MECHANISMS OF MACROPHAGE DETECTION AND CONTROL OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION

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Dedication

This thesis is dedicated to my parents, Ira and Susan Collins. Without your love and support, none of this would have been possible. I don't think words can ever fully express my love and gratitude.

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MECHANISMS OF MACROPHAGE DETECTION AND CONTROL OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION

by

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Macrophages use different mechanisms to recognize and respond to *Mycobacterium tuberculosis* infection. Macrophage recognition of *M. tuberculosis* is characterized by the production of a robust type I interferon response dependent on the activation of a cytosolic surveillance pathway by the recognition of *M. tuberculosis* DNA in the cytosol. The DNA sensor recognizing *M. tuberculosis* DNA and initiating activation of the cytosolic surveillance pathway has yet to be defined. Here we describe a role for the recently characterized DNA sensor cGAS in the detection of *M. tuberculosis* infection and initiation of the type I interferon response as well

as a role for cGAS in the targeting of *M. tuberculosis* to the autophagosome. We demonstrate that cGAS deficiency is associated with decreased survival in a mouse model of *M. tuberculosis* infection. The second part of this thesis explores how macrophages respond to *M. tuberculosis* infection. We previously showed that in mice *M. tuberculosis* infection induces the expression of the carbon monoxide producing enzyme heme oxygenase (HO1) in the macrophage and that the CO is sensed by *M. tuberculosis* to initiate a dormancy program. Mice deficient in HO1 succumb to *M. tuberculosis* infection more readily than wild-type (WT) mice. While the mechanisms used by mouse macrophages to control intracellular *M. tuberculosis* infection, including nitric oxide synthase, the respiratory burst, acidification and HO1 are well studied, how human macrophages control *M. tuberculosis* infection is less well understood. Here we show that HO1 is induced by and colocalizes with *M. tuberculosis* in both mouse and human tuberculosis lesions, and that *M. tuberculosis* induces and colocalizes with HO1 during human macrophage infection in vitro. HO1 enzymatic activity in human macrophages is necessary for inflammatory cytokine production and for control of intracellular *M. tuberculosis* replication. Finally, we find that a polymorphism in the HO1 promoter is associated with susceptibility to human tuberculosis. Thus, we demonstrate an important role for HO1 in controlling human tuberculosis.

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Prior Publications

Manzanillo PS, Ayres JS, Watson RO, **Collins AC**, Souza G, Rae CS, Schneider DS, Nakamura K, Shiloh MU, Cox JS. The ubiquitin ligase parkin mediates resistance to intracellular pathogens. Nature. 2013,501(7468): 512-516

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List of Definitions

1,25(OH)2D3 - 1,25-hydroxyvitamin D

AIM2 - Absent in Melanoma 2

APCs - Antigen presenting cells

ATG – Autophagy-related protein

BAL – Bronchioalveolar lavage

BMDM – Bone marrow derived macrophage

BSL3 – Biosafety level 3

CFU – Colony forming units

cGAMP - Cyclic GMP-AMP

cGAS - Cyclic GMP-AMP synthase

CMV – Cytomegalovirus

COX-2 - Cyclooxygenase type 2

Cpn60.1 - Chaperonin 60.1

CPN60.2 - Chaperonin 60.2

CSP – Cytosolic surveillance pathway

DC-SIGN - Dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin

DNA – Deoxyribonucleic acid

dsDNA – Double stranded deoxyribonucleic acid

DTH – Delayed type hypersensitivity

HIV – Human immunodeficiency virus

HO1 – Heme oxygenase 1

HSP65 - Heat shock protein 65

HSP70 - Heat shock protein 70

HSV-1 – Herpes Simplex Virus 1

ICAM-1 - Intracellular adhesion molecule-1

IFI16 - IFN-γ-inducible protein 16

IFN-α – Interferon alpha

IFN-β – Interferon beta

IFN-γ – Interferon gamma

IFNAR - IFN-α receptor

IL-1 – Interleukin-1

IL-10 – Interleukin-10

IL-18 – Interleukin-18

IL-1R – Interleukin-1 receptor

IL-1α - Interleukin-1α

IL-1β - Interleukin-1β

IL-23 – Interleukin-23

IL-6 – Interleukin-6

iNOS - Inducible nitric oxide synthase

IRAK - IL-1 receptor-associated kinases

IRF3 – Interferon regulatory transcription factor

IRGM1- IFNγ inducible immunity-related GTPase

ISGs – Interferon-stimulated genes

JAK/STAT - Janus kinase/signal transducer and activator of transcription

L. monocytogenes – Listeria monocytogenes

LAM – Lipoarabinomannan

LM - Lipomannan

LPS – Lipopolysaccharide

LTA4H - Leukotriene A4 hydrolase

LXA4 - Lipoxin A4

M. tuberculosis - Mycobacterium tuberculosis

MAP - mitogen-activated protein

MRC1 - Mannose receptor C type 1

MRC2 - Mannose receptor C type 2

MSMD - Mendelian Susceptibility to Mycobacterial Disease

MyD88- Myeloid differentiation primary response protein 88

NK cell – Natural killer cell

NLRP3 - (NOD)-like receptor P3

NLRs - (NOD)-like receptors

NO – Nitric oxide

NOD - Nucleotide oligomerization domain

NOD1 - Nucleotide oligomerization domain 1

NOD2 - Nucleotide oligomerization domain 2

NOS2 – Nitric oxide synthase 2

PAMPS - pathogen associated molecular patterns

PGE2 - Prostaglandin E2

PIM - Phosphatidylinositol mannoside

PPAR-ϒ - Peroxisome proliferator-activated receptor gamma

Ptges - Prostaglandin E synthase

RNA – Ribonucleic acid

RNI – Reactive nitrogen intermediates

S. pyogenes – Streptococcus pyogenes

SNP – Single nucleotide polymorphism

STING – Stimulator of interferon genes

TAK1 - TGFβ-activated protein kinase 1

TDM - Trehalose-6, 6-dimycolate

TLRs- Toll-like receptors

TNF – Tumor necrosis factor

Toll-like receptor 1 – TLR1

Toll-like receptor 2 – TLR2

Toll-like receptor 4 – TLR4

Toll-like receptor 6 – TLR6

Toll-like receptor 9 – TLR9

TRAF - TNF receptor-associated factor 6

TRIF - TIR-domain containing adapter-inducing interferon-β

WT – Wild-type

Chapter One: Introduction and Literature Review

Introduction

Mycobacterium tuberculosis (*M. tuberculosis*), the causative agent of human tuberculosis, has caused more deaths than any other bacterial pathogen in human history. Ten percent of those infected develop active disease known as primary-progressive disease[1], while the majority of infected individuals remain asymptomatic, containing but not eradicating the bacteria and developing latent disease. Of those individuals with latent disease, 5-10% will eventually progress to active disease at some point in their lifetime[2].

M. tuberculosis has persisted throughout the course of human history, with evidence of infection described in ancient Egyptian mummies, and reached epidemic levels in the 18th and 19th centuries[3]. In 1882, Robert Koch isolated and cultured *M. tuberculosis*, demonstrating for the first time that the bacterium was the cause of tuberculosis disease[4]. While efforts at eradication have led to a significant reduction in *M. tuberculosis* cases since its peak in the 1800s, *M. tuberculosis* remains a major public health concern. One third of the world's population is latently infected with *M. tuberculosis*, leading to 9 million new cases of tuberculosis every year and another 1.5 million deaths[5].

Recognition and elimination of infection by the immune system is complex, involving many different receptors, cytokines, chemokines, and effector molecules. The

objectives of this thesis are to look at both the recognition of and the response to *M. tuberculosis*. Specifically, the focus is on exploring the role of the recently described cytosolic dsDNA sensor cGAS as well as defining a role for the enzyme heme oxygenase 1 (HO1) in controlling human tuberculosis. Before delving in to either of these topics, it is important to review what is currently known about how cells sense *M. tuberculosis* and the resulting induction of protective cytokines and effector responses.

Pathology of *M. tuberculosis* infection

M. tuberculosis is spread through the inhalation of aerosolized droplets that are released in to the air when an infected individual coughs or sneezes. An infectious dose is thought to be between 1 and 200 bacilli. The bacteria are phagocytosed by resident alveolar macrophages that recognize specific pathogen associated molecular patterns (PAMPs). Following bacterial uptake, these macrophages release pro-inflammatory cytokines that recruit additional immune cells to the lung, including naïve monocytes, dendritic cells, and neutrophils. Dendritic cells that have taken up *M. tuberculosis* leave the lung and migrate to regional lymph nodes, where they drive induction of the adaptive immune response[1]. Initiation of an adaptive response leads to the formation of a granuloma surrounding the bacteria. This granuloma is composed of a central region of infected macrophages, many of which have further differentiated into multinuclear giant cells (formed via the fusion of the plasma membranes of multiple macrophages[6]), epitheloid cells, and foamy macrophages as well as neutrophils and monocytes[7]. An outer layer of B cells and T cells as well as fibroblasts that drive the development of a fibrotic capsule comprise the outer layer of the granuloma[8]. Cell lysis occurring in the center of the granuloma leads to the development of the classical caseous necrotic core seen in human tuberculosis lesions[9]. Dissemination to the regional lymph nodes via the lymphatic system or hematogenous dissemination to other organs of the body may also occur early in infection[1].

Innate recognition of *M. tuberculosis*

C-Type Lectin Receptors

C-type lectins are membrane bound or secreted calcium-dependent receptors that recognize carbohydrate containing molecules on the surface of pathogens[10]. In the immune response, they act both as cell adhesion molecules and pathogen recognition receptors[11]. Mannose receptor (MRs), DC-SIGN, Mincle, and Dectin-1 are C-type lectin receptors shown to recognize *M. tuberculosis*.

Mannose receptors

LAM and mannosylated LAM (ManLAM) are mycobacterial cell wall glycolipids recognized by mannose receptor C type 1 and 2 (MRC1 and MRC2)[12]. Phagocytosis of *M. tuberculosis* by human macrophages is mediated primarily through MRs[13], suggesting an important role for these receptors in initiating *M. tuberculosis* host cell entry. Engagement of MRs trigger the production of the anti inflammatory cytokines IL-10, IL-1R antagonist, and IL-1R type II, as well as inhibit the production of IL-12[14]. The nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-ϒ) is also activated which further inhibits the inflammatory response[15]. Additionally, MR interacting with *M. tuberculosis* inhibits phagosome-lysosome fusion in the macrophage, promoting *M. tuberculosis* infection[16]. While human polymorphisms in MRs have been associated with increased susceptibility to *M. tuberculosis* infection[17,18], MR-deficient mice control *M. tuberculosis* as well as WT mice and show similar lung pathology following aerosol infection[19].

DC-SIGN

Dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) is a type II transmembrane receptor found on the surface of both human dendritic cells and macrophages that recognizes Man-LAM on the mycobacterial surface[20]. Like MRs, DC-SIGN induces an anti-inflammatory response by inducing IL-10 production and blocking the maturation of infected dendritic cells. While there are eight genetic homologs of DC-SIGN found in mice, there is not a clear human ortholog, complicating the ability to study this protein's role in mouse models[21]. DC-SIGN promoter polymorphisms (336Aand 871G) have been associated with an increased risk of tuberculosis in South African populations[22,23]; however, no association between these promoter alterations and *M. tuberculosis* have been found in Tunisian[24], Colombian, or Chinese populations[25] indicating a need for larger population-specific studies.

Dectin-1

Dectin-1 is a type II transmembrane receptor found on the surface of macrophages, dendritic cells, and neutrophils. While the role of dectin-1 in the production of inflammatory cytokines in response to fungal β-glucans is well-characterized[26], recent studies suggest an additional role for dectin-1 in the recognition of *M. tuberculosis* in response to an as yet uncharacterized ligand. *M. tuberculosis* infection induces the expression of dectin-1 on airway epithelial cells in a TLR2-dependent manner[27], and TLR2-mediated TNF production in murine BMDM is dependent on dectin-1 following infection with avirulent *M. tuberculosis* but not virulent *M. tuberculosis*[28]. In splenic dendritic cells, dectin-1 signaling, independent of TLR2, is

important for production of IL-12p40 and IL-12p70[29]. Dectin-1 activation in monocyte-derived dendritic cells promotes a TH1/TH17 response and the production of IL-1, IL-23, TNF, and IL-6[30]; however, dectin-1$^{-/-}$ mice show no increase in susceptibility and no significant changes in inflammatory cytokine levels following *M. tuberculosis* infection when compared to WT mice, suggesting a possible redundant role in cytokine production[31].

Mincle

Mincle is expressed on the surface of macrophages, recognizes trehalose-6, 6-dimycolate (TDM, mycobacterial cord factor)[32], and is important for the production of the inflammatory cytokines and NO *in vitro*[32,33]; however, *in vivo*, Mincle-deficient mice develop a normal granulomatous response and exhibit no defect in controlling *M. tuberculosis* infection; suggesting other receptors compensate for Mincle deficiency *in vivo*[34].

Complement Receptor 3

Opsonization of *M. tuberculosis* by C3b and iC3b activates the alternative complement pathway and can lead to CR3-mediated phagocytic uptake of *M. tuberculosis*[35]. CR3 contains multiple binding sites that allow the protein to bind iC3b, intracellular adhesion molecule-1 (ICAM-1), and a variety of bacterial products including mycobacterial antigen 85C and mycobacterial oligosaccharides like LAM[36]. While CR3-deficient macrophages display decreased uptake of *M. tuberculosis*, there is no effect of

CR3 loss on the induction of anti-microbial effector mechanisms or bacterial survival *in vitro*[37]. Additionally, $CR3^{-/-}$ mice exhibit no defects in the control of *M. tuberculosis* infection[38], suggesting CR3 does not play a critical role in the immune response to the bacteria.

CD14

The ability of CD14 to bind LPS from gram-negative bacteria and lipoteichoic acids and peptidoglycan from gram-positive bacteria is well-described[39]; however, CD14 can also bind LAM[40] and chaperonin 60.1[41] to mediate uptake of *M. tuberculosis*[42]. $CD14^{-/-}$ mice do not exhibit differences in the protective response to *M. tuberculosis* up to 32 weeks post infection[43,44]; however, after 32 weeks, while no differences in bacterial numbers are observed, $CD14^{-/-}$ mice display decreased pulmonary inflammation and increased survival compared to WT control mice[44], suggesting a role for CD14 in the chronic inflammatory response seen during *M. tuberculosis* infection.

Toll-like receptors

Toll-like receptors (TLRs) recognize pathogen PAMPS. Following recognition of *M. tuberculosis* PAMPs by TLRs, the bacterium is not immediately taken up by the macrophage. Instead, this interaction initiates the induction of signaling pathways that recruit the adaptor protein myeloid differentiation primary response protein 88 (MyD88)[45]. MyD88 serves as a scaffold for the recruitment of IL-1 receptor-associated

kinases (IRAK), TNF receptor-associated factor 6 (TRAF), TGFβ-activated protein kinase 1 (TAK1), and mitogen-activated protein (MAP) kinase. This signaling complex activates multiple transcription factors such as (NF)-dB to translocate to the nucleus[46]. These transcription factors then drive the production of multiple pro-inflammatory cytokines such as TNF, IL-1β and IL-12 that stimulate IFN-γ production from NK cells and T cells. The NK cells and T cells drive macrophage activation, promote anti-mycobacterial effector mechanisms such as autophagy and the production of RNI, and inhibit bacterial growth[47].

Toll-like receptor 2 (TLR2)

TLR2 pairs with either TLR1 or TLR6 to form a heterodimer that recognizes multiple components of the *M. tuberculosis* cell wall including lipoarabinomannan (LAM), phosphatidylinositol mannoside (PIM), triacylated/diacylated lipoproteins, lipomannan (LM), 38-kDa mycobacterial glycoprotein, and 19-kDa mycobacterial glycoprotein[48-50]. *In vitro,* the production of IL-1β[51] and TNF-α[45] as well as IL-12 release from macrophages[52] in response to *M. tuberculosis* are dependent on TLR2-mediated signaling. While no differences in survival are seen, defective granuloma formation and increased bacterial numbers are observed in $TLR2^{-/-}$ mice[53] following a low dose *M. tuberculosis* infection. When a high dose infection model is used, $TLR2^{-/-}$ animals also show decreased survival compared to WT animals[43,53].

In humans, the TLR2 G2258A polymorphism causes impaired TLR2 tyrosine phosphorylation[54] and is associated with increased susceptibility to *M. tuberculosis*

infection in European and Asian populations[55-58]. However, this polymorphism has not been associated with increased susceptibility in other ethnic groups[58-60].

Toll-like receptor 4 (TLR4)

As described above, TLR4 can utilize the adaptor protein MyD88 to activate NF-kB-dependent expression of the classical pro-inflammatory cytokines TNF, IL-6 and IL-12. However, stimulation of TLR4 can also activate the MyD88-independent TIR-domain containing adapter-inducing interferon-β (TRIF) pathway resulting in responses distinct from those induced by activation of MyD88. In this pathway, TRIF up regulates IRF3 and IRF7, leading to IFN-β secretion and upregulation of interferon-stimulated genes (ISGs)[61].

Some strains of *M. tuberculosis* have been shown to activate mainly TLR2, while others also activate TLR4, resulting in different cytokine profiles characterized by different levels of type 1 interferon production and different levels of bacterial virulence[62]. *M. tuberculosis* heat shock protein 65 (HSP65), chaperonin 60.1 (Cpn60.1), and *M. tuberculosis* 50S ribosomal protein Rv0652 have been shown to mainly signal through TLR4, while heat shock protein 70 (HSP70), chaperonin 60.2 (CPN60.2), and the 38-kDA glycoprotein of *M. tuberculosis* H37Rv activates both TLR2 and TLR4-mediated signaling[63-66]. TLR4-deficient macrophages show decreased TNF production following *M. tuberculosis* infection. However, *in vivo* studies have yielded conflicting results on the importance of TLR4 in controlling *M. tuberculosis* infection. Some studies have found TLR4-deficient mice are not more susceptible to infection than wild type animals[43,67], while others have shown $TLR4^{-/-}$ animals to have decreased survival and

higher bacterial numbers compared to wild type controls[68]. While no current studies have identified polymorphisms in TLR4 associated with *M. tuberculosis* susceptibility in HIV negative individuals, a TLR4 SNP has been identified that is associated with increased susceptibility to *M. tuberculosis* in HIV-infected individuals[69].

Toll-like receptor 9 (TLR9)

TLR9 binds bacterial un-methylated CpG DNA to induce the production of pro-inflammatory cytokines[70]. TLR9 activation is important for the production of IL-12 and IFN-γ, as $TLR9^{-/-}$ mouse macrophages show defective production of both cytokines compared to wild type cells following *M. tuberculosis* infection. However, *in vivo* studies show that TLR9 knockout animals display only minor reductions in their ability to control *M. tuberculosis* infection. It is worth noting that TLR2/9 double knockout animals display significantly greater susceptibility to *M. tuberculosis* then mice singly deficient in either TLR, suggesting that TLR2 and TLR9 may cooperate for increased control of *M. tuberculosis* infection[71]. Multiple studies have found an association between TLR9 polymorphisms and susceptibility to *M. tuberculosis*[72-74].

A controversial role for TLRs in the immune response to M. tuberculosis

M. tuberculosis has been found to activate signaling through TLR2, TLR4, and TLR9. However an absolute requirement for TLR-mediated signaling in the initiation of an effective response to *M. tuberculosis* remains controversial. As described above, studies in knockout animals have yielded conflicting results. Further complicating matters, TLR2/4/9 triple knockout mice respond normally to *M. tuberculosis*, exhibiting

no defects in cytokine production, bacterial numbers, or survival[75]. This suggests TLR activation may not always be required for an effective response to *M. tuberculosis,* as it is possible additional alternative pathways exist that may compensate for the lack of TLR2/4/9 signaling in these mice[75,76]. How to reconcile the lack of phenotype in TLR2/4/9 triple knockout mice with previously described work suggesting an important role for TLR-mediated signaling in the response to *M. tuberculosis* has been difficult. Possible explanations include differences in the route or dose of infection, differences in the strains of *M. tuberculosis* used for infection and differences in the genetic backgrounds of the knockout mice[76].

Studies showing MyD88 to be a vital component of the innate response to *M. tuberculosis* has been used as evidence that TLR signaling is required in the response to *M. tuberculosis*. *M. tuberculosis*-infected MyD88$^{-/-}$ macrophages show decreased TNF, IL-12, and NO production[77], and MyD88$^{-/-}$ mice show impaired IL-12, TNF, and Th1 cytokine production as well as impaired iNOS expression and a decreased ability to control *M. tuberculosis*[77,78]. However, MyD88 is not just involved in TLR signaling pathways, but also plays an important role in signaling through the IL-1 receptor. Defects seen in MyD88$^{-/-}$ mice are due to defects in macrophage effector responses dependent on IL-1 signaling pathways[75].Much more work is still needed to definitively characterize the role of TLRs in the immune response to *M. tuberculosis*.

NOD Like Receptors

Nucleotide oligomerization domain (NOD)-like receptors (NLRs) are a cytoplasmic family of over 20 different pattern recognition receptors. NOD1 and NOD2 recognize bacterial cell wall components, leading to the NF-κB-dependent production of pro-inflammatory cytokines[79]. NOD2 recognizes the *M. tuberculosis* cell wall component mycolyarabinogalactan-peptidoglycan. *In vitro*, macrophages from NOD2-deficient mice produce decreased levels of TNF, IL-12p40, RANTES, and NO following *M. tuberculosis* infection, although no defects in control of *M. tuberculosis* replication are seen[80]. Studies in NOD2-deficient mice show no difference in bacterial numbers in the first few months after infection[80,81]. However, $NOD2^{-/-}$ mice have higher bacterial numbers six months post infection, reduced activation of antigen specific T cells, and they succumb to infection sooner then WT mice[81]. While no differences in IL-12p40 or iNOS expression are seen in NOD2-deficient animals, production of IFN-γ and TNF levels in BAL are significantly reduced[80]. Differences in lung pathology have not been consistently reported, although this may be partially due to differences in infection routes (intranasal versus aerosol)[80,81].

Knockdown of NOD2 in human monocyte derived macrophages leads to decreased levels of TNF and IL-1β following infection. While NOD2 has not been shown to be critical for control of *M. tuberculosis* in mouse macrophages *in vitro,* NOD2 silencing in human macrophages leads to increased bacterial growth[82]. Additionally, treatment with muramyldipeptide to increase levels of NOD2 activation, improves the

ability of human alveolar macrophages to control intracellular growth of *M. tuberculosis*[83].

Three non-synonymous polymorphisms in an African-American cohort have been associated with susceptibility to *M. tuberculosis*. Two of those polymorphisms may stabilize the structure of NOD2 and increase the strength of the interaction between NOD2 and *M. tuberculosis.* Both of these polymorphisms have been associated with decreased susceptibility to disease. A third is thought to correlate with defective NOD2 activity and is associated with increased susceptibility to disease[84]. Studies in a Chinese population have identified an additional NOD2 polymorphism associated with an increased risk of *M. tuberculosis*, although this polymorphism was not associated with an increased risk of disease in Uyghur or Kazak populations[85,86].

NLRP3 and AIM2

Activation of the cytoplasmic inflammasome cleaves inactive caspase-1 into an active form that then converts pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18[87]. Members of the NLR protein family can mediate inflammasome activation and NLRP3-mediated inflammasome activation has been shown to occur following *M. tuberculosis* infection[88]. Caspase-1 activation and production of IL-1β in both human and mouse macrophages is dependent on the NLRP3 *in vitro*[89], and human macrophages from humans with a gain of function mutation in NLRP3 are better able to control *M. tuberculosis* growth[90]. However, NLRP3$^{-/-}$ mice show no differences in IL-1β production, bacterial burden, or survival following *M. tuberculosis* infection compared to WT mice[89].

It remains unclear the ligand for NLRP3, and in fact, there may not be a ligand that binds directly to it. Recent work has shown that the DExD/H-box RNA helicase family member DHX33 senses viral and bacterial cytosolic RNA and then binds to and activates NLRP3[91]. Whether DHX33 is involved in the sensing of a *M. tuberculosis* ligand has yet to be determined.

Binding of the cytosolic protein Absent in Melanoma 2 (AIM2) to dsDNA can also lead to activation of the inflammasome and production of IL-1β and IL-18[92,93]. AIM2$^{-/-}$ mouse macrophages show defective IL-1β and IL-18 production and inflammasome activation. Additionally, *M. tuberculosis* DNA co-localizes with AIM2 in infected cells[94]. This conflicts with the results obtained by Shah et al. demonstrating that activation of the AIM2-inflammasome pathway is only seen following infection with avirulent *M. tuberculosis*. They report that no activation is seen when virulent strains are used due to *M. tuberculosis*-mediated inhibition of the pathway[95]. This suggests the possibility of strain specific differences between studies that needs to be further examined. However, *in vivo* work suggests a role for AIM2-inflammasome activation in the response to *M. tuberculosis*. AIM2$^{-/-}$ mice have impaired IL-1β and IL-18 production, increased bacterial burden, abnormal granuloma formation, and decreased survival compared to WT mice following infection[94].

The role of cytokines in *M. tuberculosis* infection

Interferon alpha (IFN-α)/ Interferon beta (IFN-β)

M. tuberculosis interaction with cytosolic receptors triggers the production of the type I interferons IFN-α and IFN-β. Binding of IFN-α or IFN-β to the IFN-α receptor (IFNAR) activates signaling through the JAK1-STAT pathway; leading to the production of a large number of interferon-stimulated genes (ISGs)[96].

The production of type I interferon is detrimental in the response to *M. tuberculosis* infection. IFNAR$^{-/-}$ mice as well as mice treated with an IFNαβ blocking antibody are more resistant to *M. tuberculosis* infection, with reduced bacterial burdens and increased survival compared to control mice[97,98]. *M. tuberculosis* strains that induce increased levels of type I interferon are more virulent and suppress the induction of other pro-inflammatory cytokines[62]. Further supporting a detrimental role for type I interferon, mice given exogenous IFN-α/β directly to the lung or treated with the TLR3 ligand polyinisinic-polycytidylic acid show decreased survival, increased lung pathology, and increased bacterial numbers compared to untreated *M. tuberculosis*-infected animals[98,99]. Additionally, IRF-3 knockout mice, which do not produce type I interferon following *M. tuberculosis* infection, are resistant to *M. tuberculosis* infection[100]

In human patients with active TB, when gene expression in un-stimulated whole blood leukocytes is examined, the most prominent transcripts are genes regulated by type 1 interferons and levels of these transcripts correlate with the extent of radiographic disease[101]. Additionally, hepatitis patients undergoing treatment with IFN-α treatment

have been observed to develop reactivation of latent disease after beginning treatment[102,103].

Interferon gamma (IFN-γ)

IFN-γ is a type II interferon produced by activated T cells, NK cells, and macrophages. IFN-γ production is controlled by IL-12 and IL-18 secreted by APCs following *M. tuberculosis* infection[104]. IFN-γ interacts with the IFN-γ receptor to initiate JAK-STAT signaling and upregulation of a large number of interferon responsive genes[105]. This leads to increased apoptosis, cell proliferation and adhesion, as well as activation of macrophage antibacterial mechanisms such as the production of reactive nitrogen and reactive oxygen species[106].

IFN-γ production is essential for control of *M. tuberculosis* infection. Murine macrophages pretreated with IFN-γ demonstrate increased resistance to infection and this protection is mediated by increased production of reactive RNI[107]. IFN-γ also enhances phagosome maturation[108] and activates autophagy[109] to limit *M. tuberculosis* infection. Mice defective in IFN-γ production are unable to produce RNIs, show increased bacterial burdens with widespread tissue necrosis, and succumb to infection much sooner then control mice[110,111].

In humans, defects in the IFN-γ receptor or the downstream signaling pathway are associated with increased susceptibility to mycobacterial infection[112], and treatment with exogenous IFN-γ leads to clinical improvement[113]. Inability of AIDS patients to

produce sufficient levels of IFN-γ is thought to contribute to their increased susceptibility to *M. tuberculosis* infection[114].

Interleukin-1α (IL-1α)/Interleukin-1β (IL-1β)

M. tuberculosis activation of the inflammasome induces the production IL-1α and IL-1β[115]; members of the IL-1 superfamily of cytokines that have long been known to play an important role in the host response to *M. tuberculosis*. IL-1α and IL-1β binding to the IL-1 receptor promotes inflammation via the MyD88 and NF-κB-dependent induction of multiple genes including cyclooxygenase type 2 (COX-2), inducible nitric oxide synthase (iNOS), and type 2 phospholipase. Induction of these genes leads to the production of prostaglandin-E2, NO, and platelet activating factor[116].

