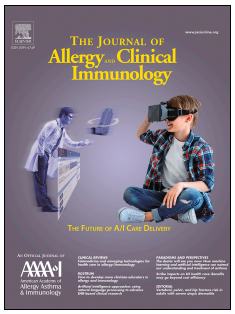
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GM-CSF instigates a dendritic cell - T cell inflammatory circuit that drives chronic asthma development

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PII: S0091-6749(21)00001-4

DOI: https://doi.org/10.1016/j.jaci.2020.12.638

Reference: YMAI 14919

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

Received Date: 7 April 2020

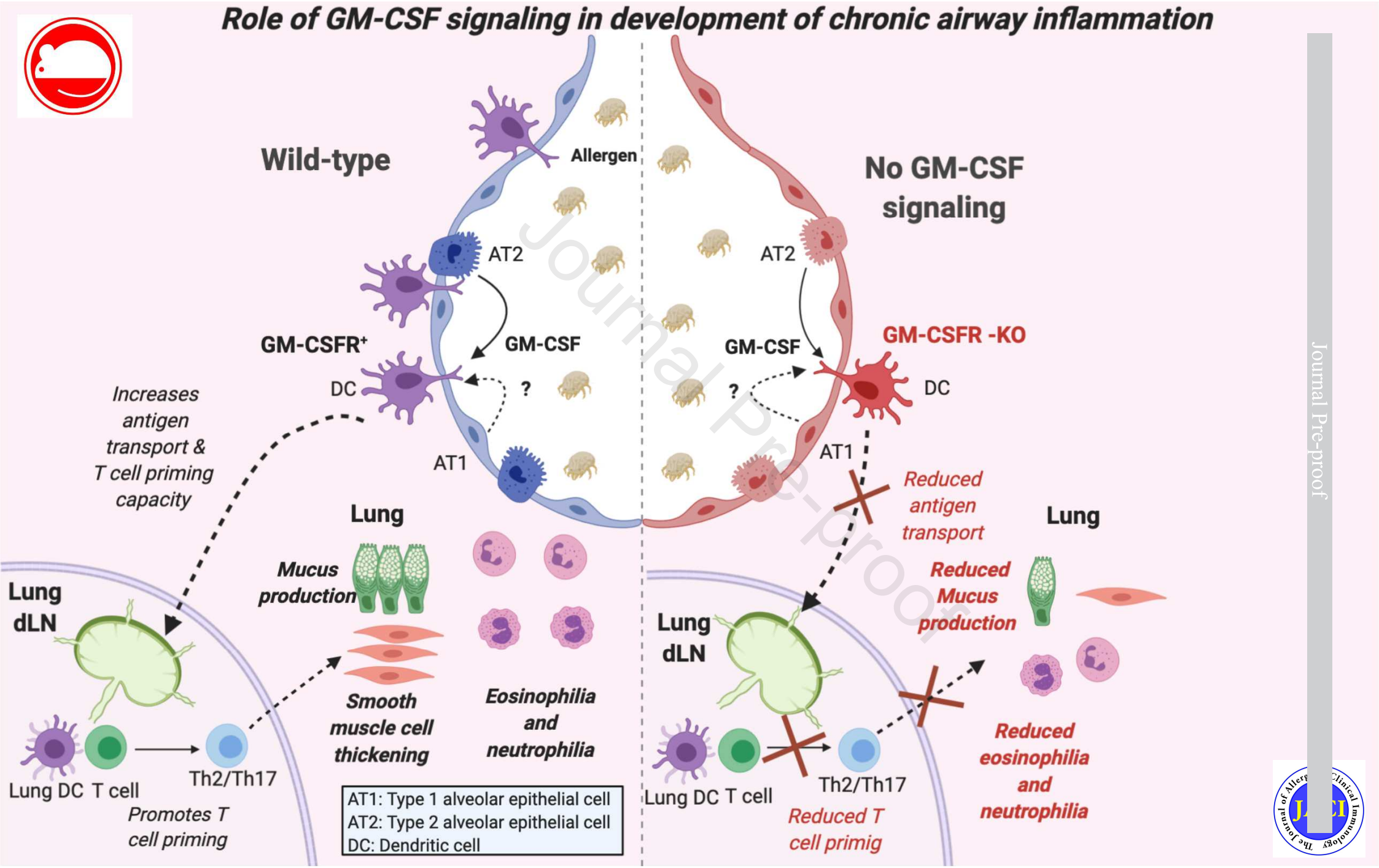
Revised Date: 6 November 2020

Accepted Date: 4 December 2020

Please cite this article as: Nobs SP, Pohlmeier L, Li F, Kayhan M, Becher B, Kopf M, GM-CSF instigates a dendritic cell - T cell inflammatory circuit that drives chronic asthma development, *Journal of Allergy and Clinical Immunology* (2021), doi: https://doi.org/10.1016/j.jaci.2020.12.638.

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GM-CSF instigates a dendritic cell - T cell inflammatory circuit that drives chronic asthma development

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Conflict of interest statement: The authors have no conflicting financial interest.

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Abstract

Background:

Steroid-resistant asthma is often characterized by high levels of neutrophils and mixed Th2/Th17 immune profiles. Indeed, neutrophils are key drivers of chronic lung inflammation in multiple respiratory diseases. Their numbers correlate strongly with disease severity and their presence is often associated with exacerbation of chronic lung inflammation.

Objective:

What factors drive development of neutrophil-mediated chronic lung disease remains largely unknown and we sought to study the role of GM-CSF as a potential regulator in chronic asthma.

Methods:

Different experimental animal models of chronic asthma were used in combination with alveolar macrophage-reconstitution of global GM-CSF receptor knockout mice as well as cell-type specific knockout animals to elucidate the role of GM-CSF signaling in chronic airway inflammation.

Results:

We identify GM-CSF signaling as a critical factor regulating pulmonary accumulation of neutrophils. We show that while being not required for intrinsically regulating neutrophil migration, GM-CSF controls lung dendritic cell function, which in turn promotes T cell-dependent recruitment of neutrophils to the airways. We demonstrate that GM-CSF regulates lung dendritic cell antigen uptake, transport and Th2/Th17 cell priming in an intrinsic fashion which in turn drives pulmonary granulocyte recruitment and contributes to development of airway hyperresponsiveness in chronic disease.

Conclusion:

We identify GM-CSF as a potentially novel therapeutic target in chronic lung inflammation, describing a GM-CSF-dependent lung conventional DC – T cell-neutrophil axis which drives chronic lung disease.

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Key Words:

Neutrophils, asthma, chronic airway inflammation, GM-CSF, dendritic cell.

Key Messages:

- GM-CSF signaling is required for neutrophil accumulation and lung inflammation in different models of chronic asthma
- GM-CSF promotes induction of Th2/Th17 cell immunity by facilitating antigen uptake, transport to the lung draining lymph node and T cell priming by lung dendritic cells
- GM-CSF signaling in lung dendritic cells is required for development of chronic allergic airway inflammation

Capsule Summary:

Neutrophils are associated with severe chronic disease in asthma and GM-CSF is required for their accumulation in the inflamed lung by promoting lung dendritic cell dependent induction of Th2/Th17 cell immunity

Abbreviations:

AM = Alveolar macrophage
DC = Dendritic cell
GM-CSF = Granulocyte-Macrophage Colony Stimulating Factor
Th2 = T helper 2
Th17 = T helper 17
HDM = House dust mite
OVA = Ovalbumin

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Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to play an important role in regulating many aberrant immune responses including autoimmunity (1) and allergy (2). Under physiological conditions in the steady-state, GM-CSF is essential for the development and maintenance of alveolar macrophages (AM) (3), (4) and has also been implicated to regulate the development of non-lymphoid tissue dendritic cells (DCs) (5). While GM-CSF is increasingly seen as a key driver of inflammation in many contexts, in the lung in particular, studying the role of GM-CSF has been obstructed by the essential role of GM-CSF in AM development. Due to the complete lack of AM in GM-CSF (4) or GM-CSF-receptor-deficient (6) animals, these mice develop strong pulmonary alveolar proteinosis (PAP), which has a profound impact on the lung micro-environment (4). This has been a strong confounding factor in previous studies looking at the role of GM-CSF in pulmonary allergic inflammation in either ligand (7) or common β-chain-deficient animals (8). In addition, neutralization of GM-CSF in a house dust mite extract (HDM) model reduced pulmonary eosinophilia and was associated with an impaired Th2 response, but the underlying mechanism was unclear (2). In this context, we could recently show that reconstitution of a normal AM compartment in GM-CSF receptor alpha deficient (*Csf2ra*$^{-/-}$) mice can be used to prevent PAP, while still allowing investigation of GM-CSF receptor importance in pulmonary disease (9). Using this approach, we identified that GM-CSF receptor signaling in eosinophils promotes their migration to the lung in a model of acute allergic asthma(9). While experimental animal models have focused on acute aspects of lung inflammation, which are driven by Th2/ILC2 responses, the long-term chronic pathology characteristic of human asthmatics remains underinvestigated(10). Indeed, also in mice chronic asthma models have indicated that apart from Th2 immunity, also Th17 responses and associated neutrophilia which are hallmarks in chronic stages of the disease (11). Neutrophilic asthma is often refractory to steroid treatment and thus also from a clinical perspective the investigation of pathophysiological mechanisms in this context is highly warranted.

In the present study, we identify a GM-CSF-driven lung DC - T cell circuit, which is critically important in driving chronic lung inflammation characterized by type 17 responses and neutrophilia. We show that GM-CSF drives antigen uptake, transport and priming of Th2/Th17 cells in a DC-intrinsic manner and this promotes airway inflammation including recruitment of granulocytes as well as airway-hyperresponsiveness (AHR). We thus identify GM-CSF as a critical novel regulator of chronic lung disease and provide a potentially new therapeutic target for chronic asthma.

Methods

Mice

C57BL/6J mice were either bred in-house or purchased from Charles River (Germany). *Csf2ra*$^{-/-}$ animals were recently described (6). *Cd11c-Cre-Csf2rb*$^{fl/fl}$ (12) and *Csf2rb*$^{-/-}$ (13) mice were provided by B. Becher, Zürich. IL-17A$^{-/-}$ animals were provided by Yoichiro Iwakura (14). OT-II mice were obtained from Jackson (15). All animals were housed in individually ventilated cages under specific pathogen free conditions at the ETH Phenomics Center (Zurich, Switzerland) and used for experiments at between 6 and 14 weeks of age unless otherwise stated with age-matched groups in every individual experiment. All animal experiments were approved by the local animal ethics committee (Kantonales Veterinaersamt Zurich) and were performed according to local rules (Swiss Animal Protection Ordinance (TschV) Zurich) and Swiss animal protection law (TschG).

Cell suspension preparations

Mice were sacrificed by i.p. injection of sodium pentobarbital. Organs were removed and then processed according to the following procedure: Spleens, and lymph nodes were minced and then digested with 2 mg/ml of type IV collagenase (Worthington) and 0.02 mg/ml DNaseI (Sigma) at 37°C for 45 minutes and subsequently passed through a 70 μm cell strainer. Lungs were digested with 1mg/ml Hyaluronidase (Sigma), 25μg/ml Collagenase XI (Sigma), 50μg/ml Liberase TM (Roche) and 0.02 mg/ml DNaseI (Sigma) at 37°C for 45 minutes and subsequently

passed through a 70 μm cell strainer. Bone marrow cells were flushed from femurs and tibia and then used directly for staining.

