DISSERTATION

THE PATHOGENESIS OF DIABETES-TUBERCULOSIS COMORBIDITY

Submitted by

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ABSTRACT

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Exposure to the bacterium, *Mycobacterium tuberculosis*, only leads to the active form of tuberculosis disease (TB) in 5-10% of infected individuals. The development of active TB, at any stage of infection, is often the result of a known TB risk factor, either intrinsic to the individual or acquired as a communicable or non-communicable disease. An association between diabetes and TB has long been recognized, but only recently was diabetes confirmed to increase the risk of developing active TB disease. The convergence of a growing diabetes epidemic on regions with endemic TB has positioned diabetes as an emerging global threat to TB control.

Of particular importance is the rapidly growing incidence of type 2 diabetes, which accounts for up to 95% of the global diabetic population. Since the potential impact of this growing comorbidity has only been recently emphasized, little is known regarding the mechanisms of dysregulated immune function and metabolism by which diabetes predisposes to active TB disease. The current understanding of this comorbidity is further limited by the lack of appropriate animal models that replicate the pathogenesis of both human type 2 diabetes and TB. The guinea pig is a well-established model of TB that replicates human pathology and disease progression. This species was emphasized in this series of studies with the goal of better understanding the impact of type 2 diabetes on TB progression and the mechanisms that may change the host response to *M. tuberculosis* infection.

In Chapter 2, we investigated the impact of hyperglycemia alone, induced as post-prandial hyperglycemia through daily administration of sucrose, on TB disease progression in non-diabetic guinea pigs. Guinea pigs receiving daily sucrose developed both higher bacterial burdens in pulmonary and extrapulmonary tissue and also more severe pathology by day 60 of

infection. This exacerbated disease manifestation was accompanied by the accumulation of advanced glycation end-products, which are inflammatory by-products of chronic hyperglycemia with known involvement in the development of diabetes-related complications. Interestingly, by monitoring glucose and lipid metabolism in these guinea pigs, we learned that TB alone leads to severe metabolic disturbances, manifesting as hyperglycemia and accumulation of circulating total free fatty acids. From this study, we were able to conclude that not only does mild post-prandial hyperglycemia worsen the course of TB disease in guinea pigs, but also, infection with *M. tuberculosis* alone induces metabolic disease resembling diabetes, similar to what has been previously reported in human TB. These conclusions rationalize the investigation of novel adjunctive therapies to restore metabolic homeostasis, which may improve the host response to infection, limit bacterial growth, and increase the efficacy of frontline antimicrobial drugs.

In Chapter 3, we developed a novel model of type 2 diabetes in the guinea pig to be used in future investigations of type 2 diabetes-TB comorbidity. Previously, the guinea pig as a diabetic model has been described only in the context of β-cell cytotoxicity with the drug, streptozotocin (STZ), but with variable efficacy. In this study, we initially optimized the dose response and STZ preparation to achieve an induction of hyperglycemia that was uniform with limited mortality. This hyperglycemic response was transient but could be stabilized through continued β-cell stress, in the form of a high fat, high sugar diet. Feeding of this modified diet led to impaired glucose tolerance and a compensatory β-cell response that could be abrogated with the use of a single optimized dose of STZ. This novel model of type 2 diabetes develops both insulin resistance and β-cell failure, which replicate the typical progression of type 2 diabetes in humans, all within a reasonable experimental timeframe. From this study, two models emerged, a type 2 diabetic guinea pig as well as a model of impaired glucose tolerance, or prediabetes, that would be used to investigate the mechanisms of diabetes-TB comorbidity.

In Chapter 4, the newly developed guinea pig models were used to investigate the overall impact of type 2 diabetes and impaired glucose tolerance on TB progression and the host immune response to *M. tuberculosis* infection. Although impaired glucose tolerance alone had limited impact on TB progression with exacerbation of disease only at chronic end points, *M. tuberculosis* infected type 2 diabetic guinea pigs closely resembled the reported manifestations of human diabetes-TB comorbidity including more severe TB disease, higher bacterial burdens, and a robust innate and cell-mediated immune response. Despite evidence of strong Th1 cell-mediated immunity, which is known to be critical for limiting bacterial growth and disease progression, diabetic guinea pigs were unable to control bacterial growth and developed damaging neutrophilic inflammation. To better understand the immune mechanisms leading to uncontrolled bacterial growth and severe disease, in Chapter 5, we investigated the innate and adaptive immune response over the course of early infection in type 2 diabetic guinea pigs. Diabetic guinea pigs were slow to develop early lesions with delayed bacterial transport to the lung draining lymph node, and a corresponding delay in antigen-specific Th1 immunity. Early alterations in cytokine expression were identified that may explain the delayed development of cell-mediated immunity and allow for substantial growth of *M. tuberculosis* in the lung of infected diabetic guinea pigs.

These data indicate that not only does type 2 diabetes increase the severity of TB but also that the chronic inflammatory process associated with TB itself may worsen diabetes. This has important implications worthy of further investigation revolving around the diagnostic criteria for diabetes when associated with TB, the impact of active TB on medical management of diabetes, and the investigation of novel therapeutic targets, both metabolic and immunological, to enhance the host immune response to infection and limit TB disease severity in diabetics.

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CHAPTER 1

Review of the Literature

Tuberculosis - Pathophysiology, Disease Diagnosis, and Treatment

Tuberculosis Epidemiology.

Tuberculosis (TB), caused by the bacterium, *Mycobacterium tuberculosis*, is currently the second leading cause of mortality due to an infectious disease, behind human immunodeficiency virus (HIV). TB has remained a global health priority since 1993, when a surge in HIV-related TB cases coincided with a rising overall TB prevalence and emergence of drug resistance[1, 2]. The TB DOTS (directly observed treatment, short-course) strategy was adopted to improve treatment success and reduce transmission and mortality among TB patients, and through demonstrated success, led to a strategy to reverse the trend of rising TB prevalence by the year 2015[3]. The overall goal is to reduce the prevalence and mortality by 50% compared to the baseline rates in 1990[4]. In 2012, progress toward this goal indicates that the prevalence of TB has been reduced by 37% since 1990 and the mortality reduced by 45%[3]. Although a slow but successful reduction in TB cases has been achieved, at approximately 2% per year, TB remains a major global health problem with 8.6 million new TB cases in 2012 and 1.3 million deaths due to TB[5].

The major factors that are contributing to the slow rate of decline in TB cases worldwide include the continual major contribution of HIV to new TB cases and mortality, the continuing expansion and inefficient detection of multidrug resistance, the lack of a vaccine that is uniformly effective across the entire population, and the emergence of new risk factors that impact TB prevalence and response to treatment[5-7]. Of the 8.6 million new TB cases and 1.3 million deaths in 2012, 1.1 million and 300,000, respectively, were associated with HIV. As such, HIV remains the leading risk factor for the development of active TB disease[5]. Globally in

2012, 3.6% of new cases, and 20.2% of previously treated TB cases were due to infection with multidrug resistant (MDR) strains of *M. tuberculosis,* which is defined as resistance to isoniazid and rifampicin, the two major frontline antimicrobials for TB treatment[5, 8]. MDR-TB is associated with a significantly lower treatment success rate of 48%, compared to 87% in drug-susceptible TB[5]. Considering the growing rate of MDR-TB cases, novel therapeutic approaches and new, well-tolerated antimicrobial compounds are urgently needed to limit this growing problem.

In addition, the true protective efficacy of BCG vaccination, the only current option for immunization against M. tuberculosis, remains uncertain[7, 9]. The vaccine is frequently implemented as a strategy to reduce childhood mortality due to TB, for which it is effective[10, 11]. However, in high burden countries, efficacy of the vaccine in the adult population is variable and lends a limited overall efficacy of 50% based on risk reduction in recent meta-analyses, which is supported by the high TB burden that remains in countries that practice uniform immunization during infancy[12, 13]. Hence, the growing rate of antimicrobial resistance, and new emerging risk factors, such as diabetes, raise concern that the current suboptimal treatment and preventive strategies are not sufficient to control the global tuberculosis burden[3, 14].

Transmission and Disease Progression in Tuberculosis.

Although TB can affect a variety of organs, this generally occurs via dissemination from the lung, which is the portal of entry in almost all cases. Exposure occurs upon encountering an infectious aerosol from a person with active pulmonary disease and capable of expelling bacteria into the upper airways. Infectious individuals are generally though to have cavitary pulmonary TB with evidence of bacilli by sputum smear[1]. Studies involving close contacts have shown an infection rate of only 25 - 50% even in the most crowded conditions[15]. These data indicate that sustained close contact and/or intrinsic host susceptibility factors are important for successful transmission of the organism. Additionally, it has become apparent that expelled organisms have adopted a specific phenotype not typical of *in vitro* culture, indicating that

organism specific factors may also play a role in transmission[16]. An individual is deemed infected if they have detectable cell-mediated immunity. Among infected individuals, only around 5-10% will develop active TB, which is the progressive form of disease and is most likely to manifest within five years after exposure[15]. The other 90-95% are assumed to have developed the latent form of TB (LTBI), where there is no detectable clinical disease and the bacteria persist within a location that is not yet fully understood; however, successful sterilization by the immune response is possible, but the frequency of this remains unclear[17]. The development of recrudescing active TB from LTBI is known as reactivation and is expected to account for the majority of active TB cases, but the contribution of reinfection after successful clearance, either by the host response or through antimicrobial treatment, is becoming increasingly recognized[18]. Most cases of active TB stem from reactivation of a latent infection state in areas with low prevalence. In areas of high endemnicity, recent exposure contributes greatly to the population with active TB disease[19, 20]. The factors that lead to LTBI reactivation or progression to active TB upon exposure are poorly understood but are at least partially attributable to acquired host risk factors[21].

Diagnosis of Tuberculosis.

Active TB can be diagnosed through a combination of radiographic evidence of active disease, detection of the bacterium in sputum by culture or molecular techniques, and acid-fast staining of the bacteria in sputum smears[22, 23]. A patient with radiographic evidence of active TB does not necessarily manifest sputum smear or culture positivity, and alternatively, culture evidence may be present without identification of bacilli by direct smear techniques. In contrast, the more frequent manifestation of LTBI poses a greater diagnostic challenge, as diagnosis often relies entirely on immunological evidence of infection, although additional strength is added by radiographic evidence of pulmonary scarring indicating previous active TB. The tuberculin skin test (TST) remains the most widely used and available test for the immunological

diagnosis of *M. tuberculosis* infection, especially in resource poor communities[24]. The TST is performed by intradermal injection of purified protein derivative (PPD), a protein preparation consisting of greater than 200 antigens precipitated from *in vitro* culture supernatant of *M. tuberculosis*[25]. In individuals that have developed cell-mediated immunity to *Mycobacterium spp*., induration develops at the site of injection due to a T cell-mediated memory recall response[26]. However, specificity of the TST is a significant issue, as people previously vaccinated with BCG will frequently be skin test positive. Additionally, environmental exposure to non-tuberculous *Mycobacteria*, which have multiple cross-reactive antigens, may also develop a TST reaction, leading to false-positive results[27, 28].

Although general rules exist to help differentiate skin test reactivity in BCG-vaccinated versus *M. tuberculosis*-infected individuals, the more recent implementation of antigen-specific blood assays have greatly improved the diagnostic specificity for TB[29]. These IFNγ release assays (IGRAs) detect the production of IFNγ by antigen-specific memory T cells against the immunodominant antigens, ESAT-6 and CFP-10, which are specific to *M. tuberculosis* and *M. bovis* (as well as *M. kansasii* and *M. marinum*)[24]. These antigens are products of the region of difference 1 (RD1) gene cluster, which is absent in *M. bovis* BCG as well as most non-tuberculous mycobacteria. Two commonly used assays exist for whole blood preparations with detection by either traditional ELISA, or by ELISPOT[30]. In culture confirmed cases of active TB, these IGRA assays demonstrate a sensitivity and specificity exceeding 90%, compared to a TST sensitivity of 77%, while BCG vaccination has a large and variable effect on TST specificity[31]. Accordingly, the IGRA has become an important tool for the diagnosis of LTBI.

Treatment of Tuberculosis.

The difficulty in the treatment of tuberculosis lies in the inherent capacity for the organism to persist within the host where it is tolerant of antimicrobial therapy, as well as the

relatively frequent emergence of genotypic antimicrobial resistance. The organism may exist within the host is essentially three states: extracellular amongst host-derived cellular debris (casseous necrosis) and actively replicating, extracellular amongst host casseous debris and in a slow or non-replicating state, or intracellular within professional phagocytes[32]. The utility of antimicrobials targeting bacteria in the intracellular environment is limited by the slow-replicating state of the organism and the acidic intracellular environment. The tolerance of bacteria is an extracellular state to antimicrobial therapy has been attributed to a non-replicating state adopted by the organism[33]. The primary target of antimicrobials may be the rapidly growing extracellular compartment, which is readily reduced during antimicrobial therapy but is also the prime site for selection of point mutations leading to genotypic resistance. It is due to this rapid emergence of resistance that monotherapy for the treatment of active TB is not utilized[34].

The first line regimens for the treatment of active disease consist of a combination of isoniazid (INH), rifampicin (RIF), and pyrazinamide (PZA), with or without ethambutol. In concert, these three drugs are bactericidal against the organism in its various locations and growth states within the host[35]. The mechanisms of action target mycolic acid synthesis, DNA-dependent RNA polymerase, and membrane transport systems for INH, RIF and PZA, respectively[34, 36, 37]. The preferred treatment method is a standard 6-month course utilizing the DOTS standards of care. The initial phase of treatment in patients with active disease, whether cavitary or not, involves 8 weeks of INH, RIF, and PZA therapy followed by a continuation phase of either 18 additional weeks with INH and RIF if sputum culture negative after initial phase, or 31 weeks if sputum culture positive[32]. The use of combination therapy and prolonged regimens are necessary both to prevent emergence of resistance and also to eliminate persistent bacterial populations, which are considered to be the source of disease relapse after incomplete therapy. The long regimens are complicated by hepatotoxicity as the major adverse effect, which contributes to poor patient compliance and early termination of therapy in some cases[38].

The Immunopathogenesis of Tuberculosis

The majority of what we know about the immune response to *M. tuberculosis* and what constitutes a favorable response to infection has been investigated in the mouse model of tuberculosis[39]. Furthermore, many of these investigations have been performed in a single inbred strain, C57BL/6, attributable to the readily available knockout and transgenic models on this background. However, important contributions have been made from the guinea pig, rabbit and non-human primate models of TB. From what has been learned in animal models, a central theory has emerged for optimal host protection, where the immune response to *M. tuberculosis* infection, particularly as chronic infection is established, is a balance between protection and pathology[40]. The inflammation that mounts in response to infection should be just enough to control bacterial growth, but not so active as to result in host tissue injury. In forming this balance during the immune response in animal models, an equilibrium between host and organism is thought to be established where the host response can contain the growth of the organism, but not eliminate it, thereby slowing the progression of disease[41]. Both the innate responses to infection as well as an appropriate adaptive T cell-mediated response are involved in the host's ability to slow disease progression.

The Innate Response to Infection.

It has long been regarded that the primary cell type infected by *M. tuberculosis* is the mature myeloid cell collectively referred to as the macrophage[42]. Although seemingly intuitive, only recently was it demonstrated that the alveolar macrophage is the first cell to be infected by this primarily pulmonary pathogen[43]. Furthermore, it is also now recognized that the catch-all term of macrophage actually extends to a diversity of cell types that are infected from the myeloid cell lineage, and that the kinetics of this is dynamic. While alveolar macrophages may be the first cell to be infected, the rate of infection is quickly exceeded by the rate of neutrophil

and dendritic cell infection[43, 44]. As the inflammatory response progresses, macrophages that are recruited from the periphery to pulmonary sites of inflammation also become infected[43, 44]. One correlate for protection in animal models is the rate by which dendritic cells become infected because the establishment of adaptive immunity is dependent on presentation of antigens by dendritic cells in the lung-draining lymph nodes[45].

While other pathogens may utilize mechanisms to evade phagocytosis as a virulence factor, *M. tuberculosis* is promiscuous in receptor binding on macrophages to encourage phagocytosis[46]. *M. tuberculosis* has been shown to bind multiple receptors to gain access to myeloid cells where bacterial-mediated inhibition of phagolysosomal fusion effectively harbors the pathogen initially within the phagosome. These receptors include complement receptors such as CR1 and CR3, transmembrane C-type lectins including the mannose receptor, and scavenger receptors including SR-A and CD36[46]. In contrast, these receptors play less of a role in infecting dendritic cells *in vitro*, which is mediated predominately by DC-SIGN, a C-type lectin that recognizes mannose molecules[47, 48]. Host innate pulmonary defense mechanisms such as surfactant D, which has an affinity for lipoarabinomannan of *M. tuberculosis* may enhance protection by promoting phagolysosomal fusion, while other surfactant proteins may enhance uptake of the bacterium[46, 49].

Within the host cell the bacterium may exist in multiple intracellular compartments. The recently recognized role of autophagy adds an additional factor to consider in the intracellular fate of the bacterium, beyond the long-accepted process of preventing phagosomal maturation[46, 50, 51]. The type VII secretion system, ESX-1, of M. tuberculosis facilitates phagosomal degradation and escape, allowing the organism to replicate in the cytosol[51, 52]. It is thought that this ESX-1 system allows for the initial recognition of the pathogen by gaining access to cytsolic pattern recognition receptors including NOD2, which occurs only if the ESX-1 system is intact[53, 54]. Organisms residing in the cytosol may become ubiquinated and sequestered further in an autophagosome, which facilitates antigen presentation, as has been

shown in M. marinum infected macrophages[55]. However, *M. tuberculosis* is able to inhibit autophagy, leading to suppressed innate immune defenses[56]. Therefore, M. tuberculosis follows a complex intracellular survival pattern that reduces host recognition of the organism and limits an effective immune response.

Some of the most recognized receptors participating in the early response to infection include NOD2, C-type lectins, and of the Toll-like receptors, TLR-2 is particularly recognized for initiating inflammation in response to *M. tuberculosis* infection. However, the critical role of the adaptor protein MyD88 in initiating the response to infection is not entirely TLR-dependent and is also heavily influenced by signaling through the IL-1 receptor[57]. MyD88 and IL-1 receptor knock-out mice are capable of generating an antigen specific response in the lung but they develop acute, severe pneumonia, a process that has been attributed to reduced bactericidal capacity of phagocytes[58]. The innate response to M. tuberculosis infection leads to synthesis of IL-12, which promotes a Th1-skewed T cell response. The production of IL-12 is critical for dendritic cell migration to the tracheobronchial lymph nodes. However, TLR signaling is linked to efficient induction of effector T cells and therefore may be involved in balancing the protection of the host[59, 60].

The production of TNFα during the innate response to infection, similar to IFNγ during the adaptive response, is an indispensable cytokine[61]. TNFα is a macrophage activation signal that synergizes with IFNγ. The induction of apoptosis by TNFα is recognized *in vitro*, and the importance of inducing this cell death mechanism *in vivo* is becoming increasingly recognized[61, 62]. Recently, it has been demonstrated in the zebra fish model of *M. marinum* infection, that optimal TNFα levels are required because TNFα in reduced or excess concentration promotes necrosis, an unfavorable mechanism of cell death, and inflammation[63]. Mice deficient in TNFα have been shown to develop a severe inflammatory response that does not necessarily correlate with high bacterial load, indicating that this cytokine has regulatory function as well[64].

The Adaptive Response to Infection.

CD4 T cells are absolutely necessary for an effective response to *M. tuberculosis* infection, and more specifically a Th1-skewed response[65]. This is most obvious in mouse models lacking IFNγ, which rapidly succumb to progressive TB, but also in human populations where a lack of CD4 T cells due to HIV infection is the major risk factor for the development of TB[65-67]. The beneficial effects of adoptively transferring T cells to deficient hosts demonstrated the requirement of T cells in a successful response[68]. Evidence from mice suggest that the development of classic casseous necrosis is dependent of the T cell population however this has been challenged in the guinea pig model where kinetic studies have demonstrated that casseous necrosis precedes the accumulation of T cells in the granuloma[69, 70]. This dependency on a T cell response is related to the generation of IFNγ because cessation of bacterial growth correlates with the arrival of IFNγ-producing T cells in the lung in multiple studies[39, 71, 72].

The generation of a functional adaptive response with antigen-specific T cells is notably delayed in tuberculosis when compared to other intracellular infections and the timing is the most critical aspect of the adaptive response to *M. tuberculosis* infection[73]. If it is too slow, bacterial growth cannot be controlled, but in the presence of an already established cell-mediated response, the host is much more successful in limiting the infection[73]. However, the efficacy of an existing antigen-specific cell-mediated response is limited in its ability to induce bactericidal activity, since the adoptive transfer of primed effector CD4 T cells still cannot control the intracellular growth of bacteria in the first seven days of infection[74]. This suggests that the organism is not recognized during this stage and provides insight into why prior BCG vaccination does not lead to sterilizing immunity even in animal models[75]. This varying process is evident within the same infected host, where early granulomata display progressive bacterial growth, while disseminated granulomata, which contain antigen specific T cells, contain much

lower bacterial loads[76, 77]. The genetic susceptibility of mice to *M. tuberculosis*, is at least in part due to the timing and strength of the adaptive response[78]. The generation of adaptive immunity is further delayed in susceptible mouse strains, when compared to resistant strains[79, 80]. This is potentially the result of failed migration of antigen presenting cells since lymph node initiation is essential and susceptible strains have delayed arrival of bacteria in the lung-draining lymph node[43, 45, 71, 78]. Inhibition of apoptosis by *M. tuberculosis* may contribute significantly to this delay since dendritic cell acquisition of the bacteria or bacterial products is greatly enhanced by apoptosis[44]. This is supported by a number of studies that have demonstrated enhanced adaptive immune function when animals are infected with pro-apoptotic strains of *M. tuberculosis* or *M. bovis* BCG[81, 82]. Recent studies suggest that the balance of cell death between apoptosis and necrosis is related to eicosanoid production, where lipoxins promote necrosis and prostaglandins promote apoptosis[83].

Although the events that occur immediately after low-dose aerosol infection remain poorly understood, the first evidence of bacterial dissemination is via the lymphatics to the lung draining lymph node by day 9 of infection at the earliest in resistant mouse strains[45, 78]. Interestingly, during this time, no mediators have been shown to accelerate the dissemination and earlier initiation of the response in the lymph node, which has been attempted with external inflammatory stimuli expected to enhance migratory activity, such as LPS[45]. However, upon antigen arrival in the lymph node, activation of naive T cells and their accumulation in the lung occurs with anticipated kinetics and therefore, it is the early innate pulmonary response that accounts for delayed priming of an adaptive response[84].

The protective effect of cell-mediated immunity has been attributed to the production of IFNγ. Mice lacking this cytokine have uncontrolled bacterial growth and rapidly succumb to progressive TB disease[66]. Part of the protective effect of IFNγ is through macrophage activation, which upregulates MHCII and CD80/86 co-stimulatory molecule surface expression

and activates microbicidal functions[85]. In murine macrophages, inducible nitric oxide synthase (NOS2) is a potent bactericidal mechanism induced by IFNγ, which leads to production of reactive nitrogen intermediates that may be directly bactericidal although recently, the bactericidal effect of nitric oxide has been attributed to the induction of apoptosis[86, 87]. However, the role of nitric oxide in human disease has been debated[88]. IFNγ also promotes phagosomal maturation and induces autophagy, which may both increase bactericidal capacity and promote antigen presentation[89, 90]. The impairment of autophagy even in the presence of IFNγ increases intracellular bacterial viability[90]. In mice lacking functional CD4 T cells, there is a tendency toward heavy granulocytic inflammation, findings that suggest an anti-inflammatory component to CD4 T cells and IFNγ production, in addition to the classical paradigm of activating infected macrophages[91]. This failure to develop the classical well developed granulomata with casseous necrosis in exchange for more neutrophil-mediated injury is also replicated in HIV-infected individuals with depleted CD4 T cell counts[92]. More recently, it was demonstrated that this anti-inflammatory property of IFNγ might result from reducing the production of IL-1β, a potent pro-inflammatory cytokine involved in the recruitment of neutrophils[93]. However, the opposite is true in lymphopenic HIV-TB patients receiving antiretroviral therapy that develop immune reconstitution inflammatory syndrome (IRIS), which is a highly suppurative process dependent on restoration of CD4 T cell help[94, 95]. In αβTCR-deletion mice infected with *M. avium,* adoptive transfer of functional antigen-specific T cells into mice with high pulmonary bacterial burden, leads to massive exacerbation of the innate cellular response resulting in injurious tissue pathology[96]. These data indicate that although T cells are generally thought to be protective, there is a balance between anti- and pro-inflammatory functions of T cells.

Although the majority of research on the adaptive response to *M. tuberculosis* heavily emphasizes the protective role of the CD4 T cell, CD8 T cells also play a critical, but independent role. Evidence suggests that CD1-mediated antigen presentation of lipids are

involved in CD8 T cell immunity against *M. tuberculosis* as well as CD1-restricted T cells[97]. To date, there is limited evidence of the importance of CD8 T cells in human TB, but multiple animal models demonstrate their importance[46]. Mice lacking CD8 T cells have reduced survival that is intermediate compared to a lack of CD4 T cells[98]. Non-human primates with antibody-mediated depletion of CD8 T cells develop more severe disease and higher bacterial burdens earlier in infection[99]. Depletion of these cells in NHPs exposed and cleared with antimicrobials resulted in a complete loss of protective immunity upon reinfection[99], results which provide rationale for targeting CD8 T cell memory during vaccination against *M. tuberculosis*. Recently, the priming of CD8 T cell immunity during infection and with BCG vaccination has demonstrated a critical role for dendritic cell cross-presentation, a process significantly enhanced by apoptosis, as indicated by pro-apoptotic mutants of both *M. tuberculosis* and *M. bovis* BCG[100, 101], data which suggests that a CD8 T cell response may be enhanced by improving the rate of apoptosis compared to necrosis of infected cells[102].

The generation of antigen-specific regulatory T cells (T_{reg}) occurs simultaneously with the onset of CD4 and CD8 effector T cell responses and subsequently, their accumulation in the lung parallels that of the Th1 effector response[103]. Regulatory T cells are expected to delay priming of newly activated T cells through several potential mechanisms including downregulation of co-stimulatory molecules on dendritic cells and macrophages, or impairment of the effector response through IL-10 or TGF-β[40]. It has been demonstrated that regulatory T cells in TB develop to specific *M. tuberculosis* antigens and that their development delays the onset of protective pulmonary immunity[104]. Elimination of this cell population can improve the effector T cell response and result in a modest reduction in bacterial burden[103, 105]. Thus, while it appears that expansion of regulatory T cells is an unfavorable natural response, the true role of this population in regulating the response to chronic infection requires further investigation. An additional regulatory cytokine prominently expressed during tuberculosis is IL-10. The propensity for production of IL-10 in the innate response in humans is associated with a

tendency toward development of active TB disease, but the strength of this association has been challenged[106, 107]. This negative impact of IL-10 is also supported by the observation that certain strains of *M. tuberculosis* susceptible mice produce higher levels of IL-10[108]. Production is not limited to regulatory T cells but the impairment of CD4 T cell response is supported by improved migration of IFNγ-producing cells to the lung in IL-10 deficient mice and an improved response to BCG vaccination[109, 110].

The production of the cytokine IL-17 represents an additional critical mediator of the response to *M. tuberculosis* but may also have a detrimental impact on disease progression when present in persistently high concentrations. IL-17 is produced very early in infection prior to the onset of the adaptive response and comes predominately from the residing γδ T cell population in the lung[111]. Neutrophils are among the first cells to arrive to the site of infection, as with any other bacterial infection, but this response is hindered in the mouse in the absence of IL-17[112]. Depleting neutrophils early in infection is known to reduce IFNγ production in organs later in disease, indicating an important role for neutrophils early in infection[113]. IL-17 production from antigen specific Th17 cells does occur later in the course of infection. It has been demonstrated that this population is important for a memory T cell response in experimental vaccine studies, which is dependent on production of IL-23 and IL-17, and that an accelerated recall response is hindered in the absence of these cells[114].

The Influence of TB Risk Factors on the Immune Response to *M. tuberculosis.*

The response to infection with *M. tuberculosis* is infrequently modeled in the context of known acquired risk factors, although these are well recognized to promote active or reactivation TB disease[115]. HIV is the leading risk factor for TB and the immune defect of low CD4 T cell counts is well-established[67]. Beyond HIV, malnutrition is among the most significant risk factors for TB and has been previously modeled in guinea pigs fed a protein-poor diet[116].

Overall, protein malnourished guinea pigs demonstrated a compromised immune response with inability to control bacterial growth in infected macrophages, impaired lymphoproliferation in response to PPD, and a reduced production of TNFα. Smoking, also a TB risk factor, has recently been evaluated in a mouse model of cigarette smoke exposure and *in vitro* in human cells. Data suggested impaired killing of bacteria in human primary macrophages and reduced tissue influx of TNFα and IFNγ producing cells. Additionally, IL-12 and TNFα production by dendritic cells was reduced in mice, which may be related to the lower IFNγ production in tissue[117]. These data indicate that cigarette smoke suppresses the protective immune response to *M. tuberculosis* infection. Diabetes, described in greater detail in the following sections, is a known risk factor for developing active TB with growing importance. Similar to other risk factors, the mechanisms that lead to increased susceptibility to TB in diabetic humans remain poorly defined and the validation of animal models for this comorbidity has been limited[14].

Diabetes Mellitus - Diagnosis and Pathogenesis

General Classification and Diagnosis of Diabetes.

Diabetes mellitus, in general, is a disease caused by a lack or dysfunction of the hormone insulin, whose most important physiologic function is to maintain glucose homeostasis. Elevated glucose, or hyperglycemia, is the hallmark feature of diabetes mellitus. As recognized by the American Diabetes Association, diabetes mellitus can generally be divided into two forms based on etiopathogenesis, either type 1 or type 2[118]. Type 1 diabetes is an autoimmune disease associated with specific destruction of β-cells, that ultimately, leads to absolute insulin deficiency, thereby requiring insulin therapy for treatment. Thus, this form has been previously referred to as insulin-dependent diabetes; however, the convention is to no longer use this terminology since it refers simply to treatment requirements rather than disease pathogenesis.

Approximately, 5-10% of the diabetic population has type 1 diabetes and in approximately 90%, one or more autoantibodies to islet cell antigens, insulin, GAD, and tyrosine phosphatases IA-2 and IA-2β can be detected concurrently with fasting hyperglycemia[118, 119].

Type 2 diabetes accounts for 90-95% of the diabetic population and has previously been referred to as non-insulin dependent diabetes, since most type 2 diabetes patients do not progress to a stage of absolute insulin deficiency that would require insulin replacement therapy. This form of the disease encompasses any range of a syndrome known as insulin resistance, along with a relative, rather than absolute, insulin deficiency. Most often, patients with this form are obese, but some type 2 diabetes patients that are not obese may have a disproportionate distribution of fat within the abdomen, as opposed to a subcutaneous location. A genetic predisposition for abdominal adiposity is recognized in Indian and Asian populations, a feature that has been attributed to the rapidly rising incidence of type 2 diabetes in these global regions[120, 121]. A recent study demonstrated a much higher prevalence of diabetes in people of Asian heritage (23%) when compared to Caucasian descent (6%), which may be a reflection of generally lower β-cell function in this population[122]. The type 2 form of diabetes is insidious in onset and often remains undiagnosed for years because classical clinical signs of diabetes do not develop with the severity that is typical of type 1 diabetes[118, 123].

Additionally, two other forms of diabetes are recognized including gestational diabetes and those attributed to specific genetic defects in insulin-producing β-cells. Gestational diabetes is a temporal state of diabetes associated with approximately 7% of pregnancies and most often resolves after birth, although it can contribute to progressive loss of beta cell function and ultimately lead to overt diabetes[118, 124]. Patients with genetic defects in β-cell function are referred to as having monogenic diabetes or maturity-onset diabetes of the young (MODY) and generally develop diabetes around the age of 20 years. Six genetic defects have been identified to date that relate to glucose sensing, or insulin production/secretion defects[118, 125].

A diagnosis of diabetes is based on criteria of glucose metabolism with evidence of hyperglycemia. The American Diabetes Association has provided guidelines for the diagnosis of overt diabetes as well as those at risk for developing diabetes, based on multiple analyses of glucose metabolism including fasting plasma glucose (FPG), oral glucose tolerance test (OGTT) and glycated hemoglobin (HbA1c), with any one or more being abnormal in a diabetic individual. The 75-gram OGTT is the most common and most sensitive of routine methods for the diagnosis of diabetes[123]. This is likely reflective of the inherent strengths of this test in evaluating post-prandial glucose utilization, a time when glucose levels are at their physiologic peak and when the functional demand of β-cells is at its highest. Additionally, an oral route of administration also evaluates the axis of glucose-sensitive incretin hormones, such as GIP and GLP-1, which are produced by the GI tract postprandially and act to increase insulin secretion and insulin sensitivity[126]. In this test, a patient is fasted overnight; glucose measured prior to initiating the test, and then administered an oral bolus of glucose solution (75 g). Glucose is measured over time through 120 minutes post glucose administration. Fasting plasma glucose (FPG) at the start of the test and at the 2-hour time point is used for diabetes diagnosis. The criteria for a diagnosis of diabetes are FPG ≥ 126 mg/dl and 2-hour OGTT ≥ 200 mg/dl[118]. More recently, it has been suggested that the 1-hour OGTT time point may also be beneficial in identifying patients at risk of developing diabetes, which can be defined as having a syndrome known as prediabetes[127].

An additional criterion has recently been set that reflects the degree of hyperglycemia over time, as measured by HbA1c[118]. This test is based on the level by which the hemoglobin protein is modified by circulating glucose, which measures the level of Amadori product formation. Amadori products are the rearrangement of Schiff bases, which is the chemical modification by a sugar molecule on a free amine group, such as that contained on the N-terminus of proteins or on amino acids with free amine groups, including lysine and arginine. Glucose present in higher circulating concentrations for an extended period of time yields more

Amadori modified hemoglobin and is the basis for this test. The HbA1c test specifically targets modifications of the N-terminal valine residue on the alpha chain and is reported most commonly as percent modified hemoglobin, with the ADA cut-off value for a diagnosis of diabetes being ≥ 6.5%. However, the measurement of A1c is only around 66% sensitive when compared to the 2-hour OGTT[123]. Arguments can be made regarding the benefit of HbA1c as a diagnostic tool but the OGTT remains the primary standard for diagnosis and it is generally recommended that HbA1c be utilized as a tool to monitor the level of hyperglycemic control in diabetic patients[128].

The Pathogenesis of Insulin Resistance and Contribution to Type 2 Diabetes.

Insulin resistance is defined as the inability of insulin to exhibit biologic activity at normal physiologic concentrations. Obesity is the greatest predictor of insulin resistance and type 2 diabetes; and insulin resistance both precedes and predicts the development of type 2 diabetes. While insulin resistance contributes directly to the progression of type 2 diabetes, it is not sufficient to induce overt diabetes when β-cells of the pancreatic islets are functioning at normal capacity. The development of type 2 diabetes represents a continuum of insulin resistance and progressive β-cell loss until compensation by insulin secretion is no longer capable of restraining diabetic levels of hyperglycemia, a process that generally spans a period of years.

In the context of glucose metabolism, insulin resistance leads to both a failure to suppress endogenous glucose production by the liver in basal and post-prandial states, as well as reduced peripheral uptake of glucose in insulin-dependent target cells such as skeletal muscle and adipose tissue[129]. Alterations in lipid metabolism in both liver and adipose make the most significant contribution to the development of systemic insulin resistance, while impaired insulin signaling in skeletal muscle makes the greatest contribution to impaired peripheral glucose clearance. The storage of lipid as triglycerides in adipose tissue is highly dependent on

a functional insulin-signaling pathway. Therefore, in states of insulin resistance, a higher basal level of adipose tissue lipolysis is present, leading to increased circulating fatty acids with downstream effects on other organs and further impairment of the insulin response[130].

The development of hepatic insulin resistance is linked to accumulation of excess fat within the liver[131]. Hepatic lipid accumulation in obesity is the result of both an increased rate of lipolysis from adipose tissue and from *de novo* synthesis in the liver, which is markedly increased in type 2 diabetic patients and is linked to excess consumption of carbohydrates[132, 133]. Recent research in the pathogenesis of type 2 diabetes has revealed that lipid accumulation in the liver is a critical mediator of insulin resistance and that when extreme hypocaloric dietary interventions are employed, there is a drastic reduction in liver fat, improved insulin production in β-cells, and a return to a basal euglycemic state comparable to non-diabetic individuals[134]. This marked and rapid improvement in glucose and lipid metabolism, and restoration of normal glucose tolerance is also consistently demonstrated in patients undergoing bariatric surgery, often with improvement over what can be achieved with medical interventions[135]. These data indicate that insulin resistance and type 2 diabetes are reversible, assuming that sufficient insulin-producing capacity remains.

It is well documented now that the activation of inflammatory pathways in adipose tissue is responsible, at least in part, for the development of systemic insulin resistance[136]. Elevated serum levels of IL-1β, IL-6 and the acute phase protein, C-reactive protein, are predictive of type 2 diabetes in humans[137, 138]. As fat mass increases, a distinct polarization of pro- and anti-inflammatory mediator expression develops with a balance shifted toward local adipose inflammation, which has systemic effects[139, 140]. Evidence for an inflammation-mediated pathogenesis of insulin resistance was first documented nearly a century ago when anti-inflammatory salicylates were noted to completely reverse hyperglycemia in diabetic patients[141]. Several studies have since documented this further, and recently have been pursued in clinical

trials as a type 2 diabetes treatment option[142]. However, the concept of an inflammatory-mediated pathogenesis was initiated by experiments that first demonstrated adipose tissue-derived TNFα as an inducer of systemic insulin resistance in mouse models of obesity[143-146].

Macrophages are a normal constituent of adipose tissue but in non-obese, metabolically healthy individuals, these are present in low numbers. However, as lipid-mediated inflammatory signals are activated, there is recruitment of additional macrophages and a shift toward the pro-inflammatory M1 phenotype of adipose-associated macrophages[147, 148]. In obesity-related insulin resistance, adipose tissue macrophages can account for up to 50% of visceral adipose mass[149, 150]. This accumulation of macrophages has been linked to production of MCP-1 (CCL2) in adipose tissue[151] and accompanies the expression of other pro-inflammatory cytokines that are produced exclusively by adipose such as leptin, known as adipokines, as well as mediators that may be produced by both adipocytes and macrophages, including IL-6, TNFα, IL-1β, and resistin[152, 153]. Anti-TNFα and IL-1β targeted therapy have been shown to restore insulin sensitivity and although the role of IL-6 has been controversial, the specific deletion of IL-6 in adipose, protects against insulin resistance specifically by promoting hepatic insulin sensitivity [136, 154-156]. In contrast, anti-inflammatory mediators produced in adipose such as the adipokine, adiponectin, and IL-10 are significantly reduced in states of obesity and insulin resistance.