IL-1 receptor knockout mice are more susceptible to *M. tuberculosis* infection, with increased mortality and increased bacterial burden. Defective granulomas containing decreased numbers of macrophages and lymphocytes and increased numbers of granulocytes are also seen in knockout animals[117,118]. *IL-1α/IL-1β* double knockout mice display a decreased ability to control bacterial growth, abnormal granuloma formation, and impaired NO production following infection[119]. Additionally, despite binding the same receptor, mice singly deficient in IL-1α or IL-1β display increased mortality and greater bacterial loads following *M. tuberculosis*, suggesting non-redundant roles for the two cytokines in response to infection[120,121].

In humans, multiple studies have demonstrated polymorphisms in IL-1 or the IL-1 receptor are associated with increased susceptibility to *M. tuberculosis*[121-123].

Tumor necrosis factor (TNF)

TNF is produced by macrophages, dendritic cells, neutrophils, and T cells following *M. tuberculosis* infection[124]. Ligation of the TNF receptor results in NF-κB-mediated production of multiple pro-inflammatory products. Additional pro-apoptotic JNK-dependent and caspase-dependent pathways are also activated following ligation of the TNF receptor[125]. During *M. tuberculosis* infection, TNF has been shown to promote the production of inflammatory chemokines and act with IFN-γ to activate macrophages and promote apoptosis of infected cells. Additionally, it is plays an important role in granuloma formation in mice and in long-term maintenance of the granuloma in both mice and non-human primates[126].

$TNF^{-/-}$ mice display increased bacterial loads and rapidly succumb to infection following widespread *M. tuberculosis* dissemination. Lungs from $TNF^{-/-}$ mice do not contain localized granulomas, but rather contain large areas of cellular infiltrates characterized by high levels of neutrophil infiltration and necrosis (although no reduction of RNI species are seen)[127].

Mice with a disruption in the TNF receptor or mice treated with anti-TNF neutralizing antibodies form similar numbers of granulomas compared to control mice, however significantly higher levels of necrosis are seen compared to control mice while

antibody treated and TNF receptor-deficient mice show increased bacterial burdens and rapidly succumb to *M. tuberculosis* infection significantly sooner then control mice[128,129]

TNF is also thought to be important in the maintenance of a latent disease state. Mice infected using a low dose infection model of TB meant to model persistent TB infection display fatal reactivation of disease characterized by granuloma disorganization, increased bacterial burden, and reduced iNOS expression following TNF neutralization[128,129].

Neutralization of TNF prior to infection in a cynomolgus macaque model leads to the rapid development of fulminant and disseminated disease. While TNF neutralized animals are able to form normal granulomas, they develop significantly increased invasive pathology. They show higher bacterial numbers and increased dissemination when compared to control animals, suggesting impairment in granuloma function and ability to contain *M. tuberculosis* spread. Additionally, TNF neutralization in latently infected animals leads to reactivation and dissemination of disease. TNF neutralized animals show normal granuloma formation but have altered chemokine receptor expression, decreased CCL4, reduced *M. tuberculosis* specific IFN-γ production in the lungs, and increased IL-12 levels compared to actively infected control animals. This suggests that disregulation of cytokines and chemokines following TNF neutralization may be responsible for disease dissemination[130].

An important role for TNF in controlling chronic infection is further supported in humans by the increased incidence of reactivation of latent TB disease in patients receiving anti-TNF therapy to treat rheumatoid arthritis or Crohn's disease[131].

Interleukin-6 (IL-6)

IL-6 is produced by macrophages, B and T cells, fibroblasts, and endothelial cells[132]. Binding of IL-6 to its receptor activates the JAK/STAT pathway resulting in the production of pro-inflammatory cytokines.

IL-6 is produced during the early stages of *M. tuberculosis* infection. Mice deficient in IL-6 display a delayed IFN-γ response and slightly increased bacterial numbers following a low dose infection with *M. tuberculosis*, but no differences in survival compared to control mice are observed[133]. However, following a high dose infection, IL-6-deficient mice are unable to control infection and succumb sooner than control mice[134].

Interleukin-12 (IL-12)

IL-12 is produced primarily by dendritic cells in response to TLR9-mediated recognition of *M. tuberculosis*. Macrophages produce significantly lower levels dependent on recognition of *M. tuberculosis* by TLR2[52]. IL-12 is found as a heterodimer (known as IL-12p70) composed of IL-12A (p35) and IL-12B (p40). The IL-12 receptor is composed of IL-12Rβ1 and IL-12Rβ2, which bind the IL-12p40 and IL-12p35 subunits respectively. Binding initiates a JAK/STAT-dependent signaling pathway leading to the production of IFN-γ, the proliferation of macrophages, NK cells, and T cells, and the differentiation of CD4+ T cells to type 1 T helper (Th1) cells[135].

IL-12 production is important both in the initial response to *M. tuberculosis*, and the control of chronic infection. IL-12p40$^{-/-}$ mice cannot control bacterial growth and succumb to infection much earlier than WT mice. They show significantly reduced levels of IFN-γ and iNOS, as well as delayed induction of TNF following infection. Knockout mice also show defective granuloma formation characterized by decreased lymphocyte numbers and highly vacuolated macrophages and are unable to mount a DTH response against mycobacterial antigens[136].

IL-12p40$^{-/-}$ mice given exogenous IL-12 for 4 weeks show no differences in control of *M. tuberculosis* infection compared to WT mice. However, once exogenous IL-12 delivery is discontinued, the IL-12p40$^{-/-}$ mice lose the ability to control infection. They show a reduction in *M. tuberculosis* specific IFN-γ producing CD4+ cells, a decrease in total CD4+ cells, breakdown of granulomas, increased bacterial numbers, and they succumb to infection significantly earlier then control mice[137].

While both IL-12p35$^{-/-}$ mice and IL-12p40$^{-/-}$ mice are defective in their ability to control *M. tuberculosis*, IL-12p40$^{-/-}$ mice show greater numbers of bacteria, fewer *M. tuberculosis* specific IFN-γ producing T cells, and greater mortality than IL-12p35$^{-/-}$ mice[138]. This increased susceptibility to infection in IL-12p40 $^{-/-}$ mice may be explained by a recently described additional role of the p40 subunit in DC migration from the lung to the draining lymph node and subsequent T cell priming independent of p35[139].

Because IL-12p70 shares a common p40 subunit with IL-23 (a heterodimer of IL-23p19 and IL-12p40) and signals through a common IL-12Rβ1 chain, IL-23p19 may be able to partially compensate for IL-12p35 deficiency[140]. While IL-23p19$^{-/-}$ mice have

lower levels of IL-17 and fewer IL-17 producing CD4+ T cells, they are effectively able to control *M. tuberculosis* infection and do not show differences in IFN- γ or IFN-γ producing CD4+ T cells compared to WT mice[141]. Although IL-23 cannot fully compensate for a lack of IL-12p70, its ability to partially compensate may explain why IL-12p40$^{-/-}$ are more susceptible to *M. tuberculosis* than IL-12p35$^{-/-}$ mice.

In humans, Mendelian Susceptibility to Mycobacterial Disease (MSMD) is a rare syndrome associate with mutations in the IL-12/IFN-γ axis. Homozygous recessive mutations in IL-12Rβ1 are the most common cause of MSMD. These mutations prevent expression of IL-12Rβ1 on the cell surface leading to an inability of T cells and NK cells to response to IL-12 and IL-23. Patients are extremely susceptible to *M. tuberculosis* infection and develop severe forms of primary disease, therefore mutations IL-12Rβ1 should be considered in children presenting with unusually severe cases of tuberculosis[141-143].

Effector mechanisms in control of *M. tuberculosis*

Reactive nitrogen intermediates (RNI)

While the production of RNI has been shown to be an important mechanism used by mouse macrophages to control *M. tuberculosis*, the role of RNI in human macrophages remains controversial.

In vitro, administration of IFN-γ and TNFα induce the expression of iNOS in mouse macrophages, leading to the production of RNI that are necessary to kill *M. tuberculosis*[107]. Activation of TLR2 by bacterial lipoproteins also leads to increased bacterial killing that is dependent on the production of RNI[50]. $NOS2^{-/-}$ mice show higher CFU values, increased necrosis in the lungs, and succumb to *M. tuberculosis* infection significantly sooner then WT or heterozygous littermates[144]. Similar results are seen when mice are treated with various chemical inhibitors to inhibit RNI production[144,145]. In multiple mouse models of latent tuberculosis, administration of an iNOS inhibitor results in reactivation of latent disease[146].

In primary human monocytes and macrophages as well as alveolar macrophages, administration of IFN-γ and TNF has no antimicrobial effect and does not lead to the production of RNI. TLR2 activation by bacterial lipoproteins does lead to increased bacterial killing, however, no significant production of RNI is observed, indicating killing is independent of the production of RNI[50]. However, in human macrophage-like cell lines, the addition of vitamin D results in upregulation of iNOS and the production of RNI, leading to increased bacterial killing that is abolished if iNOS is

inhibited[147]. Additionally, infection of human peripheral blood-derived monocytes or the U937 human macrophage like cell line leads to upregulation of iNOS and the production of RNI. The effect of iNOS inhibition is variable, with only some clinical isolates of *M. tuberculosis* showing growth enhancement following iNOS inhibition[148].

Studies of TB-infected individuals have found that NO levels in the exhaled air of patients with active disease are increased compared to uninfected individuals and upregulation of iNOS expression in both alveolar macrophages and peripheral monocytes from patients with active disease has been observed[149-151]. Additionally, polymorphisms in the NOS2A gene associated with decreased production of RNI, are associated with increased susceptibility to *M. tuberculosis* infections in populations from Brazil, Northwestern Columbia, and South Africa[152-155]. Additional work has shown the association of ten different polymorphisms with susceptibility to *M. tuberculosis* infection in African-Americans, although no associations are seen in a Caucasian population, suggesting additional population specific genetic variants that may act synergistically to increase susceptibility to infection[155,156]

Vitamin D

The active for of vitamin D, 1,25-hydroxyvitamin D (1,25(OH)2D3), has been found to play an important role in controlling *M. tuberculosis*. Human macrophages treated with 1,25(OH)2D3 up-regulate the nuclear vitamin D receptor, which then induces expression of cathelicidin LL-37, an antimicrobial peptide capable if directly killing *M. tuberculosis*[157]. TLR2 ligands can induce production of cathelicidin LL-37 in

human macrophages, however, production is dependent on sufficient levels of 1,25(OH)2D3[158]. 1,25(OH)2D3 also induces autophagy via cathelicidin LL-37 induction of Beclin-1 and Atg5 with cathelicidin LL-37 being directly recruited to phagosomes[159].

Studies in human patients further supports the important role 1,25(OH)2D3 plays in the control of *M. tuberculosis*. Patients with active TB have lower levels of 1,25(OH)2D3 compared to uninfected individuals[160,161], and 1,25(OH)2D3 deficiency is associated with an increased risk of active TB in multiple populations[162-167]. Additionally, polymorphisms in the vitamin D receptor as well as vitamin D binding protein that may decrease the downstream effects of 1,25(OH)2D3 signaling are associated with an increased risk of *M. tuberculosis* infection[162,164,167,168]

Before the development of antibiotic therapies for *M. tuberculosis*, patients were treated with vitamin D and sunlight exposure[169]. Clinical trials examining the addition of 1,25(OH)2D3 to *M. tuberculosis* treatment protocols have yielded mixed results with some studies finding 1,25(OH)2D3 improves clinical outcomes[170-172], and others finding no clinical benefit to 1,25(OH)2D3 administration[173,174]. It is important to note that these differences may be due to differences in treatment protocols between studies.

Apoptosis

Cells infected with *M. tuberculosis* may undergo apoptosis or necrosis following infection. While necrosis allows exit of the bacteria from macrophages and cell to cell spread, apoptosis maintains an intact cell membrane and has been associated with

increased control of infection[175]. Following infection of human alveolar cells, attenuated strains of *M. tuberculosis* show decreased viability as the macrophages undergo apoptosis[176], and apoptosis is induced in uninfected bystander cells as another possible mechanism used to control bacterial spread[177]. Additionally, infected macrophages are taken up by uninfected neighboring cells, a process known as efferocytosis[178]. Virulent strains of *M. tuberculosis* induce much lower levels of apoptosis following infection and do not display the same reduced viability as the avirulent strains, suggesting that apoptosis is an important effector mechanism used to control *M. tuberculosis*[176].

Eicosanoids, prostaglandin E2 (PGE2), and lipoxin A4 (LXA4) are all lipid mediators regulating if cells undergo apoptosis or necrosis following *M. tuberculosis* infection. PGE2 is pro-apoptotic while LXA4 is pro-necrotic. Virulent strains of *M. tuberculosis* have been shown to induce LXA4 and inhibit PGE2, leading to macrophage necrosis instead of apoptosis and thus resulting in increased bacterial spread [175,179-181]. Macrophages from 5-lipoxygenase knockout ($Alox5^{-/-}$) mice are unable to produce LXA4 and show increased apoptosis and reduced bacterial burden[179], while macrophages from prostaglandin E synthase knockout ($Ptges^{-/-}$) mice that are unable to synthesize PGE2, show increased levels of necrosis and higher bacterial numbers following *M. tuberculosis* infection[180].

$Alox5^{-/-}$ mice show increased survival, decreased bacterial numbers, decreased lung pathology with little evidence of necrosis, and increased levels of IL-12, IFN-γ, NOS2, and TNF compared to WT mice. This difference was lost if the $Alox5^{-/-}$ were

treated with a stable LXA4 analog[179]. Ptges$^{-/-}$ mice were found to have the opposite phenotype, with increased bacterial numbers compared to WT mice[180].

The importance of apoptosis in controlling infection is further supported by observations in human patients that polymorphisms in leukotriene A4 hydrolase (LTA4H) or Alox5 that lead to increased levels of LXA4 are associated with increased susceptibility to *M. tuberculosis* infection[182-184]

Autophagy

Autophagy, the cellular process triggered by starvation and other cellular stresses in which double-membranes vesicles called autophagosomes engulf and degrade damaged organelles and cellular proteins, also serves as an important antibacterial mechanism[185]. Greater than 30 autophagy-related proteins (ATG) organize in to complexes that control the steps of autophagy, starting with initiation and ending with fusion of the autophagosome with the lysosome[186]. Targets of autophagy are directed to the autophagosome through protein tags like polyubiquitin, adaptor proteins that recognize these molecular tags such as p62 and those involved in autophagosome membrane formation such as LC3[187]. In the case of *M. tuberculosis,* bacteria in the autophagosome are destroyed by lysosomal enzymes following fusion with the lysosome.

Many inflammatory cytokines and antimicrobial proteins induced during *M. tuberculosis* infection have been found to induce autophagy. IFN-γ has been shown to induce autophagy in human and mouse macrophages and IFN-γ-mediated autophagy

induction suppresses *M. tuberculosis* survival *in vitro*[188,189] and increases translocation of LC3 and formation of autophagosomes following infection[109]. Mice deficient in the downstream gene autophagy related gene IFN-γ inducible immunity-related GTPase (IRGM1) do not form mature autophagosomes around *M. tuberculosis* and can not control *M. tuberculosis* replication[108,190]. In humans, a polymorphism in IGRM1 is associated with protection against infection[191]. TNF also is thought to induce autophagy as the cytokine leads to increased autophagosomal maturation and TNF blockers inhibit this maturation in human macrophages[192,193].

Autophagy-mediated destruction of *M. tuberculosis* is dependent on the bacteria being tagged with ubiquitin chains that are recognized by autophagy adaptor proteins[194]. Recent work has identified the E3 ligase Parkin as pivotal in autophagy-mediated control of *M. tuberculosis.* Macrophages deficient in Parkin have decreased numbers of ubiquitin-positive *M. tuberculosis*, decreased recruitment of autophagy adaptor proteins, and increased bacterial replication and viability. Parkin-deficient mice show increased susceptibility to *M. tuberculosis* infection and human polymorphisms in the Parkin gene are associated with increased susceptibility to closely related *Mycobacterium leprae*[195].

Animal models of *M. tuberculosis* infection

Mice

Mice are the most common animal model used to study *M. tuberculosis* and they have been invaluable in furthering an understanding of the host-pathogen relationship.

The most physiologically relevant mechanism for infection in the mouse is a low dose aerosol infection delivering ~100-200 CFU per animal. Logarithmic bacterial growth occurs for the first 2-4 weeks after infection until a plateau of approximately 10^6 bacteria in the lungs is reached following initiation of the adaptive immune response. Bacterial growth is never suppressed enough for the development of latent disease. Instead the plateau is maintained and the mice enter a chronic disease state for at least 6 months until they eventually die of progressive infection[9]. The logarithmic bacterial growth seen during the first month of infection coincides with a rapid recruitment of inflammatory cells to the lung. As bacterial levels stabilize, the number of inflammatory cells continue to increase until at least 6 months after infection[196].

The mouse model of *M. tuberculosis* does have several shortcomings. Mice are more resistant to *M. tuberculosis* infection then other animals used to study infection. They can have high bacterial numbers in the lung before they show clinical signs of infection, and because they do not develop cavitary lesions or cough following infection, transmission studies are not possible[197].

Mice do not develop the same lung pathology seen in human infection. In humans, cell lysis within the granuloma leads to a hypoxic environment and the appearance of a necrotic caseous core[9]. *M. tuberculosis*-infected mice develop dense noncaseating "granulomas" consisting of large numbers of epitheloid and foamy macrophages, multinucleated giant cells, plasma cells, and lymphocytes. Formation of a fibrous capsule is not seen. Often the infected macrophages are found in the periphery, while the lymphocytes and plasma cells are found in the center of the granuloma. This cellular organization may allow more efficient interaction between infected macrophages and IFN-γ producing T cells, resulting in lower levels of necrosis then is seen in human infection[198].

Despite observed differences following infection, the mouse has been an extremely important model in the study of *M. tuberculosis*. Many parallels in the detection and response to *M. tuberculosis* have been observed and will be discussed in more detail below. The availability of a large number of genetic knockout strains of mice has proven vital in elucidating the role of many different cytokines, cell surface receptors, and immune cells. The existence of a large number of methods to study infection as well as the relative affordability of mice and the fact that they are much easier to maintain in BSL3 facilities is why the mouse continues to be the most popular animal model used in the field[199].

Guinea Pigs

The guinea pig model is thought to more closely model the response seen in humans following infection. Guinea pigs develop a strong DTH response following infection and form necrotic caseating granulomas that replicate much of the pathology of human granulomas[200]. However cavitation is rarely seen[201]. They are very susceptible to aerosol inoculation, with only a few inhaled bacteria leading to infection, making them a good model for transmission studies[202].

However, because guinea pugs develop chronic progressive disease that is primarily hematogenously disseminated, they are not a useful model to study latent infection[203]. Additionally, the limited number of reagents and genetic strains available has limited their usefulness in the study of *M. tuberculosis* infection.

Non-human primates

Nonhuman primates best replicate the human immune response to *M. tuberculosis* as they develop similar lung pathology to that seen in infected human patients. Following infection, some animals will develop active disease, while others become latently infected. Of those latently infected, only some will progress to reactivation of disease[204]. Despite the large amount of valuable information that can be obtained; the expense, as well as the lack of inbred strains and the ability of the animals to horizontally transmit disease limit the widespread use of this model[1].

Aims of this study

The broad goal of this thesis is to explore how the immune system is able to recognize and respond to *M. tuberculosis* infection. Two areas of interest were chosen; one focused on one how macrophages respond to infection and another delving in to how they sense initial infection.

The first aim of this thesis involves exploring a role for the described DNA sensor cGAS in *M. tuberculosis* infection. Only described a few years ago, mouse studies will allow an initial understanding of the role of cGAS in sensing *M. tuberculosis.* The second chapter of this thesis will explore work with cGAS-deficient mice and the characterization of their phenotype in response to *M. tuberculosis* infection.

The second aim of this thesis examines the role of human heme oxygenase 1 (HO1) as a potential anti-microbial mechanism induced by *M. tuberculosis* infection during human infection. As discussed in this introduction, mouse models are invaluable in the study of *M. tuberculosis.* Much of what is known about the human response to *M. tuberculosis* was built off of initial studies in mice. However, because there are differences in the response to infection between the species, it is important to invest in translational work to transition what is learned in mouse models to the study of human disease. The third chapter of this thesis focuses on characterizing the role of HO1 in human *M. tuberculosis* infection.

Chapter Two: A Role for cGAS in the Immune Response to *M. tuberculosis*

Introduction

Infection with *M. tuberculosis* is characterized by the production of a robust type I interferon response shortly after infection in both mice and humans[205]. While TLRs and NLRs are important mediators of type I interferon production in many types of infections, *M. tuberculosis* induction of IFNα/IFNβ is dependent on the cytosolic surveillance pathway (CSP) activated by cytosolic bacterial products[206]. *M. tuberculosis* activates the CSP by forming pores in the phagosome and allowing extracellular mycobacterial DNA to enter to cytosol[100], ultimately resulting in the IRF-3-dependent production of type I interferons and up-regulation of a large number of interferon-stimulated genes (ISGs)[207].

Activation of this pathway is mediated through STING, a cytosolic adaptor protein that activates the protein kinase TBK1 to phosphorylate IRF3[208]. Phosphorylated IRF3 translocates to the nucleus where it drives type 1 interferon production. However, mouse models suggest that in *M. tuberculosis* infection, this response is detrimental[99]. IRF3-/- knockout mice that are unable to produce type I interferon in response to cytosolic DNA are resistant to long-term infection with *M. tuberculosis*, surviving much longer then WT control mice[100]. Infection with another intracellular bacteria, *Listeria monocytogenes,* demonstrates that while beneficial in the antiviral response, type I interferons reduce the ability of macrophages to control intracellular bacterial infections. *L. monocytogenes* produce cyclic dinucleotides that can directly activate STING and the

CSP, further increasing the magnitude of the type I response and production of these cyclic dinucleotides is important for bacterial survival[209]. Mice lacking the type I interferon receptor are more resistant to infection with *L. monocytogenes*[210] and the production of IFNα suppresses the beneficial IFNγ response, increasing susceptibility to *L. monocytogenes*[211]. While *M. tuberculosis* does not produce cyclic dinucleotides that activate type I interferon production, release of bacterial DNA into the cytoplasm activates the CSP and is important for bacterial virulence[100]

The adaptor protein STING is vital in activation of the CSP as STING$^{-/-}$ mouse macrophages do not activate IRF3 translocation and type I interferon production is inhibited[100]. STING also has an additional important role in the proper induction of ubiquitin-mediated autophagy following *M. tuberculosis* infection. In response to *M. tuberculosis* infection, STING-deficient BMDMs display defective localization of ubiquitin and LC3 to the autophagosome, lysosomal targeting of bacteria is reduced, and bacterial survival increases[212]. Both STING-mediated activation of IRF3 as well as STING-dependent targeting of *M. tuberculosis* to autophagosomes require the presence of *M. tuberculosis* DNA in the cytosol[100,212]. However, while STING is an important adaptor protein in *M. tuberculosis* DNA initiated pathways, it does not directly sense dsDNA. Multiple cytoplasmic DNA sensors have been described that activate various inflammatory pathways. Several of these have been proposed to activate STING and induce type I interferon and are potential sensors of *M. tuberculosis* DNA.

As reviewed earlier, AIM2 recognition of mycobacterial DNA may be an important mechanism for inflammasome activation[94]. However, while IRF3 expression is required

for AIM2-mediated signaling[213], AIM2 deficiency has no effect on type I interferon production[214], indicating that it does not triggering the CSP.

DAI, (also called Z-DNA binding protein (ZBP-1), can activate IRF-3-mediated type I interferon production and DAI sensing is involved in the recognition of HSV-1 in microglia and astrocytes as well as CMV in human fibroblasts[215-217]. However, DAI$^{-/-}$ mice respond no differently then WT mice to *M. tuberculosis* infection and many human cells do not require DAI to sense and respond to DNA[218], suggesting a redundant role for DAI in this pathway.

IFN-γ-inducible protein 16 (IFI16) induces ASC-dependent activation of the inflammasome[219] and also is able to activate type I interferon production in response to intracellular DNA. IFI116 alternates between cytoplasmic and nuclear localization, can sense DNA in either location,[220] and induces IFN-β via activation of the IRF-3-mediated signaling pathway[220]. *M. tuberculosis* induces cytosolic translocation of IFI16, and knockdown of IFI16 in BMDM results in a partial reduction in IFN-β production[100]. However, in human THP-1 cells stimulated with DNA or infected with *L. monocytogenes*, IFI16 is not required for early IFN-β induction but is needed for proper late induction of IFN-β[221], suggesting another DNA sensor may be important in early activation of this pathway.

Cyclic GMP-AMP synthase (cGAS) binds dsDNA in the cytoplasm and then catalyzes the production of the second messenger cyclic GMP-AMP ($C_{20}H_{24}N_{10}O_{13}P_2$, cGAMP)[222]. cGAMP then binds to and induces a conformational change in STING that activates it to phosphorylate TBK1. TBK1 then activates IRF3 and triggers the

production of the type I interferon response[223]. In all cell lines tested to date, cGAS appears to be essential for DNA-mediated activation of the IRF-3 pathway[224]. While much of what is known about the role of cGAS in the immune response has focused on its role in sensing viral infection, recent work has demonstrated that intracellular bacteria in the cytoplasm activate type I interferon production in a cGAS-dependent fashion. cGAS is required for IFN-β induction in response to *Chlamydia trachamatis* infection and both human and mouse cells deficient for cGAS are unable to generate IFN-β following infection[225]. Similar results are seen in cGAS-deficient human THP1 cells infected with *Listeria monocytogenes*[221]. However, while *M. tuberculosis* can translocate into the cytoplasm, the majority of the bacteria remain localized in the phagosome[226] and secrete DNA through phagosomal pores to activate the CSP. It remains to be determined if cGAS mediates this CSP activation in response to mycobacterial DNA. Additionally, an *in vivo* role for cGAS in the response to bacterial infection has yet to be characterized. Therefore, the second focus of this thesis is to determine if cGAS is required for *M. tuberculosis*-mediated type I interferon production and to characterize the role of cGAS in a mouse model of *M. tuberculosis* infection.

Results

M. tuberculosis infection up-regulates cGAS expression

To determine the effects of M. tuberculosis infection on cGAS expression and localization, we infected THP1 cells with mCherry-labeled M. tuberculosis and then examined the expression of cGAS by confocal microscopy. Following infection, we observed increased cGAS cytoplasm of the cells (Figure 2.1A). While some cGAS could be seen in the uninfected cells, the cytoplasm of M. tuberculosis-infected cells contained many more brightly staining puncta. We observed increased accumulation of cGAS protein by western blot, confirming increased protein expression after M. tuberculosis infection (Figure 2.1B).

cGAS is expressed in tissue samples from *M. tuberculosis*-infected individuals

To determine if cGAS is expressed in human tuberculosis infection, we examined cGAS expression in lung biopsies from *M. tuberculosis*-infected patients. By immunohistochemistry, we observed high expression of cGAS in granulomas from infected individuals (Figure 2.2A). We also examined cGAS expression by immunofluorescence (Figure 2.2B). Confocal microscopy revealed cGAS puncta throughout the cytoplasm of *M. tuberculosis*-infected cells and surrounding tissue consistent with the staining pattern observed in THP-1 cells. Thus, cGAS expression is expressed in human tuberculosis infection.

cGAS is essential for IFN-β production during *M. tuberculosis* infection but not *Listeria monocytogenes* or *Streptococcus pyogenes* infection

To determine if cGAS is required for type I interferon production after *M. tuberculosis* infection, we infected BMDMs from cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice with three different MOIs of *M. tuberculosis* and determined the amount of IFN-*β* induced. Even at an MOI of 10, IFN-β production was almost abolished in both the cGAS$^{-/-}$ and STING$^{gt/gt}$ cells (Figure 2.3A). To confirm the effect seen on IFN-β induction was not due to general cytokine inhibition but rather inhibition of IRF-3-dependent cytokine production, we also examined expression of the NF-κB-dependent cytokines TNF and IL-1β. As expected, STING or cGAS deficiency did not affect the expression of either cytokine (Figure 2.3B).

Listeria monocytogenes and *Streptococcus pyogenes* (but not *M. tuberculosis*) produce cyclic dinucleotides that can directly activate STING. To determine the importance of cGAS activation following infection with cyclic dinucleotide producing species and compare that role with the required role for cGAS observed during *M. tuberculosis* infection, we infected BMDMs from cGAS$^{-/-}$, STING$^{gt/gt}$ and WT mice with *L. monocytogenes* or *S. pyogenes* and measured IFN-*β* induction. Consistent with a direct role for STING activation by cyclic dinucleotides, STING but not cGAS expression was required for IFNβ and CXCL10 induction. While some reduction of IFN-*β* and CXCL10 production was observed in cGAS$^{-/-}$ BMDMs, this was not statistically significant. As with *M. tuberculosis* infection, expression of IL-1β was not significantly affected by the loss of cGAS or STING (Figure 2.4). Thus, cGAS is vital in the production of the type I

interferon response to *M. tuberculosis*, but not *L. monocytogenes* and *S. pyogenes* in mouse BMDMs.

