ABSENCE OF COMPLEMENT RECEPTOR 3 RESULTS IN REDUCED BINDING
AND INGESTION OF *MYCOBACTERIUM TUBERCULOSIS* BUT HAS NO
SIGNIFICANT EFFECT ON THE INDUCTION OF REACTIVE OXYGEN AND
NITROGEN INTERMEDIATES OR ON THE SURVIVAL OF THE BACTERIA IN
RESIDENT AND INTERFERON-GAMMA ACTIVATED MACROPHAGES

by

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ABSTRACT

The interaction of host macrophage (M ϕ) and *Mycobacterium tuberculosis* (Mtb) is mediated by cell surface receptors and is important in establishing intracellular infection. M ϕ s are part of the mammalian defenses against microbial infection and can kill invading organisms via reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). Using a Complement Receptor 3 (CR3) knockout mouse model I have examined whether the presence of CR3 affected the binding and uptake of Mtb by resident and activated M ϕ , the survival of the ingested bacteria and the induction of ROI and RNI during this interaction.

I have shown that, although CR3 plays a definitive role in the uptake of Mtb, the receptor played no role in the subsequent survival of the bacteria. The finding held true for resident M ϕ s in the absence and presence of serum opsonins and also in Interferon-gamma (IFN- γ) activated M ϕ s. Activation of M ϕ populations with IFN- γ significantly inhibited the growth of Mtb in host M ϕ s and enhanced the respiratory burst and production of nitric oxide (NO). However, the presence of CR3 was not found to be critical in any of these mechanisms.

Furthermore, I demonstrated that the control of intracellular growth of Mtb in IFN- γ activated M ϕ s was not mediated by a direct effect of NO.

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LIST OF ABBREVIATIONS

AG	Aminoguanidine
AIDS	Acquired immune deficiency syndrome
ATCC	American type culture collection
BMM ϕ	Bone marrow-derived macrophages
BSA	Bovine serum albumin
CAT	Catalase
CFU	Colony-forming units
CR3	Complement receptor 3
DMSO	Dimethylsulphoxide
DPI	Diphenyleneiodonium chloride
DT	Doubling time
ES	Embryo-derived stem cells
FCS	Fetal calf serum
HIV	Human immunodeficiency virus
IFN- γ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
katG	<i>Mycobacterium tuberculosis</i> catalase-peroxidase gene
KO	Knockout (complement receptor 3 deficient)
L-NMMA	N ^G -monomethyl-L-arginine
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
M ϕ	Macrophage
MOI	Multiplicity of infection
NAC	N-acetyl L-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro-blue tetrazolium
NMS	Normal mouse serum
NO	Nitric oxide
PBS-Tween	Phosphate-buffered saline plus 0.1% Tween-80
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
SEM	Standard error of the mean
SOD	Superoxide dismutase
TAE	Tris-acetate-EDTA
TB	Tuberculosis
WHO	World Health Organization
WT	Wild-type (complement receptor 3 sufficient)
ZYM	Zymosan

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CHAPTER 1.

INTRODUCTION

1.1 *Mycobacterium tuberculosis*

Tuberculosis (TB), once considered a disease of the past, is growing at such a rate that in 1993 the World Health Organization (WHO) declared the epidemic a global emergency (1). TB is responsible for annual deaths amounting to more than 2 million people and is the number one cause of death due to an infectious bacterium. About 2 billion people, one-third of the world's total population, is currently infected and a person becomes newly infected with TB every second of every day. The resurgence of TB is due to a multitude of factors including HIV/AIDS, international travel and immigration via countries endemic with TB, increases in poverty and homelessness, the emergence of drug and multi drug-resistant TB and poor adherence to TB drug treatment therapies. It has been estimated that if the spread of TB is not brought under control, between the years of 2002 and 2020, "approximately 100 million people will be newly infected, over 150 million people will get sick, and 36 million will die of TB" (1). *Mycobacterium tuberculosis* (Mtb), the causative agent of disease, is an airborne disease spread through the air, like the common cold, via droplets (i.e. coughing, sneezing) from a person with active disease. It is estimated that a person suffering from active TB will infect between 10 and 15 people per year (2).

1.2 *Mycobacteria*-Host Cell Interaction

Once inside the human host, Mtb has evolved the ability to survive and grow inside the host macrophage (Mφs). Mφs are phagocytic cells of the immune system and are one of the most important subsets of immune cells involved in the elimination of pathogenic microbes.

Therefore, the interaction between host Mφs and bacteria is important for the successful establishment of infection. The initial interaction of host Mφ and tuberculosis bacilli is mediated by cell surface receptors on the outer membrane of the Mφ, including the mannose receptor, the glucan receptor, toll-like receptors, surfactant protein receptors, scavenger receptors, and the complement receptors 1, 3 and 4 (3-9). Complement Receptor 3 (CR3) is of particular interest because it has been proposed as a major route of entry for Mtb into host cells both in the absence and presence of serum (10, 11).

1.3 Complement Receptor 3

CR3 (CD11b/CD18, Mac-1, $\alpha_m\beta_2$, Mo-1) is a heterodimeric member of the β_2 - integrin family and is composed of a CD11b alpha chain non-covalently linked to a CD18 beta chain. The α chain of CR3 has a molecular mass of 165kDa while the β -chain is 95kDa (12). It is expressed on monocytes, neutrophils, Mφs, natural killer cells, eosinophils and basophils (13, 14) and to a lesser extent in some populations of T- and B-cells (15). Functions attributed to CR3 include leukocyte adhesion, hematopoietic cell migration, chemotaxis, phagocytosis and induction of the oxidative burst (13, 16, 17). CR3 possesses the key integrin family characteristic in that it is capable of undergoing an activation event in response to intracellular signalling events ("inside-out" signalling) that can change its specificity for different ligands as well as its function in mediating adhesion, phagocytosis and extracellular toxicity (18-20). The multitude of functions attributed to CR3 are controlled by these "inside-out" and "outside-in" signaling pathways that regulate the exposure of CR3 binding sites and the receptor's association with the actin cytoskeleton of the cell (21). CR3 binds a variety of ligands including the complement component iC3b, ICAM-2, ICAM-1/CD54, factor X, haemagglutinin and

fibrinogen as well as polysaccharides such as zymosan (ZYM) and lipopolysaccharide (LPS) (22-28). The multi ligand-binding capability of CR3 allows the receptor to bind a diverse repertoire of pathogens including *Bordetella pertussis*, *Ancylostoma caninum*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella* spp. (25, 29-31). Two distinct functional domains mediate the binding characteristics of CR3: the I- or A-domain, located in the α subunit, containing the site that mediates the binding and phagocytosis of iC3b-coated particles (32, 33) and the cation-independent β -glucan binding lectin site located C-terminal to the I-domain (34, 35) which is responsible for binding ZYM and polysaccharide moieties on the surface of bacteria under nonopsonic conditions.

CR3 plays a role in phagocytosis of opsonized bacteria in addition to phagocytosis via recognition of carbohydrate moieties on the surface of bacteria via two distinct mechanisms: type I phagocytosis under nonopsonic conditions and type II under opsonic conditions (36). In addition to being able to change its ligand binding capabilities CR3 can also be down- or upregulated on cell surfaces by stimulation with certain activating agents, such as interferon-gamma (IFN- γ) or phorbol 12-myristate 13-acetate (PMA), respectively (8, 11, 15, 37).

Previous work has shown that, in both the absence and presence of serum opsonins, CR3 is a major route of entry for mycobacteria (8, 38, 39). Using a CD11b knockout mouse model it was shown that approximately 40-50% of bacilli binding to host M ϕ s can be attributed to this receptor (10). Mtb can bind to CR3 in a serum and complement independent fashion via surface polysaccharides that bind directly to the CR3 β -glucan lectin site (40, 41). Differences in the polysaccharide composition between different Mtb strains play a role in the binding of Mtb to CR3 (42) and these differences may play a role in the pathogenicity of certain strains.

1.4 Macrophage Defense Mechanisms

Mφs are considered to be part of the mammalian defense system against microorganisms and, normally, resident Mφs are microbicidal when infected with any given microorganism. They act as effector cells capable of killing invading organisms via a number of different microbicidal mechanisms, of which reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) are considered to be among the most effective. Normally, internalization of a pathogen in a plasma membrane derived phagosome initiates intracellular signaling pathways that result in the activation of NADPH oxidase which, in turn, generates the superoxide anion (43) and other ROIs (hydrogen peroxide, hydroxyl radical and singlet oxygen).

Nitric oxide (NO) is produced from L-arginine by inducible nitric oxide synthase (iNOS), an enzyme that is also activated upon infection. In addition to their individual toxicities, Superoxide can also combine with NO to produce peroxynitrite, a very strong oxidant, which can inhibit the growth of pathogenic bacteria. Under certain conditions, mycobacteria are susceptible to both ROI (44) and RNI (45), but possess mechanisms that enable them to withstand Mφ defenses and survive and replicate within the Mφ (46).

The control of Mtb growth *in vivo* is mediated by Th1-immunity that consists of CD4 and CD8 T cells. These cells secrete Th1-type cytokines, including IFN-γ and IL-12, that upregulate Mφ function and enhance the ability of these cells to control mycobacterial growth (47, 48). It is thought that Mφs activated by these cytokines are able to control the intracellular replication of mycobacteria through the production of effector molecules such as ROI and RNI.

The involvement of NO in the control of Mtb is supported by the finding that iNOS knockout mice are unable to control the growth of Mtb and succumb to infection much earlier than WT mice (49, 50). Mice treated with iNOS inhibitors such as N-monomethyl-L-arginine

(L-NMMA) or aminoguanidine (AG) are also unable to control mycobacterial growth when infected with Mtb and die much sooner than untreated mice (51, 52). Furthermore, NO plays an important role in the killing of mycobacteria by human alveolar Mφs and inhibition of the NO response to infection results in a reduced ability of the Mφ to control bacterial replication (53). NO has been shown to be both bactericidal and bacteriostatic to mycobacteria and other intracellular pathogens (54-56). CR3 has been indicated to play a direct role in the production of NO from Mφs (57, 58) and microglia (59), as well as in response to infection with pathogenic bacteria (60).

ROI are also believed to play a role in the survival of mycobacteria *in vivo*, even though Mtb are relatively resistant to killing by ROIs *in vitro* (61). When infected with Mtb, *phox* knockout mice, which lack the phagocyte oxidase gene essential for superoxide production from NADPH oxidase, display enhanced bacterial growth in the lungs (62). However, this increased susceptibility is only transient and diminishes with the onset of acquired immunity (63). Ng et al determined that it is the presence of a catalase-peroxidase (*katG*) in Mtb that protects the bacteria from the deleterious effects of the respiratory burst (46) suggesting that although ROI may contribute to the control of mycobacterial growth, it is the other Mφ defense mechanisms, such as RNI, that determine the outcome of mycobacterial infection.

The observation that phagocytosis mediated by CR3 alone does not generate an oxidative burst (64-66) has resulted in the suggestion that CR3 allows pathogenic bacteria to enter the Mφ without an accompanying respiratory burst and can act as a “stealth receptor”, allowing for the successful establishment of infection.

1.5 Project Objective

To further elucidate the role that CR3 may have in the pathogenesis of Mtb this project was designed to examine the role that CR3 plays in the intracellular survival of Mtb and whether or not this receptor is critically involved in the production of ROI and RNI, two of the microbicidal mechanisms that Mφs use to control intracellular growth.

CHAPTER 2.

MATERIALS & METHODS

2.1 Mycobacteria

Mycobacterium tuberculosis strain Erdman (Trudeau Mycobacterial Collection (TMC) #107; American Type Culture Collection (ATCC) # 35811) was grown to late-log phase in modified Proskaur and Beck medium (per litre - KH_2PO_4 5.0g, asparagine 5.0g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.6g, magnesium citrate 1.0g, ferric ammonium citrate 0.2g, glucose 2.0g, 100mM sodium pyruvate 5.0ml, glycerol 20ml and 10ml of 10% Tween 80 – adjusted to pH 7.4 with NaOH or KOH) in a static culture with intermittent shaking every two-three days over a two-week period. Batch cultures were divided into 1ml aliquots and stored at -70°C for the remainder of the experiments. Viability for each batch was determined from the frozen stock; twelve representative vials were thawed, pelleted for 5 minutes at 14900xg and 0.5ml of supernatant were removed. The vials were re-spun and the remaining 0.5ml of media was removed. The pellet was resuspended in 1.0ml of Phagocytosis Medium (138mM NaCl, 8.1mM Na_2HPO_4 , 1.5mM KH_2PO_4 , 2.7mM KCl, 0.6mM CaCl_2 , 1.0mM MgCl_2 and 5.5mM D-glucose) and probe-sonicated for a ten-second burst using a VC50T 50 watt ultrasonic processor and 6mm probe (Sonics & Materials; Danbury, CT) to disperse any mycobacterial clumps. For each corresponding vial, the suspension was serially diluted (10^{-1} through 10^{-7}) into phosphate-buffered saline solution plus 0.1% Tween 80 (PBS-Tween) and plated onto Middlebrook 7H10 (DIFCO; Oakville, ON) agar plates. Plates were incubated at 37°C for 18-21 days after which colony-forming units (CFU) were enumerated. The ratio of bacteria to M ϕ present during infection (bacteria:M ϕ), termed the multiplicity of infection (MOI), was calculated for all experiments based on the average CFU/ml count for the twelve representative vials.

