IMPACT OF PULMONARY SURFACTANT ON HUMAN MACРОРОPHAGE SUSCEPTIBILITY TO Mycobacterium Tuberculosis

Dissertation
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ABSTRACT

Pulmonary tissue integrity is necessary for proper organ function but is made complicated by the continuous inhalation of environmental particles and microbes. The terminal branches of the airways, the alveolar spaces, are comprised of extremely thin tissue to enable gas exchange and are lined with a lipid-protein complex called surfactant which enables tissue expansion and prevents alveolar collapse. Alveoli are supervised by alveolar macrophages (AMs), the resident phagocytes which keep this delicate tissue space clean of inhaled detritus and additionally catabolize surfactant. Immune responses in the lung are tightly regulated to ensure the rapid removal of inhaled particulates without the initiation of strong pro-inflammatory pathways and this regulation is partially mediated through macrophage interactions with pulmonary surfactant. These homeostatic mechanisms can be exploited by pathogenic microbes, such as *Mycobacterium tuberculosis* (*M. tb*), a phenomenally successful global pathogen and the cause of tuberculosis (TB).

*M. tb* is able to colonize the alveolar spaces and replicate inside of AMs. Due to the well-documented immunosuppressive effects of surfactant, we hypothesized that the perpetual interaction between AMs and pulmonary surfactant result in an AM which is susceptible to infection by *M. tb*. Because *M. tb* has been shown to use host-derived lipids as a carbon source during infection, we were particularly interested in surfactant catabolism by AMs and hypothesized that the presence of surfactant lipids inside of
macrophages would enhance *M.tb* growth. We therefore sought to identify which receptor(s) could mediate surfactant lipid uptake by AMs and began by exploring whether surfactant could regulate the expression of several macrophage surface receptors. Among several receptors surveyed, we show that the scavenger receptor CD36 is positively regulated by surfactant at the transcript and protein levels. We further show that intracellular CD36 is redistributed to the macrophage cell membrane following exposure to surfactant components, indicating that CD36 may be of importance in the lung environment, possibly in relation to surfactant lipid uptake.

Using fluorescently-labeled surfactant lipids and siRNA knockdown approaches, we show that CD36 is required for macrophage uptake of the major surfactant lipid species dipalmitoylphosphatidylcholine (DPPC). In contrast, CD36 knockdown did not affect uptake of the second most abundant lipid in surfactant, phosphatidylglycerol (PG). Knockdown of another scavenger receptor, SR-A, increased uptake of DPPC and this was possibly due to the concomitant increase in CD36 protein levels in SR-A deficient macrophages. We next explored the hypothesis that CD36-dependent uptake of surfactant lipids would enhance intracellular *M.tb* growth. Scramble control and siCD36 macrophages were cultured in surfactant lipids prior to infection with a luciferase expressing strain of *M.tb* and bacterial growth was monitored by luminometry. We discovered that surfactant lipids augment *M.tb* growth early during infection and that the expression of CD36 is required for this surfactant-dependent growth advantage.

We speculated that the enhanced growth could be due to the immunosuppressive effects of surfactant and we therefore investigated the relative production of several cytokines. We report a significant reduction in *M.tb*-induced TNFα when macrophages were cultured in surfactant lipids, confirming that surfactant lipid-
mediated immunosuppression may impair macrophage microbicidal functions. However, this cytokine response was not altered in the absence of CD36, indicating that additional factors contribute to the *M. tb* growth advantage we observed in CD36-expressing, surfactant-exposed macrophages. We speculate that the presence of surfactant lipid metabolites likely provides a readily available carbon source for *M. tb*.

*M. tb* has been reported to induce the accumulation of lipids inside of infected macrophages, leading to foamy macrophage (FM) formation. We sought to investigate potential mechanisms by which this could occur by measuring the effects of surfactant and *M. tb* infection on the expression of macrophage lipid metabolic regulators. We hypothesized that *M. tb* would augment expression of the lipid uptake receptor CD36 and would additionally enhance expression of the lipid storage coat protein adipophilin, resulting in increased intracellular lipid content. Conversely, we hypothesized that *M. tb* would decrease expression of LAL and LPLA2, two key enzymes in the breakdown of surfactant lipids. A deficit in the expression of either LAL or LPLA2 can lead to FM formation. Finally, we hypothesized that *M. tb* would decrease expression of ABCG1, a major lipid transporter for the efflux of surfactant lipid metabolites. As expected, we report a decrease in LAL, although LPLA2 expression did not change. However, counter to our hypothesis, we observed a decrease in the expression of CD36 and adipophilin following *M. tb* infection and an increase in ABCG1 expression. We speculate that the infection-induced decrease in LAL leads to the accumulation of lipids in the cytoplasm, which cannot be packaged into lipid bodies due to the concomitant decrease in adipophilin expression. Therefore, the accumulating lipids must be exported from the cell through increased ABCG1 while a decrease in CD36 expression could serve to prevent further influx of lipids into the cell in an attempt to restore homeostasis.
Taken together, our findings indicate that CD36-dependent uptake of surfactant lipids prior to infection augments *M.tb* growth. Subsequently, the disruption of host lipid metabolism by *M.tb* leads to counter measures by the host cell to prevent lipid accumulation. This work builds our understanding of the role of the endogenous pulmonary environment in setting the stage for *M.tb* infection. In addition, the present work highlights the ability of *M.tb* to modify host lipid metabolism, and future research will answer the crucial question of whether *M.tb* can use surfactant lipids as a carbon source to rapidly establish a growth advantage within macrophages.
DEDICATION

This dissertation is dedicated to my Gran, Betty Jo Dodd.
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Major Field: Microbiology
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<tbody>
<tr>
<td>ABCG1</td>
<td>ATP-binding cassette subfamily G member 1</td>
</tr>
<tr>
<td>acLDL</td>
<td>acetylated low density lipoprotein</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end product</td>
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<tr>
<td>ALF</td>
<td>Alveolar lining fluid</td>
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<tr>
<td>AM</td>
<td>Alveolar macrophage</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>CD200</td>
<td>Cluster of differentiation 200</td>
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<tr>
<td>CD200R</td>
<td>Cluster of differentiation 200R</td>
</tr>
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<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>CD4+</td>
<td>CD 4 positive helper T cells</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CrkL</td>
<td>CT10 regulator of kinase-like protein</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalitis</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DOK</td>
<td>Docking protein</td>
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<tr>
<td>DPPC</td>
<td>Dipalmitoylphosphatidylcholine</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead Box P3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>HAMs</td>
<td>Human alveolar macrophages</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IgSF</td>
<td>Immunoglobulin superfamily</td>
</tr>
<tr>
<td>IMs</td>
<td>Interstitial macrophages</td>
</tr>
<tr>
<td>IVMs</td>
<td>Intravascular macrophages</td>
</tr>
<tr>
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<td>Interleukin 4</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-10</td>
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</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor-based tyrosine activating motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor-based tyrosine inhibitory motif</td>
</tr>
<tr>
<td>LA</td>
<td>Large aggregate</td>
</tr>
<tr>
<td>LAL</td>
<td>Lysosomal acid lipase</td>
</tr>
<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
</tr>
<tr>
<td>LB</td>
<td>Lamellar body</td>
</tr>
<tr>
<td>LD</td>
<td>Lipid droplet</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LPLA2</td>
<td>Lysosomal phospholipase A2</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>LTBI</td>
<td>Latent tuberculosis infection</td>
</tr>
<tr>
<td>MARCO</td>
<td>Macrophage-associated receptor with collagenous structure</td>
</tr>
<tr>
<td>MASP</td>
<td>MBL-associated serine protease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>-------------</td>
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</tr>
<tr>
<td>ManLAM</td>
<td>Mannosylated lipoarabinomannan</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose binding lectin</td>
</tr>
<tr>
<td>MDMs</td>
<td>Monocyte-derived macrophages</td>
</tr>
<tr>
<td>MIR</td>
<td>Monocyte/macrophage immunoglobulin-like receptor</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose receptor</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>M.tb</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>NFκβ</td>
<td>Nuclear factor kappa-light chain-enhancer of activate B cell</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligomerization domain</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidized low density lipoprotein</td>
</tr>
<tr>
<td>PDIMs</td>
<td>Pthiocercol dimycocerosates</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PIMs</td>
<td>Phosphatidylinositol mannoses</td>
</tr>
<tr>
<td>PM₂.₅</td>
<td>Particulate matter &lt;2.5 microns in diameter</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>RasGAP</td>
<td>Ras-associated GTP Activating Protein</td>
</tr>
<tr>
<td>RCD</td>
<td>Regulated cell death</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SA</td>
<td>Small aggregate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>------------------------------------------</td>
</tr>
<tr>
<td>SAP</td>
<td>Serum amyloid protein</td>
</tr>
<tr>
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<td>Surfactant protein A</td>
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<td>Scavenger receptor-A</td>
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<td>Scavenger receptor-B1</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TDM</td>
<td>Trehalose dimycolate</td>
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<td>Tfr</td>
<td>Transferrin receptor</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
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<td>TLR9</td>
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</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Tregs</td>
<td>T regulatory cells</td>
</tr>
<tr>
<td>TREM</td>
<td>Triggering receptor expressed by myeloid cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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CHAPTER 1: INTRODUCTION

1.1 General Introduction

Metazoan organisms are comprised of a fascinating menagerie of specialized cell types [1]. These cell types have been sculpted by evolution to complement each other functionally in the context of the organs and systems that they create, as well as to interact with their environment optimally to ensure the acquisition of nutrients and prevent infection or damage by foreign bodies [2]. The maintenance of homeostasis is necessary for continued function of the systems that arise through the cooperation of specialized cell types, and is therefore crucial for survival [3, 4]. The goal of this dissertation was to investigate constitutive mechanisms of pulmonary homeostasis which predispose macrophages to infection by Mycobacterium tuberculosis (M.tb). We therefore investigated the impact of pulmonary proteins and lipids on macrophage phenotype and, subsequently, the outcome of infection with M.tb. The findings presented herein provide important insights into human susceptibility to a prevalent and successful pathogen. In addition, this work merges the fields of microbiology and immunology with the study of tissue microenvironments to enhance our knowledge of pulmonary homeostasis and the exploitation of this delicate system by parasitic microbes. This introductory chapter will provide relevant background on the lung and M.tb, as well as the mechanisms by which both the pulmonary microenvironment and the bacteria modulate the behavior of macrophages.
1.2 Pulmonary Environment

The Evolution of Lungs

Life is fueled by unceasing metabolism. This process requires gas exchange for the generation of cellular energy and the subsequent expulsion of resultant metabolic by-products [5-8]. In single-celled organisms, gas exchange occurs through simple diffusion with the surrounding environment. As multi-cellular creatures evolved, specialized tissues emerged to handle the task of gas exchange. Gills were likely the first such organ to develop and enabled the exchange of gases between blood and water. As life began its terrestrial transition, rudimentary lungs emerged as an invagination of the gastrointestinal tract, which allowed early amphibious creatures to swallow air and survive outside of an exclusively aqueous environment [7]. This fascinating intermediary construct in the evolution of the pulmonary system is still utilized by African lung fishes [9]. The majority of modern amphibians have lungs and use their skin as a secondary mechanism of gas exchange. However, several amphibious species live without lungs, possibly to reduce buoyancy in aquatic environments such as streams where a small floating creature would be at risk of being swept away [10].

Reptilian lungs illustrate the next phase of pulmonary evolution [11]. Interestingly, because reptiles lack a hard palate they must stop breathing while eating. The lungs of reptiles still require the swallowing of air, while their expansion and contraction is mediated by movement of the rib cage. Although less effective than avian lungs (which are extremely efficient to accommodate the high metabolic demands of flight) mammalian lungs inspire much more effectively than reptilian lungs due to the evolution of the diaphragm. The development of the boughs, limbs and twigs of the mammalian
respiratory tree also improved upon the less stratified organ found in non-avian reptiles. The branching architecture of endotherm lungs maximizes tissue exposure to the circulatory system, enabling sufficient oxygen consumption to feed the higher metabolic rate of warm-blooded animals [7].

Structure and Function of Human Lungs

The 14,000 liters of air that humans inhale daily [12] travels first through cartilaginous bronchi, then through the less rigid and progressively narrowing bronchioles to reach the alveolar sacs where inspired oxygen is passively exchanged with carbon dioxide from the blood stream [13]. Alveoli are composed of a single layer of pulmonary epithelial cells (pneumocytes) separated from the endothelium of the vasculature by a sliver (< 1 µm) of connective tissue [14]. Type I pneumocytes provide a majority of the alveolar surface area but are extremely thin, less than 2 µm in diameter, which enables these cells to facilitate the diffusion of gases between air and blood [7]. Type II pneumocytes are cuboidal secretory cells which synthesize pulmonary surfactant [15], a lipid-protein complex which is crucial for organ function and is described in the next section. Alveolar lining fluid (ALF) includes surfactant and the aqueous hypophase below. This fluid contains complement, antibodies and multiple enzymes which function directly as antimicrobials in addition to influencing immune response mechanisms [16, 17].

The structure of alveoli is maintained by the protein fibers of the alveolar septum, which surrounds alveoli and also contains fibroblasts, blood vessels and the pores of Kohn, surfactant-filled conduits that enable the passage of cells between alveoli [18, 19]. Resident immune cells include alveolar macrophages (AMs, described in detail below)
and a few dendritic cells (DCs), which extend dendrites into the airway lumen to sample for microbes [20] and migrate through the pulmonary interstitium to deliver antigens to lymph nodes [21]. Additionally, type II pneumocytes express antigen presenting molecules and microbicidal enzymes such as lysozyme, contributing to immune responses in this tissue [19]. The space between alveoli and the bloodstream contains interstitial macrophages (IMs), which are less well characterized than AMs but are reported to be more inflammatory in nature [19]. Pulmonary interstitium is highly elastic to enable cyclic expansion and collapse of the tissue. Loss of elasticity contributes to deficits in lung contraction which characterize emphysema [22], while overgrowth of fibrotic tissue impairs inhalation during pulmonary fibrosis [23]. Arterioles in the pulmonary vasculature have thin walls and the high oxygen tension in the area encourages vasodilation, both of which reduce the systolic pressure in this tissue space to roughly 15% of the pressure exerted in systemic arteries. The concomitant vasoconstriction which accompanies regions of hypoxia ensures that blood flow is channeled towards well oxygenated regions of the lung. In addition, the low pressure in pulmonary arterioles prevents blood clots and bubbles from systemic circulation from reaching the vital arterial beds of the brain and heart [7]. The pulmonary vasculature contains intravascular macrophages (IVMs), which perform house-keeping functions in this locale [24].

The lungs are enveloped by a thin layer of tissue called the pleura, which is analogous to the pericardium surrounding the heart and the peritoneum of the abdominal cavity. The inner pleural membrane is separated by a thin layer of fluid from a second parietal pleural membrane which lines the chest cavity. The pleural fluid facilitates the movement of the lungs in conjunction with the chest wall. The diaphragm, which
separates the thoracic and peritoneal cavities, functions as a piston to enable the filling and emptying of the lungs in coordination with the two layers of intercostal muscles which line the ribs [7].

**Pulmonary Surfactant**

The evolution of vertebrate lungs was predicated on the development of pulmonary surfactant [7, 25, 26]. Surfactant is a lipid-protein complex comprised primarily of phosphatidylcholine-based lipids in addition to neutral lipids and four surfactant proteins (SP) A-D, which constitute 10% of total surfactant. The two larger, multimeric hydrophilic collectins, SP-A and SP-D, are involved in coordinating immune responses in the lung [27, 28] and will be discussed in the next section. SP-B and SP-C are relatively small and hydrophobic and interact intimately with the lipid components of surfactant to facilitate adsorption [29]. The predominant lipid species is the zwitterionic dipalmitoylphosphatidylcholine (DPPC), with lower concentrations of polar phospholipids built on glycerol, inositol, ethanolamine and serine head groups in addition to neutral lipids such as cholesterol [30]. All of these components are synthesized by alveolar type II epithelial cells and are packaged into lamellar bodies (LBs) for secretion into the alveolar space, where surfactant is converted to its functional form tubular myelin (TM), a lattice-like configuration of the lipids and proteins complexed together [15]. AMs and type II pneumocytes mediate the breakdown and recycling of surfactant components, with each cell type being responsible for approximately 50% of surfactant catabolism [31].

The saturated acyl chains of DPPC enable molecules of this lipid to associate tightly with one another, ultimately leading to the surface tension reducing function of surfactant
which is imperative for proper organ function [32]. The second most abundant phospholipid species in surfactant, phosphatidylglycerol (PG), coordinates with cholesterol to enable adsorption of surfactant at the air-liquid interface of the fluid-filled lung [25]. The precise cholesterol content of surfactant is dictated by body temperature to create a film of the appropriate fluidity and viscosity to enable surface spreading [33]. By spreading and contracting as a monolayer across the interior of the tissue, surfactant obviates the need for energy expenditure during air intake, thereby greatly facilitating pulmonary expansion during inhalation. Additionally, surfactant prevents alveolar collapse by reducing surface tension when the lungs are emptied [30]. During exhalation, tightly packed DPPC molecules “squeeze out” unsaturated phospholipids and cholesterol, forcing the ALF into a smaller volume and thereby reducing surface tension at the air-liquid interface. This prevents the alveolar walls from sticking together when the lungs are emptied of air. During inflation, the reinsertion of cholesterol and unsaturated phospholipids amongst the DPPC molecules enables respreading of the monolayer with essentially no energy consumption [25].

Due to the functions described above, surfactant is necessary for optimal lung compliance [34], which refers to the changes in volume coupled with changes in pressure during respiration. This process is made easy by the elasticity of pulmonary tissue coupled with the surface tension reduction mediated by surfactant [7]. Pre-term infants exemplify the extreme importance of surfactant in pulmonary function. Prior to 30 weeks gestation, fetal lungs have not yet produced surfactant and pre-term infants are consequently prone to respiratory distress syndrome (RDS) [35]. Surfactant replacement therapy through intratracheal installation reduces mortality from RDS by 40% [36]. In
addition to the crucial biophysical functions described above, the lipids and protein components of surfactant also contribute to immune responses in the lung [37].

**Regulation of Alveolar Macrophages and Immune Responses by Surfactant**

The interior of the lungs is consistently exposed to the external environment. Partitioned by size, a plethora of particulate and biological matter is deposited at various sites along the progressively narrow pulmonary tree. In the upper airways, mucociliary action sweeps larger debris, such as pollen, mold and dust, back up the trachea and this offal is ultimately swallowed [7]. Fine particulate matter (less than 2.5 µm in diameter, PM$_{2.5}$) generated by the combustion of organic fuels can be deposited in the alveolar spaces and cause direct damage to the tissues, as well as modify the response to subsequent infectious challenges [38-40]. Pathogenic microbes which are small enough in diameter can also be inhaled into the terminal branches of the airways, where immune responses are tightly regulated to protect tissue integrity. Patrolling phagocytes are crucial in the maintenance of alveolar sanitation through the removal of inhaled detritus.

AMs are the resident house-keeping cell of the terminal airways [41] and surfactant plays a major role in their phenotypic development and maintenance [42-48]. Studies conducted in murine models indicate that AMs are mostly derived from fetal liver macrophage progenitor cells [49-51] which arrive in the lung early during development [3] although this remains to be verified in humans [52]. The AM community is long-lived under steady state conditions, exhibiting an annual turnover rate of approximately 40%, which is accomplished by in situ differentiation [53-55]. In addition, there is evidence from animal models that following trauma or severe infection blood monocytes contribute to reseeding the AM population [56-58].
The AM phenotype is nuanced and cannot be neatly categorized into the broad classifications generally applied to macrophage subtypes [41, 59, 60]. Furthermore, the AM phenotype is also pliable to accommodate the conditions of homeostasis, infection or injury and tissue healing [61]. AMs are competent phagocytes [62], although their phagocytic capacity is less voracious than IMs [61]. The respiratory burst in AMs is attenuated [61] in part due to the SP-A mediated inhibition of NADPH oxidase assembly [43]. The expression and activity of various surface receptors is also regulated by SP-A [42, 46, 63], which often influences the intracellular fate of phagocytosed pathogens in a manner which is host detrimental [63-66] although not universally [67]. In contrast, by virtue of its four-pronged structure and ability to agglutinate microbes, SP-D encourages bacterial delivery to lysosomes [68-70]. SP-D also regulates pulmonary homeostasis through its influences on AM phenotype [71, 72] and surfactant ultrastructure [73-75]. Although historically relegated to a solely physiological role in the spreading and adsorption of surfactant, SP-C is now recognized to contribute to immune responses through interactions with AM receptors [76, 77].

Surfactant lipids also influence AM phenotype [48, 78, 79] and interactions with pathogens [48, 80, 81]. The typically anti-inflammatory effects of surfactant phospholipids manifest as divergent outcomes for the host depending on the particular pathogen. DPPC can induce expression of prostaglandin E2 (PGE2) [82], decrease the production of reactive oxygen species (ROS) [83] and prevent the entry of certain bacterial pathogens into pulmonary epithelial cells [84, 85]. PG and phosphatidylinositol (PI) prevent binding of lipopolysaccharide (LPS) to cells [86] and inhibit the subsequent inflammatory response [87, 88]. PG also selectively decreases the activity of TLR2 [89] and can prevent viral infection [81, 90]. Conversely, phosphatidylserine (PS) provides a
means for several viruses to gain cell entry through the use of molecular mimicry to take advantage of the mechanisms which mediate PS-dependent uptake of apoptotic cells [91-93]. DPPC enhances adenovirus entry [94] and surfactant phospholipids enhance the growth of several bacterial species [63, 95, 96], occasionally through pathogenic molecular mimicry tactics [97]. The fascinating yet convoluted dynamics between surfactant, AMs and microbes in the context of innate immune responses remains a robust area of investigation.

The tissue-specific phenotype of AMs extends its influence to the adaptive immune response. AMs are poor antigen presenting cells, a feature which encourages immune tolerance to the multitude of banal antigens which find their way to the alveolar spaces [98, 99]. Through the production of prostaglandins and transforming growth factor β1 (TGF-β), AMs suppress T cell activation [100]. Additionally, AMs guide T cells towards expansion of T regulatory (Treg) populations through upregulation of forkhead box p3 (FOXP3) transcription factor [101, 102] and the general suppression of T cell responsiveness in lung tissue has recently been shown to facilitate colonization by metastatic tumor cells [103]. Taken together, decades of research confirm that AMs are well suited to protect the alveolar environment from excessive inflammation. Unfortunately, host-adapted pathogens such as *M.tb* are equipped to exploit this fact.

1.3 Tuberculosis

A Historical and Contemporary Predicament

*M.tb* has been a phenomenally successful pathogen throughout human history. Evidence for skeletal TB presents frequently in 4,000 year old bones found in Egypt,
Europe and the Middle East [104]. Remains recently unearthed in Peru indicate that members of the *Mycobacterial* genus were infecting humans up to 10,000 years ago, although it is currently unknown if those species were human adapted [105]. The earliest known records of a disease which resembles TB are from Assyria in the seventh century B.C.E. Two hundred years later, the ancient Greek doctor Hippocrates wrote often of symptoms reminiscent of consumption [104]. TB became a rampant problem during the population increase incited by urban growth of the 16th and 17th centuries. From 19th century medical records, we can estimate that at least 25% of deaths in Europe were attributable to TB and at this time TB was the leading cause of death in the United States. During the early part of the 20th century, TB was so prevalent, even among the upper classes, that the fever-induced “hectic flush” and accompanying cachexia were romanticized and became fashionable [104].

Impoverished countries of the modern day still suffer from the burden of TB, with the highest incidence in Central and Southern Africa, followed by Western Russia and parts of Asia [106]. A high incidence of new infections, approximately 9 million a year, and a death rate of over 1.5 million per year, culminate in total global costs approaching 8 billion dollars annually [106]. Primary infections (pathology described below) lead to active disease in approximately 10% of patients, while the majority lack symptoms and will eliminate or successfully suppress the infection [107]. Symptoms of active TB include fever, cough, loss of appetite and cachexia, all of which are made worse by malnutrition [108], a physical state which additionally enhances the risk for reactivation TB [109].

Undeniably, TB remains a disease of poverty, with improved sanitation, infrastructure and access to healthcare being a historically tried and true method for TB
eradication [104]. Notable modern high risk populations include the homeless [110, 111], drug addicts [112], the HIV positive [113], prisoners [114, 115], miners [116] and refugees [117]. A lack of access to quality healthcare remains a grim problem for these unfortunate groups of people [118, 119] and 60% of patients with active TB will die without treatment [104]. The blooming increase in drug resistant strains of M.tb underscores the enormity of the problem posed by the smoldering reservoir of subclinical infections: the WHO estimates that nearly one third of humans harbor a latent TB infection (LTBI). For thousands of years, TB has created a global health catastrophe which is impossible to ignore.

Pathology of Infection by M.tb

M.tb spreads between hosts relatively easily, borne within a droplet nuclei expelled from the lungs of an actively infected individual. Naïve contacts who inhale the droplet nuclei can become colonized by an infectious dose of one bacillus, although fascinatingly some individuals who share a home with an active TB case will never contract the infection. M.tb is small enough in diameter (1-5 µm on droplet nuclei) to reach the alveolar spaces, where the bacteria are phagocytosed by AMs and replicate intracellularly [106]. An expanding population of infected AMs, recruited IMs and blood monocytes [120] begin aggregating into a nascent granuloma, surrounding the nidus of infection in the tissue [121]. Simultaneously, bacterial dissemination can occur through the lymphatics to establish extrapulmonary infection sites. Local production of vascular endothelial growth factor (VEGF) induces angiogenesis and early granulomas are highly vascularized structures [122]. As the macrophages are overwhelmed by M.tb growth and begin to die, recruited neutrophils provide some host defense through NADPH oxidase-
mediated bacterial killing [123], although any net positive contributions of neutrophils on
the host response during TB remains controversial [19]. DCs which become infected
[124] will transport news of the infection to the draining lymph nodes [125], priming CD4+
T cells through MHCII-mediated mycobacterial antigen presentation [126]. T cells
produce interferon gamma (IFNγ) and are therefore crucial for the activation of infected
cells [127] as well as maintenance of the granuloma throughout the life of a patient [128].
In addition, B cells are increasingly recognized to contribute to the adaptive phase of the
immune response to TB [129]. However, by the time the adaptive immune system
becomes involved 4-6 weeks after infection [130], the finalization of a mature granuloma
and suppression of bacterial replication is typically the optimal outcome [131]:
sterilization is difficult to achieve even after a complete course of antibiotic therapy and
rarely occurs spontaneously [132].

The resulting status of LTBI can persist for life, but approximately 10% of patients
will reactivate, becoming ill and contagious [106]. A lack of reliable risk factors and
biomarkers is currently a substantial hindrance for predicting which patients are most
likely to reactivate [133]. Identifying a high risk signature is greatly complicated by the
extremely disparate manifestations of LTBI. Among diverse patient populations [134],
and even within the lungs of an individual patient [135], LTBI presents as a complex and
heterogeneous condition. In addition, \textit{M.tb} subpopulations exhibit a convoluted spectrum
of metabolic and physiologic states within any given host [136, 137].

\textit{M.tb} is relatively inaccessible and recalcitrant to antibiotic treatment during LTBI
[138]. In the past, this phenomenon has been attributed to the successful imprisonment
of bacilli within granulomas, concomitant with diminished mycobacterial metabolism
leading to lower rates of bacterial growth and therefore reduced susceptibility to
antibiotics [139]. However, recent evidence shows that \textit{M.tb} resists antibiotic treatment through multiple efflux mechanisms and toxin-antitoxin modules while actively resisting host defense mechanisms through both the bacterial stringent response and the ability to use fatty acids as a carbon source [136]. Human TB granulomas are highly structured, are comprised of a variety of cell types [140] and are dependent on the cytokines tumor necrosis factor alpha (TNF\(\alpha\)) and IFN\(\gamma\), which are integral in the establishment and maintenance of granulomas, partially due to their roles in macrophage activation [141-143]. Loss of granuloma structural integrity can result in airway cavity formation and disease transmission [144]. However, LTBI is not uniformly harbored within granulomas, as bacteremia can occur prior to containment [126] and \textit{M.tb} DNA is detected in both normal lung tissue and extrapulmonary sites [145, 146]. Although LTBI is the predominant form of TB globally, the work presented herein investigates the very early stages of TB and the remainder of this dissertation will focus on interactions between AMs and \textit{M.tb} which occur within the first few days of infection.

**Lipid-Based Virulence Factors of \textit{M.tb}**

\textit{M.tb} is a slow growing bacillus [147] with a fantastically elaborate lipid-rich cell wall [148]. The mycobacterial plasma membrane is surrounded by peptidoglycan covalently linked to arabinogalactan, which in turn is covalently attached to very long chain mycolic acids. These branched, beta-hydroxy acids are found only in mycobacteria and their close relatives [149]. Together, these components complete the interior segment of the \textit{M.tb} cell wall, termed the “cell wall core”, which is insoluble [148]. The outer segment includes non-covalently attached cell wall proteins, sugar-linked mycolic acids known as trehalose-6,6'-dimycolate (TDM), the glycolipid phthiocerol
dimycoserosate (PDIM), phosphatidylmannosides (PIMs), lipomannan (LM) and lipoarrabinomannan (LAM) [148].

