THE ROLE OF CD43 IN THE GROWTH AND PATHOGENESIS OF *MYCOBACTERIUM TUBERCULOSIS* WITHIN THE MAMMALIAN HOST

by

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Abstract

Mycobacterium tuberculosis exploits various molecules on host cells to gain entry and establish a niche for survival and replication. We characterized the role of the glycoprotein CD43 in the pathogenesis of *M. tuberculosis*. Using gene-deleted mice (CD43-/-), we assessed association of the bacterium with macrophages and found that CD43 was required for optimal binding of *M. tuberculosis* strain Erdman by splenic, peritoneal, alveolar, and bone marrow-derived macrophages. Macrophages from heterozygote (CD43+/-) mice, which express 50% less CD43 than wild type (CD43+/+) mice, bound more bacteria than CD43-/- but less than CD43+/+ indicating that the surface expression of CD43 correlates with binding of *M. tuberculosis*. The role of CD43 in binding bacteria may be restricted to mycobacterial species as CD43-/- macrophages also bound less *Mycobacterium avium* and *Mycobacterium tuberculosis* H37Rv, but there was no observed role in the binding of *Salmonella typhimurium* or *Listeria monocytogenes*. Although absence of CD43 resulted in decreased binding of *M. tuberculosis*, the subsequent growth of the bacterium within CD43-/- macrophages was enhanced as illustrated by increased bacterial numbers and decreased doubling times, indicating that that the mechanism of entry may influence subsequent.

To elucidate mechanisms by which CD43 controls of growth of *M. tuberculosis*, we examined the induction of antimycobacterial activities. In response to *M. tuberculosis*, CD43-/- macrophages were deficient in the production of nitric oxide, TNF-α, and IL-12. Furthermore, *M. tuberculosis* induced less apoptosis, but more necrosis, in CD43-/- macrophages compared to CD43+/+. The enhanced growth of *M.*

tuberculosis was abrogated by IFN-γ-stimulation with whereas addition of TNF-α restored both the intracellular growth rates and amounts of apoptosis to wild type levels.

To investigate the role of CD43 *in vivo*, we infected CD43-/- and CD43+/+ mice with *M. tuberculosis* and assessed bacterial loads and organ pathology. Absence of CD43 resulted in increased bacterial loads in lungs and spleens during both acute and chronic stages of infection, and formation of granulomas occurred more quickly in CD43-/- mice. These data point to a dual role for CD43 in the uptake and subsequent growth of *M. tuberculosis* in macrophages and mice.

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List of Abbreviations

AIDS	acquired immune deficiency syndrome
AMϕ	alveolar macrophage
BCG	Bacille Calmette-Guerin
BMMϕ	bone marrow-derived macrophage
CFU	colony forming unit
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DOT	directly observed therapy
EIgG	IgG-coated erythrocytes
EIgMC'	IgM & complement-coated erythrocytes
ELISA	enzyme-linked immunosorbant assay
ESAT-6	early secreted antigenic target-6
EMB	ethambutol
GVHD	graft versus host disease
H&E	hematoxylin & eosin
HIV	human immunodeficiency virus
ICAM	intercellular adhesion molecule
IEL	intraepithelial lymphocytes
IFN	interferon
INH	isoniazid
LAM	lipoarabinomannan

LDH	lactate dehydrogenase
LPS	lipopolysaccharide
Mϕ	macrophage
mAb	monoclonal antibody
MDR	multiple drug-resistant
MHC	major histocompatibility complex
MOI	multiplicity of infection
MTB	*Mycobacterium tuberculosis*
NO	nitric oxide
OADC	oleic acid-albumin-dextrose complex
PIM	phosphatidylinositol
PMϕ	peritoneal macrophage
PPD	purified protein derivative
PZA	pyrazinamide
RA	rheumatoid arthritis
RIF	rifampin
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
SpMϕ	splenic macrophage
STR	streptomycin
Th1	T-helper cell type 1
TLR	toll-like receptor

TB	Tuberculosis
TST	Tuberculin skin test
TNF	tumour necrosis factor
WAS	Wiskott-Aldrich syndrome
WT	wild type
WHO	World Health Organization
XDR	extensively drug-resistant

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Dedication

In loving memory of Balwant Singh Randhawa, who taught me the value of education, inspired me to pursue my goals, and instilled in me the drive and determination needed to achieve them.

Chapter 1 Introduction

1.1 Tuberculosis

Tuberculosis (TB), a chronic bacterial infection caused by *Mycobacterium tuberculosis*, is the leading cause of death due to an infectious agent (1). Active TB infection is characterized by fatigue, weight loss, a chronic productive cough, fever, and night sweats. The majority of TB infections occur after inhalation of droplet nuclei expelled from an infectious host. It has been estimated that a single respiratory event, such as a cough, generates thousands of droplets containing the causative bacterium and that inhalation of less than 10 bacilli may lead to infection in a susceptible host (2). This route of infection will most often lead to deposition of the bacteria within the lungs, potentially causing pulmonary TB. Although this is the most common route of transmission, TB infection can also occur in other organs including the skin, eyes, lymph nodes, genitourinary tract, bones and joints, meninges and the gastrointestinal tract. The TB bacterium is highly contagious with a single TB case causing up to 15 new infections per year (3, 4).

Infection of immune competent individuals without prior exposure to TB results in a mild self-limiting disease in the majority of cases. Approximately 90% of individuals exposed to TB will not develop active disease, but the bacteria will remain in a latent state. Reactivation disease or secondary TB can occur years or even decades later with the same characteristics as primary TB. 5-10% of infected individuals will go on to

develop active TB; those with depressed immunity may succumb rapidly to severe systemic disease causing death within weeks.

Conventional tests for diagnosing TB have major limitations and, until recently, no major advances in diagnostics had been made for many years. A widely used tool for diagnosing clinically undeclared TB is the Tuberculin Skin Test (TST). TST measures delayed type hypersensitivity response to the purified protein derivative (PPD), a crude mixture of several mycobacterial antigens (5, 6). A major drawback of this diagnostic is the high rate of false positives due to prior BCG vaccination or previous exposure to non-tuberculous mycobacteria (7). In addition, the administration and reading of the TST results in variability and inaccuracies. Sputum smears have also been used for many decades as a reliable method of diagnosis, but not without drawbacks such as the inability to distinguish between strains. Culture of the organism has been regarded as the gold standard but has historically proven inefficient due to the slow growth of *M. tuberculosis*, although newer methods such as the BACTEC biphasic culture systems have provided faster recovery of organisms than traditional culture on solid media (8, 9). New methods for diagnosis include molecular biological tests based on nucleic acid amplification, automated sequencing analysis, and immunological assays for the rapid detection of *M. tuberculosis* antigens and antibodies to these antigens (10). In addition to challenges in diagnosing TB, there are several barriers surrounding prevention and treatment efforts, which will be explored in sections 1.1.3 and 1.1.4.

1.1.1 Prevalence & global impact of Tuberculosis

Every second of every day, someone is newly infected with *Mycobacterium tuberculosis* - the causative bacterium of TB (11). 1/3 of the world's population, or 2 billion people, are currently infected with *M. tuberculosis* and each year over 8 million new infections occur (12). With over 2 million deaths annually, TB is the most common cause of death due to an infectious agent (13, 14). The highest mortality rate due to TB infection occurs in Africa, where the epidemic is growing most rapidly due to the HIV infection rates in the region. In sub-Saharan Africa, the rates are as high as 350 cases per 100 000 people – even greater than in South-East Asia, which had the largest number of new cases in 2005 (11).

TB, along with HIV and malaria are the deadliest human pathogens today, but research funding for these devastating diseases is not proportionate to their impact on global health. Although TB affects a significant portion of the world's population, there is more funding spent on smallpox and anthrax, which have not proven to be global health threats in recent years (15). With the tremendous global impact of this disease, and the complexities of the causative organism that will be discussed in upcoming sections, it is clear that further dedication of research funding is required, and warranted.

1.1.2 Factors contributing to the resurgence of Tuberculosis worldwide

TB, once known as the white plague or consumption, became the principal cause of death during the 19th century. The incidence of TB began to decline rapidly in the

early 20th century in developed countries with improved living conditions and these trends were accelerated by the implementation of vaccination and by the development of antibiotics such as streptomycin and isoniazid. The number of reported cases of TB continued to decline until the mid 1980's when an increase in reported cases was observed and led to the World Health Organization (WHO) declaring TB a global public health emergency in 1993. The resurgence in TB cases worldwide can be attributed to a number of factors including prevention and treatment complexities, and social conditions.

Social factors that have led to the resurgence of TB include increasing populations, poverty, and homelessness, as TB thrives in crowded living conditions where people are in close contact with others with infectious TB. Individuals living in such conditions have poor access to light, fresh air, sufficient food, and clean water, predisposing them to a variety of infections. Moreover, the increase in world travel has allowed the rapid spread of infectious agents across borders and has exposed all populations to the threat of this disease.

One of the most important contributing factors to the resurgence of TB is the HIV/AIDS pandemic. The increase in TB rates began in the 1980s along with the emergence of HIV, and rates have continued to rise with the increased percentage of HIV positive individuals worldwide (1, 11, 14). It has been estimated that the risk of developing active TB among HIV/TB co-infected persons is 8% per year, compared to a 10% lifetime risk for those latently infected with *M. tuberculosis* alone (16). In addition, AIDS patients are also susceptible to infection with opportunistic mycobacteria such as the *M. avium* complex and other atypical mycobacteria (17). TB has proven to be the

principal cause of mortality among HIV positive persons in hospitals and HIV is more prevalent among TB patients than within the whole populations, indicating a tragic synergy between the two diseases (18). According to the WHO, more than 30% of the increase in TB cases since 2000 can be associated with HIV co-infection (19).

The recent emergence of multiple drug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis* are further complicating treatment of this disease. These strains require lengthy treatment with second line anti-TB drugs which are not only more expensive than first-line chemotherapeutics, but produce more severe side-effects. TB treatment and prevention efforts are both extremely complex and advancement has been confounded by a number of factors that will be explored in the next section.

1.1.3 Prevention of Tuberculosis

History has shown that TB can be controlled to some extent by improving standards of living, such as sanitation, clean drinking water and nutrition. However, these advancements are not always possible, especially in developing nations where additional measures must be taken to prevent new infections. The currently available TB vaccine, Bacille Calmette-Guerin (BCG), originated from a virulent strain of *Mycobacterium bovis* that was passaged extensively over 13 years causing loss of virulence in animal models (20, 21). BCG has been used since the 1920s in attempts to prevent TB infection, but has demonstrated a wide range of efficacy from 0-80% in randomized control trials so is no longer in use in some parts of the world, including Canada (22). Despite

variability in protecting against pulmonary TB in adults, BCG has been proven effective, and is still used for protection, against childhood TB, TB meningitis, and miliary TB with over 100 million infants vaccinated per year (23).

The range of observed efficacy of BCG against TB can be attributed to a number of factors including the great global variation in clinical TB strains, altered immunity due to previous exposure to environmental mycobacteria, and the absence of TB antigens in the vaccine strain (24-26). An ideal vaccine would prevent disease in the absence of prior exposure to *M. tuberculosis*, prevent reactivation of latent infection, and would not interfere with TB diagnosis (3, 27). Current strategies for vaccine development include attempts to boost the T-helper type 1 (Th1) immune response by using immunodominant antigens of *M. tuberculosis* such as Antigen 85A and the 6 kDa early secreted antigenic target (ESAT-6) as protein subunit or DNA vaccines (28-31). Alternatively, the current BCG vaccine could be improved by inducing expression of such immunodominant antigens (32, 33). Several groups are also attempting to produce live, attenuated *M. tuberculosis* virulence mutants, or auxotrophic mutants although the safety of live vaccines is controversial (34-36). Recently, the role of apoptosis in immunity to *M. tuberculosis* has lead to the development of pro-apoptotic mutants that elicit an enhanced immune response and may be considered as potential vaccine candidates (37, 38).

1.1.4 Treatment of Tuberculosis

Before the development of effective chemotherapy, nearly half of TB patients died within two years of developing active pulmonary disease (39). There are now five

first-line antibiotics for use in treating TB; streptomycin (STR), isoniazid (INH), ethambutol (EMB), rifampin (RIF), and pyrazinamide (PZA). STR, which works by inhibiting protein synthesis, was first discovered in the mid 1900s and was demonstrated to be effective against pulmonary TB in one of the first ever randomized clinical trials (40-42). INH was the next drug added to the treatment regimen, and was found to function by inhibiting cell wall synthesis, similarly to EMB which was discovered soon after INH (43-45). The addition of RIF, an inhibitor of RNA synthesis in *M. tuberculosis*, in 1970 allowed for a decrease in treatment duration to 9 months (46, 47). Although discovered in the 1950s, it wasn't until 1986 that PZA, an inhibitor of fatty acid synthesis, was added to the INH/RIF regimen, further reducing treatment time to 6 months (48-50).

The worldwide resurgence of TB since the 1980s and the emergence of drug-resistance prompted the WHO to implement Directly Observed Therapy (DOT), combining the use of standardized diagnostics and therapeutics along with case management in efforts to improve detection and treatment (51). The current drug regimen used in areas with high TB incidence is a two month induction phase of combined therapy of INH/RIF/PZA and either EMB or STR, followed by a continuation phase of RIF/INH for up to four additional months (47, 52). This regimen results in a success rate of greater than 85% among drug-sensitive strains compared to greater than 50% mortality if active TB is left untreated (53).

Successful treatment of TB is highly dependent upon strict adherence to prescribed drug therapy. Compliance is often compromised due to the length of treatment, drug side effects and, especially in the developing world, access to adequate

heath care services. These obstacles are even further confounded by the increased rates of multi-drug resistant and extensively drug resistant strains of *M. tuberculosis*.

1.2 *Mycobacterium tuberculosis* biology

Mycobacterium tuberculosis is a member of the genus Mycobacterium and the order Actinomycetales. Other members of the genus include non-pathogenic environmental strains such as *M. smegmatis*, opportunistic pathogens such as members of the *M. avium-intracellulare* complex, and pathogenic strains such as *M. leprae*, the cause of leprosy, and *M. ulcerans*, the cause of buruli ulcers. Other pathogenic strains that do not normally affect humans, but are important in the agricultural industry include *M. bovis*, which causes a TB-like disease in cattle and many other species, and *M. avium* ssp. *paratuberculosis*, the causative agent of Johne's disease in several species of ungulates (54).

The mycobacteria are slim, rod-shaped organisms 0.2 to 0.4 x 2 to 10 μM in size. *M. tuberculosis*, the most notorious member of the Mycobacteria, was first isolated and cultured by Robert Koch in 1882. It is a slow growing, non-motile, non-spore-forming intracellular pathogen that is highly resistant to drying and chemical disinfection. The complex cell wall of *M. tuberculosis* includes mycolic acid, a β-hydroxy fatty acid that makes the organism difficult to visualize with commonly used stains. Specialized stains such as Ziehl-Neelson, carbol fuchsin, and Auromine O must be used to stain mycobacteria which, once stained, resist decolourization with up to 3% hydrochloric acid, 95% ethanol, or both; this characteristic is referred to as acid-fastness (2, 54, 55).

The cell wall of *M. tuberculosis* is highly intricate and a major determinant of virulence for the pathogen. The plasma membrane is surrounded by the cell wall core: a layer of peptidoglycan covalently linked to arabinogalactan, which is in turn attached to the long mycolic acids. The upper segment of the cell wall structure is composed of free lipids, and fatty acid chains interspersed with the cell wall proteins. These proteins include the phosphatidylinositols (PIMs), the phthiocerol-containing lipids, lipomannan, and lipoarabinomannan (LAM) (56). One of the cell wall lipids, cord factor (trehalose 6,6'-dimycolate, TDM) is a mycolic acid containing glycolipid and is responsible for the characteristic serpentine cording often visible when *M. tuberculosis* is grown on solid media, and the clumps formed in liquid culture (56). The outer layer of the mycobacterial cell wall, or the capsule, consists of polysaccharides, proteins, and lipids and has been shown to play a role in host cell interactions

The complex cell wall is a major factor contributing to the very slow growth of this organism which has a doubling time of approximately of 10-20 hours in liquid media, or 24 hours on solid media. Slow growth poses a challenge for diagnosis and treatment of Tuberculosis; clinical specimens such as sputum samples must be incubated for several weeks to confirm diagnosis, and testing of antibiotic susceptibility is also affected, leading to severe consequences for patients infected with drug-resistant strains.

1.3 The immune response to *Mycobacterium tuberculosis*

Although infection with *M. tuberculosis* can occur at virtually any tissue site, the lung represents the chief site of infection. When droplets containing TB bacilli are

expelled by an infectious host, they can be taken up by others in the vicinity. The organisms enter the lungs where they are taken up by phagocytes, mainly alveolar macrophages (Mϕ). Mϕ and dendritic cells (DC), the main mediators of the innate response to *M. tuberculosis*, can then stimulate adaptive immunity through antigen presentation and the production of cytokines. Specific T cells stimulated in the draining lymph nodes can travel to the site of infection and induce the formation of granulomas, which contain the infection. However, eradication of the bacteria does not occur and the infection may persist or enter a state of dormancy. Several components of the immune system are involved in the response to this pathogen and will be discussed in following sections.

1.3.1 Mycobacteria:Mϕ interactions

Mϕ are most often the first cells of the immune system to encounter inhaled *M. tuberculosis* bacilli. It is well-known that these cells serve as a niche for *M. tuberculosis* and are exploited during the establishment of successful infection. The interaction of *M. tuberculosis* with Mϕ can occur in the presence or absence of serum opsonins (57, 58). In healthy individuals, the lungs are relatively free of serum opsonins so it is thought that the initial uptake of *M. tuberculosis* occurs non-opsonically; this inference is supported by the finding that alveolar Mϕ do not express high levels of receptors for serum opsonins (59, 60).

Several studies have been devoted to characterizing the Mϕ surface molecules involved in mediating the binding and phagocytosis of *M tuberculosis* (61). To date,

mycobacteria have been reported to be detected or bound by: the complement receptors, CR1, CR3 and CR4 (57, 58, 62, 63); Fcγ receptor (64); the mannose receptor (62, 65-67); scavenger receptors (61); surfactant proteins A and D (68-71); dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (72, 73); CD14 (74); CD43 (75, 76); CD44 (77); and possibly dectin-1 (78, 79) and the toll-like receptors (80-82). The relative contribution of each of these moieties to mycobacterial recognition is not completely understood, though the complement receptors appear to have a major role in binding *M. tuberculosis* in both opsonic and non-opsonic environments (58, 83).

The possible ligands on mycobacteria that bind the aforementioned Mϕ surface molecules remain unidentified, although several studies point to potential candidates. For example, lipoarabinomannan is thought to bind the mannose receptor (65, 84) and phosphatidylinositols have been shown to bind CR3 at a site distinct from the iC3b binding domain (83, 85, 86).

It is well established that Mϕ can mediate killing or inhibition of intracellular pathogens. The antimicrobial activities of Mϕ have proven to be important elements of immunity, but are not completely understood. In response to *M. tuberculosis*, Mϕ employ several effector mechanisms including the production of reactive nitrogen (RNI) and oxygen (ROI) intermediates, phagolysosome fusion leading to phagosome acidification, and the induction of apoptosis (87, 88). The intracellular survival of *M. tuberculosis* depends on the organism's ability to inhibit such antimycobacterial functions, and the bacterium has evolved several strategies to do so.

Mycobacteria have the capacity to arrest the development of the phagosome at the early endosome stage, and to hinder the fusion of the phagosome with lysosomes (64, 89,

90). This property, displayed by virulent strains of *M. tuberculosis* (91) prevents acidification of the phagosome and allows access to the extracellular milieu, thereby giving the pathogen to readily access nutrients such as iron and facilitating intracellular survival (92, 93). Residing in the early endosome may also reduce antigen processing, impeding activation of other immune cells (94). Phagosome maturation is promoted by activation of Mϕ by IFN-γ, which also stimulates other antimycobacterial functions including the production of ROI and RNI (92, 95).