High fat diet and excessive accumulation of lipid in adipocytes activates inflammation through JNK and IKKβ/NFkB pathways via signaling through pattern recognition receptors, induction of ROS and ER stress, and via activation of protein kinase C isoforms. Although this may be initiated by both adipocytes and resident adipose-associated macrophages, it is the accumulating macrophages that account for the majority of the inflammatory response as disease progresses. JNK activation mediates insulin resistance through serine phosphorylation and inactivation of insulin receptor substrate 1, a major adaptor protein required in the insulin receptor signaling pathway[139]. The mechanisms of direct NFkB induction of cellular insulin

resistance are less well understood, but the inflammatory-mediated induction of lipolysis in adipose tissue leads to free fatty acid mediated insulin resistance through similar interference with insulin receptor signaling in skeletal myocytes and hepatocytes[157, 158].

The resulting increase in circulating free fatty acids, which correlate with human obesity and insulin resistance, are known mediators of insulin resistance, particularly in skeletal muscle[130, 159]. Free fatty acids are metabolized within skeletal myocytes, leading to accumulation of diacylglycerol, which activate protein kinase C isoforms capable of serine phosphorylation, which inactivates insulin receptor substrate proteins and reduces transport of GLUT4 transporters to the cell surface[160, 161]. Free fatty acids may also directly signal through pattern recognition receptors TLR2 and TLR4 on myocytes, adipocytes and macrophages to induce JNK and IKKβ signaling pathways, both of which may inactivate IRS proteins by serine phosphorylation. The manifestation of this is reduced insulin signaling and glucose transport, which translates to systemic insulin resistance[162-164]. In the liver, downstream activation of glycogen synthase is lost, leading to decreased glycogen synthesis and failure to suppress the hepatic gluconeogenesis[159].

Beta Cell Dysfunction in Type 2 Diabetes.

Insulin resistance both precedes and coincides with type 2 diabetes; however, not all people with insulin resistance will develop type 2 diabetes. The disease pathogenesis also requires a defect in β-cell function. While previously, the defect in β-cell function was attributed to persistent stress on the cells as a result of insulin resistance, more recent evidence suggests that these two critical components likely develop independently, then synergize to promote the onset of type 2 diabetes[165]. Individuals with normal beta cell function have the capacity to fully compensate for the presence of insulin resistance and it has been shown that β-cell dysfunction can be demonstrated in people even before the development of insulin resistance, suggesting

that this is a preceding and independent process[166, 167]. β-cell dysfunction is predictive of developing type 2 diabetes, and is more likely attributable to polygenetic factors, in contrast to insulin resistance, which develops as a result of environmental influences[168, 169].

There is approximately a 50% reduction in total β-cell mass in patients with type 2 diabetes, indicating that loss of cells contributes to the pathogenesis in addition to dysfunction[170, 171]. The failure of β-cells can be attributed to a number of mechanisms, which generally derive from metabolic stress of high glucose and fatty acids, known as glucotoxicity and lipotoxicity. This process dampens the normal compensatory response of β-cells and leads to cell death. The compensatory mechanisms of β-cells, beyond hypersecretion of insulin, are to; 1) proliferate and increase in number, which occurs in humans but to a lesser degree than that seen in rodent models; or 2) through derivation of new β-cells, termed neogenesis. Proliferation is thought to contribute more than neogenesis to the compensatory response[172]. However, in response to diabetic stressors including hyperglycemia, increased fatty acids, oxidative stress and induction of cytokines, particularly IL-1β[173], the cells are reduced in their ability to proliferate and convert proinsulin to active insulin, and are also prone to undergoing apoptosis[174-176]. Apoptosis is thought to be a major contributor to the overall reduction in β-cell mass that is typical of type 2 diabetes[177]. Additionally, there is evidence in rodents that dedifferentiation of β-cells may play a role in cellular loss by transformation into glucagon-producing α-cells, which has also been demonstrated in isolated human islets from diabetic patients[178, 179]. This may further worsen metabolic defects associated with diabetes and also account for the increase in circulating glucagon that is typical of overt type 2 diabetes[180].

Treatment Options for Insulin Resistance and Type 2 Diabetes.

An abundance of metabolic targets exist as therapeutic options for the treatment of type 2 diabetes; however, those most commonly utilized as frontline therapy for glucose control in people newly diagnosed with type 2 diabetes also provide the opportunity to validate new type 2 diabetes models with these compounds. The most common frontline drugs for the treatment of type 2 diabetes include the biguanide, thiazolidinedione (TZD), sulfonylurea, and GLP-1 classes of drugs. Based on recommendations from the American Diabetes Association, the level of hyperglycemic control should be monitored through HbA1c, and monotherapy initiated with the biguanide, metformin, in addition to lifestyle interventions such as dietary restriction and increased physical activity. After 3 months, if HbA1c is not improved, dual therapy with either a sulfonylurea, TZD, or GLP-1 agonist may be added to the metformin regimen[123]. Additional drugs might be further considered if hyperglycemic control is still not achieved; however, the use of insulin in type 2 diabetes is generally reserved as a final approach to glucose control.

Metformin is the frontline drug of choice for initial therapy of type 2 diabetes and belongs to the biguanide class of compounds. The primary mechanism of action and glucose-lowering effect of metformin is through activation of AMPK in hepatocytes, leading to reduced gluconeogenesis in a manner independent of insulin[181]. The TZD class of drugs includes the most commonly used member, rosiglitazone, as well as troglitazone. These compounds are agonists for the PPARγ nuclear receptor expressed in adipocytes, in which activation leads to enhanced uptake and storage of lipids in adipose tissue[182]. The sulfonylureas, such as glipizide and glipuride, bind to the sulfonylurea receptor on β-cells and promote insulin release via potassium channel inhibition and membrane depolarization[183].

Risk for Infection as a Complication of Diabetes.

Diabetes, in general, is frequently linked to a higher risk for infection; however, due to a frequency of conflicting results, the mechanisms of this increased risk are not fully understood[184]. A recent epidemiological study demonstrated that people with both type 1 and type 2 diabetes are, in general, at greater risk of infections of the respiratory tract, urinary tract, and skin/mucous membranes[185]. Diabetic foot infection is the most common example of an infectious complication, and is often paired with additional diabetic complications of the peripheral nerves and microvasculature, indicating that the underlying mechanisms of diabetic infections likely extend beyond an impairment of the immune system alone[186]. Diabetic patients were demonstrated to be 2.17- and 1.92-times more likely to suffer hospitalization or death, respectively from an infectious etiology[187]. This increased risk is likely to be both a manifestation of host and organism specific factors that occur under diabetic conditions. The fact that hyperglycemic control greatly improves the response to treatment and outcome is evidence that elevated glucose may be among the more important diabetic factors contributing to higher risk of infection[185].

Cellular defects have been described in neutrophils, macrophages and T cells in association with diabetes. The defects in innate cell function are more consistently reported, including impaired chemotaxis, adhesion, phagocytosis, and bactericidal activity against intracellular pathogens. Neutrophils from diabetic patients are reported to display enhanced adhesion to vascular endothelium, which correlates with increased leukocyte integrin expression along with higher adhesion molecule expression on the endothelial surface[188, 189]. Despite higher adhesion, several reports of impaired neutrophil chemotaxis and transmigration *in vitro* are reported, both with and without correlation to hyperglycemia and glycation[190-192]. Similar impaired chemotaxis has also been reported in peripheral blood monocytes from patients with diabetes[193]. In contrast, in hyperinsulinemic patients, an improvement in neutrophil chemotaxis

has been demonstrated, which suggests that the glucose lowering effects of insulin therapy may not be the only benefit in improving the course of infection[194].

Contrasting results also exist among studies evaluating neutrophil phagocytic capacity. Studies have shown both defective and normal phagocytosis of opsonized bacteria in diabetes and no difference in the ability of diabetic neutrophils to phagocytize opsonized latex beads[192]. Additionally, impaired phagocytic activity has been demonstrated in monocytes and alveolar macrophages from diabetic patients as well as peritoneal macrophages in mice[195-197]. More consistently, a reduced ability to generate a respiratory burst in response to pathogens has been described, which is attributed to hyperglycemic induction of the polyol pathway[198-200]. This leads to consumption of NADPH, the necessary substrate for oxidative burst, by the enzyme aldose reductase. This defect has been confirmed by improved neutrophil superoxide generation from diabetic cells in the presence of aldose reductase inhibitors[201, 202]. However, an increase in respiratory burst in neutrophils from type 2 diabetic patients has also been observed[203].

Altered T cell function is the most commonly reported defect in the adaptive response of individuals with diabetes. In diabetic patients with relatively controlled hyperglycemia, studies have shown both impaired proliferation in response to specific antigens, as well as a robust antigen-specific T cell responses equivalent to non-diabetic patients[204, 205]. Impaired proliferation has also been demonstrated in mice with STZ-induced diabetes and in mouse lymphocytes cultured in the presence of high glucose[206]. In a separate study investigating T cell response to M. tuberculosis antigens, it was found that the antigen-specific response was unaltered but that diabetic T cells had an impaired ability to proliferate in the presence of non-specific stimulation with phytohemagglutinin[207]. Although antibody production in response to vaccination does not appear to be impaired in diabetic people, antibodies produced may be modified by non-enzymatic glycation, which parallels the level of HbA1c[208-211]. However, since

the humoral response appears to be equivalent regardless of the diabetic state, the clinical relevance of this is uncertain.

From these studies investigating mechanistic defects in immune function, it is apparent that variable and often contrasting results exist. This may be a reflection of differences in experimental conditions or the level of hyperglycemic control in the sampled populations. Additionally, there may be differences in critical pathways needed for protection against specific agents, and thus, the deviations in host response must be taken into context of what is protective for a given pathogen. It is known that virulence is enhanced in the presence of high glucose for pathogens such as *Candida* and *Klebsiella*; and *Klebsiella* and *Burkholderia pseudomallei* both have a disproportionately high prevalence in people with diabetes. This suggests that specific pathogens may take advantage of "excess nutrients" in the host and indicates that both host and pathogen response may need consideration for each disease studied. Whether this is also the case for *M. tuberculosis* under diabetic conditions is unknown.

Mechanisms of Diabetic Complications and Potential Contributions to TB Susceptibility.

Hyperglycemia is a major contributor to the onset and progression of diabetic complications, which account for the majority of diabetes-related morbidity and mortality. The pathogenesis of diabetic complications involves a combination of oxidative stress and inflammatory mediators, which promote microvascular dysfunction, the major underlying factor of these complications. The DCCT/EDIC (type 1) and UKPDS (type 2) represent large prospective studies that have followed diabetic patients in their control of hyperglycemia, split into intensively managed *versus* those conventionally managed, and the development of complications over multiple years. These studies have demonstrated that prolonged exposure to high glucose is a predictor of diabetic complications and that glucose control can significantly delay their onset[212, 213]. However, follow up in these studies indicated that despite convergence of HbA1c between previously controlled treatment groups, the emergence of complications

continued on a similar path. This indicates that there are lasting effects from previous chronic elevations in glucose, which has been termed "glycemic memory". This has important implications in the early diagnosis and management of hyperglycemia, the continued monitoring for complications even if hyperglycemia becomes well controlled, and the need for a better understanding of the underlying mechanisms of glycemic memory.

The pathobiology of diabetic complications is linked to biochemical abnormalities in the glycolysis pathway that result from elevated glucose concentrations. As described by Brownlee, five major mechanisms by which hyperglycemia causes diabetic complications are supported by an abundance of literature[214]. These five pathways include; 1) increased glucose flux in the polyol pathway; 2) Increased formation of intracellular dicarbonyl-derived advanced glycation end-products (AGEs); 3) Increased signaling through the receptor for advanced glycation end products (RAGE) and increased expression of its ligands; 4) Activation of isoforms of protein kinase C (PKC); and 5) A hyperactive hexosamine pathway. Evidence from Brownlee *et al.* supports an oxidative stress origin of all five mechanisms around the overproduction of mitochondrial superoxide due to intracellular hyperglycemia, which inhibits the glycolytic enzyme, GAPDH. As a result, metabolic by-products of glucose are shunted into alternative and detrimental pathways[215, 216].

Evidence from TB patients with diabetes suggests that, similar to the more typical diabetes complications, TB may also represent a "complication" of chronic hyperglycemia. Both human and animal studies have shown that poorly controlled and chronic hyperglycemia is an important factor in the increased risk of TB in diabetic individuals[217-219]. The unifying mechanism of oxidative stress and superoxide production in diabetic complications may also have a significant impact on immune function. Activation of protein kinase C isoforms through diacylglycerol accumulation not only impacts liver and skeletal muscle in type 2 diabetes, but also may have significant effects on T cell function[220]. Hyperglycemia-mediated oxidative stress activates the redox sensitive, pro-inflammatory transcription factor, NFkB, leading to sustained

inflammatory effects, as well as the depletion of the major intracellular antioxidant, reduced glutathione (GSH)[221]. Additionally, the consumption of NADPH in the polyol pathway due to diabetes reduces the availability of this necessary substrate for regeneration of GSH, leading to an overall intracellular depletion of GSH. This depletion has been confirmed in PBMCs of type 2 diabetic patients and is linked to reduced synthesis of the IL-12 cytokine, which is regulated by GSH[222]. Restoration of intracellular GSH in diabetic PBMCs has been shown to improve IL-12 production and improve bactericidal activity against intracellular bacteria, including *M. tuberculosis*[223].

Of the defined pathways involved in the pathogenesis of diabetic complications, RAGE receptor signaling has been the most extensively studied in leukocytes, immune function, and response to infection. The RAGE receptor is a multiligand pattern recognition receptor of the immunoglobulin superfamily and signals through binding of not only AGEs, as the name implies, but also the A8 and A9 members of S100 proteins, the cellular stress signaling protein, high mobility group box-1 (HMGB-1), and β-amyloid sheets. All of these ligands are either direct products of high glucose, or are induced by the cellular stress of hyperglycemia[216]. The formation of AGEs is of particular interest in the comorbidity of diabetes and TB because AGEs are known to accumulate to a higher level with diabetes and also in the presence of chronic inflammation, such as that which occurs in TB[224]. A recent study has demonstrated that methylglyoxal, an AGE precursor, accumulates during *M. tuberculosis* infection and contributes to macrophages apoptosis[225]. It is possible that AGEs may accumulate at a faster rate in people with *M. tuberculosis* infection and diabetes, given the convergence of hyperglycemia and chronic inflammation. AGE accumulation may alter immune cell function and lead to a sustained proinflammatory response through their activation of NFkB via RAGE receptor signaling[226, 227].

The RAGE receptor is expressed on many different immune cell types including macrophages, neutrophils and lymphocytes, but the site of greatest expression is on the

pulmonary epithelium[228]. Accordingly, multiple investigations into the role of RAGE in pulmonary infections have been reported, which may correlate with TB since it is predominately a pulmonary disease. However, it should be noted that the role of RAGE or accumulation of AGE ligands specifically in the context of diabetes-related pulmonary infection has not been previously studied. Since diabetes has a significant influence on RAGE ligand expression as well as expression of the receptor itself, the role of RAGE signaling in infection may differ under diabetic conditions.

The impact of RAGE signaling on acute bacterial infections in a non-diabetic state has been previously investigated. A lack of RAGE has been shown to attenuate the inflammatory response to both LPS stimulation and E. coli pneumonia in mice and also significantly improves survival during bacterial sepsis[229, 230]. Additionally, the infusion of AGE-modified proteins during sepsis worsens the disease course in a manner dependent on RAGE[231]. However, the evidence for RAGE involvement in *M. tuberculosis* infection is conflicting since one study has demonstrated more severe TB disease in RAGE-null mice infected with a high intranasal dose, whereas another study noted similar TB disease in RAGE-null and wild type mice infected via a more physiologically relevant low-dose aerosol route[232, 233]. In general, previous data implicate RAGE in the promotion of damaging inflammation, a process that is well recognized in diabetes, and therefore can also be rationally implicated in the comorbidity of diabetes and infectious disease.

Animal Models of Diabetes.

The mouse, rat, and non-human primate are the most commonly used models in experimental diabetes studies and generally manifest features of diabetes either through the induction of obesity or loss of β-cells. These models can be separated into those with pure hyperglycemia and type 1 diabetes, or those of diet- and obesity-induced insulin resistance and type 2 diabetes. Streptozotocin (STZ) and alloxan are glucose analog compounds with a

cytotoxic nitrosurea group that are utilized to induce acute beta cell death. Streptozotocin is used more commonly for this purpose and with a full, multiday dosing regimen, leads to complete β-cell ablation and insulin-dependent diabetes in mice. Due to absolute-insulin deficiency, this is often designated as a model of type 1 diabetes; however, these models lack the autoimmune inflammatory pathogenesis that leads to β-cell loss in type 1 diabetes and also tend to develop severe and persistent hyperglycemia requiring insulin therapy for extended survival[234]. The non-obese diabetic (NOD) mouse is the most common model of type 1 diabetes and manifests spontaneous autoimmune insulitis, however, certain strains of the rat, such as the BBdp (diabetes prone), also develop spontaneous insulitis[235].

Diet-induced obesity in mice (DIO), particularly C57/BL6, and less commonly in rats, is the most frequent method for investigating insulin resistance and β-cell responses[234, 236]. However, this model fails to progress to overt diabetes within a reasonable experimental timeframe and does not manifest the beta cell loss that is characteristic of human type 2 diabetes patients[237]. Alternative models with more severe manifestations of insulin resistance are often therefore utilized with or without a high-fat diet. The ob/ob mouse, which is obesity prone due to an inactive leptin molecule, suffers from extreme overeating. Disruption of the leptin receptor in db/db mice also targets this same obesity pathway. However, alternative polygenic models have been developed in the mice that manifest diabetic phenotypes more rapidly and are also more consistent with the typical obesity-associated form of type 2 diabetes in humans, including the NON/ShiLtJ and NONcNZO10 mice[238]. Analagous to the db/db mice, the Zucker diabetic fatty rat is a model of obesity due to a spontaneous genetic defect in the leptin receptor. A hybrid model of diet-induced insulin resistance and STZ-induced β-cell cytotoxicity has been described in the rat, which manifests both the features of impaired glucose tolerance and response to insulin, along with reduced insulin-producing capacity due to β-cell loss[239].

The guinea pig has been previously utilized as a model of diabetes, although with far less frequency, never as a diet-induced or obesity-based model of insulin resistance, and with significant variations in the utilization, route and dose of either streptozotocin or alloxan. Pure streptozotocin-induced hyperglycemia in the guinea pig is essentially the only model described in the guinea pig with any frequency. In these studies, methods describe various routes of administration including intraperitoneal, intravenous and intracardiac administration at doses ranging from 180 to 350 mg/kg. The level of mortality reported from acute toxicity is high, ranging from 50-90%[234]. Optimized methods for route of administration and effective diabetes induction while minimizing the level of toxicity are not reported in the guinea pig, as they are in the mouse and rat.

The Comorbidity of Tuberculosis and Diabetes - Current Knowledge

Risk Factors for Tuberculosis.

Many recognized risk factors exist for tuberculosis, which may be attributed to increased risk of developing active TB disease either upon exposure or as reactivation TB. Beyond host genetic factors, the majority of TB risk factors are non-communicable diseases often linked to socioeconomic factors and environmental influences. Among the greatest recognized risk factors for TB include HIV infection, immunosuppressive therapies, smoking, air pollution, alcoholism, malnutrition, chronic kidney disease, and diabetes[115]. HIV is, by far, the greatest recognized risk factor for TB, with a relative risk of nearly 30-fold over uninfected individuals[240-242]. The contribution of malnutrition as a risk factor is the result of the high global prevalence of this problem in underdeveloped countries where TB is also prevalent, leading to a high population attributable risk[240-242]. While these risk factors are well recognized, the mechanisms underlying predisposition for active TB disease are poorly understood, with the exception of HIV

infection, which may be, in part, due to the infrequent modeling of such comorbidities in animal species[116].

The Driving Epidemiology of Diabetes-Tuberculosis Comorbidity.

An association between TB and diabetes has been recognized for multiple centuries[243, 244]. However, the importance has been previously underemphasized because a convergence of diabetes on regions of high tuberculosis prevalence was not a growing concern[245]. It was not until recently that diabetes was confirmed as a significant risk factor for TB and a potential threat to public health. The emerging emphasis on this comorbidity stems from the rapidly growing diabetic population worldwide. Driven by urbanization, Western diet, and lack of physical exercise, as well as phenotypic predispositions in certain populations[246], an epidemic of diabetes is affecting predominately countries that also have endemic tuberculosis[247]. For example, India, a country with among the highest tuberculosis burden worldwide, also contains the highest prevalence of type 2 diabetes worldwide. Up to 95% of the global diabetic population is affected by type 2 diabetes, a form of the disease with a rapidly growing incidence[247]. Less than 10% of the diabetic population is affected by autoimmune, type 1 diabetes, a population that has increased but not nearly at the rate recently seen with type 2 diabetes. However, both types 1 and 2 diabetes are associated with increased risk for TB[248].

In 2012, the number of people with type 2 diabetes worldwide was estimated at 371 million individuals[249]. Approximately 80% of this population resides in developing countries where risk for exposure to *M. tuberculosis* is significantly higher. It is estimated that this population will continue to grow rapidly and will exceed 550 million affected by the year 2030, with the majority of growth occurring in densely populated developing countries. Of this population, up to 50% of diabetic patients remain undiagnosed in the most remote areas, where access to the necessary health care and diagnostic capabilities is lacking[249]. Therefore, a large proportion of the diabetic population is unknowingly affected by uncontrolled hyperglycemia and

may be at even higher risk of developing TB. In addition to patients with diagnostic criteria consistent with diabetes, it is estimated that 70% of people affected by prediabetes also reside in countries with the highest TB burdens[249].

In considering the impact that the emergence of HIV had on the incidence of tuberculosis, global TB control, and emergence of drug resistance, correlations with diabetes as a risk factor for tuberculosis highlights the urgency and importance of controlling the growing comorbidity in order to maintain a slow but steady decline in global TB incidence[5]. Although the relative risk for TB in HIV patients far surpasses that of diabetes at 30-fold compared to approximately 3-fold, respectively, the prevalence of diabetes far surpasses that of HIV, making the attributable risk for TB potentially greater than that of HIV[14]. This is reflected in the 8% of all new TB cases attributed to diabetes, which amounts to >700,000 cases[14, 250, 251]. Moreover, in 8 of the 22 countries with the highest TB prevalence, diabetes accounts for more TB cases than HIV. India is a dramatic example where between 20 and 40% of TB can be attributed to diabetes compared to 5% attributed to HIV[14, 252]. This high prevalence of comorbidity justifies the need for bidirectional screening, which would increase the rate at which new cases of either disease may be identified.

The Human Comorbidity of Diabetes and Tuberculosis.

A review of 13 appropriately conducted observational studies by Jeon and Murray has definitively linked diabetes with increased risk of TB and specifically the active form of disease, with a relative risk of 3.1[253]. The differentiation between risk in type 1 compared to type 2 diabetes has not been formally addressed, but two studies have demonstrated that insulin-dependent diabetes yields an even greater risk than non-insulin dependent diabetes, which suggests that severity of diabetes, in the form of residual β-cell function, may be a predictor of higher tuberculosis risk[254, 255]. Beyond this, it has been demonstrated that poorly controlled

diabetic hyperglycemia (HbA1c >7%) additionally increases TB risk and raises the question of the importance of chronic hyperglycemia in susceptibility to develop active TB disease[217].

However, epidemiological data suggests an increased risk from prediabetes as well based on bidirectional screening. A recent study in India by Vijay *et al.* has demonstrated a prediabetes prevalence of 24.5% in patients diagnosed with TB[256]. Because an association with TB and the presence of uncontrolled hyperglycemia has not been identified, the association of prediabetes with TB is unexpected. This may be due to altered lipid metabolism and generalized pro-inflammatory conditions that are strongly linked with the development of insulin resistance, but currently no animal models representative of human TB pathogenesis or development of insulin resistance have been pursued to investigate this susceptibility.

However, an additional confounding factor that should be considered is the impact that active TB disease has on glucose metabolism[257, 258]. As previously demonstrated, a high proportion of new diabetes diagnoses can be identified through screening for TB, but the contribution that TB might be making to impaired glucose tolerance is not yet known. Since treatment with antimicrobial therapy in diabetic patients with active TB improves glucose tolerance, a contribution from TB associated disease is implicated[262, 264]. This may be significant in the diagnosis of TB-diabetes comorbidity for adjustment of diabetes diagnostic criteria in comorbid patients. Perhaps higher thresholds on the oral glucose tolerance test or combined use with glycated hemoglobin (HbA1c) is necessary to differentiate these two mechanisms of impaired glucose tolerance but this remains to be investigated in human patients.

The importance of chronic hyperglycemia has been demonstrated based on indices of glycation as measured by HbA1c. In patients with HbA1c greater than 7%, a higher risk of active disease is recognized[217, 219]. An additional factor that remains unknown but is critical to the implementation of both diagnostic and therapeutic strategies is whether diabetes increases initial susceptibility to *M. tuberculosis* infection or whether it is more likely to develop reactivation

TB in latently infected individuals that become infected. A general lack of representative animal models of this comorbidity makes this a difficult question to investigate, but may be overcome with a model that is representative of the human diabetes-TB comorbidity.

Whether the severity of active TB is increased or the pathological manifestations of TB are altered in comorbid individuals remains to be fully elucidated. While there is evidence that radiographic features of TB disease are altered in patients with diabetes, including more frequent lower lung or multiple lobe involvement and higher frequency of cavitary disease[259-261], these are not consistent findings[262]. Additionally, pathological investigations in cases of diabetes-TB mortality are limited to a single series performed in 1934 by Root, where a manifestation of TB unique to the diabetic individuals was not appreciated[263]. However, studies suggest that death attributable to TB is more likely in comorbid individuals after initiating antimycobacterial therapy, with a reported risk 5 - 7 fold increased risk overall and 17% mortality after 1 year compared to 7% in patients with TB alone[264-266]. Additionally, failure of antimicrobial treatment in diabetic patients has been reported with frequencies of up to 40%, which are not attributable to resistance or poor adherence[267, 268], along with delayed sputum culture conversion rates[265, 269].

Mounting evidence is leaning towards a hyperactive and proinflammatory immune response profile in diabetic patients with active TB, which has been performed in antigen-stimulated and unstimulated peripheral blood mononuclear cells. Two studies in particular have demonstrated this concept in populations in Mexico and India[270, 271]. Restrepo *et al.* demonstrated that stimulated PBMCs from diabetic patients consistently produced higher levels of IFNγ, IL-1β and TNFα compared to those without diabetes, and that this change was proportionately higher in diabetic individuals with high HbA1c[270]. This study implicates, again, the potential role for uncontrolled hyperglycemia in altered response to *M. tuberculosis* infection. Similarly, Kumar *et al.* demonstrated higher frequencies of antigen responsive Th1 and Th17 CD4 T cells in comorbid patients with poorly controlled diabetes, which correlated with higher

levels of IFNγ, and IL-17 in plasma, but a reduction in the regulatory T cell population[271]. In one additional study, although no difference was noted in stimulated IFNγ production or other innate pro-inflammatory cytokines from whole blood samples of diabetic TB patients compared to those without diabetes, a shift toward a pro-inflammatory response in diabetic TB patients was suggested by reduced IL-10 production[207].

Despite evidence of unfavorable outcomes in diabetic patients, the basis for this is not yet understood. Whether higher mortality and treatment failure result from complications with TB and bacterial load, from complications with diabetes, or from a combination of both, remains to be determined. Currently, there is little information regarding the impact of glucose control on TB treatment and whether intensive diabetes management prior to initiation of combination antimicrobial therapy would improve outcome[272, 273]. Additionally, evidence suggests that pharmacokinetics of type 2 diabetes drugs would be altered and efficacy reduced particularly in the presence of rifampicin, which induces p450 enzyme activation and metabolism of T2DM hypoglycemic agents such as sulfonylureas and TZDs[274, 275]. Alternatively, it is possible that in the presence of pre-existing hepatic alterations typical of type 2 diabetes, the hepatotoxicity that is characteristic of anti-TB drugs may be worsened. Similarly, the efficacy of current preventive strategies in the face of diabetes, including BCG vaccination or isoniazid preventive therapy, is uncertain. Many questions exist regarding BCG efficacy in the non-diabetic population, and whether a protective response can be established or maintained in BCG vaccinated individuals. The influence that diabetes has on BCG efficacy will be important to address since diabetes is most prevalent in developing countries where BCG vaccination during infancy is a standard of care. Additionally, considering the higher risk of active disease in diabetic patients, IPT has been recommended for those meeting the classification of latent TB; however, the efficacy of currently recommended IPT regimens in diabetic patients has not been established. However, in order to determine IPT efficacy, it must first be known if diabetes truly leads to increased

progression from latent to active TB disease or if it leads to increased susceptibility to initial infection[250, 262, 273]. Many of these treatment questions could be effectively addressed experimentally in preclinical models that closely mimic the manifestations of type 2 diabetes and tuberculosis, particularly in models with demonstrable response to the hypoglycemic drugs used to treat type 2 diabetes, as opposed to insulin dependent models of diabetes that lack features of insulin resistance.

Animal Models of Diabetes-Tuberculosis Comorbidity.

Experimental investigations into diabetes-TB comorbidity, to this point, have been limited to studies in the rat, a species highly resistant to TB, and in mice with hyperglycemia due to absolute insulin deficiency. While this mouse model is often cited as a model of type 1 diabetes, it is more appropriate to describe this as a model of insulin-dependent diabetes, since the hyperglycemia is a reflection of total insulin deficiency and lacks the autoimmune component that is the pathognomonic feature of type 1 diabetes[234]. However, an animal model that not only more closely reflects the pathogenesis and pathology of TB in humans, but also develops the inflammatory, metabolic, and endocrine alterations typical of insulin resistance and type 2 diabetes has not been previously developed. Additionally, there is a need for a model that more closely mimics the physiologically relevant fasting and post-prandial hyperglycemia characteristic of a type 2 diabetic patient, rather than extreme and persistent hyperglycemia typical of STZ-induced insulin-dependent diabetes in mice.

The C57BL/6 mouse model of STZ-induced diabetes demonstrates more severe TB disease, which occurs in the presence of 1.5 log higher pulmonary bacterial burdens, higher production of IFNγ, IL-1β and TNFα, and increased numbers of neutrophils. In this model, the heightened susceptibility was noted only in mice infected 16 weeks after STZ treatment, with no difference in mice infected 4 weeks after induction of diabetes[218]. This requirement for persistent and chronic hyperglycemia in the susceptibility of this mouse model highlights the

importance of hyperglycemia in the pathogenesis of this comorbidity, similar to what has been demonstrated in humans with poorly controlled diabetes. Additionally, earlier in the course of infection in this mouse model, antigen specific IFNγ production is reduced during the phase of logarithmic bacterial growth[276]. Rat models of diabetes previously used to carry out diabetes-TB comorbidity studies, including STZ-treated (similar to the mouse model) and goto-kakizaki spontaneously diabetic rats, have also demonstrated a similar lower expression of IFNγ and higher bacterial burdens[277, 278]. These models demonstrate an apparent difference in Th1-related cytokine expression when compared to the previously described human studies, where human samples have demonstrated increased IFNγ production. This may be a reflection of the controlled time point of disease when samples are assayed, or a difference in the organs of primary infection sampled for animal models versus reliance on peripheral blood samples in human patients.

Alternative mouse models could be considered that are frequently used in type 2 diabetes research, however, there are some unavoidable pitfalls. The most established model of diet-induced obesity is in C57Bl6 mice, which as described above are inherently TB resistant among the characterized strains of mice. Additionally, these mice fail to develop overt type 2 diabetes within a reasonable experimental time frame. The use of mice with leptin pathway defects harbors important considerations as well since this deficient leptin signaling pathway may affect the response to *M. tuberculosis* infection even in the absence of obesity and insulin resistance. Leptin has been demonstrated to have immunomodulatory effects on response to infection and overall susceptibility to *M. tuberculosis* infection, with leptin deficiency manifesting in chronic stages of human TB[279]. The non-obese diabetic (NOD) mouse spontaneously develops autoimmune insulitis and therefore serves as a representative model of type 1 diabetes, however, it remains unknown how type 1 diabetes in the context of autoimmune inflammation impacts the response to infection with *M. tuberculosis*.

The Guinea Pig Model of Tuberculosis

Historical Significance.

The guinea pig is the oldest animal model used to study tuberculosis. If not for the guinea pig, Robert Koch would not have been able to demonstrate that tuberculosis results from an infectious etiology, fulfilling in 1882 what is now known as the Koch-Henle Postulates[280]. After this time, it was observed that growth of the infectious agent could be inhibited chemically with streptomycin and again to demonstrate the efficacy of isoniazid and ethambutol still used to this day as front line anti-tuberculosis drugs[281, 282]. Since, the guinea pig has been regarded largely as the most optimal animal species for studying the pathogenesis of tuberculosis, response to treatment, and strategies for immunization[283, 284].

General Strengths of the Guinea Pig Model of Tuberculosis.

Among the largest contributions to tuberculosis research provided by the guinea pig have come from evaluation of preventive and therapeutic strategies. The guinea pig is often regarded as the most appropriate species for vaccine investigation and validation of novel preventive measures against TB[7]. As indicated below, the guinea pig responds similar to people with *M. bovis* BCG immunization, the only currently available vaccine for TB, which fails to prevent infection and disease progression in adults but limits bacterial dissemination in young children[13]. Similar to humans, the guinea pig develops a delayed-type hypersensitivity reaction to purified protein derivative, a multi-antigen preparation derived from mycobacterial culture supernatant. Upon intradermal injection, skin induration develops at the injection site in exposed or vaccinated animals, which is also the basis of a PPD skin test in people[285].

Recently, a detailed evaluation of the guinea pig response to the frontline chemotherapy regimen of isoniazid, rifampicin, and pyrazinamide has been demonstrated in response to infection with the clinical isolate Erdman K01[286]. This prototypical response may serve as a

baseline for comparison of newly developed chemotherapeutics as well as evaluation of optimal regimens since it is likely that multiple drugs rather than a single compound are necessary for sterilizing activity to eliminate persistent bacilli and prevent relapse of infection. Also, given the exceedingly long duration of treatment required with frontline antimicrobials, there is a growing need for optimization of drug regimens that reduce the time course required for effective treatment[287, 288].

The benefits of utilizing the guinea pig for treatment and prevention strategies may be a reflection of the similarities of guinea pig and human TB disease. Utilizing the guinea pig allows for evaluation in a species that develops granulomata with morphology remarkably similar to humans and when paired with the high susceptibility of this species to biologically relevant infectious doses and route, creates an optimal model for assessing treatment interventions[289]. Additionally, the guinea pig displays disease progression similar to that of humans. In particular, the development of lymphangitis in draining lymphatic vessels in the lung leads ultimately to granulomatous lymphadenitis, a common feature of human disease particularly in infants and younger children[290]. The guinea pig is highly susceptible to M. tuberculosis infection and therefore, serves as an optimal model for experimental infection utilizing a biologically relevant experimental dose and route. The guinea pig develops uniform pulmonary infection at low dose aerosol exposure of less than 50 CFUs per animal[284]. However, the high susceptibility of this species also lends to its weaknesses as a TB model, since all experimentally-infected animals develop progressive TB disease and a true latent infection is not established in this species[291]. Additionally, the guinea pig rarely develops cavitary TB disease, the end-stage progression of human disease, which is a feature of the rabbit and non-human primate models[292].

The guinea pig also mimics some important physiological similarities to humans that are absent in other rodent species. The guinea pig shares similar lipid and cholesterol metabolism to humans with a higher ratio of the pro-atherogenic, low-density lipoproteins to high-density

lipoproteins in blood[293, 294]. The guinea pig displays high lipoprotein lipase and cholesterol transport activity similar to humans, allowing for transfer of cholesterol rich lipid particles between blood and tissue, and has been shown to respond similar to humans during experimental manipulation of dietary fat intake[294, 295]. Additionally, guinea pigs manifest metabolic disturbances including insulin resistance and hyperglycemia as a result of active TB infection[296], which have been previously described in humans[257, 258], and also develop dyslipidemia during active infection with evidence of severe lipolysis and an increase in circulating free fatty acids[296]. These metabolic features in this species may have important relevance to TB pathogenesis given the recent evidence of bacilli acquisition and utilization of host-derived fatty acids, the ability of *M. tuberculosis* bacilli to utilize host cholesterol as a source for metabolism, as well as accumulation of intracellular lipid within bacilli during states of dormancy that are expected to reflect the metabolic state of the bacteria during latency[16, 297-299]. All of these factors, combined with the evidence of dyslipidemia that mimic human related manifestations resulting from *M. tuberculosis* infection in the guinea pig make this species suitable for evaluating metabolic interventions during *M. tuberculosis* infection.

The Guinea Pig Model for Preclinical Antimicrobial Drug Development.

While the mouse has largely been used in the evaluation of new chemotherapy strategies, the guinea pig offers the strength of evaluating new approaches in a model with pathology similar to human TB. The relative benefit of assessing novel chemotherapeutic strategies in the guinea pig, in contrast to mice, relates to the development of casseous necrosis in granulomas, as described in detail below. Careful assessment of drug regimens in the guinea pig and comparison with the mouse model has revealed that responses differ significantly between species.

The importance of representative pathology in the animal model used for drug studies was highlighted using experimental compound TMC207 (now FDA-approved), which was highly

effective in nearly eradicating bacilli within granulomatous lesions[300, 301]. Importantly however, this study indicated that the location of bacteria poorly responsive to treatment was extracellular and confined to a hypoxic, acellular rim within the area of central necrosis. Further studies in guinea pigs have indicated that this small subpopulation of bacilli that survive antimicrobial therapy are not the result of emergence of genotypic antimicrobial resistance, specifically using isoniazid in this study, but rather occurs with the selection of phenotypic resistance[302]. Therefore, this has been referred to as antimicrobial tolerance, rather than resistance, and the bacteria as persistent bacilli[303].

Despite these findings, there is also evidence that residual tolerant bacilli develop in mice lacking casseous necrosis[304]. Some studies indicate that the use of the standard antituberculosis regimen in guinea pigs has greater sterilizing activity than in the mouse with reduced relapse in guinea pigs as compared to mice. These findings have suggested that casseous necrosis does not impair the sterilizing activity of RHZ[304] and has important implications in the choice of animal model for testing novel drug therapies. Differences among mouse strains in response to treatment, particularly in those which develop necrotic lesions, as well as species differences provides further support for the importance of selecting models which develop appropriate pathology when assessing preclinical efficacy of new drugs and that the use of multiple species/strains may be of particular benefit[305, 306].

A particular strength of more recent antimicrobial studies in guinea pigs has been the use of pharmacokinetics to establish human equivalent doses of antituberculosis drugs in the guinea pig. As a result, appropriate guinea pig doses have been developed for isoniazid, rifampin, pyrazinamide, moxifloxacin and streptomycin, as well as other investigational compounds[303, 307-309]. The growing incidence of drug-resistant TB cases highlights the importance of developing not only new antimicrobial compounds but also for the development of new regimens with shorter periods of administration and are better tolerated than the toxicity associated with frontline combinations including rifampicin and isoniazid. This is especially

important in the case of multidrug resistant infections requiring up to 24 months of continuous treatment. The value of the guinea pig in chemotherapy studies is reflected in the recent comparisons of efficacy between rifampicin and rifapentine. It has been suggested that rifapentine, as a substitute for rifampicin, may eliminate toxicity due to reduced activation of hepatic microsomal metabolic pathways. Studies in mice indicated more potent sterilizing activity and reduced relapse rate when treated with rifapentine compared to rifampicin[309, 310]. However, a lack of therapeutic advantage demonstrated in the guinea pig model backs up a recent evaluation in human patients also demonstrating no significant advantage of this substitution[311]. These findings suggest that although the mouse often closely resembles the success in human chemotherapy trials, the guinea pig is a reasonable model to confirm promising results that derive from the more economically feasible and statistically powerful murine preclinical studies. Guinea pigs also demonstrate differences in their response to treatment with pyrazinamide. Although there is synergy with rifampin, as is also the case in mouse and human, higher PZA doses when administered alone, provide a greater bacillary reduction in guinea pigs than is seen in mice[312]. If this translates to humans, further investigation of higher PZA dosing may be warranted.