Virulent *M. tb* is capped by mannosylated-LAM (manLAM) which can interact with the mannose receptor (MR) of macrophages to induce selective uptake of pathogenic mycobacteria and prevent phagosome-lysosome fusion [64, 150]. PDIMs are crucial for the establishment of infection in a murine model [151] and PDIM mutants are attenuated in the guinea pig [152]. The virulence mechanisms of PDIMs have not been entirely elucidated, but have been linked to resisting killing by IFNγ activated macrophages as well as intercalation into host membranes to decrease fluidity. Additionally, there is evidence that PDIMs are important for structural integrity of the cell wall and protection against environmental insults [149].

TDM, first identified as “cord factor” due to the colony morphology of virulent *M. tb* [153, 154] is sufficient to induce granuloma formation [155] and can elicit expression of pro-inflammatory cytokines and impair phagosome maturation [149]. There are three sub-classes of mycolates (α-, methoxy- and keto-mycolic acids) all of which contain at least one cyclopropane ring which seems to contribute to immunosuppression [156]. Additionally, oxygenated mycolic acids can induce lipid accumulation inside of infected macrophages [157]. *M. tb* may actively shed its cell wall lipids [158] and exocytosis of these lipids from an infected macrophage may contribute to “by-stander” effects on uninfected macrophages [159, 160]. The shedding of mycobacterial cell wall lipids may feed the CD1-dependent presentation of lipid antigens to CD1 reactive T cells [161] and it is important to note that hydrolases in the lung environment alter the mycobacterial cell wall with consequences for host-pathogen interactions [16, 17].
M. tb also produces a variety of lipoproteins which can function to promote virulence. The 19kDa lipoprotein, LpqH, interferes with antigen presentation [162], induces autophagic processes and may facilitate bacterial uptake [163]. LpRG, a secreted lipoprotein, seems to suppress immune responses, may function as a glycolipid transporter to assist in building and maintaining the mycobacterial cell wall [164] and has been shown to interact with several host cell receptors [163]. PstS-1 is an ABC transporter and is an immunodominant antigen in TB patients [165] which induces expression of TNFα and IL-6 [166]. LpqY, ModA and LppX are additional lipoproteins involved in transport or synthesis of the cell wall which have been linked to virulence [163]. The influences of M. tb cell wall lipids and virulence factors on host cell physiology, as well as the interaction of M. tb with intracellular macrophage lipids, are very intriguing areas of active research.

1.4 Macrophage Receptors in Innate Immunity and Homeostasis

Host Pathogen Interactions

The initial host-pathogen interaction occurs in the context of innate immune recognition. Amazingly, plants and animals utilize many of the same molecular mediators for dealing with pathogens, indicating that this system is evolutionarily ancient [167, 168]. The vertebrate innate immune system is comprised of macrophages, DCs, neutrophils, mast cells, eosinophils, basophils and Natural Killer (NK) cells, which express germ-line encoded receptors and an arsenal of response mechanisms which are often sufficient to thwart a pathogenic assault without the involvement of the adaptive immune system [169]. Arguably, epithelial cells and commensal microbes are
additional components of the innate immune system. Host tissue cells can present antigens and produce chemokines to signal a call to arms, as well as cytokines and additional factors to direct the immune response in a given tissue [170-172]. Commensal microbes create a physical barrier against tissue colonization by pathogens and produce immunomodulatory metabolites which not only contribute to the development of the host immune system but additionally participate in both the maintenance of homeostasis and the mediation of immune responses [173-175].

Detection is the first step in the response to any pathogen and can be perpetrated by receptors which are intracellular, secreted or surface localized [169]. Intracellular detection systems include nucleotide-binding oligomerization domain receptors (NOD1 and 2), which can detect bacterial cell wall components and initiate the subsequent activation of NFκβ [176]. *M.tb* produces an unusual glycosylated form of muramyl dipeptide (MDP) which is detected intracellularly by NOD2 [177], leading to expression of inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO) [178]. There are several intracellular members of the Toll-like receptor (TLR) family which sense nucleic acids and are therefore crucial in viral detection but also recognize unmethylated CpG motifs derived from bacterial DNA. TLR9 can detect *M.tb* CpG motifs [179, 180] and has been reported to be host protective during *M.tb* infection [181]. Additional intracellular receptors have been identified but largely mediate the response to viral infection and will not be described herein.

Soluble receptors include mannose-binding lectin (MBL), C-reactive protein (CRP) and serum amyloid protein (SAP) all of which are produced by the liver [182-184]. CRP and SAP are opsonins which can also activate the classical pathway of the complement system [185]. In previously diagnosed TB patients, elevated serum CRP correlates with
a poor prognosis, although conversely CRP is not a useful tool for the detection of undiagnosed TB [186]. Serum concentration of SAP is positively correlated with mycobacterial burden [187] although paradoxically AMs treated with SAP phagocytose lower amounts of *M. tb* [188], leading to reduced intracellular growth in a NO-dependent manner [187]. MBL is a collectin (as are SP-A and SP-D) and binds to microbial mannose moieties. The MBL-associated serine proteases (MASP1 and 2) initiate the lectin complement pathway cascade [184]. MBL increases phagocytosis of *M. bovis* BCG by neutrophils [189] and elevated serum concentrations of MBL during active TB has been linked to host susceptibility [190]. There are clearly a variety of mechanisms by which host cells detect *M. tb*; however, the remainder of this section will elaborate on immunoregulatory and homeostatic macrophage surface receptors of relevance to the present work.

**Macrophage Surface Receptors**

In order to interact with their environment, macrophages express a wide range of surface exposed molecules [191]. The inflammatory status of a tissue, as well as the tissue itself, influences the receptor repertoire which is expressed by a particular macrophage subset. In turn, this context-dependent receptor expression regulates the ability of macrophages to mediate appropriate effector functions [2]. Ligation of surface expressed receptors sparks an array of cellular responses, including cargo uptake, intracellular signaling and alterations in gene expression as well as the return to tissue homeostasis following infection or injury [191]. Several macrophage pattern recognition receptors (PRRs) of importance during TB will be briefly described, prior to a more extensive explanation of receptors which are of relevance to the present work.
Pattern Recognition Receptors

The MR is highly expressed by AMs [41] and mediates immunomodulatory and homeostatic clearance of mannose-rich material [192]. The \textit{M.tb} cell wall is highly mannosylated and uptake of pathogenic mycobacteria through the MR [193] results in bacterial survival due to a lack of phagosome-lysosome fusion [64, 150] and decreased production of NO, ROS and pro-inflammatory cytokines [192]. The cell walls of virulent strains of \textit{M.tb} contain higher amounts of mannosylated terminal residues, leading to preferential uptake of pathogenic mycobacterial species [193, 194]. Complement receptors (CRs) also contribute to the uptake of \textit{M.tb} but are less discriminatory among strains [64]. CR4 is highly expressed by AMs and may be a major route of entry by \textit{M.tb} into naïve individuals [195, 196]. CR3 mediates both opsonic and non-opsonic uptake of \textit{M.tb} by human macrophages [197], while in mice this function seems to be redundant and dispensable [198].

Despite their pro-inflammatory signaling cascades, the relative benefit for the host following \textit{M.tb} ligation of TLRs is controversial [192]. TLR2 recognizes mycobacterial PIMs, lipomannan, TDM and several of the lipoprotein antigens described above. TLR2 ligation by various cell wall components results in distinct host responses depending on the mycobacterial species and host cell types involved. Less virulent species of mycobacteria are killed by the pro-inflammatory responses induced following TLR2 ligation, while pathogenic species may use TLR2 signaling to inhibit antigen presentation to T cells [199]. TLR4 exhibits reduced function in the lung due to the immunosuppressive effects of surfactant [46] and has been less well studied during TB but does recognize mycobacterial heat shock protein (hsp) 65 [200].
**Immunoglobulin Superfamily (IgSF) Members**

IgSF receptors are grouped together due to the presence of immunoglobulin domains and therefore this family encompasses antibodies, antigen presenting molecules, cytokine and growth factor receptors, as well as leukocyte and NK cell restricted receptors [201]. The IgSF subsets expressed by macrophages typically mediate intracellular signaling responses and contain several groups which will be described briefly (in addition to a more detailed description of CD200R, which is of relevance to the present work). Triggering receptor expressed by myeloid cells (TREM) contains four proteins, of which TREM1 is an activating receptor expressed by macrophages [191]. Sialic acid-binding immunoglobulin-like lectins (Siglecs) participate in the discrimination between self and non-self and are generally inhibitory in function [202]. Monocyte/Macrophage Ig-like receptors (MIRs) associate with either immunoreceptor tyrosine-based inhibitory or activating motifs (ITIMs and ITAMs, respectively) which modulate their function [191]. SIRP-α (CD127a) is an inhibitory receptor which can bind to SP-A and SP-D to maintain AM quiescence in the absence of infection [203]. In addition, binding of SIRP-α to its main ligand, CD47, can serve as a “self” signal to prevent the unprompted phagocytosis of healthy erythrocytes [191].

**CD200R**

An inhibitory signaling receptor expressed on leukocytes, CD200R has one known ligand termed CD200 [191]. The signaling axis induced by CD200R ligation results in immunotolerance and prevention of inflammatory processes [204, 205]. Ligation of CD200R leads to tyrosine phosphorylation of the adaptor protein DOK2,
which recruits and activates RasGAP [206] to inhibit downstream RasMAPK pathways [207]. This signaling response is negatively regulated by DOK1 and CrkL [206]. CD200R is expressed on diverse subsets of tissue macrophages, mast cells and T cells, while the ligand is expressed on epithelial cells, neurons in the central nervous system (CNS), placental trophoblasts, certain kinds of tumor cells, follicular DCs, activated T cells and B cells [208]. Due to its immunosuppressive functions and wide distribution, therapeutic activation of CD200R hints at a plethora of clinical applications, such as the prevention of neurodegeneration and tissue graft rejection.

Decreased expression of the CD200:CD200R axis in the CNS has been shown to occur with aging and is thought to contribute to neurodegenerative diseases such as Alzheimer’s, Parkinson’s and multiple sclerosis (MS) [209]. CD200 knockout mice acquire experimentally induced encephalitis (EAE, a model for MS) very readily, because their microglial cells are hyperactive [210]. Maintaining expression of CD200:CD200R in the CNS during aging or neural deterioration is therefore tentatively promising avenue to alleviate the establishment and progression of neurodegenerative diseases. In addition, CD200R activation could prevent some of the negative effects mediated by macrophages during wound healing. For example, ligation of CD200R inhibits neovascularization through down-modulation of genes involved in angiogenesis. This finding was demonstrated to have translational applications as monoclonal antibodies against CD200R can prevent choroidal induced neovascularization, which is mediated by macrophages and can lead to macular degeneration [211]. Interestingly, CD200R activation can prevent graft rejection in some tissues [212] but not others [213]. In the context of cancer, blocking CD200R signaling could alleviate the tolerance induced by tumor cells which express CD200 [214].
CD200R is of particular interest in the lung environment. In comparison to IMs and other tissue macrophages, murine AMs abundantly express CD200R and the ligand is expressed by pulmonary epithelial cells [215]. Chronic interactions between the apposed AMs and pneumocytes is important for controlling AM populations and maintaining the immunosuppressive AM phenotype [41]: CD200 knockout mice have twice as many AMs, which exhibit a pro-inflammatory phenotype even in the absence of infection [215]. CD200R has been implicated in chronic, heritable diseases such as asthma [216] as well as viral and bacterial pulmonary infections.

This signaling axis is host protective to the extent that it limits pathology, as an exacerbated response to influenza challenge results in the deaths of CD200 knockout mice. The fatal pathology appears to be mediated by macrophages [215] and can be prevented by T cell depletion, indicating that T cell dependent activation of macrophages in the absence of concurrent input through CD200R can be grossly destructive [217]. Conversely, this system can be exploited by pathogens in order to establish infection and persist in mucosal tissues. During the resolution phase of an infection, apoptotic host cells which express CD200 create susceptibility to a secondary infection [218]. Amazingly, several types of viruses express CD200, which seems to enhance their success in mucosal tissues generally [219]. To our knowledge, no one has investigated any role for CD200:CD200R during TB.

**Scavenger Receptors**

A supergroup consisting of eight subclasses (A-H), scavenger receptors (SRs) share little sequence similarity across classes but recognize similar foreign and endogenous ligands, such as phospholipids, cholesterol esters, apoptotic cells,
carbohydrates, proteoglycans and lipoproteins [220]. SRs are expressed on myeloid cells and some types of endothelial and epithelial cells. Among many functions, SRs contribute to immune responses through pathogen recognition and uptake, intracellular signaling and antigen cross-linking. Additionally, SRs maintain homeostasis through the removal of modified self proteins and lipids. However, under conditions of excess, SR functions can lead to disease states, such as during atherosclerosis and Alzheimer's [221]. For the sake of brevity, only SRs of relevance to the present work are discussed herein.

**Scavenger Receptor A**

SR-A was the first member of this supergroup to be identified and was discovered in the context of modified low density lipoprotein (LDL) uptake. The three isoforms of SR-A (isoform III is non-functional) are expressed by macrophages but not monocytes and SR-A is found on mast cells, DCs, smooth muscle cells, fibroblasts and some epithelial cells. SR-A contributes to foam cell formation and mediates the binding of macrophages to the extracellular matrix of smooth muscle cells during atherosclerotic plaque development [222]. The onset of Alzheimer’s is facilitated by SR-A mediated binding of microglia to β-amyloid fibers, which leads to the neuron damaging response of ROS production [223]. SR-A also contributes to the removal of apoptotic cells and advanced glycation end products (AGE) which can cause tissue damage. Depending on the particular ligand, different intracellular signaling pathways are initiated [221].

SR-A can also scavenge pathogen-derived ligands and contributes to immune responses which are typically pro-inflammatory. SR-A binds to the lipid A moiety of LPS and also recognizes lipoteichoic acid (LTA) [224]. During polymicrobial sepsis, SR-A
pairs with TLR4 to induce the NFκβ signaling patterns which ultimately produce the fateful cytokine storm [225]. Interestingly, SR-A binds extracellular double stranded RNA (dsRNA) and internalizes and delivers this cargo to intracellular nucleic acid sensors such as TLR3 to induce the expression of interferon inducible genes [226]. SR-A typically seems to perform host protective functions and knockout mice are more susceptible to *Listeria monocytogenes* and *Staphylococcus aureus* [227]. The reverse may be true for chronic *M. tb* infection [228], although SRs have also been reported to be superfluous during TB in a murine model [229]. Due to the well established differences between humans and mice, it remains necessary to determine the relevance of SR-A during TB in humans.

**MARCO**

Many subsets of tissue macrophage exhibit constitutive expression of Macrophage Associated Receptor with Collagenous structure (MARCO), which is also upregulated during infection. Endogenous ligands for MARCO are expressed by B cells and facilitate the sequestration of marginal zone B cells in the spleen [221]. Additionally, bronchial epithelial cells express a MARCO ligand [230] which provides a source of tissue-derived anti-inflammatory signals during the resolution phase of infection [231]. MARCO can also weakly bind acLDL as well as oxLDL [221] and ozone modified lipids [232]. In the airways, MARCO is the primary receptor for unopsonized particulate matter and microbes [233] and serves to tether TDM to TLR2 and CD14, allowing for the propagation of pro-inflammatory signaling responses through NFκβ [234]. Similar to SR-A, the rapid uptake of ligands by MARCO has been shown to result in delivery of ligands to various intracellular sensors, including TLR3 and NOD-like receptors (NLRs), while
simultaneously limiting pro-inflammatory signaling through surface expressed TLR4 [235].

Given their structural homology, the “scavenging” ability of SRs has been viewed as somewhat paradoxical but can at least partially be attributed to variable locations of polarized amino acids, an explanation referred to as the electrostatic patch model. For example, SR-A and MARCO both have conserved scavenger receptor cysteine-rich (SRCR) regions but this domain serves different functions in the two proteins. MARCO binds LPS, modified LDL and bacteria with an arginine patch within its SRCR. SR-A lacks this arginine patch and uses its SRCR to interact with other membrane proteins, while ligand binding is mediated by a lysine residue in the collagen domain [236].

**SR-B1**

A class B scavenger receptor which is integral to whole body cholesterol homeostasis and reverse cholesterol transport, SR-B1 mediates the uptake of high density lipoprotein (HDL) in the liver. HDL sequesters potentially harmful free cholesterol and shuttles it to the liver for clearance; HDL is therefore protective against the development of atherosclerosis [221]. However, hepatitis C virus can exploit SR-B1 dependent HDL uptake to gain entry into host cells [237]. In addition to hepatocytes, SR-B1 is expressed on macrophages, DCs, neutrophils, adrenal glands and steroidogenic tissues. SR-B1 can also bind serum amyloid A (SAA), which leads to cholesterol efflux from cells and β–amyloid, again contributing to Alzheimer’s [221]. SR-B1 enhances LPS-mediated inflammation in the liver and kidney [238] and contributes negatively to malaria [239]. Of relevance to the lung, SR-B1 is also expressed by pneumocytes and during pneumonia SR-B1 positively regulates neutrophil migration and pro-inflammatory
responses [240]. *M.tb* can bind to SR-B1, however this receptor does not influence bacterial burden or survival in a murine model [241] and any role for SR-B1 during human TB pathology remains unknown.

**CD36**

Glycoprotein IV (GPIV) was discovered in 1989 as the protein expressed by platelets which binds to thrombospondin-1 to enable blood clotting [242]. Four years later, fatty acid translocase (FAT) was identified as a mechanism of fatty acid uptake in adipocytes [243] and simultaneously the macrophage uptake receptor for oxLDL was discovered and named CD36 [244]. The discovery of CD36 within these divergent contexts was a good indicator of the wide-ranging aptitude of this class B scavenger receptor, which functions centrally in both homeostasis and infection [245].

CD36 has two transmembrane motifs and truncated cytoplasmic tails which can interact with Src family kinases to initiate signaling cascades [246]. Additionally, these tails are post-translationally glycosylated, acetylated, ubiquitinated and/or palmitoylated to regulate CD36 expression levels and cellular location. Interestingly, binding of different ligands leads to different modifications. For example, fatty acid binding leads to ubiquitination, but insulin does not [247]. The hairpin-looped extracellular domain of CD36 is N-link glycosylated at multiple sites, bringing the molecular weight of the mature protein up to 90 kDa; however, the degree of glycosylation and therefore the molecular weight varies among tissues. Three disulfide bridges in the C-terminal half of the extracellular domain are required for membrane expression [247] and once at the cell surface CD36 can be reversibly phosphorylated, leading to alterations in binding affinity [246]. The N-terminal half of extracellular CD36 contains binding sites for fatty acids,
hexarelin, oxLDL, phospholipids, malaria infected erythrocytes and thrombospondin [247] and in coordination with several TLRs CD36 can sense microbial cell wall components [248, 249]. Additionally, as a heterotrimer with TLR4 and 6, binding of certain ligands by CD36 can result in sterile inflammation [250]. The internalization of CD36 bound ligands can occur through membrane translocation, endocytosis or phagocytosis in conjunction with FcRγ [236].

In addition to platelets, adipocytes and macrophages, CD36 is expressed by DCs, microvascular endothelium, pulmonary, retinal and mammary epithelium, smooth muscle, skeletal muscle, cardiac myocytes, microglia, taste buds and keratinocytes and can be induced in hepatocytes [246]. CD36 is thought to be a major culprit in the development of atherosclerosis by mediating unchecked uptake of lipoproteins, thereby causing foamy macrophage (FM) formation and the initiation of the atherosclerotic plaque [251]. CD36 is an evolutionarily ancient protein and the removal of oxidatively modified lipids from the circulation may be an example of an evolutionarily conserved macrophage responsibility which is ill-adapted to accommodate the Western diet. In that vein, CD36 has been implicated in several lipid metabolic diseases [246].

In the setting of a high fat diet, CD36 has been linked to insulin resistance. The CD36-dependent uptake of large amounts of fatty acids can interfere with intracellular lipid storage and cycling of GLUT4 to the cell surface [252]. Without GLUT4 at the membrane to mediate glucose uptake, insulin resistance can develop [253]. The cycle is perpetuated due to insulin-mediated prevention of CD36 ubiquitination, which has been reported to trap CD36 at the cell membrane where it can continue to import fatty acids [254]. CD36 may contribute to cardiac ischemia through its propagation of atherosclerotic plaques and its anti-angiogenic properties [246], and, similar to the other
scavenger receptors described above, CD36-dependent recognition of β-amyloid fibers has been linked to Alzheimer’s disease [236]. CD36 is also involved in many of the risk factors leading to stroke, such as hyperlipidemia and insulin tolerance [255] and has been shown to contribute to the subsequent tissue pathology following cerebral ischemia [256]. However, the etiology of these phenotypes is extremely complicated and expressly multi-factorial. For example, insulin resistance is also observed in spontaneously hypertensive rats (SHR) which are CD36-deficient [257].

Although clearly pathological in many contexts, CD36 also performs basic and beneficial functions relating to systemic health. CD36 on taste buds senses fatty acids, and interactions with afferent nerve fibers inform the brain and the gustatory nerves of the incoming fatty meal [247]. CD36 expression levels are decreased on taste buds following exposure to fatty acids, which may constitute a mechanism to prevent over-eating [258]. Disruption of this feedback mechanism has been implicated in diet-induced obesity and the resulting chronic inflammatory state of metabolic syndrome [251]. In coordination with vitronectin receptor (αvβ3 integrin) and thrombospondin, CD36 mediates the clearance of apoptotic cells, a crucial aspect of the resolution phase of inflammation [191]. However, although removal of cellular corpses is critical for a return to homeostasis, CD36 expression in the context of wound healing has been shown to enhance fibrosis. Despite the presence of non-phagocyted apoptotic cells, CD36-deficient mice develop less fibrotic matrix material during recovery from kidney injury or ischemic reperfusion [259].

CD36 also participates in a variety of pathogenic encounters, again with varying results for the host depending on the particular pathogen and the host cell type in question. Due to its scavenging abilities, CD36 on macrophages can participate in the
uptake of gram positive and gram negative bacteria [260], fungal pathogens [261] and malaria-parasitised erythrocytes [262]. Conversely, endothelial cell expression of CD36 results in tethering of parasitized red blood cells to the vasculature. This grants time for the malaria parasites to replicate by preventing infected erythrocytes from reaching the spleen, where the infection can be cleared [263]. Interestingly, HIV virions utilize CD36 to bud from infected cells [264] and CD36 blocking antibodies reduce the transfer of HIV from macrophages to T cells [265]. Somewhat paradoxically, the Nef protein produced by HIV has been reported to decrease CD36 expression in macrophages. The authors contend that the resulting decrease in bacterial uptake and reduction in pro-inflammatory TNFα may contribute to the increased susceptibility of AIDS patients to opportunistic pathogens [266]. It is worth noting that the link between CD36 and TNFα production varies by context, as increases in TNFα have been reported in CD36-deficient renal cells [259].

In keeping with the contextual dependence of CD36 function during infection, CD36 can be specifically host protective during infection with bacterial pathogens in the pulmonary environment [267]. During pneumococcal pneumonia, CD36 is necessary for tempering the inflammatory responses which damage pulmonary integrity [268]. Conversely, CD36 can partner with TLR4/6 to induce pro-inflammatory cytokine production in a c-Jun N-terminal kinase (JNK)-dependent manner [260]. CD36 also heterodimerizes with TLR2 to sense gram positive cell wall components [249] and the expression of CD36 during S. aureus infection is host protective in this capacity [248]. Recently, it was discovered that CD36 is capable of signaling without the help of TLRs. CD36-dependent signaling through Src family kinases leads to Ca\(^{2+}\) influx which activates cytoplasmic lysosomal phospholipase A2 (cLPA\(_2\)) to release arachidonic acid
(AA) from membrane phospholipids for the production of eicosinoids, specifically PGE$_2$ [247]. This is of relevance for *M.tb* infection, as increased expression of PGE$_2$ has been linked to a host susceptibility phenotype [66].

The exact contribution and relevance of CD36 during *M.tb* pathogenesis is somewhat obscure, despite having been investigated in several model systems. In guinea pigs, serum concentrations of oxLDL increase over the first few months of infection. In parallel, by day 60 post-infection levels of CD36 in the lung have increased substantially [269]. An increase in CD36 promoter activity has been shown in COS-1 cells much earlier after infection with the attenuated laboratory strain H$_{37}R$$_v$, although an actual increase in CD36 expression was not reported [270]. A murine model of infection with *M. bovis* BCG found that CD36 deficient mice exhibited lower bacterial burdens and reduced dissemination to distal organs, indicating that BCG had a more difficult time of colonizing mice which lacked CD36. However, the authors did not attribute this observation to differences in cytokine production, and ex vivo experiments using macrophages from CD36 knockout mice infected with *M.tb* showed no difference in bacterial uptake or the production of ROS [271]. Another group reported that a double knockout of CD36 and SR-A was less susceptible to initial colonization by *M.tb*, although during chronic stages of infection the lack of these two receptors did not have a noteworthy impact on disease outcome [229]. Therefore, more research is needed to determine the role of CD36 during mycobacterial infection and this prompted our undertaking of the research reported herein.
1.5 Macrophage Lipid Metabolism and Modulation by *M.tb*

The recycling or final destruction of environmental material is a paramount function of tissue macrophages [2]. AMs are crucial contributors to surfactant lipid catabolism [31, 272-278] and this duty is requisite for pulmonary performance [279-281]. The role of AMs in surfactant lipid breakdown (described in Chapter 3) results in a macrophage which contains higher amounts of intracellular lipids and metabolites under steady state conditions than macrophages which are derived from other tissue spaces [78, 79]. *M.tb* has been shown to utilize host-derived lipids as a carbon source during infection [122, 157, 282-288] and it seems likely that surfactant lipid metabolites could provide mycobacteria with nutrients from inside an AM. In addition, the continuous interaction between AMs and immunosuppressive surfactant components likely renders these cells more susceptible to *M.tb* infection [43-46, 63, 289]. *M.tb* can induce lipid accumulation inside an infected macrophage [157, 290], creating a nutrient filled cell which will ultimately undergo necrosis and contribute to caseation of the granuloma core, enabling disease transmission [122]. Therefore, the mechanisms by which *M.tb* disrupts host lipid metabolism are of great interest to the field. In the context of the relatively lipid-rich AM, it is intriguing to speculate that *M.tb* may not need to extensively manipulate host cell physiology in order to gain access to a lipid-based carbon source. Below is a description of metabolic pathways in both basal energy production and the perpetuation of immune responses, followed by a description of pathogenic interactions with host lipids.

**Metabolic Modifications for Host Defense**

During homeostasis, the β–oxidation of fatty acids can generate acetyl-coA to fuel the tricarboxylic acid (TCA) cycle within mitochondria; additionally, the glycolytic
breakdown of glucose feeds pyruvate into the TCA. The subsequent production of NADH and FADH$_2$ provides reducing power for the electron transport chain (ETC), which ultimately enables the production of ATP. This mitochondrial mechanism of energy transfer is highly fruitful for the cell and is termed oxidative phosphorylation (OXPHOS) because it requires oxygen as a terminal electron acceptor. In hypoxic conditions, glycolysis can produce lactate in the cytosol but this is a much lower energy boon for the cell [291].

During some infection scenarios, aerobic glycolysis has been reported as a mechanism to funnel metabolites towards anti-microbial functions. Infection with bacterial pathogens and TLR signaling can induce this “glycolytic reprogramming” which channels TCA intermediates towards the generation of ROS, eicosinoids and anti-microbial metabolites such as itaconate, [291] which can inhibit M.tb methylisocitrinate lyase [292]. In fact, AMs infected with M.tb cannot control bacterial growth if the metabolic shift to aerobic glycolysis is inhibited [293]. In contrast, increased β–oxidation and OXPHOS is observed in memory T cells and macrophages involved in wound healing [291]. It is therefore interesting to speculate that β–oxidation of surfactant lipids contributes to the immunoregulatory AM phenotype under steady-state conditions.

Clearly, metabolism not only provides fuel for cellular processes but the additional dividend of immunological effector molecules. The release of particular metabolic by-products or their derivatives communicates information about the health status of a cell or tissue, shaping the environment into which effector cells are recruited. Thus, in addition to mediating the primary effector purposes described above, these signals can secondarily contribute to the prevention of tissue damage and the resolution of inflammation [291]. For example, the lactate produced by aerobic glycolysis during
infection is exported from the cell and polarizes adjacent macrophages towards an anti-inflammatory phenotype [294]. Fascinatingly, tumor cell production of lactic acid has been linked to the induction of the immunosuppressive tumor-associated macrophage (TAM) phenotype, which promotes tumor growth [295].

