Tissue Specific *Cish* Expression Supports Alveolar Macrophage Homeostatic Function

by

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Abstract

Macrophages play critical roles in defense against microbes and clearance of dead cells, but also perform tissue specific functions in homeostasis. Distinct gene expression signatures in macrophages isolated from varying tissues are largely determined by environmental signals. Specifically, the lung is highly susceptible to environmental changes, such as O_2 pressure and inhalation of particulate and microbes. Alveolar macrophages are shaped by the lung environment and have critical tissue-specific functions in initiating and resolving lung inflammation, and in maintaining lung structure via surfactant and lipid catabolism. While research speculates lung specific factors form alveolar macrophage phenotype and homeostatic function, the specific signals and regulators remain largely unknown. Therefore, we sought to explore lung specific cytokine signals and downstream signaling regulators that shape homeostatic functions of alveolar macrophages.

We found Cytokine Inducible SH2 Containing Protein (*Cish*), a SOCS family member known to regulate the JAK-STAT5 pathway, is basally expressed in a tissue-specific manner in alveolar macrophages. Further, we found that the STAT5 activating cytokine GM-CSF regulates *Cish* expression in alveolar macrophages and observed reduced alveolar macrophage *Cish* expression with GM-CSF blockade in the lung. *Cish* knockout mice exhibit enlarged "foamy" alveolar macrophages, impaired surfactant metabolism, and dysregulated response to GM-CSF, all hallmarks of pulmonary alveolar proteinosis. Thus, we show alveolar macrophage *Cish*

expression is directly linked to lung specific factors, namely GM-CSF, and influences surfactant homeostasis in the lung, a critical homeostatic function of alveolar macrophages.

The lung is an especially critical site of protection as it is a barrier site that is constantly exposed to inhaled particulate and microbes and possesses a fragile structure. Alveolar macrophages act as sentinels in the lung, protecting this sensitive tissue from challenge while maintaining proper homeostasis and structure. From a public health perspective, continuing to elucidate the specific mechanisms by which alveolar macrophages mediate lung homeostasis is essential to providing cutting edge health care and to continuing to develop therapeutic treatments that can provide cures instead of simply mitigating symptoms of pulmonary disease. Here, we highlight one of many yet to be uncovered regulators of lung homeostasis.

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Preface

This project would not have been possible without the thoughtful guidance of Dr. Rachel Gottschalk. I want to thank her especially for her masterful mentorship and clever ideas. In addition, I want to thank Neha Cheemalavagu for her help with the bulk of the RNAseq analysis, as well as thoughtful discussions regarding this project in general. I also want to thank Maya Cao for her help with bench work, careful attention to detail, and for being a sounding board for ideas. I feel deep gratitude to be part of a lab full of supportive and intellectual individuals who are always there to listen.

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1.0 Introduction

1.1 Macrophages in Health and Disease

1.1.1 Macrophages in innate immunity

Macrophages play critical roles as phagocytes in defense against microbes and clearance of dead cells. Elie Mechnikoff characterized macrophages in the early 1900s based on their unique phagocytic capabilities and proposed that macrophages actively surveilled and phagocytosed foreign material to protect their host[1]. Thus, the discovery of macrophages set the stage for innate immunology and early research focused heavily on the immune functions of macrophages.

Macrophages are an essential part of the innate immune system. Constantly surveilling tissue, macrophages are one of the first immune cells to see and respond to antigens via expression of a wide variety of innate immune receptors (Figure 1), which can recognize both endogenous and exogenous danger signals. These danger signals are detected through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), complement receptors, and other scavenger receptors. Upon ligation of these receptors, macrophages become activated and initiate a pro-inflammatory response, typically through release of pro-inflammatory factors such as TNF, iNOS, IL-12, and various chemokines. This initial pro-inflammatory phenotype, typically described as "classically activated" or M1, results in recruitment of other effector immune cells and ultimately generates an adaptive immune response. These soluble factors also increase vascular permeability and enhance antimicrobial activity of macrophages and other effector cells.

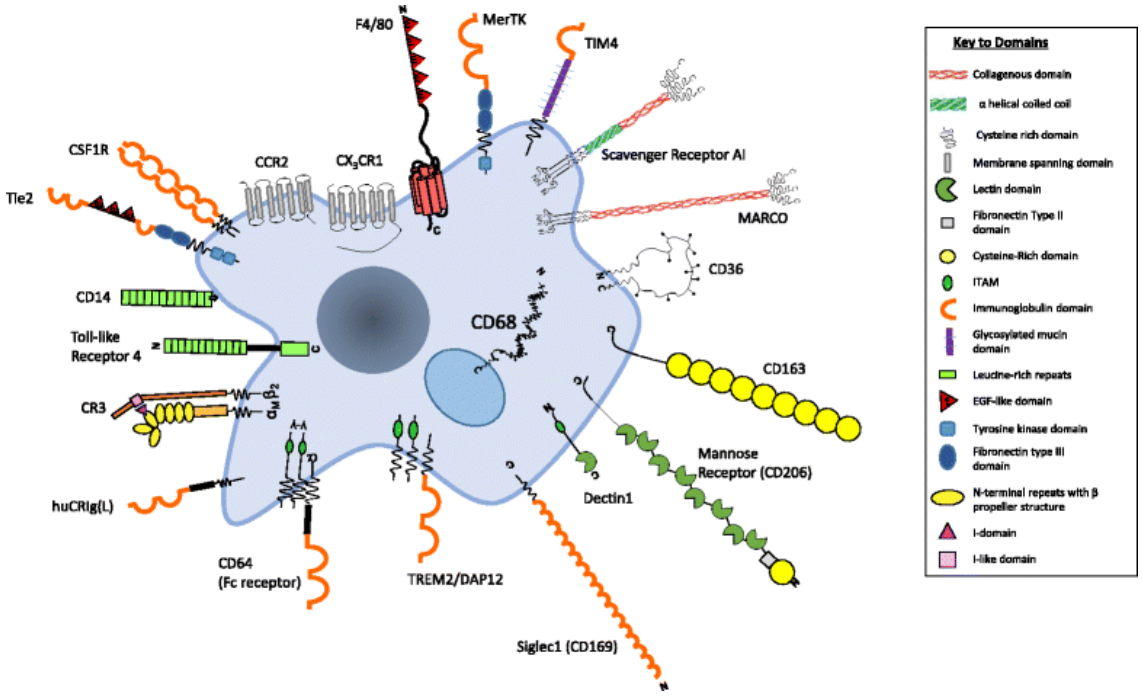

Figure 1. The diverse array of innate immune receptors expressed by macrophages.

Macrophages express a wide variety of receptors, many of which are PRRs, for sensing of microbial products or dead cells. Figure reproduced from BMC Biology[2].

While pro-inflammatory responses are critical for host defense, they can also result in tissue damage. After initial inflammation, macrophages adopt a tissue repair phenotype, typically described as M2. Classical wound healing macrophages are associated with responses to IL-4. Upon sensing IL-4, macrophages begin to upregulate anti-inflammatory factors as well as factors that promote tissue repair, such as arginase, which contributes to production of extracellular matrix.

While the binary M1/M2 classification is prevalent in the literature, it fails to account for the diverse plasticity of macrophages. For example, macrophages can take on regulatory function without contributing to tissue repair. Such regulatory macrophages produce high levels of IL-10, but lack IL-12 and arginase secretion and do not express CD80, CD86, and MHCII, so therefore do not fit neatly into the predominant M1/M2 paradigm. Thus, the field is moving towards

conceptualizing macrophage function as a spectrum between classically activated macrophages, wound-healing macrophages, and regulatory macrophages[3].

1.1.2 Tissue resident macrophages in health and disease

Macrophages' phenotypes are highly context dependent, with macrophages adopting functional states based on environmental signals. In addition to direct immune functions, macrophages are increasingly being recognized for their essential roles in tissue structure and homeostasis. In many tissues, macrophages do not only phagocytose foreign material, but also endogenous material[4]. As phagocytes, macrophages play a critical role in clearance of apoptotic cells and cellular debris from tissues. This tissue homeostasis is quiescent, either independent of immune receptors or regulated by inhibitory signals.

In addition to phagocytosis, tissue resident macrophages often perform synergistic functions specific to their tissue of residence. For example, in adipose tissue, macrophages regulate insulin sensitivity and adaptive thermogenesis[5]. Bone marrow macrophages support erythropoiesis and maintain hematopoietic stem cells in stem cell niches, while microglia play an essential role in neuronal remodeling by promoting neuronal survival and removing dead neurons[6, 7, 8]. Dysregulation of homeostasis highlights the important role of macrophages in maintaining tissue health and integrity and macrophages are implicated as key players in a number of inflammatory and autoimmune diseases. In the case of multiple sclerosis, dysregulation of communication between astrocytes and microglia is a driver of axon demyelination and resultant disease[9]. Macrophage hyperresponsiveness to gut microbiota drives the chronic inflammation that leads to Crohn's disease and inflammatory bowel disease[10, 11]. Inflammatory cytokine secretion by macrophages is often a major driver of disease progression, as evidenced in the case of macrophage

produced cytokines in the synovial fluid around joints that promotes rheumatoid arthritis[12]. These inflammatory diseases point to the importance of macrophages in tissue homeostasis, with specific emphasis on proper cross talk between macrophages and their tissue of residence.

Macrophages are extremely plastic and adapt their function and phenotype based on presence or absence of specific stimuli. The combination of input signals from these stimuli shape macrophages into an diverse range of phenotypes and drive a multitude of functions, resulting in heterogeneity across tissue resident macrophages, with well described differences in gene expression and protein expression signatures across tissue resident subsets[2, 13, 14, 15, 16]. The best described tissue specific transcription factor signatures include *Gata6* expression in peritoneal macrophages[17, 18], *Smad3* expression in microglia[19], *Lxra* expression in Kupffer cells[20] and splenic macrophages[21], and *Pparg* expression in alveolar macrophages[22, 23].