Pathology of Tuberculosis in the Guinea Pig.

A key feature of TB pathogenesis in the guinea pig is the pattern of pulmonary infection, which is followed by systemic hematogenous dissemination[76, 289]. Linking these two events is involvement of lymphatic drainage and formation of granulomatous lymphadenitis around 10 to 14 days after infection[290]. Logarithmic growth of bacteria in pulmonary lesions occurs up to 3 weeks post-infection after which, onset of adaptive immunity is associated with a stationary phase[313]. All of these processes are consistently replicated in the guinea pig, making this species an ideal model for studying kinetics of the response to *M. tuberculosis* infection. Lesion dissemination is most often demonstrated in the spleen, followed by involvement of the liver in

guinea pigs. However, more distant or less commonly involved organs, including adrenal gland, heart, pancreas and abdominal lymph nodes, may develop TB lesions during infection with highly virulent clinical strains such as HN878 or CDC1551[314].

Guinea pigs develop classical pulmonary granulomas that are characterized by central casseous necrosis and a thick wall of epithelioid macrophages[69]. A characteristic feature of these primary casseous granulomas, which occur upon initial aerogenous exposure, is the confinement of lymphocytes to a circumferential and peripheral rim around the granuloma. In primary granulomas, the majority of lymphocytes fail to penetrate the lesion [69, 315]. This is in contrast to post-primary, or otherwise described as secondary, granulomas, which occur upon hematogenous reseeding of the lung and consist of evenly admixed lymphocytes and macrophages[76]. It has been demonstrated that the formation of primary granulomas, and the associated casseous necrosis, most likely precedes the onset of adaptive immunity in the guinea pig[69]. While the secondary pulmonary lesions are readily amenable to combination antimicrobial therapy with isoniazid, rifampicin and pyrazinamide, primary lesion necrosis persists after treatment and has been shown to harbor persistent extracellular bacilli that are resistant to antimicrobial drugs[286, 300].

After initial aerogenous infection, the first cell to infiltrate pulmonary alveoli is the heterophil (neutrophil), first noticeable around day 11 of infection, and is accompanied by poorly organized infiltrates of macrophages[285]. The dynamics of macrophages types within the guinea pig lesions remains unknown. Central necrosis appears before day 21 of infection, at a time when minimal detectable CD4 or CD8 lymphocytes can be identified at the site of developing granulomata[69]. This finding challenges the hypothesis that granuloma necrosis is mediated by high IFNγ production in a delayed type hypersensitivity reaction which would require antigen-specific T cell immunity[69]. However, true demonstration of this lack of antigen specific immunity remains to be demonstrated with new and developing techniques such as the IFNγ ELISPOT[316].

Hematogenous reseeding of the lung coincides with onset of adaptive immune function around 3 weeks post-infection[317]. In contrast to primary TB granulomas, these secondary lesions contain up to 2 $\log_{10}$ fewer CFUs, likely due to a very different immune microenvironment, which forms after immune stimulation and leads to restriction of bacterial replication[318, 319]. The difference in immune function between primary and secondary TB lesions has been confirmed, and equates to a response in the pre-immune phase for primary granulomas, while secondary, consolidating lesions occur with cytokine evidence of exposure, indicating their occurrence in post-immune phase of infection[317].

One important development in particular in the guinea pig model is a growing span of studies evaluating the response of guinea pigs to infection with clinical *M. tuberculosis* isolates[314]. The contribution from these studies may significantly impact vaccine development in that efficacy of a candidate vaccine must certainly be capable of providing protection against these highly virulent and more medically relevant strains, rather than the standard H37Rv or Erdman laboratory strains frequently used in animal studies, which displays attenuated virulence[75]. Importantly, infection with clinical isolates, as opposed to the laboratory strains, imparts a wide variation in disease severity and manifestation, where clinical isolates of Beijing as well as non-Beijing lineage produced more severe pathology and extrapulmonary disseminated disease. In contrast, guinea pigs infected with MDR strains developed reduced pathology and bacillary burdens, suggesting reduced bacterial fitness of the genotypically resistant bacteria in a susceptible host species, although progressive, active TB was uniformly manifested among infected animals [320]. This is consistent with the notion that virulence is lost or reduced in drug-resistant strains of *M. tuberculosis*[321, 322]. Previously, it was indicated that the severity of disease dissemination and in particular, the degree of necrosis as measured by emerging stereologic methods, is a better predictor of strain virulence than recovery of viable bacilli counts from tissue. These findings support the use of area quantification in pathology assessment, in addition to CFU assays or survival measurements, for the identification of new

chemotherapeutic or vaccine candidates with improved efficacy[314]. Because disease manifestations from clinical isolates are so variable, it remains important to evaluate new vaccine candidates against these isolates to determine if the level of protection provided by a vaccine is equivalent to that from a laboratory strain challenge[7]. Additionally, evaluation of organs infrequently assessed for dissemination, including heart, adrenal gland, and distant lymph nodes may provide an advantage in evaluating vaccine efficacy against clinical isolates.

Because the pathology of TB in the guinea pig so closely mimics that of human TB, this species has also been utilized to evaluate bacterial proteomics in early and chronic stages of disease as well as host metabolomics during infection with clinical W-Beijing strains of *M. tuberculosis*. Importantly, hypoxia is feature of the guinea pig lesions, which is presumed to trigger metabolic changes in bacilli associated with latency[323-325]. Using the guinea pig, it is apparent that bacilli alter their protein expression significantly between acute and chronic infection. Bacilli isolated from granulomas on day 30 or day 90 of infection had only 28% and 27% overlap of identified proteins at each time point, respectively[326]. These methods have identified the most abundant classes of proteins expressed during infection as those involved in cell wall biosynthesis and intermediary metabolism and respiration, and importantly, have identified some potential significant differences between day 30 and 90 of infection that support metabolic adaptation of *M. tuberculosis* over time. This in vivo approach has allowed for identification of a large protein population, which can be mined for potential therapeutic and vaccine targets against the bacterium.

Evaluation of the metabolomic profile in the guinea pig also identified distinct metabolic patterns in guinea pigs that were able to differentiate naive from infected animals and also those that change with progression of infection[327, 328]. These findings have important implications toward identification of biomarkers with predictive or diagnostic potential that can be further investigated from this defined pool of molecules. Additionally, these techniques could

potentially be applied to antimicrobial treated or vaccinated guinea pigs to predict correlates of protection or effective treatment.

Manifestations of Tuberculosis in Guinea Pigs Under Natural Exposure Conditions.

Until recently, the true susceptibility of an individual to TB exposure had been extrapolated from the downstream disease manifestations in people because all studies utilizing animal models had been performed with experimental infections. While this has obvious benefits for controlled experimental conditions, the majority of animal models of experimental infection fail to replicate the diverse manifestations of TB that are characteristic of naturally occurring human TB. A new platform combining the controlled conditions of timed and evenly distributed exposure by aerosol route with the source of exposure being infected human patients has recently been developed. Although the highly susceptible guinea pig develops uniformly progressive TB disease after experimental aerosol infection, it has recently been shown that this is not the case when guinea pigs are naturally exposed to infectious aerosols. In a hospital setting where airflow from a multi-drug resistant human TB ward is routed to individual guinea pig cages, guinea pigs developed a spectrum of TB disease responses similar to what is expected in a human population.

In guinea pigs exposed over a period of 4 months to air exhausted from an inpatient MDR/XDR-TB ward, the majority of animals became skin test positive. Importantly, however, TST responses were diverse with many guinea pigs reverting to TST negative status only later to display positive TST recurrence. This high level of TST reactivity over the course of the experiment is indicative of a high rate of exposure to viable M. tuberculosis bacilli. However, unlike with experimental infections largely accompanied by a 100% rate of infection and progressive, active TB, only a minority of guinea pigs actually developed active TB disease. Given an unlikely role of establishing latency in these non-progressors that maintained TST reactivity since corticosteroid-induced immunosuppression failed to reactivate disease, these

findings suggest the ability to clear infection. The loss of TST reactivity followed by recurrence suggests that first, even in a uniformly susceptible model, not all exposures lead to progressive disease and that spontaneous sterilization by host immunity is possible, and second, that recurrence of TST reactivity are likely due to reexposure and reinfection events.

Importantly, guinea pigs manifesting the greater delayed-type hypersensitivity response, and likely the strongest T cell response, as measured by a TST of >19mm, were the animals with the greatest rate of protection and clearance of infection. Histologic analysis of the lungs from these guinea pigs confirmed infection but lesions in these animals were predominated by healing indicators such as fibrosis with reduced active inflammation and dystrophic calcification. Whether the development of a robust TST response represents a protective correlate is worthy of further investigation.

Perhaps one of the most intriguing findings from this study is the apparent role of reinfection, suggesting that clearance of the bacillus may actually occur with greater frequency than previously assumed but also indicating that the immune response acquired from initial infection is insufficient to prevent infection upon reexposure. The fact that a uniformly susceptible species failed to develop active disease also suggests that experimental infection doses may be relatively high compared to what is naturally transmitted, in addition to the possibility of significant differences in the state of host-transmitted and artificially cultivated bacteria[16, 329]. The concept that a waning TST response may indicate a failure to initiate long-term memory T cell function poses an intriguing question regarding the true efficacy of BCG vaccination, which previously has only been assessed under experimental conditions. Little is understood about how the BCG vaccine actually works and what the protective correlates are, making it difficult to interpret the significance of responses to novel vaccine candidates[7, 75]. This natural exposure setting may provide an optimal environment for better understanding the BCG mechanisms of protection and also as a strategy for testing new vaccines in general. The guinea pig may also be valuable in evaluating methods for reducing transmission of the bacilli.

A recent study from this same group has indicated that simply wearing surgical masks significantly reduces the transmission of *M. tuberculosis* as evidenced by reduced guinea pig tuberculin skin test reactivity as a marker of exposure[330].

The Immune Response to *Mycobacterium tuberculosis* Experimental Infection by Aerosol Exposure.

Due to limited reagent availability for cytokine analysis in the guinea pig species, the majority of investigations into cytokine kinetics have been measured through relative gene expression. An advantage to this approach is that the measured responses are lesion specific due to the employment of laser capture microdissection[317, 331]. Innate cytokines dominate the early cytokine response at 3 weeks of infection with overwhelming expression of TNFα that persists through 6 weeks of infection in non-vaccinated guinea pigs. In contrast, BCG-vaccinated guinea pigs develop an early type 1 response at 3 weeks of infection, with high IFNγ and IL-12p40 expression which transitions to a response dominated by the antiinflammatory cytokine TGFβ.

Further investigations into the cytokine response by McMurray *et al.* have taken advantage of the primary and secondary lesion manifestations in the guinea pig. A very similar expression profile to BCG vaccinated guinea pigs in microdissected secondary lesions indicates that hematogenous reseeding of the lung does occur in the post-immune phase of infection as was anticipated by previous studies[69]. In contrast, primary TB lesions are dominated by TNFα with lesser contribution from IL-12p40 and barely detectable IFNγ expression[317]. This indicates that the initial granulomas formed are dominated by an innate pro-inflammatory immune response.

The development of flow cytometry methods using guinea pig surface markers for specific immune cell types and fine tuning of appropriate gating strategies has allowed for

characterization of the cellular immune response to infection in the guinea pig[315, 332]. Although CD4 and CD8 T cells can be identified early in infection in low numbers, their appearance does not peak until around day 30 of infection and are generally confined to the rim of the granuloma based on immunohistochemistry. In contrast, the lesion necrosis, which precedes lymphocyte accumulation, has no apparent association with the adaptive response, suggesting innate mechanisms that lead to the development of lesion necrosis. Further investigations also employing flow cytometry have indicated that granulocytes are the first immune cell type to increase in the guinea pig lung and that their infiltration may be associated with the development of lesion necrosis[315]. Using CD45 high surface expression as an indicator of activation, it is also apparent that while activated CD4+ and CD8+ lymphocytes peak around day 30 of infection, this state is not maintained and there is a significant reduction in the number activated T cells through day 90 of infection, suggesting loss of an ongoing T cell-mediated response[315].

In evaluating the cytokine response to infection and modulation by BCG vaccination in the guinea pig, TNFα is the most well studied to date. McMurray et al. have evaluated the role of TNFα in the guinea pig using recombinant guinea pig TNFα as well as anti-guinea pig TNFα neutralizing antibodies. Ex vivo studies from isolated guinea pig macrophages, either alveolar or peritoneal, have revealed the antimicrobial properties of this cytokine, where neutralization of TNFα in BCG vaccinated animals leads to enhanced bacterial growth[333]. Additionally, from *ex vivo* studies it is apparent that TNFα acts synergistically with the T cell response, enhancing the proliferative capacity of T cells as well as increasing IL-12p40 and IFNγ expression[333-335]. Additional studies have indicated that neutrophils may be an important source of TNFα early in infection, leading to enhanced macrophage activation[336]. However, this cytokine may also be detrimental through enhancing bacterial growth in non-vaccinated guinea pigs as well as through perpetuating an active and destructive inflammatory response[337].

In recent development of guinea pig reagents, a custom micro-array with targets limited to immune evaluation (86 transcripts) has been developed as a method for evaluating response to vaccination[338]. With this microarray, a protective response was most associated with Th1-related cytokines. Even more recently, a larger microarray has allowed for determination of an immune transcriptome response signature to infectious agents in the guinea pig, using *M. tuberculosis* as the model infectious agent. Further use of these new assays will aid in identification of better protective correlates that can be measured in vaccine efficacy trials. Additionally, the ability to study and better understand TB pathogenesis in this highly relevant species will be significantly improved by the growing number of immunologic reagents available for the guinea pig. In particular, McMurray and colleagues have made a significant contribution to the reagent pool, initially through the use of relative gene expression but more recently by the generation of eukaryotic recombinant guinea pig cytokines and chemokines including IL-17, MCP-1, IL-10, TNFα, and IL-4[339-342]. Also, recent evidence that guinea pig macrophages displaying functional pinocytotic and phagocytic activity can be derived from bone marrow using human M-CSF (but not GM-CSF or either murine counterpart), will promote *in vitro* studies using infected cells of guinea pig origin[343].

Although genetic investigations into the guinea pig are continually growing, the studies performed to date have identified a number of important immunological similarities between this species and humans[284]. Several important similarities in the guinea pig should be noted. The sequence encoding for both CXCL8 (IL-8) and its receptor CXCR1, both of which are absent in the mouse, are present in the guinea pig[344, 345]. Additionally, although the mouse lacks all CD1 proteins except for the group 2 homologue, CD1d, the guinea pig genome encodes homologues of group1 CD1b and c, as well as group 2 CD1d and intermediary, CD1e[346, 347]. CD1 molecules are similar to MHC but are restricted to presentation of lipid antigen and these serve to present mycobacterial lipid antigens including mycolic acids and LAM[348, 349]. The guinea pig is the most suitable small animal model for studying the role of lipid antigen presentation as well as CD1-

restricted T cell function during TB because mycobacterial lipid antigens are presented by group 1 rather than group 2 CD1 molecules[346].

The Guinea Pig Model for Evaluation of New Tuberculosis Vaccines.

BCG vaccination remains the only available option for protection against tuberculosis but still does not protect adults from infection of active TB disease. Therefore, all new vaccines being developed must display a significant improvement in protection exceeding that of BCG in preclinical models in order to proceed to human clinical trials. The two strategies for immunization improvements are either the replacement of BCG with a more effective vaccine or a modification of the BCG vaccine regimen with a recombinant form or a boosting immunization that improves upon the protection[9, 75]. Lessons learned from the most recent human efficacy trials of a new vaccine BCG-MVAAg85a performed in infants, which failed to demonstrate significant improvement upon BCG-only immunization, indicate a strong need for identification of protective correlates in animal models that mirror human data as well as the more careful design of preclinical studies to improve statistical power and a demonstration of efficacy equivalent to that expected from vaccines in human clinical trials[350]. Despite the results of the phase II trials, some protective efficacy from this vaccine was apparent in guinea pigs in terms of enhancing survival, while evaluation at a fixed end-point and with a lower challenge dose did not indicate a protective advantage over BCG[351, 352]. The guinea pig has been utilized to evaluate the efficacy of other vaccine candidates in the form of attenuated live mutants of *M. tuberculosis* such as the SO2 mutant with a disrupted phoP gene and reduced virulence as well as a pro-apoptotic secA2 deletion mutant[7, 353].

One of the important criteria in evaluation of new vaccine candidates in preclinical models is the challenge strain that is used. The majority of all new vaccine candidates are tested in animals against the laboratory strains H37Rv or Erdman. However, the virulence of these strains is not representative of clinical TB isolates, and therefore the use of clinical strains

may provide a more stringent basis for evaluation of vaccines and their improvement upon BCG[75]. The two criteria used for evaluation of vaccine efficacy in the guinea pig (as well as other animal model species) is a reduction in bacterial load after the acute phase of bacterial growth and the improvement of TB disease as indicated by longer survival intervals in vaccinated animals[75, 354, 355].

The guinea pig has been a useful model for evaluating the disease features associated with more relevant clinical strains[320]. Previous studies have indicated that guinea pigs infected with both Beijing and non-Beijing clinical strains develop rapidly more progressive pulmonary and extrapulmonary disease. Additional studies have indicated variability among Beijing sublineages with those of higher pathogenicity tending toward greater induction of regulatory T cell responses and reduced T cell activation over the disease course[356]. Although reduced efficacy of BCG against Beijing clinical strains has been observed in mice during establishment of chronic disease, the impact of the Beijing strains on BCG vaccine efficacy in guinea pigs is unknown[357]. Evidence that reduced efficacy from BCG vaccination against clinical strains is evident however from infection studies with Erdman K01[358]. The emergence of regulatory T cells seen in mice, which may counteract protective immunity conferred by BCG, has also been suggested in guinea pigs infected with W-Beijing strains, which demonstrated elevated expression of the Foxp3 transcription factor[359].

As an alternative to BCG vaccination, the use of non-tuberculous mycobacteria for immunization has been evaluated for protective effects in the guinea pig. Specifically, evaluation of M. indicus pranii was demonstrated to provide a heightened Th1 response as compared to the BCG group, as well as reduced bacterial burden and improved pathology[360]. Evaluation of immunization through aerosol route was shown to provide superior protection and overall, data suggested better protection with MIP over BCG.

Another facet of vaccine evaluation is post-exposure prophylaxis, rather than pre-exposure immunization. Post-exposure immunization with immunodominant antigens Ag85a

and ESAT6 as well as fusion protein F36, consisting of TLR2 agonist fused to ESAT6, was able to provide early protection by day 40 of infection but this effect was transient with no significant improvement in survival intervals[361]. More recently, further investigations into post-exposure therapeutic vaccination, using a TLR4 and TLR9 agonist adjuvant, have indicated that although a reduction in bacterial burden is not achieved with these methods, a large reduction in consolidating secondary lesions resulted in significantly improved pulmonary pathology in guinea pigs infected with clinical isolates. An improvement in survival could be achieved using a priming immunization followed by a single boost[362].

Response to BCG Vaccination in the Guinea Pig.

The response of guinea pigs to BCG vaccination closely replicates the efficacy of this vaccine in humans. BCG effectively prolongs survival in experimentally infected guinea pigs and because this vaccine is sufficiently protective, it can be used for comparison to candidate vaccines. Although immunization slows the progression of pulmonary and extrapulmonary TB disease, this vaccine does not prevent infection nor does it prevent dissemination of the bacilli to commonly involved organs. Also, BCG vaccination in guinea pigs does not prevent the development of casseous necrosis in granulomas. These findings indicate that regardless of timing of adaptive immune function, whether pre-existing or not, this response cannot prevent the classical primary granuloma formation that is characteristic of the guinea pig model[358]. In contrast, the primary purpose of BCG vaccination in infants is to prevent severe multi-organ dissemination, to which young children are predisposed, but this is less effective in the guinea pig model. In addition, it was recently shown that BCG vaccination does not prevent reactivation of latent or persistent TB disease in a guinea pig model of reactivation TB[358]. Whether the response to BCG vaccination accurately reflects the efficacy seen in humans under the previously described natural exposure conditions remains to be evaluated[363].

The immune response in BCG vaccinated guinea pigs differs significantly from that of non-vaccinated guinea pigs. Vaccination significantly increases antigen specific IFNγ production in ex vivo stimulation assays by 10 weeks post-vaccination and leads to higher IFNγ expression, but reduced TNFα expression, post-infection in the lungs[364]. There is also evidence that BCG vaccination promotes a functional T cell response to infection in the lungs based on higher numbers of CD4, but not CD8, positive T cells with low CD62L surface expression, as an indicator of T cell activation[364]. Additional evidence CD4 T cell activation is enhanced over the course of disease by BCG vaccination indicated by upregulated surface expression of CD45[365] and homing receptor CT4[358]. BCG also reduces heterophil/neutrophil influx into the lungs and is associated with an earlier B cell response[365]. This primed response in the lung has been confirmed by laser capture microdissection of lesions from lungs of BCG vaccinated guinea pigs. Based on qRT-PCR methods for relative gene expression in laser-capture microdissected pulmonary lesions, primary lesions in BCG vaccinated guinea pigs are dominated by the anti-inflammatory cytokine TGFβ, with lesser contributions from IFNγ and IL-12p40, and have a marked reduction in the level of TNFα expression[317, 366].

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CHAPTER 2

Non-diabetic hyperglycemia exacerbates disease severity in *Mycobacterium tuberculosis* infected guinea pigs[1]

Rationale: Diabetes is a poorly understood but recognized risk factor for TB and the incidence is rising at an epidemic rate globally. Hyperglycemia is the hallmark feature of diabetes and epidemiological evidence suggests that hyperglycemia may be an important contributor to diabetic susceptibility to TB because diabetic individuals with adequate blood glucose control have a risk of developing TB equivalent to that of non-diabetic individuals. Chronic hyperglycemia leads to non-enzymatic glycation of host macromolecules, which serves as the basis for glycated hemoglobin (HbA1c), an indicator of blood glucose levels over time. In the presence of chronic hyperglycemia, advanced glycation end products (AGEs) are formed, which are inflammatory molecules linked to the development of diabetic complications, and also may influence the chronic inflammatory response to infection. Previously we identified that sucrose increases the severity of TB disease based on the results from carrier control guinea pigs in a study utilizing sucrose as a carrier solution for antimicrobial drug administration, indicating that the guinea pig may be an ideal model to study the impact of hyperglycemia and diabetes as a risk factor for TB. Because little is understood regarding the pathogenesis of diabetes-TB comorbidity, we used the sucrose-fed guinea pig model to investigate the impact of hyperglycemia alone on progression of TB disease in association with the formation of AGEs.

Hypothesis: Hyperglycemia, induced by daily feeding of sucrose, worsens TB disease in guinea pigs in association with increasing the formation of AGEs.

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Summary.

Hyperglycemia, the diagnostic feature of diabetes also occurs in non-diabetics associated with chronic inflammation and systemic insulin resistance. Since the increased risk of active TB in diabetics has been linked to the severity and duration of hyperglycemia, we investigated what effect diet-induced hyperglycemia had on the severity of *Mycobacterium tuberculosis* infection in non-diabetic guinea pigs. Post-prandial hyperglycemia was induced in guinea pigs on normal chow by feeding a 40% sucrose solution daily or water as a carrier control. Sucrose feeding was initiated on the day of aerosol exposure to the H37Rv strain of *M. tuberculosis* and continued for 30 or 60 days of infection. Despite more severe hyperglycemia in sucrose-fed animals on day 30, there was no significant difference in lung bacterial or lesion burden until day 60. However the higher spleen and lymph node bacterial and lesion burden at day 30 indicated earlier and more severe extrapulmonary TB in sucrose-fed animals. In both sucrose- and water-fed animals, serum free fatty acids, important mediators of insulin resistance, were increased by day 30 and remained elevated until day 60 of infection. Hyperglycemia mediated by *M. tuberculosis* infection resulted in accumulation of advanced glycation end products (AGEs) in lung granulomas, which was exacerbated by sucrose feeding. However, tissue and serum AGEs were elevated in both sucrose and water-fed guinea pigs by day 60. These data indicate that *M. tuberculosis* infection alone induces insulin resistance and chronic hyperglycemia, which is exacerbated by sucrose feeding. Moreover, *M. tuberculosis* infection alone resulted in the accumulation tissue and serum AGEs, which are also central to the pathogenesis of diabetes and diabetic complications. The exacerbation of insulin resistance and hyperglycemia by *M. tuberculosis* infection alone may explain why TB is more severe in diabetics with poorly controlled hyperglycemia compared to non-diabetics and patients with properly controlled blood glucose levels.

Introduction.

Historically the risk factors most frequently linked to TB are HIV infection, tobacco use, extremes in age, alcoholism, malnutrition, chronic kidney disease and both type 1 and type 2 diabetes[1]. How these conditions increase the susceptibility to *M. tuberculosis* infection is poorly understood but is generally thought to be associated with altered cellular immune responses. Even though diabetes has been known for centuries to increase the risk of active TB[2, 3], the current diabetes epidemic has the potential to significantly hamper efforts to control the spread of TB especially in parts of the world where *M. tuberculosis* is endemic[4, 5]. It is estimated that 80% of the global diabetic population is concentrated in developing countries with the incidence estimated to increase from 285 million currently to 439 million by the year 2030[6]. In regions where the incidence of diabetes surpasses HIV the cumulative TB risk associated with diabetes has the potential to equal or exceed that linked to HIV infection despite the lower relative risk of TB in diabetic individuals compared to HIV[1, 7].

The link between diabetes and TB susceptibility has been recently reaffirmed through retrospective review of clinical and observational studies, which show that regardless of study design and population, diabetes increases the risk of active TB[8]. These clinical studies have revealed that diabetic patients with active TB have more frequent radiographic evidence of multilobar disease [9], an increased incidence of cavitary lesions[10, 11], increased risk of extrapulmonary dissemination[12, 13], increased sputum bacterial counts[14], and more frequent TB drug treatment failures and higher mortality compared to those without diabetes[15, 16].

Recent studies have shown that despite the numerous metabolic derangements associated with type 2 diabetes, chronically elevated blood glucose (hyperglycemia) especially, is linked to the increased risk of active TB. Patients with glycated hemoglobin (HbA1c) levels exceeding 7%, indicating poorly regulated blood glucose, have a higher risk of developing TB than diabetic patients with properly controlled blood glucose levels[17, 18]. However

hyperglycemia, the diagnostic feature of both type-1 and type-2 diabetes, can also occur in the absence of diabetes and can presumably also increase the risk of active TB. The relationship between non-diabetic hyperglycemia and the susceptibility to *M. tuberculosis* has not been adequately investigated yet is an important consideration since it is estimated that the pre-diabetic population worldwide currently exceeds 280 million people and is rapidly growing[19]. Non-diabetic hyperglycemia as a TB risk factor was prioritized as important and in need of more research by a panel of experts commissioned to develop a research agenda addressing how the growing diabetes epidemic impacts TB treatment responses and thus global TB control measures[20].

In individuals with type 2 diabetes or pre-diabetes, hyperglycemia is due in part to decreased responsiveness of tissues to the hormone insulin, a phenomenon known as insulin resistance. How hyperglycemia or insulin resistance increases the susceptibility to TB whether due to diabetes or not is unknown[21]. One possibility may be related to the chemical interaction of reducing sugars including glucose, with proteins and other host macromolecules known as the Maillard reaction. The non-enzymatic and therefore unregulated binding of sugar residues to proteins (glycation) is associated with the normal aging process but is exacerbated by chronic elevations in blood glucose in individuals with poorly controlled diabetes. Glycation and the intermediate formation of Amadori products is a reversible reaction, which precedes the formation of irreversibly modified adducts referred to as advanced glycation end products (AGEs)[22]. The accelerated accumulation of AGEs during diabetes is implicated in the pro-inflammatory responses directly linking hyperglycemia to debilitating and life-threatening complications including nephropathy, neuropathy, retinopathy and atherosclerosis[23-25].

Because diabetes is an important TB risk factor and little is known about how hyperglycemia alone increases the risk of *M. tuberculosis* infection, we investigated the impact diet-induced hyperglycemia had on the severity of experimental *M. tuberculosis* infection and AGE accumulation in non-diabetic guinea pigs. The design of this study was based on the

hypothesis that the consequences of hyperglycemia and TB share a common pathogenesis involving chronic inflammation and the formation of AGEs and that combining the two conditions exacerbates TB severity even in hyperglycemic, non-diabetic guinea pigs.

Materials and Methods.

Animal treatments.

A total of 60 guinea pigs (Charles River Laboratories; North Wilmington, MA) were randomly assigned to 4 treatment groups split between two separate experiments: *M. tuberculosis* infected and sucrose-fed (n=20), *M. tuberculosis* infected and water-fed (n=20), uninfected and sucrose-fed (sucrose control, n=10) and uninfected and water-fed (uninfected control, n=10). All animals treated with sucrose were given 400 mg of sucrose as a 40% w/v solution *per os* daily beginning on the day of infection based on an unpublished observation that this dose of sucrose increases the severity of TB in guinea pigs when used as a carrier control in anti-TB drug treatment studies. In addition, a slightly lower dose of sucrose induces post-prandial hyperglycemia and insulin resistance in the rat [26]. This 400 mg dose effectively increases post-prandial blood glucose levels with a peak average of 2.14 fold above baseline in the guinea pig (Figure S1). The mock-treated, water-fed animals were given an equivalent volume of water *per os* daily also beginning on the day of infection.

Aerosol infection of guinea pigs with *M. tuberculosis.*

Culture stocks of *Mycobacterium tuberculosis* strain H37Rv (TMC #102, Trudeau Institute; Saranac Lake, NY) collected at mid-log phase of growth in Proskauer-Beck liquid medium containing 0.05% Tween-80 were diluted to 1×10^6 CFU/ml and delivered by low-dose aerosol infection using a Madison chamber aerosol generation device calibrated to deliver approximately 20 bacilli to each animal.

Euthanasia and Sample Collection.

At days 30 and 60 of infection, 10 guinea pigs from each infected treatment group and 5 guinea pigs from each uninfected group were anesthetized by intramuscular injection of ketamine (20 mg) and diazepam (1 mg) prior to humane euthanasia with an overdose of sodium pentobarbital by intraperitoneal injection (1.5 ml/kg). Lung, spleen and mediastinal lymph node was collected either for histopathology and fixed in 4% paraformaldehyde or for Mycobacterial culture and weighed prior to homogenization and plating of serial dilutions. All paraformaldehyde fixed tissue was removed after 72 hours and placed in 70% ethanol for storage.

Tissue CFU Quantification.

Bacterial burden in the lung, spleen, and mediastinal lymph nodes was determined by plating serial dilutions of tissue homogenates on nutrient 7H11 agar followed by counting colony-forming units after incubation at 37°C for 3-6 weeks. Data was expressed as CFUs per gram of tissue.

Histopathology and lesion analysis.

Either on day 30 or day 60 of infection, lung, spleen, and mediastinal lymph node were collected and fixed in buffered 4% paraformaldehyde for 3 days then stored permanently in 70% ethanol. Tissues were paraffin embedded, sectioned at 5 µm and stained with hematoxylin and eosin by standard methods.

Total lung, lymph node and spleen tissue area for each organ was quantified using the Stereo Investigator software version 10.02 (MBF Bioscience; Williston, VT) and the Nikon Eclipse 80i microscope. Total lesion area was compared to total tissue area of lung, lymph node or spleen respectively to yield total lesion burden expressed as a percentage of lesion to

total tissue area using the area fraction fractionator as previously described[27]. Similarly, area ratio quantification was performed comparing necrosis to lesion area.

Serum glucose and oral glucose tolerance test.

Serum was collected from guinea pigs at the time of euthanasia on either day 30 or day 60 of infection. Glucose was measured using the glucose oxidase enzymatic method (Cayman Chemical; Ann Arbor, MI) at an absorbance of 500 nm on a microplate spectrophotometer. The oral glucose tolerance test was performed on the animals after 12 hours of overnight fasting. At time 0 each guinea pig was administered an oral dose of D-glucose (Sigma-Aldrich; St. Louis, MO) of 1g/kg. Percutaneous whole blood obtained from an ear pinna prick site was used for sequential glucose measurements performed at 0, 60, and 120 minutes post-glucose administration with a handheld glucometer (Freestyle Lite, Abbott Diabetes Care; Alameda, CA) validated against the glucose oxidase assay.

Immunohistochemistry for AGEs.

Immunohistochemistry was performed on paraformaldehyde-fixed, paraffin-embedded 5 µm sections of lung from the sucrose-fed and water-fed guinea pigs targeting AGEs. Slides containing tissue sections were deparaffinized and rehydrated followed by antigen retrieval (PT Module Buffer 4, Thermo Scientific; Rockford, IL) at an incubation temperature of 95°C for 30 minutes. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide treatment for 10 min, rinsed in Tris-buffered saline with 1% Tween-20 (TTBS), and followed by two blocking steps: (i) 15 min incubation with 0.15 mM glycine in PBS, and (ii) 30 min incubation with 10% fetal bovine serum and 1% bovine serum albumin. The slides were then incubated for 30 minutes with rabbit polyclonal antibody to AGE (ab23722, AbCam; Cambridge, MA) or rabbit polyclonal IgG (ab27478, AbCam; Cambridge, MA) at a 1:400 or 1:100 dilution, respectively, in blocking buffer. After rinsing, the slides were incubated for 30 minutes with biotinylated goat-

anti rabbit IgG antibody (Vector Laboratories; Burlingame, CA). Horseradish peroxidase was added followed by diaminobenzidine substrate to visualize bound antibody. Hematoxylin was used as a counterstain.

Evaluation of immunohistochemical reactivity within TB lesions of lung tissue was based on a 4-point scoring system and performed by a pathologist blinded to the study groups. Areas of lung containing primary lesions with or without necrosis were separated from lesion-free lung and scored based on the following 2 criteria: (i) Percent of lesion inflammatory cells displaying immunoreactivity: 0- No immunoreactivity is evident, 1- Up to 25% of cells are reactive, 2- Up to 50% of cells are reactive, 3- Up to 75% of cells are reactive, 4- Greater than 75% of cells are reactive. (ii) Intensity of immunoreactivity was assessed as follows: 0- none, 1- mild, 2- moderate, 3- severe. The maximum possible score for AGE IHC is 7.

The specificity of anti-AGE antibodies for AGE-modified guinea pig proteins was validated by competitive inhibition of tissue binding utilizing AGE-modified bovine serum albumin (ab51995, Abcam; Cambridge, MA) at an antibody:antigen ratio of 1:100 prior to adding the primary antibody to each tissue section. Specificity was confirmed by loss or lack of immunoreactivity in tissue sections indicating effective blocking of anti-AGE antibodies to guinea pig lung tissue. Purified polyclonal rabbit IgG was applied as a primary antibody for a negative control.

Quantification of serum AGE levels.

Protein content of serum was measured using the BCA assay (Thermo Scientific; Rockford, IL) and samples were diluted to a concentration of 10 µg/ml. AGE levels were measured in diluted serum samples by ELISA (Cell Biolabs; San Diego, CA). Serum samples were analyzed on a standard curve of AGE-modified bovine serum albumin as directed by the manufacturer and expressed as the mass of AGE-modified serum protein per 10 µg analyzed.

This assay detects AGE structures formed on proteins in the presence of glycolaldehyde including two of the most prevalent, carboxymethyllysine and pentosidine.

Quantification of serum free fatty acid levels.

Serum free fatty acid levels were measured by fluorescence in an assay utilizing a coupled enzymatic reaction (Cayman Chemical; Ann Arbor MI). Briefly, acyl CoA synthetase catalyzes fatty acid acylation of coenzyme A. The acyl CoA generated is oxidized by acyl CoA oxidase to generate hydrogen peroxide, which in the presence of horseradish peroxidase and 10-aceyl-3,7-dihydroxyphenoxazine (ADHP) generates fluorescence that is measured spectrophotometrically at an excitation wavelength of 530 nm and an emission wavelength of 585 nm.

Data Analysis.

Statistical analyses and graphic expression of the data was by the use of the statistical package in GraphPad Prism 5. Bacterial tissue burden was $\log_{10}$ transformed and normalized to per gram of tissue prior to analysis to ensure approximate normal distribution with a common variance. Comparison of paired observations based on a single treatment was performed by paired t test. The differences between treatment groups of the remaining data was compared using a two-way ANOVA followed by Bonferroni post-test for pair-wise comparison of means with significance set at $P \leq 0.05$.

Results.

Serum glucose and glucose tolerance tests.

At day 30 of infection, serum glucose levels (Fig 2.1) were elevated with a mean value of 132.6 mg/dl in infected water-fed control guinea pigs but this increase was not statistically significant ($p>0.05$). In contrast, hyperglycemia was further exacerbated by sucrose feeding at 30 days of infection with a mean value of 147.6 mg/dl ($p\leq0.01$). Interestingly, similar hyperglycemia was evident by 60 days of infection in both the water- and sucrose-fed groups with mean values of 196.3 and 197.4 mg/dl, respectively ($p\leq0.01$).

Oral glucose tolerance tests (OGTT) were performed prior to euthanasia at both days 30 (Appendix Fig 2A) and 60 (Appendix Fig 2B) of infection to confirm that sucrose feeding or infection alone or in combination did not induce a diabetic type response to oral glucose challenge. A response similar to that of normal water-fed controls was present in all groups independent of sucrose feeding or infection with a peak glucose concentration of approximately 2 fold at 60 minutes and a return to <1.5 fold by 120 minutes post-challenge.

Histopathology.