Lipid flux specifically is crucial for cellular energy, maintenance of membrane integrity and generation of molecular messengers. Dysregulation of lipid metabolism and homeostasis through excess dietary fats or genetic disorders leads to several inflammatory pathologies, which may predispose those individuals to infection [291]. Type 2 diabetes is now recognized as a risk factor for TB [296, 297], partially due to increased production of IL-10 [298] and an extended delay in the onset of adaptive immunity followed by an over-aggressive Th1 response [291]. In mice, hyperlipidemia has been linked to augmented myelopoiesis, Alzheimer’s and spontaneous inflammatory remodeling of the lungs. When given a high cholesterol diet, hyperlipidemic mice are very susceptible to M.tb due to intensified pathology and escalated bacterial dispersion [299]. Conversely, a cholesterol-rich diet in mildly hyperlipidemic humans correlates with M.tb containment within granulomas [300]. M.tb mutants which cannot import [282] or metabolize [287] cholesterol do not survive within murine in vivo or in vitro models. Studies using human macrophages have shown that cholesterol is important for M.tb uptake [301] and that M.tb phagosomes merge with infection-induced lipid droplets (described below), which contain triacylglycerol (TAG) and esterified cholesterol. In this context M.tb has been shown to acquire host TAG [157], but to our knowledge the acquisition of host cholesterol from human macrophages has not been explored and merits investigation.
There is a current controversy as to whether lipid accumulation in macrophages is inherently pro-inflammatory [302] or whether external signals from the tissue environment secondarily lead to the production of cytokines and ROS which are associated with the progression of lipid overload conditions such as atherosclerosis [303, 304]. In the case of infection, there is a balance between attempting to regain homeostasis and obliterating the pathogen. The activity of lipid uptake receptors such as CD36 can have both pro- and anti-inflammatory affects in this context, for example leading to the release of TNFα while promoting the subsequent transition to an immunoregulatory macrophage phenotype [303]. The interplay between lipid metabolic pathways and host defense mechanisms is integral in immunological responses, but can be overwhelmed by substrate excess or infection, leaving macrophages with no choice but to contain lipids within organelles which accumulate in the cytosol [291].

**Lipid Droplets**

Excess lipids inside of cells are stored as lipid droplets (LDs), dynamic organelles which provide a readily accessible, energetically-dense fuel source [305]. In addition, LDs contain cytokines, enzymes [306] and the precursors for lipid mediators [307] which participate in immune responses [308]. These organelles are present in few cell types during homeostasis and are predominantly found in adipocytes as a lipid storage site. The pathology of several kinds of cancer involves lipid dysregulation and the abnormal formation of LDs. Interestingly, tumor cells release soluble factors which induce LD accumulation in non-cancerous cells [308]. Infection with eukaryotic parasites [309, 310], bacteria [157, 311, 312] and viruses [313, 314] can induce cytoplasmic accumulation of
LDs and is commonly viewed to be beneficial for the pathogen. For example, replication of many viruses and some bacterial species depends on LDs [312, 314].

LDs are predominantly composed of TAG and cholesterol esters (CE), surrounded by LD-associated coat proteins [305, 315]. LDs are formed in the endoplasmic reticulum and bud into the cytosol in a manner analogous to the formation of aqueous-cored vesicles from the endosomal system [316]. Perilipin and adipophilin are constitutively associated with LDs and regulate the availability of LD content. When not associated with LDs, these two coat proteins are degraded in proteasomes. Perilipin is heavily expressed in adipocytes and maintains extremely stable LDs, enabling long-term fat storage [317]. In contrast, adipophilin has a broad expression pattern and does not guard as firmly against hydrolysis, rendering the content of LDs in other tissues more bioavailable [318]. The exchangeable coat proteins, Tip47, S3-12 and OXPAT, are preformed and stable in the cytosol and associate with nascent LDs to accommodate rapid intake of dietary fats [316].

The ability of mycobacteria to induce LD accumulation has been reported for several species and occurs in a variety of host cells. *M. leprae* induces the accumulation of LDs inside of Schwann cells, contributing to peripheral neuropathy and enhancing bacterial survival [311]. *M. marinum* causes LD formation in the amoeboid Dictyostelium model [319], which augments bacterial numbers through the metabolism of host lipids [320]. Lung macrophages from BCG-infected guinea pigs display a foamy phenotype [269] and human macrophages infected with *M.tb* or oxygenated mycolic acids from virulent *M.tb* accumulate LDs which can be transferred to the bacteria through fusion with phagosomes [157]. A particular mechanism by which *M.tb* can induce lipid retention has recently been attributed to miR-33 dependent inhibition of cholesterol efflux
However, we speculate that during pulmonary infection *M.tb* capitalizes on the endogenous existence of cytoplasmic lipid inclusions inside of AMs.

### 1.6 Goals for the Dissertation

**Central Hypothesis and Specific Aims**

Macrophages are integral mediators of internal homeostasis and defenders against the external environment. Decades of research have solidified the notion that tissue resident macrophages are shaped by their locale, resulting in an array of macrophage subsets which are astonishingly diverse in their specializations. Our central hypothesis was that pulmonary surfactant skews AMs to a phenotype which is susceptible to infection by *M.tb*. Specifically, we hypothesized that the scavenger receptor CD36 mediates the uptake of surfactant lipids and that the CD36-dependent presence of those lipids inside of a macrophage would augment *M.tb* growth. To this end, the research presented herein explored the role of pulmonary surfactant in the expression of macrophage surface receptors and lipid metabolic regulators in the context of infection with *M.tb*. The ultimate goal of this research was to aggrandize our appreciation of the endogenous pulmonary environment in host susceptibility to *M.tb* colonization and was undertaken in the pursuit of three specific aims. The first aim is described in Chapter 2 and sought to determine macrophage surface receptors of relevance to TB which are regulated by surfactant. The second aim was to evaluate the receptor(s) involved in surfactant lipid uptake by macrophages. These findings, presented in Chapter 3, constitute the first description of a receptor mediating the uptake of a specific surfactant lipid species by macrophages. The third aim investigated the
impact of receptor-dependent surfactant lipid uptake on the intracellular growth of *M.tb* and the modulation of lipid storage, catabolism and efflux mechanisms by surfactant and *M.tb* infection.
CHAPTER 2: PULMONARY SURFACTANT AND M. TB INFECTION

REGULATE THE EXPRESSION OF SCAVENGER RECEPTOR

CD36

2.1 Summary

*Mycobacterium tuberculosis* (*M. tb*) predominantly causes pulmonary disease [107] and AMs are the primary host cell for this pathogen [322]. Due to the long and exclusive evolutionary history between *M. tb* and human beings, as well as this pathogen’s continued global success [323], it is likely that the endogenous pulmonary environment into which *M. tb* is inhaled is amenable to colonization. The very nature of this tissue space may facilitate TB disease and AMs seem to be particularly hospitable to *M. tb* growth [289, 324]. Therefore, characterizing the tissue-specific mechanisms which regulate AM physiology will lend new insights into TB pathogenesis. Building our knowledge of the endogenous pulmonary environment during homeostasis, which is the context for the initial interactions between *M. tb* and its host, will enable us to understand how *M. tb* is so successful in establishing a lifelong pulmonary infection. We therefore decided to investigate aspects of the lung environment which may steer macrophages toward a phenotype which is susceptible to infection by *M. tb*.

Pulmonary surfactant is a crucial mechanistic component of proper lung function [30] and is additionally important for regulating tissue-appropriate behaviors within the alveolar space due to its influence on AM phenotype [37, 41, 47]. The lung is a unique organ, inherently delicate to enable gas exchange yet consistently exposed to particulate
and biological matter from the environment. By inhibiting macrophage ability to proceed with robust pro-inflammatory signaling pathways and responses, surfactant serves to protect pulmonary tissue integrity despite chronic environmental insult. Our previous work has shown that blood monocyte-derived macrophages (MDMs) which have been exposed to SP-A exhibit diminished signaling through TLR2 [46] and are unable to assemble the NADPH oxidase complex [43]. Additionally, we have shown that surfactant lipids are able to block M.tb-induced TNFα release [63] as well as inhibit NFκβ activity while increasing IL-10 expression following macrophage exposure to LPS [44]. Ex vivo experiments using AMs have also demonstrated the immunosuppressive effects of surfactant [45] and AMs isolated from the lungs of SP-A knockout mice are restored to wild type phenotype following exposure to exogenous SP-A [325]. Based on these results, we hypothesized that surfactant-induced features of the immunoregulatory AM phenotype result in susceptibility to M.tb.

AMs are responsible for keeping the alveolar airspaces free of both inhaled debris and the detritus generated by immune responses [326]. This function is mediated by the expression of various surface receptors and we sought to determine which macrophage receptors may be regulated by pulmonary surfactant and/or M.tb infection. We first elected to investigate CD200R, an immunosuppressive signaling receptor [207] which is constitutively expressed by AMs and serves to limit the extremity of inflammatory responses in the lung [215]. We conducted experiments to determine whether CD200R could be an aspect of the AM phenotype which is regulated by surfactant and whether expression of this receptor would be affected by M.tb infection. Ultimately, we show that CD200R is not regulated by either surfactant or M.tb infection in human macrophages.
Subsequently, we selected several scavenger receptors (MARCO, SR-A, SR-B1 and CD36) which are important in the maintenance of pulmonary homeostasis [236, 327] in order to ultimately explore the hypothesis that one or more of these receptors could contribute to surfactant lipid uptake. We began our investigations by cataloguing the effects of surfactant and *M. tb* on receptor expression because all four receptors have been linked to TB in cell lines or animal models. MARCO is known to mediate the removal of non-opsonized particles [328] and microbes [233] from the lung, as well as cooperate in the signaling response to components of the mycobacterial cell wall [234]. SR-A mediates uptake of cholesterol [329] and participates in host responses to a variety of pulmonary pathogens, although the role of this receptor is not always host protective [330, 331]; in fact, SR-A contributes negatively to the chronic stages of TB in a murine model [228]. SR-B1 functions in cholesterol transport as well as pathogen recognition [332, 333], and contributes to the uptake of *M. tb* in a murine model, but does not impact host survival [241]. CD36 is well characterized to mediate lipid uptake into a variety of tissues [334, 335] and negatively influences the early host response to mycobacterial infection [63, 271]. Among these four scavenger receptors, we found that CD36 alone is positively regulated by surfactant and, counter to our hypothesis, CD36 expression is decreased by *M. tb* infection of human macrophages.
2.2 Results

CD200R expression in human macrophages is not regulated by surfactant exposure or *M. tb* infection

CD200R is highly expressed by AMs relative to other tissue macrophages and is thought to contribute to the immunoregulatory AM phenotype [215]. We sought to determine whether human macrophage expression of CD200R could be regulated by surfactant and began our investigation by exploring the effects of SP-A on *CD200R* transcript levels during the differentiation of monocytes into macrophages. As shown in figure 1A, the addition of SP-A on freshly isolate (day 0) monocytes results in decreased expression of *CD200R*, followed by a transient increase in *CD200R* transcripts in day 1 and 2 monocytes. However, transcript levels recede below baseline expression when SP-A is administered on subsequent days during the differentiation process (Fig 1A). Since the goal of our studies was to understand how surfactant components affect receptor expression on mature macrophages as opposed to blood monocytes, we decided to focus on differentiated macrophages (day 6 MDMs) for the remainder of our investigation.

In order to evaluate the optimal dose and timing of SP-A for differentiated macrophages, we conducted a dose-response experiment using increasing concentrations of SP-A. There is a trend towards inhibition of *CD200R* transcript levels 6h after exposure, regardless of SP-A concentration. 24h after exposure, there is a trend towards increased expression, with the lowest concentration of SP-A having the most pronounced effect. By 48h, *CD200R* expression has returned to baseline, regardless of
SP-A concentration (Fig 1B). Based on these results, we decided to use 10 µg/mL SP-A for future experiments.

The majority of surfactant is composed of various lipid species [30] and we therefore incorporated surfactant lipids in the form of Survanta (commercially available bovine-derived surfactant lacking the major immunomodulatory proteins) into our experiments. We first sought to determine whether short term surfactant exposure could induce CD200R expression and exposed MDMs to SP-A (Fig 1C) or Survanta (Fig 1D) for various times. CD200R expression levels were similar to baseline at all time points tested. Although we found a three-fold increase in CD200R expression following exposure to 10 µg/mL SP-A in our dose-response experiment (Fig 1B), we did not observe a similar increase in CD200R expression under those conditions in repeat experiments and attribute the initial results to donor variation. We next investigated whether longer duration of exposure to surfactant components (48-96h) would induce CD200R expression. All conditions tested decreased CD200R expression at the 48h time point, with expression returning to baseline levels at subsequent time points (Fig 1E-G). Based on these results, we concluded that CD200R expression in human macrophages is not regulated by surfactant exposure under the conditions of our assay.

Due to the immunosuppressive effects of CD200R on macrophage response mechanisms, we hypothesized that expression of this receptor would be beneficial for M.tb and would therefore be increased following infection. As mentioned above, exposure of MDMs to surfactant lipids is a model system used to recreate some aspects of the AM phenotype. Although we had not seen any surfactant-dependent regulation of CD200R, the goal of our next experiment was to determine how M.tb would affect expression of CD200R in AMs, and we therefore exposed our cells to surfactant lipids
prior to infection. Similar to previous experiments, surfactant lipids alone had little to no effect on CD200R transcript levels. Counter to our hypothesis, M.tb infection further decreased CD200R transcript levels 24h after infection. Although there was an upward trend 48h after infection in surfactant-cultured MDMs, we concluded that this was largely due to donor variation, with the response averaging at around 0.5-fold increase among donors (Fig 1H).

Finally, we investigated CD200R transcript abundance in resting and M.tb infected HAMs, with the expectation that resting HAMs would have high levels of CD200R transcripts that would decrease following infection. Freshly isolated HAMs from six independent donors were left resting or were infected with M.tb H37Rv, and transcript abundance for CD200R (Fig 1I) and it’s only known ligand CD200 (Fig 1J) were determined. Counter to our hypothesis, HAMs express very low levels of CD200R transcripts which were only slightly increased following M.tb infection. Overall, our results lead us to conclude that CD200R is not significantly regulated by M.tb infection at the level of transcription.

**CD200R protein levels are not significantly regulated by surfactant**

To complete a thorough analysis of possible regulation of CD200R by surfactant, we next investigated CD200R protein levels in surfactant-exposed and resting MDMs. 24h after exposure to surfactant lipids, there is a two-fold increase in CD200R protein levels relative to resting control. Due to the lack of surfactant-mediated increase in CD200R transcript levels, we hypothesize that the increase in protein is due to inhibition of the normal degradation pathways for CD200R and/or post-translational modifications which increase protein stability. Interestingly, SP-A appeared to prevent this
phenomenon from occurring, since all conditions which included SP-A did not show a similar doubling in CD200R at 24h (Fig 2A and B). We next tested whether increasing the concentration of SP-A would have an effect on CD200R protein levels and observed no change regardless of SP-A concentration (Fig 2C and D). We speculated that the process of adhering MDMs to tissue culture plates may be preventing upregulation of CD200R. We therefore cultured MDMs in Teflon wells, a material to which macrophages do not adhere tightly, and added SP-A directly to the Teflon well for 24 or 48h. SP-A also does not alter CD200R protein levels in non-adherent MDMs (Fig 2E, F).

We hypothesized that high basal levels of CD200R may be obscuring any possible contribution of surfactant in the expression of this receptor. We therefore allowed MDMs a three day rest period after plating in tissue culture dishes in an attempt to deplete the endogenous pool of CD200R prior to surfactant exposure. We hypothesized that in the absence of pre-existing intracellular CD200R, surfactant would induce upregulation of this receptor. IL-4 has been reported to induce CD200R expression [205] and was included as a positive control. No increase in CD200R was observed following surfactant or IL-4 exposure (Fig 2E, F). The lack of induction in the IL-4 group is likely due to differences in experimental approaches and model systems.

We next investigated whether surfactant had any effect on the location of CD200R. Day 6 MDMs were exposed to surfactant components or IL-4 for 24-72h and cell membranes were isolated and probed for CD200R protein. As shown in figure 2G and H, no increase in CD200R surface localization was observed relative to resting MDMs. Finally, because day 12 MDMs are more stable in their phenotype and in their responses to stimuli, we repeated the membrane localization experiment using day 12 MDMs exposed to surfactant or IL-4 for 24 or 48h. No increase in CD200R protein was
observed in the membrane fraction of surfactant-cultured day 12 MDMs relative to resting cells (Fig 2I, J). Taken together, our results indicate that CD200R protein levels and location are not regulated by surfactant in human macrophages.

**CD200R protein levels are not regulated by mycobacterial infection or PPARγ in human macrophages**

After observing a slight decrease in CD200R transcript levels following infection of MDMs with *M. tb*, we hypothesized that CD200R protein levels would be decreased by mycobacterial infection. Day 6 MDMs were infected with *M. tb* (Fig 3A) or BCG (Fig 3B) and CD200R protein levels were evaluated. We observed no change in CD200R protein following infection with *M. tb*; however, BCG induced a slight increase in CD200R protein 48h after infection. We wondered whether pre-exposure of macrophages to SP-A would affect the ability of BCG to augment CD200R protein levels. Similar to the inhibitory effect of SP-A on the Survanta-induced doubling of CD200R which we had observed previously, SP-A pre-treatment diminished the induction of CD200R 48h after BCG infection (Fig 3C, D). Based on these and our previous results, we conclude that SP-A may be mildly inhibitory to expression of CD200R in the context of certain infections, possibly to circumvent the immunosuppressive effects of CD200R signaling and enable a more effective host response. Finally, we evaluated CD200R protein levels in resting or *M. tb* infected HAMs. We determined that CD200R is present in HAM whole cell lysates, does not decrease over time and is not affected by *M. tb* infection (Fig 3E, F). Thus, *M. tb* infection does not appear to impact CD200R protein levels in human macrophages.
PPARγ is a transcription factor which regulates aspects of AM phenotype [336] and is required for development of AMs from embryonic progenitor cells [337]. In addition, PPARγ has been reported to induce CD200R expression in murine microglial cells [338]. We therefore investigated whether PPARγ may regulate the expression of CD200R in macrophages independently of surfactant. MDMs were exposed to the PPARγ agonist PGJ2 or DMSO vehicle control and no difference in CD200R protein levels were observed between treatment or control groups (Fig 3G, H). We next investigated the effects of PPARγ activation in the context of mycobacterial infection. MDMs were exposed to PGJ2 or DMSO for 24h prior to BCG infection and again no differences were detected across conditions (Fig 3I, J).

Although we did not observe an increase in CD200R following activation of PPARγ, we wondered whether the absence of PPARγ would decrease CD200R expression. MDMs were transfected with scramble control or PPARγ siRNA and allowed to rest overnight prior to IL-4 exposure. No difference in CD200R protein occurred following PPARγ knockdown or in the presence or absence of IL-4 (Fig 3K, L), consistent with our previous findings. Taken together, our results indicate that CD200R is not regulated by surfactant, PPARγ or mycobacterial infection in human macrophages under the conditions of our assay.

**MARCO expression in human macrophages is inhibited by surfactant exposure and M.tbc infection**

MARCO is critical in the removal of unopsonized particles and microbes that are inhaled into the alveolar space [233, 328] and has been shown to mediate signaling responses to mycobacterial antigens [234]. We therefore investigated whether MARCO
expression is regulated by surfactant and began by determining the effects of SP-A on MARCO transcript levels during the differentiation of monocytes into macrophages. Regardless of the day of SP-A administration, we observed a decrease in MARCO transcript levels relative to resting cells (Fig 4A). We concluded that SP-A inhibits the expression of MARCO during the differentiation of monocytes into macrophages and focused on differentiated macrophages for the remainder of our experiments.

We next investigated whether increasing concentrations of SP-A would have any effect on MARCO expression and found that even the highest concentration of SP-A induced only a modest two-fold increase in MARCO expression (Fig 4B). Because higher concentrations of SP-A did not yield a robust result and SP-A is costly and time-consuming to purify, we decided to use 10 µg/mL SP-A for future investigations. We moved on to investigate the short-term effects of SP-A and Survanta exposure on MARCO expression in MDMs. As shown in figure 4C and D, MARCO expression remains around baseline levels following exposure to surfactant components out to 24h. Upon exposing MDMs to SP-A (Fig 4E), Survanta (Fig 4F) or both (Fig 4G) for several days, we again observed a decrease in MARCO transcript levels relative to resting cells. Based on these cumulative findings, we concluded that surfactant components have an inhibitory effect on MARCO expression in human macrophages.

MARCO has been implicated in host-protective signaling responses following exposure to trehalose dimycolate (TDM) the major component of the outer M.tb cell wall [234]. We therefore investigated whether MARCO expression would be increased following M.tb infection, again using the surfactant-cultured macrophage model to recapitulate aspects of the AM phenotype. Similar to previous experiments, surfactant had an inhibitory effect on MARCO expression. M.tb infection alone did not increase
MARCO transcript levels above baseline and surfactant lipids continued to further suppress MARCO expression following infection. Finally, we investigated MARCO transcript abundance in resting and *M.tb* infected HAMs. Freshly isolated HAMs express MARCO, which decreases over time regardless of whether or not the cells are infected (Fig 4H, I). Overall, our findings lead us to conclude that *M.tb* infection does not affect MARCO expression, while surfactant appears to have an inhibitory effect on the expression of MARCO in human macrophages. Due to our observation that HAMs contain MARCO transcripts and the published evidence indicating the relevance of MARCO in the lung, it is likely that other aspects of this tissue microenvironment regulate MARCO expression.

**MARCO protein levels are not affected by surfactant but receptor clustering occurs at the cell surface following exposure to surfactant lipids**

To complete our analysis of the effects of surfactant on MARCO expression, we next evaluated protein levels following surfactant exposure. We began by investigating the effects of SP-A on MARCO protein levels during the differentiation of monocytes into macrophages. Although there is slightly more protein relative to resting from day 2 onward (Fig 5A, B), the deviation from baseline is minimal and we elected to focus on differentiated macrophages for the remainder of our protein studies.

We next investigated MARCO protein levels following MDM exposure to surfactant components for 24-72h (Fig 5C). Although donor variation was observed, MARCO protein levels did not deviate significantly from baseline (Fig 5D). We wondered whether increasing concentrations of SP-A would have any effect on MARCO protein levels and observed no alteration in the relative amount of MARCO regardless of SP-A
concentration (Fig 5E, F). We speculated that the process of macrophage attachment to tissue culture dishes may interfere with the ability of surfactant to induce MARCO expression. We therefore cultured MDMs in Teflon wells and added SP-A directly to the wells for 24 or 48h. No striking differences in MARCO levels were observed in SP-A-exposed non-adherent MDMs (Fig 5G, H). We reasoned that high basal levels of MARCO may obviate the ability of surfactant to affect MARCO levels. We therefore allowed plated MDMs three days to rest in order to deplete any intracellular protein prior to surfactant exposure. As shown in Fig 5I and J, surfactant does not have any effect on MARCO levels in rested MDMs.

Although surfactant was not having an effect on total levels of MARCO protein, we hypothesized that surfactant exposure would increase surface localization. Using the more stable day 12 MDM model, we isolated the membrane protein fraction from MDMs which had been exposed to surfactant components or IL-4 for 24 or 48h. IL-4 was included because to our knowledge no one had investigated the effects of IL-4 on expression of MARCO in human macrophages. No alterations in the amount of membrane localized MARCO were observed in any condition (Fig 5K, L). Finally, using confocal microscopy we visualized the surface distribution pattern of MARCO in non-permeabilized macrophages exposed to surfactant components for 24 or 48h. SP-A inhibits the surface localization of MARCO (Fig 5M, N). Although exposure to surfactant lipids does not increase the total amount of MARCO at the cell surface, receptor clustering was observed at both time points examined. We concluded that although surfactant does not alter the amount of MARCO protein found in human macrophages, MARCO may be responsive to the presence of surfactant lipids. Therefore, it is possible
that MARCO activity is regulated by surfactant components, although we did not pursue this further.

**MARCO protein levels are not regulated by mycobacterial infection or PPARγ in human macrophages**

We did not observe any alterations to MARCO transcript levels following infection with *M. tb*, but to complete our analysis of the effects of mycobacterial infection on MARCO expression we elected to investigate protein levels. MDMs were infected with *M. tb* H37Rv (Fig 6A, B) for 24 or 48h and no differences in MARCO levels were observed following infection. Although a slight decrease was detected 48h after BCG infection (Fig 6C, D), because there was no difference following *M. tb* infection we chose to discontinue the exploration of mycobacterial infection of MDMs in relation to MARCO expression. Resting HAMs contain MARCO protein following isolation from the lung, which decreases overtime regardless of *M. tb* infection (Fig 6E, F).

To our knowledge, no one had investigated the role of PPARγ in the expression of MARCO in human macrophages. We therefore investigated the affects of the PPARγ agonist PGJ2 on MARCO protein levels and observed no differences in MARCO levels between treatment and control groups (Fig 6G, H). Taken together, we conclude that surfactant has an inhibitory effect on the expression of MARCO and that *M. tb* infection and PPARγ activation do not alter expression of this receptor in human macrophages under the conditions of our assay.
Surfactant does not significantly alter \textit{MSR1} or \textit{SCARB1} transcript levels in human macrophages

SR-A (gene name \textit{MSR1}) contributes to both host protective [331] and detrimental immune responses in the lung [330] and we hypothesized that expression of SR-A is regulated by surfactant. SR-A has also been implicated in perpetuating detrimental aspects of the chronic phase of \textit{M.tb} infection in a murine model [228]; however, we did not investigate the effect of \textit{M.tb} infection on expression of SR-A herein. Following short-term exposure of day 6 MDMs to SP-A (Fig 7A) or Survanta (Fig 7B), \textit{MSR1} transcript levels exhibit an oscillating expression pattern. An initial decrease is observed 1h after exposure to surfactant components, followed by an approximate doubling at 6h and a return to baseline levels after 12h (Fig 7A, B). Longer exposure to surfactant components does not alter \textit{MSR1} transcript levels (Fig 7C-E). Based on these results, we concluded that expression of \textit{MSR1} is variably regulated by surfactant in human macrophages and the role of surfactant in SR-A expression was not explored further.

SR-B1 (gene name \textit{SCARB1}) is important in lipid uptake [332], a key function of AMs, leading us to hypothesize that SR-B1 expression is regulated by surfactant. SR-B1 is also involved in pathogen recognition [333] and, importantly, this function of SR-B1 includes the uptake of, but not the host response to, \textit{M.tb} in a murine model [241]. However, we did not explore the effects of \textit{M.tb} on the expression of SR-B1 herein. MDMs were exposed to SP-A (Fig 7F) or Survanta (Fig 7G) and \textit{SCARB1} transcript levels were analyzed. Similar to \textit{MSR1}, a transient doubling was observed 6h after exposure to SP-A or Survanta. Although the increase in \textit{SCARB1} transcript levels following Survanta is sustained temporally, the increase was not statistically significant.
relative to resting MDMs. We concluded that expression of *SCARB1* is potentially mediated by surfactant to a small extent. However, given the magnitude of this response, we did not further pursue the possibility of surfactant contribution to SR-B1 expression.

**CD36 transcript and protein levels are increased by surfactant lipids**

Scavenger receptor CD36 is expressed by AMs [63, 339, 340] and contributes to lipid uptake [63, 335], the removal of gram positive and gram negative bacterial pathogens [260] and phagocytosis of apoptotic host cells [341]. All of these are pivotal processes for pulmonary homeostasis which are known to be mediated by AMs [41]. We therefore hypothesized that CD36 expression in human macrophages is regulated by surfactant components. Similar to our analysis for the receptors described above, we began our investigation into the role of surfactant in CD36 expression by evaluating the effects of surfactant exposure during the differentiation of monocytes into macrophages. Monocytes exposed to SP-A on day 0 exhibit a three-fold increase in *CD36* transcript levels which drops down to basal levels over time (Fig 8A). Although monocytes have been shown to upregulate *CD36* during differentiation into macrophages [339], we conclude that SP-A does not further augment the expression of *CD36* during this time and we focused on differentiated macrophages for the remainder of our studies with CD36.

In an attempt to determine the optimal concentration of SP-A for induction of CD36, we performed a dose-response experiment using increasing concentrations of SP-A. No differences in *CD36* transcript levels were observed regardless of SP-A concentration (Fig 8B). At 24h, the time point at which whole cell lysates were collected,
no differences in \textit{CD36} protein levels were observed despite increasing concentrations of SP-A (Fig 8C, D). We therefore decided to use 10 µg/mL SP-A for future experiments.