2.2 Mice

Both complement receptor 3 expressing (WT) and complement receptor 3 deficient (KO) mice were used for this project. Two distinct sources were used to provide the mice needed for experiments.

Early experiments were performed using mice sourced through a collaboration with Glaxo-Wellcome UK. Mice derived from this source are herein referred to as Glaxo mice. Glaxo knockout mice are of C57BL/6 background and are deficient in the CD11b protein portion of the CR3 heterodimer, and therefore do not express functional CR3 on cell surfaces. The CD11b gene was disrupted using homologous recombination in embryo-derived stem (ES) cells (10). In brief, a portion of CD11b exons 13 and 14 were eliminated by introducing a neomycin resistance gene that included a translational stop signal. This insertion truncates CD11b at the C-terminal/transmembrane end of the protein and therefore prevents insertion and expression of the molecule at the cell surface. HM-1 ES cells were transfected with a targeting vector and resistant clones were screened using Southern Hybridization. Clones with the CD11b gene knocked out were injected into C57BL/6J blastocysts and were subsequently injected into foster mothers, creating CD11b null offspring. Male null offspring were mated with C57BL/6J wild-type females, producing heterozygote progeny later used to produce offspring of +/+, +/- and -/- genotypes. Six Glaxo CD11b heterozygous (CD11b +/-) breeding pairs were obtained and bred to obtain both CD11b+/+ and CD11b-/- mice.

Midway through the project, due to difficulties maintaining a breeding colony and the inability to directly purchase the mice needed, the mouse source was changed to Jackson Laboratories (Bar Harbor, ME). Homozygous breeding pairs (+/+ and -/-) were purchased but we were unable to continue breeding these pairs in house and both WT and KO mice were

subsequently purchased directly from Jackson Laboratories for the remainder of the project. KO mice (strain B6.129S4-*Itgam*^{tm1Myd}, stock number 003991, deposited by donating investigator Tanya N. Mayadas) have a targeted mutation in the CD11b gene, also called integrin alpha M (itgam), located on chromosome 7, cytoband F4 and are herein referred to as Itgam mice (16). Briefly, a targeting vector was used to replace a portion of the *Itgam* gene exon that encodes the translational initiation codon and 15 amino acids of the signal peptide. A neomycin resistant construct was electroporated into 129S4/SvJae-derived ES cells, which were subsequently injected into C57BL/6 blastocysts producing chimeric animals. These B6:129S4 chimeras were backcrossed to C57BL/6 for a minimum of ten generations resulting in an Itgam KO on a C57BL/6 background. Itgam KO mice do not express the CD11b protein portion of the CR3 heterodimer on cell surfaces and are therefore functionally CR3 deficient. WT mice (strain C57BL/6J, stock number 000664) were purchased at the same time as the KO mice and were age- and sex-matched for each set of experiments.

All mice used for experiments were maintained in a specific pathogen-free animal housing facility following Canadian Committee on Animal Care Guidelines.

2.3 Murine Genotyping

Glaxo mice were genotyped at the age of 4-6 weeks to determine the CD11b gene profiles of the offspring of the heterozygous breeding partners. Itgam mice ordered from Jackson Laboratories are genetically identified at source.

2.3.1 DNA Extraction

DNA samples were obtained from skin samples of the ear punch used to visually identify the offspring. To extract the DNA, the ear punch sample was placed in 150 μ l of digestion buffer (100mM NaCl, 10mM Tris-HCl (pH8), 25mM EDTA (pH8), 0.5% SDS, 0.1mg.ml proteinase K) in a 1.5ml sterile Eppendorf tube and incubated overnight (approx 18 hours) at 55°C with constant tube rotation. The resulting digest was centrifuged at 14900xg to remove any undigested pieces and/or hair and the clear supernatant was transferred to a new sterile tube. Fifteen μ l (1/10 the volume of the cleared supernatant) of 3.0M potassium acetate was added to the tube and mixed. This mix was then thoroughly extracted using an equal total volume (165 μ l) of phenol/chloroform/isoamyl alcohol and spun at 14900xg for 10 min in a microfuge. The top (aqueous) layer was transferred to a new sterile tube and ½ volume (approximately 70 μ l) of isopropanol was added. The aqueous/isopropanol solution was left to stand for 0.5-1.0 hours at room temperature (or 15 min at -20°C) at which time the tube was centrifuged at 14900xg for 5 minutes to pellet out the DNA. The supernatant was removed, the pellet washed with 70% ethanol, centrifuged again under the same conditions followed by careful removal of the ethanol using a pipette. The tube was covered with parafilm punched with small holes and the clean DNA pellet was allowed to air dry at room temperature. This DNA pellet was then resuspended in 20 μ L of 10mM Tris-HCl pH 8.0 and the yield and purity of the DNA was assessed using a spectrophotometer ($A_{260/280}$ comparison).

2.3.2 PCR Genotyping

DNA samples from the Glaxo mice were analyzed using Polymerase Chain Reaction (PCR) to amplify the regions of the genome that correspond to both the CD11b WT and KO genotypes.

The PCR primers used were as follows:

Primer 856 5' - TAT CTT CTA GTG ATT TCC CCA GTA - 3'

Primer 2506 5' - CCC ACC CCT TCC CAG CCT CGA GC - 3'

Primer 2924 5' - TGT AGA CAG CGC CCT GAT TCT CCT - 3'

Sterile, Dnase/RNase-free 0.5ml ultra-thin walled PCR tubes were used for all PCR reactions. Two μ l of ear punch-extracted DNA was aliquotted into the tube followed by 5 μ l of a primer mix (primer mix was an equimolar combination of all three CD11b-specific primers; 856, 2924 and 2506 which, as aliquotted, provided a final amount of 25 pmol of each primer per PCR reaction). Thirty μ l of a PCR master mix was then added to each tube. The master mix contained 25 μ l 100 μ M dNTPs, 13 μ l 50mM MgCl₂, 52 μ l 10x GIBCO PCR buffer (Invitrogen; Burlington, ON), 215 μ l sterile distilled/deionized (ddH₂O) water and 5 μ l of GIBCO Taq Polymerase (master mix prepared for batches of tubes in multiples of ten). PCR controls were performed by repeating the same conditions, replacing the DNA with sterile ddH₂O.

PCR was performed using a Bio-Rad Gene Cyclor (Bio-Rad; Mississauga, ON) under the following conditions: a denaturation step of 5 minutes at 94°C, followed by 35 cycles of [1 minute at 94°C, 1 minute at 60°C, 1 minute at 72°C], finished with 5 minutes at 72°C. Reaction tubes were removed from the thermal cyclor immediately upon reaction completion and were stored at 4°C until examination.

2.3.3 Agarose Gel Electrophoresis

PCR products were analyzed using 1.5% agarose gels (0.75g agarose in 50ml TAE). Fifteen μ l of the finished PCR reaction was loaded onto the gel and electrophoresed for 60-90 minutes at 80V. Bands were visualized by staining the gel with ethidium bromide followed by UV illumination and photographic record using a Bio-Rad gel documentation (GelDoc) system. Known standards (100 base-pair ladder; Invitrogen) were loaded onto the gel along with the PCR samples so that the size of the PCR products could be determined. Expected size products are 600 bp for the WT genotype and 400 bp for the CD11b KO genotype.

2.4 Bone Marrow-Derived Macrophages

Bone marrow-derived macrophages (BMM ϕ) were used for all experiments and were isolated from mice using the following procedure. Mice were sacrificed by anaesthetization followed by cervical dislocation. Using aseptic technique, the femur, tibia and humerus were removed, cleaned and surface sterilized with 70% ethanol, both ends cut from the bones and using a 25-gauge needle, the bone marrow was flushed into a petri-dish using RPMI 1640 medium plus 10% L-conditioned media (see below), 10% heat-inactivated Fetal Bovine Serum (GIBCO Burlington, ON), 10mM L-glutamine (GIBCO) and 10mM sodium pyruvate (GIBCO) (Supplemented RPMI). Bone marrow washes were pooled and dispersed by repeated passage through an 18-gauge needle. The resulting suspension was pelleted at 1000xg for 5 minutes and resuspended in 5 ml 0.17M ammonium chloride (pH 7.2) for 5 minutes to lyse red blood cells. The cells were pelleted again, resuspended in 25 ml of supplemented RPMI, transferred to a 150 cm² tissue culture flask and incubated for three hours at 37°C, 5% CO₂ to deplete non-stem cells.

After 3 hours the non-adherent stem cells were removed, counted and plated on glass coverslips in 24-well tissue culture plates at a concentration of 2.5×10^5 cells in 1ml of supplemented RPMI. Cells were incubated for 7 days at 37°C in 5% CO₂ adding an additional 0.5ml of supplemented media to each well on Day 5. Cells were used after 7 days of differentiation *in vitro*.

2.4.1 L-conditioned Media

As a source of M ϕ -colony stimulating factor (M-CSF), L929 cells (ATCC #CCL-1) were grown in cRPMI (RPMI 1640 medium containing 10% heat-treated FCS, 10mM L-glutamine and 10mM sodium pyruvate). Cells at 5×10^3 per ml in a total volume of 125ml were seeded into a 150 cm² flask and incubated at 37°C, 5% CO₂. On Day 7, or when the monolayer becomes confluent, whichever was later, the supernatant was collected and filtered through a 0.22 μ M filter and stored at -20°C until use.

2.5 *In Vitro* Growth of *Mycobacterium tuberculosis* in Bone Marrow-Derived Macrophages

The growth of Mtb was followed over 7 days in BMM ϕ from WT and KO mice with or without prior activation with IFN- γ . Following the maturation of BMM ϕ (above) the overlay of supplemented RPMI was replaced with 0.5 ml cRPMI. Immune-activated M ϕ s were prepared by the addition of 250 units/ml of murine recombinant IFN- γ (Research Diagnostics Inc; Flanders, NJ). IFN- γ was stored at -70°C as a stock solution at 2500U/ μ L in 0.5x PBS pH 8.0 plus 1mg/ml bovine serum albumin (BSA) (Roche Diagnostics; Laval, QC), a carrier protein used to stabilize IFN- γ and prevent absorption to vial walls. Resident BMM ϕ were therefore maintained by changing the supplemented media to cRPMI and adding BSA at the same concentration as

that added with IFN- γ . BMM ϕ were left for 16 hours at 37°C, 5% CO₂ before infection with Mtb.

Prior to adding to BMM ϕ , Mtb was pelleted and resuspended in 1.0ml of Phagocytosis Medium and clumps were dispersed by syringing the suspension up and down 10 times using a 25-gauge needle and 1 ml syringe. The Mtb suspension was diluted to the appropriate concentration using Phagocytosis Medium. As needed, LPS (*E.coli* serotype 026:B6. SIGMA; St Louis, MO), at a final concentration of 4 μ g/ml, was added to the monolayer immediately prior to the addition of Mtb. Mtb was then added to the BMM ϕ monolayers in 100 μ L aliquots at the concentration needed to obtain each reported multiplicity of infection (MOI). Following addition of the mycobacteria, the monolayers were incubated at 37°C, 5% CO₂ on a Clay Adams Brand nutating platform (Becton Dickinson; Sparks, MD) for 1 hour followed by stationary incubation for 2 hours. After the 3-hour infection, the supernatant was aspirated off and unbound mycobacteria were removed by washing the monolayers twice with 0.5 ml Phagocytosis Medium. Three replicate coverslips were transferred to 14 ml polystyrene tubes containing 2 ml of PBS-Tween for processing. Remaining coverslips were transferred to new tissue-culture plates containing 1 ml of fresh cRPMI, with or without supplemental IFN- γ and LPS as appropriate, and re-incubated prior to processing on subsequent days. For processing infected BMM ϕ monolayers, the coverslips were crushed in the tubes containing PBS-Tween, followed by probe sonication for 10 seconds to disrupt M ϕ s and break up any clumps of mycobacteria. The mycobacterial suspension was then serially diluted in PBS-Tween and plated in duplicate on 7H10 quad plates. Plates were incubated at 37°C for 18-21 days after which CFU were enumerated.

Processing BMM ϕ monolayers on Days 4 and 7 was essentially the same except that the overlay and the 2 washes from the monolayer were transferred to a 14ml polystyrene tube along with the corresponding coverslip. This ensured that the total contents of the well were assessed and not just the bacteria present in the remaining adherent cells of the monolayer. Preliminary studies have shown that, as infections progress, M ϕ s detach from the monolayer but still contain viable intracellular bacteria (38). The coverslip was again broken, the contents of the tube probe sonicated for 10 seconds, serially diluted and plated in duplicate on 7H10 plates. CFU were again enumerated after 18-21 days.

