Title: **Lung Megakaryocytes are Immune Modulatory Cells that Present Antigen to CD4$^+$ T cells**

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Abstract

While platelets are the cellular mediators of thrombosis, platelets are also immune cells. Platelets interact both directly and indirectly with other cells, impacting immune cell activation and differentiation, as well as all phases of the immune response. Megakaryocytes (Mks) are the cell source of circulating platelets and until recently Mks were typically only considered as bone marrow (BM) resident cells. However, platelet producing Mks also reside in the lung, and lung Mks express increased levels of immune molecules compared to BM Mks. We therefore sought to define the immune functions of lung Mks. Using single cell RNA-Seq of BM and lung myeloid enriched cells, we found that lung Mks (Mk_L) have gene expression patterns that are similar to antigen presenting cells (APC). This was confirmed using imaging and conventional flow cytometry. The immune phenotype of Mks is plastic and driven by the tissue immune environment as evidenced by BM Mks having a Mk_L like phenotype under the influence of pathogen receptor challenge and lung associated immune molecules such as IL-33. Our *in vitro* and *in vivo* assays demonstrated that Mk_L internalized and processed both antigenic proteins and bacterial pathogens. Furthermore, Mk_L induce $CD4^+$ T cell activation in a MHC II dependent manner both *in vitro* and *in vivo.* These data indicate that Mks in the lung have key immune regulatory roles dictated in part by the tissue environment.

Introduction

Platelets are commonly described as megakaryocyte (Mk) derived cellular mediators of thrombosis. Mk differentiation and platelet production have been extensively studied in the bone marrow (BM) environment, but recent research has expanded our prior limited view of both platelet origins and functions. Platelets are increasingly appreciated as having diverse inflammatory and immune regulatory roles (1), and despite studies going back to 1893 by Aschoff demonstrating that Mks reside in the lungs, lung Mks were only recently shown to be a significant source of circulating platelets (2-6). These novel emerging concepts of platelet functions and origins are rapidly re-shaping how we view platelets in both health and disease.

Upon activation, platelets either express or secrete abundant inflammatory and immune molecules that recruit and activate leukocytes, both at sites of platelet deposition and systemically (7-12). Platelets and platelet derived immune mediators contribute to the initiation or acceleration of inflammatory diseases such as atherosclerosis and asthma, as well as responses to bacterial and viral infection (13-15). The large number of circulating platelets, and the diversity of their inflammatory molecules, including platelet factor 4 (PF4), CCL5, CD154, and transforming growth factor beta (TGF-β), give platelets important and under-appreciated immune functions relevant in many disease contexts (2, 11, 16-18). We have shown that platelets initiate, accelerate, and regulate of all phases of the immune responses, including platelet induction of an acute phase response (19), regulation of monocyte trafficking and differentiation (17), induction and maintenance of T helper cell differentiation (20), and platelets processing and presentation of antigen to $CD8^+$ T cells (21). Others have also demonstrated that Mks can cross present antigen *in vitro* (22). These studies, and those by many other labs, have demonstrated a central role for platelets and Mks in both innate and acquired immune responses.

Recent studies demonstrated that intravascular lung Mks produce circulating platelets and also identified non-platelet producing, sessile Mks in lung interstitial tissue (2). Bulk RNA-Seq data compared BM and lung Mks and found that lung Mks were enriched with mRNAs associated with immune regulatory functions, including mRNA for many immunoreceptors, chemokines, and cytokines (2). The role of Mks as immune regulatory cells is poorly understood in general, but particularly so with lung Mks. Lung Mks increase in number during pulmonary and cardiovascular diseases, further suggesting that lung Mks may be dynamic and responsive to inflammatory states (23).

Lungs and BM are very different tissue environments. Compared to the lung, the BM faces few pathogen challenges, is relatively hypoxic, and is an immune suppressive environment. In contrast, the lung has a microbiome, high O_2, and the lung tissue environment is primed to induce immune cell activation (24-26). Cells in the lung, such as airway epithelial cells (AEC), produce cytokines in response to pathogen or immune challenge that regulate immune cell differentiation, including the maturation of lung dendritic cells (27). Like all hematopoietic derived immune cells, Mks come from hematopoietic stem cells (HSCs) and develop under the influence of thrombopoietin (TPO). Because Mks have only been studied in the immune quiescent BM environment, it is not known whether Mks in other tissues have a different immune functional phenotype. Our data now demonstrate that lung and BM Mks have distinct immune phenotypes and functions; lung Mks secrete inflammatory cytokines and express molecules that are similar to antigen presenting cells (APCs), and lung Mks process live intact bacteria and present bacteria-derived antigen to $CD4^+$ T cells both *in vitro* and *in vivo*. Our *in vivo* data suggest that lung Mks have important roles in the early activation of T cell responses to pulmonary pathogen challenge, identifying a novel immune regulatory role for lung Mks.