In MDMs exposed to SP-A (Fig 8E), Survanta (Fig 8F) or both (Fig 8G) for 1-5h, no changes in \textit{CD36} transcript levels were observed. However, following a longer exposure to surfactant components, we identified a reproducible increase in \textit{CD36} transcript levels. 72h after exposure to SP-A MDMs contain twice as many \textit{CD36} transcripts relative to resting MDMs (Fig 8H). Surfactant lipids induce a larger increase in \textit{CD36} transcript levels 48 and 72h after exposure, peaking at a three-fold increase (Fig 8I). Exposure to both SP-A and Survanta yielded the strongest and most sustained effect, with a maximum increase of four-fold 96h after exposure (Fig 8J). Based on these results, we conclude that surfactant components positively regulate \textit{CD36} transcript levels in human macrophages.

We next queried whether the major lipid species in surfactant, DPPC, would be sufficient to augment \textit{CD36} transcript levels. MDMs were exposed to DPPC or DMSO control for 48, 72 or 96h. DPPC induces a three-fold increase in \textit{CD36} transcript levels 72h after exposure (Fig 8K), indicating that DPPC may be the component of Survanta which mediates the observed increase in \textit{CD36} transcript levels.

After confirming that surfactant components are capable of increasing \textit{CD36} transcript levels, we investigated the effects of surfactant on \textit{CD36} protein abundance. MDMs were exposed to SP-A, Survanta, both or left resting as a control out to 72h and whole cell lysates were probed for \textit{CD36}. Consistent with the transcript data, no increase in \textit{CD36} protein occurred at these time points (Fig 8L, M). Following the surfactant-mediated increase in \textit{CD36} transcript levels 72h after exposure, there is a five-fold increase in total \textit{CD36} protein 96h after surfactant lipid exposure (Fig 8N, O). Twice as
much CD36 protein is present at the cell membrane 96h after surfactant lipid exposure (Fig 8O). Taken together, our results indicate that CD36 transcript and protein levels are regulated by surfactant in human macrophages.

**CD36 transcript and protein levels are decreased following *M. tb* infection of human macrophages**

Previous in vivo work has indicated that expression of CD36 is host-detrimental during mycobacterial colonization [229, 271] and our data suggest that CD36-dependent uptake of surfactant lipids is potentially responsible for this negative impact on the host early during *M. tb* infection [63]. CD36 expression has been reported to increase in lung lesions 60 days after infection with *M. tb* in a guinea pig model [269] and the attenuated laboratory strain H$_{37}$R$_{v}$ has been shown to induce CD36 gene expression in the COS1 cell line [270]. However, to our knowledge no one has investigated the effects of *M. tb* infection on the expression of CD36 in human macrophages. Based on the previous publications cited above, we hypothesized that *M. tb* infection would increase CD36 expression. MDMs were exposed to Survanta or left resting for two days prior infection, which, counter to our hypothesis, decreased CD36 transcript levels. In fact, *M. tb* infection obviated the surfactant-mediated increase in CD36 expression (Fig 9A). Due to the augmentation of *M. tb* growth predicated on CD36-dependent uptake of surfactant lipids, we speculate that the decrease in CD36 following infection is a host defense mechanism.

We next investigated CD36 transcript abundance in HAMs. Freshly isolated HAMs contain low levels of CD36, apparently in contrast to the presence of CD36 protein which has previously been reported [63, 339, 340]. CD36 is known to be recycled
through the endosomal pathway and is a relatively long-lived protein [245, 342], potentially accounting for the low levels of transcripts found in HAMs. CD36 transcript levels increase over time following isolation from the lungs and *M.tb* infection mildly inhibits this phenomenon (Fig 9B). Based on these findings, we conclude that *M.tb* infection decreases CD36 transcript levels in human macrophages.

We next evaluated the impact of *M.tb* infection on CD36 protein levels 24 and 48h after infection. As shown in figure 9C, there is a 50% reduction in CD36 protein 48h after *M.tb* infection. A similar decrease in CD36 protein levels was observed 72h after infection in HAMs (Fig 9D). Despite this *M.tb*-induced decrease in overall protein levels, we sought to determine whether *M.tb* co-localizes with CD36 in infected MDMs, with the hypothesis that *M.tb*’s exploitation of CD36-mediated lipid uptake would result in co-localization of *M.tb* with CD36. MDMs were infected with mCherry and no co-localization was observed 24 or 48h after infection, although we did observe a decrease in total CD36 protein in keeping with our Western blot data (Fig 9G-J). Taken together, our results show for the first time that *M.tb* infection of human macrophages exerts an early inhibitory effect on CD36 expression. As stated above, we speculate that this response constitutes a host-defense mechanism to prevent further influx of CD36-dependent lipids into the cell.

2.3 Discussion

The initial interaction between an intracellular pathogen and its host cell determines whether the invader is destroyed or a parasitic relationship ensues [343] and this early encounter is typically mediated by a receptor on the host cell surface [169]. In the specialized and delicate pulmonary space, tissue-specific signals strongly influence
the receptor repertoire expressed by resident macrophages [41, 289]. Herein, we investigated the role of pulmonary surfactant, an indispensable contributor to proper organ function and immune responses [30, 48, 344-346], in the expression of several macrophage receptors. These receptors were selected due to their known expression by AMs, their appreciable roles in pulmonary immune responses and, for the scavenger receptors, their possible mediation of surfactant lipid uptake.

We began our investigation with CD200R, which has been linked to the maintenance of the immunosuppressive AM phenotype [204, 215]. The only known ligand for this receptor, CD200, is found on cell types and tissues throughout the body and is structurally identical to its receptor, other than having a truncated cytoplasmic tail which lacks signaling capabilities [347]. In mast cells, CD200R-mediated signaling responses culminate in the inhibition of pro-inflammatory RasMAPK pathways [207]. Whether or not this specific signaling paradigm holds true for other members of the myeloid lineage remains to be determined.

CD200R contributes to an array of immunological responses and mediates both positive and negative outcomes for the host. Studies investigating CD200 knockout mice reported more robust rejection of allografted organs relative to wild type mice, mediated by unchecked inflammatory responses which prevented transplant acceptance [212]. However, the same knockout mouse strain is resistant to breast cancer metastasis, due to cellular activation in response to tumor antigens and the subsequent ability to control the spread of cancerous cells [214]. Clearly, the CD200:CD200R signaling axis plays an important role in a variety of disease contexts, with the common theme being inhibition of responses to foreign or harmful biological material.
The inhibitory activity of CD200R may be of particular importance in the lung. Following intranasal infection, CD200 knockout mice succumbed to a typically sub-lethal dose of influenza virus. The modality of death was excessive inflammatory responses mediated by AMs, due to the lack of suppressive input which is ordinarily mediated by CD200:CD200R ligation [215]. This paper provided strong evidence that CD200R activity constitutes an integral aspect of immunoregulatory AM functions and led us to hypothesize that surfactant increases expression of CD200R and promotes the permissive AM phenotype which is exploited by M.tb. As described above, our results to date disprove our hypothesis and we conclude that CD200R expression is not regulated by surfactant (Fig 1A-G and Fig 2A-L) or mycobacterial infection in human macrophages (Fig 1H-J and Fig 3A-F). To our knowledge, these findings represent the first investigation into CD200R expression during mycobacterial infection. However, they certainly do not constitute the end of the story. One deficit in our experimental design was the lack of CD200 ligand, which prevented us from examining CD200R activity in the context of M.tb infection. However, because our studies focused on evaluating receptor expression, we do not feel that the lack of CD200 in our system impacted the results reported herein. Due to our negative findings regarding expression of CD200R following surfactant exposure or infection with M.tb, we elected not to pursue any functional role of this receptor during M.tb infection.

We next investigated the expression of several scavenger receptors, due to their well-defined contributions to tissue homeostasis and innate immune responses. Scavenger receptors are a diverse family and mediate the uptake of a variety of cargo, from modified self-molecules to microbes, and are separated into eight classes based on structural similarity [236]. Through their propensity to promiscuously associate with
binding partners, scavenger receptors have the ability to perpetuate both pro- and anti-inflammatory macrophage functions. For example, by forming a complex with tyrosine kinase MER (MERTK) SR-A contributes to tissue repair and the removal of apoptotic cells [348], but initiates pro-inflammatory signaling responses to LPS in conjunction with TLR4 [349]. Possessing the ability to function in conjunction with an array of co-receptors is an integral aspect of the “scavenging” ability of this family of receptors [236] and generally obviates their classification as either pro- or anti-inflammatory contributors to immune responses. The function of a given scavenger receptor is therefore highly dependent on cell and tissue context.

Herein, we studied the regulation of scavenger receptor expression by pulmonary surfactant in order to fill a gap in our knowledge regarding macrophage-mediated homeostatic regulation of the lung microenvironment. Additionally, in pursuit of our hypothesis that M.tb has evolved to exploit the endogenous pulmonary environment, we sought to identify scavenger receptors which contribute to pulmonary homeostasis and are regulated by surfactant with the goal of ultimately determining whether M.tb could exploit the endogenous function of those receptors early during infection.

For our studies we investigated two prominent members of class A (MARCO and SR-A) and class B (CD36 and SR-B1). MARCO has been shown to mediate AM removal of unopsonized particulate [328] and biological matter [233] as well as ozone-modified surfactant lipids [232]. Additionally, MARCO tethers mycobacterial TDM to CD14 and TLR2 to enable host-protective signaling responses through NFκβ and the production of pro-inflammatory cytokines [234]. The above studies indicated that MARCO contributes to pulmonary homeostasis and the response to mycobacterial infection and we therefore investigated the role of surfactant and M.tb infection in MARCO expression.
We report an inhibitory effect of surfactant components on MARCO transcript levels (Fig 4A-G) and no alteration to MARCO protein levels (Fig 5A-L). However, we do show that MARCO clustering occurs at the cell surface following exposure to surfactant lipids (Fig 5M, N), indicating that MARCO is functionally responsive to the presence of surfactant although MARCO expression is not positively regulated concurrently. Additionally, we show that *M. tb* infection does not affect the expression of MARCO in human macrophages (Fig 4H, I and Fig 6A-F), which, to our knowledge, constitutes the first report of the effects of *M. tb* infection on MARCO expression. Although we observed no alteration in MARCO levels following *M. tb* infection, these findings do not contradict previous observations that MARCO participates in the cellular response to *M. tb* exposure [234].

SR-A is active in immunological responses in the pulmonary environment [330, 331], and contributes to lipid uptake in other tissues [350], leading us to hypothesize that SR-A expression would be positively regulated by surfactant components. As described in the results section, no change in transcript levels were observed following surfactant exposure (Fig 7A-E) and SR-A was not explored further in this context. SR-B1 is expressed by AMs and mediates cholesterol and lipid flux in a variety of macrophage sub-populations in addition to participating in pathogen recognition. Although we did observe a two-fold increase in *SCARB1* transcript levels following MDM culture in surfactant lipids (Fig 7F), this result was not significant and SR-B1 was not pursued further. However, SR-A and SR-B1 merit additional investigation in the context of *M. tb* infection. AMs from SR-A /- mice produce significantly more TNFα in response to mycobacterial TDM [351], indicating that SR-A may inhibit inflammatory responses to *M. tb*, although the in vivo role of SR-A during TB remains controversial [228, 229]. SR-
B1 contributes to the uptake of *M. tb* and although ultimately host survival was not altered by the absence of SR-B1 [241], it would be interesting to explore the intracellular fate of *M. tb* following SR-B1-mediated cell entry.

CD36 exerts a wide influence in the human body during homeostasis and the pathogenesis of multiple diseases, ranging from Alzheimer’s and stroke to atherosclerosis and diabetes [246]. CD36 is additionally involved in both the inflammatory and resolution stages of infection. For example, in conjunction with TLR4 in LPS-primed cells, CD36 contributes to *M. tb* ManLAM-induced production of TNFα [352] and conversely mediates the removal of apoptotic cells during wound healing [341]. Due to the well established role of CD36 in the uptake of fatty acids and cholesterol [245, 334, 335, 342] (described further in Chapter 3) as well as the observation that CD36 is detrimental during the early stages of mycobacterial infection [63, 229, 271], it seemed likely to us that the expression of CD36 would be regulated by surfactant and *M. tb* infection. We show that CD36 transcript and protein levels are regulated by surfactant (Fig 8H-K, N, O), particularly the lipid components and possibly DPPC specifically (Fig 8K). Since no surfactant-mediated increases were observed for other scavenger receptors, CD36 may be particularly important in the lung environment and is likely involved in the maintenance of pulmonary homeostasis through the uptake of surfactant lipid components as described in Chapter 3.

Based on the publications cited above which indicated that CD36 expression is detrimental to the host during infection with *M. tb*, we hypothesized that CD36 expression would be augmented following mycobacterial infection. As shown in figure 9, *M. tb* infection decreases CD36 transcript and protein levels in both MDMs and HAMs. We speculate that this decrease is a host response which is aimed at preventing the
continued exploitation of CD36 function by *M. tb*. In Chapter 4, we show that the *M. tb*-induced decrease in CD36 is accompanied by inhibition of several other mediators of lipid storage and degradation, indicating widespread disruption of host lipid uptake, storage and metabolic pathways following infection with *M. tb*. A number of studies indicate that interference with the uptake and processing of host lipids is one of the many cruxes of *M. tb* infection [66, 122, 157, 288].

Another reason that we elected to focus on the above scavenger receptors is their conserved ability to recognize and mediate the uptake of lipid-based substrates [232, 332, 334, 350]. The *M. tb* cell wall is a strikingly complex construction of layered lipids, studded with structural sugars and proteins [149]. The mycolic acids of the *M. tb* cell wall are virulence factors necessary for survival in a host [151] and are capable of influencing host physiology on a cellular level [157]. During infection, *M. tb* actively sheds components of its cell wall [149], which serves to interfere with host cellular processes but also creates potential antigens for receptors such as scavenger receptors. We hypothesized that not only would surfactant regulate the expression of the scavenger receptors described above, but that *M. tb* infection would further enhance scavenger receptor expression due to macrophage interaction with lipid components of the mycobacterial cell wall. To our surprise, every receptor surveyed in the context of *M. tb* infection exhibited no change or a decrease in expression. As implied above, these expression results do not rule out participation of these receptors during mycobacterial infection.

Other macrophage surface receptors of relevance to *M. tb* infection include TLRs and C-type lectins [343]. TLRs are leucine-rich repeat proteins which are conserved among eukaryotes and function to initiate signaling responses following their detection of
pathogens through the recognition of pathogen-associated molecular patterns (PAMPs). Of the surface expressed TLRs, \textit{M}. \textit{tb} is known to predominantly ligate TLR2, but also interacts with TLR4 [353, 354]. However, because our previous work indicates that surfactant, specifically SP-A, is inhibitory to TLR2/4 function [46], we did not pursue their expression in the context of surfactant exposure. C-type lectins recognize microbial sugars and also participate in cell adhesion [343]. In the context of macrophage infection with \textit{M}. \textit{tb}, the mannose receptor (MR) is the most relevant C-type lectin and has been extensively explored in our previous work, which showed that SP-A increases surface activity of the MR. Engagement of the MR by \textit{M}. \textit{tb} initiates bacterial uptake [64] and prevents fusion of the \textit{M}. \textit{tb} phagosome with lysosomes [150]. Additionally, MR activation during \textit{M}. \textit{tb} inhibits host-protective responses by inducing the expression of IL-10 [355] and PPARγ [66].

PPARγ is a transcription factor which has been established as necessary for AM development [337] as well as maintenance of their phenotype [336]. During the course of our studies, we therefore investigated the possibility that PPARγ contributes to the expression of two of our chosen receptors, CD200R and MARCO. PPARγ has been shown to mediate the expression of CD200R in microglial cells [338] and to our knowledge no one has investigated its role in the expression of MARCO. Under our experimental conditions, PPARγ activation did not alter protein levels of either CD200R (Fig 3G-J) or MARCO (Fig 6G, H), nor did the absence of PPARγ affect protein levels of CD200R (Fig 3K, L). Although we did not explore transcript levels of these receptors under this experimental paradigm, we conclude that CD200R and MARCO protein levels are not regulated by PPARγ activity in human macrophages.
Evaluated as a whole, our findings indicate that surfactant components do not regulate the expression of CD200R, SR-A or SR-B1 and may decrease MARCO expression. In contrast, CD36 is positively regulated by surfactant at both the transcript and protein level. PPARγ activation does not appear to influence protein levels of CD200R or MARCO although as noted above our analysis does not rule out transcriptional regulation. We further show that *M. tb* infection does not affect expression of CD200R or MARCO and decreases CD36 transcript and protein levels. Although we did not further explore the role of CD200R or MARCO in *M. tb* infection, the implications of the observed decrease in CD36 are elaborated on in Chapter 4.

### 2.4 Materials & Methods

**Reagents**

RPMI 1640 +L-Glutamine was purchased (Life Technologies, Carlsbad, CA, USA) and supplemented with donor autologous serum for cell culture or with 20mM HEPES buffer (Sigma Chemical Co, St. Louis, MO, USA), pH 7.2 and 1 mg/mL human serum albumin (HSA) (Calbiochem Corp., La Jolla, CA, USA) during *M. tb* infection. Goat anti-human CD200R polyclonal (Q-15, 1:200) and rabbit anti-human CD36 polyclonal (H-300, 1:500) were purchased from Santa Cruz (Santa Cruz, CA, USA). Rabbit anti-human MARCO polyclonal (AF7586, 1:500) was purchased from R&D Biosystems (Minneapolis, MN, USA). For confocal microscopy, mouse IgM anti-human CD36 (sc-7309, Santa Cruz) Ab was used, followed by AlexaFluor (AF) 488-conjugated goat-anti-mouse IgM (Invitrogen, Waltham, MA, USA). Isotype control was mouse IgM ( Ancell Corporation, Stillwater, MN, USA). Survanta (bovine-derived surfactant lipids lacking SP-
A and SP-D) was from Abbott pharmaceuticals (Abbott Park, IL, USA). SP-A was purified from the bronchoalveolar lavage of alveolar proteinosis patients as described previously [356].

**Human monocyte-derived macrophage and alveolar macrophage isolation and cultivation**

Human peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood of healthy donors on a Ficoll-Hypaque (Amersham, Pittsburgh, PA, USA) cushion as described [64]. PBMCs were then cultured in Teflon wells in RPMI 1640 + 20% donor autologous serum for 5 days at 37°C, 5% CO\(_2\) [64]. During this time, monocytes differentiate into monocyte-derived-macrophages (MDMs). Experiments were conducted in duplicate or triplicate wells using MDM monolayers in tissue culture plates. Human alveolar macrophages (HAMs) were isolated from the BAL of healthy human donors [65]. PBMC and HAM protocols were approved by The Ohio State University (OSU) IRB.

**Macrophage infection with *M. tb***

*M. tb* H\(_{37}\)R\(_v\) (ATCC #25618) wild type or a strain expressing the mCherry reporter were used [64]. Day 6 MDMs (4 x 10\(^5\)/ml) were seeded in a tissue culture plate and either exposed to Survanta (100 µg/ml) for 48h prior to infection or left resting during that time. MDMs were infected with *M. tb* H\(_{37}\)R\(_v\) or mCherry (MOI 1:1 or 5:1 as indicated per experiment) at 37°C with 5% CO\(_2\) on a platform shaker for 30 min, followed by 90 min incubation without shaking. Monolayers were washed, repleted with RPMI containing 2% autologous serum and incubated up to 96h.
Quantitative real-time PCR

Macrophage RNA was isolated using TRIzol reagent (Invitrogen Life Technologies) [357]. RNA purity and quality were determined using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific). Total RNA was reverse transcribed to cDNA using SuperScriptIII reverse transcriptase (Invitrogen Life Technologies) [357]. Quantitative real-time PCR (qRT-PCR) was conducted using human CD200R1, MARCO, MSR1, SCARB1 and CD36 TaqMan gene expression systems (Applied Biosystems). Negative controls consisted of no-reverse transcriptase and no-template reactions. All samples were run in triplicate using a cfx96 real-time system (Bio-Rad) and analyzed using the threshold cycle (2^{-ΔΔct}) method [358]. Gene expression was normalized against β–actin.

Preparation of protein lysates and Western blot analysis

MDM monolayers were incubated in TN1 lysis buffer at 4℃ on a platform rocker for 10 min to create whole cell lysates then centrifuged at 18,000 × g at 4℃. Supernatants were transferred to a new tube and stored at -20℃. To isolate membrane fractions, MDM monolayers were lysed and processed with Pierce Cell Surface Protein Isolation Kit (ThermoFisher Scientific) according to the manufacturer’s instructions. Protein concentrations were determined via Pierce BCA-protein assay kit (ThermoFisher Scientific) according to the manufacturer’s instructions. Lysates were separated by SDS-PAGE, transferred onto nitrocellulose membranes and blocked with 5% BSA for 3h and incubated with primary antibodies at 4℃ overnight on a rocker. Membranes were washed three times in TBS-T and secondary antibodies were added for 1h at room
temperature, followed by an additional three washes in TBS-T. Membranes were developed using ECL (GE Healthcare) and band densitometry was determined using Image J software to subtract background intensity and normalize to β-actin.

**siRNA transfection of MDMs**

Using the Amaxa Nucleofector as directed by the manufacturer (Lonza AG, Basel, Switzerland) total PBMCs containing MDMs were subjected to nucleofection and transfected with 25 nM PPARγ or control siRNA [177]. PBMCs were plated in tissue culture dishes and incubated in RPMI plus 10% autologous serum for 2 h at 37°C in 5% CO₂, at which time monolayers were washed to remove lymphocytes. The remaining transfected MDMs were incubated overnight in RPMI plus 10% serum at 37°C in 5% CO₂ before experimentation.

**Confocal microscopy**

MDMs (1.5 x 10⁵) adhered to glass coverslips in 24-well tissue culture plates were exposed to Survanta (100 µg/mL) for 96h. Monolayers were fixed with 4% PFA for 10 min in the dark at room temperature, washed and left intact or permeabilized by a one minute methanol exposure [359]. Coverslips were blocked overnight at 4°C (PBS + 5% BSA + 10% FBS) and labeled with anti-CD36 (1:200 for 1h at room temperature) primary Ab and AF secondary Ab (1:500 for 1h at room temperature). Coverslips were mounted on glass slides using ProLong Gold AntiFade Mounting media plus DAPI (Invitrogen Life Technologies) and viewed using a FluoView 1000 Laser Scanning Confocal microscope (Olympus). The mean fluorescence intensity (MFI) of random confocal images was quantified using pixel intensity measurement (NIH Image J
program). The MFI was calculated for approximately 150 MDMs per coverslip, from duplicate slides for each experiment.

**Statistical analysis**

Experiments were conducted from a single biological replicate (Western blots), duplicates (confocal) or triplicates (gene expression) using MDMs from a minimum of three different donors. Prism-5 software (Version 5.04; GraphPad) was used to determine the statistical significance of differences in the means of experimental groups using an unpaired, one-tailed Student t-test. p values < 0.05 were considered significant.
**FIGURE 1. CD200R expression in human macrophages is not altered by surfactant exposure or *M. tb* infection.** (A) CD200R transcript levels following SP-A exposure during the differentiation of monocytes into macrophages. Day 0 monocytes were plated in tissue culture dishes and SP-A (10 µg/mL) was added at day 0, 1, 2, 3 or 4. Monolayers were lysed 24h after SP-A exposure and CD200R transcript levels were evaluated by qRT-PCR. (B) Higher concentrations of SP-A do not increase CD200R transcript levels. Day 6 MDMs were exposed to 10, 20 or 40 µg/mL of SP-A for 6, 24 or 48h and transcript levels were analyzed. (C, D) No change in CD200R transcript levels early after exposure to surfactant components. MDMs were exposed to (C) SP-A (10 µg/mL) for 30 mins, 1-6h, 12h or 24h or (D) Survanta (100 µg/mL) for 1, 6, 12 or 24h and CD200R transcript levels were evaluated. (E-G) Prolonged exposure to surfactant components does not increase CD200R transcript levels. MDMs were exposed to (E) SP-A (10 µg/mL), (F) Survanta (100 µg/mL) or (G) both for 48-96h and CD200R transcript levels were analyzed. (H) *M. tb* infection does not increase CD200R transcript levels. MDMs were exposed to Survanta (100 µg/mL) for 48h prior to infection with *M. tb* H37Rv at an MOI of 1:1 for 24 or 48h and CD200R transcript levels were measured. (I, J) Freshly isolated HAMs were left resting or were infected with *M. tb* (MOI 5:1) for 2, 24 or 72h. Total transcripts were isolated and transcript reads per million were analyzed by AmpliSeq for abundance of transcripts for CD200R (I) or its ligand CD200 (J). Graphs show fold change in transcript abundance relative to resting MDMs at the corresponding time points (±SEM) from one (A, B) or three (C-H) independent experiments conducted in triplicate or cumulative data from six (I, J) independent experiments conducted in duplicate.
FIGURE 2. CD200R protein levels are not increased by surfactant exposure. (A) MDMs were exposed to SP-A (10 µg/mL), Survanta (100 µg/mL), both or left resting as a control. Whole cell lysates were collected and CD200R protein levels were analyzed by Western blot. Representative blot of three independent experiments. (B) Cumulative densitometric analysis of three independent experiments. (C) Increasing SP-A concentration does not affect CD200R. MDMs were exposed to 10, 20 or 40 µg/mL SP-A for 24h. Whole cell lysates were collected and CD200R protein levels were evaluated by Western blot. (D) Densitometric analysis of increasing SP-A concentrations on CD200R protein levels. (E) Non-adherent MDMs exposed to SP-A do not increase CD200R protein levels. MDMs were cultured in Teflon wells and exposed to SP-A (10 µg/mL) for 24 or 48h or left resting as a control. Whole cell lysates were collected and CD200R protein levels were analyzed. (F) Densitometric analysis of CD200R protein levels in non-adherent MDMs. (G) MDMs were plated and allowed to rest for 72h to reduce basal protein levels prior to exposure to surfactant or IL-4 (50 ng/mL). Whole cell lysates were collected and CD200R protein levels were evaluated. (H) Densitometric analysis of the blot shown in G. (I) Surfactant does not induce redistribution of CD200R to the cell membrane in day 6 MDMs exposed to SP-A, Survanta, both or IL-4 for 24-72h. Cell membrane fractions were isolated and CD200R levels were analyzed by Western blot. (J) Densitometric analysis of blot shown in J. (K) Surfactant does not induce redistribution of CD200R to the cell membrane in day 12 MDMs. After exposure to SP-A, Survanta, both or IL-4 for 24 or 48h, cell membranes were isolated and CD200R levels were analyzed. (L) Densitometric analysis of blot shown in K. Graphs show fold change in actin-normalized protein levels relative to resting MDMs at the corresponding time points (±SEM). Data were collected from one (C-L) or three (A, B) independent experiments.
Figure 2

A. Resting, SP-A, Survanta, SP-A + Survanta

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)

B. Time after SP-A (10μg/mL)

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)

C. [SP-A] μg/mL

Resting, 10, 20, 40

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)

D. SP-A, Survanta

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)

E. Resting, SP-A

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)

F. Rested 3d prior to stimulation

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)

G. R, SP-A, Sur, +Sur, IL-4

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)

H. Rested 3d prior to stimulation

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)

I. 24h, 48h, 72h

Resting, SP-A, Survanta, IL-4

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)

J. Day 5 MDMs

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)

K. 24h, 48h

Resting, SP-A, Survanta, IL-4

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)

L. Day 12 MDMs

IB: CD200R (60 kDa)

