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Stat5 is required for CD103+ Dendritic Cell and Alveolar Macrophage Development and Protection from Lung Injury

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Abstract

We tested the role of Stat5 in dendritic cell (DC) and alveolar macrophage homeostasis in the lung using CD11c-cre mediated deletion ($Cre^{+}5^{f/f}$). We show that Stat5 is required for $CD103^{+}$ dendritic cell (DC) and alveolar macrophage development. We found that fetal monocyte maturation into alveolar macrophages was impaired in $Cre^{+}5^{f/f}$ mice, and we also confirmed impaired alveolar macrophage development of progenitor cells using mixed chimera experiments. In the absence of Stat5 signaling in alveolar macrophages, mice developed alveolar proteinosis with altered lipid homeostasis. In addition, loss of Stat5 in $CD11c^{+}$ cells was associated with exaggerated LPS-induced inflammatory responses and vascular leak. In $Cre^{+}5^{f/f}$ mice, there was loss of immune-dampening effects on epithelial cells, a key source of CCL2 that serves to recruit monocytes/macrophages. These findings demonstrate the critical importance of Stat5 signaling in maintaining lung homeostasis and underscore the importance of resident macrophages in moderating tissue damage and excess inflammation.

Introduction

Pulmonary alveolar proteinosis (PAP) is an uncommon pulmonary disorder characterized by intra-alveolar accumulation of surfactant proteins, impaired lipoprotein processing by macrophages, and increased susceptibility to pulmonary infections (1, 2). Studies in mice led to the discovery of the critical role of GM-CSF and more specifically, the macrophage in disease pathogenesis. Deletion of GM-CSF or its receptor, GM-CSFR, in mice recapitulates the human disease,(3–6) and GM-CSF receptor mutations and autoantibodies to GM-CSF are associated with the clinical disease (7–9).

GM-CSF drives expression of several transcription factors including PU-1 and PPAR-γ. PU-1 is critical for macrophage differentiation and function, and macrophage-specific

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deletion of PPAR-γ leads to PAP (10, 11). More recently, both GM-CSF and PPAR-γ have been shown to promote the differentiation of fetal monocytes to mature alveolar macrophages further clarifying alveolar macrophage ontogeny (11, 12).

GM-CSF can activate both STAT3 and STAT5 to promote DC and macrophage differentiation via pleiotrophic effects on downstream transcription factors, including IRF8, Spi-B, IRF7, PU-1, and PPAR-γ (13, 14). After cellular maturation, Stat5 can be activated by many additional cytokines, including TSLP, IL-2, and IL-5. We have previously shown using mice lacking Stat5 in CD11c+ cells, a significant role for Stat5 in TSLP-dependent DC activation and Th2 but not Th1 responses (15).

Here, we tested the consequence of CD11c-cre mediated deletion of Stat5 in DC and alveolar macrophage homeostasis in the lung. We show that Stat5 is required for CD103+ DC and alveolar macrophage development. In the absence of Stat5 signaling in alveolar macrophages, mice developed alveolar proteinosis, similar to that reported in the GM-CSF knock-out mouse, with altered lipid homeostasis that could not be rescued with PPAR-γ agonists. In addition, loss of Stat5 in $CD11c^{+}$ cells was associated with exaggerated inflammatory responses and vascular leak to LPS-induced sterile lung inflammation suggesting the critical importance of Stat5 signaling in maintaining lung homeostasis.

Material and Methods

Mice

Cd11c-Cre on the BALB/c background were crossed with loxP-flanked Stat5 alleles (*Stat5*$^{f/f}$) on a BALB/c background as described (15). Mice were maintained under specific pathogen free conditions. Animal procedures were approved by the Institutional Animal Care and Use Committees at the University of Washington and Benaroya Research Institute.

Genotyping and DNA deletion

Mice were genotyped for the presence of the Cre recombinase allele and floxed *Stat5* alleles as previously described (16). The detection of the floxed Stat5 and deleted floxed Stat5 in DNA was performed as previously described to determine the deletion efficiency (17).

LPS Challenge

Mice under isoflurane anesthesia received *E.coli* LPS (1.5 μg/g mouse weight; Sigma-Aldrich, St. Louis, MO) via oropharyngeal aspiration. Mice were weighed and monitored daily until harvest days 2–5. For cell sorting experiments, mice were harvested at day 1.

Bone Marrow chimera

CD45.1/CD45.2 BALB/C host mice (8 weeks old) were irradiated with 900 rads followed by injection of 5×10^{6} bone marrow cells from CD45.1 WT BALB/C or CD45.2 $Cre^{+}5^{f/f}$ BALB/C donor mice. Additional mice received a 50:50 mix of WT and $Cre^{+}5^{f/f}$ BALB/C donor cells. Mice were harvested 3 months post-bone marrow transplant for analysis.

Rosiglitazone

Neonate $Cre^{+}5^{f/f}$ and $Cre^{-}5^{f/f}$ mice were injected daily with rosiglitazone (10 mg/kg; Sigma-Aldrich, St. Louis, MO) in 5% DMSO in sterile PBS. Control mice received 5% DMSO in sterile PBS. Injections were given IP in a volume of 10 μl/g mouse weight from DAB2-DAB10, and lungs were harvested on DAB11 for analysis by flow cytometry. Additional $Cre^{+}5^{f/f}$ and $Cre^{-}5^{f/f}$ mice were fed standard chow diet compounded with Rosigiltazone Maleate (20 mg/kg) or standard chow diet alone starting at day 21 for up to 4 weeks.

Tissue Processing

Murine lungs were serially lavaged with 0.8, 0.7, and 0.7 ml PBS + 5 mM EDTA. Bronchoalveolar lavage (BAL) cells were pelleted by centrifugation for analysis. The vascular compartment was perfused with PBS after cutting the hepatic vein, and the lung was removed and stored on ice. Lung tissue was dissociated by chopping and digested using Liberase TM (1 mg/ml; Roche, Indianapolis, IN) and DNase I (1 mg/ml; Sigma-Aldrich) for 20 minutes at 37°C then filtered through a 70uM cell strainer (Fisher Scientific, Hanover Park, IL). Red blood cells were lysed with RBC Lysis Buffer (eBioscience, San Diego, CA) for 8 min at room temperature. Lavage cells and lung digests were washed with 1% FBS+ 2 mM EDTA in PBS and counted on a Cellometer Auto 200 (Nexelcom Bioscience, Lawrence, MA).

Cytospins and Oil Red O Stain

Cytospins were prepared by centrifuging BAL cells onto a slide at 750g for 5 min. Cells were then stained in Diff Quik (Siemens, Malvern, PA) following the manufacture's protocol. For lipid staining, cells were fixed with 60% isopropanol and stained with Oil Red O (Sigma-Aldrich) for 15 min. Slides were then washed twice with 60% isopropanol for 1 minute, rinsed with water and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL).

Flow Cytometry

All stains were performed in 96 well u-bottom plates in a volume of 100 μl. 1×10^{5}–1×10^{6} cells were plated and blocked with Fc receptor block (BD, Biosciences, San Jose, CA) for 15 min at 4°C and subsequently incubated with antibodies for 1 h at 4°C. Cells were then washed and fixed in 5% formalin for 15 min at room temperature. Flow cytometry was acquired using a FACSCanto RUO system (BD Biosciences). Data analysis was performed using FlowJo (Treestar, Ashland OR). Antibodies included: Siglec F PE (BD), Ly6G APC, Ly6G FITC, CD11b PE-Cy7, CD11c PB, CD71 PerCP Cy5.5, CD206 BV605, ICAM1 FITC, CD45 APC-Cy7, MHCII BV510, CD103 APC (Biolegend).

In vitro LPS Stimulation

Murine lungs were serially lavaged with 0.8, 0.7, and 0.7 ml 5 mM EDTA in PBS, and cells were then spun down, suspended in growth medium (RPMI 1640; Gibco, ThermoFisher Scientific) containing 50 μg/ml Penicillin-Streptomycin (Corning, Corning, NY) and 10% FBS (GE Healthcare, Logan, UT) and plated in 96-well flat bottom tissue culture treated plates (Corning, Corning, NY). Alveolar macrophages were allowed to adhere for 1 h at

37°C and non-adherent cells were washed away with medium. Adherent cells were then stimulated with 10 ng/ml *E.coli* LPS (Sigma-Aldrich) for 4 h at 37°C, washed with warm PBS and then harvested for RNA purification.