Results

To verify the presence and distribution of Mks in the lung, immunohistochemistry (IHC) with anti-CD42c antibody was performed on lung tissue isolated from wild-type (WT) and Mk deficient thrombopoietin receptor knockout ($TPOR^{-/-}$)(28) mice as a negative control. Macaque monkey lungs were also immunostained with anti-CD41 antibody. IHC demonstrated that Mks were diffusely distributed throughout both WT mouse and primate lungs (Fig 1A). Unlike BM Mks, lung Mks resembled leukocytes and were smaller than BM Mks with a primarily 2N ploidy distribution (Fig 1B), potentially explaining why the presence of lung Mks has been largely overlooked. Using collagen digested lung tissue and flow cytometry to assess Mk and DC numbers, Mks were approximately 2-3% of the total lung cell population, versus lung DCs ($CD103^{+}$, $CD11b^{+}$), which were approximately 6-7% (Fig 1C). To gain a more complete analysis of gene expression differences between BM and lung Mks in relation to other hematopoietic cells in each tissue, we performed single cell RNA sequencing (scRNA-seq) and integrated cluster analysis on myeloid enriched BM and lung cell isolates. Mks identified by common Mk markers in the BM and lungs clustered as genetically distinct populations, with lung Mks clustering more closely to both BM and lung DCs (Fig 1D). Further gene expression analysis indicated that lung Mks expressed numerous genes typical of immune related molecules, including many associated with DCs (Fig 1E and Supplementary S1-S3). These data were validated in part using imaging flow cytometry to rule out the potential for analyzing myeloid cells with adherent platelets. Lung Mks, but not BM Mks, expressed MHC II and DC markers such as CD11c (Fig 2A and Supplementary S4). Mks were further phenotyped for APC-like markers including MHC II, CD80, CD40, ICAM-1, LFA-1, and CCR7 (Fig 2B and Supplementary S5), which were all expressed at higher levels on lung Mks relative to BM Mks. Additionally, macaque primate lung Mks had a similar APC-like, immune phenotype as mouse lung Mks (Fig 2C and Supplementary S6). Taken together, these data indicate that unlike BM Mks, lung Mks,

that we now term Mk_L, have an immune phenotype with several characteristics typical of an APC.

Lungs and BM are very different tissue environments; BM is sterile and hypoxic, while lungs face constant pathogen/antigen challenges and high O_2 exposure. Newborn post-natal day 0 (P0) mouse lungs have not had prolonged O_2 exposure or antigenic challenges and P0 lungs have an immature immune cell population (25). To begin to determine whether the tissue environment influences Mk_L differentiation, we compared P0 neonatal and adult mouse lung Mks. MHC II and ICAM1 were each expressed at much higher levels on adult compared P0 mouse Mk_L, suggesting that the Mk immune phenotype may be post-natal environmentally regulated (Fig 3A). To demonstrate the potential for Mk immune plasticity, BM Mks were isolated using a negative selection protocol (purity of isolation protocol confirmed by imaging flow cytometry, Supplementary S7), BM Mks were fluorescently labeled and then delivered via an oropharyngeal (OP) route to recipient mice (control mice given buffer only via OP route). Two days later transferred cells were recovered and assessed by flow cytometry. Transferred BM Mks acquired a Mk_L immune phenotype in the lung (Fig 3B) indicating that the lung environment may dictate Mk_L immune differentiation.

To explore a potential role for O_2 in Mk_L immune differentiation, isolated BM Mks were exposed to hypoxic or normoxic (5% or 21% O_2) conditions *in vitro* for 48 hrs. MHC II expression was not changed by incubation in hypoxic conditions indicating that O_2 is unlikely to regulate Mk immune differentiation (Supplementary S8). To investigate whether pathogen associated and/or cytokine mediated stimulation induced BM Mks to acquire a Mk_L-like phenotype, BM Mks were isolated and stimulated with pathogen receptor agonists or with interferon gamma (IFNγ). LPS, IFNγ, and CpG each increased BM Mk MHC II expression, further demonstrating BM Mk immune plasticity (Figure 3C, Supplementary S9). The cellular composition and cytokine milieu also differ between lungs and BM. Tissue-resident cells in the lung, such as airway epithelial cells (AEC),

secrete cytokines that promote DC maturation, including IL-33, IL-13, and TSLP (29). BM Mks incubated with IL-33, either alone or with the other lung DC maturation cytokines, increased MHC II and ICAM1 expression (Fig 3D). Together these data demonstrate that the Mk phenotype is 'plastic' and can be altered by immune stimuli.

