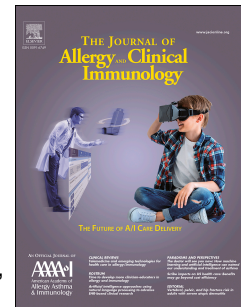


Journal Pre-proof

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PII: S0091-6749(20)31634-1

DOI: <https://doi.org/10.1016/j.jaci.2020.09.041>

Reference: YMAI 14848

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 21 November 2019

Revised Date: 5 June 2020

Accepted Date: 2 September 2020

Please cite this article as: Dipl-Biol AH, Happle C, Grychtol RM, Skuljec J, Busse M, Dalüge K, Obernolte H, Sewald K, Braun A, Meyer-Bahlburg A, Hansen G, Regulatory B cells control airway hyperreactivity and lung remodeling in a murine asthma model, *Journal of Allergy and Clinical Immunology* (2020), doi: <https://doi.org/10.1016/j.jaci.2020.09.041>.

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Regulatory B cells control airway hyperreactivity and lung remodeling in a murine asthma model

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Funding

This research was supported by the German Center for Lung Research (DZL; BMBF, Federal Ministry of Education and Research), Cluster of Excellence RESIST (EXC 2155, DFG, German Research Foundation), Hannover Medical School, Hannover, Germany and by the Dörenkamp Foundation.

Conflict of interest disclosure

The authors declare no commercial or financial conflict of interest.

Abstract

Background: Asthma is a widespread, multifactorial chronic airway disease. The influence of regulatory B cells (Bregs) on airway hyperreactivity (AHR) and remodeling in asthma is poorly understood.

Objective: We analyzed the role of B cells in a house dust mite (HDM)-based murine asthma model.

Methods: By employing wildtype (WT) and B cell-deficient (μ MT) mice and transfer of IL-10-proficient and IL-10-deficient B cells to μ MT mice, the influence of B cells on lung function, tissue remodeling and the immune response was analyzed.

Results: After HDM-sensitization, both WT and μ MT mice developed AHR, but AHR was significantly stronger in μ MT mice as confirmed by two independent techniques: invasive lung function measurement *in vivo* and precision-cut lung slices *ex vivo*. Moreover, airway remodeling was significantly increased in allergic μ MT mice as shown by enhanced collagen deposition in the airways, while regulatory FoxP3⁺ and FoxP3⁻ IL-10-secreting T cells (Tregs) were reduced. Adoptive transfer of IL-10-proficient, but not IL-10-deficient B cells into μ MT mice prior to HDM-sensitization, attenuated AHR and lung remodeling. In contrast, FoxP3⁺ Tregs were equally upregulated by transfer of IL-10-proficient as well as IL-10-deficient B cells.

Conclusion: Our data in a murine asthma model illustrate a central role of Bregs in the control of lung function and airway remodeling, and may support future concepts for B cell targeted prevention and treatment strategies for allergic asthma.

Key Messages

- B cell-deficiency leads to increased airway hyperreactivity, airway remodeling, and reduced FoxP3⁺ and FoxP3⁻ IL-10-secreting regulatory T cells (Tregs) in an HDM-based experimental murine asthma model
- Adoptive transfer of IL-10-proficient but not IL-10-deficient B cells improves lung function and attenuates airway remodeling in HDM-sensitized B cell-deficient mice, while FoxP3⁺ Treg numbers are equally affected by transfer both cell types

Capsule Summary

IL-10-proficient but not IL-10-deficient B cells are crucial for the control of lung function and airway remodeling in experimental asthma, while FoxP3⁺ Treg expansion is equally affected by both B cell types.

Key words

experimental asthma, allergy, house dust mite, airway hyperreactivity, lung remodeling, regulatory B cells, IL-10, B cell transfer, precision-cut lung slices

Abbreviations

AHR: airway hyperreactivity, **BALF:** bronchoalveolar lavage fluid, **C:** compliance, **LN:** lymph nodes, **Bregs:** B cells with a regulatory function, **E:** elastance, **FM:** follicular mature, **GC:** germinal centre, **H&E:** haematoxylin and eosin, **IL-10:** Interleukin-10, **i.n.:** intranasal, **MCh:** methacholine, **μMT:** B cell-deficient mice, **NaCl:** sodium chloride, **PAS:** periodic acid-Schiff, **PB/PC:** plasmablasts/plasma cells, **PCLS:** precision-cut lung slices, **R:** resistance, **Th2:** T helper type 2, **Tregs:** regulatory T cells, **Tr1:** type 1 regulatory T, **WT:** wildtype.

Introduction

Asthma is a chronic respiratory disease affecting more than 300 million people worldwide.¹ It is caused by an inadequate immune response towards otherwise harmless antigens and is characterized by airway hyperreactivity (AHR) with reversible airway obstruction, mucus hypersecretion, airway inflammation, and remodeling.²⁻⁴ Today, it is the most common chronic disease in children.⁵

One key objective of current research approaches is to better understand the mechanisms of immunological tolerance in order to develop effective prevention and treatment strategies. In the last decades, T cells have been in the primary focus of immune tolerance research, and numerous experimental and clinical studies clearly demonstrated their key role in the pathogenesis and regulation of allergic diseases.^{6, 7} However, more recently, B cells have been recognized as important mediators in allergy and tolerance.⁸⁻¹¹ One important B cell population are Interleukin-10 (IL-10)-producing regulatory B cells (Bregs)¹² which have the capacity to reduce activation of T cells and block the production of pro-inflammatory cytokines.¹³ The regulatory and anti-inflammatory potential of *in vitro* and *in vivo* activated and tolerogenic B cells has been demonstrated in numerous studies over the last years: their adoptive transfer alleviated the disease phenotype at least partly in different murine models of chronic organ inflammation such as food allergy,¹⁴ allergic airway disease,^{15, 16} and skin allograft transplantation tolerance.¹⁷ Recently, data from human asthma patients and murine asthma models have confirmed the relevance of IL-10-positive Bregs in allergic airway inflammation and their contribution to long-term tolerance towards allergens.¹¹

To elucidate the role of B cells in the development of allergy and immunological tolerance, we used an experimental asthma model based on sensitization with the clinically relevant allergen house dust mite (HDM).^{18, 19} Our results in B cell-deficient (μ MT) mice show that lack of B cells leads to significantly increased AHR and airway remodeling compared to the wildtype (WT) situation. While the transfer of WT B cells significantly improved lung function and airway remodeling in allergic B cell-deficient mice, the transfer of IL-10-deficient (IL-10^{-/-}) B cells failed to do so. However, transfer of both cell types boosted regulatory FoxP3⁺ T cell (Treg) numbers in recipient animals. These data in a murine asthma model, illustrate the central role of Bregs in the regulation of allergic asthma.

Materials and methods

Animals

B6.129S2-Ighm^{tm1Cgn}/J (B cell-deficient/ μ MT) mice were obtained from Jackson (Bar Harbor, ME, USA), C57BL/6J WT mice from Charles River (Sulzfeld, Germany), B6.SJL-Ptprc^aPepc^b/BoyJ and B6.129P2IL10^{tm1Cgn}/J (IL10 KO/IL10^{-/-}) mice from André Bleich, Hannover Medical School. Mice were gender and age matched between groups and housed under specific pathogen-free conditions. All experiments were approved by the committee of animal welfare.

Experimental model of HDM-induced asthma

Mice were treated as described previously.²⁰ In brief, WT and B cell-deficient mice were immunized once per week by intranasal (i.n.) application of 100 μ g *Dermatophagoides pteronyssinus* extract (HDM, Greer Laboratories, Lenoir, NC, USA) in 0.9% sodium chloride (NaCl, Fig 1A). Control mice received NaCl i.n. only (NaCl). Lung function measurements were performed three days after the last HDM application. Subsequently, mice were sacrificed.

B cell isolation and adoptive transfer

Splenic B cells from naïve WT and IL-10^{-/-} mice were enriched by CD43 microbeads according to the manufacturer's recommendations (Miltenyi Biotec, Bergisch Gladbach, Germany) yielding 94-97% pure CD19⁺ B cells. About 16x10⁶ B cells were injected intravenously (i.v.) into the tail vein within 24 hours prior to the first HDM-immunization.

Lung function

Invasive lung function was measured as described previously.^{8, 21} In brief, mice were anesthetized and AHR was measured in response to increasing methacholine concentrations (maximum 30mg/ml MCh, Sigma-Aldrich, Steinheim, Germany). Resistance (R), compliance (C) and elastance (E) are presented as percentage of baseline.

Bronchoconstriction and cytokine production of precision-cut lung slices (PCLS)

PCLS were performed as described previously.²² For contraction measurements, a lung slice with airway was placed in DMEM/F12 medium (Gibco/ThermoFisher Scientific, Waltham, MA, USA) including 1% penicillin/streptomycin, 15mM HEPES (Biochrom, Berlin, Germany) and used for videomicroscopy. Increasing MCh concentrations (10⁻¹⁰-10⁻³M) were

added and images were captured in 10-second-intervals for a total of 2 minutes/concentration by stereo microscope Zeiss Discovery V8 and digital video camera AxioCam Icm1 (Zeiss, Oberkochen, Germany). Airway area quantification was performed employing ImageJ software and bronchoconstriction is presented as percentage of initial airway area. For cytokine production, two slices/well were placed in DMEM/F12 medium and stimulated with 10µg/ml HDM and medium in triplicates for one day. Total protein content was determined as described elsewhere.²² IL-10 was detected by cytometric bead array according to the manufacturer's manual (LEGENDplex, BioLegend, Fell, Germany) and results were normalized to protein content (Fig E1 in the Online Repository).

Analysis of bronchoalveolar lavage fluid (BALF)

Preparations of BALF and cell differentiation were performed as previously described.²³

Lung histology

Preparation of lung sections, haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining were performed as described elsewhere.²⁴ Collagen fibers were stained by Masson Goldner (HTX-Weigert (Merck, Darmstadt, Germany); Ponceau-Säurefuchsin (Waldeck, Münster, Germany; Sigma-Aldrich), Orange G, Lichtgrün (both Merck)), and Elastika-van-Gieson staining (Resorcin-Fuchsin, Picrofuchsin (Waldeck; Sigma-Aldrich), HTX-Weigert (Merck)). Lung cross sections were scanned using Keyence BZ 9000 microscope (Keyence, Osaka, Japan) and NanoZoomer S210 Digital slide scanner C13239 (Hamamatsu Photonics, Hamamatsu, Japan). Pulmonary inflammation, mucus production and collagen fibers were quantified by an experimenter-independent in-house developed software.²⁵

Sircol soluble collagen assay

Freshly generated acid- and pepsin-soluble collagen was measured according to the manufacturer's instructions (Biocolor, Carrickfergus, United Kingdom). Briefly, right post-caval lung lobe was lysed in 0.1mg/ml pepsin (Sigma-Aldrich) and 0.5M acetic acid (J.T.Baker/Thermo Fisher Scientific) overnight. 1ml Sircol dye reagent/100µl supernatant was added and formed collagen-dye complex was resuspended in 1ml alkali reagent. Absorbance was measured in duplicates at 540nm (Synergy2 Multidetector microplate reader, BioTec Instruments, Winooski, VT, USA). Collagen content was calculated using a standard curve.

