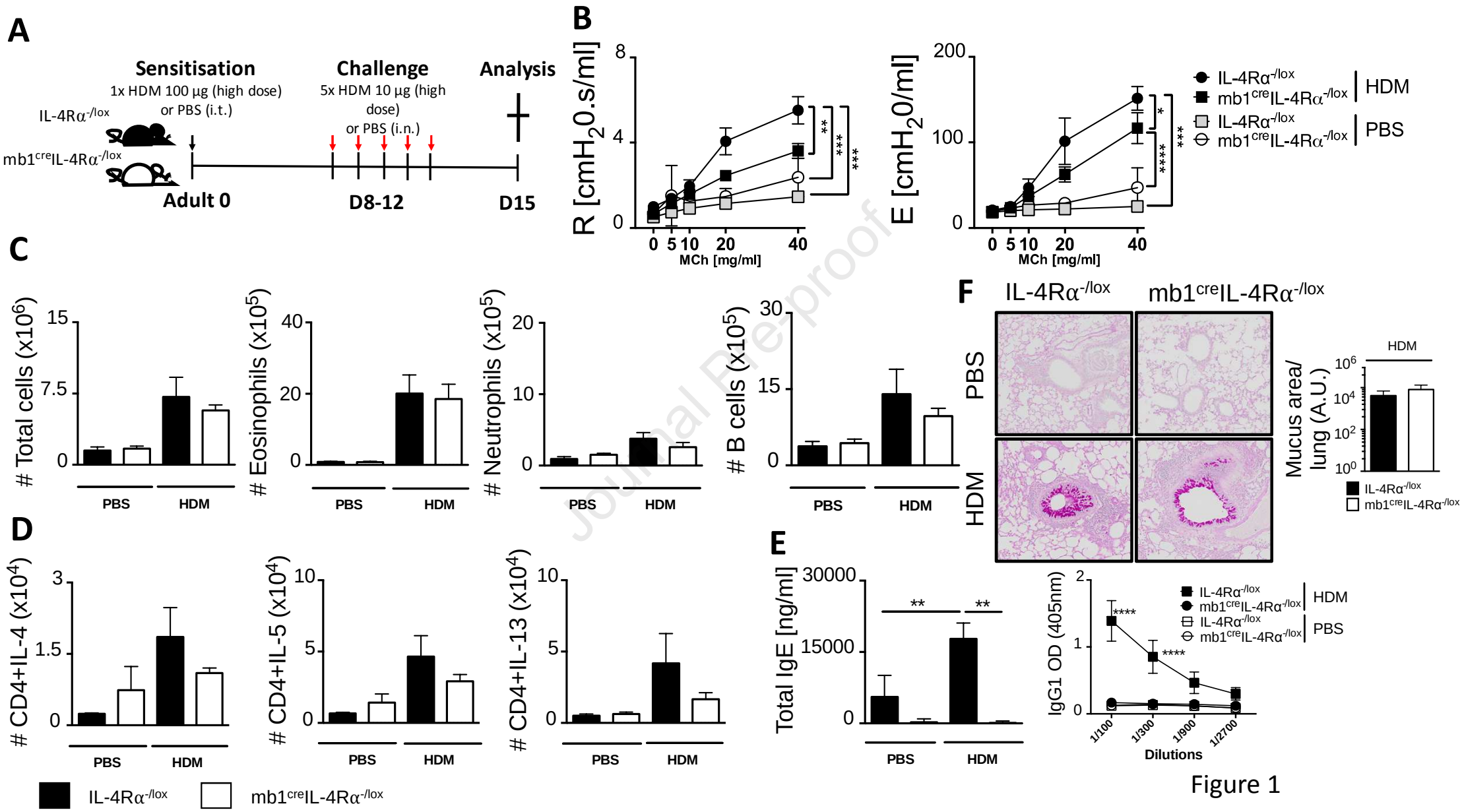

A
Sensitisation
1x HDM 100 µg (high dose)
or PBS (i.t.)
Challenge
5x HDM 10 µg (high dose)
or PBS (i.n.)
Analysis
IL-4Rα-/lox
mb1creIL-4Rα-/lox
Adult 0
D8-12
D15
B
R [cmH20.s/ml]
E [cmH20/ml]
MCh [mg/ml]
IL-4Rα-/lox
mb1creIL-4Rα-/lox
HDM
PBS
C
Total cells (x10^6)
Eosinophils (x10^5)
Neutrophils (x10^5)
B cells (x10^5)
PBS
HDM
D
CD4+IL-4 (x10^4)
CD4+IL-5 (x10^4)
CD4+IL-13 (x10^4)
IL-4Rα-/lox
mb1creIL-4Rα-/lox
E
Total IgE [ng/ml]
IgG1 OD (405nm)
Dilutions
1/100
1/300
1/900
1/2700
F
IL-4Rα-/lox
mb1creIL-4Rα-/lox
PBS
HDM
Mucus area/ lung (A.U.)

Figure 1

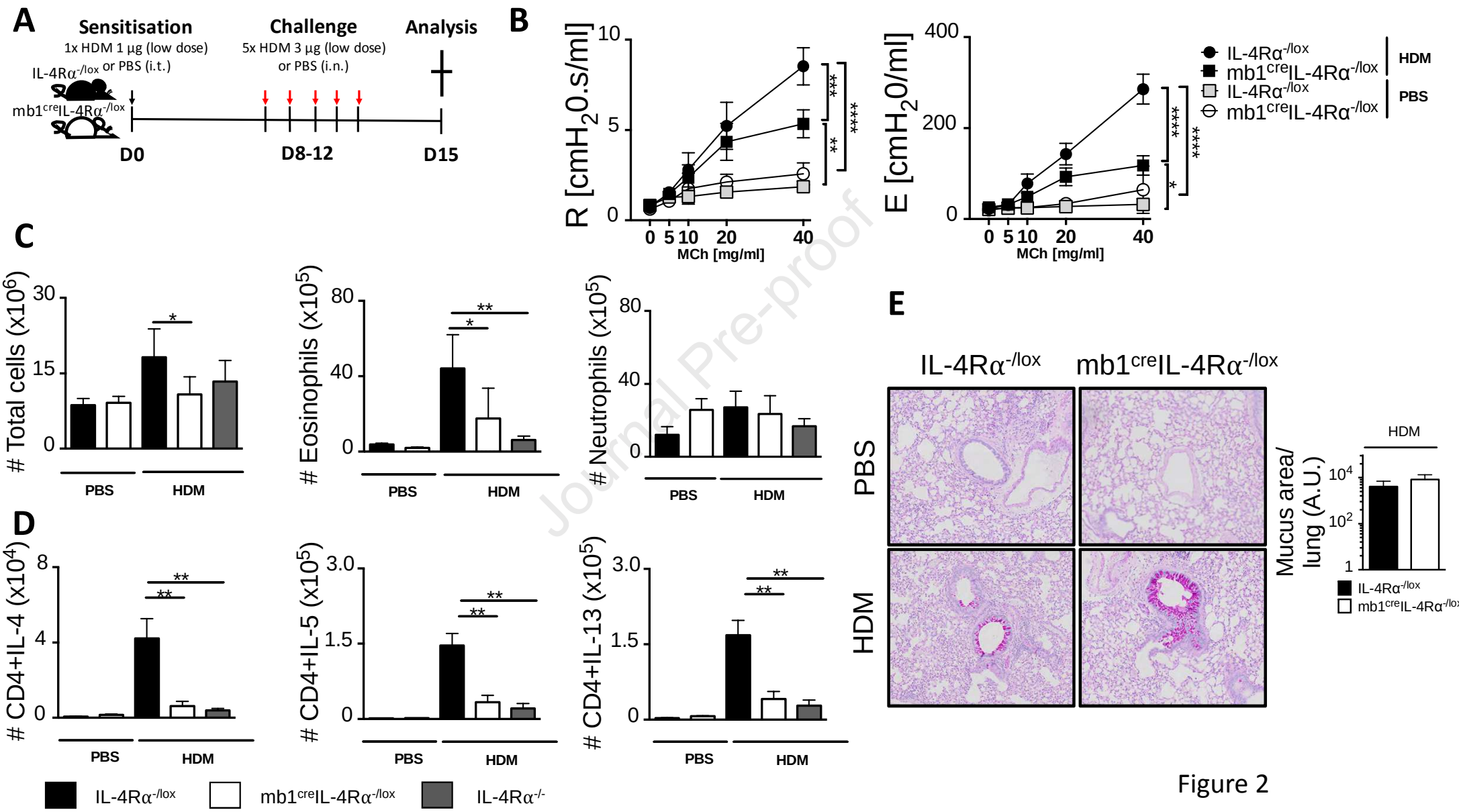

A
Sensitisation
1x HDM 1 µg (low dose)
IL-4Rα-/lox or PBS (i.t.)
mb1creIL-4Rα-/lox
Challenge
5x HDM 3 µg (low dose)
or PBS (i.n.)
Analysis
D0
D8-12
D15
B
R [cmH2O.s/ml]
E [cmH2O/ml]
MCh [mg/ml]
IL-4Rα-/lox
mb1creIL-4Rα-/lox
HDM
PBS
C
Total cells (x10^6)
Eosinophils (x10^5)
Neutrophils (x10^5)
PBS
HDM
D
CD4+IL-4 (x10^4)
CD4+IL-5 (x10^5)
CD4+IL-13 (x10^5)
IL-4Rα-/lox
mb1creIL-4Rα-/lox
IL-4Rα-/-
E
IL-4Rα-/lox
mb1creIL-4Rα-/lox
PBS
HDM
Mucus area/ lung (A.U.)