To explore whether IL-33 regulates Mk_L immune differentiation *in vivo*, mice were treated with Mk-lineage depleting antibody or control IgG to deplete lung Mks (Supplementary Fig S10). 24 hours later mice were treated with ST2/IL-33R-Fc chimera protein (ST2-Fc) to block IL-33 during the Mk_L recovery. Recovered Mk_L were then assessed on d4 post-depletion and found to express higher levels of MHC II compared to control mouse Mk_L (Fig 3E). However, blocking IL-33 greatly reduced MHC II expression on the recovering Mk_L (Fig 3E). To further demonstrate a potential role for IL-33 in lung Mk_L immune differentiation, neonatal mice were treated with control IgG or ST2-Fc on P1 and P4, and Mk_L immune phenotype determined on day P7. Blocking IL-33 reduced Mk_L MHC II and ICAM1 (Fig 3F). Taken together, these data suggest an important role for IL-33 in Mk_L immune differentiation.

We next asked whether primary Mk_L and BM Mks differ in their responses to immune stimuli. To begin to address this question, we isolated Mk_L and BM Mks and Mks were stimulated overnight with LPS (10 ng/mL) or control buffer. As a control, BM derived dendritic cells (BMDC) were prepared and also treated with buffer or LPS. A cytokine membrane array was performed on the supernatants. Compared to BM Mks, Mk_L secreted more immune molecules that were similar to the molecules secreted by LPS challenged BMDC (Fig. 4A). These data were confirmed by measuring KC secretion which was found to be much higher in the supernatant of LPS stimulated Mk_L than BM Mks (Fig 4B).

For a cell to function as an APC it must internalize and process an antigen in order to present the antigen to $CD4^+$ T cells. To determine whether Mks process antigen, we cultured primary BM Mks and Mk_L with DQ-Ovalbumin (Ova, 200 µg/mL) and cell fluorescence was determined by flow cytometry 30 minutes later as a measure of Ova processing (DQ-Ova only

fluorescent following cleavage). BM and lung Mks similarly processed Ova antigen *in vitro* (Fig 4C). To determine whether Mk_L acquire and process antigen *in vivo,* PF4 reporter mice ($Pf4^{Cre}$-Rosa26-LSL-tdTomato mice) were challenged with LPS (0.5 mg/kg) then DQ-Ova was delivered via an oropharyngeal (OP) route and the lungs live imaged for 80 minutes. $PF4^+$ cells acquired and processed DQ-Ova (Fig 4D and online video). DQ-Ova was also noted in Mk_L 24 hrs after OP delivery (Fig 4E). To compare the ability of Mk_L and BM Mks to take up bacteria, isolated Mks were co-cultured with live GFP^+ *E. coli* for 30 minutes and imaging flow cytometry used to measure intracellular bacteria. While Mk_L and BM Mks both phagocytosed *E. coli*, Mk_L internalized more *E. coli* (Fig 4F). GFP^+ *E. coli* were also delivered to mice via an OP route and 3 hrs later the number of GFP^+ Mk_L and DCs were similar (Fig 4G). These data demonstrate that Mk_L are phagocytic and can acquire inhaled antigens and pathogens.

Because Mk_L cells expressed MHC II and acquired antigen, we next asked whether Mk_L present antigen to $CD4^+$ T cells *in vitro*. Isolated Mk_L and BMDCs were LPS stimulated, incubated with Ova and co-cultured with OTII T cells that recognize Ova presented in the context of MHC II for 3 days. Mk_L activated OTII T cells in an Ova dependent manner (Fig 5A). To begin to determine whether Mk_L present antigen *in vivo,* OTII T cells were transferred to WT and $TPOR^{-/-}$ mice ($TPOR^{-/-}$ mice have greatly reduced numbers of Mks and circulating platelets (28)). 24 hrs after OTII cells were infused, mice were given Ova expressing *E. coli* (*E. coli*^{Ova}) via an OP route. OTII cell activation in the lungs and mediastinal lymph nodes (mLN) was then determined on d3 by flow cytometry. Infected WT and $TPOR^{-/-}$ mice had similar post-infection weight loss, indicating similar infections (Supplementary S11), however, OTII T cells in the lungs and mLN of WT infected mice were more activated compared to those in $TPOR^{-/-}$ mice (Fig 5B and Supplementary S12). These data indicate that Mk_L may regulate $CD4^+$ T cell responses to lung infection.