Flow cytometry

Multi-parameter analysis was performed on a LSR Fortessa (BD) and analyzed with FlowJo software (Tree Star). Fluorochrome-conjugated or biotinylated monoclonal antibodies specific to mouse CD11c (N418), CD11b (M1/70), Siglec-F (E50^{-}2440, BD Biosciences), CD45 (30-F11), CD45.2 (104), CD4 (GK1.5), CD8 (53-6.7), CD3e (145-2C11), IL-4 (11B11), IL-5 (TRFK5), IFNγ (XMG1.2), T1/ST2 (DIH9), Ly-6G (1A8-Ly6g), CD127 (A7R34), CD172a (P84), CD19 (1D3/CD19), CD24 (M1/69), NKp46 (29A1.4, Thermofisher), CD49b (HMa2), MHC class II (M5/114.15.2, Thermofisher), CD64 (X54^{-}5/7.1), CD19 (6D5), CD90.2 (30-H12), TCR-b (H57-597) NK1.1 (PK136, eBioscience) ST2 (DIH9), CD25(PC61), FcεRIα (MAR-1, Thermofisher), XCR1 (ZET), IL-5 (TRFK5), GATA3(TWAJ, Thermofisher), RORγt (AFKJS-9, Thermofisher), IL-17A (Thermofisher eBio17B7), IL-13 (eBioscience, eBio13A), F4/80 (BM8), Ly-6C (HK1.4), TCRγδ (GL3), were purchased from Biolegend unless otherwise stated. Dead cells were excluded using the live/dead marker eFluor780 (Thermofisher). Prior to all stainings, FcγIII/II receptors were blocked by incubation with homemade anti-CD16/32 (2.4G2). For intracellular staining of transcription factors cells were fixed and stained using the intracellular fixation and permeabilization kit (eBioscience) according to the manufacturer's instructions. For DNA staining Fx-cycle violet stain (Thermofisher) was used according to the manufacturer's instructions. Cells were identified the following way: CD4^{+}T cells as CD45^{+}TCRb^{+}CD4^{+}, CD8^{+} T cells as CD45^{+}TCRb^{+}CD8^{+}, ILC2s as CD45^{+}CD90.2^{+}TCRb^{-}CD19^{-} NK1.1^{-}CD11b^{-}ST2^{+}, ILC1/3s CD45^{+}CD90.2^{+}TCRb^{-}CD19^{-}NK1.1^{-}CD11b^{-}ST2^{-}, B cells as CD45^{+}CD19^{+}TCRb^{-} cells, eosinophils as CD45^{+}CD11c^{-}CD11b^{+}Siglec-F^{+}SSC-A^{high}, neutrophils as CD45^{+}CD11c^{-}CD11b^{+}Ly-6G^{+} cells, lung cDC1s as Siglec F^{-}MHCII^{+}CD11c^{+}XCR1^{+}, lung cDC2s as Siglec-F^{-}MHCII^{+}CD11c^{+}XCR1^{-}CD11b^{+}CD64^{-} and lung moDCs as Siglec-F^{-} MHCII^{+}CD11c^{+}XCR1^{-}CD11b^{+}CD64^{+} cells, lung AM Siglec-F^{+}CD11c^{+}CD64^{+}, dLN cDC2 as

174 $CD45^+$autofluorescent$^-CD11c^+MHCII^{high}$ $XCR1^+$, lung dLN cDC1 as $CD45^+$autofluorescent$^-$
175 $CD11c^+MHCII^{high}$ $XCR1^-CD11b^+CD64^-$ and Ly-$6C^{high}$ monocytes as Siglec-F^-Ly-$6G^-$
176 $CD11b^+$Ly-$6C^{high}$.
177
178 **Airway-hyperresponsiveness**
179 Airway hyperresponsiveness was measured using unrestrained whole body plethysmography
180 (Buxco). Animals were placed in individual chambers and were then left to adjust to their new
181 environment for 15mins before being exposed to increasing concentrations of methacholine 0-
182 50mg/ml. The PenH was measured as a readout of changes in breathing due to airway
183 restriction.
184
185 **DC-T cell co-culture**
186 cDC1 and cDC2 were sorted from naïve lungs of WT or AM-reconstituted *Csf2ra-/-* animals.
187 $CD4^+$ T cells were obtained with CD4 MACS-bead (Miltenyi) pre-enrichment and subsequent
188 FACS-sorting from splenocytes from OT-II mice. $CD4^+$ T cells were sorted as $CD4^+CD11c^-$
189 aufluorescent-negative cells. T cells and DCs were then cultured together for 5 days in complete
190 IMDM medium (Life Technologies) with 100nM $OVA_{323-339}$ peptide (Mimotopes Australia).
191 Cells were restimulated for 4h with PMA (Sigma), ionomycin (Sigma) and monensin (Sigma).
192
193 **Murine models of airway inflammation**
194 For the chronic OVA model, animals received 10µg OVA with 1µg TNF i.t. and were then
195 challenged with 50µg OVA as indicated in the scheme in figure 2. For the chronic HDM model
196 animals received 10µg HDM 3 times/week during 4 consecutive weeks and were then analyzed
197 3 days after the last challenge. For the acute HDM model mice received 10µg HDM on D0 for
198 sensitization and were then challenged daily with 10µg HDM on days 7-11 with analysis on
199 D14.
200
201 **Bronchoalveolar lavage**

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For collection of BAL after euthanization of the animal the trachea was punctuated using a syringe and the lungs were flushed twice with 0.8µl of PBS.

Transfer of alveolar macrophage precursors into neonates

Lungs from E17.5 embryos from CD45.1$^+$ WT animals were harvested, minced and digested with collagenase IV as described above. CD45$^+$ cells were then purified using MACS beads (Miltenyi) and cells were then transferred i.n. into d1-7 p.b. neonates to allow reconstitution of a WT AM compartment.

BMDC culture

Femurs and tibias were collected from front and back leg of donors. They were flush with FACS buffer, erythrocytes were lysed with ACK for 3 min and BM cells were then cultured with with 300 ng/mL Flt3L (home-made) at 2x10^6 cells/ml in 10ml of BMDC media (RPMI-1640 (Invitrogen), 10% heat inactivated FCS, 100 U/ml Penicillin; 100µg/ml Streptomycin solution (Invitrogen), 200 µM L-Glutamine (Invitrogen, in 10cm petri dish. On Day 3 and 5 5 ml of fresh BMDC media with 300 ng/mL Flt3L were added.

Transfer of HDM-pulsed BMDCs

After 9 days of culture, BMDCs were pulsed with 100µg/ml HDM for 4h. Pulsed and un-pulsed cells were then transferred i.t. into recipient mice. Each mouse received 4x10^5 cells. Animals were then challenged daily with 10µg HDM on days 7-11 with analysis on D14.

DC-mediated antigen uptake and transport

Animals received 100µg Ovalbumin-AlexaFluor488 (Invitrogen) and 100µg HDM (Greer) i.t. Lungs and dLNs were collected 24h p.i. and analysed using flow cytometry.

Histology

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Lungs from animals were removed and fixed in 4% formalin. Thereafter, the lungs were processed and then stained with either hematoxylin and eosin (H&E) or periodic acid–Schiff (PAS) and lungs were evaluated in a blind manner.

Statistical Analysis

Mean values, SD, SEM, Student's t test (unpaired) were calculated with Prism (GraphPad Software, Inc) or R software. p-values and Hedges' g are indicated for all significant comparisons. p <= 0.05 (*), p <= 0.01 (**), p <= 0.001 (***).

Results

GM-CSF signalling controls development of HDM-induced chronic allergic lung inflammation

In order to address the role of GM-CSF receptor signalling in the pathogenesis of chronic airway inflammation *in vivo*, we employed GM-CSF receptor α-deficient (*Csf2ra*$^{-/-}$) mice, which were transplanted with wild-type CD45^{+} cells from E17.5 embryonic lungs neonatally to allow establishment of an intact AM compartment and thus prevent development of PAP as recently described (9). PAP leads to low-grade inflammation(6) and could therefore confound interpretation of experimental results. After full reconstitution of the AM compartment and development into adulthood, *Csf2ra*$^{-/-}$ mice were treated three times a week over the course of 4 weeks with house dust mite extract (HDM), representing a common human allergen and a clinically relevant model of chronic asthma (Fig 1A) (16). Due to the well-established role of Th17 immunity in mediating exacerbation of both acute (17) and chronic pulmonary allergic inflammation (11) by promoting neutrophilia and structural changes in the lung (11), we included IL-17A-deficient mice as a comparison to *Csf2ra-/-* animals. Evaluating the inflammatory infiltrate after 4 weeks of chronic HDM-exposure revealed that the numbers of both neutrophils (p <= 0.0001, Hedges' g (H) = 5.2594) and eosinophils (p = 0.0386, H =

1.3938) were strongly reduced in *Csf2ra-/-* mice in the BAL and lungs compared to wild-type controls (Fig 1B, C and D and Fig S1). IL-17A-deficient mice exhibited a strong decrease in neutrophils ($p = 0.0002$, $H = 4.4321$), and no defect in eosinophilia (Fig 1B, C and D). No differences were observed in innate aspects of the immune response such as the number of AM and $Ly6C^{high}$ monocytes (Fig S2A) or the number of innate lymphoid cell (ILC) subsets (Fig S2B).

However, the number of both conventional dendritic cell subsets cDC1 ($p= 0.0011$, $H = 2.6509$) and cDC2 ($p = 0.002$, $H = 2.4286$) as well as monocyte-derived DCs ($p = 0.0001$, $H = 3.7278$) were significantly lower in Csf2ra KO mice (Fig 1E). This deficiency in lung DCs was associated with a lower accumulation of both $CD4^+$ ($p = 0.0064$, $H = 2.011$) and $CD8^+$ T cells ($p = 0.001$, $H = 2.6728$) (Fig 1F). Re-stimulation of $CD4^+$ T cells revealed a strong defect in Th17 immunity in the absence of functional GM-CSF receptor signalling ($p = 0.0022$, $H = 2.3989$) (Fig 1G). This was accompanied by a trend towards a reduction in some Th2 cytokines, while Th1 polarization appeared to be more potent in *Csf2ra-/-* mice ($p = 0.0006$, $H = 2.8531$) (Fig 1G). To corroborate the strong deficiency in Th17 immunity we quantified the number of effector and regulatory $CD4^+$ T cells expressing RORγt, the key transcription factor for Th17 polarization (18). It was clearly evident that the number of $ROR\gamma t^+$ effector T cells was significantly reduced in *Csf2ra-/-* mice ($p <= 0.0001$, $H = 5.0985$) (Fig 1H). Interestingly, $ROR\gamma t^+$ Tregs were also reduced ($p <= 0.0001$, $H = 5.496$) (Fig 1H), while only a minor effect could be seen on the general $FoxP3^+$ regulatory T cell compartment in the BAL ($p = 0.0007$, $H = 2.8107$) and lung ($p = 0.0138$, $H = 2.5108$) (Fig 1I). In order to evaluate the physiological consequences of GM-CSF receptor deficiency on lung function, airway hyperresponsiveness (AHR) was measured. *Csf2ra-/-* animals were found to be largely protected from AHR development ($p = 0.0387$, $H = 1.3926$ at 50mg/ml), while IL-17A-deficiency had no effect (Fig 1J). In addition, we found a decrease in the levels of HDM-specific IgG1 (Fig 1K), consistent with impaired $CD4^+$ T cell help. Overall, these results indicated a potent deficiency in chronic lung inflammation in the absence of functional GM-CSF receptor signalling.

GM-CSF is essential for airway responses and neutrophil dominated inflammation following chronic ovalbumin exposure.

In order to corroborate our results from the chronic HDM model, we wanted to evaluate the role of GM-CSF signalling in a second animal model of chronic asthma based on ovalbumin (OVA)/TNF-α sensitization and subsequent chronic challenge with OVA (11) (Fig 2A). The inflammatory infiltrate at day 53 post sensitization was characterized by flow cytometry as depicted in Fig S1 and contained very few eosinophils and abundant neutrophils in lung and BAL, whose presence was strongly dependent on intact GM-CSF ($p = 0.0003$, $H = 3.3835$) and IL-17A signalling ($p = 0.0001$, $H = 4.6682$) (Fig 2B-D). No major differences could be observed in lymphocyte infiltration, although the number of $CD8^+$ T cells was slightly reduced in *Csf2ra*-/- mice ($p = 0.0124$, $H = 1.8868$) (Fig 2E). As seen in the chronic HDM model, the frequency of $CD4^+IL\text{-}17A^+$ cells, was considerably reduced in the absence of *Csf2ra*, ($p = 0.0004$, $H = 3.2865$) and to a lesser extent also $IL\text{-}5^+$ ($p = 0.0031$, $H = 2.4282$) and $IL\text{-}4^+$ ($p = 0.0179$, $H = 1.7496$) cells (Fig 2F). Supporting these results, the number of $ROR\gamma t^+$ $CD4^+$ effector ($p = 0.0005$, $H = 3.2026$), and regulatory T cells ($p = 0.001$, $H = 5.2277$) was also significantly reduced in *Csf2ra-/-* animals (Fig 2G, H). Indeed, all major differences observed in infiltration of immune cells were dependent on OVA challenge and were not visible with PBS alone (Fig S2C-F). Moreover, *Csf2ra*-/- mice were protected from AHR following chronic airway exposure to OVA, similar to the chronic HDM model (Fig 2I). Overall, these results supported the general notion that GM-CSF signaling is critical for the development of chronic inflammation in asthma.