To determine the role of cGAS in human cells, we infected THP-1 cells containing a stable shRNA-mediated knockdown of STING or cGAS or a scramble control with *M. tuberculosis*. Consistent with our observations in BMDMs, we observed decreased expression of IFN-β as well as the interferon-stimulated gene CXCL10 in both STING and cGAS-deficient cells (Figure 2.5A), whereas TNF production was not affected by cGAS or STING deficiency (Figure 2.5B). An essential role for cGAS in the response to human tuberculosis was further confirmed by a lack of IFN-β production in cGAS-deficient BJ cells following *M. tuberculosis* infection (Figure 2.5C). Thus, similar to mouse BMDMs, human cell lines are dependent on cGAS activity for type I interferon production following *M. tuberculosis* infection.

M. tuberculosis-dependent cGAMP production is abolished in cGAS-deficient cells

It is expected that cGAMP should be produced following cGAS recognition of *M. tuberculosis* DNA. To determine the optimal time after infection to measure cGAMP, WT BMDM were infected with *M. tuberculosis* for 1, 2, 4, 8, and 24 hours and cGAMP production analyzed. Optimal cGAMP levels were obtained at 8 hours post infection (Figure 2.6A).

BMDMs from cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice were then infected for 8 hours and cGAMP production analyzed. As expected, robust cGAMP production was detected in BMDMs from both WT and STING$^{gt/gt}$ mice but was abolished in BMDMs from cGAS$^{-/-}$ animals (Figure 2.6B). Relative intensities were compared to an internal reference standard of C-13-labeled cGAMP and the number of cells plated used to calculate the total number of cGAS/cell. Thus, *M. tuberculosis* induces the cGAS-dependent production of cGAMP in BMDMs.

cGAS deficiency is associated with early CFU defects but does not affect *M. tuberculosis* survival over a 7-day time course

To determine if the interferon defects observed early during infection would effect *M. tuberculosis* intracellular survival, we infected shScramble, shSTING, and shcGAS THP-1 cells and quantified the number of bacteria 24 hours after infection (Figure 2.7A). We observed higher CFU values in shcGAS cells indicating that cGAS-deficient cells were less able to control infection. There was a trend towards STING-deficient cells being less able to control infection, but this was not significant.

After determining that there was a CFU defect in the shcGAS THP-1 cells 24 hours after infection, we infected shScramble, shSTING, and shcGAS THP-1 cells and quantified the number of bacteria 3 and 7 days after infection to determine if the CFU defect seen in the shcGAS cells at 24 hours would persist over a 7-day time course. We did not observe any CFU differences 3 or 7 days post infection (Figure 2.7B). Thus,

cGAS deficiency is associated early CFU defects in THP-1 cells, but differences in *M. tuberculosis* survival do not persist over a 7-day time course.

cGAS is required for *M. tuberculosis* targeting to autophagosomes

In addition to its role in initiating the type I interferon response, cGAS also interacts with the autophagy protein Beclin-1. cGAS binding to Beclin-1 releases the autophagy protein Rubicon to induce autophagy[227]. To determine if cGAS is important for the autophagy response in *M. tuberculosis* infection, BMDMs from cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice expressing GFP-LC3 were infected with mCherry-labeled *M. tuberculosis*. LC3 is a marker for the autophagosome. Colocalization of GFP-LC3 and *M. tuberculosis* identified bacteria in the autophagosome. We quantified the percentage of *M. tuberculosis* colocalizing with GFP-LC3 for each genotype. Cells deficient in cGAS showed significantly lower levels of colocalization, indicating a decrease of *M. tuberculosis* targeting to the autophagosome. While cells deficient in STING also showed decreased targeting of *M. tuberculosis* to the autophagosome, cGAS deficiency induced a greater defect in autophagosome targeting than STING deficiency (Figure 2.8). Thus, cGAS deficiency inhibits autophagy response in macrophages.

STING$^{-/-}$ mice display decreased bacterial numbers following an extremely high dose *M. tuberculosis* infection

While waiting to obtain a sufficient number of cGAS$^{-/-}$ mice for *in vivo* studies, we performed a pilot high dose infection using STING$^{gt/gt}$ mice to determine if STING deficiency alters the ability of mice to control *M. tuberculosis* infection. While a standard low dose infection delivers 100-200 bacteria per mouse, mice in this study received approximately 5400 CFU/mouse (Figure 2.9A). We sacrificed mice 14 days after infection and measured CFU values in the lung, liver, and spleen as well as cytokine levels in the lung. An additional group of mice was monitored for survival. STING$^{gt/gt}$ mice showed less weight loss than WT mice 14 days after infection (Figure 2.9B). While there was not a difference in the numbers of bacteria in the liver or spleen, STING$^{gt/gt}$ mice showed decreased bacterial burden in the lung (Figure 2.10A). We found reduced levels of IFN-β, TNF, and IL-1β expression in the lungs of the STING$^{gt/gt}$ mice, however the difference was not statistically significant (Figure 2.10B). With such a large initial infectious dose, all of the mice succumbed to infection by day 16 and there was no significant difference in survival between WT and STING$^{gt/gt}$ mice (Figure 2.11). Thus, while STING$^{gt/gt}$ mice are better able to control *M. tuberculosis* infection following a high dose infection, this does not result in increased survival.

Survival following a low dose *M. tuberculosis* infection is reduced in cGAS-deficient mice

To determine if defects in type I interferon production and autophagy with would affect the outcome of *M. tuberculosis* infection *in vivo*, we infected cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice using a low dose aerosol model of infection. Mice sacrificed at day 0 confirmed the mice received an infectious dose of ~200 bacteria/mouse (Figure 2.12). We sacrificed cohorts of mice at day 21 and day 42 and quantified the number of bacteria in the liver, lung, and spleen. We also analyzed cytokine levels in the lung, liver, spleen, and serum and collected tissue sections for histology. We monitored the remaining mice for survival differences.

We did not observe differences in values at day 21 (Figure 2.13A) or day 42 (Figure 2.13B) in the lung, liver, or spleen. We examined cytokine expression by qPCR analysis and did not find any differences in any of the cytokines measured in the lung (Figure 2.14A), spleen (Figure 2.14B), or liver (Figure 2.14C). ELISA analysis of in the lung and sera confirmed the qPCR results demonstrating no defect in cytokine production in STING$^{gt/gt}$ or cGAS$^{-/-}$ mice at day 21 (Figure 2.15A) and no defects in the sera or lungs at day 42 (Figure 2.15B). We used a bioassay to examine IFN-β levels in the serum at day 42 and did not observe any differences between the genotypes (Figure 2.15C). We quantified the percent inflammation in H&E sections from lungs collected at day 42. While all of the lungs showed a large amount of inflammation, we did not observe differences in the inflammation levels between the cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice (Figure 2.16). Serum collected from mice in the survival study at day 95 was

analyzed for cytokine differences and we observed no differences in the levels of IFN-β, IFN-γ, IL-1β, IL-12p40, IL-12p70, or TNF (Figure 2.17). We did find the cGAS-/- mice to be more susceptible to infection then the $STING^{gt/gt}$ or WT mice (Figure 2.18), while there was no significant difference in survival between the $STING^{gt/gt}$ mice and the WT mice. We collected lungs from the mice at time of death to determine CFU values (Figure 2.19), and did not observe differences in terminal CFU values. Thus, while no differences in CFUs, cytokine production, or inflammation are seen, $cGAS^{-/-}$ mice are more susceptible to *M. tuberculosis* infection as indicated by decreased survival time following infection.

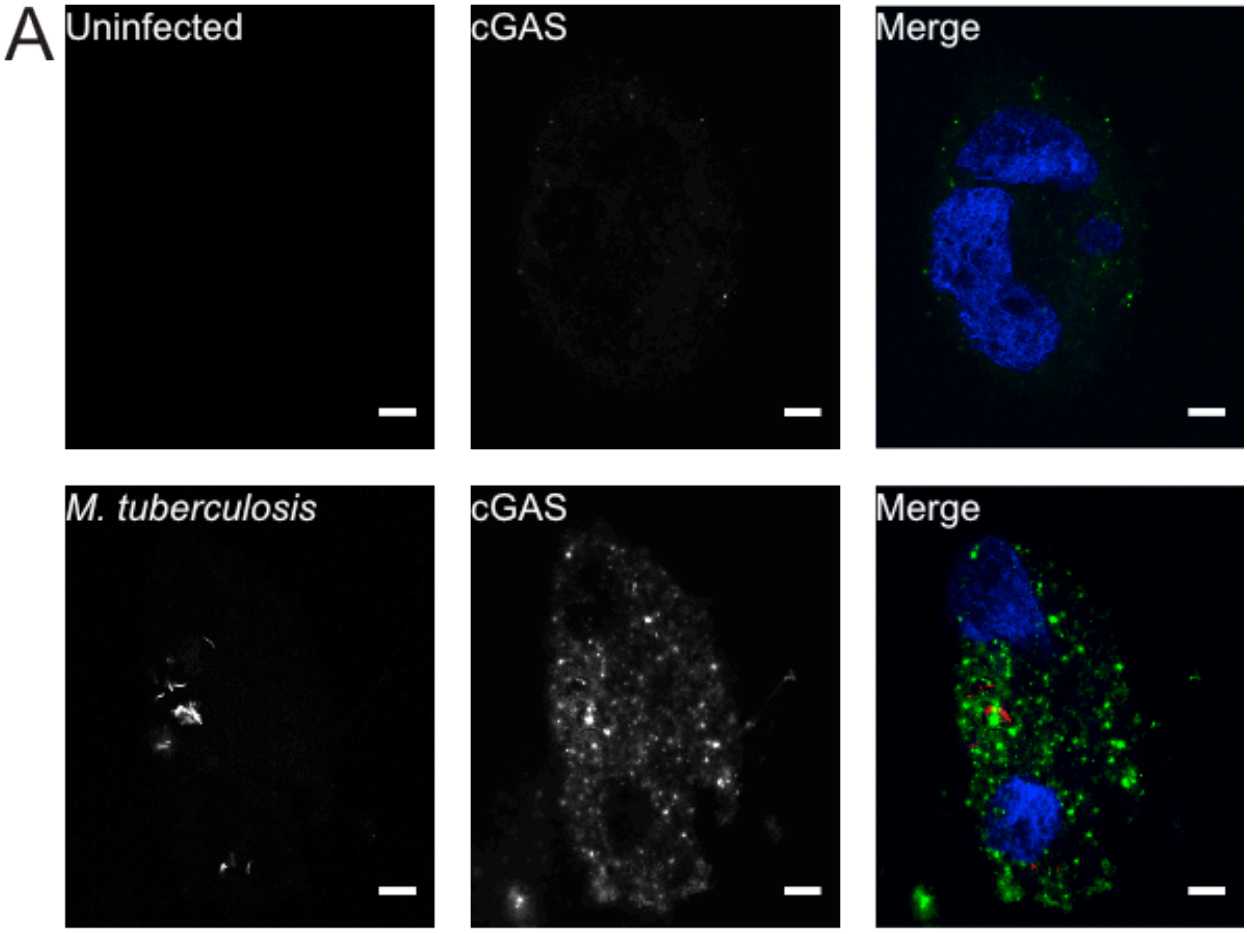
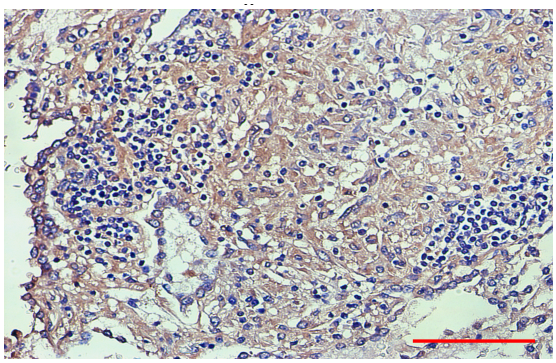

A
Uninfected
cGAS
Merge
M. tuberculosis
cGAS
Merge
B
M. tuberculosis — +
cGAS
Actin

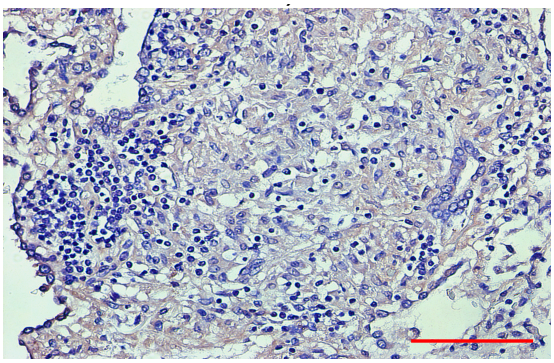
Figure 2.1: *M. tuberculosis* infection upregulates cGAS expression.

(A) THP-1 cells were infected with mCherry-labeled *M. tuberculosis* for four hours, fixed in 4% paraformaldehyde and stained with a rabbit polyclonal anti-cGAS antibody or an isotype-matched control followed by a donkey anti-rabbit secondary. Staining was amplified by the addition of biotin-conjugated tyramide followed by Alexa 488 conjugated streptavidin. Nuclei were stained with Hoechst stain and cells were visualized by immunofluorescence. Results are representative of >3 independent experiments. Scale bars are 10μm. **(B)** THP-1 cells were infected at an MOI of 10 for 4 hours and lysed in RIPA buffer. Lysates were analyzed by western blotting for cGAS expression. Results are representative of 2 separate experiments.

A

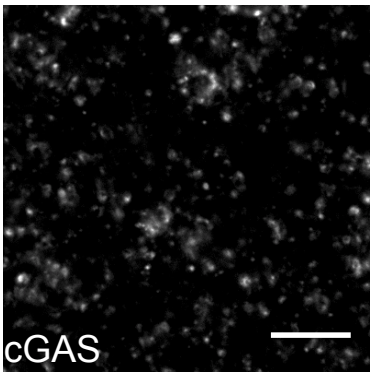


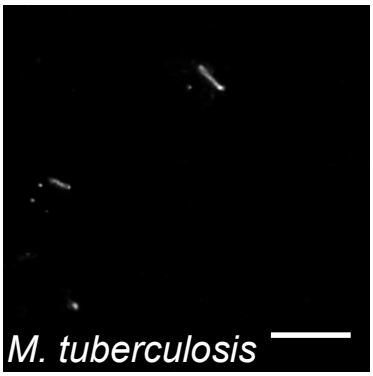
cGAS

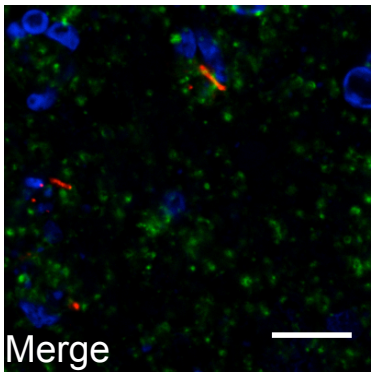


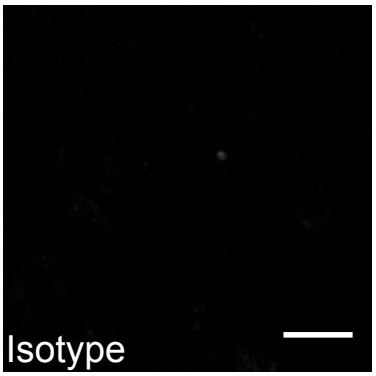
Isotype

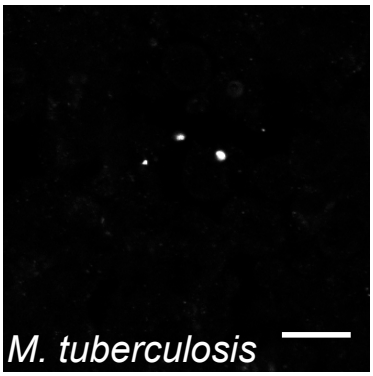
B











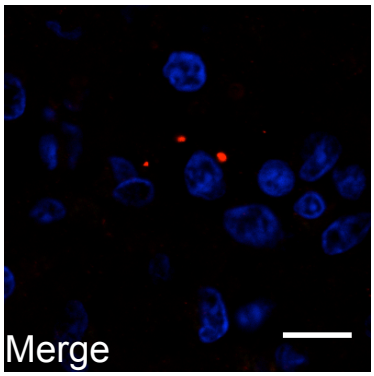
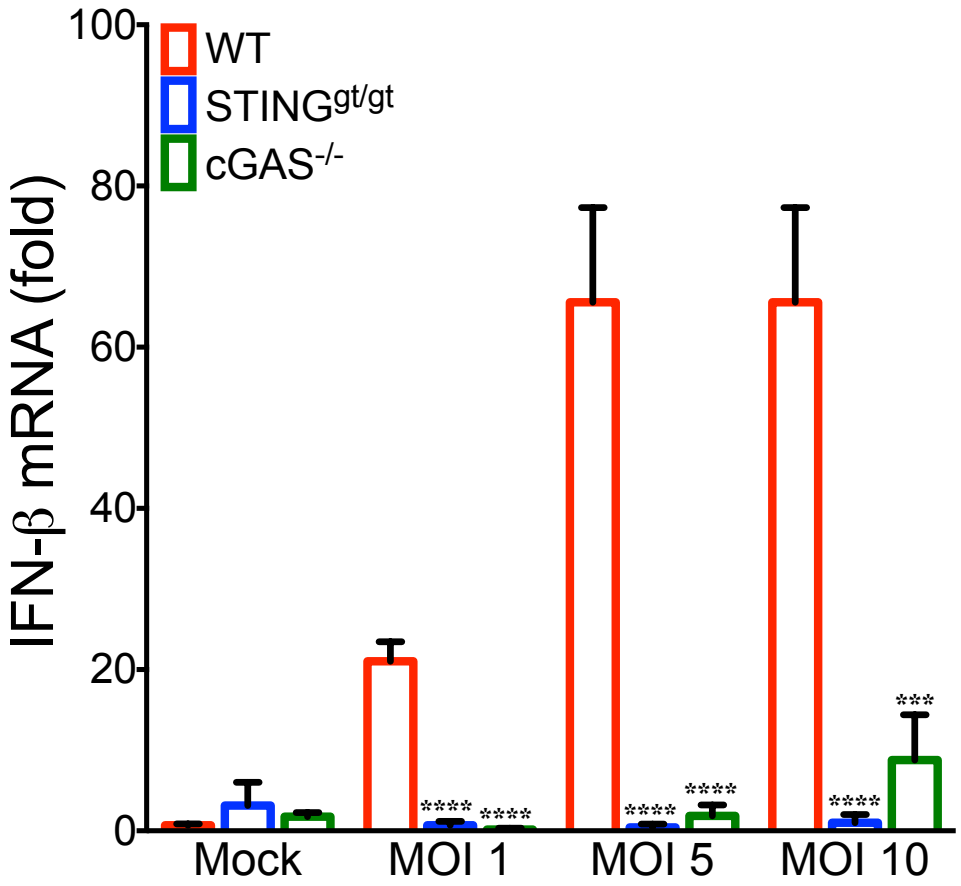


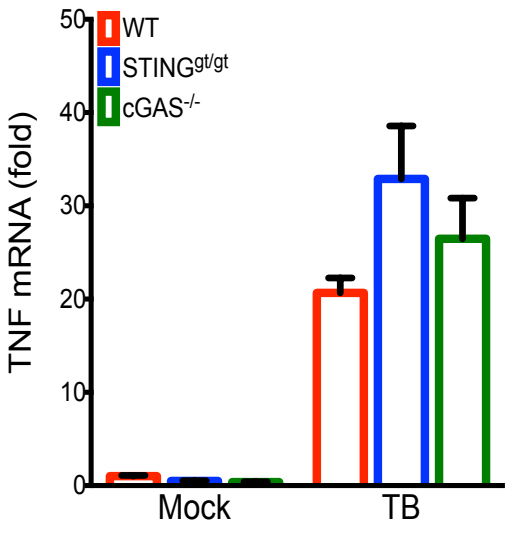
Figure 2.2: cGAS is expressed in tissue samples from *M. tuberculosis*-infected individuals

(A) Paraffin embedded tissue samples from *M. tuberculosis* individuals were deparaffinized and subjected to heat-mediated antigen retrieval. Specimens were treated with a rabbit polyclonal anti-cGAS antibody or isotype control followed by treatment with an HRP conjugated donkey anti-rabbit secondary. Staining was amplified using AB reagent and detected using DAB reagent. Scale bars are 100μm. **(B)** Specimens from *M. tuberculosis*-infected individuals were deparaffinized as in (A). Slides were costained using a rabbit anti-cGAS antibody or isotype control and a guinea pig anti-*M. tuberculosis* antibody. Slides were imaged for immunofluorescence. Scale bars are 10μm.

A


IFN-β mRNA (fold)
100
80
60
40
20
0
WT
STINGgt/gt
cGAS-/-
****
****
****
****
***
Mock
MOI 1
MOI 5
MOI 10

B


TNF mRNA (fold)
50
40
30
20
10
0
WT
STINGgt/gt
cGAS-/-
Mock
TB

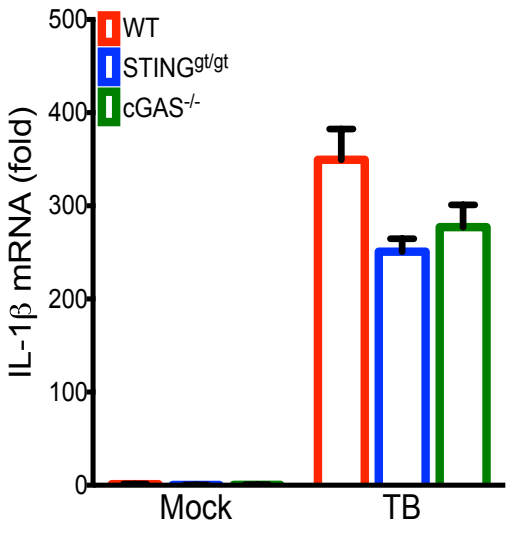
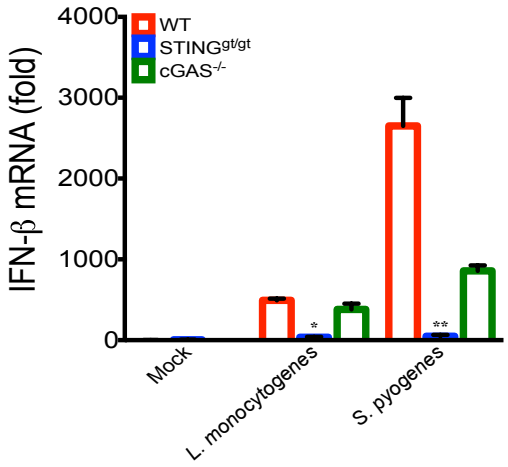
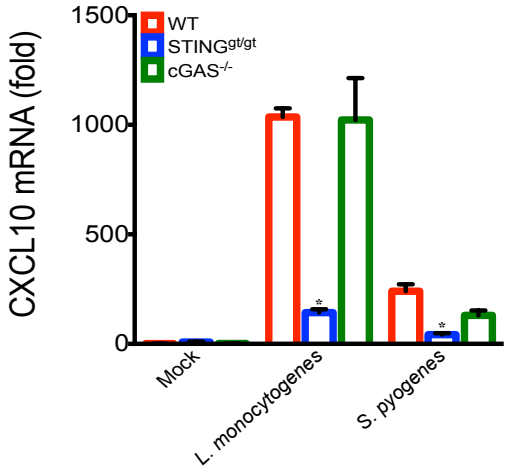

IL-1β mRNA (fold)
500
400
300
200
100
0
WT
STINGgt/gt
cGAS-/-
Mock
TB

Figure 2.3: cGAS is required for IFN-β production by BMDMs during *M. tuberculosis* infection.

(A) BMDMs from cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice were infected with *M. tuberculosis* at MOIs of 1, 5, and 10. RNA was collected for qPCR analysis and IFN-*β* induction determined. **(B)** Samples infected at an MOI of 10 were further analyzed for TNF and IL-β induction. (A) is representative of 2 separate experiments while (B) are representative of 3 separate experiments. ***$p \leq 0.0005$; ****$p \leq 0.00005$; compared to WT-infected cells. (One-Way ANOVA).


4000
3000
2000
1000
0
IFN-β mRNA (fold)
WT
STINGgt/gt
cGAS-/-
*
**
Mock
L. monocytogenes
S. pyogenes


1500
1000
500
0
CXCL10 mRNA (fold)
WT
STINGgt/gt
cGAS-/-
*
*
Mock
L. monocytogenes
S. pyogenes

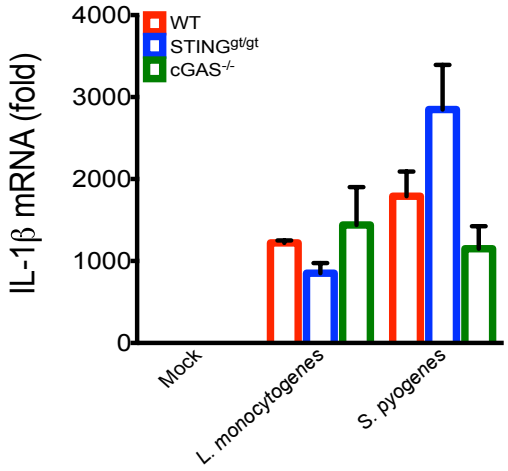

4000
3000
2000
1000
0
IL-1β mRNA (fold)
WT
STINGgt/gt
cGAS-/-
Mock
L. monocytogenes
S. pyogenes

Figure 2.4: cGAS is not required for IFN-β production by BMDMs during *L. monocytogenes* or *S. pyogenes* infection

BMDMs from cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice were infected with *L. monocytogenes* or *S. pyogenes.* RNA was collected for qPCR analysis and the induction of IFN-β, CXCL10, and IL-1β determined. Results are representative of 3 separate experiments. *$p \leq 0.05$; **$p \leq 0.005$; compared to WT-infected cells. (One-Way ANOVA)

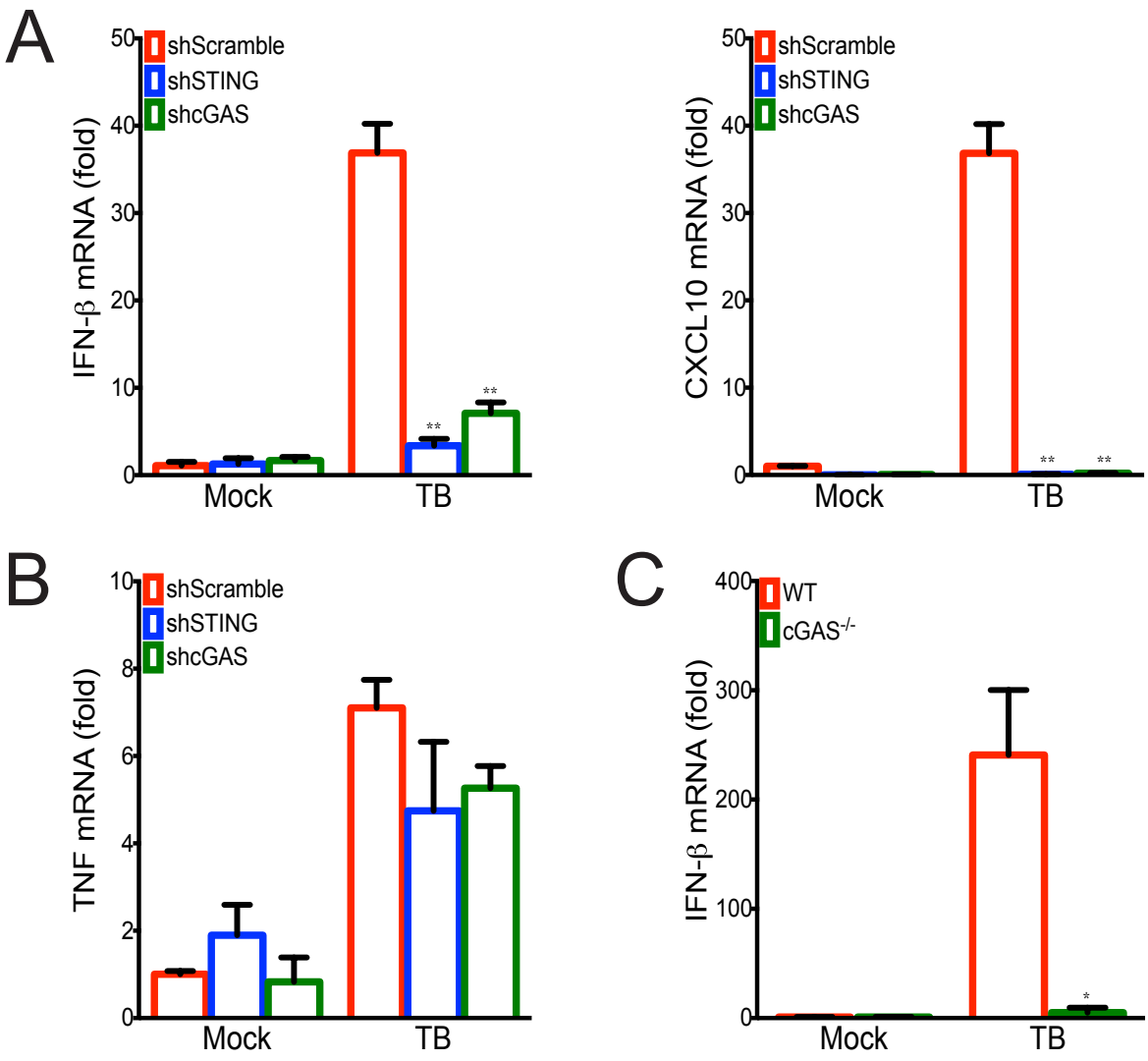
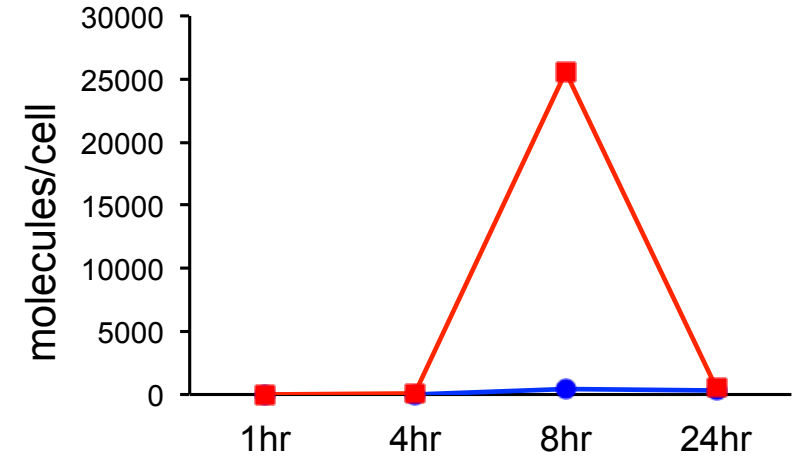


Figure 2.5: cGAS is required for IFN-β production by THP-1 and BJ cells in response to *M. tuberculosis* infection.