The activation of Mϕ by cytokines such as TNF-α and IFN-γ results in the initiation of intracellular signaling pathways that lead to the activation of NADPH oxidase and the generation of ROI such as superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen, and the production of nitric oxide from L-arginine by inducible nitric oxide synthase (96). In the murine model, RNI have been shown to have a protective role in both the acute and chronic phases of *M. tuberculosis* infection (97, 98). Moreover, high levels of nitric oxide synthase expression has been detected in alveolar lavage fluid from patients with active TB (99). The importance of ROI, however, is not as as clearly described (100), although hydrogen peroxide produced by activated Mϕ has been shown to be mycobacteriocidal (101). Mycobacteria are capable of evading these toxic metabolites by mechanisms such as the production of a catalase peroxidase, KatG, which has been shown to be required for growth of *M. tuberculosis in vivo* (102) and counteracts the toxic effects of hydrogen peroxide *in vitro* (103).

An additional innate defense mechanism employed by Mϕ is the induction of apoptosis, which allows for controlled killing of the infected cell without eliciting an inflammatory response (104). A role for apoptosis in restricting the growth of *M.*

tuberculosis has been well-evidenced and is thought to function by depriving the bacterium of a niche and leading to enhanced cross-priming of antigen-specific CD8+ T cells (37, 105-108). Evasion of apoptosis by mycobacteria has also been described as an additional means of intramacrophage survival (105, 109, 110) and is thought to occur by the upregulation of anti-apoptotic pathways by the pathogen (111).

1.3.2 Adaptive immunity

Cell-mediated immunity to *M. tuberculosis* develops 2 to 6 weeks after infection following the secretion of cytokines and chemokines by infected phagocytes including Mϕ and DCs. These cells can also travel to the draining lymph nodes where they will present mycobacterial antigens to circulating T cells (88, 112). The T cell subsets involved in mounting a protective response against *M. tuberculosis* include primarily CD4+ and CD8+ T cells (88, 113, 114), although there is increasing evidence of a role for CD1 restricted T cells (108, 115, 116) and αβ/γδ T cells (117-119) may also play a role (120). Expansion of antigen-specific T cells is followed by the development of the granulomas at the site of infection; such lesions contain activated B and T lymphocytes, monocytes, fibroblasts, and multi-nucleated giant cells (88, 121). Granuloma formation occurs in an attempt to sequester and prevent dissemination of the infection, but mycobacteria exploit this environment to establish a latent infection that can eventually re-activate. Such re-activation of infection will cause increased bacterial growth and caseation of the granulomas, leading to eruption of the lesion and release of the bacteria into the airways thereby facilitating transmission to a new host (88, 121, 122).

1.3.3 Latency/persistence

A feature of *M. tuberculosis* central to its pathogenesis is its unique ability to persist in the granulomas of an asymptomatic host. In these situations, the immune response is able to restrict and contain the pathogen, but not completely eradicate it. Viable *M. tuberculosis* have been isolated from granulomatous lesions in the lungs of individuals with clinically inactive TB, suggesting that the bacteria can survive in a latent state for several years (123, 124). It is not known whether the bacteria are actually metabolically dormant or if they continue to replicate during inactive disease. Attempts to investigate these questions are made difficult by the lack of animal models that accurately represent human latent TB, though *in vitro* and *in vivo* systems have been developed and have contributed to the current knowledge (125-129). Further investigation is necessary, given the difficulties surrounding treatment of latent TB.

1.4 CD43

CD43 (gpL115; leukosialin; sialophorin) is a sialoglycoprotein that is abundantly expressed on cells of haematopoietic origin including monocytes/macrophages, granulocytes and T cells (130-132). The molecule is a member of the mucin-type O-glycoprotein family, which are characterized by the linkage of N-acetylgalactosamine to serine or threonine residues.

CD43 has been shown to have a role in number of biological functions including cell activation, regulation of apoptosis, lymphocyte homing, and cell signaling (133, 134). It is proposed that CD43 has dual function in cell-cell interaction as there is evidence for it acting as both an adhesion and a barrier molecule (133, 135-142).

CD43 knockout mice (CD43-/-) have been generated by homologous recombination via embryonic stem cell chimeras. Genetic deletion of CD43 results in a mild phenotype with normal T cell development and normal haematopoiesis (135, 143). Although preliminary attempts at knocking out the CD43 gene in mice resulted in a hyperproliferative T cell phenotype (135), this was likely due the 129 genetic background of the mice used, as it has since been shown that CD43-/- mice backcrossed onto the C57Bl/6 background do not display this hyper-responsive phenotype (143).

1.4.1 CD43 expression

The human CD43 gene is found on chromosome 16p11.2, while in mice it is located on chromosome 7 (144-146). In both species, the gene is encoded by two exons, the first encoding for a 5'-untranslated sequence whereas the second exon encodes the entire polypeptide backbone (145, 147, 148). Furthermore, CD43 in lymphoid cell lines has been found to be encoded by two mRNA species with diverse polyadenylation signals, that are encoded by the same CD43 exon and translated to a single gene product (144, 147). It is thus evident that the molecular weight heterogeneity of the CD43 molecule is not due to alternative gene splicing, but post-translational modifications (131).

As previously stated, CD43 expression has been found on most haematopoietic cells, with the exception of mature B cells. CD43 was originally described on K562 human erythroleukemic cells (149), but has since been found to be expressed on a number of cell types in humans, mice, rats and rabbits (131, 132, 138, 150-152). Tissue specific expression patterns of CD43 isoforms have been observed on granulocytes, monocytes, platelets, pre-cursor and mature T cells, B cell pre-cursors and antibody-secreting plasma cells (131, 153-155). It is proposed that the level of CD43, and the prevalent gycoforms expressed on these cell types varies with cell lineage, or maturation/activation state of the cells (156).

Although CD43 is thought to be a lymphohaematopoietic cell surface marker, it has also been found on colon cell adenomas carcinomas, even though normal epithelial

cells in the colon do not express CD43 (157, 158). The kinetics of increased CD43 expression in these studies suggests a role for the molecule in tumour development.

The regulation of CD43 expression is thought to occur by several mechanisms. The mucin-like extracellular region of CD43 has been found in human plasma so there appears to be some means of shedding (159). There is evidence for the molecule being proteolytically cleaved from neutrophils, T cells and mast cells (160-162), and in neutrophils this can be induced by antibody ligation or by stimulatory molecules such as TNF-α (163). In dendritic cells, antibody ligation of CD43 causes internalization of the molecule by endocytosis (164).

1.4.2 CD43 structure

CD43 is a single chain type I transmembrane glycoprotein of 400 amino acids in humans or 395 in mice including a leader peptide (19aa) followed by the extracellular region (234aa in humans, 228aa in mice), a transmembrane domain (22aa) and an intracytoplasmic domain (123aa in humans, 124aa in mice) (145, 165). The overall composition of the molecule has been found to be 60% carbohydrate and 40% protein (165, 166). CD43 has a significant number of sialic acid residues, which impart a net negative charge on the molecule and may be responsible for some of its anti-adhesive properties (167). The extracellular mucin-like region extends 45nm from the cell surface and is a rigid carbohydrate covered polypeptide backbone rich in serine, threonine, and proline residues (144, 168). Proteolytic cleavage of the extracellular region of CD43 can

occur, and has been found in a soluble form known as galactoglycoprotein in blood plasma (169).

There are two major isoforms of CD43 that differ in the glycosylation of the extracellular portion leading to molecular weight heterogeneity. The 115kDa glycoform has been shown to be predominant on resting T cells and monocytes and is characterized by tetrasaccharide side chains, whereas neutrophils and activated T cells carry mainly hexasaccharide side chains and express the 135kDa CD43 glycoform. Some cell types, including macrophages, are thought to express a mixture of both isoforms (170, 171). The branching of the O-glycans on CD43 is attributed to the activity of the glycosyltransferase Core 2-β−1,6-N-acetylglucosaminyl transferase (C2GnT), which catalyzes the transfer of N-acetylglucosamine residues on O-glycans (171-174). Monoclonal antibodies (mAb) can be used to distinguish the two CD43 glycoforms. For example, the rat mAb 1B11 recognizes only the 135kDa hexasaccharide form, whereas the lower molecular weight tetrasaccharide glycoform can be identified by the rat mAb S7. In contrast, the mAb S11 has been shown to recognize CD43 independent of glycosylation and can thus be utilized as a pan-CD43 antibody (174-176).

It has been demonstrated that the molecular weight heterogeneity of CD43 is due to post-translational modifications and not alternative exon splicing (131). Furthermore, heterogeneity among species is largely due to differences in the extracellular regions, as the transmembrane and cytoplasmic domains are highly conserved among humans, mice and rats. Mouse and human CD43 transmembrane and cytoplasmic domains share, respectively, 65% and 72% sequence identity while the extracellular region shows only

42% identity (146, 152, 177). Despite differences in the extracellular region, the heavily glycosylated mucin characteristics remain similar across species.

1.4.3 Described functions of CD43

Several distinct functions have been described for CD43 including roles in cell activation and proliferation, and signal transduction. In cell-cell interactions, CD43 is proposed to have a dual function as there is evidence for both adhesive and anti-adhesive properties (133). Moreover, CD43 has been implicated in regulating cell death through its role in apoptosis Evidence for these suggested functions of CD43 will be discussed in this section.

Cell Activation

CD43 has been shown to be involved in the activation of T cells, monocytes and neutrophils. Several studies showed that anti-CD43 mAb (L10) could induce proliferation in T cells comparable to that induced by anti-CD3 (178, 179). It was later shown that additional anti-CD43 antibodies could amplify T cell proliferative responses induced by the known T cell mitogens Concanavalin A and leukoagglutinin (138). Sperling et al. found that an anti-CD43 mAb (R2/60) could co-stimulate T cells in the absence of accessory cells in a fashion similar to CD28, suggesting a role for CD43 as a T cell receptor (180).

Anti-CD43 mAb L10 has also been implicated in activation of monocytes, as measured by hydrogen-peroxide producing capacity, at levels comparable to activation

by IFN-γ (181), and in activation of neutrophils leading to increased adhesiveness and aggregation (182).

Cell signaling

Although there are no definitive ligands identified for CD43, antibody ligation of the molecule has been shown to induce cell signaling events. It is likely this process is mediated by the cytoplasmic tail of CD43, as this region displays properties which indicate signaling capabilities, such as the presence of conserved serine and threonine residues that could potentially be phosphorylated, and has been found to be required for CD43 to generate intracellular signals (183-186). Park *et al*. found the cytoplasmic domain of CD43 to be hyper-phosphorylated during T cell activation, and others have shown constitutive phosphorylation in response to tumour promoting phorbol esters and ligation of the molecule with anti-CD43 antibodies (183, 186, 187). CD43 engagement in human T cells leads to tyrosine phosphorylation and recruitment of Src family kinases to the cytoplasmic tail of CD43, and phosphorylation of the adapter proteins Shc and SLP-76 leading to several downstream signaling events (180, 188, 189). Furthermore, CD43-mediated activation of T cells can result in activation of the mitogen-activated protein kinase pathway, causing recruitment of transcription factors including AP-1, NF-AT, and NF-κB, ultimately leading to regulation of gene expression (189, 190). Despite the evidence for signaling capabilities, and identification of some components of CD43-mediated signaling cascades, the mechanisms by which CD43 leads to these events are not completely understood. It should be noted that CD43 has also been implicated in

signaling events leading to cell death by apoptosis, evidence for which will be discussed in the proceeding section.

Apoptosis

Numerous experimental findings have pointed to a role for CD43 in the regulation of apoptosis. Both pro- and anti-apoptotic roles have been described as ligation of CD43 on T cells and haematopoietic progenitor cells can induce cell death (191-194), but high levels of CD43 expression on T cells can inhibit T cell receptor (TCR)/CD3 mediated apoptosis (195). Recently, it was found that CD43 can modulate Fas-induced cell-death in a human T lymphoblastic cell line pointing to a potential role in activated-induced cell death (196). CD43 expression decreases during neutrophil apoptosis and has been shown to parallel, but not result from, membrane blebbing, with the molecule being released on microvesicles distinct from blebs (197, 198). Moreover, transient capping of CD43 has been observed on Jurkat cells undergoing apoptosis and that this correlated with the ability of macrophages to recognize these cells, suggesting that CD43 capping is responsible for the generation of ligands for recognition of dying cells by the immune system (199). The mechanisms by which CD43 modulates apoptosis are unclear, but it could possibly occur by direct receptor-ligand interactions during adhesion, or CD43 itself may be a transducer of apoptotic signals.

Adhesion vs. Anti-adhesion

The role of CD43 in cell-cell interactions has been heavily debated, as there is contradictory evidence whether the molecule has an adhesive or anti-adhesive role (133,

134). The large size of CD43, and its heavily glycosylated and negatively charged extracellular region would suggest a tendency for the molecule to repel cell contact. In agreement with these physical properties, it has been shown that CD43 expression causes decreased cell adhesion and that targeted disruption of the molecule increases cell-cell contact (135-137, 200, 201).

Conversely, there is also substantial evidence pointing to a pro-adhesive role for CD43. Although the specific *in vivo* interactions are unknown, several ligands have been proposed for CD43, including intercellular adhesion molecule-1 (ICAM-1) (202), complement component C1q (203), E-selectin (204, 205), galectin-1 (206), sialoadhesin (207), and major histocompatibility complex class 1 molecules (MHC-1) (141). Additionally, anti-CD43 mAbs are able to inhibit cell interaction, further supporting adhesive functions of CD43 (139, 140, 208).

The structural properties discussed earlier, and the experimental evidence for the diverse roles of CD43 suggest that the function of the molecule may depend on the specific interactions in play. Clearly, there is evidence supporting a dual-functionality of CD43 that is integral to regulation of cell interactions.

1.4.4 CD43 & disease

Dysregulation of CD43 expression has been shown to have a potential role in a number of disease processes including graft-versus-host disease (GVHD), Wiskott-Aldrich Syndrome (WAS), Human Immunodeficiency Virus/Acquired Immune

Deficiency Syndrome (HIV/AIDS), and Rheumatoid Arthritis. The evidence for the involvement of CD43 in these conditions will be further discussed in this section.

Graft-versus-host Disease

GVHD is a syndrome characterized by pathology in several organs including the skin, liver, lungs and gastrointestinal tract. This disease is a common complication of allogenic stem cell transplantation and can be classified as acute or chronic. Acute GVHD is mediated by immunocompetent donor T cells migrating to lymphoid tissues after infusion, where they recognize host alloantigens and become activated upon interaction with host antigen-presenting cells (209). These activated donor T cells display differential expression of the CD43 glycoforms. In GVHD patients, activated donor T cells show increased expression of 135 kDa CD43 (210). In the mouse model of GVHD, it has been shown that both 115 kDa and 135 kDa structures are upregulated on CD4+ T cells in the spleen. Interestingly, on CD8+ cells, CD43 135 kDa was found to be upregulated while CD43 115 kDa was down regulated. Additionally, other activation markers such as IL-2α/β and ICAM-2 were found to be upregulated on these cells, lending further evidence for 135 kDa CD43 being characteristic of activated T cells (211). Moreover, a diverse population of small intestine epithelial T cells called intraepithelial lymphocytes (IEL), which normally express either 115 kDa or 135 kDa CD43, express both CD43 glycoforms in GVHD patients (212). These reports link CD43 to effector-like activities in GVHD that are related to the glycosylation of the molecule.

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS) is an inherited immunodeficiency syndrome characterized by thrombocytopenia, eczema, recurrent infections, and increased incidence of autoimmune diseases and malignancies (213, 214). Several studies have found abnormal glycoforms of CD43 exhibiting altered glycosylation patterns in WAS patients (170, 172, 215, 216). Further, circulating T cells in WAS patients were found to express the high MW glycoform of CD43, which is normally only associated with activated T cells (216). The activity of the enzyme responsible for the branching of O-glycans on CD43, C2GnT, was found to be increased in resting T cells, B cells and platelets of WAS patients (216). The abnormal branching on CD43 and increased activity of C2GnT indicates a dysregulation of T cell activation in WAS patients, and it has been proposed that this causes escape of self-reactive T cells into the periphery leading to autoimmunity. In addition, T cells from WAS patients show reduced proliferation in response to immobilized CD43 antibody and produce less IL-2, leading to further immune defects (217). A role for CD43 has also been proposed in eczema seen in WAS patients, as altered T cell binding to glycoproteins in the epidermis has been linked to CD43 and atopic dermatitis and other forms of cutaneous inflammation (218-220).

HIV/AIDS

Acquired immune deficiency syndrome (AIDS) is caused by one of two lentiviruses termed human immunodeficiency viruses (HIV-1 and HIV-2), which disable the immune system, thus predisposing patients to an array of opportunistic infections. Auto-antibodies to CD43 have been found in HIV patients' T cells (221). Furthermore, T

cells from these patients appear to be sulphated and hyposialated, which could impair CD43-mediated homotypic aggregation and cause the formation of auto-antibodies, which may accelerate disease progression (222, 223). Such auto-antibodies may affect the viability of T cells in HIV patients as CD43 ligation has been shown to induce apoptosis (191, 192). In addition, one group found that HIV patients that have progressed to AIDS expressed a partially sialated CD43 in the activation-associated form (135 kDa), whereas an asymptomatic HIV patient expressed the 115 kDa glycoform on T cells, consistent with healthy controls suggesting that MW of CD43 increased with disease progression (224, 225).

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease primarily characterized by chronic inflammation of the joints and surrounding tissue leading to destruction and functional disability, but it can also affect other organs leading to systemic illness. T cells in the synovial joint fluid of RA patients have been shown to have higher than normal levels of the high MW CD43 135 kDa. It has also been demonstrated that adhesion molecules, crucial to inflammation, are expressed in neutrophils of RA patients' joint tissue, and that the integrin CD11b is upregulated, while L-selectin, CD43 and CD44 are down-regulated (226). Despite this decreased expression of CD43, the interaction of these neutrophils with the endothelium remains intact. CD43 shedding was found to be enhanced by a neutrophil elastase that is present in high quantities in synovial joint fluid of RA patients (167). The significance of these findings to RA pathology remains to be elucidated.

1.5 Introduction to the thesis

The information presented in this chapter validates the importance of research into TB pathogenesis. It is quite apparent that novel preventative and therapeutic strategies are required and thus we must broaden our knowledge of the disease processes from both the perspective of host defenses and bacterial evasion of such defenses. The interactions that occur between the pathogen and host are pertinent to our understanding of mycobacterial biology and TB pathogenesis. The initial event in infection, recognition of *M. tuberculosis* by host Mϕ, is a critical step in the establishment of infection and therefore merits thorough investigation.

Numerous Mϕ receptors have been identified as having a role in the initial association with *M. tuberculosis* but the differences in the outcome of infection depending on the route of bacterial entry are not fully understood. A study in 2000 by Fratazzi et al. was the first evidence of a potential role for CD43 in mycobacterial infections (75). CD43 was found to be required for efficient binding of mycobacteria by macrophages and CD43-transfected HeLa cells, and addition of soluble human CD43 restored binding of *M. avium* to splenic macrophages from CD43 deficient mice. The authors suggested that CD43 is important in promoting a stable interaction between mycobacteria and host cells. Although no specific evidence was given for *in vivo* mycobacterial disease, this was the first description of a possible role for CD43 in an infectious process, and further investigation was indeed warranted.

In addition to this important evidence that CD43 may be involved in the host response to mycobacteria, it has been shown to be involved in a number of

immunological events and disease processes as previously mentioned. The described roles of CD43 in cell migration, activation, signaling and apoptosis led us to hypothesize that the role of CD43 in mycobacterial pathogenesis may extend beyond initial Mϕ-bacteria interactions and that CD43 may be a significant feature of the innate response to TB infection. We tested our hypotheses by first confirming the findings of Fratazzi, et al. and by extending those findings to additional cell types and mycobacterial strains, as outlined in Chapter 2. Subsequently, we sought to determine whether CD43 was involved in the growth and pathogenesis of *M. tuberculosis* in the mammalian host, using a mouse model of infection. Chapter 3 describes the second part of the investigation, where we enquired whether CD43 affects the survival and growth of *M. tuberculosis* in Mϕ, and the mechanisms by which mycobacterial growth is controlled in this model.