GM-CSF receptor signalling does not intrinsically control granulocyte accumulation or cytoplast formation in chronic airway inflammation.

Given the potent effect on T cell immunity in the absence of GM-CSF receptor, in particular on Th17 polarization, we wanted to gain a deeper understanding of the underlying molecular mechanisms. As cytoplast formation after neutrophils extracellular trap (NET) formation has

been shown to be a potent inducer of Th17 immunity in the lung (19) and GM-CSF has been suggested to promote NETosis *in vitro (20)*, we wanted to investigate a potential role of GM-CSF signalling in directly regulating neutrophil cytoplast generation *in vivo*. Cytoplasts can be identified by harbouring low amounts of DNA (Fig 3A) and indeed we could detect neutrophil as well as eosinophil-derived cytoplasts in chronic allergic lung inflammation (Fig 3A). However, no effect on the frequency of DNA^{low} cells could be observed in *Csf2ra*$^{-/-}$ mice (Fig 3B). Nonetheless, the overall number of cytoplasts was reduced due to the lower number of granulocytes present in lungs of *Csf2ra*$^{-/-}$ mice (for neutrophils $p = 0.0153$, $H = 1.9417$; for eosinophils $p = 0.0213$, $H = 1.8061$) (Fig 3C). In order to exclude a potentially intrinsic role of GM-CSF signalling in cytoplast formation 50:50 bone marrow (BM) chimeras were generated, where WT animals received a 1:1 mixture of either a mixture of WT (CD45.1):WT(CD45.2) or (CD45.1): *Csf2ra-/-* (CD45.2) BM. This revealed that GM-CSFR signalling was not intrinsically required for cytoplast formation *in vivo* (Fig 3D). Interestingly, normal recruitment of *Csf2ra*-deficient neutrophils to the bronchoalveolar space in mixed chimera indicates that neutrophil intrinsic GM-CSFR signalling is not required for lung migration and accumulation in chronic inflammation (Fig 3E), while wild-type eosinophils exhibited a competitive advantage compared to *Csf2ra*-deficient counterparts ($p = 0.0348$, $H = -1.4092$) (Fig 3F). Taken together these results suggested that the reduction in chronic lung inflammation in *Csf2ra*-deficient mice was not due to cytoplasts or granulocyte-intrinsic roles of GM-CSF signalling but largely due to other extrinsic factors.

GM-CSF receptor signalling controls dendritic cell activation, antigen uptake and transport to the lung draining lymph node

Having observed a strong deficiency in T cell immunity and a reduction in DC numbers, we hypothesized that one potential explanation could be a role for GM-CSF signalling in DC-mediated induction of T cell responses in chronic asthma. Lung DCs can be grouped into at least three distinct subsets including the well-described BATF3 (21) and PI3Kγ-dependent (22) type I, the IRF4-dependent type 2 conventional DCs (23) (cDC1 and cDC2) as well as monocyte-

derived DCs (moDCs) (24). Confirming previous reports (9), (5), we found a significant reduction of lung DCs in naive AM-reconstituted *Csf2ra-/-* mice (Fig S3A). Indeed 50:50 BM chimeras revealed that this was due to an intrinsic role of GM-CSF signalling in lung DCs (Fig S3B). We next wanted to assess the impact of GM-CSF receptor deficiency on DC function as a potential explanation for the poor induction of T cell immunity. Antigen uptake and transport are key abilities of DCs to initiate T cell immunity and we therefore aimed to assess these functions in the absence of functional GM-CSF receptor signalling. For this purpose, we injected wild-type and AM-reconstituted *Csf2ra-/-* mice with fluorescently labelled OVA together with HDM as an adjuvant and analysed the lung and lung draining lymph node (dLN) 24h later. The total number of cDC1s ($p = 0.045$, $H = 1.109$), cDC2s ($p = 0.0361$, $H = 1.1679$) as well as OVA^+ cDC1s ($p = 0.0435$, $H = 1.1186$) and as OVA^+ cDC2s ($p = 0.0417$, $H = 1.1297$) in the lung dLN was strongly reduced in the absence of GM-CSF receptor (Fig 4A-C). The frequency of OVA^+ cells among cDC1 and moDCs in dLN was comparable in *Csf2ra-/-* and wild-type mice, while it was moderately reduced in cDC2s ($p = 0.002$ $H = 6.859$) (Fig 4D). In the lung both conventional DCs subsets lacking *Csf2ra* exhibited a reduced antigen uptake (cDC1 $p = 0.001$ $H = 6.195$; cDC2 $p = 0.002$ $H = 4.294$) while OVA uptake was unaffected in *Csf2ra*-deficient moDCs (Fig 4E). In order to evaluate a possible impact of *Csf2ra*-deficiency on expression of costimulatory molecules, we measured a number of well-known markers in the lung and the lung dLN (Fig 4F-M). It was clearly evident, that surface expression (i.e. MFI) of CD80 (cDC1: $p = 0.043$, $H = 1.1819$; cDC2: $p = 0.009$, $H = 1.6156$) and CD86 (cDC1: $p = 0.011$, $H = 3.408$; cDC2: $p = 0.028$, $H = 4.487$) on conventional DCs in lung dLN of *Csf2ra*$^{-/-}$ animals was considerably lower than WT controls (Fig 4F, G). No differences could be observed in other molecules such as CD40 (Fig 4H). Similarly, the conventional DC subsets in the lung exhibited a much lower MFI in CD80 (cDC1: $p = 0.0126$, $H = 1.5242$; cDC2: $p = 0.0081$, $H = 1.6454$) and CD86 (cDC1: $p = 0.0252$, $H = 1.3329$; cDC2: $p = 0.0194$, $H = 1.4046$) (Fig 4K, L) but not CD40 (Fig 4L). Interestingly IL-33 receptor ST2 (also known as IL1RL1) expression on dLN cDC2 was lower in HDM-treated animals compared to naïve controls ($p = 0.050$, $H = 1.4761$) (Fig 4I), but no effect could be observed on lung DCs (Fig 4M). Overall,

these results indicated that GM-CSF receptor signalling was required for efficient antigen uptake and transport to the dLN by DCs, as well as their expression of key surface costimulatory molecules.

GM-CSF receptor signalling intrinsically controls DC-mediated T cell immunity

Having observed a pronounced deficiency of DC-mediated antigen uptake, transport and costimulation, we wanted to functionally evaluate the impact of GM-CSF signalling on DC-T cell interactions. For this purpose, lung DCs were sorted from naïve WT and AM-reconstituted *Csf2ra*-/- mice and were co-cultured with $CD4^+$ T cells (from OTII tg mice) and 100nM of OVA-peptide. Quantification of the number of T cells after 5 days of culture revealed that *Csf2ra*-deficient DCs were less potent at inducing $CD4^+$ T cell expansion (for cDC1 $p = 0.0051$, $H = 2.0637$; for cDC2 $p = 0.0017$, $H = 3.9342$) (Fig 5 A). Lung cDC1s and cDC2 predominantly induced Th2 and Th1 differentiation, respectively, while Th17 differentiation was poorly induced by both DC subsets under neutral (non-polarizing) culture conditions (Fig 5B). Interestingly, while GM-CSF receptor was dispensable in cDC1s and cDC2 for their ability to induce $CD4^+$ T cell subset characteristic cytokine production (Fig 5C), it was required for the capacity of cDC1s ($p = 0.0011$, $H = 2.6236$) and cDC2s ($p = 0.0015$, $H = 4.0346$) to induce the Th2 and Th17 polarizing transcription factors GATA-3 and RORγt, respectively (Fig 5D). The differences may be explained by brief PMA/Ionomycin stimulation prior measurement of intracellular cytokine production. Overall these results indicated a potent intrinsic role for GM-CSF signalling in lung DCs-T cell interactions and Th2 and Th17 but not Th1 polarization.

Dendritic-cell intrinsic GM-CSF receptor signalling is required for potent induction of chronic lung inflammation and T cell immunity *in vivo*

After seeing a potent effect of global *Csf2ra*-deficiency on lung DC function *in vivo* and a DC intrinsic requirement of *Csf2ra* for $CD4^+$ T cell expansion and upregulation of the transcription factors RORγt and GATA3, *in vitro*, we wanted to address whether selective absence of *Csf2ra*

in DCs affects development of chronic asthma. For this purpose, we crossed *Csf2rb*$^{fl/fl}$ to *Cd11c-Cre* animals to generate mice with a GM-CSF receptor deficiency in DCs and CD11c^{+} AM. To overcome the defect in AM, these animals were then reconstituted with AM to prevent PAP development and were subsequently subjected to chronic HDM exposure. Evaluating chronic inflammation after 4 weeks of treatment indicated that infiltration of both eosinophils ($p = 0.0229$, $H = 1.7756$) as well as neutrophils ($p = 0.0403$, $H = 1.5458$) was strongly reduced in the BAL of *Csf2rb*$^{fl/fl}$*-Cd11c-Cre* animals compared to *Csf2rb*$^{fl/fl}$ littermate controls (Fig 6A). BAL CD4^{+} T cells (Fig 6B), and innate immune cell populations such as NK cells and ILCs were unaffected (Fig 6C). Notably, evaluation of DC numbers (Fig6E, F) revealed a strong decrease in lung ($p = 0.0001$, $H = 5.6369$) and lung dLN cDC2s ($p = 0.0001$, $H = 4.3726$) , and to a lesser extent cDC1s in both naïve and asthmatic mice. Furthermore, the frequencies of GATA-3^{+} ($p = 0.0442$, $H = 1.5082$,) and RORγt^{+} CD4^{+} T cells ($p = 0.0109$, $H = 2.0851$) (Fig 6G) as well as cells producing cytokines IL-17A ($p = 0.0003$, $H = 3.9036$), IL-5 ($p = 0.0349$, $H = 1.6047$,) and IL-13 ($p = 0.0016$, $H = 2.9666$), were significantly reduced in animals that lacked functional GM-CSF receptor signalling in DCs (Fig 6H). To corroborate our results from flow cytometry we evaluated lung inflammation in HDM-challenged animals using histological analysis. Consistent with our FACS results, histology revealed that both chronic immune infiltration as well as the mucus producing goblet cells were reduced in *Csf2rb*$^{fl/fl}$*-Cd11c-Cre* animals (Fig 6I) Overall, these results identified a critical intrinsic requirement for GM-CSF receptor in lung DCs for the potent induction of T cell immunity driving lung inflammation in a model of chronic asthma.

GM-CSF responsiveness in DCs is sufficient to drive allergic airway inflammation

In order to further demonstrate that GM-CSF signalling in DCs was sufficient to drive HDM-induced airway inflammation, we generated bone-marrow-derived DCs using Flt3L from WT and *Csf2rb*-deficient mice for subsequent transfer *in vivo*. No differences could be observed in the phenotype of cultured cells (FigS3C). After 9 days of culture DCs were pulsed with HDM and were then transferred to WT recipients. Subsequent HDM challenge and flow cytometric

analysis of the mice revealed that HDM-pulsed WT DCs induced more potent eosinophilia than *Csf2rb*-deficient cells, which was strongly dependent on the pulsing with HDM ($p = 0.0365$, $H = 1.731$) (Fig7A, B). Furthermore, HDM-pulsed WT DCs induced a more pronounced infiltration of $CD4^+$ T cells, compared to un-pulsed controls ($p = 0.0165$, $H = -2.4056$), which was not observed in GM-CSF signalling deficient cells (Fig7C). Interestingly, transfer of pulsed WT DCs induced a stronger accumulation of cDC2s in the lungs of recipient mice compared to that of KO DCs ($p = 0.0064$, $H = -2.5761$) (Fig7D). Furthermore, T cell polarization was found to selectively affected by the presence of GM-CSF receptor on transferred DCs. The frequency of both GATA3-expressing ($p = 0.0135$, $H = 2.1992$) (Fig7E) and type 2 cytokines IL-4 ($p = 0.0213$, $H = 1.9812$), IL-5 ($p = 0.005$, $H = 2.6991$) and IL-13-producing ($p = 0.0227$, $H = 1.9516$) $CD4^+$ T cells (Fig7F) were reduced in mice which had received pulsed KO DCs. Interestingly, although there was a trend for IL-17A in the same direction, this was found to be not significant ($p = 0.174$, $H = 1.015$) (Fig7F). Taken together, these results corroborated a direct and important role of GM-CSF signalling in modulating the capacity of DCs to drive HDM-induced airway inflammation.