Figure 2

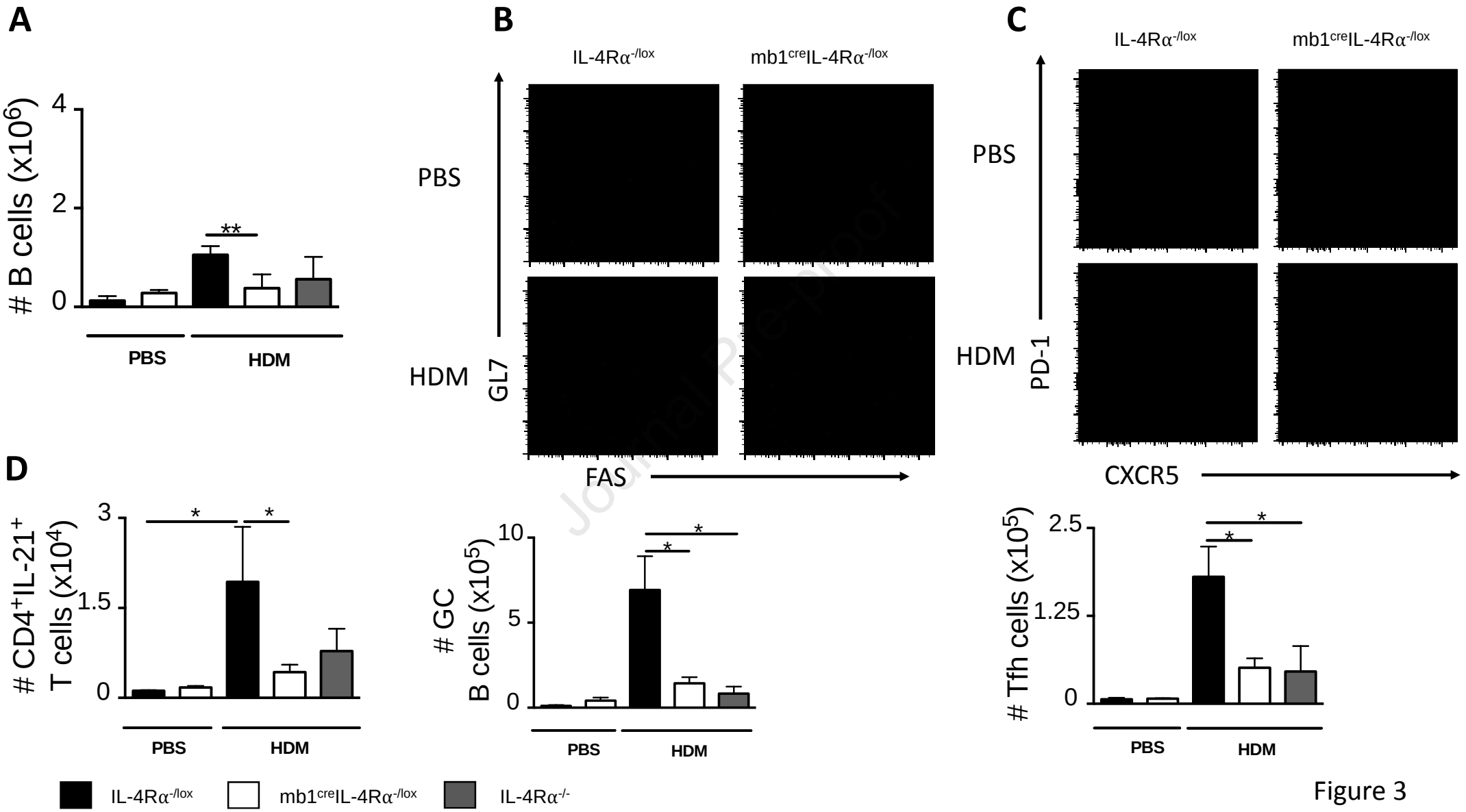


Figure 3

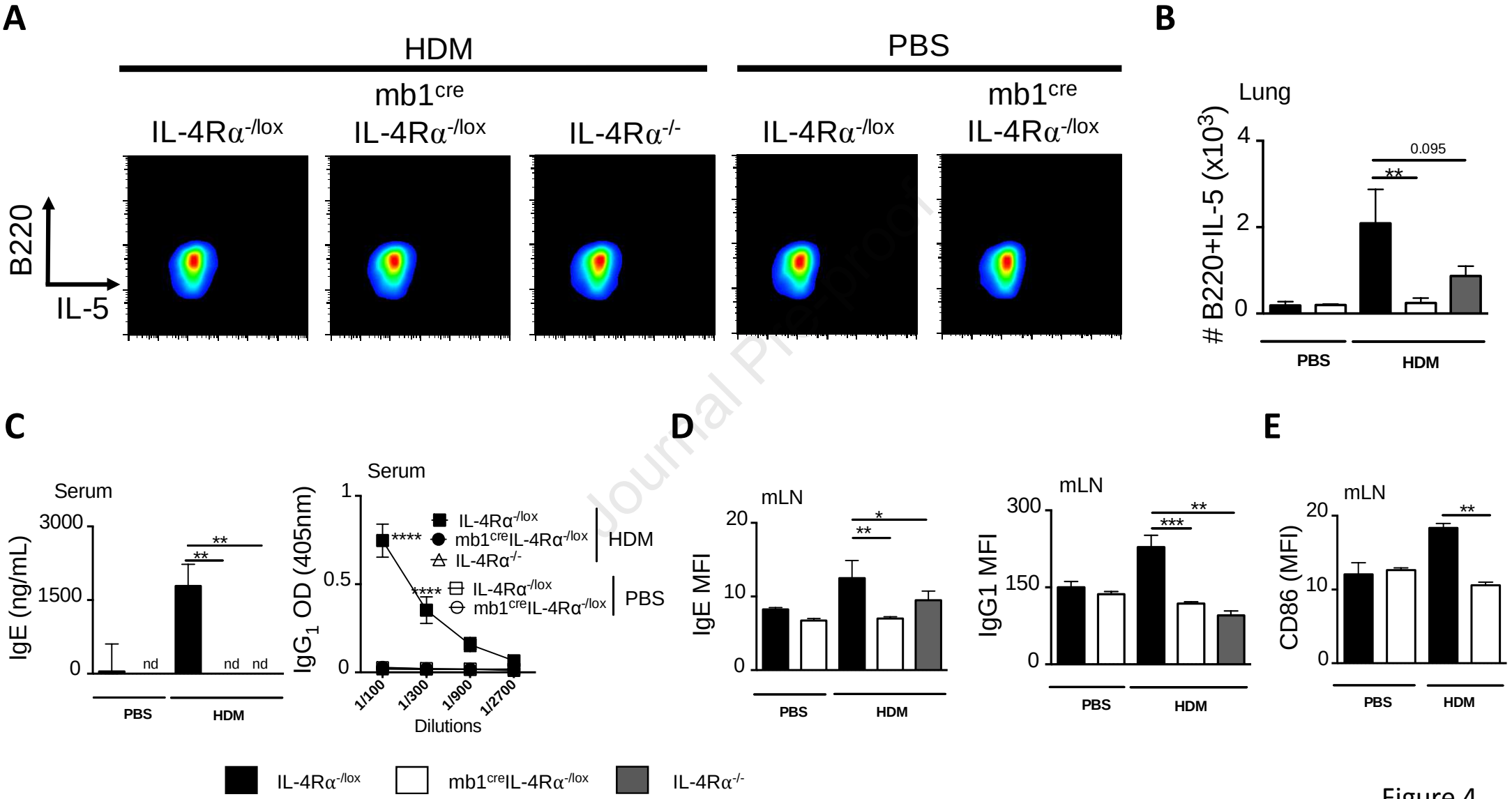


Figure 4

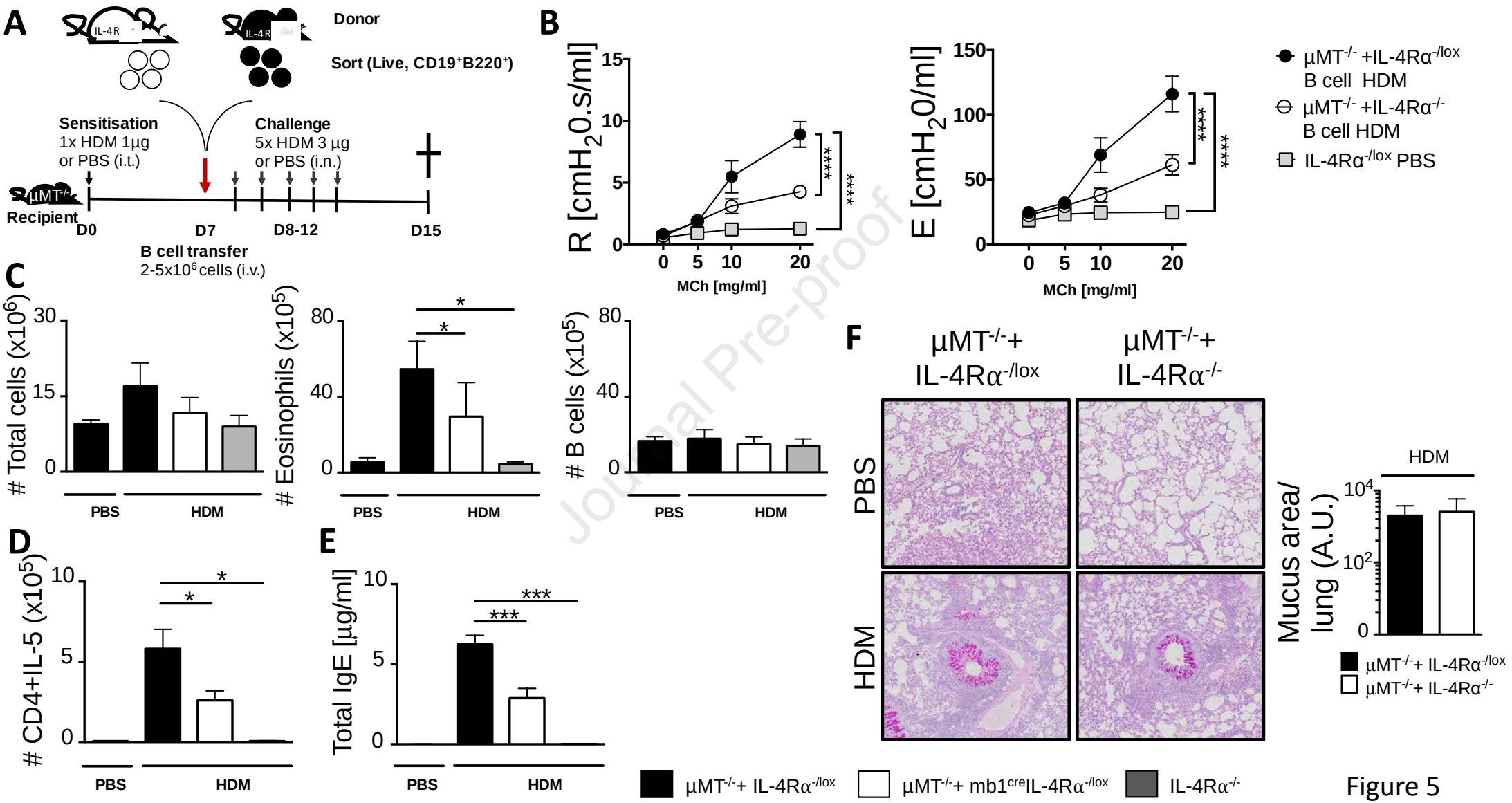


Figure 5

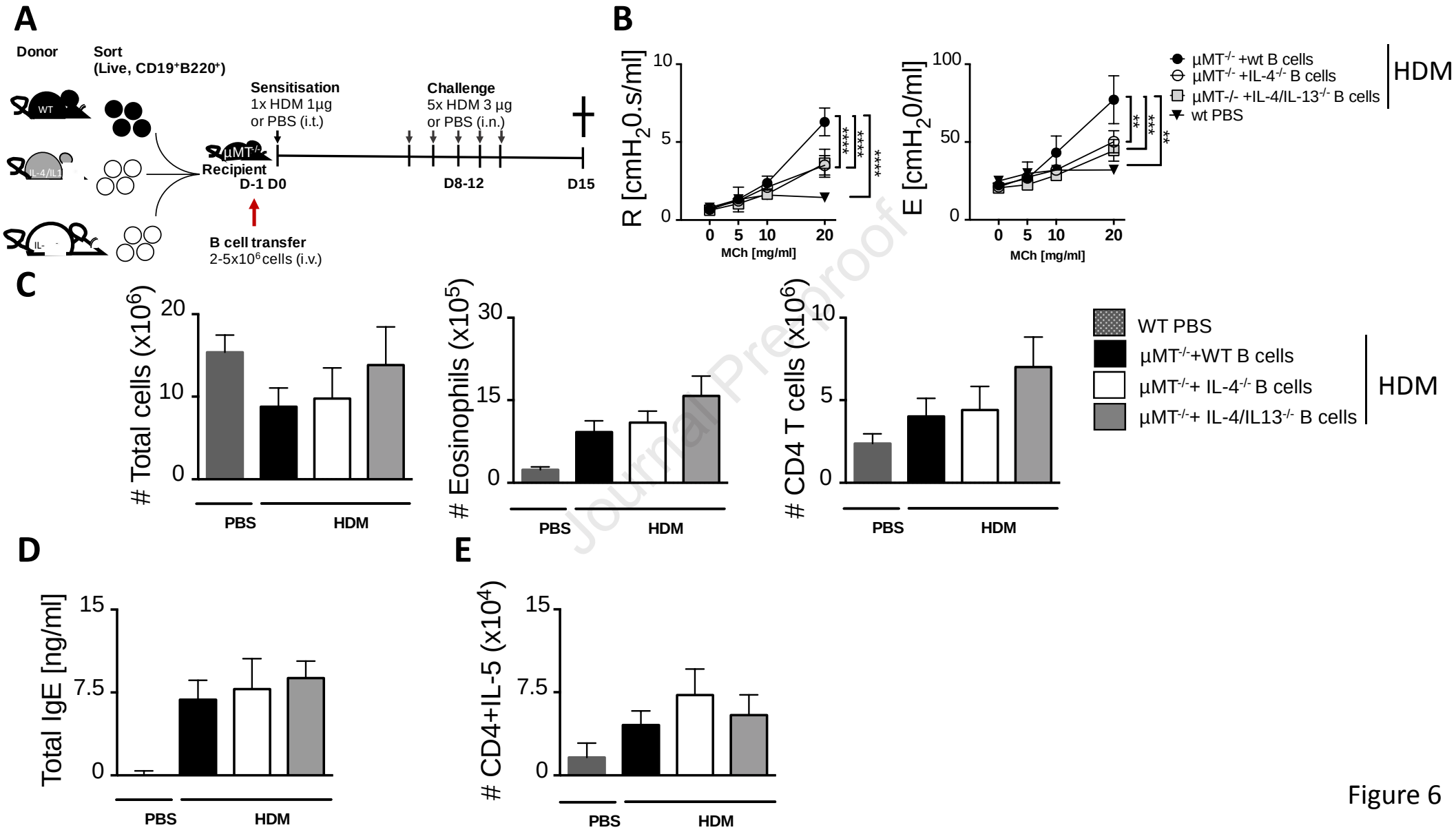

A
Donor
Sort
(Live, CD19+B220+)
WT
IL-4/IL13
IL-
μMT-/-
Recipient
D-1 D0
Sensitisation
1x HDM 1μg
or PBS (i.t.)
Challenge
5x HDM 3 μg
or PBS (i.n.)
D8-12
D15
B cell transfer
2-5x10^6 cells (i.v.)
B
R [cmH2O.s/ml]
E [cmH2O/ml]
MCh [mg/ml]
μMT-/- +wt B cells
μMT-/- +IL-4-/- B cells
μMT-/- +IL-4/IL-13-/- B cells
wt PBS
HDM
C
Total cells (x10^6)
Eosinophils (x10^5)
CD4 T cells (x10^6)
PBS
HDM
WT PBS
μMT-/-+WT B cells
μMT-/-+ IL-4-/- B cells
μMT-/-+ IL-4/IL13-/- B cells
D
Total IgE [ng/ml]
E
CD4+IL-5 (x10^4)