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Chapter 2 CD43 is required for optimal growth inhibition of *Mycobacterium tuberculosis* in macrophages and in mice[1]

2.1 Summary

We explored the role of macrophage (Mϕ) CD43, a transmembrane glycoprotein, in the pathogenesis of *M. tuberculosis*. Using gene-deleted mice (CD43-/-), we assessed the association of the bacterium with distinct populations of Mϕ and found that CD43-/- Mϕ bound less *M. tuberculosis* than CD43+/+ Mϕ. Increased infective doses did not abrogate this difference. However, reduced association due to the absence of CD43 could be overcome by serum components. Mϕ from heterozygote mice, which express 50% of wild type CD43, bound more bacteria than CD43-/- but less than CD43+/+ proving that the gene dose of CD43 correlates with binding of *M. tuberculosis*. Furthermore, the reduced ability of CD43-/- Mϕ to bind bacteria was restricted to mycobacterial species. We also found that the survival and replication of *M. tuberculosis* within Mϕ was significantly enhanced in the absence of CD43, making this the first demonstration that the mechanism of mycobacterial entry influences its subsequent growth. Most importantly, we show here that the absence of CD43 in mice aerogenically infected with *M. tuberculosis* results in an increased bacterial load during both the acute and chronic stages of infection and more rapid development of granulomas, with greater lung involvement and distinctive cellularity.

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2.2 Introduction

Mycobacterium tuberculosis infects over 8 million people and causes nearly 2 million deaths annually, making it the deadliest human pathogen. The World Health Organization estimates that between 2002 and 2020, 1 billion people will become infected with *M. tuberculosis* and over 36 million people will die of Tuberculosis (TB) if the rise in incidence is not controlled.

A critical step in the pathogenesis of *M. tuberculosis* is the initial interaction of the pathogen with the host macrophage (Mϕ). This interaction is mediated by several Mϕ receptors in association with ligands on the bacterium including the complement receptors CR1, CR3, and CR4, (1-6), Fcγ receptor (7), mannose/glucan receptors (1, 8), CD14, (9, 10), scavenger receptors (4) and surfactant protein receptors A (11, 12) and D (13). It has also been shown that CD43 (leukosialin; sialophorin) may be important in promoting a stable interaction of mycobacteria with Mϕ (14) .

CD43 is a negatively charged transmembrane sialoglycoprotein expressed on most hematopoietic cells (15). The function of this molecule has been the subject of debate; it has been shown that CD43 on T cells and B cells acts as a barrier molecule restricting cell-cell contact (16-19) but that it can also have a pro-adhesive quality (20-22). Thus, it has been proposed that CD43 may play a dual role in intercellular contact (23, 24). Involvement of CD43 in leukocyte homing and tissue infiltration, possibly due to its adhesive or anti-adhesive properties, has been shown in several studies (19, 25, 26). It has also been demonstrated that CD43 can regulate cell survival (27, 28) and is involved in the apoptosis of T cells and hematopoietic progenitor cells (29-32).

Fratazzi et al. first described a role for CD43 in mycobacterial pathogenesis when they found that splenic Mϕ from CD43-/- mice could not bind *M. tuberculosis* or *Mycobacterium avium in vitro* but that the ability to bind *M. avium* could be restored by addition of the extracellular region of CD43. They also found that CD43-transfected HeLa cells bound *M. avium* but not other bacteria, and that CD43 was required for TNF-α production by Mϕ in response to infection with *M. avium* (14).

In this study, we further explore the role of CD43 in the binding and uptake of *M. tuberculosis* by Mϕ and to determine the role of CD43 in *M. tuberculosis* pathogenesis using a gene-deleted mouse model that lacks expression of CD43 (33).

2.3 Materials and methods

Bacteria

M. tuberculosis strain Erdman (TMC #107; ATCC #35801), *M. tuberculosis* strain H37Rv (TMC #102, ATCC #27294), and *M. avium* (TMC #724, ATCC #25291) were grown to late log phase in Proskauer & Beck medium supplemented with 0.05% Tween 80. Cultures were stored and tested for viability as previously described (2). *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and *Listeria monocytogenes* were grown to mid-log phase in tryptic soy broth (Difco) and washed in PBS before use.

Mice

Wild-type (WT) control mice (CD43+/+), CD43-/-, and CD43+/- mice backcrossed 7 generations on C57Bl/6 background (33) were housed in a specific pathogen-free animal facility in micro isolator cages. Experiments were done in accordance with the standards set by the Canadian Council on Animal Care. For all experiments, mice were age- and sex-matched and controls were littermates.

Macrophage monolayers

Resident alveolar, peritoneal, and bone marrow-derived Mϕ (AMϕ, PMϕ and BMMϕ, respectively) were obtained from CD43-/- and CD43+/+ mice as previously described (2, 34, 35). Splenic Mϕ (SpMϕ) were obtained by gently disrupting spleens into single cell suspensions, washing in PBS, and resuspending cells in supplemented RPMI (RPMI 1640 medium with 10% fetal calf serum, 10 mM L-glutamine, and 10 mM

sodium pyruvate, all from Life Technologies, Inc., Grand Island, NY) at a concentration of $2x10^6$ cells per mL. Cells were plated in 1 mL aliquots onto 13 mm acid-washed, sterile glass coverslips in 24 well plates and incubated at 37°C/5% CO_2 for 24 hours, at which point the non-adherent cells were removed and 1 mL of fresh media added to each well. The cells were incubated for an additional 4 days before use.

Particles for probing macrophage receptors

The function of FcγR was examined using sheep red blood cells (SRBC) coated with IgG (EIgG), complement receptors were investigated using SRBC coated with IgM and iC3b (EIgMC'), while lectin-like receptors were probed using zymosan particles, all as previously described (2). Latex beads (Diameter 1.07 μm, Polybead® polystyrene microspheres, Polysciences, Inc., Warrington, PA.) were used to investigate non-specific phagocytosis.

Flow Cytometry

AMϕ, PMϕ, BMMϕ and SpMϕ from CD43-/- and control mice were isolated as described above and plated onto sterile petri dishes (bacteriologic plastic was used to facilitate subsequent removal of adherent cells). Following incubation at 37°C/5% CO_2, non-adherent cells were removed by washing with RPMI. Adherent cells were removed by cooling and scraping, washed with DMEM (Life Technologies) and processed for flow cytometry. Cells were stained with mAb S11-FITC (rat anti-pan-CD43, kindly supplied by Dr. John Kemp, University of Iowa), (36, 37) and/or rat anti-mouse Mϕ F4/80 (Caltag Laboratories, Burlingame, CA) at 2mg/mL, or with secondary antibody

alone (Streptavidin-cychrome). After staining, cells were washed twice with HBSS (Life Technologies), and analyzed on a FACScan IV flow cytometer (Becton Dickinson, Mountain View, CA).

In vitro assay for binding of particles to macrophages

Mϕ monolayers on coverslips in 24 well plates were washed twice with binding medium (138 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.6 mM CaCl2, 1 mM $MgCl_2$, and 5.5 mM D-glucose) (38). A 500 μL aliquot of binding medium was added to each well, and the cells were acclimatized for 10 min at 37°C/5% CO_2. For non-opsonic studies, the particles to be tested were diluted to the desired concentration in binding medium and added to monolayers. In studies of opsonic binding, 1% normal, or 1% heat-inactivated, CD43-/- or CD43+/+ mouse serum was added prior to addition of bacteria. For experiments with mycobacteria or particles, monolayers were infected for 1 hour rocking (Nutator, Becton Dickinson, Mountain View, CA) followed by 2 hours stationary at 37°C/5% CO_2, whereas in experiments with *S. typhimurium* and *L. monocytogenes*, monolayers were infected for 40 min at 4°C. Monolayers were then washed 3 times, fixed, stained with Kinyoun's Carbol Fuchsin and malachite green for mycobacteria-infected Mϕ, or Giemsa for other bacteria and control particles. Binding was quantified microscopically by counting 100 Mϕ per coverslip and assessing the percentage of Mϕ that bound at least 1 bacterium and the average number of bacteria that were associated with each infected Mϕ.

In vitro survival and replication of *M. tuberculosis* following phagocytosis by CD43-/- and CD43+/+ Mϕ

Intracellular growth assays were conducted in BMMϕ from CD43-/- and WT CD43+/+ mice. Monolayers were infected with *M. tuberculosis* Erdman at a multiplicity of infection (MOI) of 20:1 (bacteria:Mϕ). Because preliminary studies showed that fewer bacteria were able to infect CD43-/- Mϕ, these were also infected at 30:1. Monolayers were infected according to the procedures described above. To eliminate extracellular bacteria from the infected Mϕ monolayer, coverslips were washed three times in binding medium after infection and transferred to new 24 well plates containing 1 mL of supplemented RPMI per well. At this time (day 0), and on days 1, 4 and 7 post-infection, coverslips and supernatants were processed to assess the CFU, as previously described (39).

Growth and pathogenesis of *M. tuberculosis* in mice

CD43-/- and CD43+/+ mice were infected with a low dose of *M. tuberculosis* Erdman (50-100 CFU) using an inhalation exposure chamber (Glas-Col, Terre Haute, IN). On specific days post-infection, 4-6 mice in each group were euthanized, and lungs, livers and spleens were aseptically removed and weighed. A portion of each tissue was removed and fixed in 10% buffered formalin for histopathological examination while the remainder (lungs and spleens only) was homogenized in PBS to assess bacterial loads. Serial dilutions of tissue homogenates were plated on 7H10 agar supplemented with OADC. Plates were incubated at 37^{o}C and CFU counted after 21-25 days. Another group

of mice was infected intravenously with *M. tuberculosis* by injecting 1 x 10^5 bacteria into the tail vein. Bacterial loads were determined as described above.

Histopathology

Livers, spleens, and lungs from mice infected with *M. tuberculosis* (as described above) were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with Hematoxylin and Eosin (H&E) for histopathological examination using standard techniques. The population of cells in areas of pulmonary inflammation was quantified by classifying cells as either (i) typical Mϕ (including epithelioid cells), (ii) "foamy" Mϕ - cells having highly vacuolated cytoplasm, which gave a foamy appearance or (iii) lymphocytes. The proportion of each cell type in representative areas of inflammation was determined by counting 100 cells, and the results expressed to the nearest 5% to allow for a degree of variation between areas.

Statistics

Data are expressed as mean ± SEM. Student's *t* test for independent means was used; $p < 0.05$ was considered significant unless otherwise noted.

2.4 CD43-/- Mϕ of different origins bind *M. tuberculosis* less readily than CD43+/+ Mϕ at various multiplicities of infection

We first determined if CD43 affected the non-opsonic binding of *M. tuberculosis* by using SpMϕ, PMϕ, BMMϕ AMϕ from CD43-/- and WT CD43+/+ mice. SpMϕ, PMϕ and BMMϕ were infected at an MOI of 20:1 while AMϕ were infected at a higher MOI of 500:1 as it has been shown that these cells do not bind *M. tuberculosis* very well *in vitro* (35). After a 3 hour infection, we found that SpMϕ and PMϕ bound bacteria more efficiently than did BMMϕ and AMϕ, as assessed by both the percentage of the Mϕ population infected (data not shown) and the number of bacteria binding to individual infected Mϕ (Figure 2.1A). All CD43-/- Mϕ phenotypes bound significantly less bacteria than WT CD43+/+ Mϕ ($p<0.01$); CD43+/+ SpMϕ, PMϕ, BMMϕ and AMϕ bound, respectively, 117%, 31%, 162% and 55% more bacilli than did CD43-/- derived Mϕ (Figure 2.1A). We chose to use BMMϕ as a model in subsequent studies as they can be obtained in large numbers and represent a recently differentiated Mϕ, such as those that may be found entering sites of infection during the course of pathogenesis of *M. tuberculosis*.

Infection of BMMϕ with increasing numbers of *M. tuberculosis* demonstrated that reduced association of the bacteria with CD43-/- Mϕ was consistent over a wide range of MOI. At ratios of 20:1, 40:1, 60:1, 100:1 and 200:1 (Figure 2.1B) bacteria:Mϕ, CD43-/- Mϕ associated with significantly less bacteria than did CD43+/+ Mϕ ($p<0.05$). The reduction in non-opsonic binding of *M. tuberculosis* by CD43-/- BMMϕ is

manifested not only as a reduction in the number of bacteria binding to the Mϕ population, but also a reduction in the percentage of Mϕ binding at least one bacillus (data not shown).

2.5 Opsonization of bacteria overcomes the impaired ability of CD43-/- Mϕ to bind *M. tuberculosis*

CD43+/+ and CD43-/- BMMϕ were infected with *M. tuberculosis* in the absence or presence of 1% normal- or heat inactivated (HI) (56°C for 30 min) mouse serum at a MOI of 40:1 (Figure 2.2). For these experiments, only serum autologous to the Mϕ source was used. In the unopsonized control 27.8 ± 1.4% of CD43+/+ Mϕ had associated with bacteria while only 9.3 ± 1.3% of CD43-/- Mϕ were infected ($p<0.001$). In the presence of serum opsonins, a higher percentage of both Mϕ populations bound bacteria and there was no significant difference between them (51.0 ± 1.6% of CD43+/+ and 47.3 ± 4.8% of CD43-/- Mϕ bound bacteria). However, when monolayers were infected with *M. tuberculosis* in the presence of HI serum, the difference in binding of bacteria to the two Mϕ populations was restored (32.5 ± 2.5% of CD43+/+ Mϕ; 20.8 ± 1.9% of CD43-/- Mϕ associated with bacteria, $p<0.01$). Interestingly, the level of binding observed with CD43-/- Mϕ in the presence of HI serum was higher than that seen in the absence of any serum.

2.6 CD43 is involved in binding other mycobacteria but its absence does not abrogate binding of *S. typhimurium* or *L. monocytogenes* by BMMϕ

In order to determine if the reduced ability of CD43-/- Mϕ to bind *M. tuberculosis* strain Erdman was unique, we infected BMMϕ from CD43-/- and CD43+/+ mice with the virulent strain, H37Rv, with the opportunistic pathogen *M. avium* and with representative Gram-positive (*L. monocytogenes*) or –negative (*S. typhimurium*) intracellular bacteria (Figure 2.3). For *M. tuberculosis* and *M. avium,* BMMϕ were infected at MOIs of 40:1 and 20:1, respectively, for 3 hours. For non-mycobacteria, monolayers were infected at a MOI of 10 bacteria per Mϕ for 40 min at 4ºC. Less CD43-/- Mϕ were able to bind the mycobacterial species than could CD43+/+ Mϕ. However, there was no difference between CD43-/- and CD43+/+ Mϕ in their ability to bind the other bacteria.

2.7 CD43 deficiency does not affect Mϕ non-specific uptake or phagocytosis via FcγR and complement receptors, but does enhance binding via lectin-like receptors

To investigate whether CD43 deficiency affects the function of other Mϕ receptors, we measured binding of several different control particles that are commonly used to study receptor-ligand interactions of phagocytes (Figure 2.4). EIgG were used to investigate the function of FcγR and EIgMC' were used to examine complement receptors. Zymosan, a yeast cell wall preparation containing polysaccharides, was used

to probe for lectin-like receptors while latex beads were used to examine non-specific interactions. When assessing the actual number of particles associated with Mϕ, there was no difference in the ability of the CD43-/- Mϕ to phagocytose EIgG, EIgMC', or latex spheres when compared to the CD43+/+ controls (Figure 2.4). However, CD43-/- Mϕ bound significantly more zymosan particles than WT Mϕ (20.4 ± 0.8 vs. 14.13 ± 1.1, respectively, $p<0.01$).

2.8 The level of CD43 surface expression differs between Mϕ phenotypes

CD43 cell surface expression was monitored using mAb S11 (37). The epitope recognized by anti-CD43 mAb S11 is not affected by changes in CD43 glycosylation (40), thus mAb S11 cell surface binding reflects the level of CD43 protein expression. As expected, all Mϕ populations from CD43-/- mice did not express CD43 above background. Although all cell types from CD43+/+ mice expressed low levels of CD43 compared to T cells (41), levels on BMMϕ were higher than those on SpMϕ, PMϕ, or AMϕ (Figure 2.5). The mean fluorescence intensity for AMϕ was 20% of that seen in BMMϕ. In addition, a much lower percentage of the AMϕ population expressed CD43.

2.9 CD43 gene dose correlates to the ability of BMMϕ to bind *M. tuberculosis*

To ascertain whether the amount of CD43 expressed by Mϕ affects their ability to bind *M. tuberculosis*, we compared the binding of the bacterium to Mϕ that were heterozygous (+/-) for the CD43 gene (and express 50% less CD43 than do CD43+/+)

(33) with CD43-/- and CD43+/+ Mϕ (Figure 2.6). We found that the CD43+/- Mϕ population associated less with bacteria than did the CD43+/+ Mϕ (19.0 ± 2.5% and 37.3 ± 2.11%, respectively). In contrast, only 11.8 ± 1.6% CD43-/- Mϕ had associated bacilli (Figure 2.6A). When the actual numbers of bacteria per Mϕ were assessed, CD43 gene dose again correlated with the amount of associated bacteria, as CD43+/+ cells bound 2.07 ± 0.24, while CD43+/- bound 1.08 ± 0.24, and CD43-/- bound 0.56 ±0.11 bacilli per Mϕ (Figure 2.6B).

2.10 The survival and replication of *M. tuberculosis* within CD43-/- Mϕ is enhanced

BMMϕ from CD43+/+ and CD43-/- mice were infected with *M. tuberculosis* and the subsequent growth of the bacteria was measured by determining CFU over 7 days. As shown in Table 1, at a MOI of 20:1, CD43+/+ Mϕ phagocytosed more bacteria than CD43-/- Mϕ on day 0 ($p<0.001$) but by day 7 there were comparable amounts in the two populations. However, when CD43-/- Mϕ were infected at a MOI of 30:1, the same amount of bacteria were taken up as in CD43+/+ Mϕ at 20:1 on day 0 and by day 7 post-infection there were twice as many bacteria in the CD43-/- population ($p<0.001$). Moreover, the doubling times of *M. tuberculosis* in CD43-/- Mϕ were significantly less than in wild type Mϕ, where it took 27.69 ± 0.26 hours for one doubling compared to 24.04 ± 0.18 and 24.12 ±0.39 hours in CD43-/- Mϕ infected at 20:1 and 30:1 respectively ($p<0.001$).

2.11 CD43 deficient mice have a reduced ability to control *M. tuberculosis* growth during the acute and chronic phases of infection following aerosol inhalation of bacteria

To determine the role of CD43 on the *in vivo* growth and pathogenesis of *M. tuberculosis*, we infected CD43-/- and CD43+/+ mice aerogenically with a low dose of the bacterium (50-100 bacilli). There appeared to be no differences in bacterial load in either the lung or spleen during the first two weeks of infection (Figure 2.7). However, by day 28 there was a significantly higher bacterial load in both the lungs and spleens of mice lacking CD43. After this initial peak, there was a period of host control of growth in the CD43-/- mice resulting in the bacterial load being reduced to levels similar to those in the control mice. Subsequently, bacterial loads remained relatively constant in CD43+/+ mice whereas in CD43-/- mice, bacterial loads steadily increased until the termination of the experiment. There were no differences in the survival of mice (data not shown). A comparable experiment following infection over a shorter time period (84 days) gave similar results (data not shown).

To determine whether the greater bacterial load in the spleens of CD43-/- mice during the acute phase of infection (up to day 28) was due to a greater susceptibility of splenic Mϕ in CD43-/- mice or to greater seeding of the spleen with bacteria from the lung, we infected CD43-/- and CD43+/+ mice with *M. tuberculosis* via the intravenous route. This ensured equal numbers of bacteria were deposited into the spleens and lungs of both mouse strains. No differences were seen in the growth rate of *M. tuberculosis* over 6 weeks (Figure 2.8).

2.12 Organ pathology is exacerbated in CD43 deficient mice

Histopathologic assessment of organs from *M.tuberculosis*-infected mice revealed that pathology in CD43-/-mice was more severe and developed more rapidly than in CD43+/+ mice. By day 1 post-infection, lymphoid hyperplasia was evident in the spleens of CD43-/- mice and by day 56 these mice displayed multifocal granulomatous inflammation affecting nearly 50% of the lung (Table II). This level of pathology did not appear in the CD43+/+ control mice until day 85. By the final experimental time point, CD43-/- mice had granulomatous inflammation affecting more than 50% of the lung sections, severe lymphoid hyperplasia in the spleen, and vascular, perivascular, and interstitial infiltrates of lymphocytes and neutrophils in the liver. At various time points CD43-/- mice also showed an increased number of foamy Mϕ in lung sections. Although granulomas in CD43+/+ mice also contained some foamy Mϕ, these were only seen during the chronic stages of infection and decreased in numbers towards the end of the experiment whereas in CD43-/- mice foamy Mϕ were present from day 56 onwards and in greater numbers (Figure 2.9). Overall, granuloma formation in CD43-/- mice occurred more rapidly, more extensively, affected a greater proportion of the lung, and included more foamy macrophages.