Preparation of serum and lung supernatant

Serum preparation was described elsewhere.²¹ Right middle lobe was mechanically crushed on ice by micropestle (Eppendorf, Hamburg, Germany), syringe and needles of decreasing diameter, centrifuged and supernatant was frozen at -80°C until use.

Cell isolation

Isolation of spleen, lymph node (sum of mandibular, superficial cervical and bronchial lymph nodes) and lung cells were performed as described earlier.⁸ In some experiments splenic T cells were isolated by CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's recommendations or by sorting of CD19⁻CD3⁺CD4⁺ T cells using FACS Aria IIu and FACS Aria Fusion (both BD, Heidelberg, Germany). Lung B cells were sorted as CD3⁻CD19⁺ B cells. Sorting strategy and representative reanalysis plots are shown in Fig E4, E and G in the Online Repository.

Gene expression

RNA was extracted by RNeasy Mini/Micro plus Kit (Qiagen, Hilden, Germany). Reverse transcription and quantitative PCR were performed as described previously with minor modifications.²⁶ 20-100ng RNA were used for PCR with following primers: Il10 (Mm00439614_m1), Tgfb1 (Mm01178820_m1), Ebi3 (Mm00469294_m1), Il12b (Mm01288989_m1), Il12a (Mm00434169_m1), Il2b (Mm00434256_m1), Lag3 (Mm00493071_m1), Il6 (Mm00446190_m1), Ifng (Mm01168134_m1), Il4 (Mm00445259_m1), Tnf (Mm00443258_m1), Hpvt (Mm01545399_m1, all Thermo Fisher Scientific). Gene expression was normalized using 2^{-ΔΔCt} algorithm.²⁷

In vitro cytokine production

Cells were restimulated *in vitro* with HDM (10μg/ml) in RPMI+ (RPMI 1640 medium including 10% FBS, 1% penicillin/streptomycin (all Biochrom), 1% HEPES (Gibco, Thermo Fisher Scientific), 100μM β-mercaptoethanol (Sigma-Aldrich) and RPMI+ for three days. Cytokines in supernatants were measured via DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA) or cytometric bead array (LEGENDplex, BioLegend) according to manufacturer's manuals. In some experiments cells were restimulated *in vitro* with HDM (10μg/ml) in RPMI+ for 25 hours. IL-10-secreting T and B cells were analyzed by IL-10 secretion assay according to the manufacturer's manual (Miltenyi Biotec).

Flow cytometry and antibodies

Flow cytometry was performed as described previously^{6, 23} using the following antibodies (clones): CD19-PerCP-Cy5.5 (1D3), CD19-BV510 (1D3), CD4-PE (RM4-5), CD8-PerCP-Cy5.5 (53-6.7), CD25-PE-Cy7 (PC61), CD138-APC (281-2), CD4-PerCP-Cy5.5 (RM4.5; all BD, Heidelberg, Germany), CD21/CD35-PE (7E9), CD24-FITC (M1/69), Ki-67-APC (16A8), B220-PerCP-Cy5.5 (RA3-6B2), CD21-PerCP-Cy5.5 (7E9), CD3e-APC-Cy7 (145-2C11), CD4-Pacific Blue (GK1.5), CD19-APC-Cy7 (6D5), CD44-APC-Cy7 (IM7), CD24-Pacific Blue (M1/69), CD25-BV510 (PC61), CD49b-APC-Cy7 (DX5), LAG-3-PE-Cy7 (C9B7W), 7-AAD Viability Staining; all BioLegend), CD23-PE-Cy7 (B3B4), MHCII-APC (M5/114.15.2), Foxp3-APC (FJK-16s), ICOS-FITC (C398.4A), IgM-PE-Cy7 (II/41), CD38-PE (90; all eBioscience, Frankfurt, Germany), PNA-FITC (Vector Laboratories, Peterborough, United Kingdom).

Immunoglobulin measurements

Immunoglobulins were detected by cytometric bead array (LEGENDplex) and ELISA (IgE, both BioLegend) according to the manufacturer's manuals.

Statistical analysis

For all statistical analyses, GraphPad Prism software was employed (GraphPad Software, La Jolla, CA, USA). Statistically significant differences (95% confidence interval) between two groups were determined using unpaired two-tailed Student's *t*-testing or nonparametric Mann-Whitney test. To compare more than two groups, two-way ANOVA (Tukey's multiple comparison post-test) or one-way ANOVA (Tukey's or Sidak's multiple comparison post-test or Kruskal-Wallis test combined with Dunn's multiple comparison post-test for data without Gaussian distribution) were used. Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

HDM-sensitized B cell-deficient mice develop significantly increased AHR, but display diminished airway inflammation compared to WT mice

To analyze the influence of B cells on local and systemic immune responses during experimental asthma, WT and B cell-deficient mice were referred to our well-established asthma protocol (Fig 1, A). In both, HDM-treated WT and B cell-deficient mice, this led to increased AHR compared to their respective controls (Fig 1, B-D). However, sensitized B cell-deficient mice displayed a significantly stronger AHR compared to their sensitized WT counterparts (Fig 1, B-D). To test this unexpected observation, we employed a second, independent technique for AHR measurement. We used PCLS and assessed airway constriction after methacholine provocation *ex vivo*.²² In line with our *in vivo* observation these measurements confirmed the stronger bronchoconstriction in lung tissue of HDM-sensitized B cell-deficient versus WT mice (Fig 1, E).

Analysis of BALF revealed significantly increased total cell counts and influx of eosinophils in both sensitized mouse strains compared to their respective controls. However, absolute cell numbers were significantly higher in sensitized WT compared to B cell-deficient mice, most prominently with regard to eosinophils (Fig 1, F, left), while percentages of eosinophils and neutrophils were similar in both strains (Fig 1, F, right). Investigator-independent quantification of H&E-stained lung tissues revealed significantly reduced airway inflammation in HDM-sensitized B cell-deficient versus WT mice (Fig E2, A in the Online Repository). Thus our analyses demonstrate that B cell-deficiency exacerbates AHR in HDM-sensitized mice.

Pulmonary collagen deposition is augmented in sensitized B cell-deficient mice compared to WT mice

Mucus production was assessed by PAS-staining of lung sections. HDM-sensitization induced mucus hypersecretion in both strains without significant difference between the genotypes (Fig 2, A-B).

Reduced lung compliance can be associated with fibrotic tissue remodeling in airway diseases.^{28, 29} We therefore assessed the collagen content in the lung. HDM-sensitized B cell-deficient but not WT mice showed an increase of collagen deposition as quantified by positive Masson Goldner (Fig 2, C-D) and Elastika-van-Gieson stains (Fig E2, B in the Online Repository), and of newly synthesized collagen (Fig 2, E). Taken together, these results demonstrate that B cells are central in controlling HDM-mediated airway remodeling.

Tregs and IL-10 are reduced in sensitized B cell-deficient mice compared to WT mice

To evaluate the HDM-induced T cell response under the control of B cells, we investigated antigen-specific cytokine responses *in vitro*. IL-10 production was significantly reduced in splenic and LN cells as well as in PCLS cultures from HDM-sensitized B cell-deficient compared to WT mice (Fig 3, A, Fig E3, C in the Online Repository). LN cells from HDM-sensitized WT mice produced significantly higher levels of IL-5 and IL-13 compared to those from allergic B cell-deficient mice (Fig 3, B), while IL-5 and IL-13 levels were comparable in spleen and lung of both strains (Fig E3, A-B in the Online Repository). The analysis of splenic T cell subsets demonstrated fewer T helper and cytotoxic T cells in naïve and allergic B cell-deficient mice compared to their WT counterparts (Fig E4, A-C in the Online Repository). Also, allergic B cell-deficient mice displayed significantly fewer spleen and LN FoxP3⁺ and FoxP3⁺ICOS⁺ Tregs (Fig 3, C-D) and decreased FoxP3 mRNA expression of splenic CD4⁺ T cells (Fig 3, F). Pulmonary Treg numbers were comparable between both mouse strains (Fig E4, D in the Online Repository). To test if Tregs are involved in the overall low IL-10 levels in secondary lymphoid organs of B cell-deficient mice we analyzed IL-10 mRNA expression and IL-10 secretion by splenic T cells. CD4⁺ T cells from allergic B cell-deficient mice expressed less IL-10 mRNA compared to those from WT mice (Fig 3, G). Additionally, in HDM-sensitized B cell-deficient mice the total number of IL-10-secreting CD4⁺ T cells, FoxP3⁺IL-10⁺, FoxP3⁺ICOS⁺IL-10⁺ Tregs, and FoxP3⁺CD49b⁺LAG-3⁺ IL-10⁺ type 1 regulatory T (Tr1) cells was significantly reduced after HDM-restimulation *in vitro* (Fig. 3, H-I, Fig E4, F in the Online Repository). However, IL-10 secretion of FoxP3⁺ and FoxP3⁺ICOS⁺ Tregs was comparable between both mouse strains, whereas FoxP3⁺CD49b⁺LAG-3⁺ Tr1 cells from B cell-deficient mice secreted less IL-10 as their WT counterparts (Fig 3, J). Taken together, our data illustrate significantly impaired T cell responses and disturbed IL-10 production in HDM-sensitized B cell-deficient mice.