While tissue derived factors are thought to drive these distinct tissue resident macrophage signatures, only a few tissue specific factors have been well described as driving tissue specific signatures in macrophages. Retinoic acid present in the peritoneal omentum drives *Gata6* expression in peritoneal macrophages, which is essential for localization of peritoneal macrophages as well peritoneal macrophage regulation of gut IgA responses[17]. GM-CSF in the lung drives *Pparg* expression, which is critical for perinatal development of alveolar macrophages. GM-CSF deficiency results in "foamy" alveolar macrophages, which are unable to properly maintain surfactant homeostasis in the lung[23]. Splenic macrophages rely on oxysterol in the spleen, which drives expression of *Lxra*[21]. *Lxra* deficient splenic macrophages fail to fully mature, resulting in improper spleen structure and impaired pathogen clearance[20]. The strong TGF-β signal in the brain drives *Smad3* expression essential for proper microglia phenotype and function[24]. Loss of TGF-β signal results in a hyperinflammatory microglia phenotype which causes unnecessary

tissue damage in the brain[25, 26]. While these examples strongly support the notion of tissue specific factors driving unique tissue resident macrophage signatures, they only account for a few of the many tissue specific factors that potentially impact tissue resident macrophage signatures.

1.2 Alveolar Macrophages and Lung Homeostasis

1.2.1 Alveolar macrophages immune function

The lungs are a barrier site not only with a large surface area and fragile structure, but also undergo constant environmental changes, such as fluctuations in gas exchange, hypoxia, changes in the lung microflora, and inhalation of particulate and other substances[27]. Further, damage to the lungs leads to improper gas exchange, easy access of microbes to the bloodstream, or potential edema. Alveolar macrophages are one of the most abundant immune cells to occupy this unique tissue and must tolerate and adapt to these constant environmental changes, while conferring heavy protection with minimal tissue damage[28].

In homeostasis, alveolar macrophages clear dead cells, particulate, and microbes with minimal initiation of pro-inflammatory responses in order to prevent unnecessary damage to the alveolar epithelium. The lung environment is known to dampen alveolar macrophage pro-inflammatory responses through both cell-to-cell contact and soluble factors. Alveolar macrophages remain closely adhered to alveolar epithelial cells (AECs) through surface receptors and form gap junction channels with AECs, which transduce Ca^{2+} signals and allow direct communication between the alveolar epithelium and alveolar macrophages to modulate immune responses[29]. Alveolar macrophages also communicate with AECs through a number of surface

receptors such as CD200R, MARCO, TREM2, TGF-βR, and SIRPa, which serve immunomodulatory roles in order to prevent unwarranted airway inflammation[27].

Alveolar macrophages also directly contribute to the immunosuppressive environment in the steady state lung. Alveolar macrophages have been reported to secrete SOCS1 and SOCS3 in exosomes to suppress inflammation in other cells in the lung[30]. Close contact of alveolar macrophages with AECs induces activation of TGF-β, a major regulator of immune function in the steady state lung[31]. This localized TGF-β signal is one of many immunomodulatory factors present in the lung that allow alveolar macrophages to silently surveil the lung. Alveolar macrophages also actively suppress adaptive immune responses, regulating both alveolar dendritic cell function and T cell activation[32]. Depletion of alveolar macrophages leads to inflammatory reactions to otherwise harmless challenges, demonstrating their critical role in lung homeostasis[33].

Upon invasive infection, alveolar macrophages serve as one of the lung's first lines of defense. Alveolar macrophages must respond to invasive challenge in a way that eliminates challenges quickly and efficiently, while limiting inflammatory damage to the sensitive structure of the lung in order to maintain proper gas exchange in the tissue. Signals that override the homeostatic anti-inflammatory signals are required to initiate a pro-inflammatory response. Once an alveolar macrophage receives a large enough danger signal through PRR stimulation, alveolar macrophages rapidly lose contact with the alveolar epithelium, removing the brakes from a pro-inflammatory response. Loss of contact with AECs shuts down active TGF-β signaling and induces fluxes in Ca^{2+} signaling[29]. Crosstalk between recruited lymphocytes and alveolar macrophages results in resolution of inflammation through restoration of contact of alveolar macrophages with the alveolar epithelium as well as active TGF-β production (Figure 2). Thus, alveolar macrophages

play an essential role in limiting tissue damage by maintaining and restoring proper structure of the lung.

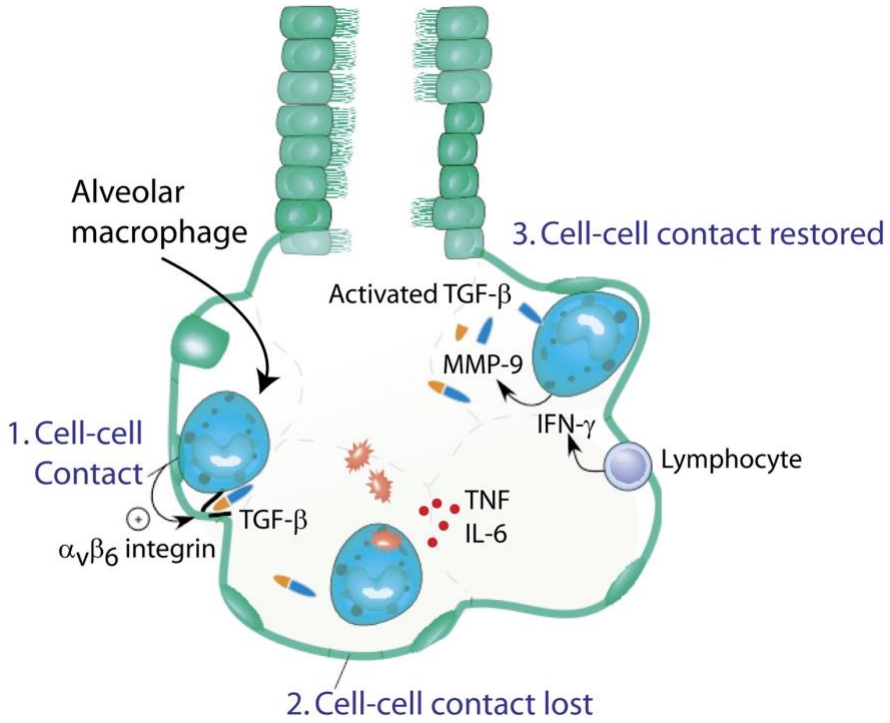

Figure 2. TGFb dependent contact of alveolar macrophages with alveolar epithelium.

Contact of alveolar macrophages with the alveolar epithelium plays an important role in response to challenge. Figure reproduced with permission from Immunity[34], copyright Elsevier Inc.

1.2.2 Lung specific stimuli drive alveolar macrophage gene expression and homeostasis

Alveolar macrophages are well known to be shaped by factors present in the lung environment. The best described lung specific signal essential for alveolar macrophage phenotype is GM-CSF. The alveolar epithelium is a major source of GM-CSF, which signals primarily through the STAT5 pathway[35, 36]. GM-CSF is been shown to be required for the differentiation of alveolar macrophages by driving expression of the transcription factor specific for alveolar macrophages, PPAR-γ, together with transcription factor PU.1[23]. Alveolar macrophages that fail to get a proper GM-CSF signal remain stuck in a pre-alveolar macrophage state, failing to

upregulate CD11c, proliferate properly, and remaining functionally deficient compared to fully matured alveolar macrophages.

Another tissue specific regulator of alveolar macrophage function is TGF-β. TGF-β is not only a potent anti-inflammatory signal maintained through communication between alveolar macrophages and the alveolar epithelium, but also critical for proper alveolar macrophage development. Similar to GM-CSF, TGF-β signal in the lung results in upregulates PPAR-γ, a transcription factor that is critical for alveolar macrophages. Alveolar macrophages that fail to receive a TGF-β signal exhibit reduced expression of PPAR-γ and remain stuck in an underdeveloped state, much like alveolar macrophages that fail to receive a proper GM-CSF signal[37].

GM-CSF and TGF-β are tissue specific factors that are critical for the unique phenotype of alveolar macrophages, but other lung specific factors are also likely involved. Culture of alveolar macrophage *ex vivo* results in "deprogramming" in which gene expression and regulatory signaling thresholds are altered due to loss of lung specific stimuli. Simply culturing "deprogrammed" macrophages in the presence of known tissue specific factors, such as tissue derived omentum, is not sufficient to retain a "programmed" macrophage signature[14].

Further, differentiated tissue resident macrophages can be "reprogrammed" simply by relocation to a different tissue context[16]. Specifically, peritoneal macrophages transferred into the alveolar cavity for 15 days upregulated alveolar macrophage-specific genes, including the critical alveolar macrophage transcription factor, *Pparg*, while downregulating peritoneal macrophage-specific genes, such as *Gata6*. In fact, approximately 70% of the highly differentially expressed genes in these transferred macrophages switched from a peritoneal macrophage expression signature to an alveolar macrophage expression signature. While transferred peritoneal

macrophages retained ~30% of their original tissue signature and failed to downregulate CD11b, PCA analysis confirmed these transferred macrophages more closely resembled alveolar macrophages than peritoneal macrophages, highlighting both the phenotypic plasticity of macrophages and the importance of tissue specific microenvironment in tuning specific macrophage signatures.

1.2.3 Lung surfactant homeostasis and pulmonary alveolar proteinosis

Alveolar macrophages are vital for surfactant homeostasis in the lung. Pulmonary surfactant is a mixture of phospholipids surfactant protein A, B, C, and D (SP-A, SP-B, SP-C, and SP-D) secreted by primarily by AECs[38]. Surfactant coats the alveoli, facilitating proper gas exchange by reducing surface tension and liquid interface to allow the lung tissue to expand and contract easily with breathing. Surfactant also plays a role in host defense through opsonization of microbes and particulate, as well as modulation of pulmonary immune cell function, causing upregulation of PRRs and regulating cytokine secretion[39 40, 41]. Further, surfactant has been shown to possess direct microbicidal activity against bacteria and fungi[42]. In healthy individuals, alveolar macrophages maintain proper surfactant levels through lipid catabolism. In cases where alveolar macrophage surfactant catabolism is dysregulated, individuals develop pulmonary alveolar proteinosis (PAP), in which surfactant builds up in the lung, causing difficulty breathing and pulmonary failure if left untreated[43, 44]. Alveolar macrophages mediate surfactant homeostasis in the lung and drive disease in a dysregulated state.