(A) shScramble, shSTING, and shcGAS THP-1 cells were infected with *M. tuberculosis* at an MOI of 10. RNA was collected for qPCR analysis and IFN-β, CXCL10, and TNF **(B)** induction determined. Results are representative of greater then 3 separate experiments. **(C)** cGAS-/- and WT human BJ cells were infected with *M. tuberculosis* at an MOI of 10. RNA was collected for qPCR analysis and IFN-β induction determined. Results are representative of 2 separate experiments. *p ≤ 0.05; **p ≤ 0.005; compared to shScramble (THP-1) or WT(BJ)-infected cells. (One-Way ANOVA)

A


30000
25000
20000
15000
10000
5000
0
molecules/cell
1hr
4hr
8hr
24hr

B

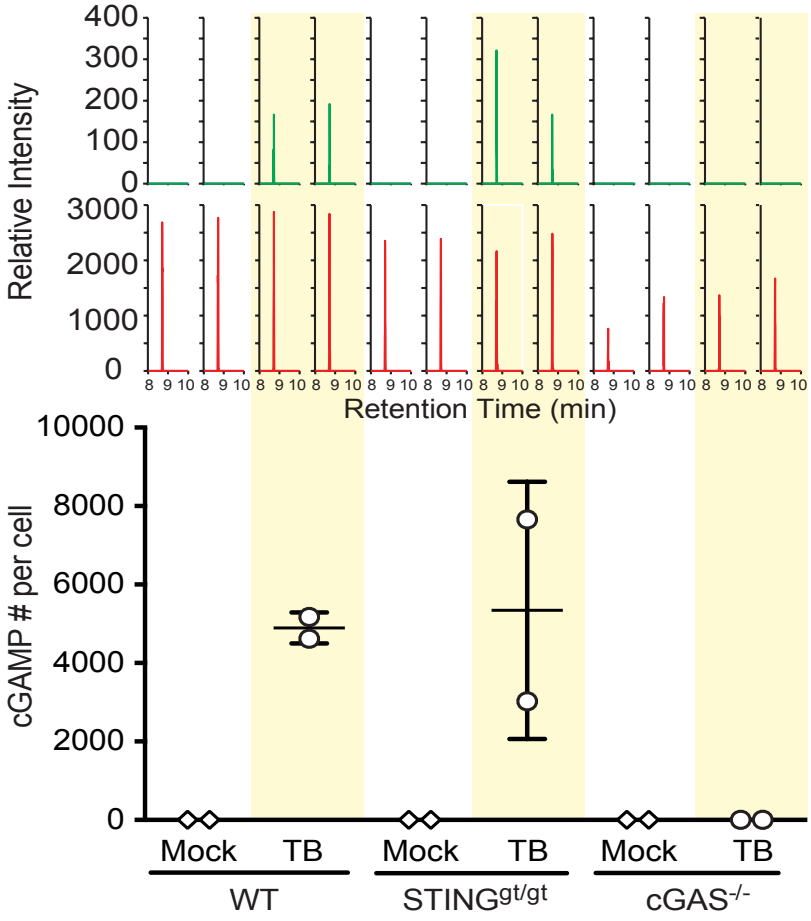

400
300
200
100
0
3000
2000
1000
0
Relative Intensity
8 9 10
Retention Time (min)
10000
8000
6000
4000
2000
0
cGAMP # per cell
Mock
TB
WT
STINGgt/gt
cGAS-/-

Figure 2.6: cGAS-dependent cGAMP production in response to *M. tuberculosis* infection.

(A) To determine the point of maximum cGAMP production during *M. tuberculosis* infection, WT BMDMs were infected at an MOI of 10 and cGAMP levels determined 1, 4, 8, and 24 hours after infection. Cells were lysed in cold 2% acetic in methanol and analyzed using mass spectrometry. **(B)** BMDMs from cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice were infected at an MOI of 10, cells lysed 8 hours after infection as in (A), and cGAMP levels determined by mass spectrometry. Red bars represent a C-13-labeled cGAMP internal standard. Green bars represent biological samples. Based on the number of cells plated, the number of cGAMP/cell was then calculated.

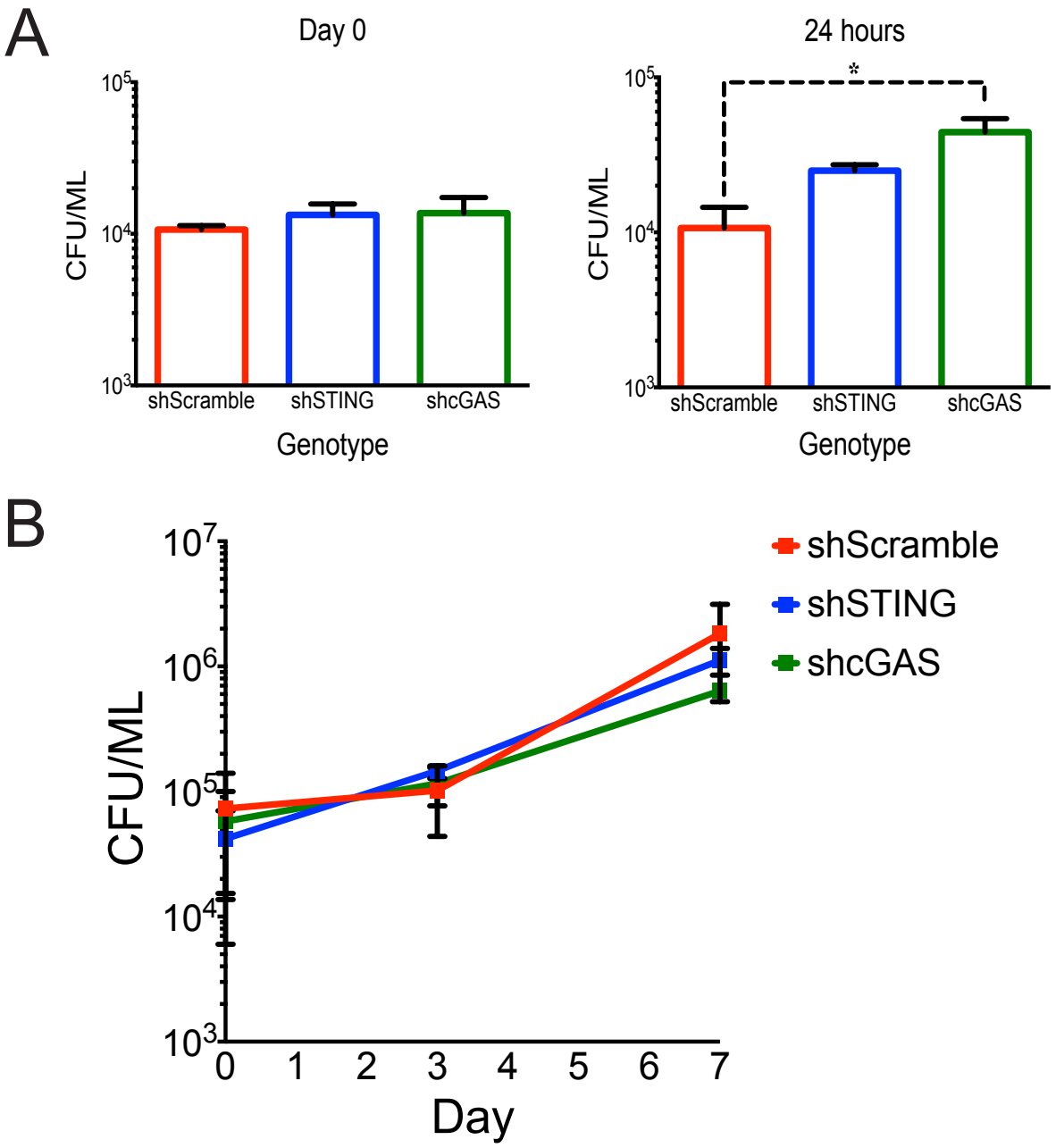
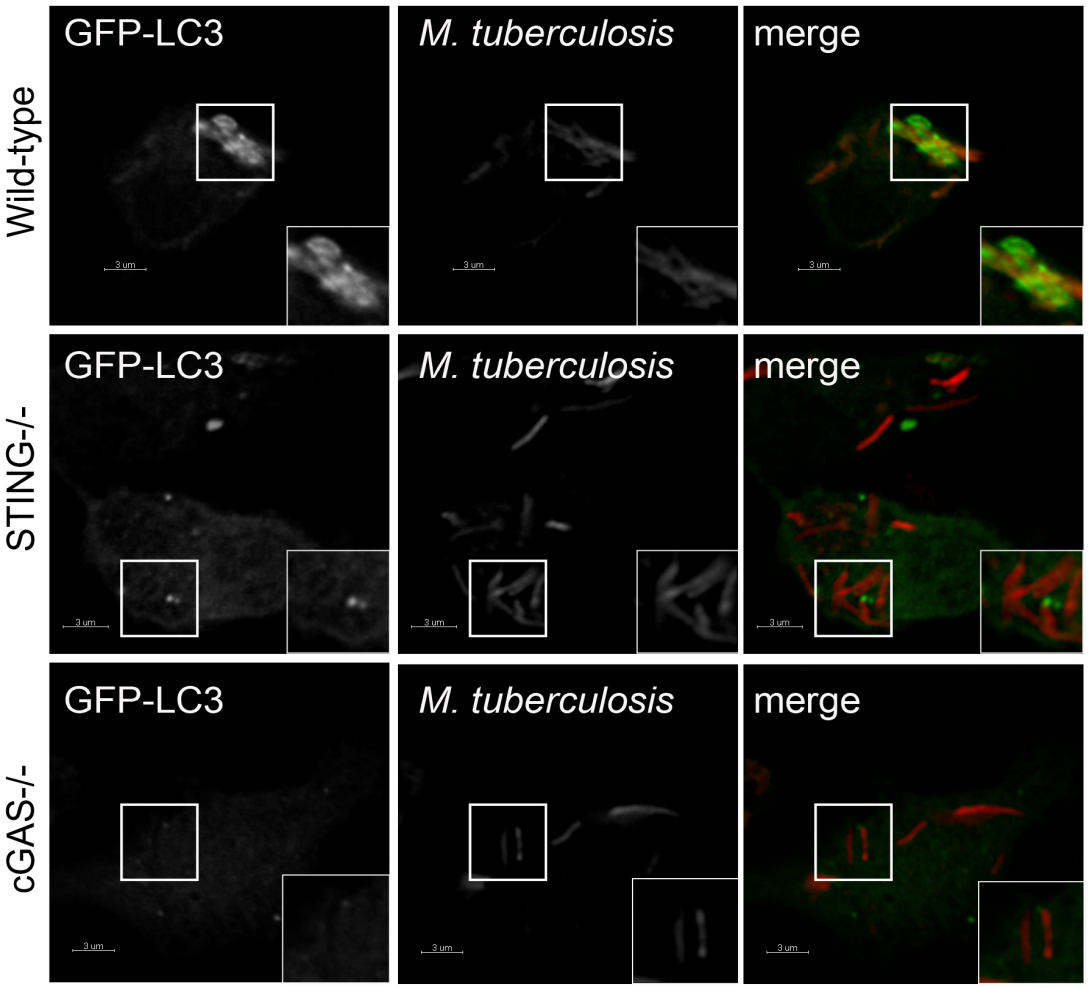
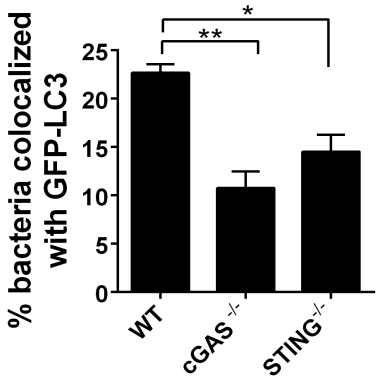


Figure 2.7: *In vitro* infection of cGAS and STING-deficient macrophages

(A) shScramble, shSTING, and shcGAS THP-1 cells were infected with *M. tuberculosis* at an MOI of 0.1. Cells were lysed and CFU values obtained at day 0 and 24 hours after infection **(B)** shScramble, shSTING, and shcGAS THP-1 cells were infected as in (A) with an MOI of 0.1. Cells were lysed and CFU values obtained at days 0, 3, and 7. Results are the combined results from 2 independent experiments. *$p \leq 0.05$. (One-way ANOVA)





* P<0.05; ** P<0.01 (unpaired t test)

Figure 2.8: cGAS is required for *M. tuberculosis* targeting to autophagosomes

BMDMs from cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice transfected with a GFP-LC3 expressing lentivirus were infected with mCherry-labeled *M. tuberculosis* and the percentage of bacteria colocalized with LC3 quantified.

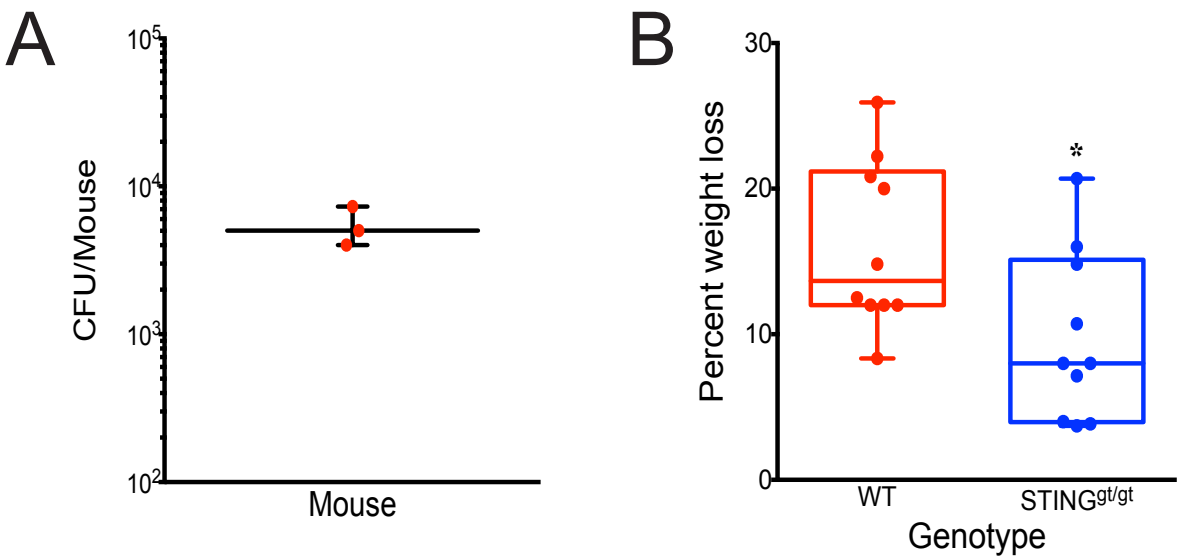


Figure 2.9: High dose *M. tuberculosis* aerosol infection of STING$^{gt/gt}$ mice.

(A) WT and STING$^{gt/gt}$ mice were infected with the Erdman strain of *M. tuberculosis*. Mice received an inoculum of ~5400 CFU/mouse via an aerosol infection. On day 0, 3 additional WT mice were infected and the lungs collected to determine the initial inoculum. Mice were sacrificed 14 days after infection (n=10 for each genotype) and **(B)** percentage weight loss from maximum weight was determined for each mouse. *$p \leq 0.05$

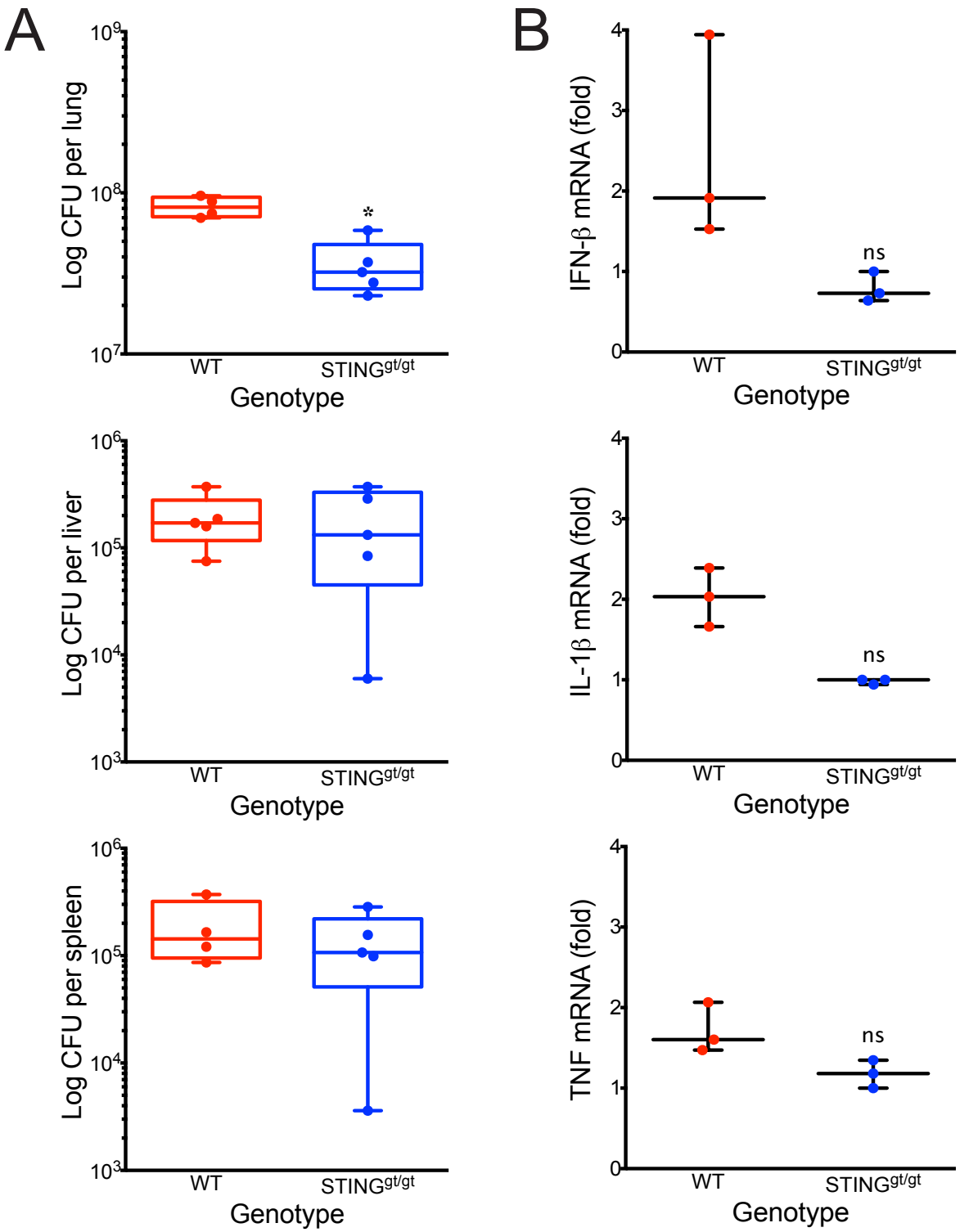

A
B
Log CFU per lung
Log CFU per liver
Log CFU per spleen
IFN-β mRNA (fold)
IL-1β mRNA (fold)
TNF mRNA (fold)
*
ns
WT
STINGgt/gt
Genotype

Figure 2.10: STING$^{gt/gt}$ mice have decreased CFU values but show no differences in cytokine production following a high dose *M. tuberculosis* infection

(A) CFU values from the lung, liver, and spleen of WT and STING$^{gt/gt}$ mice were determined 14 days after a high dose infection with *M. tuberculosis*. **(B)** IFN-β, IL-1β, and TNF levels in the lung were determined by qPCR. *$p \leq 0.05$ (Mann Whitney-U test)

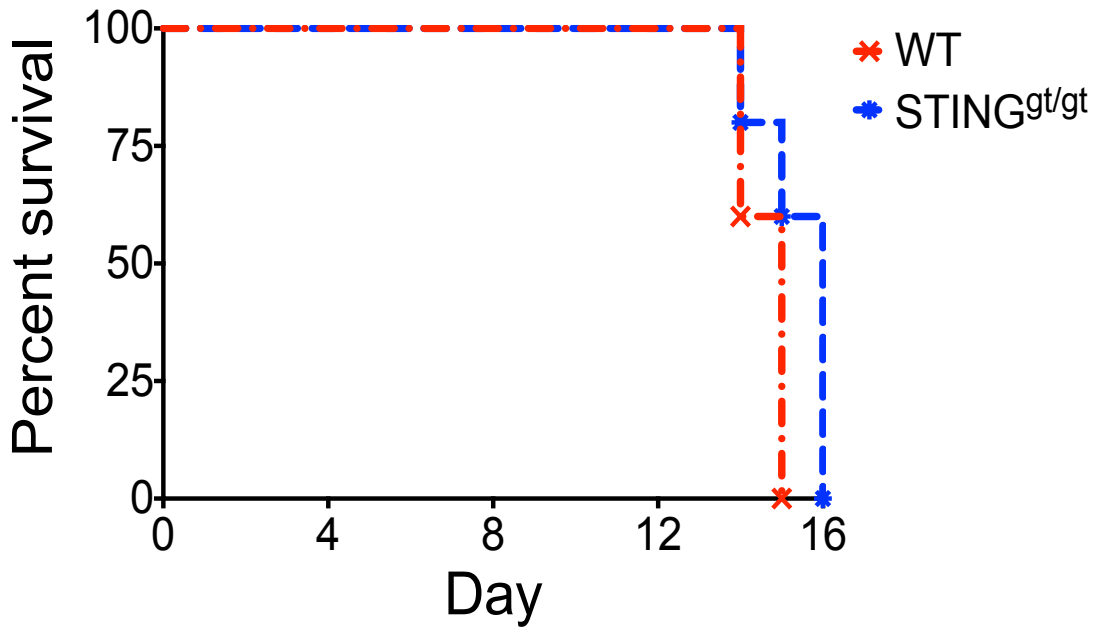


Figure 2.11: STING$^{gt/gt}$ mice show no differences in survival following a high dose *M. tuberculosis* infection

WT and STING$^{gt/gt}$ mice were infected with a high dose *M. tuberculosis* infection and monitored for survival. Mice were euthanized when they lost 15% of their maximum body weight. n=5 for each genotype.

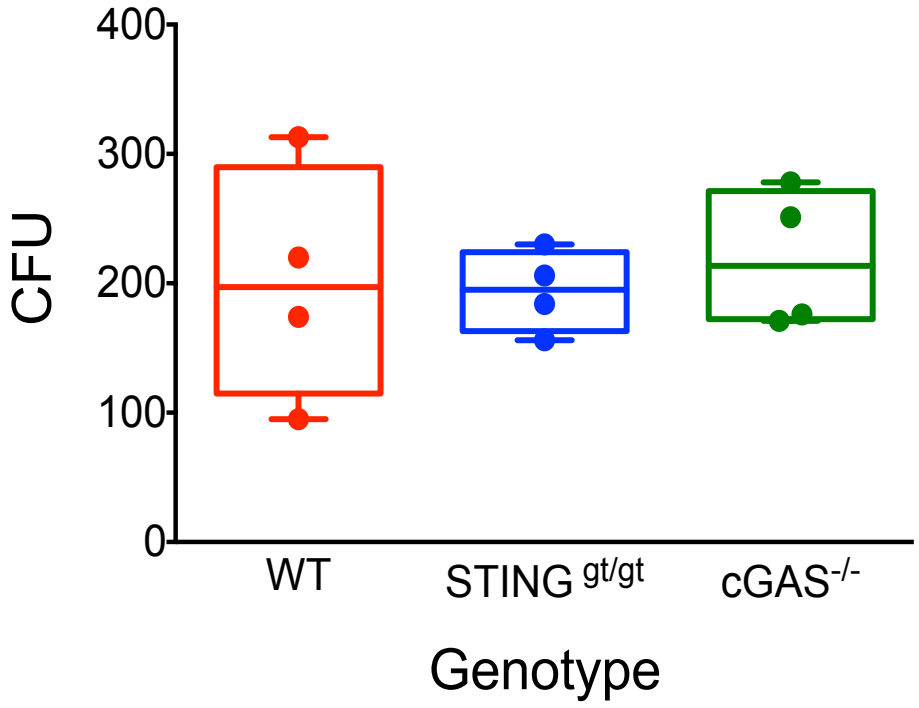


Figure 2.12: Low dose *M. tuberculosis* aerosol infection of cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice

cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice were infected with a low dose aerosol infection of the Erdman strain of *M. tuberculosis.* Mice sacrificed at D0 confirmed that each mouse received an infectious dose of ~200 bacteria (n=5 of each genotype).