HDM-immunization induces a strong B cell response in the lung

With the aim to analyze local B cell responses, we first characterized B cells from HDM-sensitized and naïve WT mice. Total B cell numbers in the lung of HDM-sensitized mice were significantly increased with a specifically high number of follicular mature (FM) B cells, germinal centre (GC) B cells and plasmablasts/plasma cells (PB/PC) (Fig 4, A-D). FM B cells expressed elevated levels of MHCII and CD23, indicating their activated status (Fig 4,

E). Consistent with these findings, we detected elevated levels of IgM, tolerogenic IgA, as well as Th2-related IgE, IgG1, IgG2b, and Th1-related IgG2c in sensitized lungs (Fig 4, F). Moreover, the expression of immunosuppressive IL-10, LAG-3, and Epstein-Barr virus (EBV)-induced gene 3 (Ebi-3, β -chain subunit of IL-35), but not TGF- β was significantly increased in lung B cells from allergic mice (Fig 4, G-H). The expression of multifunctional and pleiotropic IL-6 was significantly increased, while TNF- α , IFN- γ and IL-12a expression was comparable between both groups (Fig 4, H). Expression of Th2-related IL-2, IL-4, and IL-12b was not detectable (data not shown). Taken together, our data demonstrating a significant increase of differentiated B cell subsets and upregulation of IL-10, LAG-3 and Ebi-3 as well as IgM, IgA, IgE, IgG1, IgG2b, and IgG2c support a critical role of B cells in the local immune response to allergen challenge.

IL-10-secreting B cell subsets are increased in lungs of allergic WT mice

Since lungs from sensitized mice exhibited elevated B cell numbers and B cell-specific IL-10 expression, we analyzed their potential to secrete IL-10 after HDM-restimulation *in vitro*. Consistently, HDM-sensitization resulted in increased relative and absolute numbers of IL-10-secreting B cells in the lung (Fig 5, A). Next, we elucidated the involvement of lung FM, GC B cells and PB/PC in IL-10 production. All three B cell subsets derived from HDM-sensitized mice produced more IL-10 as those derived from naïve mice. The highest IL-10 amount was produced by FM B cells, followed by GC B cells and PB/PC (Fig 5, B-D). Our data hint to an important role of IL-10-secreting B cells in the lung in response to allergen challenge.

Transfer of WT but not of IL-10^{-/-} B cells significantly reduces AHR in allergic B cell-deficient mice

Based on the observation that IL-10, FoxP3⁺ and FoxP3⁺IL-10⁺ Tregs were significantly reduced in HDM-sensitized B cell-deficient compared to WT mice, we hypothesized that IL-10 secreting B cells may be key in the regulation of AHR. To test this hypothesis, we performed adoptive B cell transfer experiments employing naïve WT and IL-10^{-/-} mice as B cell donors. Purified CD19⁺ B cells from WT and IL-10^{-/-} mice were transferred i.v. into B cell-deficient mice prior to HDM-sensitization (Fig 6, A, Fig E5, A in the Online Repository). Importantly, both distribution of transferred B cell subpopulations and B cell engraftment of WT and IL-10^{-/-} B cells were comparable in B cell-deficient recipients (Fig E5, B-D in the Online Repository). However, while transfer of WT B cells significantly alleviated the

increase of AHR in sensitized B cell-deficient mice as demonstrated by improvement of all analyzed lung function parameters, the transfer of IL-10^{-/-} B cells had no such effect (Fig 6, B-D). Furthermore, WT but not IL-10^{-/-} B cell transfer led to an increase in total BALF cell numbers, without affecting the distribution of inflammatory BALF cell types (Fig 6, E). In accordance with the improved lung function, airway remodeling in allergic B cell-deficient mice was clearly ameliorated upon WT, but not IL-10^{-/-} B cell transfer, as demonstrated by decreased collagen deposition (Fig 7, A-B). No difference of freshly generated collagen or mucus hypersecretion was seen (Fig E6, A-C in the Online Repository). Taken together, these data illustrate that IL-10-proficient B cell transfer significantly improves AHR and airway remodeling in HDM-induced asthma.

Both WT and IL-10^{-/-} B cell transfer impact antigen specific cytokine production, FoxP3⁺ Treg expansion and IgE secretion

Adoptive transfer of WT B cells to B cell-deficient mice resulted in a normalization of splenic IL-10 levels and T cell subsets including Treg numbers (Fig 8, A-C, Fig E7, B-C in the Online Repository). Additionally, the production of antigen-specific IL-5 and IL-13 was similarly impacted (Fig E7, A in the Online Repository). However, all these effects appeared to be independent of B cell-derived IL-10, as no significant differences occurred between recipients of WT and IL-10^{-/-} B cells (Fig 8, D-H, Fig E7, D-F in the Online Repository).

Since GC B cells and PB/PC of HDM-sensitized WT mice secreted significantly more IL-10 as those derived from naïve mice, we analyzed the impact of IL-10-competent and IL-10-deficient B cells on IgE production. We found that transfer of IL-10^{-/-} B cells compared to WT B cells resulted in significantly elevated IgE levels in the BALF of recipient mice (Fig 8, I).

Discussion

The present study investigated the impact of B cells on allergy and immunological tolerance in a HDM-based murine asthma model. Our data demonstrate that local allergen-sensitization boosted the pulmonary B cell response, especially IL-10-secreting B cells. In contrast, HDM-sensitized B cell-deficient mice displayed significantly stronger AHR and airway remodeling, decreased IL-10 levels, and diminished Treg numbers in secondary lymphoid organs. The transfer of IL-10-proficient WT B cells into B cell-deficient recipients prior to HDM-sensitization substantially counteracted these features, especially AHR and pulmonary collagen deposition. By contrast, transfer of IL-10^{-/-} B cells failed to improve AHR and lung remodeling, while FoxP3⁺ Tregs and cytokine levels were similar in recipients of WT or IL-10^{-/-} B cells. Our findings indicate that AHR and airway remodeling are regulated by IL-10-producing B cells while other B cell mediated effects like FoxP3⁺ Treg expansion appear to be IL-10-independent and impacted by other B cell functions.

To the best of our knowledge, we here demonstrate for the first time that airway hyperreactivity and remodeling, two hallmark features of allergic asthma, are increased in B cell-deficient mice in experimental HDM-based asthma. This increased remodeling may have contributed to the development of poor lung function³⁰ and airway obstruction³¹ in our model. Our finding on the relevance of B cells in lung remodeling is in line with previous work by Arras and colleagues, who showed that B cells contribute to the protection against murine lung fibrosis induced by silica particles.³² Moreover, B cell-deficiency was shown to be associated with hepatic fibrosis and increased granuloma formation after *Schistosoma mansoni* infection.³³

A detailed phenotypical analysis of pulmonary B cells from HDM-sensitized compared to naïve WT mice revealed an increase of overall B cells as shown by elevated FM B cells, GC B cells and PB/PC. Additionally, lung B cells showed strengthened IL-10 secretion and B cell-deficiency resulted in decreased IL-10 production. We therefore directly compared the role of IL-10-competent versus IL-10^{-/-} B cells in AHR and pulmonary remodeling using adoptive cell transfer experiments. Of note, although comparable engraftment of IL-10-competent and IL-10^{-/-} B cells did not fully restore the B cell compartment in recipient mice, we demonstrated that both hallmark features of allergic asthma are centrally regulated by IL-10-competent but not IL-10^{-/-} B cells. This observation is in line with recent work showing that the supply of recombinant IL-10 into murine lung can prevent and reverse pulmonary fibrosis³⁴ and that B cell-derived IL-10 restrains excessive monocyte infiltration into the

liver, thereby alleviating hepatic inflammation and fibrosis in murine *Schistosoma* infection.³⁵ The central influence of B cells on AHR has been controversially discussed.^{36,37,38} We therefore used invasive lung function measurement, the gold standard to assess AHR *in vivo*. Additionally, we verified our results by PCLS *ex vivo*.²²

Generally, the effect of B cell-derived IL-10 can be either direct or indirect.³⁹⁻⁴¹ Several studies have shown that B cell-derived IL-10 can directly induce Treg expansion and inhibition of antigen-presenting function of dendritic cells (DC) and monocyte-derived TNF- α *in vitro*.⁴²⁻⁴⁴ The suppression of DC-derived proinflammatory cytokines subsequently prevents T helper cell differentiation.^{45, 46} IL-10 can also impact B cells in a para- and autocrine way. High IL-10 expression by B cells can directly mediate an immunoregulatory B cell phenotype by upregulation of suppressor of cytokine signaling 3 (SOCS3), glycoprotein a repetitions predominant (GARP), CD25 and programmed cell death ligand 1 (PD-L1),⁴⁷ as well as the enrichment of IL-10-producing Bregs,⁴⁸ and the preferential differentiation into IgG4⁺ plasma cells.⁴⁹

B cell-deficiency resulted in both, reduced FoxP3⁺ Treg and IL-10-secreting CD4⁺ T cell pools in secondary lymphoid organs. Although absolute numbers of IL-10-secreting FoxP3⁺ and FoxP3⁺CD49b⁺LAG-3⁺ Tr1 cells were diminished, only Tr1 cells showed reduced capacity to secrete IL-10. This finding suggests that IL-10 is reduced in B cell-deficient mice partly because of absent B cell-derived IL-10, decreased Treg numbers and Tr1-derived IL-10 secretion. Indeed, in human allergen-specific immunotherapy (IT), increased IL-10-secreting Tr1 cells were associated with diminished clinical symptoms.⁵⁰ Furthermore, in experimental asthma models, IT decreased AHR and pulmonary structural changes and induced Tr1 cells, which crucially contributed to IT effectiveness,⁵¹ and when transferred, reduced AHR and airway inflammation.⁵²

It remains to be determined, if IL-10⁺ and IL-10^{-/-} B cells similarly boost functional IL-10 production by Tregs in our model. Given that IL-10^{-/-} B cell mice develop exacerbated collagen-induced arthritis and reduced IL-10-secreting Tr1 cells⁵³ and that IL-10-producing LAG-3⁺ Treg-of-B cells alleviates AHR in an OVA-based model,⁵⁴ it is tempting to speculate that IL-10-competent B cells could improve Tr1 cell function in allergic B cell-deficient recipients more effective than IL-10^{-/-} B cells, which in turn could modulate the pulmonary outcome in our model.

Another IL-10-dependent mechanism could be the decrease of IgE-production and -function. It is well known, that IgE-mediated mast cell degranulation leads to reversible airway

obstruction.³⁰ Previous studies showed B cell-dependent suppression of IgE- and antigen-mediated mast cell activation in a IL-10-dependent manner *in vitro* and in an anaphylaxis model⁴⁸ as well as diminished IgE production by IL-10-overexpressing B cells and PBMC cultured with IL-10 *in vitro*.^{47, 55} Since our results exhibit elevated numbers of IL-10-secreting FM B cells, GC B cells and PB/PC in lungs from allergic WT mice and increased IgE levels in the BALF of IL-10^{-/-} B cell recipients, one can speculate that higher IgE production and/or disability to produce IL-10 could partly lead to strengthened mast cell degranulation and airway obstruction in allergic IL-10^{-/-} B cell recipients. Therefore, further studies are needed to clarify the exact mechanism(s) by which B cell-derived IL-10 improves the pulmonary outcome.