Considering that GM-CSF is a critical lung-specific regulator of alveolar macrophage development and function, it is not surprising that alveolar macrophage-mediated surfactant homeostasis is dependent on GM-CSF[43]. Loss of GM-CSF results in dysregulated surfactant

maintenance and development of primary PAP. Although PAP is a disease of heterogenous origins, over 90% of clinical cases of primary PAP result due to disruption of GM-CSF signaling in the lung[45]. This disruption of GM-CSF signal is typically due to either autoimmune antibodies that neutralize GM-CSF or genetic mutations that impair the GM-CSF receptor. Disruption of the GM-CSF signal results in dysregulation of surfactant metabolism in alveolar macrophages[43]. Alveolar macrophages develop a "foamy" phenotype in which deposits of surfactant accumulate intracellularly but fail to be properly catabolized (Figure 3). As PAP progresses, surfactant accumulates in the alveoli, continuing to be produced at the same levels by AECs, but failing to be properly cleared by alveolar macrophages[44].

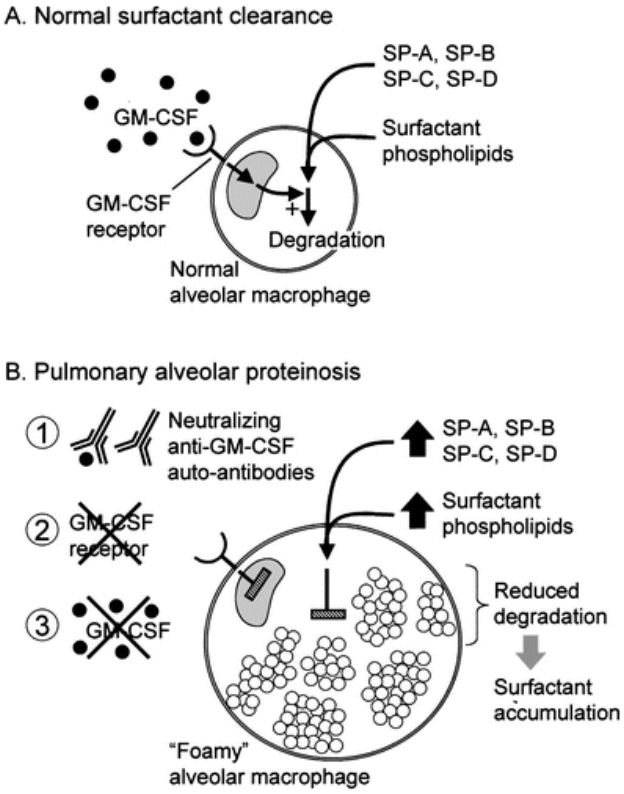

Figure 3. GM-CSF signaling in alveolar macrophages mediates surfactant clearance.

In a healthy lung, surfactant is taken up and degraded by alveolar macrophages, mediated by a GM-CSF signal. Loss of this GM-CSF signal results in reduced surfactant degradation by alveolar macrophages, and thus intracellular and extracellular accumulation of surfactant. Figure reproduced with permission from Annual Reviews of Physiology[43], copyright Annual Reviews.

The discovery that GM-CSF deficient mice accurately recapitulate PAP pathology was a breakthrough that facilitated today's understanding and treatment of PAP. Mice with deletions in either GM-CSF or the GM-CSF receptor both develop PAP, in which alveolar macrophages are severely impaired in their ability to mature and the few that do exhibit reduced functionality and a foamy phenotype[46, 47]. These GM-CSF deficient mice exhibit higher susceptibility to pulmonary infections as well as increased mortality from pulmonary infections, highlighting the importance of alveolar macrophage surfactant homeostasis in host defense[48, 49]. Simple replacement of GM-CSF in GM-CSF deficient mice restores alveolar macrophage phenotype and function, surfactant homeostasis, and susceptibility to infection[50]. Therefore, GM-CSF is the major clinical target in PAP patients, with many primary PAP therapies involving restoration of proper GM-CSF signaling in the lung.

GM-CSF serves as a prime example of a tissue specific factor exerting a critical on macrophage phenotype and function. Further, loss of this tissue specific factor results in impaired alveolar macrophage function, resulting in tissue dysregulation, and progressive pathology. Taken together, surfactant homeostasis mediated through alveolar macrophages via tissue-specific GM-CSF emphasizes the importance of understanding how macrophages communicate with their tissue of residence in order to maintain tissue homeostasis, host protection, and overall health.

1.2.4 *Cish* in regulation of signaling in alveolar macrophages

Cytokine Inducible SH2 Containing Protein (CISH), the first described member of the Suppressor of Cytokine Signaling (SOCS) protein family, is induced by GM-CSF and other JAK-STAT5 pathway activating cytokines[51, 52, 53, 54]. CISH binds to the phosphorylated tyrosine residues on cytokine receptors through its SH-2 domain, blocking sites for STAT5 to dock, and thus

inhibiting downstream cytokine signaling. In T cells, CISH also directly interacts with a principle TCR signaling component, PLCγ, targeting it for proteasomal degradation thus negatively regulating TCR signaling[55, 56]. PLCγ is directly involved in regulation of calcium flux after TCR stimulation, suggesting a potential role of *Cish* in calcium signaling.

Although the exact molecular mechanisms of regulatory properties of *Cish* are unclear, *Cish* is directly implicated as important for immune responses in cancer and infections. Analysis of *Cish* polymorphisms revealed that specific variants of *Cish* are associated with increased susceptibility to infections such as malaria, tuberculosis, and bacteremia[57]. Further, one specific SNP CISH variant is significantly associated with susceptibility to hepatitis B virus infection[58]. Although *Cish* is best described in T cells, emerging research shows *Cish* is an important regulator of immune responses in macrophages. Two recent publications show that *Cish* mediates immune response in bone marrow derived macrophages (BMDM) in *M. tuberculosis* infection *in vitro*. Studies found dysregulation of cytokine secretion and decreased bacterial burden in *Cish-/-* BMDM infected with *M. tuberculosis*, compared to WT BMDM. Macrophage expression of CISH was induced by STAT5 activation through the GM-CSF signaling in response to *M. tuberculosis* infection. *Cish* was shown to directly regulate endosome acidification, identifying a novel role of *Cish* in regulation of immune response[59]. Considering the previously unappreciated function of *Cish* in macrophages and its regulation by GM-CSF, additional research is needed to elucidate the tissue-specific function of *Cish* in alveolar macrophages.

2.0 Statement of the Project

In this project, we seek to investigate tissue specific signals that drive specialized functions in tissue resident macrophages. Specifically, we will examine the influence of cytokines present in the lung microenvironment on alveolar macrophage gene expression and function. We will test the hypothesis **that lung specific cytokines induce lung specific signaling genes that regulate specialized functions in alveolar macrophages.**

2.1 Aim 1: Investigate Tissue Specific Regulation of *Cish* in Alveolar Macrophages

Tissue resident macrophages have distinct gene expression signatures corresponding to tissue specific functions. We describe high basal *Cish* expression specifically in alveolar macrophages, compared to other macrophage subsets, and elucidate lung specific factors that regulate alveolar macrophage *Cish* expression.

2.2 Aim 2: Investigate Tissue Specific Function of *Cish* in Alveolar Macrophages

Considering the high level of *Cish* expression in alveolar macrophages, we hypothesize that *Cish* plays role in lung homeostasis. We utilize a *Cish* deficient mouse model to examine *Cish*-dependent changes in alveolar macrophage phenotype and function, and in overall lung homeostasis.

3.0 Materials and Methods

3.1 Mice

Wild type and *Cish* knockout mice, generously provided by Dr. Nicholas Restifo[56], were all from a wild-type C57BL/6J background (Jackson Laboratories). Mice were maintained in specific-pathogen-free conditions and all procedures were approved by the Institutional Animal Care Committee of the University of Pittsburgh (IACUC).

3.2 Cells

3.2.1 Mouse bone marrow derived macrophages (BMDM)

Bone marrow progenitors isolated from mice were differentiated into BMDM during a 6 day culture in complete Dulbecco's modified Eagle's medium (DMEM + 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-Glutamine, 20 mM HEPES) supplemented with 60 ng/ml recombinant mouse M-CSF (R & D systems). One day prior to stimulation, cells were rinsed with cold PBS, then scraped from plates using a cell lifter. Cells were then plated in the appropriate tissue-culture-treated plate in complete DMEM and allowed to rest overnight at 37°C, 5% CO_2, 95% relative humidity prior to stimulation.

3.2.2 BMDM stimulation

BMDM were plated at 1 x 10^4 cells/well on 96-well black imaging plates (Greiner Bio-One) for immunofluorescent phagocytosis assays and 2 x 10^5 cells/well on 48-well plates (Greiner Bio-One) for GM-CSF stimulation. For immunofluorescent phagocytosis assays, BMDM were stimulated with (concentration) fluorescent S. aureus bioparticles (Invitrogen). Following stimulation, cells were fixed and stained for immunofluorescent imaging analysis. For GM-CSF stimulation, BMDM were stimulated with 20 ng/mL of GM-CSF (Biolegend). Following stimulation, cells were lysed in TRIzol Reagent (Ambion) and RNA was isolated for gene expression analysis.

3.2.3 Alveolar macrophage harvest and sorting

Lungs were harvested into gentleMACS C tubes (Miltenyi Biotec) containing lung digestion buffer (1X PBS, 0.5% FBS, 10µg/mL Liberase TM, and 10U/mL DNase I). Lungs were minced to 1mm^3 size pieces and moved to a shaker for 30 minutes at 37° C. Lungs were then further dissociated using program "m_lung_02_01" on the gentleMACS Dissociator (Miltenyi Biotec). Lung cell homogenates were filtered through a 70 µm filter and red blood cells were lysed with ACK Lysing Buffer (Lonza). Cells were stained with CD11c-BV421 (Biolegend) and Siglec-F-PE (BD Biosciences). CD11c+, Siglec-F+ cells were sorted directly into TRIzol Reagent (Ambion) using the BD FACSAria II (BD Biosciences).