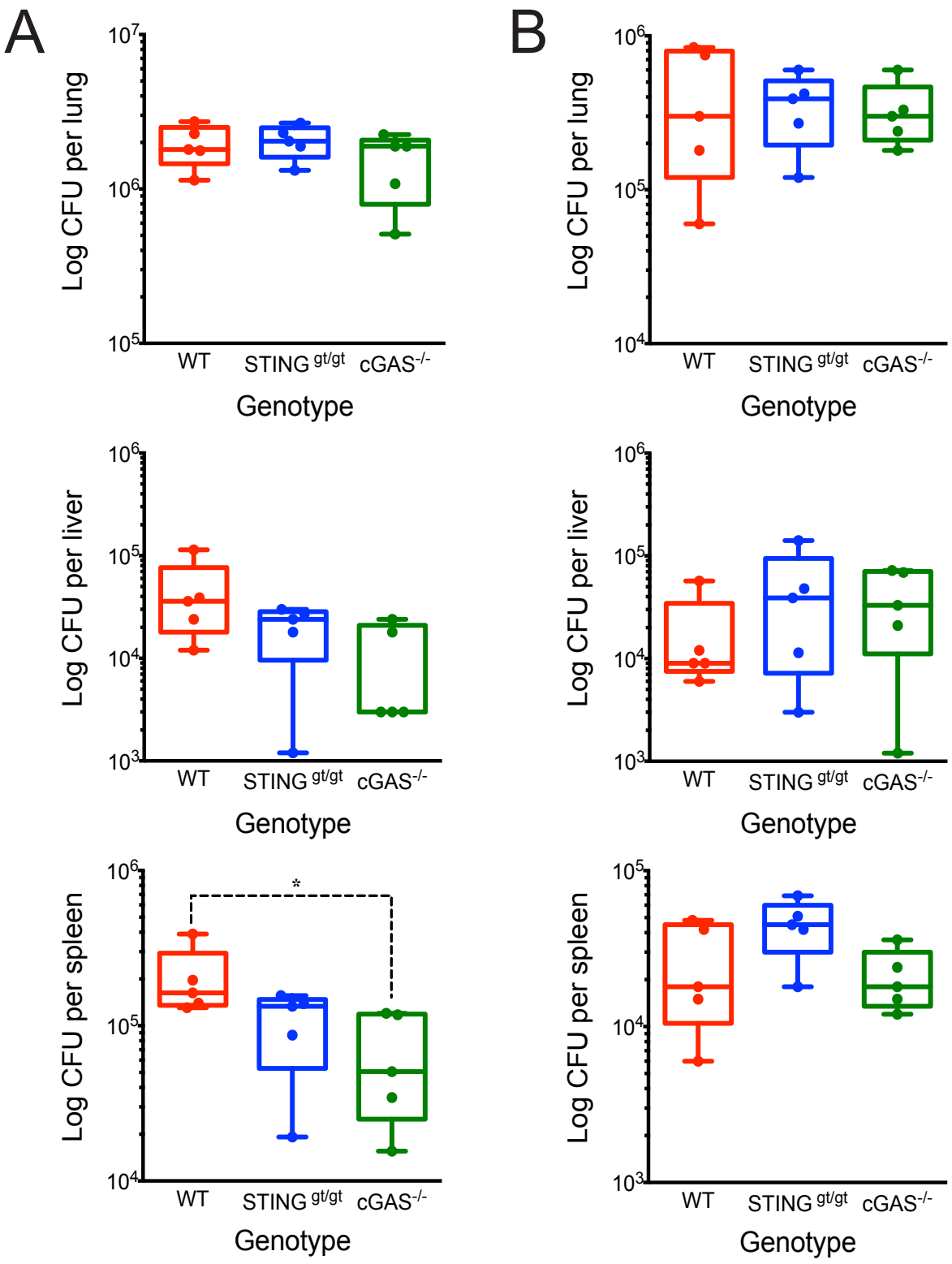

A
B
Log CFU per lung
Log CFU per liver
Log CFU per spleen
WT
STING gt/gt
cGAS-/-
Genotype
*

Figure 2.13: CFU values in the lung, liver, and spleen of cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice 21 and 42 days after a low dose *M. tuberculosis* infection

cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice were infected with a low dose aerosol infection of the Erdman strain of *M. tuberculosis*. Mice were sacrificed and the lungs, liver, and spleen collected for CFU analysis at days 21 **(A)** and 42 **(B).** *$p \leq 0.05$; (One-Way ANOVA), n=5 of each genotype

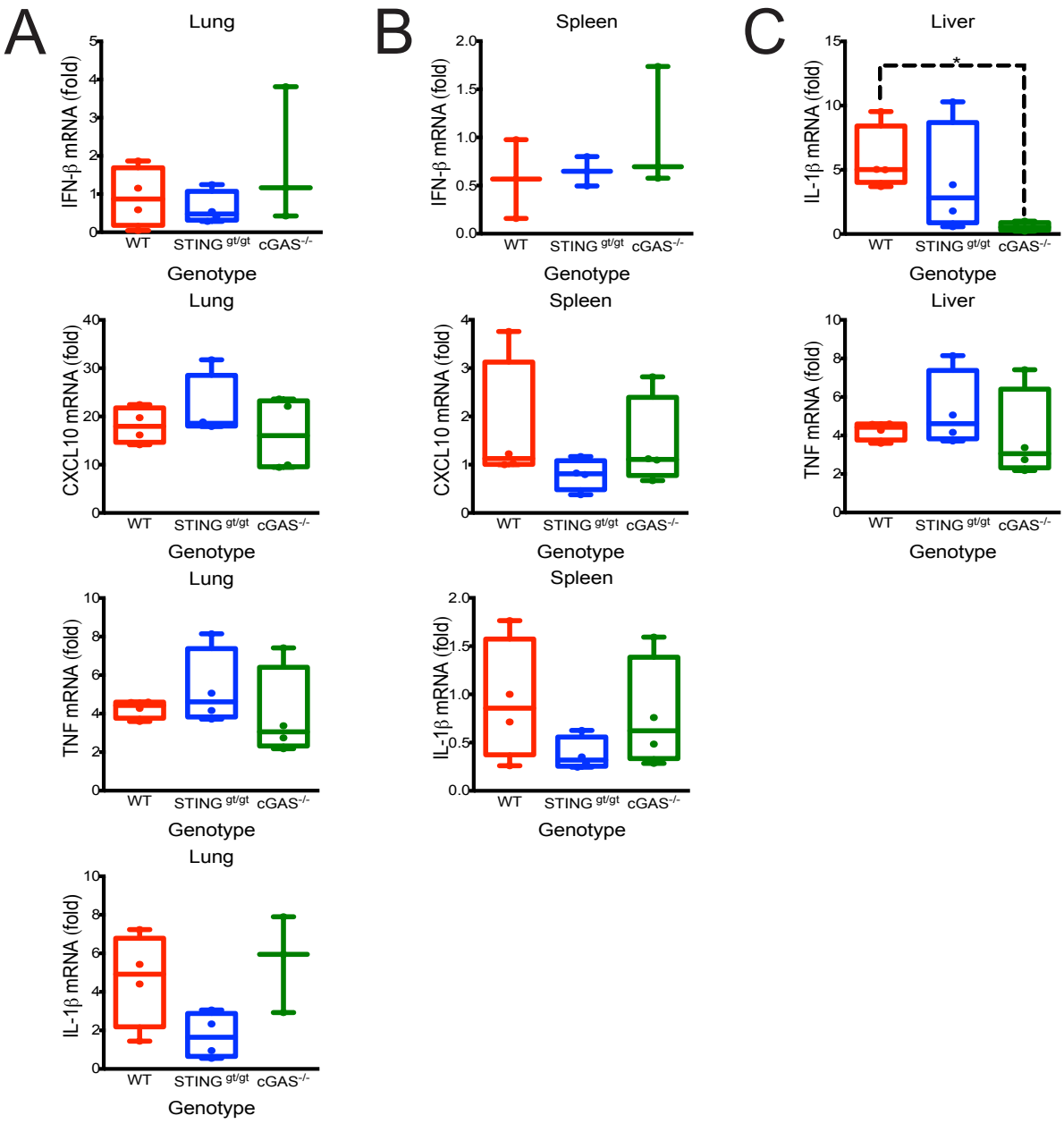


Figure 2.14: Inflammatory cytokine induction in cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice 21 days after a low dose *M. tuberculosis* infection

Inflammatory cytokine production in the lungs (A), spleen (B), and liver (C) of cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice were determined 21 days after a low dose *M. tuberculosis* infection by qPCR. *p ≤ 0.05; (One-Way ANOVA)

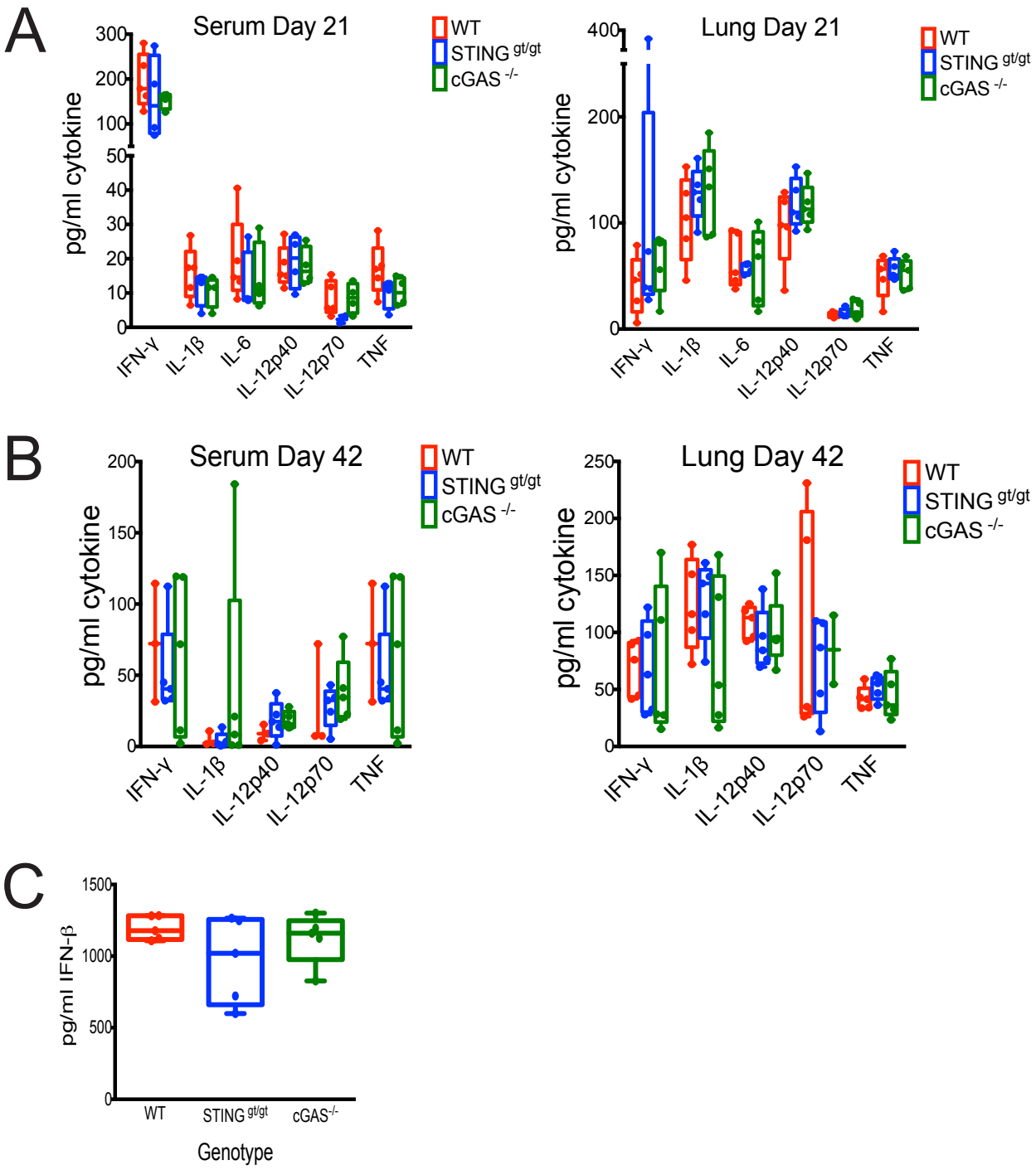

A
Serum Day 21
pg/ml cytokine
300
200
100
50
40
30
20
10
0
IFN-γ
IL-1β
IL-6
IL-12p40
IL-12p70
TNF
WT
STING gt/gt
cGAS -/-
Lung Day 21
pg/ml cytokine
400
200
100
0
IFN-γ
IL-1β
IL-6
IL-12p40
IL-12p70
TNF
WT
STING gt/gt
cGAS -/-
B
Serum Day 42
pg/ml cytokine
200
150
100
50
0
IFN-γ
IL-1β
IL-12p40
IL-12p70
TNF
WT
STING gt/gt
cGAS -/-
Lung Day 42
pg/ml cytokine
250
200
150
100
50
0
IFN-γ
IL-1β
IL-12p40
IL-12p70
TNF
WT
STING gt/gt
cGAS -/-
C
pg/ml IFN-β
1500
1000
500
0
WT
STING gt/gt
cGAS-/-
Genotype

Figure 2.15: Inflammatory cytokine production in cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice 21 and 42 days after a low dose *M. tuberculosis* infection

Inflammatory cytokine levels in lung homogenates and serum from cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice 21 **(A)** and 42 **(B)** days after a low dose *M. tuberculosis* infection were quantified using a multiplex ELISA. **(C)** IFN-β levels were quantified using an IFN-β specific bioassay from serum taken 42 days after infection.

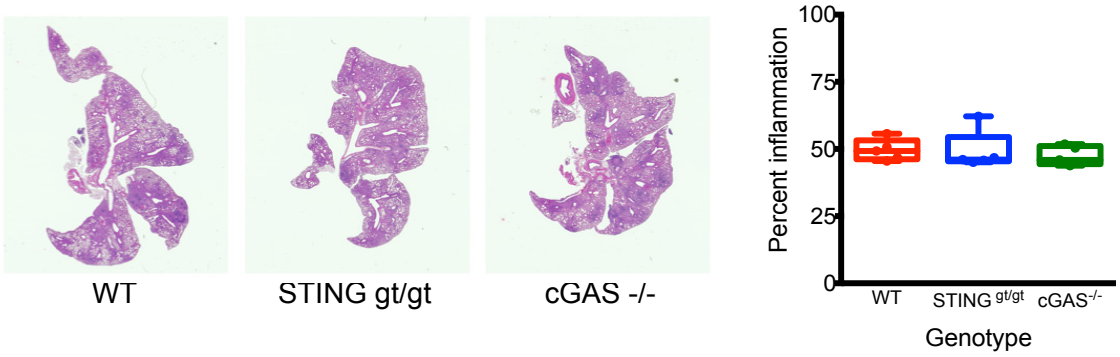


Figure 2.16: Lung inflammation 42 days after a low dose *M. tuberculosis* infection of cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice

Lung sections from cGAS$^{-/-}$, STING$^{gt/gt}$, WT and mice sacrificed 42 days after a low dose *M. tuberculosis* infection were paraffin embedded and sectioned for H&E staining to quantify the percentage of inflammation present. Histology images are representative examples of the staining observed for each genotype. n=5 mice for each genotype.

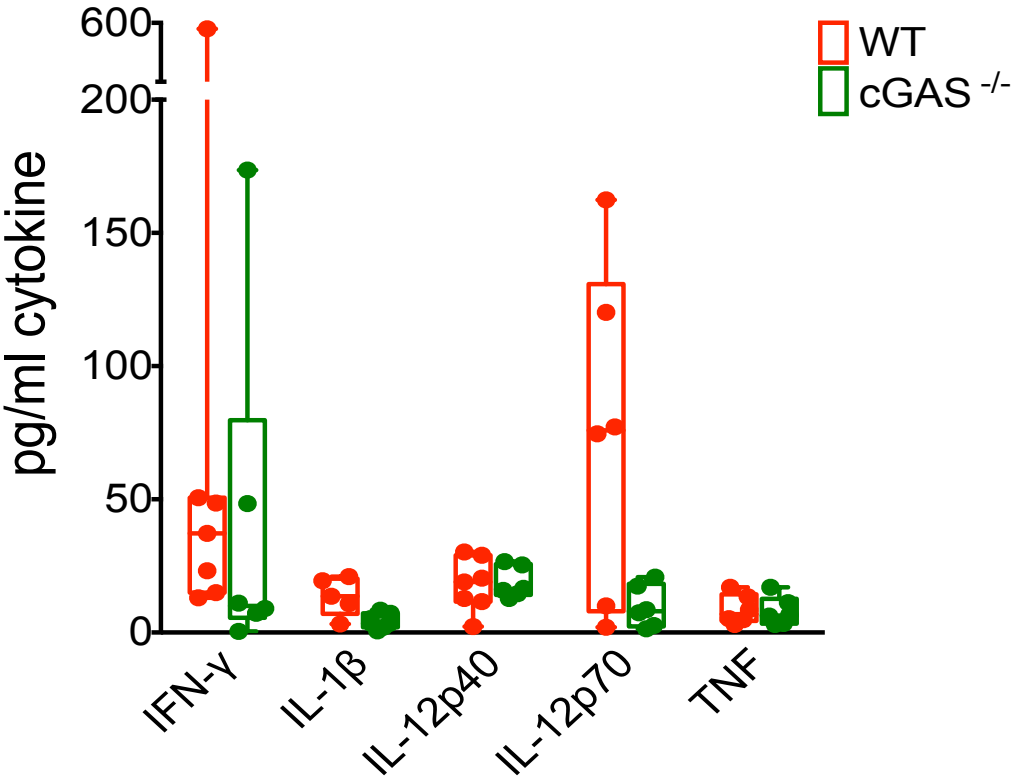


Figure 2.17: Inflammatory cytokine production 95 days after a low dose *M. tuberculosis* infection of cGAS$^{-/-}$ and WT mice

Sera collected from cGAS$^{-/-}$, and WT mice 95 days after a low dose infection with *M. tuberculosis* was analyzed for inflammatory cytokine production by ELISA.

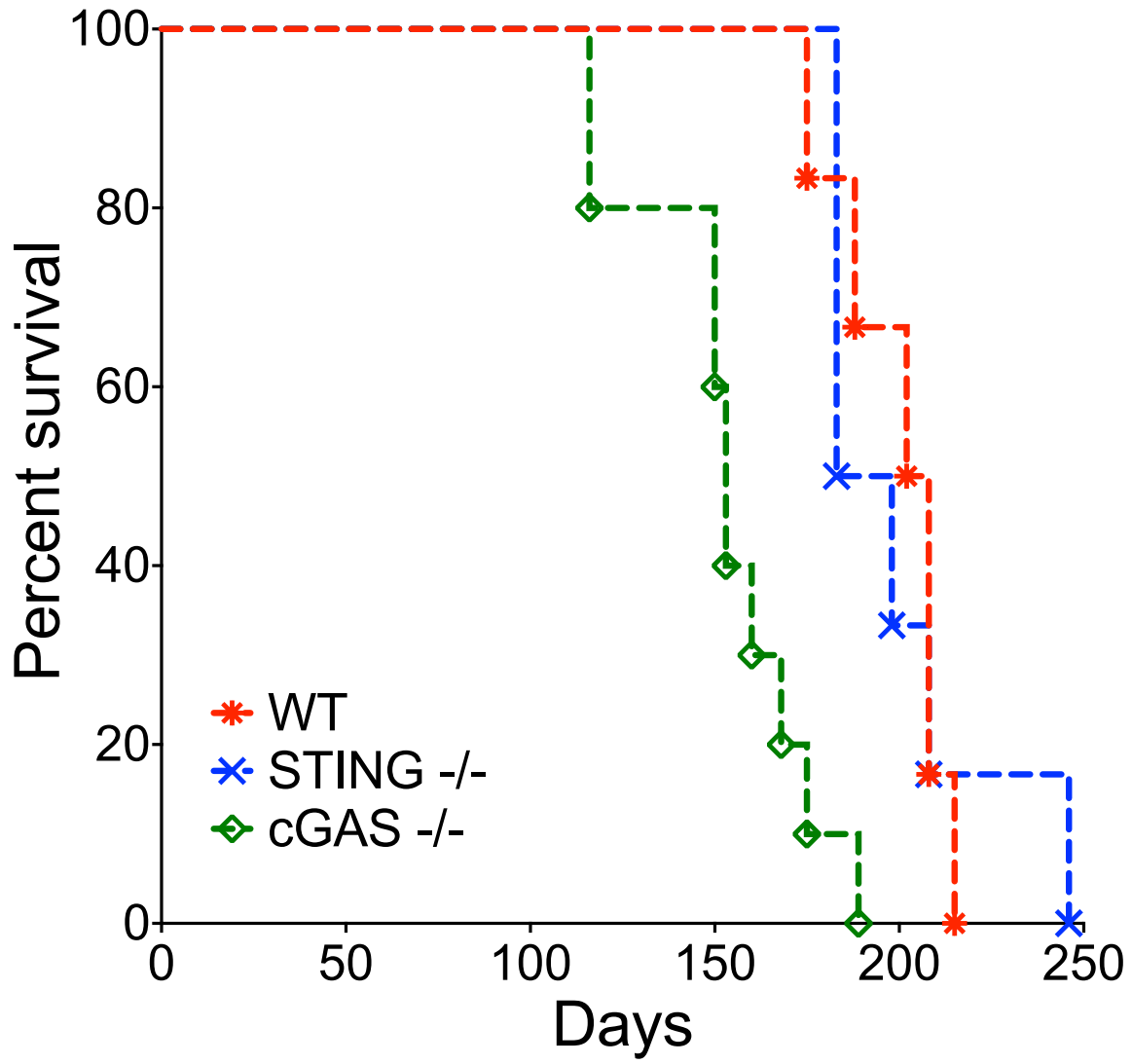


Figure 2.18: cGAS$^{-/-}$ mice display decreased survival following a low dose aerosol infection with *M. tuberculosis*

cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice were infected with a low dose *M. tuberculosis* infection and monitored for survival. Results are representative of 1 of 2 experiments. Kaplan-Meier analysis was performed to determine statistical significance.

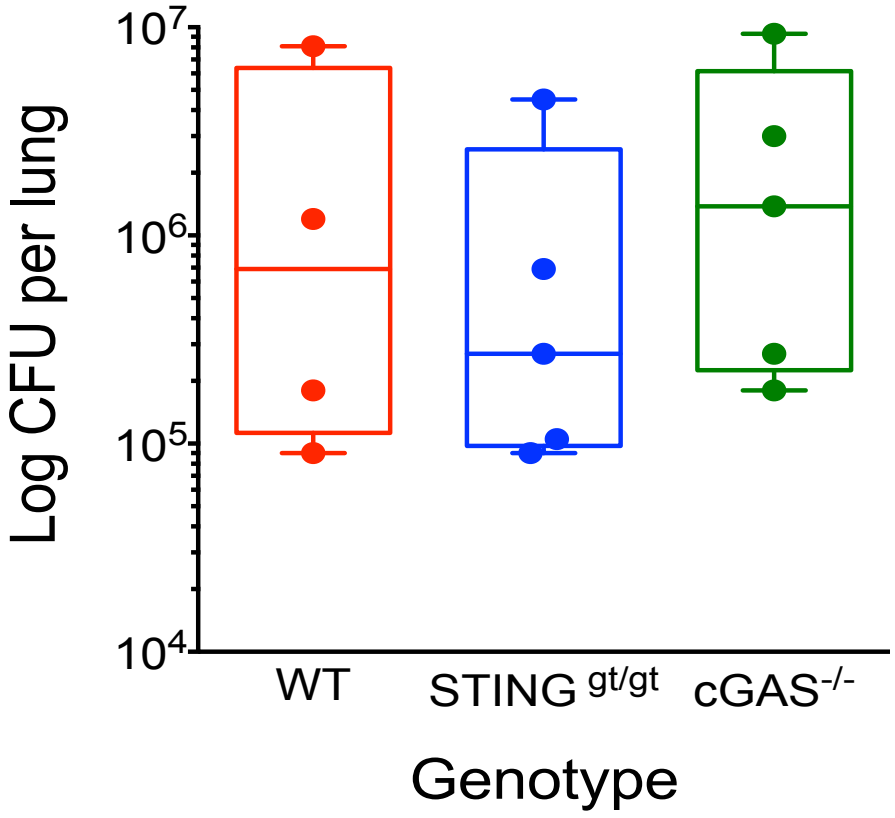


Figure 2.19: Terminal CFU values from cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice following a low dose *M. tuberculosis* aerosol infection

For mice enrolled in a survival study, lungs from cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice infected with a low dose *M. tuberculosis* infection were collected at the time of death and terminal CFU values obtained.

Discussion

An essential role for STING as an adaptor molecule involved in activation of the CSP and the induction of type I interferon production in *M. tuberculosis* infection is well-described[100]. However, the cytosolic DNA sensor initiating activation of this pathway has remained unclear. The recent recognition of cGAS as a cytoplasmic DNA sensor that functions upstream of STING[222], and is vital for type I interferon production following viral infection[228], led us to explore a potential role for cGAS in *M. tuberculosis*-induced activation of the CSP.

Here, we demonstrate that *M. tuberculosis* infection induces upregulation of cGAS that is visualized as punctate staining throughout the cytoplasm of *M. tuberculosis*-infected cells. Transfection of DNA is known to induce similar punctate staining, and transfected DNA can be colocalized with cGAS in many of these areas of cGAS aggregation[222]. As *M. tuberculosis* DNA is detected in the cytosol of infected cells[100], it is likely that *M. tuberculosis* DNA colocalizes to some of these areas of increased cGAS expression, allowing cGAS-mediated activation of the CSP.

Increased cGAS staining is also visualized in adjacent non-infected cells, an effect most likely due to paracrine type I interferon signaling from adjacent *M. tuberculosis*-infected cells[229]. Type I interferon signals through the type I interferon receptor in both an autocrine and paracrine fashion, to up-regulate a large number of interferon-stimulated genes (ISGs)[229]. Recent identification of cGAS as an ISG[230] supports a model where interferon production from *M. tuberculosis*-infected cells

induces cGAS expression in neighboring uninfected cells via paracrine-mediated type I interferon signaling. Additionally, cGAMP produced in one cell can be transferred via gap junctions to a neighboring cell where it will activate STING and the production of type I interferon[231]. This type I interferon will upregulate ISGs such as cGAS. This suggests that transfer of cGAMP from *M. tuberculosis*-infected cells to neighboring cells may represent another mechanism for the upregulation of cGAS observed in uninfected cells.

To determine if cGAS is required for the induction of type I interferon after *M. tuberculosis* infection, we infected both human and mouse cGAS-deficient cells and found that in all cell types tested, cGAS is required for *M. tuberculosis*-mediated activation of the CSP. This is consistent with recent findings demonstrating a requirement for cGAS in the type I interferon response to both *L. monocytogenes* infection in human macrophages[221] and *C. trachomatis* infection of human and mouse cells[225].

We also confirm that activation of this pathway by *M. tuberculosis* is not due to the production of STING activating cyclic nucleotides as has been observed with *L. monocytogenes* infection of mouse cells[209]. We demonstrate that while type I interferon production is abolished in both cGAS- and STING-deficient mouse BMDMs following infection with *M. tuberculosis*, type I interferon production is only abolished in the STING-deficient cells following infection with cyclic dinucleotide-producing *L. monocytogenes* or *S. pyogenes*. While our findings suggest that cGAS is not required for type I interferon production in response to *L. monocytogenes* infection of mouse

BMDM, recent work suggests that type I interferon production in human cells infected with *L. monocytogenes* is dependent on activation of cGAS[221]. Human STING is less responsive to bacterial cyclic dinucleotides than mouse STING[232-234], suggesting species specific differences are responsible for the differences seen in cGAS-deficient human and mouse cells following *L. monocytogenes* infection.

We next examined cGAMP production in cGAS- and STING-deficient cells following *M. tuberculosis* infection. While cGAS-dependent cGAMP upregulation is known to occur following viral infection[223], a similar increase following bacterial infection has yet to be reported. Using cGAS- and STING-deficient mouse BMDMs, we quantified cGAMP production to show for the first time that *M. tuberculosis* infection induces the production of cGAMP in a cGAS-dependent fashion.

Because cGAS deficiency results in an impaired type I interferon response following *M. tuberculosis* infection, we next wanted to determine what effect this defect would have on the ability of macrophages to control *M. tuberculosis* infection. We infected shScramble, shSTING, and shcGAS THP-1 cells with *M. tuberculosis*, and while no differences were seen over a 7-day time course, we did find cGAS-deficient cells were less able to control *M. tuberculosis* in the first 24 hours after infection. These observations suggests that cGAS is important in the early control of *M. tuberculosis*. Additional cGAS-independent antimicrobial mechanisms, such as the production of TNF and IL-1β, also contribute to the control of *M. tuberculosis.* These additional mechanisms may help compensate for early defects in the control of *M. tuberculosis*

associated with cGAS deficiency, resulting in no defect in the control of *M. tuberculosis* over a 7-day time course.

We next wanted to look at potential mechanisms for the observed CFU defect seen in cGAS-deficient cells 24 hours after infection. Recent work demonstrates that cGAS interacts with Beclin-1 to release Rubicon and induce autophagy following sensing of cytosolic DNA[227]. To determine if cGAS is important in the induction of autophagy following *M. tuberculosis* infection, we transfected WT, STING$^{gt/gt,}$ and cGAS$^{-/-}$ BMDMs with a lentiviral construct expressing GFP-LC3 and quantified the amount of LC3 colocalization with *M. tuberculosis*. We found decreased colocalization in both the STING- and cGAS-deficient cells, indicating decreased targeting of *M. tuberculosis* to the autophagosome. This defect in autophagy represents a possible mechanism behind the early CFU defects observed in cGAS-deficient cells. It is plausible that this observed autophagy defect is due to a lack of cGAS-mediated release of Rubicon. However, more work still needs to be done to elucidate the mechanism behind our observation that cGAS deficiency leads to decreased targeting of *M. tuberculosis* to the autophagosome and to determine if this decreased targeting is cause of the observed early survival defects in cGAS-deficient cells. Additionally, while we observe decreased bacterial colocalization with LC3, the total number of autophagosomes formed was not quantified. This quantification should be included in further work to confirm that the differences in colocalization are due to decreased autophagosome targeting and not caused by an overall decrease in autophagosome formation.

Based on our *in vitro* results, we next wanted to determine if cGAS would be important for the control of *M. tuberculosis in vivo.* However, we first ran a pilot experiment using an extremely high dose of *M. tuberculosis* to infect WT and STING$^{gt/gt}$ mice to see if there would be differences between the genotypes in the ability to control infection. While type I interferon production is thought to be decrease control of *M. tuberculosis*, autophagy increases bacterial control and it wasn't clear if one effect or the other would be more important in the *in vivo* control of *M. tuberculosis.* Our observation that STING$^{gt/gt}$ mice have decreased bacterial numbers 14 days after infection in the lung, is consistent with the hypothesis that type I interferon signaling is detrimental in the control of *M. tuberculosis*. Mice deficient in IRF-3 or IFNAR are better able to control *M. tuberculosis*[97,100], administration of exogenous IFN-α/β to *M. tuberculosis*-infected mice increases the bacterial burden[98], and more virulent strains of *M. tuberculosis* are associated with increased induction of type I interferon[99]. A high dose infection such as the one given in our experiment, would induce a large amount of type I interferon. The STING$^{gt/gt}$ mice cannot activate IRF3 and this may lead to decreased levels of type I interferon in these mice and possible better control of *M. tuberculosis*. While there was not a significant difference in IFN-β induction at 14 days between the WT and STING$^{gt/gt}$ mice, we did not examine IFN-β induction in the first few days after infection. Activation of other pathways, such as those mediated by TLR9, can also induce type I interferon and may partially compensate for the lack of IRF3 activation. However, with such a large inoculum, early differences in type I interferon may be significant and may lead to the differences in bacterial numbers we observed. While no survival differences were

observed, such a high dose of *M. tuberculosis* is rapidly fatal, with all the mice succumbing to infection in a 2-day time span and making an accurate determination of possible survival defects unlikely.