Figure 6

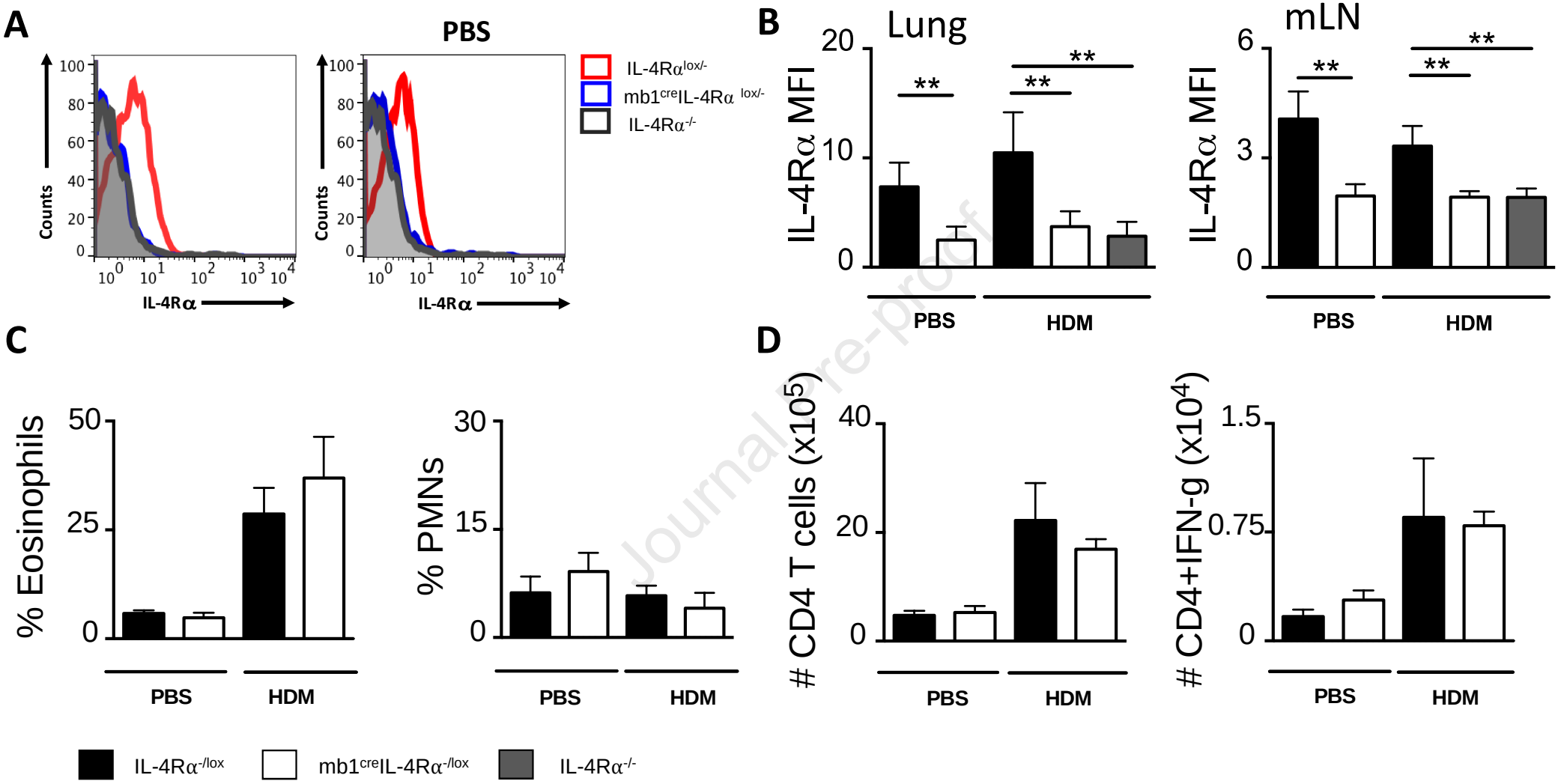


Figure E1

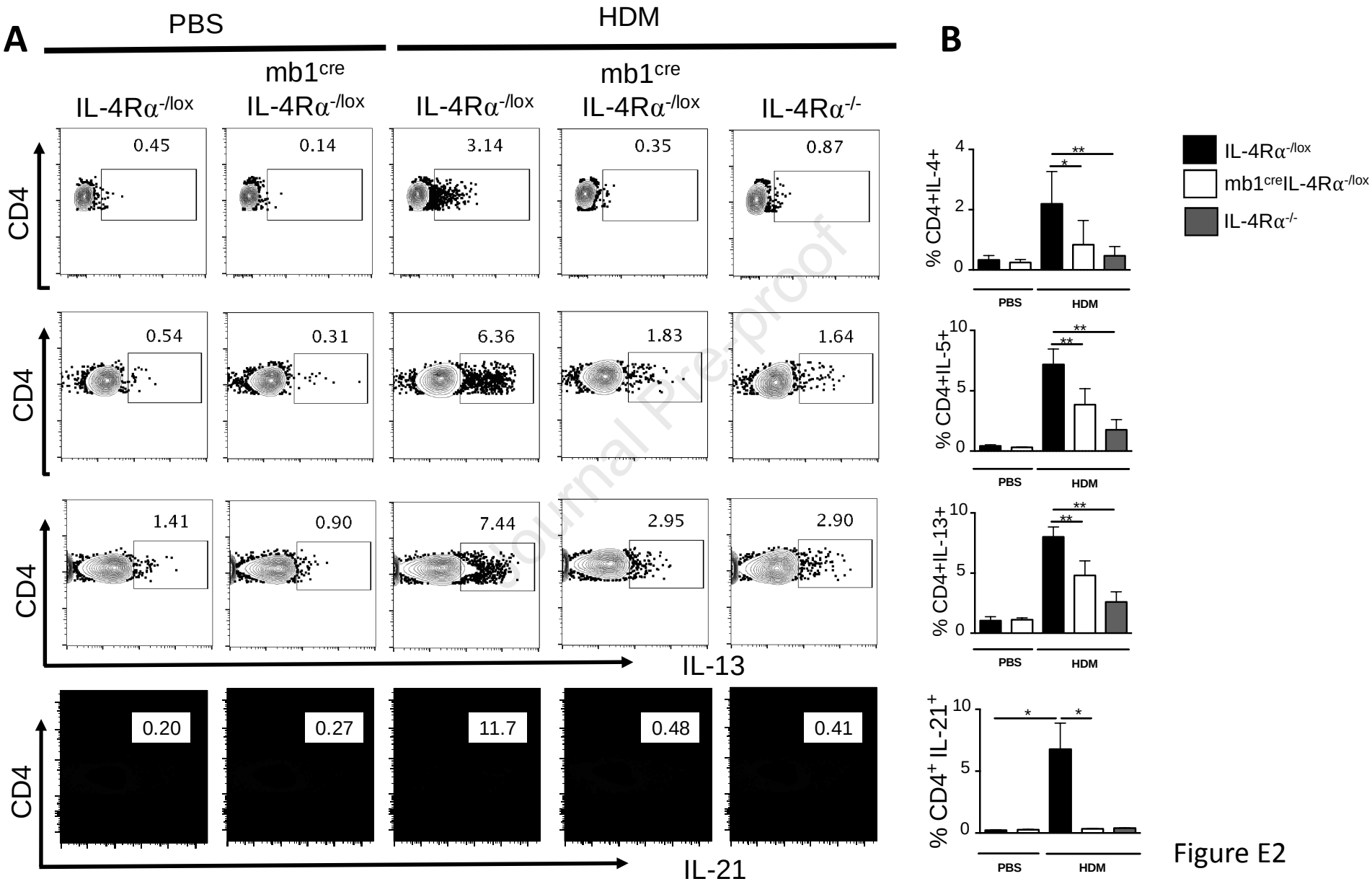


Figure E2

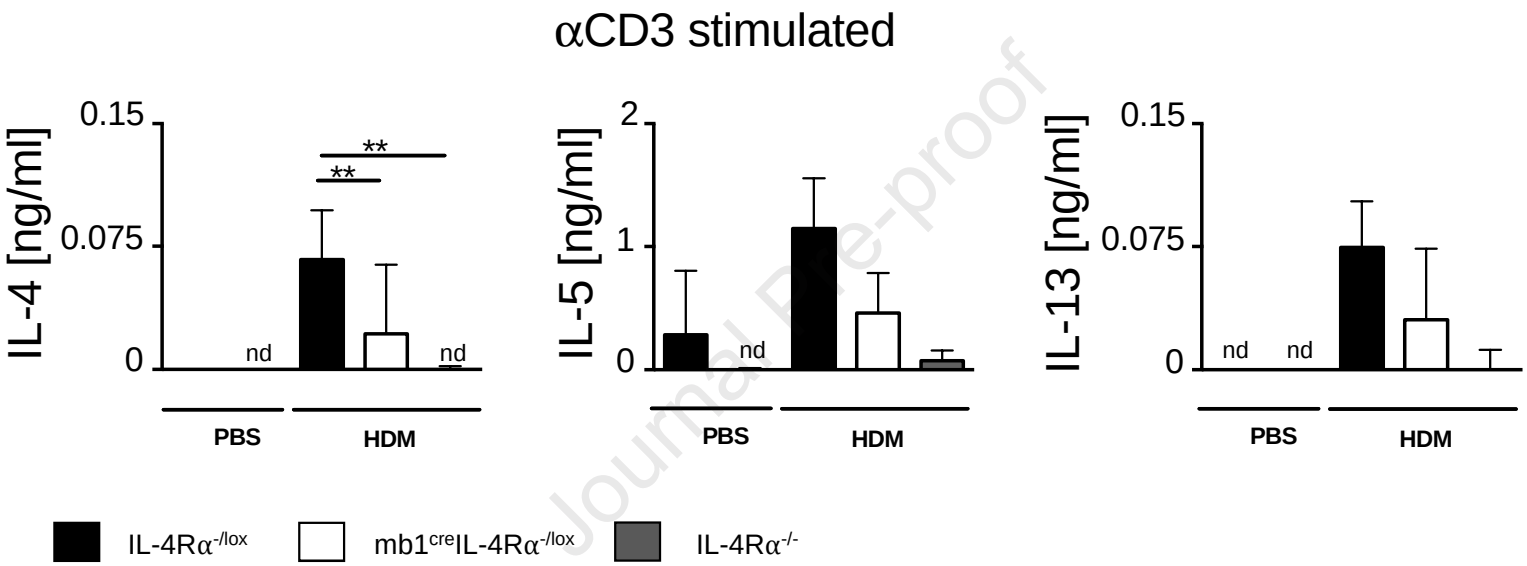


Figure E3

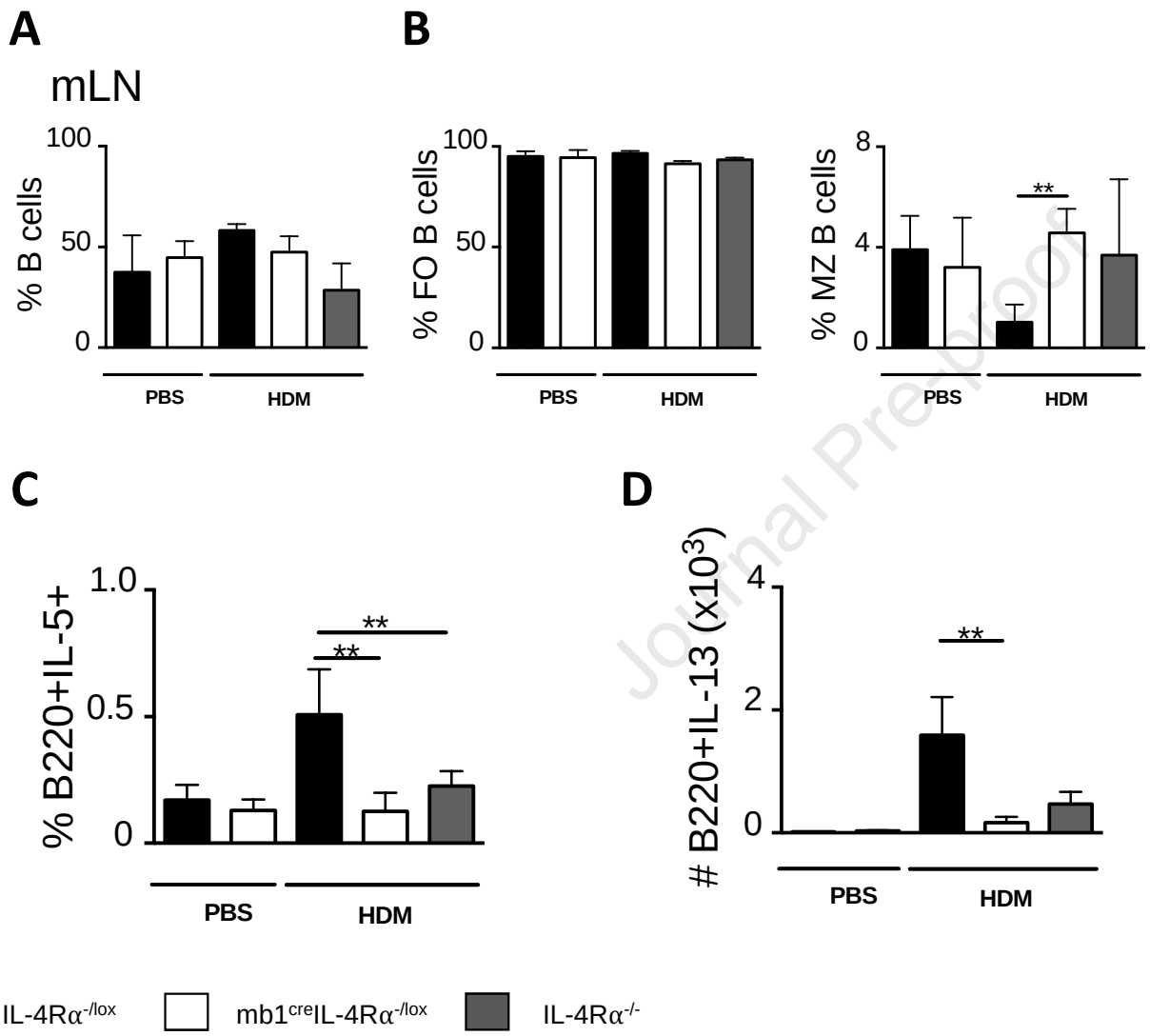


Figure E4

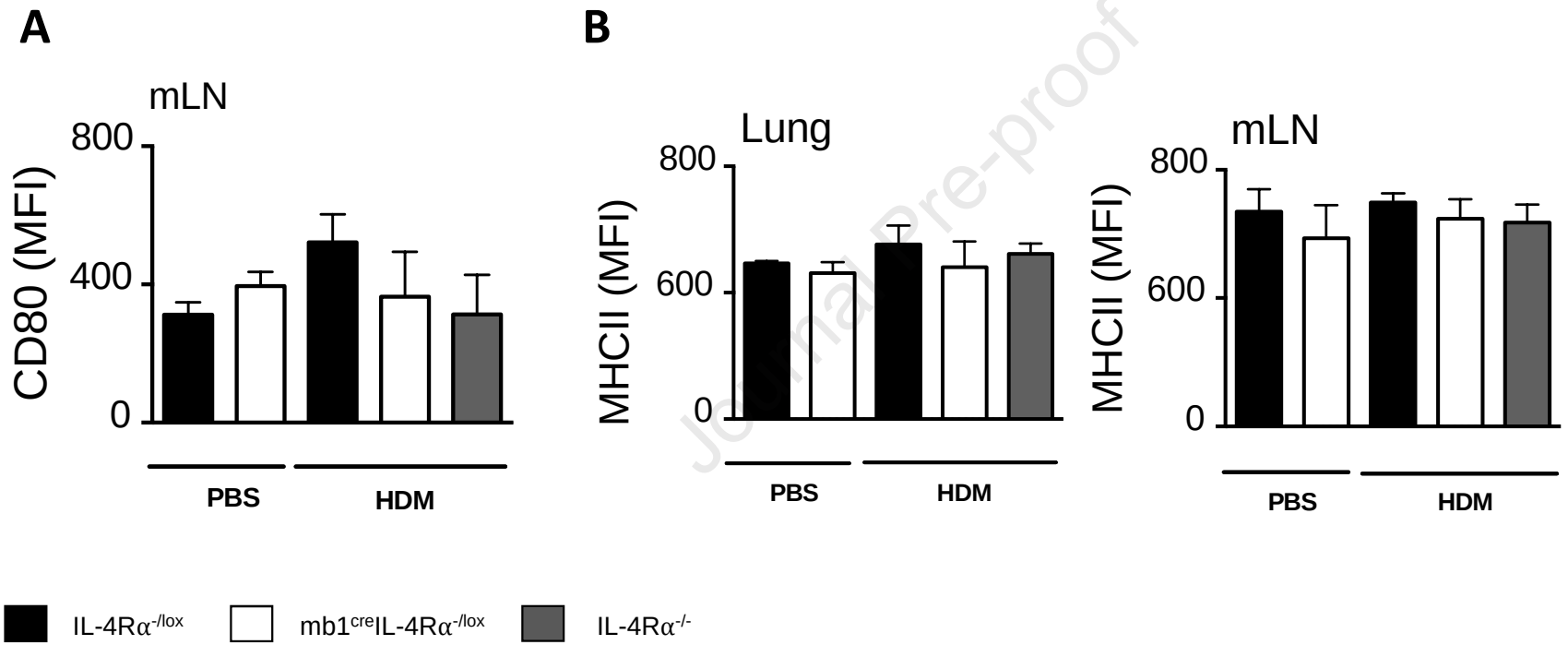


Figure E5

Full title: Deletion of IL-4Rα 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-4Rα) 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-4Rα in B cells is not clearly defined.

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

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

Results: We found that IL-4Rα signalling on B cells was important for optimal TH2 allergic immune responses mainly when the load of antigen is limited. IL-4Rα 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 in other parameters.

Conclusion: IL-4Rα 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-4Rα; TH2 cells; B cells; germinal centre; T follicular helper cell; B effector 2 cells

Abbreviations used:

IL-4Rα: interleukin 4 receptor alpha

BAL: Bronchoalveolar lavage

GC: Germinal Centre

HDM: House dust mite

i.n.: Intranasal

i.t.: intratracheal

medLN: Mediastinal lymph node

TFH: T follicular helper

AHR: airway hyperresponsiveness

Be2: B effector 2 cells

Key Messages

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

92 **CAPSULE SUMMARY**

93 B cells expressing high level IL-4Rα are important in class switching to IgE in GC. Targeting IL-

94 4Rα signalling on B cells has clinical benefit in allergic asthma even in high IgE setting.

95

96

97

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 ($\mu MT^{-/-}$) 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

asthma [14]. Furthermore, the load of antigen seems to be critical in the involvement of B cells in HDM-induced allergic asthma [14]. 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 responses [15].