Another interesting finding was the impact of B cells on antigen-specific T cell cytokine production and Treg distribution. An imbalanced Th2 response is characteristic of allergic asthma.¹⁰ Antigen-specific Th2 cytokine production of LN cells was significantly reduced in HDM-treated B cell-deficient compared to WT mice. This is in line with findings showing the importance of LN T cell:B cell zone for initial Th2 cell development, and influence of B cells on IL-4-producing T cells,⁵⁶ which may have been a cause for reduced Th2 cytokines in our observations.

Furthermore, B cell transfer significantly induced FoxP3⁺ Tregs and IL-10 production, demonstrating that B cells are necessary to induce an adequate regulatory lymphocyte pool to counteract allergic inflammation. This tolerogenic effect, lasting at least 3.5 weeks post-transfer in our model, is in line with their immediate influence in acute allergic airway inflammation: short term transfer of CD9⁺ B cells from asthmatic into HDM-immunized WT mice led to improved plethysmography-based airway function, reduced allergen-specific serum IgE, and a favorable Treg/T effector ratio two days after cell transfer.⁵⁷ However, transfer of both WT and IL-10-deficient B cells induced comparable numbers of FoxP3⁺ Tregs and similar IL-10 production by splenic and LN cells in our model, supporting the notion that FoxP3⁺ Treg induction may not have been the decisive mechanism influencing AHR and remodeling in our experiments. Consistent with these data, previous studies showed that adoptive transfer of WT and IL10^{-/-} B cells induced comparable numbers of FoxP3⁺ Tregs in experimental autoimmune encephalomyelitis *in vivo*.⁵⁸ Furthermore, OVA-pulsed IL-10^{-/-} B cells were able to effectively induce Tregs *in vitro*,⁵⁹ suggesting that B cell-derived IL-10 is not the only prerequisite for B cell-mediated induction and/or maintenance of FoxP3⁺ Tregs.⁵⁸

Next to IL-10, B cells can also exert immunosuppressive effects via TGF- β , IL-35,^{40, 41} thrombospondin (TSP1)⁶⁰ and CD1d.⁶¹ Our data demonstrated increased Ebi-3, but reduced TGF- β expression in lung B cells from allergic mice, suggesting a possible involvement of Ebi-3, rather than TGF- β . Ebi-3 can form the heterodimers IL-35 (together with IL-12b), IL-27 (together with IL-27p28) or regulate independently the immune response.⁶² Indeed, transferred IL-35-stimulated B cells subsequently produced IL-35 and ameliorated experimental autoimmune uveitis.⁶³ Furthermore, mice lacking IL-35⁺ B cells lost their ability to recover from experimental autoimmune encephalomyelitis.⁶⁴ Additionally, IL-35 application during OVA-sensitization attenuated AHR, local DC accumulation, conversion of recruited monocytes into inflammatory DCs and T cell proliferation.⁶⁵ Recently, Ayasoufi et al showed IL-27 expression by B cells,⁶⁶ which is critically involved in the suppression of OVA-induced experimental asthma.⁶⁷ Moreover, our data show that lung B cells from sensitized mice upregulated the expression of inhibitory receptor LAG-3. It has recently been shown that LAG-3 is expressed on immunosuppressive natural plasma cells in an infection model⁶⁸ and that Treg-derived LAG-3 can suppress DC maturation and immunostimulatory capacity.⁶⁹ Additional studies will answer, which IL-10-independent B cell function(s) caused the observed regulatory effects.

B cells are key in allergy and immunological tolerance, and a plethora of recent work supports the role for B cells in regulating immune reactions.⁷⁰ The production of anti-inflammatory molecules by Bregs is crucial in immunosuppression, and we here show that IL-10-producing B cells are central in HDM-induced allergy and regulation of AHR and remodeling. Moreover, B cells are crucial to induce Tregs and modulate antigen-specific cytokine responses in this process. This may be interesting in the development of further treatment or prevention strategies for asthma. For example, IT has been shown to induce Bregs in human patients.¹² As such, we hope our data may help to pave the way for B cell-targeted prevention and treatment strategies for asthma and allergies in human patients in the future.

In summary, we here show a central role of Bregs in the modulation of lung function and airway remodeling as well as induction of Tregs in a murine asthma model. Our data may support future concepts for B cell targeted prevention and treatment strategies.

Acknowledgements

The authors thank Jana Bergmann, Christin Albrecht, Heike Selina Grundmann, Anika Dreier, Michaela Bartsch and Beate Junk for excellent technical assistance; Institute for Pathology at Hannover Medical School for staining tissue slides; PD Dr. Susanne Rittinghausen, Fraunhofer Institute for Toxicology and Experimental Medicine Hannover, for excellent scans of lung cross sections; and Dr. Matthias Ballmaier, Central research facility cell sorting at Hannover Medical School for his excellent guidance and help concerning cell sorting.

This research was supported by the German Center for Lung Research (DZL; Federal Ministry of Education and Research), by the Cluster of Excellence RESIST (EXC 215) by the Deutsche Forschungsgemeinschaft (DFG) and by the Dörenkamp Foundation.

Author contributions

AH, AMB, GH designed the research and wrote the manuscript; AH, CH, JS, MB, KD, HO, RG performed experiments; AH, MB, HO analyzed data; all authors contributed to data interpretation, commented on the manuscript and approved it.

Conflict of interest disclosure

The authors declare no commercial or financial conflict of interest.

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Figure Legends

Fig 1. Allergic B cell-deficient (μ MT) mice develop increased airway hyperreactivity (AHR) compared to WT mice. **A**, Mouse model of house dust mite (HDM)-induced experimental asthma. Mice were sensitized intranasally (i.n.) four times with sodium chloride solved HDM (HDM/NaCl 0.9%). Control mice received NaCl 0.9% only. Mice were sacrificed three days after the last HDM-application. **B-D**, Lung function parameters resistance (R, *B*), compliance (C, *C*) and elastance (E, *D*) of allergic (HDM) and control (NaCl) WT and B cell-deficient mice were measured during methacholine (MCh) provocation and are presented as percentage of baseline respectively (n=10-14/group, three independent experiments). **E**, *Ex vivo* measurement of bronchoconstriction by precision-cut lung slices (PCLS). The graph displays relative airway area of initial area during stimulation with increasing MCh concentrations (n=3-4/group, seven preparations). * WT_{NaCl} versus WT_{HDM} , # μMT_{NaCl} versus μMT_{HDM} , § WT_{HDM} versus μMT_{HDM} . **F**, Cell differentials of the bronchoalveolar lavage fluid (BALF) are presented as total (*left*) and relative (*right*) numbers. Data are presented as mean $\pm$ SEM (n=7-9/group, two independent experiments). Macrophages (Mac), eosinophils (Eos), lymphocytes (Lym), neutrophils (Neu), * § $P < .05$, ** ### $P < .01$, *** $P < .001$, and ***** ##### $P < .0001$

Fig 2. HDM-induced allergic asthma elevates pulmonary collagen deposition in B cell-deficient (μ MT) mice. **A-B**, Lung cross sections (Fig 2, *A*) were stained with periodic acid-Schiff (PAS) and positive pixel were quantified by in-house software HistoClick (Fig 2, *B*, n=9-10/group, two independent experiments). **C-D**, Lung cross sections (Fig 2, *C*) were stained with Masson Goldner and positive pixel were quantified by HistoClick (Fig 2, *D*, n=9-12/group, three independent experiments). **E**, Newly synthesized acid- and pepsin-soluble collagen amount in the right post caval lung lobe of HDM-sensitized and naïve mice (Fig 2, *E*, n=8-11/group, two independent experiments). Data are presented as mean $\pm$ SEM. ns not significant, * $P < .05$, ** $P < .01$, *** $P < .001$, and ***** $P < .0001$. Magnification = x200, scale bar = 100 μ m.

Fig 3. Allergic B cell-deficient (μ MT) mice show reduced IL-10 levels and regulatory T cell numbers. **A** and **B**, Cytokines were measured in the supernatants of HDM-restimulated cells (Fig 3, *A-B*, spleen: n=9-15, lymph nodes (LN): n=7-12, three independent experiments respectively). **C-D**, FoxP3⁺ regulatory T cells (Tregs) and ICOS⁺FoxP3⁺ Tregs in spleens and

LN_s were measured by flow cytometry (Fig 3, C, n=6-14/group, three independent experiments; D, n=3-8/group, two independent experiments). E, Flow cytometry data were used to calculate Teff/Treg ratio (Fig 3, E, n=6-14/group, three independent experiments). F-G, Expression of FoxP3 and IL-10 mRNA was measured in splenic CD4⁺ T cells by quantitative PCR (Fig 3, F-G; n=6-8/group, two independent experiments) and normalized by 2^{-ΔΔCt} algorithm. H-J, Splenic IL-10-producing Treg subsets were measured after HDM-restimulation *in vitro* via flow cytometry (CD4⁺IL-10⁺, FoxP3⁺IL-10⁺ Tregs: n=12-17/group, three independent experiments; FoxP3⁺ICOS⁺IL-10⁺ Tregs, FoxP3⁺CD49b⁺LAG-3⁺IL-10⁺ type 1 regulatory T (Tr1) cells: n=9-10/group, two independent experiments). Data are presented as mean ± SEM. ns not significant, * *P* < .05, ** *P* < .01, *** *P* < .001, and **** *P* < .0001.

Fig 4. HDM-immunization induces a strong B cell response in the lung. A-E, Total lung B cells and B cell subsets were analyzed via flow cytometry (Fig 4, A, n=9-17/group, four independent experiments). Follicular mature (FM) B cells (Fig 4, B), germinal centre (GC) B cells (Fig 4, C, n=11-15/group, three experiments respectively) and plasmablasts/plasma cells (PB/PC, Fig 4, D, n=8-16/group, four independent experiments) as well as CD23 and MHCII expression on FM B cells were analyzed in HDM-immunized and control WT mice (Fig 4, E, n=6-7, two independent experiments). F, Total immunoglobulin isotypes were measured in the lung (Fig 4, F, n=9-14/group, two independent experiments) by cytometric bead array and ELISA. G-H, mRNA expression was analyzed in isolated lung B cells by quantitative PCR and was normalized by 2^{-ΔΔCt} method (Fig 4, G-H, n=9-14/group, three till four independent experiments). Data are presented as mean ± SEM. MFO Fluorescence minus one control, ns not significant, * *P* < .05, ** *P* < .01, *** *P* < .001, and **** *P* < .0001.