2.13 Discussion

While recent studies have shown that the interaction of *M. tuberculosis* with Mϕ does not necessarily result in uptake of the bacteria, certain Mϕ populations can and do ingest them (2, 35, 38). Understanding how *M. tuberculosis* enters, survives and establishes an infection in these Mϕ populations is crucial to comprehending the pathogenesis of mycobacterial infections.

Recently, Fratazzi et al. described a role for CD43 in mycobacteria-Mϕ interactions (14). Their results suggested that CD43 may play a role in promoting a stable interaction of mycobacteria with receptors on host cells and that this interaction regulated TNF-α production by the Mϕ. To advance our understanding of this interaction, we further characterized the role of CD43 in mycobacterial infections by analyzing the association of *M. tuberculosis* with different Mϕ phenotypes, by using other bacterial species and control particles, by studying the relationship between CD43 expression and mycobacterial binding, and by monitoring the growth of *M. tuberculosis* in Mϕ monolayers and in CD43 knockout mice.

We confirm that CD43 is involved in the ability of Mϕ to bind and engulf mycobacteria, as Mϕ from CD43 knockout mice were less able to phagocytose *M. tuberculosis*. Our studies extend this observation and show that this reduction of *M. tuberculosis* binding depends upon various factors. Firstly, Mϕ of distinct origins varied in their ability to bind *M. tuberculosis* and showed different levels of reduction in binding when CD43 was absent. While the previous study of CD43-TB interactions focused on SpMϕ (14) we show here that BMMϕ, PMϕ, and AMϕ also had an impaired

association with *M. tuberculosis* in the absence of CD43. Although AMϕ are the cell that first encounters *M. tuberculosis in vivo*, new mononuclear phagocytes arrive at the site of infection during the course of the disease, where they may differentiate and encounter bacteria. BMMϕ are an acceptable model for these elicited Mϕ and are commonly used in studies of *M. tuberculosis*. We therefore used these cells as a model for our studies.

Within a single population of Mϕ (BMMϕ), the level of expression of CD43 directly correlated with binding of *M. tuberculosis* (Figure 2.6). However, flow cytometry of different Mϕ populations showed that expression of CD43 did not directly correlate with binding of *M. tuberculosis*. AMϕ expressed the lowest level of CD43 and bound *M. tuberculosis* poorly. However, BMMϕ bound *M. tuberculosis* at levels lower than did SpMϕ and PMϕ, yet expressed the highest levels of CD43 (Figure 2.5). It is possible that the expression of CD43 on the surface of Mϕ may not reflect its functional state, as has been seen with other Mϕ receptors (2). Alternatively, CD43 may act in conjunction with other Mϕ receptors to mediate uptake of *M. tuberculosis*. Thus, variation in expression of these other receptors would explain differences in binding capacity of the various Mϕ populations. This contention is supported by the observation that binding of mycobacteria to CD43-/- Mϕ can be restored by the addition of the extracellular portion of CD43 (14). That CD43 is critical for optimal association of *M. tuberculosis* with these secondary receptors is shown by the direct correlation of binding with CD43 expression within a single Mϕ population.

Further evidence that CD43 was not the only surface moiety involved in binding *M. tuberculosis* was that as the MOI was increased, both CD43-/- and WT CD43+/+ Mϕ bound higher numbers of bacteria. However, at no point did CD43-/- BMMϕ bind the

same number of bacteria as did CD43+/+ Mϕ and it was calculated that CD43 was accountable for up to 40-50% of *M. tuberculosis* binding by Mϕ.

We show that a heat-labile component of serum can overcome the reduction in binding due to the absence of CD43. It has been previously demonstrated that the extracellular mucin region of CD43 is present in, and can be isolated from, plasma (42) and that this molecule can increase binding of mycobacteria by CD43-/- Mϕ (14). Therefore it appears, in agreement with previous findings, that soluble CD43 present in serum may potentiate mycobacteria-Mϕ interactions. It is also very likely that complement is responsible for at least some of the enhanced binding of the bacterium in the presence of serum, as it has been implicated in facilitating uptake of mycobacteria by Mϕ (1, 3, 5, 6) and heat inactivated serum lacks the capacity for complement activation (43). However, other heat-resistant opsonins may contribute to enhanced binding. Even in the presence of heat-inactivated serum, both CD43-/- and CD43+/+ Mϕ showed increased binding of *M. tuberculosis* compared to non-opsonic binding. This suggests that some component of serum that is heat stable can also mediate binding to Mϕ. Thus, our studies show that CD43 mediated binding of *M. tuberculosis* depends upon the Mϕ phenotype, the number of infecting bacteria, the presence of serum opsonins and the amount of CD43 expression.

Binding studies with control particles demonstrated that the absence of CD43 does not affect non-specific phagocytosis by Mϕ, or the function of complement receptors and FcγR. Interestingly, CD43-/- Mϕ had an increased affinity for zymosan. This could be due to the removal of factors impeding the interaction of zymosan binding, such as the large negative charge of CD43 sialic acid residues or steric hindrance created

by the large size of CD43. The effect of CD43 on bacterial binding to Mϕ also had a level of specificity, as representative gram negative and positive bacteria did not require the presence of CD43 to bind to Mϕ. However, three strains of mycobacteria all required the presence of CD43 for optimal binding. This supports the contention that CD43 binds specifically to a mycobacterial moiety.

The intracellular growth of *M. tuberculosis* was significantly enhanced in CD43-/- Mϕ even though the bacteria are less readily phagocytosed by the Mϕ. This increased growth rate was independent of the number of bacteria initially ingested. The higher rate of growth could be due to the fact that CD43-/- Mϕ have an impaired ability to initiate TNF-α production (14), which is known to be involved in controlling intracellular growth *of M. tuberculosis* (44-47). Other cytokines have also been shown to be involved in stimulating the release of the chemokines RANTES and macrophage inflammatory protein-1 (48), which could also affect the intracellular growth of *M. tuberculosis*. Additionally, CD43-/- Mϕ could be selectively phagocytosing the more virulent bacteria within the innoculum, or uptake of the bacterium in the absence of CD43 may lead to altered phagosome maturation, which is known to be associated with the survival of intracellular mycobacteria (49-53). Moreover, the induction of killing mechanisms by Mϕ may differ in CD43-/- and +/+ cells. For example, it has been demonstrated that CD43 is involved in apoptotic signaling pathways that would affect the fate of mycobacteria-infected Mϕ (27, 29-31).

CD43 appears to have a significant role in controlling the growth of *M. tuberculosis* in the murine host. When infected via aerosol, CD43-/- mice had increased bacterial loads in the lung and spleen during the acute phase of infection up to day 28.

This increased growth of *M. tuberculosis* in CD43-/- mice may be attributed to the enhanced growth in CD43-/- Mϕ we demonstrated *in vitro*. Alternatively, it may be due to differences in the type and/or number of cells recruited to sites of infection. In H&E stained tissue samples, lymphoid hyperplasia was seen in the spleens of CD43-/- mice as early as day 1 post-infection and there were more granulomas in the lungs by day 28 compared to CD43+/+ controls. The difference in bacterial growth during the acute phase of infection in CD43+/+ and CD43-/- mice was more pronounced in the spleens. As this effect was not seen in mice infected intravenously with *M. tuberculosis*, we can conclude that this is not just because of enhanced bacterial growth in CD43-/- SpMϕ but is more likely due to increased dissemination from the lungs of CD43-/- mice.

Following the development of the adaptive immune response (around day 28) the mice were able to control the infection for a period of time. However, following this period the CD43-/- mice also failed to control bacterial growth during the chronic stage of infection. It is possible this difference can also be ascribed to the increased susceptibility of CD43-/- Mϕ. However, the adaptive immune response was able to reduce the bacterial load in CD43-/- mice between days 28 and 56. This suggested that CD43-/- Mϕ were capable of being activated to kill intracellular *M. tuberculosis* just as effectively as CD43+/+ Mϕ during this stage of the infection. During the chronic stage of the infection, an effective immune response must be maintained with the corresponding maintenance of granulomata to contain the bacteria. We have shown that during this late stage of infection, bacterial growth is not controlled in CD43-/- mice and histological findings show that normal granuloma formation is impaired in these mice and may account for increased bacterial loads. Other published roles of CD43, including

involvement in T cell activation and differentiation, and the recruitment of lymphocytes to sites of infection (19, 25, 26) could also explain the inadequacies in the immune response against *M. tuberculosis*.

It is critical to note that the ability of CD43-/- mice to control infection with *M. tuberculosis* depended upon the route of infection. While significant differences were seen between CD43-/- and CD43+/+ mice infected aerogenically with *M. tuberculosis*, there was no significant difference when mice were infected via an intravenous injection with the same bacterium. It has been previously shown that *M. tuberculosis* may have increased virulence when administered aerogenically as opposed to intravenously (54) and that the pathogenesis of the organism is affected by route of delivery (55). This emphasizes the importance of utilizing experimental infection procedures that most closely mimic natural exposure in order to obtain results that are most physiologically relevant.

In summary, this study establishes that CD43 is involved in the binding, uptake and subsequent growth of *M. tuberculosis* in murine Mϕ and *in vivo*. These results support the theory that CD43 has a dual function in cell-cell interactions (23, 24, 56) and that the nature of particles interacting with CD43 can dictate its function. Additional studies are necessary to determine the biology of *M. tuberculosis*-CD43 interactions, to identify potential mycobacterial ligands for CD43, and to understand mechanisms of cell recruitment in CD43 knockout mice.

Mϕ type (MOI)	DAY 0	DAY 1	DAY 4	DAY 7	Doubling Time (h)
CD43+/+ (20:1)	4.76×10^4 (7.85×10^2)	4.01×10^4 (9.35×10^2)	1.63×10^5 (2.85×10^3)	3.19×10^6 (5.46×10^4)	27.69 (0.256)
CD43-/- (20:1)	*2.58×10^4 (4.42×10^2)	*2.31×10^4 (7.38×10^2)	1.68×10^5 (3.85×10^3)	3.31×10^6 (4.91×10^4)	*24.04 (0.177)
CD43-/- (30:1)	4.81×10^4 (8.08×10^2)	3.96×10^4 (9.30×10^2)	*2.20×10^5 (4.25×10^3)	*6.13×10^6 (9.59×10^4)	*24.12 (0.387)

Table 2.1 Intracellular survival and replication of *M. tuberculosis* is enhanced in CD43-/- BMMϕ

CD43-/- and CD43+/+ control BMMϕ were incubated with *M. tuberculosis* Erdman at 20:1 bacteria:Mϕ and CD43-/- Mϕ were also infected at 30:1. Average CFU/mL and doubling times are shown for day 0 and days 1, 4 and 7 post-infection. Results are expressed as mean ± SEM for 3 independent experiments, each with 3 coverslips, plated in duplicate at each time point.

				Infiltrating cell types (%)		
Time Post-infection	**Mouse Type**	**Granuloma formation**	**Area of Lung affected (%)**	**Normal Mϕ**	**Foamy Mϕ**	**Lymphocytes**
Day 56	CD43+/+	Single area	<25%	50	0	50
	CD43-/-	**Multifocal**	**~50%**	**20**	**30**	**50**
Day 85	CD43+/+	Single area	<25%	25	25	50
	CD43-/-	**Multifocal**	**~50%**	**0**	**50**	**50**
Day 127	CD43+/+	Multifocal	<25%	20	30	50
	CD43-/-	**Multifocal**	**~50%**	**0**	**25**	**75**
Day 168	CD43+/+	Multifocal	~50%	25	25	50
	CD43-/-	**Multifocal**	**>50%**	**0**	**25**	**75**
Day 210	CD43+/+	Multifocal	~50%	25	5	70
	CD43-/-	**Multifocal**	**>50%**	**0**	**25**	**75**

Table 2.2 Granuloma formation in CD43-/- mice is more severe and has altered morphology

At the indicated times post-infection H&E-stained sections of lung from CD43-/- and CD43+/+ mice infected with *M. tuberculosis* were evaluated for the number and type of granulomas present (multifocal = numerous granulomas throughout the lung), the amount of the section affected (%), and the dominant cell types present in the granulomatous region (%), assessed as described in Materials & Methods. Organ sections are from the same mice for which bacterial loads were assessed in Figure 2.7.

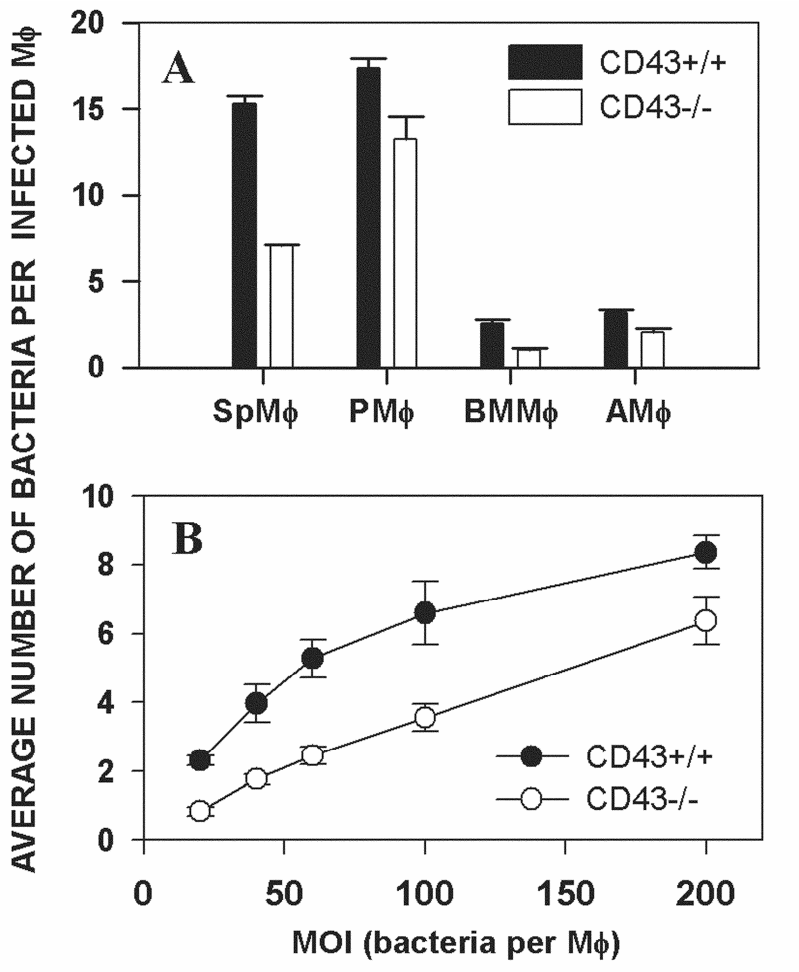

Figure 2.1 ***M. tuberculosis*** **has a reduced ability to associate with CD43 deficient Mϕ**

A) SpMϕ, PMϕ and BMMϕ from CD43-/- and CD43+/+ mice were infected with *M. tuberculosis* Erdman at 20:1 bacteria:Mϕ, while AMϕ were infected at 500:1. The average number of bacteria per infected Mϕ was assessed microscopically for 100 randomly chosen Mϕ.

B) CD43-/- and CD43+/+ BMMϕ were infected with *M. tuberculosis* Erdman at MOIs of 20:1, 40:1, 60:1, 100:1 and 200:1 and associated bacteria were quantified as above.
For both A) and B) The mean ± SEM from 2 independent experiments, each with 3 coverslips, is shown.

All CD43-/- values are statistically less than WT values ($p<0.05$).

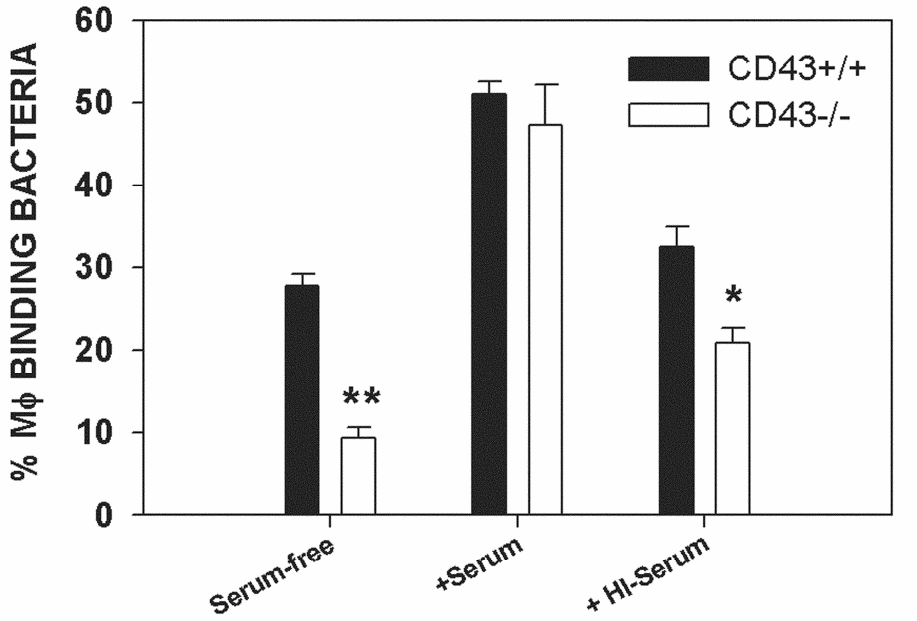

Figure 2.2 Heat-labile serum opsonins overcome the reduced binding of *M. tuberculosis* to CD43-/- Mϕ

BMMϕ from CD43+/+ and CD43-/- mice were infected with *M. tuberculosis* Erdman in the absence (Serum-free) or presence of 1% normal (+ Serum) or heat-inactivated (+ HI-Serum) mouse serum at a MOI of 40:1. The percentage of Mϕ binding at least 1 bacillus is shown. Values represent the mean ± SEM from 3 independent experiments, each with 3 coverslips.

** = p <0.001 and * = p <0.01 when compared to CD43+/+ control.

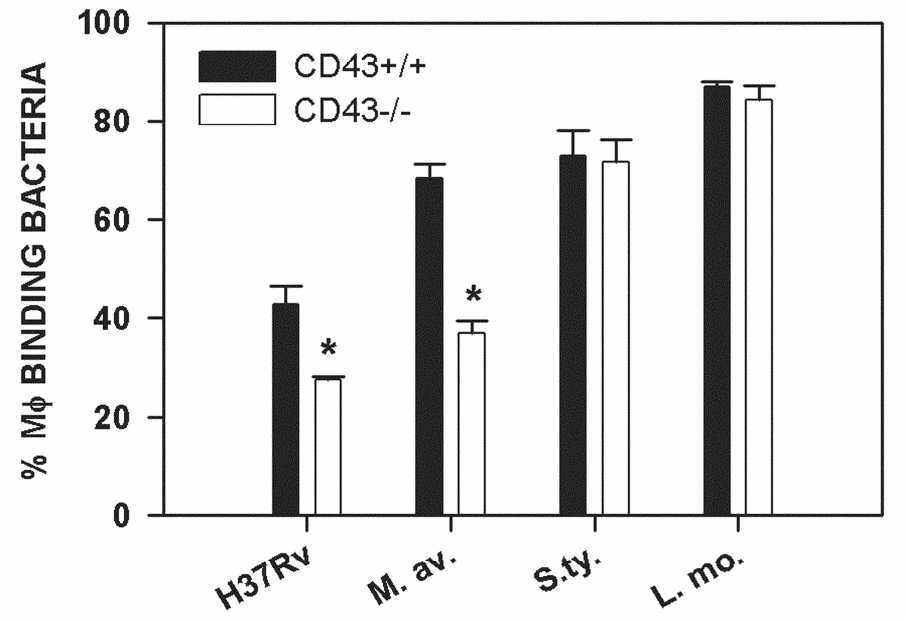

Figure 2.3 CD43 is involved in Mϕ binding of other mycobacterial species but not other intracellular bacteria

CD43-/- and CD43+/+ BMMϕ were infected with *Mycobacterium tuberculosis* H37Rv (H37Rv) or *Mycobacterium avium* (M. av) at MOI of 40:1 and 20:1, respectively, for 3 hours, or with *Salmonella enterica* serovar Typhimurium (S. ty) or *Listeria monocytogenes* (L. mo) at 10:1 for 40 min. The mean ± SEM percent of Mϕ binding at least 1 bacillus is shown for 3 independent experiments, each with 3 coverslips.