Pulmonary pathology showed no statistically significant quantitative differences between the sucrose-fed animals compared to water-fed controls on day 30 of infection ($p>0.05$) but was more severe in the sucrose-fed group by day 60 ($p\leq0.05$) (Fig 2.2A). More striking was the increase in size and frequency of the extrapulmonary lesions in the spleen (Fig 2.2B) and lymph node (Fig 2.2C) of sucrose-fed guinea pigs compared to the water-fed controls. In the spleen, the lesion burden was significantly higher in sucrose-fed guinea pigs by day 30 of infection ($p\leq0.05$) with an even greater difference in severity by day 60 ($p\leq0.05$). At day 30 of infection, the lesion burden in mediastinal lymph nodes was significantly higher in the sucrose-fed group ($p\leq0.05$), which progressed in both groups such that there were no differences between water

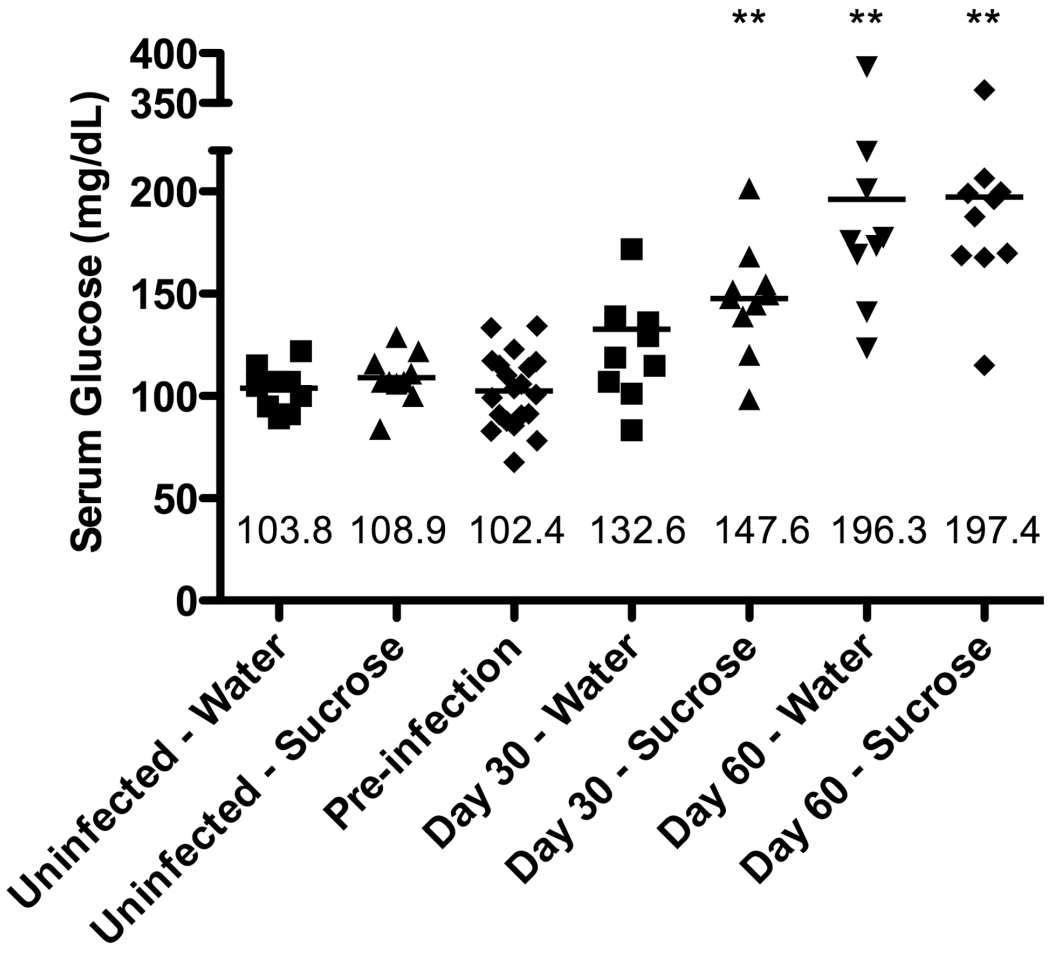

Figure 2.1. Hyperglycemia resulting from *M. tuberculosis* infection was exacerbated early by sucrose treatment. Random sampling of serum glucose values were compared to the mean glucose level of guinea pigs prior to sucrose feeding or infection (Pre-infection, n=20) and served as the normal reference value. Serum glucose values of uninfected guinea pigs are similar to normal pre-infection values. Despite a mild increase in serum glucose associated with *M. tuberculosis* infection in the water-fed controls, significant exacerbation of hyperglycemia was only induced in sucrose-fed guinea pigs on day 30 of infection (n=10). However, this difference was independent of sucrose feeding by day 60 (n=9) of infection since persistent hyperglycemia was present in both sucrose- and water-fed groups. **p≤0.01.

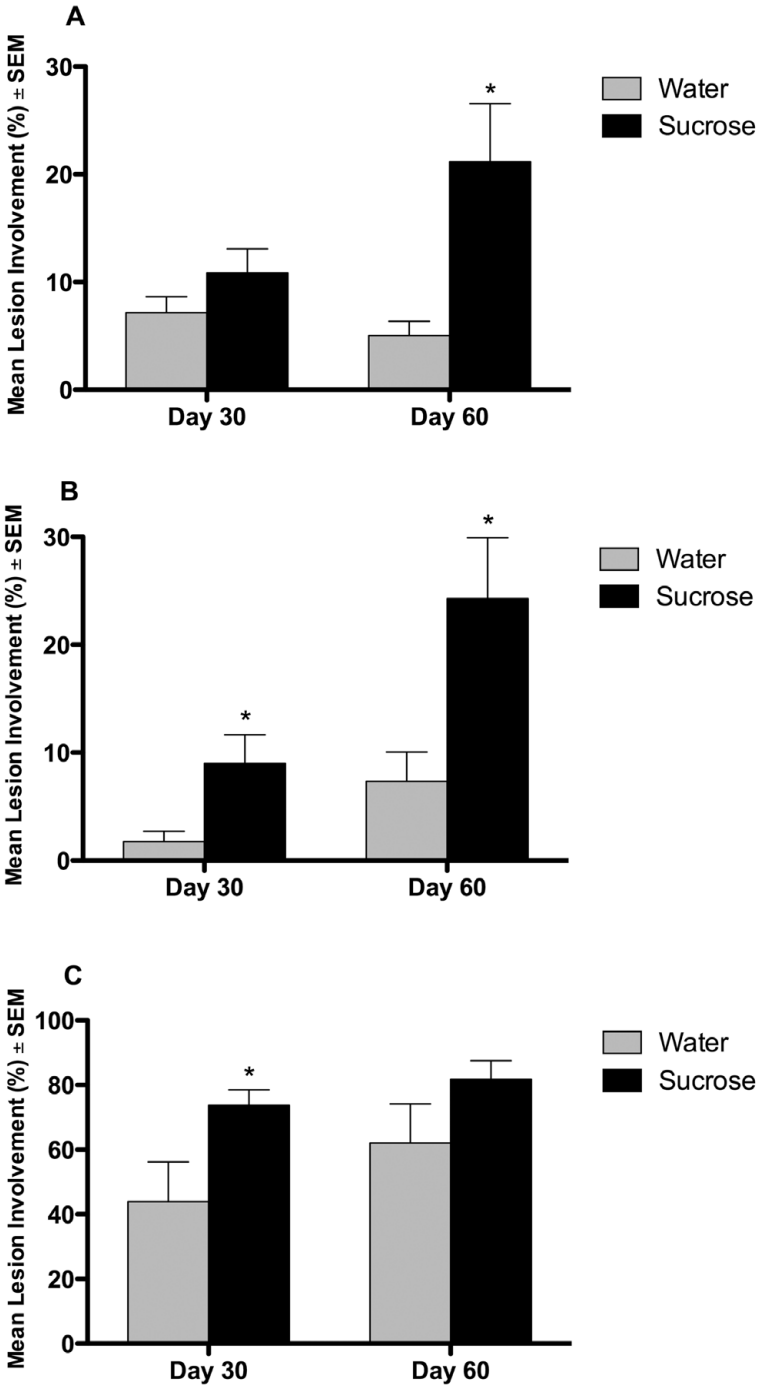

Figure 2.2. Sucrose fed guinea pigs had significantly higher lung and extrapulmonary TB lesion burden. The lung and lesion area was determined from hematoxylin and eosin stained tissue sections for each animal and the data expressed as mean percent involvement for each treatment group. The sucrose-fed guinea pigs had a significantly higher lesion burden compared to the water-fed control group in the lung (A) on day 60 of infection, spleen (B) on days 30 and 60 of infection, and mediastinal lymph node (C) on day 30 of infection. n=10, *p≤0.05.

and sucrose-fed groups by day 60 of infection (Fig 2.2C). The degree of necrosis, measured as percent necrosis area to lesion area, was not significantly different between water and sucrose-treatment in any organ at either day 30 or day 60 of infection ($p \leq 0.05$) (Appendix Fig 3).

The qualitative differences of lesions for each treatment group are depicted in representative photomicrographs of lung in Figure 2.3 and spleen in Figure 2.4. Individual lesions were represented by well-delineated granulomas consisting of epithelioid macrophages and scattered multinucleated giant cells with fewer lymphocytes in sections of lung, lymph node and spleen of both treatment groups by day 30 of infection with necrosis and central accumulations of infiltrating granulocytes. By day 60 of infection the granulomas had expanded in size, as did the extent of central lesion necrosis, peripheral lymphocytic infiltration, circumferential fibrosis and central dystrophic mineralization. Non-necrotic secondary pulmonary lesions indicative of hematogenous reinfection of the lung in sucrose-fed animals were more pronounced by day 30 of infection and progressed in size and number by day 60 of infection. Based on histologic examination the cellular constituents were similar between treatment groups however the size and frequency of lesions were more severe in the sucrose-fed groups.

Tissue bacterial burden.

On day 30 of infection similar numbers of viable bacilli were cultured from the lungs of water- and sucrose-fed groups ($p > 0.05$, data not shown). There was a significant increase in the lung and spleen bacterial burden by day 60 of infection ($p \leq 0.05$) (Fig 2.5). Bacterial numbers in the mediastinal lymph node on day 60 of infection were not statistically different between sucrose- and water-fed, infected groups ($p > 0.05$). While the difference in bacterial numbers in the lung of sucrose-fed animals was significant, the differences between treatment groups as reflected by spleen bacterial numbers were even greater.

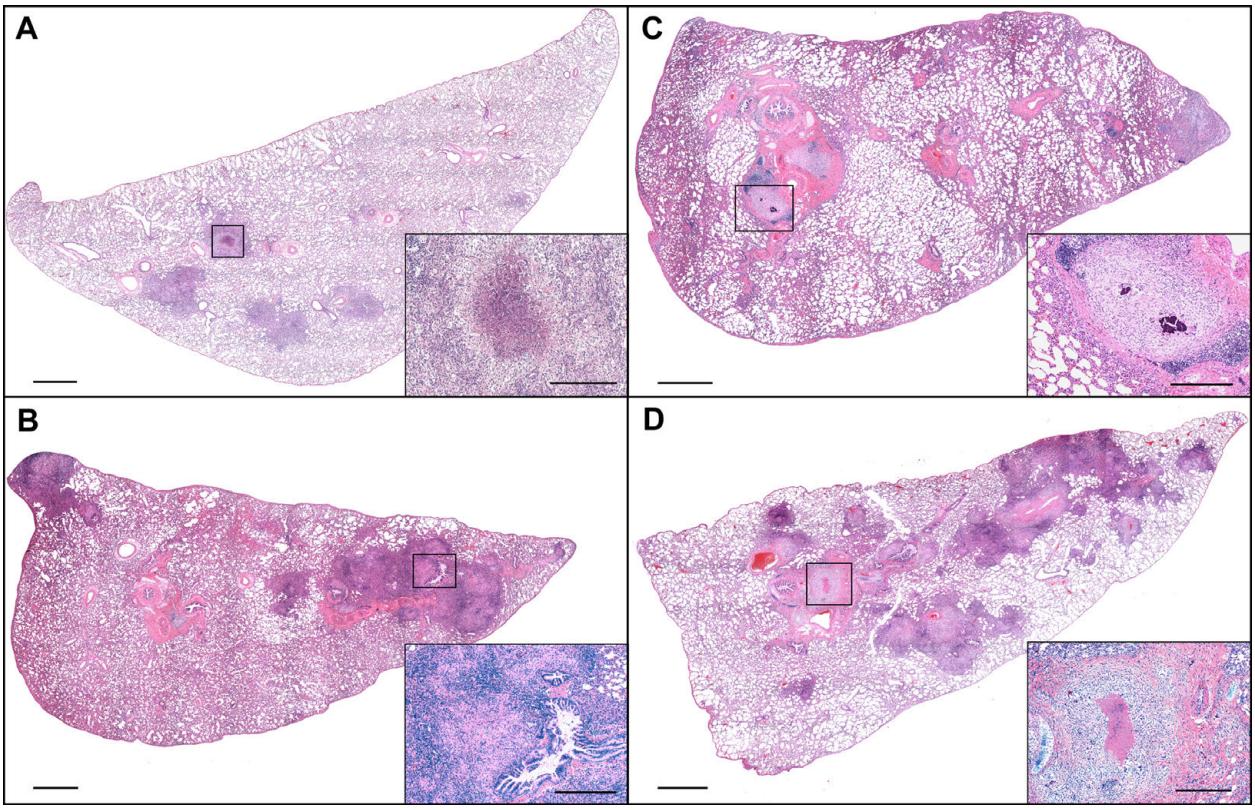

Figure 2.3. Sucrose feeding resulted in a more severe pulmonary lesion burden in *M. tuberculosis* infected guinea pigs. Images represent the animals closest to the mean values for severity of lesion burden as determined by measuring lesion and normal lung area using morphometric analysis (see Figure 2). Pulmonary lesion severity was similar between water- (A) and sucrose-fed (B) guinea pigs at day 30 of infection but was more severe in the sucrose-fed animals by 60 days of infection (D) compared to the water-fed controls (C). Bar = 1000µm. Hematoxylin and eosin stain. *Insets*: High magnification views of the TB lesions delineated on the subgross views of A-D; Bar = 100µm.

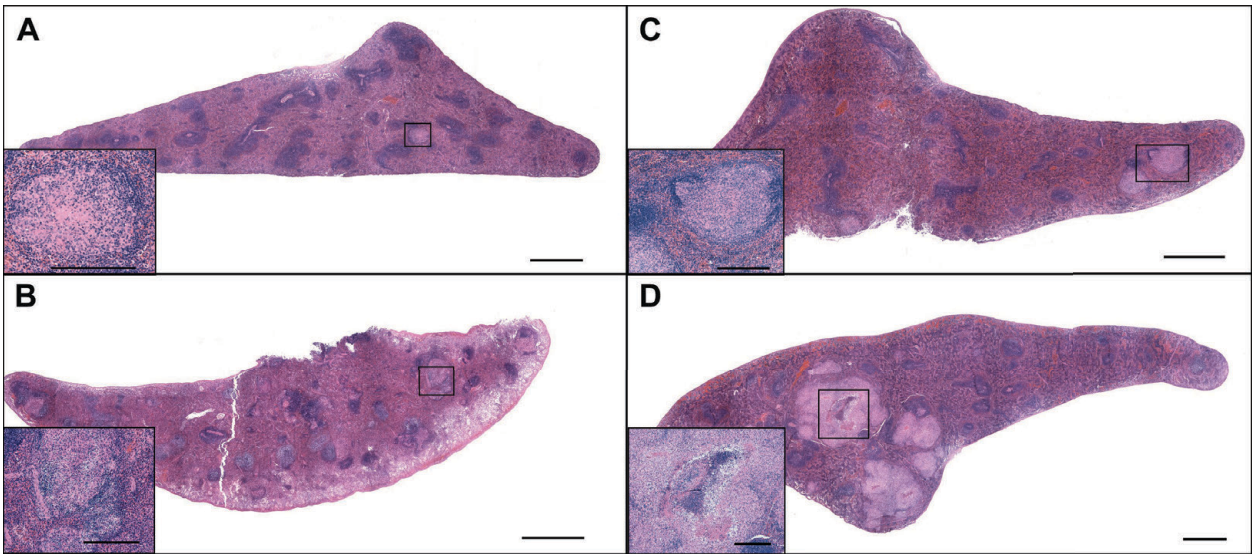

Figure 2.4. Spleen lesions from extrapulmonary dissemination of *M. tuberculosis* were more severe and more numerous in sucrose-treated guinea pigs. Images represent the animals closest to the mean severity of lesion burden as determined by measuring lesion and normal spleen area using morphometric analysis (see Figure 2). Splenic involvement was mild in the sucrose-fed guinea pigs at 30 days of infection (A) which was even less in the water-fed controls (B). However, splenic lesions were significantly more severe in the sucrose-fed animals by day 60 of infection (D) compared to the water-fed control group (C). Bar = 1000µm. Hematoxylin and eosin stain. *Insets*: High magnification views of the TB lesions delineated on the subgross views of A-D; Bar = 100µm.

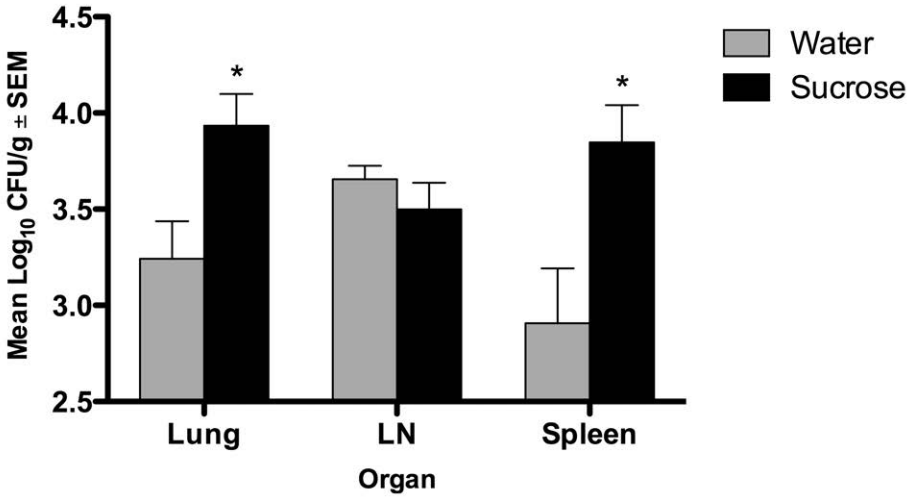

Figure 2.5. Sucrose treatment resulted in higher bacterial burden in lung and spleen of *M. tuberculosis* infected guinea pigs. Yield of viable bacilli from the lung was significantly higher in sucrose-fed guinea pigs on day 60 of infection compared to water-fed control animals. Even higher yields of bacilli were recovered from the spleens of sucrose-fed guinea pigs on day 60 of infection. No significant differences were observed in the mediastinal lymph nodes (LN). n=10, $p \leq 0.05$.

Serum Free Fatty Acid Levels.

Total free fatty acids were measured in serum from guinea pigs of all treatment groups at days 30 and 60 of infection (Fig 2.6). Elevated mean FFA levels were present in both water- and sucrose-fed, *M. tuberculosis* infected groups at both days 30 and 60 of infection ($p \leq 0.001$). Compared to mean values of 102.8 µM and 107.2 µM at day 30 of infection in uninfected sucrose- and water-fed guinea pigs, respectively, FFAs were elevated with mean values of 207.6 µM and 204.3 µM in the infected groups. Serum FFAs remained elevated at day 60 of infection with mean values of 205.4 µM and 202.3 µM in sucrose- and water-fed, infected guinea pigs, respectively while uninfected mean values were minimally reduced at 67.2 µM and 65.03 µM. Sucrose feeding alone in uninfected guinea pigs did not result in alterations of serum FFAs compared to water-fed controls ($p > 0.05$).

Serum AGE Levels.

The differences in serum AGEs between treatment groups at days 30 and 60 of infection are illustrated in Figure 2.7. Elevated serum AGEs were present in a single guinea pig in the sucrose-fed, infected group at day 30 of infection with no reflective increase in mean serum AGEs for that group ($p > 0.05$). All remaining guinea pigs of sucrose- and water-fed, infected groups were similar to uninfected, water-fed controls. In contrast, by day 60 serum AGEs were similarly elevated in both the water- and sucrose-fed infected groups compared to the water-fed uninfected control group ($p \leq 0.05$). There was no evidence of increased AGEs due to sucrose feeding alone when compared to water-fed, uninfected controls ($p > 0.05$).

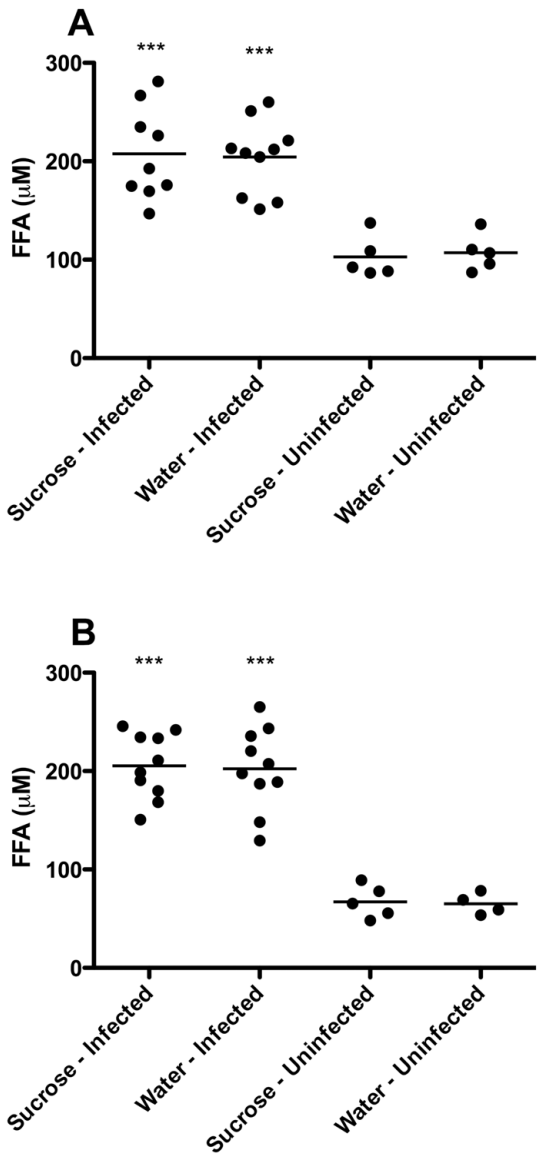

Figure 2.6. Elevated total serum free fatty acids occurred as a result of *M. tuberculosis* infection and not due to sucrose treatment. Serum free fatty acids are similarly elevated between sucrose- (n=9) and water-fed (n=10) *M. tuberculosis* infected guinea pigs at day 30 of infection (A). The levels remain similarly elevated in sucrose- (n=10) and water-fed (n=10) *M. tuberculosis* infected guinea pigs at day 60 of infection with no increase in the mean values over time (B). Serum free fatty acid levels in sucrose-fed uninfected animals (day 30, n=5; day 60, n=5) are comparable to water-fed controls (day 30, n=5; day 60, n=4) at both time points. ***$p \leq 0.001$.

AGE Immunohistochemistry.

Immunoreactivity for AGEs was seen specifically in the pulmonary TB lesions, within the serum and lymph fluid of all blood and lymphatic vessels and associated with extracellular matrix surrounding airways and blood vessels. There was no immunoreactivity seen in other normal microanatomic structures of the lung parenchyma. In both sucrose- and water-fed, uninfected animals, immunoreactivity was limited to intravascular serum, lymph fluid and extracellular matrix and were not significantly different. Immunoreactivity specifically colocalized with cells of the pulmonary TB lesions and most strikingly within the areas of central necrosis of the primary lesions. Occasionally, AGE-positive macrophages were in the lumen exudate, which frequently accumulates within bronchioles in the chronic stages of *M. tuberculosis* infection.

The number and immunostaining intensity of macrophages within lesions was significantly different between treatment groups (Fig 2.8). The sucrose-fed guinea pigs had a marked accumulation of AGEs within lesions on both days 30 and 60 of infection compared to water-fed guinea pigs ($p \leq 0.001$ and $p \leq 0.05$, respectively) with elevated but little difference in the sucrose-fed group between days 30 and 60. Interestingly, pulmonary AGEs increased in the water-fed group between days 30 and 60 of infection but did not increase to a level comparable to that of the sucrose-fed animals. However, this increase in the water-fed group was not statistically significant ($p > 0.05$). The differences in IHC scores were due to morphologic differences in macrophage or necrosis localization, frequency of immunoreactivity, and signal intensity in lungs between sucrose- and water-fed guinea pigs (Fig 2.9). Lungs from sucrose-fed guinea pigs had greater numbers of inflammatory cells with more intense immunoreactivity compared to water-fed guinea pigs. Additionally, there was increased intensity of immunoreactivity present within areas of lesion necrosis in the sucrose-fed compared to water-fed guinea pigs.

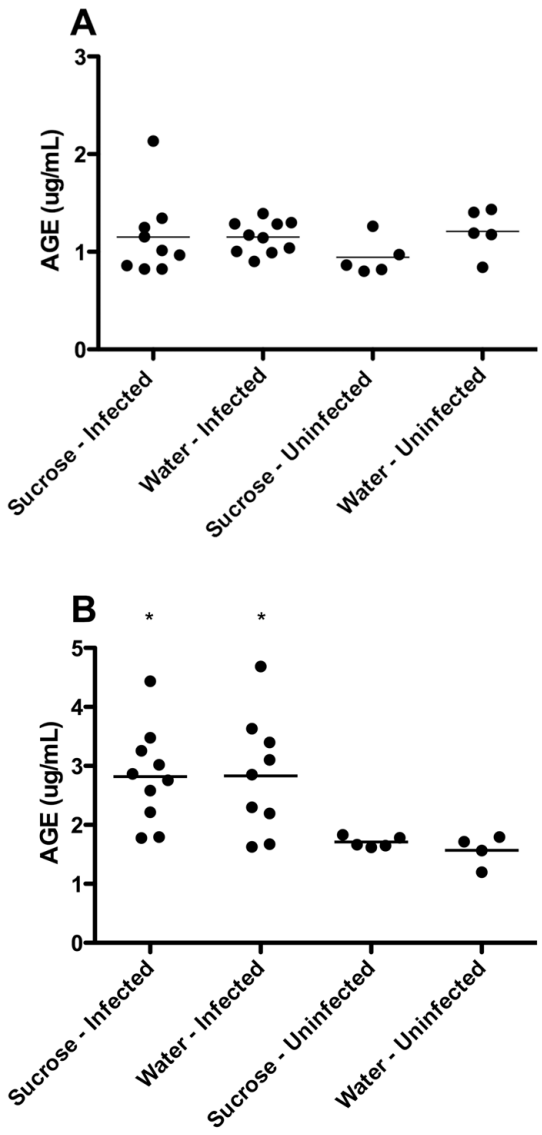

Figure 2.7. *M. tuberculosis* infection induces the formation and accumulation of AGEs in serum from guinea pigs independent of sucrose feeding. Levels of serum AGEs were analyzed in sucrose- or water-fed guinea pigs with and without *M. tuberculosis* infection at both 30 and 60 days of infection. At 30 days of infection (A), mean serum AGEs are not elevated by either sucrose feeding or *M. tuberculosis* infection with levels remaining similar to uninfected controls. However, elevations in serum AGEs were present at 60 days due to *M. tuberculosis* infection (B) (sucrose-infected n=10, water-infected n=9) and were not exacerbated by sucrose feeding. AGE levels in uninfected, sucrose- and water-fed controls (n=5 and n=4, respectively) remain similar to levels at day 30. *$p \leq 0.05$.

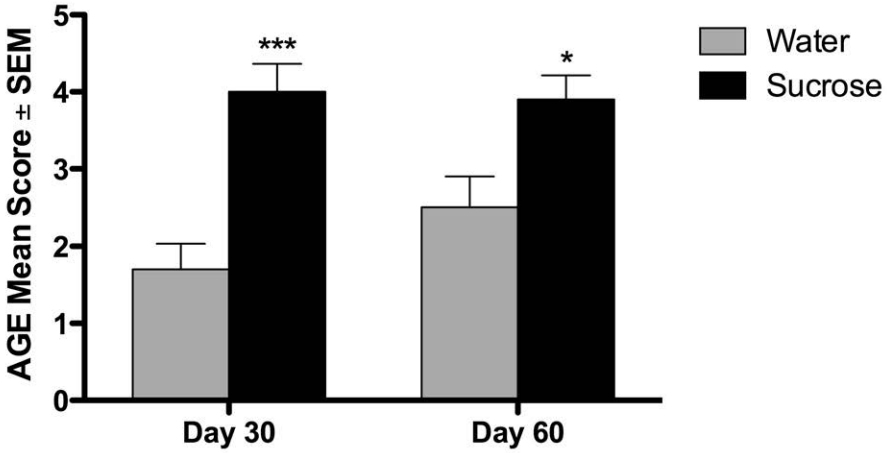

Figure 2.8. Sucrose feeding of *M. tuberculosis* infected guinea pigs significantly increased tissue AGEs by day 30 of infection. The mean score of AGE immunohistochemistry on lung is depicted in water- and sucrose-fed guinea pigs on days 30 and 60 of infection. Significantly increased AGE formation and accumulation was present within TB lesions by day 30 of infection in the sucrose-fed group compared to the water-fed controls. AGE levels at day 30 were similar to day 60 in the sucrose-fed group but additional AGEs accumulated within lesions by day 60 of the water-fed controls, which was not statistically significant. n=10, *$p \leq 0.05$,***$p \leq 0.001$.

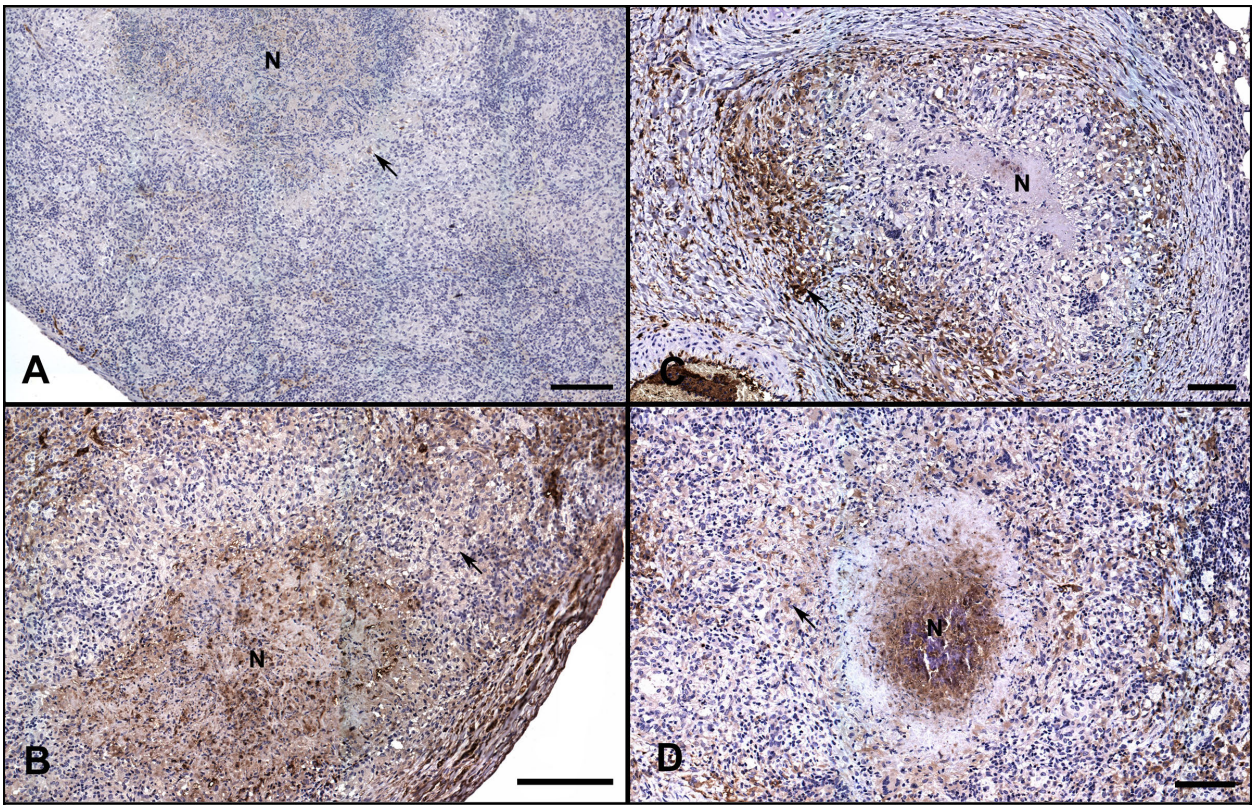

Figure 2.9. Sucrose feeding of *M. tuberculosis* infected guinea pigs increased lesion associated AGEs on day 30 and 60 of infection. AGEs are evaluated by immunohistochemistry on lung tissue sections of *M. tuberculosis* infected guinea pigs. The majority of strong immunoreactivity was associated with TB lesions. Immunoreactivity was evident within the cytoplasm of macrophages forming granulomatous lesions and was also strongly present within central necrosis (N) of primary TB pulmonary lesions. (A) Minimal reactivity was confined to the serum, rare macrophages in the lesions (arrow) and minimally within areas of necrosis (N) of the water-fed control group at 30 days of infection. (B) In contrast, at 30 days of infection, the sucrose-fed animals had strong immunoreactivity in the majority of lesion macrophages (arrow) and in areas of necrosis (N). (C) By day 60 of infection, AGEs began to accumulate within macrophages (arrow) in the water-fed controls but minimal reactivity was present in areas of necrosis (N). (D) The sucrose-fed animals had strong immunoreactivity within the majority of macrophages (arrow) and in areas of necrosis (N) at day 60 of infection. All tissue sections depicted are representative of the mean AGE IHC score for each treatment group at each time point (see Figure 8). Bar = 100 µm

Discussion.

In this study we show that daily feeding of a relatively small amount of sucrose in non-diabetic guinea pigs exacerbated *M. tuberculosis* infection-associated hyperglycemia, which significantly increased TB disease severity. Most notable was not only increased lung lesion and bacterial burden but also earlier hematogenous dissemination of bacilli in sucrose-fed animals, which resulted in earlier and more severe extrapulmonary TB. These studies were initiated following a fortuitous observation showing that the use of sucrose as an oral anti-TB drug carrier in guinea pigs resulted in more severe disease compared to non-sucrose fed controls. The importance of these data is that inducing post-prandial hyperglycemia in guinea pigs by supplementing a normal chow diet with sucrose had a significant negative impact on the severity of experimental *M. tuberculosis* infection. These data are significant since it has been shown that hyperglycemia is a TB risk factor and diabetics with uncontrolled hyperglycemia are at greater risk for active TB compared to those who have successfully maintained near normal blood glucose levels[17, 18]. What remains unknown however, is whether hyperglycemia in non-diabetic humans also increases the risk and severity of TB as suggested by these data.

Invariably preceding the development of overt type 2 diabetes, there is a decrease in systemic insulin sensitivity referred to as insulin resistance, which is also a feature of chronic inflammatory conditions in non-diabetic individuals. Our results show that hyperglycemia in *M. tuberculosis* infected guinea pigs fed sucrose was largely driven by infection associated inflammation and sucrose feeding contributed to hyperglycemia in the early stages of infection. Therefore, these data suggest that the persistent hyperglycemia was more likely due to insulin resistance associated with chronic *M. tuberculosis* infection. However, sucrose feeding lengthened the duration and severity of hyperglycemia as was evident by increased clinical disease severity and the formation and accumulation of tissue and serum AGEs.

One possible explanation for how chronic *M. tuberculosis* infection increases blood glucose levels is through the elevation of pro-inflammatory cytokines, specifically TNF-α. TNF-α rapidly induces insulin resistance by stimulating free fatty acid (FFA) synthesis and secretion, which interferes with post insulin receptor signaling via altered phosphorylation of insulin receptor substrates[28-30]. Our data are consistent with FFA mediated insulin resistance in *M. tuberculosis* infected guinea pigs since FFA levels were elevated in both the water- and sucrose-fed animals, with similar elevations present at both days 30 and 60 of infection.

Although significant glucose intolerance was not demonstrated by OGTT, significant post-prandial hyperglycemia was confirmed 60 minutes after administration of 1 g/kg of glucose administered during the test. However, even a short interval of post-prandial hyperglycemia is known to result in significant oxidative stress in humans[31]. Additionally, oral supplementation of sucrose has been shown to model insulin-resistance in rats as early as 2 weeks after initiation of treatment while persistent hyperglycemia does not manifest until 9 weeks after treatment[26]. In this study, a comparatively low dose of 400mg of sucrose daily was utilized to mimic the original observation made when sucrose was used as an anti-TB drug carrier. Compared to water-fed controls, this treatment induced more severe hyperglycemia by 30 days when combined with *M. tuberculosis* infection. We showed, however, that combining sucrose feeding and *M. tuberculosis* infection did not induce diabetes since oral glucose tolerance tests did not show any evidence of elevated fasting glucose or glucose intolerance typical of type 2 diabetes.

The degree of hyperglycemia, in particular non-diabetic hyperglycemia, has not been previously investigated as a risk factor for TB in humans and experimentally in animal models. In this study, non-diabetic guinea pigs with hyperglycemia had more severe and earlier onset of extrapulmonary TB as evident by higher lesion burden in the mediastinal lymph nodes at 30 days of infection followed by an increased bacterial burden and increased severity of spleen lesions on both days 30 and 60 of infection. Immunosuppression has been implicated as the mechanism by which diabetes and other TB risk factors increase the susceptibility to *M.*

tuberculosis infection or exacerbate active TB disease[1]. While suppression of the adaptive immune response potentially explains the increased numbers of viable bacilli isolated from both lung and spleen of sucrose-fed animals on day 60 of infection there are alternative explanations. The delay in the adaptive immune response as demonstrated in diabetic mouse models of *M. tuberculosis* infection combined with a pro-inflammatory innate response associated with hyperglycemia could also explain our findings[32]. In addition, chronic hyperglycemia and free fatty acids may represent a readily available carbon source for rapidly replicating bacilli[33].

The extrapulmonary spread of bacilli to the regional lymph nodes is a consistent feature of the experimental *M. tuberculosis* infections in animal models including the guinea pig[34]. The rate of dissemination and severity of extrapulmonary lesions is an indicator of increased susceptibility of the host or increased virulence of the challenge strain of *M. tuberculosis*[35, 36]. Studies performed in our laboratory and others have demonstrated an increased resistance to extrapulmonary TB in guinea pigs vaccinated with BCG prior to challenge, but few studies have demonstrated a decreased resistance in this model[34, 36]. Disseminated extrapulmonary TB in humans is associated with HIV infection and the risk increases proportionally with severity of immunosuppression[37, 38]. Similarly, diabetes, a condition known to alter immune function, is associated with increased risk of developing extrapulmonary dissemination of *M. tuberculosis* in humans[12, 13]. In this study, we show that non-diabetic hyperglycemia had a significant negative impact on disease progression resulting in earlier and more severe dissemination of bacilli. A potential mechanism leading to more extensive *M. tuberculosis* dissemination may be related to microvascular damage, a direct consequence of hyperglycemia, which is central to the pathogenesis of diabetic complications[39].

A potential link tying together high carbohydrate diet and a maladaptive pro-inflammatory state is the increased formation of circulating and tissue-associated AGEs. The presence of increased AGEs is tied to both a pro-oxidative and pro-inflammatory state where oxidative stress mediates the persistent inflammatory response[40, 41]. In patients with poorly controlled

diabetes and increased glycated hemoglobin (HbA1c), peripheral blood leukocytes express increased innate and type 1 cytokines indicative of a pro-inflammatory phenotype[42, 43]. In this study, sucrose-fed guinea pigs developed more severe pulmonary and splenic inflammation, which corresponded to increased AGE formation in the TB lesions. The specific localization and accumulation of AGEs in TB lesions even in water-fed controls implicates AGEs as a potential driving force for the pro-inflammatory state in TB as well, which is exacerbated by diabetic or non-diabetic hyperglycemia. In part, this may be due to increased oxidative stress, which we have shown to be important in the pathogenesis of TB. Interestingly, this pathogenic mechanism is further supported by our findings that treatment of *M. tuberculosis* infected guinea pigs with the antioxidant drug N-acetyl cysteine significantly reduces disease burden including the extent of extrapulmonary dissemination[44].