Cell Sorting

Lung cells were blocked with Fc block (BD Biosciences) for 20 min 4°C and subsequently incubated with CD45-biotin (eBioscience). Cells were washed with FACS buffer, incubated with anti-biotin microbeads (Miltenyi Biotech, San Diego, CA) and run through magnetic columns per manufacture's protocol. The $CD45^{pos}$ fraction was collected for RNA. The $CD45^{neg}$ flow through was stained with CD326-FITC, CD31-PE (eBioscience), and CD45-APC-Cy7 (BD Biosciences) antibodies. Cells were sorted using an Aria cell sorter (BD Biosciences).

For sorting of macrophage and DC populations, lung cells were blocked with Fc block and incubated with antibodies to CD45, MHCII, Siglec F, Ly6G, CD11b, CD11c, CD103. Cells were then washed twice with FACS buffer and sorted directly into 500μl RA1 Lysis Buffer (Clontech) on an Aria Cell Sorter (BD). Cell populations were defined by the following surface expression patterns. "CD103+ DCs" were defined as $CD45^{+}/Ly6G^{-}/SigF^{-}/CD11c^{+}/CD103^{+}$, aAMs" as $CD45^{+}/Ly6G^{-}/SigF^{int}/CD11c^{int}/CD11b^{high}$, and "mAMs" were defined as $CD45^{+}/Ly6G^{-}/SigF^{high}/CD11c^{high}/CD11b^{low}$, and "CD11b DCs" defined as $CD45^{+}/Ly6G^{-}/CD11c^{+}/CD103^{neg}/CD11b^{high/}MHCII^{high.}$

qRT-PCR

RNA was collected and purified using a NucleoSpin RNA kit (Clontech, Mountain View, CA) and RNA quantity and quality was measured with a spectrophotometer (NanoDrop Inc., Wilmington, DE). Reverse transcription was performed using High Capacity Reverse Transcription (Applied Biosciences, Carlsbad, CA), following manufacturers instructions. Real-time PCR was performed using Sensimax II Hi-ROX kit (Bioline, Taunton, MA) according to manufacturers instructions and run on a 7900HT thermal cycler (Applied Biosystems). The threshold cycle (Ct) was obtained from duplicate samples and averaged. The ΔCt was the difference between the average Ct for the target gene and the housekeeping gene, *Hprt*. The ΔΔCt was the average ΔCt for a given sample point minus the average ΔCt of control samples. The data are expressed as relative quantification calculated as $2^{-\Delta\Delta Ct}$.

Protein Measurements

Cytokines and chemokines concentrations for CXCL1/KC, CXCL2/MIP-2α, CCL2/JE, CCL4/MIP-1β, CCL5/RANTES, IP-10, IL-12, and G-CSF were measured in BAL samples using a magnetic bead Luminex assay per manufacture instructions (R&D Systems, Minneapolis, MN) and analyzed on Bioplex 200 (Bio-Rad Laboratories, Raleigh, NC). BAL surfactant D levels were determined by ELISA (Mouse SP-D Quantikine ELISA Kit; R&D systems, Minneapolis, MN), following manufacturers instructions. IgM levels were determined using an IgM ELISA Quantification Kit (Bethyl Labs, Montgomery, TX) following the manufacturer's protocol.

Electron Microscopy

Murine lungs were inflated with 0.8ml ½ Karnovsky's fixative (2% Paraformaldehyde/2.5% Glutaraldehyde buffered in 0.2 M Cacodylate buffer) and immersed in fixative overnight. Lungs were then washed in 0.1M Cacodylate buffer and incubated in 2% aqueous OsO_4/0.2 M Cacodylate buffer. Lungs were dehydrated through an ethanol gradient and incubated overnight in 1:1 Propyleneoxide/Epon 812 resin solution. The following day fresh resin was applied for 2 hours and then sections were embedded in fresh resin. 70-nm sections were cut and put on grids then stained with uranyl acetate for 2 h and lead citrate for 5 min. Microscopy and imaging was performed on a JEM-1400TEM microscope (Joel, Peabody, MA) with a Ultrascan 1000XP 2K × 2K camera (Gatan, Pleasanton, CA).

Statistics

Results are expressed as means ± SEM. Statistical significance was determined using Student's t-test after passing normality assumption (Shapiro-Wilk normality test), or Mann Whitney test for non-normally distributed data using Prism software (GraphPad Software, Inc, La Jolla, CA). Differences were considered significant if the *P*-value was <0.05.

Results

Stat5 is required for dendritic cell homeostasis and alveolar macrophage development

To investigate the role of Stat5 in lung homeostasis, we first measured the effect of CD11c-cre mediated deletion of $Stat5^{f/f}$ in lungs of CD11c-Cre- × $Stat5^{f/f}$ ($Cre^{-}5^{f/f}$) and CD11c-Cre + × $Stat5^{f/f}$ ($Cre^{+}5^{f/f}$) on pulmonary immune cells in naïve mice. We collected alveolar and lung compartment cells from $Cre^{-}5^{f/f}$ and $Cre^{+}5^{f/f}$ mice (ages 8–12 weeks) and assessed cell counts and myeloid populations by FACS. In the alveolar compartment, there was an overall reduction in leukocytes (CD45+ cells) in $Cre^{+}5^{f/f}$ mice, which was due largely to a marked reduction in alveolar macrophages ($CD11c^{high}SigF^{high}CD11b^{low}$) (Fig 1A,B). However, in $Cre^{+}5^{f/f}$ mice, there was an additional $F4/80^{+}$ population characterized by $CD11c^{int}SigF^{int}CD11b^{high}$ expression ($CD11b^{high}$ cells) (Fig 1A–C). In $Cre^{-}5^{f/f}$ mice, alveolar cells were homogeneous in size and morphology (Fig 1D). In contrast, alveolar cells from $Cre^{+}5^{f/f}$ mice showed heterogeneity in size, granularity and morphology, containing a population of strikingly large and foamy macrophages (Fig 1D). The larger cells expressed markers of alveolar macrophages, whereas the smaller cells were $CD11b^{high}$ cells (not shown). In both populations of macrophages, efficiency of Stat5 deletion was > 90% (Supplemental Figure 1).

In the $Cre^{+}5^{f/f}$ lung digests, we found a similar reduction in tissue macrophages and an increase in $CD11b^{high}$ cells within the $CD11c^{+}SigF^{+}$ gate (Fig 2A,B). Since Stat5 has been reported to be important in DC development, we investigated the changes to $CD103^{+}$ and $CD11b^{+}$ DC populations in the naïve lung (Fig 2A). We found a significant reduction in $CD103^{+}$ DCs ($2.3\pm0.2 \times 10^5$ versus $0.4\pm0.1 \times 10^5$ cells in Cre^{-} and Cre^{+} lungs; p value < 0.005) and a no change in $CD11b^{+}$ DCs ($2.5\pm0.3 \times 10^5$ versus $2.4\pm0.1 \times 10^5$ cells in Cre^{-} and Cre^{+} lungs) suggesting Stat5 is required for $CD103^{+}$ DC development but not that of other DC populations. In CD11b+ DCs and CD103+ DCs isolated from the lung from $Cre^{+}5^{f/f}$ mice, efficiency of Stat5 deletion was 100% (Supplemental Figure 1).

Stat5-deficiency leads to early failure of alveolar macrophage development

Since GM-CSF is required for maturation of alveolar macrophages from fetal monocytes, we assessed macrophage maturation in post-natal lungs (PND11), when fetal monocytes differentiate to alveolar macrophages. We found reduced alveolar macrophage maturation and an increase in arrested alveolar macrophages (aAM) in the $Cre^{+}5^{f/f}$ lungs (Fig 3). At PND11, aAM represented 40.3 ± 7.6% of $F4/80^{+}CD11c^{+}$ cells in $Cre^{-}5^{f/f}$ mice and 60.9 ± 1.2% in $Cre^{+}5^{f/f}$ mice. In contrast, mature alveolar macrophages (mAM) represented an increased proportion in $Cre^{-}5^{f/f}$ compared to $Cre^{+}5^{f/f}$ mice (51.6 ± 7.9% vs. 30.6 ± 1.1%, respectively.) In 30 day old mice, there was complete maturation of fetal monocytes in $Cre^{-}5^{f/f}$ lungs, whereas they remained in significant numbers in $Cre^{+}5^{f/f}$ lungs (Fig 3B).