Fig 5. IL-10-secreting B cell subsets are increased in lungs of allergic WT mice. A-D, Relative and total numbers of IL-10-secreting B cells (Fig 5, A, n=9, two independent experiments), follicular mature (FM) B cells (Fig 5, B), germinal centre (GC) B cells (Fig 5, C) and plasmablasts/plasma cells (PB/PC, Fig 5, D, n=5/group, one representative experiment of two respectively) were detected after HDM-restimulation *in vitro* via flow cytometry. Representative flow cytometry plots are shown. Data are presented as mean ± SEM. * *P* < .05, ** *P* < .01, and *** *P* < .001.

Fig 6. Transfer of WT B cells improves poor lung function of allergic B cell-deficient (μ MT) mice. **A**, CD19⁺ B cells from WT and IL-10-deficient (IL-10^{-/-}) mice were purified and transferred into B cell-deficient (μ MT) mice prior to the first intranasal (i.n.) HDM-immunization (HDM/NaCl). Mice were sacrificed three days after the last HDM-application. **B-D**, Lung function parameters resistance (R, Fig 6, B), compliance (C, Fig 6, C) and elastance (E, Fig 6, D) were measured during methacholine (MCh) provocation and are presented as percentage of baseline respectively (n=11-19/group, two till five independent experiments). **E**, Differentiation of bronchoalveolar lavage fluid (BALF) cells is presented as total (Fig 6, E top) and relative (Fig 6, E bottom) numbers (n=3-11/group, two independent experiments). Results are expressed as mean $\pm$ SEM. Macrophages (Mac), eosinophils (Eos), lymphocytes (Lym), neutrophils (Neu), * $P < .05$, *** $P < .001$, and **** $P < .0001$

Fig 7. Transfer of WT B cells reduces pulmonary collagen deposition in HDM-immunized B cell-deficient (μ MT) mice. **A** and **B**, Lung cross sections (Fig 7, A) were stained with Masson Goldner and positive pixel were quantified by in-house software HistoClick (Fig 7, B, n=4-13/group, two independent experiments). Magnification = x200, scale bar = 100 μ m. Data are presented as mean $\pm$ SEM. ns not significant, * $P < .05$, and ** $P < .01$

Fig 8. IL-10-proficient B cells do not significantly influence cytokine production and FoxP3⁺ Treg priming. **A-C**, After adoptive transfer of WT B cells and HDM-immunization, IL-10 levels in the supernatant of HDM-restimulated cells (Fig 8, A, spleen: n=11-16/group, lymph nodes (LN): n=9-14/group), splenic FoxP3⁺ Tregs (Fig 8, B, n=11-14/group, three independent experiments respectively), and FoxP3⁺ICOS⁺ Tregs (Fig 8, C, n=8-11/group, two independent experiments) were measured. **D-F, I**, IL-10 production upon *in vitro* HDM-restimulation (Fig 8, D, n=6-13/group), total amounts of FoxP3⁺ Tregs, FoxP3⁺ICOS⁺ Tregs (Fig 8, E-F, n=6-13/group, two independent experiments respectively), and IgE level in serum and bronchoalveolar lavage fluid (BALF; Fig 8 G, serum, n=11-19/group, two till five independent experiments; BALF, n=11-13/group, two till three independent experiments), were measured after WT and IL-10-deficient (IL-10^{-/-}) B cell transfer and HDM-immunization in allergic B cell-deficient (μ MT) mice. Data are expressed as mean $\pm$ SEM. MFI mean fluorescence intensity, ns not significant, * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$

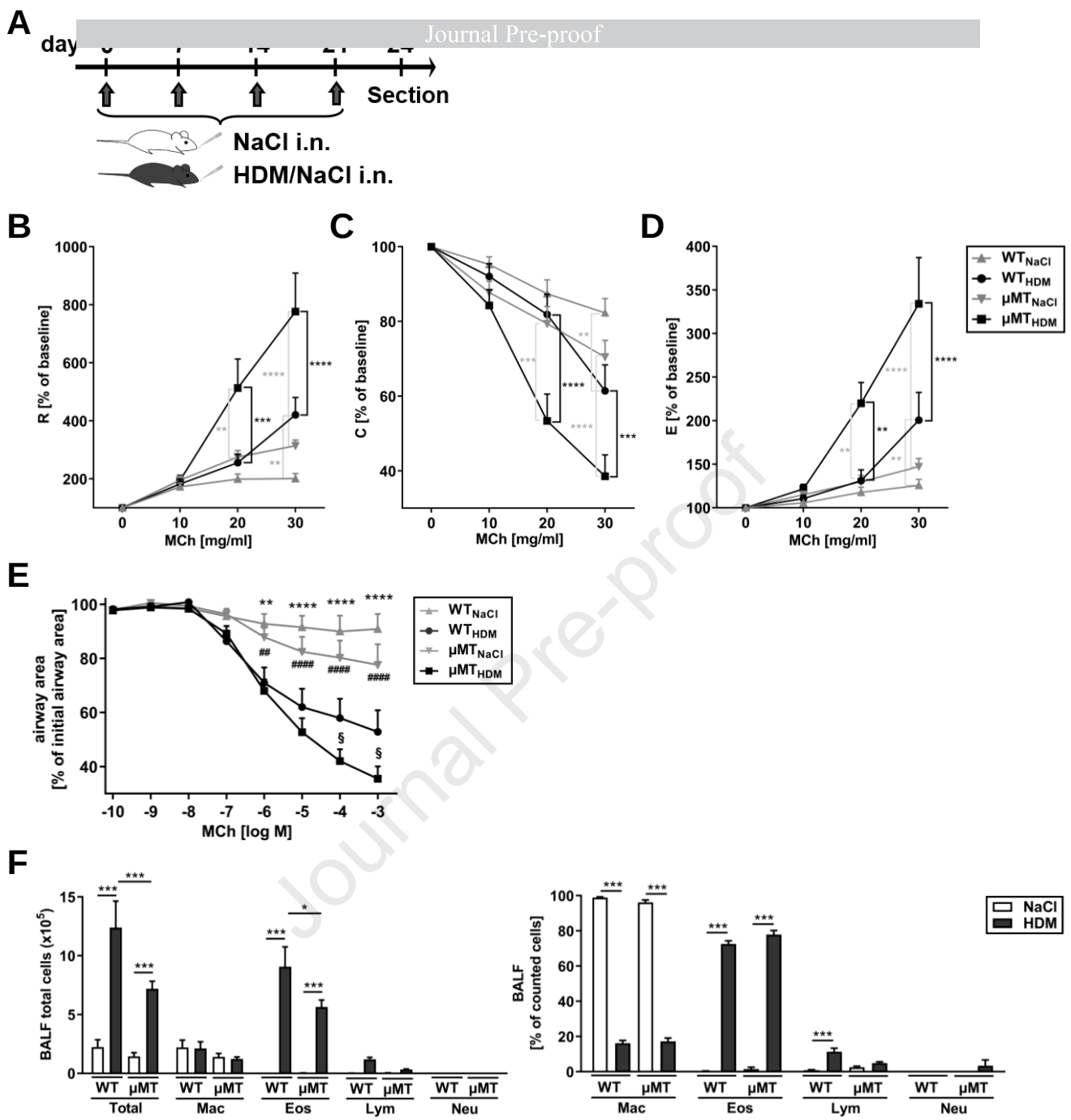
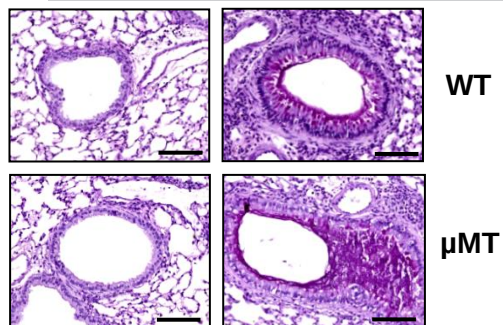
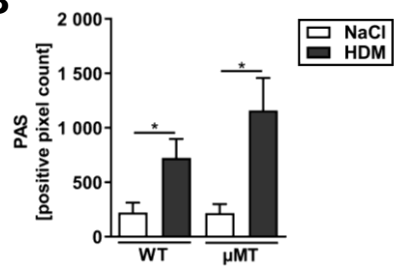
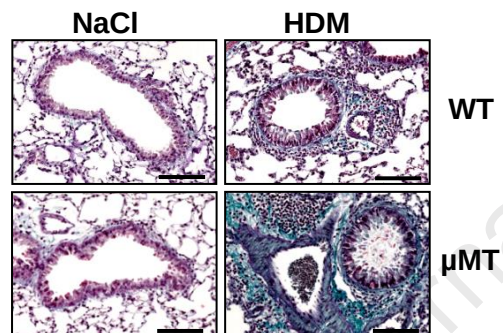
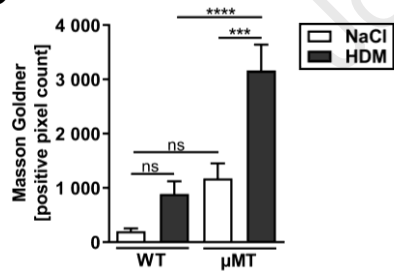
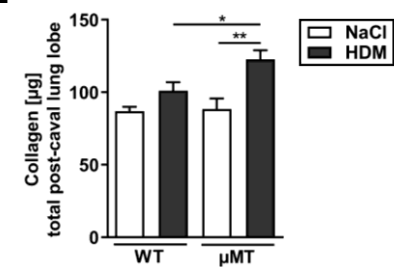


FIGURE 1.

