Cracking the Macrophage Code in Immunity to TB: Ontogeny and Metabolism

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Abstract

Mycobacterium tuberculosis (*Mtb*) remains one of the most successful pathogens worldwide and it is a leading cause of death due to infectious disease. *Mtb* initially infects pulmonary macrophages, which orchestrate a complex innate and adaptive immune response to transit into a latent stage of chronic infection. Studies have shown that some people can clear the infection prior to the onset of adaptive immunity, as determined by a negative tuberculin skin test (TST), potentially due to an effective innate immunity. Thus, the early events following exposure to *Mtb* could potentially tip the balance in favor of either clearance of infection or establishment of a latent infection. While alveolar macrophages (AM) are the first immune cells to encounter *Mtb*, bone marrow-derived monocytes are quickly recruited to the lungs to combat the infection. Interestingly, fate-mapping studies have demonstrated that AM are of prenatal origin and can self-renew without the need for replacement by bone marrow-derived macrophages (BMDM).

In this study, we aimed to better understand the poorly-defined roles of embryonic AM compared to recruited BMDM during the early events of mycobacterial infection. Our central hypothesis is that AM are more permissive to mycobacterial infection than BMDM. To investigate this, we used an *in vitro* model of AM along with 'M1'-like BMDM to model recruited macrophages and 'M2'-like BMDM to model tissue resident macrophage populations. Since immune cell metabolism has been shown to reflect the functional capacity of the cell, we sought to characterize the link between metabolism and anti-mycobacterial function of AM and BMDM. We determined that AM have a unique oxidative metabolism and failed to shift to a more glycolytic, inflammatory phenotype following infection. Consequently, they were unable to produce critical anti-mycobacterial cytokines and, thus, were not effective in controlling bacterial growth. In contrast, BMDM efficiently switched to a glycolytic phenotype to produce critical cytokines for controlling the

growth of *Mtb*. In addition, we expanded our *in vitro* observations to *in vivo* by using a mouse model lacking AM. Mice lacking AM were more protected from H37Ra infection, and upon challenge with H37Rv, had a less disseminated infection. Therefore, we conclude that AM are more susceptible than BMDM and overall detrimental to host protection. Targeting alveolar macrophages at the early time points of infection might be a novel avenue for the development of anti-mycobacterial treatments.

Résumé

Les infections par *Mycobacterium tuberculosis* (*Mtb*) représentent l'une des principales causes de mortalité due à des maladies infectieuses dans le monde. La pathogénicité de *Mtb* repose sur une infection initiale des macrophages, puis sur la transition de l'infection vers une phase latente ou chronique. Des études ont montré que certains individus étaient capables d'éliminer l'infection avant la mise en place de l'immunité adaptative, illustré par un test cutané à la tuberculine négatif. Ces études ont montré que ce phénomène était potentiellement du a une réponse immunitaire innée efficace. Ainsi, les évènements précoces suivant l'infection par *Mtb* pourraient potentiellement faire pencher la balance en faveur de l'élimination de la bactérie ou de l'établissement d'une infection latente. Les macrophages alvéolaires (MA) sont les premières cellules immunitaires rencontrées lors de l'infection pulmonaire par *Mtb*. Ensuite, les monocytes provenant de la moelle osseuse sont rapidement recrutés dans les poumons pour aider à combattre l'infection. Il est intéressant de noter que des études de 'fate-mapping' ont montré que l'origine des MA était prénatale et qu'ils avaient la capacité de s'auto-renouveler, sans remplacement par les macrophages issus de la moelle osseuse (BMDM).

Dans ce projet, nous avons pour but de comparer le rôle des MA embryonnaires à celui des BMDM recrutés dans les phases précoces après infection par *Mtb*. Nous avons émis l'hypothèse que les MA étaient plus permissifs à l'infection par *Mtb* que les BMDM. Afin de tester notre hypothèse, nous avons utilisé un modèle in vitro de MA ainsi que de BMDM-M1, modèle de macrophages recrutés, ou de BMDM-M2, pour modéliser les macrophages résidents du poumon. Nous avons également utilisé un modèle in vivo avec les souris *csf2rb*$^{-/-}$, dépourvues de MA. Étant donné qu'il a été montré que le métabolisme des cellules immunitaires reflète les capacités fonctionnelles de la cellule, nous avons cherché à caractériser le lien entre le métabolisme et les fonctions anti-

mycobacteriennes des MA et des BMDM. Nous avons déterminé que les MA ont un métabolisme oxydatif unique et n'ont pas la capacité de passer à un phénotype glycolytique, inflammatoire, après infection par *Mtb*. Par conséquent, les MA ne peuvent pas produire de cytokines anti-mycobacteriennes essentielles au contrôle de la croissance bactérienne. Au contraire, les BMDM passent efficacement à un phénotype glycolytique après infection et produisent les cytokines clés pour contrôler la croissance de *Mtb*. Les souris dépourvues MA sont mieux protégées contre l'infection par H37Ra, et après infection par H37Rv, présentent moins de dissémination bactérienne. Nous avons conclu que les MA sont plus susceptibles que les BMDM à l'infection par *Mtb* et compromettent les mécanismes de protection de l'hôte. Le ciblage des macrophages alvéolaires dans les étapes précoces suivant l'infection par *Mtb* pourrait représenter un nouvel axe de recherches pour le développement de traitements anti-mycobactéries.

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Preface and Contribution of Authors

MD conceived the study. MD and LM designed the experiments and LM performed the experiments with technical assistance from NK, EK or JoD. LM performed the data analysis and LM and MD wrote the thesis.

List of abbreviations

AM	-alveolar macrophages
BAL	-bronchoalveolar lavage
BCG	-Bacillus Calmette-Guérin
BMDM	-bone marrow-derived macrophages
CCL2	- chemokine ligand 2
CCR2	-CC-chemokine receptor 2
CFU	-Colony-forming units
CLRs	-C-type lectin receptors
DC	-dendritic cell
ECAR	-extracellular acidification rates
ELISA	- enzyme-linked immunosorbent assay
FAO	-fatty acid oxidation
FCCP	-fluorocarbonyl cyanide phenylhydrazone
HIV	-human immunodeficiency virus
IFNγ	-interferon-γ
IL	-interleukin

LAM	-lipoarabinomannan
LN	-lymph nodes
LPS	-lipopolysaccharide
ManLam	-mannosylated LAM
MDR-TB	-multidrug-resistant TB
MOI	-multiplicity of infection
Mtb	-*Mycobacterium tuberculosis*
MyD88	-myeloid differentiation primary response gene 88
NK	-natural killer
NLRs	-nucleotide-binding oligomerization domain-like receptors
NO	-nitric oxide
NOS2	-nitric oxide synthase
NOX2	-NADPH oxidase
OCR	-oxygen consumption rate
OXPHOS	-oxidative phosphorylation
PAMPS	-pathogen-associated molecular patterns
PRR	-pattern recognition receptor

RNI	-reactive nitrogen intermediates
ROI	-reactive oxygen intermediates
RR-TB	-rifampicin-resistant TB
SRC	-spare respiratory capacity
TB	-tuberculosis
TLR	-Toll-like receptor
TNFα	-Tumor necrosis factor α
TST	-tuberculin skin test
XDR-TB	-extensively drug-resistant TB

Chapter I: Introduction

1.1 Tuberculosis Disease and Epidemiology

Despite the world-wide application of the Bacillus Calmette-Guérin (BCG) vaccination and other anti–*Mycobacterium tuberculosis* (*Mtb*) interventions, *Mtb* remains one of the most successful human pathogens. Annually two million people die of tuberculosis (TB), with eight to ten million new cases of active TB occurring each year due to the large reservoir of asymptomatic people with *Mtb* infection [2]. Approximately one third of the world's population belongs to this latent reservoir, defined as the absence of clinical symptoms but the presence of an immune response against *Mtb*, which is evaluated by the tuberculin skin test (TST). A positive TST is an indication of previous exposure to live bacilli, as it induces a T-cell-mediated delayed-type hypersensitivity reaction, causing redness and swelling. During latent infection, the bacteria are in a relatively dormant state, with a reactivation rate of 5-10% per year [3]. Over 95% of deaths due to TB occur in low- and middle-income countries in sub-Saharan Africa and Southeast Asia [4]. In fact, 60% of the total burden falls on India, Indonesia, China, Nigeria, Pakistan and South Africa [4]. In Canada, most cases arise in foreign-born individuals within 5 years of arrival, while the highest incidence rate occurs in Aboriginal people, particularly amongst the Inuit [5].

Symptoms of pulmonary tuberculosis–although not always present –include coughing, fever, chest pain, weight loss, night sweats, loss of appetite and general malaise [6]. TB treatment relies mainly on a drug regimen first developed in the 1960s: a cocktail of isoniazid, rifampicin, ethambutol and pyrazinamide [7]. Thus, it is of no surprise that drug-resistant variants of *Mtb* have emerged, such as multidrug-resistant (MDR)-TB, which is resistant to the two most powerful TB drugs, isoniazid and rifampicin. MDR-TB is responsible for approximately half a million cases of TB each year,

with another 100,000 cases due to rifampicin-resistant (RR) TB [2]. Even more worrisome is extensively drug-resistant (XDR)-TB, defined as MDR-TB which has also developed resistance to fluoroquinolones and at least one of the three injectable second-line drugs (kanamycin, amikacin or capreomycin). Not only are these strains more difficult to treat due to drug resistance, but the treatment regimens are also longer and more expensive [7]. The increased frequency of highly drug-resistant strains of *Mtb* underscores the urgent need for a novel therapy for TB.

Upon exposure to pulmonary *Mtb*, there are two possible outcomes: immediate clearance or primary infection, which leads to either the establishment of a latent infection or to the progression of active disease. However, it remains largely unknown as to what dictates the outcome of exposure to *Mtb* in healthy hosts. Arguably, the latent reservoir of asymptomatic people, with the capacity to reactivate and progress to active disease, remains the largest obstacle for the control and, ultimately, eradication, of TB.

The ability of *Mtb* to persist in individuals with apparently normal immune systems implies that *Mtb* has developed strategies to evade both the innate and adaptive immune system. Undoubtedly, an effective, preventative vaccine is essential for control of the epidemic, and yet a recent study [8] revealed another failure of a large randomized human vaccine trial in infants. Importantly, present vaccine efforts[8-10], centered around boosting the current BCG vaccine, are mainly focused on T-cell-mediated immunity, despite both pre-clinical and clinical outcomes of this approach being very discouraging [8]. Certainly, a novel approach is needed to develop an effective vaccine against *Mtb* to combat the global burden of disease and emerging drug resistant strains.

1.2 Immune system of the lung

The lungs are protected by a multifaceted immune system, comprised of physical barriers and both humoral and cellular components which *Mtb* must overcome to establish an infection. Many physical structures of the airways are essential in host defense, including the branching of the respiratory tree, ciliary beat, mucus clearance and cough response [11]. If a pathogen overcomes these physical barriers, it encounters humoral immunity in the form of non-specific secreted factors like defensins and complement, amongst others [12]. Finally, if a pathogen reaches the lower airways (alveolar space), it faces the resident macrophage of the airways (alveolar macrophage).

Macrophages are the quintessential innate immune cell, with high functional plasticity. They are a heterogeneous population acting as the primary phagocytic cell type responsible for the removal of foreign particulate matter (including microbes), in addition to cellular debris. They engulf particles through phagocytosis, subsequently digesting and removing them from the system. The local micro-environment influences both their morphology and functional characteristics [13].

Macrophages are commonly classified as M1-like, inflammatory, or M2-like, anti-inflammatory/regulatory. An M1 phenotype can be induced *in vitro* by stimulation of macrophages with Toll-like receptor (TLR) ligands or bacterial pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS, a gram-negative bacterial product), and/or by inflammatory cytokines such as interferon-γ (IFNγ) [14]. This stimulation will cause a shift in arginine metabolism, promoting the production of nitric oxide (NO), a molecule with important anti-microbial activity. On the other hand, M2 macrophages are stimulated by type 2 cytokines such as interleukin (IL)-4 or IL-13 [15]. In these macrophages, arginine is metabolized into ornithine, which promotes cell proliferation as well as tissue remodeling and repair [15]. In addition, it limits the amount of arginine available for NO production, thereby reducing the

contribution of the macrophage to inflammation. Due to the functional plasticity of macrophages, the most widely accepted paradigm is that macrophages exist on a spectrum of activation from M1 to M2, rather than strictly as M1/M2 [16]. Typically, tissue-resident macrophages, like alveolar macrophages, are characterized as regulatory, maintaining homeostasis while limiting inflammation caused by foreign particles to preserve tissue function [17]. However, during infection, there is an influx of M1-like macrophages to clear and limit the spread of the infection.