Because the $TPOR^{-/-}$ mice also have greatly reduced platelet counts, we could not rule out a platelet mediated OTII response mechanism. Therefore, we next asked whether Mk_L can directly present antigen to $CD4^+$ T cells in a MHC II dependent manner. Mk_L were isolated from

WT and complete MHC $II^{-/-}$ mice and co-cultured with OTII T cells and Ova. Naïve OTII T cell activation was assessed 3 days later and found to be greatly reduced with MHC $II^{-/-}$ Mk_L co-incubation (Fig 5C). In separate experiment, OTII T cells were co-cultured with WT Mk_L, MHC $II^{-/-}$ Mk_L, or BMDC and Ova for 8d and IL-2 production determined by ELISA. IL-2 increased in WT, but not MHC $II^{-/-}$ Mk_L and BMDC co-cultures (Fig 5D). These data indicate that Mk_L activate $CD4^+$ T cells in a MHC II dependent manner.

To more specifically determine whether Mk_L mediated lung $CD4^+$ T cell responses are MHC II dependent, we made $PF4^{cre}$-MHC $II^{flox/flox}$ (Mk-MHC $II^{-/-}$) mice. WT and Mk-MHC $II^{-/-}$ mice had similar immune cell development and platelet activation (Supplementary S13-17), and Mk_L, but not DCs, had reduced MHC II expression (Supplementary S18). WT and Mk-MHC $II^{-/-}$ mice each received OTII T cell transfer and *E. coli*^{Ova} infection (Supplementary S19). On d3 post-infection, OTII T cells in WT mouse lungs had increased CD25 and more proliferation compared to Mk-MHC $II^{-/-}$ mice (Fig 5E). OTII cells in mLNs were similar in both WT and Mk-MHC $II^{-/-}$ mice (Supplementary S20), demonstrating that $CD4^+$ T cell activation in the lung is at least in part Mk MHC II dependent.

Discussion

Our data indicate that Mk_L have novel and prior unknown immune regulatory roles, including *in vitro* and *in vivo* APC-like functions. Our scRNA-seq data indicated that Mk_L and BM Mks are genetically distinct populations with Mk_L expressing a number of key genes in common with DCs. We have also demonstrated that Mk_L immune phenotype is at least in part shaped by their tissue environment, as the lung is continuously exposed to pathogens and lung cells secrete cytokines, such as IL-33, that contribute to Mk_L immune differentiation. We demonstrate the ability of Mk_L to mediate host responses to pathogenic challenge suggesting that Mk_L and BM Mks have distinct immune functions and that Mk_L may have roles in pulmonary immune responses, including in common diseases such as asthma and allergy, lung infections, and pulmonary hypertension, providing potential new avenues for future therapeutic development.

The developmental origin of lung Mks is not yet clear. There are HSCs in the lung and lung Mks can re-populate the BM and vice versa (2). When combined with our current data, this suggests that similar to other immune cells, the tissue environment, rather than an inherent genetic program during site specific development, has a major effect on their immune phenotype. However, at this point it is unclear whether lung Mks are primarily derived from lung HSCs or are seeded from a bone marrow source, and whether the limited ploidy of lung Mks is directly associated with their immune differentiation. MHC II is expressed on burst-colony-forming-unit Mk progenitors, but its expression is lost with Mk maturation typified by increased Mk ploidy (30). Without the maturation stimuli found in the BM, lung Mks may be 'less mature' and retain the expression of MHC II.

Our studies may lead to a better understanding of the pathogenesis of lung diseases ranging from infectious pneumonias, to asthma, and pulmonary hypertension. While DCs clearly have a major role in $CD4^+$ T cell responses, therapies solely aimed at DCs may be missing other relevant immune activating cells, including Mks, as our data indicates that a reduction in Mk MHC II in the lung results in decreased $CD4^+$ T cell activation in an antigen specific manner. $TPOR^{-/-}$ mice

deficient in Mks and platelets had only limited $CD4^+$ T cell activation. This implies that lung Mks or circulating platelets support $CD4^+$ T cell responses to lung pathogens by mechanisms beyond directly presenting antigen. This likely includes the secretion of lung Mk cytokines or chemokines that help to drive immune responses and development beyond direct antigen presentation. Although we have focused on this intriguing T cell activating function for lung Mks, they are likely to have important roles in all phases of lung immune responses. Mk_L may also be important in immune homeostasis, as we have shown that platelets maintain both basal T helper cell and monocyte immune phenotypes. Mk_L may therefore have key roles in lung immune quiescence in healthy conditions that remain to be discerned.