There are multiple potential mechanisms for AGE formation in this study. Hyperglycemia may induce AGEs via direct interactions between glucose and serum or extracellular matrix proteins. Alternatively, increased intracellular glucose under hyperglycemic conditions promotes rapid formation of dicarbonyl intermediates of glycolysis including glyoxal, 3-deoxyglucasone and methylglyoxal, all potent inducers of AGE formation[45-47]. Accumulation of AGEs associated with chronic inflammation has been shown to occur[48] and methylglyoxal[49], as well as its precursor dihydroxyacetone[50], have been identified in association with TB lesions. This demonstrates the potential for at least localized AGE formation even in euglycemic animals infected with *M. tuberculosis*. AGE formation associated with *M. tuberculosis* infection was a prominent feature in this study where AGE accumulation increased in TB lesions between day 30 and 60 of infection even in water-fed guinea pigs. Additionally, elevated serum AGEs at day 60 of infection were equally elevated in both sucrose-fed and water-fed guinea pigs. Recently AGE formation and secretion by bacteria has been shown *in vitro* and may represent a third source of AGEs in *M. tuberculosis* infection[51]. However, the significant increase in tissue AGEs, which resulted from sucrose feeding, suggests that while bacteria and local tissue metabolism

may be a source for AGEs during *M. tuberculosis* infection, the accumulation is greatly exacerbated by hyperglycemia.

The importance of understanding the impact diabetes and hyperglycemia have on TB pathogenesis and treatment has been highlighted as a global priority[7]. Aside from overt clinical diabetes, the results herein indicate the potential for hyperglycemia in a non-diabetic state to significantly worsen active TB. These data emphasize the potential impact of stringent screening of glycemic responses in TB patients at the time of diagnosis even if diagnostic criteria for diabetes are not met[20, 52]. Our data indicate that inflammation-associated insulin resistance during *M. tuberculosis* infection may be an additional factor contributing to hyperglycemia. Based on our data, impaired control of *M. tuberculosis* infection, amplification of the inflammatory response and exacerbated extrapulmonary dissemination are evident in association with hyperglycemia-mediated AGE accumulation, which may represent a central mechanism in the pathobiology of diabetes-tuberculosis comorbidity.

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CHAPTER 3

Developing a Model of Diet-Induced Glucose Intolerance and Type 2 Diabetes in the Guinea Pig

Rationale: An overall goal of this project is to advance the current understanding of the relationship between DM and TB with an animal model that accurately reflects the pathogenesis of both diseases in humans. The guinea pig is well regarded as a tuberculosis model due to remarkable similarities to human granuloma development and morphology. As such, the guinea pig has been relied upon for the preclinical evaluation of both new therapeutic and preventive strategies for TB. However, the use of the guinea pig species as model of diabetes, in general, is limited. Since the overwhelming majority of diabetes cases worldwide, as well as diabetes-associated cases of TB are due to type 2 diabetes, a model of type 2 diabetes is critical for understanding the mechanisms of this rapidly emerging comorbidity. This chapter describes the development of the first model of diet-induced insulin resistance and type 2 diabetes in the guinea pig. In the chapters to follow, the use of this novel diabetes model will represent the first use of a representative animal model of type 2 diabetes and TB to study to study the two diseases combined.

Hypothesis: A guinea pig model that closely replicates the human pathogenesis of type 2 diabetes can be generated through a two-stage process first involving induction of insulin resistance by dietary manipulation and second, through β-cell decompensation by subtotal cytotoxic β-cell depletion.

Summary.

The goal of this study was to develop a model of type 2 diabetes in outbred guinea pigs that closely replicates the typical pathogenesis of human type 2 diabetes associated obesity and insulin resistance. Previously, the guinea pig has never been used as a model of type 2 diabetes and has infrequently been reported as a diabetic model using chemical-mediated β-cell cytotoxicity with the compound streptozotocin (STZ), but with significant variability and a high level of mortality. To optimize the dose and efficacy of hyperglycemic induction, while minimizing mortality with STZ, guinea pigs were treated with a range of STZ doses either by subcutaneous or intraperitoneal route, with or without pretreatment with the α2 adrenergic antagonist, yohimbine, and with STZ prepared as an anomer equilibrated solution. In this study, guinea pigs responded to STZ doses of 200 and 250 mg/kg while minimizing mortality and anomer-equilibration was found to have a similar hyperglycemic induction rate of 25-50%. Complete hyperglycemic conversion occurred in all animals when administered yohimbine prior to STZ at a dose of 250 mg/kg. However, this hyperglycemic effect was transient and diminished along with glucose intolerance by 21 days after treatment. To induce insulin resistance, guinea pigs were fed a high fat, high carbohydrate diet, which led to impaired glucose tolerance within eight weeks alongside compensatory hyperinsulinemia and normal fasting glucose. To eliminate the capacity for compensation, guinea pigs were treated with an optimized STZ treatment of 200 mg/kg after yohimbine administration, which yielded a reduction in plasma insulin and a stable level of glucose intolerance consistent with type 2 diabetes. In conclusion, we have demonstrated that the effect of STZ alone in the guinea pig is transient and that a steady diabetic state can be achieved through dietary induction of glucose intolerance and an optimized STZ treatment protocol to reduce β-cell mass, methodology that mimics the typical progression of human type 2 diabetes.

Introduction.

The development of type 2 diabetes requires the presence of both peripheral insulin resistance and impaired β-cell function[1]. During a state of insulin resistance, insulin-mediated glucose disposal is impaired and leads to a compensatory hypersecretion of insulin by pancreatic β-cells to maintain a state of euglycemia[2]. The typical progression of obesity-associated, polygenic type 2 diabetes spans a period of years where impaired glucose tolerance, detectable by an oral glucose tolerance test, progressively worsens to a diabetic state, as the β-cell compensatory response is lost and often, the severity of insulin resistance increases[3]. Consequently, the lower circulating insulin concentrations and associated reduced β-cell mass of type 2 diabetes patients is unable to maintain a euglycemic state[4]. To better mimic the growing trend in poor diet, reduced physical activity and obesity, this typical pathogenesis involving insulin resistance and hyperinsulinemia is often replicated in rodents through dietary manipulation.

A number of well-characterized rodent models of type 2 diabetes exist involving either high fat diet-induced glucose intolerance in mice and rats[5, 6] as well as spontaneous or targeted mutations either directly disrupting insulin signaling or leading to alterations in lipid metabolism[4]. Many of the models involving genetic defects develop severe obesity and metabolic disturbances, which typically mimic the most severe manifestations of diabetes. In contrast, the use of high-fat diet in guinea pigs specifically for the induction of glucose intolerance and insulin resistance has not been previously described. Guinea pigs fed a high carbohydrate diet or a diet high in saturated fat; however, have been emphasized as models of dietary-associated inflammation, dyslipidemia and atherosclerosis[7, 8]. The cardiovascular risks and atherogenic susceptibility of this species can be attributed to lipid metabolism that is comparable to humans. Unlike all other laboratory rodent species that have serum lipoprotein profiles dominated by

high-density lipoproteins, the majority of guinea pig lipoproteins are the low-density type, similar to humans[9-11]. Guinea pigs fed a high carbohydrate diet develop evidence of systemic inflammation including high TNFα and IL-6 production[12, 13], which are also characteristic features of the inflammatory pathogenesis of insulin resistance and type 2 diabetes in humans[14, 15]. Therefore, guinea pigs are a suitable species for studying the impact of dietary-induced alterations in lipid metabolism, the primary factor contributing to the development of insulin resistance.

Previously, induction of diabetes in the guinea pig has been limited to the use of the β-cell cytotoxic compound streptozotocin (STZ) alone, with the goal of obtaining a model of absolute insulin deficiency that is more reflective of insulin-dependent or type 1 diabetes. The use of STZ to induce diabetic hyperglycemia in guinea pigs has been reported with variable efficacy, dose, and route of administration, and often involves significant levels of mortality in the acute stages after STZ administration[16-20]. However, the degree of mortality associated with STZ toxicity, compared to severe diabetic hyperglycemia has not been specifically addressed and an optimized model for the use of STZ in guinea pigs has not been developed. Furthermore, previous studies have suggested that the guinea pig is resistant to the hyperglycemic effects of β-cell cytotoxins, including both STZ and alloxan[21]. As a result, the degree of variability in the literature lends limited credibility to the guinea pig as a suitable model of chemical-induced, insulin-dependent diabetes.

In this study, we have developed a guinea pig model that mimics the two stages typical of human type 2 diabetes, initial insulin resistance followed by reduced beta cell function. A high fat, high carbohydrate diet was used initially to induce glucose intolerance and followed by STZ treatment to induce a partial decline in insulin secretory capacity by reducing overall β-cell mass. The goal of combining STZ with diet-induced glucose intolerance was to abolish the hyperinsulinemic compensatory response and unmask hyperglycemia consistent with type 2

diabetes. Herein, we describe a guinea pig model that demonstrates evidence of insulin resistance and β-cell loss, the two principle components in the natural progression of type 2 diabetes in humans.

Materials and Methods.

Animal care and sample collection.

Female, outbred Dunkin-Hartley guinea pigs, weighing between 250 and 300 grams, were purchased from Charles River Laboratories and diabetogenic treatments were initiated at a weight of approximately 300 grams in mixed gender guinea pigs. Guinea pigs with various dietary and diabetogenic interventions were monitored by weight change, water intake and food consumption. For collection of serum, guinea pigs were anesthetized via isoflurane inhalation, placed in dorsal recumbency, and blood collected from the cranial vena cava percutaneously at the junction of the manubrium and the first rib. Fasting was performed by removal of food overnight for a period of 12 hours. Glucose was measured routinely using the Freestyle Lite glucometer (Abbot, Alameda, CA) from a skin prick site adjacent to the most peripheral vein on the pinna. At the time of euthanasia, guinea pigs were administered 40 mg of ketamine and 0.5 mg of diazepam via intramusclar route for anesthetic induction. Anesthetized guinea pigs were administered a 750 mg dose of pentobarbital via intraperitoneal route for euthanasia.

Preparation of Streptozotocin (STZ) solution for injection.

All STZ preparations were dissolved in sodium citrate buffer at a pH of 4.5. Sodium citrate buffer for injection was prepared initially as a 40mM citric acid solution (pH 2.9). A solution of 40mM sodium citrate was titrated into the 40mM citric acid until a pH of 4.5 was reached. STZ, >75% α-anomer purity (Sigma, St. Louis, MO), was dissolved in the freshly

prepared sodium citrate buffer at a concentration of 100 mg/ml. The STZ solution was passed through a 0.22 micron filter to sterilize and administered either freshly prepared or as an equilibrated solution.

Optimization of STZ treatment for induction of diabetic hyperglycemia.

Various doses of STZ and routes of administration were evaluated for diabetogenic efficacy and level of mortality in guinea pigs initially fed a normal diet and subsequently in guinea pigs with diet-induced impaired glucose tolerance. Dunkin-Hartley guinea pigs (n=4 or 5) were administered STZ via subcutaneous or intraperitoneal route, at doses of 300 mg/kg, 250 mg/kg, or 200 mg/kg; or as multiple daily consecutive doses of 50 mg/kg for four or six days. In a separate experiment, STZ was prepared either fresh in citrate buffer or allowed to equilibrate between α and β anomers for two hours at 4°C and protected from light. Fresh or anomer-equilibrated STZ was administered via subcutaneous route at a dose of 200 mg/kg. Additionally, the diabetogenic efficacy of STZ was investigated for enhancement by pre-treating guinea pigs with 0.5 mg/kg of yohimbine, and α_2 adrenergic receptor antagonist, intramuscularly 20-30 minutes prior to STZ injection to increase the induction of hyperglycemia. For the depletion of β-cell mass in HFHC-fed guinea pigs, individuals with evidence of impaired glucose tolerance were administered a single 200 mg/kg dose of anomer-equilibrated STZ after pre-treatment with 0.5 mg/kg yohimbine administered by intramuscular route.

Custom Diet Formulation.

Guinea pigs were fed a custom formulated high fat and high carbohydrate (HFHC) diet (Dyets Inc., Bethlehem, PA). The custom formulated diet consisted of total calories as 30% fat, 52% carbohydrate, and 18% protein, where calories from fat were derived equally from Primex vegetable shortening and beef tallow, and carbohydrate calories were composed of 55%

fructose and 45% sucrose. For comparison, control guinea pigs were fed a conventional guinea pig diet containing 3% fat, 18% protein and 55% carbohydrates as fiber (Harlan-Teklad #2040).

Evaluation of insulin resistance and impaired glucose tolerance.

To evaluate the glucose-lowering effects of insulin in guinea pigs fed either normal or HFHC diet, regular acting human recombinant insulin (Humulin-R, Eli Lilly, Indianapolis, IN) was administered as a subcutaneous injection at a dose of 0.5 units/kg. At the time of injection, glucose was measured in unfasted guinea pigs using the Freestyle Lite glucometer then measured at 25, 50, 75, and 100 minutes after administration. To determine glucose tolerance in normal diet control, HFHC-fed and HFHC/STZ diabetic guinea pigs, a standardized oral glucose challenge consisting of a 2 g/kg bolus of D-glucose (0.5g/ml) was administered after a 12-hour fasting period (oral glucose tolerance test, OGTT). Glucose levels were measured at times 0, 60, 90, 120 or 150 minutes post-administration with the Freestyle Lite glucometer validated for accuracy in the guinea pig against the glucose oxidase method for quantification of glucose in serum.

Quantification of lipid parameters.

Total serum free fatty acid levels were measured by fluorescence in an assay utilizing a coupled enzymatic reaction (Cayman Chemical; Ann Arbor MI). Briefly, acyl CoA synthetase catalyzes fatty acid acylation of coenzyme A. The acyl CoA generated is oxidized by acyl CoA oxidase to generate hydrogen peroxide, which in the presence of horseradish peroxidase and 10-aceyl-3,7-dihydroxyphenoxazine (ADHP) generates fluorescence that is measured spectrophotometrically at an excitation wavelength of 530 nm and an emission wavelength of 585 nm. Total serum triglycerides were quantified by sequential enzymatic conversion with lipoprotein lipase, glycerol kinase and glycerol phosphate oxidase followed by peroxidase

mediated colorimetric change (Cayman Chemical, Ann Arbor, MI) measured spectrophotometrically at an absorbance of 450 nm.

Quantification of serum insulin.

The C-terminal decapeptide of the guinea pig insulin beta chain, amino acid sequence DDGFFYIPKD, was conjugated to the c-terminus of bovine serum albumin through custom synthesis (Bio-Synthesis Inc., Lewisville, TX). Four Balb/C mice were immunized with 100 µg of this conjugated protein initially in TiterMax adjuvant (Sigma, St. Louis, MO), then twice additionally at three-week intervals in Freund's Incomplete adjuvant (Sigma, St. Louis, MO). Serum titers were then evaluated by direct ELISA. Briefly, high binding polystyrene 96-well plates were coated with 50 µg of the target insulin peptide. Serum from each mouse, collected via submandibular vein, was diluted in a 2-fold serial dilution, beginning with a dilution of 1:8 and ending at 1:32,768. Specific serum antibodies were detected with HRP-conjugated, goat anti-mouse IgG and 3,3,5,5-Tetramethylbenzidine (TMB) substrate. All mice produced antibody at a titer of at least 16,384. Mice were euthanized and serum was collected. For the detection of guinea pig insulin, blood was collected from fasted guinea pigs, corresponding fasting glucose levels measured, and serum was coated on high-binding polystyrene plates overnight at 4°C at a 1:10 dilution in sodium bicarbonate buffer (pH 9.0). Guinea pig insulin was detected with pooled polyclonal mouse anti-serum (1:1000), followed by HRP-goat anti-mouse IgG antibody (1:1000) and TMB substrate. This assay was optimized with a detectable linear range of 0.1 - 7 ng/ml of insulin (APPENDIX Figure 3).

Treatment with oral antihyperglycemic therapy.

Beginning at 3 weeks after STZ treatment, diabetic guinea pigs were treated with a combination of metformin (25 mg daily) and glipizide (0.25 mg daily) in combination. Efficacy of

treatment was assessed after 14 days of daily therapy based on improvement in glucose tolerance by OGTT. Guinea pigs, either normal non-diabetic controls, diabetic mock-treated, or diabetic and treated with metformin and glipizide in combination, were followed for survival for 140 days with daily treatment five days per week.

Histology and area morphometry of guinea pig pancreas.

Tissues were removed at necropsy and fixed in 4% buffered paraformaldehyde. Sections of pancreas were sampled from either end of the pancreatic limbs or at the center of the organ. The tissues were paraffin embedded and 5 μm sections were stained with hematoxylin and eosin for histopathologic evaluation using routine methods. Morphometric analysis was performed using a Nikon 80i Eclipse microscope and StereoInvestigator software, version 10.02 (MBF bioscience, Williston, VT) with tissue area estimated using the area fraction fractionator method. The frequencies of islets within the quantified area were counted to determine a ratio of islets per mm^2 of pancreatic tissue.

Immunofluorescent detection of insulin in pancreatic tissue.

Paraffin embedded 5 μm sections of pancreas were deparaffinized and rehydrated in serial ethanol washes followed by antigen retrieval by boiling in target retrieval buffer (Dako, Carpinteria, CA) for 30 minutes. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 and blocking performed in 0.15mM glycine and 10% FBS, 1%BSA in PBS. Tissue sections were incubated with anti-proinsulin antibody, clone K36AC10 (Abcam, Cambridge, MA), at a 1:500 dilution overnight at 4°C. After 3 washes in tween-TBS, tissue sections were incubated with HRP conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA) for 60 minutes at room temperature, washed 3 times in tween-TBS, and the signal amplified with the tyramide signal amplification kit (Life Technologies, Grand Island, NY) employing AlexaFluor

488-labeled tyramide, as directed by the manufacturer. Purified murine IgG1 was applied as a primary antibody for a negative isotype control.

Results.

Optimized STZ dosing minimizes toxicity-associated mortality in the guinea pig model.

In developing a guinea pig model of STZ-induced hyperglycemia, three parameters were identified as being critical for a successful response to STZ without adverse effects, including dose, route, and preparation of the STZ-solution. We evaluated a range of STZ doses from 100 to 300 mg/kg, administered either IP or SC, and as a single dose or multiple daily injections of 50 mg/kg. In guinea pigs receiving 300 mg/kg, regardless of SC or IP route, or single *versus* multiple dosing, a high level mortality occurred within days after completing the treatment. Mortality in 300 mg/kg dosed guinea pigs ranged from 80% in those receiving a single dose by SC route (n=5), 75% in those receiving a single dose by IP route (n=4), and 100% in those receiving six consecutive daily doses of 50 mg/kg IP (n=4), using a multidose schedule shown to be effective in mice[22] (Fig 3.1).

At the time of euthanasia, morbidity was often accompanied by hypoglycemia and only a single guinea pig out of the 13 receiving a cumulative dose of 300 mg/kg ever developed evidence of hyperglycemia (Table 3.1). Evaluation of histopathology revealed that mortality in guinea pigs was the result of acute STZ-mediated toxicity. The most severe and frequent manifestation of acute STZ toxicity was acute necrosis of the intestinal mucosal epithelium followed by acute necrosis of renal tubular epithelium (Fig 3.2). There was no evidence of differences in toxicity-related pathology between route of administration and frequency of dosing in those receiving a total of 300 mg/kg.

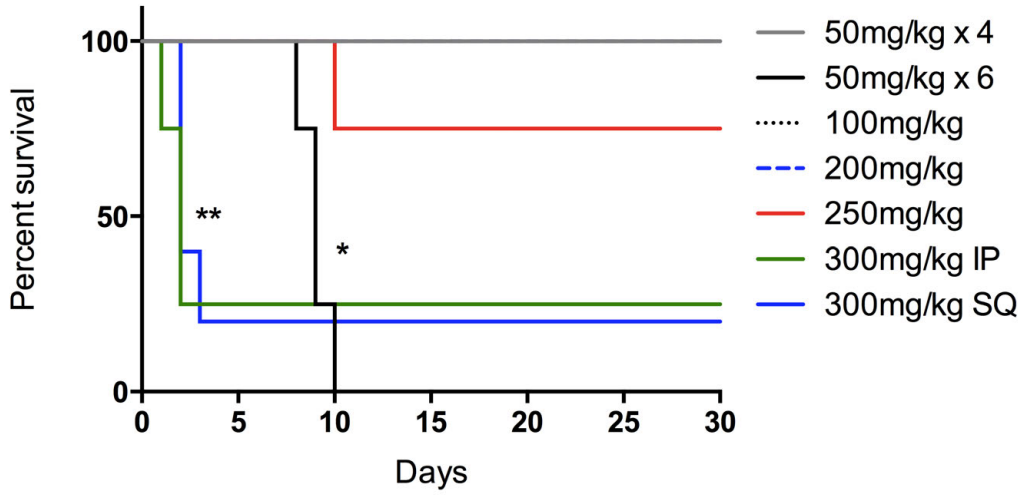

Figure 3.1. A Dose-related trend in mortality is associated with higher doses of STZ. The greatest proportion of survival is observed at doses of STZ equal to 200 mg/kg, while still achieving some hyperglycemic effect in the guinea pig. Intermediate survival is observed at a dose of 250 mg/kg but high levels of mortality occur at a dose of 300 mg/kg, whether administered as a single dose or a combination of multiple 50mg/kg doses. 100% survival was observed at doses of 50mg/kg x 4, 100mg/kg, and 200mg/kg. *P<0.05, **P<0.01

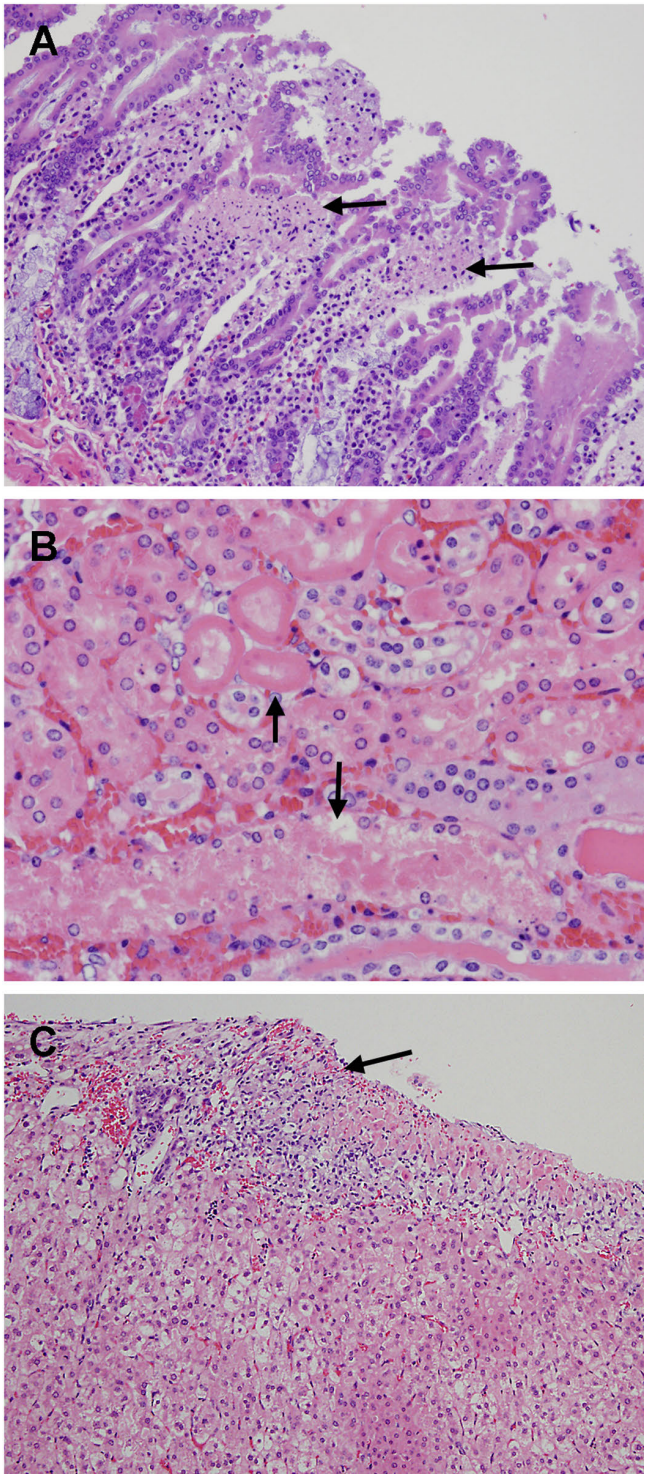

Figure 3.2. Acute toxicity associated with STZ in guinea pigs involves acute necrosis of GLUT2-expressing cell types. Necrosis of the surface enterocytes in the small intestine of a guinea pig receiving a 300 mg/kg dose of STZ (A). Acute renal tubular necrosis is multifocally present in the cortex of the kidney from a guinea pig receiving an STZ dose of 300 mg/kg (B). Associated with IP administration of STZ, there is capsular fibrosis and mild inflammation at the surface of the liver (C). Lesions are indicated by arrows, H&E stain.

Table 3.1. Summary of survival and rate of hyperglycemia with various STZ treatments.
AE = anomer-equilibrated, α_2= yohimbine, SQ = subcutaneous, IP = intraperitoneal

Dose (mg/kg)	Route	Sample Size	% Survival	%Hyperglycemic
100	IP	4	100%	0%
300	SQ	5	20%	20%
300	IP	4	25%	0%
50 x 6	IP	4	0%	0%
200	SQ	4	100%	50%
50 x 4	SQ	4	100%	0%
250	SQ	4	75%	25%
250	SQ	5	80%	40%
250 AE	SQ	5	100%	40%
200 AE	SQ	5	100%	20%
200 AE + α_2	SQ	5	100%	100%

While no significant differences in mortality were observed between IP and SC routes of administration, guinea pigs that received IP STZ in citrate buffer (pH 4.5) developed mild peritonitis and exuberant peritoneal and serosal fibrosis on visceral organs, precluding further use of this route of administration (Fig 3.2).

In contrast, guinea pigs receiving a single or multiple daily doses amounting to a total of 200 mg/kg displayed much improved tolerance and survival, with 100% survival out to 30 days post-injection (Fig 3.1). However, the hyperglycemia response rate was unacceptably low at this dose, with no conversion at 4 doses of 50 mg/kg (n=4) and only 50% with a single dose (n=4). This rate of response was not improved upon by a slightly higher single dose of STZ at 250 mg/kg (Table 3.1). Coinciding with a low rate of STZ-induced hyperglycemia at 300 mg/kg, there was no histological evidence of beta cell death within pancreatic islets observed at this high dose. However, acute cellular necrosis was evident within the islets of guinea pigs treated with 200 and 250 mg/kg doses of STZ and corresponded with aggregates of insulin protein presumably released from β-cell necrosis (Fig 3.3 and 3.10).

Because it is well demonstrated that the α-anomeric form of STZ is more biologically active and consequently, more likely to induce adverse toxicity, we evaluated the preparation of STZ as an equilibrated solution of α- and β-anomers previously shown to contain approximately 56% β- and 44% α-anomer composition[23-25]. The goal was to determine if, at an equivalent dose, the equilibrated solution of STZ is capable of producing a similar hyperglycemic response. At a dose of 250 mg/kg, both freshly prepared and anomer-equilibrated STZ induced non-fasting hyperglycemia in 40% of treated guinea pigs (n=5 each), indicating a similar efficacy between both preparations (Table 3.1). In these two groups, survival was minimally improved by administration of anomer-equilibrated STZ with 100% survival to day 21 in guinea pigs receiving anomer-equilibrated STZ compared to 80% in those receiving freshly prepared STZ.

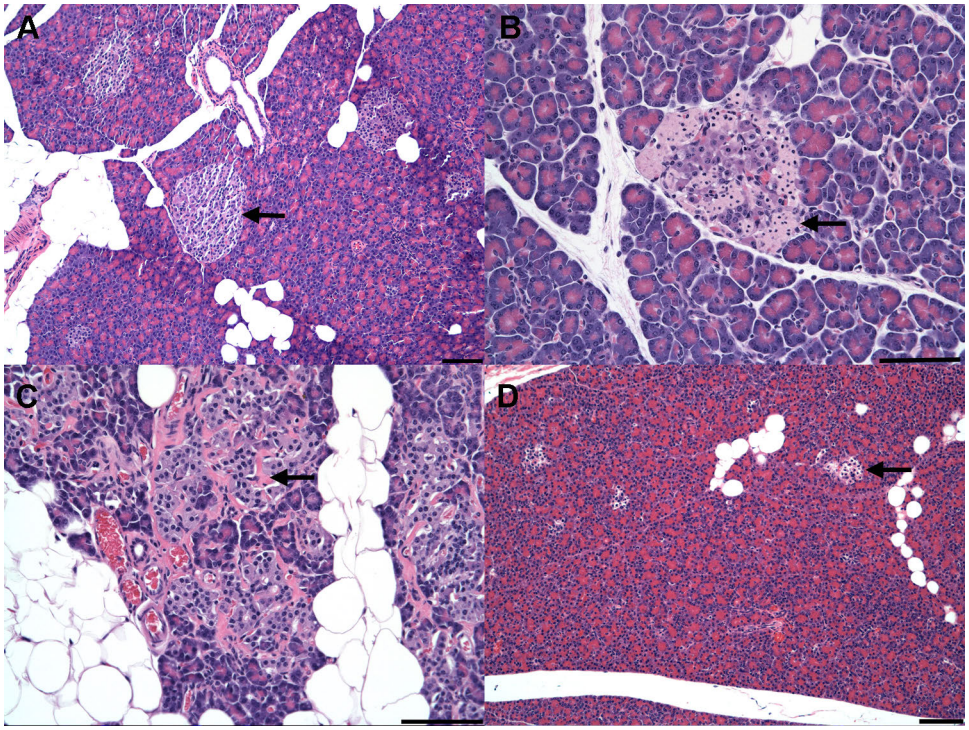

Figure 3.3. Pancreatic islet pathology is consistent with acute and chronic phases post-STZ and after long-term HFHC diet. (A) Normal guinea pig pancreatic islet, arrow. (B) Example of an islet from a guinea pig 24 hours after receiving a 250 mg/kg dose of STZ. There is selective necrosis of some islet cells (arrow) while others remain morphologically viable, presumably representing the selective toxicity of STZ for β-cells over other islet cell populations. (C) Pancreatic islet from a guinea pig allowed to consume HFHC-diet for six consecutive months without receiving STZ. The islets are confluent and are dissected by fibrosis (arrows). (D) Pancreatic islets of a guinea pig after 11 weeks of HFHC-diet and three weeks after receiving 250 mg/kg of STZ. The islets are shrunken, irregular and indistinct (arrow). Bar = 100µm.

Rate of STZ-induced hyperglycemia is enhanced with antecedent use of an α2-adrenergic receptor antagonist.

Since the success rate of achieving hyperglycemia in guinea pigs at mid-range doses of STZ remained low regardless of dose or route of administration (Table 3.1), additional adjunctive treatment was pursued to enhance the specific targeting of pancreatic β-cells. Previously, the α2-antagonist yohimbine was demonstrated to enhance the diabetogenic effects of STZ in mice[26]. We evaluated the rate of hyperglycemia from anomer-equilibrated STZ treatment at 200 mg/kg in guinea pigs either pre-treated with yohimbine or mock-treated with normal saline. Guinea pigs that were pre-treated with yohimbine (n=5) were 100% responsive to the STZ treatment as indicated by hyperglycemia present for the following seven-day period (Table 3.1, Fig 3.4). In contrast, of the guinea pigs mock-treated with saline prior to administration of STZ (n=5), only 20% developed hyperglycemia. Acute mortality did not occur at a dose of 200 mg/kg of anomer-equilibrated STZ.

STZ-induced hyperglycemia in guinea pigs is transient with recovery from glucose intolerance in the absence of HFHC diet.

At STZ doses of 200 and 250 mg/kg (with or without yohimbine), where hyperglycemia was manifested in 25-100% of treated guinea pigs, elevated glucose was evident within 48 hours. In the course of daily random glucose sampling, non-fasted hyperglycemia persisted in the range of 200 to 400 mg/dl, for 7 to 10 days. There was a consistent steady decline in the degree of hyperglycemia beginning around day 4 and extending through day 14 post-STZ treatment. The reduction in glucose over time was best demonstrated in yohimbine pre-treated guinea pigs, which yielded hyperglycemia success rate of 100% (Fig 3.4). This transient development of hyperglycemia was uniform across all guinea pigs that developed

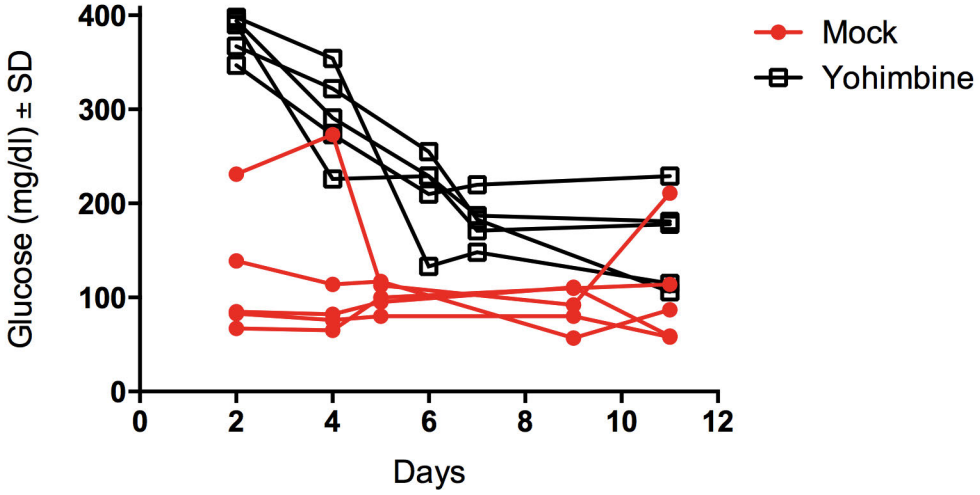

Figure 3.4. Random sampled hyperglycemia induced by STZ is transient. The average blood glucose trend measured without fasting is plotted through 11 days after STZ-treatment in guinea pigs treated subcutaneously with 250mg/kg of STZ and either mock pre-treatment or yohimbine pre-treatment. Regardless of the efficacy of the particular STZ-treatment in inducing hyperglycemia, random non-fasting glucose decreases over time. (n=5)

hyperglycemia from STZ treatment. Evaluation of glucose tolerance by OGTT in guinea pigs treated with STZ alone after 14 and 21 days demonstrated that glucose tolerance returned to normal over time, when compared to OGTT performed on day 7 after treatment where severe glucose intolerance was present (Fig 3.5).

Guinea pigs fed an HFHC-diet are not obese but do develop dyslipidemia.

Weight change was monitored over time in guinea pigs fed either a normal guinea pig diet or the HFHC diet, and also for the three weeks following STZ treatment of HFHC-fed guinea pigs. After eight weeks of consuming the HFHC diets, these guinea pigs weighed less than control guinea pigs fed a normal diet. This difference in weight was apparent in the transition between normal and HFHC diets, within the first week of initiating the HFHC feeding (Fig 3.6). Subsequently, HFHC-fed guinea pigs gained weight at a rate consistent with, but, on average, did not exceed the weight of normal diet controls. These data are consistent with the additional observation that HFHC-fed guinea pigs consumed a nearly equivalent caloric intake on a per weight, per day basis when compared to normal diet controls (Table 3.2). However, HFHC/STZ guinea pigs with diabetic levels of glucose intolerance showed steady weight loss over the three-week period following STZ administration. Average weights after the 11-week period are listed in Table 2. After five weeks of initiating the HFHC diet, increased water consumption was observed on average in the HFHC-fed guinea pigs (Table 3.2), but in contrast to the HFHC/STZ-treated guinea pigs where all animals had detectable glucose in urine, glucosuria was present in only one out of the five urine samples evaluated from this group. Both HFHC-fed and HFHC/STZ guinea pigs had elevated fasting serum triglyceride levels. However, elevations in total serum free fatty acids were present only in the HFHC/STZ guinea pigs (Fig 3.6).