Until recently, it was thought that all macrophages arose from monocytes, which themselves originate from hematopoietic stem cells in the bone marrow. Monocytes would subsequently circulate throughout the body, entering the tissues and differentiating into macrophages to replenish tissue resident macrophages as needed [18]. Although bone marrow-derived monocytes are the main source of pulmonary macrophages during inflammatory settings, recent fate-mapping studies have demonstrated that under steady state conditions, resident tissue macrophages from the brain, spleen, peritoneal cavity, and lung are all of prenatal origin and are not dependent on bone marrow-derived macrophages for self-renewal [19-21]. Therefore, during infection/inflammation, pulmonary macrophages have two distinct origins (**Figure 1.**): alveolar macrophages, the tissue resident population of the lung arising from the yolk sac [19], and monocyte-derived macrophages, recruited from the bone marrow.

Bone marrow-, or monocyte-, derived macrophages have several distinct qualities compared to alveolar macrophages. In contrast to alveolar macrophages, recruited bone marrow-derived macrophages arise from circulating monocytes, which have their origins in the bone marrow. Monocytes emigrate from the bone marrow through CC-chemokine receptor 2 (CCR2)-mediated signaling and can then differentiate into macrophages or dendritic cells [22]. During infection, monocytes expressing high levels of CCR2 and Ly6C are termed 'inflammatory monocytes', and represent the major source of recruited macrophages [22]. As macrophages are important contributors to the immune response during *Mtb* infection, mice that lack CCR2 expression are extremely susceptible to *Mtb* and rapidly succumb to infection [23]. Although the recruitment of inflammatory monocytes/macrophages is essential for host resistance against pulmonary infection, this response also needs to be tightly regulated, to minimize lung immunopathology and injury. In an acute model of infection, it has been shown that bone marrow-derived inflammatory macrophages are removed from the lung via Fas-dependent apoptosis when the infection is cleared [24]. BMDM express high levels of Fas receptor while resident alveolar macrophages do not, allowing for the selective removal of recruited

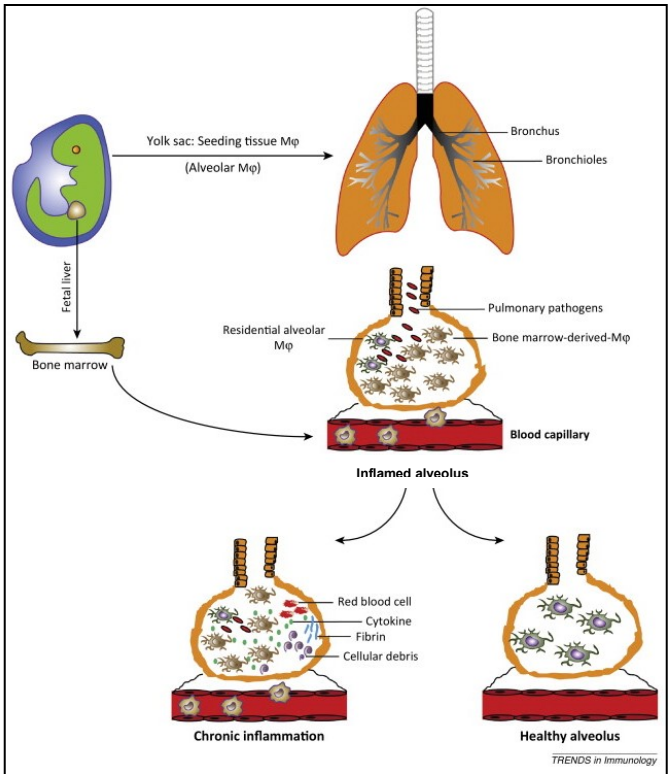

Figure 1. The origin of monocytes/macrophages and their potential contribution to pulmonary *Mtb* infection. *Trends in Immunology, 2015* [1]

inflammatory macrophages [24]. This is an essential step in the resolution of inflammation, as persistent inflammation hinders tissue repair processes.

1.3 Immunity to *Mtb*

Mtb infects humans via the aerosol route, when a person with active pulmonary TB coughs and aerosolizes the bacteria. The biologically relevant infectious dose has remained hard to determine in humans, as the range of bacteria in a single droplet is between 1 to 200 bacteria [25]. These aerosolized bacteria are then inhaled by uninfected individuals and bypass the lung's physical barriers (e.g. branching of the respiratory tree, ciliary beat, mucus clearance, and cough response [11]) to reach the lower respiratory tract, where *Mtb* encounters alveolar macrophages. During *Mtb* infection, if innate immunity–pulmonary macrophages– becomes insufficient to eliminate the pathogen, the host initiates the adaptive immune system. If the combination of both innate and adaptive effector cells (e.g. T-cells) fail to kill *Mtb*, the host is forced to form granulomas, which are a mixed response of both innate and adaptive immune cells, to isolate the pathogen. *Mtb* certainly brings host immunity to its knees by inducing the formation of granulomas.

1.3.1 Innate immune response

It is well documented that among close household contacts of highly active TB patients, 50% of exposed individuals are completely resistant to TB since they are tuberculin skin test (TST) negative, indicating *Mtb* can be eliminated by a robust innate immune response [26, 27]. These observations suggest that the only window of opportunity for the complete elimination of *Mtb* might be limited to the early phase of infection when the bacteria are still in the airways, prior to gaining access to the lung interstitium, activating adaptive immunity, as well as granuloma formation.

Following a successful infection of the alveolar macrophage, the innate immune phase of infection begins, during which monocytes are recruited to the site of infection in a CCR2-dependent manner [22], along with neutrophils and natural killer (NK) cells. In this phase of infection, macrophages play a critical role. Not only are they the first immune cell to encounter the pathogen, but they are also the main site of bacterial replication, seemingly favoring the establishment of infection. Monocyte-derived macrophages make up the majority of the macrophages present during infection [22], and their constant replenishment from the bone marrow give the bacteria a continuous supply of host cells in which to replicate.

Mtb is actively taken up by macrophages through receptor-mediated phagocytosis, when pattern recognition receptors (PRRs) recognize conserved pathogen-associated molecular patterns (PAMPs) on the cell surface of *Mtb.* Various PRRs have been shown to be involved in the recognition and uptake of *Mtb*, including: TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and complement receptors, among others. TLRs and NLRs are thought to be more involved in downstream cytokine production and host protection, whereas CLRs and complement receptors seem to benefit the pathogen.

1.3.1.1 Macrophage recognition and uptake of Mtb

1.3.1.1a Toll-like receptors

TLRs are the canonical PRR and play a critical role in innate immunity. They are type 1 transmembrane proteins which, upon binding to their ligands, homo- or heterodimerize to begin the signal transduction pathway. So far, there are 10 TLRs that have been discovered in humans and 13 in mice. They all signal through myeloid differentiation primary response gene 88 (MyD88), with the exception of TLR3, which signals through Toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF). Importantly, each TLR recognizes a

different ligand, allowing for the specific recognition of a broad range of bacterial antigens. The main TLRs involved in immunity to *Mtb* are TLR2, TLR4 and TLR9 [28].

TLR2 senses bacterial lipopeptides, and heterodimerizes with TLR6 or TLR1 to recognize two separate types of lipopeptides: diacylated lipopeptides or triacylated lipopeptides, respectively [29]. Several lipoproteins produced by *Mtb* are recognized by TLR2, including LpqH, LprA, LprG and PhoS1[30]. It has been shown that recruited macrophages/monocytes express lower levels of TLR2 than alveolar macrophages, making them hyporesponsive to mycobacterial lipoproteins [30]. Activation of TLR2 leads to downstream production of tumor necrosis factor α (TNFα), interleukin (IL)-12 and IL-1β [31]. Additionally, TLR2 is important in anti-mycobacterial responses *in vivo*, as mice lacking this receptor are highly susceptible to infection [32, 33].

TLR4 has also been implicated in host protection to *Mtb*, and though it is best known for sensing LPS, in the case of mycobacteria, it is activated by heat shock protein 60/65 (Hsp60/65) and other secreted proteins. Activation of TLR4 leads to macrophage activation and upregulation of costimulatory molecules, the production of TNFα, as well as to the recruitment of monocytes through the production of chemokine ligand 2 (CCL2) [34]. However, its role *in vivo* has yet to be confirmed as there have been conflicting results regarding the susceptibility of TLR4-deficient mice [33, 35].

Though TLR2 and TLR4 both play major and well characterized roles in immunity to *Mtb*, TLR9 has also been recently implicated [36]. TLR9 recognizes unmethylated CpG motifs in bacterial and viral DNA and, unlike TLR2 and TLR4 that are found on the cell membrane, is expressed intracellularly, within the endosome. TLR9 has been shown to be important for IL-12 production by both macrophages and dendritic cells; however, the implications *in vivo* are less striking [36].

While TLR9-deficient mice were shown to be more susceptible than wild type mice following high-dose aerosol *Mtb* infection, only the double knockout of TLR2/TLR9 were more susceptible at the physiological low-dose [36].

Interestingly, a study showed that a triple knockout of TLR2/TLR4/TLR9 had no increase in susceptibility to *Mtb* [37], questioning the contributions of TLRs in host resistance to *Mtb* infection. Interestingly, while MyD88-, an important adaptor molecule in TLR signaling, deficient mice were found to be highly susceptible to *Mtb* [37], MyD88-deficient humans have shown no susceptibility to *Mtb* and many other pulmonary pathogens [38]. Moreover, macrophage TLRs have also been shown to be involved in tissue repair in the lung [39]; thus, their roles may differ based on the cytokine milieu and stage of infection. Clearly the roles of TLRs and other potential pathways which may activate MyD88 in *Mtb* infection require further study.

1.3.1.1b NOD-like receptors

NOD-like receptors are cytoplasmic proteins with leucine-rich repeats at their C-terminus, which recognize bacterial PAMPs. Of interest during *Mtb* infection is NOD2, a cytoplasmic sensor recognizing bacterial cell wall components such as muramyl dipeptide (MDP), which is expressed by epithelial cells and immune cells such as macrophages and dendritic cells [40]. Activation of NOD2 leads to downstream activation of NF-κB and pro-inflammatory cytokine production [41], thus aiding in pathogen clearance. While MDP is present in many bacterial cell walls in the N-acetyl form, *Mtb* produces N-glycolyl MDP, which has been shown to be more immunogenic [42]. Importantly, NOD2 is required for an adequate innate and adaptive immune response to *Mtb* [43], indicating that this pathway is important for host protection against *Mtb*.

1.3.1.1c C-type lectin Receptors

CLRs are PRRs that specialize in the recognition of polysaccharide structures on pathogens. The prime example of a C-type lectin is the macrophage mannose receptor (MR), also known as CD206, which is a transmembrane protein that binds to mannose residues on the surface of pathogens. MRs are expressed on the cell surface of both tissue resident macrophages, such as alveolar macrophages, and monocyte-derived macrophages; however, they are not expressed by monocytes [44]. In the case of *Mtb*, MR binds to mannose residues on lipoarabinomannan (LAM) or mannosylated LAM (ManLAM) on virulent strains of *Mtb* (H37Rv), but not avirulent strains (H37Ra)[45]. Importantly, binding of MR to ManLAM, and subsequent uptake of the bacteria, inhibits phagolysosome maturation [46], allowing the bacteria to thrive inside of the macrophage. It also leads to production of IL-4 and IL-13 while inhibiting IL-12, nitric oxide and reactive oxygen species production [31], favoring bacterial growth.