To calculate the rate at which mycobacteria grow in the monolayers, the doubling times (DT) for 7-day growth of Mtb in BMM ϕ were calculated using the equation $N_t = N_0 e^{kt}$ where N_t = number of bacteria at time t (7 days), N_0 = number of bacteria at time 0 (after 3 hour infection), k = growth rate constant, t = time in hours and e = natural logarithm.

2.6 Nitric Oxide Production from Bone Marrow-Derived Macrophages

The Griess Assay was used to measure nitrite (NO_2^-), a stable end product of NO production (67). Samples were taken from the same wells used to monitor Mtb growth in M ϕ s. Control groups were comprised of either Phagocytosis Medium alone (negative control) or LPS alone (positive control).

Following 1, 4 or 7 days after infection of BMM ϕ , 100 μ l aliquots of supernatant were removed from each of the culture wells and transferred to a 96-well plate to which 50 μ l of Griess Reagent 1 (1% (w/v) sulphanilamide dissolved in 2.5% phosphoric acid (H_3PO_4)) and 50 μ l of Griess Reagent 2 (0.1% (w/v) naphthylethylenediamine dihydrochloride dissolved in 2.5% phosphoric acid (H_3PO_4)) were added sequentially and mixed. It is recommended that cell-free

supernatants be used for the Griess Assay, however preliminary experiments showed that removing cells from the supernatant prior to addition of the Griess reagents had no effect on assay outcome, so the step was eliminated. Replicate wells containing fresh cRPMI were included to calculate background values for each plate. Standards were prepared from a 2 mM stock of NaNO_2 in cRPMI (0.014 g dissolved in 100 ml freshly made media) to give a range of 1 μM to 128 μM . After a 10-minute incubation at room temperature, the absorbance values of all samples were read using a microplate reader (Bio-Rad; model: Benchmark) with a measurement wavelength of 550 nm and a reference filter of 655 nm.

The data was analyzed against a standard curve plotted in Excel using the trendline function and setting the y-intercept to the background value.

2.7 Superoxide Production from Bone Marrow-Derived Macrophages

The respiratory burst from BMM ϕ was measured using Nitro-Blue Tetrazolium (NBT), a formazan salt that is reduced in the presence of superoxide to form an insoluble blue deposit inside the M ϕ (68).

NBT (SIGMA) was freshly prepared at a concentration of 1.25 mg/ml and pre-warmed to 37°C prior to use. BMM ϕ monolayers were used following 16h incubation with or without IFN- γ (see above). The media was aspirated off, monolayers were washed 2 times with 1 ml of Phagocytosis Medium and 300 μl of NBT solution was added to the well followed by 100 μl of the trigger being tested; Mtb, PMA at 250 ng/ml, ZYM at an MOI of 50:1 or Phagocytosis Medium as a negative control. PMA is a member of the phorbol ester family and elicits a generalized respiratory burst when added to M ϕ s. ZYM is a yeast cell wall preparation known to bind to M ϕ and induce a respiratory burst. LPS was also added to some experimental groups at a

concentration of 4 $\mu\text{g/ml}$. BMM ϕ were incubated with the different agents for 1 hour rotating, 2 hours stationary. The supernatant solution was then aspirated off, the monolayers washed 2 times with 1 ml of Phagocytosis Medium, followed by 2 washes with 1 ml of methanol (to ensure removal of all unreduced NBT) and air drying. The reduced NBT deposits were solubilized by the addition of 120 μl /well of 2M KOH followed by 140 μl /well dimethyl sulphoxide (DMSO). After mixing, the resultant blue solution was quantitated at $\text{OD}_{570\text{nm}}$ using a Bio-Rad microplate reader. NBT background values were determined by incubating M ϕ monolayers with the NBT solution but without a trigger. A standard curve was obtained by solubilizing 1 mg of powdered NBT in 1 ml of 1.2:1.4 KOH:DMSO, which reduced the powder to the blue color, and serially diluting the solution through to 0.98 $\mu\text{g/ml}$. Replicate wells of each standard solution were added to each 96-well plate at the time of reading. The data were again analyzed against a standard curve plotted in Excel using the trendline function and setting the y-intercept to the background value.

Diphenyleneiodonium chloride (DPI) (SIGMA), an NADPH oxidase inhibitor, was used to determine the actual amount of NBT reduction due strictly to the metabolic products of the respiratory burst of the M ϕ s (i.e. used to determine background). DPI was stored as a stock solution (10 mg/ml in DMSO) and prior to infection the DPI stock was diluted 1:100 in cRPMI and added to the wells. DPI-control wells were incubated with 10 μM DPI during the 3-hour infection and the NBT readings from these wells were subtracted from the corresponding experimental well to reflect only the DPI-inhibitable value of superoxide production for each condition.

2.8 Intracellular *Mycobacterium tuberculosis* Growth in the Presence of Reactive Nitrogen and Oxygen Intermediate Inhibitors

The *in vitro* growth of Mtb in M ϕ monolayers was assessed in the presence of RNI inhibitors to determine the role that RNI play in the inhibition of Mtb growth. N^G-monomethyl-L-arginine (L-NMMA) is an arginine analogue that inhibits the production of NO from arginine by binding to the active site of inducible nitric oxide synthase (iNOS) (69-71). Stock L-NMMA (SIGMA) was prepared at a concentration of 25-50 mg/ml in PBS and stored at -20°C until use. Following preparation of BMM ϕ monolayers (see above), L-NMMA was added to the wells, to a final concentration of 1 μ M or 5 μ M, immediately prior to addition of the mycobacteria as necessary. The presence of L-NMMA was maintained in tissue culture after initial infection and throughout complete experiments.

To inhibit ROI, DPI (10 μ M), N-acetyl L-cysteine (NAC) (SIGMA) (5 and 10 μ M) or superoxide dismutase (SOD) (SIGMA) (150 U/ml) plus catalase (CAT) (Roche) (6000 U/ml) was added to the BMM ϕ monolayer immediately prior to the addition of Mtb. The antioxidants remained in the wells for the entire 7 days of growth, blocking ROI-production accordingly. Coverslips plus supernatant were processed as described above.

2.9 Statistics

One or two-way ANOVA followed by Tukey's multiple comparison test was performed, as appropriate, using GraphPad PRISM statistical analysis software. Differences were considered significant with a *P* value < 0.05.

CHAPTER 3.

RESULTS

3.1 Growth of *Mycobacterium tuberculosis* in Resident Macrophages

To determine whether CR3 plays a role in the *in vitro* growth of Mtb, resident BMM ϕ from both Glaxo (Fig.1A) and Itgam (Fig.1B&C) background mice, were infected with Mtb at two different MOIs, which were arbitrarily designated High (50:1 bacteria:M ϕ) (Fig.1A and B) and Low (20:1) (Fig 1C). Because previous work in our lab had indicated that 40-50% of binding of Mtb to resident M ϕ s is due to CR3 (Melo 2000), I also infected KO M ϕ s at double the MOI for both the High and Low MOIs to compensate for the deficiency in binding in the KO M ϕ s. CFU/ml were enumerated immediately after a 3-hour infection (DAY 0) and subsequently at 4 and 7 days post-infection (In preliminary experiments CFU/ml were also enumerated 24 hours after infection (Day 1) for the High MOI groups, but results did not contribute any additional information so were not carried out for Low MOI and are not shown).

For the Glaxo Mice:

Uptake of viable Mtb at Day 0, measured by CFUs, in the KO M ϕ s infected at 50:1 was lower ($3.82e^5 \pm 6.3e^3$) than the WT M ϕ s ($4.46e^5 \pm 1.2e^4$) when infected at the same MOI, however, this observation was not statistically significant ($P>0.05$) (Fig 1A). Uptake of Mtb in KO M ϕ s infected at 100:1 ($6.07e^5 \pm 2.6e^4$) was significantly higher ($P<0.001$) than WT M ϕ s infected at 50:1 ($4.46e^5 \pm 1.2e^4$), indicating that doubling the MOI to try correct for the expected 40-50% binding deficiency overcompensates for the difference in binding due to the absence or presence of CR3. Uptake in KO M ϕ s infected at 100:1 was significantly higher than uptake in KO 50:1 M ϕ s ($P<0.001$). At Day 4, KO 50:1 ($4.30e^6 \pm 2.9e^5$) was significantly lower than WT

Figure 1. Non-Opsonic Growth of Mtb in Resident CR3 WT and CR3 KO Mφs. BMφs derived from WT and KO mice were infected with Mtb Erdman 107 *in vitro*. The ability of Mtb to grow within resident Mφs was examined in Glaxo (A) and Itgam (B+C) mice at two different sets of MOIs; High/50:1 (A + B) and Low/20:1 (C). Data represented are mean ± SEM from A) n=3, B) n=5 and C) n=3 separate experiments, each performed with coverslips in triplicate. Doubling Times calculated using the formula described in Materials and Methods.

50:1 ($6.44e^6 \pm 2.5e^5$) ($P < 0.001$) and KO 100:1 ($6.33e^6 \pm 1.4e^5$) was no longer significantly higher than WT 50:1, suggesting that the bacteria in the KO Mφs may not be growing as quickly as those in the WT Mφs. By Day 7 the pattern of Mtb uptake remained, however there was no longer any statistical difference between CFUs from WT 50:1 and KO 50:1 or 100:1 ($1.26e^7 \pm 6.8e^5$, $1.03e^7 \pm 8.5e^5$ and $1.30e^7 \pm 5.5e^5$, respectively). Overall, KO 50:1 was lower and KO 100:1 was the same or slightly higher than WT 50:1 over the week infection, with varying statistical significance. KO 50:1 was statistically lower ($P < 0.001$) than KO 100:1 at each time point sampled during infection.

For the Itgam Mice:

Uptake of Mtb at DAY 0 in the KO 50:1 Mφs was lower ($1.81e^5 \pm 2.3e^4$) than the WT 50:1 Mφs ($1.94e^5 \pm 2.0e^4$), however, like that seen in Glaxo BMMφ, this observation was not statistically significant ($P > 0.05$) (Fig 1B). Similarly, uptake of Mtb in KO Mφs infected at 20:1 ($1.96e^4 \pm 1.7e^3$) was lower than WT Mφs infected at 20:1 ($2.33e^4 \pm 1.6e^3$), but again not significantly ($P > 0.05$) (Fig 1C). Uptake of Mtb in KO Mφs infected at 100:1 ($3.00e^5 \pm 3.4e^4$) was significantly higher ($P < 0.01$) than WT Mφs infected at 50:1, mirroring the effect seen in the Glaxo mice which indicated that doubling the MOI overcompensates for the difference in binding between WT and KO Mφs. Uptake of Mtb in KO Mφs infected at 40:1 ($3.33e^4 \pm 3.2e^3$) was also higher ($P < 0.05$) than WT Mφs infected at 20:1. Uptake of Mtb at the High MOI was approximately 10 times higher than uptake at the Low MOI for both WT and KO Mφs at DAY 0. At Days 4 and 7 the pattern seen at Day 0 was still observed: Mtb CFU counts in the KO wells were lower (but not significantly, $p > 0.05$) than those in the WT wells infected at the same MOI,

while doubling the MOI resulted in higher counts than in the WT M ϕ for both High and Low MOIs.

Uptake in the Glaxo-derived M ϕ s was significantly higher ($P<0.0001$) than Itgam in the populations infected at the same MOI, demonstrating that the different B6 backgrounds and/or differences in the CR3 gene knockout have an effect on mycobacterial uptake.

Because it was not possible to infect WT and KO M ϕ with exactly the same numbers of bacteria, making it hard to make direct comparisons between groups, I also assessed the growth of Mtb *in vitro* using the bacterial doubling time (DT) as a measure of growth (Fig.1). The DT examines the relationship between the number of bacteria at Day 0 and the number of bacteria at Day 7. There was no significant difference ($P>0.05$) in the DT between Glaxo WT 50:1, KO 50:1, and KO 100:1 (34.9 ± 0.9 , 35.7 ± 1.7 and 38.1 ± 1.6 hours, respectively) or Itgam WT 50:1, KO 50:1, and KO 100:1 (30.2 ± 1.5 , 29.9 ± 1.0 and 33.0 ± 1.1 hours, respectively) nor between the DT in Itgam WT 20:1, KO 20:1 and KO 40:1 with DT of 25.1 ± 0.5 , 24.8 ± 0.8 and 24.8 ± 0.8 hours, respectively. Overall, DT in the Glaxo groups were longer than DT in the Itgam groups infected at the same MOI and DT at the High MOI were longer than in the Low MOI group, indicating that the bacteria replicated more slowly, perhaps as a result of the initial higher load of bacteria in the M ϕ . In both Glaxo and Itgam M ϕ populations, the DT in the KO M ϕ s infected at a MOI of 100:1 were slightly, although not significantly, longer than in M ϕ s infected at 50:1. This further supports the conclusion that the difference in DT was a result of the high bacterial load in the M ϕ and not due to CR3 deficiency.