IB: β-actin (44 kDa)
FIGURE 3. CD200R protein levels are not regulated by mycobacterial infection or PPARγ in human macrophages. MDMs were infected with (A) *M. tb* H₃⁷R₅, or (B) BCG at an MOI of 5:1 for 24 or 48h. Whole cell lysates were collected and CD200R protein levels were evaluated by Western blotting. (C) BCG infection and SP-A do not increase CD200R protein levels. MDMs were left resting, infected with BCG (MOI 5:1) or were exposed to SP-A (10 µg/mL) for 2h prior to infection with BCG, or cultured in SP-A alone for 24 or 48h. Whole cell lysates were collected and CD200R protein levels were analyzed. (D) Densitometric analysis of the blot shown in C. (E) HAMs express CD200R protein, which is not affected by *M. tb* infection. HAMs were plated and lysed after 2h, or left resting or infected with *M. tb* H₃⁷R₅ (MOI 5:1) for 24h. Whole cell lysates were evaluated by Western blot for CD200R protein. (F) Cumulative densitometry from three independent experiments. (G) PPARγ agonist PGJ2 does not increase CD200R protein levels relative to DMSO control. MDMs were exposed to PGJ2 (2 µg/mL) or DMSO vehicle control (1:1000) for 24-72h. Whole cell lysates were collected and CD200R protein levels were analyzed. (H) Densitometric analysis of the blot shown in G. (I) CD200R protein levels are not increased by a combination of BCG infection and PGJ2 exposure. MDMs were exposed to PGJ2 or DMSO for 24h prior to infection with BCG (MOI 5:1) for 24h and CD200R protein levels were analyzed. (J) Densitometric analysis of the blot shown in I. (K) PPARγ knockdown does not affect CD200R protein levels. MDMs were transfected with SC or anti-PPARγ siRNA via Amaxa nucleofection. IL-4 was added for 24h and CD200R protein levels were analyzed by Western blot. (L) Densitometric analysis of CD200R protein levels in SC or siPPARγ MDMs. Shown are results of one (A-D, G-L) or three independent experiments (E, F) (± SEM).
Figure 3
FIGURE 4. MARCO expression in human macrophages is inhibited by surfactant exposure or *M. tb* infection. (A) MARCO transcript levels are decreased by SP-A during the differentiation of monocytes into macrophages. Day 0 monocytes were plated in tissue culture dishes. SP-A (10 µg/mL) was added at day 0, 1, 2, 3 or 4 after plating. Monolayers were lysed 24h after SP-A exposure and MARCO transcript levels were evaluated by qRT-PCR. (B) Exposure to higher concentrations of SP-A for 48h results in a two-fold increase in MARCO transcript levels. MDMs were exposed to 10, 20 or 40 µg/mL or SP-A for 6, 24 or 48h and transcript levels were evaluated by qRT-PCR. (C, D) Short-term exposure to surfacetant components does not affect MARCO transcript levels. Day 6 MDMs were exposed to (C) SP-A (10 µg/mL) for 30 mins, 1-6h, 12h or 24h or (D) Survanta (100 µg/mL) for 1, 6, 12 or 24h and MARCO transcript levels were evaluated by qRT-PCR. (E-G) Long-term exposure to surfactant components decreases MARCO transcript levels. Day 6 MDMs were exposed to (E) SP-A (10 µg/mL), (F) Survanta (100 µg/mL) or (G) both for 48-96h and MARCO transcript levels were evaluated by qRT-PCR. (H) *M.tb* infection decreases MARCO transcript levels. Day 6 MDMs were exposed to Survanta (100 µg/mL) for 48h prior to infection with *M.tb* H37Rv at an MOI of 1:1 for 24 or 48h and MARCO transcript levels were evaluated by qRT-PCR. (I) Freshly isolated HAMs were left resting or were infected with *M.tb* (MOI 5:1) for 2, 24 or 72h. Total transcripts were isolated and transcript reads per million were analyzed by AmpliSeq for abundance of MARCO transcripts. Graphs show fold change in MARCO transcript levels relative to resting MDMs at the corresponding time points (±SEM) from one (A, B) or three (C-H) independent experiments conducted in triplicate, or cumulative data from six (I) independent experiments conducted in duplicate.
Figure 4

A

MARCO transcript fold change (Relative to resting MDMs)

Monocyte/MDM (Days after SP-A)

B

MARCO transcript fold change (Relative to resting MDMs)

[SP-A] μg/mL

C

MARCO transcript fold change (Relative to resting MDMs)

1h 6h 12h 24h

D

MARCO transcript fold change (Relative to resting MDMs)

1h 6h 12h 24h

E

MARCO Transcript Change (Relative to resting MDMs)

SP-A

48h 72h 96h

F

MARCO Transcript Change (Relative to resting MDMs)

Survanta

48h 72h 96h

G

MARCO Transcript Change (Relative to resting MDMs)

SP-A + Survanta

48h 72h 96h

H

MARCO transcript fold change (Relative to resting MDMs)

Survanta

M.tb

Survanta + M.tb

24h 48h

I

MARCO Reads per Million

Resting Infected

24h 48h 24h 48h 24h 72h
FIGURE 5. MARCO protein levels are not altered by surfactant but receptor clustering occurs at the cell surface following Survanta exposure. (A) Day 5 MDMs were exposed to SP-A (10 µg/mL), Survanta (100 µg/mL), both or left resting as a control for 24-72h. Whole cell lysates were collected and MARCO protein levels were analyzed by Western blotting. Shown is a representative blot of three independent experiments. (B) Densitometric analysis of three independent experiments. (C) Increasing the concentration of SP-A does not increase MARCO protein expression. Day 6 MDMs were exposed to 10, 20 or 40 µg/mL SP-A for 24h. Whole cell lysates were collected and MARCO protein levels were evaluated by Western blotting. (D) Densitometric analysis of the blot shown in C. (E) Non-adherent MDMs exposed to SP-A do not increase MARCO protein levels. MDMs were cultured in Teflon wells and exposed to SP-A (10 µg/mL) for 24 or 48h or left resting as a control. Whole cell lysates were collected and MARCO protein levels were analyzed by Western blotting. (F) Densitometric analysis of MARCO expression in non-adherent MDMS. (G) Day 6 MDMs were plated and allowed to rest for 72h to deplete basal protein levels prior to exposure to SP-A (10 µg/mL), Survanta (100 µg/mL), both or IL-4 (50 ng/mL) for 24 or 48h. Whole cell lysates were collected and MARCO protein levels were evaluated by Western blotting. (H) Densitometric analysis of the blot shown in G. (I) Surfactant does not induce redistribution of MARCO to the cell membrane in day 12 MDMs. Day 12 MDMs were exposed to SP-A (10 µg/mL), Survanta (100 µg/mL), both or IL-4 (50 ng/mL) for 24-48h. Cell membranes were isolated and MARCO levels were analyzed by Western blotting. (J) Densitometric analysis of MARCO protein in the cell membranes of day 12 MDMs. (K) Surfactant lipids induce MARCO clustering at the cell membrane 24h after exposure. Day 6 MDMs were plated on coverslips and exposed to SP-A (10 µg/mL) or Survanta (100 µg/mL) for 24-48h. Monolayers were fixed, labeled with anti-MARCO antibody and surface expression was analyzed by confocal microscopy. (J) Fold change in mean fluorescence intensity of MARCO protein at the cell membranes of MDMs exposed to surfactant up to 48h. Shown are results of one (A, B, E-N) or three (C, D) independent experiments (±SEM).
FIGURE 6. MARCO protein levels are not regulated by mycobacterial infection or PPARγ in human macrophages. (A) Day 6 MDMs were infected with *M. tb* H₃⁷Rₛ at an MOI of 5:1 for 24 or 48h. Whole cell lysates were collected and MARCO protein levels were evaluated by Western blotting. (B) Densitometric analysis of the blot shown in A. (C) Day 6 MDMs were infected with BCG at an MOI of 5:1 for 24 or 48h. Whole cell lysates were collected and MARCO protein levels were evaluated by Western blotting. (D) Densitometric analysis of the blot shown in C. (E) HAMs express MARCO protein, which is decreased over time and by *M. tb* infection. HAMs were plated and lysed after 2h, left resting for 24h or infected with *M. tb* H₃⁷Rₛ MOI 5:1 for 24h. Whole cell lysates were evaluated by Western blot for MARCO protein. (F) Cumulative densitometry from three independent experiments. (G) PPARγ agonist PGJ2 does not increase MARCO protein levels relative to DMSO control. Day 6 MDMs were exposed to PGJ2 (2 µg/mL) or DMSO vehicle control (1:1000) for 24-72h. Whole cell lysates were collected and MARCO protein levels were analyzed by Western blotting. (H) Densitometric analysis of the blot shown in G. Shown are results of one (A-D, G, H) or three independent experiments (E, F) (± SEM).
Figure 6

A. 24h 48h
R M.tb R M.tb
IB: MARCO (53 kDa)
IB: β-actin (44 kDa)

B. IB: MARCO (53 kDa)
IB: β-actin (44 kDa)

C. Resting BCG
24h 48h 24h 48h
IB: MARCO (53 kDa)
IB: β-actin (44 kDa)

D. Resting BCG
24h 48h 24h 48h
IB: MARCO (53 kDa)
IB: β-actin (44 kDa)

E. Resting M.tb
2h 24h 24h
IB: MARCO (53 kDa)
IB: β-actin (44 kDa)

F. IB: MARCO
IB: β-actin

G. Resting PGJ2 DMSO
24h 48h 72h 24h 48h 72h
IB: MARCO (53 kDa)
IB: β-actin (44 kDa)

H. PGJ2 DMSO
24h 48h 72h 24h 48h 72h
IB: MARCO (53 kDa)
FIGURE 7. *MSR1* and *SCARB1* transcript levels are not significantly increased by surfactant in human macrophages. (A, B) Short term exposure to surfactant components does not affect *MSR1* transcript levels. Day 6 MDMs were exposed to 10µg/mL SP-A (A) or 100µg/mL Survanta (B) for 1, 6 or 12h and *MSR1* transcript levels were evaluated by qRT-PCR. (C-E) Long term exposure to surfactant components does not affect *MSR1* transcript levels. Day 6 MDMs were exposed to (C) SP-A (10 µg/mL), (D) Survanta (100 µg/mL) or (E) both for 24-96h and *MSR1* transcript levels were evaluated by qRT-PCR. (F, G) *SCARB1* transcript levels are not regulated by surfactant. Day 6 MDMs were exposed to (F) SP-A (10 µg/mL) or (G) Survanta (100 µg/mL) for 1, 6, 12, 24 or 48h. *SCARB1* transcript levels were analyzed by qRT-PCR and no significant differences were observed. Shown are cumulative data from three independent experiments (± SEM) conducted in triplicate.
Figure 7
FIGURE 8. Surfactant lipids increase CD36 transcript and protein levels in human macrophages. CD36 transcript levels are increased by SP-A during the differentiation of monocytes into macrophages. (A) Day 0 monocytes were plated in tissue culture dishes. MDMs were exposed to SP-A (10 µg/mL) at day 0, 1, 2, 3 or 4 after plating. 24h after SP-A exposure, monolayers were lysed and CD36 transcript levels were evaluated by qRT-PCR. (B) Exposure to increasing concentrations of SP-A for up to 48h does not alter CD36 transcript levels in differentiated macrophages. Day 6 MDMs were exposed to 10, 20 or 40 µg/mL SP-A for 6, 24 or 48h and transcript levels were evaluated by qRT-PCR. (C) Exposure to higher concentrations of SP-A does not increase CD36 protein levels. MDMs were exposed to 10, 20 or 40 µg/mL SP-A for 24h and whole cell lysates were probed for CD36 protein. (D) Densitometric analysis of the blot shown in C. (E-G) Short term exposure to surfactant components does not increase CD36 transcript levels. Day 6 MDMs were exposed to (E) SP-A (10 µg/mL), (F) Survanta (100 µg/mL), or (F) both for 1-5h and CD36 transcript levels were analyzed by qRT-PCR. (H-J) Long-term exposure to surfactant components increases CD36 transcript levels. Day 6 MDMs were exposed to (H) SP-A (10 µg/mL), (I) Survanta (100 µg/mL), or (J) both for 24-96h and CD36 transcript levels were analyzed by qRT-PCR. (K) DPPC alone increases CD36 transcript levels. Day 6 MDMs were exposed to DMSO control or 20 µg/mL DPPC for 24-72h and CD36 transcript levels were analyzed by qRT-PCR. (L) Surfactant does not increase CD36 protein levels up to 72h. MDMs were cultured in SP-A, Survanta, both or left resting as a control for 24-72h. Whole cell lysates were probed for CD36 protein levels by Western blot. (M) Cumulative densitometric analysis of three independent experiments, representative blot shown in L. (N) Culturing MDMs in surfactant lipids for 4 days increases CD36 protein levels 5-fold. Representative image of MDMs on coverslips exposed to 100 µg/mL Survanta for 96h, fixed with 4% PFA, permeabilized with methanol (or left non-permeabilized, image not shown) and labeled with anti-CD36 antibody for confocal microscopy. (O) Fold change in CD36 MFI in non-permeabilized and permeabilized Survanta-cultured MDMs relative to resting MDMs. Shown is data from one (A-G), three (K-O) or four (H-J) independent experiments (mean ± SEM where applicable) conducted in duplicate (L-O) or triplicate (A-K).
Figure 8

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

Resting

Survanta

Permeabilized

96h
FIGURE 9. CD36 transcript and protein levels are decreased following *M.tb* infection of human macrophages. (A) Day 6 MDMs were exposed to Survanta (100 µg/mL) for 2 days prior to infection with *M.tb* H₃₇R₅ at an MOI of 1:1. Monolayers were lysed 24-96h after infection and CD36 transcript levels were analyzed by qRT-PCR. (B) Freshly isolated HAMs express low levels of CD36 transcripts which increase over time and are inhibited by *M.tb* infection. HAMs were left resting or were infected with *M.tb* H₃₇R₅ (MOI 5:1) for 2, 24 or 72h. Total transcripts were isolated and transcript reads per million were analyzed by AmpliSeq for abundance of CD36 transcripts. (C) *M.tb* infection decreases CD36 protein levels. Day 6 MDMs were infected with *M.tb* H₃₇R₅ (5:1) for 24 or 48h and whole cell lysates were probed for CD36 protein. (D) Densitometric analysis of the blot shown in C. (E) *M.tb* infection decreases CD36 protein in HAMs. Freshly isolated HAMs were left resting for 2 or 24h or were infected with *M.tb* H₃₇R₅ (5:1) for 24h. Whole cell lysates were analyzed for CD36 protein. (F) Densitometric analysis of the blot shown in E. Shown are data from one (C, D), three (A, E, F), or six (B) independent experiments (±SEM).
CHAPTER 3: CD36 MEDIATES THE UPTAKE OF THE MAJOR PULMONARY SURFACTANT LIPID SPECIES DIPALMITOYLPHOSPHATIDYLCHOLINE

3.1 Summary

As detailed in Chapter 2, surfactant is an intimate regulator of AM phenotype and behavior. In addition, AM-dependent maintenance of homeostatic levels of functional surfactant is crucial for proper pulmonary function [272-274, 360, 361]. Although about 50% of surfactant lipid recycling and catabolic processes are carried out by type II alveolar epithelial cells [31], AM degradative capabilities are required for surfactant homeostasis [278-280] and several pulmonary pathologies can be attributed to defects in AM surfactant catabolism [277, 362]. Surfactant breakdown by AMs is an integral component of AM-mediated pulmonary homeostasis and the cellular pathways involved seem to be closely connected to AM response mechanisms following infectious challenge [277, 281, 363].

Due to the essentiality of surfactant catabolism by AMs, we sought to fill a major gap in our knowledge regarding this important pulmonary process: the mechanism(s) of surfactant lipid uptake by AMs. Although macrophage uptake of surfactant lipids is receptor-mediated [364], the receptor(s) involved have yet to be identified and we therefore investigated which receptor(s) contribute to surfactant lipid uptake by human macrophages. Based on the literature and our previous results (described in Chapter 2),
we elected to focus on scavenger receptor CD36 as a possible regulator of surfactant lipid uptake by macrophages.

CD36 mediates approximately 70% of fatty acid uptake in cardiomyocytes [365] and is the major receptor for the uptake of oxidized low density lipoprotein (oxLDL), thereby contributing to the development of foamy macrophages (FM) during atherosclerosis [251]. Notably, CD36 mediates uptake of palmitate by type II pneumocytes [334], the cells which synthesize surfactant [15] and CD36 is expressed on AMs [63, 339, 340]. Surfactant is primarily composed of phosphatidylcholine-based lipids and about 40% of total surfactant is dipalmitoylphosphatidylcholine (DPPC), with smaller amounts of phosphatidyglycerol (PG), other phospholipids and cholesterol [30]. In addition to numerous other ligands, CD36 recognizes phospholipids [247], making it a viable candidate for the uptake of surfactant lipids.

Preformed CD36 protein is stored in intracellular depots and is redistributed to the cell membrane following the application of an appropriate stimulus, a phenomenon which has been carefully studied in cardiac muscle. In cardiomyocytes, insulin induces phosphatidylinositol 3 kinase (PI3K)-dependent redistribution of CD36, while electrically-induced cell contractions result in PI3K-independent CD36 relocation [366-368], indicating that CD36 can be stored in distinct cellular compartments which are responsive to divergent stimuli. Furthermore, the abrogation of both insulin and contraction-induced fatty acid uptake following administration of CD36-specific inhibitors confirms that CD36 is required for stimulant-induced fatty acid uptake, at least in cardiomyocytes [367]. These observations led us to begin our analysis of CD36-dependent surfactant lipid uptake by investigating whether surfactant could induce redistribution of preformed CD36 to the cell membrane of MDMs.
The intracellular fate of CD36-dependent cargo varies depending on the uptake stimulus and concomitant alterations in the expression of lipid processing enzymes [369]. Additionally, following substrate uptake CD36 can be degraded, recycled to the cell surface or returned to intracellular storage sites [245], further adding to the complexity of intracellular CD36 trafficking. Characterizing the fate of surfactant lipids which enter the cell through CD36, as well as the fate of CD36 itself following surfactant lipid uptake, was beyond the scope of the present work. However, we did investigate the possibility that CD36 is trafficked through the recycling endosomal pathway following surfactant lipid exposure.

Herein, we show that surfactant lipids and SP-A increase CD36 surface expression by inducing translocation of preformed CD36 to the cell membrane. We further show that CD36 knockdown macrophages have a significantly diminished ability to acquire DPPC, the most abundant lipid in surfactant, but not phosphatidylglycerol (PG), the second most abundant lipid in surfactant. Exposure of macrophages to surfactant lipids enhances CD36-dependent uptake of DPPC, indicating that increased surface expression of CD36 following surfactant exposure has a functional impact on macrophage lipid uptake. SR-A knockdown increases DPPC acquisition (possibly due to the elevated CD36 expression which we observed in SR-A deficient cells) lending further support to the notion that CD36 specifically contributes to DPPC uptake. Finally, although we have confirmed through cycloheximide experiments that preformed CD36 is being trafficked to the cell membrane independently of protein synthesis following surfactant exposure, we did not observe co-localization of CD36 with known components of the recycling endosomal pathway. Therefore, the mechanism of surfactant-induced redistribution of CD36 remains to be determined.
3.2 Results

CD36 surface localization is transiently increased following short term exposure to surfactant lipids

As a membrane localized receptor, CD36 mediates the uptake of an array of lipid substrates. We observed limited surface expressed CD36 in resting MDMs (Fig 10A), although these cells contain an abundance of intracellular CD36 (Fig 10E). Preformed CD36 is known to translocate to the cell surface in response to an appropriate stimulus [335, 342, 366-368] and we hypothesized that surfactant would induce redistribution of CD36 in macrophages. MDMs were cultured in SP-A, Survanta or both for up to 4h, fixed with PFA, labeled with CD36 antibody and surface localization was evaluated by confocal microscopy. We observed a transient two-fold increase in CD36 surface localization 30 min after exposure to surfactant lipids (Fig 10A, C). Conditions which included SP-A somewhat inhibited this phenomenon (Fig 10A, B, D) and surface localization generally returned to baseline over time (Fig 10B-D). As expected based on our previous results, total levels of CD36 protein remain constant following exposure to SP-A (Fig 10F) or SP-A plus Survanta (Fig 10H) out to 4h. Total levels of CD36 appear to fluctuate in MDMs exposed to Survanta alone (Fig 10G), although we speculate that this is likely due to an artifact of the fixation and/or permeabilization process which would normalize if the experiment was repeated.
Preformed intracellular CD36 is redistributed to the cell membrane following prolonged culture in surfactant.

Although a transient increase in CD36 surface localization was detected 30 min after exposure to surfactant lipids, our previous results (reported in Chapter 2) indicated to us that a longer exposure to surfactant may be necessary to effect a more robust change on the location of CD36. We therefore investigated the cellular distribution of preformed CD36 in MDMs following exposure to surfactant components for up to 48h. Resting MDMs demonstrate low level CD36 surface localization at all time points tested. Following surfactant exposure, CD36 surface localization is increased by roughly 2-fold at 6h and peaks at approximately 3-fold at the 24h time point before returning to basal levels after 48h (Fig 11A, B). Regardless of exposure to surfactant, MDMs in tissue culture at the time points tested contain similar levels of preformed intracellular CD36 as determined by quantifying CD36 abundance in permeabilized MDMs (Fig 11C, D). This is in keeping with our previous finding that CD36 protein levels do not increase until 96h after surfactant exposure. Interestingly, DPPC alone is sufficient to induce surface relocation of CD36 (Fig 11E, F), further supporting our results in Chapter 2 which indicated that DPPC was the component of Survanta to which CD36 was responsive. Cyclohexamide treatment [42] does not impair Survanta-induced redistribution of CD36 to the cell surface (Fig 11G, H) confirming that protein synthesis is not needed. Based on these results, we conclude that surfactant induces trafficking of preformed CD36 to the plasma membrane of human macrophages.
**PPARγ knockdown and *M. tb* infection do not have a significant effect on CD36 surface localization**

CD36 expression can be regulated by PPARγ [370] and we wondered whether the absence of PPARγ would prevent surfactant-induced redistribution of CD36. MDMs were transfected with SC or anti-PPARγ siRNA prior to exposure to SP-A for 6 (Fig 12A, B), 24 (Fig 12C, D) or 48h (Fig 12E, F) and CD36 surface expression was evaluated by confocal microscopy. Although PPARγ knockdown actually slightly increased SP-A induced surface localization of CD36 relative to SC MDMs, the change was minimal (less than 2-fold). We concluded that PPARγ knockdown does not substantially affect CD36 surface expression following surfactant exposure, indicating that CD36 activity is regulated independently of any role for PPARγ in CD36 transcription.

We next queried whether *M. tb* infection would affect surfactant-mediated CD36 redistribution. Based on our hypothesis that *M. tb* exploits CD36 function during pulmonary infection (described in Chapters 2 & 4), we predicted that *M. tb* infection would enhance CD36 surface localization. MDMs were exposed to SP-A for 24h prior to infection with *M. tb* H37Rv at an MOI of 1:1. Monolayers were fixed 2 or 24h after infection and CD36 surface localization was evaluated in non-permeabilized MDMs. 2h after infection, *M. tb* alone induced a modest (0.5 fold) increase in CD36 surface expression (Fig 12G, E) which returned to baseline 24h after infection (Fig 12G, F). For this donor, SP-A alone had no effect on CD36 membrane localization and, when combined with *M. tb*, there was a slight inhibitory effect 24h after infection. We conclude that *M. tb* infection does not have a significant effect on CD36 surface localization. Although we obtained negative data from our experiments investigating the role of PPARγ knockdown
and *M.tb* infection in the location of CD36, these results do lend credence to the specificity of our findings with surfactant (Fig 11).

**CD36 mediates the uptake of DPPC, but not PG, in human macrophages**

After observing that the cellular location of CD36 could be affected by the presence of surfactant, we next investigated whether CD36 contributes to the uptake of surfactant lipids by macrophages. We first optimized CD36 knockdown using siRNA, which is 80% effective relative to SC transfected MDMs (Fig 13A, B) and significantly inhibits Dil-oxLDL uptake (Fig 13C, D). We next investigated the acquisition of DPPC, which comprises ~40% of the total surfactant lipids [30] and was therefore chosen as a starting point for determining the relevance of CD36 in surfactant lipid uptake by macrophages. siCD36 or SC MDMs were exposed to DPPC for 30 min and uptake was determined by confocal microscopy. As shown in figure 13E and F, CD36 knockdown MDMs acquire five-fold less DPPC relative to SC cells. To determine whether this phenomenon is specific to DPPC uptake, we evaluated the acquisition of PG, the second most abundant lipid in surfactant [30]. CD36 knockdown and SC MDMs acquire comparable levels of PG, indicating that CD36 specifically imports DPPC into macrophages.

**Survanta exposure augments CD36-dependent uptake of DPPC**

Due to our observation that surfactant lipids increase CD36 surface localization and the discovery that CD36 mediates the uptake of DPPC, we hypothesized that culturing macrophages in surfactant lipids for 24h prior to DPPC exposure would augment DPPC uptake through increased surface localization of CD36. MDMs were left
resting or were exposed to Survanta for 24h prior to a 30 min incubation with DPPC. As shown in figure 14A and B, surfactant-cultured MDMs take up three times as much DPPC as resting MDMs. DPPC seems to accumulate within the surfactant-cultured macrophages as evidenced by dense regions of fluorescence in the cells. These results suggest that the surfactant-mediated increase in CD36 surface expression has a functional impact on the uptake and processing of surfactant lipids.

We next wondered whether the Survanta-induced increase in DPPC uptake was CD36-dependent. MDMs were transfected with SC or anti-CD36 siRNA and cultured in Survanta for 24h prior to a 30 min DPPC exposure. Similar to our previous results (Fig13E, F), resting siCD36 MDMs acquire substantially less DPPC than SC macrophages (Fig 14C, D). Survanta-cultured SC MDMs take up and accumulate more DPPC, again seen aggregating as fluorescent-rich regions within the cells. However, in CD36 knockdown MDMs there is only a minimal increase in surfactant-induced DPPC uptake. Furthermore, there is essentially no accumulation of DPPC in the aggregates we observed in SC MDMs (Fig 14E, F). These results indicate that the surfactant-mediated increase in DPPC uptake is largely dependent upon CD36. The slight increase in DPPC uptake in Survanta-cultured CD36 knockdown cells is likely accounted for by increased surface localization and activity of the residual 20% of CD36 and/or the activity of additional lipid uptake receptors.

Scavenger receptor A knockdown increases DPPC acquisition and reduces PG uptake to a small extent

To confirm that DPPC uptake by human macrophages is mediated by CD36 and not scavenger receptors in general we next knocked down SR-A (Fig 15A, B), which is
also expressed by AMs [267] and mediates uptake of acetylated (ac)-LDL [371, 372]. As a positive control, we verified that SR-A knockdown significantly reduces macrophage acquisition of acLDL (Fig 15C, D). In contrast to the results following CD36 knockdown, DPPC acquisition is increased after SR-A knockdown (Fig 15E, F). CD36 and SR-A are known to have compensatory expression profiles [373] and we observed a 40% increase in CD36 expression following SR-A knockdown (Fig 15G, H) which may account for the increase in DPPC acquisition. Finally, we tested the ability of SR-A knockdown MDMs to acquire PG in order to determine whether SR-A could have a role in the uptake of other surfactant lipids. PG uptake is decreased to a small extent in SR-A knockdown cells (Fig 15I, J). However, the results were not significant and any contributions by SR-A to the uptake of less abundant surfactant components was not evaluated further.

**Surfactant lipids induce translocation of the mannose receptor to the cell surface**

Because surfactant is known to regulate aspects of the AM phenotype, we hypothesized that other macrophage receptors would be relocated to the cell surface by surfactant exposure, regardless of whether or not they were involved in lipid uptake. We investigated the effects of surfactant exposure on the cellular location of the mannose receptor (MR, CD206), which is expressed by AMs [374] and contributes to immune responses in the lung [27], including during M.tb infection [64-66, 150, 355]. MDMs were left resting or were exposed to Survanta for 6 or 24h and MR surface expression was evaluated on non-permeabilized cells. 6h after exposure to surfactant lipids, there is a 3-fold increase in MR surface levels relative to resting MDMs (Fig 16A, C). After a 24h exposure to surfactant lipids, MR surface expression is increased by 4-fold (Fig 16B, C). During this time, total levels of MR do not change (Fig 16D-F), indicating that surfactant
lipids are inducing redistribution of the MR to the cell membrane. This observation is in keeping with our lab's previous findings, which show SP-A induced translocation of the MR to the cell surface [42]. Our results lend further credence to the decades old assertion that surfactant is able to regulate prominent aspects of macrophage phenotype.

**CD36 does not co-localize with transferrin receptor or Rab11 following surfactant exposure**

As described in the introduction to this chapter, CD36 can traffic through the recycling endosomal pathway [369]. We wondered if the redistribution of CD36 following surfactant exposure was occurring through the trafficking of recycling endosomes. We therefore investigated the extent of co-localization of CD36 with two well known constituents of the recycling endosomal pathway, transferrin receptor (Tfr) and Rab11 [375-377]. Because endocytic recycling can occur very rapidly [378] and we had not investigated the effects of surfactant exposure at time points prior to 30 min in previous CD36 experiments, we chose to conduct this experiment very early after surfactant lipid exposure.

MDMs were exposed to Survanta for 5, 15 or 30 min and labeled with anti-Tfr (Fig 17A-C) or anti-Rab11 (Fig18A-C) and the possibility of cytoplasmic co-localization with CD36 was investigated in permeabilized MDMs. Tfr and CD36 did not co-localize at any of the time points tested regardless of the presence or absence of Survanta, although the cellular distribution of Tfr appears to migrate towards the perinuclear region of the recycling endosomal compartment [377] 30 min after surfactant lipid exposure (Fig 17C). Minimal co-localization of CD36 with Rab11 is seen 15 min after the addition of
Survanta (Fig18B), although due to the high amounts of CD36 in the cell the legitimacy of the co-localization is questionable. The high level of perinuclear labeling of Rab11 at all time points is in keeping with the previously observed perinuclear localization of this protein [376]. Our results indicate that CD36 is not stored in the same intracellular compartment as Tfr or Rab11 and is unlikely to be trafficked in vesicles which also contain Tfr or Rab11 in response to surfactant exposure.