Next, we used a more physiologically relevant *M. tuberculosis* low dose aerosol infection model to infect WT, STING$^{gt/gt,}$ and cGAS$^{-/-}$ mice. We observed a survival defect in cGAS-deficient mice, but this defect was not associated with cytokine disregulation at days 21, 42, or 95 or increased bacterial burden at days 21 or 42. Additionally, no differences were seen in levels of lung inflammation. We initially hypothesized that subtle differences in autophagy would lead to defective control of bacteria over time and that CFU differences would manifest later in infection. However, terminal CFU values taken as the mice died showed no differences that could explain the survival defects we observed.

It is possible that cytokine defects are present in the cGAS$^{-/-}$ mice. We have looked at cytokines associated with a Th1 response, however, we have not examined IL-17 and IL-1α levels. Mice deficient in ATG5 display significant defects in autophagy and increased mortality following *M. tuberculosis* infection[235]. As autophagy also inhibits the production IL-17 and IL-1α, ATG5-deficient mice are hyperinflammatory, with hypersecretion of IL-17 and IL-1α as well as increased neutrophil infiltration[235]. Because cGAS deficiency is associated with defects in autophagy, it is possible that IL-17 and IL-1α are elevated in the cGAS$^{-/-}$ mice. This may lead to increased neutrophil accumulation in the lungs. While we looked at inflammation on a gross level, we have not yet determined if the cGAS$^{-/-}$ mice display microscope differences in inflammation, such as

the cellular composition of the inflammatory areas or other pathologic differences such as differences in fibrosis. Autophagy-mediated differences in IL-17 and IL-1α leading to changes in the predominant cell types found in inflammatory regions is one possible mechanism behind the increased mortality following *M. tuberculosis* infection observed in cGAS-deficient mice.

Even if additional cytokine studies do not find any cytokine differences between WT and cGAS-/- mice, the composition of the inflamed lung tissue should be further explored and a more detailed analysis undertaken to determine more precise levels of inflammation in the tissue. CD14-knockout mice are more resistant to *M. tuberculosis* than WT mice, showing increased survival after infection[44]. This survival difference is not associated with differences in bacterial burden, and there are only small differences in inflammatory cytokine production that the authors of the study caution may be biased due to differences between the mice in lung weight[44]. Using a more detailed method to measure lung inflammation than we used, it was determined that the CD14-knockout mice display less inflammation. Additionally, they have decreased leucocyte recruitment to the lungs and this is associated with increased survival despite lack of CFU differences[44]

A survival defect without any associated CFU or cytokine defects is also seen in mice deficient in the inflammasome component PYCARD following *M. tuberculosis* infection[87]. PYCARD-deficient mice also show no differences in gross inflammation after *M. tuberculosis* infection when compared to WT mice[87]. However, knockout mice appear to have defects containing the bacteria within granuloma in the lungs as well as defects

in granuloma formation[87] and it is these defects that are hypothesized to lead to increased mortality.

While the mechanisms behind the increased mortality in PYCARD$^{-/-}$ mice or decreased mortality in the CD14$^{-/-}$ mice are not yet clear, a comparison of granuloma structure and composition as well as *M. tuberculosis* localization in infected lung tissue from cGAS$^{-/-}$ and WT mice may reveal differences between the genotypes and provide a starting point for determining a mechanism behind the observed survival defects in cGAS$^{-/-}$ mice.

In conclusion, we describe an important role for cGAS in both DNA sensing and induction of type I interferon production as well as in autophagy in response to *M. tuberculosis* infection. Additionally, we characterize a survival defect in cGAS-deficient mice in response to *M. tuberculosis* infection, providing the first *in vivo* evidence supporting an important role for cGAS during intracellular bacterial infection.

Materials and methods

Immunohistochemistry and immunofluorescence of human specimens:

Paraffin embedded specimens were deparaffinized in xylene, subjected to heat-mediated antigen-retrieval in 10mM sodium citrate (pH 6.0), permeabilized in 0.2% Triton-100 (Sigma) and blocked in 5% donkey sera. For immunohistochemistry, human sections were stained with rabbit anti-cGAS (1:100, SIGMA) or rabbit anti-GFP (1:100) as an isotype control. HRP conjugated secondary antibodies were obtained from Jackson Immunochemicals and used at 1:250. Staining was amplified with AB reagent (Vecta-stain) and detected using DAB reagent (Thermo Scientific). Images were acquired using a Zeiss Axioplan 2 microscope. For immunofluorescence in THP-1 cells and human tissue, cGAS was identified using rabbit anti-cGAS (Sigma 1:100) and an HRP conjugated donkey anti-rabbit secondary (1:100, Jackson Immunochemicals) followed by amplification with tyramide (1:50, Perkin Elmer). *M. tuberculosis* was identified using guinea pig anti-*M. tuberculosis* (1:25, BEI) and an Alexa 488 conjugated donkey anti-guinea pig secondary (1:100, Jackson Immunochemicals). Hoechst stain was used to visualize nuclei. Sections were mounted using Prolong Gold, allowed to cure for 24 hours in the dark, and then visualized. Images were acquired using a Leica TCS SP5 confocal microscope. This study was approved by the Institutional Review Board at the University of Texas Southwestern Medical Center at Dallas.

Mice

cGAS$^{-/-}$[228] and STING$^{gt/gt}$[236]mice were provided by Dr. Zhijian Chen. Both strains are on a C57BL/6J background. WT C57BL/6J mice were obtained from the UT Southwestern Mouse Breeding Core Facility. Mice were housed under specific pathogen-free conditions and experiments were carried out according to experimental protocols approved by Institutional Animal Care and Use Committee and the University of Texas Southwestern Medical Center at Dallas.

Strains and media

The Erdman wild type strain of *M. tuberculosis* was grown in Middlebrook 7H9 medium or on Middlebrook 7H11 agar plates supplemented with 10% oleic acid-albumin-dextrose-catalase. Tween-80 was added to liquid medium to a final concentration of 0.05%

Isolation of mouse bone marrow derived macrophages

Bone marrow cells were collected from the femurs and tibias of cGAS$^{-/-}$, STING$^{gt/gt}$, and WT mice and cultured in DMEM (Gibco) containing 10% FBS (Gibco) and 30% conditioned media from L929 cells for 7-8 days. Cells were then collected, re-suspended in DMEM containing FBS and L-glutamine, and plates on 6 or 24 well plates.

Cell maintenance and macrophage differentiation

THP-1 cells containing a stable shRNA knockdown of cGAS, STING, or a scrambled control were grown in suspension RPMI media (Gibco) supplemented with 10% FBS, 0.05% beta-mercaptoethanol (Sigma), and Pen/strep. For all experiments, cells were treated with PMA and plated at a density of 1 million cells/ml. Cells were kept at 37 degrees for 2 days at which time the media was changed to antibiotic free media. Cells were kept at 37 degrees for an additional 2 days before *M. tuberculosis* infection.

***In vitro M. tuberculosis* infections**

For macrophage infections, *M. tuberculosis* cultures were centrifuged and washed twice with PBS. Cultures were then re-suspended in a small volume of PBS and centrifuged at 500xg for 5 minutes to collect any clumps. The supernatant was transferred to a fresh tube and sonicated twice (7 seconds, 90% power). The bacteria were re-suspended in RPMI media plus 10% horse serum. Bacterial dilutions or plain media for uninfected controls were added to the macrophages and plates centrifuged for 10 minutes at 1500 RPM. Macrophages were washed with PBS and fresh macrophage media applied. Cells were incubated at 37 degrees.

Quantitative PCR

THP1 cells or BMDMs were infected at an MOI of 10. At 4 hours, infected cells were lysed using Triazol and RNA purified. cDNA was prepared using the iScript cDNA

synthesis kit (Bio-Rad). For qPCR analysis, Fast Sybr Green (Life Technologies) and an Applied Biosystems 7500 Real Time PCR machine used with gene specific primers.

Western blotting

THP-1 or BMDMs were infected at an MOI of 10. At 4 and 24 hours post infection, infected cells were lysed in 0.1% Triton. Lysates were filtered twice through a 0.2 μm filter (Light Labs) prior to removal from the bsl3 facility. cGAS was detected using a polyclonal rabbit anti-cGAS antibody (Abcam) at 1:1000. Donkey anti-rabbit HRP was used at 1:5000. Mouse anti-β-actin HRP (Santa Cruz) at 1:5000 was used as a loading control. Supersignal West Femto Chemiluminescent Substrate was used for detection of cGAS. Supersignal West Pico Chemiluminescent Substrate was used to detect β-actin.

Survival in macrophages

THP-1 macrophages were infected at an MOI of 0.1 and spun at 1500 rpm for 10 minutes. The macrophages were washed and medium without antibiotics added. Cells were washed with PBS daily and fresh medium added. THP-1 cells were lysed at 24 hours, 3 days, and 7 days post infection. Cells were lysed using 1% Triton X-100 (Sigma) and serial dilutions on to 7H11 plates (BD) made to determine CFU values.

In vitro cGAS imaging

THP-1 cells were plated on glass coverslips and infected with mCherry-labeled *M. tuberculosis* at an MOI of 1 for 4. Cells were fixed in 4% paraformaldehyde for 1 hour then transferred to PBS. Cells were fixed in cold methanol for 5 minutes, washed in PBS and blocked with Super Block for 1 hour at room temperature. Rabbit anti-cGAS antibody (Sigma) or a rabbit anti-GFP isotype matched control antibody were used at a concentration of 1:100 in blocking buffer. Coverslips were incubated overnight in primary antibody at 4 degrees Celsius. Donkey anti-rabbit HRP at a concentration of 1:500 was used as a secondary antibody. A 1:50 solution of biotin tyramide (Perkin Elmer) was applied for 10 minutes for signal amplification followed by treatment with streptavidin-488 at a concentration of 1:250 for 30 minutes. Nuclei were visualized using Hoecsht dye. Slides were mounted using Prolong Gold, protected from light and cured for 24 hours at room temperature. Images were acquired using a Leica TCS SP5 confocal microscope

GFP-LC3 lentiviral transfection and autophagy analysis

BMDMs were transfected with a GFP-LC3 plasmid. Cells were infected with mCherry-labeled *M. tuberculosis* for 4 and 15 hours, fixed with 4% paraformaldehyde, and transferred to PBS. Co-localization of LC3 with mCherry-labeled *M. tuberculosis* were then quantified.

In vivo M. tuberculosis infections

Bacteria were administered to mice via nebulization for 15 minutes. Serum collected from infected animals was filtered twice for removal from the BSL3 and cytokine levels analyzed. Lungs, liver, and spleen were collected from infected animals, homogenized in PBS, and plated on 7H11 agar plates. The remaining homogenate was centrifuged and the supernatant filtered twice for removal from the BSL3 for cytokine analysis. Sections of liver, lung, and spleen were collected and homogenized in Triazol for RNA analysis. Additional sections of lung were collected and treated with 10% normal buffered formalin for 48 hours for histology. Following formalin treatment, tissues were transferred to PBS and submitted to the Molecular Pathology Core Facility at UT Southwestern for paraffin embedding, sectioning, and H&E staining. For survival studies, mice were euthanized when they reached 15% loss of their maximum body weight[237].

Cytokine measurements

Mouse IFN-β was detected using B16 IFN-α/β reporter cell line (Invivogen). Briefly, B16 cells were grown in RPMI (Gibco) supplemented with glutamine, 10% FBS (Gibco), penicillin/streptomycin, and normocin (Invivogen).

All other cytokine levels were measured using a magpix multiplex cytokine assay.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. For *in vitro* studies, Analysis of Variance (ANOVA) tests were used for experiments with multiple comparisons. For experiments with single comparisons, two-tailed unpaired student's T tests were used. Kaplan-Meier analysis was used to analyze survival studies.

Chapter Three: Heme Oxygenase 1 as a Potential Effector Mechanism in *M. tuberculosis* infection

Introduction

M. tuberculosis is spread from person to person through aerosolized droplets[238] that are inhaled and bacteria are taken up by alveolar macrophages found in the terminal alveoli of the lungs[239]. Cytokines produced by these macrophages then recruit a large number of immune cells to the lung, including dendritic cells, B cells, T cells and additional monocytes. In the majority of cases bacterial replication is restricted, leading to latent infection[240,241]. However, in about 5% of early cases, bacterial replication is not controlled, leading to active disease. Importantly, how the host immune system and in particular what mechanisms are used by human macrophages to restrict *M. tuberculosis* growth remains poorly understood.

As discussed in the introduction, the mouse model of *M. tuberculosis* infection has been extremely valuable in furthering the understanding of macrophage anti-mycobacterial mechanisms. While there are differences in human and mouse mechanisms used to control *M. tuberculosis*, much of what is known regarding how human macrophages fight infection was first described in mouse models. However, it is still unclear if many of the sensing pathways and effector responses described in mice are important in human tuberculosis. The role of heme oxygenase 1 (HO1) in controlling *M. tuberculosis* infection is one such example of an enzyme involved in the mouse

response to TB that has yet to have a role in the human response to *M. tuberculosis* described.

HO1 catalyzes the breakdown of heme into carbon monoxide, biliverdin, and iron, with biliverdin being further broken down into bilirubin by the action of biliverdin reductase[242,243]. *In vitro* infection of mouse macrophages results in production of HO1 and *M. tuberculosis* infection in mice induces the production of HO[243,244], HO1 accumulation is observed within the macrophages in the lungs of these infected animals[243]. Additionally, HO1-knockout mice show increased susceptibility to *M. tuberculosis* infection[245,246] and *M. tuberculosis* sensing of carbon monoxide (CO), one of the products produced by HO1, can alter the bacterial transcriptional program[243,247]. While it is clear that HO1 plays a role the mouse response to *M. tuberculosis*, very little is known about its action during human infection. Based on what has been observed in mice, it was hypothesized that HO1 would be produced by human macrophages in response to *M. tuberculosis* infection and that HO1 production would be important for controlling *M. tuberculosis*.

Additionally, the level of HO1 is variable in the population due to polymorphisms within the *HO1* promoter. The number of GT repeats found in the HO1 promoter correlates with HO1 expression, with a greater number of repeats resulting in less HO1 expression and activity, and these polymorphisms are associated with outcomes of a variety of diseases[248]. Therefore, it was also hypothesized that if HO1 is important for controlling *M. tuberculosis* infection, individuals with HO1 promoter polymorphisms leading to less HO1 production would be more susceptible to *M. tuberculosis* infection.

The second part of this thesis focuses on the role of HO1 in human tuberculosis and results showing that HO1 was robustly expressed both in human tuberculosis lesions and in human macrophages in response to *M. tuberculosis* infection, and HO1 directly co-localized with *M. tuberculosis* inside infected macrophages. Moreover, HO1-regulated inflammatory cytokine production and the control of infection such that HO1 inhibition during macrophage infection led to enhanced intracellular growth of *M. tuberculosis*. Furthermore, genetic differences in the HO1 promoter were associated with increased likelihood of having active tuberculosis. Thus, individuals whose promoters contain a greater number of GT repeats, and express lower levels of HO1, were more likely to have active tuberculosis than individuals with fewer GT repeats. Together these results suggest a vital role for HO1 in controlling *M. tuberculosis* infection by mediating inflammatory cytokine expression and restricting intracellular growth.

Results

HO1 expression colocalizes with *M. tuberculosis* during chronic aerosol infection

Previous work demonstrated that HO1 is induced 10 days after intravenous infection of mice with *M. tuberculosis*[243]. To determine if HO1 expression is induced during a more physiologically relevant *M. tuberculosis* infection, a low dose aerosol infection model was used. BALB/c mice received a low dose of *M. tuberculosis* Erdman (200 CFU/mouse) and lungs were collected 21, 42, 77 and 112 days after infection. HO1 and *M. tuberculosis* expression were assessed by immunohistochemistry and immunofluorescence microscopy. By immunohistochemistry it was observed that HO1 was modestly expressed at 21 days, and expression gradually increased over time so that at 112 days it was robustly expressed within characteristic foamy macrophages (Figure 3.1). To show that HO1 and *M. tuberculosis* exist within the same cellular organelles, confocal microscopy was used to demonstrate that within infected cells, HO1 and *M. tuberculosis* co-localized, with HO1 expression often surrounding individual bacteria (Figure 3.2). Thus, in mice, HO1 expression appeared to increase over the course of infection, and HO1 and *M. tuberculosis* co-localized to the same cells and organelles.

HO1 is expressed in CD68+ cells within tissue from *M. tuberculosis*-infected individuals

To determine the role of HO1 in human tuberculosis, we first examined HO1 expression by immunohistochemistry and immunofluorescence microscopy in lung biopsies from eight *M. tuberculosis*-infected patients. All samples demonstrated an accumulation of HO1, while no HO1 expression was observed in autopsy specimens from uninfected individuals (Figure 3.3A). Staining with secondary antibody alone or with an isotype matched control also showed minimal HO1 expression. To determine if HO1 expression was increased in *M. tuberculosis*-infected macrophages, we stained serial sections for HO1, *M. tuberculosis* or the macrophage marker CD68 and found that HO1 and *M. tuberculosis*-positive cells were also CD68-positive (Figure 3.3B). By confocal microscopy we found that, similar to our observations of *M. tuberculosis*-infected mouse tissue, HO1 colocalized to *M. tuberculosis*-infected cells in lungs from patients with active tuberculosis (Fig. 3.4). Furthermore, HO1 enveloped individual bacteria in the same staining pattern as *M. tuberculosis*-infected mouse lungs (Figure 3.2). Thus, in human tuberculosis, HO1 was expressed in *M. tuberculosis*-infected, CD68+ macrophages.

M. tuberculosis infection is sufficient to induce HO1 expression in human macrophages

To ask if *M. tuberculosis* infection alone is sufficient to induce HO1 in human macrophages, we measured the regulation of HO1 by *M. tuberculosis* infection in the human macrophage cell line U937. We observed that *M. tuberculosis* infection increased *HO1* transcription by using quantitative PCR (Figure 3.5A) and resulted in an accumulation of HO1 protein by Western blotting (Figure 3.5B). We did not observe HO1 induction in mock-infected cells, while robust HO1 expression was induced when cells were treated with heme, a compound known to induce HO1 expression. We next infected cells with mCherry expressing *M. tuberculosis*, and found by confocal microscopy that *M. tuberculosis*-infected U937 cells induced HO1 expression in a very focal distribution compared to cells treated with heme (Figure 3.6). Whereas heme treated cells demonstrated diffuse HO1 staining throughout the cell, HO1 induction following *M. tuberculosis* infection co-localized with *M. tuberculosis*. In particular, HO1 staining surrounded individual bacteria in infected macrophages (Figure 3.6). Thus, *M. tuberculosis* infection of human macrophages *in vitro* up-regulated HO1 expression, and HO1 was targeted to *M. tuberculosis*-containing organelles.

HO1 inhibition prevents inflammatory cytokine production by *M. tuberculosis*-infected macrophages

To explore the functional significance of HO1 activity during *M. tuberculosis* infection, we infected U937 macrophages in the presence or absence of the HO1 inhibitor tin protoporphyrin (SnPP) and determined the effect on inflammatory cytokine production. SnPP blocks the active site of HO1 leading to competitive inhibition with a nanomolar Ki[249]. As has been previously observed, *M. tuberculosis* infection of human macrophages induced a number of inflammatory cytokines including TNFα, IL-1β, and IL-6 (Figure 3.8). Surprisingly, inhibition of HO1 activity significantly decreased the accumulation of many inflammatory cytokines (Figures 3.7, 3.8, 3.9). In particular, IL-1β, TNFα, and IL-6 showed the greatest fold reduction following inhibition (Figure 3.8). Many of the additional cytokines we tested were not significantly induced by *M. tuberculosis* infection (Figure 3.9). Thus, we conclude that HO1 expression is important for inflammatory cytokine production following *M. tuberculosis* infection.

HO1 activity is required to restrict *M. tuberculosis* growth within infected macrophages

Because HO1-deficient mice are hyper-susceptible to *M. tuberculosis* infection[245,246] and *M. tuberculosis* expresses a gene that protects against CO toxicity[250], we hypothesized that HO1 in human macrophages might have antimicrobial

activity. We first confirmed that SnPP alone had no direct effect on *M. tuberculosis* viability by growing the bacteria with and without SnPP added to the media and performing CFU assays over the course of 6 days. No significant growth difference was seen between bacteria treated with SnPP and untreated controls (not shown). We next infected U937 cells with *M. tuberculosis* in the presence or absence of SnPP and quantitated CFU from infected macrophages at days 3 and 6 post-infection. We found that SnPP treatment led to approximately 3 fold higher CFU 6 days after infection (Figure 3.10). Thus, we conclude that HO1 activity plays a role in controlling *M. tuberculosis* infection of human macrophages.

HO1 promoter polymorphism influences susceptibility to active tuberculosis

HO1 expression in response to external stimuli varies in humans due to polymorphism of a GT-rich region within the promoter[248]. Promoters with fewer GT repeats demonstrate increased HO1 expression whereas promoters with longer GT repeats express less HO1[251,252] (Figure 3.11A). To test the hypothesis that HO1 expression affects susceptibility to active tuberculosis, we sequenced the GT-rich promoter region from individuals with active tuberculosis and their disease-free close contacts from the Houston Tuberculosis Initiative [253-256]. The later cohort included individuals with either latent infection (PPD+, CXR-) or without infection (PPD-). We classified alleles by the number of GT repeats present as “short” (<27 repeats), “medium” (27-33 repeats), or “long” (>33 repeats) as has been reported previously [257] (Figure 3.11B).

There were between 21 and 43 GT repeats in the population of individuals studied. In line with previously published studies[258,259], a tri-modal distribution was observed with peaks at 23, 30 and 39 repeats (Figure 3.11B). In uninfected individuals, 23.5% of alleles were “S”, 48.2% were “M”, and 28.3% were “L”. In the *M. tuberculosis*-infected population, 22.8% of the alleles were “S”, 36.2% were “M”, and 40.9% were L. The frequency of the “L” allele was significantly higher in *M. tuberculosis*-infected individuals (N= 113, 40.9%), when compared to uninfected individuals (N=77, 28.3%) ($p<0.005$). The odds ratio of *M. tuberculosis*-infected individuals with the L allele was 1.76 (95% CI, 1.23-3.5) (Table 1).

Based on the “S-M-L” classification of alleles, six genotypes were defined (SS, SM, SL, MM, ML, and LL). The proportion of L-allele carriers was significantly higher among the *M. tuberculosis*-infected individuals. Thus, presence of the “L” allele is associated with an increased susceptibility to *M. tuberculosis* infection (Figure 3.11C, 3.11D).

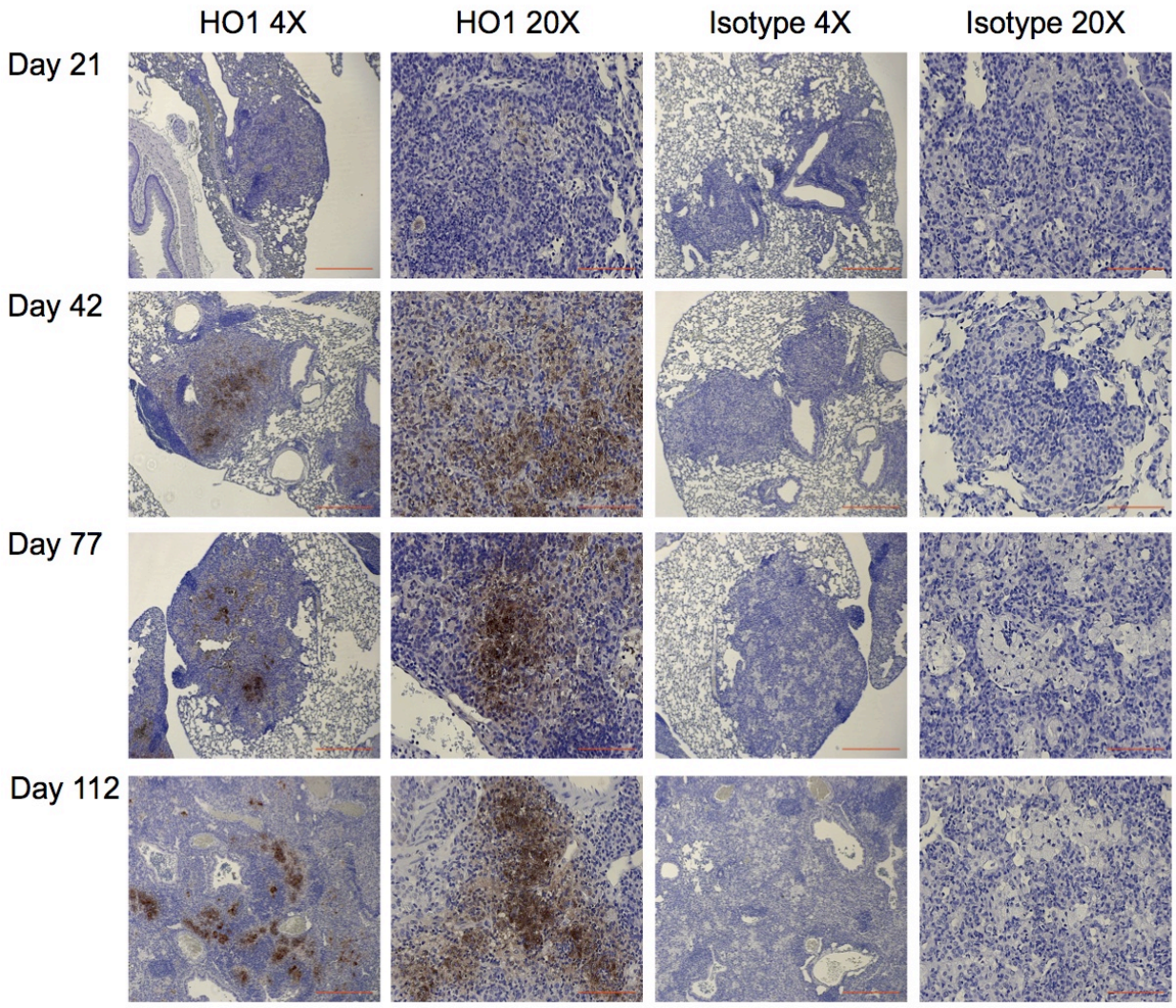


Figure 3.1: HO1 expression in *M. tuberculosis*-infected mouse lung

BALB/c mice were infected with *M. tuberculosis* via a low dose aerosol infection and lungs harvested at days 21, 42, 77, and 112. Shown is immunohistochemistry of paraffin sections with anti-HO1 (brown) and counterstained with hematoxylin (purple). Scale bars are 500um for 4x images and 100 um for 20x images.