Since PPAR-γ deficient $CD11c^{+}$ cells develop a similar phenotype of impaired maturation of monocytes into alveolar macrophages,(11) we assessed if treatment with a PPAR-γ agonist could rescue alveolar macrophage development. To test this hypothesis, we administered the PPAR-γ agonist, rosiglitazone, from PND2-11 via intraperitoneal injection or from 2–4 weeks via diet supplementation. In both groups, rosiglitazone had no effect on alveolar macrophage development (not shown). These negative findings suggest that either PPAR-γ levels are too low for rescue by an agonist or there are other Stat5-dependent mechanisms required for macrophage maturation.

As another assessment of the ability of Stat5-deficient cells to reconstitute alveolar macrophages in the lung, we generated chimeras using CD45.1 $Cre^{+}5^{f/f}$, CD45.2 $Cre^{-}5^{f/f}$ or 50:50 mixed donor marrow into CD45.1/CD45.2 wild type recipients. At 3 months, we assessed the BAL cell populations by FACS (Supplemental Fig 2). Mice that received 100% $Cre^{+}5^{f/f}$ bone marrow failed to reconstitute alveolar macrophage with donor cells, with the majority of the alveolar macrophages representing remaining host cells (Supplemental Fig 2). In contrast, mice that received wildtype bone marrow had nearly entire reconstitution of their alveolar macrophages with donor cells. Recipients of the 50:50 mixed marrow demonstrated that a majority of alveolar macrophages were derived from WT cells (78%), with fewer than 1% of $Cre^{+}5^{f/f}$ origin (Supplemental Fig 1B). In mice reconstituted with either 50% or 100% $Cre^{+}5^{f/f}$ donor cells, there was a population of $CD11c^{+}$ cells representing a population of immature macrophages (Supplemental Fig 2C).

$Cre^{+}5^{f/l}$ alveolar macrophages have increased lipid accumulation and altered transcriptional profiles

It is well established that GM-CSF is required for phospholipid processing by alveolar macrophages and disruption of this pathway leads to pulmonary alveolar proteinosis.(18, 19) Since GM-CSF signals via Stat5, we investigated if loss of Stat5 in alveolar macrophages would cause pulmonary alveolar proteinosis. By 12 weeks of age, there was a significant increase in lipid-loaded macrophages and material in the alveolar fluid (Fig 4A-C), and surfactant protein D was markedly increased in the alveolar fluid from $Cre^{+}5^{f/f}$ mice consistent with development of pulmonary alveolar proteinosis (Fig 4D).

Given these marked increases in lipid-loaded macrophages, we assessed the gene expression of several genes involved in lipid homeostasis in the macrophage populations from $Cre^{-}5^{f/f}$

and $Cre^{+}5^{f/f}$ mice. We sorted $CD11c^{high}SigF^{high}CD11b^{low}$ (mAM) and $CD11c^{int}SigF^{int}CD11b^{high}$ (aAM) from $Cre^{+}5^{f/f}$ lungs and $CD11c^{high}SigF^{high}CD11b^{low}$ (AM) from $Cre^{-}5^{f/f}$ lungs. Using qRT-PCR, we found that aAM from $Cre^{+}5^{f/f}$ mice expressed less *Abcg1* and *Pparg* but greater levels of *Apoe* and *Abca1* compared to $Cre^{+}5^{f/f}$ mAM and $Cre^{-}5^{f/f}$ AM (Fig 4E). $Cre^{+}5^{f/f}$ mAM gene expression more closely resembled that of $Cre^{-}5^{f/f}$ AM, with similar levels of *Pparg* and *Abcg1,* but with increased *Lxra* and *Apoe* expression (Fig 4E). We also assessed if loss of Stat5 in CD103+DCs and $CD11b^{+}$ DCs altered *Pparg* expression. Unlike that observed for aAM, the expression of *Pparg* from $Cre^{+}5^{f/f}$ and $Cre^{-}5^{f/f}$ CD103+ DCs and CD11b+ DCs was similar (Fig 4F,G) indicating that Stat5 effects on *Pparg* are cell-specific. Overall, the absence of Stat5 was associated with altered pathways involving lipid homeostasis, most notably in the aAM population.

Inflammatory responses in the lung are increased in $Cre^{+}5^{f/f}$ mice

To determine the consequences of Stat5 deletion on inflammatory responses in the lung, we challenged mice with LPS delivered via oropharyngeal aspiration post-LPS.

At the peak of inflammation on day 2, $Cre^{+}5^{f/f}$ mice lost more weight than $Cre^{-}5^{f/f}$ mice (Fig 5A), and there was a greater increase in recruited PMNs and $CD11b^{+}$ macrophages in $Cre^{+}5^{f/f}$ compared to $Cre^{-}5^{f/f}$ along with a persistent reduction in alveolar macrophages (Fig 5B). In addition, pro-inflammatory cytokines and chemokines were markedly higher in LPS-treated $Cre^{+}5^{f/f}$ mice (Fig 5C).

By day 5, $Cre^{+}5^{f/f}$ mice started to recover their weights (not shown) and BAL neutrophilia was less (~ 50 fold) at d5 as compared to d2, suggesting resolution of lung injury. However, compared to LPS-treated $Cre^{-}5^{f/f}$ mice, there were increased numbers of neutrophils and reduced numbers of resident alveolar macrophages in the alveolar compartment (Fig 6A, B). Most striking was a marked increase in vascular leak with elevated IgM that was detected at baseline (day 0) with markedly greater increases at days 2 and 5 post-LPS in $Cre^{+}5^{f/f}$ mice compared to control $Cre^{-}5^{f/f}$ mice (Fig 6C). Along with greater IgM levels, increased RBCs in BALF were also observed in LPS-treated $Cre^{+}5^{f/f}$ mice (not shown). In the lung compartment, there was also increased numbers of neutrophils and reduced numbers of resident alveolar macrophages and CD103+ DCs in $Cre^{+}5^{f/f}$ mice (Fig 7A,B). Together, these results suggest that perturbations in DC or alveolar macrophage function are critical for tempering the induction of lung inflammation and for regulating vascular integrity at both baseline and during acute lung injury.

Increased Inflammatory responses in $Cre^{+}5^{f/f}$ lung are localized to leukocytes and epithelial cells

Given the early inflammatory changes to LPS, we hypothesized that the source of increased inflammatory cytokines/chemokines (Fig 5C) was the macrophage. We removed the alveolar cells from mice and stimulated these cells with LPS to assess the inflammatory cytokine expression by qRT-PCR. In contrast to what we observed *in vivo*, the cells from $Cre^{+}5^{f/f}$ mice were less responsive to LPS and generated less *Cxcl1, Tnfa, and Il1b* mRNA (Fig 8A), suggesting that other cells sources are responsible for the increased levels observed *in vivo* and may highlight an indirect anti-inflammatory role for either CD103+ DCs, CD11b+ DCs,

or alveolar macrophages. To test this hypothesis, we sorted CD31+ endothelial cells, CD326+ epithelial cells, and CD45+ leukocytes from the lung at 24 h post-LPS treated $Cre^{-}5^{f/f}$ and $Cre^{+}5^{f/f}$ mice. We found that endothelial cells had similar expression of inflammatory cytokines/chemokines across both genotypes (Fig 8B). However, epithelial cells from $Cre^{+}5^{f/f}$ mice expressed significantly more CCL2 and G-CSF (Fig 8C), and leukocytes from $Cre^{+}5^{f/f}$ mice expressed significantly more CXCL1, G-CSF, and MIP-1β (Fig 8D). These results indicate that there was a loss of immune-dampening effects on epithelial cells, a key source of CCL2 that serves to recruit monocytes/macrophages. In addition, there was evidence of altered leukocyte activation or composition that contributes to enhanced expression of neutrophil chemokines.