3.3 Discussion

Maintenance of pulmonary surfactant homeostasis in the alveolus is crucial but the mechanisms are incompletely characterized [31]. Many cellular factors contribute to this complex process, which seems to be inter-related with macrophage host defense activities: in addition to regulating lung compliance and enabling respiration, surfactant lipids and proteins modulate immune responses [44, 63, 81, 345]. Therefore, defining the means of balancing between surfactant synthesis and degradation (ie, the mediation of surfactant homeostasis) is crucial for understanding immune activation and resolution in the lung. We therefore endeavored to identify macrophage receptor(s) which mediate the uptake of surfactant lipids, a key aspect of the degradative arm of surfactant homeostasis.

Surfactant is a complex mixture of lipids and proteins which ultimately adsorbs to the air-liquid interface between inhaled gases and the alveolar hypophase. Surfactant is synthesized by cuboidal type II alveolar epithelial cells, where it is packaged into lamellar bodies (LBs) and stored intracellularly until secretion. Following their release into the alveolar space, LBs unravel into tubular myelin (TM), a lattice-like structure which replenishes the adsorbing surfactant monolayer [15]. During the respiratory cycle,
surfactant spreads and contracts, undergoing a conversion from large aggregates (LAs) during inhalation to unilamellar small aggregates (SAs) during exhalation. LAs are the surface active and complete form of surfactant, being comprised of both lipids and proteins. SAs are composed exclusively of lipids and lack the ability to adsorb. Therefore, SAs cannot reduce surface tension [379] but can be reincorporated into LAs, recycled by type II cells for incorporation into newly synthesized surfactant, or taken up by macrophages for degradation [31].

Complete surfactant is approximately 10% protein [15, 30]. SP-B and SP-C are small hydrophobic proteins which associate with the phospholipid monolayer and contribute to adsorbance at the air-liquid interface [29]. SP-B is necessary for lung compliance and acute respiratory failure ensues in its absence [34]. SP-C knockout mice exhibit low levels of intrinsic inflammation and SP-C has been shown to inhibit the immune response to LPS by blocking TLR4-dependent cytokine induction [77] through a direct interaction with LPS and CD14 [76]. SP-B and SP-C are included in Survanta and were therefore potential contributing factors in any results generated through exposure to Survanta, although their precise contributions were not explored herein.

The larger hydrophilic proteins, SP-A and SP-D, are well known to regulate immunological processes and surfactant homeostasis [346]. SP-A has been shown to enhance the uptake of surfactant lipids by macrophages [272], although SP-A knockout mice have normal lung physiology and only minor abnormalities in surfactant structure [380]. In our own work, we observed less robust regulation of CD36 cellular location following SP-A exposure relative to surfactant lipids (Fig 10A, B; Fig 11A, B; Fig 12).

SP-D is necessary for the maintenance of pulmonary integrity as well as surfactant homeostasis. SP-D knockout mice display pooled surfactant and constitutive
edema [73] due to spontaneous upregulation of metalloproteases through unchecked NFκβ activity [71]. The defect in surfactant catabolism in SP-D -/- mice is attributed to altered surfactant structure, characterized by ultradense LAs and abnormal, multilamellated SAs. These changes to SA structure prevent their uptake by alveolar type II cells, leading to surfactant pooling in pulmonary tissue [75]. Because the defects in surfactant catabolism following SP-D knockout did not involve AMs [74], SP-D was not explored in the present work.

Surfactant lipids are destroyed in the macrophage lysosome, primarily by the enzymes lysosomal acid lipase (LAL) and lysosomal phospholipase A2 (LPLA2), which catalyze the degradation of surfactant cholesterol and phospholipid components, respectively [273, 274, 381, 382]. GM-CSF, PPARγ, ABCG1 and PU.1 have also been shown to be necessary for effective surfactant catabolism by AMs [275, 276, 278-281]. GM-CSF is a cytokine which regulates hematopoiesis, terminal differentiation of macrophages and inflammatory processes [383]. Deficiency or dysregulation of GM-CSF leads to pulmonary alveolar proteinosis (PAP) [384], a disorder characterized by accumulation of excess surfactant due to defects in AM surfactant catabolic pathways [280]. GM-CSF regulates the expression of PPARγ, a transcription factor which mediates the β-oxidation and storage of lipids as well as the inhibition of inflammatory processes in a variety of tissues [385]. Although surfactant-induced redistribution of CD36 is not dependent on PPARγ (Fig 12A-D), PPARγ is required for surfactant lipid homeostasis through regulation of AM lipid metabolic processes and the expression of ABCG1 [278, 279, 386].

ABCG1 mediates the efflux of cholesterol and phospholipids from macrophages and is required for intracellular lipid homeostasis [387]. ABCG1 knockout mice acquire a
progressive pulmonary lipidosis disorder and ex vivo experiments using macrophages from these mice show accumulation of lipid droplets following surfactant exposure [275], indicating that the ability to efflux surfactant degradation products is necessary for surfactant homeostasis. Finally, GM-CSF also regulates the expression of PU.1, a transcription factor which mediates lineage-specific differentiation of immune cells [388] and the expression of cell surface receptors which enable cellular interactions with their environment [389]. The role of GM-CSF in PU.1 expression was discovered through the observation that GM-CSF knockout mice are deficient for PU.1. Interestingly, retrovirus-mediated expression of PU.1 in GM-CSF knockout mice restored not only AM surfactant degradative capabilities but also innate immune functions such as TLR expression and phagocytosis [281], again emphasizing the interconnected aspects of AM surfactant catabolism and host defense [277, 281, 363].

The most prevalent lipid species in surfactant is DPPC, which is responsible for the reduction in surface tension. PG is the second most abundant surfactant lipid and promotes DPPC adsorption [32]. Minor phospholipids in surfactant include phosphatidylinositol, phosphatidylserine and sphingomyelin. The precise functions of the relatively minor components of surfactant have not been determined [32] and their uptake by macrophages was not investigated herein. The neutral lipids in surfactant have also not been extensively investigated, but cholesterol is the most abundant neutral lipid and helps to regulate appropriate fluidity of surfactant [15, 30, 32]. Because CD36 is already known to mediate the uptake of cholesterol [329, 390, 391], this was not pursued in the present work.

As described in the introduction to this chapter, CD36 surface expression is induced by the redistribution of intracellular CD36 following cellular stimulation. Herein,
we show for the first time that preformed CD36 is trafficked to the cell membrane following exposure to surfactant components (Fig 10A, B and Fig 11A, B). DPPC is sufficient to induce CD36 surface expression (Fig 11E, F) and CD36 specifically mediates the uptake of DPPC (Fig 13E, G and Fig 14E, F), constituting the first description of homeostatic surfactant lipid uptake by a specific macrophage receptor. Furthermore, culturing macrophages in surfactant lipids increases DPPC uptake in a CD36-dependent manner (Fig 14E, F), supporting our hypothesis that in the pulmonary environment CD36 surface expression is upregulated by surfactant to facilitate homeostasis. Although we were unable to identify the mechanism of surfactant-induced CD36 redistribution (Fig 17 and 18), the finding that CD36 is likely to be functional in the surfactant-rich alveoli as the mediator of macrophage DPPC uptake greatly enhances our knowledge of the endogenous pulmonary space.

3.4 Materials & Methods

Reagents

RPMI 1640 +L-Glutamine was purchased (Life Technologies, Carlsbad, CA, USA) and supplemented with donor autologous serum for cell culture. For confocal microscopy, mouse IgM anti-human CD36 (sc-7309, Santa Cruz), goat anti-human SR-A (AB5486, EMD Millipore), mouse IgG anti-human MR (59511, InvivoGen), rabbit anti-human Tfr (ab84036, Abcam) and rabbit anti-human Rab11(ab3612, Abcam) Abs were used, followed by AlexaFluor (AF) 488- or 647-conjugated goat-anti-mouse IgM or IgG, AF647-conjugated donkey-anti-goat or AF568-conjugated goat-anti-rabbit IgG (Invitrogen, Waltham, MA, USA). Isotype controls were mouse IgM or IgG, goat IgG and
rabbit IgG (Ancell Corporation, Stillwater, MN, USA). Survanta (bovine-derived surfactant lipids lacking SP-A and SP-D) was from Abbott pharmaceuticals (Abbott Park, IL, USA). SP-A was purified from the bronchoalveolar lavage of pulmonary alveolar proteinosis patients as described [356]. Dil oxLDL was from Alta Aesar (Haverhill, MA, USA), Dil acLDL from ThermoFisher Scientific (Waltham, MA, USA) and nitrobenzoxadiazole-conjugated DPPC and nitrobenzoxadiazole-conjugated PG from Avanti Polar Lipids (Alabaster, AL, USA).

Human monocyte-derived macrophage isolation and cultivation

Human peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood of healthy donors on a Ficoll-Hypaque (Amersham, Pittsburgh, PA, USA) cushion as described [64] and approved by The Ohio State University (OSU) IRB. PBMCs were then cultured in Teflon wells in RPMI 1640 + 20% donor autologous serum for 5 days at 37°C, 5% CO₂ [64]. During this time, monocytes differentiate into monocyte-derived-macrophages (MDMs). Experiments were conducted in duplicate using MDM monolayers in tissue culture plates.

Confocal microscopy

MDMs (1.5 x 10⁵) adhered to glass coverslips in 24-well tissue culture plates were exposed to SP-A (10 µg/mL), Survanta (100 µg/mL) or both for various times. Monolayers were fixed with 4% PFA for 10 min in the dark at room temperature, washed and left intact or permeabilized by a one minute methanol exposure [359]. Coverslips were blocked overnight at 4° (PBS + 5% BSA + 10% FBS) and labeled with anti-CD36 (1:200), anti-SR-A (1:50), anti-MR (1:200) anti-Tfr (1:200) or anti-Rab11 (1:400) primary
Abs for 1h at room temperature followed by AF secondary Abs (1:500) for 1h at room temperature. Coverslips were mounted on glass slides using ProLong Gold AntiFade Mounting media plus DAPI (Invitrogen Life Technologies) and viewed using a FluoView 1000 Laser Scanning Confocal microscope (Olympus). The mean fluorescence intensity (MFI) of random confocal images was quantified using pixel intensity measurement (NIH Image J program). The MFI was calculated for approximately 150 MDMs per coverslip, from duplicate slides for each experiment.

**Transfection of human macrophages and exposure to surfactant lipids**

Day 6 MDMs were incubated with 25 nM of CD36, SR-A, PPARγ or scramble control siRNA (SMARTPool, Dharmacon, Lafayette, LA, USA) in Mirus TransitX2 transfection solution (Mirus Bio LLC, Madison, WI, USA) per the manufacturer’s protocol. Knockdown efficacy was determined by confocal microscopy or Western blot. MDMs were washed once with warm RPMI and exposed to 10 µg/mL Dil oxLDL or Dil acLDL, or 20 µg/mL NDB-DPPC or NBD-PG in RPMI + 2% donor autologous serum for 30 min, followed by fixation and labeling for confocal microscopy.

**Statistical analysis**

Experiments which were analyzed for statistical significance were conducted in duplicate using MDMs from a minimum of three different donors. Prism-5 software (Version 5.04; GraphPad) was used to determine the statistical significance of differences in the means of experimental groups using an unpaired, one-tailed Student t-test. *p* values < 0.05 were considered significant.
Figure 10. CD36 surface localization is transiently increased following short-term exposure to surfactant lipids. (A) Representative images of MDMs which have been left resting, exposed to SP-A (10 µg/mL), Survanta (100 µg/mL) or both for 30 min prior to fixation with PFA and labeling with anti-CD36 antibody (green) or DAPI (blue). MFI of time course for MDMs exposed to (B) SP-A, (C) Survanta or (D) both for 30 min, 1h, 2h, 3h or 4h. (E) Representative images of permeabilized MDMs either left resting or exposed to SP-A, Survanta or both for 30 min. Monolayers were fixed with PFA and permeabilized with methanol prior to labeling with anti-CD36 antibody. MFI of time course for permeabilized MDMs exposed to (F) SP-A, (G) Survanta or (H) both for 30 min, 1h, 2h, 3h or 4h. Images are representative of two independent experiments conducted in duplicate (A, E). Graphs show fold change relative to resting MDMs and are cumulative data from two independent experiments (30 min, 1h, 2h) or data from one experiment (3h, 4h) conducted in duplicate. Scale bar is 50 microns.
Figure 10

A

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<th>Resting</th>
<th>SP-A</th>
<th>Survanta</th>
<th>SP-A + Survanta</th>
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B

- **SP-A**
  - CD36 MRI fold change (Relative to untreated MDMs)
  - 30 min, 1h, 2h, 3h, 4h

C

- **Survanta**
  - CD36 MRI fold change (Relative to untreated MDMs)
  - 30 min, 1h, 2h, 3h, 4h

D

- **SP-A + Survanta**
  - CD36 MRI fold change (Relative to untreated MDMs)
  - 30 min, 1h, 2h, 3h, 4h

E

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<tr>
<th></th>
<th>Resting</th>
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<th>Survanta</th>
<th>SP-A + Survanta</th>
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<td><img src="image8" alt="Image" /></td>
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F

- **SP-A**
  - CD36 MRI fold change (Relative to untreated MDMs)
  - Permeabilized

G

- **Survanta**
  - CD36 MRI fold change (Relative to untreated MDMs)
  - Permeabilized

H

- **SP-A + Survanta**
  - CD36 MRI fold change (Relative to untreated MDMs)
  - Permeabilized
Figure 11. Preformed intracellular CD36 is redistributed to the cell membrane following prolonged exposure to surfactant. (A) Representative images showing surface localization of CD36 following culture in surfactant components for 24h. MDMs were exposed to SP-A (10 µg/mL), Survanta (100 µg/mL) or both, fixed with PFA, labeled with CD36 antibody (green) and DAPI (blue) and imaged by confocal microscopy. (B) Fold change in CD36 MFI from non-permeabilized MDMs exposed to surfactant components for 6 or 24h. (C) Representative images showing total CD36 in permeabilized MDMs. MDMs were cultured in SP-A, Survanta, both or left resting for 24h, fixed with PFA and permeabilized with methanol prior to labeling with anti-CD36 antibody. (D) Fold change in total CD36 MFI from permeabilized MDMs cultured in surfactant components for 6 or 24h. (E) DPPC is sufficient to induce trafficking of CD36 to the cell surface. MDMs were exposed to DPPC (20 µg/mL) or DMSO control (1:1000) for 24h and surface localization of CD36 was evaluated in non-permeabilized cells. (F) Fold increase in MFI of DPPC exposed MDMs relative to DMSO control. (G) Pre-treatment with CHX does not prevent surface redistribution of CD36. MDMs were exposed to CHX (10 µg/mL) for 30 min prior to addition of Survanta for 24h. MDMs were fixed with PFA and surface localization of CD36 was determined by confocal microscopy. (H) MDMs were exposed to CHX prior to Survanta, fixed and permeabilized to evaluate total CD36 levels. Shown are cumulative data from three (C-H) or four (A, B) independent experiments conducted in duplicate (±SEM). Scale bar is 50 microns. Statistical significance was determined using an unpaired, one-tailed student’s t test with a p-value <0.05 considered significant.
Figure 12. PPARγ knockdown and *M. tb* infection do not have a significant effect on CD36 surface localization. MDMs on coverslips were transfected with SC or siPPARγ siRNA and left resting or exposed to SP-A (10 µg/mL) for 6 (A), 24 (C) or 48h (E). Monolayers were fixed with PFA and labeled with anti-CD36 antibody (green) to evaluate surface localization by confocal microscopy. Fold change in surface localized CD36 MFI from scramble control MDMs + SP-A relative to scramble control alone, and siPPARγ MDMS + SP-A relative to siPPARγ alone at 6 (B), 24 (D) or 48h (F). (G) MDMs were infected with *M. tb* H37Rv at an MOI of 5:1 for 24h, fixed with PFA and CD36 surface localization was evaluated by confocal microscopy. (H) Fold change in CD36 MFI of MDMs left resting or infected with *M. tb* for 2 or 24h. Images shown are representative from one experiment conducted in duplicate. Graphs show MFI from approximately 300 cells per condition. Scale bar is 50 microns.
Figure 13. CD36 mediates the uptake of DPPC, but not PG, by human macrophages. (A) Day 6 MDMs on coverslips were transfected with SC or anti-CD36 siRNA via MirusX2 for 72h. Monolayers were fixed with PFA and permeabilized with methanol. Cells were labeled with anti-CD36 antibody (green) and DAPI (blue) and the efficacy of CD36 knockdown was evaluated by confocal microscopy. (B) anti-CD36 siRNA reduces CD36 protein by 80%. (C) CD36 knockdown by siRNA prevents the uptake of oxLDL. MDMs were transfected with SC or anti-CD36 siRNA prior to exposure to fluorescent oxLDL (10 µg/mL) for 30 min. Monolayers were fixed with PFA and labeled with anti-CD36 antibody to verify knockdown. (D) oxLDL MFI in SC or siCD36 MDMs. (E) CD36 knockdown prevents uptake of DPPC. SC or siCD36 MDMs were exposed to fluorescent DPPC (20 µg/mL, purple) for 30 min. Monolayers were fixed with PFA and labeled with anti-CD36 antibody (green). (F) DPPC MFI in SC or siCD36 MDMs. (G) CD36 knockdown does not affect the uptake of PG. SC or siCD36 MDMs were exposed to PG (20 µg/mL, cyan) for 30 min. Monolayers were fixed with PFA and labeled with anti-CD36 antibody (green). (H) PG MFI in SC or siCD36 MDMs. Shown are representative images and graphs from seven (A, B), four (C-F), or three (G, H) independent experiments conducted in duplicate (±SEM). Scale bar is 50 microns. Statistical significance was determined using an unpaired, one-tailed student’s t test with a p-value <0.05 considered significant.
Figure 14. Survanta exposure augments CD36-dependent uptake of DPPC. (A) MDMs were cultured in Survanta (100 µg/mL) or left resting for 24h prior to a 30 min exposure to fluorescent DPPC (20 µg/mL, magenta). Monolayers were fixed with PFA, mounted on coverslips and examined by confocal microscopy. (B) Relative amounts of DPPC acquired by resting or Survanta-cultured MDMs. (C) SC or siCD36 transfected MDMs were left resting for 24h. Fluorescent DPPC (purple) was added for 30 min, monolayers were fixed and labeled with anti-CD36 antibody and DPPC acquisition was evaluated by confocal microscopy. (D) DPPC MFI in SC and siCD36 MDMs. (E) SC or siCD36 MDMs were cultured in Survanta for 24h. Monolayers were exposed to DPPC for 30 min, fixed with PFA and labeled with anti-CD36 antibody (green) and DPPC uptake (purple) was analyzed. (F) DPPC MFI in SC and siCD36 MDMs exposed to Survanta for 24h. Shown are the results of single experiments conducted in duplicate. Scale bar is 50 microns.
Figure 14

A  
DPPC alone  |  Survanta + DPPC

30 min

B  
DPPC MFI (a.u.)

DPPC alone  |  Survanta + DPPC

30m DPPC Exposure

C  
SC  |  siCD36

DPPC alone

D  
DPPC MFI (a.u.)

SC  |  siCD36

No Survanta

E  
Survanta + DPPC

F  
DPPC MFI (a.u.)

SC  |  siCD36

Survanta [100 μg/mL] 24h
Figure 15. Scavenger receptor A knockdown increases DPPC acquisition and inhibits PG uptake to a small extent. (A) Day 6 MDMs were transfected with SC or anti-SR-A siRNA via MirusX2 for 72h. Monolayers were fixed, permeabilized with methanol, labeled with anti-SR-A antibody (red) and knockdown efficacy was evaluated by confocal microscopy. (B) SR-A knockdown is 60% effective. (C) SC or siSR-A MDMs were exposed to fluorescent acLDL (10 µg/mL, cyan) for 30 min and uptake was evaluated by confocal microscopy. (D) acLDL MFI in SC and siSR-A MDMs. (E) Control or SR-A knockdown MDMs were given fluorescent DPPC (20 µg/mL, purple) for 30 min and uptake was evaluated by confocal microscopy. (F) DPPC MFI in SC and siSR-A MDMs. (G) CD36 protein levels are increased in SR-A knockdown macrophages. SC or SR-A knockdown MDMs were fixed, permeabilized and labeled with anti-CD36 antibody. (H) CD36 MFI in SC and siSR-A MDMs. (I) SC or siSR-A MDMs were exposed to fluorescent PG (20 µg/mL, green) for 30 min and uptake was measured by confocal. (J) PG MFI in SC or siSR-A MDMs. Shown are the results of three independent experiments (±SEM). Statistical significance was determined using an unpaired, one-tailed student’s t test with a p-value <0.05 considered significant.
Figure 16. Surfactant lipids induce translocation of the mannose receptor to the cell surface. MDMs were left resting or were exposed to Survanta (100 µg/mL) for 6 (A) or 24h (B). Monolayers were fixed with PFA and labeled with anti-mannose receptor antibody (red) and DAPI (blue). Surface localization was evaluated by confocal microscopy. (C) Fold increase in surface exposed mannose receptor on MDMs which were cultured in surfactant lipids. MDMs were left resting or were exposed to Survanta for (D) 6 or (E) 24h. Monolayers were fixed and permeabilized with methanol to determine total mannose receptor content of the cells. (F) Mannose receptor MFI in Survanta-cultured MDMs relative to resting MDMs. Shown are the results of one experiment conducted in duplicate. Scale bar is 50 microns.
Figure 16

A

B

C

D

E

F

Non-permeabilized

Resting

Survanta

Resting

Survanta

Non-permeabilized

24h

6h

MR MFI fold change (relative to resting)

MR MFI fold change (relative to resting)

Non-permeabilized

Permeabilized

6h

24h

4

2

1

0

1

2

4

5
Figure 17. CD36 does not co-localize with transferrin receptor following surfactant lipid exposure. MDMs were left resting or were exposed to Survanta (100 µg/mL) for (A) 5, (B) 15 or (C) 30 min. Monolayers were fixed with PFA, left intact or permeabilized with methanol and labeled with anti-CD36 (green) and anti-transferrin receptor (red) antibodies. Shown are representative images from two independent experiments conducted in duplicate. Scale bar is 50 microns.
Figure 17
Figure 18. CD36 does not co-localize with Rab11 following surfactant lipid exposure.

MDMs were exposed to Survanta (100 µg/mL) for (A) 5, (B) 15 or (C) 30 min or left resting as a control. Monolayers were fixed with PFA and left intact or permeabilized with methanol. MDMs were labeled with anti-CD36 antibody (green) and anti-Rab11 (red) and co-localization was evaluated by confocal microscopy. Results are representative of two independent experiments conducted in duplicated. Scale bar is 50 microns.
Figure 18
CHAPTER 4: PULMONARY SURFACTANT REGULATES LIPID METABOLISM PATHWAYS AND ENHANCES THE INTRACELLULAR GROWTH OF M.TB IN A CD36-DEPENDENT MANNER

4.1 Summary

The feud between Mycobacterium tuberculosis (M.tb) and humanity has been unfolding for millennia [323] and tuberculosis (TB) remains a prominent global health threat, causing a human death every twenty seconds. In conjunction with this alarming death rate, M.tb’s escalating ability to resist antibiotic treatment adds urgency to the delineation of host responses during TB. M.tb is an undeniably successful pathogen, residing latently within a third of the world’s population and circulating effectively among humans by airborne transmission [106]. Thus, characterizing endogenous lung processes which are encountered by M.tb upon inhalation into the alveolar spaces is crucial, because homeostatic mechanisms of the healthy lung are likely exploited by M.tb to enhance its success early during infection. Furthermore, due to the prominent role of surfactant in shaping the AM immune response repertoire and the contributions of AMs to pulmonary homeostasis, it is necessary to incorporate aspects of the lung environment, such as surfactant, into experiments addressing macrophage responses to M.tb, a predominantly pulmonary pathogen. We therefore investigated the role of surfactant and M.tb infection in the expression of several mediators of AM surfactant
catabolism and homeostasis: adipophilin (gene name PLIN2), lysosomal acid lipase (LAL, gene name LIPA), lysosomal phospholipase A2 (LPLA2), ABCG1 and PPARγ.

Although we had already explored the influence of surfactant and \textit{M}.\textit{tb} on CD36 expression (Chapter 2), we hypothesized that surfactant entry via CD36 could contribute to TB pathogenesis. CD36 has been implicated in the establishment and progression of TB in several animal models [269, 271], although data indicating that CD36 is dispensable for long-term control of TB in the murine model have been reported as well [229]. In contrast to potentially enabling pulmonary colonization by \textit{M}.\textit{tb}, CD36 seems to provide a host-protective function during infections with other pathogens [249, 267, 268, 392]. This discrepancy between the roles of CD36 during TB relative to other infectious diseases led us to hypothesize that CD36 performs a homeostatic function in the lung which \textit{M}.\textit{tb} capitalizes on early during airborne infection. Based on the literature and our CD36-dependent surfactant uptake data (Chapter 3), we hypothesized that CD36 mediates constitutive surfactant lipid uptake by macrophages and that CD36-dependent surfactant lipids would augment \textit{M}.\textit{tb} growth.

As described in Chapters 2 and 3, AM phenotype and behavior are influenced by surfactant exposure, which has major implications for AM-mediated immune responses in pulmonary tissue. Previous work has shown that surfactant lipids can inhibit the production of pro-inflammatory cytokines [44, 45] and we hypothesized that CD36-dependent uptake of surfactant lipids could potentially enhance \textit{M}.\textit{tb} growth by dampening macrophage microbicidal responses through inhibition of pro-inflammatory cytokine signaling. Additionally, CD36 is known to serve as a co-receptor for several TLRs to mediate their location and cellular signaling responses during both infection and sterile inflammation [248, 250, 393, 394]. We therefore also investigated TLR2-
dependent cytokine production in CD36 knockdown macrophages exposed to surfactant lipids, to determine whether the absence of CD36 would affect TLR2-dependent cytokines in surfactant cultured macrophages. Finally, we conducted a series of experiments to determine whether \textit{M.tb} could induce accumulation of DPPC and/or total surfactant phospholipids as a means of exploring the functional outcome of the infection-induced modulation of macrophage mediators of lipid storage, catabolism and efflux.

Herein, we show that \textit{PLIN2} transcripts are up-regulated by surfactant lipids, a phenomenon which is inhibited by \textit{M.tb} infection. Surfactant lipids do not increase adipophilin protein levels but \textit{M.tb} infection again has a negative effect on protein abundance. Surfactant lipids do not affect \textit{LIPA} expression but \textit{M.tb} completely abrogates LAL protein 72h after infection. Surfactant and \textit{M.tb} do not affect \textit{LPLA2} expression but SP-A and \textit{M.tb} do augment \textit{ABCG1} expression. Any alterations in expression levels reported herein are not linked to increased expression of \textit{PPAR}_{\gamma}, although this does not rule out increased activity of this transcription factor, which is known to be regulated independently of expression [395]. We further show that surfactant does not regulate expansion of peroxisomes or autophagosomes.

In addition, we show that surfactant lipids increase macrophage uptake of \textit{M.tb} and augment intracellular growth in a CD36-dependent manner. The increased growth may be partially attributed to surfactant-mediated inhibition of TNF\textalpha expression and release. We further show that TLR2-induced IL-10 and IL-6 are partially dependent on CD36 but independent of surfactant lipids. Conversely, we show that surfactant lipids augment TLR2-induced TNF\textalpha independently of CD36. Finally, we show that \textit{M.tb} is capable of inducing the accumulation of DPPC but not total surfactant phospholipids in
human macrophages, indicating that *M.tb* infection may disrupt processing of specific lipid substrates.

### 4.2 Results

**Surfactant increases PLIN2 transcript levels and *M.tb* infection decreases adipophilin protein**

Adipophilin is required for the maintenance of lipid droplets (LDs) [316, 396], dynamic organelles which function as storage sites for excess cholesterol and triacylglycerol (TAG) in addition to containing enzymes, signaling molecules and cytokines [305]. Adipophilin is expressed in healthy pulmonary tissue but is abundantly over-expressed in tissue adjacent to caseating human granulomas [155]. Furthermore, excessive accumulation of LDs is a hallmark of lipid-laden “foamy” macrophages (FMs) a host susceptibility phenotype during TB [122, 157]. We first observed that freshly isolated human alveolar macrophages (HAMs) contain lipid bodies (Fig 19A), as well as abundant PLIN2 transcripts. We therefore investigated whether surfactant regulates the expression of adipophilin in human macrophages and whether *M.tb* augments adipophilin expression, potentially as a mechanism to induce FM formation.

We found that *M.tb* infection does not alter PLIN2 transcript levels in HAMs, nor does PLIN2 transcript abundance decrease over time out of the lung in uninfected cells (Fig 19B). We next queried whether surfactant could regulate the expression of PLIN2 and found that SP-A increases PLIN2 expression modestly after 72h. The addition of Survanta (bovine-derived surfactant lipids lacking SP-A and SP-D) resulted in an oscillating expression pattern peaking at approximately a four-fold increase (Fig 19C).
We conclude that surfactant lipids positively regulate \( PLIN2 \) transcript levels in monocyte-derived macrophages (MDMs).