* = p <0.01 when compared to control.

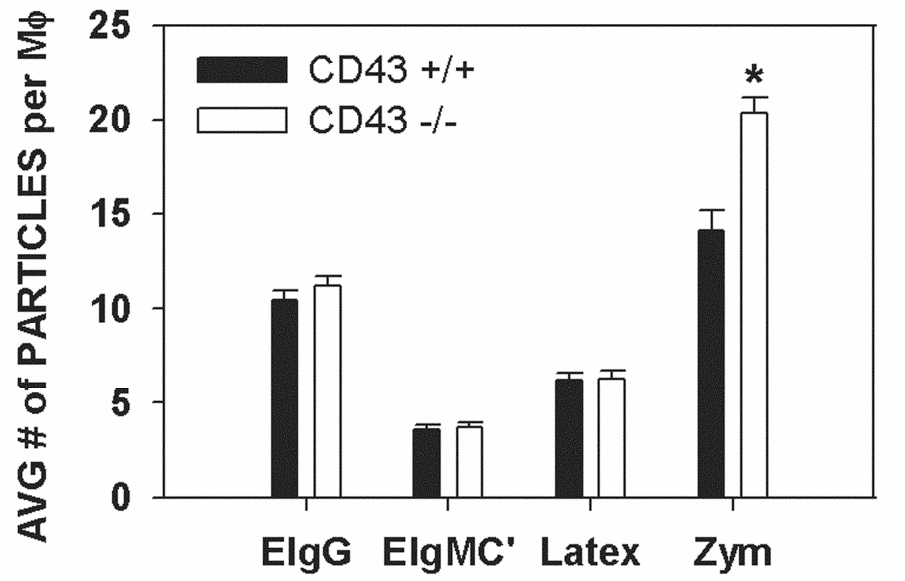

Figure 2.4 The absence of CD43 on Mϕ does not affect phagocytosis via complement receptors, FcγR, or non-specific uptake, but enhances uptake of zymosan

CD43-/- and CD43+/+ BMMϕ were incubated with test particles at the following MOI: EIgG (50:1), EIgMC' (50:1), latex spheres (25:1), or zymosan (25:1). After 3h, the association of particles was assessed microscopically. The average number of particles bound per Mϕ is shown. Results are expressed as the mean ± SEM of 3 independent experiments, each with 3 coverslips.

* = $p<0.01$ when compared to control.

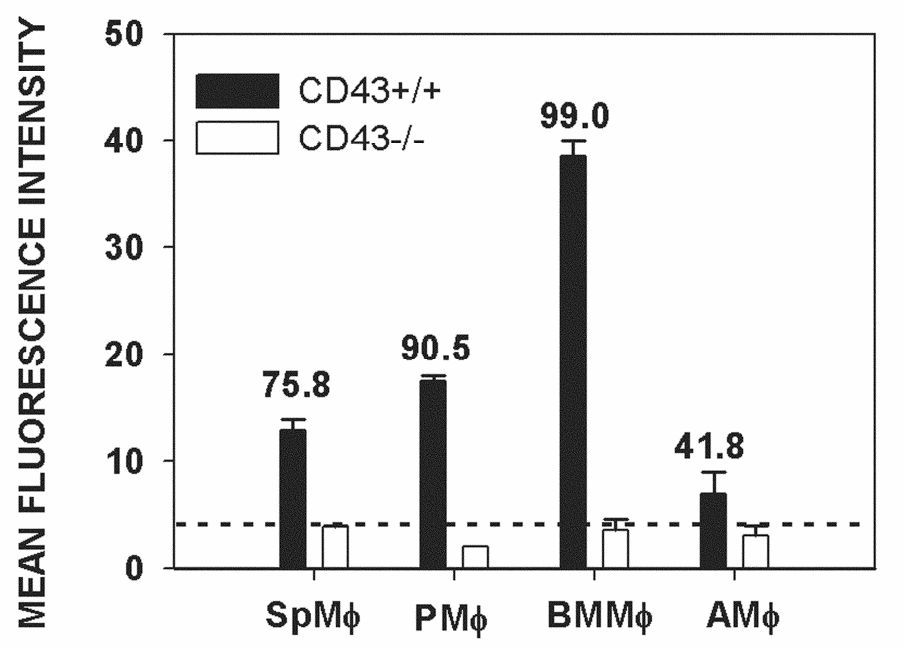

Figure 2.5 Surface expression of CD43 varies on different Mϕ phenotypes

CD43-/- and CD43+/+ SpMϕ, PMϕ, BMMϕ and AMϕ were stained with MAb S11 (anti-pan-CD43). The mean fluorescence intensity ± SEM from 2 independent experiments is shown. Numbers above the bars represent the percentage of the Mϕ population that expressed CD43. The dotted line represents background levels of anti-CD43 mAb binding

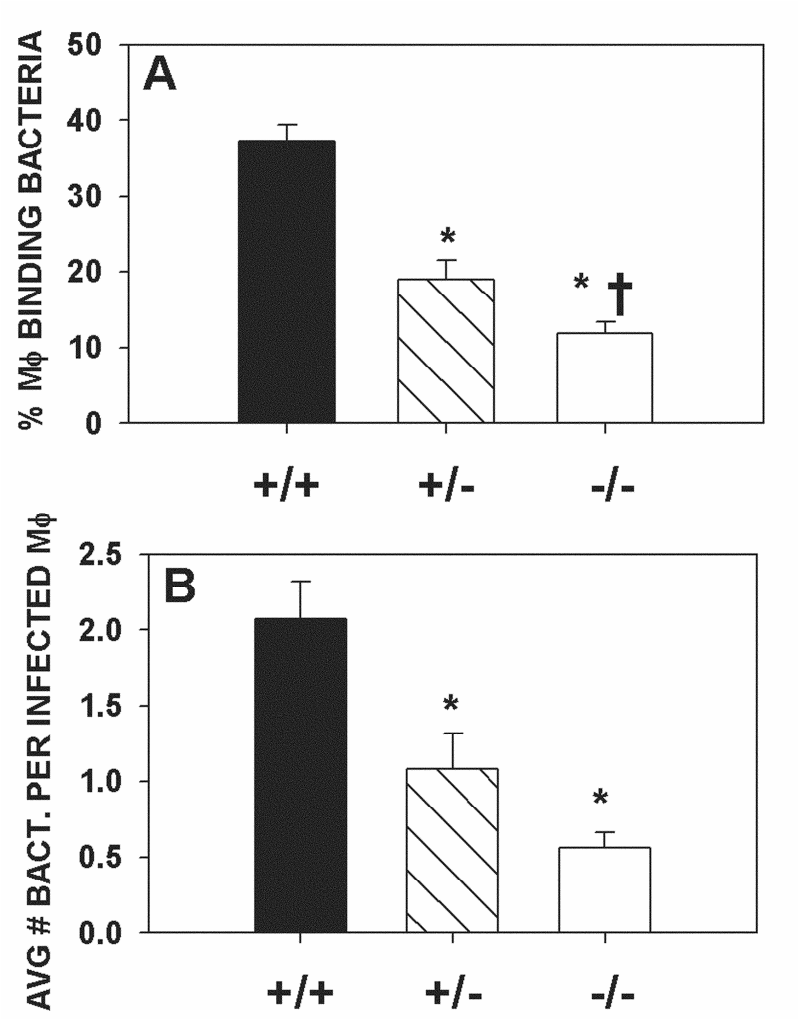

Figure 2.6 ***M. tuberculosis*** **binding to BMMϕ is dependent on CD43 gene dose**

BMMϕ from WT (+/+), CD43 knockout (-/-) and CD43 heterozygous (+/-) mice were incubated with *M. tuberculosis* Erdman at a ratio of 40 bacteria per Mϕ.
A) The percentage of Mϕ binding at least 1 bacillus is shown. **B)** The average number of bound bacteria per infected Mϕ is shown. Figures represent the mean ± SEM from 2 experiments, each with 3 coverslips

* = p <0.001 when compared to CD43+/+, † = p <0.05 when compared to CD43+/-.

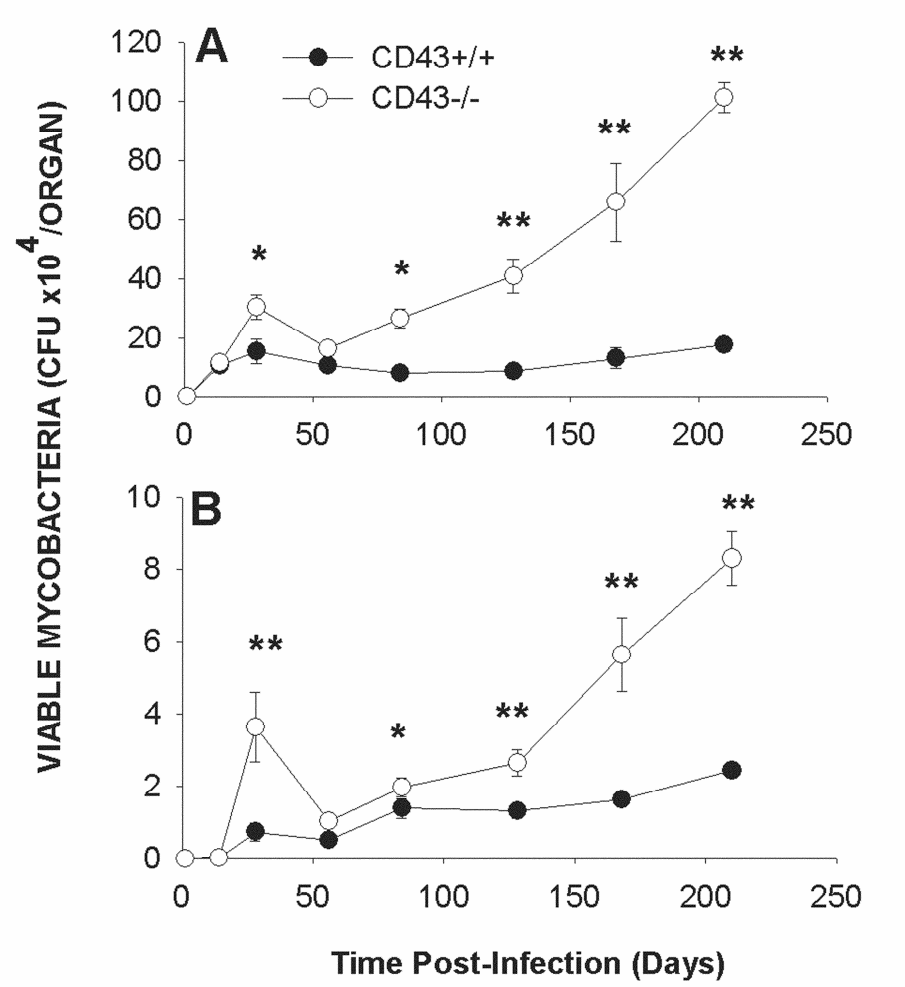

Figure 2.7 CD43 is necessary for the control of *M. tuberculosis* growth during both the acute and chronic phase of infection in mice

CD43-/- (open circles) and CD43+/+ (filled circles) mice were infected aerogenically with a low-dose of *M. tuberculosis*. The bacterial loads in the **A)** Lung and **B)** Spleen are shown as the mean CFU/Organ x 10^4 ± SEM for 4-6 mice per experimental group at each time point.

* = p <0.05, ** = p <0.01,when compared to control.

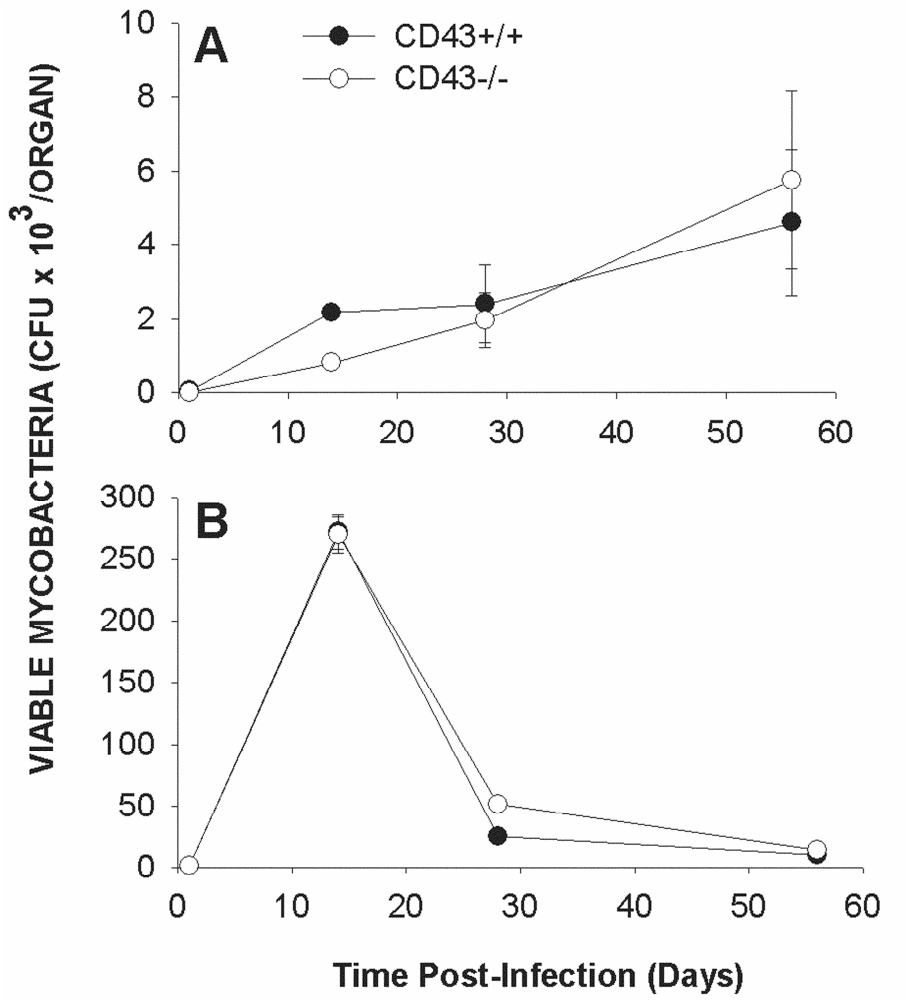

Figure 2.8 **CD43 deficient mice infected intravenously with *M. tuberculosis* do not show impaired control of bacterial growth during the acute phase of infection**

CD43-/- (open circles) and CD43+/+ (filled circles) mice were infected intravenously with *M. tuberculosis*. The bacterial loads in the **A)** Lung and **B)** Spleen are shown as the mean CFU/Organ x 10^3 ± SEM for 5 mice per group at each time point.

No significant difference was found between the experimental groups.

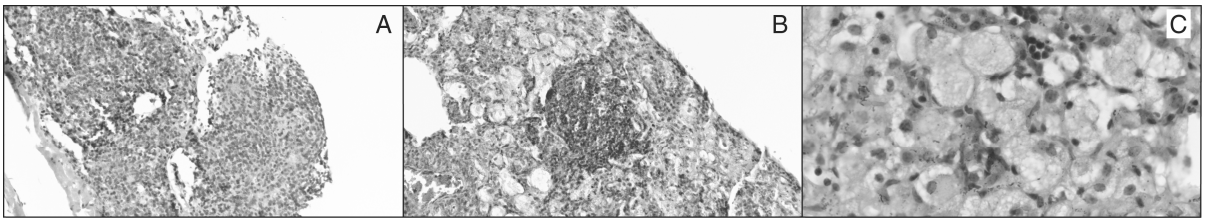

Figure 2.9 Lung pathology is exacerbated in CD43 deficient mice infected with *M. tuberculosis*

Representative granuloma from **A.)** CD43+/+ and **B.)** CD43-/- mice 210 days post-infection with *M. tuberculosis* via aerosol exposure, 200X magnification. A predominantly lymphocytic infiltration is seen in the CD43+/+ mouse, whereas numerous foamy macrophages can still be seen surrounding lymphocytes in the granuloma of the CD43-/- mouse. **C.)** Foamy macrophages are shown 600X magnification.

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Chapter 3 CD43 controls intracellular growth of *Mycobacterium tuberculosis* through the induction of TNF-α-mediated apoptosis[2]

3.1 Summary

Establishment of Tuberculosis infection begins with the successful entry and survival of the pathogen within macrophages. We previously showed that macrophage CD43 is required for optimal uptake and growth inhibition of *Mycobacterium tuberculosis* both *in vitro* and *in vivo*. Here, we explore the mechanisms by which CD43 restricts mycobacterial growth in murine macrophages. We found that although *M. tuberculosis* grows more readily in resting CD43-/- macrophages, when these cells were primed with IFN-gamma the bacterial growth rate was similar to that seen in CD43+/+ cells. To discern the mechanisms by which *M. tuberculosis* exhibits enhanced growth within resting CD43-/- macrophages, we assessed the induction of inflammatory mediators in response to infection. We found that the absence of CD43 resulted in reduced production of nitric oxide, TNF-alpha, IL-12 and IL-6 by *M. tuberculosis*-infected macrophages. We also found that infected resting, but not activated CD43-/- cells showed decreased levels of apoptosis and increased levels of necrosis. Addition of TNF-alpha to CD43-/- macrophages restored control of *M. tuberculosis* growth and induction of apoptosis to CD43+/+ levels. We thereby propose that CD43 is involved in the inflammatory response to *M. tuberculosis* and can regulate apoptosis through the induction of pro-inflammatory mediators to control intracellular growth of the organism.

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[2] This chapter forms part of a manuscript in preparation.

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3.2 Introduction

Despite treatment and prevention efforts, *Mycobacterium tuberculosis* remains the most-common cause of infection-related mortality worldwide (1, 2). Each year, nearly 2 million deaths occur due to Tuberculosis and over 8 million people are newly infected with the causative bacterium, *M. tuberculosis* (3). A central feature of the pathogenesis of this organism it the ability to infect and persist within the macrophage (Mϕ). Following inhalation of the bacteria, Mϕ serve as the first line of host immune defense against infection and act as the primary host cell of *M. tuberculosis*. For these reasons, it is crucial that we fully understand the events that occur during the initial encounter between the Mϕ and the bacterium, and the dynamics of this relationship during the intracellular survival of *M. tuberculosis*.

Several receptors on phagocytic cells have been shown to be involved in the initial detection of *M. tuberculosis*, including the complement receptors, the mannose receptor, surfactant protein A receptor, scavenger receptors, dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN), mannose-binding lectin, dectin-1, and Toll-like receptor 2 (4-14).

Recent studies have also shown that CD43 is involved in the binding and uptake of *M. tuberculosis* by Mϕ (15, 16). CD43 is a transmembrane glycoprotein that is abundantly expressed on lymphohemopoietic cells (17). It appears that CD43 may have a dual function in cell-cell interactions as it has been shown to act as a barrier molecule that can restrict contact between cells (18-20), but has also displayed a role in adhesion (21, 22). In addition, we have shown that the presence of CD43 on Mϕ enhances binding

of *M. tuberculosis*, whereas it inhibits binding of zymosan (a mannan-rich yeast cell wall product) (16). It is clear that this molecule has a number of physiological functions as it has also been shown to play a role in the activation of T cells (23-26), monocytes (27), and neutrophils (28), leukocyte homing and tissue infiltration (29, 30) and regulation of apoptosis (31-37).