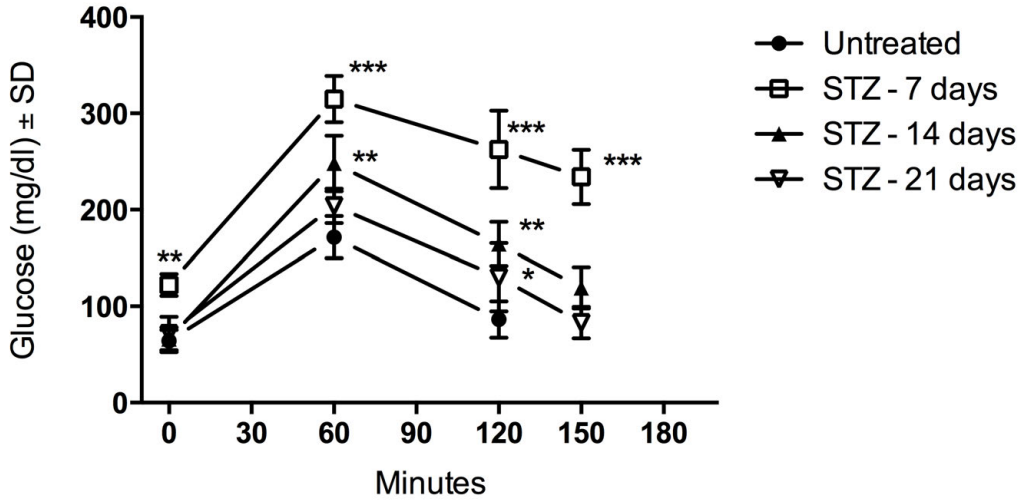

Figure 3.5. Diabetic glucose tolerance progressively wanes in STZ-only guinea pigs after a period of 21 days. Glucose intolerance measured by OGTT in guinea pigs treated with yohimbine prior to a 250 mg/kg dose of STZ. Severe glucose intolerance is evident seven days after STZ but steadily decreased over time and returned to near normal levels by 21 days after treatment. *P<0.05 , **P<0.01, ***P<0.001 (n=5)

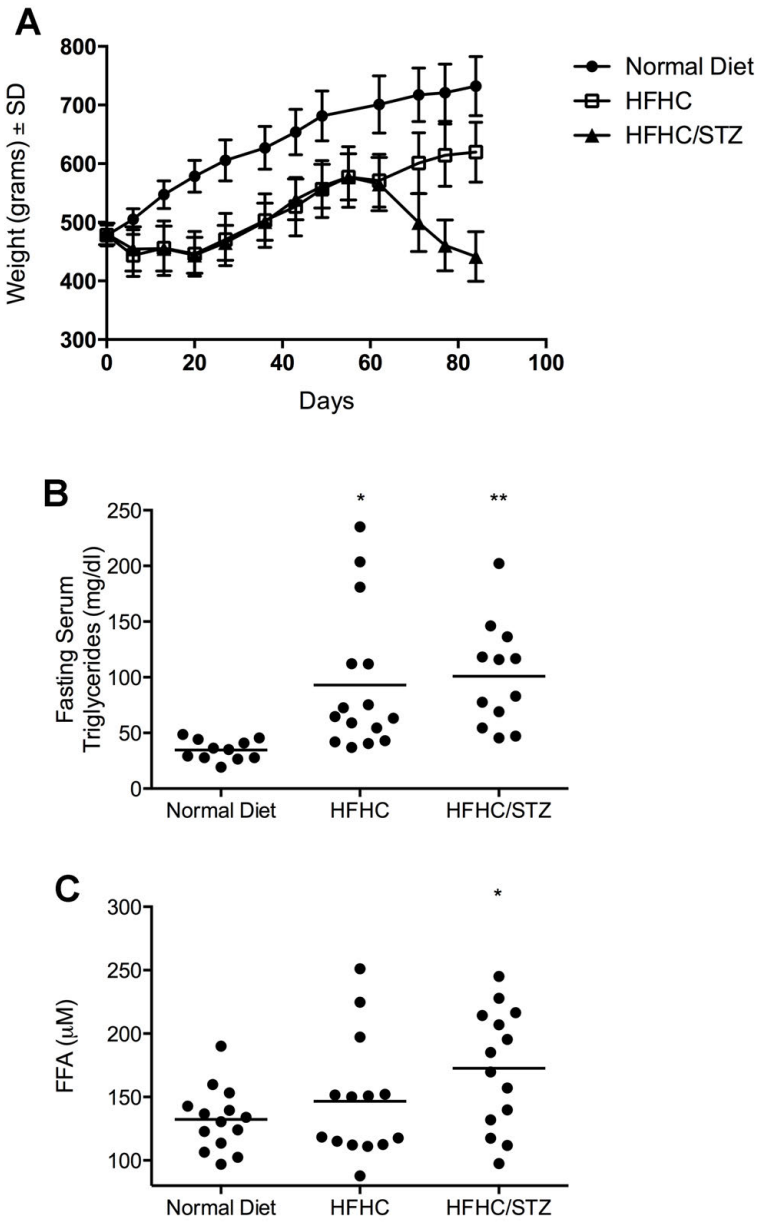

Figure 3.6. HFHC Fat Fed guinea pigs are not obese but do develop dyslipidemia. (A) Weight trends of normal diet control guinea pigs compared to HFHC-fed and HFHC/STZ groups are compared (n=20). After an initial brief period of weight loss, the rate of weight gain is similar between normal diet controls and HFHC-fed guinea pigs, but HFHC-fed guinea pigs do not exceed normal diet controls. However, significant weight loss occurs in the three-week period following induction of overt diabetes with STZ treatment. (B) After a six-hour fasting period, guinea pigs fed HFHC diet (n=15) and HFHC/STZ type 2 diabetic guinea pigs (n=12) demonstrate hypertriglyceridemia, while (C) elevated total serum free fatty acids are increased significantly only in HFHC/STZ diabetic guinea pigs (n=14 per group). *P<0.05 ,**P<0.01

Table 3.2. Clinical parameters from guinea pigs subjected to diabetogenic treatments.
ND = not detected, n/a = not measured, NS = not significant, BW = body weight (n=5)

	Chow-Fed	HFHC-Fed	HFHC/STZ	P-value
Weight *(g)*	732.4 ± 50.5	619.6 ± 51.1	441.6 ± 42.4	<0.0001
Water Consumption *(ml)*	65.3 ± 8.6	161 ± 19.6	n/a	0.001
Diet Consumption *(kcal/100g BW/day)*	40 ± 3.7	44.1 ± 5.2	n/a	NS
Urine Glucose *(mg glucose per g creatinine)*	ND	16.3 ± 36.5	90.2 ± 98.5	NS

HFHC-fed guinea pigs demonstrate impaired glucose tolerance and response to insulin with glucose intolerance reaching diabetic levels in HFHC/STZ guinea pigs.

HFHC-fed guinea pigs were evaluated for evidence of insulin resistance based on oral glucose tolerance test. No significant difference in the glucose response was observed between normal diet controls and HFHC-fed guinea pigs after two or four weeks on the diet. However, after 8 weeks of consuming the HFHC diet, impaired glucose tolerance was evident as increased average blood glucose at the 60-minute time point of the OGTT (n=20) (Fig 3.7). Significantly elevated fasting blood glucose was not ever observed in guinea pigs fed HFHC diet alone. The development of fasting hyperglycemia and severely impaired glucose tolerance was only evident in HFHC/STZ guinea pigs. These guinea pigs demonstrated significantly elevated blood glucose levels during OGTT, which persisted at 120 and 150 minutes after glucose administration (Fig 3.7).

HFHC-fed guinea pigs display compensatory hyperinsulinemia, which is eliminated in HFHC/STZ guinea pigs.

Fasted serum insulin concentrations were compared along with fasting blood glucose from the same blood sample to determine if a compensatory response was present in HFHC-fed and HFHC/STZ guinea pigs. Compared to normal diet controls, hyperinsulinemia was present in HFHC-fed guinea pigs after consuming the diet for 8 weeks (Fig 3.8). However, the fasting glucose concentrations did not differ from those of normal diet controls. Three weeks after STZ-treatment, the serum insulin concentrations of HFHC guinea pigs were decreased to a level comparable to those of the normal diet controls (Fig 3.8).

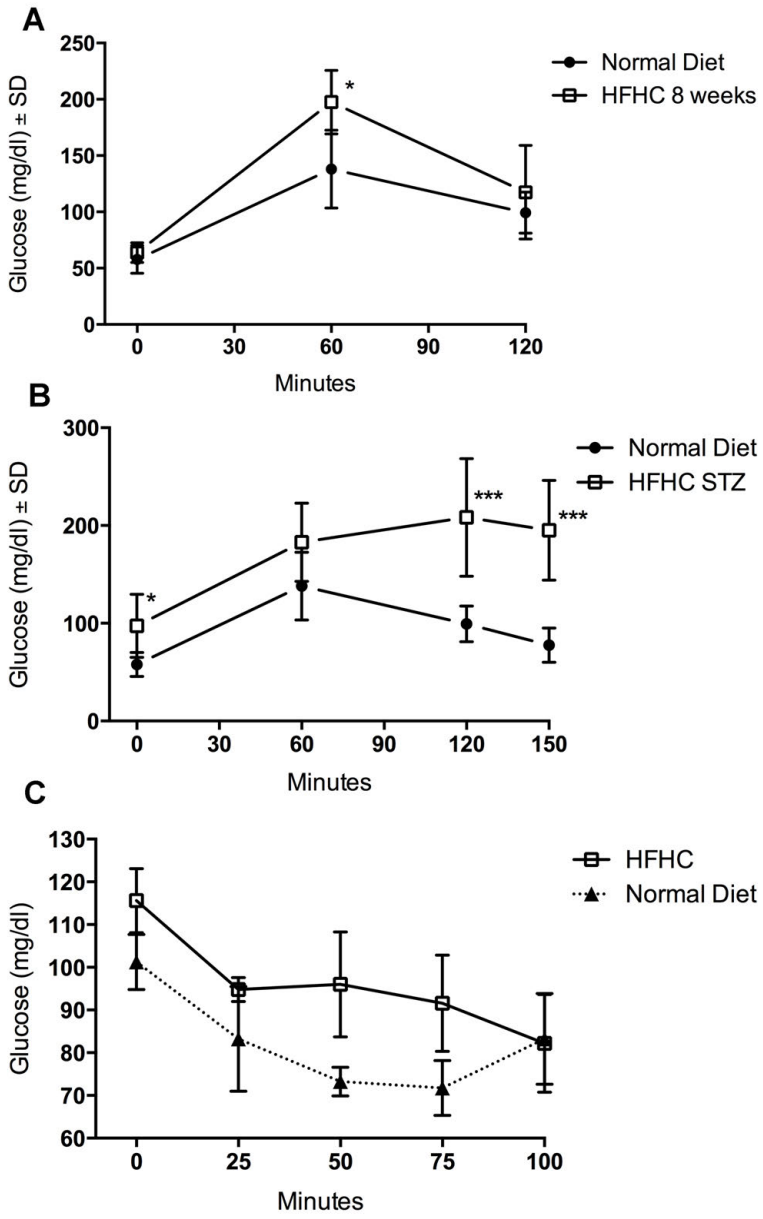

Figure 3.7. HFHC-fed guinea pigs are glucose intolerant and insulin resistant. (A) Oral glucose tolerance test performed in HFHC-fed guinea pigs reveals delayed glucose clearance at 60 minutes post-glucose administration in the OGTT after eight weeks of HFHC feeding (n=20). (B) HFHC guinea pigs were treated with 200 mg/kg STZ after eight weeks on the diet, and OGTT performed 20 days after STZ treatment. A diabetic level of glucose intolerance is retained in these guinea pigs 20 days post-STZ treatment (n=20). (C) Insulin tolerance test performed in HFHC guinea pigs after 6 weeks of HFHC feeding. Exogenous insulin failed to reduce blood glucose at a level comparable to normal diet controls (n=5). *P<0.05 ,***P<0.001

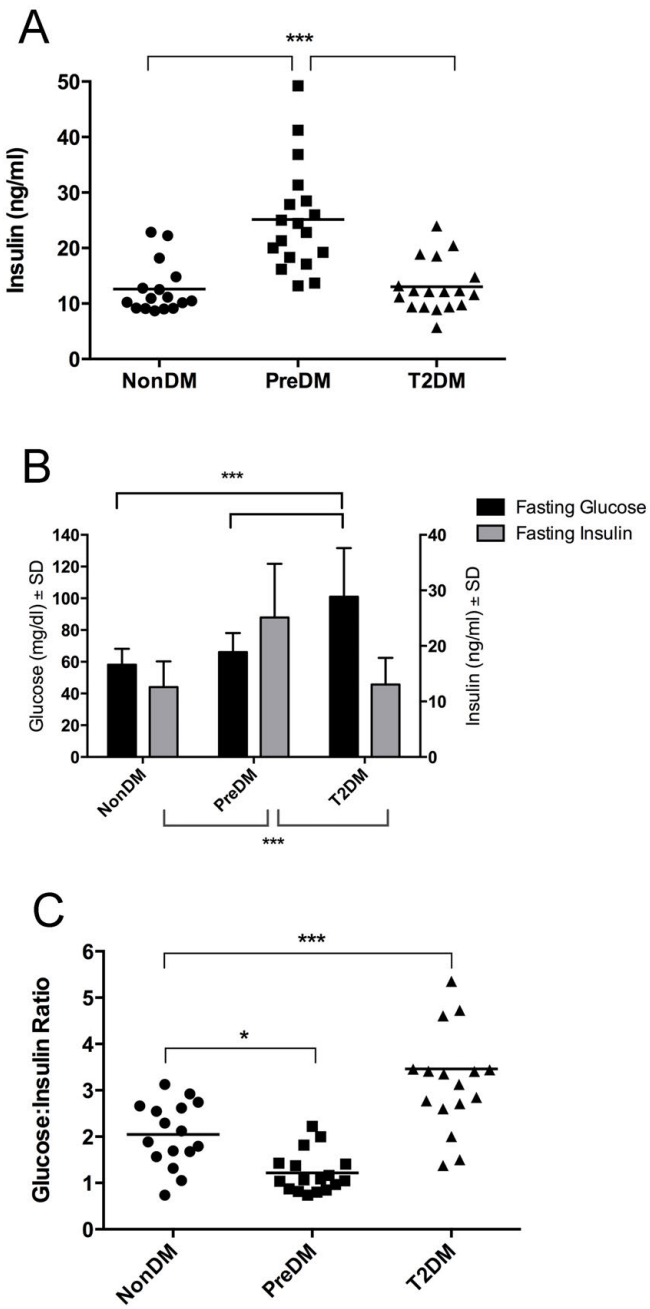

Figure 3.8. Guinea pigs fed HFHC diet for 8 weeks develop compensating hyperinsulinemia, which is lost after treatment with STZ. (A) Fasting serum insulin concentrations, measured by direct ELISA, are significantly higher in HFHC (PreDM) guinea pigs compared to normal diet controls (NonDM) and HFHC/STZ guinea pigs (T2DM). (B) Similar fasting glucose concentrations in HFHC guinea pigs compared to normal diet controls indicating a compensatory response by elevated insulin in HFHC guinea pigs. In contrast, compensation for insulin resistance is no longer present after STZ-induced reduction in insulin production, leading to fasting hyperglycemia. These fasting insulin and glucose levels are expressed as a ratio of glucose:insulin for each individual guinea pig to demonstrate elevated insulin and lower glucose in HFHC guinea pigs compared to lower insulin and elevated glucose typical of diabetic STZ/HFHC guinea pigs. *P<0.05, ***P<0.001(n=16 per group)

Islet cell hyperplasia accompanies HFHC-induced hyperinsulinemia and insulin-production is retained in HFHC/STZ guinea pigs.

The pancreatic islets of HFHC-fed guinea pigs were, in general, larger in size and quantitatively more numerous per mm^2 of total evaluated pancreatic tissue when compared to normal diet controls (Fig 3.9). Immunofluorescent detection of proinsulin in the HFHC-guinea pigs revealed a high frequency of insulin producing cells in all islets, including those that were significantly enlarged (Fig 3.10). In contrast, islet morphology was altered in HFHC/STZ guinea pigs with generally reduced islet size, and indiscernible, irregular margins (Fig 3.3). Immunofluorescent detection of proinsulin in the HFHC/STZ guinea pigs revealed a retention of insulin-producing β-cells, but these were significantly reduced in frequency compared to the normal diet controls and HFHC-fed guinea pigs (Fig 3.10).

HFHC/STZ guinea pigs are non-insulin dependent and responsive to oral antihyperglycemic therapy.

Prior to initiation of oral antihyperglycemic therapy, three weeks after STZ treatment and 11 weeks total of HFHC feeding, HFHC/STZ guinea pigs displayed a degree of glucose intolerance consistent with overt diabetes. Complete reversal of diabetes-related glucose intolerance was evident in guinea pigs treated with a combination of both metformin and glipizide after 14 days of treatment (Fig 3.11). Guinea pigs treated with metformin and glipizide in combination (n=4) displayed 100% survival out to 120 days while only a 25% survival rate was evident among mock-treated diabetic controls (n=4) during this time (Fig 3.11).

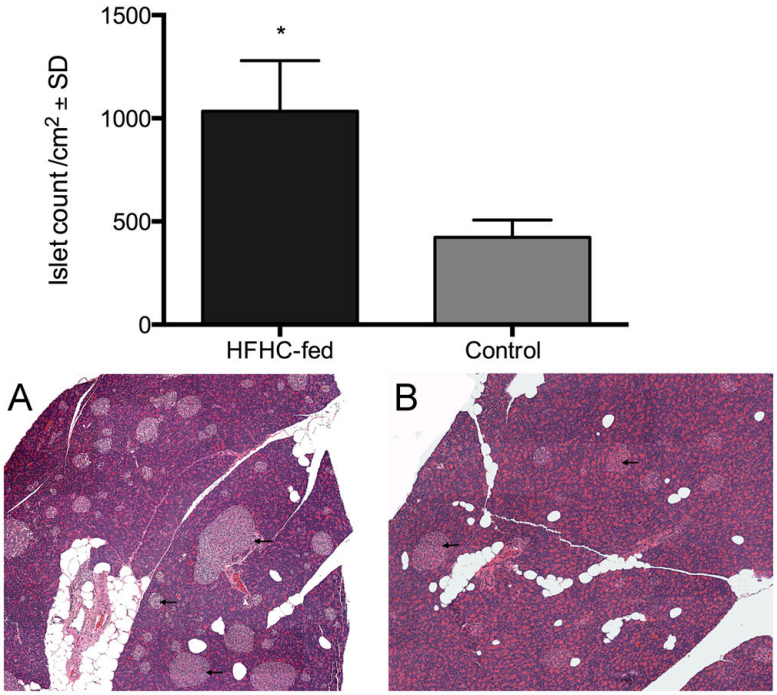

Figure 3.9. Islet cell hyperplasia is present in guinea pigs fed an HFHC diet. Count of absolute numbers of pancreatic islets in HFHC-fed guinea pigs is, on average, 2.4-fold higher per cm2 compared to normal diet controls (upper panel). (A) High frequency of pancreatic islets (arrows) in HFHC-fed guinea pigs with significant variability in size. (B) Histology of the pancreas from normal diet control guinea pigs demonstrates frequency of pancreatic islets (arrows). *P<0.05 (n=5 per group)

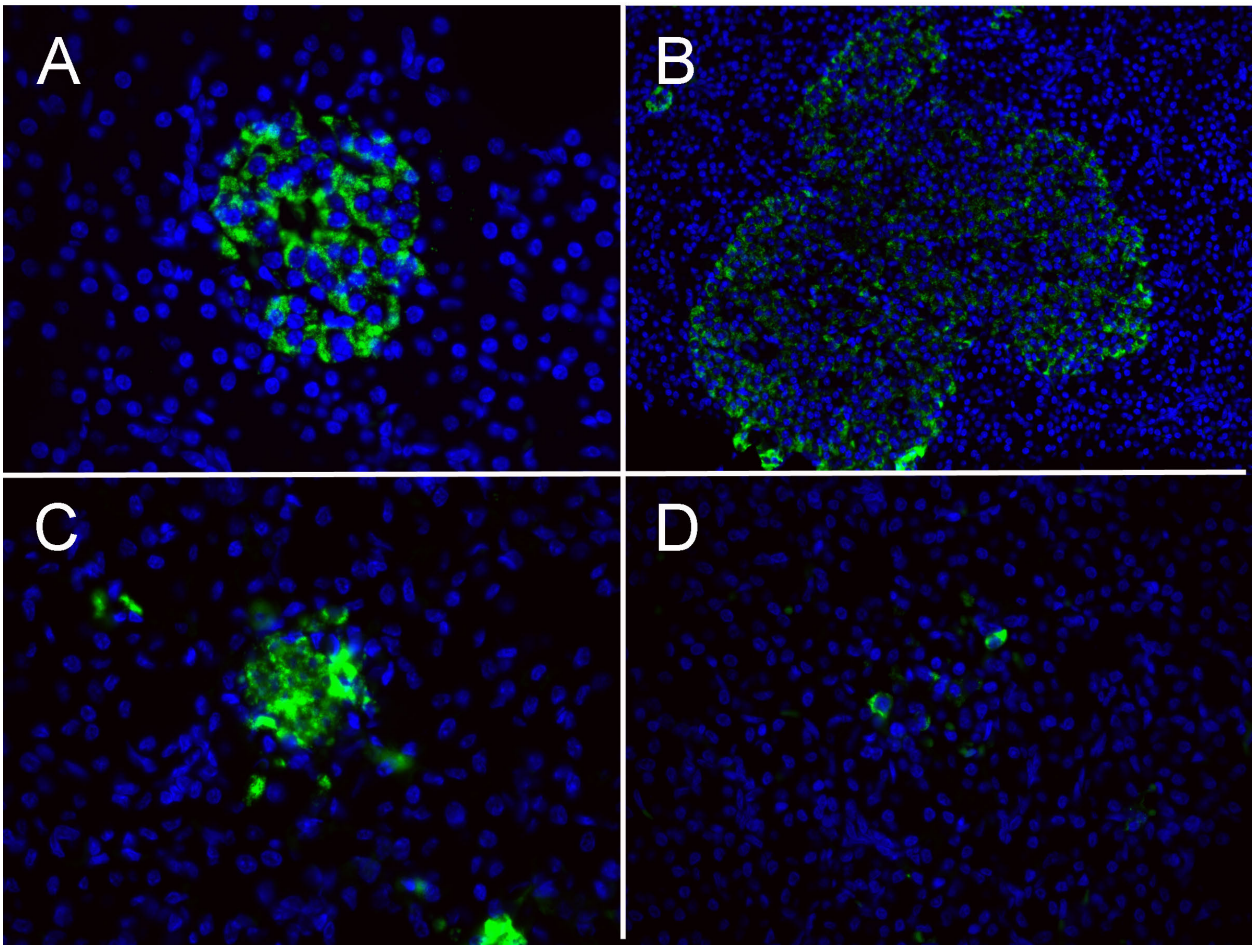

Figure 3.10. Insulin immunoreactivity is frequent in enlarged islets of HFHC-guinea pigs and retained in HFHC/STZ guinea pigs. Representative photomicrographs of insulin immunoreactivity of pancreatic tissue after various diabetogenic treatments. (A) Frequency of insulin producing cells in the islets of a normal diet control guinea pig. (B) Insulin producing cells are frequent within a significantly enlarged islet of an HFHC-fed guinea pig. (C) Destruction of normal morphology and aggregation of insulin in an islet 24 hours after receiving 250 mg/kg of STZ. (D) Insulin producing cells are significantly reduced but remain present in an HFHC guinea pig treated with 200 mg/kg of STZ. *green = insulin, blue = Hoechst nuclear counterstain.*

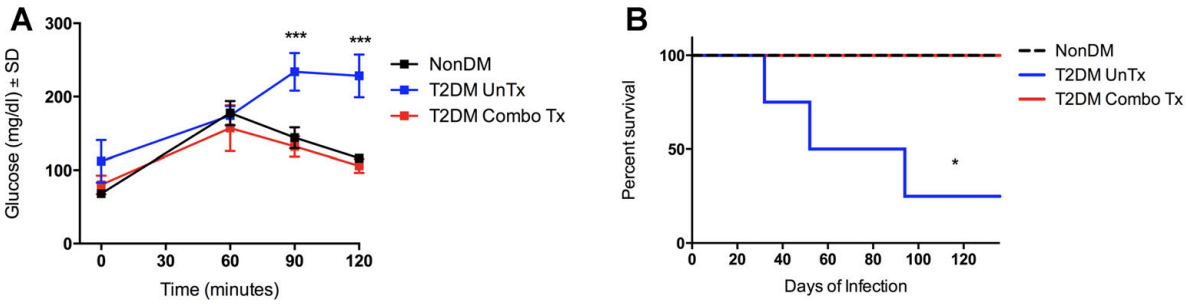

Figure 3.11. Oral antihyperglycemic therapy improves diabetic glucose intolerance to a level equivalent to non-diabetic controls. (A) Guinea pigs with type 2 diabetes were treated with either 25 mg/kg of metformin and 0.25 mg glipizide *per os* or mock-treated with oral water. An oral glucose tolerance test was performed on diabetic guinea pigs 14 days after treatment and compared to untreated, non-diabetic control guinea pigs. Treatment with metformin and glipizide in combination improves glucose tolerance in diabetic HFHC/STZ (T2DM) guinea pigs to a level comparable to normal non-diabetic controls (NonDM). (B) Combination therapy with metformin and glipizide greatly improves survival in T2DM HFHC/STZ guinea pigs compared to diabetic guinea pigs left uncontrolled. (NonDM, n=2; T2DM, n=4 per group) *P<0.05, ***P<0.001

Discussion.

In this study, we have developed a novel model of type 2 diabetes in the guinea pig through a combination of diet-induced glucose intolerance and subtotal β-cell cytotoxicity. In the process, we have identified critical factors necessary for improved survival, induction of hyperglycemia, and sustainable diabetic glucose intolerance induced with STZ in the guinea pig model. The use of STZ to establish insulin-dependent hyperglycemia in mice and rats is well established. The protocols for use of STZ in these rodents and in comparison to what has been identified in our guinea pig model demonstrates the wide variability in the response of multiple rodent species to STZ. The rat model requires only a single low dose of STZ for nearly complete β-cell cytoxicity, while five consecutive doses of 50 mg/kg is generally accepted as the most effective dosing schedule for mice[27]. Comparatively, we have demonstrated that the guinea pig generally requires higher doses of STZ as a single bolus.

It is quite apparent from this study that regardless of route of administration or if administered as a single or multiple doses of 50 mg/kg, a total dose of 300 mg/kg is not tolerated, yielding high mortality by day 10 post-administration. In contrast, a much lower dose of 100 mg/kg is well tolerated by IP and SQ routes of administration for at least 30 days after administration, but this dose does not produce sustained hyperglycemia or glucose intolerance. Paradoxically, hyperglycemia also was not noted with the highest dose of 300 mg/kg while midrange doses of 200 and 250 mg/kg did produce hyperglycemia at varying levels. The lack of hyperglycemia is further supported by an additional lack of islet lesions in these animals. This could be explained by the level of acute toxicity seen at such a high dose, which precluded the establishment of diabetes due to failure to selectively target β-cells for cytotoxicity. Additionally, the rapid clinical decline and mortality may have occurred at a rate preceding diabetogenic effects detectable by random glucose sampling or glucose tolerance test.

Regardless, the pathological lesions identified in guinea pigs suffering mortality post-STZ treatment are most consistent with acute toxicity especially since all lesions were present in the major cell types expressing the GLUT2 solute transporter, including intestinal epithelium and kidney tubular epithelium, as well as pancreatic β-cells[28]. The STZ molecule, a nitrosurea glucose analogue, enters cells through the GLUT2 transporter, which dictates it's selective cytotoxicity of β-cells, and induces DNA alkylation leading to cell death[29, 30]. This also further supports the suggestion that guinea pig β-cells are similar to other rodent species, which are dominated by surface GLUT2 receptors and unlike human β-cells, which recently have been shown to utilize GLUT1 transporters in glucose sensing[30, 31].

Although dose appears to significantly impact STZ tolerance and the development of hyperglycemia in the guinea pig, we have also demonstrated similar efficacy between anomer-equilibrated STZ solution and STZ that is freshly prepared. It is well demonstrated that rapid decomposition of STZ occurs due to instability in solution, leading to a reduction in α-anomer forms of the molecule and a higher proportion of β-anomer forms. It has been suggested with frequency that the α-anomeric form is more diabetogenic than the β-anomer and, for this reason, it is often cited that STZ must be prepared fresh and administered immediately[32, 33]. However, a recent study demonstrated that an equilibrium is established between α- and β-anomeric forms of STZ within two hours of dissolution, and this preparation leads to consistent hyperglycemia in mice with a significant reduction in mortality[22, 34-36]. These findings are recapitulated in our guinea pig model, which demonstrates that anomer-equilibrated STZ is equally effective in inducing hyperglycemia in guinea pigs compared to an equivalent dose of freshly prepared STZ. This study therefore provides additional support for the adoption of anomer equilibration in STZ chemical-induced animal models of diabetes in an attempt to improve reproducibility and reliability of studies between laboratories.

The guinea pig is reportedly resistant to the diabetogenic effects of both STZ and alloxan. However, previous studies administering alloxan, a β-cell toxin similar to STZ, directly into the pancreatic circulation have yielded uniform hyperglycemia in guinea pigs, in contrast to those receiving systemic administration, which were largely insensitive[26]. Thus, the reported level of resistance to both alloxan and STZ in the guinea pig may not be a direct reflection of the β-cell itself, but rather the availability of the compound to the β-cells after systemic administration. This is further supported by the fact that isolated pancreatic islets from the guinea pig are sensitive to the actions of both compounds in vitro[37].

Protection against STZ-induced β-cell death by non-metabolizable glucose analogs indicates that competitive prevention of STZ uptake can prevent the toxic effects in other rodent species, but this protection is not afforded by the presence of high glucose[33]. Previously it has been reported that extended fasting prior to STZ administration yields improved β-cell cytotoxicity in the guinea pig[33]. However, this may not be due to the glucose lowering effects of starvation but rather, may be related to low insulin secretion present at the time of STZ administration or counter-regulatory mechanisms to prevent severe hypoglycemia.

In line with reports of resistance of guinea pigs to STZ, we experienced a rate of diabetes induction at doses of 200 and 250 mg/kg that was variable and incomplete[33]. However, also in line with the previously suggested difficulty associated with systemic administration, we were able to markedly improve the rate of STZ-induced hyperglycemia by treating guinea pigs with the α_2-adrenergic receptor antagonist, yohimbine, prior to administering STZ. This increased rate of hyperglycemia was associated with obvious selective islet cell death in islets of guinea pigs analyzed within 48 hours post-STZ administration. It has been previously demonstrated that STZ-induced diabetes could be improved through the use a specific α_2-adrenergic receptor antagonist but not through blockade of α_1 receptors[38, 39]. It is known that α_2-adrenergic receptors are involved in glucose-sensing and insulin secretion from

the pancreatic β-cell[27, 40], and that reduced circulating insulin in yohimbine-treated rats coincides with higher efficacy of STZ[41]. Our data indicates that yohimbine also improves sensitivity of guinea pig β-cells to STZ; however, if this is due to manipulation of insulin secretion remains to be evaluated with our recently developed ELISA assay.

Previous use of STZ in the spontaneously hypertensive rat (SHR) model, which develops hyperinsulinemia and insulin resistance in the absence of obesity, demonstrated that hyperglycemia results from doses of STZ that have no impact on glucose metabolism in normal wild-type rats[40]. This suggests that in the face of metabolic stress, compensating β-cells may be more susceptible to the cytotoxic effects of STZ. This is further supported by our data, which demonstrates transient hyperglycemia and glucose intolerance with STZ alone but a greater and persistent impact when combined with pre-existing diet-induced glucose intolerance. The transient hyperglycemic effect of STZ alone in guinea pigs suggests a significant compensatory or regenerative β-cell response in this species. Under otherwise normal β-cell physiology without the stress of dyslipidemia-associated insulin resistance both rodent and human β-cells have substantial regenerative capacity[42]. Therefore, this potential regenerative response in the guinea pig is not unexpected. However, the persistent diabetic glucose intolerance generated in STZ-treated guinea pigs after diet-induced glucose intolerance indicates that the metabolic stress of the high fat, high carbohydrate diet may limit the β-cell recovery potential. It is well documented that in the presence of insulin resistance, β-cells are limited in their proliferative capacity and are more prone to cell death[43]. Additionally, the islet cell hyperplasia and hyperinsulinemia present in HFHC-fed guinea pigs in this study is in line with the typical response of other rodent models of type 2 diabetes and demonstrates further the proliferative and compensatory capacity of guinea pig β-cells[44-46].

The primary amino acid sequence of guinea pig insulin, and insulin of other hystricomorph rodents, is highly divergent, having only approximately 65% homology to human

insulin. In contrast, this sequence is generally highly conserved among other mammalian species[47, 48]. As a result, our ability to detect insulin by commercial ELISA-based assays against mouse, rat or human was limited in the guinea pig (data not shown) and we have accordingly developed a direct insulin ELISA for the purpose of validating this model. Measurement of serum insulin with this ELISA assay showed significantly higher production of insulin in guinea pigs fed an HFHC diet, and when considered in combination with the normal fasting glucose concentrations in these animals, is consistent with a successful compensatory response to peripheral insulin resistance. However, after treatment with STZ, HFHC guinea pigs had serum insulin concentrations similar to those of normal diet controls and developed both fasting hyperglycemia and severe glucose intolerance. These data indicate that treatment with STZ was able to reduce β-cell insulin production sufficiently to eliminate the compensatory response, but did not completely abolish the β-cell population.

The STZ-induced models of diabetes are often regarded as an insulin-dependent form of diabetes due to an absolute insulin deficiency. In contrast, we have shown that guinea pigs first fed an HFHC diet then subsequently treated with STZ develop hyperglycemia largely due to the presence of insulin resistance. This is based on the fact that fasting hyperglycemia was evident only after the compensating hyperinsulinemia of HFHC-fed guinea pigs was abolished through STZ treatment. We have also confirmed that insulin production and secretion is retained but at lower levels in HFHC/STZ guinea pigs both within the pancreatic islets and in serum. Therefore, we had hypothesized that HFHC/STZ guinea would not only respond to the oral antihyperglycemic therapy frequently used to treat type 2 diabetes and insulin resistance in humans, but also that this therapy would improve survival without requiring the use of exogenous insulin therapy. We have demonstrated here that this model of type 2 diabetes in the guinea pig is not insulin-dependent because extended survival occurs in guinea pigs treated

with a combination of metformin and glipizide, at doses capable of reversing diabetic glucose intolerance.

Previously, the guinea pig model of STZ-induced hyperglycemia has been difficult to interpret due to a significant degree of variability in the literature regarding effective dose and route, responsiveness as indicated by hyperglycemic response, and high levels of reported mortality. In summary, we recommend the induction of acute STZ-induced hyperglycemia in the guinea pig through use of yohimbine pretreatment and anomer-equilibrated STZ solution at doses of 200 or 250 mg/kg administered by subcutaneous route. This combination of methods will minimize mortality and maximize the hyperglycemic response rate. For persistent diabetic levels of impaired glucose tolerance, the guinea pig species requires an additional component of β-cell stress, which we have demonstrated can be successfully accomplished with a high fat, high carbohydrate diet.

This study describes the development a novel guinea pig model of type 2 diabetes through careful consideration of the hyperglycemic response of guinea pigs to STZ, identification of optimal dosing ranges and adjunctive methods for improving β-cell loss and hyperglycemic induction in the guinea pig, and also through enhancing the relative stability of the diabetic state in the guinea pig beyond what is induced with STZ alone. Furthermore, we show that guinea pigs respond with more consistent and long-lasting diabetes when combined with an initial β-cell stressor, in this case a high-fat, high-carbohydrate diet, with the goal of replicating a natural progression in the typical pathogenesis of type 2 diabetes. Overall, this guinea pig model demonstrates important similarities to human type 2 diabetes including glucose intolerance that precedes diabetic levels of hyperglycemia, altered lipid metabolism and reduced compensatory β-cell capacity. The induction of diabetes in this model, which closely replicates the pathogenesis of human type 2 diabetes, will improve the future use of this species as a model of diabetes.

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CHAPTER 4

Increased Severity of Tuberculosis in Guinea Pigs with Type 2 Diabetes: A Model of Diabetes-Tuberculosis Comorbidity[2]

Rationale: The association of TB with type 2 diabetes is becoming increasingly recognized in TB endemic countries. In some regions, up to 30% of new TB diagnoses may be attributable to type 2 diabetes. However, the mechanisms by which diabetes, in general, increases the susceptibility to TB are poorly understood and an appropriate model of type 2 diabetes that can be used to better understand the pathogenesis of this comorbidity has not been developed. In the previous chapter, we developed a novel model of type 2 diabetes in the guinea pig, which develops the two characteristic features of type 2 diabetes, insulin resistance and reduced insulin secretory capacity. We propose that this model will aid in better understanding the immunological and metabolic mechanisms that may lead to more severe TB disease in people with type 2 diabetes. In this chapter, we use this guinea pig model of type 2 diabetes to characterize the impact on TB progression, bacterial growth and cellular immune response.

Hypothesis: Overt type 2 diabetes will further impair the control of bacterial growth and worsen TB disease, the impact of which will be more severe than that of non-diabetic hyperglycemia or diet-induced glucose intolerance and pre-diabetes.

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Summary.

Impaired glucose tolerance and type 2 diabetes was induced in guinea pigs to model the emerging comorbidty of *Mycobacterium tuberculosis* (*M. tuberculosis*) infection and diabetes. Non-insulin dependent diabetes was induced by low dose streptozotocin in guinea pigs rendered glucose intolerant by first feeding a high fat, high carbohydrate diet prior to *M. tuberculosis* exposure. *M. tuberculosis* infection of diabetic guinea pigs resulted in severe and rapidly progressive tuberculosis (TB) with a shortened survival interval, more severe pulmonary and extrapulmonary pathology and higher bacterial burden compared to glucose intolerant and non-diabetic controls. Compared to non-diabetics, diabetic guinea pigs with TB had an exacerbated pro-inflammatory response with more severe granulocytic inflammation and higher gene expression for the cytokines/chemokines IFNγ, IL-17A, IL-8 and IL-10 in the lung as well as IFNγ, TNFα, IL-8 and MCP-1 in the spleen. TB disease progression in guinea pigs with impaired glucose tolerance was similar to non-diabetic controls in the early stages of infection, but was more severe by day 90. The guinea pig model of type 2 diabetes-TB comorbidity mimics important features of the naturally occurring disease in humans. This model will be beneficial in understanding the complex pathogenesis of combined TB and diabetes and to test new strategies to improve TB and diabetes control when the two diseases occur together.

Introduction.

Host susceptibility to *Mycobacterium tuberculosis* (*M. tuberculosis*) is influenced by a variety of chronic communicable and noncommunicable diseases, which increase the risk of infection and the development of active tuberculosis (TB) disease. Moreover, *M. tuberculosis* infected patients with concurrent diseases often have more severe TB, which further complicates treatment responses to conventional antimicrobial drugs. The risk factors most frequently linked to *M. tuberculosis* susceptibility are HIV infection, malnutrition, tobacco use, air pollution, alcoholism, extremes in age, chronic kidney disease and diabetes. The highest relative risk for TB is associated with profound immunosuppression associated with HIV infection. However, recent epidemiological evidence suggests that, in the face of a growing diabetes epidemic, the population-attributable risk of diabetes may be equivalent to or exceed that of HIV/AIDS. In countries with the highest TB and diabetes incidence, type 2 diabetes in particular may account for up to 20% of active TB cases whereas less than 5% may be attributable to HIV[1, 2].

Type 2 diabetes accounts for approximately 95% of diabetes cases and is associated with obesity, poor diet, and a sedentary lifestyle, all of which are often linked to urbanization. An estimated 371 million people were diagnosed with type 2 diabetes in 2012, with the overwhelming majority residing in low- and middle-income countries. Moreover, there is an additional 280 million people that are prediabetic, many of which have undiagnosed insulin resistance with non-diabetic hyperglycemia and impaired glucose tolerance[3]. Furthermore, the global incidence of type 2 diabetes is projected to rise to approximately 552 million by 2030[4]. Evidence in human studies suggests that glucose control, more than any other feature of altered metabolism in diabetic patients, influences the susceptibility to *M. tuberculosis* infection, highlighting the importance of uncontrolled hyperglycemia in TB risk[5]. The increased difficulty in controlling blood glucose levels in diabetic patients with TB, combined with poor responses to

antimicrobial drug treatment, has the potential to further hamper current TB control efforts worldwide[6]. An animal model that more closely mimics the pathogenesis of this comorbidity in humans is essential for identifying more effective strategies for antimicrobial drug treatment and for control of blood glucose levels in diabetics with TB. In addition, the influence impaired glucose tolerance and insulin resistance has on TB susceptibility and pathogenesis has not been adequately investigated.

Currently, the animal models most often used to study the pathogenesis of TB concurrent with diabetes are inbred strains of mice treated with high doses of the cytotoxic drug streptozotocin[7, 8]. These models mimic chronic hyperglycemia resulting from total insulin deficiency as in human type 1 diabetes. While this strategy has provided valuable information on how absolute insulin deficiency and persistent hyperglycemia impact active TB disease, it fails to take into account a wide variety of other metabolic defects associated with impaired glucose tolerance and type 2 diabetes through dietary manipulation. Unlike murine models, lipid metabolism in the guinea pig more closely resembles that of humans, making this species ideal for studying cardiovascular disease risks and other consequences of altered glucose and lipid metabolism associated with type 2 diabetes[9]. Moreover, the majority of mouse strains used to model TB, including those previously used in diabetes-TB comorbidity studies, fail to respond to *M. tuberculosis* infection with the development of pulmonary and extrapulmonary granulomata with caseous necrosis as is typical in humans, as well as guinea pigs, non-human primates and rabbits[10]. The value of the guinea pig TB model has been further validated recently in studies of natural transmission of *M. tuberculosis* from human TB patients to guinea pigs. A subpopulation of guinea pigs exposed to aerosols from TB patients developed active TB disease and an array of clinical and pathological responses, which more accurately reflects the clinical variation of naturally occurring *M. tuberculosis* infection in humans[11].