1.3.1.1d Complement Receptors

Structurally, complement receptors occur in two forms: as a monomeric transmembrane protein, such as CR1 that binds C3b and C4b, or as heterodimeric proteins like CR3 and CR4, which belong to the integrin family and can also bind C3bi [44]. Interestingly, alveolar macrophages express high levels of CD11c, which combines with CD18 to form CR4, whereas recruited macrophages/monocytes express high levels of CD11b, which combines with CD18 to form CR3 [44, 47]. *Mtb* activates the alternative pathway of complement activation, leading to opsonization with C3b and C3bi. CR1, CR3 and CR4 are then able to bind to the bacteria and lead to the uptake of the pathogen. Of the three receptors, CR3 appears to be the most important, as deleting this receptor abrogates 70-80% of the phagocytosis of both virulent and avirulent *Mtb* [28]. Uptake through CR3 has also been shown to inhibit the respiratory burst [48], indicating that this form of

uptake is perhaps beneficial to the bacteria. Additionally, *Mtb* actively recruits C2a, leading to the downstream production of active C3b and opsonization [49], again hinting at potential benefits for the pathogen.

1.3.1.2 Macrophage-mediated killing of Mtb

Macrophages usually excel at eliminating the pathogens they ingest through several mechanisms, including phagosome maturation and acidification, and the production of reactive oxygen and nitrogen intermediates. However, *Mtb* has evolved to reside and replicate inside of the macrophage phagosome, subverting macrophage immunity in many cases [50]. Interestingly, the ability of some housemates of infected individuals to initially eliminate the infection and thus remain TST negative [26] indicates that the alveolar macrophages of some individuals are able to directly kill the bacteria. Furthermore, macrophages activated by T-cell-derived IFNγ are also able to kill mycobacteria [51].

Macrophage-mediated killing is achieved through phagolysosome fusion, when the phagosome in which the bacteria reside becomes increasingly acidified and merges with lysosomes [51]. Following pathogen uptake, *Mtb* is able to inhibit the fusion of lysosomes with the phagosome, preventing phagolysosome maturation and generating a protected niche for replication [52]. In inactivated resting macrophages, secreted soluble factors such as protein tyrosine phosphatase A (PtpA) and secreted acid phosphatase M (SapM) from *Mtb* interfere with host trafficking processes to arrest phagosome-lysosome fusion [52]. However, macrophages activated through the action of IFNγ or TNFα are able to drive phagosome maturation and control the infection [51].

Activated macrophages are also major producers of NO and reactive oxygen intermediates (ROI). Using arginine and oxygen, the nitric oxide synthase (NOS2) system produces NO which then

diffuses through the phagosomal membrane [51]. NADPH oxidase (NOX2) is also activated to produce ROI, and together with NO, these highly oxidative intermediates can destroy bacterial membrane lipids, proteins, carbohydrates and nucleic acids [53]. *Mtb* has evolved mechanisms to cope with these toxic intermediates, such as the production of the *katG* product catalase-peroxidase to convert anti-mycobacterial H_2O_2 into water and oxygen; however, at high doses of ROI, *Mtb* is killed [54]. While the role of ROI is not fully understood in host defense mechanisms against *Mtb*, mice deficient in NO succumb rapidly to infection [55], demonstrating its importance in the murine model of *Mtb* infection. In humans, although macrophages produce NO in response to mycobacterial infection, its protection effects are less striking compared to murine macrophages [56, 57].

1.3.1.3 Cytokine production by macrophages during Mtb infection

IL-12

IL-12 is a key cytokine in host defense against *Mtb* produced by macrophages, among other cell types. Following activation via bacterial uptake or IFNγ, macrophages produce IL-12 which helps in the development of a type 1 immune response and the further production of IFNγ [50]. Additionally, IL-12 is required for the maturation of dendritic cells and for their migration to the draining lymph node, where they prime naïve T-cells [58]. Both mice and humans deficient in IL-12 are extremely susceptible to mycobacterial infection [59, 60].

TNFα

TNFα is a pro-inflammatory cytokine produced by macrophages, monocytes and dendritic cells, which signals in both a paracrine and autocrine manner. Together with IFNγ produced by T-cells, it activates macrophages to upregulate production of both IL-1 and IL-6, as well as NO and ROI

[50]. Mice lacking the TNFα receptor or those treated with anti-TNFα are more susceptible to *Mtb* infection [61], possibly due to the crucial role of TNFα in maintaining granuloma structure and integrity [62]. Interestingly, it has also been shown that TNFα may have some anti-inflammatory roles during mycobacterial infection, controlling the type 1 response and limiting immunopathology in the lung [63, 64].

IL-6

IL-6 acts as both a pro-inflammatory and an anti-inflammatory cytokine during *Mtb* infection. It is produced early in infection by macrophages and is important for cytotoxic T-cell differentiation [50]. *In vivo* studies have shown that mice lacking IL-6 are more susceptible to *Mtb* infection compared to WT mice [65], mainly due to the profound differences in T-cell responses. Paradoxically however, IL-6 has also been shown to limit the production of TNFα and IL-1β [66] and to decrease responsiveness of uninfected macrophages to IFNγ [67], thus acting in a detrimental manner.

IL-10

IL-10 is an anti-inflammatory cytokine produced by macrophages following stimulation with mycobacterial LAM or phagocytosis of *Mtb* [68]. It downregulates the pro-inflammatory response by inhibiting the production of IFNγ, TNFα and IL-12 [68], which are important in mounting a protective anti-mycobacterial immune response. It has also been shown that IL-10 arrests phagosomal maturation and acidification in infected macrophages [69] and limits the production of ROI and NO, thus enhancing *Mtb* growth [70]. While IL-10 is often thought to maintain tissue integrity and contribute to host tolerance during infection, it seems to be preferentially supporting mycobacterial growth, since mice lacking IL-10 revealed decreased bacterial burdens and

accelerated T-cell responses [71, 72]. One report has shown a positive role for IL-10 in the maintenance of tolerance during the chronic stage of *Mtb* infection [73].

1.3.1.4 Macrophage cell death mechanisms

Macrophages infected with *Mtb* have two primary cell death modalities: apoptosis or necrosis. Apoptosis is the last line of defense for the macrophage, as it is a way in which to contain the bacteria at the expense of host cell viability. However, in addition to modulating its own uptake and adapting to the intracellular environment of the macrophage, *Mtb* is able to control the cell death program, which is an important part of its virulence. Virulent strains of *Mtb*, like H37Rv, are able to induce necrosis of the macrophage [74], compromising the plasma membrane of the cell by preventing membrane repair [75] and allowing for bacterial dissemination into the surrounding tissue and bystander cells. On the other hand, avirulent strains, like H37Ra, induce apoptosis in macrophages [74], allowing for containment of the infection and the uptake of apoptotic vesicles by dendritic cells, which increases cross-presentation of mycobacterial antigens to enhance anti-mycobacterial adaptive immunity [76].

1.3.2 Adaptive immune response

The bacteria are able to survive intracellularly, multiply and initially evade detection by the immune system by preventing maturation of the phagolysosome into the early/late endosome, thereby diminishing entry of bacterial proteins into the class II MHC antigen processing pathway [77]. In the mouse model, bacterial dissemination to the regional lymph nodes (LN) precedes initiation of the anti-*Mtb* T-cell response [78]. The dogma is that dendritic cells (DCs) or monocytes transport bacteria to the pulmonary LN [79, 80]. However, it is not certain whether *Mtb* directly infects pulmonary DCs, or whether the death of infected macrophages (as discussed above) leads to secondary uptake of free bacteria or apoptotic macrophages by DCs [76, 80].

Nevertheless, immature DCs form a network in non-lymphoid tissues and, following exposure to microbial antigens or pro-inflammatory cytokines, they mature and traffic to the draining LN. Here, they activate naïve T-cells in an antigen-specific manner, a phenomenon referred to as T-cell priming. DCs are critical for priming *Mtb*-specific $CD4^+$ and $CD8^+$ T-cells *in vivo* [81, 82]. Once activated, both antigen-specific $CD4^+$ and $CD8^+$ T-cells undergo three distinct phases: clonal expansion (proliferative phase), clonal contraction (death phase), and memory. T-cell production of cytokines, including IFNγ, is critical in the activation of macrophages, which leads to the induction of nitric oxide synthase and the production of reactive nitrogen intermediates (RNI), which kill intracellular *Mtb* [53]. Ultimately, an intense inflammatory response occurs, leading to the development of granulomas, which serve to wall off infectious foci.

1.3.3 Granulomatous response

Since the host immune system is unable to completely eradicate the bacteria, it contains *Mtb* in a granuloma, which is comprised of a center core predominated by macrophages and surrounded by lymphocytes and a fibrous cuff. It serves to limit the spread of the bacteria, which enter a long-lived stationary phase known as latency [83]. These few organisms can remain viable for years and further replication of the bacteria is likely prevented by active immunity. This results in an asymptomatic, non-transmissible infection. Sustained activation of macrophages plays an important role in the maintenance of the granuloma, as macrophages are constantly recruited and have a high turnover rate. They are present in many forms, including epithelioid cells, foamy cells and multinucleated giants cells [50]. If impairment of T-cell-mediated immunity occurs, caused by AIDS, malnutrition, or cancer [84, 85], the likelihood of developing reactivated tuberculosis is

increased, since T-cells and macrophage-activating cytokines are critical for the immune system to control and contain the infection in both humans and in rodent models of tuberculosis [86].

1.4 Immunometabolism

One of the lesser mentioned symptoms of tuberculosis is cachexia (wasting) of the TB patient, which may play a larger role in mortality than originally thought. Interestingly, a study examining *Mycobacterium marinum* infection in the fruit fly *Drosophila melanogaster* showed that increased mortality is independent of bacterial load and is mediated by altered host metabolisms and increased body wasting [87]. This study hypothesizes that the wasting is due to sustained energy relocation towards fighting off the infection, since activated immune cells have been shown to increase glucose uptake and metabolism in response to infection [88, 89]. This indicates that metabolism plays an enormous role in the response to infection and may be hijacked by pathogens to increase virulence. As macrophages are important players in *Mtb* infection, their metabolism is critical for optimal anti-mycobacterial responses.

1.4.1 Host-pathogen metabolic interactions

Mtb has been shown to be able to modulate macrophage metabolism. For example, foamy cells, which are induced by *Mtb*, are a hallmark of the granuloma. These cells are macrophages which have dysregulated lipid metabolism and accumulate lipid droplets following interactions with mycobacterial long chain fatty acids [90]. The lipid droplets contain high levels of nutrients and are thought to be used as the main energy source for engulfed mycobacteria persisting in the granuloma in a dormant state, since mycobacteria-containing endosomes can merge with these droplets [90].

On the other hand, macrophages can also shift their metabolisms to aid in pathogen clearance. Activated/inflammatory macrophages, display a metabolic program similar to Warburg metabolism, utilizing aerobic glycolysis for ATP and metabolic intermediate production [91]. It has recently been shown that the switch to aerobic glycolysis by human macrophages is required for control of bacterial growth in mycobacterial infection [92].

1.4.2 M1 macrophage metabolism

The dynamic cellular metabolism of macrophages reflects their roles and the current bioenergetic demands of the cell [93]. M1-like macrophages are generated in the context of inflammation, and constitute some of the first cells to be recruited to inflammatory lesions; thus, they require a rapid source of energy. Although aerobic glycolysis is much less efficient at generating ATP compared to oxidative metabolism, it allows the cell to produce the many metabolic intermediates needed to carry out their inflammatory functions, like cytokine production [94]. The aerobic glycolysis program also allows for the formation of many reactive intermediates (such as ROI or NO), increasing the microbicidal activity of the macrophage.

Upon activation of the cell, via TLR stimulation by bacterial PAMPS for example, genes involved in glycolysis as well as inflammatory cytokine production are upregulated, a process which is highly reliant on HIF-1α [94]. The macrophage increases the uptake of both glucose and glutamine, leading to a rapid flux of metabolites through glycolysis and glutaminolysis and generating NADPH through the pentose phosphate pathway (PPP) or the malate-asparate shuttle and $NADP^+$-dependent malate dehydrogenase, respectively [91]. NADPH is then used to produce ROI through the NADPH oxidase (NOX) system. In addition to the ROI generated by NOX, mitochondrial ROI (mROI) is also generated and can aid in the killing of intracellular pathogens. In fact, it has been

shown that macrophage stimulation through TLR-1, TLR-2 or TLR-4 leads to the recruitment of mitochondria to the phagolysosome and increases mROI production [95].