Discussion

We tested the role of Stat5 in DC and alveolar macrophage homeostasis in the lung using CD11c-cre mediated deletion. Here, we show that Stat5 is required for alveolar macrophage development in the lung. We found that fetal monocyte maturation into alveolar macrophages was impaired in $Cre^{+}5^{f/f}$ mice, and we also confirmed impaired alveolar macrophage development of progenitor cells using mixed chimera experiments. These findings are consistent with the known role of GM-CSF in alveolar macrophage development,(12) which is known to signal through Stat5,(14) and further confirms dependency on Stat5 as the key transcription factor involved in their development.

In addition to alveolar macrophage development, we also found a role for Stat5 in pulmonary $CD103^{+}$ DC development. In hematopoietic *Stat5*$^{-/-}$ mice, tissue development of $CD103^{+}$ DC has been shown to be Stat5-dependent,(13) and GM-CSF has been shown to control the development of dermal $CD103^{+}$ DCs but not that of other DC populations (20). Interestingly, previous work in these mice revealed that Stat5 was dispensable in DC homeostasis in other tissues,(15) and these differences likely reflect the unique cytokine milieu in the lung. To our knowledge, deficiency of $CD103^{+}$ DCs has not been reported in other murine models of pulmonary alveolar proteinosis (PAP) or in human PAP; however, $CD103^{+}$ DC development has been shown to be dependent on GM-CSF (21).

In the absence of Stat5 in alveolar macrophages, mice developed pulmonary alveolar proteinosis as evidenced by increased surfactant protein in the alveolar compartment and presence of lipid-laden macrophages. We also observed altered expression of genes involved in lipid homeostasis, including reduced PPAR–γ expression in aAM. Reduced PPAR-γ has been reported in alveolar macrophages from patients with PAP,(22) and deletion of PPAR-γ in $CD11c^{+}$ cells results in a similar phenotype to $Cre^{+}5^{f/f}$ mice in terms of impaired alveolar macrophage maturation and development of pulmonary alveolar proteinosis (11). Hence, our findings implicate Stat5 as being an upstream regulator of PPAR-γ in progenitors of alveolar macrophages. Since PPAR–γ was reduced in aAM from $Cre^{+}5^{f/f}$ mice, we tested whether a PPAR-γ agonist could rescue alveolar macrophage development. Despite two delivery strategies, we were unable to pharmacologically rescue alveolar macrophage development, suggesting that additional pathways may be involved.

Despite effective deletion of Stat5 in both arrested and mature alveolar macrophage populations in the $Cre^{+}5^{f/f}$ mice, we cannot explain why some alveolar macrophages mature and others remain in an arrested state. However, *Pparg* levels were unaffected by *Stat5* deletion in mAMs, suggesting that these cells may escape the early effects of *Stat5* deletion on alveolar macrophage maturation.

We also tested the inflammatory responses in $Cre^{+}5^{f/f}$ mice using a sterile model of inflammation. Loss of Stat5 in $CD11c^{+}$ cells was associated with exaggerated inflammatory responses and vascular leak to LPS-induced acute lung injury. Although the overall inflammatory response was increased in $Cre^{+}5^{f/f}$ mice compared to $Cre^{-}5^{f/f}$ mice at days 2 and 5, there was a significant decrease in neutrophils over time in $Cre^{+}5^{f/f}$, suggesting that resolution was intact. These findings may reflect the fact that $Cre^{+}5^{f/f}$ mice were still able to recruit macrophages from the circulation that participate in resolution of lung injury.

More impressive was the prominent vascular leak observed by both increased RBCs and IgM in the alveolar compartment. By scanning EM, we did not observe any defects in endothelial cell morphology in naïve mice (not shown), although there was a small increase in IgM at baseline suggesting some degree of endothelial cell injury. It is compelling to hypothesize that there is a population of macrophages in the lung that helps support endothelial cell health. In $CD11b^{+}$DTR mice, ablation of macrophages in the neonate led to a marked vascular phenotype, suggesting there may exist populations that directly modulate endothelial cell biology (23).

The increased recruitment of neutrophils and macrophages in LPS-treated $Cre^{+}5^{f/f}$ mice was likely due to augmented expression of CXCL1 and CCL2 chemokines. Although inflammatory cytokines and chemokines were markedly increased in $Cre^{+}5^{f/f}$ mice, we found no evidence that $Cre^{+}5^{f/f}$ macrophages were directly contributing, as their *in vitro* response to LPS was not enhanced. Similarly, we found no evidence that the pulmonary DCs were contributing, as sorted $CD103^{+}$ and $CD11b^{+}$ DCs from LPS-treated Cre^{+} and Cre^{-} mice did not differ in their cytokine expression (not shown). These findings suggest either of two possibilities -there is loss of critical cell populations that have anti-inflammatory effects, or other sequelae, such as aberrant lipid accumulation, may lead to low-level injury and susceptibility to acute lung injury.

CD103 DCs have known roles in resolution of lung injury; (21, 24) however, findings of an early difference at 2 days post-LPS challenge does not reflect a resolution phenotype. To our knowledge, CD103+ DC's role in modulating initial inflammatory responses in the lung have not been shown; however, in a colitis model, selective depletion of $Clec9A^{+}CD103^{+}CD11b^{-}$ DCs resulted in more severe disease with increased neutrophil recruitment and rectal bleeding (25). There was also decreased epithelial integrity and protein leak, and the authors found that $Clec9A^{+}CD103^{+}CD11b^{-}$ DCs increased lymphocyte expression of IFN-γ that then resulted in IFN-γ inducible gene expression in the epithelium.

However, dissecting a role for CD103+ DCs in $Cre^{+}5^{f/f}$ is more challenging given the other perturbations in these mice, and rescue experiments by adoptive transfer of such small populations of cells remain a limitation. In addition, alveolar macrophages have been shown

to dampen inflammatory responses of epithelial cells to LPS (26). Hence, loss of alveolar macrophages or aberrant macrophage function may lead to over-exuberant inflammatory responses by the epithelium. Lastly, others have shown that *Pparg* deletion in CD11c cells led to defects in mitochondrial H_2O_2 and increased NF-κβ activation in response to inhaled antigens (27). However, we found that *Pparg* expression was intact in CD103+ DCs, CD11b + DCs, and mAMs from $Cre^{+}5^{f/f}$ mice. Although there are similarities between mice that lack *Pparg* in CD11c+ cells (Cre^{+} *Pparg*$^{f/f}$) and $Cre^{+}5^{f/f}$ mice, such as defects in alveolar macrophage development, *Pparg* expression does not appear to be Stat5-dependent in all cell types. Given these findings, it is not surprising that these mice would also differ in phenotypes. In fact, the $Cre^{+}5^{f/f}$ mice have reduced Th2 responses in the lung, (15) whereas Cre^{+} *Pparg*$^{f/f}$ mice have impaired tolerance and increased pro-inflammatory cytokines in CD11c+ cells (27, 28). Since we show that *Pparg* expression is unaltered in DCs and mature AMs in Cre^{+} *Pparg*$^{f/f}$, there may be other factors or cell sources contributing to augmented inflammation in $Cre^{+}5^{f/f}$ mice.

To dissect out the cellular source of the increased pro-inflammatory cytokines/chemokines in LPS-treated $Cre^{+}5^{f/f}$ mice, we compared gene expression of CXCL1/KC, G-CSF, CCL2/MCP-1, RANTES, and CCL4/MIP-1β from sorted leukocytes, epithelial, and endothelial cells. We found that loss of Stat5 resulted in greater epithelial expression of CCL2/MCP-1, consistent with our finding of increased macrophage recruitment in these mice. In contrast, the leukocytes were the source of greater CXCL1/KC, G-CSF, and CCL4/MIP-1β, and the endothelial cells did not contribute to cytokine differences between genotypes. These findings suggest one consequence of Stat5 deletion is loss of an immune-dampening effect on the epithelium. Since LPS-treated alveolar macrophages from $Cre^{+}5^{f/f}$ mice did not express more pro-inflammatory cytokines compared to control alveolar macrophages, it is likely that the increased neutrophil chemokine expression by $Cre^{+}5^{f/f}$ pulmonary leukocytes may reflect changes in leukocyte recruitment and/or activation.