3.2.4 BAL alveolar macrophage stimulation

Alveolar macrophages were harvested by bronchoalveolar lavage (BAL). Red blood cells were lysed using ACK Lysing Buffer (Lonza) and cells were resuspended in complete DMEM and plated at approximately 1 x 10_4 cells per well (200 µL/well) in a 96 well plate. After resting cells for 30 minutes, cells were stimulated with 20 ng/mL of recombinant mouse GM-CSF (Biolegend).

3.3 Broncholaveolar Lavage

3.3.1 Bronchoalveolar lavage harvest

Lungs were lavaged with 1 mL phosphate buffered saline (PBS) containing 5% FBS (Corning) and 100uM EDTA (Teknova). BAL cells were then spun down, supernatant was saved, and BAL cells were used for further experimentation.

3.3.2 SP-D ELISA

BAL supernatant SP-D concentration was assessed using the Mouse SP-D ELISA Kit (Abcam), according to manufacturer's directions. Plates were read using the Epoch Microplate Spectrophotometer (BioTek Instruments).

3.4 Gene Expression

3.4.1 qPCR

Samples were lysed in TRIzol Reagent (Ambion). RNA was isolated used Directzol RNA MicroPrep kit (Zymo Research), according to manufacturer's directions. RNA was quantified using a Nanodrop. RNA was reverse transcribed to using cDNA was generated using qScript cDNA SuperaMix (Quantabio). qPCR was performed using TaqMan probes (Thermo Scientific) on the CFX96 Real-Time System (BioRad). Analyses were performed using the ΔΔCt method comparing genes of interest to a *Gapdh* control.

3.4.2 RNAseq

Alignment and raw read counts were generated using Rsubread and then normalized using DESeq2. DESeq2 was then used to calculate the log fold changes between sample groups. Low expression genes were removed along with genes that were not differentially expressed (adjusted p-value ≤ 0.05) in at least one group. Remaining DESeq2 normalized reads were then z-score normalized. Genes were then hierarchically clustered, using complete linkage and correlation as a distance metric. Pathway specific heat maps were generated using gene sets from PathCards (Weizmann Institute of Science).

3.5 Imaging

3.5.1 Immunofluorescent staining and imaging

After stimulation, cells were fixed with 4% PFA for 10-15 minutes. Cells were then blocked with Blocking Buffer (1X PBS with 5%goat serum, 0.3% Triton™ X-100). After blocking, cells were stained with primary antibody diluted in antibody dilution buffer (1X PBS, 1% BSA, 0.3% Triton X-100) overnight at 4° C. BMDM were stained with the primary antibodies CD11b, followed by secondary antibodies: alveolar macrophages were stained with the following primary antibodies: hamster anti-mouse CD11c (1:200, Biolegend) and rabbit anti-mouse pSTAT5 (1:200, Cell Signaling Technology), followed by secondary antibodies: anti-hamster Cy3 (1:500, Jackson ImmunoResearch) and anti-rabbit AlexFluor 488 (1:500, Invitrogen). All cells were stained with Hoescht 3342 (ThermoFisher Scientific). After staining cells were washed extensively with PBS and imaged using the Cellnsight CX5 High Content Screening Platform (ThermoFisher Scientific).

3.5.2 Oil Red O staining and imaging

BAL alveolar macrophages were plated on a 96 well black imaging plate and fixed with 10% Formalin for 1 hour. After fixation cells were washed with 60% isopropanol and allowed to dry completely. Cells were then stained with Oil Red O for 10 minutes and washed extensively with H_2O. Images were acquired using the IX83 Inverted Microscope (Olympus).

3.6 Western Blot Analysis

Lung tissue was lysed on ice (10 mM TrisHCl, 140 mM NaCl, 2 nM EDTA, 1% NP40 lysis buffer containing Roche PhosSTOP and cOmplete ULTRA phosphatase and protease inhibitors). Protein concentration was determined using Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Samples were diluted using 4X Laemeli Sample buffer and heated to 99°C for 10 minutes. Equal amounts of protein were then loaded into a pre-cast 4-20% TGX gel (BioRad). Protein was transferred to a nitrocellulose membrane (BioRad) and then blocked in 5% milk for 1 hour and probed with p-STAT3 (1:1000, Cell Signaling Technology), p-STAT5 (1:1000, Cell Signaling Technology), followed by anti-rabbit HRP-conjugated secondary antibody (1:3000, Cell Signaling Technology). Blots were developed with FluorChem M (ProteinSimple).

4.0 Results

4.1 Aim 1: Investigate Tissue Specific Regulation of *Cish* in Alveolar Macrophages

4.1.1 *Cish* expression is tissue specific

Using the Immgen RNAseq Gene Skyline to compare *Cish* expression across various tissue resident macrophages, we found high *Cish* expression is unique to alveolar macrophages (Figure 1A). We next sought to determine if *Cish* expression in alveolar macrophages is dependent on factors present in the lung. We analyzed *Cish* expression in alveolar macrophages freshly harvested versus cultured *ex vivo* in media and found dramatic reduction of *Cish* in alveolar macrophages after removal from the lung (Figure 1B). This is consistent with the hypothesis that alveolar macrophage *Cish* expression is dependent on factors present in the lung environment. We also investigated tissue specific expression of *Cish* using publish RNAseq data[16]. Peritoneal macrophages transferred into the lung for 15 days increased expression of *Cish* to expression levels comparable to alveolar macrophages (Figure 1C), further suggesting *Cish* expression is dependent on lung specific factors.

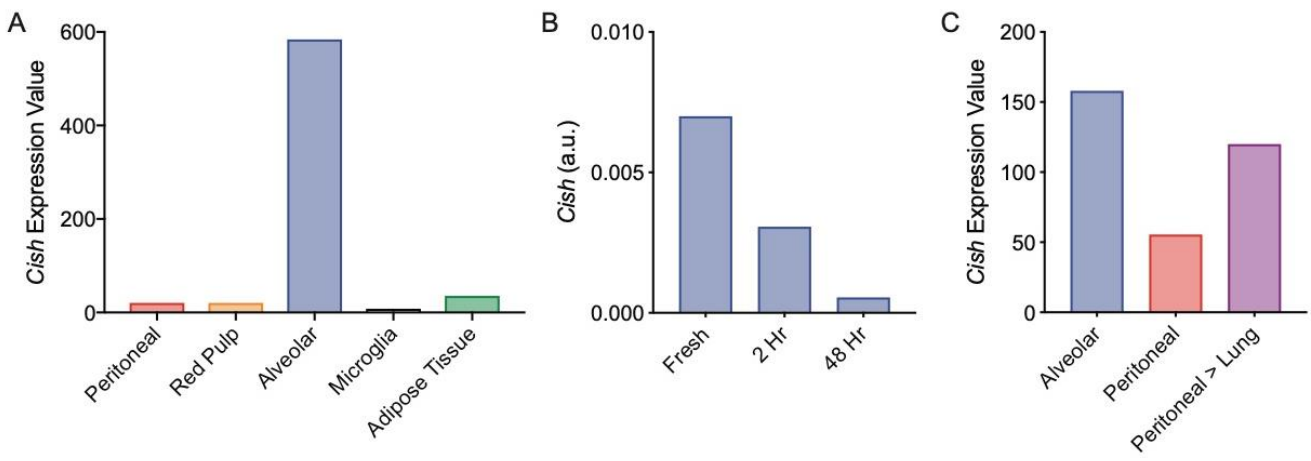

Figure 4. *Cish* expression in macrophages is lung specific.

(A) Expression of *Cish* across subsets of tissue resident macrophages (Immgen). (B) *Cish* expression in BAL macrophage either fresh from the lung, or cultured in media for 2 or 48 hours quantified by pPCR. (C) Expression of *Cish* in alveolar macrophages, peritoneal macrophages, or peritoneal macrophages transferred into the lung after 15 days.

4.1.2 GM-CSF regulates *Cish* expression

Cish is well described to regulate and be regulted by STAT5[53, 54], leading us to investigate STAT5 inducing cytokines present in the lung. GM-CSF not only signals through the STAT5 pathway[36], but is also highly expressed in the lung and known to shape alveolar macrophage development and phenotype[23]. We first tested the ability of GM-CSF to induce *Cish* expression in bone marrow derived macrophages (BMDM) and found GM-CSF stimulation resulted in strong induction of *Cish* (Figure 5A).

To better understand the relationship between GM-CSF and *Cish* expression *in vivo*, we compared whole lung *Csf2* expression to alveolar macrophage *Cish* expression across individual WT mice, and found a significant correlation (R^2= 0.828, p=0.0017) between the two (Figure 5B). This suggested to us that GM-CSF in the lung plays a role in alveolar macrophage *Cish* expression *in vivo*. We next tested the effect of blocking GM-CSF in the lung by administering either anti-

GM-CSF or isotype control antibodies intranasally over the course of three days. We found that GM-CSF blockade in the lung resulted in significant (p=0.0327) reduction of *Cish* expression in alveolar macrophages (Figure 5B). To assess the efficacy of our blockade, we also measured expression of *Mrc1* and *Pparg*, both described to be regulated GM-CSF in alveolar macrophages[23, 60]. We found a modest, bu significant reduction of *Mrc1*, but non-significant reduction of *Pparg*, suggesting only partial GM-CSF blockade in the lung (Figures 5D & 5E).