1.4.3 M2 macrophage metabolism

While M1-like macrophages are extensively studied in the context of inflammation or infection, little is known about M2-like or regulatory macrophages, especially alveolar macrophages. M2-like macrophages play a role during the long resolution phase of inflammation, thus requiring a more stable and efficient means of energy production. They utilize oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) as their primary means of ATP production, which also limits ROI and NO production [91].

M2-like macrophages are stimulated by type 2 cytokines like IL-4 or IL-13. The switch to FAO and OXPHOS is primarily mediated through signal transducer and activator of transcription 6 (STAT6), by upregulating metabolic genes involved in the aforementioned pathways as well as mitochondrial biogenesis [96]. Additionally, STAT6 induces the expression of PPARγ, PPARδ, and PGC-1β, which synergize with STAT6 to help sustain the switch to oxidative metabolism [94]. PGC-1β plays an especially important role, as it's often thought to be one of the main triggers of the metabolic shift seen in M2-like macrophages. It induces mitochondrial respiration, mitochondrial biogenesis and drives the production of key mitochondrial components like cytochrome C and ATP synthase [91]. On the other hand, PPARγ, PPARδ are thought to mainly be involved in the maintenance of the M2 phenotype [91].

Interestingly, deleting genes involved in the regulation of metabolism, or inhibiting aerobic glycolysis or OXPHOS pathways pharmacologically showed that the development of an M1-like or M2-like macrophage phenotype, respectively, can be blocked [96, 97]. Thus, if a pathogen can

alter the phenotype of immune cells by influencing the expression of genes involved in metabolism, it can also control the response for its survival. As seen in the *D. melanogaster* model, the ability of the host to overcome infection cannot only be evaluated by investigating the direct immune responses against the pathogen. Certainly, evaluation of host metabolomics can lead to a deeper understanding of the mechanisms involved in host protection against *Mtb*.

1.5 Central Hypothesis and Aims

The central hypothesis of the current study is that **alveolar macrophages are more permissive than recruited bone marrow-derived macrophages to *Mtb* infection**.

To test our hypothesis, we have designed three specific aims:

1. To determine the metabolism of alveolar macrophages compared to bone marrow-derived macrophages in steady state and upon mycobacterial infection
2. To investigate the impact of macrophage metabolism on their anti-mycobacterial functions
3. To investigate the protective capacity of alveolar versus bone marrow-derived macrophages against mycobacterial infection *in vitro* and *in vivo*

Chapter II: Material and Methods

2.1 Mice

Six- to ten- week old C57BL/6 mice were bred in-house. *csf2rb*$^{-/-}$ mice were a kind gift from Dr. Clinton Robbins at the University of Toronto, Canada, and bred in-house. All experiments were conducted in accordance with the guidelines of the animal research ethics board of McGill University.

2.2 Bacterial strains and *in vivo* infection

Virulent *Mycobacterium tuberculosis* strain H37Rv and attenuated strain H37Ra were grown in a liquid culture of Middlebrook 7H9 medium (Difco) containing 0.2% glycerol (Fisher), 0.05% Tween-80 (Sigma-Aldrich) and 10% albumin-dextrose-catalase. For pulmonary aerosol infection, ~100-200 bacteria were delivered via the aerosol route using a nose-only exposure unit (Intox Products). Initial infectious dose was measured by enumeration of bacteria from the lungs of animals 24 hours post-infection. For intratracheal infection, $0.5x10^6$ bacteria were deposited into the lungs intratracheally. Colony-forming units (CFUs) were determined by plating serial dilutions of lysates in PBS-Tween-80 (0.05% Tween-80) on Middlebrook 7H10 agar (Difco) plates containing 0.5% glycerol (Fisher), 10% oleic acid-albumin-dextrose-catalase and PANTA (BD Biosciences). Colonies were counted 3 weeks post-plating.

2.3 Generation of bone marrow-derived macrophages (BMDM)

Femurs and tibias from 6-10 week old C57BL/6 mice were harvested aseptically. Bones were flushed with R10% (RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), both from Wisent), and whole bone marrow was plated in 9 mL BMDM medium (RPMI-1640 supplemented with 10% heat-inactivated FBS, 2% HEPES, 1mM sodium pyruvate, 1%

essential amino acids, 1% non-essential amino acids and 100U/mL penicillin/streptomycin, all from Wisent) containing 30% L929 cell- (ATCC) conditioned medium at a density of 5-10x10^6 cells per petri dish and cultured at 37°C, 5% CO_2. After 3 days, 9 mL of fresh BMDM medium containing 30% L929 media was added to each dish. On day 6, non-adherent cells were aspirated and adherent macrophages were washed once with PBS prior to harvesting using Cell Stripper (Corning).

2.4 Stimulation of BMDM

After differentiation, BMDM were plated at 1x10^6/well in a 6-well plate or 1x10^5/well in a Seahorse Xf96 assay plate in BMDM medium and allowed to adhere to 24 hours prior to stimulation with 100 ng/mL LPS and 20 ng/mL IFNγ to produce M1-like macrophages or 20 ng/mL IL-4 to produce M2-like macrophages. Macrophages were stimulated for 24 hours prior to further use.

2.5 Collection of alveolar macrophages

Alveolar macrophages were harvested and pooled from the bronchoalveolar lavage (BAL) of six- to ten- week old C57BL/6 mice. Briefly, mice were euthanized with CO_2 and the intact lung and trachea complex was carefully removed. A cannula was inserted into the trachea and firmly secured using surgical string. Lungs were then lavaged *ex vivo* five times with 1 mL of ice cold sterile PBS while gently massaging the tissue to maximize recovery. Purity was greater than 95% as assessed by flow cytometry, and non-adherent cells were washed away 4h after plating in BMDM medium.

2.6 *In vitro* macrophage infection

Bacteria were grown to log phase, as determined by an optical density of 0.3 to 0.8, prior to infection. For H37Rv, cells were infected at a multiplicity of infection (MOI) of 1, whereas for H37Ra macrophages were infected at an MOI of 2.5. Macrophages were cultured for at least 4 hours in BMDM medium without Pen/Strep prior to infection. 4 hours post-infection, macrophages were washed 3 times with R10% and cultured in 1 mL BMDM medium without Pen/Strep. At 4h, 24h, 48h and 72h post-infection, cells and supernatants were harvested and centrifuged for 5 minutes at 15,000 rpm in a microcentrifuge to pellet down the cells and bacteria. Supernatant was then collected and filtered for downstream assays. The cell pellet was resuspended in 500 µL of sterile water and lysed by incubating for 5 minutes at room temperature. Following cell lysis, 500 µL of PBS-Tween-80 (0.05%) was added and CFUs were determined as described above.

2.7 Extracellular flux analysis

Real-time oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) of macrophages were measured in XF media (non-buffered DMEM containing 2mM L-glutamine, 25mM glucose and 1mM sodium pyruvate) using a Seahorse XFe 96 Analyzer (Agilent Technologies). For the mitochondrial stress test, mitochondrial inhibitors oligomycin (1µM, Sigma), fluorocarbonyl cyanide phenylhydrazone (FCCP, 1.5µM, Sigma), antimycin A (0.5µM, Sigma) and rotenone (0.5µM, Sigma) were used. Briefly, macrophages were seeded at a density of 100,000 cells per well and 3 basal measurements were taken. Following this, 2 consecutive measurements were taken following each injection of oligomycin, FCCP, and antimycin A with rotenone. Basal respiration was determined as the last basal measurement minus the non-mitochondrial respiration rate, as determined by the last reading following antimycin A/ rotenone injection (**See diagram 1 below**). ATP production was determined as the decrease in OCR

following oligomycin injection and coupling efficiency is determined by the ratio of ATP production to basal respiration. Finally, spare respiratory capacity was determined as the absolute increase in OCR after FCCP injection compared to basal respiration. All measurements were normalized to cell number using a crystal violet dye extraction assay. Oxygen consumption curves were generated using Wave Desktop 2.3 (Agilent Technologies).

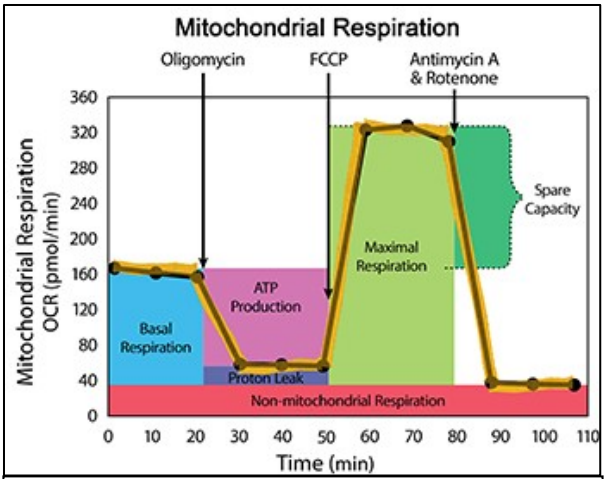

Diagram 1. Mitochondrial stress test (Agilent Technologies)

2.8 Cytokine production

The production of IL-6, IL-12p40, TNFα, and IL-10 was measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D systems) according to the manufacturer's instructions.

2.9 Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated from macrophages using TRIzol reagent (Invitrogen) and purified using the RNeasy mini kit (Qiagen). 200ng of RNA was reverse transcribed to cDNA using 5X All-In-One RT MasterMix (Applied Biological Materials Inc.) according to manufacturer's instructions, using the S1000 Thermal Cycler (Bio-Rad). cDNA was then used in the qPCR reaction with EvaGreen qPCR MasterMix (Applied Biological Materials Inc.) with the following primers: *gapdh*-forward: 5'-GGTCCTCAGTGTAGCCCAAG -3'; *gapdh*-reverse: 5'-AATGTGTCCG TCGTGGATCT-3'; a*rg1*-forward: 5'-ACATTGGCTTGCGAGACGTA-3'; *arg1*-reverse: 5'-

ATCACCTTGCCAATCCCCAG-3'; *inos*-forward: 5'-GGTGAAGGGACTGAGCTGTT-3'; *inos*-reverse: 5'-ACGTTCTCCGTTCTCTTGCAG-3'. Cq values were obtained on the CFX96 Real-Time PCR Detection System (BioRad) and were analyzed using the formula $2^{-\Delta Cq}$. *arg1* and *inos* expression were normalized to *gapdh*.

2.10 Flow Cytometry

For BMDM staining, macrophages were harvested from 6-well plates using 1mL of Cell Stripper (Corning). Cells were then washed and incubated for 10 minutes with anti-CD16/32 in FACS buffer (0.5% BSA/PBS) at 4°C to block non-specific antibody interaction with Fc receptors. They were then stained for 30 minutes at 4°C with the following antibodies (ebioscience or BD unless otherwise stated): anti-CD11b PE-Cy7, anti-F4/80 APC-efluor780, anti-CD86 V450, anti-CD206 APC (Biolegend). For lung staining, a single-cell suspension was obtained by incubating the tissue with 300 units of collagenase IV in 2mL of media for 1 hour at 37°C and passing it through a 40µM cell strainer. Red blood cells were lysed for 1 minute and samples were washed to remove lysis buffer prior to staining. $3x10^6$ cells were first incubated with viability dye in efluor506 (ebioscience) for 30 minutes at 4°C, followed by a wash and an incubation with anti-CD16/32 for 10 minutes in FACS buffer at 4°C. They were then stained for 30 minutes at 4°C with the following antibodies (antibodies from ebioscience or BD unless otherwise stated): anti-CD11b BUV395, anti-CD11c BV421, anti-CD45.2 APC, anti-F4/80 APC-efluor780, anti-Ly6C FITC, anti-Ly6G AlexaFluor700, anti-NK 1.1 BV650 and anti-Siglec F PECF594. All samples were fixed in 1% paraformaldehyde prior to acquisition using a BD LSRFortessa X-20 flow cytometer (BD Biosciences) and FACSDiva software (BD Biosciences). Data analysis was performed using FlowJo v.10 (TreeStar).

2.11 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 7.02 (GraphPad Software, San Diego, California, USA). All data are presented as mean ± SEM and differences were considered significant if $p<0.05$. 1-way ANOVA followed by Tukey comparison or 2-way ANOVA followed by either Bonferroni or Dunnetts comparison was used, as specified in each figure.