These studies demonstrate an important immune function for Mks in the lung and represent a very novel concept - Mk_L can internalize, process, and present antigen to $CD4^+$ T cells. While more work needs to be done to determine the disease relevance of these studies and the ontogeny of Mk_L, these data lay the foundation for better understanding Mk_L functions and origins.

Methods

Mice. All experiments were performed using institutional animal care and use committee (IACUC) approved protocols. All mice used in our studies were on a C57BL/6J background. Mice that were not bred in-house were purchased from Jackson Labs. All mice used were 8 to 12 weeks of age and a mix of males and females in our *in vivo* experiments. I-AB-flox mice (stock number 003584) and PF4-cre mice were purchased from Jackson Labs. Thrombopoietin receptor knockout ($TPOR^{-/-}$) were used in prior studies by our laboratory(17, 20).

Single cell suspension for cell culture, ImageStream flow cytometry, flow cytometry, and scRNA-seq. Whole lungs were removed and put into complete DMEM with 1 mg/mL of collagenase type II (Fisher Scientific; Catalog #NC9693955). Complete DMEM consisted of 10% FBS (Invitrogen), Penicillin/streptomycin (Invitrogen; Cat #15-140-122), vitamins (Invitrogen; Cat #11120052), Glutamax (Invitrogen; Cat #10566016), non-essential amino acids (Invitrogen; Cat #11140050), and sodium pyruvate (Invitrogen; Cat #11-360-070). Lungs were incubated at 37˚C for 30 minutes and then mashed through a 100 µm cell strainer (Thermo Fisher Scientific; Cat #08-771-19). ACK Lysing Buffer (Thermo Fisher Scientific; Cat #A1049201) was added to the single cell suspension and this mixture was spun down at 1,200 RPM for 5 minutes. The ACK was washed out with 50 mL of isolation buffer. Isolation buffer consisted of 1 mM EDTA and 2.5% FBS in PBS.

For Mk isolation, a negative selection protocol was used that included biotinylated anti-CD11b (BioLegend; Cat #NC0200884), B220 (BioLegend; Cat #NC0200885), CD3ε (BioLegend; Cat #100304), and CD146 (BioLegend; Cat #134716) antibodies and incubating in a 5 mL polystyrene tube (Laboratory Product Sales; Cat #L285601) with 50 µL/mL of rat serum. All antibodies were 5 µg/mL final concentration and streptavidin beads (STEMCELL Technologies; Cat #19860) at 75 µL/mL of sample (under 1×10^9 cells) concentration added. Each sample was

then added to a magnet and incubated for 3-5 minutes and non-bound cells transferred to a 15 mL tube with pre-warmed complete DMEM.

Cells from tibias and femurs were isolated by flushing the bone marrow with isolation buffer using a 20-gauge needle (BD Biosciences, Cat# 14-826D). If Mks were used for experimentation, the same procedure as the lungs were used for the BM. For bone marrow derived dendritic cells we followed the protocol provided by Abcam (https://www.abcam.com/protocols/bmdc-isolation-protocol).

Flow cytometry and ImageStream reagents. Anti- CD41 (MWReg30, BioLegend), MHC II (M5/114.15.2, BioLegend), CD54 (YN1/1.7.4, BioLegend), CD69 (H1.2F3, BioLegend), Brilliant Stain Buffer (BD Biosciences, Cat #BDB563794), CD4 (GK1.5, BioLegend), CD205 (NLDC-145, BioLegend), CD207 (4C7, BioLegend), CD8α (53-6.7, BioLegend), CD326 (G8.8, BioLegend), CD103 (2E7, BioLegend), CD105 (SN6h, BioLegend), CD252 (RM134L, BioLegend), Ly-6C (HK1.4, BioLegend), CD62P (RMP-1, BioLegend), CD3 (17A2, BioLegend), CD86 (GL-1, BioLegend), CD19 (MB19-1, BD Bioscience), CD19 (1D3/CD19, BioLegend), CCR7 (4B12, BioLegend), Cell Activation Cocktail (without Brefeldin A) (BioLegend, Cat #50-712-273), CD25 (3C7, BioLegend), OneComp eBeads (Fisher Scientific, Ca t# 50-112-9031), CD11b (M1/70, BioLegend), TER-119 (TER-119, BioLegend), Corning™ Falcon™ Round-Bottom Polystyrene Tubes (Corning, Cat #352054), H-2Db (KH95, BioLegend), DRAQ5™ Fluorescent Probe Solution (Thermo Fisher Scientific, Cat #PI62251), CD45 (PI62251, BioLegend), IFN-γ (XMG1.2, BioLegend), and Leukocyte Activation Cocktail, with BD GolgiPlug™ (Fisher Scientific, Cat #BDB550583).