Although there was a quantitative difference between the numbers of Mtb in infected resident monolayers, the patterns of growth observed between Glaxo and Itgam, High and Low MOI groups and WT and KO M ϕ s were the same and indicated that although CR3 plays a role in

the initial binding of the tuberculosis bacilli to the M ϕ , it had no significant effect on the growth of Mtb once inside resident M ϕ s. Higher bacterial load in the resident monolayer affected the DT of the bacteria, but did not change the finding that CR3 played no role in the growth of Mtb in resident M ϕ s.

3.2 The Role Of Serum Opsonins in the Growth Of *Mycobacterium tuberculosis* in Resident Macrophages

Data in Fig.1 showed that CR3 played no role in the growth of Mtb following non-opsonic uptake by resident M ϕ s. Because CR3 has two different binding sites (12), and uptake via the lectin-like binding site of CR3 had no effect on the growth of Mtb in resident M ϕ s, I was interested in determining if binding and uptake via the second, iC3b, binding site on CR3 would result in a different outcome for the bacteria. As seen in Fig.2, resident BMM ϕ from the Itgam background were infected with Mtb in the presence of 1% normal mouse serum (NMS) to coat the mycobacteria with complement proteins, again at both High and Low sets of MOIs. In the High MOI group, KO 50:1 took up 25% less bacteria than WT 50:1 and KO 100:1 took up 10% less than WT 50:1, although neither reduction was determined to be statistically significant ($P>0.05$). At a Low MOI, KO 20:1 took up 50% less than WT 20:1 ($P<0.001$) and KO 40:1 took up 15% less than WT 20:1 ($P>0.05$). Unlike non-opsonic conditions, doubling the MOI was not able to completely restore the deficiency of Mtb uptake via CR3 KO M ϕ s under opsonic conditions. By Day 7 WT 50:1 CFU counts were not significantly different from either KO 50:1 or KO 100:1 in the High MOI group ($1.41e^7 \pm 1.5e^6$, $1.38e^7 \pm 8.7e^6$ and $1.37e^7 \pm 1.1e^6$, respectively). At Low MOI the CFU values for WT 20:1, KO 20:1 and KO 40:1 ($1.17e^7 \pm 1.1e^6$, $1.07e^7 \pm 2.3e^6$ and $1.53e^7 \pm 2.1e^6$, respectively) were also not statistically different at Day 7.

Figure 2 - The Effect of Serum Opsonization on the Growth of Mtb in Resident Mφs. CR3 WT and KO Mφs were infected with Mtb Erdman 107 in the presence of 1% NMS, *in vitro*. The ability of opsonized Mtb to grow within resident Mφs was examined in Itgam-derived BMMφs at two different sets of MOIs; High/50:1 (A) and Low/20:1 (B). Data represented are mean ± SEM from A) n=3 and B) n=2 separate experiments, each performed with coverslips in triplicate. Doubling Times calculated using the formula described in Materials and Methods.

Again, I looked at the bacterial growth rates of Mtb in WT and KO Mφs to further determine whether CR3 plays a role in growth of serum in resident Mφs under opsonic conditions. The DT for WT 50:1, KO 50:1 and KO 100:1 (31.5 ± 3.1 , 29.4 ± 1.4 and 30.8 ± 0.5 , respectively) were not statistically different. The calculated DT for WT 20:1, KO 20:1 and KO 40:1 (30.0 ± 1.0 , 26.7 ± 2.2 and 27.0 ± 0.9 , respectively) were also not statistically different from each other. The collective data represented in Figure 2 indicates that in the presence of serum opsonins, the initial bacterial burden in Mφs affected the growth rate of intracellular Mtb and, as for non-opsonic binding, CR3 played a role in uptake but not the growth of Mtb in resident Mφs for either of two different MOIs.

3.3 The Role Of Complement Receptor 3 in Immune-Activated Macrophages

In Mtb resistant WT mice, such as C57BL/6, the growth of Mtb is controlled starting approximately 20 days into infection (72). The inhibition of Mtb growth *in vivo* is controlled by acquired T-cell mediated immunity which is responsible for the subsequent production of IFN- γ and other Th1-type cytokines (47) which activate Mφ functions that control the growth of Mtb. To determine if CR3 possibly plays a role in infection after the onset of acquired immunity I looked at the growth of Mtb in BMMφ that had been activated by exposure to IFN- γ and IFN- γ plus LPS (Table 1). I examined growth of Mtb in immune-activated Mφs in Glaxo BMMφ infected at High MOI and Itgam BMMφ at Low MOI.

Firstly it was observed, in both Glaxo and Itgam backgrounds, that treatment of BMMφ with IFN- γ had a slight inhibitory effect on the uptake of Mtb at Day 0. IFN- γ treatment resulted in 0-5% fewer bacteria being taken up in Glaxo BMMφ and 15-40% fewer bacteria in Itgam BMMφ during the initial infection in both WT and KO Mφs, however neither reduction in uptake

Table 1 – Effect of IFN- γ Activation and the Addition of LPS on the Growth of Mtb in BM Φ in the Absence and Presence of CR3

GLAXO						ITGAM					
Resident M Φ s	DAY 0 (CFU/ml)	DAY 4 (CFU/ml)	DAY 7 (CFU/ml)	Doubling Time (h)		Resident M Φ s	DAY 0 (CFU/ml)	DAY 4 (CFU/ml)	DAY 7 (CFU/ml)	Doubling Time (h)	
WT 50:1	4.46x10⁵ $\pm 1.2 \times 10^4$	6.44x10⁶ $\pm 2.5 \times 10^5$	1.26x10⁷ $\pm 6.8 \times 10^5$	34.9 ± 0.9		WT 20:1	2.33x10⁴ $\pm 1.6 \times 10^3$	2.46x10⁵ $\pm 2.5 \times 10^4$	2.45x10⁶ $\pm 2.4 \times 10^5$	25.1 ± 0.5	
KO 50:1	3.82x10⁵ $\pm 6.3 \times 10^3$	4.30x10⁶ $\pm 2.9 \times 10^5$	1.03x10⁷ $\pm 8.5 \times 10^5$	35.7 ± 1.7		KO 20:1	1.96x10⁴ $\pm 1.7 \times 10^3$	2.20x10⁵ $\pm 2.9 \times 10^4$	2.35x10⁶ $\pm 3.1 \times 10^5$	24.8 ± 0.8	
KO 100:1	6.07x10⁵ $\pm 2.6 \times 10^3$	6.33x10⁶ $\pm 1.4 \times 10^5$	1.30x10⁷ $\pm 5.5 \times 10^5$	38.1 ± 1.6		KO 40:1	3.33x10⁴ $\pm 3.2 \times 10^3$	3.98 x10⁵ $\pm 3.8 \times 10^4$	3.66x10⁶ $\pm 3.3 \times 10^5$	24.8 ± 0.8	
IFN- γ Activated M Φ s						IFN- γ Activated M Φ s					
WT 50:1	4.29x10⁵ $\pm 2.3 \times 10^4$	1.06x10⁶ $\pm 1.5 \times 10^5$	5.43x10⁶ $\pm 5.1 \times 10^5$	47.1 ± 4.4		WT 20:1	1.80x10⁴ $\pm 9.6 \times 10^2$	9.66x10⁴ $\pm 1.2 \times 10^4$	4.18x10⁵ $\pm 3.5 \times 10^4$	37.4 ± 1.5	
KO 50:1	3.67x10⁵ $\pm 5.6 \times 10^3$	8.17x10⁵ $\pm 1.1 \times 10^5$	4.07x10⁶ $\pm 3.9 \times 10^5$	49.8 ± 4.2		KO 20:1	1.41x10⁴ $\pm 8.0 \times 10^2$	7.68x10⁴ $\pm 1.1 \times 10^4$	3.80x10⁵ $\pm 2.7 \times 10^4$	35.6 ± 1.6	
KO 100:1	6.34x10⁵ $\pm 1.6 \times 10^4$	1.32x10⁶ $\pm 1.7 \times 10^5$	4.90x10⁶ $\pm 3.8 \times 10^5$	58.4 ± 5.2		KO 40:1	2.89x10⁴ $\pm 1.4 \times 10^3$	1.34x10⁵ $\pm 1.9 \times 10^4$	7.25x10⁵ $\pm 8.9 \times 10^4$	37.4 ± 3.5	
IFN- γ Activated M Φ s plus LPS						IFN- γ Activated M Φ s plus LPS					
WT 50:1	3.63x10⁵ $\pm 2.4 \times 10^4$	8.32x10⁵ $\pm 7.8 \times 10^4$	4.33x10⁶ $\pm 2.2 \times 10^5$	47.0 ± 3.1		WT 20:1	1.14x10⁴ $\pm 4.9 \times 10^2$	6.69x10⁴ $\pm 7.0 \times 10^3$	3.92x10⁵ $\pm 2.5 \times 10^4$	33.1 ± 1.6	
KO 50:1	3.08x10⁵ $\pm 1.3 \times 10^4$	5.61x10⁵ $\pm 7.5 \times 10^4$	2.83x10⁶ $\pm 3.0 \times 10^5$	55.8 ± 8.2		KO 20:1	8.78x10³ $\pm 5.6 \times 10^2$	5.20x10⁴ $\pm 5.9 \times 10^3$	4.12x10⁵ $\pm 4.8 \times 10^4$	30.7 ± 1.3	
KO 100:1	5.32x10⁵ $\pm 3.4 \times 10^4$	1.39x10⁶ $\pm 2.4 \times 10^5$	4.66x10⁶ $\pm 5.0 \times 10^5$	55.1 ± 5.1		KO 40:1	1.71x10⁴ $\pm 8.1 \times 10^2$	1.19x10⁵ $\pm 1.3 \times 10^4$	7.24x10⁵ $\pm 8.8 \times 10^4$	31.7 ± 2.1	

Data expressed as mean $\pm$ SEM of n=9 replicates comprising 3 separate experiments performed in triplicate for both Glaxo and Itgam.
Doubling Times calculated using the formula described in Materials and Methods.

was statistically significant ($P>0.05$). Addition of LPS to the IFN- γ activated M ϕ s at time of infection resulted in a statistically significant reduction ($P<0.001$) of bacterial uptake in both Glaxo and Itgam-derived BMM ϕ , with approximately 20% and 50% less bacteria being taken up, respectively, in both resident WT and KO M ϕ s.

The pattern of Mtb uptake I observed in resident M ϕ s was also seen in IFN- γ activated M ϕ s; in the Glaxo-derived M ϕ s, uptake of Mtb at Day 0 in IFN- γ activated KO M ϕ s infected at an MOI of 50:1 was lower than IFN- γ activated WT M ϕ s infected at an MOI of 50:1, however the difference was not statistically significant. Uptake in IFN- γ activated KO M ϕ s infected at an MOI of 100:1 was significantly higher ($P<0.001$) than IFN- γ activated WT M ϕ s infected at an MOI of 50:1, indicating that CR3 was indeed responsible for partial uptake of Mtb into the M ϕ , but Mtb can also invade the M ϕ via other cell surface receptors. The pattern of uptake in IFN- γ activated Itgam-derived M ϕ s was qualitatively the same as that seen in Glaxo-derived M ϕ s. At Day 0 uptake of Mtb in KO 20:1 M ϕ s was lower, but not statistically different, than WT 20:1 M ϕ s. Uptake in KO 40:1 M ϕ s was significantly higher ($P<0.001$) than WT 20:1 M ϕ s, again showing that CR3 was not essential for Mtb invasion. The same pattern of Mtb uptake at Day 0 was also mirrored in IFN- γ activated M ϕ s infected in the presence of LPS; in Glaxo-derived M ϕ s KO 50:1 was lower than WT 50:1, whereas KO 100:1 was higher, with only the latter being significant ($P<0.001$). In Itgam background M ϕ s KO 20:1 was lower than WT 20:1, whereas KO 40:1 was higher however, in this instance, neither difference was statistically significant. Four days after infection, in both IFN- γ activated and IFN- γ activated plus LPS M ϕ populations, the CFU patterns were maintained in both the Glaxo and Itgam, WT and KO monolayers; KO 50:1 and KO 20:1 lower than WT 50:1 and WT 40:1, respectively, and KO 100:1 and KO 40:1

higher, respectively, but the differences at this timepoint were not statistically different. At Day 7, the number of bacteria in the IFN- γ activated or IFN- γ activated plus LPS M ϕ monolayers with respect to the presence of CR3 follows the pattern revealed by Day 4; there was no statistical difference in the numbers of Mtb in WT or KO monolayers in M ϕ s derived from either Glaxo or Itgam backgrounds.

Seven days post infection the effect of immune-activation on the growth of Mtb in M ϕ monolayers was quite apparent. IFN- γ activated M ϕ s were much more efficient at controlling Mtb growth, reflected in bacterial loads from IFN- γ activated WT and KO M ϕ monolayers that were 2-4 fold lower in the Glaxo-background M ϕ s and 5-6 fold lower in the Itgam background, than that of the matched resident monolayer. The same decrease in bacterial load applied to monolayers infected in the presence of IFN- γ and LPS, when compared to resident M ϕ s.