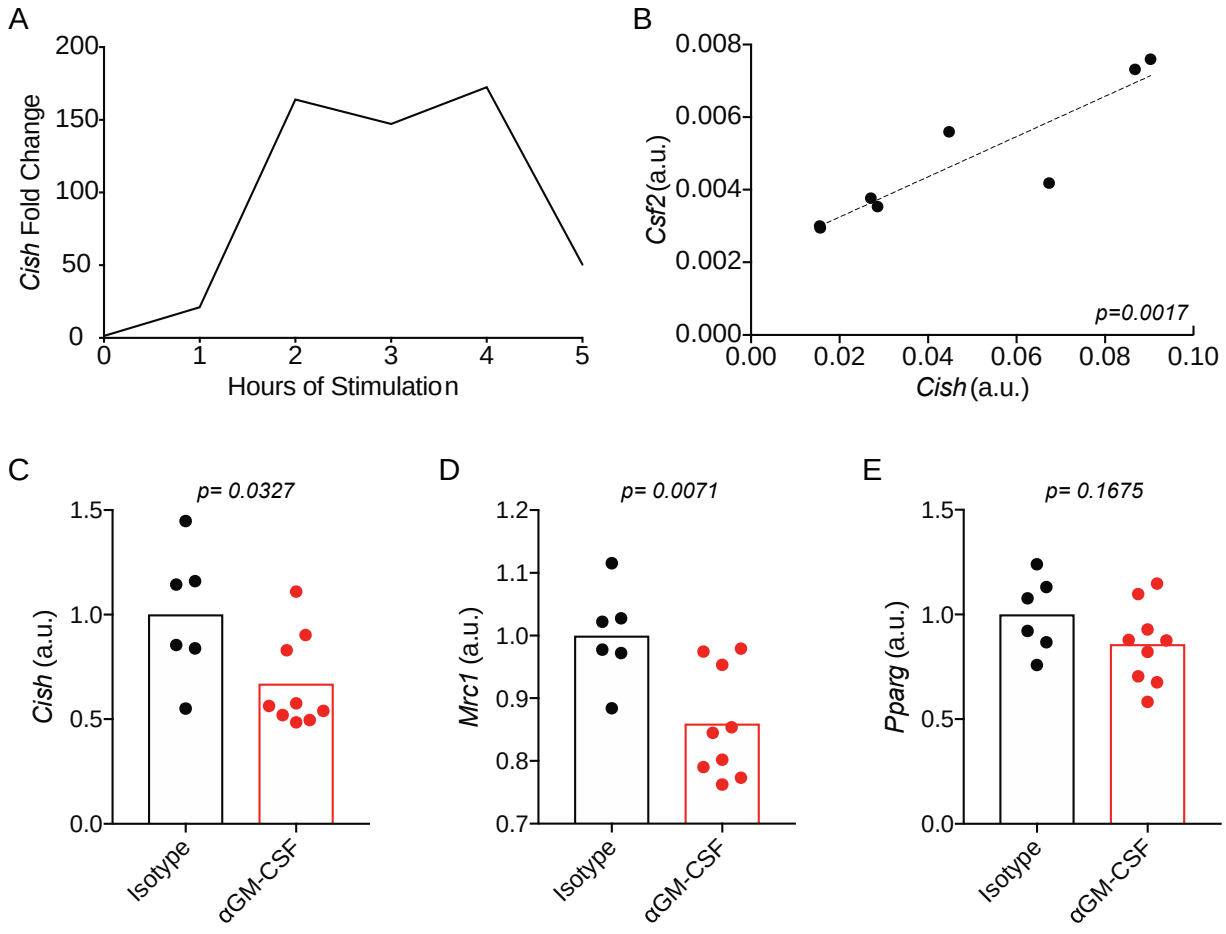

Figure 5. GM-CSF regulates *Cish* expression.

(A) *Cish* expression in BMDM after stimulation with GM-CSF (20 ng/mL) quantified by qPCR. (B) Whole lung *Csf2* expression versus BAL alveolar macrophage *Cish* expression in WT BL6 mice quantified by qPCR. Expression of (C) *Cish*, (D) *Mrc1*, and (E) *Pparg* in alveolar macrophages (CD11c+, Siglec-F+) sorted from WT BL6 mice after three day intranasal administration of anti-GM-CSF (red) or isotype control (black) antibodies.

4.2 Aim 2: Investigate Tissue Specific Function of *Cish* in Alveolar Macrophages

4.2.1 Dysregultion of lung homeostasis in *Cish* KO mice

A previous study reported that *Cish* KO mice develop spontaneous lung disease, noting consolidation of airways due to deposit of proteinaceous material and mononuclear infiltrates, with disease worsening with age[61]. This study focused on the role of *Cish* in T cells using a model of induced lung inflammation, failing to further characterize the spontaneous disease or to assess potential contribution of alveolar macrophages, which express high levels of *Cish*. Therefore, we sought to investigate *Cish* KO lung disease, with specific focus on *Cish* in alveolar macrophages. We compared lung samples from 4 month old (young) and 12 month old (old) *Cish* KO mice to age matched WT controls. To assess cytokine signaling on the protein level, we examined whole lung lysates by Western blot and we found increased STAT3 and STAT5 activation in Cish KO mice compared to WT controls (Figure 6A). This suggested dysregulation of cytokine signaling in lungs of *Cish* KO mice. To better assess cytokine regulation at a gene expression level, we assessed whole lungs by qPCR analysis. We found a trend of increased expression of proinflammatory chemokines and cytokines, *Cxcl1*, *Ccl2*, *Ccl3*, *Il6*, and *Csf2* in the lungs of old *Cish* KO mice (Figure 6B-F). In addition, we observed a trend of decreased expression of *Stat5a* in the lungs of *Cish* KO mice compared to WT mice in both the young and the old groups (Figure 6G), further evidence suggesting dysregulation of the STAT5 network *Cish* KO lungs.

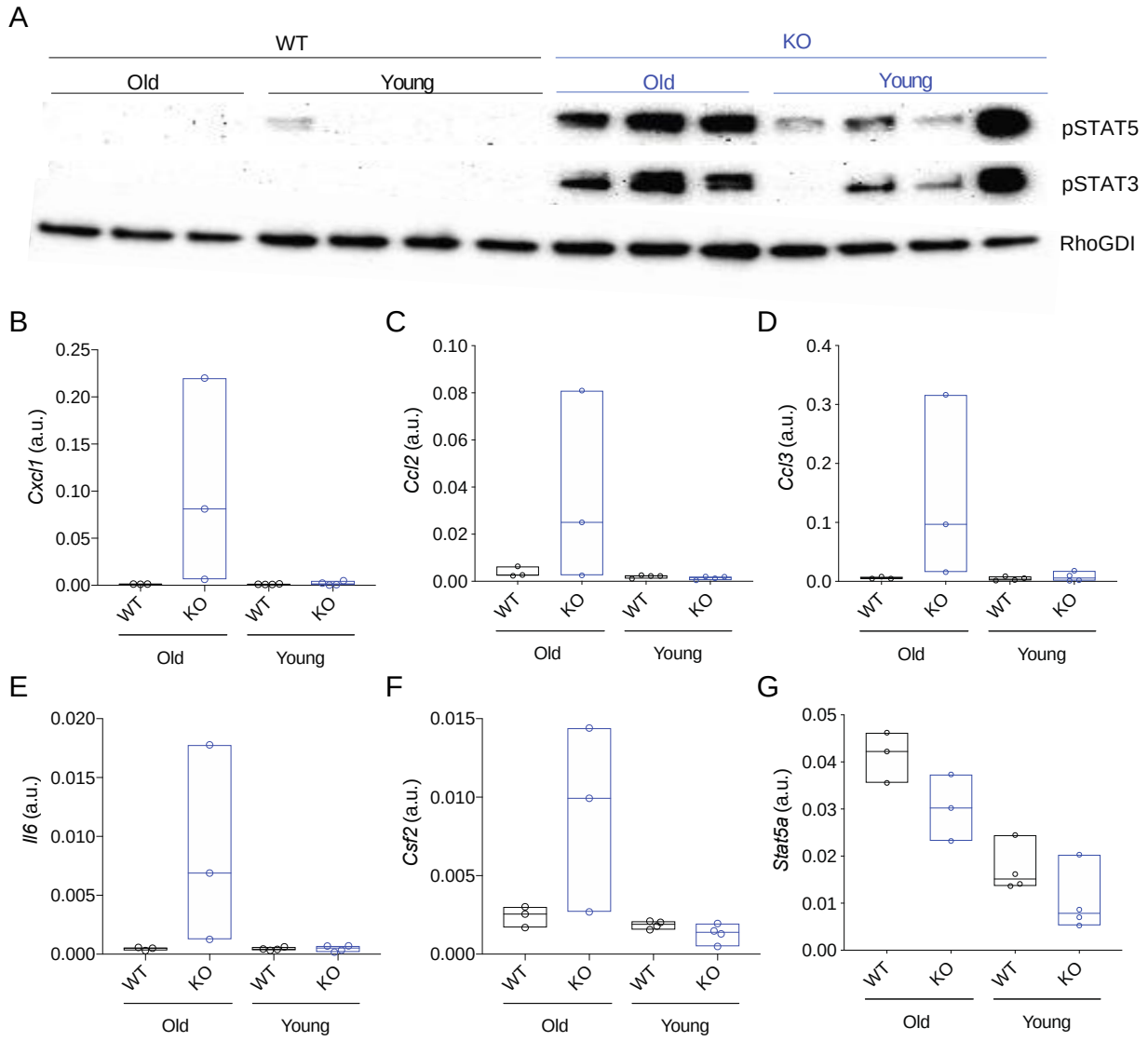

Figure 6. *Cish* KO mice exhibit cytokine dysregulation in the whole lung.

(A) Western blot analysis of the whole lung in young (4 month) and old (1 year) WT and *Cish* KO mice. RhoGDI was used as a loading control. (B-G) qPCR analysis of the whole lung in young (4 month) and old (1 year) WT and *Cish* KO mice.

Based on our finding that GM-CSF regulates *Cish* expression in alveolar macrophages (Figure 5) and observation of whole lung dysregulation of GM-CSF/STAT5 signaling in *Cish* KO mice exacerbated by age (Figure 6), we sought to further investigate *Cish* in the context of GM-CSF/STAT5 related alveolar macrophage dysfunction. Aberrant GM-CSF signaling in alveolar

macrophages results in pulmonary alveolar proteinosis (PAP), in which alveolar macrophages fail to adequately degrade surfactant in the absence of a proper GM-CSF signal[43, 44, 47, 50]. When we assessed levels of surfactant in the BAL, we found elevated levels of surfactant protein D (SP-D) in the *Cish* KO mice (Figure 7A). Further, we noticed increased granularity (SSC-A) in alveolar macrophages from old *Cish* KO mice, as well as a trend of increased size (FSC-A) in alveolar macrophages from the old and young *Cish* KO mice, compared to WT controls (Figure 7B), consistent with the foamy alveolar macrophage phenotype seen with dysregulation of surfactant homeostasis[62].