Single cell RNA-seq. Cell suspensions were loaded on a Chromium Single-Cell Instrument (10x Genomics, Pleasanton, CA, USA) to generate single-cell Gel Bead-in-Emulsions (GEMs).

Libraries were prepared using Chromium Single-Cell 3′ Library & Gel Bead Kit (10x Genomics). The beads were dissolved and cells were lysed per manufacturer's recommendations. GEM reverse transcription (GEM-RT) was performed to produce a barcoded, full-length cDNA from poly-adenylated mRNA. After incubation, GEMs were broken and the pooled post-GEM-RT reaction mixtures were recovered and cDNA was purified with silane magnetic beads (DynaBeads MyOne Silane Beads, PN37002D, ThermoFisher Scientific). The entire purified post GEM-RT product was amplified by PCR. This amplification reaction generated sufficient material to construct a 3' gene expression library. For 3' gene expression library, enzymatic fragmentation and size selection was used to optimize the cDNA amplicon size and indexed sequencing libraries were constructed by End Repair, A-tailing, Adaptor Ligation, and PCR. Final libraries contain the P5 and P7 priming sites used in Illumina bridge amplification. Paired-end reads were generated for each sample using Illumina's NextSeq550v2.

Single cell RNA-seq analysis. Raw sequencing was processed and aligned to the *Mus musculus* genome assembly (mm10) using Cell Ranger software (v3, 10x Genomics). Subsequent quality control and secondary analysis steps were carried out using Seurat and cells with high mitochondria content (10% of total reads) were removed. Cells with very high RNA or gene content (doublets) were also excluded from downstream analysis. Technical variations such as sequencing depth, proportion of mitochondrial transcripts, differences in cell cycle states (dividing versus non-dividing) were regressed out during data normalization and scaling. For each sample, cells with similar transcriptomic profiles were grouped into specific clusters by a shared nearest neighbor (SNN) modularity optimization-based clustering algorithm. We assigned cell type identities to clusters of interest based on canonical markers.

Sample from all three captures (one from bone marrow, two from lungs) were integrated to investigate shared cell states across multiple data sets. Differentially expressed genes for identity classes were identified using Wilcoxon rank sum test (Seurat FindMarkers default). Markers that are specific to each identity were then submitted to enrichR(31, 32) for gene ontology analysis.

Lung Intravital Imaging. Lung imaging was done after oropharyngeal (OP) challenge with LPS and intratracheal dosing with DQ-Ova. Mice were Pf4-Cre Rosa26-LSL-tdTomato. Evans blue dye was used as a blood plasma label. Imaging started around 30 mins after DQ-OVA delivery and continued for approximately 80 mins with each time frame taking around 95 secs to cycle. Lung imaging used the set-up procedure two-photon microscopy techniques, and image analysis are previously described(2).

Immunohistochemistry. Lungs were fixed in 60% methanol, 10% acetic acid, 30% H_2O solution and 5 μM unstained sections produced by standard methodology. Slides were stained with anti-CD42c (Emfret Analytics, Cat# M050-0) at a concentration of 1:500 and were counterstained after secondary staining with a mouse anti-rat antibody. Macaque lungs were stained with anti-CD41 (Abcam, Cat# ab63983; DAB brown chromagen) at a concentration of 1:50 on the Leica Bond RX auto-staining machine.

Blood collection, Complete Blood Counts (CBCs), Platelet activation, and Plasma Isolation. Complete blood counts (CBC) were performed using an Abaxis VetScan HM5 on mouse blood collected in EDTA tubes (VWR, Cat# 95057-293) from a retro-orbital bleed. Plasma was isolated from blood spun at 3,000 RPMs for 10 minutes. Plasma not used immediately was stored at -20˚C. Platelets were activated as previously described(17).

ELISA. ELISAs were performed following manufacturer's instructions. ELISAs included: OVAL High Sensitivity ELISA Kit (Chicken) (Aviva Systems Biology, Cat#: OKCD01353), Mouse TNF-alpha DuoSet ELISA (Fisher Scientific, Cat#: DY41005), Mouse IL-6 DuoSet ELISA (Fisher Scientific, Cat#: DY40605), Mouse IL-4 DuoSet ELISA (Fisher Scientific, Cat#: DY40405), Mouse IL-2 DuoSet ELISA (Fisher Scientific, Cat#: DY40205), Mouse IL-10 Quantikine ELISA Kit (Fisher Scientific, Cat#: M1000B), and Mouse IFNγ DuoSet ELISA (Fisher Scientific, Cat#: DY485-05). The Proteome Profiler Mouse Cytokine Array Kit, Panel A (Fisher Scientific, Cat#: ARY006) was used to compare BM and Lung Mk cytokine expression. The methods used for the Cytokine Array were provided by the manufacturer. Lippopolysaccaride (LPS) (Sigma-Aldrich, Cat#: L6529-1mg) was used as a stimulus in many of the Mk activation experiments.