We next investigated whether \( M. tb \) infection would augment the effects of surfactant on \( PLIN2 \) transcript levels in our model system. MDMs were exposed to Survanta for 48h prior to infection with \( M. tb \) and \( PLIN2 \) transcript abundance was evaluated 24-96h after infection. \( PLIN2 \) transcript levels are significantly upregulated after a 72h exposure to Survanta, a phenomenon which is inhibited by \( M. tb \) infection (Fig 19D). This is in contrast to our results in HAMs, where we observed no change in \( PLIN2 \) following infection (Fig 19B). No deviation from basal expression levels was observed in any condition at subsequent time points and we concluded that \( M. tb \) is able to negatively regulate the expression of \( PLIN2 \) early after infection in MDMs.

Subsequently, we explored the effects of surfactant lipids and \( M. tb \) infection on adipophilin protein levels. MDMs were cultured in surfactant lipids for two days prior to infection and protein levels were quantified. 24 and 48h after infection, \( M. tb \) decreases adipophilin protein levels by ~50%, while surfactant lipids do not alter adipophilin protein abundance (Fig 19E, F). Based on these data, we conclude that although surfactant lipids can increase \( PLIN2 \) transcript levels this does not lead to an increase in protein under the conditions of our assay. Counter to our expectations based on the literature, \( M. tb \) infection decreases adipophilin expression. We speculate that the decrease in adipophilin would have the effect of decreasing lipid body stability, although this was not investigated herein.
M.\textit{tb} infection decreases expression of LAL and does not affect LPLA2

LAL (gene name \textit{LIPA}) and LPLA2 are the enzymes which mediate the majority of surfactant lipid breakdown by AMs [273, 382] and inhibition of either enzyme leads to the accumulation of intracellular lipids and FM formation [274, 397]. LPLA2 is crucial for an effective host response to \textit{M.tb} in a murine model [398], however nothing is known about the role of LAL during \textit{M.tb} infection in vivo, or the role of surfactant in expression of either enzyme. Because LAL is an important mediator of surfactant catabolism, we hypothesized that exposure to surfactant components would increase macrophage expression of LAL. Conversely, based on previous publications indicating that the absence of LAL contributes to FM formation [382], we hypothesized that \textit{M.tb} infection would result in decreased expression of LAL.

We found that both surfactant lipids and \textit{M.tb} have a mildly inhibitory effect on \textit{LIPA} transcript levels during the first two days after infection. No differences relative to resting MDMs were observed at subsequent time points (Fig 20A). Interestingly, in surfactant cultured MDMs there is a relative increase in LAL protein 2h after infection (Fig 20B, C). We speculate that this result may indicate increased stability of LAL protein early after infection. Over time, \textit{M.tb} infection decreases LAL protein to undetectable levels. Although surfactant lipids alone do not alter LAL levels, they do prevent the \textit{M.tb}-mediated decrease which we observed in the infection alone condition. However, as the infection proceeds, LAL levels are decreased substantially by \textit{M.tb} and no protein is detected in either infection condition by 72h, regardless of surfactant exposure. We conclude that \textit{M.tb} infection decreases LAL protein abundance in human macrophages. Furthermore, although surfactant lipids do not increase LAL abundance relative to
resting MDMs, surfactant may contribute to LAL protein stability as indicated by prevention of the \textit{M.tb}-induced decrease at earlier time points after infection.

Due to the importance of LPLA2 in the breakdown of surfactant phospholipids \cite{273}, we hypothesized that LPLA2 expression would be increased following surfactant exposure. We further hypothesized that \textit{M.tb} infection would decrease LPLA2 expression, based on literature reporting that the absence of LPLA2 not only leads to FM formation \cite{274} but also increases \textit{M.tb} growth in a murine model due to defective cell-mediated responses \cite{398}. In fact, we observed no differences in \textit{LPLA2} expression following either surfactant exposure, \textit{M.tb} infection or both (Fig 20D) and we concluded that \textit{LPLA2} expression is not regulated by surfactant lipids or \textit{M.tb}.

\textit{M.tb} infection increases expression of \textit{ABCG1} in human macrophages

\textit{ABCG1} is a lipid efflux transporter which is necessary for surfactant homeostasis \cite{275} and we hypothesized that surfactant exposure would increase \textit{ABCG1} expression. Furthermore, a decrease in \textit{ABCG1} expression has been implicated in mycobacterial-induced FM formation \cite{270}. However, that study was conducted using attenuated \textit{M.tb} in COS-1 cells and we therefore investigated the effects of virulent \textit{M.tb} infection on the expression of \textit{ABCG1} in human macrophages. SP-A increases \textit{ABCG1} transcript levels, in contrast to surfactant lipids which have an inhibitory effect on SP-A induced expression (Fig 21A). We hypothesize that the divergent regulatory effects of these surfactant components enable the expression of appropriate levels of \textit{ABCG1} in the lung. Interestingly, \textit{M.tb} infection augments expression of \textit{ABCG1}, with surfactant lipids again serving to somewhat inhibit this phenomenon (Fig 21B). Conversely, although \textit{M.tb} infection alone did not alter \textit{ABCG1} protein levels, we observed an increase early
after infection in surfactant-cultured macrophages, followed by a decrease in ABCG1 protein over time (Fig 21C, D). We conclude that \textit{M.tb} infection positively regulates ABCG1 expression early after infection in human macrophages.

**Surfactant and \textit{M.tb} infection do not significantly influence PPAR\(\gamma\) expression and surfactant lipids do not affect pmp70 levels**

Finally, we investigated the role of surfactant and \textit{M.tb} infection in the expression of PPAR\(\gamma\), a transcription factor which serves as a crucial regulator of lipid homeostasis [270, 278, 279, 386] and can regulate the expression of ABCG1 [386]. PPAR\(\gamma\) expression during murine development is necessary for AM differentiation [337] and is needed for surfactant homeostasis [278]. Additionally, several groups, including ours, have shown that PPAR\(\gamma\) activity is important during mycobacterial infection [66, 270]. However, to our knowledge no one has investigated the effects of surfactant exposure or \textit{M.tb} infection on PPAR\(\gamma\) transcript levels. We therefore conducted a time course experiment to determine whether surfactant components and/or \textit{M.tb} infection would augment PPAR\(\gamma\) expression. SP-A approximately doubles PPAR\(\gamma\) transcript levels 72h after exposure, while surfactant lipids do not appear to have an effect (Fig 22A). Similarly, \textit{M.tb} infection does not appear to regulate the expression of PPAR\(\gamma\) (Fig 22B).

Because our group has previously shown that \textit{M.tb} infection enhances PPAR\(\gamma\) protein levels and activity [66], we conclude that \textit{M.tb} may increase rates of translation or protein stability independently of transcript abundance.

PPAR\(\gamma\) was discovered through its role in mediating the expansion of peroxisomes, organelles which mediate fatty acid \(\beta\)-oxidation [399, 400]. Because PPAR\(\gamma\) is a crucial regulator of AM phenotype and little is known about the effects of
surfactant on peroxisomes abundance, we explored the effects of surfactant exposure on the expression of pmp70, a peroxisome membrane protein which increases in abundance during peroxisome expansion [401]. We also labeled the cells with Oil Red O (ORO) to determine if surfactant lipids alter the intracellular lipid content of MDMs and whether surfactant lipids co-localize with pmp70, which would indicate that peroxisome activity could contribute to surfactant lipid breakdown. As shown in Fig 22C and D, surfactant lipids do not accumulate inside of MDMs, pmp70 expression is not increased by surfactant exposure and pmp70 does not co-localize with the ORO stain. Our results indicate that peroxisomes activity does not contribute to the breakdown of surfactant lipids.

**Surfactant does not influence LC3 puncta formation**

The majority of surfactant lipid catabolism is known to be mediated by lysosomal activity [361] and the results described in the previous section indicate that peroxisomes do not contribute to surfactant lipid breakdown. However, nothing is known about the influence of surfactant components on the regulation of autophagy, a constitutive cellular process which is crucial for homeostasis [402]. The downstream efflux of lipid metabolites derived from LAL activity is mediated by autophagy-dependent mechanisms [403] and autophagic processes are known to be important during immune responses in the lung [404]. The impairment of autophagy disrupts pulmonary function and contributes to idiopathic fibrosis and chronic obstructive pulmonary disease [405]. We therefore elected to investigate the hypothesis that exposure to surfactant lipids and protein would increase autophagic activity in human macrophages, possibly as a mechanism for delivery of surfactant components to the lysosome or efflux of metabolites [403].
Autophagy has previously been shown to be an important aspect of the host response to *M. tb* infection [406-408] and was not explored in the context of TB herein.

To evaluate surfactant-mediated effects on autophagy, we utilized the autophagosomal marker LC3, which aggregates into puncta during autophagosomal expansion [409]. We first conducted a time course using various concentrations of pp242, a molecule which inhibits the negative regulator of LC3, mTOR [402, 410]. We observed transient LC3 puncta formation 60 min after exposure to all concentrations of pp242 tested (Fig 23A-F). We therefore fixed the pp242 exposure time at 60 min, although we continued to use a range of pp242 concentrations for experiments involving surfactant. MDMs were exposed to SP-A, Survanta or both for 6 (Fig 23G) or 48h (Fig 23H) prior to exposure to 1, 10 or 100 µM pp242 for 60 min and LC3 puncta formation was evaluated. A two-fold increase relative to DMSO control was observed in cells exposed to 1 µM pp242 48h after surfactant components (Fig 23H). However, no alterations to LC3 puncta were observed in any of the other conditions and we concluded that surfactant does not significantly regulate autophagy in human macrophages.

**Surfactant lipids increase *M. tb* uptake and augment intracellular growth in a CD36-dependent manner**

We postulated that upon deposition in the alveolar space, *M. tb* likely encounters surfactant prior to being taken up by macrophages. We therefore incubated *M. tb*-lux in RHH plus surfactant lipids or RHH alone as a control prior to infecting resting macrophages. We observed a 90% increase in the amount of *M. tb* taken up by macrophages if the bacteria were incubated in surfactant lipids (Fig 24A), indicating that
surfactant exposure may facilitate bacterial uptake. We reasoned that the increased uptake could potentially be due to bacterial clumping in the surfactant-treated condition. We therefore repeated the experiment using mCherry and looked for bacterial clumps by confocal microscopy. As shown in Fig 24B, the majority of bacteria between conditions are single bacilli, with low levels of clumping occurring sporadically. Interestingly, a similar percentage of macrophages become infected regardless of whether the bacteria were exposed to surfactant (Fig 24C).

We hypothesized that exposure of macrophages to surfactant lipids prior to infection would augment intracellular *M.tb* growth, either due to inhibition of pro-inflammatory response pathways or through the availability of surfactant lipid metabolites as a carbon source for the bacteria. MDMs were cultured in surfactant lipids or left resting for two days prior to infection with *M.tb*-lux and bacterial growth was monitored by luminometry. *M.tb* growth is increased by roughly 50% when MDMs are cultured in surfactant lipids prior to infection (Fig 24D). In CD36 knockdown MDMs, this surfactant-mediated growth advantage is abolished (Fig 24E). The difference in growth is not due merely to the absence of CD36 but is predicated on the presence of surfactant lipids, as SC and siCD36 MDMs contain comparable bacterial burdens in the absence of Survanta (Fig 24F). Finally, we verified that *M.tb* uptake is not affected by transfection, a lack of CD36, or exposure to surfactant lipids prior to infection; similar bacterial burdens are observed across conditions immediately following infection (Fig 24G). We conclude that CD36-mediated uptake of surfactant lipids is beneficial for *M.tb* during the early stages of human macrophage infection.
Surfactant lipids inhibit *M.tb* induced TNFα and IL-10 release

We hypothesized that the surfactant lipid-dependent growth advantage could be due to a suppression of macrophage microbicidal responses. Based on previous publications [45, 411, 412], we hypothesized that Survanta would inhibit the release of TNFα and IL-6, pro-inflammatory cytokines which are induced by *M.tb* infection [413, 414]. In addition, we evaluated the expression of IL-10, an anti-inflammatory cytokine which is also induced by *M.tb* infection [415] and has previously been shown to be augmented by surfactant lipids following LPS challenge [44]. We investigated the effects of surfactant lipid exposure on the ability of macrophages to produce these cytokines in response to infection and found that culturing MDMs in Survanta prior to infection significantly inhibits release of *M.tb*-induced TNFα and IL-10 (Fig 25A, B). Our results are in keeping with previous publications which indicate that, depending on the stimulus, the expression patterns of TNFα and IL-10 are linked, with a decrease in TNFα leading to inhibition of IL-10 production [416]. TNFα is crucial for *M.tb* control, as therapeutics designed to block TNFα can lead to both TB reactivation and increased susceptibility to mycobacterial infection [417]. In contrast, *M.tb*-induced IL-6 has been shown to be beneficial for the bacteria [414] and IL-6 levels were unaffected by surfactant lipids (Fig 25C). Therefore, surfactant-mediated inhibition of TNFα potentially contributes to the enhanced bacterial growth that we observed.

**Surfactant lipids decrease TNFα and IL-6 transcript levels following *M.tb* infection**

We wanted to determine at which level of regulation the surfactant-mediated alteration in cytokine production is occurring. We therefore investigated transcript abundance following surfactant exposure and *M.tb* infection. Similar to our cytokine data, *M.tb* begins to increase TNFα expression 72h after infection and MDMs which were
cultured in surfactant lipids prior to infection express lower levels of TNFα transcripts (Fig 26A), indicating that surfactant lipids have an inhibitory effect on TNFα at the level of transcription. Interestingly, IL-10 transcript levels did not deviate from baseline expression following surfactant exposure or *M. tb* infection (Fig 26B), indicating that the inhibitory effects of surfactant are perpetrated beyond the level of transcription. Finally, the ability of *M. tb* to increase IL-6 transcript levels is inhibited by surfactant (Fig 26C), although interestingly similar levels of IL-6 release were observed between conditions (Fig 25C), indicating that additional levels of regulation are involved.

**TLR2-induced IL-10 and IL-6 is partially dependent on CD36 and surfactant lipids augment TLR2-induced TNFα independent of CD36**

TNFα, IL-10 and IL-6 can all be induced through TLR2 [248, 393, 394], *M. tb* expresses TLR2 ligands [418] and CD36 has been shown to contribute to host responses in conjunction with TLR2 [248, 393, 394]. We therefore investigated whether the absence of CD36 affects the TLR2-dependent release of IL-10, IL-6 and TNFα and whether surfactant lipids have an impact on the production of TLR2-dependent cytokines. MDMs were transfected with SC or anti-CD36 siRNA and were left resting or cultured in Survanta for two days prior to exposure to the TLR2 ligand Pam3Cys. Interestingly, we found that Pam3Cys induction of both IL-10 and IL-6 is partially dependent on CD36 (Fig 27A-D). Early after Pam3Cys exposure, Survanta is generally inhibitory to cytokine release (Fig 27A, C, E), although there are larger amounts of variation between donors one hour after Pam3Cys exposure. By 24h it is clear that Pam3Cys induces less IL-10 (Fig 27B) and IL-6 (Fig 27D) in the absence of CD36.
Survanta does not have an effect on the TLR2-dependent production of these cytokines at 24h (Fig 27B, D).

Conversely, the absence of CD36 does not affect the magnitude of TLR2-induced TNFα released 1 (Fig 27E) or 24h (Fig 27F) after Pam3Cys exposure. However, surfactant lipids augment TLR2-induced TNFα 24h after the addition of Pam3Cys (Fig 27F). Based on this finding, we conclude that TLR2 does not contribute to the signaling responses which lead to surfactant-mediated inhibition of \textit{M}.\textit{tb}-induced TNFα.

Furthermore, because a lack of CD36 did not impact TLR2-induced TNFα, it is possible that CD36 does not contribute to the surfactant-dependent reduction in TNFα following \textit{M}.\textit{tb} infection (Fig 25A). However, a role for CD36 in downstream cytokine signaling following \textit{M}.\textit{tb} infection cannot be completely ruled out, because \textit{M}.\textit{tb} and CD36 both interact with multiple TLRs [246, 353] and the effects of CD36 knockdown on \textit{M}.\textit{tb}-induced TNFα were not explored specifically.

\textbf{\textit{M}.\textit{tb} can induce accumulation of DPPC but not total surfactant lipids}

Finally, we investigated whether \textit{M}.\textit{tb} infection could be altering the trafficking and breakdown of surfactant lipids. We hypothesized that \textit{M}.\textit{tb} would induce surfactant lipid accumulation, potentially as a means of sequestering a carbon-based nutrient source. We began by evaluating the effects of the timing of lipid exposure on the ability of \textit{M}.\textit{tb} to manipulate intracellular lipid distribution. We hypothesized that \textit{M}.\textit{tb} would co-localize with surfactant lipids which are inside of macrophages prior to infection (ie, the \textit{M}.\textit{tb} phagosome would fuse with lipid bodies as has been previously shown [288]) but would not co-localize with lipids which entered the cell after infection due to the defects in intracellular trafficking which occur following \textit{M}.\textit{tb} infection [419]. We further
hypothesized that infection would result in the accumulation of lipids which entered the cell at any time either before or after infection. When macrophages were exposed to DPPC for 24h prior to infection with mCherry *M.tb*, no accumulation and no co-localization were observed (Fig 28A). However, when MDMs were exposed to DPPC for 30 min prior to infection, DPPC accumulated within macrophages but again did not co-localize with *M.tb* (Fig 28B). These results indicate that *M.tb* can affect the intracellular fate of lipid cargo which has recently been acquired by macrophages prior to infection, possibly before substantial processing of the lipids has occurred (Fig 28A).

Interestingly, when macrophages were exposed to DPPC 2h after infection and fixed 30 min later, no lipid accumulation occurs (Fig 28C). We speculate that infection may interfere with the uptake of lipids at this early time point. Again, we observed no co-localization. When DPPC was added 24h after infection and the cells were fixed a day later, we observed robust accumulation of DPPC, although no co-localization (Fig 28D). When combined with our data indicating that *M.tb* infection decreases expression of the enzymes required for surfactant catabolism (Fig 19, 20), we conclude that the resulting inability of macrophages to process and degrade surfactant lipids leads to their accumulation within the cytoplasm. Across conditions, no DPPC accumulation was observed in the absence of infection.

We next wondered whether total surfactant phospholipids in the form of Survanta would accumulate within MDMs and whether *M.tb* would have an effect. MDMs were exposed to Survanta for 48h prior to infection and lipid accumulation was evaluated 24 and 72h after infection via Bodipy staining. We observed no increase in lipid content across conditions (Fig 28E, F) and a similar percentage of macrophages are infected
regardless of Survanta pre-exposure (Fig 28G). Taken together, we conclude that \textit{M.tb} infection specifically interferes with the processing of DPPC in human macrophages.

4.3 Discussion

Understanding aspects of host cell physiology which render AMs endogenously susceptible to infection by \textit{M.tb} will ultimately enhance our ability to develop novel therapeutic strategies effective in the pulmonary environment. In the absence of infection, AMs indispensably contribute to surfactant catabolism to maintain lung homeostasis. We contend that this function of AMs creates a host cell which is naturally susceptible to \textit{M.tb} infection due to both the immunosuppressive properties of surfactant and the presence of surfactant lipid metabolites as a readily available carbon source for this host-adapted pathogen.

We hypothesized that throughout the intimate evolutionary history shared by humans and \textit{M.tb} [323], the bacteria have developed the ability to exploit and possibly augment endogenous features of the pulmonary environment. We are particularly interested in this concept as it relates to mechanisms of surfactant homeostasis and therefore investigated the possible contributions of surfactant and \textit{M.tb} infection to the regulation of several proteins which are integral to surfactant turnover. We hypothesized that surfactant would increase the expression of mediators of lipid storage, catabolism and efflux which are of relevance in surfactant homeostasis. We speculated that \textit{M.tb} would induce a decrease in lipid catabolism and efflux regulators as a possible mechanism for FM formation.

We began by investigating adipophilin, the expression of which increases in pulmonary tissue adjacent to necrotic granulomas at later stages of progressive TB
Adipophilin is a coat protein associated with LDs and we first show that HAMs contain both LDs and PLIN2 transcripts (Fig 19A, B). We next show that surfactant components are able to increase PLIN2 transcript levels (Fig 19C, D), although this did not translate into an increase in adipophilin protein (Fig 19E, F). Counter to our hypothesis that M.tb would augment adipophilin expression, M.tb infection actually blocked the ability of surfactant lipids to increase PLIN2 expression (Fig 19D) and reduced adipophilin protein abundance by half 24 and 48h after infection (Fig 19E, F).

We speculate that decreased adipophilin expression and the resultant destabilization of LBs may render carbon sources such as TAG more available for the bacteria, although this was not directly investigated.

We next explored the regulation of two lysosomal enzymes which are necessary for surfactant catabolism, LAL and LPLA2. Surfactant lipids do not increase LIPA transcript (Fig 20A) or LAL protein levels (Fig 20B, C). M.tb infection does not affect LIPA expression (Fig 20A), but does steadily decrease LAL protein levels over days (Fig 20B, C). Interestingly, we observed that surfactant lipids prevent the M.tb-induced decrease in LAL protein (Fig 20B, C). In fact, very early after infection there is an increase in LAL protein levels in surfactant-cultured MDMs (Fig 20B, C). Due to the rapidity of this event, we speculate that there is an increase in protein stability or prevention of the normal degradative pathways as opposed to new protein synthesis. However, by 72h after infection M.tb completely abrogates LAL protein regardless of surfactant exposure and we conclude that M.tb infection decreases LAL expression. LPLA2 expression does not appear to be regulated by surfactant or M.tb (Fig 20D).

A decrease in the expression of LAL has been shown to lead to lipid accumulation [382], which could perhaps explain the increase in expression of ABCG1
efflux transporter which we observed after infection (Fig 21B-D). We speculate that the concurrent decrease in adipophilin precludes the packaging of excess lipids in to LDs and therefore an increase in lipid efflux could be a response by the host cell to attain lipid homeostasis. We observed divergent regulation of ABCG1 by SP-A and Survanta (Fig 21A) and we hypothesize that in the lung this balance would lead to appropriate expression levels of ABCG1.

PPARγ has been linked to the expression of adipophilin [420], ABCG1 [386] and LPLA2 [421]. The promoter for LAL lacks a PPAR response element but there is evidence that in certain cell types LAL expression can be augmented slightly by PPARγ agonists [397, 422]. Because our own work had identified an important role for PPARγ in the host response to M.tb [66], we explored PPARγ expression levels following exposure to surfactant or M.tb. Although we found no alterations in PPARγ expression levels (Fig 23A, B) this does not rule out a functional role for this transcription factor in the surfactant-dependent increase in PLIN2 (Fig 19A) and M.tb-induced ABCG1 (Fig 22B-D).

PPARγ activity also regulates peroxisomes, which are known to contribute to the synthesis of surfactant phospholipids. Approximately 60% of phosphatidic acid, a key precursor in surfactant synthesis, is generated by the activity of peroxisomal enzymes [423]. There is evidence that peroxisomes could hypothetically contribute to surfactant degradation [424] although to our knowledge this has not been tested experimentally. We investigated the effects of surfactant exposure on peroxisome activity through immunolabeling of pmp70, a peroxisomal membrane protein which increases during peroxisome expansion [401] and observed no surfactant-mediated increase in pmp70 expression (Fig 22C, D). Additionally, we labeled total intracellular lipids with ORO to
investigate the possibility that surfactant lipids would co-localize with pmp70 and we observed no co-localization under these conditions. An iteration of this experiment which could yield more precise results would be the use of fluorescent surfactant lipids to investigate their co-localization with peroxisomal markers, although this was not investigated herein.

We also explored the possibility that surfactant could influence the induction of autophagy, potentially as a mechanism for delivering surfactant components to the lysosome for destruction or as a contributing factor to the efflux of surfactant lipid metabolites [403]. Using LC3 puncta formation as a readout, we observed no significant alterations to autophagosome activity following exposure to surfactant lipids (Fig 23G, H). Although both peroxisomal and autophagic pathways contribute to surfactant and pulmonary homeostasis, we did not find evidence that surfactant regulates activity of these metabolic pathways.

Surfactant lipids can bind directly to pathogens such as respiratory syncytial virus (RSV) and prevents their interaction with host cells [81]. We therefore investigated whether surfactant lipids could affect entry of M.tb into macrophages and show a 90% increase in bacterial uptake when the bacteria are coated in surfactant lipids (Fig 24A). Interestingly, similar numbers of macrophages become infected (Fig 24C), indicating that a subset of macrophages ingest higher numbers of bacteria. This is not due to increased bacterial clumping following surfactant exposure (Fig 24B).

We further show that culturing macrophages in surfactant lipids prior to infection enhances bacterial growth (Fig 24D) in a manner at least partially attributable to the immunosuppressive properties of surfactant (Fig 25A), which are well documented in the literature. Surfactant proteins and lipids not only function in maintaining lung
homeostasis [34, 48, 67, 72], but also contribute significantly to host-pathogen interactions [65, 68-70] in addition to being directly microbicidal [425]. The role of tissue factors which are unique to the lung environment (e.g., surfactant components) is an aspect of \textit{M.tb} infection which is often overlooked in experimental studies yet they are likely to play defining roles in TB pathogenesis. We report suppression of \textit{M.tb}-induced TNFα by surfactant lipids (Fig 25A), a host response which may augment bacterial growth. This possibility is in keeping with the well-known effects of TNFα inhibitors on TB reactivation [417]. CD36 knockdown did not alter TLR2-dependent TNFα release (Fig 27E, F). However, because TLR2 recognizes an array of established ligands [418], we cannot rule out the possibility that CD36 is involved in the TLR2 response to \textit{M.tb}.

The relevance of CD36 during TB pathogenesis in the mouse model remains somewhat obscure, as conflicting data have been reported [229, 271]. However, temporal discrepancies in the experiments may partially explain this dissonance. Court \textit{et al} found that a double knockout of SR-A and CD36 did not impact the control of chronic \textit{M.tb} infection, while Hawkes \textit{et al} demonstrated that CD36 knockout mice are less susceptible to mycobacterial colonization and distal organ dissemination early during infection. Additionally, the exact role of CD36 during infection remains to be revealed, since \textit{ex vivo} experiments using macrophages from CD36 knockout mice did not exhibit differences in phagocytosis or reactive oxygen species production. Furthermore, the authors did not attribute the differential growth to the cytokine response during infection [271]. Although CD36 may not be regulating cytokine production during mycobacterial infection, the present study provides evidence that CD36-dependent uptake of surfactant lipids by human macrophages dampens protective cytokine production and facilitates intracellular \textit{M.tb} growth. These findings enhance our
knowledge regarding the contributions of the lung environment during the establishment of *M. tb* infection.

The possibility remains that surfactant lipid metabolites provide a readily available carbon source to *M. tb* following entry into a macrophage. During *in vitro* infection, *M. tb* up-regulates genes involved in lipid metabolism [359, 426] and utilizes host lipids as a carbon source [157, 286, 288]. However, the source of the host lipids used by the bacteria remains less clear. There is evidence that the ability to metabolize cholesterol contributes to intracellular growth [282, 287, 427] although this may be more crucial during persistent stages of infection [282, 283]. Cholesterol metabolism by *M. tb* is not required to establish infection in the lung [282] and there is some evidence that cholesterol is not a necessary carbon source for survival within a host [428]. Therefore, identifying essential nutrient sources for the bacteria during early infection remains of great importance. Future studies with Survanta should incorporate cholesterol, because Survanta does not contain cholesterol and the combination merits investigation.

*M. tb* must be able to metabolize fatty acids and cholesterol for long-term virulence in an animal model [282, 284] and, interestingly, *M. tb* is found in the adipose tissue of TB patients [429]. The ability to metabolize host-derived lipids may contribute to the bacterium’s success as a persister [157, 286, 288, 426] and post-primary reactivation TB has historically been described as lipid pneumonia [430], illustrating the disruption of pulmonary lipid homeostasis during TB progression. In vitro infection experiments have shown that *M. tb* alone or cell wall lipids from virulent *M. tb* can induce a lipid-laden foamy macrophage (FM) phenotype [122, 157] and the inundation of an infected macrophage with lipids halts *M. tb* replication [157, 290]. During this period of dormancy, the bacteria incorporate host-derived TAG into intracytoplasmic lipid
inclusions (ILI) which are rapidly metabolized during the resumption of replication [290, 431]. The high number of lipid processing enzymes encoded by the M.tb genome underscores the importance of lipid metabolism for the bacterium [432]. We therefore speculate that surfactant lipids likely serve as an initial carbon source for M.tb, as well as the substrate for FM formation as the infection proceeds.