Chapter III: Results

3.1 Macrophage phenotyping

We initially established an *in vitro* M1/M2 model to investigate how AM and BMDM would be placed within these two extremities of the spectrum of macrophages. As monocyte-derived macrophages are mainly recruited to the site of infection, we initially speculated that M1-like macrophages are representative of recruited macrophages and M2-like macrophages are representative of alveolar macrophages (AM), which are thought to be more regulatory. We generated M1-like (M1) and M2-like (M2) macrophages and collected alveolar macrophages from naïve mice **(Figure 2A)**. Prior to running functional experiments, we phenotyped the different subsets of macrophages by flow cytometry to ensure that they were displaying the M2 marker CD206 **(Figure 2B and 2C)** or the M1 marker CD86 **(Figure 2E and 2F)**. M2 macrophages showed significant upregulation of CD206 and higher expression of *arg1* transcripts when compared to AM or M1 macrophages **(Figure 2B, 2C, 2D).** In contrast, M1 macrophages showed significant upregulation of CD86 and higher expression of *inos* transcripts compared to M2 and AM **(Figure 2E, 2F, 2G)**. Surprisingly, AM did not resemble either M1 or M2 macrophages in terms of expression of surface markers or classical M1/M2 transcriptional genes.

3.2 Macrophage metabolism

Next, we investigated the metabolic signature of the cells at baseline to gain insight into the resting functions of the different subtypes of macrophages. Extracellular acidification of the cell media is linked to the glycolytic rate of the cell, since aerobic glycolysis generates lactate and H^+, which acidify the media, to regenerate reducing equivalents needed to fuel glycolysis. Additionally, since oxidative phosphorylation (OXPHOS) consumes O_2, the level of oxygen in the media can be

measured and serves as a proxy for OXPHOS. We measured extracellular acidification rate (ECAR), and oxygen consumption rate (OCR) of M1-BMDM, M2-BMDM and AM at baseline and following the addition of oligomycin, FCCP and both antimycin A and rotenone **(Figure 3)**. At baseline, prior to injections of mitochondrial inhibitors, we observed that M2-BMDM and AM have similar metabolisms **(Figure 3A-3D),** having higher OCR **(Figure 3C)** and lower ECAR **(Figure 3D)** than M1-BMDM. Additionally, they showed a lower ECAR/OCR ratio when compared to M1-BMDM (**Figure 3B)**. Following baseline measurements, oligomycin was injected to inhibit ATP synthase and measure how efficiently oxygen consumption is coupled to ATP production **(Figure 3A, 3F)**. While we found that M2-BMDM and AM had a higher OCR, there was no difference in the proportion of consumed oxygen being used for ATP generation (coupling efficiency) when compared to M1-BMDM. Next, we injected FCCP, an uncoupling agent which dissipates the proton gradient and allows for maximal oxygen consumption in the cell **(Figure 3A)**. We then calculated the spare respiratory capacity (SRC), a measure of cell fitness which is linked to the capacity of the cell to meet energetic demands as well as cell survival [98, 99]. While AM and M2-BMDM showed similar basal metabolisms, there was a significant difference in SRC between them, though both were higher than M1 **(Figure 3E)**. Thus, although AM closely resemble M2-BMDM at baseline, they have a unique metabolic signature, which perhaps reflects a pro-survival phenotype and their long half-life [100].

3.3 Macrophage metabolism after mycobacterial infection

We then investigated the metabolism of M1-BMDM, M2-BMDM and AM following infection with mycobacteria (H37Ra). Macrophages were infected for 24hrs at an MOI of 2.5 followed by measurement of their extracellular flux **(Figure 4A)**. Prior to the injection of mitochondrial inhibitors, both M1- and M2-BMDM had higher ECAR/OCR ratios than AM, indicating that they

have a higher glycolytic rate **(Figure 4B)**. Interestingly, as it is shown in **Figure 4A**, the SRC of AM remains high while the mitochondria of M1- and M2-BMDM are now operating at maximum capacity (**Figure 4C)**, indicating that AM consistently maintain a pro-survival phenotype, even upon infection. In addition, while all macrophages showed decreased OCR after infection (**Figure 4D)**, M1- and M2-BMDM increased their ECAR, indicative of a shift to a more inflammatory phenotype, while AM did not **(Figure 4E)**. These data collectively indicate that following mycobacterial infection AM fail to shift to glycolytic metabolism and adopt an inflammatory phenotype (**Figure 4F)**. Furthermore, the high levels of SRC in AM, which potentially explains their enhanced survival, might lead *Mtb* to preferentially infect AM for replication.

3.4 Macrophage function *in vitro*

Since the switch to a glycolytic phenotype is a hallmark of activated inflammatory macrophages, we sought to investigate the impact of the failure of AM to become glycolytic on their anti-mycobacterial cytokine production following mycobacterial infection. We infected M1-BMDM, M2-BMDM and AM with H37Rv at an MOI of 1, and measured cytokine production over 72hrs. We found that AM failed to produce cytokines, including IL-12p40 **(Figure 5B)**, IL-6 **(Figure 5C)** and IL-10 **(Figure 5D)**. The same trend was observed following infection with H37Ra (MOI=2.5) **(Figure 5F, 5G, 5H)**. However, the production of TNFα in response to both infections **(Figure 5A, 5E)** was intact in AM, which has been previously shown to increase bacterial growth in macrophages [101]. We next investigated the protective capacity of the macrophages *in vitro* by measuring the bacterial load in the cells over 72hrs. AM had a significantly higher bacterial load compared to M1- or M2-BMDM at both 48hrs and 72hrs following H37Rv **(Figure 6A)** or H37Ra **(Figure 6B)** infection, indicating that they are more permissive to mycobacterial infection than BMDM.

3.5 Macrophage function *in vivo*

To examine if the increased permissiveness of AM *in vitro* would translate to decreased protection *in vivo*, we used *csf2rb*$^{-/-}$ mice, which lack the β-chain of the GM-CSF receptor. GM-CSF is a critical growth factor for AM development and maintenance; thus, *csf2rb*$^{-/-}$ mice lack mature, functional AM [102]. Initially, we infected wild-type (WT) or *csf2rb*$^{-/-}$ mice with H37Ra intratracheally, and assessed the bacterial burden at the early time points post-infection. Surprisingly, but in line with our *in vitro* data supporting AM as an enhanced replicative niche for mycobacteria, we found that *csf2rb*$^{-/-}$ mice were more protected from H37Ra infection at these early time points, particularly at day 12 post-infection **(Figure 7A)**.

We next sought to reconstitute the lungs of *csf2rb*$^{-/-}$ mice with either WT alveolar macrophages, or WT bone marrow-derived macrophages, to investigate if we could recapitulate our *in vitro* observation **(Figure 6A)**, and if the mice given BMDM instead of AM would be more protected against *Mtb* compared to WT mice. After culturing the BMDM *in vitro* for 6 days, we adoptively transferred 1x10^6 BMDM or AM (collected via BAL) intratracheally to *csf2rb*$^{-/-}$ mice **(Figure 7B)** 1 day prior to aerosol infection with H37Rv at a dose of ~200 CFU. At the peak of infection (day 30), mice were sacrificed and the bacterial burden was assessed in both lungs and spleens. We also assessed the frequencies of inflammatory monocytes **(Figure 7C)** and alveolar macrophages **(Figure 7D)** by flow cytometry in the lung, and found that while the *csf2rb*$^{-/-}$ mice lack AM, they recruited more inflammatory monocytes to the lungs after infection than WT mice. No differences in monocytes nor AM were found between the *csf2rb*$^{-/-}$ mice groups which had received macrophages prior to infection and those which did not. Finally, we determined the bacterial load in the spleen **(Figure 7E)** and lungs **(Figure 7F)**. While there were no significant differences in

the bacterial load in the lungs **(Figure 7F)**, WT mice had a higher bacterial load in the spleen, indicating that bacterial dissemination was better controlled in *csf2rb*$^{-/-}$ mice compared to WT mice. These results suggest that the presence of AM in WT mice impairs host protection during pulmonary *Mtb* infection. However, whether this impairment is predominantly mediated by direct support of pulmonary *Mtb* replication or by promoting bacterial dissemination requires further validation.

Chapter IV: Discussion

Mycobacterium tuberculosis is an ancient pathogen, as the footprint of the bacterial DNA was identified in ancient Egyptian mummies [103]. Recently, it has been suggested that *Mtb* has coevolved with humans since our earliest evolutionary days: the initial migration out of Africa approximately 70,000 years ago [104]. This parallel evolution has allowed *Mtb* to specifically target the human immune system and develop strategies to survive in the hostile environment of the host driven by both innate and adaptive immune responses. Since *Mtb* is spread by the aerosol route and, thus, rapidly enters the lungs, the alveolar macrophage is ideally situated as the first immune cell to respond to the invading pathogen. Consequently, *Mtb* has evolved to specifically target alveolar macrophages for infection, survival and growth.

Alveolar macrophages are a unique macrophage subtype in the airways both at steady state and during infection due to their specific ontogeny. Fate mapping studies reveal that alveolar macrophages are seeded in the lungs during embryonic development, initially from the yolk sac and later by monocytes derived from the fetal liver [20]. Interestingly, their population is maintained by self-renewal with minimal to no contribution from circulating monocytes derived from the bone marrow throughout adulthood [19]. However, during infection, monocyte-derived

macrophages must be recruited from the bone marrow to the site of infection to aid in pathogen clearance [22]. These macrophages are derived from inflammatory monocytes, which emigrate from the bone marrow in a CCR2-dependent manner, and make up the majority of the macrophages present during infection [22]. Thus, the macrophages present in the lungs during infection have two distinct ontogenies: long-lived embryonically-seeded alveolar macrophages, with the ability to self-renew, and bone marrow-derived macrophages, short-lived inflammatory cells which are constantly replenished.

Because of the chronicity of TB, studies often focus on later timepoints after infection, once adaptive immunity is active in fighting the infection. Given the importance of adaptive immunity in controlling *Mtb* infection as well as the memory capacity of cellular compartments of adaptive immune responses (e.g. T- and B- cells), the majority of vaccines currently in development are designed to elicit a rapid and robust antigen-specific T-cell response. However, neither pre-clinical nor clinical trials of vaccines targeting T-cell mediated immunity have been successful [8]. Interestingly, the currently available vaccine, BCG, derived from *Mycobacterium bovis*, does not provide protective immunity in adults, though it is effective in preventing milliary TB in early childhood [105, 106]. Importantly, the vaccine does not generate T-cell-mediated immunity against the main *Mtb* antigens ESAT-6 and CFP-10, as they are not expressed by BCG [107]. Additionally, BCG-specific T-cell responses have also not been directly linked to protective immunity against *Mtb* [108], and therefore, the protection afforded by the vaccine may be mainly dependent on innate immunity. In this regard, there have been several recent studies indicating that BCG can 'train' innate immune cells via epigenetic reprogramming of monocytes/macrophages via altering their metabolism to reflect a 'primed' state [109, 110]. With the failure of vaccines targeting adaptive immunity [8] and the newly found existence of trained innate immunity [111],

we envision that the initial innate immune response presents an interesting target for anti-mycobacterial therapy and, thus, warrants further investigation.

The ability of many pulmonary pathogens to cause disease depends on the initial immunity of macrophages. However, few studies have aimed to characterize the initial events following *Mtb* infection. In *Mtb* infection, macrophages, which normally excel at destroying the biological particles they engulf by phagocytosis, are instead killed by *Mtb*, enabling the bacteria to spread to nearby cells and propagate the infectious process. The success of *Mtb* is, therefore, largely dependent on its ability to avoid the microbicidal capabilities of macrophages. To this end, we anticipate that macrophages present a unique target for future anti-mycobacterial therapy and vaccine development, and aim to further understand their critical role in immunity against *Mtb* infection. Given the longstanding evolutionary history of *Mtb* and the different ontogenies of macrophages present in the lungs during *Mtb* infection, we hypothesized that alveolar macrophages are more permissive than recruited bone marrow-derived macrophages to *Mtb* infection.