Oropharyngeal Treatment. Mice were anesthetized with isoflurane and suspended using dental floss to allow oral access. The tongue was gently encouraged out of the mouth and held in place with a pipette tip to ensure the mouse was unable to swallow. 50 µL of control or treatment was delivered into the trachea via a standard lab pipette (20-200 µL). The mouse was held in position until the breathing of the mouse returned to normal.

Mk-lineage depletion and IL-33 depletion experiments. Mice were given 4 µg/g of α-CD42b or the appropriate IgG control during the first day of the experiment. On the following day the mice were given Fc-ST2 5 µg/mouse. On D4 the mice were sacrificed. For the IL-33 depletion experiments, pups were given either Fc-ST2 or IgG (200 ng/pup) subcutaneous injections within hours of birth and again on D4. On D7 the pups were sacrificed with 10 minutes of CO_2 and then decapitation. After sacrificing mice for each experiment flow cytometry was used for data analysis

BM Transfer Experiment. To determine whether the lung environment is one of the mechanisms behind the differences in the BM and lung Mks we wanted to see if BM Mks transferred into the lung would have an altered phenotype. BM Mks were isolated from naïve WT mice and were stained with CellTrace Violet (Thermo Fischer, Cat#: C34557) using the instructions from the manufacturer. Three million cells were transferred to naïve WT mice via OP route with either PBS or GFP *E. Coli.* The mice were sacrificed 2 days later for flow cytometry.

***E. coli* culture and CFU count.** Mice were given either *Escherichia coli*$^{GFP+}$ or *E. coli*$^{GFP+}$ (ATCC: The Global Bioresource Center, Cat# 25922GFP) expressing full length Ovalbumin via an oropharyngeal (OP) administration. The ovalbumin plasmid was obtained from AddGene (Cat#: 25099). Mice were given 5 x10^7 *E. coli*. GFP *E. coli*. Mouse weight was monitored daily.

CFU was determined using a plate count protocol. Frozen bacterial stocks were taken from the -80˚C freezer, after which a 100 µL tip was passed three times through the bacterial stock and then the whole tip was added to a flask with LB Broth (Thermo Fisher Scientific, Cat#: BP1427-500) and 100 mg/mL of ampicillin (Thermo Fisher Scientific, Cat#: BP1760-5). The cells were shaken at 37˚C and 200 RPMs for 19 hours. Dilutions of the bacteria were plated on LB nutrient agar plates and incubated overnight. At the end of the incubation time the plates that had below 30 colonies or above 300 colonies were discarded due to concerns about accuracy. Plates with colonies between those numbers were counted and the following equation was used to determine CFU/mL:

$$\frac{\text{number of colonies (CFUs)}}{\text{dilution X amount plated}} = \text{\# of bacteria/ml}$$

Data Analysis. ELISAs were quantified using the BMG Fluostar OPTIMA and MyAssays. Flow cytometers were an Accuri C6 and BD LSR II. All flow cytometry samples were analyzed by FlowJo Version 10.0.7 or Version 8.7. Imagestream data was analyzed using IDEAS.

Statistics. Statistics were performed using GraphPad Prism Version 7. Based on the experimental design either a *t*-test (2-tailed) or One-way ANOVA using Bonferroni's correction were used. A P-value less than 0.05 was considered significant. The value of the stars are indicated as *P = 0.01 to 0.05, **P = 0.001 to 0.01, ***P = 0.0001 to 0.001, and ****P < 0.0001. All data are shown as mean ± SEM.

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Figure Legends

Figure 1. Lung and BM Mks are phenotypically distinct. A) Mks are present in the lung. Lung sections from WT mice, TPOR$^{-/-}$ mice, and macaque primates were immunostained with anti-CD42c antibody. Mks were noted in WT mouse and primate lungs (representative images). B) BM Mk ploidy is greater than lung Mk. BM and lung Mk ploidy determined by flow cytometry. C) Integration of bone marrow and lung scRNA-seq data. BM Mks and lung Mks had distinct mRNA expression patterns. D) Venn diagram and dot plot from scRNA-seq analysis. Venn diagram demonstrates common and specific markers for Mks and DCs. Dot plot indicates the average expression and proportion of cells expressing genes of interest. (± SEM; *P<0.05, **0.01, ***0.001).