4.4 Materials & Methods

Reagents

RPMI 1640 +L-Glutamine was purchased (Life Technologies, Carlsbad, CA, USA) and supplemented with donor autologous serum for cell culture or with 20mM HEPES buffer (Sigma Chemical Co, St. Louis, MO, USA), pH 7.2 and 1 mg/mL human serum albumin (HSA) (Calbiochem Corp., La Jolla, CA, USA) during M.tb infection. For confocal microscopy, rabbit polyclonal anti-pmp70 (ABT12, EMD Millipore, Billerica, MA, USA) and rabbit polyclonal anti-LC3 (D3U4C, Cell Signaling Technology, Danvers, MA, USA) Abs were used, followed by AlexaFluor (AF) AF488-conjugated goat-anti-rabbit IgG (Invitrogen, Waltham, MA, USA). Isotype control was rabbit IgG (Ancell Corporation, Stillwater, MN, USA). Survanta (bovine-derived surfactant lipids lacking SP-A and SP-D) was from Abbott pharmaceuticals (Abbott Park, IL, USA). SP-A was purified from the bronchoalveolar lavage of alveolar proteinosis patients as described [356]. NBD-DPPC was from Avanti Polar Lipids (Alabaster, AL, USA), Bodipy was from Thermo Fisher Scientific (Waltham, MA, USA) and ORO was from Sigma-Aldrich (St. Louis, MO, USA). Pam3Cys was purchased from Calbiochem (EMD Biosciences, La Jolla, CA, USA).
Human anti-TNFα, anti-IL-10 and anti-IL-6 DuoSet ELISA development kits were purchased from R&D Systems (Minneapolis, MN, USA).

**Human monocyte-derived macrophage and alveolar macrophage isolation and cultivation**

Human peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood of healthy donors on a Ficoll-Hypaque (Amersham, Pittsburgh, PA, USA) cushion as described [64]. PBMCs were then cultured in Teflon wells in RPMI 1640 + 20% donor autologous serum for 5 days at 37°C, 5% CO₂ [64]. During this time, monocytes differentiate into monocyte-derived-macrophages (MDMs). Experiments were conducted in duplicate or triplicate wells using MDM monolayers in tissue culture plates. Human alveolar macrophages (HAMs) were isolated from the BAL of healthy human donors [42]. PBMC and HAM protocols were approved by The Ohio State University (OSU) IRB.

**Confocal microscopy**

MDMs (1.5 x 10⁵) adhered to glass coverslips in 24-well tissue culture plates were left resting or were exposed to Survanta (100 µg/mL) for various times. Monolayers were fixed with 4% PFA for 10 min in the dark at room temperature, washed and left intact or permeabilized by a one minute methanol exposure [359]. Coverslips were blocked overnight at 4° (PBS + 5% BSA + 10% FBS) and labeled with anti-pmp70 (1:200 for 1h at room temperature) or anti-LC3 (1:200 for 1h at room temperature) primary Abs and AF secondary Abs (1:500 for 1h at room temperature). Coverslips were mounted on glass slides using ProLong Gold AntiFade Mounting media plus DAPI (Invitrogen Life
Technologies) and viewed using a FluoView 1000 Laser Scanning Confocal microscope (Olympus). For MDM experiments, the mean fluorescence intensity (MFI) of random confocal images was quantified using pixel intensity measurement (NIH Image J program). The MFI was calculated for approximately 150 MDMs per coverslip, from duplicate slides for each experiment. To evaluate intracellular lipid content, HAMs (1 x 10^5) and MDMs on coverslips were labeled with ORO per the manufacturer’s instructions or 2.5 µg/mL Bodipy (Life Technologies) for 30 min in the dark at room temperature and imaged by confocal microscopy.

**CD36 knockdown in human macrophages and exposure to surfactant lipids**

Day 6 MDMs were incubated with 25 nM of CD36 or scramble control siRNA (SMARTPool, Dharmacon, Lafayette, LA, USA) in Mirus TransITX2 transfection solution (Mirus Bio LLC, Madison, WI, USA) per the manufacturer’s protocol for 72h. Knockdown efficacy was determined by confocal microscopy and Western blotting (WB). MDMs were washed twice with warm RPMI and infected with *M.tb* for growth assays as described below, or washed once with warm RPMI and exposed to 20 µg/mL NDB-DPPC in RPMI + 2% donor autologous serum for various times before or after infection, followed by fixation for confocal microscopy.

**Quantitative real time PCR**

Macrophage RNA was isolated using TRIzol reagent (Invitrogen Life Technologies). RNA purity and quality were determined using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific). Total RNA was reverse transcribed to cDNA using SuperScriptIII reverse transcriptase (Invitrogen Life Technologies)[44].
Quantitative real-time PCR (qRT-PCR) was conducted using human PLIN2, LIPA, LPLA2, ABCG1, PPARγ, TNFα, IL-10 and IL-6 TaqMan gene expression systems (Applied Biosystems). Negative controls consisted of no-reverse transcriptase and no-template reactions. All samples were run in triplicate using a cfx96 real-time system (Bio-Rad) and analyzed using the threshold cycle \( (2^{-\Delta\Delta ct}) \) method [358]. Gene expression was normalized against β–actin.

**M. tb strains and macrophage infection**

*M. tb* H₃⁷Rᵥ (ATCC #25618), a luciferase expressing reporter strain (*M. tb*-Lux) containing the plasmid pMV306hsp+Lux [433] or an mCherry expressing reporter strain were used [64]. Day 6 MDMs (4 x 10⁵/ml) were seeded in a 24 well tissue culture plate and either exposed to Survanta (100 µg/ml) for 48h prior to infection or transfected with scramble or CD36 siRNA for 48h and then exposed to Survanta for 24h. MDMs were infected with *M. tb* H₃⁷Rᵥ, *M. tb*-Lux or mCherry *M. tb* at 37°C with 5% CO₂ on a platform shaker for 30 min, followed by 90 min incubation without shaking. Monolayers were washed, repleted with RPMI containing 2% autologous serum and incubated up to 72h. Bacterial growth was measured by luciferase activity [433] or supernatants were collected for ELISAs.

**ELISAs**

Day 6 MDMs were exposed to Survanta (100 µg/mL) or left resting for 48h prior to infection with *M. tb* H₃⁷Rᵥ at an MOI of 1:1 as described above. Cell free supernatants were collected 24, 48 and 72h after infection. Alternatively, day 6 MDMs were transfected with SC or anti-CD36 siRNA via the MirusX2 transfection system for 72h.
Monolayers were washed and exposed to Pam3Cys (100 ng/mL) and cell free supernatants were collected after 1 or 24h. ELISAs were conducted to evaluate TNFα, IL-10 and IL-6 release per the manufacturer’s protocol (R&D Systems) using triplicate supernatants from a minimum of three independent experiments.

Statistical analysis

Experiments were conducted in duplicate (confocal, infections) or triplicate (gene expression, ELISAs) using MDMs from a minimum of three different donors. Prism-5 software (Version 5.04; GraphPad) was used to determine the statistical significance of differences in the means of experimental groups using an unpaired, one-tailed Student t-test. p values < 0.05 were considered significant.
Figure 19. Surfactant increases PLIN2 transcript levels and *M. tb* infection decreases adipophilin protein in human macrophages. HAMs contain lipid bodies. (A) Freshly isolated HAMs were plated on coverslips and labeled with Bodipy to visualize lipid bodies by confocal microscopy. HAMs contain PLIN2 transcripts and expression is not altered by infection. (B) Total RNA was extracted from resting and infected HAMs and submitted for AmpliSeq analysis to quantify PLIN2 transcripts. Surfactant increases PLIN2 transcript levels. (C) MDMs were exposed to SP-A (10 µg/mL), Survanta (100 µg/mL) or both for 6-96h and PLIN2 transcript levels were evaluated relative to resting MDMs. *M. tb* infection blocks surfactant lipid-induced increase in PLIN2 and *M. tb* infection decreases adipophilin protein levels. MDMs were exposed to Survanta (100 µg/mL) for 48h prior to infection with *M. tb* (MOI 1:1). Infection proceeded out to 96h and PLIN2 transcript levels were measured (D) or adipophilin protein levels were immunoblotted (E) and quantified by densitometry (F). Results shown are from one (A, C), four (D) or six (B) independent experiments conducted in triplicate (± SEM) or from two (E, F) independent experiments (± SD).
Figure 19

A

B

C

D

E

F

IB: Adipophilin (48 kDa)

IB: β-actin (44 kDa)
Figure 20. *M. tb* infection decreases expression of LAL and does not affect LPLA2. (A) MDMs were cultured in surfactant lipids for 2 days prior to infection with *M. tb* for up to 96h and *LIPA* transcript levels were evaluated. (B) LAL protein abundance was analyzed in MDMs cultured plus or minus surfactant for 48h prior to infection with *M. tb*. Shown is a representative Western blot from two independent experiments (C) Densitometric analysis of actin-normalized LAL protein levels graphed as fold change relative to resting MDMs. (D) MDMs were cultured in surfactant lipids for two days prior to infection with *M. tb* and *LPLA2* transcript levels were analyzed 24, 48, 72 and 96h after infection. Results are cumulative from three (A) independent experiments conducted in triplicate (± SEM) or two (B, C, D) independent experiments (± SD).
Figure 21. SP-A and *M.tb* infection increase expression of ABCG1 in human macrophages. 

(A) MDMs were cultured in SP-A (10 µg/mL), Survanta (100 µg/mL) or both for 72 or 96h and *ABCG1* transcript levels were evaluated. (B) MDMs were cultured in surfactant for 2 days prior to infection with *M.tb* and the infection proceeded out to 96h. Graphs show fold change relative to resting MDMs at the corresponding time point. (C) Representative blot showing ABCG1 protein levels in MDMs cultured in surfactant lipids for two days prior to infection with *M.tb* (MOI 5:1). Whole cell lysates were collected 2, 24, 48 or 72h after infection. (D) Cumulative densitometric analysis from two independent experiments. Results are from one (A) or three (B) independent experiments conducted in triplicate (± SEM) or two (C, D) independent experiments.
**Figure 22.** Surfactant and *M. tb* infection do not influence PPARγ expression and surfactant lipids do not regulate pmp70 levels. (A) MDMs were cultured in surfactant components for 6-96h and PPARγ transcript levels were analyzed. Graphs show fold change relative to resting MDMs at the corresponding time points. (B) MDMs were cultured in surfactant lipids or left resting for two days prior to infection with *M. tb* and PPARγ transcript levels were evaluated. (C) MDMs on coverslips were left resting or exposed to surfactant lipids for 30 min, 6, 24 or 48h. MDMs were fixed, permeabilized and labeled with ORO and anti-pmp70 antibody. (D) Fold change in pmp70 MFI over time in culture with surfactant lipids relative to resting MDMs. Results are from one (A, 6-48h), two (B) or three (A, 72-96h) independent experiments conducted in triplicate (± SEM) or one experiment (C, D) conducted in duplicate.
Figure 22

A

B

C

D

30 min  6h  24h  48h

Untreated

Survanta

Fold change in PMH mRNA (relative to untreated)

Time after Survanta
Figure 23. Surfactant does not influence LC3 puncta formation. MDMs were exposed to DMSO (A) or increasing concentrations of pp242 (B-E) for 60, 90 or 120 mins. MDMs were fixed, permeabilized and labeled with anti-LC3 antibody for evaluation by confocal microscopy. (F)
Quantification of LC3 puncta induction following exposure to increasing concentrations of pp242 for 60 min. (G, H) Surfactant does not augment pp242-induce LC3 punctation. MDMs were left resting or were exposed to SP-A (10 µg/mL), Survanta (100 µg/mL) or both for 6 (G) or 48h (H) prior to exposure to DMSO (1:1000) or increasing concentrations of pp242 for 60 mins. Monolayers were fixed, permeabilized and labeled with anti-LC3 antibody. Results are from one experiment conducted in duplicate (A-E) or two (F, G, H) experiments conducted in triplicate (± SD).
Figure 24. Surfactant lipids enhance *M.tb* uptake and augment intracellular growth of *M.tb* in a CD36-dependent manner. (A) *M.tb*-lux was incubated in RHH plus or minus Survanta (100 µg/mL) overnight at 4° on a nutator and MDMs were infected at an MOI of 1:1. Uptake at 2h was assayed by luminometry and bacterial growth was measured every 24h subsequently. (B) mCherry *M.tb* was incubated in RHH plus or minus Survanta overnight at 4° on a nutator and MDMs on coverslips were infected at an MOI of 5:1. Monolayers were fixed after 24h and the prevalence of single bacilli was evaluated by confocal microscopy. (C) Percentage of infected MDMs 24h after infection with Survanta-coated mCherry. (D) MDMs were left resting or were cultured in surfactant lipids for two days prior to infection with *M.tb*-lux at an MOI of 1:1 and bacterial growth was measured by luminometry. (E) SC or siCD36 MDMs were cultured in surfactant lipids for 24h prior to infection with *M.tb*-lux and bacterial growth was measured by luminometry. Graph shows percent increase in growth in surfactant-cultured infected macrophages relative to resting infected macrophages. (F) Bacterial growth in SC or siCD36 MDMs without Survanta. (G) *M.tb* uptake at 2h is similar in SC and siCD36 MDMs regardless of the addition of surfactant lipids. Results are from two (A-C), three (D, G) or four (E, F) independent experiments conducted in duplicate or triplicate (± SEM). Differences in the means of experimental groups were determined using a one-tailed Student *t* test with a *p*-value < 0.05 considered significant.
Figure 24

A

% increase in RLU

M.tb + Survanta relative to M.tb alone

2h 24h 48h 72h

B

mcherry MOI 5:1

mcherry + Survanta MOI 5:1

C

% MDMs infected

24h 24h

D

% increase in RLU Relative to M.tb alone

M.tb 48h 72h

E

% Change in RLU

48h 48h 72h 72h

F

Relative Light Units (RLU)

48h 48h 72h 72h

G

Relative Light Units

2h 2h 2h 2h

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Figure 25. Surfactant lipids inhibit *M. tb*-induced TNFα and IL-10 release. MDMs were cultured in Survanta (100 µg/mL) or left resting for 48h prior to infection with *M. tb* at an MOI of 1:1. Cell free supernatants were collected 24, 48 and 72h after infection. The effects of surfactant lipids and *M. tb* infection on MDM release of (A) TNFα, (B) IL-10 and (C) IL-6 were evaluated by ELISA. Results are cumulative from three (B, C) or four (A) independent experiments conducted in triplicate (± SEM). Statistical differences in the means of experimental groups were determined using a one-tailed Student t test with a p-value < 0.05 considered significant.
Figure 25

A

B

C

24 h p.i. (MOI 1:1)

48 h p.i. (MOI 1:1)

72 h p.i. (MOI 1:1)
Figure 26. Surfactant lipids decrease TNFα and IL-6 transcript levels following *M.tb* infection. MDMs were left resting or were cultured in Survanta (100 µg/mL) for two days before infection with *M.tb* at an MOI of 1:1. RNA was collected 24, 48 and 72h after infection and the effects of surfactant lipids and *M.tb* infection on MDM expression of (A) TNFα, (B) IL-10 and (C) IL-6 was evaluated by qRT-PCR. Results are from one experiment conducted in triplicate.
Figure 26

A

B

C
Figure 27. TLR2-induced IL-10 and IL-6 is partially dependent on CD36 and surfactant lipids augment TLR2-induced TNFα independent of CD36. SC or siCD36 MDMs were left resting or were exposed to Survanta (100 µg/mL) for 24h prior to exposure to TLR2 ligand Pam3Cys (100 ng/mL) for 1 or 24h. Cell free supernatants were collected and (A) IL-10, (B) IL-6 and (C) TNFα release were evaluated by ELISA. Results are cumulative from three independent experiments conducted in triplicate (±SEM). Differences in the means of experimental groups were analyzed by one-tailed Student t test and a p-value < 0.05 was considered significant.
Figure 28. *M. tb* infection induces accumulation of DPPC but not total surfactant phospholipids. (A) MDMs were exposed to NBD-DPPC for 24h prior to infection with mCherry *M. tb* (MOI 5:1) for 24h. Monolayers were fixed and co-localization was investigated by confocal microscopy. (B) MDMs were exposed to NBD-DPPC for 30 min prior to infection with mCherry *M. tb* (MOI 5:1) and monolayers were fixed 24h after infection. (C) MDMs were infected with mCherry *M. tb* (MOI 5:1) for 2h and NBD-DPPC was added for 30 min. Monolayers were fixed and evaluated by confocal microscopy. (D) MDMs were infected with mCherry *M. tb* for 24h prior to addition of NBD-DPPC. Monolayers were fixed 24h later and the extent of co-localization was analyzed. (E) MDMs were left resting or were cultured in Survanta for 48h prior to infection with *M. tb*. Monolayers were fixed, labeled with Bodipy (2.5 µg/mL) and intracellular lipid content was analyzed by confocal microscopy. (F) Fold change in Bodipy MFI relative to resting MDMs. (G) The percentage of infected MDMs is the same regardless of surfactant lipid exposure. Results are from one (A-D) or two (E-G) experiments conducted in duplicate (± SD).
Figure 28

A. DPPC 24h before infection
B. DPPC 30 min before infection
C. DPPC 2h after infection
D. DPPC 24h after infection

E. Resting, Survanta, M.tb, Survanta + M.tb

24h p.i.

F. Comparison of fluorescence intensities

G. Comparison of Mtb and Survanta + M.tb

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CHAPTER 5: SYNTHESIS

The ambient air is rife with inert and biological particulates. Dust, fuel combustion by-products, pollen, mold spores and bacteria hasten into the lungs with each inspiration. The smallest particles are exhaled while the largest of the inhaled debris is cleared by mechanical mechanisms, such as the mucociliary escalator. Particles of the appropriate size will reach the alveolar spaces, the end point of the terminal airways where gas is exchanged between the air and blood. The adult human lung has approximately 300 million alveoli and each is a microcosm where homeostasis must be maintained to enable continued respiration [7]. The impetus for inflammation must therefore cross a high threshold in order to incite an immune response, and the majority of potential antigens are removed by resident phagocytes (alveolar macrophages, AMs) without incident. When a pulmonary pathogen is encountered, the response to infectious challenge must be executed as innocuously as possible and must be resolved quickly [41, 289, 434].

Tissue-specific factors contribute to polarizing the immune cells in this microenvironment [19]. Alveolar type I epithelial cells are thin and flat to enable gas diffusion and constitute the majority of alveolar lining cells. Alveolar type II epithelial cells are cuboidal secretory cells which produce pulmonary surfactant, a lipid-based lubricant which lines the interior of the alveolar spaces [7]. Surfactant spreads and contracts to enable the cyclic expansion and collapse of alveoli, which allows the filling and emptying
of the lungs with minimal effort. AMs assist in the maintenance of homeostatic levels of functional surfactant by continuously catabolizing the surfactant protein and lipids around them [31, 273]. This is indispensable for pulmonary function [277-279, 281] and has the side effect of creating a macrophage population which contains relatively more intracellular lipids during conditions of homeostasis than macrophage populations in other tissue spaces [78, 79].

Surfactant also contributes to the tissue-specific phenotype of AMs by regulating their expression of inflammatory mediators [289]. Additionally, by interacting with microbial surfaces, the proteins and lipids in surfactant influence how macrophages detect and process invaders [86]. The pulmonary pathogen *Mycobacterium tuberculosis* (*M.tb*) may get an additional boon from the presence of surfactant lipids within AMs, its primary host cell. *M.tb* has been shown to acquire host lipids as a carbon source during infection [157, 282, 288], although the source of those lipids during a pulmonary infection has not been explicitly determined. *M.tb* is a global health scourge as the perpetrator of tuberculosis (TB), a disease which kills over a million people annually and negatively impacts millions more [106]. We speculate that part of *M.tb*’s success may be due to the ready availability of surfactant lipids as a carbon source within AMs, as well as the immunosuppressive consequences of AM-surfactant interactions.

This dissertation explored the general hypothesis that macrophage exposure to surfactant results in a phenotype of susceptibility to infection by *M.tb*. The concept of the immunosuppressive pulmonary environment is supported by decades of research from our lab and others [17, 42, 44-46, 65]. We specifically hypothesized that the constitutive duty of surfactant catabolism by macrophages is exploited by *M.tb* early during infection. Although surfactant lipid uptake by AMs has been shown to be receptor-mediated [364],
no one had yet identified any receptor(s) involved in this process. We therefore sought to identify which receptor(s) are involved in surfactant lipid uptake by human macrophages in order to fill a key gap in our knowledge of the pulmonary environment. We began by investigating whether surfactant could regulate the expression of several macrophage surface receptors: the immunomodulatory signaling receptor CD200R, as well as the scavenger receptors MARCO, SR-A, SR-B1 and CD36 and found that CD36 alone is positively regulated by surfactant components (Fig 8). CD36 is capable of recognizing and importing a variety of lipids into macrophages [245] and also contributes to the immune response to a variety of pathogens [246]. Due to previous reports in mice showing that the expression of CD36 was detrimental to the host during the establishment of mycobacterial infection [271], we hypothesized that CD36 likely performs a function in the lung, such as constitutive surfactant lipid uptake, which M.tb capitalizes on to gain an early growth advantage in the lung.

Consistent with literature reporting the stimulus-dependent redistribution of CD36 to the cell membrane [366-368], we report the novel finding that preformed CD36 in monocyte-derived macrophages (MDMs) is trafficked to the cell surface following exposure to surfactant components (Fig 10, 11). After observing that CD36 expression and location are regulated by surfactant, we conducted a series of experiments to determine whether CD36 contributes to surfactant lipid uptake (Fig 13-15). Herein, we show that CD36 mediates the uptake of the major phospholipid species in surfactant, dipalmitoylphosphatidylcholine (DPPC). To our knowledge, this constitutes the premier report of a particular receptor regulating the uptake of a specific surfactant lipid species by macrophages. We next hypothesized that the CD36-dependent uptake of surfactant lipids would augment intracellular M.tb growth. We show that macrophages cultured in
surfactant lipids prior to infection harbor significantly more *M.tbc*, but only when CD36 is present (Fig 25). We further show that this growth advantage is likely to be partially attributable to the immunosuppressive effects of surfactant, as indicated by decreased production of the pro-inflammatory cytokine TNFα (Fig 26). However, this surfactant-mediated immunosuppression occurred independently of CD36 expression (Fig 28).

We additionally report *M.tbc*-induced alterations to the expression of several mediators of lipid uptake, storage and catabolism consistent with metabolic reprogramming of macrophages [291]. As described in Chapter 4, *M.tbc* infection of human alveolar macrophages has been shown to induce enhanced carbon flux through aerobic glycolysis, which enables the cells to better control *M.tbc* growth [293].

Interestingly, we found that pre-exposure of macrophages to surfactant lipids results in a delay of *M.tbc*-induced alterations to the expression of macrophage metabolic mediators (Fig 19-22). This surfactant-mediated delay in the "switching time" [434] to host protective aerobic glycolysis could contribute to the increased intracellular growth which we observed in surfactant-cultured macrophages (Fig 25). Taken together, our findings have great implications for our understanding of the exploitation of the endogenous pulmonary environment by *M.tbc* early during infection.

*M.tbc* is able to acquire and metabolize host derived cholesterol [282] and phospholipids [288]. These carbon sources have been demonstrated to be relevant in both vivo and in vitro models. Although the precise origin of host derived cholesterol and phospholipids have not been identified, the inner leaflet of the phagosomal membrane has been postulated as a source [157, 283]. Additionally, *M.tbc* infection has been linked to accumulation of lipid droplets (LDs), which are primarily composed of cholesterol.
esters (CE) and triacylglycerol (TAG). Infection-induced LDs have therefore also been proposed to be a possible source of carbon-based nutrients for *M.tb* [288].

Cholesterol and phospholipids are both components of surfactant and due to the predilection of *M.tb* for AMs, where surfactant metabolites are abundant [78], it seems likely to us that *M.tb* would utilize cholesterol and phospholipids derived from surfactant. We therefore undertook a series of experiments to determine whether *M.tb* would induce the accumulation of Survanta (total surfactant phospholipids) or isolated DPPC inside of MDMs. As reported in Fig 28, we did not observe accumulation of total surfactant phospholipids within MDMs, in either the presence or absence of infection (although DPPC alone did accumulate under certain conditions of our infection assay). However, the Survanta used in those experiments does not include cholesterol or other neutral lipids. We hypothesize that the addition of cholesterol to Survanta will influence the packaging of the phospholipids, thereby potentially enhancing the accumulation of surfactant lipids inside of MDMs following infection.

The next step in these studies should directly investigate the possibility that *M.tb* is using surfactant lipid metabolites as a carbon source during infection. Growth curves comparing the rates of *M.tb* growth in rich media versus minimal media supplemented with surfactant lipids would be a good starting point. Subsequent iterations of this experiment should explore whether or not *M.tb* can grow on individual surfactant lipids or whether total surfactant provides a better energy source for the bacteria. This set of growth experiments would answer the question of whether or not *M.tb* can grow on native surfactant lipids as a carbon source. However, we hypothesize that macrophage processing of surfactant lipids will be necessary for *M.tb* to derive a growth advantage from surfactant. Therefore, the next iteration of these experiments should incorporate
macrophage lysates as the sole carbon source in minimal media. Macrophages would be left resting as a control or cultured in individual surfactant lipids, Survanta alone or Survanta plus cholesterol. Monolayers would be lysed at various times after lipid exposure using a freeze-thaw approach to prevent lipid degradation. These macrophage lysates would then be added to minimal media to investigate the mycobacterial growth response. We hypothesize that \textit{M.tb} will grow robustly in the presence of lysates derived from macrophages which were cultured in surfactant lipids. Finally, CD36 knockdown should be performed prior to culturing macrophages in surfactant and collecting lysates. This would assist in determining whether CD36-dependent uptake of surfactant lipids is required for \textit{M.tb} to obtain a growth advantage from the presence of macrophage-derived surfactant metabolites.

These experiments would answer several questions. First, it is possible that upon entry into the pulmonary space \textit{M.tb} could grow extracellularly, using the abundant surfactant lipids in the alveolus as a carbon source. However, due to the obligatory intracellular lifestyle of \textit{M.tb}, we hypothesize that \textit{M.tb} will not be able to grow on native surfactant lipids as a carbon source. We anticipate a growth advantage only when the surfactant lipids have been metabolically processed by macrophages prior to addition to the minimal media. Furthermore, we hypothesize that lysates from resting macrophages will confer no growth advantage. Additionally, consistent with our previous work (Fig 13, 25), we hypothesize that CD36 expression will be necessary for normal uptake of surfactant lipids by macrophages and we therefore hypothesize that CD36 knockdown will decrease any bacterial growth advantage on surfactant-cultured macrophage lysates. However, due to the redundancy in scavenger receptor function it is likely that CD36 knockdown will not completely abrogate macrophage uptake of surfactant lipids.
and it would be informative to investigate additional receptors in this context. These experiments would lend evidence to the hypothesis that M.tb is able to acquire and use the metabolic by-products of surfactant lipids which are abundantly present inside of AMs [78, 79].

Taking a closer look at the specific metabolites derived from surfactant within macrophages would be informative in regards to the potential energy substrates available for M.tb in the lung. Delineating the break-down products of surfactant generated by human macrophages could lead to the identification of the particular carbon source(s) used by M.tb during infection. The next set of experiments in this line of research could therefore use mass spectrometry approaches to characterize surfactant lipid metabolic profiles of both human alveolar macrophages (HAMs) and MDMs. This experiment would serve the additional purpose of further validating the surfactant-cultured MDM model as a tractable surrogate for the use of HAMs, as lipids extracted from freshly isolated HAMs would serve as the comparator for the MDM metabolite profile. MDMs would be left resting or cultured in deuterated individual surfactant lipids, total surfactant phospholipids in the form of Survanta, or Survanta supplemented with cholesterol for various times, ranging from very brief exposure (5 min) out to days. Lipids would be extracted and submitted for analysis by mass spectrometry to determine the kinetics of surfactant lipid breakdown and the lipid metabolite species produced.

Identifying physiologically relevant lipid metabolic substrates for M.tb could ultimately lead to the development of novel antibiotics. Targeting mycobacterial metabolic pathways which are vital for growth within pulmonary macrophages is a promising avenue for the discovery of tissue-appropriate therapeutics. Alternatively,
continuing to characterize endogenous and constitutive AM functions which are exploited by *M. tb* could reveal possibilities for host-directed therapeutics. Due to the increasing and alarming rates of antibiotic resistance reported for TB, host-directed therapies could be more fruitful than the introduction of new classes of antibiotics, against which the bacteria is likely to eventually gain resistance mechanisms. The work presented herein has identified the first receptor involved in the uptake of surfactant lipids by macrophages, CD36, and links this function of CD36 to increased intracellular *M. tb* growth within the first days of infection. Therefore, ascribing this pulmonary-specific role for CD36 lends insight into the host resistance phenotype reported in CD36 knockout mice infected with mycobacteria. Continuing to characterize surfactant lipid metabolism during homeostasis and the perturbation of these endogenous pathways by *M. tb* will not only deepen our understanding of host-pathogen interactions in the lung but also has the potential to reveal aspects of host cell and mycobacterial metabolism which could be targeted therapeutically.
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