Interleukin 4 receptor alpha (IL-4Rα) is central in TH2 allergic airway asthma[16–18] and other type 2 diseases [19,20]. In allergic asthma, we and others have shown temporal[21] and cell-specific requirement of IL-4Rα in DCs [22], T cells[23] and epithelial cells[24] and also redundant role of this IL-4/IL-13 signalling receptor in macrophages[25] 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-4Rα 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-4Rα responsive B cells in allergic asthma. We found that although mice lacking IL-4Rα on B cells ($mb1^{cre}$IL-4R$\alpha^{-/lox}$) 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$\alpha^{-/lox}$ mice had reduced AHR, type 2 responses, eosinophilia, T_{FH} and ability to

produce TH2 cytokines. By adoptively transferring naïve IL-4Rα-deficient B cells into $\mu MT^{-/-}$ mice sensitised to low dose HDM, we demonstrated the importance of IL-4Rα 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-4Rα 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-4Rα only on B cells ($mb1^{cre}$IL-$4R\alpha^{-/lox}$), we intercrossed homozygous $mb1^{cre}$ mice[31] with IL-$4R\alpha^{-/-}$ [32] on Balb/c background. We then further mated $mb1^{cre}$ IL-$4R\alpha^{-/-}$ mice with homozygous IL-$4R\alpha^{lox/lox}$ mice[19] to generate hemizygous $mb1^{cre}$IL-$4R\alpha^{-/lox}$ [33] which were backcrossed up to 10 generations in Balb/c background. Hemizygous littermates (IL-$4R\alpha^{-/lox}$) expressing single functional IL-4Rα 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 mice[14]. 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 and sensitised intratracheally (i.t.) on day 0 with 100 μg of HDM (Stellergens Greer Laboratories, Lenoir, U.S.A.) and intranasally challenged with 10ug HDM on days 8, 9, 10, 11 and 12. For low dose treatment , mice were challenged with 1 μg and sensitised with 3 μg of HDM . 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 40μm strainer to obtain single cell suspensions. Cells were stained with FITC-B220 and APC-CD19 for 30min at 4^{o}C. A dead cell exclusion dye (7AAD) was added before sorting on BD FACS Aria I to at least 96% purity. 2-5 x 10^{6} 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 previously[25] 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-