To better assess whether this increased size and granularity was due to intracellular accumulation of surfactant in alveolar macrophages, we performed an Oil Red O stain, which stains neutral lipids. BAL macrophages from *Cish* KO mice exhibited notably increased staining with Oil Red O compared to WT controls (Figure 7C), further suggestive of a foamy alveolar macrophage phenotype, consistent with PAP[63, 64]. We then looked specifically at expression of gene related to surfactant metabolism (PathCards, Weizmann Institute of Science) in alveolar macrophages sorted from young and old *Cish* KO and WT mice. We noted dysregulation of surfactant metabolism gene expression in the *Cish* KO alveolar macrophages, compared to WT mice, exacerbated with age (Figure 7D). Taken together, these results are highly suggestive of a PAP like pathology developing in *Cish* KO mice. *Cish* KO mice exhibit the hallmarks of PAP, in which improper surfactant homeostasis results in accumulation of surfactant in the BAL and foamy alveolar macrophages with increase in size and granularity due to improper intracellular surfactant metabolism[43, 44, 47, 50, 63, 64, 65].

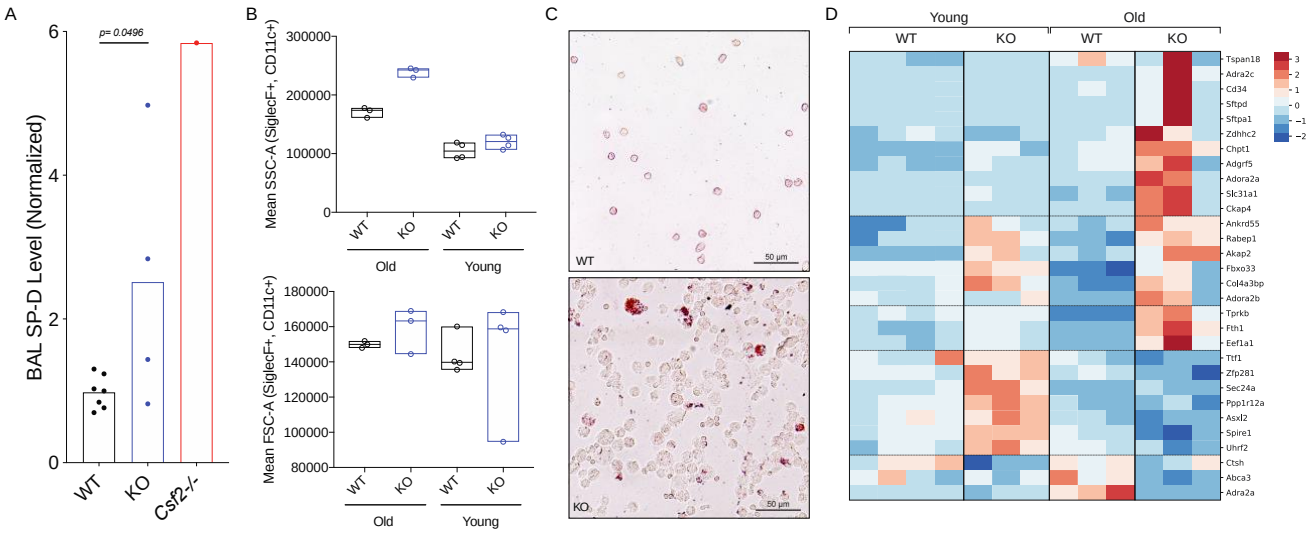

Figure 7. *Cish* KO mice develop PAP-like phenotype.

(A) Levels of SP-D in BAL supernatant from 4 month old mice quantified by ELISA, with individual experiments internally normalized to WT controls. An age matched *Cs2f-/-* mouse was included as a PAP control (B) Mean SSC-A (top panel) and mean FSC-A (bottom panel) in alveolar macrophages flow sorted on Siglec-F+, CD11c+ cells from lungs of young (4 month) and old (1 year) *Cish* KO and WT mice. (C) Representative Oil Red O stain from 4 month old *Cish* KO and WT mice. (D) RNAseq heat map for surfactant metabolism from sorted alveolar macrophages (same as B).

4.2.2 Cell intrinsic implications of *Cish* deficiency in alveolar macrophages

We sought to explore the mechanism by which this PAP-like phenotype develops in *Cish* KO mice. Due to numerous studies reporting that PAP is driven by loss of a proper GM-CSF signal to alveolar macrophages[43, 47, 50], we investigated the response of *Cish* KO alveolar macrophages to GM-CSF stimulation. We harvested alveolar macrophages from *Cish* KO and WT mice by BAL, stimulated them *ex vivo* with GM-CSF, and quantified pSTAT5 by immunofluorescence. We first noted that unstimulated *Cish* KO alveolar macrophages displayed significantly reduced average pSTAT5 intensity compared to WT (Figure 8A), consistent with dysregulated STAT5 signaling *in vivo*. When stimulated with GM-CSF, we noticed that average pSTAT5 signal remained markedly

reduced in *Cish* KO alveolar macrophages compared to WT alveolar macrophages (Figure 8B). However, when plotted as a fold change, we noticed that the average pSTAT5 signal appeared more sustained at later timepoints in *Cish* KO alveolar macrophages compared to WT alveolar macrophages (Figure 8C). Though preliminary, these data suggest a failure to properly turn off the STAT5 signal, consistent with loss of *Cish*, a negative regulator of STAT5 signaling.

To gain insight into the impact of *Cish* deficiency in regulation of GM-CSF signaling in alveolar macrophages *in vivo*, we plotted a gene expression for a GM-CSF signaling gene set (PathCards, Weizmann Institute of Science) across sorted young and old *Cish* KO and WT alveolar macrophages (Figure 8E). In this heat map, we noticed distinct gene expression patterns across *Cish* KO and WT alveolar macrophages, with the most robust differences in the old *Cish* KO alveolar macrophages. This is consistent with aberrant GM-CSF mediated STAT5 signaling in *Cish* KO alveolar macrophages.

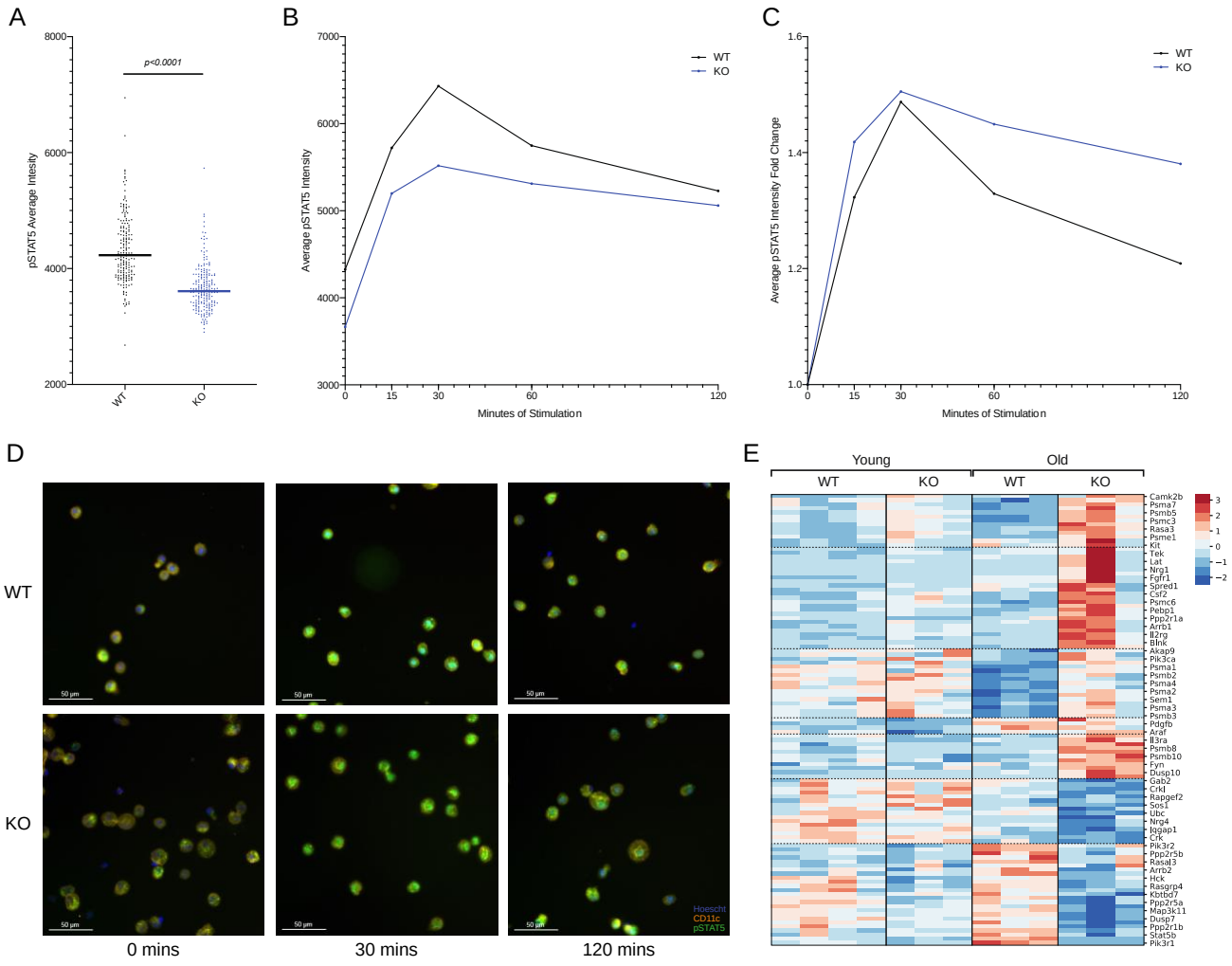

Figure 8. *Cish* KO alveolar macrophages exhibit dysregulated response to GM-CSF.