The DT of Mtb in IFN- γ activated WT and KO M ϕ monolayers were significantly longer ($P < 0.0001$) than in resident M ϕ s for both Glaxo and Itgam M ϕ s, reflecting the decreased bacterial numbers in the monolayers by Day 7. LPS was also added to enhance the IFN- γ activation of the M ϕ monolayers to help control the growth of Mtb, and although the numbers of bacteria at Day 7 were similar to those of M ϕ s activated with IFN- γ alone, when the decreased uptake of Mtb at Day 0 was taken into consideration LPS did not enhance the control of bacterial growth over IFN- γ treatment alone. In the Itgam M ϕ s in particular, the addition of LPS was actually detrimental to the control of bacterial growth by IFN- γ activated M ϕ s, decreasing the DT so that, although they still replicate significantly more slowly ($P < 0.001$) than bacteria in resident M ϕ s, the DT in the presence of IFN- γ and LPS was shorter than in the presence of IFN- γ

alone ($P < 0.05$). In the Glaxo IFN- γ activated M ϕ monolayers, there was no significant difference in the doubling times between WT 50:1, KO 50:1 and KO 100:1 in the absence of LPS (47.1 ± 4.4 , 49.8 ± 4.2 and 58.4 ± 5.2 , respectively) or in the presence of LPS (47.0 ± 3.1 , 55.8 ± 8.2 and 55.1 ± 5.1 , respectively), however in both groups of activated Glaxo M ϕ s the DT in the KO M ϕ s were somewhat longer than the DT in WT M ϕ s. Within the Itgam IFN- γ activated M ϕ monolayers, there was no significant difference in the doubling times between WT 20:1, KO 20:1 and KO 40:1 (37.4 ± 1.5 , 35.6 ± 1.6 and 37.4 ± 3.5 hours for IFN- γ activated M ϕ s, and 33.1 ± 1.6 , 30.7 ± 1.5 and 31.7 ± 2.1 for IFN- γ activated M ϕ s plus LPS, respectively). These results indicated that CR3 does not play a role in the growth of Mtb in IFN- γ activated M ϕ s.

3.4 Complement Receptor 3 Does Not Contribute To The Respiratory Burst Of Macrophages

The respiratory burst consists of the production of superoxide radicals and other toxic oxygen metabolites and it is widely reported that the respiratory burst is an effective antimicrobial mechanism used by M ϕ s to control intracellular bacterial replication. Also of interest is the possibility that CR3 allows pathogenic bacteria to enter the M ϕ without an accompanying respiratory burst (65, 73). The ability of resident and IFN- γ activated M ϕ s to reduce NBT was examined using three distinct triggers: PMA, ZYM and Mtb in comparison to M ϕ s incubated with no trigger at all (Fig.3). Both Itgam and Glaxo M ϕ s demonstrated basal NADPH-dependent NBT reduction in the absence of any trigger, which increased when the cells were incubated with IFN- γ prior to stimulation.

In Glaxo-derived M ϕ s, superoxide production was slightly lower but not significantly different between resident WT and KO (3.0 ± 0.5 vs 2.3 ± 0.3) or IFN- γ activated WT and KO

(9.2 ± 0.7 vs 8.6 ± 1.1) M ϕ s triggered with PMA. NBT reduction elicited by ZYM by WT and KO resident (5.8 ± 0.4 vs 4.6 ± 0.6 , respectively) and IFN- γ activated M ϕ s (12.7 ± 0.3 vs 11.5 ± 0.3 , respectively) were also not significantly different within each group. The superoxide response to Mtb was not significantly different between WT or KO M ϕ s in either resident or IFN- γ activated M ϕ populations. There was a small increase in the reduction of NBT in KO M ϕ s incubated with Mtb at 100:1 MOI over WT and KO 50:1, suggesting that higher Mtb levels may be able to elicit a superoxide response however, this result was not statistically significant so I am unable to make definitive conclusions about this observation. Also of note was the observation that Mtb did not elicit a superoxide response above basal levels (no trigger) in either the resident or IFN- γ activated M ϕ populations. In resident M ϕ s, the respiratory burst from KO M ϕ s infected at an MOI of 50:1 (1.9 ± 0.8) did not differ significantly from WT M ϕ s infected at an MOI of 50:1 (1.8 ± 0.6). KO 100:1 (2.5 ± 0.4) was slightly higher but, also not significantly different from WT 50:1. The pattern was repeated in IFN- γ activated WT and KO M ϕ s. Neither KO 50:1 nor KO 100:1 were significantly different from WT 50:1 (4.7 ± 0.4 , 5.7 ± 0.6 and 4.4 ± 0.9 , respectively). In the resident population in particular, the NBT reduction elicited by incubation with Mtb was lower, but not significantly, than that of untriggered M ϕ s indicating that Mtb may actually reduce or inhibit the superoxide response from host M ϕ s.

In Itgam-derived M ϕ s the superoxide production elicited in response to PMA treatment was also not significantly different between resident WT and KO M ϕ s (6.9 ± 0.4 vs 5.1 ± 0.6) or between IFN- γ activated WT and KO M ϕ s (11.5 ± 0.8 vs 10.5 ± 1.1 , respectively) although again, in both cases, the KO M ϕ s had slightly lower respiratory bursts. Levels of NBT reduction elicited by ZYM between resident WT and KO M ϕ s (4.6 ± 0.4 vs 3.9 ± 0.7) or between IFN- γ

activated WT and KO Mφs (7.5 ± 0.7 vs 7.0 ± 0.7 , respectively) were also slightly lower in the KO Mφs but still not significantly different. The ability of Mφs to produce superoxide in response to infection with Mtb did not differ significantly between resident WT and KO Mφs or between IFN- γ activated WT and KO Mφs. In resident Mφs, the respiratory burst from KO 50:1 (2.0 ± 0.4) or KO 100:1 (1.7 ± 0.3) did not differ significantly from WT 50:1 (1.7 ± 0.3). In IFN- γ activated WT and KO Mφs, neither KO 50:1 nor KO 100:1 were significantly different from WT 50:1 (4.4 ± 0.7 , 5.2 ± 0.6 and 5.0 ± 0.5 , respectively). Also, as seen in the Glaxo-derived Mφs, Mtb did not elicit a respiratory burst significantly above background levels in either the resident or IFN- γ activated Mφ populations.

IFN- γ activation significantly increased ($P < 0.001$) the overall ability of the Mφ to produce a respiratory burst in both the Glaxo and Itgam WT and KO populations for the other triggers tested (PMA and ZYM). Addition of LPS prior to Mtb infection was tested but did not affect the production of the respiratory burst and therefore data was not shown.

Except for a higher response to PMA in resident Itgam BMMφ and significantly stronger ROI production in response to ZYM in IFN- γ activated Glaxo BMMφ, there were no major differences in ROI production from Glaxo or Itgam-derived BMMφ, in the absence or presence of CR3.

3.5 Nitric Oxide Production from Resident and Immune-Activated Macrophages

To explore whether CR3 plays a role in another Mφ defense mechanism, I measured the production of NO from WT and KO resident and immune-activated Mφs (Table 2). As a function of NO production, nitrite levels were tested from culture supernatant at 1, 4 and 7 days

Table 2 – NO Production From WT and KO Mφ Monolayers During Intracellular Replication of Mtb.

Strain	MOI	DAY 4 Nitrite Values (μM) ^a	DAY 7 Nitrite Values (μM) ^a		Strain	MOI	DAY 4 Nitrite Values (μM) ^a	DAY 7 Nitrite Values (μM) ^a
Resident					Resident			
Glaxo WT	50:1	1.0 ± 0.1	2.6 ± 0.3		Itgam WT	20:1	1.6 ± 0.2	2.2 ± 0.1
Glaxo KO	50:1	0.8 ± 0.2	2.8 ± 0.4		Itgam KO	20:1	1.2 ± 0.1	1.7 ± 0.3
Glaxo KO	100:1	2.0 ± 0.6	4.5 ± 1.1		Itgam KO	40:1	1.0 ± 0.2	1.6 ± 0.2
IFN-γ Activated					IFN-γ Activated			
Glaxo WT	50:1	24.3 ± 1.4	27.5 ± 1.8		Itgam WT	20:1	11.4 ± 0.8	20.2 ± 1.2
Glaxo KO	50:1	27.9 ± 1.8	33.0 ± 1.6		Itgam KO	20:1	9.7 ± 1.2	14.9 ± 0.9
Glaxo KO	100:1	26.8 ± 2.4	30.0 ± 1.9		Itgam KO	40:1	15.6 ± 1.1	25.4 ± 1.4
IFN-γ Activated plus LPS					IFN-γ Activated plus LPS			
Glaxo WT	50:1	21.4 ± 1.5	20.0 ± 0.9		Itgam WT	20:1	38.2 ± 2.3	41.2 ± 3.3
Glaxo KO	50:1	21.8 ± 0.8	21.0 ± 0.5		Itgam KO	20:1	32.9 ± 2.1	37.4 ± 2.8
Glaxo KO	100:1	26.8 ± 2.4	20.7 ± 1.9		Itgam KO	40:1	30.2 ± 2.8	34.4 ± 3.5

^a The nitrite value expressed is μM in the tissue-culture supernatant from a well containing 2.5×10^5 cells at the time of Mtb infection. Data shown for Nitrite values are mean ($\pm$ SEM) for n=6-9 replicates from 2-3 separate experiments performed in triplicate (Glaxo) or n=16-18 replicates from 6 separate experiments performed in duplicate or triplicate (Itgam).

post-infection. Nitrite is stable in solution and levels accumulate over the 7 day period representing the total NO response of the Mφs due to Mtb infection. NO production from resident BMMφ remained at the lower end of assay detection over 7 days and was not significantly different between KO and WT Mφs at either 4 or 7 days post-infection. IFN-γ activation significantly increased the NO response from Mφ monolayers ($P<0.0001$) when compared to resident cells. The addition of LPS to IFN-γ activated cells decreased the production of NO from Glaxo-derived Mφs ($P<0.0001$) but increased the NO response above that of IFN-γ activation alone ($P<0.0001$) in the Itgam populations.

At both Day 4 and Day 7, NO production from the IFN-γ activated Glaxo-derived Mφs were not significantly different between WT and KO, regardless of the MOI. The same pattern appeared in IFN-γ activated Mφs in the presence of LPS. There was not a significant difference in the production of NO from WT and KO Mφs. NO production from these monolayers was lower with the addition of LPS in both the WT and KO Mφs, suggesting that, in the Glaxo monolayers, LPS may have played a role in reducing the NO response to Mtb.

The production of NO from Itgam-derived Mφs was slightly different from Glaxo-derived Mφs. At Day 4 nitrite detected from IFN-γ activated KO 20:1 Mφs was slightly lower but not significantly different from the nitrite levels in WT 20:1 IFN-γ activated Mφs. NO production from KO 40:1 was significantly higher than NO production from WT 20:1 ($P<0.05$) and KO 40:1 was also significantly higher than KO 20:1 ($P<0.001$), indicating that Mtb infection of Mφs may have had a dose effect on NO production from these Mφs. By day 7, in these immune activated populations, NO production from KO 20:1 Mφ monolayers was significantly lower ($P<0.01$) than that of WT 20:1 Mφ monolayers and NO from KO 40:1 infected

M ϕ monolayers remained significantly higher than production from WT 20:1 ($P<0.01$) and KO 20:1 infected M ϕ s ($P<0.001$), suggesting that CR3 did play a role in NO production from Itgam-derived BMM ϕ . In the Itgam M ϕ s, LPS treatment in addition to IFN- γ activation during Mtb infection universally increased the NO response in both WT and KO. At both days 4 and 7 post-infection NO production from IFN- γ activated M ϕ s treated with LPS in conjunction with Mtb was 2-3 times higher than IFN- γ activated M ϕ s infected with Mtb alone. There was no significant difference in NO production between WT and KO M ϕ s treated with IFN- γ and LPS at either Day 4 or Day 7, indicating that, in the Itgam monolayers, the addition of LPS elicited maximal CR3-independent NO production.

In preliminary experiments NO production measured at Day 0 (3 hours after infection) was not detectable and at Day 1 (24 hours after infection) was very low. There were also no significant differences between NO produced from resident and IFN- γ activated M ϕ s at either of these times points (data not shown).