Overall, these findings implicate the importance of Stat5 on alveolar macrophage and pulmonary CD103+DC development. In the absence of Stat5, there was aberrant accumulation of surfactant protein in the alveolar compartment, and alveolar macrophages accumulated excess lipids with marked alteration of pathways involving lipoproteins and cholesterol efflux. At baseline there was evidence of mild vascular injury, and post-LPS, there was enhanced inflammation and more prominent vascular injury, highlighting novel roles for Stat5 in lung homeostasis and acute injury responses. Our studies also support an immune-dampening role via epithelial-mediated expression of CCL2. Future studies will be needed to determine the relationship of myeloid cell populations in vascular integrity and immune-dampening properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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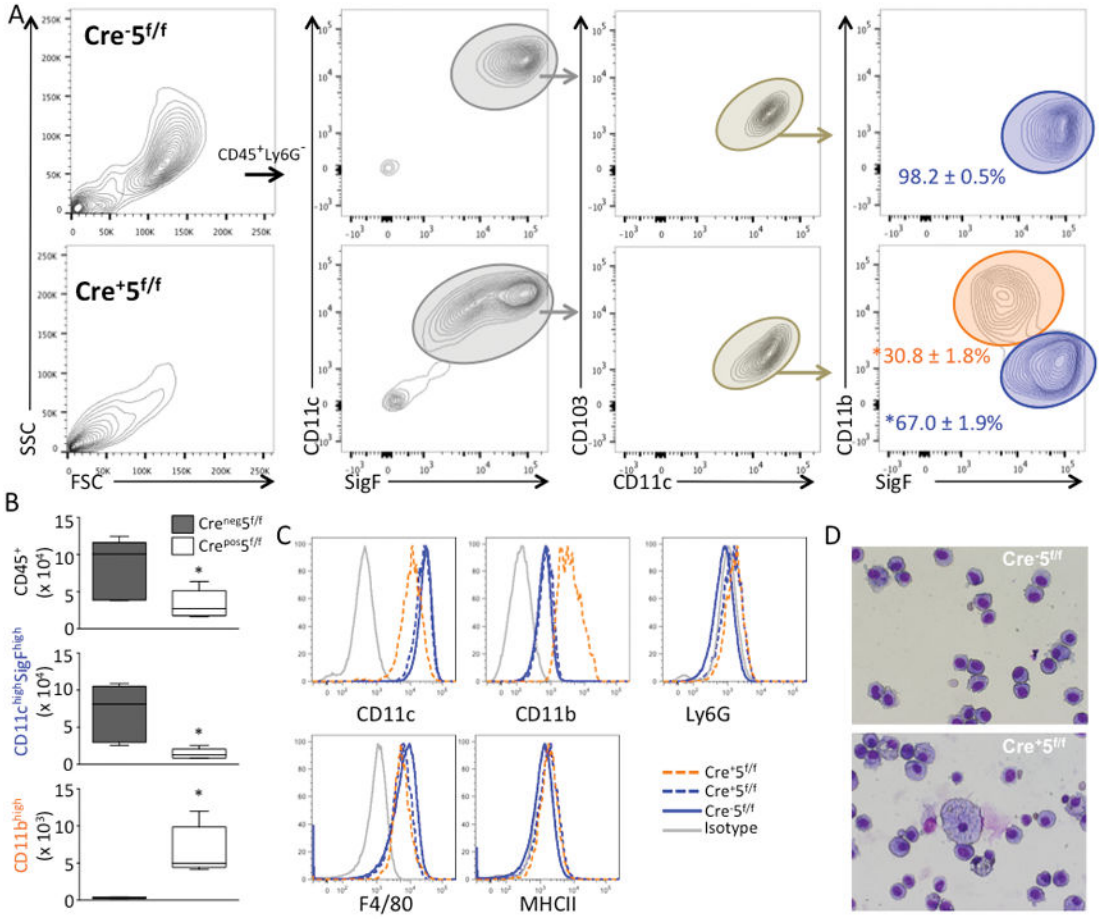

Figure 1. Loss of Stat5 in CD11c+ cells impairs alveolar macrophage development

Naïve $Cre^{-}5^{f/f}$ and $Cre^{+}5^{f/f}$ murine lungs were lavaged and cell counts, morphology, and differential assessed. (A) FACS gating strategy of BAL cells from $Cre^{-}5^{f/f}$ (top row) and $Cre^{+}5^{f/f}$ (bottom row) mice. In $Cre^{+}5^{f/f}$ mice, there was a significant shift in macrophage populations, with a reduction of alveolar macrophages (blue gate) and a new population of $CD11b^{high}$ cells (orange gate). Results presented as % ± SEM of cells within the $CD11c^{+}CD103^{-}$ sub-gate. (B) Quantification of the total leukocytes ($CD45^{+}$) and alveolar macrophages ($CD11c^{high}SigF^{high}$), and $CD11c^{int}CD11b^{high}$ cells. (C) Histograms demonstrating differential expression of CD11c, CD11b, similar expression of F4/80, and negative staining for MHCII and Ly6G across the three populations (solid blue: $Cre^{-}5^{f/f}$ alveolar macrophages; solid orange: $Cre^{+}5^{f/f}$ alveolar macrophages; dashed orange: $Cre^{+}5^{f/f}$ $CD11b^{high}$ cells). (D) Diff-Quick stained cytospins demonstrating heterogeneity of $Cre^{+}5^{f/f}$ BAL cells (bottom) compared to $Cre^{-}5^{f/f}$ cells. *p value <0.05 (n=5 mice/genotype; 3–5 experimental replicates).

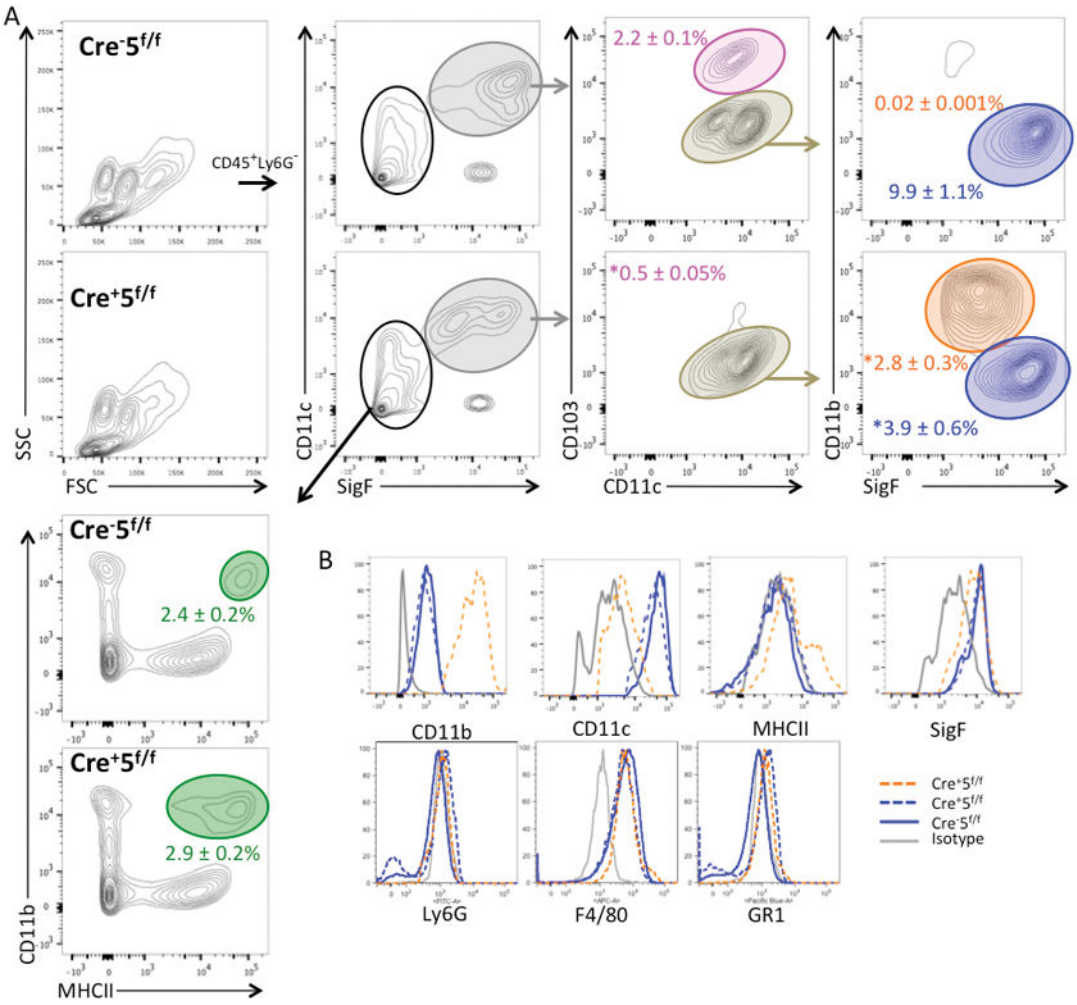

Figure 2. Loss of Stat5 in CD11c+ cells reduces alveolar macrophage and CD103+ DC populations in the lung