GM-CSF signalling in DCs also promotes lung inflammation in acute allergic asthma

After having established an important role of GM-CSF signalling in lung DCs in driving chronic allergic airway inflammation we wanted to test the broader relevance of our findings and determine whether GM-CSF signalling in lung DCs was generally required for allergen-induced lung inflammatory disease. For this purpose, we employed a model of acute allergic lung inflammation induced by HDM as described previously (9) using AM reconstituted *Csf2rb* $^{fl/fl}$*-Cd11c-Cre* mice. These experiments revealed that infiltration of eosinophils in particular ($p = 0.0086$, $H = 2.185$) and to some extent also neutrophils ($p = 0.1047$, $H = 1.1571$) was reduced in *Csf2rb* $^{fl/fl}$*-Cd11c-Cre* as opposed to floxed littermate controls (Fig 8A, B). This deficiency in granulocyte accumulation was associated with a reduction in the number of cDC2s ($p = 0.0297$, $H = 1.6702$) and moDCs ($p = 0.0085$, $H = 2.194$) (Fig 8C) as well as a lower number of $CD4^+$ T cells ($p = 0.050$, $H = 1.4519$) (Fig 8D). These significant

differences in various key immune cells could be also seen in histological sections where lungs of DC-intrinsic GM-CSF receptor deficient animals exhibited lower overall inflammation (Fig 6E). Finally, this reduction in HDM-induced respiratory inflammation was associated with a lower production of IL-4 ($p = 0.0032$, $H = 2.9511$), IL-5 ($p = 0.0205$, $H = 2$) and IL-17A ($p = 0.001$, $H = 6.2763$) (Fig 8E) in $CD4^+$ T cells in animals which lacked functional GM-CSF signalling in DCs. Taken together these findings highlighted the general importance of GM-CSF in potentiating the function of lung DCs to drive allergic lung inflammation.

Discussion

GM-CSF has been implicated to play a critical role in lung immunity in multiple contexts including infection(4) and acute allergic responses (9). However, its role in regulating chronic lung inflammation remains largely unclear. Here we show that in the context of chronic asthmatic inflammation it is critically involved in driving recruitment of disease-exacerbating neutrophils and eosinophils, development of AHR and chronic airway inflammation. We identify a GM-CSF-dependent inflammatory circuit where GM-CSF-triggered lung DC-mediated priming of Th17 and Th2 immunity drives chronic lung disease. While these findings identify GM-CSF as a critical player in development of chronic allergic lung inflammation, they also suggest potential roles of this cytokine in other chronic inflammatory lung conditions such as COPD and lung fibrosis. Indeed in both these diseases neutrophils are also seen as important drivers of lung inflammation (25) (26) and it is likely that GM-CSF, by promoting induction of T cell immunity or potentially by direct recruitment of neutrophils, also contributes to disease exacerbation in these contexts. Furthermore, GM-CSF was recently shown to exacerbate disease in COVID-19-related pneumonia (27). One mechanism which is key for neutrophils exacerbating disease is the formation of cytoplasts after NETosis, which were shown to directly

promote T cell immunity (19). Indeed, in chronic allergic inflammation it is likely that GM-CSF is driving a positive feedback loop where Th17-mediated recruitment of neutrophils leads to accumulation of cytoplasts. This in turn, further promotes Th17 immunity and therefore more accumulation of neutrophils. Furthermore, while neutrophil-derived cytoplasts have recently been investigated (19), the role of eosinophil-derived cytoplasts as a disease exacerbating factor in this context remains completely unclear. In addition, our study also highlights the importance of using animal models which truly represent chronic lung inflammation to study the pathophysiological mechanisms of human chronic lung disease rather than extending findings from models with more acute types of inflammation as is commonly done in asthma research (28). This is exemplified by the findings of our own recent study where we found an important intrinsic role for GM-CSF in recruitment of eosinophils in acute allergic airway inflammation (9). It is therefore no surprise that, early efforts using neutralizing GM-CSF in asthma in generally did not show much success (29), likely due to lack of patient stratification and a focus on individuals with neutrophilic inflammation.

Interestingly, on a mechanistic level we found potentially distinct roles of GM-CSF in promoting the Th2- and Th17-polarizing capacity of cDC1s and cDC2s respectively. This would be consistent with some indications in the literature, which have suggested cDC1s as more potent inducers of Th2 (30) and cDC2s of Th17 responses (23). Furthermore, apart from promoting expression of key costimulatory molecules such as CD80 and CD86 it is likely that GM-CSF affects further aspects of lung DC function including cytokine secretion. For example IL-6 presentation by SIRPα^{+} DCs was recently shown to be crucial for priming of pathogenic Th17 cells *in vivo* (31) and it is possible that in chronic asthma the same mechanism is driving Th17 immunity. Interestingly, Th1 immunity is completely unaffected or even enhanced by the absence of GM-CSF signalling in DCs in the asthma models shown here and upon influenza virus infection (4) . This would suggest that GM-CSF actually specifically promotes Th2 and Th17 responses in DCs or that Th1 immunity is rescued by compensatory mechanisms such as NK cell/ILC1-derived IFNγ, which overcome the requirement for GM-CSF for Th1-cell induction. Furthermore, it appears that GM-CSF is important regardless of where antigen uptake

and DC activation occurs, as induction of airway inflammation by transfer of HDM-pulsed DCs was still dependent on sensing GM-CSF. Interestingly, the capacity of BMDCs to induce Th17 immunity was much less affected by GM-CSF receptor deficiency, suggesting that lung DCs in situ potentially receive additional signals *in situ* to potential their Th17 priming functionality. Another likely explanation for the reduction of Th17 cells is the reduced number of lung DCs in GM-CSFR-deficient animals. This is not the case in the BMDC model, where in all conditions the recipient animals received the same number of DCs. In addition, GM-CSF receptor-deficient animals did not just exhibit a reduced number of RORγt$^+$ effector T cells, but also a significantly reduced frequency of RORγt$^+$ Tregs. This suggests that potentially similar mechanisms promote Th17 and Treg differentiation in this setting. Whether these cells play any role in the pathophysiology of chronic asthma remains to be investigated in the future. Taken together with the reduction of the number of lung DCs in GM-CSF receptor deficient animals, these results suggest that GM-CSF critically regulates the quantity and quality of DC-mediated induction of T cell immunity in the lung, which in turn drives chronic lung inflammation in asthma and likely in other respiratory disease contexts. Interestingly, in influenza virus infection antiviral T and B cell immunity was unimpaired in GM-CSF deficient animals (4). Immunity against influenza virus is characterized by a potent Th1 response (4) and indeed the fact that GM-CSF is dispensable for the induction of adaptive immunity is consistent with our findings that Th1 immunity is unimpaired in chronic allergic inflammation. Furthermore, in this context induction of CD8$^+$ T cell immunity by cDC1s is particularly important (32) and these cells play a less important role in chronic airway inflammation.

Overall, our results identify GM-CSF as a key orchestrator of a neutrophil-DC-Th17 circuit driving chronic lung disease, not just providing a potentially new therapeutic target in chronic asthma but suggesting new avenues of research into the underlying molecular mechanisms of other chronic lung pathologies.

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Author contributions: S.P.N., L.P., F.L. and M.Ka. performed experiments, S.P.N., L.P. and M.K. designed experiments, B.B provided valuable reagents. S.P.N., L.P. and M.Ka analyzed data and S.P.N and M.K wrote the manuscript.

Acknowledgments:

This study was funded by SNSF and ETH Zurich

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Figure Legends

Figure 1: GM-CSF signalling controls development of HDM-induced chronic allergic lung inflammation

(A) AM-reconstituted *Csf2ra-/-* mice were sensitized and challenged with HDM, as indicated in the scheme. (B) Shown are representative FACS dot plots and (C-D) numbers (mean ± SEM) of BAL and lung eosinophils and neutrophils. (E) Number of lung dendritic cell subsets and (F) BAL $CD4^+$ and $CD8^+$ T cells. (G) BAL cells were restimulated for 4h with PMA/ionomycin and indicated cytokines were stained intracellularly. Shown is the frequency of cytokine-producers among $CD4^+$ T cells. (H) Frequency of $ROR\gamma t^+$ among $Foxp3^+$ $CD4^+$ Tregs and $Foxp3^-$ effector $CD4^+$ T cells. (I) Frequency of $FoxP3^+$ Treg among $CD4^+$ T cells in indicated tissues. (J) AHR in response to increasing concentrations of methacholine (n=5-7/group). (K) HDM-specific IgG1 determined by ELISA. Shown is OD_{405} value. The data is representative of 3 independent

experiments. The Student's t test (unpaired) was used: p <= 0.05 (*), p <= 0.01 (**), p <= 0.001 (***).

Figure 2: Ovalbumin-based chronic asthma model also demonstrates GM-CSF receptor requirement for development of chronic lung inflammation.

(A) *Csf2ra-/-* mice (CD45.2) reconstituted with alveolar macrophages (CD45.1) were sensitized and challenged with ovalbumin as shown in the scheme. (B) Dot plots of BAL cells (from a representative individual of indicated groups of mice) gated on $CD11b^+CD45.2^+$ cells with Siglec-F^+Ly-$6G^-$ eosinophils and Ly-$6G^+$ Siglec-F^- neutrophils. (C-H) Quantitation of flow cytometry analysis showing number of (C-D) eosinophils and neutrophils in BAL and lung, (E) BAL $CD4^+$ T cells and $CD8^+$ T cells. (F) BAL cells were stimulated for 4h with PMA/ionomycin and intracellular cytokine staining performed. Shown is the frequency of IL-4, IL-5, IFNγ and IL-17A-producers among $CD4^+$ T cells. (G) Number of lung RORγt^+ and RORγt^- effector $CD4^+$ T cells. (H) Number of RORγt^+ and RORγt^- Treg cells in the lung. (I) AHR in response to increasing concentrations of methacholine (n=5-7/group), All graphs show (mean ± SEM) (n=5-6/group) The data is representative of 2 independent experiments. The Student's t test (unpaired) was used: p <= 0.05 (*), p <= 0.01 (**), p <= 0.001 (***).

Figure 3: GM-CSF receptor signalling does not intrinsically control granulocyte accumulation or cytoplast formation in chronic allergic lung inflammation

(A) AM-reconstituted *Csf2ra-/-* mice were sensitized and challenged with HDM, as indicated in figure 1. Shown are representative FACS dot plots of DNA low cytoplasts as well as a summary of the FACS data in terms of (B) frequency of parent and (C) total number of eosinophils and neutrophils. (D) CD45.1+CD45.2+ WT animals were irradiated and subsequently reconstituted with a 50:50 mix of $CD45.1^+$ WT:$CD45.2^+$WT or $CD45.1^+$WT:$CD45.2^+$*Csf2ra-/-* BM. After 8 weeks mice were exposed to HDM as described in figure 1. Shown is a summary of FACS data of DNA low cells normalized to the parent frequency of $CD45.2^+$ neutrophils or eosinophils respectively. (E-F) Show is the frequency of $CD45.2^+$ cells normalized to blood B cells. All

graphs show (mean ± SEM). The data is representative of 2 experiments. (n=6-7/group) The Student's t test (unpaired) was used: p <= 0.05 (*), p <= 0.01 (**), p <= 0.001 (***).

Figure 4: GM-CSF signaling controls lung dendritic cell activation, antigen uptake and transport to the draining lymph node

WT and AM-reconstituted *Csf2ra-/-* mice were injected intratracheally with 100µg OVA-AF488 and 100µg HDM and sacrificed 24h later for analysis of the lung and the dLN by flow cytometry. Shown are (A) the gating strategy in the lung dLN as well as representative FACS dot plots of naïve and treated animals, and the total numbers of (B) dLN DCs and (C) OVA-AF488$^+$ DCs in the lung dLN, (D, E) as well as the frequencies of OVA-AF488$^+$ DCs in lung dLN and lung. (F-M) Shown are the MFIs of indicated DC activation markers for lung and lung dLN DC subsets as a summary of FACS data. All graphs show (mean ± SEM). The data is representative of 2 independent experiments. (n=3-6/group) The Student's t test (unpaired) was used: p <= 0.05 (*), p <= 0.01 (**), p <= 0.001 (***).