3.6 Nitric Oxide Does Not Play a Role in the Growth Inhibition of *Mycobacterium tuberculosis* by Interferon- γ Activated Macrophages

As there appeared to be a correlation between the induction of NO and the control of Mtb growth in IFN- γ activated M ϕ s, I examined the growth of Mtb in both resident and IFN- γ activated Itgam M ϕ s in the presence of the iNOS inhibitor, L-NMMA. To determine the direct correlation between NO production and growth, nitrite levels were sampled from the same wells that CFUs were quantitated. The presence of 5mM L-NMMA during infection had no effect on the uptake of Mtb at Day 0 in either the resident or IFN- γ activated M ϕ populations. The absence or presence of L-NMMA did not significantly affect the growth of Mtb in resident M ϕ

as measured by CFU at Day 7 or by DT (Table 3). As expected, NO levels were at the lower end of assay detection and were not significantly different between resident M ϕ s with or without L-NMMA treatment. As reported above, IFN- γ activation significantly ($P<0.0001$) decreased the ability of Mtb to grow (lengthened the DT) and significantly increased the production of NO from M ϕ monolayers ($P<0.0001$). The addition of 5mM L-NMMA to IFN- γ activated monolayers reduced the production of NO to baseline/resident M ϕ level but did not influence the ability of IFN- γ activated M ϕ s to control mycobacterial growth (Table 3). DT of Mtb in these monolayers remained unaffected when L-NMMA was added and the CFU counts were not significantly different between monolayers treated with IFN- γ or with IFN- γ plus L-NMMA (Table 3). Of particular interest, is the observation that the presence of L-NMMA greatly reduced the loss of M ϕ from the monolayers left untreated or treated with IFN- γ alone (data not shown). In the latter groups, the M ϕ were found to detach from the coverslips and remain suspended in the supernatant. These detached cells contain viable Mtb and account for the vast majority of the CFU present in the supernatant (38).

Preliminary experiments had been initially carried out using 1mM L-NMMA. However, inhibition of NO production in IFN- γ activated M ϕ was incomplete at this concentration presenting the possibility that the residual RNI production could explain the continued anti-mycobacterial activity of IFN- γ activated M ϕ s (data not shown).

3.7 The Role Of The Respiratory Burst in Macrophage Control of Mycobacterial Growth

In an attempt to determine if ROI play a role in the control of mycobacterial growth I analyzed intracellular Mtb growth in the presence of various antioxidants. Unfortunately, all the antioxidants tested were either toxic to the M ϕ s or were unable to inhibit the intracellular

Table 3 - Effect of the NO Inhibitor L-NMMA on the Growth Of Mtb in Resident and IFN- γ Activated M ϕ s Relative to the Presence of CR3.

Strain	MOI	Treatment	DAY 0 (CFU/ml)	DAY 7 (CFU/ml)	DOUBLING TIME (h)	Nitric Oxide Levels (μ M)
ITGAM WT	20:1	Resident	2.41×10^4 $\pm 7.2 \times 10^2$	9.89×10^5 $\pm 1.0 \times 10^5$	31.4 ± 1.3	2.6 ± 0.2
ITGAM KO	20:1	Resident	1.88×10^4 $\pm 1.2 \times 10^3$	1.03×10^6 $\pm 1.6 \times 10^5$	29.8 ± 2.6	2.7 ± 0.3
ITGAM KO	40:1	Resident	3.84×10^4 $\pm 2.4 \times 10^3$	2.00×10^6 $\pm 2.1 \times 10^5$	29.9 ± 2.2	2.0 ± 0.2
ITGAM WT	20:1	Resident + 5 mM NMMA	2.34×10^4 $\pm 8.6 \times 10^2$	1.24×10^6 $\pm 1.7 \times 10^5$	29.5 ± 1.9	2.4 ± 0.3
ITGAM KO	20:1	Resident + 5 mM NMMA	1.99×10^4 $\pm 1.4 \times 10^3$	1.02×10^6 $\pm 1.5 \times 10^5$	30.4 ± 2.8	1.9 ± 0.2
ITGAM KO	40:1	Resident + 5 mM NMMA	3.97×10^4 $\pm 2.8 \times 10^3$	2.21×10^6 $\pm 3.0 \times 10^5$	29.4 ± 2.0	2.4 ± 0.3
ITGAM WT	20:1	IFN- γ Activated	2.39×10^4 $\pm 1.3 \times 10^3$	3.42×10^5 $\pm 3.2 \times 10^4$	43.6 ± 3.0	19.1 ± 1.6
ITGAM KO	20:1	IFN- γ Activated	1.67×10^4 $\pm 9.9 \times 10^2$	3.13×10^5 $\pm 4.0 \times 10^4$	40.1 ± 3.5	16.0 ± 1.8
ITGAM KO	40:1	IFN- γ Activated	3.37×10^4 $\pm 1.9 \times 10^3$	7.35×10^5 $\pm 1.0 \times 10^5$	38.2 ± 2.3	26.2 ± 2.3
ITGAM WT	20:1	IFN- γ Activated + 5 mM NMMA	2.64×10^4 $\pm 1.0 \times 10^3$	4.29×10^5 $\pm 6.5 \times 10^4$	42.6 ± 5.0	2.7 ± 0.3
ITGAM KO	20:1	IFN- γ Activated + 5 mM NMMA	2.03×10^4 $\pm 1.0 \times 10^3$	3.81×10^5 $\pm 4.6 \times 10^4$	40.0 ± 3.1	2.4 ± 0.2
ITGAM KO	40:1	IFN- γ Activated + 5 mM NMMA	3.84×10^4 $\pm 1.7 \times 10^3$	7.12×10^5 $\pm 9.7 \times 10^4$	40.3 ± 3.3	3.6 ± 0.5

NOTE : nitrite values sampled from same wells as CFU were enumerated

Data shown for both CFU/ml and Nitrite values are mean $\pm$ SEM for n=9 replicates from 3 separate experiments performed in triplicate.

respiratory burst. DPI, used in the NBT assays to successfully inhibit ROI over 3 hours, exhibited toxicity starting 24 hours after infection, and by Day 4 of growth the M ϕ monolayer had deteriorated completely. Another antioxidant, NAC, also exhibited toxicity starting 24 hours after infection. NAC was also tested as an alternative to DPI in the NBT assays, however, it was found to be a strong reducing agent and therefore unusable in experiments designed to measure the respiratory burst. Finally, the combination of SOD + CAT was tested, however this was not able to inhibit the deposition of the formazan salt in the NBT assay nor did it have any effect on Mtb replication when added to the M ϕ monolayers during Mtb growth assessment (all data not shown) so it was determined to be inadequate for my experimental procedures.

CHAPTER 4.

DISCUSSION

The purpose of the work reported here was to elucidate the role of CR3 in the pathogenesis of Mtb. CR3 has been suggested as a major player in the infection of Mφs due to its binding capabilities and attributed roles in the formation of the respiratory burst and the production of RNI. Using two genetically different, but phenotypically similar CR3 KO mice I have examined the role that CR3 plays in the pathogenesis of Mtb. I observed quantitative, but few major qualitative differences between the two mouse sources, which indicated that although the different genetic KO background affected some of the observations made, it did not make a difference in the overall conclusion I was able to make.

Interaction of CR3 with its ligand can promote rapid phagocytosis and subsequent degradation of the particle once internalization has occurred (74). Previous work in our lab has shown a 40% decrease in non-opsonic binding and 50% decrease in opsonic binding of Mtb binding to Mφs in the absence of CR3 (10). In the current study I observed a similar decrease in the number of bacteria taken up by resident KO Mφs but could not replicate the 40% decrease reported previously for non-opsonic binding, in either the Glaxo or Itgam backgrounds. There are a number of possible explanations for the difference: 1.) it is known that Mφ phenotype determines the non opsonic binding of Mtb to murine Mφs (8). BMMφ were used in the current study whereas the previous study had utilized alveolar and peritoneal Mφs. Thus the difference in binding and uptake may be a function of the different Mφ types used in each set of experiments. 2.) An additional explanation may lie in the different techniques used to assess binding of Mtb. In the current study bacterial numbers were assessed by measuring CFU associated with the Mφ population. In the earlier study (10), bacterial association with Mφ was

assessed microscopically, a method that cannot distinguish live and dead bacteria. As all mycobacterial cultures contain a percentage of dead bacteria there is a possibility that CR3 binds dead bacteria more efficiently than other receptors, resulting in a relatively higher percentage of dead bacteria binding to WT Mφs than KO Mφs. This would result in a large difference in Mtb binding between WT and KO Mφs when assessed microscopically but when CFU are enumerated, which can only count live bacteria, the difference would no longer be apparent. Regardless of the quantitative differences between this body of work and previous data I was still able to conclude that CR3 plays a role in binding of Mtb to Mφs in the absence of serum.

There was a clear difference in non-opsonic binding and uptake between the Glaxo and Itgam BMMφ, indicating that the genetic background did play a role in the uptake of Mtb in the absence or presence of CR3. Glaxo BMMφ took up approximately 2 times more bacteria than Itgam BMMφ at the same MOI. There may be a difference in the surface receptor activities between Glaxo and Itgam-derived Mφs that could account for the difference in binding and uptake between the two Mφ sources.

Itgam BMMφ infected at the High MOI took up almost 10 times more bacteria than Itgam BMMφ infected at the Low MOI. This shows that MOI played an important role in determining the amount of mycobacterial uptake by BMMφ. The difference in MOI between the High and Low MOI groups, however, was only 2.5-fold so I did not expect to see a 10-fold difference in binding between the two groups. The observation that the increased binding to BMMφ was not directly proportional with respect to the increase in bacterial number present in the well suggests that the efficiency of Mtb uptake was enhanced with increasing bacterial numbers, probably due to increased Mtb-Mφ contact. Another explanation is that different

batches of Mtb may exhibit different binding properties with BMM ϕ , as the Mtb used for the High and Low MOIs were grown as separate cultures.

Whereas the DT between WT and KO were not statistically different within experiments, there was a noticeable difference in the DT between Glaxo BMM ϕ infected at the High MOI and Itgam BMM ϕ infected at High and Low MOI. The difference in DT appeared to be related to the observed pattern of Mtb uptake between the groups. As the uptake of Mtb decreased between the BMM ϕ populations the DT also decreased; Glaxo High MOI DT > Itgam High MOI DT > Itgam Low MOI DT, indicating that high bacterial numbers were detrimental to intracellular Mtb replication. In both the Glaxo and Itgam M ϕ populations, the KO 100:1 DT was slightly longer than KO 50:1 DT which further supports the suggestion that the difference in DT was a result of higher bacterial load in the M ϕ .

The role of CR3 in mycobacterial uptake became clearer in the presence of serum opsonins. I observed a 50% reduction of Mtb binding in the absence of CR3 at a LOW MOI and a 30% reduction in Mtb binding in the absence of CR3 at High MOI. With the presence of serum in the M ϕ monolayers I could not completely restore KO M ϕ binding of Mtb to WT levels at either MOI tested, matching results from Cywes et al who observed that Mtb bound to CR3 in, at least partly, a serum independent manner (41). I was able to restore Mtb binding to near WT levels by doubling the MOI in the CR3 KO M ϕ but because I was unable to fully restore binding, or observe overcompensation as was seen for non-opsonic uptake, the additional uptake in KO M ϕ s was most likely due to complement-mediated binding to other complement receptors, such as CR1 and CR4, and I therefore conclude that CR3 was a dominant receptor for Mtb binding in the presence of serum opsonins. These results indicated that CR3 played a major (although not exclusive) role in Mtb uptake under opsonic conditions but, under non-opsonic conditions,

alternative cell-surface receptors were better able to compensate for the reduced uptake of Mtb in the absence of CR3. The difference in uptake seen following infection with High and Low MOIs, both in the absence and presence of serum, also indicated that increasing the number of Mtb available for infection could at least partially overcome the loss of binding due to CR3 deficiency and that other cell surface receptors were sufficient in allowing Mtb invasion of host Mφs.

Because the initial interaction between Mtb and host cells in the lung is in a relatively serum-free environment (75), non-opsonic uptake of Mtb probably plays a more important role in the initial interaction of CR3 and Mtb and so I chose to focus the rest of my studies on the role of CR3 in the absence of serum.

Whereas I have confirmed that CR3 has a significant role in the binding and uptake of Mtb by resident Mφs, I also showed that CR3 does not play a significant role in the subsequent survival and replication of the bacteria. Mtb was able to replicate in CR3-deficient Mφs at rates comparable to those seen in WT Mφs, irrespective of which of the two CR3 knockouts was utilized, the MOI used or whether serum was present during uptake. I have shown that Mtb does not gain any selective advantage, nor a significant disadvantage, by entering resident Mφs via CR3.