A**B****C****D****E****FIGURE 2.**

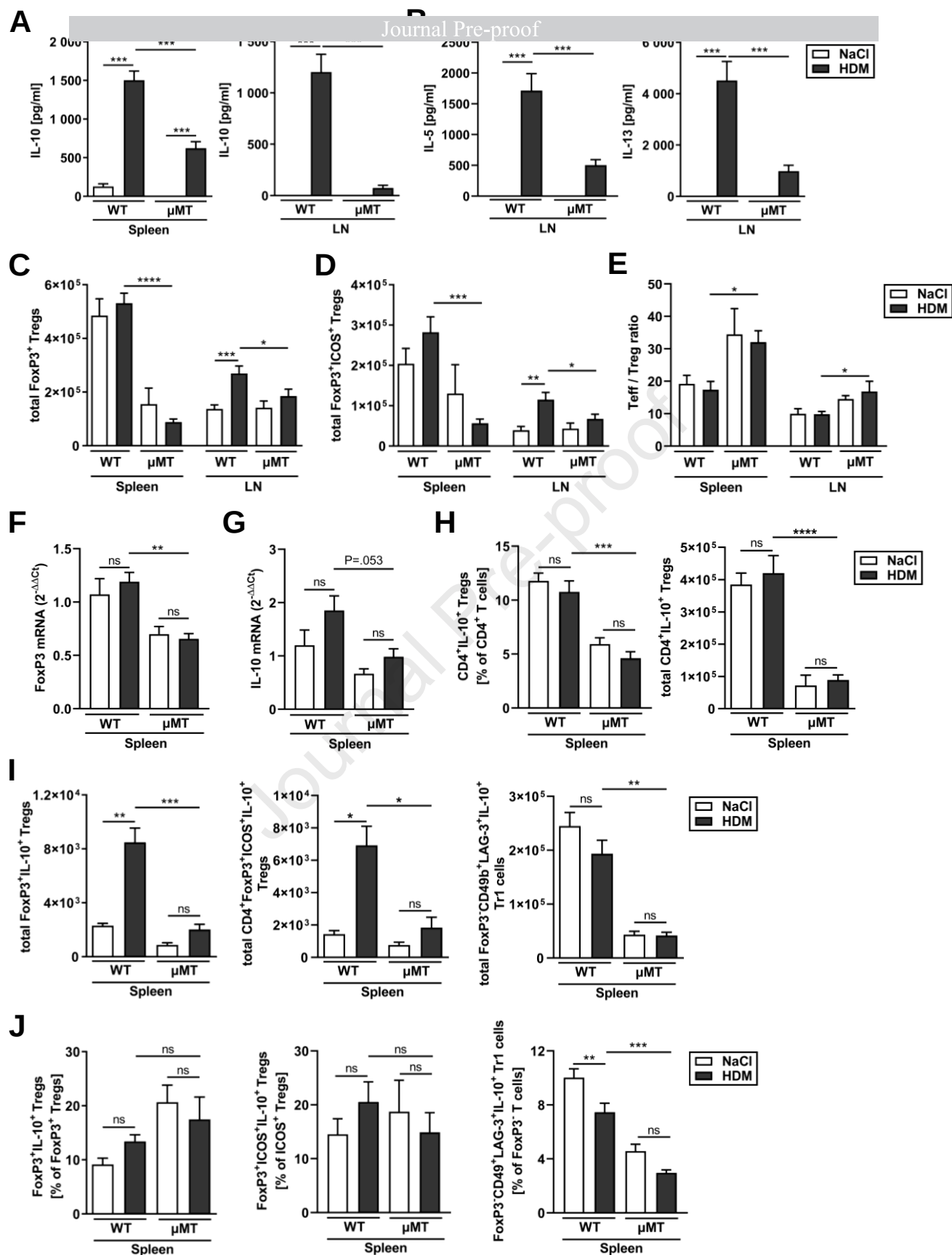


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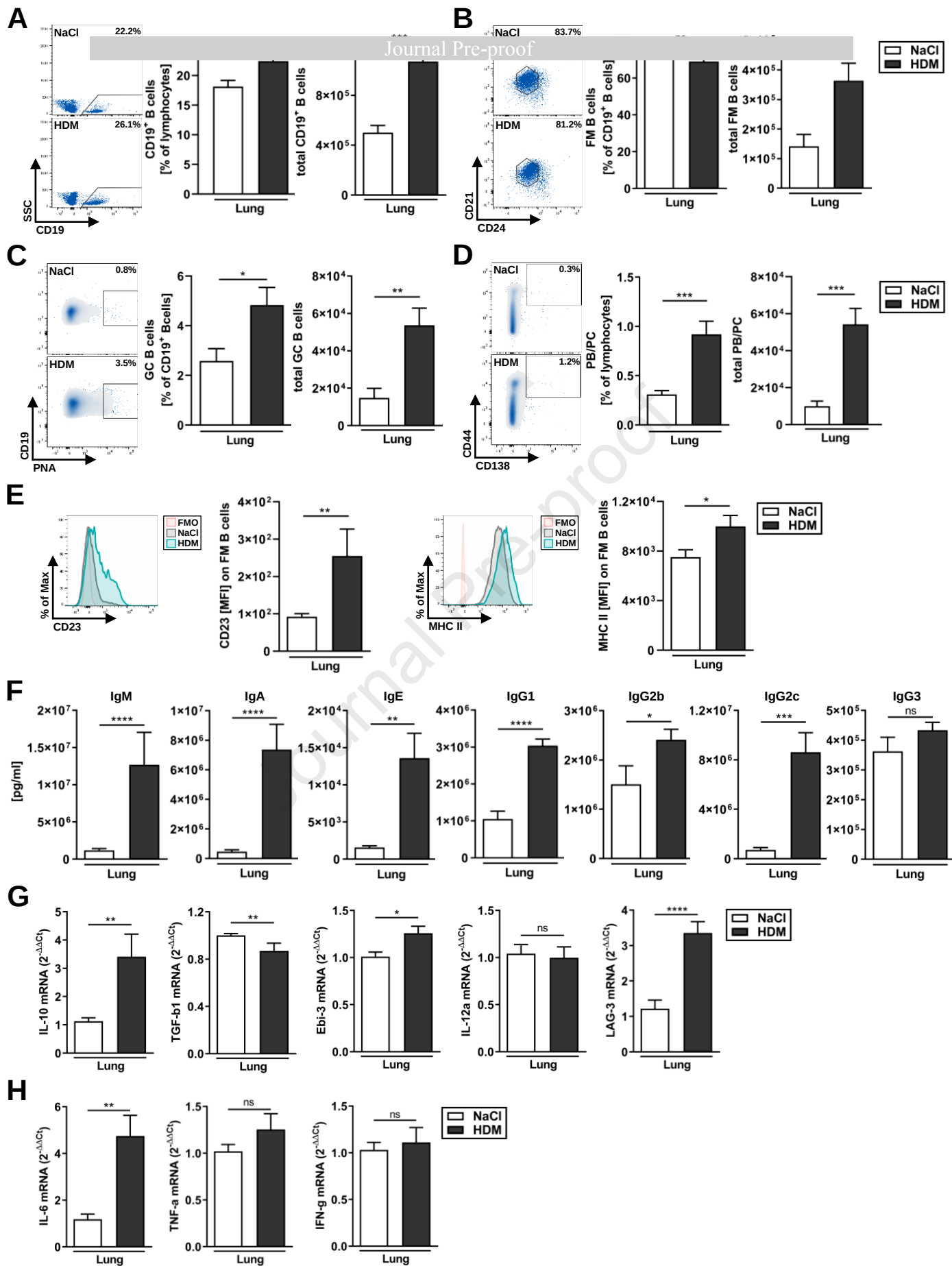


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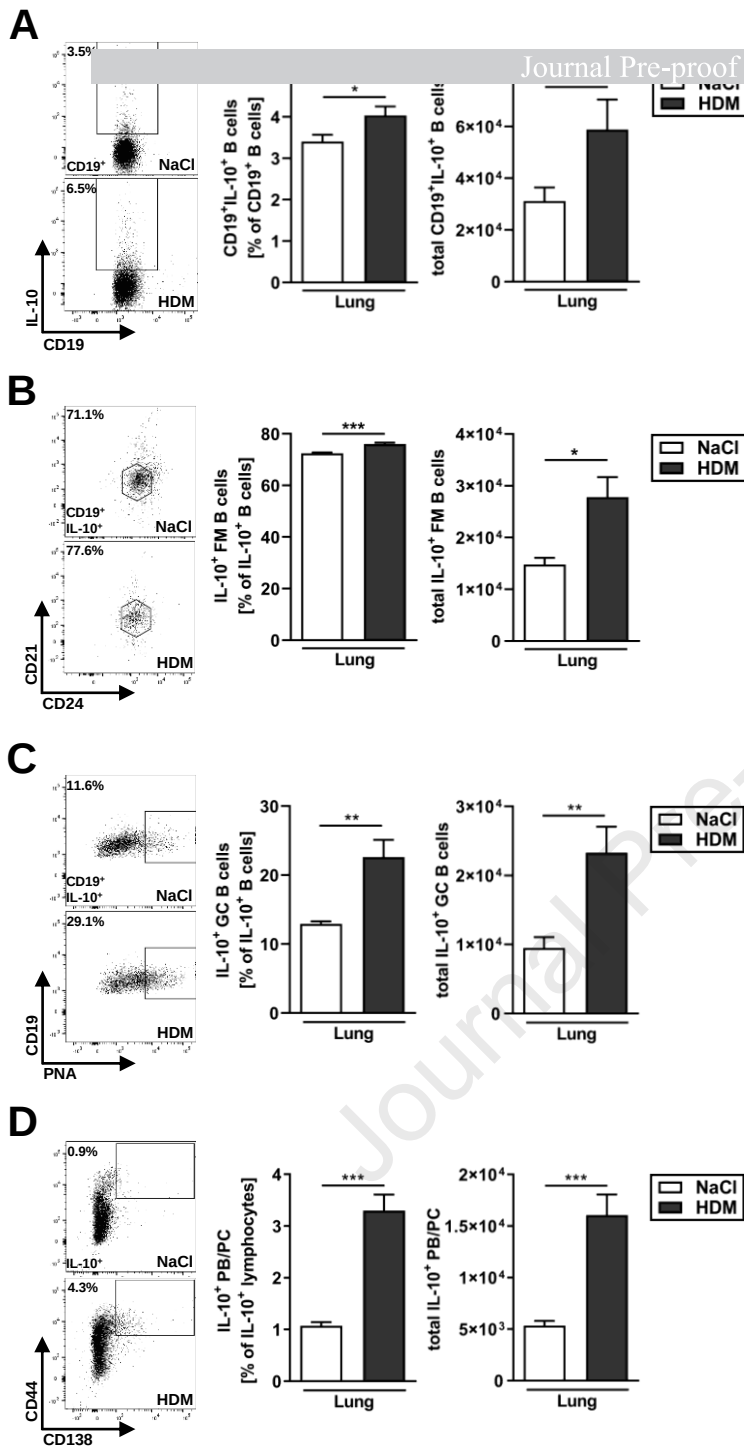