194 test. Only mice with acceptable measurements for all doses (coefficient of determination
195 >0.90) were included in the analysis.
196
197 **Flow cytometry**
198 Bronchoalveolar lavage (BAL) fluid cells were obtained as previously described[26]. Single-cell
199 suspensions were prepared from lymph nodes in Roswell Park Memorial Institute (RPMI)
200 media (Gibco, Paisley, United Kingdom) by passing them through 100µm strainer. To obtain
201 single cell suspensions from lung tissues, a left lobe was digested for 1 hour at 37^{o}C in RPMI
202 containing 13 mg/mL DNase I (Roche, Randburg, South Africa) and 50 U/mL collagenase IV
203 (Gibco, Waltham, Massachusetts) and passed through 70µm strainer. Antibodies used in
204 these experiments included, phycoerythrobilin (PE)- conjugated anti-Siglec-F (clone, E50-
205 2440), anti-CD124 (IL-4Rα, clone, M-1), anti-IL-5 (clone, TRFK5), anti-CD44 (clone, KM114),
206 FITC- conjugated anti-Gr-1 (clone, RB6-8C5), CD45 (clone, 30-F11), IL-4 (clone, 11B11), PerCP
207 Cy5.5- conjugated anti-Ly6C (clone, AL-21), -CD45.1 (clone, A20), anti-IL-17 (clone, TC11-
208 18H10), Allophycocyanin (APC)- conjugated anti-CD11c (clone, HL3), anti-FoxP3 (clone,
209 MF23), V450 conjugated anti-CD11b (clone, M1/70), anti-CD62L (clone, MEL-14), anti-IgG1
210 (clone, A110-1), AlexaFlour 700- conjugated anti-CD3ε (clone, 145-2C11) -anti IFN-γ, V500-
211 anti-CD4 (clone, RM4-5) and anti- B220 (clone, RA3-6B2), APC-Cy7-conjugated anti-CD19
212 (clone, 1D3) and anti-CD8 (clone, 53-6.7), BV786 conjugated anti-IgE (clone, R35-72) and
213 anti-IL-33R (ST2) (clone, U29-93), biotin-CD25 (clone, 7D4) were purchased from BD
214 Pharmingen (San Diego, CA). PE-Cynanine7 anti-F4/80 (clone, BM8), anti-IL-13 (clone,
215 eBio13A), AlexaFlouro 700- conjugated anti-MHC II (clone, M5/114), APC- conjugated anti-
216 IL-21 (clone, FFA21) and Live/dead Fixable Yellow stain (Qdot605 dead cell exclusion dye)
217 were purchased from eBiosciences. Biotin-labelled antibodies were detected by Texas Red

conjugated PE (BD Biosciences). For staining, cells (1 x 10^6) were stained and washed in PBS, 3% FCS FACS buffer. For intracellular cytokine staining, cells were restimulated with phorbal 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 described[26] 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 ($2x10^5$ cells, in 200µL) 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 (30µg/mL) or anti-CD3 (10µg/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-4Rα-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 cells[14]. We used a standard high dose of 100 μg HDM to sensitise mice at day 0 and challenged with a reduced dose of 10 μg 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-4Rα expression in both lung and mediastinal lymph nodes (mLNs) in mice lacking IL-4Rα on B cells (mb1^{cre}IL-4Rα$^{-/lox}$) when compared to littermate (IL-4Rα$^{-/lox}$) control mice or IL-4Rα-deficient mice (Suppl. Figure 1, A-B). We found that mice lacking IL-4Rα 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-4Rα$^{-/lox}$ 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 phorbal 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-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ and littermate control IL-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ mice when compared to mb1^{cre}IL-4Rα$^{-/lox}$ challenged with high dose HDM (Figure 1, E). We observed no differences in mucus production and inflammation between mb1^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice (Figure 1, F). Overall, these results demonstrated that IL-4Rα on B cells plays a minimal role in the development of allergic asthma after a challenge with high dose HDM.

IL-4Rα-responsive B cells play an essential role in low dose HDM induced allergic asthma. B cell-deficient mice (μMT$^{-/-}$) 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 μg reduced influx of eosinophils, proliferation of Derp-1-specific T cells and type 2 cytokine production when compared to wild type mice[14]. 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-4Rα$^{-/lox}$ sensitised and challenged with low dose HDM when compared to littermate IL-4Rα$^{-/lox}$ mice (Figure 2, B). Mb1^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ littermates compared to mb1^{cre}IL-4Rα$^{-/lox}$ or global IL-4Rα-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-4Rα$^{-/lox}$ littermate mice when compared to mb1^{cre}IL-4Rα^{-}

$^{/lox}$ and IL-4Rα$^{-/-}$ 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-4Rα B cell-deficient mLNs 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-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ 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-4Rα on B cells contributes significantly to the development of allergic asthma and Th2-type lung inflammation.

IL-4Rα-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 disease [13]. Firstly, we measured percentages and number of B cells in the mLNs and found frequencies to be intact (Suppl. Figure 4, A). However, total numbers were significantly reduced when comparing mb1^{cre}IL-4Rα$^{-/lox}$ to IL-4Rα$^{-/lox}$ littermate mice and global IL-4Rα-deficient mice (Figure 3, A). We then compared germinal centre (GC) B cells in mLNs and found comparably low levels between mb1^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ littermate mice when compared to mb1^{cre}IL-4Rα$^{-/lox}$ 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 mLNs 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 lung [37]. 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-4Rα-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-4Rα-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-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ when compared to IL-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ when compared to IL-4Rα$^{-/lox}$ 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-4Rα$^{-/lox}$ to IL-4Rα$^{-/lox}$ littermate mice (Figure 4, D). B and T cell engagement through CD86 and CD28 in T cell zones is essential for T_{FHs} generation and class switching to IgE [39]. 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-4Rα signalling on B cells is essential for Be2 function and class switching to IgE and this contributes to overall TH2 responses.

IL-4Rα-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 sensitisation [13]. We asked whether IL-4Rα-responsive B cells were only important at sensitisation stage. We sensitised B cell deficient μMT$^{-/-}$ mice with low dose HDM and a day before challenge, we transferred naïve B cells either sufficient or lacking IL-4Rα (Figure 5, A). We found that μMT$^{-/-}$ with B cells from mb1^{cre}IL-4Rα$^{-/lox}$ had significantly reduced resistance and elastance when compared to μMT$^{-/-}$ receiving B cells from IL-4Rα$^{-/lox}$

littermate mice (Figure 5, B). This reduced AHR in mb1^{cre}IL-4Rα$^{-/lox}$ 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 μMT$^{-/-}$ mice that received mb1^{cre}IL-4Rα$^{-/lox}$ B cells when compared to μMT$^{-/-}$ mice receiving IL-4Rα$^{-/lox}$ B cells (Figure 5, D). We then measured total IgE by ELISA and found significantly increased IgE in μMT$^{-/-}$ mice that received IL-4Rα$^{-/lox}$ B cells compared to μMT$^{-/-}$ mice that received mb1^{cre}IL-4Rα$^{-/lox}$ B cells (Figure 5, E). We observed comparable mucus area between μMT$^{-/-}$ mice receiving IL-4Rα$^{-/lox}$ B cells or mb1^{cre}IL-4Rα$^{-/lox}$ 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-4Rα 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 μMT$^{-/-}$ 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 μMT$^{-/-}$ mice that received IL-4-deficient or IL-4/IL-13-double deficient B cells when compared to μMT$^{-/-}$ 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).

400 The number of B cells were lower in μMT$^{-/-}$ mice that were adoptively transferred with B
401 cells from various strains compared to control mice, however, no differences were observed
402 between mice that were challenged with a low dose of HDM (Figure 6, C). Total IgE
403 measured by ELISA was not changed between groups of μMT$^{-/-}$ mice adoptively transferred
404 with B cells, sensitised and challenged with low dose HDM (Figure 6, D). We also did not
405 observe any changes in type 2 cytokines produced by CD4 T cells in the absence of type 2
406 cytokines produced by B cells when comparing μMT$^{-/-}$ mice that received WT B cells to those
407 that received IL-4-deficient or IL-4/IL-13-double deficient B cells (Figure 6, E). Taken
408 together, these results suggested that although B cells producing type 2 cytokines are
409 essential in airway hyperresponsiveness, they play minimal role in airway inflammation.
410

411 **DISCUSSION**

412 B cells secrete IgE are important in activating mast cells and basophils degranulation, which
413 initiates a cascade of inflammatory signals. However, contradictory findings on the
414 requirement of B cells exists in studies using mice lacking B cells. More recent evidence
415 suggested that antigen load determines the importance of B cells, particularly in
416 interactions with T helper cells and generation of T follicular helper cells. IL-4 is critical in
417 class switching of B cells to generate IgE[40], however, whether signalling through the IL-4Rα
418 on B cells is required for generation of T_{FH} cells and IgE have not been investigated in the
419 context of allergic asthma. Here, we showed that IL-4Rα responsive B cells are important
420 mainly when the load of HDM antigen is limiting. We further showed that IL-4Rα responsive
421 B cells regulate AHR, IgE secretion, T_{FH} cells generation and that B cells derived type 2
422 cytokines are required for optimal TH2 responses.
423
424 We first challenged mice with high dose HDM and found IL-4Rα responsive B cells to be
425 important in AHR and IgE production, but not for eosinophil recruitment or type 2 cytokine
426 production. This is consistent with previous studies where B cell-deficient mice showed
427 similar levels of eosinophilia and type 2 cytokines when sensitised and challenged with high
428 dose HDM[14]. B cell-deficient mice have significantly increased AHR at high dose HDM, a
429 phenotype different to what we observe in IL-4Rα-deficient B cells, which suggested that IL-
430 4Rα responsive B cells may be involved in driving AHR during allergic asthma. Differences in
431 diseases susceptibility is also observed between $\mu MT^{-/-}$ mice and $mb1^{cre}$IL-$4R\alpha^{-/lox}$ mice
432 during chronic *Schistosoma mansoni* infection, which is attributed to differences in
433 immunomodulatory IL-10 production seen in $\mu MT^{-/-}$ mice, but not in $mb1^{cre}$IL-$4R\alpha^{-/lox}$ mice
434 [28,29].