In bacterial infections, it has been shown that CD43 on Mϕ is involved in the binding of *M. avium* and *M. tuberculosis* in a dose dependent manner, but does not appear to associate with *Shigella flexneri*, *Salmonella typhimurium* or *Listeria monocytogenes* (15, 16). Fratazzi et al. also showed that re-addition of the extracellular portion of the molecule can restore binding of *M. avium* to CD43-/- Mϕ. As with its role in cell-cell interactions, it appears that CD43 may play a dual role in mycobacterial infections as it was not only found to be involved in initial binding of the bacteria, but also plays a role in the subsequent growth within Mϕ, as *M. tuberculosis* grew more readily in CD43-/- BMMϕ (16). The enhanced growth of *M. tuberculosis* due to absence of CD43 was also observed *in vivo*; CD43-/- mice infected aerogenically with *M. tuberculosis* displayed increased bacterial loads in lungs and spleens during both the acute and chronic stages of infection and also had altered granuloma formation (16).

In this study, we further explored the role of CD43 in mycobacterial pathogenesis. To discern the mechanisms by which CD43 controls intracellular growth of *M. tuberculosis*, we compared the growth of *M. tuberculosis* within resting and IFN-gamma (IFN-γ) activated CD43-/- and +/+ Mϕ and also examined killing mechanisms employed by Mϕ in response to *M. tuberculosis*.

3.3 Materials and methods

Bacteria

M. tuberculosis strain Erdman (Trudeau Mycobacterial Collection #107; ATCC #35801), was grown to late log phase in Proskauer & Beck medium supplemented with 0.05% Tween 80 under static culture conditions with intermittent agitation (every 2 days over a 2-3 week period). Cultures were stored at -80°C and assessed for colony forming units (CFU) as previously described (6).

Mice

Wild type (CD43+/+) and CD43 knockout (CD43-/-) mice backcrossed at least 10 generations on C57Bl/6 background (38) were housed in a specific pathogen-free animal facility in micro isolator cages. Mice were age- and sex-matched, and wherever possible, wild type controls were littermates from CD43 heterozygote (+/-) matings. All experiments were carried out in accordance with the standards set by the Canadian Council on Animal Care.

Culture of bone marrow-derived Mϕ

Bone marrow-derived macrophages (BMMϕ) were isolated from CD43+/+ and CD43-/- mice as previously described (6, 39). Mice were euthanized and dissected to remove femurs, tibiae, and humeri. The ends of the bones were cut and using a 25-gauge needle, the marrow was flushed out with supplemented RPMI (RPMI 1640 medium with 10% fetal calf serum, 10mM L-glutamine, and 10mM sodium pyruvate, all from GIBCO

Burlington, ON). Bone marrow washes were pooled and red blood cells were lysed using 0.17M ammonium chloride (NH_4Cl, pH 7.2). After washing, cells were resuspended in supplemented RPMI and incubated in tissue culture treated flasks (Becton Dickinson (BD) Labware, Franklin Lakes NJ) for 3 hours at 37ºC, 5% CO_2 to deplete non-stem cells by adherence. Non-adherent cells were then washed, resuspended in bone-marrow media (supplemented RPMI plus 10% L-cell conditioned media, prepared as described previously (39)), counted, plated in 24-well plates (BD), and allowed to differentiate for 7 days at 37ºC, 5% CO_2. Media was replenished on day 5. Wherever indicated, Mϕ were primed for 16 hours with 100 U/mL murine recombinant IFN-γ (R&D Systems Inc., Minneapolis MN) prior to infection. IFN-γ was supplemented every 72 hours during *in vitro* growth assays.

Intracellular growth of *M. tuberculosis*

The growth of *M. tuberculosis* within Mϕ monolayers was evaluated by infecting BMMϕ and determining bacterial CFU counts over a 7 day period, as previously described (16, 40). Briefly, BMMϕ were cultured on 13 mm acid-washed sterile glass coverslips in 24 well plates for 7 days, at which point media was replaced with 0.5 mL supplemented RPMI +/- 100 U/mL recombinant IFN-γ, and left overnight. Frozen *M. tuberculosis* stocks were thawed, pelleted, and resuspended in phagocytosis medium (138 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.5 mM D-glucose, pH 7.4). Bacteria were passed through a 25-gauge needle 10 times to disperse the bacterial clumps characteristic of mycobacteria, and diluted to the required concentration using phagocytosis medium. The overlay was removed from

BMMϕ monolayers and replaced with *M. tuberculosis* in phagocytosis medium at multiplicities of infection (MOI) of 20:1 or 30:1 (bacteria:Mϕ). Infected Mϕ were incubated for 1 hour rocking (Nutator, Becton Dickinson, Mountain View, CA) followed by 2 hours stationary at 37°C/5% CO_2. Following infection, monolayers were washed twice with phagocytosis medium and coverslips were transferred to new 24 well plates containing 1 mL supplemented RPMI alone or with 100 U/mL IFN-γ. To determine bacterial counts, well contents were sonicated (VC50T 50W probe sonicator, Sonics & Materials, Danbury, CT) for 10 seconds to lyse Mϕ and break up clumps of bacteria, serially diluted in PBS + 0.1% Tween 80, and plated in duplicate on 7H10 agar (Difco) supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose complex (OADC). OADC was prepared as described previously (41). Plates were incubated in sealed bags for 18-21 days at 37ºC, after which CFU were enumerated. To allow for direct comparison of findings, intracellular growth assays were completed in conjunction with cytokine, nitric oxide, cytotoxicity, and apoptosis assays.

Cytokine detection by enzyme-linked immunosorbant assay (ELISA)

The production of TNF-α, IL-12, IL-6, and IL-10 by BMMϕ was measured using OptEIA™ ELISA Sets (BD) according to manufacturer's instructions. In short, 96 well plates were coated with capture antibody, and incubated overnight at 4°C. Plates were washed, blocked, and experimental samples and standards were added. After 2 hours incubation at room temperature, plates were washed and subsequently, detection antibodies and substrates were applied. Absorbance readings (450nm, reference 570nm) were determined using a microplate reader (Bio-Rad Benchmark, Mississauga ON)

Measurement of nitric oxide production by Mϕ

The production of nitric oxide (NO) by BMMϕ was measured using the Griess Assay, as previously described (39). Briefly, at various time points 100 μL aliquots of culture supernatant were removed from uninfected or *M. tuberculosis*-infected Mϕ monolayers. Serial dilutions of $NaNO_2$ in supplemented RPMI were used to obtain a standard curve. Samples and standards were mixed with 50 μL Griess Reagent 1 (1% w/v sulphanilamide in 2.5% phosphoric acid (H_3PO_4)) and 50 μL Griess Reagent 2 (0.1% w/v naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4) for 10 minutes after which absorbance was read (550nm, reference 655nm).

Lactate dehydrogenase assay

Levels of cellular necrosis were assessed by measuring the release of lactate dehydrogenase (LDH), a stable cytosolic enzyme, from BMMϕ monolayers using the CytoTox96® non-radioactive cytotoxicity assay (Promega, Madison WI). 50 μL of culture supernatant was used for this enzymatic assay, which results in the conversion of a tetrazolium salt into a red formazan product, measurable by OD. The amount of product is proportional to the number of lysed cells in the sample. Background levels were determined by the amount of LDH released from untreated control Mϕ, whereas detergent-lysed uninfected Mϕ served as a positive control for maximum amount of LDH released. The relative percentage of cytotoxicity in experimental samples was calculated by: [release of LDH from infected cells (OD_{490})/maximum LDH release (OD_{490})] x 100.

Quantification of histone-associated DNA fragments

Apoptosis levels were compared using the Cell Death Detection ELISA^{PLUS} kit (Roche) according to manufacturer's instructions. Briefly, CD43+/+ and CD43-/- BMMϕ were infected as described above. After 4 days of incubation, plates were centrifuged to pellet contents including any cells that may have lifted off the monolayer, and incubated with lysis buffer. Cell lysate was centrifuged to pellet intact nuclei and the cytoplasmic fraction (supernatant) samples retained for ELISA. Sandwich ELISA was performed by placing samples into a streptavidin-coated 96 well plate and incubating with anti-histone-biotin and anti-DNA-peroxidase. Subsequently, unbound antibodies were washed and the amount of peroxidase retained was determined by absorbance (OD_{405}), with ABTS as the substrate. 5 μM staurosporine (STS, Sigma-Aldrich, Oakville ON) was used as a positive control for induction of apoptosis by incubating with uninfected Mϕ for 4 hours.

Measurement of Caspase-3 activity

Caspase-3 activity was measured using the colorimetric Caspase-3 substrate Ac-DEVD-pNA (Calbiochem, San Diego CA). CD43+/+ and CD43-/- BMMϕ were infected with *M. tuberculosis* as described above. On day 4 of infection, 24 well plates were centrifuged to pellet any cells that may have lifted off the monolayer and the overlaying medium was removed. STS was used to induce apoptosis in positive controls as described above. Cells were then incubated with 50 μL lysis buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 0.2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 10 μg/mL aprotinin, 2 μg/mL leupeptin, and 1 mM PMSF, all from Sigma-Aldrich) for 10 min at 4°C. Lysates were

centrifuged at high speed for 10 min to pellet cellular debris, the supernatant transferred to microfuge tubes and kept at 4°C. 100 μg of protein lysate, as quantified using the Bradford Assay, was incubated at 37°C for 1 hour with reaction buffer (50 mM HEPES, pH 7.4, 20% glycerol, 2 mM EDTA, and 10 mM DTT added immediately before use) and 1 mM Caspase-3 substrate. A standard curve was produced by diluting a stock solution of pNA (Sigma-Aldrich) from 200 mM to 25 mM and absorbance measured by determining OD at 405nm.

TNF-α neutralization/reconstitution

For TNF-α neutralizing/reconstitutions assays, 100 ng/mL recombinant TNF-α or 10 ng/mL anti-TNF-α antibody (both from R&D Systems) were added along with bacteria at the time of infection and supplemented every 48 hours. *In vitro* growth assays and apoptosis ELISA were performed as described above.

Statistical analysis

Data are expressed as mean ± SEM. Student's *t* test for independent means was used; $p < 0.05$ was considered significant unless otherwise noted.

3.4 IFN-γ activation abrogates the enhanced growth of *M. tuberculosis* in CD43-/- Mϕ

In previous studies, we found that *M. tuberculosis* grows more readily in CD43-/- BMMϕ (16). In those experiments, the cells used were representative of resident tissue Mϕ. To determine whether cells representing T-cell-activated Mϕ require CD43 to control the intracellular growth of *M. tuberculosis*, we compared the growth of the bacterium in wild type (CD43+/+) and knockout (CD43-/-) Mϕ with or without prior IFN-γ stimulation. Because previous studies (16) have shown that Mϕ deficient in CD43 bind mycobacteria less efficiently than wild-type Mϕ, we infected CD43-/- BMMϕ at MOIs of 20:1 and 30:1, in addition to the 20:1 MOI used for CD43+/+ cells in order to ensure equal uptake of the bacteria by both populations as well as equal MOIs.

When resting (not stimulated with IFN-γ) BMMϕ (Figure 3.1A-B) were infected at 20:1, CD43-/- Mϕ phagocytosed approximately 40% less mycobacteria than CD43+/+ Mϕ on day 0 (Figure 3.1A), as expected. Adding 30:1 bacteria per Mϕ to CD43-/- cells resulted in uptake comparable to CD43+/+ at 20:1 (Figure 3.1A), but resulted in significantly greater bacterial load after 7 days (Figure 3.1B, $p<0.0001$), in agreement with previous findings (16). In assessing growth within IFN-γ-stimulated Mϕ (Figure 3.1C-D), both CD43+/+ and CD43-/- Mϕ showed reduced bacterial loads by day 7 compared to resting Mϕ. Bacterial uptake remained significantly less when CD43 was not expressed (Figure 3.1C, $p=0.002$) but there were no statistical differences in the growth rate of the bacteria in CD43+/+ and -/- IFN-γ activated Mϕ, as illustrated by bacterial load after 7 days (Figure 3.1D). Thus, it appears that although CD43 is required

for restricting the growth of *M. tuberculosis* in resting BMMϕ, activation of the cells with IFN-γ overcomes the difference in growth due to lack of CD43. This is illustrated by both the bacterial load (Figure 3.1) and also by the doubling time of the bacteria (Table 3.1). In untreated Mϕ, *M. tuberculosis* grows significantly faster when CD43 is not expressed (doubling time of ~24 hours) compared to within wild-type Mϕ (doubling time of ~28 hours). However, in IFN-γ treated Mϕ, there is no significant difference in the growth rate of the bacteria regardless of CD43 expression. As expected, the growth rate of *M. tuberculosis* within IFN-γ activated Mϕ is less than within resting Mϕ, irrespective of whether CD43 is expressed or not.

3.5 CD43 is required for greatest induction of pro-inflammatory mediators by *M. tuberculosis*-infected Mϕ

In order to explore the mechanisms by which CD43 restricts intracellular growth of *M. tuberculosis*, we compared the induction of various Mϕ defense mechanisms that are often employed in the response to this bacterium. Firstly, we examined the production of various cytokines by CD43+/+ and CD43-/- Mϕ after infection with *M. tuberculosis* (Figure 3.2). It has been shown that IFN-γ is essential for protective immunity against Tuberculosis infection in the mammalian host, but several studies have also shown that IFN-γ alone is insufficient for full protection (42-46). TNF-α has also been shown to play an important role in restricting mycobacterial pathogenesis, particularly in regulating the formation of granulomas and regulation of Th1 cytokine expression (45, 47-51). In addition, the IL-12 family of cytokines including IL-12 itself

and IL-23, which shares the IL-12p40 subunit, have been shown to be induced by *M. tuberculosis* and have proven important in restricting mycobacterial growth in mice likely due to their role in inducing IFN-γ, and activation of antigen-specific lymphocytes (52-55).

In comparing the production of TNF-α, and IL-12p40, we discovered that CD43-/- Mϕ produced significantly less of both cytokines than did CD43+/+ cells (Figure 3.2A-B). These results were similar irrespective of pre-treatment of Mϕ with IFN-γ before infection with *M. tuberculosis*. Furthermore, it was noted that the amount of bacteria added to CD43-/- Mϕ (20:1 or 30:1 bacteria per Mϕ) did not affect the resultant cytokine production.

IL-6, commonly regarded as an indicator of general inflammation, may also play a role in the acute response to *M. tuberculosis* (56). We found that CD43-/- Mϕ were also deficient in production of this cytokine regardless of IFN-γ activation and again, that MOI was not a factor in cytokine production (Figure 3.2C). We also examined the production of IL-10, which has been shown to play an anti-inflammatory role in response to *M. tuberculosis* (57-59). Neither CD43+/+ nor CD43-/- Mϕ produced large amounts of IL-10 in response to *M. tuberculosis* and, because some levels were below the detection limit of the assay (50pg/mL), statistical comparison was not possible (Figure 3.2D).

Production of reactive nitrogen intermediates (RNI) such as nitric oxide is a well-known antimicrobial mechanism employed by Mϕ (60), and has been shown to be important in host defense against *M. tuberculosis* (61-63). We thus compared the production of NO by *M. tuberculosis*-infected CD43+/+ and CD43-/- BMMϕ. Figure 3.3 shows the amount of RNI (measured as nitrite (NO_2^-) – a stable end product of NO

production) produced by CD43+/+ and CD43-/- IFN-γ-activated BMMϕ in response to infection with *M. tuberculosis*. It should be noted that in our studies, resting Mϕ that were not stimulated with IFN-γ did not produce detectable amounts of RNI (data not shown). We found that throughout the course of infection, CD43 deficient Mϕ produced significantly less RNI than CD43+/+ Mϕ at both MOI tested (Figure 3.3). As observed with cytokine production, the amount of bacteria added was unrelated to the amount of RNI produced by CD43-/- Mϕ. In both cell types, the production of RNI seemed to reach maximum levels by approximately day 4 of the experiment.

3.6 Enhanced growth of *M. tuberculosis* in CD43-/- Mϕ is associated with increased levels of necrosis but decreased induction of apoptotic pathways

TNF-α and NO have been linked to the induction of apoptosis (64-66) and CD43 has recently been shown to also play a role in regulating cell death (31-37). Due to this documented involvement of CD43 in regulating apoptosis, and the apparent role we observed for CD43 in the production of TNF-α and NO, we inquired whether the absence of CD43 on BMMϕ affected the level and/or type of cell death induced in response to *M. tuberculosis* infection.

To compare overall levels of cytotoxicity in wild-type and CD43-/- Mϕ, we measured the release of lactate dehydrogenase (LDH), a stable cytosolic enzyme released upon cell lysis. In uninfected cell cultures, we did not notice any measurable differences in levels of cell death between CD43+/+ and CD43-/- Mϕ over 4 days (Figure 3.4). However, when infected with *M. tuberculosis*, we found that the amount of cytotoxicity

was higher in CD43-/- Mϕ, as measured by the amount of LDH released into the medium. This was true for both MOI tested but only in resting Mϕ (Figure 3.4A). In contrast, there were no measurable differences in the amount of cytotoxicity induced by *M. tuberculosis* in CD43-/- and CD43+/+ Mϕ that were pre-stimulated with IFN-γ (Figure 3.4B).

Next, we measured apoptosis levels using a colorimetric enzyme assay that allows for quantification of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induction of apoptosis (Figure 3.5). Staurosporine (STS), a potent inducer of apoptosis, produced similar results in both CD43-/- and CD43+/+ Mϕ, at various doses regardless of IFNγ stimulation. When infected with *M. tuberculosis*, resting CD43+/+ Mϕ displayed a significantly greater concentration of histone-associated DNA fragments than did CD43-/- Mϕ (Figure 3.5A). When Mϕ were stimulated with IFN-γ prior to infection, this difference was no longer present (Figure 3.5B). Thus, it appears that CD43 plays a role in the induction of apoptosis in resting BMMϕ infected with *M. tuberculosis*.

Apoptosis can occur via several distinct pathways. Some of the most well-characterized pathways for the induction of apoptosis are the Caspase-mediated pathways, although the involvement of Caspases in *M. tuberculosis* infection has been debated (67, 68). To determine the mechanism of apoptosis in this model, we assessed the level of activity of Caspase-3, a downstream component in the Caspase pathway. We found that Caspase-3 was indeed active in staurosporine-induced and *M. tuberculosis*-infected samples (data not shown). Furthermore, in agreement with our preceding apoptosis assay, we found that resting CD43-/- Mϕ had significantly less Caspase-3

activity than did CD43+/+ Mϕ (Figure 3.6A) but that IFN-γ treatment partially restored Caspase-3 levels in CD43-/- Mϕ to levels not significantly different from CD43+/+ Mϕ (Figure 3.6B).

3.7 Enhanced growth of *M. tuberculosis* and dysregulation of apoptosis in CD43-deficient Mϕ is partially regulated by induction of TNF-α

As previously stated, TNF-α has been shown to be crucial in restricting the growth of *M. tuberculosis* and is also implicated in the initiation of apoptosis. Since CD43-/- Mϕ displayed increased susceptibility to intracellular *M. tuberculosis* growth and showed lower levels of *M. tuberculosis*-induced apoptosis, we hypothesized that this could be due to the observed deficiency in TNF-α production. To investigate this further, we infected CD43+/+ and CD43-/- Mϕ with *M. tuberculosis* in the presence of either recombinant TNF-α (rTNF-α) or an anti-TNF-α monoclonal antibody (anti-TNF-α) and assessed both intracellular growth (Figure 3.7) and induction of apoptosis (Figure 3.8).

Over a 7 day period, treatment of CD43+/+ Mϕ with anti-TNF-α resulted in enhanced survival and growth of *M. tuberculosis* to levels significantly higher ($p=0.02$) than in control CD43+/+ Mϕ (Figure 3.7A) reaching levels comparable ($p=0.17$) to untreated CD43-/- Mϕ (Figure 3.7B). This difference was reflected in the levels of apoptosis in these Mϕ, with anti-TNF-α treatment reducing apoptosis in CD43+/+ Mϕ to levels significantly less than in untreated cells ($p=0.007$) and not significantly different ($p=0.39$) than untreated CD43-/- Mϕ (Figure 3.8). In contrast, anti-TNF-α had no effect on CD43-/- Mϕ (Figures 3.7 & 3.8).