When we initially phenotyped the various macrophage subsets using qPCR and flow cytometry, it was surprising to see that AM, which are typically known for having a high expression of the M2 marker CD206 [112], in fact expressed it at levels lower than both BMDM groups. Additionally, they had a lower expression of the M2 gene *arg1* and the M1 markers CD86 and *inos*. Thus, our data agrees with more recent hypotheses that AM do not fit precisely into either M1-like nor M2-like categories [17]. However, as an *ex vivo* macrophage instead of an *in vitro*-derived and polarized macrophage, the expression profiles of AM and BMDM may differ too greatly to directly compare them to each other phenotypically. Interestingly, AM basal metabolism was similar to M2 macrophages. Both subtypes were more oxidative when compared to M1 macrophages;

however, AM had a substantially higher spare respiratory capacity (SRC), a measure which has been linked to increased cell fitness and survival [98, 99]. The SRC was significantly higher even following infection with H37Ra, which together with the lack of shift towards a glycolytic, inflammatory phenotype indicates that *Mtb* can effectively hide in AM and replicate. Interestingly, a recent study showed that human AM undergo a shift in metabolism following *Mtb* infection [92], conversely to what we have observed in the murine model. However, their study differs from ours in several aspects. Firstly, irradiated H37Rv was used to examine ECAR and OCR instead of live bacteria. They also used lactate production as a metabolic indicator with live bacteria but did not concurrently measure oxygen consumption. Additionally, they did not investigate the protective capacity of AM.

We next sought to investigate the impact of macrophage metabolism on their anti-mycobacterial functions by measuring cytokine production. The lack of metabolic shift was mirrored by a lack of cytokine response to both virulent and avirulent *Mtb*, since AM did not produce IL-6 or IL-12, both crucial cytokines in anti-mycobacterial immunity. However, both M1- and M2-BMDM were able to switch metabolic phenotypes to produce important cytokines. One possible explanation for this is that *Mtb* has evolved to specifically target, infect and hide within the AM without triggering a response from its host cell.

Our last aim was to investigate the protective capacity of alveolar versus bone marrow-derived macrophages against mycobacterial infection *in vitro* and *in vivo*. When we examined the impact of the complacency of AM *in vitro* compared to M1- and M2-BMDM in terms of the bacterial load, we found that AM were much more permissive to both avirulent H37Ra and virulent H37Rv infection. We also observed that they had less control of the infection over 72hrs.

Next, we investigated whether we could translate our *in vitro* findings to an *in vivo* model. Since we are focused on understanding the early time points, we began by looking at the time points prior to the onset of adaptive immunity using an avirulent strain of mycobacteria. Since AM were more permissive *in vitro*, we used *csf2rb*$^{-/-}$ mice, which lack mature AM, and compared them to WT mice. We found that the *csf2rb*$^{-/-}$ were more protected following infection with H37Ra, which would indicate that AM are in fact detrimental in mycobacterial infection and supports our hypothesis. Interestingly, a group in the Netherlands found similar results using clodronate liposomes to deplete airways macrophages [113]. On the other hand, another study found that *csf2*$^{-/-}$ (mice lacking GM-CSF as opposed to its receptor), were equally susceptible to avirulent mycobacteria [114]. However, this study only had one time point, prior to the onset of adaptive immunity, and used BCG instead of H37Ra. This study also found that *csf2*$^{-/-}$ mice were more susceptible to H37Rv infection. Thus, we infected WT and *csf2rb*$^{-/-}$ with H37Rv, and looked at day 30, where they noted high mortality in the knockout mice. While the differences in lung CFUs were not significant, surprisingly, *csf2rb*$^{-/-}$ mice had significantly less bacterial dissemination, as indicated by lower spleen CFUs. Thus, perhaps the permissiveness of AM allows for early dissemination of the bacteria, but later control of infection in the lungs by optimally recruiting protective antigen-specific T-cells. Although there is a clear difference between GM-CSF-deficient and GM-CSF receptor-deficient hosts, these studies collectively indicate that more research needs to be performed to fully understand the contributions of AM in immunity to *Mtb*.

We had also included 2 more groups of knockout mice whose lungs had been reconstituted with either WT-AM or WT-BMDM, with the idea that *csf2rb*$^{-/-}$ given WT-AM would recapitulate the WT phenotype, while *csf2rb*$^{-/-}$ given WT-BMDM would have a decreased bacterial load compared to WT. However, no differences were noted between the groups of mice which had received either

AM or BMDM and the control mice which did not receive macrophages. We anticipate that this experiment failed and certainly requires further optimization. For instance, one of the important roles of AM is the maintenance of homeostasis in the lungs and clearance of surfactant, so the *csf2rb*$^{-/-}$ mice have a buildup of debris and surfactant in their lungs. Thus, following macrophage transfer, it is possible that the macrophages are filled with surfactant and debris, and that the kinetics of infection may differ once these macrophages are exposed to *Mtb*. We envision that following optimization of this experimental protocol, we will have more insight into the exact roles of different macrophage subtypes *in vivo*.

While the *csf2rb*$^{-/-}$ mice help to provide some insight into the roles of AM *in vivo*, a limitation of this model is the impact that GM-CSF signaling has on other immune cells. *csf2rb*$^{-/-}$ mice are able to maintain a relatively normal immune system; however, they are deficient in eosinophils [115]. Eosinophils are a crucial innate immune cell type during helminth infection, but in *Mtb* infection they play a minor role, with very few studies addressing their functions. It has been shown, however, that an eosinophil-derived enzyme, eosinophil peroxidase (EPO), has anti-mycobacterial effects when administered directly to the bacteria [116]. In contrast, IFNγ-deficient mice showed that eosinophils play a detrimental role during mycobacterial infection, as eosinophil accumulation was noted in the granulomas and anti-IL-5 treatment was able to reduce both eosinophil accumulation and bacterial load [117]. Thus, to address the limitations of our model, we will need to generate a lung-chimeric mouse. The lungs of the *csf2rb*$^{-/-}$ mouse would be protected by a lead shield during irradiation and the bone marrow would be reconstituted with WT donor cells. This technique was pioneered by Janssen et al. [118], and will allow us to study *Mtb* in lungs which lack AM but have normal cell recruitment during infection. This model would also be more appropriate for the translation of our *in vitro* results to an *in vivo* model.

Overall, our *in vitro* results along with preliminary *in vivo* results with H37Ra and H37Rv indicate that AM are in fact detrimental to host protection during *Mtb* infection, and that M1-BMDM, which model recruited macrophages, are better equipped to control the infection. AM, as the sentinel cells of the lungs, are tasked with removing debris and inhaled particulate matter without initiating an inflammatory response and causing tissue damage, since the lungs filter over 10,000 L of air per day. Consequently, AM provide an ideal intracellular environment for the growth of *Mtb,* which has co-evolved with humans, and as such, has adapted to specifically target AM. This is done by inhibiting the metabolic shift, thereby hindering the production of cytokines and impairing bacterial control, leading to increased host susceptibility **(Figure 8)**. Our lab is currently investigating trained innate immunity and the role of recruited educated monocytes during *Mtb* infection (Kaufmann et al., 2017, in revision). We found that BCG-educated monocytes are able to better control mycobacterial infection, thus, the education of AM could also aid in stopping the ability of *Mtb* to initially infect the host, but requires further investigation.

Chapter V: Conclusion

When a complex organism like the human species becomes the only host of a complex microorganism like *M. tuberculosis*, a Darwinian framework is necessary to understand millennia of host-pathogen interactions. The genus of *Mycobacterium* originated more than 150 million years ago and is known as the "Captain of all these men of death", and it is still "the captain" of 2 billion infections worldwide with nearly 1.5 million death annually [119], indicating that our current strategy in vaccine development against TB must be drastically changed. Although since 1960, the field of T-cell biology has exponentially exploded and tremendously broadened our understanding of T-cells in infectious diseases—including their critical role in helping B-cells for generating antibodies against pathogens—the direct evidence of protective T-cells in all current human vaccines is very little. Furthermore, the human observation indicates that among close household contacts of highly active TB patients, 50% of exposed individuals do not convert their TST response, suggesting that these individuals were able to eliminate *Mtb* during the early stage of infection via innate immunity [26, 27]. Therefore, further understanding of alveolar macrophage biology during the initial stage of infection offers insight into how *Mtb* is able to avoid the innate immune system to establish a chronic infection. Identifying the imprint of the protective innate immunity will provide a novel approach for generating vaccines against TB and potentially other persistent infectious diseases.

Figures and Figure Legends

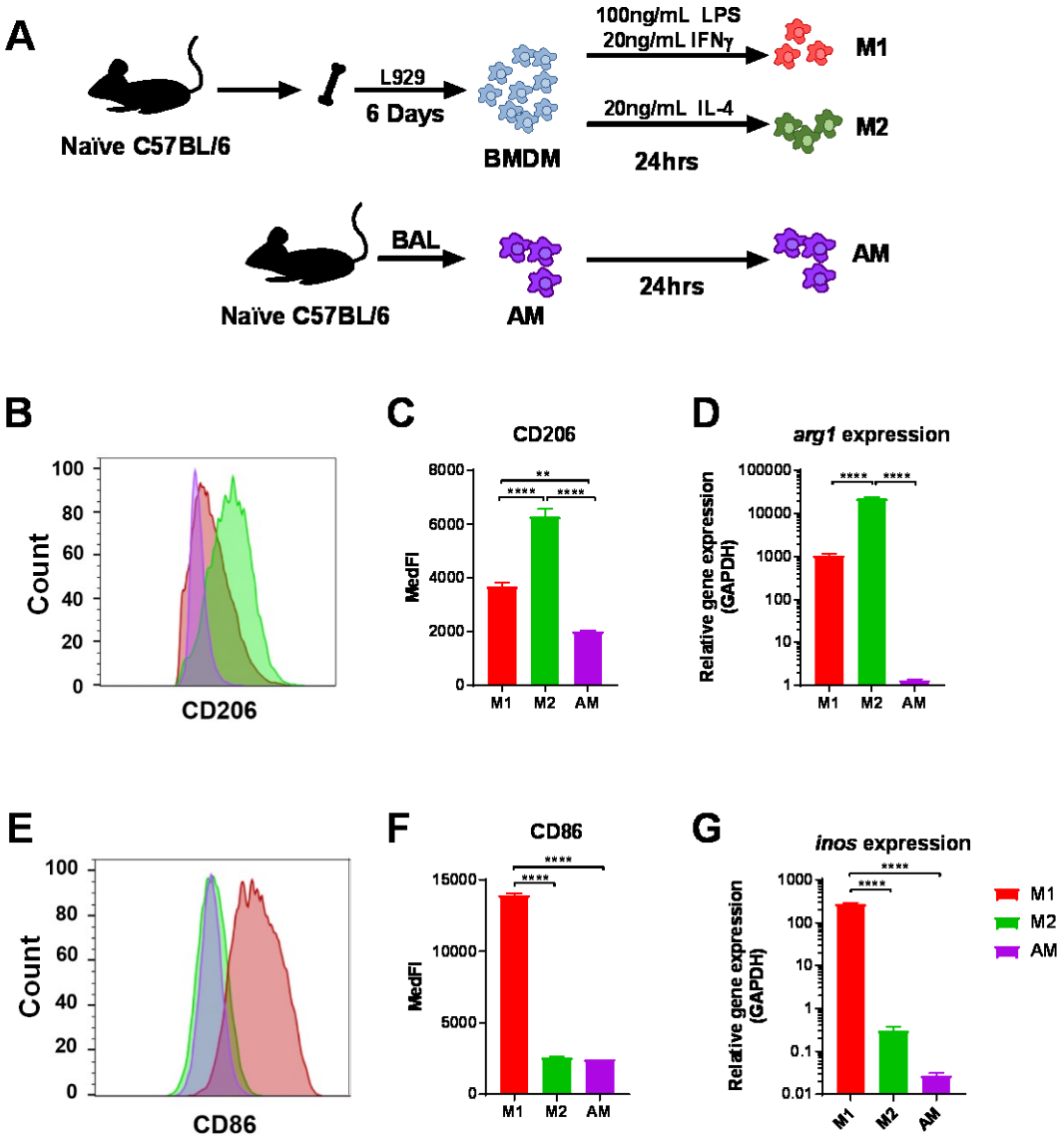

Figure 2. *In vitro* derivation and phenotyping of M1, M2 and alveolar macrophages. (A) Bone marrow was harvested from naïve C57BL/6 mice and cultured in the presence of L929-conditioned media for 6 days prior to polarization with either LPS and IFNγ (M1), or IL-4 (M2) for 24hrs. Alternatively, alveolar macrophages (AM) were harvested from naïve mice. (B) Histogram depicting the expression of CD206 as measured by flow cytometry following 24hr polarization. (C) Quantification of median fluoresence intensity (MedFI) of CD206 expression following 24hr polarization. (D) Relative gene expression of *arg1* compared to GAPDH. (E) Histogram depicting the expression of CD86 as measured by flow cytometry following 24hr polarization. (F) Quantification of MedFI of CD86 expresion following 24hr polarization. (G) Relative gene expression of *inos* compared to GAPDH. Three sample replicates were individually analyzed and the data are given as mean ± SEM. ** p<0.01; **** p<0.0001 (1-way ANOVA followed by Tukey comparison).