Figure 5: GM-CSF receptor signalling in lung cDC1s and cDC2s differentially controls Th subset polarization

Lung cDC1 and cDC2 from naive WT and AM-reconstituted *Csf2ra-/-* mice were isolated and co-cultured with naïve OT-II CD4$^+$ T cells in the presence of 100nM OVA peptide for 5 days. Shown is data pooled from three experiments. (A) The total number of CD4$^+$ T cells normalized to the WT mean (B-D) Cells were then restimulated for 4h with PMA/ionomycin. Shown are representative FACS dot plots as well as the frequency of indicated cytokines and transcription factors. All graphs show (mean ± SEM) as a summary of FACS data. The Student's t test (unpaired) was used: p <= 0.05 (*), p <= 0.01 (**), p <= 0.001 (***).

Figure 6: DC intrinsic GM-CSF receptor signalling is required for potent induction of chronic lung inflammation and Th2 and Th17 cell immunity *in vivo*

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AM-reconstituted *Csf2rb*$^{lox/lox}$-*Cd11c-Cre* mice were sensitized and challenged with HDM, as indicated in figure 1. Shown are (A) representative dot plots and (B) total cell numbers (mean ± SEM) of BAL eosinophils and neutrophils, (C) BAL CD4$^+$ and CD8$^+$ T cells, (D) Lung innate lymphocytes, (E) Lung DC subsets, and (F) DC subsets in lung dLN as determined by flow cytometry. (G) Frequencies of lung CD4$^+$ T cells expressing indicated transcription factors. (H) BAL cells were restimulated for 3h with PMA/ionomycin and cytokine production determined. Shown is the frequency (mean ± SEM) of IL-4, IL-5, IL-13, IL-17A and IFNγ-producing CD4$^+$ T cells (n=6-11/group) (I) Shown are representative histological H&E and PAS-stained sections of HDM-challenged lungs. The data is representative of 2 independent experiments. The Student's t test (unpaired) was used: p <= 0.05 (*), p <= 0.01 (**), p <= 0.001 (***).

Figure 7: DC intrinsic GM-CSF receptor signalling is required for potent induction of allergic airway inflammation

WT mice received 4x10^5 Flt3L-derived WT or GM-CSFR-deficient HDM-pulsed or un-pulsed BMDCs. Animals were then challenged daily with 10ug HDM on days 7-11 with flow cytometric analysis on day 14. Shown are (A-B) representative dot plots of total numbers of eosinophils and neutrophils in the BAL (mean ± SEM) of (C) BAL CD4$^+$ and CD8$^+$ T cells (D) Lung DC subsets, (E) Frequencies of lung CD4$^+$ T cells expressing indicated transcription factors. (F) BAL cells were restimulated for 3h with PMA/ionomycin and cytokine production determined. Shown is the frequency (mean ± SEM) of IL-4, IL-5, IL-10, IL-13, IL-17A and IFNγ-producing CD4$^+$ T cells (n=3-5/group) The data is representative of 2 independent experiments. The Student's t test (unpaired) was used: p <= 0.05 (*), p <= 0.01 (**), p <= 0.001 (***).

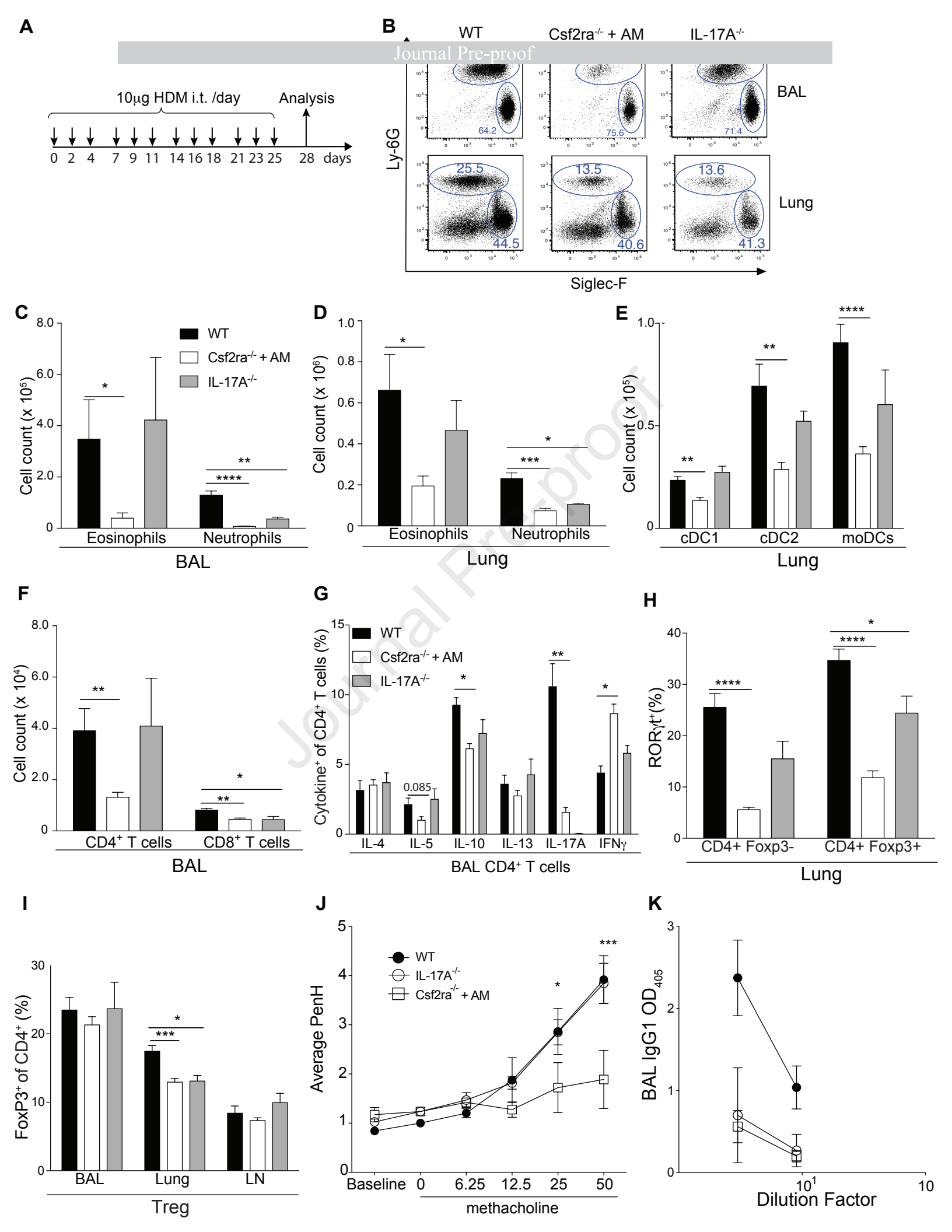
Figure 8: DC intrinsic GM-CSF receptor signalling promotes lung inflammation in acute allergic asthma

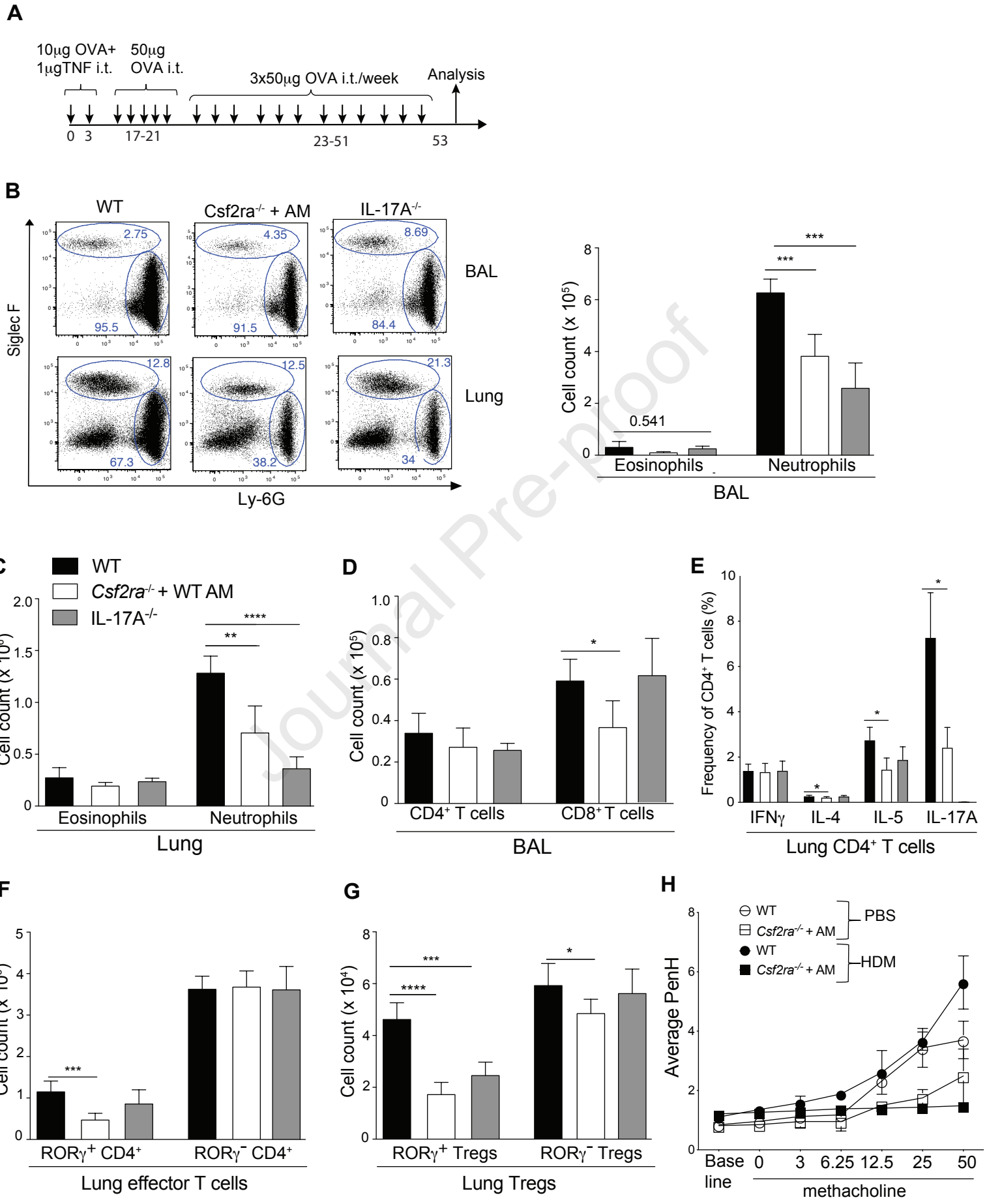
AM-reconstituted *Csf2rb*$^{lox/lox}$-*Cd11c-Cre* mice were sensitized with 10ug HDM on day 0 and were then challenged with 10µg of HDM daily on days 7-11 with flow cytometric analysis after

14 days. (A-B) Representative dot plots of BAL and total numbers (mean ± SEM) of eosinophils and neutrophils, (C) Lung DC subsets (D) BAL $CD4^+$ and $CD8^+$ T cells, (E) I) Representative histological H&E sections of HDM-challenged lungs (F) BAL cells were restimulated for 3h with PMA/ionomycin and cytokine production determined. Shown is the frequency (mean ± SEM) of IL-4, IL-5, IL-10, IL-17A and IFNγ-producing $CD4^+$ T cells (n=3-5/group) The data is representative of 2 independent experiments. The Student's t test (unpaired) was used: p <= 0.05 (*), p <= 0.01 (**), p <= 0.001 (***).