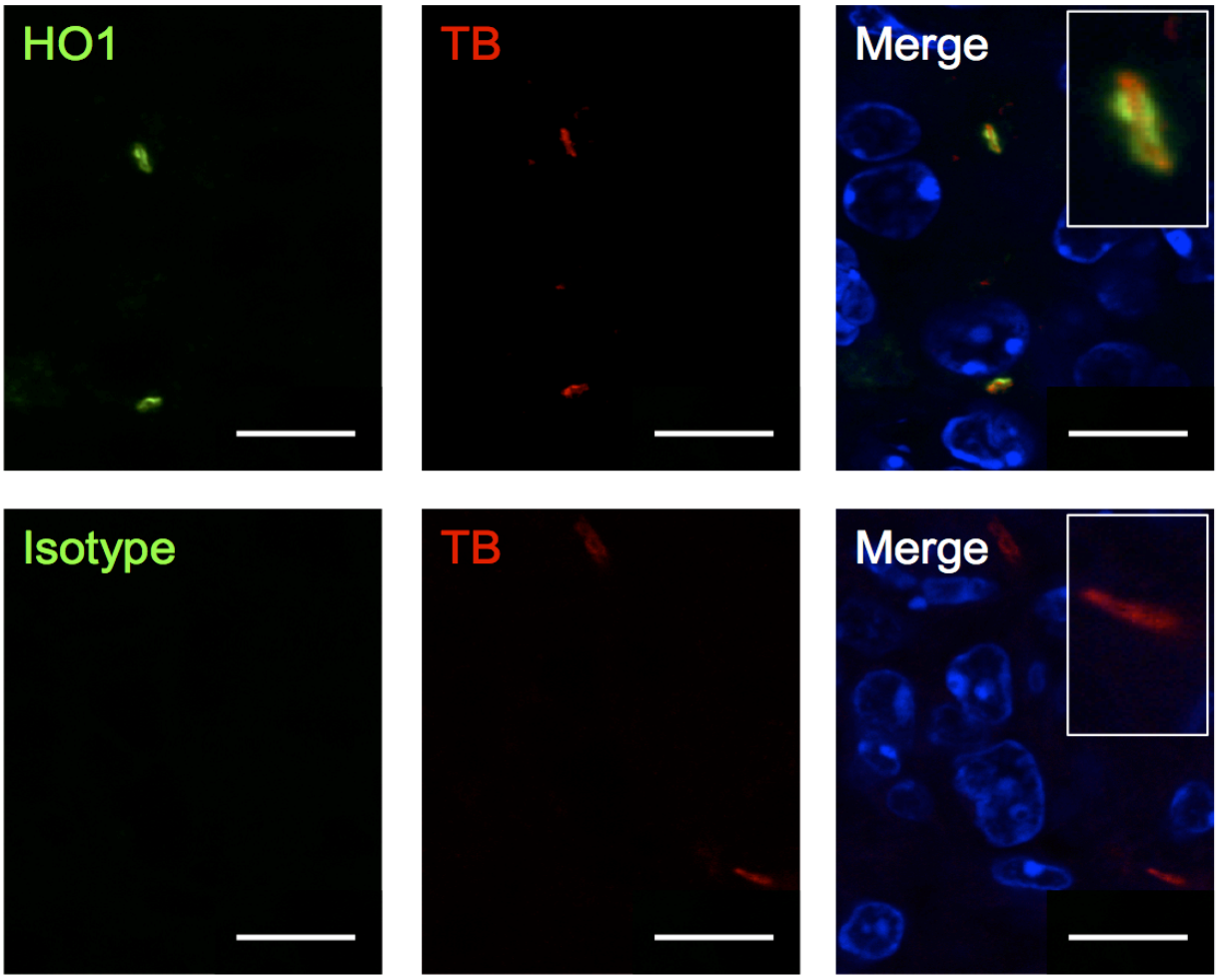


Figure 3.2: **HO1 colocalizes to *M. tuberculosis*-infected macrophages in an aerosol model of infection.**

Paraffin embedded tissue samples from *M. tuberculosis* individuals were deparaffinized and subjected to heat-mediated antigen retrieval. Slides were costained using a rabbit anti-cGAS antibody or isotype control and a guinea pig anti-*M. tuberculosis* antibody. Slides were imaged for immunofluorescence. Scale bars are 10μm.

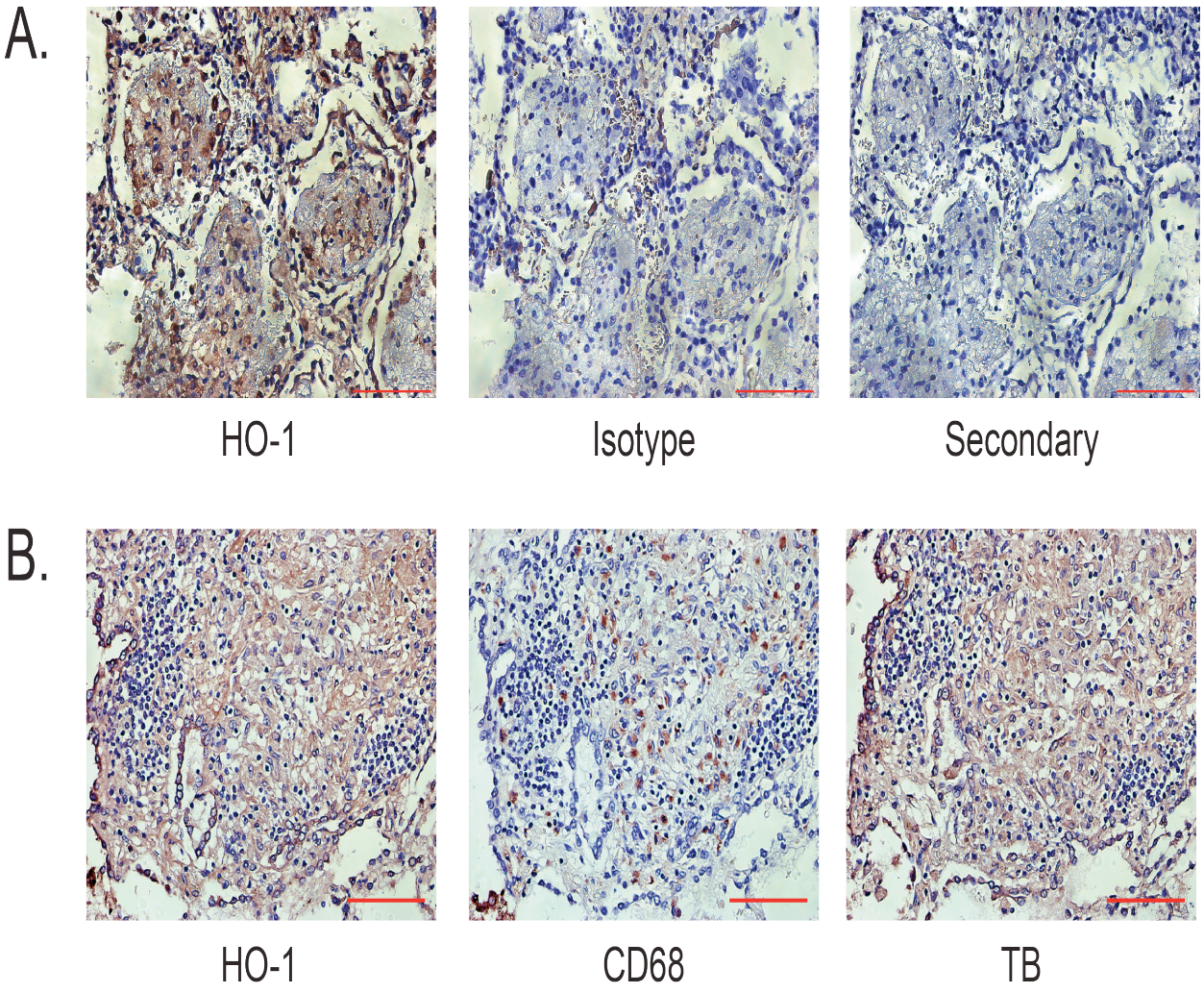


Figure 3.3: HO1 is expressed in *M. tuberculosis*-infected CD68+ human specimens.

(A) Immunohistochemistry was performed on paraffin embedded tissue samples from *M. tuberculosis*-infected individuals using anti-HO1 antibody and DAB detection (brown). Sections were counterstained with hematoxylin. Control specimens were treated with either isotype control or secondary antibody alone. Scale bars are 100 μm. **(B)** Serial sections were stained with either anti-HO1, anti-CD68, or anti-*M. tuberculosis* as in A.

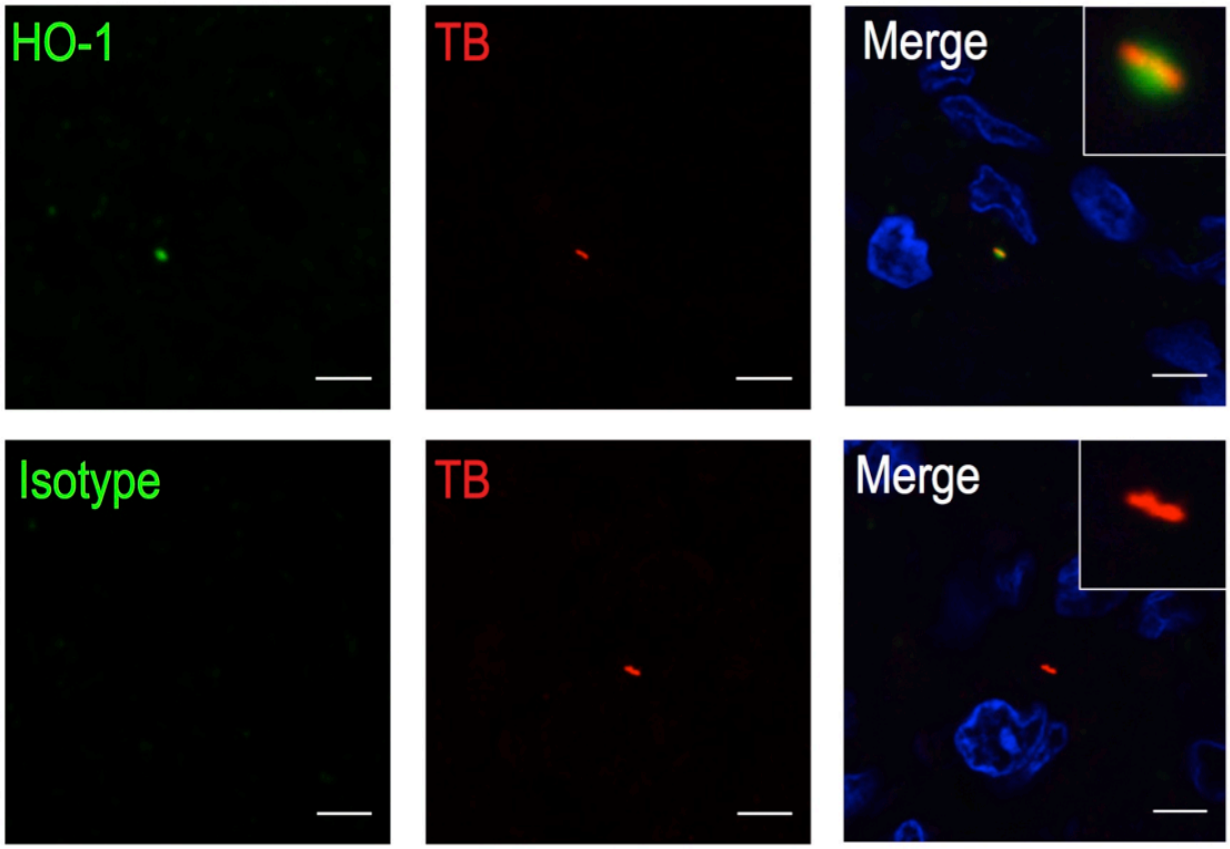


Figure 3.4: **HO1 colocalizes with *M. tuberculosis* in infected human tissue samples.**

Paraffin embedded tissue samples from *M. tuberculosis* individuals were deparaffinized and subjected to heat-mediated antigen retrieval. Slides were costained using a rabbit anti-cGAS antibody or isotype control and a guinea pig anti-*M. tuberculosis* antibody. Slides were imaged for immunofluorescence. Scale bars are 10μm.

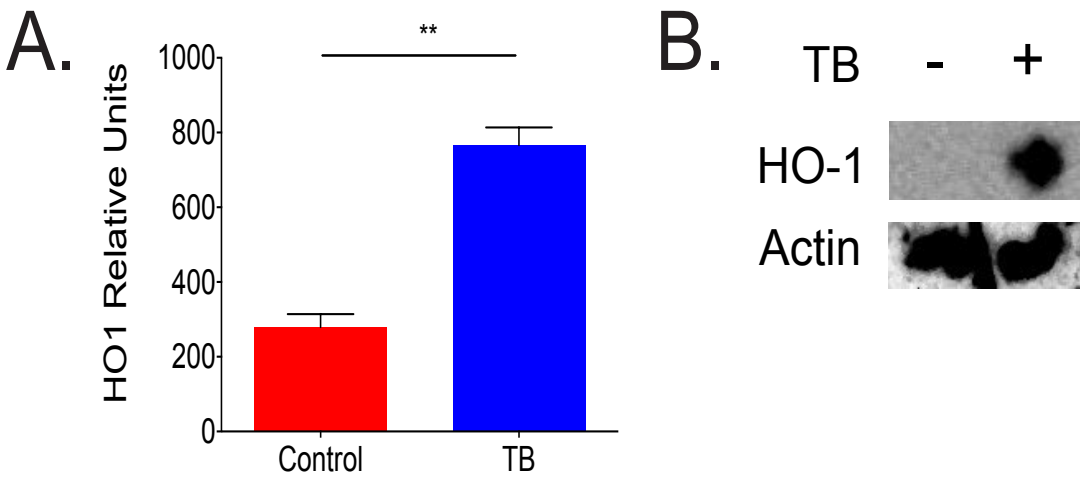


Figure 3.5: *M. tuberculosis* infection is sufficient to induce HO1 expression in human macrophages.

(A) qPCR analysis of HO1 gene expression in human U937 cells following *M. tuberculosis* infection. Cells were infected for 24 hours with the Erdman strain of *M. tuberculosis*. RNA was collected from each sample and qPCR analysis was performed using Sigma KiCqStart primers specific for HO1. **P = < 0.005. **(B)** Western blot of lysates from human U937 cell infected for 24 hours with the Erdman strain of *M. tuberculosis* or mock infected with PBS were probed with anti-HO1 or anti-actin antibody.

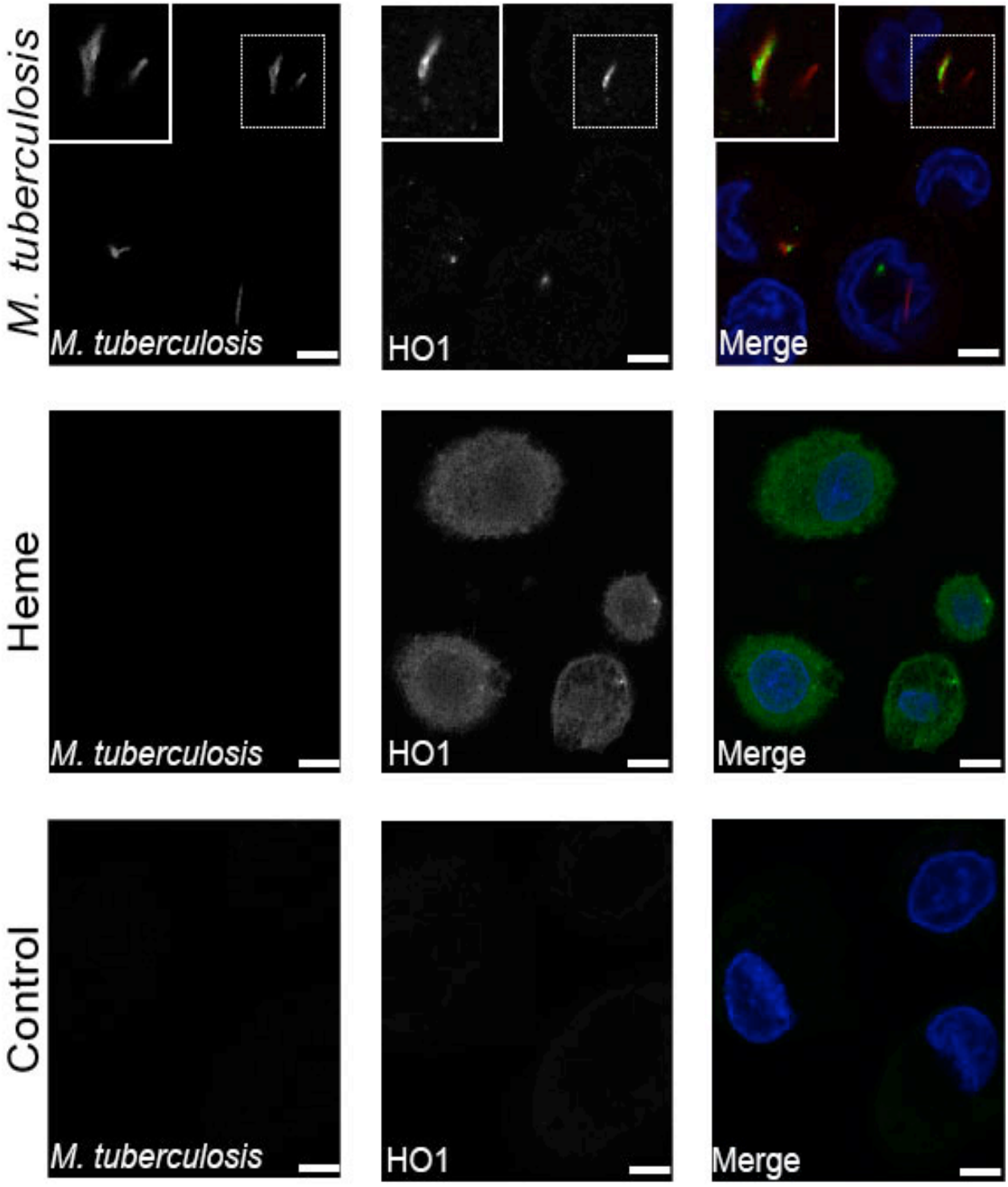

M. tuberculosis
Heme
Control
M. tuberculosis
HO1
Merge
M. tuberculosis
HO1
Merge
M. tuberculosis
HO1
Merge

Figure 3.6: HO1 colocalizes with *M. tuberculosis* after infection of U937 cells.

U937 cells were infection with mcherry expressing *M. tuberculosis* for 24 hours. Cells were fixed in 4% paraformaldyhyde and transferred to PBS. HO1 was detected using anti-HO1 antibody (green) and nuclei were staining using Hoechst dye. Scale bars are 10 μm

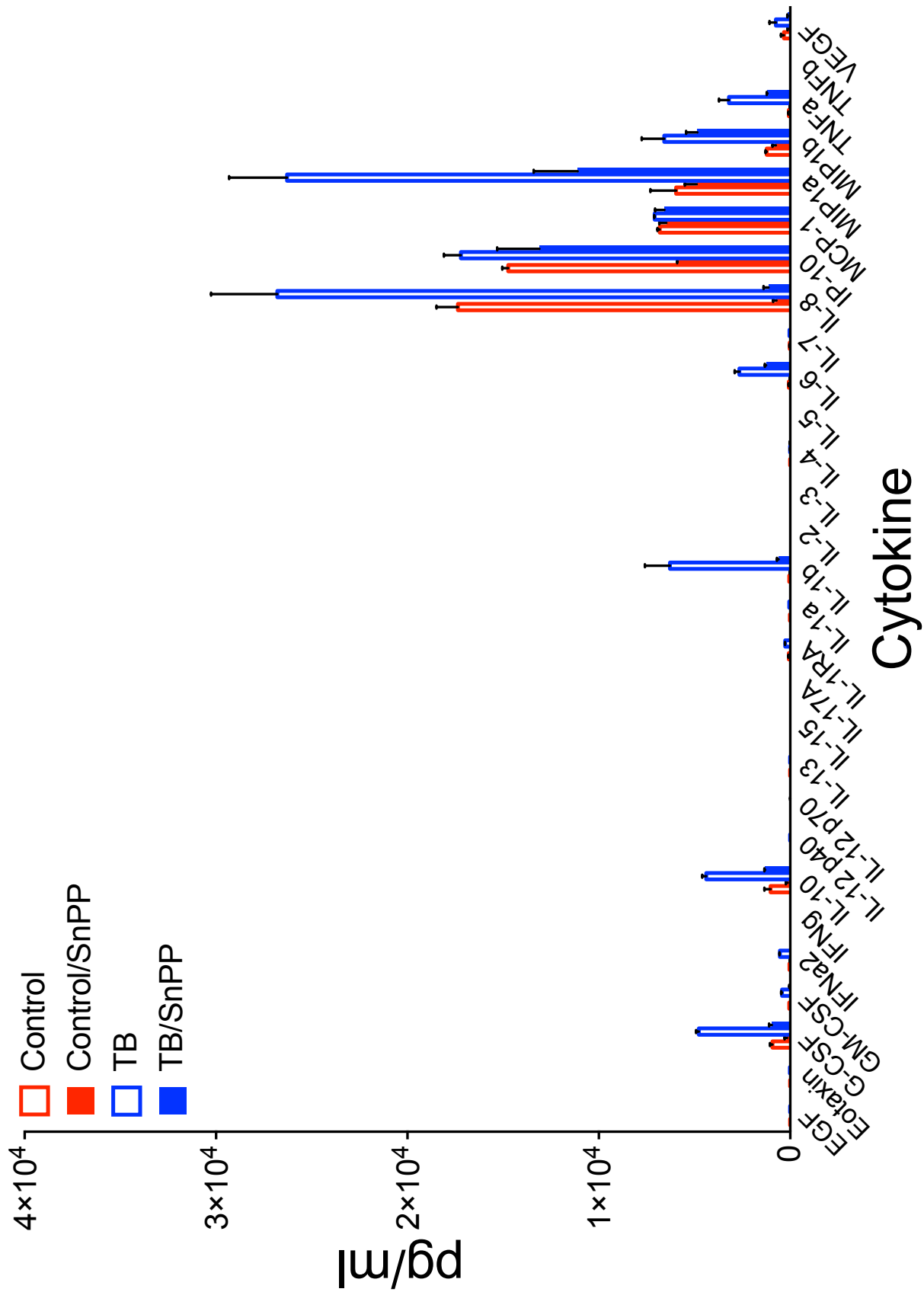
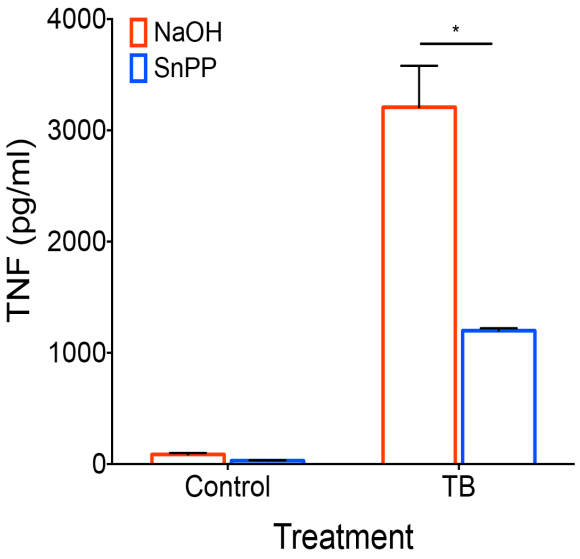
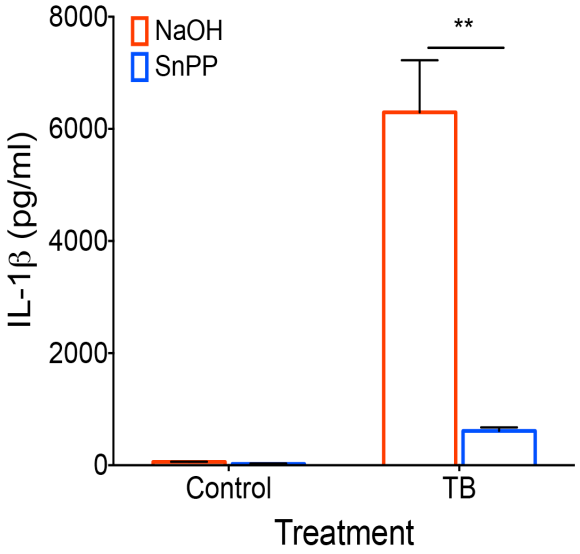

Control
Control/SnPP
TB
TB/SnPP
pg/ml
4×10^4
3×10^4
2×10^4
1×10^4
0
Cytokine
EGF
Eotaxin
G-CSF
GM-CSF
IFNa2
IFNg
IL-10
IL-12p40
IL-12p70
IL-13
IL-15
IL-17A
IL-1RA
IL-1a
IL-1b
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IP-10
MCP-1
MIP1a
MIP1b
TNFa
TNFb
VEGF

Figure 3.7: HO1 inhibition reduces inflammatory cytokine production following *M. tuberculosis* infection of U937 macrophages

U937 cells were infected with Mtb or mock infected with PBS with and without the presence of tin protoporphyrin (SnPP) for 24 hours. Supernatants were collected and assayed for a panel of inflammatory cytokines using Magpix multiplexing technology. HO1 inhibition reduced expression of 20 of the 29 cytokines tested.


4000
3000
2000
1000
0
TNF (pg/ml)
NaOH
SnPP
*
Control
TB
Treatment


8000
6000
4000
2000
0
IL-1β (pg/ml)
NaOH
SnPP
**
Control
TB
Treatment

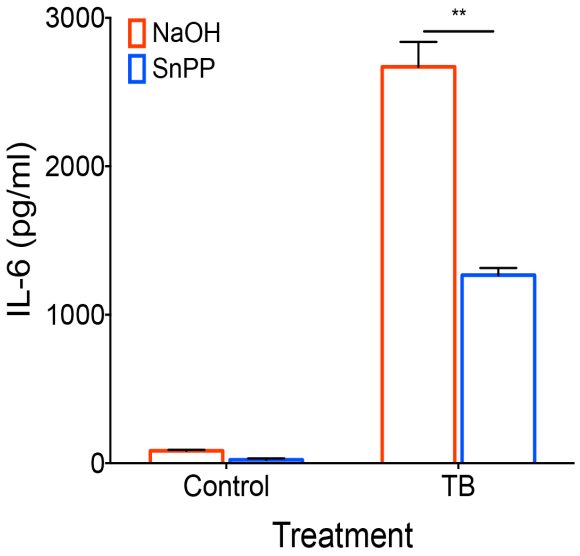

3000
2000
1000
0
IL-6 (pg/ml)
NaOH
SnPP
**
Control
TB
Treatment

Figure 3.8: HO1 inhibition reduces production of IL-6, TNFα, and IL-1β following infection with *M. tuberculosis.*

U937 cells were infected with Mtb or mock infected with PBS with and without the presence of tin protoporphyrin (SnPP) for 24 hours. Supernatants were collected and assayed for a panel of inflammatory cytokines using Magpix multiplexing technology. IL-6, TNFα, and IL-1β showed the greatest fold reduction following inhibition. *$p = < 0.05$. **$p = < 0.005$.

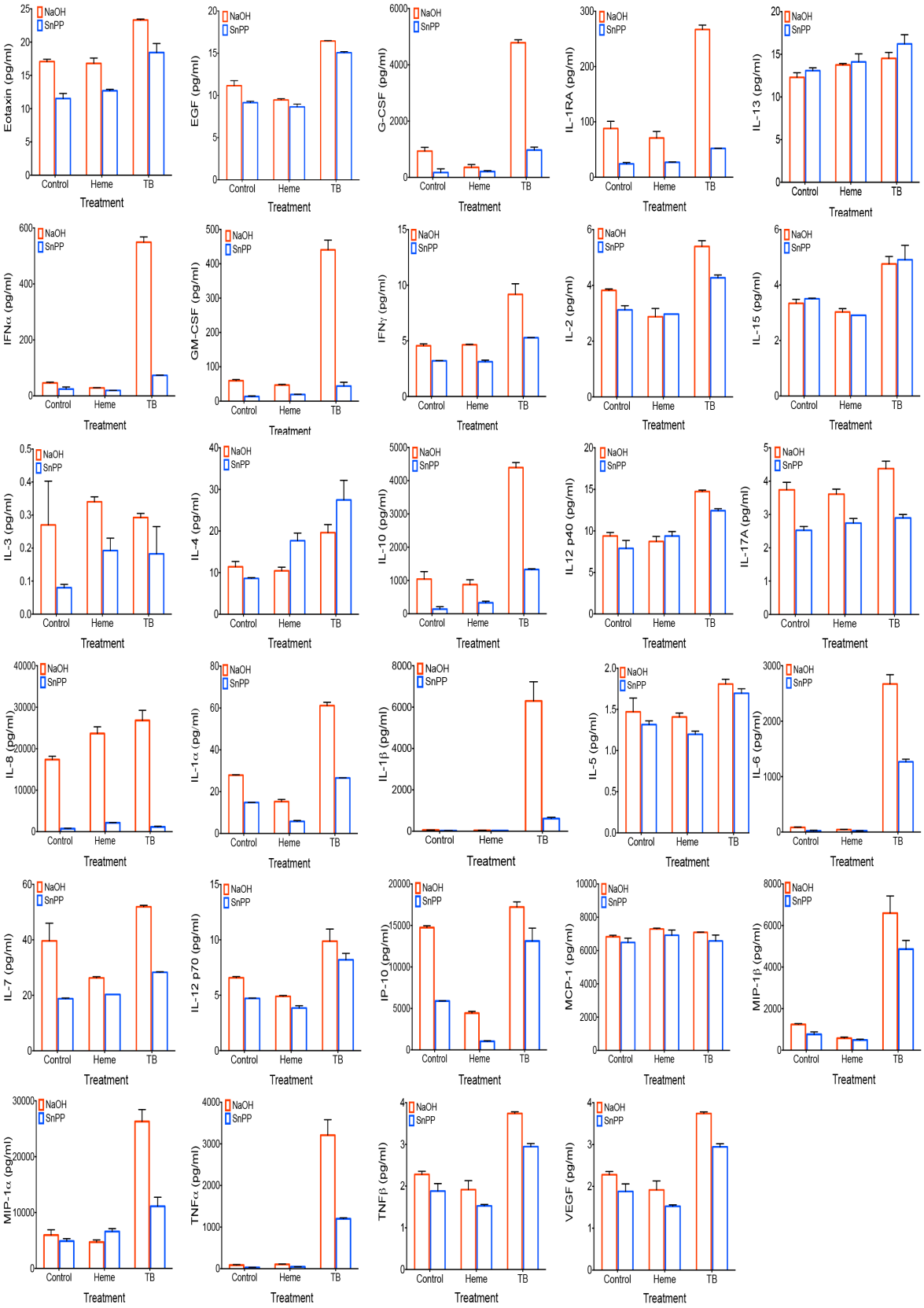


Figure 3.9: Individual results for all cytokines examined in figure 3.7.