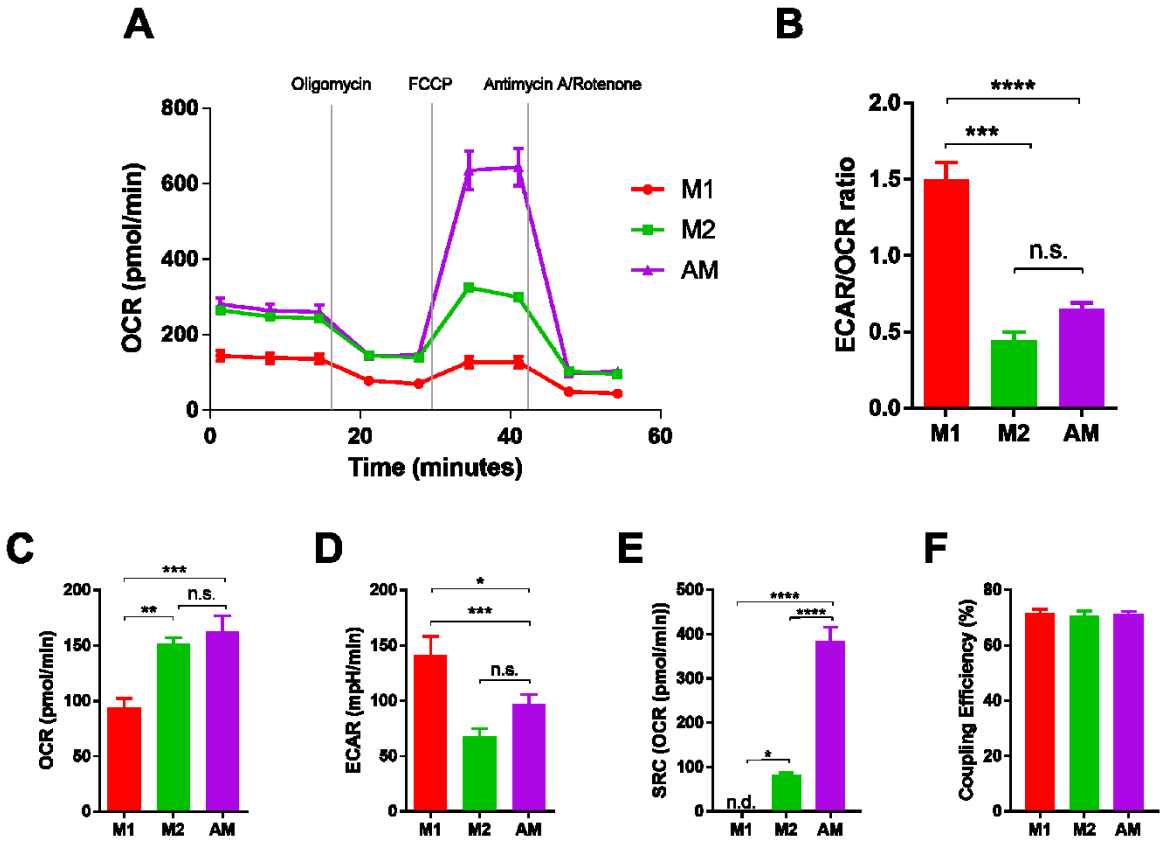

Figure 3. Alveolar macrophages have a unique metabolic signature, distinct from M2 macrophages. M1 and M2 bone marrow-derived and naïve alveolar macrophages were subjected to a Seahorse assay to measure cellular metabolism. (A) Oxygen consumption rate (OCR) curve following sequential injections of mitochondrial inhibitors oligomycin, FCCP and antimycin/rotenone (Ant Rot). (B) Ratio of extracellular acidification rate (ECAR)/OCR. (C) Quantification of basal levels of oxygen consumption, (D) ECAR, (E) spare respiratory capacity (SRC) and (F) coupling efficiency. 6 sample replicates were individually analyzed and the data are given as mean ± SEM. The results are representative of 2 independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$ (1-way ANOVA followed by Tukey comparison).

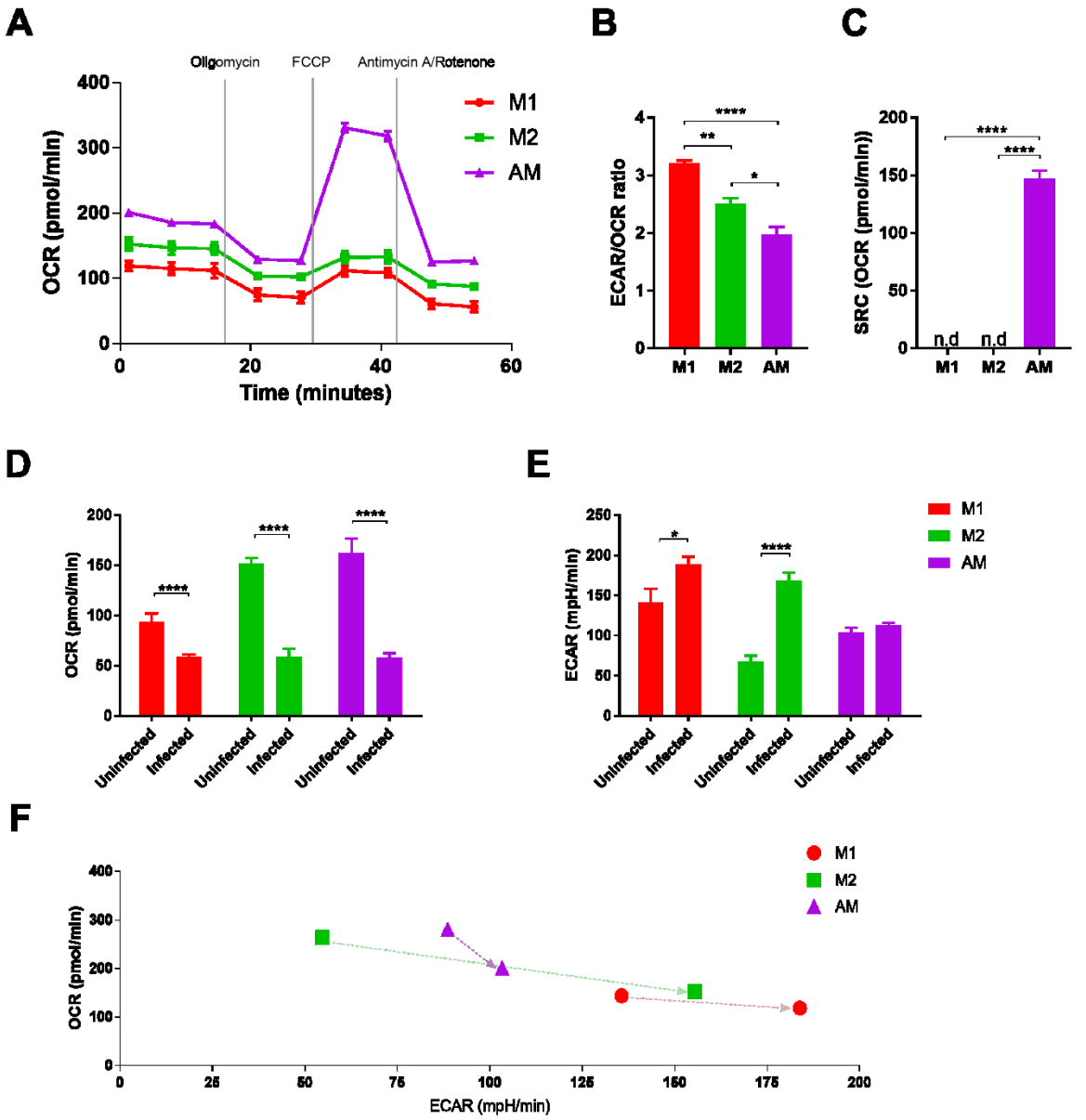

Figure 4. Alveolar macrophages do not switch to a glycolytic phenotype following mycobacterial infection. M1 and M2 bone marrow-derived and naïve alveolar macrophages were subjected to a Seahorse assay to measure cellular metabolism 24hr following H37Ra infection at an MOI of 2.5. (A) Oxygen consumption rate (OCR) curve of infected macrophages following sequential injections of mitochondrial inhibitors oligomycin, FCCP and antimycin/rotenone. (B) Ratio of extracellular acidification rate (ECAR)/OCR of infected macrophages. (C) Quantification of spare respiratory capacity of infected macrophages. Shift in (D) OCR and (E) ECAR following infection, illustrated in (F). 6 sample replicates were individually analyzed and the data are given as mean ± SEM. The results are representative of 2 independent experiments.
* $p<0.05$; ** $p<0.01$; **** $p<0.0001$ (1-way ANOVA followed by Tukey comparison (B), (D,E) 2-way ANOVA followed by Bonferroni comparison).

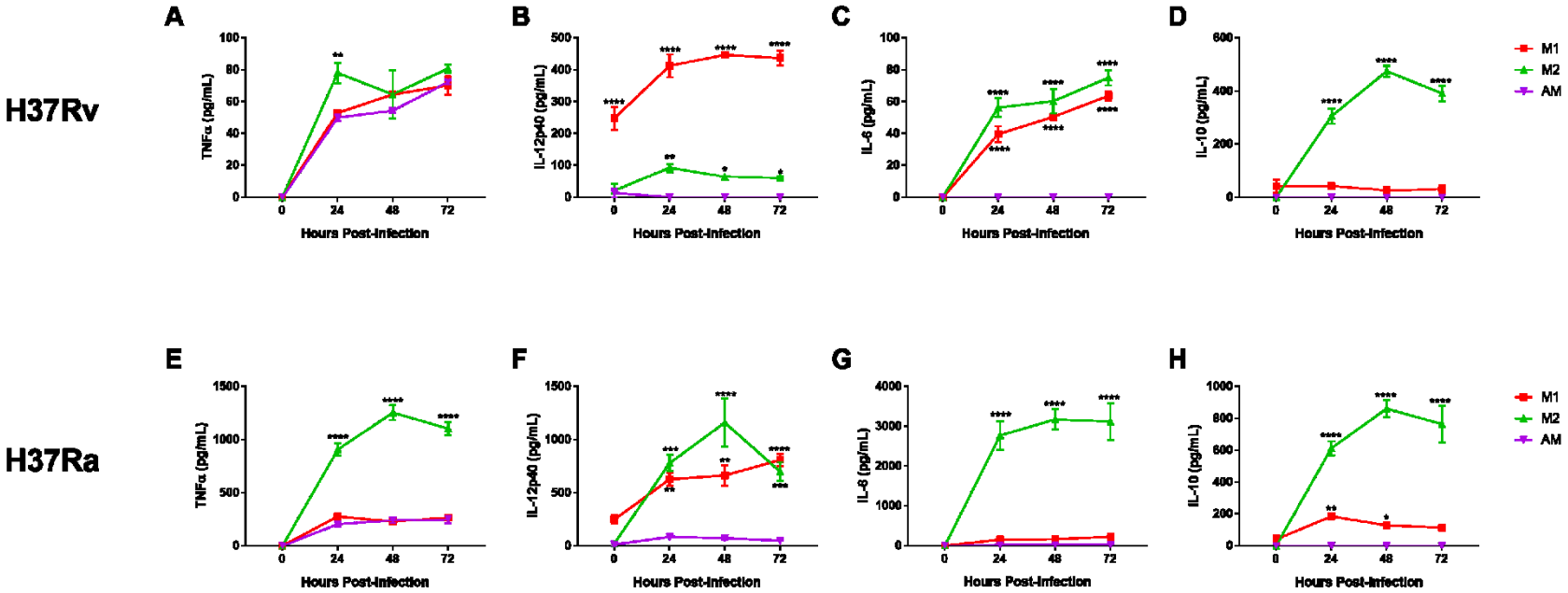

Figure 5. Alveolar macrophages fail to produce critical anti-mycobacterial cytokines following infection. M1 and M2 bone marrow-derived and naive alveolar macrophages were infected at MOI 1 with H37Rv (A-D) or at MOI 2.5 with H37Ra (E-H). Kinetics of cytokine release by infected macrophages of (A,E) TNFα, (B,F) IL-12p40, (C,G) IL-6 and (D,H) IL-10 as measured by ELISA. 3 sample replicates were individually analyzed and the data are given as mean ± SEM. The results are representative of 3 (A-D) or 2 (E-H) independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$ compared with AM (2-way ANOVA followed by Dunnetts comparison).