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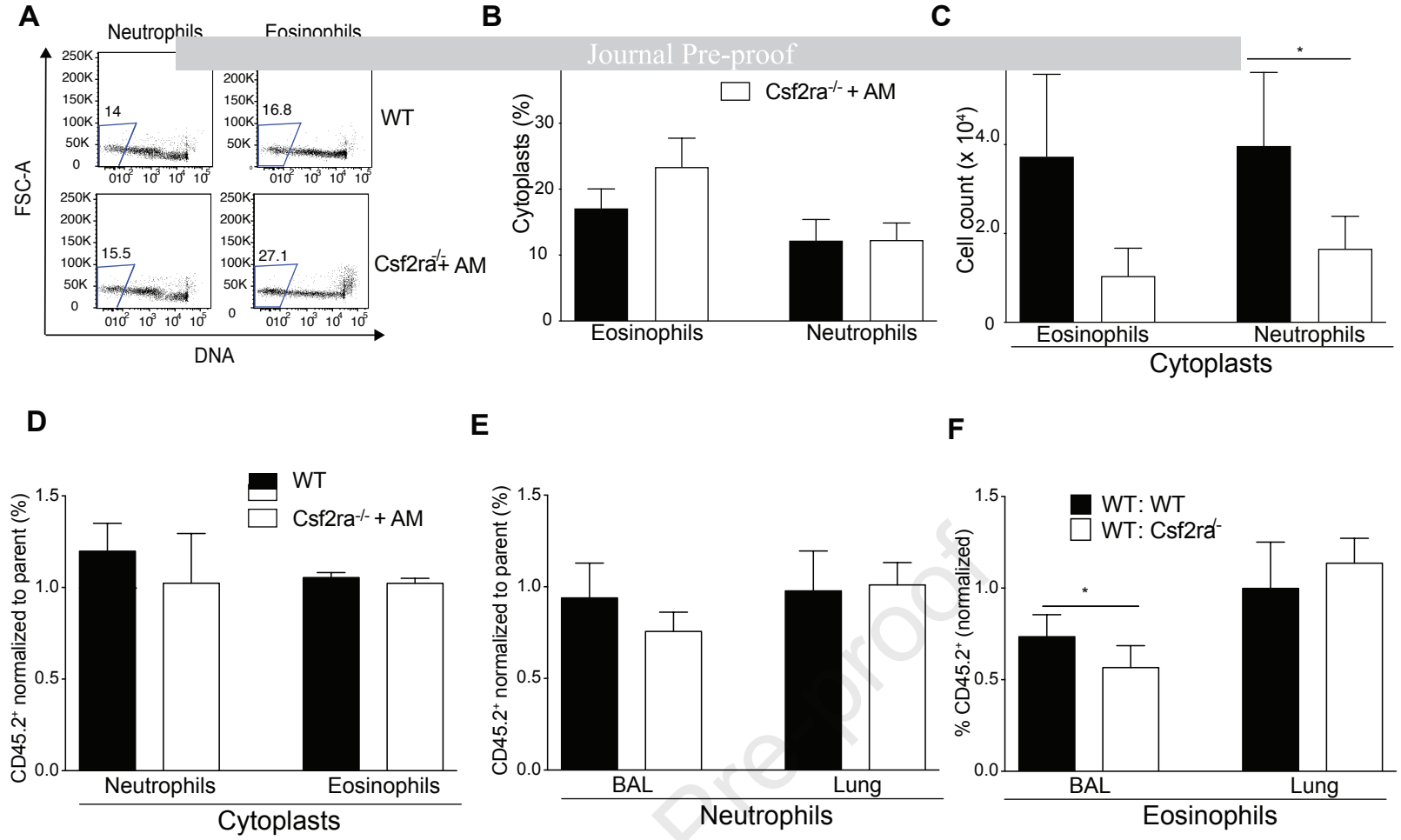
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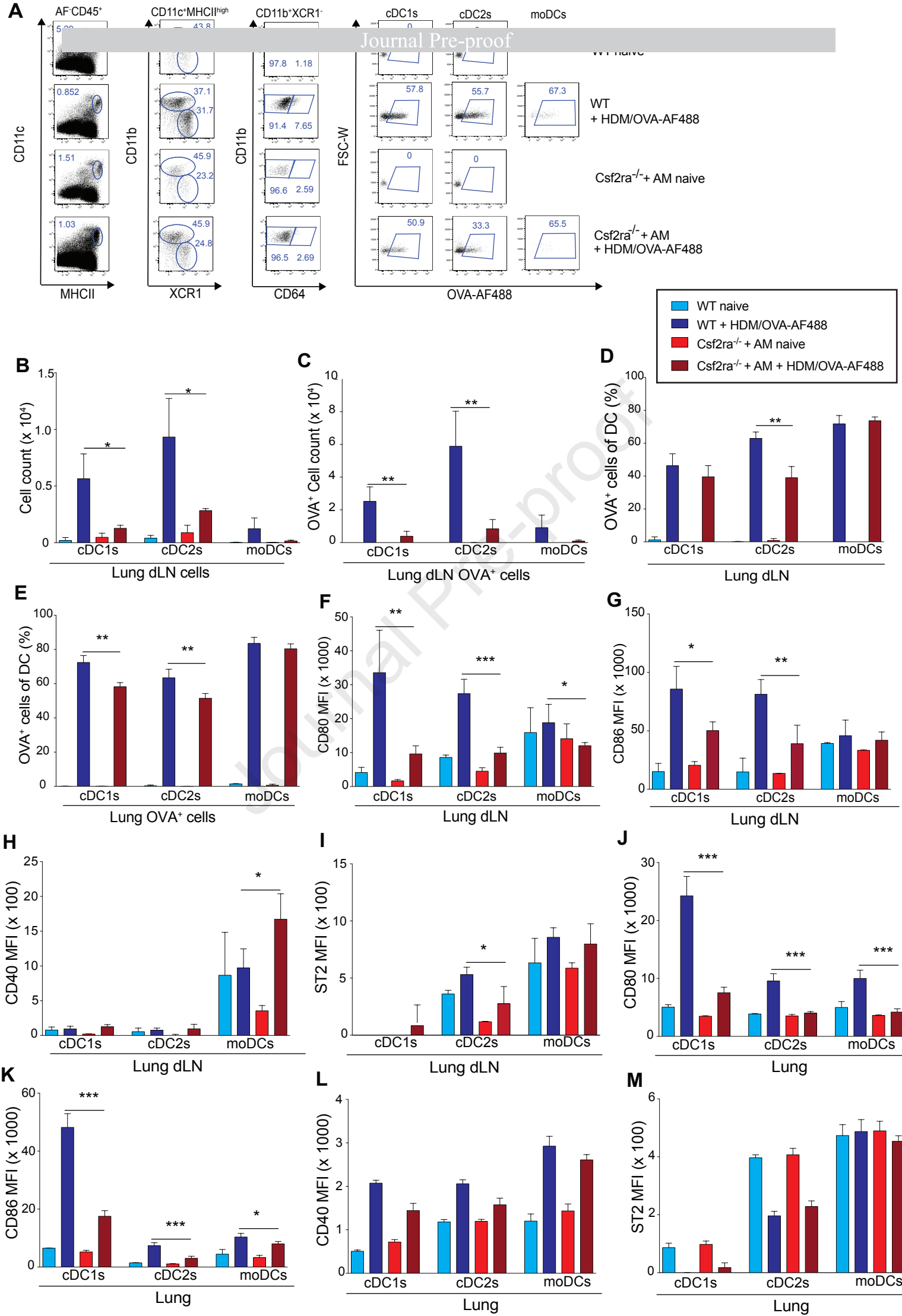
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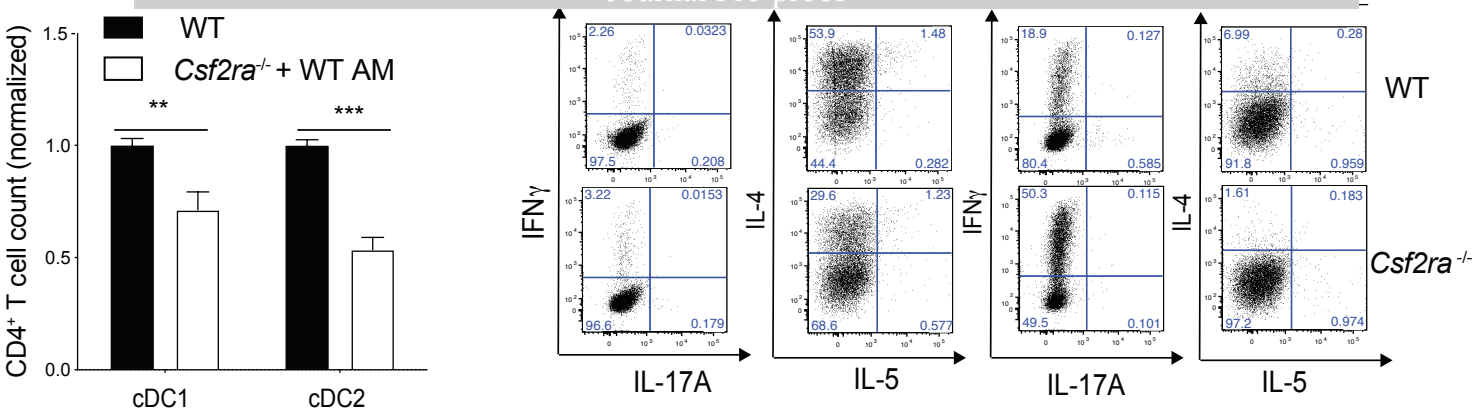


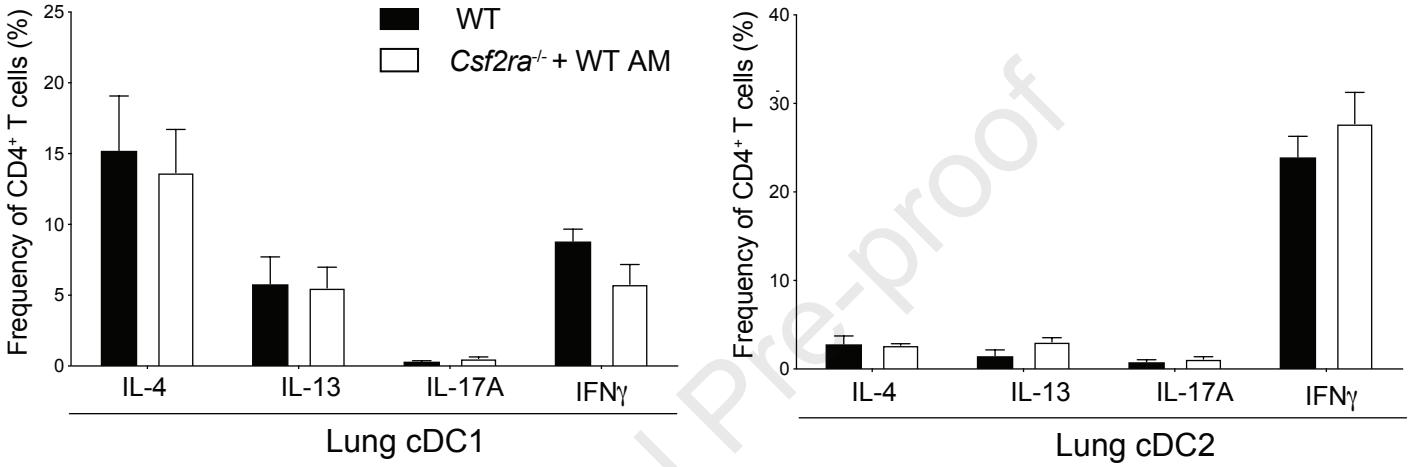
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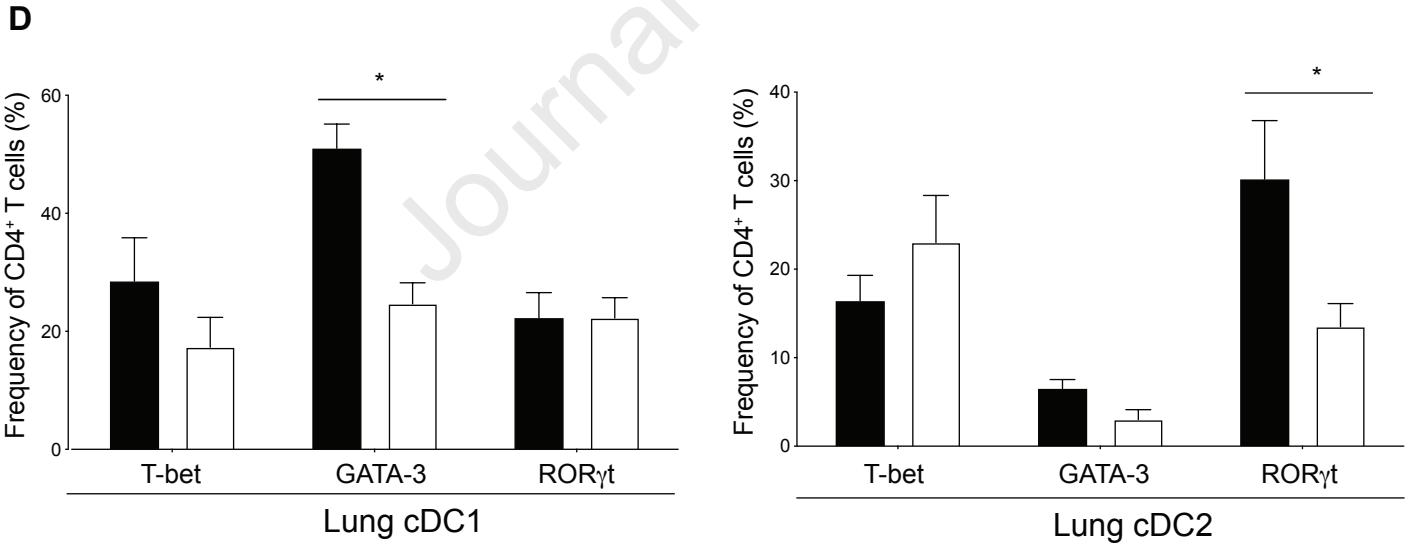
Journal Pre-proof