Similarly to what was reported by Dullaers et al.[14], mb1^{cre}IL-4Rα$^{-/lox}$ mice that were sensitised with 1 μg HDM and challenged with 3 μg HDM displayed a great reduction in AHR, eosinophil recruitment and type 2 cytokine production compared to littermate IL-4Rα$^{-/lox}$ 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-4Rα B cells help contribute to the overall type 2 immune response output. This is consistent with previous studies, where IL-4Rα 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-4Rα signalling on B cells [33,41]. Although, IgE can directly be generated from IgM particularly with low antigen load[42], we think that in our model, lack of IL-4Rα 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-4Rα 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,

459 which was attributed to redundant role of B cells at this stage. This is in contrast with recent
460 findings where blocking B cells with anti-CD20 before HDM challenge, significantly reduced
461 TH2 airway responses [15]. To consolidate these findings, we transferred IL-4Rα-deficient B
462 cells after HDM sensitisation and before challenge. Our data showed that IL-4Rα-responsive
463 B cells are required at the challenge stage, as we observed reduced AHR, TH2 cytokines and
464 total IgE when we transferred B cells lacking IL-4Rα into recipient mice. Both previous
465 studies had used a similar dose of 20 μg of HDM to sensitise and challenge and we used 3
466 μg of HDM to challenge. It is likely that the choice of method used to target B cells or their
467 function might be a major contributing factor between the two studies and not necessarily
468 the load of HDM antigen. In our studies, we transferred naïve B cells a day before challenge,
469 whereas Ballestos-Tato et al.[13] had blocked T_{FH} cells using BLC6 inhibitor and Wypych et al.[15]
470 targeted B cells using anti-CD20 monoclonal antibody. All in all, our data demonstrated that
471 IL-4Rα on B cells is important in TH2 allergic asthma at both sensitisation and challenge
472 stages and contributes to overall TH2 responses when the antigen load is limited.
473
474 Previous studies have shown a controversial role of IL-21 in T_{FHs} that eventually developed
475 into committed TH2 cells. Ballesteros-Tato et al.[13] suggested that lung TH2 cells were direct
476 descendants of IL-21^+Bcl6^+ T_{FH} cells and developed 6 days after multiple sensitisation with
477 25 μg of HDM exposure. In contrast, Coquet et al.[37] found that IL-21^+ T_{FH} cells did not
478 differentiate efficiently into $ST2^+$ TH2 cells and migrated into the lung without all key
479 features of T_{FH} cells such as CXCR5 expression. This idea was recently supported by Tibbitt et
480 al.[48], where a trajectory single cell analysis of differentiating TH2 cells up until day 10,
481 suggested that naïve CD4 T cells acquired many features of T_{FH} cells but did not express Bcl6
482 or CXCR5 which suggested that TH2 cells did not descend directly from GC T_{FH} cell

precursors. In our study, the absence of IL-4Rα 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-4Rα and require presence of intact TH2 cells[28,38,49]. Since we had observed that IL-4Rα 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 $\mu MT^{-/-}$ 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-4Rα, 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 secretion[15]. 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-4Rα-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 burdens[30]. We did not observe any changes in CD80 or MHCII expression on IL-4Rα signalling deficient B cells when compared to IL-4Rα 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 potentiation[39]. 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-4Rα signalling on B cells.

In conclusion, we showed that IL-4Rα-responsive B cells play a non-redundant role in allergic asthma in an antigen load dependent manner. We further showed that IL-4Rα 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-4Rα 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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REFERENCES

1. Lambrecht BN, Hammad H. The immunology of asthma. Nat Immunol [Internet]. 2014;16:45–56. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25521684
2. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol [Internet]. 2008;8:183–92. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18274560
3. Lloyd CM, Hessel EM. Functions of T cells in asthma: more than just TH2 cells. Nat Rev Immunol. 2010;10:838–48.
4. Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. Nat Rev Immunol [Internet]. 2003;3:721–32. Available from: https://pubmed.ncbi.nlm.nih.gov/12949496
5. Gould HJ, Ramadani F. IgE responses in mouse and man and the persistence of IgE memory. Trends Immunol [Internet]. 2015;36:40–8. Available from: https://doi.org/10.1016/j.it.2014.11.002
6. Plantinga M, Guilliams M, Vanheerswynghels M, Deswarte K, Branco-Madeira F, Toussaint W, et al. Conventional and Monocyte-Derived CD11b+ Dendritic Cells Initiate and Maintain T Helper 2 Cell-Mediated Immunity to House Dust Mite Allergen. Immunity [Internet]. 2013;38:322–35. Available from: http://dx.doi.org/10.1016/j.immuni.2012.10.016
7. Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MAM, Kool M, et al. Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. J Exp Med [Internet]. 2010;207:2097–111. Available from: http://jem.rupress.org/content/207/10/2097.short

8. Archambault AS, Carrero JA, Barnett LG, McGee NG, Sim J, Wright JO, et al. Cutting Edge: Conditional MHC Class II Expression Reveals a Limited Role for B Cell Antigen Presentation in Primary and Secondary CD4 T Cell Responses. J Immunol [Internet]. 2013;191:545 LP – 550. Available from: http://www.jimmunol.org/content/191/2/545.abstract

9. McKnight CG, Jude JA, Zhu Z, Panettieri RA, Finkelman FD. House Dust Mite–Induced Allergic Airway Disease Is Independent of IgE and FcεRIα. Am J Respir Cell Mol Biol [Internet]. 2017;57:674–82. Available from: https://doi.org/10.1165/rcmb.2016-0356OC

10. Korsgren M, ält JSE, Korsgren O, Sundler F, Persson CGA. Allergic Eosinophil-rich Inflammation Develops in Lungs and Airways of B Cell–deficient Mice. J Exp Med [Internet]. 1997;185:885–92. Available from: https://doi.org/10.1084/jem.185.5.885

11. MacLean JA, Sauty A, Luster AD, Drazen JM, De Sanctis GT. Antigen-induced airway hyperresponsiveness, pulmonary eosinophilia, and chemokine expression in B cell-deficient mice. Am J Respir Cell Mol Biol [Internet]. 1999;20:379–87. Available from: https://pubmed.ncbi.nlm.nih.gov/10030835

12. Hamelmann E, Takeda K, Schwarze J, Vella AT, Irvin CG, Gelfand EW. Development of Eosinophilic Airway Inflammation and Airway Hyperresponsiveness Requires Interleukin-5 but Not Immunoglobulin E or B Lymphocytes. Am J Respir Cell Mol Biol [Internet]. 1999;21:480–9. Available from: https://doi.org/10.1165/ajrcmb.21.4.3659

13. Ballesteros-Tato A, Randall TD, Lund FE, Spolski R, Leonard WJ, León B. T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite. Immunity. 2016;44:259–73.

14. Dullaers M, Schuijs MJ, Willart M, Fierens K, Van Moorleghem J, Hammad H, et al.

House dust mite–driven asthma and allergen-specific T cells depend on B cells when the amount of inhaled allergen is limiting. J Allergy Clin Immunol [Internet]. 2017;140:76-88.e7. Available from: http://dx.doi.org/10.1016/j.jaci.2016.09.020

15. Wypych TP, Marzi R, Wu GF, Lanzavecchia A, Sallusto F. Role of B cells in THcell responses in a mouse model of asthma. J Allergy Clin Immunol. 2018;141:1395–410.

16. Tomkinson A, Duez C, Cieslewicz G, Pratt JC, Joetham A, Shanafelt M-C, et al. Inhibition of the IL-4/IL-13 receptor system prevents allergic sensitization without affecting established allergy in a mouse model for allergic asthma. J Allergy Clin Immunol [Internet]. 2001;166:1361–9. Available from: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.166.9.5792

17. Grünig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 Independently of IL-4 in Experimental Asthma. Natl Institutes Heal. 1998;282:2261–3.

18. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. Science. 1998;282:2258–61.

19. Herbert DR, Hölscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, et al. Alternative Macrophage Activation Is Essential for Survival during Schistosomiasis and Downmodulates T Helper 1 Responses and Immunopathology. Immunity. 2004;20:623–35.