Naïve $Cre^{-}5^{f/f}$ and $Cre^{+}5^{f/f}$ murine lungs were mechanically and enzymatically digested for FACS analysis of leukocyte subsets. (A) FACS gating strategy of lung cells demonstrating a reduction of alveolar macrophages (blue gate) and CD103+ DCs (pink gate), and an increase in $CD11b^{high}$ cells (orange gate), and no change in CD11b+ DCs (green gate). Percentages shown represent % of $CD45^{+}$ cells. *p value <0.05 (n=5 mice/genotype; 3–5 experimental replicates).

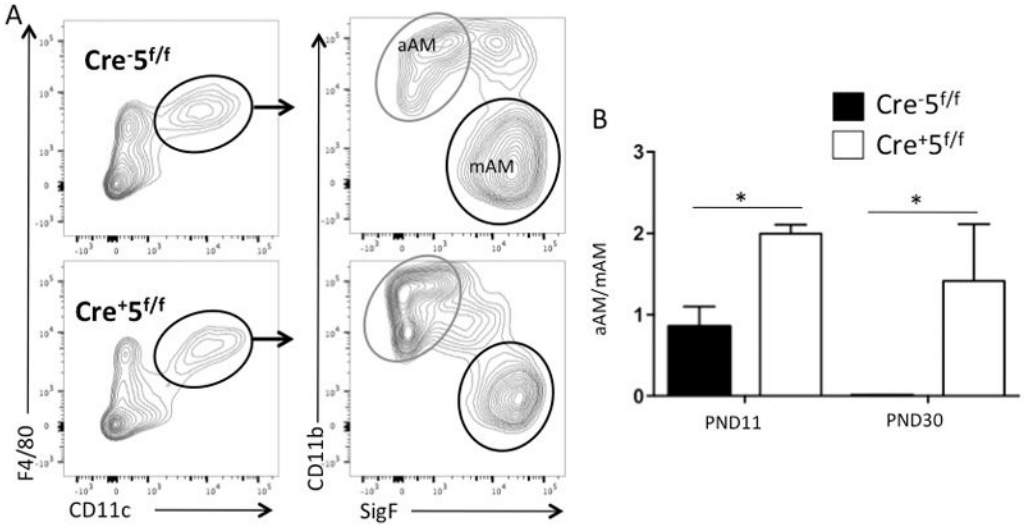

Figure 3. Stat5 is required for alveolar macrophage development from fetal monocytes

$Cre^{-}5^{f/f}$ (top row; black bar) and $Cre^{+}5^{f/f}$ (bottom row; white bar) murine lungs were harvested at PND11 and PND30 for analysis of alveolar macrophages maturation by FACS. (A) $F4/80^{+}/CD11c^{+}$ cells were identified and among those cells, mature alveolar macrophages (mAM) identified as $CD11b^{-}$/Siglec F^{+} and arrested alveolar macrophages (aAM) identified as $CD11b^{+}$/Siglec F^{-}. (B) Expressed as a ratio of mAM/aAM, there was impairment of fetal monocyte maturation in $Cre^{+}5^{f/f}$ mice. *p value <0.05 (n=3–5 mice/genotype).

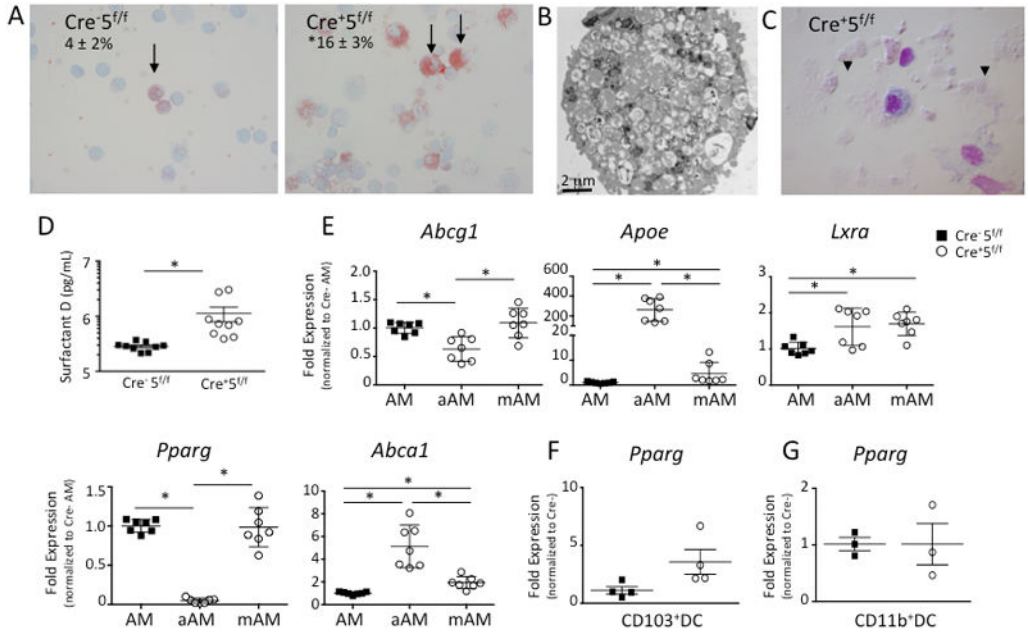

Figure 4. Loss of Stat5 results in pulmonary alveolar proteinosis and abnormal lipid clearance and homeostasis

Naïve $Cre^{-}5^{f/f}$ and $Cre^{+}5^{f/f}$ murine lungs were lavaged for surfactant protein D measurements and assessment of cells by cytospins. (A) Cytospins of BAL cells were stained with Oil Red O revealing increased percentage of stained cells (arrows) from $Cre^{+}5^{f/f}$ mice. (B) Electron microscopy of a $Cre^{+}5^{f/f}$ alveolar macrophage demonstrating extensive lipid loaded vacuoles. (C) Representative Cytospin from $Cre^{+}5^{f/f}$ mice revealing amorphous, acellular material (arrow heads). (D) Surfactant protein D levels were increased in the BAL fluid of $Cre^{+}5^{f/f}$ mice. *p value <0.05 (n=5–9 mice/genotype; 2 experimental replicates). (E) *Abcg1, Apoe, Lxra, Pparg,* and *Abca1* gene expression was assessed from sorted aAM and mAM from $Cre^{+}5^{f/f}$ lungs and AM from $Cre^{-}5^{f/f}$ lungs. (F,G) *Pparg* gene expression was determined from sorted CD103+DCs and CD11b+ DC from both genotypes. *p value <0.05 (n=3–4 mice/genotype; 2 experimental replicates).