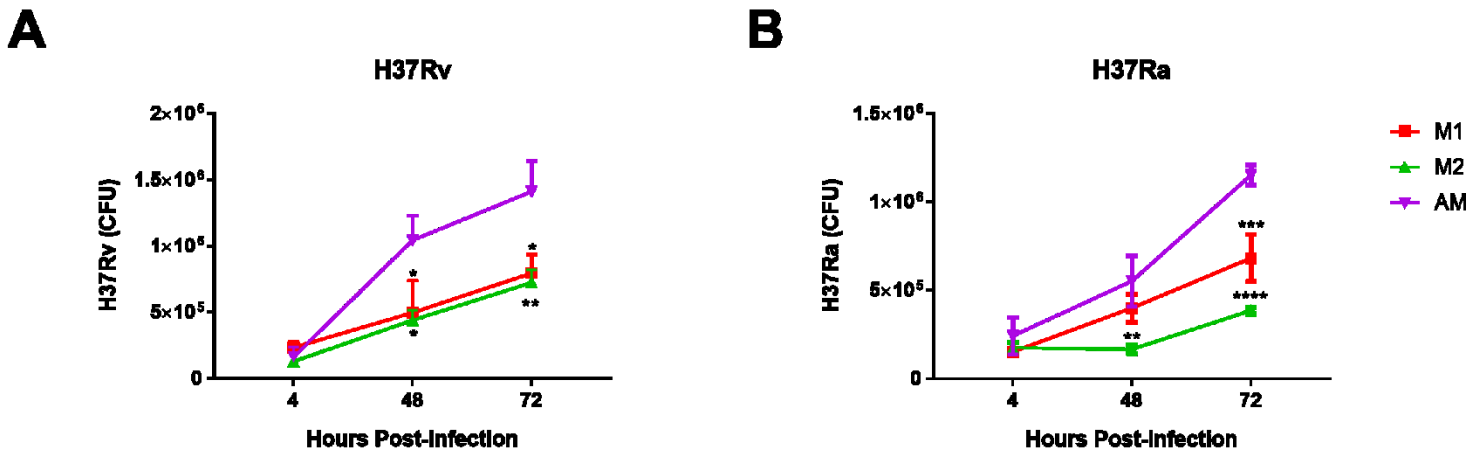

Figure 6. Alveolar macrophages are more permissive to bacterial growth than bone marrow-derived macrophages. M1 and M2 bone marrow-derived and naive alveolar macrophages were infected (A) at MOI 1 with H37Rv or (B) at MOI 2.5 with H37Ra and subjected to CFU assays. 3 sample replicates were individually analyzed and the data are given as mean ± SEM. The results are representative of 3 (A) or 2 (B) independent experiments. *** $p<0.001$; **** $p<0.0001$ compared with AM (2-way ANOVA followed by Bonferroni comparison).

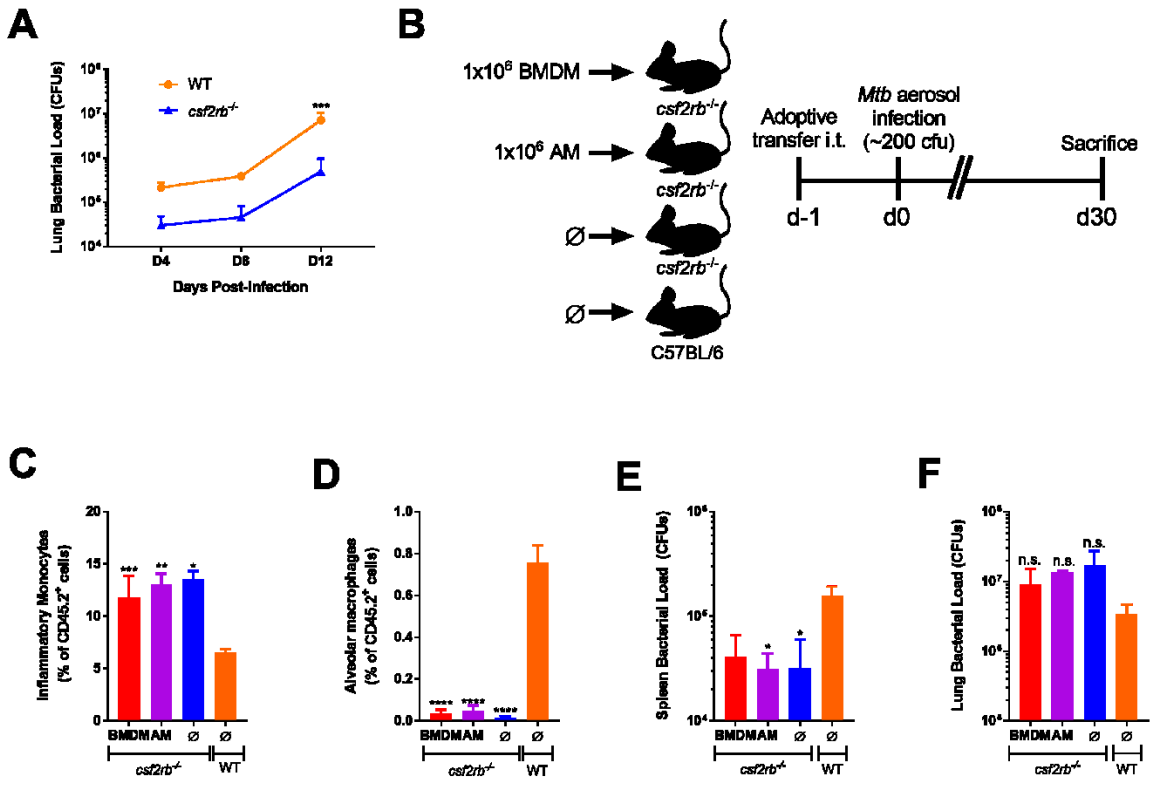

Figure 7. Permissivity of alveolar macrophages contributes to reduced host protection during mycobacterial infection. (A) Bacterial burden (colony-forming units, CFUs) in the lungs of *csf2rb*$^{-/-}$ and wild-type (WT) mice infected intratracheally (i.t.) with 0.5x10^6 H37Ra. (B) Experimental model for (C-F).(C) Frequency of inflammatory monocytes (CD45$^+$SiglecF$^-$NK1.1$^-$CD11b$^+$Ly6G$^-$ Ly6C^{hi}) as a percentage of CD45$^+$ cells. (D) Frequency of alveolar macrophages (CD45$^+$SiglecF$^+$CD11c$^+$) as a percentage of CD45$^+$ cells. (E) H37Rv CFUs detected in the spleen. (F) H37Rv CFUs detected in the lungs. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001, 2-way ANOVA followed by Bonferroni comparison (A) and 1-way ANOVA followed by Tukey comparison, compared to WT (C-F). (A-F) n=3-5 mice per group.

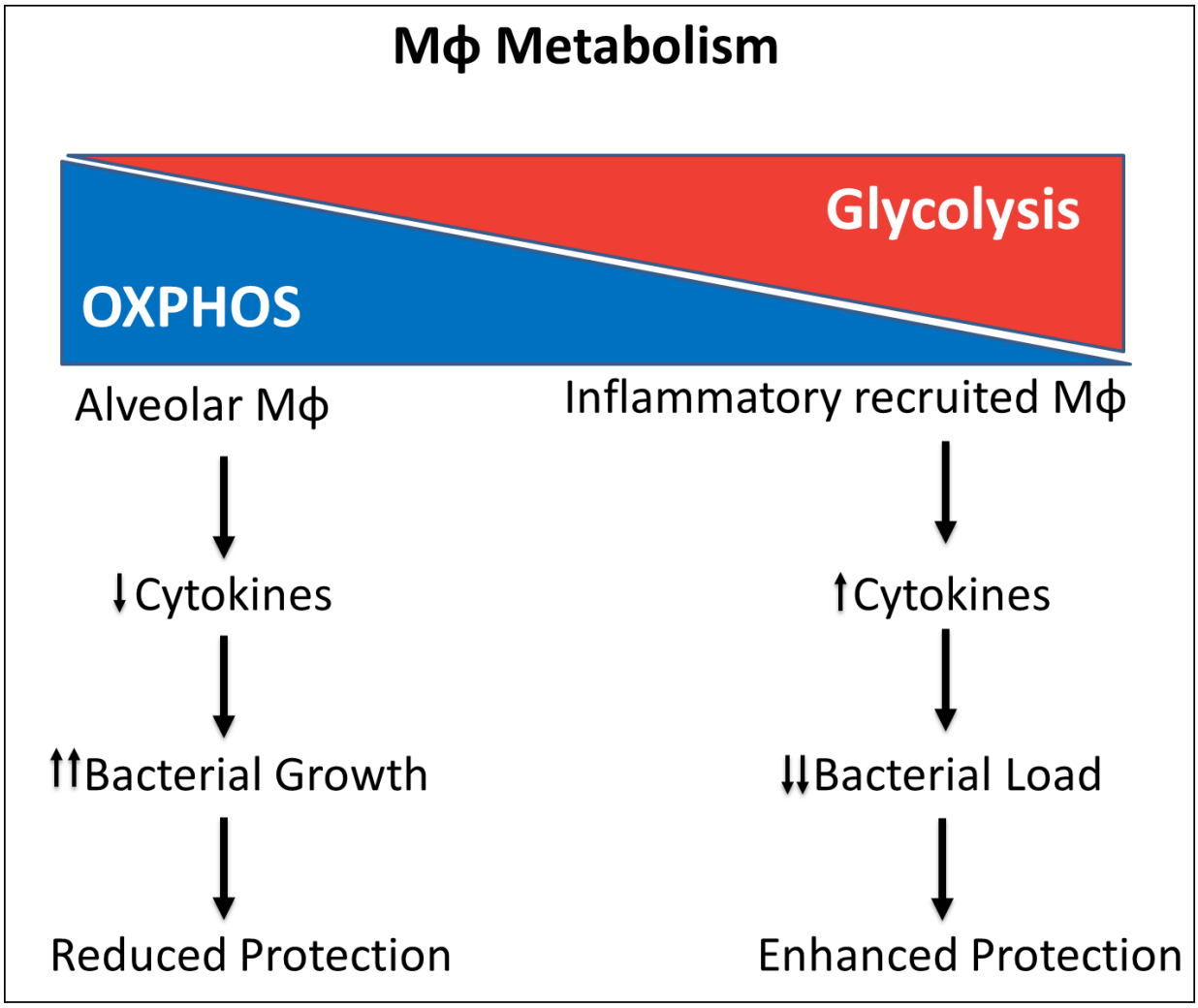

Figure 8. Working model of alveolar macrophage permissivity to mycobacterial infection. Macrophages originating in the bone marrow are recruited in an inflammatory manner and are able to switch to a glycolytiv phenotype following mycobacterial infection. This leads to increased production of cytokines, decreased bacterial load and overall enhanced host protection. However, alveolar macrophages are unable to make the glycolytic shift, thus produce low levels of cytokines and promote bacterial growth, leading to reduced host protection.

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