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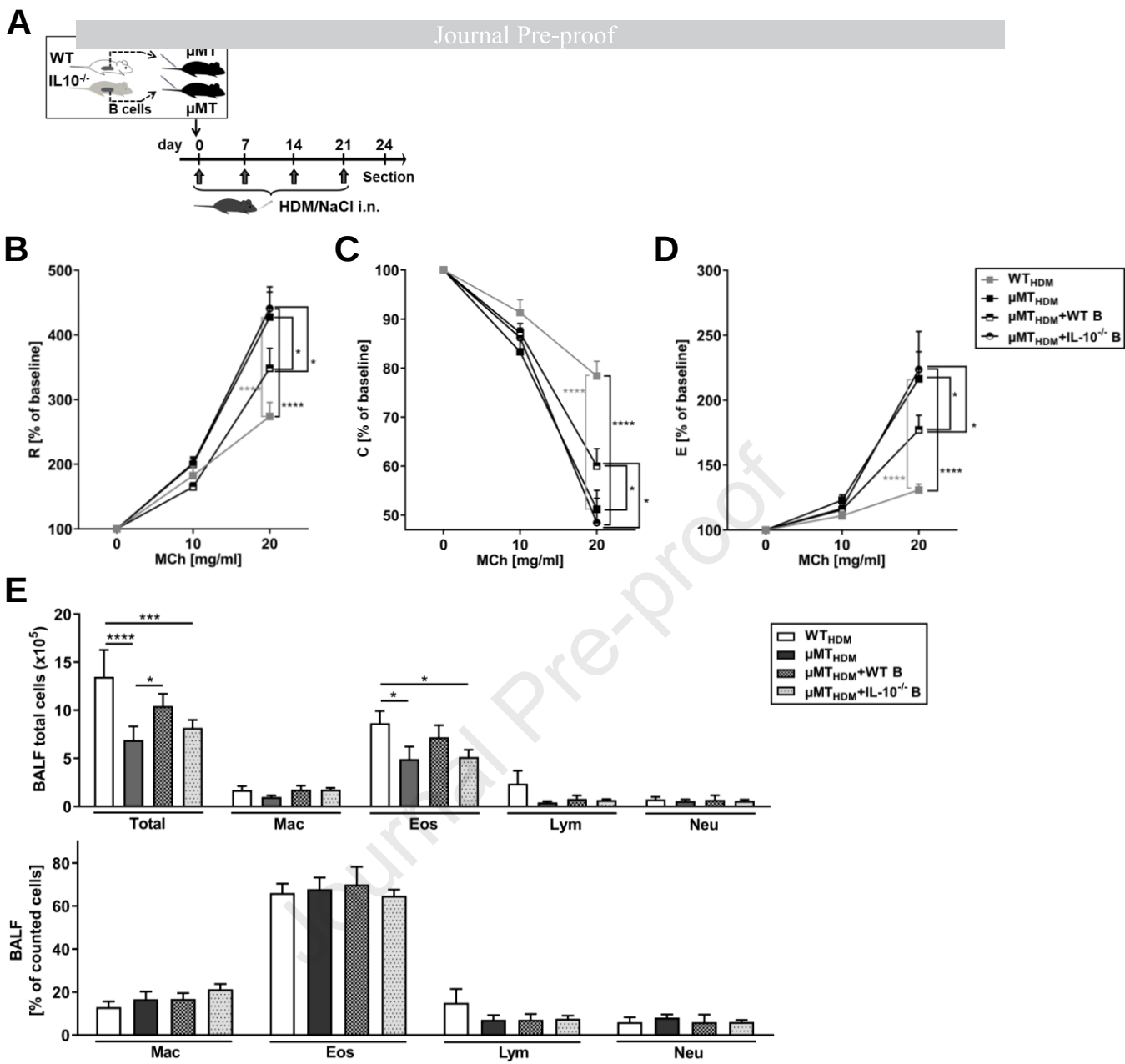
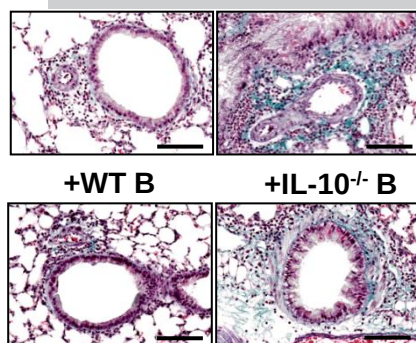
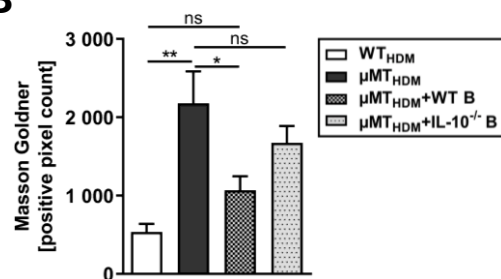


FIGURE 6.

A**B****FIGURE 7.**

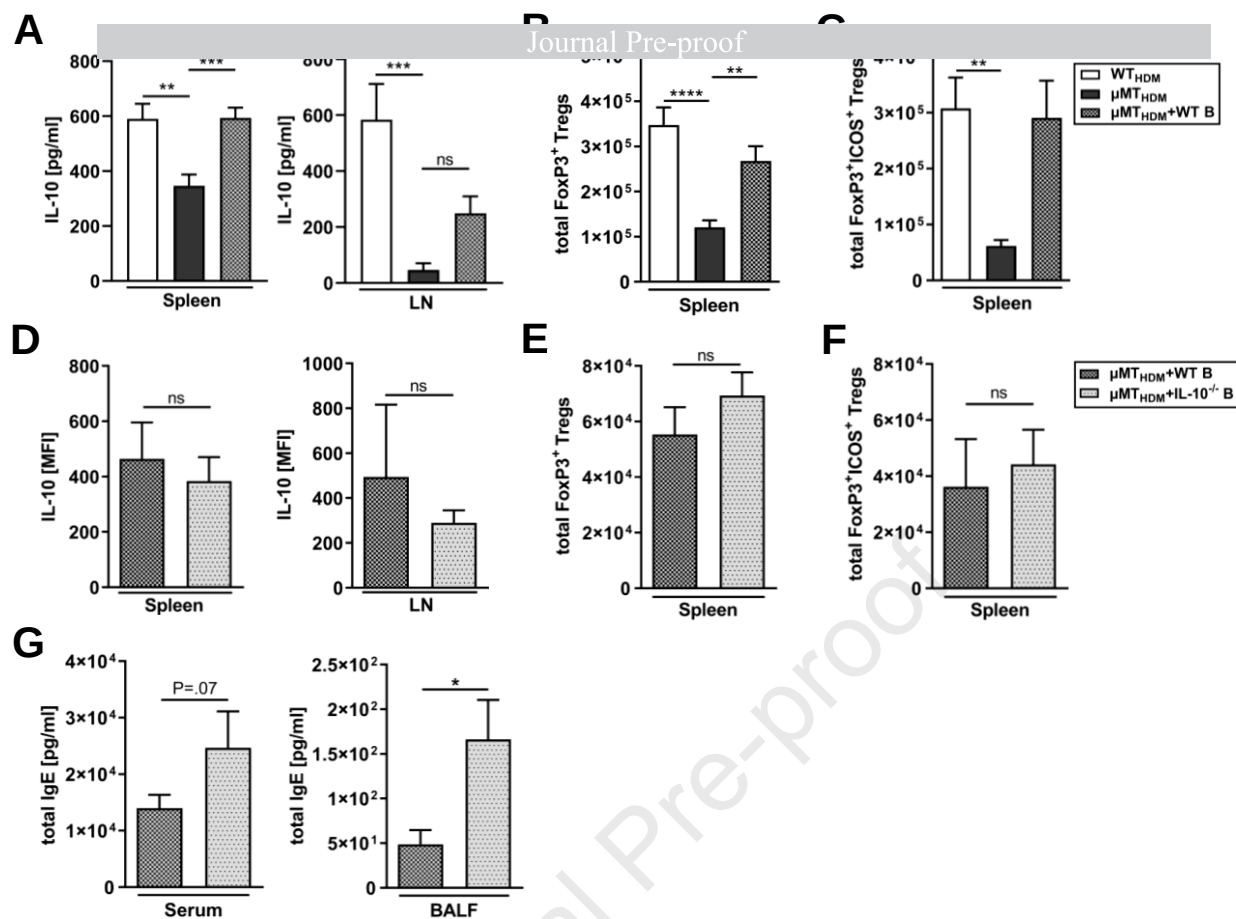
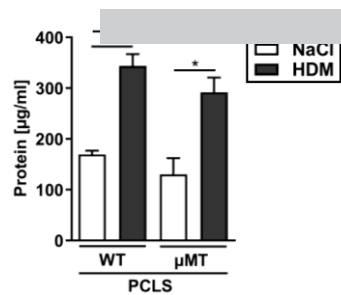
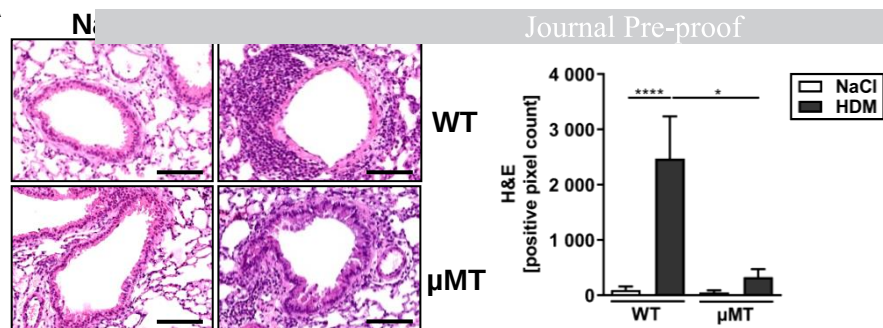
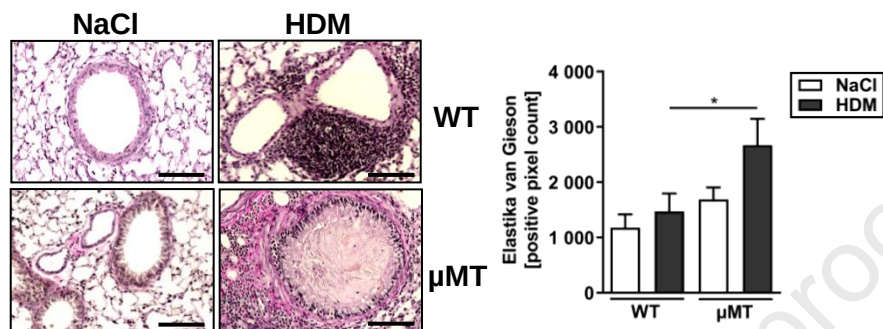
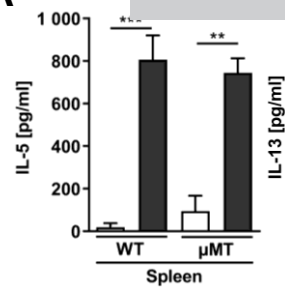
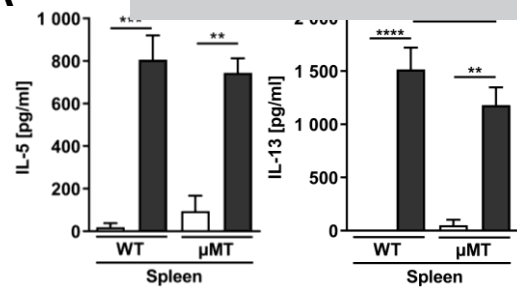
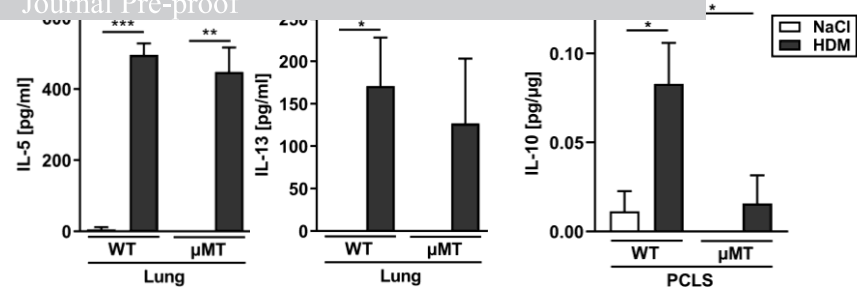