Journal Pre-proof

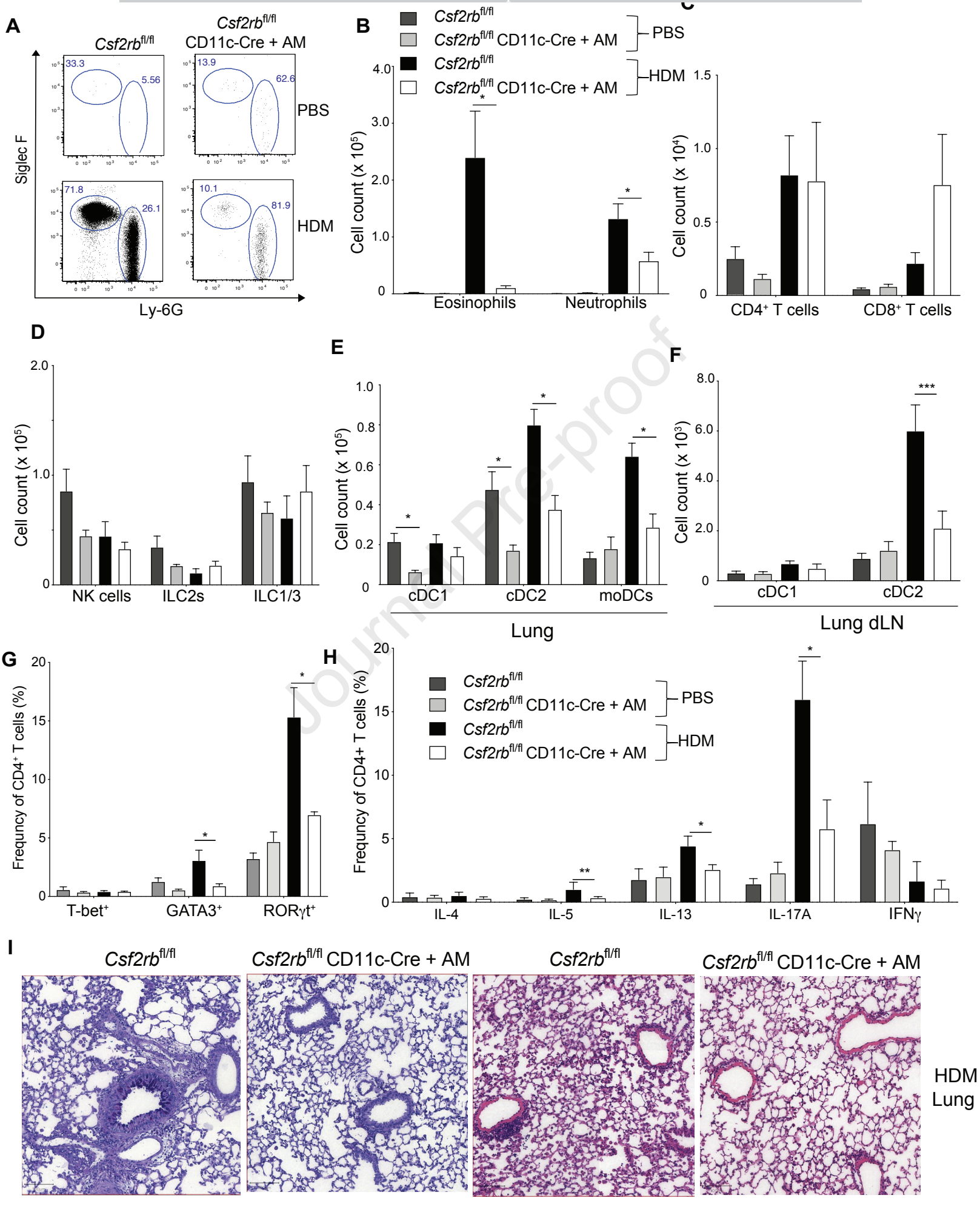




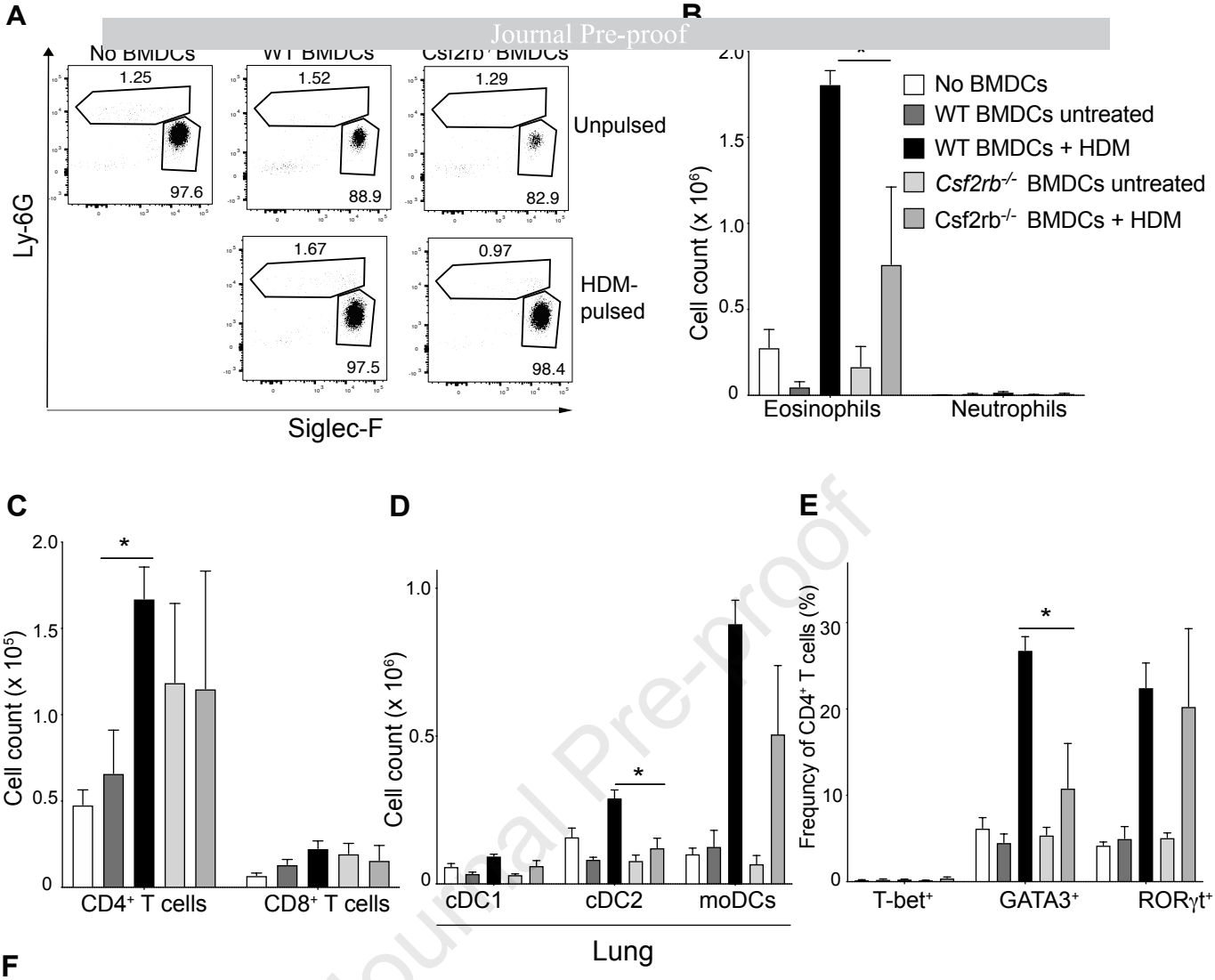


Journal Pre-proof

Journal Pre-proof



Journal Pre-proof



Journal Pre-proof

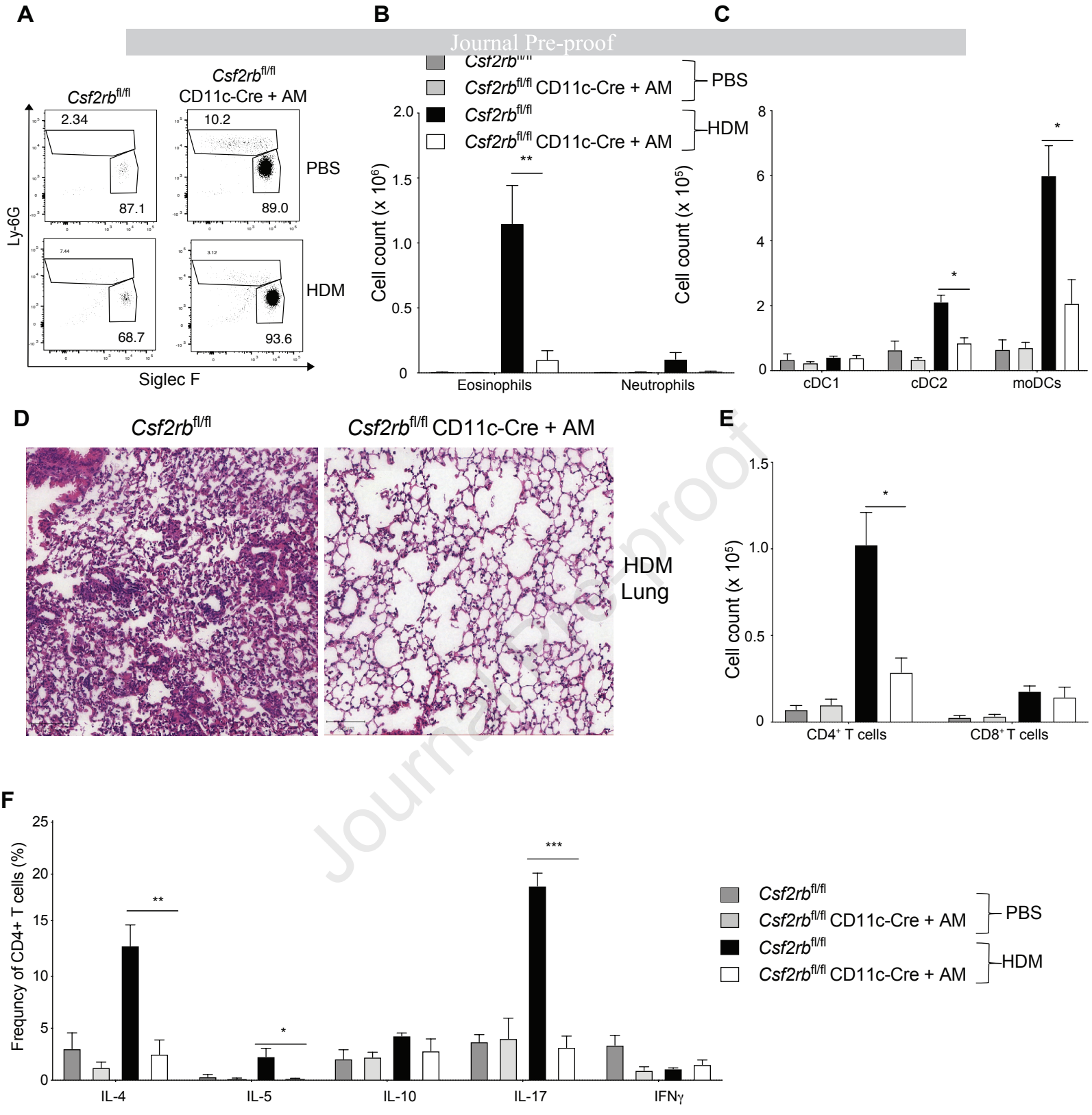
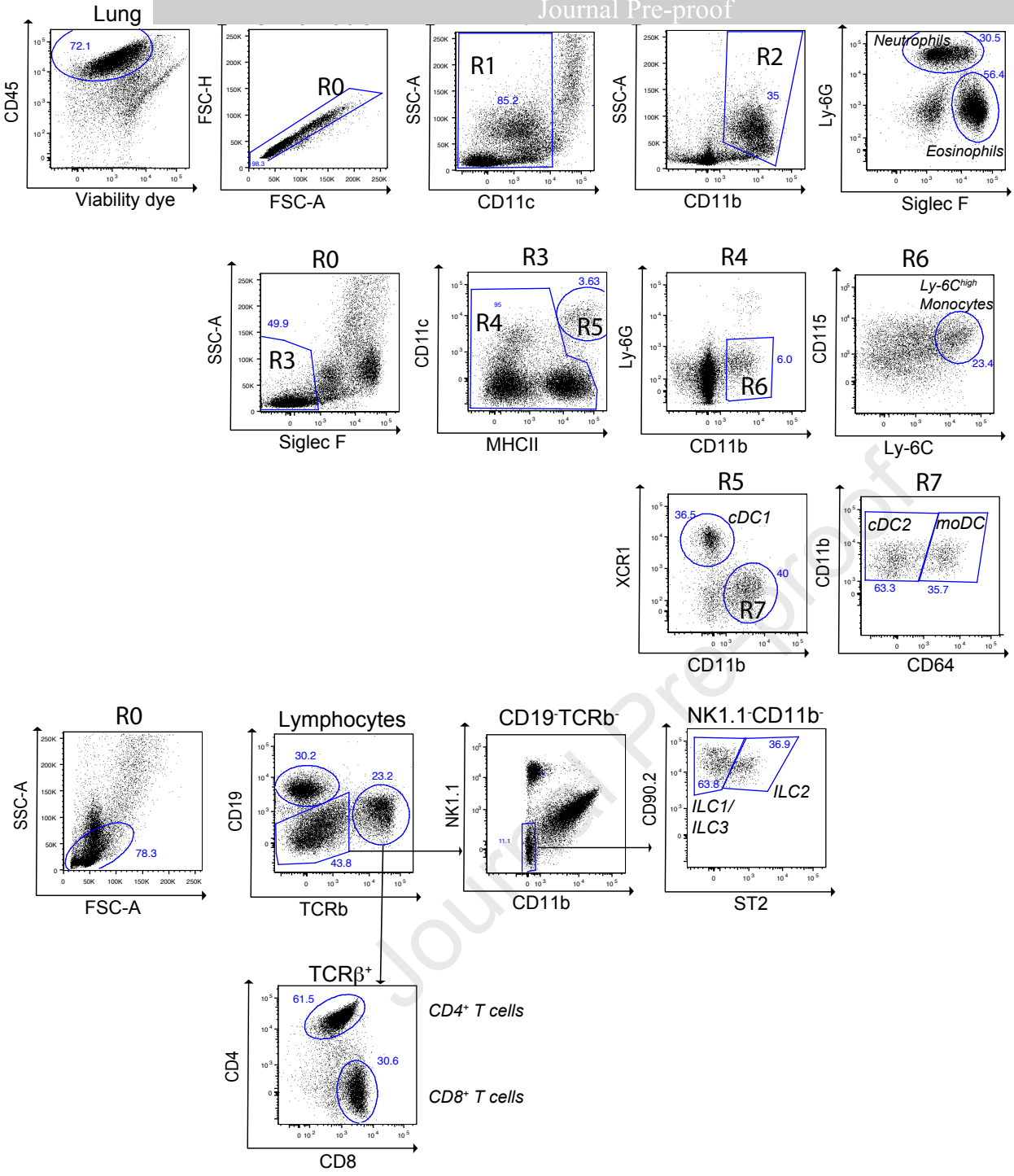
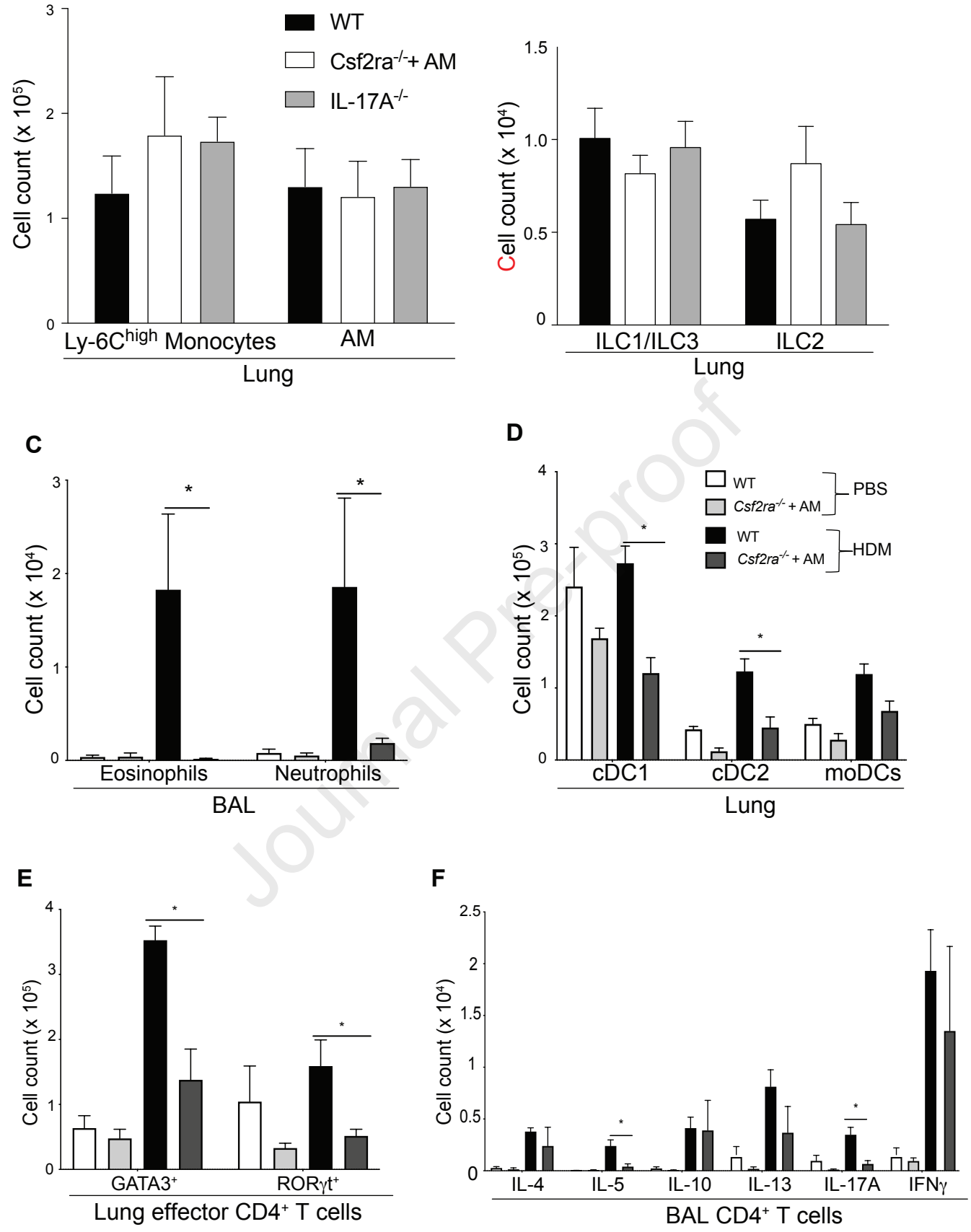


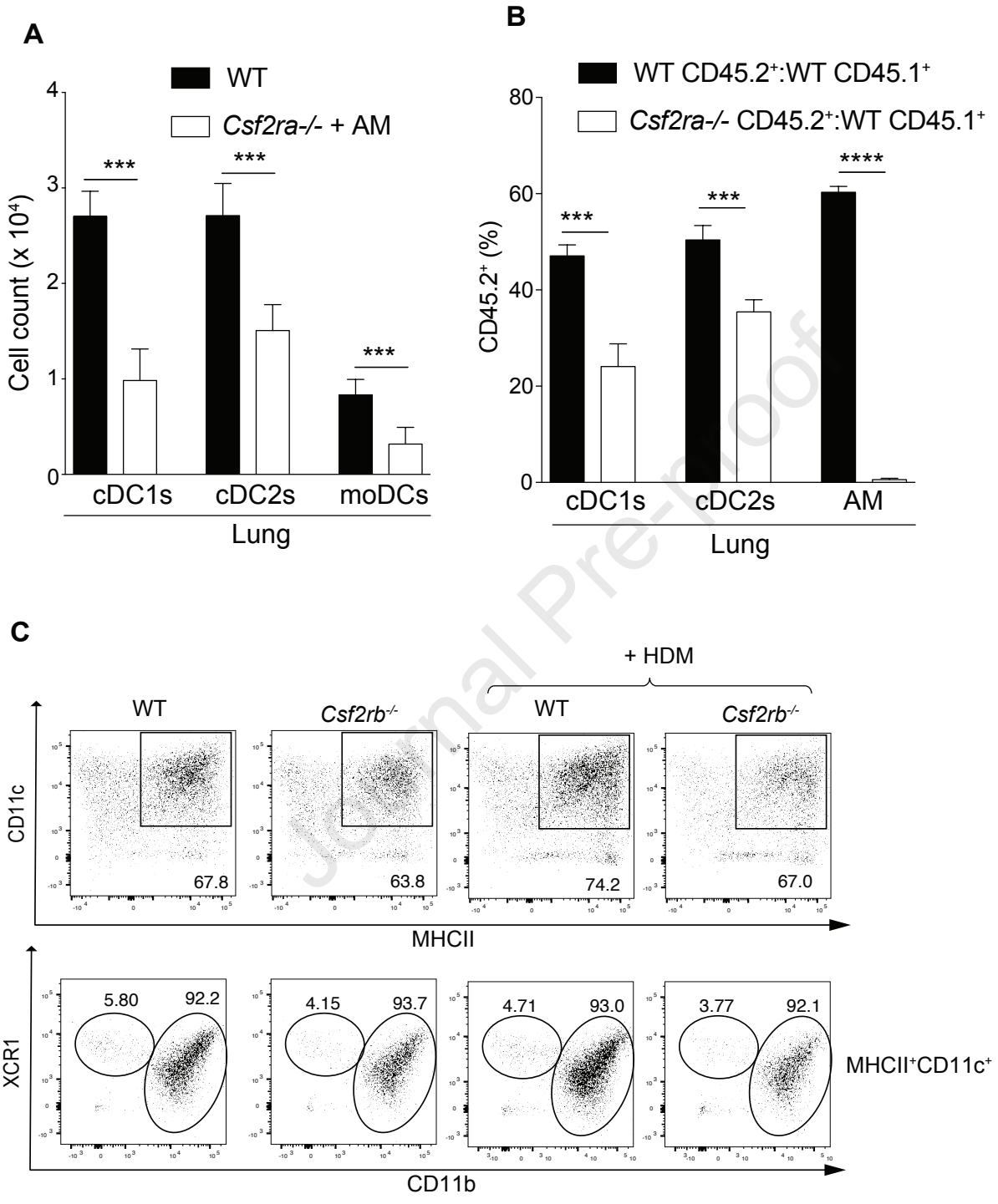
Figure S1

Journal Pre-proof



Journal Pre-proof




A
WT
Csf2ra-/- + AM
Cell count (x 10⁴)
cDC1s
cDC2s
moDCs
Lung
B
WT CD45.2⁺:WT CD45.1⁺
Csf2ra-/- CD45.2⁺:WT CD45.1⁺
CD45.2⁺ (%)
cDC1s
cDC2s
AM
Lung
C
+ HDM
WT
Csf2rb-/-
WT
Csf2rb-/-
CD11c
MHCII
67.8
63.8
74.2
67.0
XCR1
CD11b
5.80
92.2
4.15
93.7
4.71
93.0
3.77
92.1
MHCII⁺CD11c⁺