Immunofluorescent imaging analysis of *Cish* KO and WT alveolar macrophages from 4 month old mice with (A) quantified pSTAT5 average intensity unstimulated and *ex vivo* GM-CSF (20 ng/mL) stimulation over time quantified by (B) average pSTAT5 intensity and (C) pSTAT5 average intensity fold change. (D) Representative immunofluorescent images. (E) RNAseq heatmap of GM-CSF signaling pathway of sorted alveolar macrophages (CD11c+, Siglec-F+) sorted from young (4 month) and old (1 year) *Cish* KO and WT mice.

We next sought to further explore cell intrinsic functions of *Cish* in relation to macrophage function. Considering that *Cish* KO alveolar macrophages already exhibited impaired clearance of surfactant, we decided to test if *Cish* plays a role in phagocytosis of microbes, a critical function of all macrophages, especially at barrier sites such as the lung. We first quantified *Cish* KO and

WT BMDM internalization of fluorescent *S. aureus* bioparticles via immunofluorescent imaging. We found that *Cish* KO BMDM exhibited impaired phagocytosis of *S. aureus* bioparticles at later timepoints compared to WT BMDM (Figure 9A). This must be tested in alveolar macrophages to draw meaningful conclusions about *Cish* function in the context of the macrophages in the lung.

Although *Cish* is best described as a regulator of JAK/STAT5 signaling, *Cish* has also been described to regulate calcium signaling in T cells[55]. Phagocytosis in macrophages is mediated by a calcium signal[66, 67, 68, 69], and thus dysregulated phagocytosis in *Cish* KO BMDM could be related to improper regulation of calcium signaling. We speculated that *Cish* KO alveolar macrophages could also have dysregulated calcium signaling, in addition to impaired STAT5 regulation. When we plotted calcium signaling gene expression across alveolar macrophages sorted from young and old *Cish* KO and WT mice, we indeed found different gene expression patterns in *Cish* KO alveolar macrophages compared to WT macrophages, with the most robust difference appearing the old *Cish* KO alveolar macrophages (Figure 9C). This is consistent with our hypothesis that *Cish* plays a role in alveolar macrophage calcium signaling, but requires further investigation.

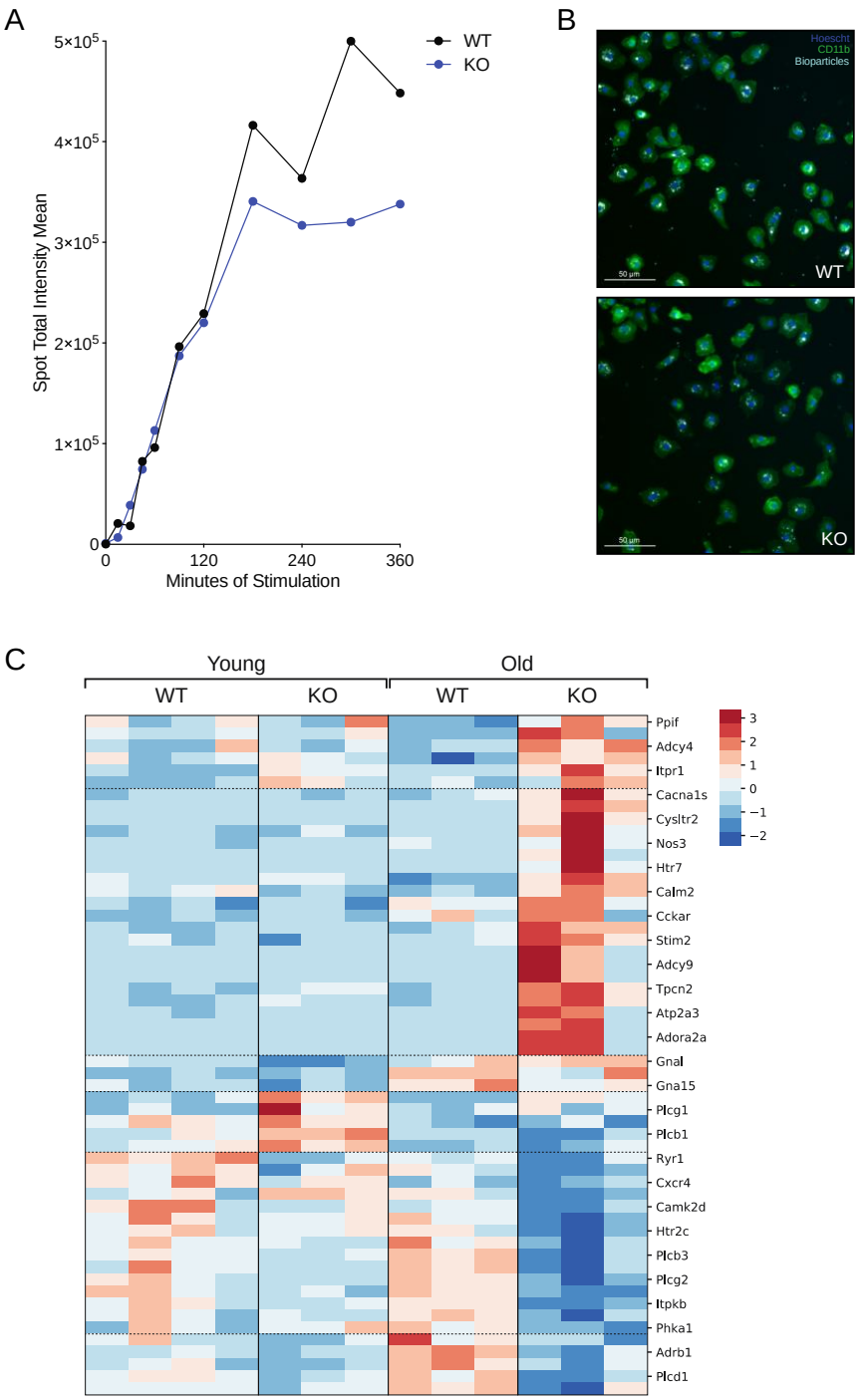

Figure 9. *Cish* KO macrophages exhibit potential calcium dysregulation.

(A) Immunofluorescent imaging analysis of *Cish* KO and WT BMDM (CD11b+ cells, green) phagocytosis of fluorescent *S. aureus* bioparticles (light blue) quantified by mean fluorescent intensity of *S. aureus* bioparticles within BMDM. (B) Representative images of immunofluorescent phagocytosis assay at the 5 hour time point. (C) RNAseq heatmap of calcium signaling pathway of sorted alveolar macrophages (CD11c+, Siglec-F+) sorted from young (4 month) and old (1 year) *Cish* KO and WT mice.

5.0 Discussion

Macrophages reside in nearly every organ of the body, where they perform a variety of functions that extend beyond simple immune surveillance of their host. These various functions typically coincide with their tissue of residence, where they crosstalk with other cells present in the tissue to maintain overall tissue homeostasis. For example, microglia work to remodel neurons, by eliminating dead neurons and stimulating growth of new ones[8, 24]. Bone marrow macrophages play a pivotal role in generation of new red blood cells[6] and red pulp macrophages remove old red blood cells from circulation[21]. Increasingly, tissue resident macrophages are being appreciated for their communication with their tissue of residence and their role in tissue homeostasis.

In addition to possessing distinct roles across different tissues, tissue resident macrophages have also been shown to have distinct gene expression patterns[16, 17, 18]. Many speculate that tissue specific factors are responsible for these driving distinct gene expression signatures, and in turn phenotypes and tissue specific functions. While there is emerging evidence to support this idea, the tissue specific signals responsible for informing these unique signatures remain poorly defined. In addition, the link between tissue specific gene expression and tissue specific function remains relatively vague, with few concrete examples. Therefore, in this project we sought to uncover lung specific drivers of alveolar macrophage gene expression and in turn lung specific function, with specific emphasis on cytokine signals present in the steady state lung microenvironment.

We indeed found that expression of *Cish*, a SOCS regulator of STAT5 signaling[53], is specific to alveolar macrophages and is dependent on factors present in the homeostatic lung microenvironment (Figure 4). Further, we found that regulation of *Cish* in alveolar macrophages is dependent on GM-CSF (Figure 5), which is well described to signal through the STAT5 pathway

and highly expressed in the steady state lung environment[35, 36]. However, while GM-CSF blockade in the lung was sufficient to significantly decrease *Cish* expression in alveolar macrophages, we speculate there are likely additional signals present within the lung microenvironment that drive expression of *Cish* in alveolar macrophages.

In addition to addressing the regulation of this alveolar macrophage specific gene, *Cish*, we also sought to investigate the function of *Cish* in alveolar macrophages by studying the effect of *Cish* deficiency in a mouse model. Based on previous reports of *Cish* KO mice developing spontaneous pulmonary disease with age[61], we posited that *Cish* deficiency results in dysregulated lung homeostasis which worsens with age and is mediated by alveolar macrophages, an abundant immune cell in the lung expressing high levels of *Cish*. Therefore, we aged *Cish* KO mice and WT controls to better define the pulmonary disease and specifically examine the contribution of *Cish* deficiency within alveolar macrophages to disease development. We recapitulated the previous finding of *Cish* KO mice developing lung disease which worsened with age. Elaborating on this study, we found that this lung disease closely resembled pulmonary alveolar proteinosis, with alveolar macrophages as major drivers of this disease phenotype.

Because our primary focus was on steady state cytokine signals in the lung, we first examined the impact of loss of a tissue specific cytokine regulator, *Cish*, on lung cytokine homeostasis. As mice aged, we found that *Cish* KO mice exhibited dysregulated cytokine balance in the whole lung, specifically with increased activation of STAT3 and STAT5, as well as trends towards increased production of pro-inflammatory chemokines and cytokines. We also noticed trends towards increased *Csf2* expression and decreased *Stat5a* expression in the lungs of *Cish* KO mice (Figure 6). Taken together, these results pointed to aberrant GM-CSF/STAT5 signaling in

the lung in *Cish* KO, which was not surprising considering previous reports describing *Cish* as a STAT5 regulator[36].