FIGURE 8.

**FIGURE E1.**

A**B****FIGURE E2.**

A**B****C****FIGURE E3.**



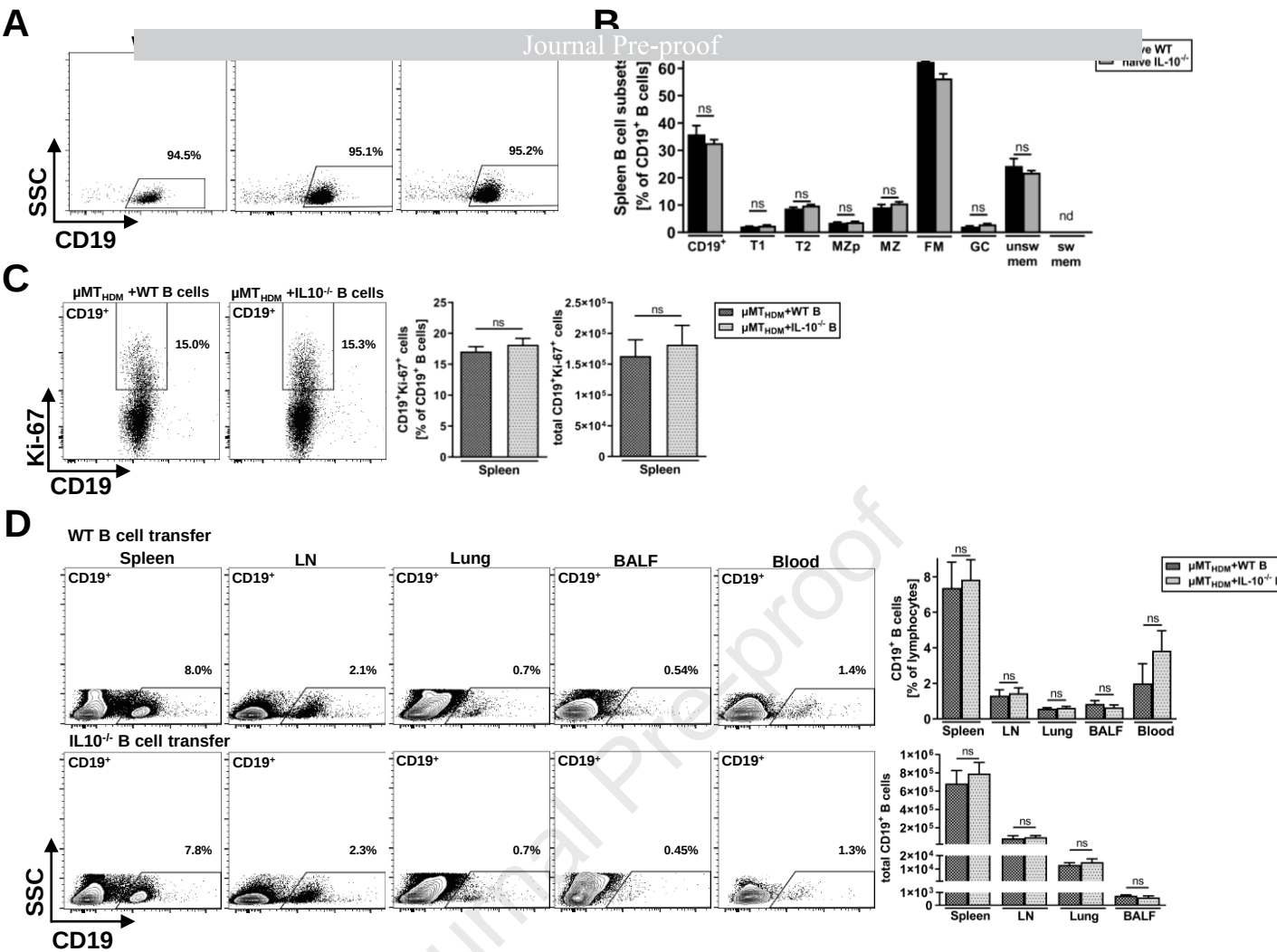


FIGURE E5.

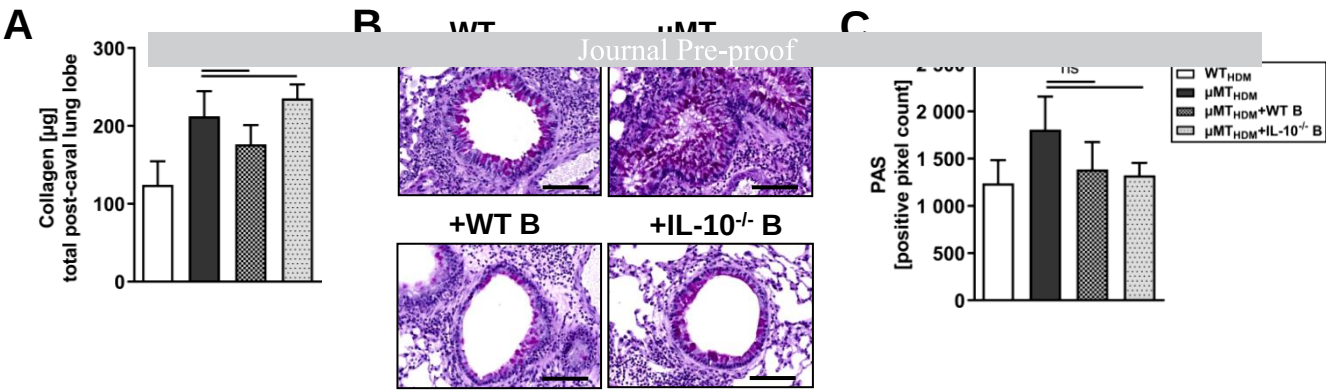


FIGURE E6.

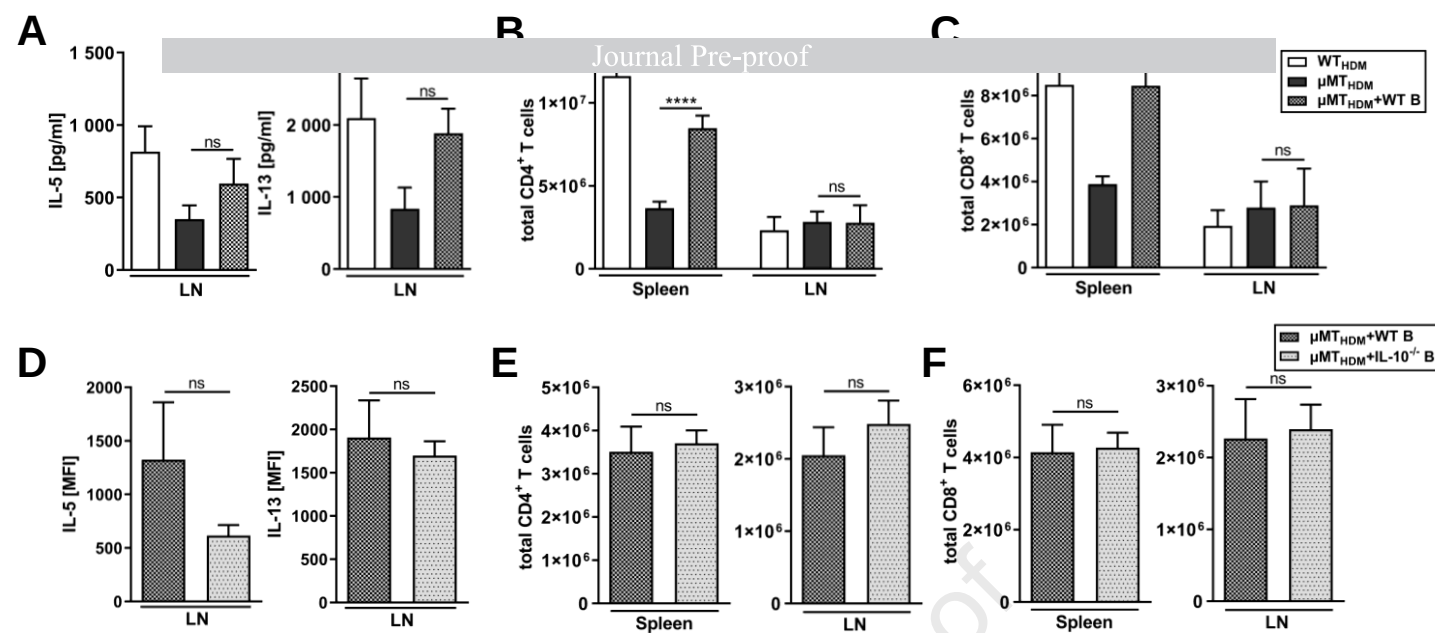


FIGURE E7.