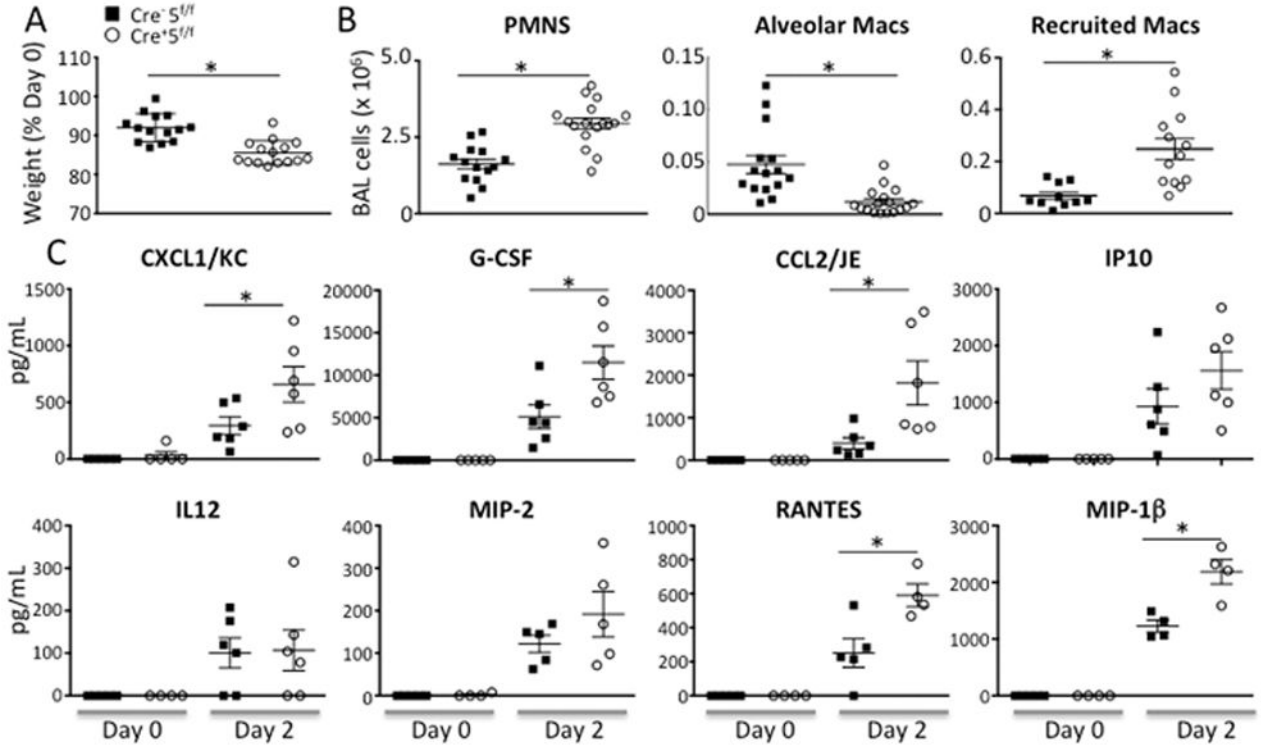

Figure 5. Acute inflammatory responses are increased in Stat5-deficient mice

$Cre^{-}5^{f/f}$ and $Cre^{+}5^{f/f}$ mice received LPS via oropharygeal aspiration, and the inflammatory cells and cytokines in the alveolar compartment were quantified at day 2. (A) There was greater weight loss in LPS-treated $Cre^{+}5^{f/f}$. (B) Number of recruited neutrophils and $CD11b^{high}CD11c^{low}SigF^{low}$ macrophages (recruited Macs) were increased in LPS-treated $Cre^{+}5^{f/f}$ mice compared to control. Resident alveolar macrophage remained reduced in $Cre^{+}5^{f/f}$ mice. (C) Several pro-inflammatory cytokines and chemokines were increased in LPS-treated $Cre^{+}5^{f/f}$ mice compared to LPS-treated control mice. *p value <0.05 (n=4–6 mice/genotype x 4 experimental replicates).

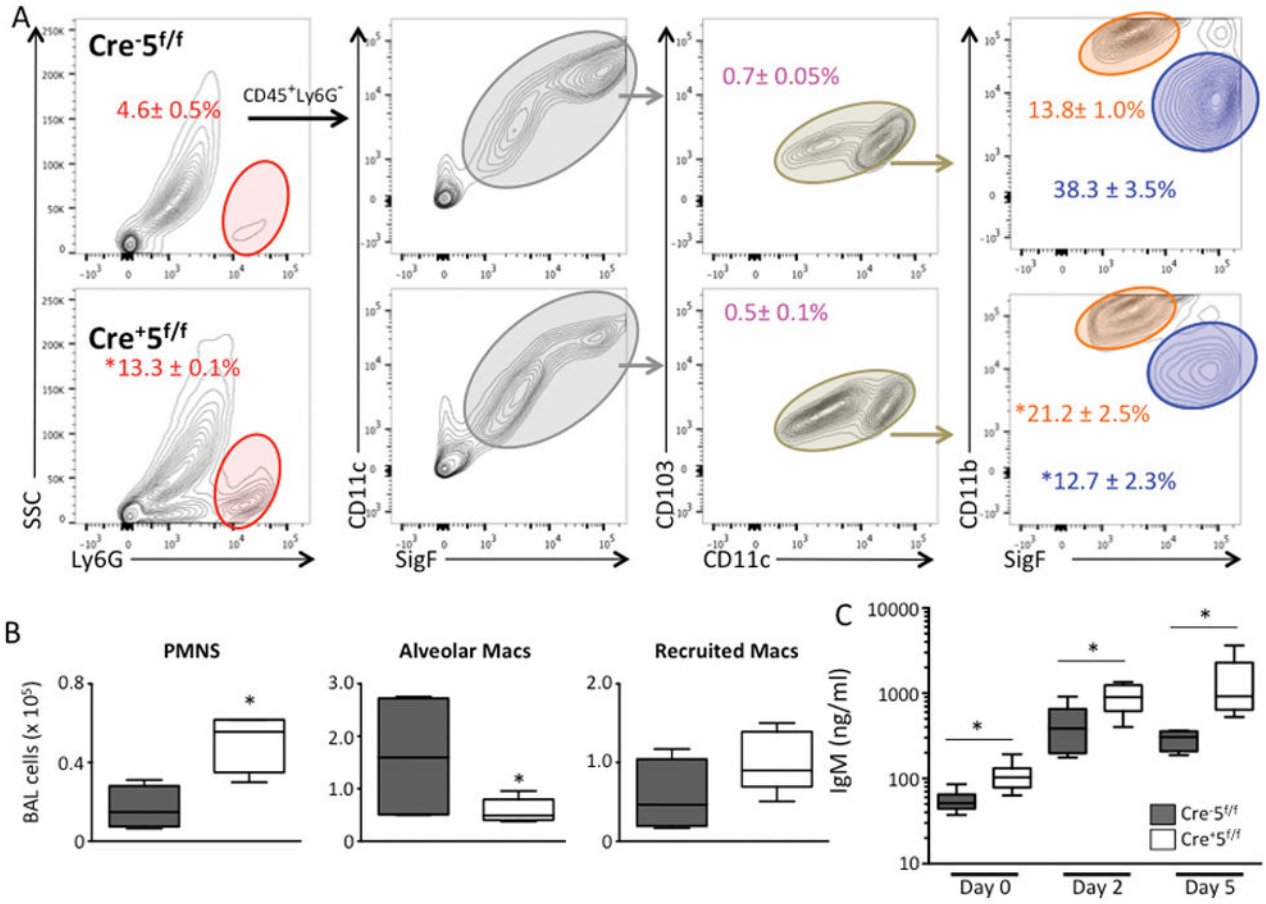

Figure 6. Late inflammatory responses and lung injury are increased in Stat5-deficient mice

$Cre^{-}5^{f/f}$ and $Cre^{+}5^{f/f}$ mice received LPS via oropharygeal aspiration, and at day 5, the inflammatory cells in the alveolar compartment were quantified and analyzed by FACS. (A) Gating strategy to identify leukocyte subpopulations in BAL. (B) Quantification of BAL leukocytes demonstrating an increase in BAL neutrophils and a reduction in alveolar macrophages in $Cre^{+}5^{f/f}$ mice compared to control $Cre^{-}5^{f/f}$ mice. (C) BAL IgM concentrations from BALF from day 0 (uninjured), and days 2 and 5 post-LPS demonstrating a significant increase in $Cre^{+}5^{f/f}$ mice compared to $Cre^{-}5^{f/f}$ mice at all time points. *p value <0.05 (n=4–6 mice/group; 3 experimental replicates).