We sought to more closely examine the implications of this aberrant GM-CSF/STAT5 signaling on overall lung homeostasis mediated by alveolar macrophages. Literature review revealed that GM-CSF signaling is not only critical for alveolar macrophage development[23], but also for alveolar macrophage mediated pulmonary surfactant homeostasis[43]. In people, loss of a GM-CSF signal to alveolar macrophages in the lung, most typically through autoimmune antibodies against GM-CSF, results in pulmonary alveolar proteinosis, in which alveolar macrophages fail to properly degrade surfactant, leading to accumulation of surfactant in the lung[45]. Due to our initial finding of improper GM-CSF/STAT5 signaling in the whole lung in *Cish* KO mice in addition to the previous finding of *Cish* KO developing spontaneous lung disease in which filling of the alveoli with proteinaceous material was observed, we sought to examine the possibility that *Cish* KO mice develop PAP, mediated by improper GM-CSF/STAT5 signaling in alveolar macrophages.

When we examined the broncheoavleolar lavage fluid we noticed elevated levels of surfactant protein in *Cish* KO mice. Additionally, we noted "foamy" alveolar macrophages in *Cish* KO mice, with increased size and granularity, consistent with the phenotypic hallmark of PAP, as alveolar macrophages in PAP increase in size and granularity as surfactant accumulates intracellularly, failing to be properly metabolized[62]. We stained for intracellular lipid accumulation, and also saw increased intracellular lipid deposition in *Cish* KO alveolar macrophages, another hallmark of PAP[63, 64]. Further, when we looked at a gene set for surfactant metabolism, we noticed differences in gene expression in *Cish* KO alveolar macrophages

compared to WT macrophages, supporting the notion that alveolar macrophages *Cish* deficiency results in improper pulmonary surfactant homeostasis (Figure 7).

To further address the cell intrinsic mechanisms by which *Cish* deficiency results in dysregulated alveolar macrophage mediated surfactant homeostasis, we tested alveolar macrophage GM-CSF mediated STAT5 signaling *ex vivo*. Although preliminary results, we noticed that alveolar macrophages from *Cish* KO mice contained lower basal levels of pSTAT5 compared to WT alveolar macrophages, suggesting lack of responsiveness to GM-CSF in *Cish* KO alveolar macrophages. When stimulated with GM-CSF *ex vivo*, *Cish* KO alveolar macrophages activated STAT5 at levels consistently less than WT alveolar macrophages. In addition, pSTAT5 signaling remained at more sustained levels at later timepoints in *Cish* KO alveolar macrophages compared to WT alveolar macrophages, consistent with loss of a negative regulator of STAT5 signaling (Figure 8). Additional experimentation is required to further explore these differences.

We next sought to explore immune implications of *Cish* deficiency in macrophages, specifically in relation to response of microbes. We tested the ability of *Cish* KO macrophages to phagocytose microbes, as alveolar macrophages from other PAP models have been reported to exhibit defects in phagocytosis[47, 65]. Although our results are preliminary and only tested so far in BMDM, we noticed significant impairment of phagocytosis of *S. aureus* bioparticles in *Cish* KO BMDM (Figure 9A). We suspect that this impaired phagocytosis is due to improper regulation of a calcium signal mediated by *Cish*, as *Cish* has been implicated in regulation of calcium signaling[55]. Although this requires further examination in alveolar macrophages, this inspired us to look specifically at expression of genes in calcium signaling pathways in our sorted alveolar macrophage RNAseq dataset. We found that *Cish* KO alveolar macrophages exhibited notably

different gene expression patterns compared to WT alveolar macrophages, especially in the old *Cish* KO alveolar macrophages (Figure 9C), further suggesting a potential role for *Cish* in calcium signaling in alveolar macrophages, independent of STAT5 signaling.

In this study, we show that *Cish* is expressed in a lung specific manner in alveolar macrophages and that Cish expression is regulated by GM-CSF present in the lung. Further, *Cish* expression in alveolar macrophages is important for their homeostatic role in the lung, namely surfactant homeostasis. While previous studies have hinted at tissue specific regulators being expressed in a tissue specific manner and informing tissue specific functions of macrophages, this study provides concrete evidence to support this idea.

6.0 Future Directions

These results highlight the tissue specific function of *Cish* in alveolar macrophages, in which *Cish* deficiency results in apparent dysregulation of lung homeostasis, namely through improper cytokine balance and improper surfactant clearance. While we present a strong case for *Cish* deficiency leading to PAP, as evidenced foamy alveolar macrophages and increased levels of pulmonary surfactant in *Cish* deficient mice, this study could be strengthened by lung histology showing PAP as well. Architectural issues with initial histology prevented us from showing these results with confidence, but our initial histological results were supportive of development of PAP in *Cish* KO mice (data not shown). We are undergoing further optimization of lung histology in order to definitively address this.

Additionally, while we show imbalance of cytokines present in the lungs of *Cish* KO mice, future studies addressing the mechanisms with which this occurs over time would provide more insight into how alveolar macrophage *Cish* expression tunes proper cytokine signaling in the lung. To address this, we would need to carefully examine the balance of cytokine expression and secretion in the lungs of *Cish* KO mice as they age. Better understanding the dynamics by which cytokines are dysregulated in the lungs of *Cish* KO mice would provide insight into the complex crosstalk of cells in the lungs as well as inform further experimentation into additional implications of improper cytokine homeostasis in the lung.

While we are beginning to further explore cell intrinsic effects of *Cish* deficiency in alveolar macrophages, such as response to GM-CSF and calcium signaling, additional research is required to explore the mechanisms by which *Cish* regulates these cell intrinsic functions within alveolar macrophages. Our preliminary results suggest that *Cish* KO alveolar macrophages have

impaired responses to GM-CSF/STAT5 signaling, but the mechanism with which *Cish* regulates this signal in alveolar macrophages needs to be further addressed. Based on our initial findings, we speculate that *Cish* deficiency results in a prolonged STAT5 signal, failing to properly shut off due to lack of *Cish*.

Based on preliminary findings, we also speculate that *Cish* likely plays a role in regulating calcium signals in alveolar macrophages. While we focused on phagocytosis in this study, calcium signaling is also important for a number of cellular functions, such as cell adhesion, cytokine production, and response to oxygen[70, 71, 72]. Thus improper regulation of calcium signaling could have a wide range of effects for alveolar macrophage mediated lung homeostasis and protection. We are beginning to investigate calcium signaling in response to various stimuli in *Cish* KO and WT alveolar macrophages to address whether *Cish* regulates calcium signals in alveolar macrophages.

Further, we wish to understand the impacts of these cell intrinsic functions of *Cish* for overall lung homeostasis and protection. Based on preliminary data, we suspect that alveolar macrophage *Cish* deficiency results in worsened outcome upon bacterial challenge in the lung, with decreased survival and increased weight loss in *Cish* KO mice (data not shown). These results would be consistent an SNP analysis showing that specific *Cish* variants have increased susceptibility to pulmonary infections, suggesting proper regulation of immune response mediated by *Cish* plays an important role in protection of the lung[57, 58]. These results would also be consistent with other PAP models, in which alveolar macrophages in PAP have been shown to exhibit impaired immune responses to various microbes[47, 65].

We predict that alveolar macrophage *Cish* deficiency would lead to defective immune responses to pulmonary pathogens, namely through dysregulated cytokine responses, leading to

overall worsened outcome in pulmonary infections. We also speculate that calcium signaling, shown to be important in communication between alveolar macrophages especially in the context of infection, could be dysregulated in a *Cish* KO model. Mechanisms by which *Cish* regulates responses to microbes in alveolar macrophages and implications for outcome of lung infection need to be addressed experimentally.

In addition to worsened outcome in instances of pulmonary infection, we also predict that *Cish* deficient alveolar macrophages have irregular responses to microbes present in the lung in the steady state. The lung has been shown to harbor its own unique microflora and alveolar macrophages typically detect these microbes quiescently, without generating pro-inflammatory responses[73]. We speculate that *Cish* KO alveolar macrophages would be either hyper or hypo-responsive to microbes in the steady state lung environment, either causing unnecessary lung damage or allowing opportunistic infections respectively.

One major shortcoming of this project is use of a global *Cish* KO mouse model. Although this model is sufficient for showing tissue specific regulation and function of *Cish* in alveolar macrophages, a handful of other immune cells including as CD8+ T cells can express *Cish*. To address this shortcoming, moving forward the ideal model to use would be a conditional *Cish* KO, likely under transcriptional control of *Cx3cl1*, which is uniquely highly expressed by alveolar macrophages. This conditional *Cish* KO model would be able to address whether *Cish* deficiency in other cell types in the lung further contribute to the phenotype we describe in this study, as well as in future experimentation.

7.0 Public Health Significance

Our study specifically found a spontaneous PAP like phenotype driven by *Cish* deficiency, highlighting the importance of tissue specific regulatory signaling in maintenance of tissue homeostasis. This loss of proper lung homeostasis has numerous implications for the host, from proper protection of the lung to simple ability to breathe in severe cases of disease, a critical barrier site. From a public health perspective, continuing to elucidate the specific mechanisms by which alveolar macrophages mediate lung homeostasis is essential to providing cutting edge health care and to continuing to develop therapeutic treatments that can provide cures instead of simply mitigating symptoms of pulmonary disease.

From a human health standpoint, SNP analysis across *Cish* variants found that specific variants of *Cish* are more highly susceptible to various infections, such as malaria, tuberculosis, bacteremia, and hepatitis B, further emphasizing the importance of signaling regulators, specifically *Cish* in this case, in host protection[57, 58]. Further, although PAP is a relatively rare disease, individuals with PAP are more susceptible to various pulmonary and systemic infections, such as tuberculosis[45]. In addition to being the major drivers of PAP, alveolar macrophages in PAP have also been shown to exhibit dysregulated immune responses, such as defects in expression of PRRs, secretion of cytokines, phagocytosis, and microbial killing[47, 65].

This study emphasizes that alveolar macrophage function has implications not only for overall lung structure and function, but also that impaired lung homeostasis can lead to impaired tissue protection. Elucidating the signals that are responsible for proper lung homeostasis is vital to better understand the complex crosstalk between cells in the lung and to understand how these signals get dysregulated in order to create better targets in treatment of various pulmonary diseases.

Therefore, better understanding the mechanisms with which alveolar macrophage mediate lung homeostasis is critical for continuing to provide the best health care and treatments to individuals with pulmonary disease or infection.

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