20. Horsnell WGC, Vira A, Kirstein F, Mearns H, Hoving JC, Cutler AJ, et al. IL-4Rα-responsive smooth muscle cells contribute to initiation of TH2 immunity and pulmonary pathology in Nippostrongylus brasiliensis infections. Mucosal Immunol [Internet]. 2011;4:83–92. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20737001

21. Khumalo J, Kirstein F, Scibiorek M, Hadebe S, Brombacher F. Therapeutic and prophylactic deletion of IL-4Rα signaling ameliorates established ovalbumin-induced allergic asthma. Allergy [Internet]. 2019;n/a. Available from: https://doi.org/10.1111/all.14137

22. Nieuwenhuizen NE, Kirstein F, Hoving JC, Brombacher F. House dust mite induced allergic airway disease is attenuated in CD11ccreIL-4Rα−/l°x mice. Sci Rep [Internet]. 2018;8:885. Available from: http://www.nature.com/articles/s41598-017-19060-9

23. Kirstein F, Nieuwenhuizen NE, Jayakumar J, Horsnell WGC, Brombacher F. Role of IL-4 receptor α–positive CD4+ T cells in chronic airway hyperresponsiveness. J Allergy Clin Immunol. 2015;

24. Kuperman DA, Huang X, Nguyenvu L, Holscher C, Brombacher F, Erle DJ. IL-4 Receptor Signaling in Clara Cells Is Required for Allergen-Induced Mucus Production. J Immunol [Internet]. 2005;175:3746–52. Available from: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.175.6.3746

25. Nieuwenhuizen NE, Kirstein F, Jayakumar J, Emedi B, Hurdayal R, Horsnell WGC, et al. Allergic airway disease is unaffected by the absence of IL-4Rα-dependent alternatively activated macrophages. J Allergy Clin Immunol [Internet]. 2012;130:743-750.e8. Available from: http://dx.doi.org/10.1016/j.jaci.2012.03.011

26. Kirstein F, Horsnell WGC, Kuperman D a, Huang X, Erle DJ, Lopata AL, et al. Expression of IL-4 receptor alpha on smooth muscle cells is not necessary for development of experimental allergic asthma. J Allergy Clin Immunol. 2010;126:347–54.

27. Perkins C, Yanase N, Smulian G, Gildea L, Orekov T, Potter C, et al. Selective stimulation of IL-4 receptor on smooth muscle induces airway hyperresponsiveness in mice. J Exp Med. 2011;208:853–67.

640 28. Hurdayal R, Ndlovu HH, Revaz-Breton M, Parihar SP, Nono JK, Govender M, et al. IL-
641 4–producing B cells regulate T helper cell dichotomy in type 1- and type 2-controlled
642 diseases. Proc Natl Acad Sci U S A. 2017;114:E8430–9.
643 29. Ndlovu H, Nono JK, Abdel Aziz N, Nieuwenhuizen NE, Brombacher F. Interleukin-4
644 Receptor Alpha Expressing B Cells Are Essential to Down-Modulate Host
645 Granulomatous Inflammation During Schistosomasis [Internet]. Vol. 9, Frontiers in
646 Immunology . 2018. p. 2928. Available from:
647 https://www.frontiersin.org/article/10.3389/fimmu.2018.02928
648 30. Horsnell WGC, Darby MG, Hoving JC, Nieuwenhuizen N, McSorley HJ, Ndlovu H, et al.
649 IL-4Rα-Associated Antigen Processing by B Cells Promotes Immunity in
650 Nippostrongylus brasiliensis Infection. PLoS Pathog. 2013;9:1–12.
651 31. Hobeika E, Thiemann S, Storch B, Jumaa H, Nielsen PJ, Pelanda R, et al. Testing gene
652 function early in the B cell lineage in mb1-cre mice. Proc Natl Acad Sci [Internet].
653 2006;103:13789 LP – 13794. Available from:
654 http://www.pnas.org/content/103/37/13789.abstract
655 32. Mohrs M, Ledermann B, Köhler G, Dorfmüller A, Gessner A, Brombacher F.
656 Differences between IL-4- and IL-4 receptor alpha-deficient mice in chronic
657 leishmaniasis reveal a protective role for IL-13 receptor signaling. J Immunol.
658 1999;162:7302–8.
659 33. Hoving JC, Kirstein F, Nieuwenhuizen NE, Fick LCE, Hobeika E, Reth M, et al. B Cells
660 That Produce Immunoglobulin E Mediate Colitis in BALB/c Mice. Gastroenterology
661 [Internet]. 2012;142:96–108. Available from:
662 http://www.sciencedirect.com/science/article/pii/S0016508511013709
663 34. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust

mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med [Internet]. 2009;15:410–6. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2789255&tool=pmcentrez&rendertype=abstract%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/19330007%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2789255

35. Debeuf N, Haspeslagh E, van Helden M, Hammad H, Lambrecht BN. Mouse Models of Asthma. In: Current Protocols in Mouse Biology. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2016. p. 169–84.

36. Vroman H, Bergen IM, Li BWS, van Hulst JAC, Lukkes M, van Uden D, et al. Development of eosinophilic inflammation is independent of B–T cell interaction in a chronic house dust mite-driven asthma model. Clin Exp Allergy. 2017;47:551–64.

37. Coquet JM, Schuijs MJ, Smyth MJ, Deswarte K, Beyaert R, Braun H, et al. Interleukin-21-Producing CD4^{+} T Cells Promote Type 2 Immunity to House Dust Mites. Immunity [Internet]. 2015;43:318–30. Available from: https://doi.org/10.1016/j.immuni.2015.07.015

38. Wojciechowski W, Harris DP, Sprague F, Mousseau B, Makris M, Kusser K, et al. Cytokine-Producing Effector B Cells Regulate Type 2 Immunity to H. polygyrus. Immunity [Internet]. 2009;30:421–33. Available from: http://dx.doi.org/10.1016/j.immuni.2009.01.006

39. Jeannin P, Delneste Y, Lecoanet-Henchoz S, Gauchat J-F, Ellis J, Bonnefoy J-Y. CD86 (B7-2) on Human B Cells: A FUNCTIONAL ROLE IN PROLIFERATION AND SELECTIVE DIFFERENTIATION INTO IgE- AND IgG4-PRODUCING CELLS . J Biol Chem [Internet]. 1997;272:15613–9. Available from: http://www.jbc.org/content/272/25/15613.abstract

40. Shimoda K, van Deursent J, Sangster MY, Sarawar SR, Carson RT, Tripp RA, et al. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted State6 gene. Nature. 1996;380:630–3.

41. Mandler R, Finkelman FD, Levine AD, Snapper CM. IL-4 induction of IgE class switching by lipopolysaccharide-activated murine B cells occurs predominantly through sequential switching. J Immunol [Internet]. 1993;150:407 LP – 418. Available from: http://www.jimmunol.org/content/150/2/407.abstract

42. SUDOWE S, RADEMAEKERS A, KÖLSCH E. Antigen dose-dependent predominance of either direct or sequential switch‘qc in IgE antibody responses. Immunology [Internet]. 1997;91:464–72. Available from: https://doi.org/10.1046/j.1365-2567.1997.00268.x

43. Wu YL, Stubbington MJT, Daly M, Teichmann SA, Rada C. Intrinsic transcriptional heterogeneity in B cells controls early class switching to IgE. J Exp Med [Internet]. 2016;214:183–96. Available from: https://doi.org/10.1084/jem.20161056

44. Turqueti-Neves A, Otte M, Schwartz C, Schmitt MER, Lindner C, Pabst O, et al. The Extracellular Domains of IgG1 and T Cell-Derived IL-4/IL-13 Are Critical for the Polyclonal Memory IgE Response In Vivo. PLOS Biol [Internet]. 2015;13:e1002290. Available from: https://doi.org/10.1371/journal.pbio.1002290

45. Crawford G, Hayes MD, Seoane RC, Ward S, Dalessandri T, Lai C, et al. Epithelial damage and tissue γδ T cells promote a unique tumor-protective IgE response. Nat Immunol [Internet]. 2018;19:859–70. Available from: https://doi.org/10.1038/s41590-018-0161-8

46. Ramadani F, Bowen H, Upton N, Hobson PS, Chan Y-C, Chen J-B, et al. Ontogeny of human IgE-expressing B cells and plasma cells. Allergy [Internet]. 2017;72:66—76.

Available from: https://europepmc.org/articles/PMC5107308

47. Yanagihara Y, Ikizawa K, Kajiwara K, Koshio T, Basaki Y, Akiyama K. Functional significance of IL-4 receptor on B cells in IL-4-induced human IgE production. J Allergy Clin Immunol. 1995;96:1145–51.

48. Tibbitt CA, Stark JM, Martens L, Ma J, Mold JE, Deswarte K, et al. Single-Cell RNA Sequencing of the T Helper Cell Response to House Dust Mites Defines a Distinct Gene Expression Signature in Airway Th2 Cells. Immunity. 2019;51:169-184.e5.

49. Harris DP, Goodrich S, Mohrs K, Mohrs M, Lund FE. Cutting Edge: The Development of IL-4-Producing B Cells (B Effector 2 Cells) Is Controlled by IL-4, IL-4 Receptor α, and Th2 Cells. J Immunol [Internet]. 2005;175:7103 LP – 7107. Available from: http://www.jimmunol.org/content/175/11/7103.abstract

FIGURE LEGENDS

Figure 1: IL-4Rα-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-$4R\alpha^{-/lox}$) and wild type littermate control (IL-$4R\alpha^{-/lox}$) were sensitised with HDM 100μg intra-tracheally on days 0 and challenged with HDM 10μg 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 ±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-4Rα-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 1µg intra-tracheally on days 0 and challenged with HDM 3µg 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 ±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-4Rα 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 in 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 ±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-4Rα 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 μMT$^{-/-}$ mice were sensitised with HDM 1μg intra-tracheally on days 0 and naïve B cells (live^{+}B220^{+}CD19^{+}) from mb1^{cre}IL-4Rα$^{-/lox}$ or IL-4Rα$^{-/lox}$ mice (2-5 x10^{6} cells) were adoptively transferred intravenously a day before challenge with HDM 3μg 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 ±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 x10^{6} cells) were adoptively transferred intravenously into µMT$^{-/-}$ mice a day before sensitisation with HDM 1µg intra-tracheally on days 0 and challenged with HDM 3µg 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 ±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,

834 ****p< 0.0001