Clinical studies have shown that the increased susceptibility of people with diabetes to *M. tuberculosis* is accompanied by an altered host response to infection. Diabetic patients with

active TB have higher bacterial burdens based on sputum culture and are refractory to first-line antimicrobial combination therapy with longer time to sputum conversion, and higher mortality rates during therapy[12-14]. Diabetic patients often have atypical radiographic findings with more frequent involvement of lower lung lobes and some studies indicate a higher rate of cavitary disease, consistent with more severe pulmonary pathology[15, 16]. However, the impact diabetes has on the development of extrapulmonary TB in humans is conflicting with some studies indicating a relative risk similar to, or less than that of non-diabetic individuals while others show a predisposition for extrapulmonary and even miliary disease patterns[5, 17, 18]. In patients with TB and poorly controlled type 2 diabetes, an exaggerated innate and type 1 cytokine response has been demonstrated clinically[19]. However, the impact insulin resistance alone has on the response to *M. tuberculosis* infection is unknown. The goals of this study were to develop an animal model that more closely mimics the clinical and immunologic manifestations of diabetes-TB comorbidity in humans.

Materials and Methods.

Induction and confirmation of glucose intolerance and type 2 diabetes.

Sixty outbred Dunkin-Hartley guinea pigs, weighing between 300-400 g, were obtained from Charles River Laboratories (Wilmington, MA) and maintained in individual housing. Guinea pigs were divided into three groups; non-diabetic, impaired glucose tolerance, and type 2 diabetic (n=20 per group). Eleven weeks prior to infection with *Mycobacterium tuberculosis,* guinea pigs were fed a custom-formulated high fat, high carbohydrate (HFHC) diet (Dyets Inc., Bethlehem, PA) *ad libitum* to induce impaired glucose tolerance. The diet consisted of 18% protein, 30% fat, and 52% carbohydrate with the carbohydrate portion consisting of 45% sucrose and 55% fructose. The dietary fat composition consisted of equivalent kCal/kg of beef tallow and Primex vegetable shortening creating a fatty acid composition of 42% saturated, 50%

monounsaturated, and 8% polyunsaturated fatty acids. After 8 weeks of HFHC diet, type 2 diabetes was induced in half of the guinea pigs with impaired glucose tolerance with a single subcutaneous injection of streptozotocin (STZ) to induce subtotal beta cell cytotoxicity[20]. STZ treatment consisted of an optimized single dose of 200 mg/kg in citrate buffer (pH 4.5) after anomer equilibration at 4°C for 2 hours[21] and 20 minutes after pre-treatment with an intramuscular injection of 0.5 mg/kg of the α2-adrenergic receptor antagonist, yohimbine[22]. A standardized oral glucose challenge (oral glucose tolerance test, OGTT) was used to assess the severity of glucose intolerance by administering a 2 g/kg dose of D-glucose (0.5g/ml) after a 12-hour fasting period and measuring glucose levels at times 0, 60, 90, 120 and 150 minutes post-administration with the Freestyle Lite glucometer (Abbot, Alameda, CA) validated for accuracy in the guinea pig against the glucose oxidase method, as previously described[23].

Infection with *M. tuberculosis* and euthanasia.

Low dose aerosol exposure of guinea pigs to *M. tuberculosis* was performed using the Madison chamber aerosol generation device calibrated to deliver approximately 20 bacilli of the H37Rv strain *of M. tuberculosis* (TMC102, Trudeau Institute, Saranac Lake, NY) isolated during log phase growth in Proskauer-Beck media. The course of infection was evaluated in non-diabetic, glucose intolerant and diabetic guinea pigs at the predetermined endpoints of TB disease progression, days 30, 60 or 90 of infection or to humane endpoints then euthanized (n=5 per group, per time point). At day 30 of infection, 7 out of 20 diabetic guinea pigs remained and were euthanized due to declining clinical condition. Euthanasia was performed by anesthetic induction with ketamine (40 mg) and diazepam (1 mg) prior to intraperitoneal injection of an overdose of sodium pentobarbital (1.5ml/kg).

Histopathology and determination of lesion burden.

To determine the impact impaired glucose tolerance and diabetes had on TB disease progression, histopathology was evaluated and lesion burden quantified on days 30, 60 and 90 of *M. tuberculosis* infection. Tissues were fixed in 4% buffered paraformaldehyde. Standardized sampling of the lung was performed by midsaggital sectioning of the left caudal lung lobe from each guinea pig at a predetermined anatomic location, irrespective of visible lung lesions. The head of the spleen from each animal was also uniformly sectioned for histopathologic evaluation. The tissues were paraffin embedded and 5 μm sections were stained with hematoxylin and eosin for histopathologic evaluation using routine methods. Morphometric analysis was performed using a Nikon 80i Eclipse microscope and StereoInvestigator software, version 10.02 (MBF bioscience, Williston, VT) with tissue area and lesion area estimated using the area fraction fractionator method and expressed as a percent ratio of lesion to total tissue area, as previously described[24].

Quantification of tissue bacterial burden.

Lung, spleen, and liver were homogenized in 1 ml of PBS and plated in serial dilutions on nutrient 7H11 agar and incubated at 37°C for 3-6 weeks. Colony forming units (CFUs) were log transformed and expressed as CFUs/g of tissue. In addition, the intrathoracic trachea was removed at necropsy, flushed with 1 ml of PBS, and plated on 7H11 agar undiluted. Data was expressed as either positive or negative for *M. tuberculosis* growth.

Quantification of serum lipids.

Total serum triglycerides were quantified by sequential enzymatic conversion with lipoprotein lipase, glycerol kinase and glycerol phosphate oxidase followed by peroxidase mediated colorimetric change (Cayman Chemical, Ann Arbor, MI) measured spectrophotometrically at an absorbance of 450 nm. Total serum free fatty acids (FFAs) were

quantified by sequential enzymatic conversion with ascorbate oxidase and acyl CoA oxidase followed by peroxidase mediated generation of fluorescence (Cayman Chemical, Ann Arbor, MI) measured spectrophotometrically at excitation 530 nm, and emission 585 nm, as previously described[23].

Quantification of tissue and serum advanced glycation end-products.

Lung tissue was mechanically homogenized in 1ml of PBS, centrifuged to remove insoluble material, and the supernatant used for AGE detection. Serum and cell lysate from lung homogenate were assayed for total protein by the BCA method and diluted to 10 µg/ml for AGE detection by ELISA (Cell Biolabs, San Diego, CA). Quantification was performed by interpolation from a standard curve derived from AGE-modified BSA and data expressed as µg of AGE-modified protein per ml. Antibodies utilized in this assay detect glycolaldehyde-derived AGEs including two of the most biologically prevalent, carboxymethyllysine (CML) and pentosidine.

RNA isolation from lung tissue.

The pulmonary circulation was perfused with RNasin RNase inhibitor (Promega, Madison, WI) (40 units/ml) in Hank's balanced salt solution (HBSS) at the time of necropsy. Perfused lung was collected in incomplete DMEM and maintained at 4°C unless otherwise indicated. Tissues were digested with collagenase D (0.7 mg/ml) and DNaseI (100 units) at 37°C for 30 minutes then pushed through a 70 µm cell strainer. Viable cells were separated from necrotic cell debris using Ficoll-Paque Premium (GE Life Sciences, Pittsburg, PA), washed twice in HBSS, and 1ml of Trizol reagent (Life Technologies, Grand Island, NY) added to the recovered viable cells. RNA isolation with Trizol was performed as instructed by the manufacturer followed by treatment with 10 units of DNaseI and recovery with an additional

phenol/chloroform separation and sodium acetate-ethanol precipitation. RNA integrity was assessed using the Agilent Technologies RNA 6000 nano chip (Santa Clara, CA) and samples with an RNA integrity number (RIN) greater than five utilized for relative gene expression.

Relative gene expression by quantitative RT-PCR.

The influence of impaired glucose tolerance and diabetes on the immune response to *M. tuberculosis* infection was measured by relative gene expression of selected key cytokines. Following cDNA synthesis, quantitative RT-PCR was performed with SYBR green detection (BioRad, Hercules, CA) using the BioRad CFX-96 real time thermal cycler per manufacturer's instructions with each reaction containing 0.2 µM of each primer and 50 ng of cDNA template. Under these reaction conditions, primer design was optimized for equivalent amplification efficiencies. Gene expression was normalized to two reference genes, hypoxanthine-guanine phosphoribosyltransferase (HPRT) and TATA-box binding protein (TBP), both of which were validated for consistent expression under these experimental conditions. The HPRT, IL-12p40, IFNγ, TNFα, IL-1β, TGFβ, and FoxP3 primer sequences used in this study are previously published [25-27]. TBP, IL-17A, IL-23, MCP-1 and IL-10 primers were designed using transcripts from the EMBL Ensembl annotated genome browser[28] for the guinea pig with the aid of Primer3 primer design software[29] and primers targeting IL-4 were designed from the recently published sequence[30]. The primer sequences are as follows: TBP (ENSCPOT00000001200) Forward 5'-CCAAGCGGTTTGCTGCTGTA, Reverse 5' GGCTCCTGTGCACACCATCTT; IL-10 (ENSCPOT00000009023) Forward 5'-GCCTTTGGCAGGGTGAAGAC, Reverse 5'-GGCTTGGCASACCCAGGTAAC; IL-17A (ENSCPOT00000010600) Forward 5'-AATGCCGTTACTCGGGCTGT, Reverse 5'-AGCGGGCAGTTCTGAGGTTC; MCP-1 (ENSCPOT00000013601) Forward 5'-AGCAGCAGGTGTCCCCAAGA, Reverse 5'-TCTCTGGTCCAGTTTGGCAATG; IL-23 (ENSCPOT00000015072) Forward 5'-

GCAACCACCACACCTTGCAAGAAA, Reverse 5'-ATCAGCAAAGACGTCCGTGACCAGC; IL-4 (NCBI NM_001257263) Forward 5'-GCAACCACCACACCTTGCAAGAAA, Reverse 5'-ATCAGCAAAGACGTCCGTGACCAGC. RNA isolates without reverse transcription were amplified for each animal to ensure a lack of contaminating genomic DNA and all assays performed included no-template controls. Data was expressed as normalized fold expression on a $\log_2$ scale.

Quantification of leukocyte phenotype in tissue by flow cytometry.

Single cell suspensions from lung, tracheobronchial lymph node, and spleen were prepared as previously described[31]. Cell suspensions were labeled with anti-guinea pig CD4-RPE, CD8-FITC, Pan T-cell-APC, CD45-FITC, MIL4 (granulocytes), MR1 (macrophages) and MHC-II antibodies (AbD Serotec, Raleigh, NC) as previously described[32]. Unconjugated antibodies were detected with a secondary anti-mouse IgG antibody conjugated with RPE fluorochrome for CD45 detection on CD8 T-cells, MHC-II and MIL4 antibodies; and FITC fluorochrome for the MR1 antibody. Data acquisition was performed with the BD Biosciences LSR II flow cytometer and analyzed with FACSDiva software (San Jose, CA) using a minimum of 100,000 events. Compensation for spectral overlap was performed as previously described[32].

Data analysis.

Analysis was performed with SAS 9.3 software. A one-factor analysis of variance was used for glucose tolerance test, morphometric microscopy, tissue bacterial culture and cytokine data with log transformation to correct for unequal variance and Tukey HSD used for pairwise comparisons. A two-factor analysis was used on the log transformed flow cytometry data and Tukey HSD used for pairwise comparisons between groups at each time point. To determine

differences between survival curves a Logrank test with Wilcoxon adjustment for multiple comparisons was utilized. Significance was set at $p \leq 0.05$.

Results.

HFHC diet impairs glucose tolerance in guinea pigs.

Guinea pigs fed a high fat, high carbohydrate diet for 8 weeks consistently developed impaired glucose tolerance as determined by OGTT. Delayed glucose utilization resulted in abnormally elevated blood glucose levels as early as 60 minutes after glucose challenge as expressed as a significant increase in total area under the curve (Figure 4.1, C and D) when compared to OGTT prior to initiating the diet (Figure4.1, A and B).

HFHC diet combined with streptozotocin induces non-insulin dependent diabetes in guinea pigs.

OGTT performed two weeks after STZ treatment of guinea pigs fed the HFHC diet showed fasting hyperglycemia and further exacerbation of impaired glucose tolerance with glucose levels remaining high at 150 minutes after glucose challenge (Figure 4.1, C and F) criteria consistent with human type 2 diabetes[33]. The impact of diabetogenic treatments on serum biochemical parameters after a 12-hour fasting period is summarized in Table 4.1. Guinea pigs with impaired glucose tolerance fed the HFHC diet only for 11 weeks, as well as guinea pigs with 3 weeks of uncontrolled diabetes both had elevated fasting triglycerides prior to *M. tuberculosis* infection. FFAs were significantly elevated only in the diabetic guinea pigs prior to *M. tuberculosis* infection.

Infection with *M. tuberculosis* impairs glucose tolerance in non-diabetic, HFHC fed only and diabetic guinea pigs.

At day 30 of infection, TB induced impaired glucose tolerance in non-diabetic guinea pigs with significantly higher levels at 120 minutes after glucose administration, and an overall increase in area under the curve compared to pre-infection glucose tolerance (Figure 4.2, A and B). Impaired glucose tolerance was more severe in HFHC fed animals and diabetic guinea pigs following *M. tuberculosis* infection with total area under the curve exceeding pre-infection, post-diabetogenic treatment levels (Figure 4.2, A and B). While impaired glucose tolerance persisted in HFHC fed alone guinea pigs through day 60 and 90 of infection, total area under the curve was not significantly different from pre-infection glucose tolerance in non-diabetic guinea pigs at day 60 and 90 of infection (Figure 4.2, C-F). However, despite minimal difference in total area under the curve, impaired glucose tolerance was evident at the endpoint of OGTT in non-diabetic guinea pigs (Figure 4.2, E and F). Infection with *M. tuberculosis* also markedly increased serum FFAs in all groups. Serum FFAs were highest at day 30 of infection and subsided as chronic infection was established (Table 4.1).

AGEs accumulate when diabetes and TB are combined.

Serum AGEs were elevated above preinfection levels by approximately 3-fold at day 30 of infection only in diabetic guinea pigs while no difference was evident between non-diabetic and guinea pigs with impaired glucose tolerance at days 30, 60, or 90 of *M. tuberculosis* infection (Table 4.1). Increased pulmonary tissue AGEs were present in *M. tuberculosis*-infected non-diabetic, glucose intolerant and diabetic guinea pigs to a similar degree (Table 4.1) at approximately 3-fold over uninfected non-diabetic lung (data not shown).

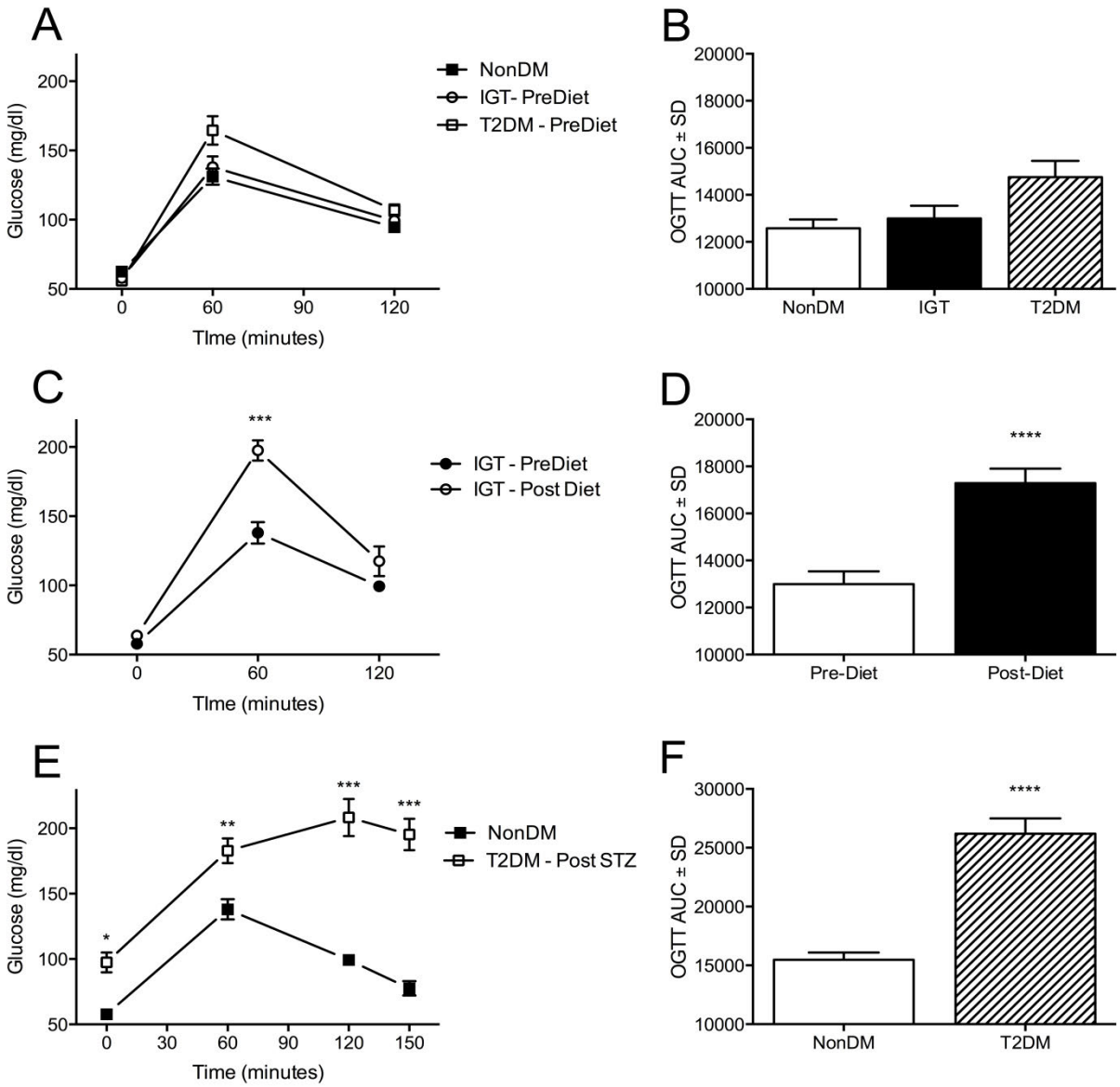

Figure 4.1. Glucose tolerance is impaired in guinea pigs with prediabetic impaired glucose tolerance and type 2 diabetes. Normal glucose tolerance in non-diabetic guinea pigs prior to initiating HFHC diet by oral glucose tolerance test (OGTT) (A) and corresponding area under the curve (AUC) (B). Feeding of high fat, high carbohydrate diet impairs glucose tolerance in guinea pigs fed the HFHC diet alone with reduced glucose disposal at 60 minutes of OGTT (C) and overall increase in AUC (D), compared to the same guinea pigs prior to initiating the diet. A diabetic level of impaired glucose tolerance in diabetic guinea pigs 11 weeks after combined HFHC/STZ diabetogenic treatment (E) with marked increase in AUC (F). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; NonDM = non-diabetic, IGT = impaired glucose tolerance, T2DM = diabetic

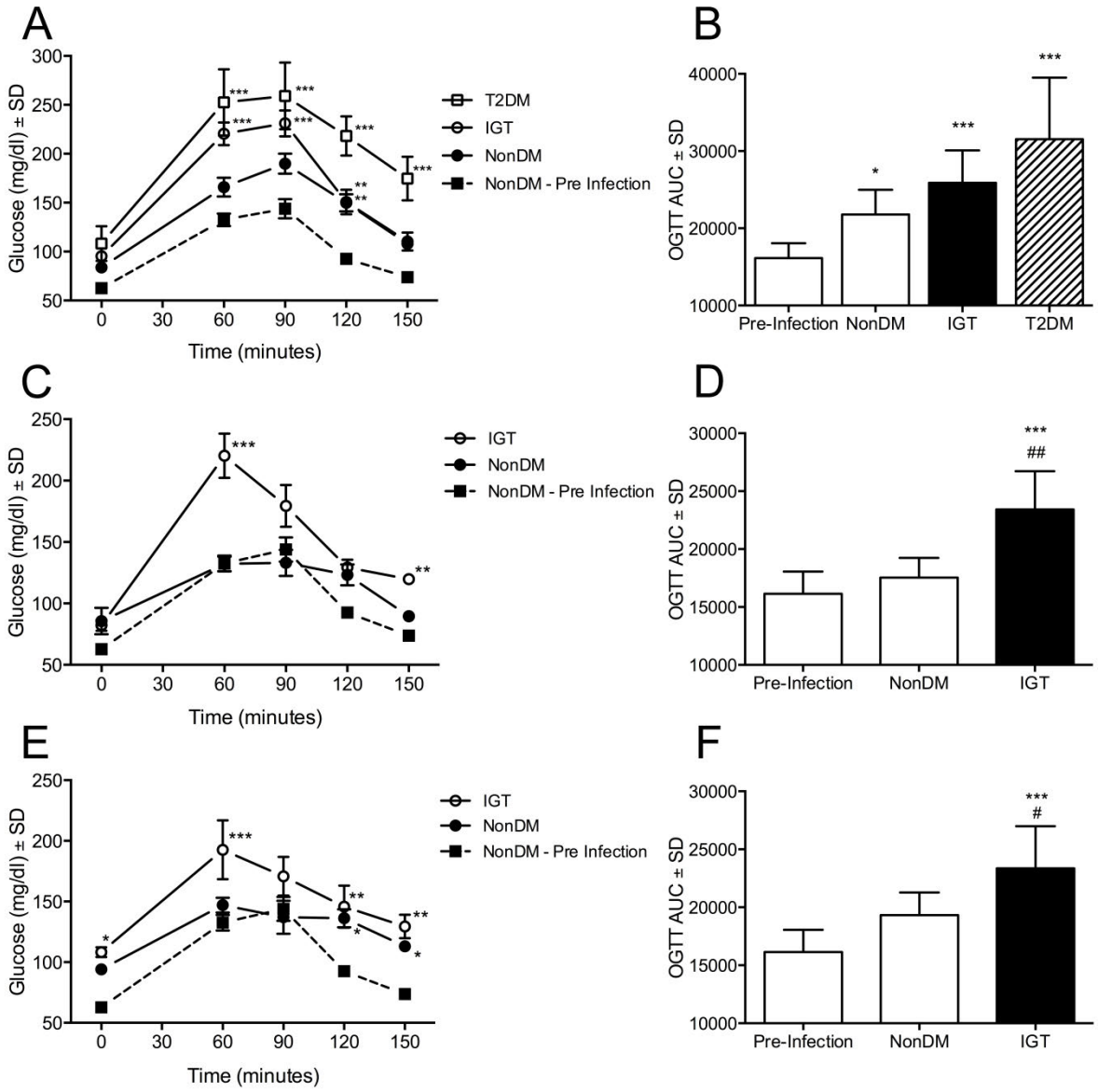

Figure 4.2. Infection with *M. tuberculosis* further impairs glucose tolerance in non-diabetic, HFHC fed only and diabetic guinea pigs. OGTT at day 30 of infection (A) shows amplification of impaired glucose tolerance in non-diabetic, HFHC fed only and diabetic guinea pigs due to *M. tuberculosis* infection with increased AUC (B) compared to pre-infection values. At day 60 of infection (C) glucose tolerance is comparable to pre-infection levels in non-diabetic guinea pigs while impaired glucose tolerance and increased AUC (D) remains in guinea pigs with pre-existing impaired glucose tolerance. Similarly, impaired glucose tolerance persists at day 90 of infection (E) in guinea pigs with pre-existing impaired glucose tolerance as indicated by elevated AUC (F) while overall glucose tolerance is comparable to pre-infection levels in non-diabetic controls although blood glucose remains elevated in this group 2 hours post-administration. */† $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; NonDM = non-diabetic, IGT = impaired glucose tolerance, T2DM = diabetic

Table 4.1. Metabolic parameters from non-diabetic, glucose intolerant and diabetic guinea pigs prior to infection with *M. tuberculosis* (Day 0) and on days 30, 60 and 90 after infection. TG = triglyceride, FFA = free fatty acid, AGE = advanced glycation end product, ND = not determined; * = compared to non-diabetic, † = compared to pre-infection levels, */† p≤0.05, ††† p≤0.001, †††† p≤0.0001

Day	0	30	60	90
TG (mg/dl)				
NonDM	34.7 ± 9.4	58.4 ± 16.3	40.59 ± 19.3	ND
IGT	93.1 ± 63.7*	66.9 ± 44.1	77.1 ± 42.1	ND
T2DM	101 ± 47*	52.2 ± 13.9	ND	ND
FFA (µM)				
NonDM	132.3 ± 24.8	380 ± 46.2††††	260.6 ± 8.6††	252.7 ± 25.4††
IGT	146.6 ± 47.5	406.1 ± 84††††	256.9 ± 19.2††	240.3 ± 16.6††
T2DM	172.6 ± 47.4*	454.2 ± 90.7††††	ND	ND
AGE (µg/ml)				
NonDM	1.079 ± 0.181	1.266 ± 0.188	1.125 ± 0.179	0.956 ± 0.122
IGT	0.973 ± 0.011	1.186 ± 0.273	1.007 ± 0.094	0.987 ± 0.14
T2DM	1.163 ± 0.168	2.522 ± 0.532†††	ND	ND

Diabetic guinea pigs develop rapidly progressive TB disease.

Progression of TB was rapid in diabetic guinea pigs with 65% mortality before day 30 of infection and a median survival time of 25 days (Figure 4.3A). Guinea pigs with impaired glucose tolerance showed intermediate, but not statistically significant, mortality compared to non-diabetic controls. Mortality in guinea pigs with impaired glucose tolerance was 50% before termination of the study at day 145 with a median survival time of 95 days. Guinea pigs with impaired glucose tolerance and infected with M. tuberculosis were euthanized due to respiratory distress and had developed marked cranial mediastinal and tracheobronchial lymph node enlargement with compression of primary bronchi. No mortality occurred in non-diabetic guinea pigs prior to the terminal endpoint at day 145 of infection.

***M. tuberculosis* infection in guinea pigs with diabetes accelerates bacilli dissemination and exacerbates TB disease severity.**

Pulmonary lesion burdens were markedly increased in diabetic guinea pigs with a 2.7-fold increase in mean percent involvement over non-diabetic controls. While pulmonary lesions were higher in guinea pigs with impaired glucose tolerance at day 30, this difference was not statistically significant and no significant differences were present on days 60 and 90 of infection between glucose intolerant and non-diabetic guinea pigs (Figure 4.3B). In contrast to typical well-structured granulomas of non-diabetic and guinea pigs with impaired glucose tolerance (Figure 4.3D), diabetic guinea pigs had marked granulocytic inflammation that disrupted lung granuloma architecture often resulting in airway erosions (Figure 4.3E). Splenic lesion burden, as a measure of extrapulmonary TB disease, was increased in diabetic guinea pigs at day 30 of infection, reaching as high as 97% involvement (Figure 4.3C). In addition to extensive involvement, guinea pigs with diabetes had altered lesion morphology characterized by widely disseminated, yet smaller and often coalescing aggregates of macrophages (Figure 4.3G),

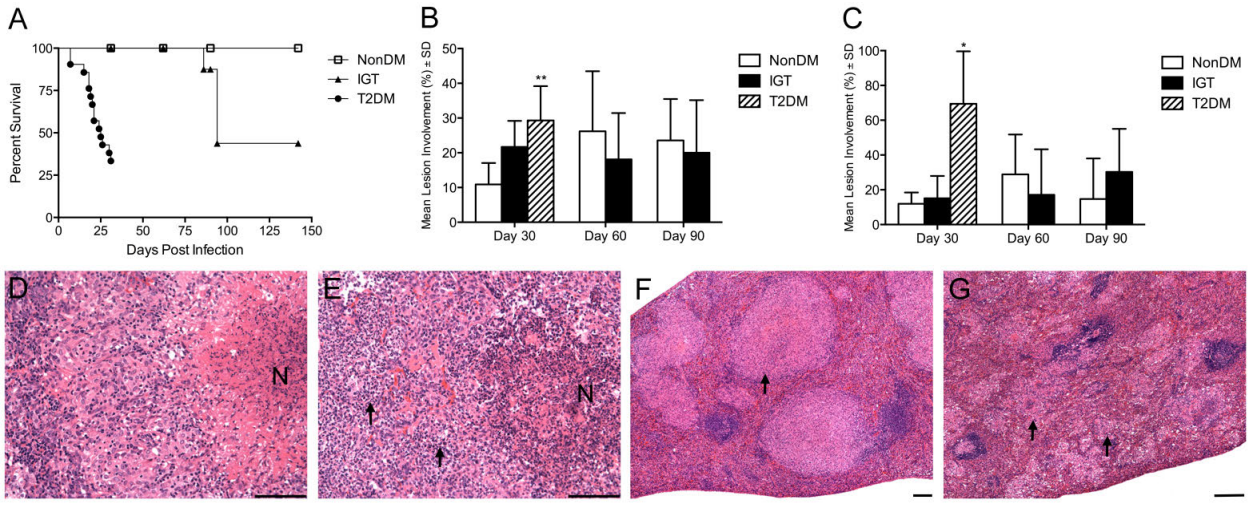

Figure 4.3. TB is more severe in guinea pigs with type 2 diabetes. Markedly reduced survival in diabetic guinea pigs and intermediate, but insignificant, susceptibility seen in guinea pigs with impaired glucose tolerance (A). Approximately 3-fold increase in lung disease severity in diabetic guinea pigs on day 30 of infection as measured by lesion burden while no significant differences were present between glucose intolerant and non-diabetic controls (B). Markedly increased spleen lesion burden in diabetic guinea pigs on day 30 of infection while no significant differences were present between glucose intolerant and non-diabetic controls (C). When compared to normal granuloma morphology in the lung of non-diabetic guinea pigs (D), there was much higher granulocyte infiltration (arrows) and disruption of granuloma architecture in diabetic guinea pigs (E) (H&E, bar = 100μm). When compared to typical large and discrete granulomas (arrow) in the spleen of non-diabetic guinea pigs (F), diabetic guinea pigs had a widespread miliary pattern of lesion dissemination in the spleen consisting of small and coalescing granulomas (arrows) (G) (H&E, bar = 100μm). * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$; NonDM = non-diabetic, IGT = impaired glucose tolerance, T2DM = diabetic

when compared to non-diabetic controls (Figure 4.3F). Progressive lesions developed within the lymph nodes draining the lungs in all guinea pigs regardless of their diabetic state.

Diabetic guinea pigs have higher bacterial burden.

At day 30 of infection, diabetic guinea pigs had approximately 2 $\log_{10}$ higher bacterial burdens in all tissues evaluated compared to non-diabetic controls and guinea pigs with impaired glucose tolerance (Figure 4.4A). While no significant differences were present at day 60 of infection (Figure 4.4C), the bacterial burden was lower in spleen and liver of non-diabetic guinea pigs at day 90 compared to guinea pigs with impaired glucose tolerance whose extrapulmonary burden remained elevated (Figure 4.4E). The higher lung bacterial burden in diabetic guinea pigs was accompanied by increased shedding of bacilli into the upper airways. While *M. tuberculosis* could not be isolated from tracheal wash fluid of any non-diabetic controls (n=4) on day 30 of infection, *M. tuberculosis* was cultured from two of four guinea pigs with impaired glucose tolerance and five of eight diabetic guinea pigs (Figure 4.4B). As chronic infection was established, the frequency of viable bacilli cultured from tracheal wash of guinea pigs with impaired glucose tolerance was reduced. Tracheal washes were culture positive from one non-diabetic and glucose intolerant guinea pig on day 60 of infection (Figure 4.4D) but all were negative by day 90 (Figure 4.4F).

Guinea pigs with impaired glucose tolerance and diabetes respond to *M. tuberculosis* infection with altered pro- and anti-inflammatory cytokine profiles.

At day 30 of infection, elevations in cytokine and chemokine expression in the lungs of diabetic guinea pigs included IL-12p40 (2.3 fold), IFNγ (4.7 fold), IL-17A (4.3 fold), IL-8 (5.6 fold), and IL-10 (12.6 fold) compared to non-diabetic controls (Figures 4.5-7). In contrast, non-diabetic guinea pigs and those with impaired glucose tolerance had similar cytokine profiles early in infection and through day 60 (Figures 4.5-7), with the exception of a 4.0- and 12.6-fold

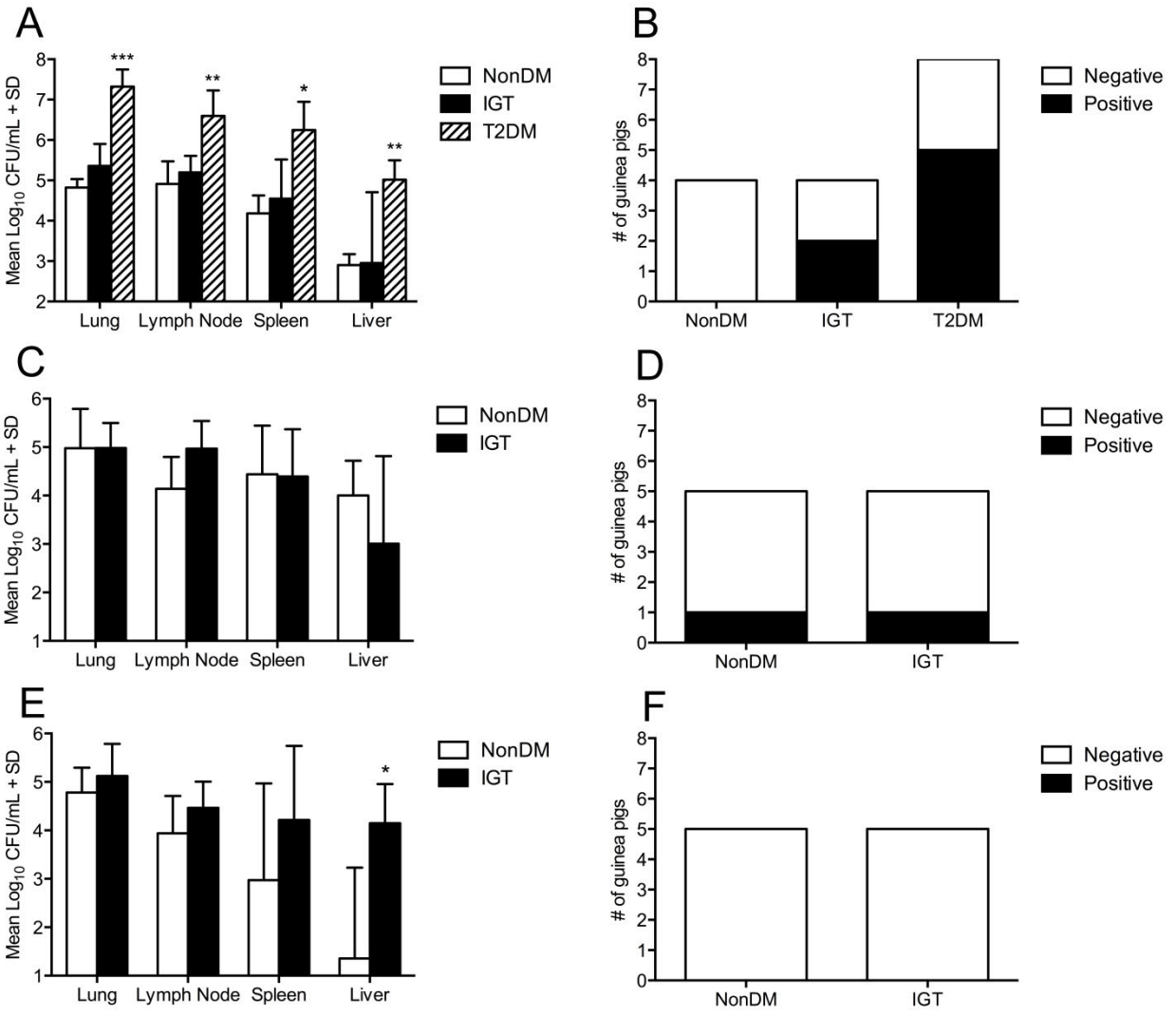

Figure 4.4. Guinea pigs with diabetes have a higher bacterial burden. Diabetic guinea pigs at day 30 of infection have much higher bacterial burden in pulmonary and extrapulmonary organs (A). Higher frequency of *M. tuberculosis* growth from tracheal wash fluid of diabetic guinea pigs, exceeding positive samples from guinea pigs with impaired glucose tolerance as compared to no growth in non-diabetic controls (B). No significant differences in tissue bacterial burden on day 60 of infection between glucose intolerant and non-diabetic guinea pigs (C) and similar tracheal wash culture results, which have declined in guinea pigs with impaired glucose tolerance (D). By day 90 of infection, extrapulmonary burden has declined in non-diabetic guinea pigs while it is persistently elevated in guinea pigs with impaired glucose tolerance (E). All tracheal wash cultures from non-diabetic and glucose intolerant guinea pigs are negative at day 90 of infection (F). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; NonDM = non-diabetic, IGT = impaired glucose tolerance, T2DM = diabetic