Because I showed that the lack of CR3 did not significantly affect the growth of Mtb within resident Mφs I wanted to examine the possibility that entry via CR3 may play a role after the onset of acquired immunity in the host. The onset of T-cell mediated immunity in the host is characterized by the activation of host Mφs by T-cells via cytokines such as IFN- γ and TNF- α . To mimic the host events that take place during acquired immunity I treated Mφ monolayers with IFN- γ . As expected, treatment of BMMφ with IFN- γ successfully activated the Mφs so that they

were better able to control Mtb growth (45, 76, 77). IFN- γ treatment also resulted in fewer bacteria being taken up during the initial infection in both Glaxo and Itgam WT and KO M ϕ s, confirming earlier reports that IFN- γ plays a key role in host immunity against intracellular pathogens and has been shown to alter the expression of cell surface receptors (37, 78-80). IFN- γ treatment has been specifically shown to reversibly inactivate CR3 and reduce adherence and ingestion of mycobacteria (11, 37) and other particles (81), however this explanation for the reduction of Mtb binding in the presence of IFN- γ is only applicable in WT BMM ϕ . IFN- γ treatment also down-regulates the function of other complement receptors in addition to CR3 (11) and it was therefore more likely that the reduction of Mtb binding in the presence of IFN- γ was due to an overall down-regulation of the receptors used for Mtb entry. The effect of IFN- γ on Mtb binding was less obvious in the Glaxo BMM ϕ populations than in the Itgam BMM ϕ populations, perhaps because of the higher MOI, and therefore increased binding, of Mtb to M ϕ s which in turn, diminished the effects of IFN- γ activation receptor binding. An alternative explanation is that M ϕ s derived from the Glaxo background were less susceptible to the effects of IFN- γ on surface receptor expression.

LPS was also used in conjunction with IFN- γ to enhance the activation of M ϕ s. This treatment significantly reduced the binding of Mtb to both Glaxo and Itgam M ϕ s. LPS has been shown to reduce the capability of CR3 on murine M ϕ s to bind and phagocytose complement opsonized particles (82) and CR3 has been shown to act as a receptor for LPS (28) through a site distinct from the iC3b site. It was possible, therefore, that addition of LPS to the monolayers inhibited binding of Mtb by competing for the same lectin binding site or that LPS causes a conformational change in CR3 that changes the binding capabilities of CR3, rendering it less

efficient in binding Mtb. However, because a decrease in binding in the presence of LPS was also seen in KO Mφs lacking CR3 it is more likely that LPS enhances Mφ activation by IFN-γ thus increasing the inhibition of binding seen by IFN-γ through increased down regulation of receptors. LPS has also been shown to inhibit the expression of Mφ surface receptors such as the scavenger receptor and it may be via LPS-mediated down-regulation of this and other receptors that the decrease in Mtb binding is seen (83).

Although uptake of Mtb was affected by the absence of CR3 in activated Mφ populations, I did not observe any concomitant differences in intracellular replication. In both IFN-γ and IFN-γ plus LPS treated Itgam Mφs there was a slight, but statistically insignificant, reduction in the DT of Mtb in KO Mφs infected at the same MOI as WT, suggesting that entry via CR3 may inhibit replication of the bacteria in the host cell. When the MOI was increased in the KO Mφs, however, this difference was diminished or eliminated altogether, indicating that the slower replication in the WT Mφ was more likely due to the presence of additional Mtb in the cell and was not related to the absence or presence of CR3. However, in Glaxo BMMφ, the opposite phenomenon was observed. In IFN-γ plus LPS treated Glaxo Mφs there was a slight, but statistically insignificant, increase in the doubling time of Mtb in KO Mφs infected at the same MOI as WT, suggesting that entry via CR3 may enhance the replication of the bacteria in the host cell. The DT in KO Mφs were somewhat longer than the DT in WT Mφs indicating that, in the Glaxo Mφ populations, entry via CR3 improved bacterial replication and was advantageous to Mtb. In the IFN-γ activated Glaxo KO BMMφ increasing the MOI in the KO Mφs from 50:1 to 100:1 further increased the DT, again suggesting that an increase in the initial Mtb load can have a negative impact on the subsequent growth of intracellular mycobacterial growth.

However, as there was no statistical difference between Mtb growth in resident or activated WT or KO Mφs at any MOI, or in either the Glaxo or Itgam populations, I therefore conclude that entry via CR3 did not play a significant role in the intracellular survival of Mtb and it was not the absence, or presence of CR3 alone that effected bacterial replication.

I examined whether CR3 played a role in the respiratory burst of Mφs and if this receptor does indeed act as a “stealth receptor” in either resident or immune-activated Mφs. CD11b^{-/-} mice have been shown to be deficient in oxygen radical formation and neutrophils have been used to show that a striking increase in oxygen radical generation accompanies CD11b/CD18 mediated phagocytosis (16). More specifically, CR3 has been shown to play a dominant role in the stimulation of a neutrophil superoxide burst mediated by ZYM and β-glucan particles (84). Further evidence in the role of CR3 in the respiratory burst is the observation that patients with a genetic deficiency in CR3 have neutrophils that do not have a respiratory response to ZYM (85).

I examined whether CR3 played a role in the induction of the respiratory burst during Mtb invasion of Mφs. Using PMA, a potent receptor-independent stimulator of the respiratory burst, I found that KO Mφs were not inherently deficient in the production of an internal respiratory burst and as expected, treatment of the cells with IFN-γ significantly increased the respiratory burst from both WT and KO Mφs. ZYM was used as a receptor-mediated particle control and contrary to earlier reports (10, 16) I found no significant difference in the ability of KO Mφs to produce a respiratory burst in either resident or IFN-γ activated Mφs. I think that the differences found in my current study were due, in part, to differences in the Mφ types examined (BMMφ in this study versus peritoneal Mφ and neutrophils) and the assay utilized (intracellular production of ROI in this study versus extracellular production of ROI). Of note, in both

resident and activated PMA and ZYM stimulated Mφs, the KO cells exhibited a slightly lowered respiratory burst, however, the differences were not statistically significant.

I found no significant difference in the production of superoxide measured between resident WT and KO Mφs or between IFN-γ activated WT and KO Mφs when triggered with Mtb. My results indicated that Mtb alone did not elicit a respiratory burst in either resident or activated Mφs. Mtb possess a multitude of virulence factors that may allow them to evade the respiratory burst, such as superoxide dismutase (sodB) and catalase (katG) that can detoxify oxygen radicals (86, 87), and lipoarabinomannan that can scavenge ROI (88, 89), therefore, it is likely that Mtb are resistant to the effects of any ROI produced from IFN-γ activated Mφs (90). Consequently, it has been found that oxygen radical scavengers have no effect on the antimycobacterial activity of Mφs and mice deficient in the NADPH-oxidase complex are still able to control mycobacterial infection indicating that ROI probably do not play a significant role in the killing of Mtb (47, 61, 91). Other reports have also suggested that mycobacteria may invade Mφs without eliciting a respiratory burst (92, 93). I believe that this is because one or more of the above listed virulence factors either breaks down or inhibits the production of intracellular ROIs during phagocytosis. More recently, it has been shown that the production of ROI plays no role in controlling the growth of Mtb unless the bacteria is specifically deficient in the mechanism used (katG) to counteract this host defense mechanism (46).

Although CR3 has been shown previously to play a role in the respiratory burst I have shown that, under certain conditions, alternative phagocytic receptors allowed particle uptake into the Mφ and an accompanying internal respiratory burst in the absence of CR3. However, in the case of Mtb, this was most likely redundant because the bacteria were not shown to specifically elicit a respiratory burst and would be resistant to it if there was one. It is also

probable that different cell types or different M ϕ populations may have different respiratory burst responses, as it has been shown that mycobacteria are phagocytosed by different receptors depending on the M ϕ type encountered (7). Also, of interest is the observation that superoxide production in response to nonopsonic uptake of ZYM and mycobacteria depends on which of the binding epitopes of the receptor is engaged (35, 94). There is also evidence from neutrophil populations that phagocytosis is triggered by CR3 via simultaneous recognition of the iC3b site in conjunction with polysaccharide binding via the lectin site which induces a primed receptor state (95), indicating that more than one trigger is needed to produce a respiratory burst. Uptake of ZYM also occurs via the mannose (96) and β -glucan receptors (97) including dectin-1 (98) and it may be via these receptors or in cooperation with these receptors that the accompanying internal respiratory burst occurs. The differences in contribution of CR3 to the respiratory burst may also be due to the activation state of the receptor. CR3 exists in two distinct conformational states, active and non-active, that regulate the binding capabilities of the receptor (99). In response to certain agonists CR3 undergoes a rapid activation that exposes new binding epitopes on the cells surface (8, 100) and it may be the activation state of the receptor that determines the M ϕ response/production of ROI. It has also been shown that engagement of different epitopes of CR3 determines whether a respiratory burst will accompany phagocytosis (19, 35, 101). CR3 has also been shown to interact with Fc γ receptors to facilitate neutrophil functions (102) and it has been shown that cooperation between CR3 and the Fc γ receptors receptor is required for the generation of a synergistic respiratory burst (66). Binding of specific peptide motifs have been shown to indirectly influence the binding activity of CR3 most likely through interactions with other surface receptors (103). Finally, it has also been shown that mycobacteria can enter the M ϕ via the mannose receptor without an accompanying respiratory burst and it may be that this

receptor is a better candidate for the silent entry of mycobacteria into Mφs (104). There are a multitude of receptors accompanying various signaling mechanisms to account for the production of the respiratory burst in the absence of CR3.

An interesting observation made when examining the role of ROI on the survival of Mtb in the host Mφ was the toxicity of some of the most common antioxidants used to assess the role of ROI in host defenses. I found, with both DPI and NAC, that Mφ cell death in the monolayer was evident at 24 hours after infection. In the presence of DPI the monolayers had completely deteriorated and the Mtb CFU counts were almost undetectable by Day 4 of growth *in vitro*. The toxicity of these compounds to the Mφs means that previous information on bacterial growth, in relation to ROI, obtained by examining growth in the presence of these compounds, is now subject to questioning. With Mtb in particular, the presence of an intact, live Mφ is necessary to facilitate intracellular growth of the bacteria. If the antioxidant being used to examine the role of ROI is toxic to the Mφ one cannot be sure if any results obtained were due strictly to the inhibition of ROI or if it is simply the death of the Mφ that is responsible for the outcome. For example, if I did not consider the role of cell death due to the presence of the antioxidant, I could conclude from my results that ROI were essential for the survival of Mtb as, in the absence of ROI, rapid mycobacterial clearance was observed. It is imperative that future examinations on the role of ROI on intracellular mycobacterial survival refrain from using compounds that are toxic to the Mφ to ensure that only the role of ROI is determined.

It has been concluded that NO is essential for the control of mycobacterial growth in the host, based on studies using both iNOS KO mice and the use of iNOS inhibitors. I performed two sets of experiments related to this phenomenon: the examination of NO production in relation to the presence of CR3 and the role NO plays in the control of intracellular Mtb growth.

Treatment of Mφs with IFN-γ alone prior to infection has been shown to result in a significant increase in production of NO from the cells (105), which is potentiated by infection with Mtb (77) or the addition of LPS (106). I found that NO production from Mφs, in response to Mtb, was significantly enhanced by pre-treatment of the cells with IFN-γ prior to infection. In Itgam BMMφ, the production of NO was further increased by the addition of LPS to the IFN-γ activated monolayers at infection, confirming that LPS and IFN-γ can act synergistically to induce iNOS (107). Mφs synthesize large amounts of IL-1 and TNF-α in response to LPS (108) which in turn, upregulate the activity of iNOS and the production of NO (109, 110). IFN-γ treatment and the subsequent TNF-α induction induces the production of NO from LPS-treated murine Mφs (111, 112). CR3 can also interact with GPI-anchored proteins such as CD14, the receptor for LPS, providing the signal transduction machinery (31) for transmembrane signalling capabilities. It may be via this interaction, and through the transcriptional regulator NFκB, that IFN-γ and LPS in conjunction with Mtb provides such a significant production of NO after infection (113). In contrast to the Itgam BMMφ populations, addition of LPS to IFN-γ activated Glaxo BMMφ monolayers did not result in a significant increase in NO production from these cells. This difference in NO response to Mtb indicated that the Glaxo BMMφ did not respond to LPS, perhaps due to a defective CD14 or Toll-4 receptor or because of a deficiency in downstream receptor signaling pathways.

By Day 7 after infection, I saw very little NO in the supernatants from infected resident Mφ populations and significant production of NO from IFN-γ activated Mφs corresponding to the inhibition of Mtb growth in these populations. The levels of NO from the supernatants from IFN-γ activated Itgam KO Mφs infected at an MOI of 20:1 were significantly lower than NO

levels from WT 20:1 on Day 7 indicating that CR3 may play a role in the production of NO from Mφs. The decrease in NO production, however, was annulled by the addition of more Mtb, indicating that, much like the loss of binding due to the absence of CR3, the loss of NO production in CR3 KO Mφs could be overcome through alternative surface receptors. Even though there was a significant difference in the levels of NO produced between WT and KO monolayers infected at the same MOI, there was no indication that this difference had any effect on the growth of Mtb, as DT between WT and KO IFN-γ activated Mφs were not found to be significantly different. This may be because the mycobactericidal effect of NO has a threshold effect, i.e. only a certain level is needed to achieve growth inhibition and anything more does not significantly affect the end point. Alternatively, it may be that NO is not directly responsible for the killing of mycobacteria in IFN-γ activated Mφs and so no difference in Mtb growth was observed. Therefore, I conclude that although CR3 may play a small role in the production of NO from host Mφs, it played no significant role in the control of Mtb replication.