Addition of rTNF-α to CD43-/- Mϕ (Figure 3.7B) significantly reduced the survival and growth of *M. tuberculosis* (p=0.003) to levels comparable to CD43+/+ Mϕ (p=0.19, Figure 3.7A). Again, this difference was mirrored by an increase in apoptosis in CD43-/- Mϕ treated with rTNF-α to levels significantly higher (p=0.0003) than in untreated CD43-/- Mϕ (Figure 3.8B) and not significantly different (p=0.64) than in CD43+/+ control Mϕ (Figure 3.8A). Overall, the results demonstrated that Mϕ CD43 is critical for control of intracellular *M. tuberculosis* through the action of TNF-α mediated apoptosis.

3.8 Discussion

Understanding how the antimicrobial activities of host Mϕ can affect the intracellular growth of *Mycobacterium tuberculosis* is of great importance to our knowledge of this devastating pathogen, and could be key to developing novel therapeutic or preventative measures. It has been shown that Mϕ CD43 plays a role in the uptake of *M. tuberculosis* (15, 16) and previous work in our lab has shown that this molecule is also involved in restricting the growth of the bacterium in Mϕ and in mice (16). In this study, we have described the mechanisms by which CD43 controls intracellular growth of *M. tuberculosis*.

We confirmed our previous finding that, in resting Mϕ, *M. tuberculosis* grows more readily in the absence of CD43, but also showed that this enhanced growth is abrogated when Mϕ are stimulated with IFN-γ. These results correlate with our *in vivo* data demonstrating that CD43-/- mice were more permissive to bacterial growth during the acute phase of infection (first 3-4 weeks following infection) when only resident tissue Mϕ and other innate immune mechanisms would be present. However, following the induction of adaptive immunity, when IFN-γ activated Mϕ are thought to become involved, growth of bacteria was controlled (16). Thus, it appears that the defense mechanisms employed by Mϕ differ depending on the activation state of the cells. Moreover, our findings do not support the common supposition that resting Mϕ cannot control the growth of *M. tuberculosis*. We have shown that resting Mϕ can control mycobacterial growth to some extent, and that this is dependent upon the presence of CD43.

The differences seen in the growth of the bacterium in resting versus IFN-γ activated Mϕ are likely due to differences in the killing mechanisms employed by these cells, of which not all will require the involvement of CD43. It is well established that Mϕ have several ways of resisting intracellular growth of *M. tuberculosis*, many of which are circumvented by the bacterium (45). Some of these antimycobacterial effector functions include the induction of apoptosis and the production of reactive oxygen and reactive nitrogen intermediates (ROI/RNI), which are enhanced by cytokines such as IFN-γ and TNF-α.

We examined the function of CD43 in these processes and found a role for it in the *M. tuberculosis*-induced production of RNI, production of pro-inflammatory cytokines TNF-α, IL-12, and IL-6, and regulation of apoptosis. However, the activity of these mediators and the associated involvement of CD43 seems to be more pivotal in resting Mϕ than in IFN-γ activated Mϕ with regards to control of *M. tuberculosis* growth. Again, this is possibly due to the type of antimycobacterial responses employed by resting versus activated Mϕ. For example, it has been shown that resting Mϕ do not produce significant levels of ROI/RNI in response to *M. tuberculosis* (39) so these mechanisms are seemingly irrelevant in these cells but may be critical in IFN-γ activated Mϕ. Our results suggest that processes such as the production of protective cytokines and induction of apoptosis are likely more fundamental in the response of resting Mϕ to *M. tuberculosis*.

TNF-α has been shown to play a central role in not only host defense against mycobacteria, but in the initiation of apoptotic pathways. Several studies have linked these findings and shown that TNF-α mediated apoptosis may be a fundamental host

defense mechanism in mycobacterial infections (59, 69-74) and our data support this assertion, at least for resting Mϕ. CD43-/- Mϕ were deficient in TNF-α production and showed lower levels of Caspase-3 mediated apoptosis. When TNF-α was added to CD43-/- Mϕ, apoptosis levels increased and growth of *M. tuberculosis* was controlled to levels seen in CD43-expressing cells. Moreover, the neutralization of TNF-α in CD43+/+ cell cultures resulted in decreased levels of apoptosis and increased growth of *M. tuberculosis*. These results clearly indicate that TNF-α mediated apoptosis is key to controlling the intracellular growth of *M. tuberculosis* and that CD43 plays a regulatory role in this process.

As mentioned previously, apoptosis is emerging as a vital host defense mechanism in controlling *M. tuberculosis* infection; a number of studies have shown that virulent mycobacteria can inhibit the induction of apoptosis and in doing so, thrive within Mϕ (74-77). Circumventing apoptotic pathways allows the pathogen to evade bactericidal effector functions (72, 78) and avoid T cell priming (79-81), leading to a favorable environment for persistence. Recent studies have begun to elucidate mycobacterial gene products that may be involved in averting apoptosis (77, 81). The *secA2* gene, which encodes a component of a virulence-associated protein secretion pathway, has been found to be required for the inhibition of apoptosis in *M. tuberculosis*-infected Mϕ (81). Previous studies investigating the growth of a Δ*secA2* gene-deletion mutant of *M. tuberculosis* uncovered results very similar to ours, but examined parameters from the perspective of the bacterium instead of the host (82). The Δ*secA2* mutant was defective for growth within Mϕ in vitro and elicited greater amounts of RNI, TNF-α, and IL-6. These findings support the contention that these inflammatory

mediators, in conjunction with Mϕ apoptosis, are important antimycobacterial effectors. In addition, the aforementioned study found that the Δ*secA2* mutant showed decreased growth in mice during early stages of an aerosol infection and that the enhanced growth within Mϕ was abrogated with IFN-γ stimulation, similar to our results. Thus, it appears that apoptosis-related defense mechanisms may be of greater significance in resident tissue Mϕ than in activated cells.

In parallel, the lack of a clear role for CD43 in the IFN-γ activated Mϕ may be due to the presence of additional host defense mechanisms such as RNI/ROI that compensate for the loss of TNF-α mediated apoptosis in resting CD43-/- Mϕ. Indeed, both RNI and ROI are known to induce apoptosis, so it is possible that the similar levels of apoptosis in IFN-γ activated Mϕ from CD43+/+ and CD43-/- Mϕ may be mediated by these mechanisms. Furthermore, the observation that CD43-/- IFN-γ activated Mϕ had comparable levels of cytokine inhibition as resting Mϕ, yet restricted mycobacterial growth better, suggests that these cytokines have no role in IFN-γ mediated killing.

Our results in this study, taken together with our previous findings, indicate a multifunctional role for CD43 in the growth and pathogenesis of *M. tuberculosis*. We have shown that CD43 is involved in the binding and uptake of *M. tuberculosis* and other mycobacteria (16), and that CD43 is necessary for optimal control of intracellular replication of *M. tuberculosis* by resting Mϕ. We have also shown that CD43 is involved in the induction of RNI by IFN-γ activated Mϕ infected with *M. tuberculosis*. Additionally, we have demonstrated a regulatory role for CD43 in the production of proinflammatory cytokines including TNF-α, which mediates apoptosis in *M.*

tuberculosis-infected Mϕ and is known to be important for granuloma formation in *M. tuberculosis*-infected mice (47).

Together, these results suggest that CD43 may act as either a pattern recognition receptor or perhaps a co-receptor to facilitate the phagocytosis of *M. tuberculosis* and regulate downstream effector functions. We are currently undertaking studies to further explore the mechanisms of this phenomenon including comparing signaling capacities of CD43-/- and +/+ Mϕ in response to mycobacteria. Further inquiry is also warranted to analyze other antimycobacterial functions of Mϕ that may be affected by CD43 such as phagolysosome fusion, and production of reactive oxygen species, chemokines, or other cytokines important in the host response to *M. tuberculosis.*

	MOI	IFN-γ	DT ±SEM (h)
CD43+/+	20:1	-	27.71 ±0.15
CD43 -/-	20:1	-	*24.82 ±0.20
CD43 -/-	30:1	-	*24.07 ±0.19
CD43+/+	20:1	+	77.27 ±6.72
CD43 -/-	20:1	+	87.91 ±3.73
CD43 -/-	30:1	+	83.01 ±5.97

Table 3.1 ***M. tuberculosis*** **grows more readily in resting but not IFN-γ activated CD43-/- Mϕ than in CD43+/+ Mϕ.**

Bone marrow-derived Mϕ from CD43 knockout (CD43-/-) or control (CD43+/+) mice were infected with *M. tuberculosis* at 20:1 or 30:1 (MOI) with or without overnight stimulation with 100U/mL IFN-γ. The doubling time of the bacteria was calculated by comparing bacterial loads over 7 days. Results are shown as doubling time (DT) in hours ±SEM for 3 independent experiments, each with 3 replicates plated in duplicate.
* Indicates $p<0.05$ when compared to CD43+/+ group.

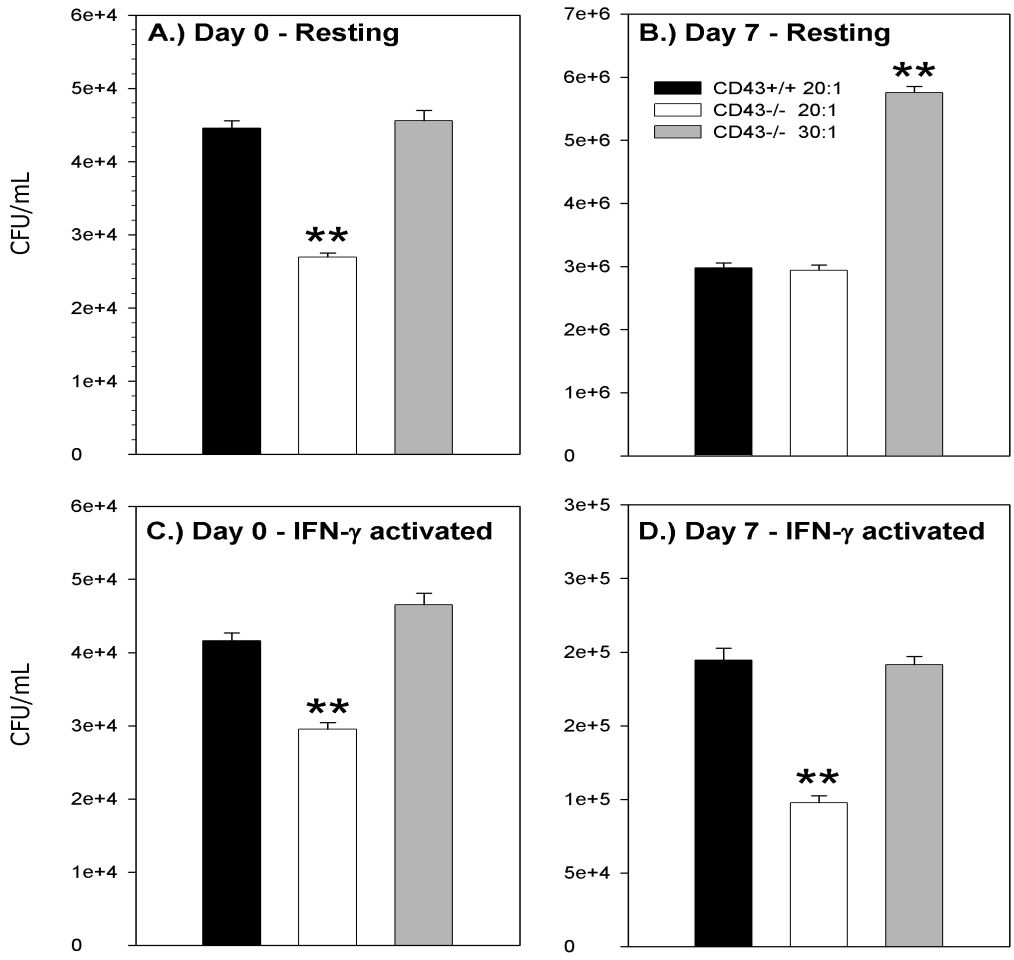

Figure 3.1 IFN-γ stimulation overcomes enhanced growth of *M. tuberculosis* in CD43-/- Mϕ.

CD43-/- and +/+ Mϕ were infected with *M. tuberculosis* at 20:1 (CD43+/+ black bar, CD43-/- white bar) or 30:1 (CD43-/- grey bar) for 3 hours. Monolayers were washed to remove unbound bacteria and infection was monitored for 7 days. Bacterial loads (CFU/mL) are shown ±SEM for 3 independent experiments, each with 3 replicates, plated in duplicate. (**) indicates statistical significance ($p<0.01$) compared to CD43+/+ control. (A) and (B) show bacterial loads in resting Mϕ, while in (C) and (D), Mϕ were primed overnight with 100 U/mL IFN-γ before infection.

NOTE: y-axis scales are not equivalent.

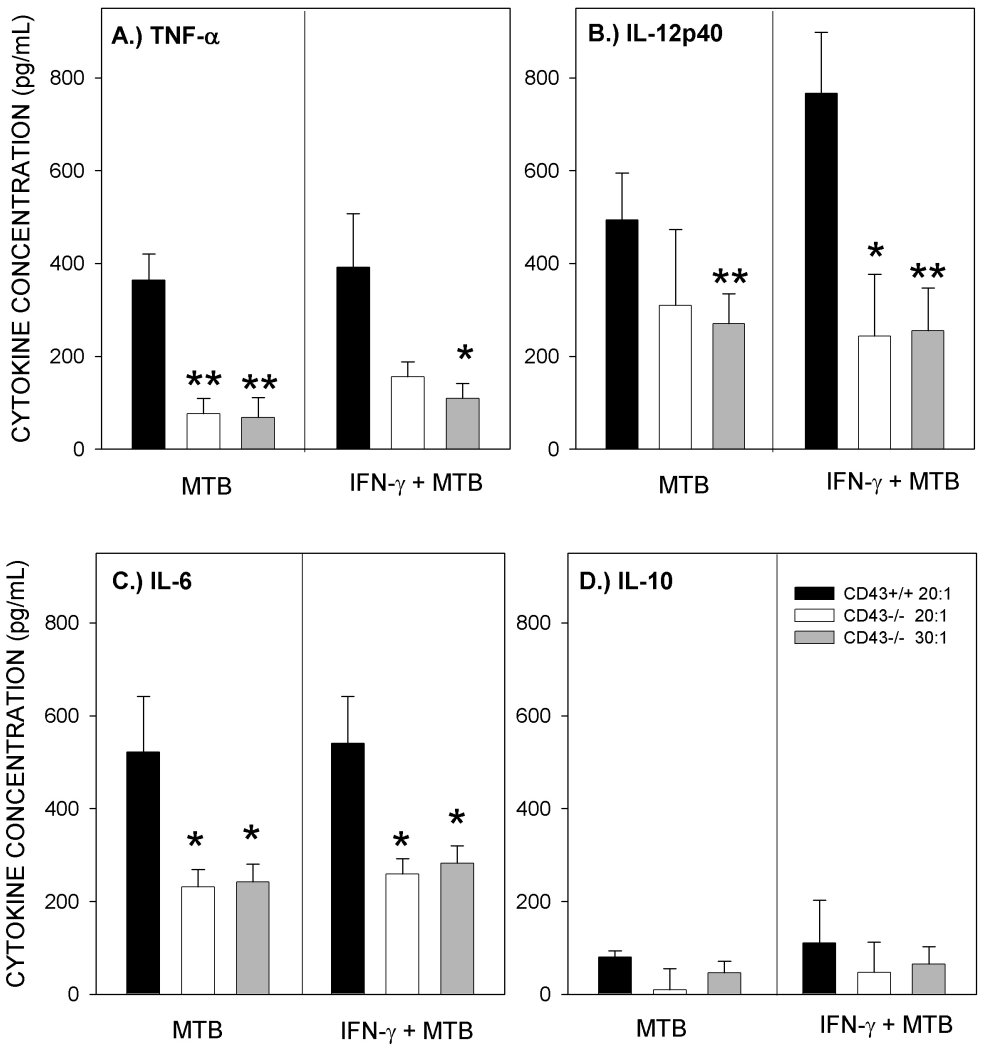

Figure 3.2 CD43-/- Mϕ are deficient in pro-inflammatory cytokine production in response to *M. tuberculosis* infection.

Resting or IFN-γ activated BMMϕ were infected with *M. tuberculosis* for 3 hours. Control CD43+/+ Mϕ were infected at 20:1 (black bars), while CD43-/- Mϕ were infected at both 20:1 (white bars) and 30:1 (grey bars) to equalize bacterial uptake. After infection, the monolayers were washed to remove unbound bacteria and re-incubated for 24 hours, at which point 100 μL of culture supernatant was removed for cytokine detection by ELISA as described in the text. For (A) TNF-α and (B) IL-12p40 results shown are the mean ±SEM for 5 independent experiments, each with 3 replicates, whereas for (C) IL-6 and (D) IL-10 results are the mean ±SEM for 3 independent experiments, each with 3 replicates. Asterisks indicate significant difference compared to CD43+/+ control where (*) indicates $p<0.05$ and (**) $p<0.001$.

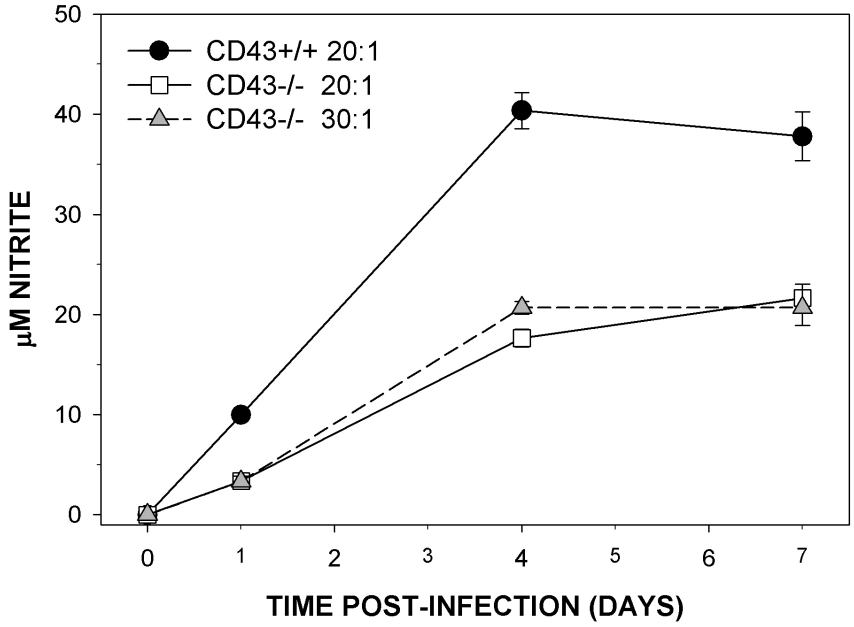

Figure 3.3 IFN-γ activated CD43-/- Mϕ infected with *M. tuberculosis* produce less RNI than do CD43+/+ Mϕ

IFN-γ activated BMMϕ were infected with *M. tuberculosis* for 3 hours, after which unassociated bacteria were removed by washing, and cells were re-incubated for 7 days. At the onset of infection, and on days 1, 4, and 7 post-infection, 100 µL of culture supernatant was removed from each well to measure RNI production using the Griess Assay as outlined in the text. Control CD43+/+ Mϕ were infected at 20:1 bacteria per Mϕ (filled circles), while knockout CD43-/- Mϕ were infected at MOIs of 20:1 (open squares) and 30:1 (shaded triangles). Results are expressed as the mean ±SEM for 3 independent experiments, each with 3 replicates. Where error bars are not visible, SEM was not beyond the graphic symbol. At days 1, 4, and 7, all CD43-/- values are significantly lower than CD43+/+ controls ($p<0.001$).