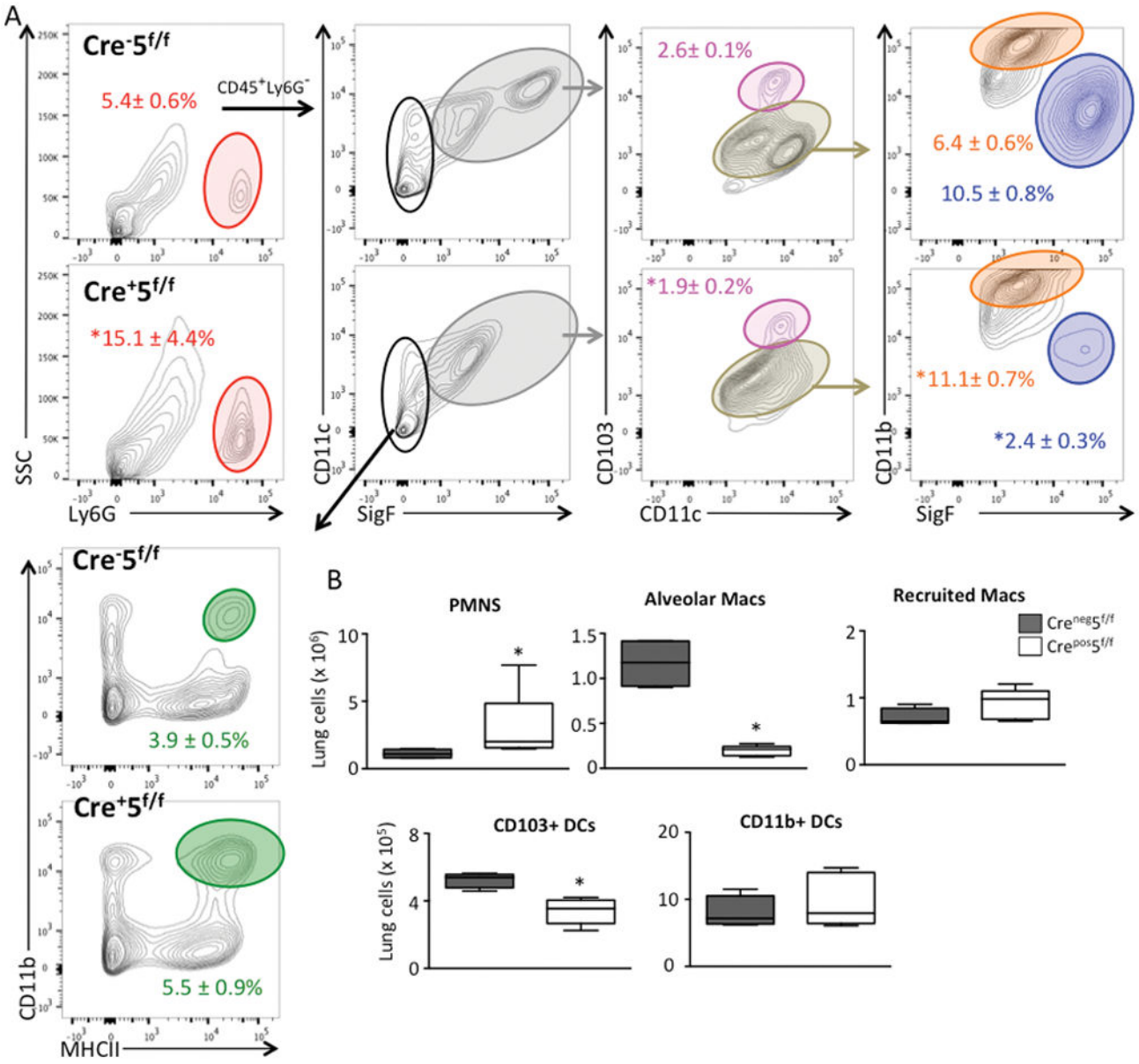

Figure 7. Recruitment of CD103+ DCs is diminished in Stat5-deficient mice

$Cre^{-}5^{f/f}$ and $Cre^{+}5^{f/f}$ mice received LPS via oropharygeal aspiration, and at day 5, the inflammatory cells in the lung compartment were quantified and analyzed by FACS. (A) Gating strategy to identify leukocyte subpopulations. (B) Quantification of lung leukocytes demonstrating an increase in neutrophils, intact recruitment of $CD11b^{+}$ macrophages, a reduction in alveolar macrophages and CD103+ DCs in $Cre^{+}5^{f/f}$ mice compared to control $Cre^{-}5^{f/f}$ mice.

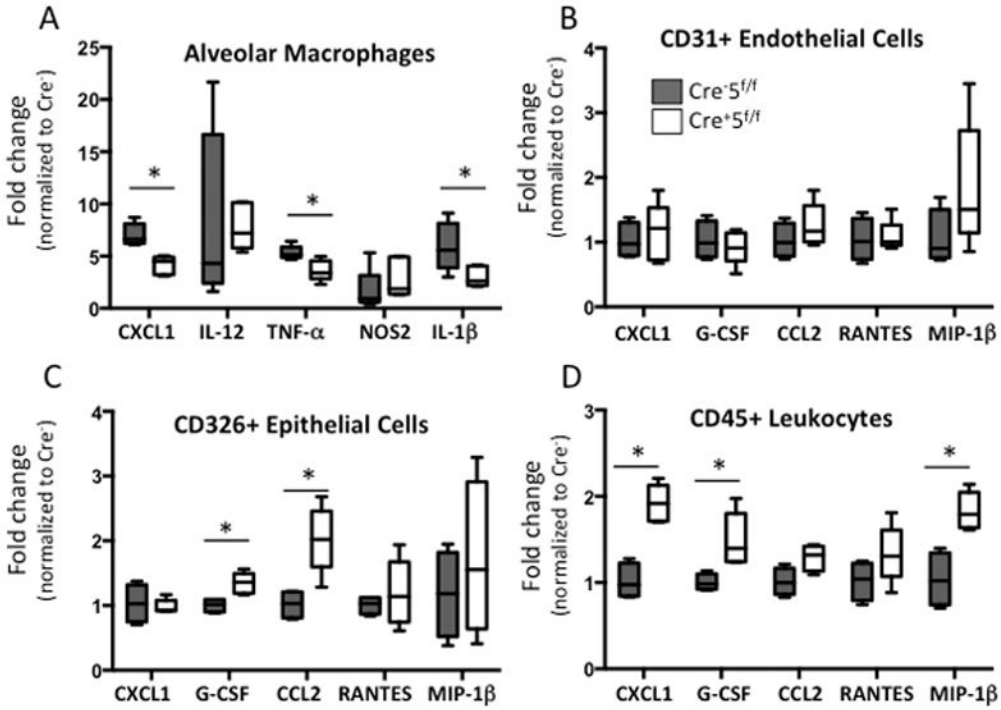

Figure 8. Inflammatory cytokine expression in cellular populations reveals immune-dampening roles of Stat5-expressing cells

(A) Alveolar macrophages from uninjured $Cre^{-}5^{f/f}$ and $Cre^{+}5^{f/f}$ mice were isolated and stimulated with LPS *in vitro*. Gene expression for *Cxcl1, Il12, Tnfa, Nos2,* and *Il1b* was measured using qRT-PCR and shown as fold change over untreated $Cre^{-}5^{f/f}$ cells. There was a significant blunting of *Cxcl1, Tnfa, and Il1b* expression in alveolar macrophages from $Cre^{+}5^{f/f}$ mice. *p value <0.05 (n=4–6 mice/group; 2 experimental replicates). (B–D) CD31+ endothelial, CD326+ epithelial cells, and CD45+ leukocytes from lung digests were isolated by magnetic bead selection and FACS sorting 24 h post LPS treatment. Gene expression for *Cxcl1, Gcsf, Ccl2, Rantes, Mip1b* were determined and expressed as fold change relative to $Cre^{-}5^{f/f}$ cells. (B) Endothelial cell inflammatory responses were similar between genotypes. (C) Epithelial cells from $Cre^{+}5^{f/f}$ mice had significantly greater *Gcsf* and *Ccl2* expression, and (D) CD45+ leukocytes from $Cre^{+}5^{f/f}$ mice expressed higher levels of *Cxcl1, Gcsf*, and *Mip1b*. *p value <0.05 (n=5–6 mice/group).