Figure 2. Lung and Mk immune molecule expression. A) ImageStream Flow Cytometry demonstrated that lung Mks expressed more CD11c and MHC II compared to BM Mks.
B) Comparison of mouse lung and BM Mk APC related molecule expression by flow cytometry. Lung Mks express more APC associated molecules. C) Comparison of primate lung and BM APC related molecule expression. Lung Mks express more APC associated molecules. (± SEM; *P<0.05, **0.01, ***0.001).

Figure 3. Lung Mk immune phenotype is environmentally regulated. A) MHC II and ICAM1 expression on Mks from P0 and adult mice. Neonatal Mks had reduced MHC II and ICAM1 compared to adult lung Mks. B) BM Mk phenotype plasticity *in vivo*. BM Mks were isolated, CFSE labeled and OP delivered to control mice. 2 days later transferred BM Mk MHC II and CCR7 were determined. BM Transferred BM Mks had increased immune molecule expression in the lung environment. C) BM Mks increased MHC II expression in response to immune stimuli. BM Mks were incubated with immune stimuli for 48 hrs and MHC II expression determined. D) Lung derived immune modulatory cytokines induced BM Mk immune differentiation. BM Mks were

incubated with IL-33 or IL-33 in combination with other common lung cytokines. 48 hrs later immune differentiation was determined. E) IL-33 promoted lung Mk immune differentiation *in vivo*. Mice were treated with Mk depleting antibody or control IgG. Mice were then treated with either Fc-ST2 as an IL-33 blocking agent or control IgG. Recovering Mks had increased MHC II that was greatly attenuated by IL-33 blocking. F) P0 mice were treated with IgG or Fc-ST2 and on P7 Mk_L immune phenotype determined. IL-33 blocking reduced post-natal Mk_L immune differentiation. (± SEM; *P<0.05, **0.01, ***0.001).

Figure 4. Lung Mks Process and Present Antigen. A-B) Lung Mks had greater LPS induced inflammatory molecule production compared to BM Mks. Mks were incubated for 24 hrs with control buffer or LPS. Inflammatory molecules were identified by a A) cytokine array and B) KC in the supernatant measured by ELISA. C) Antigen processing *in vitro*. Mks were incubated with DQ-Ova for 30 mins and fluorescence was determined by flow cytometry. D-E) MKs internalize antigen *in vivo*. D) Mice were treated with control buffer or DQ-Ova via an OP route. 80 mins later real-time *in vivo* lung imaging was performed (representative image). E) 24 hrs post DQ-Ova lungs were also isolated to quantify fluorescent Mks. Lung Mks internalized antigen. F) Lung Mks are more phagocytic than BM Mks. BM and lung Mks were incubated with control buffer or GFP-*E Coli* and 30 mins later fluorescence was determined by ImageStream (representative images). G) Lung Mks take up *E. coli in vivo. E*. *coli* was delivered via OP route and 3 hrs later *E. coli* positive lung Mks and DCs were quantified by flow cytometry. (± SEM; *P<0.05, **0.01, ***0.001)

Figure 5. Lung Mks Present Antigen to T Cells. A) Lung Mks activated OTII T cells *in vitro*. T cells were co-cultured with lung Mks or splenocytes and on d3 T cell activation was determined. B) Mice lacking Mks had reduced antigen specific T cell responses *in vivo*. WT and $TPOR^{-/-}$ mice were given OTII T cells and 24 hrs later mice were OP treated with *E. coli*^{OVA}. OTII T cell activation

was determined on d3. WT, but not TPOR$^{-/-}$ mice, had OTII T cell activation in the lungs and reduced OTII T cell activation in mLN. C-D) Lung Mks present antigen in the context of MHC II *in vitro*. WT and MHC II$^{-/-}$ lung Mks were incubated with OTII T cells and Ova/LPS. On d3 T cell activation was determined by flow cytometry. C) WT lung Mks induced more T cell activation than did MHC II$^{-/-}$ lung Mks on d3 and D) more IL-2 production on d8. E) Mk_L present antigen in the context of MHC II *in vivo*. WT and Mk specific MHC II$^{-/-}$ mice were given OTII T cells and Ova/LPS via an OP route. On d3 OTII T cell activation was determined by flow cytometry. WT mice had more CD25 positive OTII cells and OTII T cell proliferation compared to Mk-MHC II$^{-/-}$ mice. (± SEM; *P<0.05, **0.01, ***0.001)