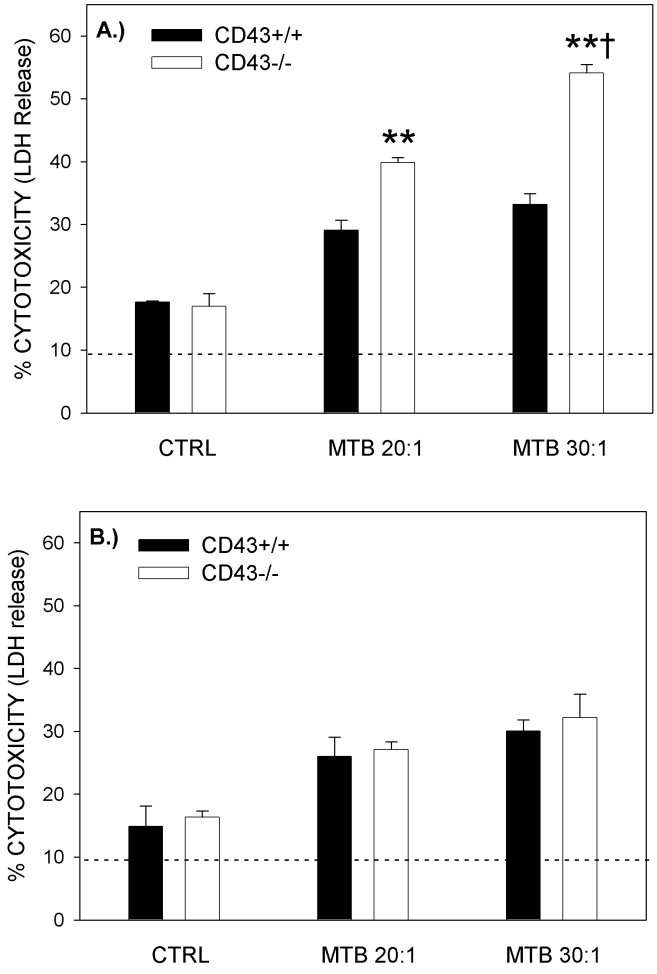

Figure 3.4 ***M. tuberculosis*** **infection causes greater cytoxicity in CD43-/- Mϕ than CD43+/+ control Mϕ**

Resting (A) and IFN-γ activated (B) CD43+/+ (black bars) and CD43-/- (white bars) BMMϕ were left uninfected (CTRL) or infected with *M. tuberculosis* (MTB) at 20:1 and 30:1 bacteria per Mϕ as described. After 4 days of infection, levels of necrosis were assessed by measuring the release of lactate dehydrogenase (LDH) into the culture supernatant. Cytotoxicity was measured as a percentage compared to a fully lysed positive control (100% cytotoxicity). Results are expressed as the mean ±SEM % cytotoxicity for 3 independent experiments, assayed in duplicate. (*) indicates $p<0.01$ compared to CD43+/+ control at same MOI, whereas (†) indicates a statistical difference of $p=0.0003$ between CD43-/- 30:1 and CD43+/+ 20:1. Dotted line indicates background levels in media alone.

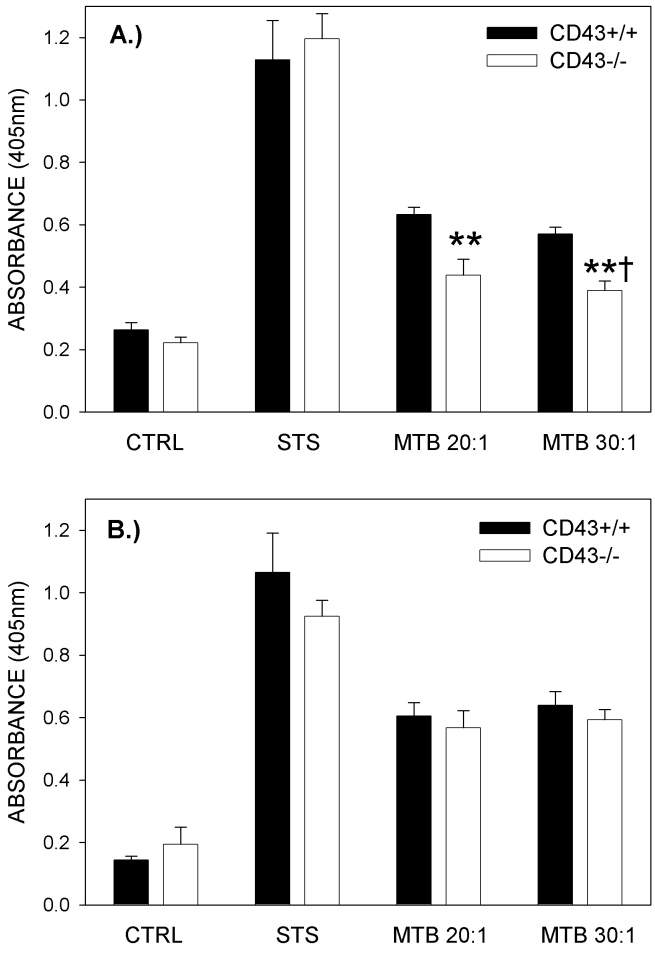

Figure 3.5 CD43 is required for optimal induction of apoptosis in *M. tuberculosis*-infected Mϕ

Apoptosis was measured in resting (A) and IFN-γ activated (B) CD43+/+ (black bars) and CD43-/- (white bars) BMMϕ after 4 days of infection with *M. tuberculosis* by determining the concentration of histone-associated DNA fragments as outlined in Materials & Methods. Uninfected cells (CTRL) served as a negative control while the apoptosis inducer staurosporine (STS) was used as a positive control. Results are expressed as the mean ± SEM OD for 3 independent experiments assayed in duplicate. (**) indicates $p<0.01$ compared to CD43+/+ control at same MOI, whereas (†) indicates a statistical difference of $p=0.007$ between CD43-/- 30:1 and CD43+/+ 20:1.

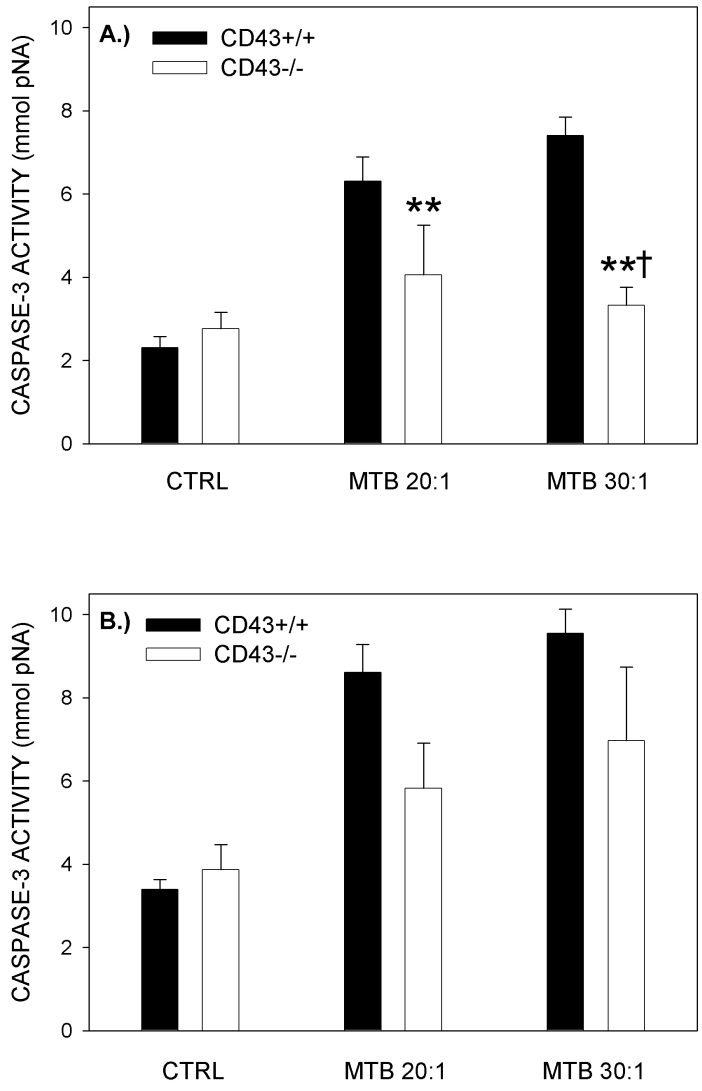

Figure 3.6 Absence of CD43 results in decreased activity of Caspase-3 in *M. tuberculosis*-infected Mϕ

Caspase-3 activity was measured by an enzymatic assay using samples from resting (A) and IFN-γ activated (B) CD43+/+ (black bars) and CD43-/- (white bars) BMMϕ after 4 days of infection with *M. tuberculosis*. Results are shown as mean ±SEM enzyme activity for 3 independent experiments assayed in duplicate. (**) indicates $p<0.01$ compared to CD43+/+ control at same MOI, whereas (†) indicates statistical difference between CD43-/- 30:1 and CD43+/+ 20:1 ($p<0.01$).

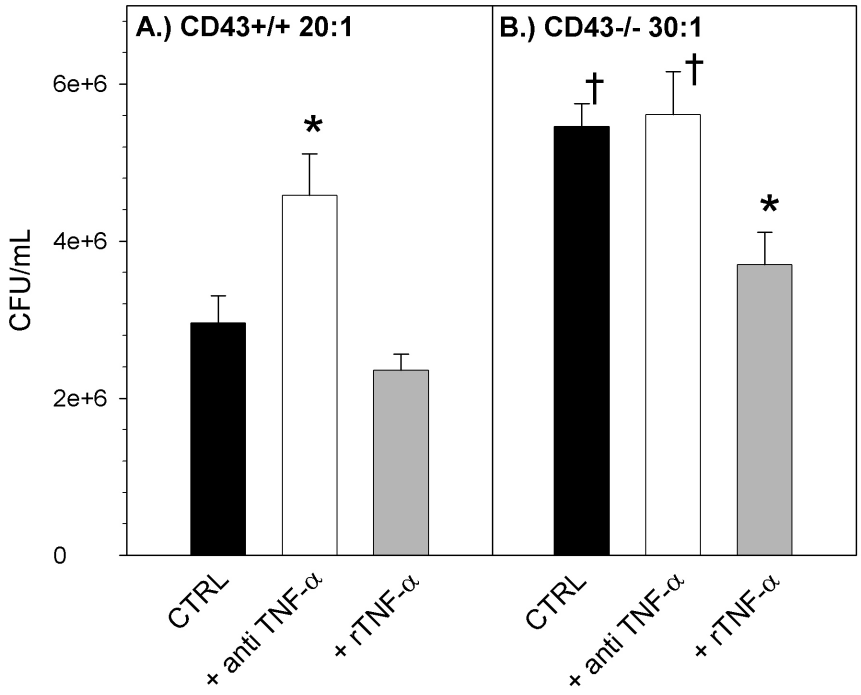

Figure 3.7 Enhanced growth of *M. tuberculosis* in CD43-/- Mϕ is due to deficient TNF-α production

Growth of *M. tuberculosis* was measured in CD43+/+ (A) and -/- (B) BMMϕ to compare the effect of 10 ng/mL anti-TNF-α antibodies (white bars) or 100 ng/mL recombinant TNF-α (rTNF-α, grey bars). Reagents were added along with bacteria at the time of infection and supplemented every 48 hours. Bacterial loads after 7 days of infection are shown as mean CFU/mL ±SEM for 3 independent experiments, each with 3 replicates plated in duplicate. (*) indicates $p<0.05$ compared to control within the same group, whereas (†) indicates statistical difference of $p<0.001$ for the CD43-/- group (B) compared to CD43+/+ control (A).

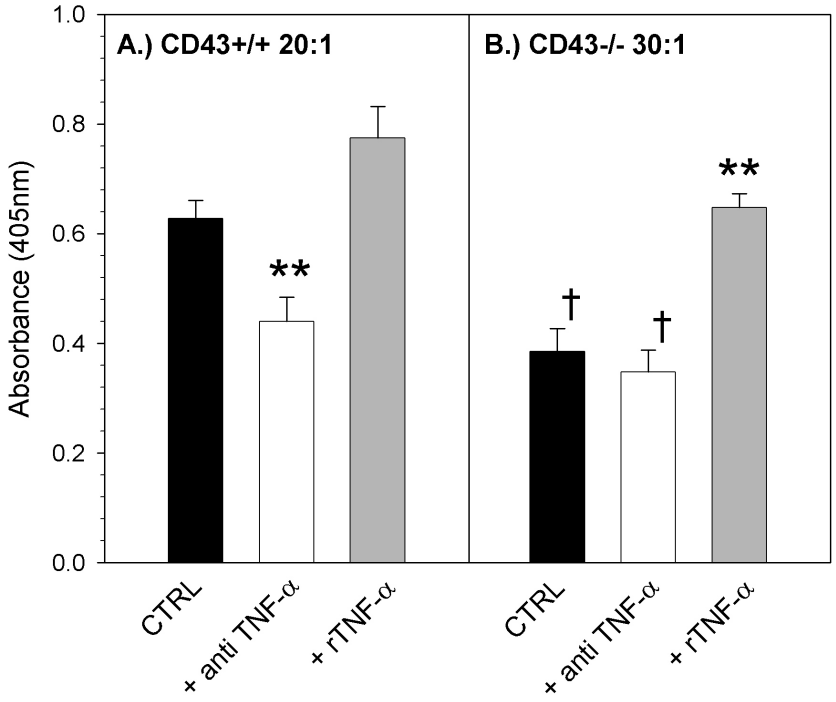

Figure 3.8 *M. tuberculosis*-induced apoptosis in CD43-/- Mϕ is TNF-α dependent

Apoptosis was measured in CD43+/+ (A) and -/- (B) *M. tuberculosis*-infected BMMϕ to compare the effect of 10ng/mL anti-TNF-α antibodies (white bars) or 100ng/mL recombinant TNF-α (rTNF-α, grey bars). Reagents were added along with bacteria at the time of infection and supplemented every 48 hours. Apoptosis was compared by measuring histone-associated DNA fragments after 4 days of infection as in Figure 3.5. Results are expressed as mean ±SEM OD for 3 independent experiments (**) indicates $p<0.01$ compared to control within the same group, whereas (†) indicates $p<0.01$ for the CD43-/- group (B) compared to the CD43+/+ control (A).

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Chapter 4 Discussion & future directions

4.1 Discussion

Tuberculosis remains a serious threat to global health. With 1/3 of the population infected, over 8 million active cases, and the emergence of multiple- and extensively-drug-resistant strains of *M. tuberculosis*, it is clear that improved vaccines and therapeutics are imperative (1). Several factors including its ease of transmission, complex biological structure, efficient evasion of host immune responses, and treatment complexities make *M. tuberculosis* a successful pathogen. These characteristics have also posed a number of challenges for clinicians and researchers seeking novel approaches to combat TB disease.

There are several approaches to studying infectious organisms in efforts to understand pathogenesis including examining bacterial virulence mechanisms and/or host defense strategies. In these studies, we focused on the initial interaction of *M. tuberculosis* with host cells and explored how the dynamics of this interaction can affect the subsequent progression of infection. Mϕ, the primary host cells for *M. tuberculosis*, serve as the first line of defense against infection and can dictate the subsequent innate and adaptive responses, and ultimately the outcome of infection. Optimally, Mϕ eliminate intracellular mycobacteria using a number of defense mechanisms. However, *M. tuberculosis* has successfully evolved mechanisms to evade such defenses and survive and replicate in these cells

As mentioned in the introductory chapter, there are several Mϕ surface components that have been shown to interact with *M. tuberculosis* but none of these have been shown to affect the successive control of bacterial growth. CD43 is the first and only receptor whose presence has been shown to affect the survival of *M. tuberculosis*. This evidence indicates that there is clearly a functional link between the binding of *M. tuberculosis* by CD43-mediated processes and the subsequent events. This supposition is further supported by our findings that CD43 regulates the production of inflammatory mediators and the induction of apoptosis in response to *M. tuberculosis.*

Apoptosis, or programmed cell death, plays an important role in immunity to pathogens so it is not surprising that many intracellular pathogens exhibit anti-apoptotic mechanisms (2-4). Although some studies have shown that *M. tuberculosis* may induce host cell apoptosis under certain conditions (5-8), there is considerable evidence suggesting that virulent strains of the bacterium actually inhibit apoptosis (9-12). Moreover, it has recently been shown that the differential susceptibility of various mouse strains to *M. tuberculosis* infection is linked to the induction of necrosis versus apoptosis in infected Mϕ (13). In that study, it was shown that cells that became apoptotic upon infection were more resistant to bacterial growth, while cells with a necrotic phenotype were extremely susceptible to the intracellular growth of *M. tuberculosis* or *Listeria monocytogenes* (13). This hypothesis that *M. tuberculosis* evades apoptosis to allow for increased replication and spread within the host is supported by our findings reported here.

Our results also suggest that the controlled growth of *M. tuberculosis* in Mϕ via induction of apoptosis is mediated mainly by TNF-α. Consistent with other published

studies (14), we found that neutralization of TNF-α resulted in lower levels of apoptotic cell death and enhanced bacterial survival. The significant role for TNF-α in host defense against Tuberculosis infection has been established by several studies using neutralizing antibodies, over expression of TNF receptors, or knockout mice (14-17) but the mechanism by which TNF-α mediates host defense is not fully understood. Our data suggest that TNF-mediated apoptosis is an important process employed by host cells to restrict mycobacterial growth. Furthermore, this may be of significance in the development of granulomas in *M. tuberculosis*-infected individuals as not only has TNF-α been shown to be important in the formation of granulomas, but the CD43-/- mice showed inadequate containment of mycobacterial infection, likely due to their deficiency in TNF-α production. This remains to be elucidated, as other factors may be responsible for the abnormal organ pathology seen in CD43-/- mice infected with *M. tuberculosis*. For instance, CD43 has been shown to be important in activation of T cells and in lymphocyte homing (18, 19).

The preliminary study by Fratazzi et. al implied a potential role for CD43 in the recognition of mycobacteria. This evidence, along with the published roles for CD43 in cell activation, migration and signaling events led us to hypothesize that it may have a significant function in *M. tuberculosis* infection. We confirmed this hypothesis by showing that CD43 plays a number of roles including the initial binding and uptake of the bacterium, and the production of inflammatory mediators and antimycobacterial effector functions of Mϕ. We contend that CD43 is required for the optimal control of *M. tuberculosis* infection, especially during early stages of infection, and suggest it is a critical component of the innate immune response to Tuberculosis.

4.2 Future directions

Several avenues for future research in the area of CD43 interactions with *M. tuberculosis* exist that were beyond the scope of the studies presented in this manuscript. We have successfully established that CD43 is an important host cell molecule that interacts with the mycobacterial surface. The nature of this interaction is yet to be established and the question of whether CD43 acts as a pattern recognition receptor or as a co-receptor will needs to be resolved in future studies. Perhaps there are other molecules that cooperate with CD43 in binding to the bacterial surface and the characterization of these will aid our understanding of this interaction.

To date, no definitive ligands have been identified on the bacterium that may bind to cell surface receptors, but evidence by other researchers in our lab indicates that the outer layer of the cell wall envelope of *M. tuberculosis* modulates the interaction of the bacterium with Mϕ (20). Identification of specific ligands and the mechanisms of ligand-receptor interactions will be central to our understanding of mycobacterial pathogenesis as it is becoming clear that such interactions can determine the induction of innate immune mechanisms and the subsequent outcome of infection.

Our results in the present study point to the involvement of CD43 in cell signaling processes in response to *M. tuberculosis*. Intra- and intercellular signaling events potentiate effector cell activation and are important components of the immune response against invading pathogens, thereby bridging the innate and adaptive branches of immunity. We have begun comparing gene expression using gene array technology based on the findings of this project. These studies are ongoing and the results will be

analyzed using bioinformatics tools that will allow comparison of gene expression by CD43-/- and CD43+/+ Mϕ in response to *M. tuberculosis* infection and may lead to the identification of components of interest for further study.

CD43 is not only present in high concentrations on Mϕ, but on nearly all hematopoietic cell types (18, 21-23) and the role of CD43 on these cells and their interaction with *M. tuberculosis* will be of interest for future studies. For example, DCs are vital in the progression of innate to adaptive immunity to many pathogens including *M. tuberculosis*, and the presence of CD43 on these cells may have a function in this role. Additionally, further inquiry into the adaptive immune response to *M. tuberculosis* is warranted, given our results that granuloma formation is impaired in CD43 deficient mice. Because CD43 has been associated with regulating the homing of lymphocytes to sites of inflammation, analyzing the composition of granulomas and evaluating the infiltrating cell types may offer insight into how this process develops.

In summary, studies that elucidate mechanisms involved in *M. tuberculosis* infection and the innate immune response of infected individuals will lead to a thorough understanding of the molecular mechanisms of disease and ultimately allow identification of therapeutic targets or vaccine candidates.

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