The addition of LPS to the Itgam monolayers resulted in a large increase in the levels of NO in IFN-γ activated supernatants, but did not show a corresponding increase in inhibition of Mtb growth in the host. This result further strengthened the above conclusion that NO may either have had an effective threshold that is needed to control mycobacterial growth or played no role in controlling growth in Mφ.

The production of NO from the Mφ has been shown to play an important role in the inhibition of intracellular growth of mycobacteria both *in vivo* and *in vitro*. Work with inhibitors of the production of NO has shown that NO plays an important role in the antimycobacterial effect of Mφs (62, 114) and that the use of chemical inhibitors of NO is an effective tool to examine the role of NO in biological systems by preventing its production. The addition of L-

NMMA or other iNOS inhibitors to M ϕ populations inhibits the production of NO from the cell (115). It has been shown that L-NMMA accelerated the growth of Mtb H37Rv inside IFN- γ activated murine peritoneal M ϕ s (116) and of *Mycobacterium bovis* in IFN- γ activated BMM ϕ (91) indicating an essential role of NO production in the inhibition of mycobacterial replication. I therefore expected that the addition of L-NMMA to Mtb infected IFN- γ activated M ϕ s would abrogate the inhibition of Mtb growth in these M ϕ s (117). However, I did not observe any effect of the NO-inhibition on the survival of intracellular Mtb.

The data available on the contribution of NO to the control of mycobacterial growth is currently diverse and contradictory. Whereas many researchers have established the link between the production of NO and killing of mycobacteria, other groups have also found that NO may not play the key role it has been assigned. Inhibition of NO with L-NMMA has no effect on the growth of *M. bovis* (BCG) within human monocytes and M ϕ s, suggesting that NO does not play a role in growth in these cells (118). Inhibition of mycobacterial growth in an IFN- γ plus LPS activated M ϕ cell line could not be completely abrogated in the presence of L-NMMA (119). The growth of *M. leprae* was not increased in iNOS KO mice and growth could be controlled in the absence of NO (120). Other groups have found that iNOS KO mice as well as IFN- γ activated M ϕ s from these animals are still able to control growth of *M. avium*, underscoring the lack of a role for RNI in the control of this organism as well (121, 122). It has been suggested that under stringent conditions, RNI can kill Mtb, but that under less harsh, more physiological conditions, the effects of RNI range from partial to negligible (123).

An interesting possibility to explain these differences is that the susceptibility of Mtb to RNI and the ability of L-NMMA to reverse the suppression of growth of Mtb in M ϕ s may be a phenomenon related to the strain of Mtb being used for experimentation. It is known that strains

of Mtb vary in their susceptibility to RNI generated *in vitro* (124) and in IFN- γ activated M ϕ s (123). I used Mtb strain Erdman for the current study and so it may be that the observations I made were limited specifically to this strain, though Erdman is neither particularly resistant or susceptible to RNI generated *in vitro* (123).

I also consider that the methodology used to examine the role of RNI on mycobacterial growth in M ϕ influences the results obtained. In the current study I infected M ϕ monolayers with Mtb and monitored the growth of the bacteria and NO production from the same well. In addition, the enumeration of Mtb in infected M ϕ included the contents of the entire well; i.e. the monolayer plus the cells that have lifted over the course of the experiment into the culture medium. Other studies have reported Mtb growth only in the intact monolayer that is remaining after the reported period of intracellular growth. Previous work has shown that, over time, M ϕ infected with Mtb detach from the coverslip yet still contain viable bacteria (38). These bacteria still remain part of the closed *in vitro* system and should be included in any quantitation of CFU present in that experimental group. Quantitating bacteria in just the monolayer does not reflect the true consequences of any treatment added to the well and may result in under- or overestimating the CFU present in the well. For instance, I observed that L-NMMA treatment greatly reduced the loss of M ϕ from the monolayer when compared to IFN- γ treated M ϕ . Enumeration of CFU in the monolayers alone could have erroneously led to the conclusion that L-NMMA resulted in enhanced viability of the Mtb. However, by assessing the viable bacteria present in both adherent and non-adherent M ϕ I found that there was no significant difference in the CFU present in M ϕ treated with IFN- γ alone or IFN- γ plus L-NMMA.

Pathogenic mycobacteria have been shown to be able to inhibit the production of NO from IFN- γ activated M ϕ s (125, 126) as well as have the capacity to resist toxic nitrogen radicals

(127) and it may be in this capacity that virulent mycobacteria evade the M ϕ immune response. The ability of other intracellular pathogens, such as *Salmonella typhimurium* and *Candida albicans*, to suppress the NO response from IFN- γ activated M ϕ s has also been reported (128, 129). Furthermore, virulent mycobacteria have also been shown to be resistant to RNI *in vitro* (130) and have evolved potential virulence factors that protect themselves from RNI (131). The presence of phthiocerol dimycocerosates in the mycobacterial membrane have been shown to protect the bacteria from the cidal activity of RNI released by activated M ϕ s (132). Recently, it has been shown that iNOS does not colocalize to the mycobacterial phagosome, suggesting that the mycobacteria may inhibit iNOS recruitment to phagosomes during M ϕ infection as a means to protect themselves from intracellular NO exposure (133).

While my results showed that RNI produced by M ϕ s infected with Mtb was not directly mycobactericidal, my data does not contradict the observations that iNOS mice are more susceptible to Mtb. An alternative explanation for why RNI may be essential for optimal control of mycobacterial infections independent of enhanced M ϕ killing lies in the ability of the host to develop RNI-expressing granulomas (49, 134). iNOS KO mice have large, bacteria-filled granulomas at time of death instead of the small compact granulomas that are normally seen in WT mice. Adams et al. were able to abrogate the inhibition of mycobacterial growth in IFN- γ activated peritoneal M ϕ s by the addition of the iNOS inhibitor AG but also found that in the lungs of iNOS knockout mice, growth of Mtb was the same compared to WT controls early in infection (up to day 28) and only after this time point was a role of NO evident in these mice (62). In iNOS KO mice infected with Mtb via low-dose aerosol infection (mimics the path by which humans are infected) bacterial growth is also not different in early infection than in WT mice infected via the same route (135). Results such as these may indicate that, under certain

conditions and with certain M ϕ types in an artificial *in vitro* system, RNI can play a direct role in the control of mycobacterial growth. However, *in vivo*, the role that RNI play may be limited to the effects they have on the formation of a tight granuloma to control mycobacterial growth. Evidence suggests that NO production may be a double-edged sword in the fight against mycobacteria. On one hand the molecule may be able to directly inhibit the growth of mycobacteria, but on the other, its detrimental role in inflammation/granuloma formation and the T-cell response may be what allows mycobacteria to survive in the host. As suggested by work with a NOS2 KO mouse, it is probably not the direct role of NO that plays in the killing of mycobacteria but the important role it plays in the development of the immune response (135). NO still plays a important role in the cellular response to infection, and it is the cellular response that is important in regulating granuloma formation and the dissemination of mycobacteria throughout the tissues. Without NO, granulomas do not form properly and bacterial spread is not controlled, the bacteria can spread throughout the body where they grow more readily in the absence of NO, especially in the liver (135). In addition to poor granuloma formation, the lack of NO allows tissue infiltration of T-cells and other lymphocytes that would not normally happen in the presence of NO, resulting in damage to the lung tissues. It is possible that the increased murine death rate in NOS KO mice is not seen simply because NO is needed to kill the bacteria but is due to increased lymphocyte infiltration and damage to the lungs, followed by the inability to control bacterial growth once the pathogen spreads to the surrounding tissues.

NO may still play a role in the control of mycobacterial growth however, based on my observations, this role cannot be as a direct toxicity to the bacteria inside the M ϕ . It is more likely that NO plays a role in regulating other immune events that are responsible for the control of mycobacterial growth *in vivo*. Perhaps most interestingly, NO has been shown to have both

pro-and anti-apoptotic effects related to the concentration of NO elicited under physiological and pathophysiological conditions: high concentrations tend to trigger apoptosis and low, inhibits apoptosis (136). NO has been shown to play a role in inducing T-cell apoptosis (137) as well as having the ability to protect cells from TNF- α induced apoptosis (138). As apoptosis has been shown to be a mechanism by which the host controls the replication of Mtb (139, 140), this presents the possibility that NO is inhibiting the growth of Mtb via the apoptotic killing of infected cells. Support for the suggestion that NO plays an important role in the induction of apoptosis was seen in BMM ϕ monolayers with and without L-NMMA. In BMM ϕ monolayers treated with IFN- γ , which induces NO production and consequently apoptosis, I observed a progressive detachment of Mtb infected M ϕ s from the coverslip into the supernatant over the course of infection. In the presence of L-NMMA, however, the M ϕ monolayers remained attached to the coverslip throughout the infection, indicating that the elimination of NO prevents the induction of M ϕ apoptosis.

The absence of gross abnormalities or lack of immune compromise in CD11b deficient mice also supports my conclusions that CR3 does not play a specific role in the success of Mtb infection (16). In addition, no discernable effect of the loss of CR3 on the disease course of Mtb in either resistant or susceptible mouse strains has been observed (10, 141). Data from experiments using *M. avium* and CD18-deficient mice also support the indication that mycobacteria can enter tissue M ϕ s through receptors other than CR3 and that CR3 may not play as important a role in the course of TB infection as previously thought (142). The mannose receptor has been named as a receptor that may play an important role in mediating M ϕ adherence of virulent strains of Mtb (3, 143) and CD14 has been suggested via its interactions with mycobacterial lipoarabinomannan (144). Looking at the role of CR3-mediated uptake from

the point of view of the bacilli, also supports the idea that CR3 does not play a role in the control of intracellular growth. It has also been found, using selective receptor blockade, that intracellular survival and replication of mycobacteria were the same no matter which cell surface receptor was used for binding and phagocytosis, indicating that the mechanisms used by mycobacteria to evade killing are independent from the receptor route of entry (6).

The initial association of Mtb with M ϕ s in the relatively serum-free lung would be an important step in the infection of the host. However, as resident alveolar M ϕ s poorly express CR3 and do not efficiently bind mycobacteria in the absence or presence of serum (39) this suggests that the role CR3 plays in Mtb pathogenicity is, at best, limited to the later stages of infection.

My current results further demonstrate that the role of CR3 in the pathogenesis of Mtb is minimal.

CHAPTER 5.

CONCLUSION

Together, the data presented indicated that although CR3 played a definitive role in the non-opsonic and opsonic binding and uptake of Mtb by murine BMM ϕ *in vitro*, the presence of this cell surface receptor was not essential for the successful establishment of host infection. Furthermore, entry via CR3 did not contribute to the intracellular survival of Mtb in either resident or IFN- γ activated M ϕ s. IFN- γ activation of the host M ϕ s significantly inhibited intracellular mycobacterial growth in addition to increasing the induction of the respiratory burst and the production of RNI, however, CR3 was not shown to be essential for any of these M ϕ functions. Of particular interest is the finding that NO could not be shown to play a direct role in the control of intracellular Mtb growth. Finally, whereas differences were found between BMM ϕ derived from mice with distinct genetic knockouts of the same gene, the utilization of different KO backgrounds did not change the conclusions made with respect to the function of CR3 in the pathogenesis of Mtb and the M ϕ defense mechanisms examined.

Because a stronger role for Mtb uptake via CR3 was evident in the presence of serum opsonins, a role for CR3 in the pathogenesis of Mtb may still be found upon further examination of both WT and KO M ϕ s under opsonic conditions. Whereas a role for CR3 could not be shown in resident M ϕ s under opsonic and non-opsonic conditions, or in IFN- γ activated M ϕ s under non-opsonic conditions, it is still of interest to look at intracellular Mtb growth, the induction of the respiratory burst and the production of RNI under opsonic conditions in IFN- γ activated M ϕ s. It may be under these conditions that a role for CR3 can be established.

Most important is further examination of the role of NO in the control of Mtb. While the use of iNOS KO mice have shown a definitive role for the production of NO in host control of

Mtb, I have shown that its effects cannot be attributed to direct toxicity of NO to the bacteria. It would be of interest to examine if the difference between my results and those reported by other groups, in terms of direct toxicity of NO, is due simply to examination of whole culture wells versus only the intact monolayer. Alternatively, *in vitro* examination of the growth of Mtb in Mφs in the presence of alternative NO inhibitors, such as AG, could be performed to confirm that the observation that NO does not play a direct role in mycobacterial growth inhibition is not limited to the effects of L-NMMA. Also, comparison of the induction of apoptosis in resident and IFN-γ activated Mφs with and without the presence of L-NMMA, *in vitro*, would help to determine if the production of NO by Mφs triggers the induction of apoptosis as a means to control intracellular Mtb growth. Finally, future experiments should focus on how NO contributes to the host control of Mtb *in vivo*, specifically, the role that NO plays in the induction of host Mφ apoptosis and granuloma formation and how these relate to mycobacterial killing.

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