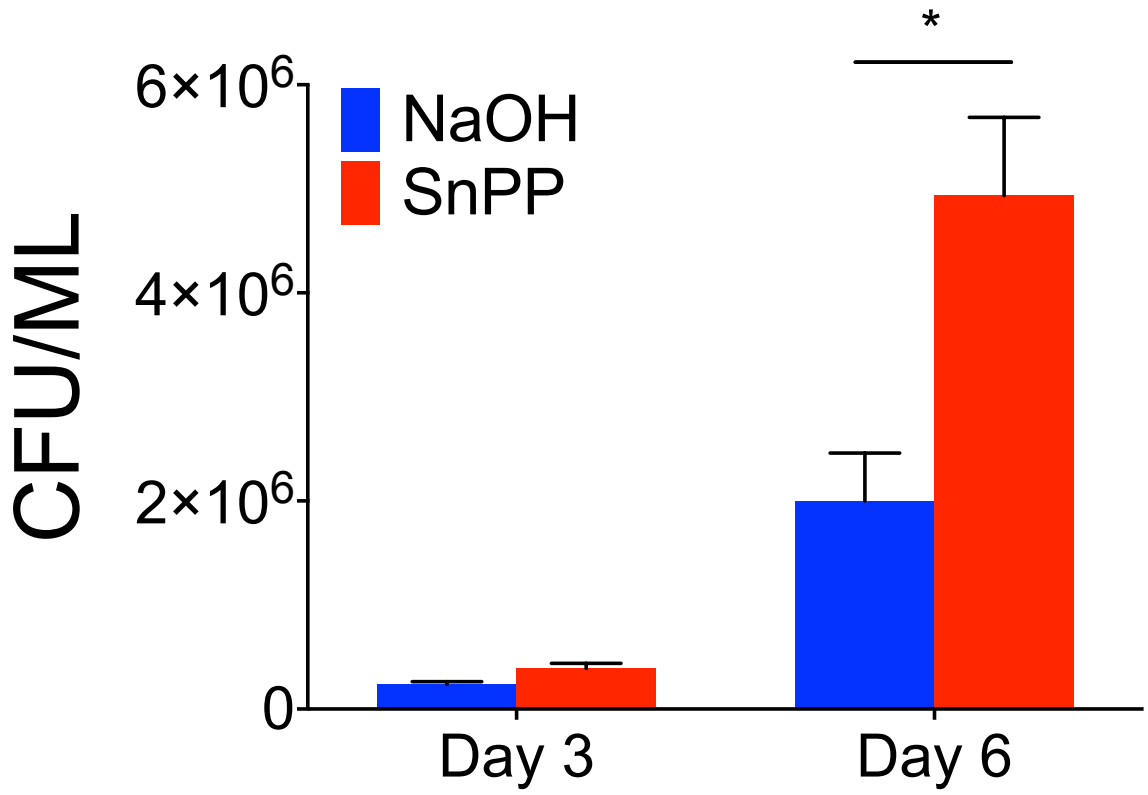


Figure 3.10: HO1 inhibition is associated with decreased control of *M. tuberculosis in vitro.*

U937 cells were infected with Mtb at an MOI of 0.1 for 2 hours at 37°C. Macrophages were washed and fresh media added. Macrophages were lysed on days 3 and 6 post infection and intracellular Mtb enumerated on 7H10 plates. *p = < 0.05.

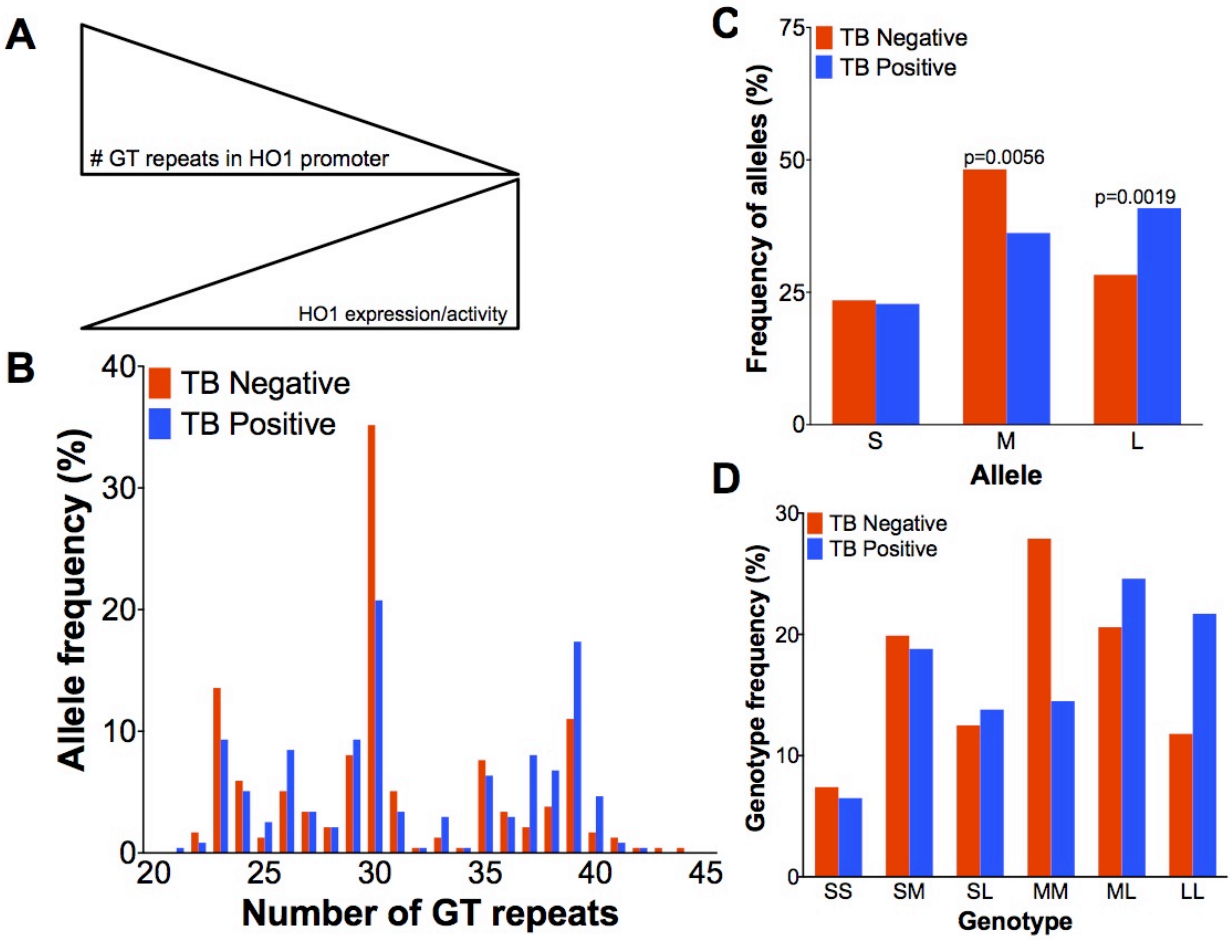


Figure 3.11: Longer GT repeats and presence of the "L" allele in the HO promoter are associated with an increased incidence of active *M. tuberculosis* infection.

(A) There is an inverse relationship between the number of GT repeats in the HO1 promoter and HO1 expression (ref) **(B)** Frequency distribution of the number of GT repeats in the HO1 promoter in Mtb positive and Mtb negative individuals. **(C)** Each allele was classified by the number of GT repeats present as "short" (<27 repeats), "medium" (27-33 repeats), or "long" (>33 repeats) as reported previously (ref) and the frequency of each allele was determined. **(D)** Based on the "S-M-L" classification of alleles, the distribution of the six possible genotypes (SS, SM, SL, MM, ML, and LL) in Mtb positive and Mtb negative individuals was determined.

Table 1. Association between Mtb status and the different alleles for the GT repeat region of the HO1 promoter

Allele Class	TB Negative	TB Positive	OR (95% CI)	p-value
S (<27 repeats)	64 (23.5%)	63 (22.8%)	0.93 (0.62-1.38)	0.7622
M (27-33 repeats)	131 (48.2%)	100 (36.2%)	0.61 (0.43-0.86)	0.0056
L (>33 repeats)	77 (28.3%)	113 (40.9%)	1.76 (1.23-2.51)	0.0019

Discussion

We, and others, have shown previously that HO1 is induced shortly after *M. tuberculosis* infection in mice[243,244]. HO1-deficient mice are less able to control mycobacterial infection compared to wild type mice as indicated by increased bacterial numbers and mortality following infection[245,246]. Here we demonstrate that the production of HO1 continued throughout the course of a chronic low dose mouse infection. Modest HO1 expression was observed at day 21 and increases over time so that by day 120, robust levels of HO1 were observed. Additionally, HO1 colocalized with *M. tuberculosis* within mouse macrophages, appearing to surround the bacteria in what is likely the phagosome. Taken together this data further supports an important role for HO1 in controlling *M. tuberculosis* in mice.

Human tuberculosis and experimental murine tuberculosis infection differ in many ways, including how infected macrophages kill *M. tuberculosis*[260,261], what cell types are found in experimental infections[262], how *M. tuberculosis* antigens are presented to the adaptive immune system[263,264] and the architecture of infected tissue [9]. The experimental models used in mice such as intravenous infection could potentially explain some of these differences. However, even the aerosol mouse model, while more physiological, still differs significantly from human infection[265,266]. Thus, it is important to test if the functions of host genes identified in mouse models are shared in humans.

To determine the role of HO1 in human tuberculosis, we examined tissue samples from individuals infected with *M. tuberculosis* and determined that HO1 is

expressed in human tuberculosis lesions. Similar to its localization in the lungs of mice infected with tuberculosis, HO1 colocalized with *M. tuberculosis* in human granulomas both at the cellular and subcellular level. The observation that HO1 is expressed within infected human lungs is consistent with the recent finding that plasma HO1 levels are elevated in human tuberculosis patients, and that HO1 levels return to baseline following successful treatment [267].

Because HO1 is known to be up-regulated by a variety of stimuli, we wanted to ask if *M. tuberculosis* infection alone is sufficient to induce HO1. We found that that in both human macrophage-like cell lines and primary macrophages, HO1 is increased following infection and colocalizes with *M. tuberculosis* to the same subcellular organelle.

HO1 is important to control a variety of infections in mice, including *M. tuberculosis*[246],*Listeria monocytogenes*[268], *Plasmodium falciparum*[269] and *Toxoplasma gondii* [270]. In human cells, HO1 can inhibit the replication of viruses such as HIV[271], Ebola[272] and HCV[273]. However, a direct role for HO1 in the control of *M. tuberculosis* in human infection has not previously been established. We found that treatment of *M. tuberculosis*-infected human macrophages with an HO1 inhibitor reduced their ability to restrict intracellular *M. tuberculosis* growth, supporting the hypothesis that HO1 activity is necessary for human macrophages to control *M. tuberculosis* infection. This is also consistent with our recent finding that *M. tuberculosis* encodes for a CO resistance gene that when mutated results in attenuated virulence in mice[250].

Previously, HO1 was found to regulate expression of multiple cytokines[242]. In purely inflammatory disease models, HO1 is anti-inflammatory [274,275]. Splenocytes from HO1-knockout mice secrete increased levels of pro-inflammatory cytokines following mitogenic stimulation with LPS or anti-CD3/anti-CD28 antibodies [276]. Additionally, HO1 transgenic mice that constitutively express HO1 in the lung show a significant reduction in the production of pro-inflammatory cytokines and chemokines in response to hypoxia[277]. However, in microbial infectious disease models, recent studies suggest HO1 may be involved in the induction of inflammatory cytokines. In mice, conditional deletion of HO1 from myeloid cells results in impaired IFNβ production as well as decreased production of IRF-3-dependent target genes such as RANTES, IP-10, and MCP-1 in peritoneal macrophages in the setting of both viral and bacterial infection[278]. Additionally, HIV infection of LPS-activated primary human monocyte-derived macrophages leads to increased HO1 expression and increased levels of the inflammatory cytokines MIP1α and MIP1β which are attenuated following treatment with the HO1 inhibitor SnPP[271]. In this this study we found that inhibition of HO1 concurrent with *M. tuberculosis* infection suppressed the accumulation of multiple inflammatory cytokines following infection. This supports the hypothesis that in the context of microbial infection, HO1 acts as an important early component of the innate immune response leading to the production of pro inflammatory cytokines.

M. tuberculosis-infected macrophages produce a variety of inflammatory cytokines including IFN-β, IL-1β, IL-6, and TNF[279]. While we demonstrate that the production of these cytokines was significantly reduced following HO1 inhibition, what

remains unclear is the mechanism behind this inhibition. One possibility is that HO1-dependent production of pro-inflammatory cytokines is mediated through one of the products of HO1, such as iron, biliverdin or CO. Alternatively; cytokine induction may be due to direct interaction of HO1 with other host molecules. For example, *M. tuberculosis* can directly activate the cytoplasmic DNA surveillance pathway resulting in IRF-3 phosphorylation and IFN-β production by infected macrophages [100]. Interestingly, HO1 directly interacts with IRF3, and HO1-deficient macrophages show a reduction in the expression of IFNβ and other IRF3 target genes [278]. Whether inhibition of HO1 activity would also alter binding to, and nuclear translocation of, IRF-3 is unknown. Thus, the reduction in inflammatory cytokine production following HO1 inhibition may be due to decreased IRF3 activation, resulting in a decreased type 1 interferon response.

A number of genetic determinants for *M. tuberculosis* susceptibility have been described[280,281] including polymorphisms in the genes for TNFα[282,283], IL-1β[284], and IL-6[285]. Furthermore, TNFα blockade is an established risk factor for developing active tuberculosis[286]. Interestingly, we found that HO1 activity mediates the production of these key cytokines by infected macrophages and suggests that in addition to a potential antimicrobial role for HO1 derived CO, another role for HO1 is via regulation of pro-inflammatory cytokines.

The promoter for HO1 is polymorphic, containing a region of GT repeats ranging from 11-40. It has consistently been shown that higher numbers of GT repeats are associated with higher levels of HO1 expression[251,252,287]. The number of GT repeats in the promoter has been associated with susceptibility to bacterial pneumonia, with

individuals with a greater number of GT repeats, and thus lower levels of HO1 expression, showing increased susceptibility to infection[257]. Additionally, greater numbers of GT repeats have been associated with higher viral levels in HIV-infected individuals[288]. Consistent with its role in regulating inflammatory cytokines and intracellular control of *M. tuberculosis* infection, we found that a longer promoter containing a higher number of GT repeats and thus less HO1 predicts increased likelihood of active tuberculosis. Intriguingly, we also found that individuals with an intermediate GT repeat (M-allele) seemed to be protected from active tuberculosis. This observation is reminiscent of the recent report of improved survival from tuberculous meningitis in individuals heterozygous for a promoter polymorphism in leukotriene A(4) hydrolase (LTA4H), which regulates the balance between pro- and anti-inflammatory eicosanoids [289]. Patients homozygous for either high or low LTA4H activity were less likely to survive tuberculous meningitis than heterozygotes owing to lack of bacterial killing or excessive inflammation respectively [289]. Thus, HO1 expression may function similarly, facilitating appropriate expression of inflammatory cytokines in the setting of human tuberculosis.

In conclusion, we show that HO1 is an important host molecule in human tuberculosis that functions to mediate pro-inflammatory cytokine production and restrict intracellular bacterial replication. Individuals that have a promoter polymorphism associated with reduced HO1 expression are at increased risk of developing active tuberculosis. Chemical induction of HO1 may therefore be a novel host-directed therapeutic approach in the treatment of tuberculosis.

Materials and methods

Strains and media

The Erdman wild type strain of Mtb was grown in Middlebrook 7H9 medium or on Middlebrook 7H10 agar plates supplemented with 10% oleic acid-albumin-dextrose-catalase. Tween-80 was added to liquid medium to a final concentration of 0.05%.

Cell maintenance and macrophage differentiation

U937 cells were grown in suspension in RPMI media supplemented with 10% FBS and pen/strep. For all experiments cells were treated with PMA and plated at a density of 1 million cells/ml. Cells were kept at 37°C for 2 days at which time the media was changed to antibiotic free media. Cells were kept at 37°C for an additional 2 days before Mtb infection.

Mouse infections

We infected BALBc mice (Jackson Laboratories) using a Glas-Col aerosol exposure chamber to deliver ~200 bacilli per mouse. Prior to aerosolization, bacteria were washed repeatedly and sonicated to generate a single-cell suspension. At day zero, we plated total organ homogenates from both lungs (5 mice per group) to determine the initial inoculum. At subsequent time points, lungs from infected animals

were fixed in formalin for 24 hours and paraffin embedded. Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas (UT) Southwestern.

Immunohistochemistry and immunofluorescence of tissue specimens

Sections from paraffin embedded mouse and human lung were deparaffinized in xylene, subjected to heat-mediated antigen-retrieval in 10 mM sodium citrate (pH 6.0), permeabilized in 0.2% Triton-100 (Sigma) and blocked in 5% donkey sera. For immunohistochemistry, sections were stained with rabbit anti-HO1 (1:50, ADI-SPA-896-F Enzo Life Sciences), rabbit anti-HA (1:50, sc-805 Santa Cruz), or mouse anti-CD68 (1:100, ab955 Abcam). HRP conjugated secondary antibodies were obtained from Jackson Immunochemicals and used at 1:250. Staining was amplified with AB reagent (Vectastain) and detected using DAB reagent (Thermo Scientific). Images were acquired using a Zeiss Axioplan 2 microscope. For immunofluorescence, HO1 was identified using rabbit anti-HO1 (1:50) and an HRP conjugated donkey anti-rabbit secondary (1:100, 711-035-152 Jackson Immunochemicals) followed by amplification with tyramide (1:50, Perkin Elmer). Mtb was identified using guinea pig anti-Mtb (1:25, NR-13818, NR-13823 BEI) and an Alexa 488 conjugated donkey anti-guinea pig secondary (1:100, 706-545-448 Jackson Immunochemicals). Images were acquired using a Leica TCS SP5 confocal microscope.

Immunofluorescence of macrophages infected in vitro

U937 cells were differentiated as described above. Cells were infected at an MOI of 10 or treated with 200 um hemin (Sigma). At 24 hours after infection, cells were washed with PBS and fixed in 4% paraformaldehyde (Alfa Aesar). Cells were permeabilized with methanol and blocked with SuperBlock Blocking Buffer (Thermo Scientific). HO1 was identified using rabbit anti-HO1 (1:100) and an HRP conjugated donkey anti-rabbit secondary (1:500, 711-035-152 Jackson Immunochemicals) followed by amplification with tyramide (1:100, Perkin Elmer). Mtb was identified using guinea pig anti-Mtb (1:100, NR-13818, NR-13823 BEI) and an Alexa 488 conjugated donkey anti-guinea pig secondary (1:500, 706-545-448 Jackson Immunochemicals). Images were acquired using a Leica TCS SP5 confocal microscope.

Western blotting

U937 were infected at an MOI of 10 or treated with 200 um hemin (Sigma). At 24 hours after infection, cells were lysed in 0.5% Triton-100X (Sigma) plus protease inhibitors (Roche) and all samples boiled for 30 minutes. Blotting was performed with rabbit anti-HO1 polyclonal antibody (1:1000, Enzo Life Sciences) or anti-β actin antibody (Santa Cruz) and donkey anti-rabbit secondary (1:5000, Jackson Immunobio). Supersignal West Femto Chemiluminescent Substrate (Pierce) were used for signal detection.

Analysis of $(GT)_n$ repeats in the HO1 promoter

Clinical samples were obtained from the Houston Tuberculosis Initiative and included 138 Mtb infected (culture positive) individuals or 136 of their close contacts that were Mtb (culture) negative. DNA samples were extracted from whole blood using Nucleon DNA extraction and purification kits (Amersham International). The 5'-flanking region of the HO-1 promoter region was amplified by PCR with a fluorescently labeled primer (5′-FAM-CCAGCTTTCTGGAACCTTCTG) and an unlabeled antisense primer (5′-GAAACAAAGTCTGGCCATAGGA). Results were confirmed using a second a fluorescently labeled primer (*5′*-FAM-AGAGCCTGCAGCTTCTCAGA- 3′*)* and an unlabeled antisense primer *(*5′-ACAAAGTCTGGCCATAGGAC-3′*).* Both primer sets were designed using published sequences [252,290]. The total number of GT repeats present at each allele was determined for each patient. Using classifications found in the current literature, each allele was classified by the number of GT repeats present as "short" (<27 repeats), "medium" (27-33 repeats), or "long" (>33 repeats) and the frequency of each allele was determined. Based on the "S-M-L" classification of alleles, six genotypes were defined (SS, SM, SL, MM, ML, and LL). Distribution of genotypes in Mtb positive and Mtb negative individuals was then determined.

Quantitative PCR

U937 or THP-1 cells were infected at an MOI of 10. At 24 hours, infected cells were lysed using Triazol and the RNA purified. cDNA was prepared using the iScript

cDNA synthesis kit (Bio-Rad). For qPCR analysis, Fast Sybr Green (Life Technologies) was used. Primers were from Sigma. qPCR was performed on an Applied Biosystems Vii7 using the following primers:

HO-1 forward (5'-CAACAAAGTGCAAGATTCTG-3'),

HO-1 reverse (5'-TGCATTCACATGGCATAAAG-3'),

IFN-β forward (5'CGCTGCGTTCCTGCTGTGCTT-3'),

IFN-β reverse (5'-AGGTGAGGTTGATCTTTCCATTCA-3').

Magpix cytokine analysis

U937 cells were infected at an MOI of 10 for 2 hours. Cells were washed with PBS and fresh medium +/- SnPP. At 24 hours, conditioned medium was collected and filtered 2 times through a 0.22 micron filter. Cytokine levels were measured using a 29-plex Magpix cytokine assay (Millipore HCYTMAG-60K-PX29).

Survival in macrophages

U937 macrophages were infected at an MOI of 0.1 for 2 hours in 10% horse serum in RPMI. Cells were washed and macrophage medium without antibiotics (± 50 uM SnPP) was then replaced. To avoid the need for antibiotics, cells were washed with PBS daily and fresh medium ± SnPP added. On days 3 and 7, cells were lysed using 1% Triton X-100 (Sigma) and serial dilutions performed to determine CFU values.

Human studies

The Institutional Review Boards of the University of Texas Southwestern and Methodist Hospital approved all research using human specimens.

Statistical analysis

Quantitative PCR was analyzed by unpaired Student's t-test. Cytokine data from conditioned media was first analyzed by a two way ANOVA then by Tukey's multiple comparisons test. Genotype associations were via Chi-square analysis. All analyses were performed using Prism 6 (GraphPad Software, Inc.).

Chapter Four: Conclusions and Future Directions

Summary of research findings - I

Results presented here explore how macrophages are able to recognize and respond to *M. tuberculosis* infection. The first portion of this thesis characterizes the role of the recently described DNA sensor cGAS in *M. tuberculosis* infection. We describe a role for cGAS both as a cytoplasmic DNA sensor, and as a regulatory protein in autophagy as well as demonstrate that cGAS deficiency is associated with decreased early control of *M. tuberculosis* infection *in vitro*. Additionally we demonstrate that cGAS deficiency is associated with decreased survival following *M. tuberculosis* infection. To our knowledge, we are the first to describe an *in vivo* role for cGAS in the control of bacterial infection. This work provides the framework for future work to further characterize the role of cGAS as both a DNA sensor and an autophagy regulatory protein.

Potential interactions of cGAS with other DNA sensors

cGAS is one of several cytoplasmic proteins identified that may sense DNA. While some of these sensors such as AIM2, appear to induce a different group of cytokines then cGAS, others such as IFI16 are thought to also be involved in induction of the type I interferon response. These don't appear to be redundant mechanisms, as cGAS deficiency eliminates induction of the type I interferon. However, IFI16 deficiency also leads to large defects in type I interferon production[100]. Work in *L. monocytogenes* suggests that IFI16 may not be necessary for the early induction of type I interferon, but may be required for late induction[221], and recent work has demonstrated that IFI16 knockdown attenuates the ability of THP-1 cells to respond to cGAMP[291]. This suggests a potential role for IFI16 in amplification of cGAS-mediated type I interferon induction. Our *in vivo* studies did not find long term differences in type I interferon between WT and $cGAS^{-/-}$ mice. It is plausible that another DNA sensor such as IFI16 can compensate for the lack of cGAS during an extended course of infection. While both cGAS and IFI have proposed roles in the response to *M. tuberculosis*, potential interactions between the two have not been explored.

Human polymorphisms in cGAS

Many of the receptors involved in the recognition of *M. tuberculosis* have well characterized polymorphisms associated with increased or decreased activity. Population studies have demonstrated that polymorphisms in many of these genes are associated with increased or decreased susceptibility to *M. tuberculosis* infection. While polymorphisms in STING have been described[292], so far no polymorphisms affecting cGAS expression have been identified. It would be advantageous to attempt to identify polymorphisms in cGAS. If these polymorphisms could be associated with either increased or decreased susceptibility to tuberculosis, it would help clarify the role of cGAS in human *M. tuberculosis* infection.

Summary of research findings – II

The second part of this work focuses on how macrophages respond to *M. tuberculosis.* We describe a novel role for HO1 in the response to human *M. tuberculosis*, show that inhibition of HO1 results in decreased inflammatory cytokine production following *M. tuberculosis* infection, and demonstrate that HO1 inhibition is associated with CFU defects *in vitro.* Additionally, we show that genetic polymorphisms associated with decreased production of HO1 are associated with increased susceptibility to *M. tuberculosis.* These findings lead to many new questions and many future directions to further determine the role of HO1 in human *M. tuberculosis.*

Genetic knockdown of HO1

For the studies described in this thesis, the chemical HO1 inhibitor tin protoporphyrin was used. Although no effects were seen in control samples, there is always a concern about off target effects anytime an exogenous chemical is added. Therefore, confirmation of our results with a genetic knockdown of HO1 would be advisable in future work. Genetic knockdown has an additional advantage of allowing the role of HO1 enzymatic activity to be further explored. SnPP inhibits HO1 by binding to the active site, but there is still accumulation of HO1 protein in the cell. With a genetic knockdown, it would be possible to add back a catalytically inactive form of HO1 to the HO1-deficient cells. This would allow us to confirm that the role of HO1 in *M. tuberculosis* infection is mediated by its enzymatic activity.

Potential roles for CO, biliverdin and iron

This thesis explores the role of HO1 in the response to *M. tuberculosis* infection. However, HO1 catalyzes the breakdown of heme into biliverdin, CO, and iron. Inhibition of HO1 alters the production of these products in the cell. It is possible the effects of HO1 inhibition on cytokine production are not due to a direct interaction with HO1, but are mediated through interactions with one of these products. If this is the case, it may be possible to “rescue” the cytokine defects associated with HO1 inhibition by adding back one of these products.

In vitro effects of HO1 promoter polymorphisms

As described in chapter 3, genetic polymorphisms in the HO1 promoter that result in decreased HO1 production are associated with increased susceptibility to *M. tuberculosis* infection. To further explore this association, primary human macrophages can be obtained from human subjects. Macrophages obtained from individuals with few GT repeats should express higher levels of HO1. These macrophages can be compared to macrophages from groups of individuals with greater numbers of GT repeats, as those macrophages should express lower levels of HO1. Macrophages can be infected with *M. tuberculosis* to determine if genetic differences result in differences in cytokine production or control of *M. tuberculosis.*

Putting it all together

While this thesis has focuses separately on the roles of cGAS and HO1 in *M. tuberculosis* infection, it is important to mention a possible link between the two. We have described a role for cGAS in the targeting of *M. tuberculosis* to the autophagosome. A potential role for HO1 in the induction of autophagy has been described in response to multiple stimuli, including LPS and sepsis[293,294]. Additionally, this HO1-mediated autophagy is inhibited following treatment with SnPP[294]. CO has also been shown to potentially up-regulate autophagy[295]. It is possible that HO1 functions downstream of cGAS in the induction of autophagy or there may be some interaction between the two. It is possible that HO1 levels could be altered in cGAS-deficient cells if HO1 induction following *M. tuberculosis* infection is affected by cGAS activity. As both these proteins affect macrophage control of *M. tuberculosis,* and both are involved in autophagy, examining the effects of cGAS deficiency on the function of HO1 is a logical step to try and link the two stories together.

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