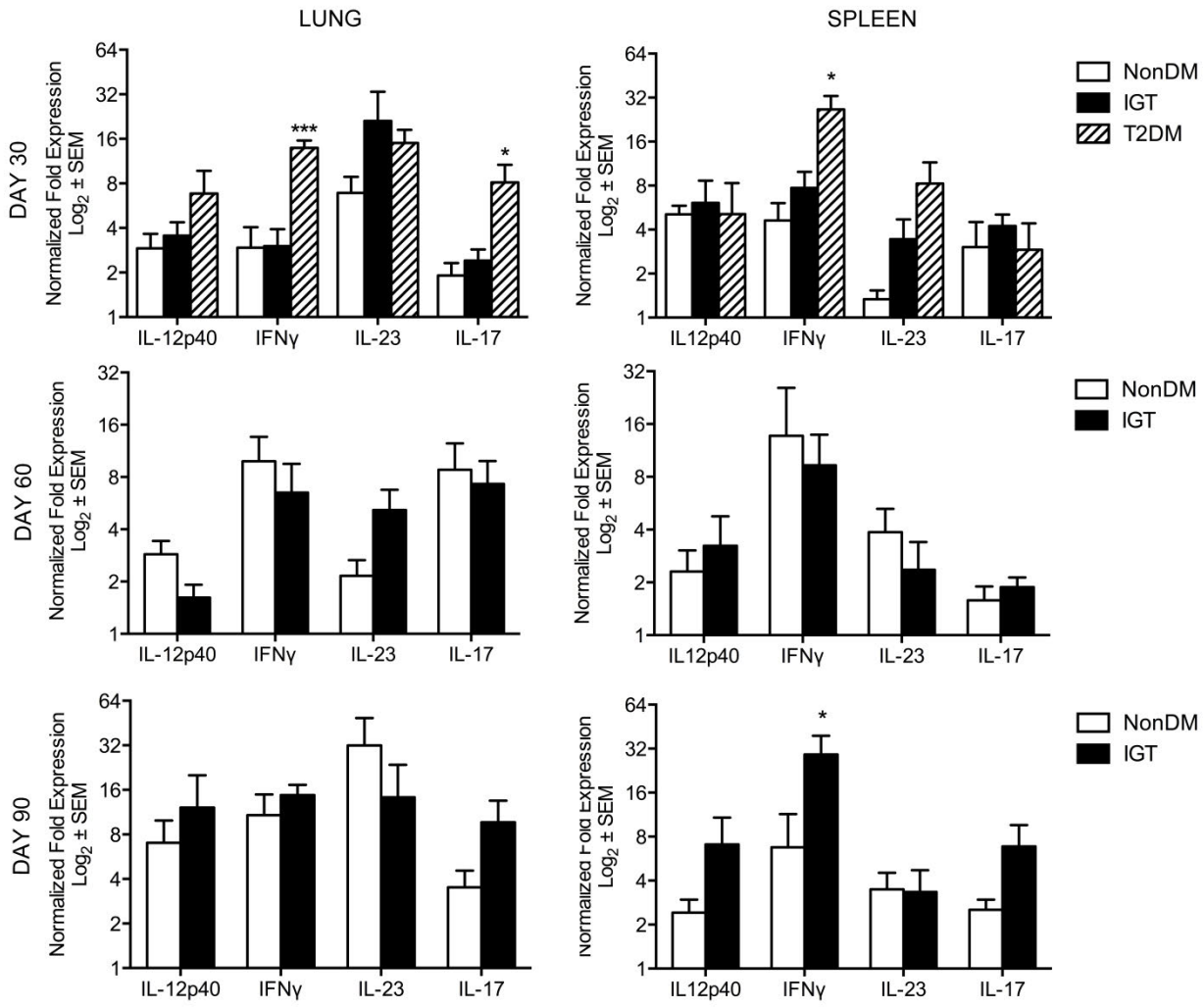

Figure 4.5. Guinea pigs with type 2 diabetes respond to *M. tuberculosis* infection with a more robust type 1 cytokine response. Relative gene expression of cytokines promoting a Th1-biased T cell response was measured by qRT-PCR. Lung IFNγ and IL-17 expression as well as spleen IFNγ was higher in diabetic guinea pigs infected with M. tuberculosis on day 30 of infection. Elevated IFNγ was not seen in guinea pigs with impaired glucose tolerance until day 90 of infection. No significant differences were observed in IL-12p40 or IL-23 expression at any time point evaluated. * $P \leq 0.05$, *** $P \leq 0.001$; NonDM = non-diabetic, IGT = impaired glucose tolerance, T2DM = diabetic

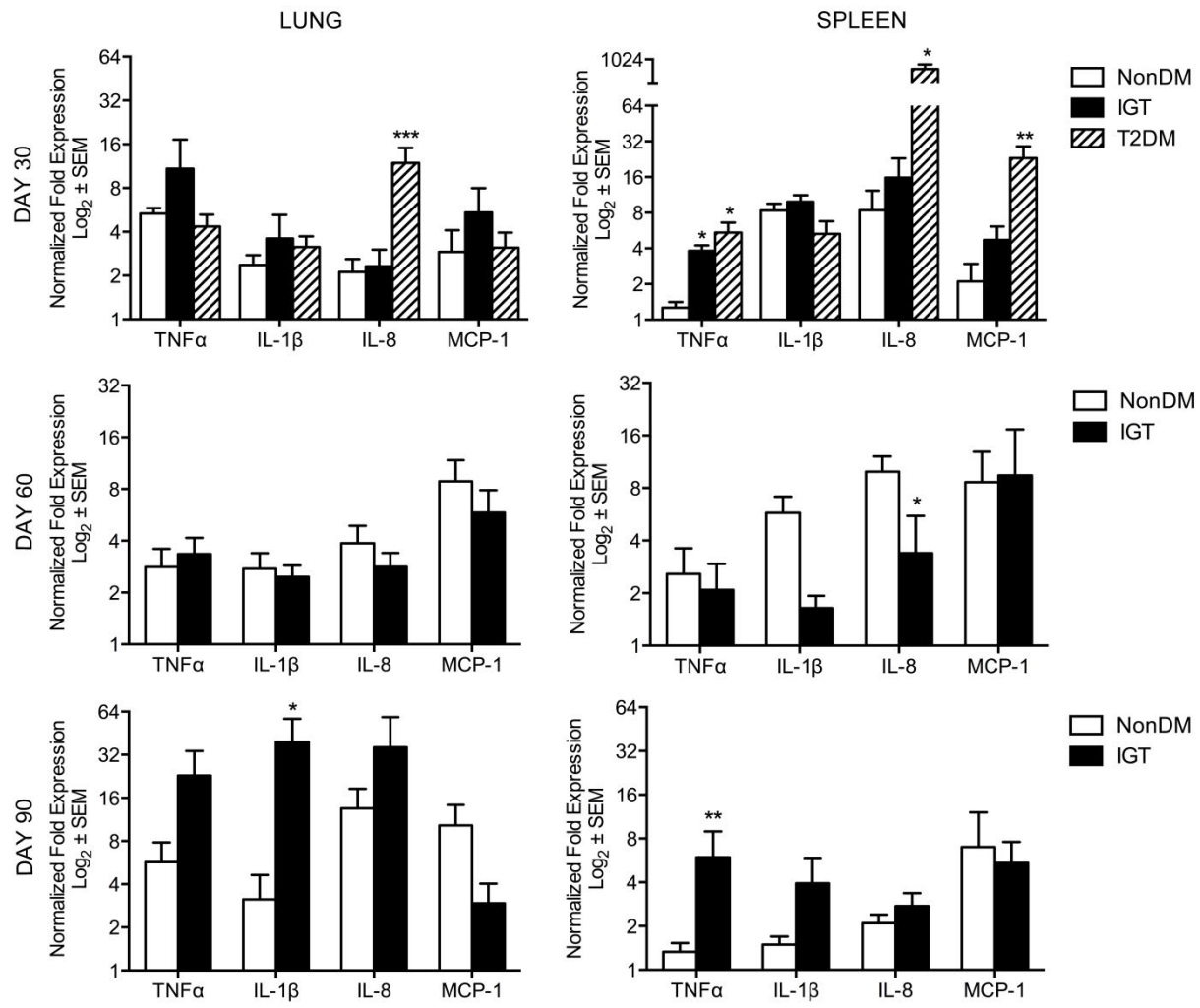

Figure 4.6. Guinea pigs with type 2 diabetes respond with an elevated innate chemokine/cytokine response early in *M. tuberculosis* infection. Relative gene expression of innate cytokines and chemokines most involved in TB pathogenesis were measured by qRT-PCR. Elevated expression of neutrophil and macrophage chemokines IL-8 and MCP-1, respectively, as well as splenic TNFα expression are significantly elevated in guinea pigs with type 2 diabetes on day 30 of infection. In contrast, elevated expression of IL-1β and TNFα occurred only at day 90 of infection in guinea pigs with impaired glucose tolerance. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; NonDM = non-diabetic, IGT = impaired glucose tolerance, T2DM = diabetic

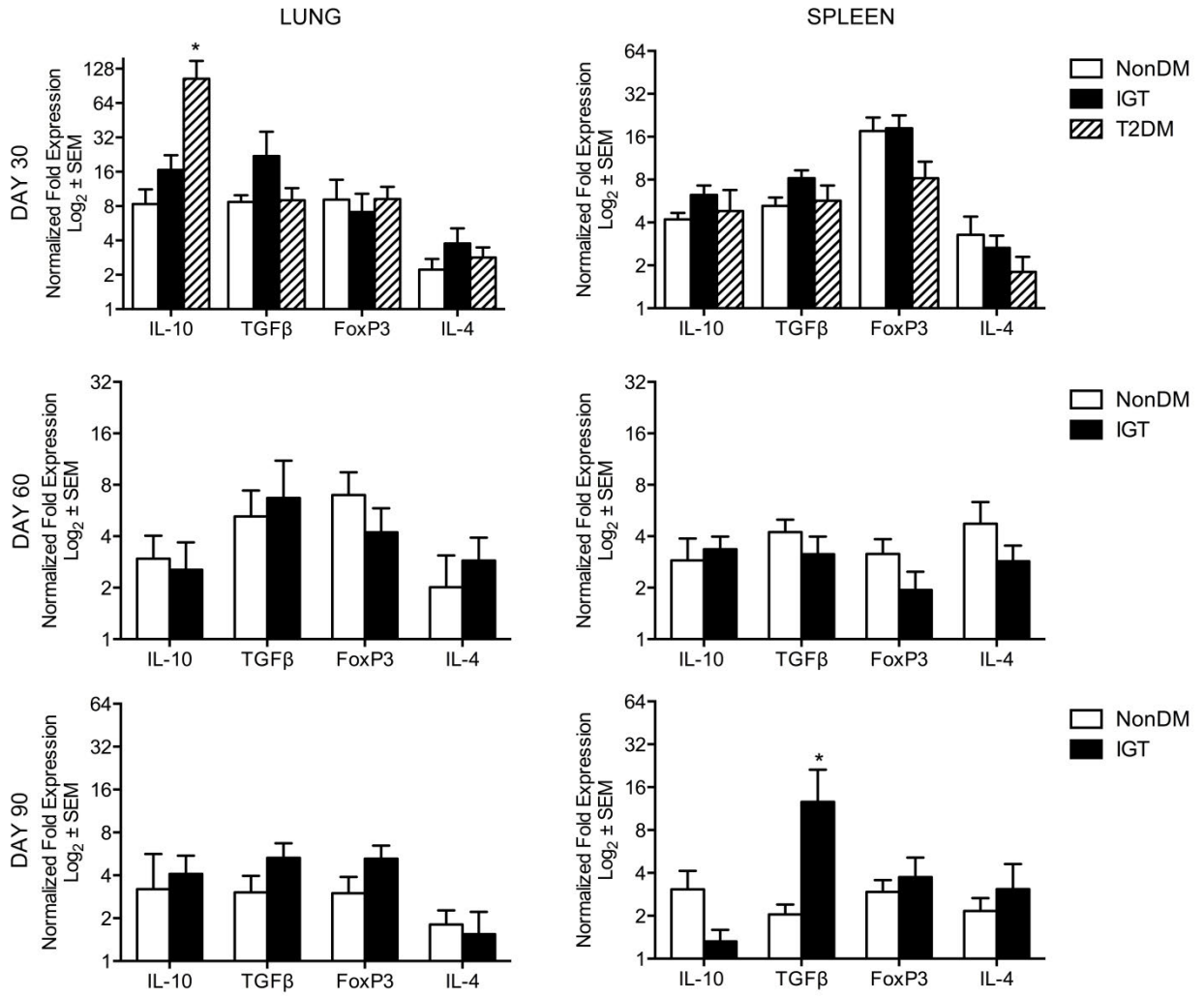

Figure 4.7. Increased IL-10 expression does not impair the inflammatory response in type 2 diabetic guinea pigs. Relative gene expression of cytokines and transcription factors that oppose the cell-mediated response during TB were measured by qRT-PCR. A productive type 1 cytokine response persisted in diabetic guinea pigs despite elevated IL-10 expression on day 30 of infection with *M. tuberculosis.* IL-4 expression, as an indicator of Th2 T cell differentiation, or FoxP3 expression, as an indicator of regulatory T cell differentiation, did not differ between non-diabetic, insulin resistant or diabetic states at any time point evaluated. * P≤ 0.05; NonDM = non-diabetic, IGT = impaired glucose tolerance, T2DM = diabetic

increase in TNFα (p=0.1) and IL-1β, respectively, at day 90 of infection in guinea pigs with impaired glucose tolerance compared to non-diabetic controls (Figure 4.6). Diabetic guinea pigs had elevated IFNγ (5.8 fold), TNFα (4.3 fold), and MCP-1 (11.0 fold) in the spleen at day 30 of infection while guinea pigs with impaired glucose tolerance had elevated TNFα (3.0 fold), compared to non-diabetic controls (Figures 4.5 and 4.6). Differences in cytokine expression in the spleen at day 90 of infection were present in guinea pigs with impaired glucose tolerance with elevated IFNγ (4.4 fold), TNFα (4.5 fold), and TGFβ (6.2 fold) (Figures 4.5-7). Expression of FoxP3, and IL-4 did not differ between any groups at any point throughout the study (Figure 4.7).

Guinea pigs with diabetes have higher levels of macrophage activation and neutrophil infiltration early in disease while innate immune cell phenotypes increase late in disease in guinea pigs with impaired glucose tolerance.

Absolute quantification of immune cell phenotypes per gram of lung, lymph node and spleen is depicted in Table 4.2. Diabetic guinea pigs responded to *M. tuberculosis* infection with higher total numbers of activated MHCII^{high} macrophages in lung, lymph node and spleen (Table 4.2) as well as a greater proportion of activated macrophages out of all macrophage cells recovered from lung and lymph node compared to non-diabetic and glucose intolerant controls (Figure 4.8, G and H). This correlated with higher total numbers of activated CD45^{high} CD4 T cells from the lymph node of diabetic guinea pigs although the percentage of activated CD45^{high} T cells out of total CD4 T cells recovered did not differ (Figure 4.8, A-C). In addition, diabetic guinea pigs had a higher proportion of granuloyctes in the lung at day 30 of infection compared to non-diabetic controls (Figure 4.8J). In contrast, guinea pigs with impaired glucose tolerance had lower cell numbers of all phenotypes recovered from lymph node at day 30 of infection, but

Table 4.2. Immune cell phenotypes in absolute number as measured by flow cytometry in lung, lymph node, and spleen on days 30, 60 and 90 of infection. Results are expressed at 10^5 cells and normalized to per gram of tissue. * = Compared to IGT, # = Compared to T2DM, */# P≤ 0.05, **/## P ≤ 0.01, ***/### P≤ 0.001; MAC = macrophage, Gran = granulocyte, NonDM = non-diabetic, IGT = glucose intolerant, T2DM = diabetic

		Lung			Lymph Node			Spleen		
	DAY	30	60	90	30	60	90	30	60	90
NonDM	**CD4 TCR**	3.09 ± 0.58	8.13 ± 1.04	5.31 ± 1.12	16.32 ± 1.53**	52.10 ± 9.48	21.71 ± 7.07	6.86 ± 0.84	29.73 ± 8.56	10.09 ± 1.74
IGT		3.24 ± 0.33	9.92 ± 1.65	8.05 ± 0.83	5.75 ± 1.33$^{\#\#}$	46.30 ± 8.92	19.86 ± 5.22	7.65 ± 1.43	28.20 ± 5.01	13.59 ± 5.25
T2DM		2.00 ± 0.22			24.59 ± 4.55			6.30 ± 0.67		
NonDM	**CD4 TCR CD45^{high}**	2.20 ± 0.41	0.88 ± 0.26	4.03 ± 0.94	11.38 ± 0.87*	17.96 ± 3.85	13.61 ± 3.81	3.08 ± 0.44	1.85 ± 0.72	6.10 ± 1.23
IGT		2.24 ± 0.22	1.08 ± 0.19	5.79 ± 0.70	3.96 ± 1.18$^{\#\#}$	15.50 ± 4.26	13.69 ± 4.21	4.07 ± 0.99	2.12 ± 0.81	8.06 ± 3.31
T2DM		1.47 ± 0.16			15.31 ± 3.14			3.42 ± 0.65		
NonDM	**CD8 TCR**	2.42 ± 0.58	2.35 ± 0.22	3.06 ± 0.92	11.91 ± 1.94*	12.29 ± 3.11	8.49 ± 1.95	6.95 ± 0.85	16.90 ± 4.63	18.84 ± 5.03
IGT		2.53 ± 0.42	3.91 ± 0.66	5.34 ± 0.74	3.92 ± 0.73$^{\#\#\#}$	16.29 ± 4.00	13.60 ± 4.48	7.00 ± 1.41	17.49 ± 5.53	17.78 ± 7.01
T2DM		1.78 ± 0.16			21.38 ± 3.97			5.76 ± 1.68		
NonDM	**CD8 TCR CD45^{high}**	1.07 ± 0.27	0.60 ± 0.09*	0.91 ± 0.23	6.81 ± 1.18	25.06 ± 13.60	6.36 ± 1.98	0.81 ± 0.09	2.66 ± 0.93	2.62 ± 1.24
IGT		1.15 ± 0.24	1.11 ± 0.18	2.12 ± 0.38	2.45 ± 0.42$^{\#\#\#}$	10.53 ± 2.37	11.20 ± 3.92	0.96 ± 0.19	2.60 ± 1.04	2.63 ± 0.84
T2DM		0.60 ± 0.06			14.01 ± 2.64			0.73 ± 0.34		
NonDM	**MHCII^{low} MAC**	51.00 ± 5.32	47.08 ± 7.02	84.16 ± 13.95	145.9 ± 16.14	157.5 ± 24.26	150.1 ± 46.24	58.92 ± 7.71	106.3 ± 23.43	115.9 ± 23.42
IGT		45.86 ± 3.28	49.64 ± 8.02	154.20 ± 14.36	100.1 ± 10.94$^{\#}$	113.9 ± 17.38	199.8 ± 17.44	68.00 ± 7.18	109.6 ± 21.43	185.7 ± 57.52

T2DM		35.96 ± 3.87			167.1 ± 12.11			93.03 ± 15.16		
NonDM	**MHCII^{High} MAC**	4.62 ± 1.63$^{\#}$	8.36 ± 1.88	9.73 ± 2.79	47.87 ± 18.57	89.46 ± 32.07	26.70 ± 6.62	6.42 ± 1.99	20.50 ± 5.38	15.12 ± 2.35
IGT		5.32 ± 0.59	9.07 ± 1.40	19.14 ± 6.24	22.42 ± 6.47$^{\#}$	56.28 ± 4.97	86.68 ± 8.30	5.28 ± 1.34$^{\#}$	17.43 ± 3.30	22.50 ± 5.05
T2DM		8.44 ± 1.44			103.6 ± 31.98			19.18 ± 6.75		
NonDM	**MIL4 Gran**	11.39 ± 1.20	8.08 ± 1.55	14.91 ± 2.99*	133.0 ± 25.87	92.22 ± 31.12**	34.54 ± 10.69	3.49 ± 0.47	6.24 ± 1.78	5.47 ± 0.70
IGT		12.83 ± 1.26	8.70 ± 2.05	25.68 ± 4.58	92.42 ± 17.41$^{\#}$	35.22 ± 15.65	35.22 ± 15.65	3.71 ± 0.79	5.94 ± 1.31	8.45 ± 3.02
T2DM		15.86 ± 2.71			178.3 ± 33.21			5.98 ± 1.41		
NonDM	**B cell**	41.20 ± 5.20	42.94 ± 6.99	74.74 ± 11.33**	91.46 ± 13.54	163.3 ± 39.14	123.1 ± 37.71	28.10 ± 2.56	85.10 ± 19.44	94.06 ± 12.57
IGT		41.92 ± 2.54	47.38 ± 7.30	138.4 ± 14.74	55.70 ± 8.90$^{\#}$	101.6 ± 7.24	188.0 ± 25.59	40.70 ± 9.42	89.16 ± 20.30	149.5 ± 48.68
T2DM		27.96 ± 2.29			106.3 ± 14.21			60.40 ± 9.78		

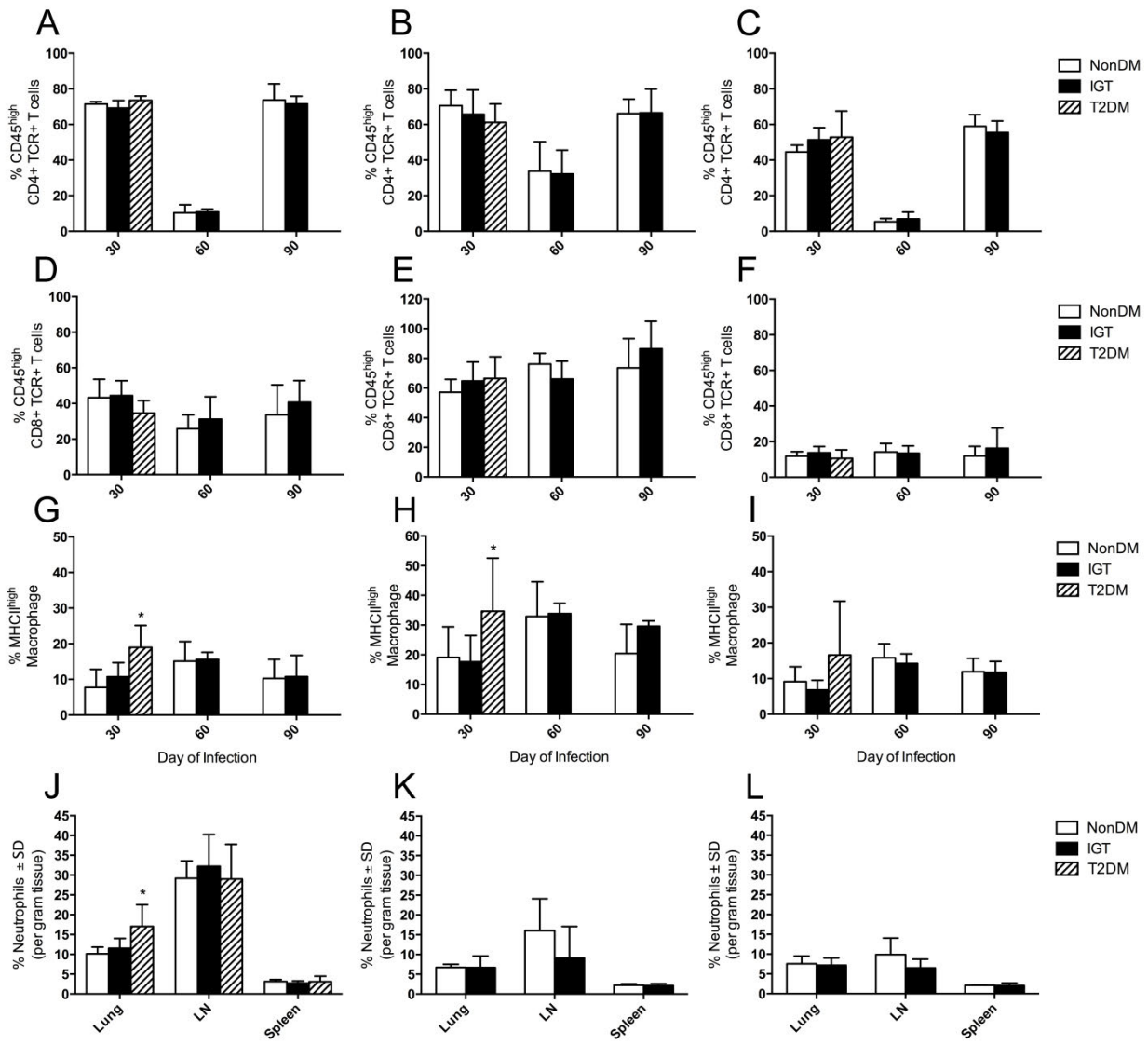

Figure 4.8. Activated macrophages and granulocytes are increased in diabetic guinea pigs at day 30 of *M. tuberculosis* infection. Total $CD4^+$ or $CD8^+$ TCR+ T cells and MHCII low or high expressing macrophages were quantified by flow cytometry and normalized to per gram of tissue in lung, lymph node and spleen then expressed as percent activated based on proportions of $CD45^{high}$ $CD4^+$ and $CD8^+$ lymphocytes and $MHCII^{high}$ macrophages. The proportion of activated CD4 T cells decreases in non-diabetic and glucose intolerant guinea pigs at day 60 of infection but is equally elevated at day 90 of infection in all organs in non-diabetic and glucose intolerant guinea pigs (A-C). No differences in CD8 T cell activation at any time point between non-diabetic, glucose intolerant and diabetic guinea pigs (D-F). Higher proportions of activated macrophages are present in diabetic guinea pigs in lung and lymph node on day 30 of infection (G-I). Granulocytes as a proportion of total leukocytes counted per gram of tissue are increased in the lung of diabetic guinea pigs (J-L). * $P \leq 0.05$; NonDM = non-diabetic, IGT = impaired glucose tolerance, T2DM = diabetic

higher numbers of activated MHCII^{high} macrophages as well as granulocytes were present at day 90 of infection. An overall decrease in the proportion of activated CD45^{high} CD4 T cells occurred in all organs in both non-diabetic guinea pigs and those with impaired glucose tolerance between day 30 and 60 of infection, but increased again at day 90 of infection (Figure 4.8, A-C).

Discussion.

This study represents both the first model of non-insulin dependent diabetes in the guinea pig as well as the first description of *M. tuberculosis* infection in an animal model with diet-induced impaired glucose tolerance or type 2 diabetes. The guinea pig model was chosen to better mimic the comorbidity of diabetes and TB in humans, based on the guinea pig response to *M. tuberculosis* infection and similarities to human glucose and lipid metabolism compared to mice or rats. The most common indicator of systemic insulin resistance in human patients is delayed utilization of glucose, referred to as impaired glucose tolerance, as measured by OGTT. In this study, guinea pigs fed a HFHC diet developed impaired glucose tolerance evidenced by an elevated 1-hour post-load blood glucose during OGTT, a measurement recently shown to predict early insulin resistance and beta cell dysfunction in human patients[34]. In addition, guinea pigs developed serum lipid alterations, including elevated fasting triglycerides and FFAs, consistent with insulin resistance similar to that seen in humans[35]. In humans, the progression from prediabetic, impaired glucose tolerance and insulin resistance to type 2 diabetes is gradual and can take years[36]. Consequently, the low-dose treatment with the cytotoxic drug STZ in these studies was included to accelerate the rate of beta cell loss in guinea pigs with impaired glucose tolerance. The combination of the HFHC diet and low-dose STZ consistently induces fasting hyperglycemia and blood glucose levels

exceeding 200 mg/dl in a 2-hour OGTT as early as 14 days after STZ treatment, data consistent with the diagnostic standards established for humans with type 2 diabetes[33].

Severe systemic inflammation in non-diabetic individuals is often associated with hyperglycemia and impaired glucose tolerance mediated in part by proinflammatory cytokines, especially TNFα. The pathogenesis of cytokine-mediated hyperglycemia and impaired glucose tolerance is directly linked to the release of FFAs, which are potent mediators of insulin resistance[37-39]. In this study, impaired glucose tolerance was induced by *M. tuberculosis* infection alone even in non-diabetic guinea pigs, as we have previously described[23]. *M. tuberculosis* infection in diabetic as well as non-diabetic guinea pigs fed the HFHC diet only, further exacerbated impaired glucose tolerance. This finding paralleled the marked elevations in serum FFAs in infected guinea pigs, which exceeded the modest elevations associated with diabetes induction prior to infection. In addition, elevations in FFAs reflected increased cytokine expression during the early stages of infection, which subsided at day 60 of infection when innate cytokine expression was low. The relationship between cytokine expression and FFA concentrations was also reflected at day 90 of *M. tuberculosis* infection in guinea pigs with impaired glucose tolerance, which also correlated with increased TNFα and IL-1β expression. Impaired glucose tolerance and insulin resistance with hyperglycemia has been previously reported in human patients with active TB, which may be explained by elevated proinflammatory cytokines consistent with these data. The clinical significance of impaired glucose tolerance and hyperglycemia induced by *M. tuberculosis* infection is that it further complicates both the diagnosis and treatment of diabetes in patients with TB [40-43].

Overt diabetes had a profound impact on TB progression in this study. An overall increase in disease severity, characterized by a significantly higher bacterial load and more severe and rapidly progressive pulmonary and extrapulmonary disease contributed to decreased survival in diabetic guinea pigs with TB. These findings are consistent with reports of

more severe pulmonary TB in human diabetic patients[15, 16]. Despite the conflicting evidence in human patients[5, 10, 17, 18], more severe and rapidly progressing extrapulmonary disease in diabetic guinea pigs was a consistent finding. The altered pattern of lesion dissemination in the spleen of diabetic guinea pigs resembles miliary TB, a pattern of disease that is associated with increased mortality especially in severely immunocompromised human TB patients[44]. *M. tuberculosis* infection in diabetic guinea pigs also resulted in increased shedding of bacilli into the tracheal lumen at the predetermined endpoints. *M. tuberculosis* was cultured from tracheal washes of diabetic guinea pigs with greater frequency than non-diabetic guinea pigs and those with impaired glucose tolerance. The low frequency of animal-to-animal transmission of *M. tuberculosis* in normal guinea pigs is likely related to the failure to routinely develop open cavitary lesions, which typically harbor large numbers of bacilli[10, 45]. Our data indicate that diabetes induction increases the frequency of airway shedding of *M. tuberculosis*, even in the absence of cavitation, which may be related to higher pulmonary bacterial load and/or an alteration in the diabetic airway microenvironment[46, 47]. With the increasing interest in natural *M. tuberculosis* transmission from humans to animals or between animals[11], the diabetic guinea pig may represent a viable animal-to-animal transmission model, which warrants further investigation.

In contrast, impaired glucose tolerance in guinea pigs appeared to have little impact on TB disease progression in the early stages of disease. Although the initial pulmonary burden was higher in guinea pigs with impaired glucose tolerance, the differences were not statistically significant and the number of activated $CD45^{high}$ lymphocytes in the lung as well as cytokine expression was comparable to non-diabetic controls. Differences in response to *M. tuberculosis* infection in guinea pigs with impaired glucose tolerance were not seen until the chronic stages of infection. On day 90, TNFα and IL-1β expression as well as the extrapulmonary bacterial burden in guinea pigs with impaired glucose tolerance was higher than non-diabetic controls. Recently, an increased prevalence of impaired glucose tolerance and insulin resistance has

also been recognized in TB patients, however it is unknown whether these metabolic alterations are a consequence of systemic insulin resistance mediated by *M. tuberculosis* infection[48]. These results provide the first experimental evidence that impaired glucose tolerance similar to a prediabetic state also increases TB disease severity, a finding that warrants further investigation in animal models and humans[49]. While guinea pigs with impaired glucose tolerance did not develop TB disease that progressed as rapidly as in diabetics, active inflammation persisted, which accounted for more severe clinical disease in the chronic stages of infection. In this study, the more severe TB disease in guinea pigs with hyperglycemia as a result of impaired glucose tolerance are similar to our previous studies showing that TB disease severity is increased even in non-diabetic guinea pigs with repeated post-prandial hyperglycemia [23]. Moreover, our results are similar to that described in diabetic humans and STZ treated mice with chronic hyperglycemia, which suggest that poor glycemic control is an important determinant of TB disease risk[7, 19].

Aside from the increased severity of disease, the pathological features of *M. tuberculosis* infection in diabetic guinea pigs differed from that of non-diabetic controls. These changes may be related to the altered expression of particular cytokines and subsequent cellular response to *M. tuberculosis* infection. Increased IL-17, also described in humans with type 2 diabetes and TB[50], as well as elevated IL-8 expression may be linked to more granulocytic infiltration and pathology seen in diabetic guinea pigs infected with *M. tuberculosis*. A persistent IL-17 response during TB contributes to excessive inflammation and is generally limited by IFNγ production although was not evident in diabetic guinea pigs in this study[51, 52]. We have shown that the accumulation of advanced glycation end products (AGEs) occurs as a result of *M. tuberculosis*-mediated inflammation but is further increased when combined with diabetes. This response was reflected by the marked increase in serum AGEs only in diabetic guinea pigs with TB. These byproducts of chronic hyperglycemia, combined with oxidative stress, induce a pro-inflammatory response and may have contributed to the more severe inflammation and TB

disease in guinea pigs with type 2 diabetes[53-55]. A significant increase in MCP-1 expression in diabetic guinea pigs with TB correlated with the pattern of coalescing foci of tissue macrophages without granuloma formation in the spleen. Increased MCP-1 expression in human TB patients is a feature of pulmonary TB, however, the increased expression of this cytokine in diabetic guinea pigs may explain the more severe, miliary pattern of extrapulmonary spread to the spleen, which is prevalent in this species[56].

It is generally accepted that a Th1-biased cytokine response is critical for protection during *M. tuberculosis* infection. Guinea pigs with type 2 diabetes displayed higher macrophage activation, an appropriate response to the increased production of IFNγ. Despite this seemingly favorable response to *M. tuberculosis* infection, diabetic guinea pigs failed to limit bacterial growth. A balance between the ability to limit bacterial growth and the development of damaging inflammation during active TB, may explain the increase in anti-inflammatory cytokines. The increased anti-inflammatory cytokine expression is likely a reciprocal response intended to prevent host injury from the exacerbated innate response to *M. tuberculosis* infection seen in diabetic guinea pigs. In this study, the increase in IL-10 occurred despite, the counter regulatory effects of high IFNγ expression. These findings suggest possible IFNγ-mediated induction of IL-10 as has been previously described associated with a high *M. tuberculosis* burden[57, 58]. Previously, a delayed onset of IFNγ production in response to *M. tuberculosis* infection was shown in STZ-treated mice with hyperglycemia, which also fail to control bacterial growth[8]. The contribution of rapid disease progression prior to onset of adaptive immunity to *M. tuberculosis* in diabetic guinea pigs will need to be determined in future studies that focus on the very early stages of diabetes-TB comorbidity. Previous studies have shown that the accumulation of regulatory T cells expressing FoxP3 during *M. tuberculosis* infection parallels the activation of a Th1-biased adaptive immune response and along with the Th2 cytokine, IL-4, is thought to impair a productive Th1 lymphocyte response[59]. However, in this

study, no differences were noted in FoxP3 or IL-4 expression suggesting that the over-expression of immunosuppressive T cell subsets are not a major determinant in the increased severity of TB in guinea pigs with type 2 diabetes.

From this and other studies investigating diabetes-TB comorbidity, it is evident that the diabetic state impairs *M. tuberculosis* host defenses resulting in uncontrolled bacterial growth. In addition, altered cytokine expression and more severe pulmonary pathology indicative of a proinflammatory response to *M. tuberculosis* infection are consistent with features described in diabetes-TB comorbidity of humans. Moreover, these studies demonstrate that *M. tuberculosis* infection alone is associated with altered host lipid and glucose metabolism, which reveals the possibility of using antiglycemic drugs as an adjunct with antimicrobial drugs to treat TB. Because of the shared features of *M. tuberculosis* infection in diabetic humans and guinea pigs, this model will be valuable in testing new TB treatment and diabetes control strategies when both diseases occur together.

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CHAPTER 5

Impaired Transition from Innate to Adaptive Immunity in the Guinea Pig Model of Diabetes-Tuberculosis Comorbidity

Rationale: In the previous chapter, we demonstrated that more severe TB disease in diabetic guinea pigs presents as an exacerbated adaptive and innate immune response, which is accompanied by very high bacterial growth in lung and extrapulmonary tissue. However, the mechanisms preventing the host from controlling this bacterial growth at a level similar to non-diabetic animals is not yet understood. In the typical pathogenesis of TB, it is well demonstrated that the ability of the host to orchestrate a successful transition from innate to adaptive immunity is essential for controlling the infection. This process is highly dependent on timing since cells harboring bacteria early during infection have much more successful bactericidal capacity in the presence of a cell-mediated response. Therefore, if the adaptive transition is delayed, effective control of bacterial growth may be hindered. An early hyperactive innate response similar to what has been identified at day 30 of infection in diabetic guinea pigs, may lead to early host injury and impair successful T cell help at the site of pulmonary infection.

Hypothesis: The early immune response in diabetic guinea pigs is skewed toward production of inflammatory innate cytokines in the presence of rapid bacterial growth that promote a robust granulocytic response to infection, damaging inflammation, and reduced cell-mediated immunity.

Summary.

Diabetes increases the risk of developing active tuberculosis (TB) disease and clinical studies suggest that TB is more severe in diabetic individuals. We have developed a guinea pig model of type 2 diabetes-TB comorbidity, which closely replicates these features in the human comorbidity. By day 30 of infection, diabetic guinea pigs have high bacterial growth and damaging neutrophilic inflammation despite the presence of cell-mediated immunity expected to control these manifestations. Therefore, in this study, we evaluated the early response to *Mycobacterium tuberculosis* infection in diabetic guinea pigs, in order to identify innate and cell-mediated factors that may influence the host's ability to contain bacterial growth and prevent progressive TB disease. By day 10 of *M. tuberculosis* infection, diabetic guinea pigs demonstrated limited pulmonary inflammation, reduced inflammation of the pulmonary lymphatics and impaired transport of bacteria to lung draining lymph nodes, the site for development of a cell-mediated response. Correlating with this, diabetic guinea pigs demonstrated a reduced antigen-specific IFNγ response and lower overall production of IFNγ in lung and lymph node on day 19 of infection. However, bacterial growth did not differ between diabetic and non-diabetic guinea pigs at either day 10 or day 19 of infection. This evidence of impaired antigen transport and delayed production of an antigen specific immune response may be related to the impact of early increased expression of the cytokine IL-6, which is known to impair Th1 immunity and migratory ability of antigen presenting cells by reducing the expression of the critical chemotactic receptor CCR7, which was also present in diabetic guinea pigs. These data indicate that bacterial growth is not enhanced in diabetic guinea pigs early in infection and that a delayed antigen-specific Th1 cell-mediated response may represent a central mechanism leading to the inability of diabetic guinea pigs to contain bacterial growth later in the course of disease.

Introduction.

Diabetes is a recognized risk factor for tuberculosis (TB) and the growing epidemic of type 2 diabetes, particularly in countries where TB is endemic, accounts for the majority of diabetes-associated TB[1, 2]. Currently, 95% of the 262 million diabetic people worldwide have type 2 diabetes, and of these, up to 80% reside in TB endemic countries[3]. Moreover, with a rapidly rising incidence of type 2 diabetes worldwide, it is estimated that this population will nearly double by the year 2030[4]. Although a number of altered disease manifestations and immune response elements have been identified, the underlying mechanisms that make people with diabetes more likely to develop active TB disease remain poorly understood[5-7].

Recently, we have described a guinea pig model of type 2 diabetes, which is preceded by progressive dietary-induced insulin resistance followed by reduced β-cell capacity, the two defining features of type 2 diabetes pathogenesis in humans[8]. In response to infection with *Mycobacterium tuberculosis*, these diabetic guinea pigs develop more severe TB disease that is dominated by neutrophilic inflammation, higher bacterial burdens, and an exacerbated innate and cell-mediated immune response[8]. These finding are in line with reported features in human clinical studies in type 2 diabetic patients with TB including radiographic evidence of more severe disease, higher bacterial burdens based on sputum culture, and higher innate and type 1 cytokine production from stimulated peripheral blood mononuclear cells, including TNFα, IL-17, IL-10 and IFNγ[9-14]. These findings are also consistent with previous studies in a mouse model of diabetes characterized by total insulin deficiency and absolute hyperglycemia, where more severe disease manifests in the presence of high bacterial loads and inflammatory cytokines[15].

Interestingly, despite a greater expression of the Th1 cytokine IFNγ and associated macrophage activation, both of which are linked to bactericidal activity in macrophages, diabetic guinea pigs were unable to contain the growth of *M. tuberculosis*. However, the alterations in

the immune response early in infection that allow for high bacterial growth are unknown. Many alterations in immune function have been reported in diabetic patients, the most common of which include reduced phagocytosis and microbial killing through oxidative burst in neutrophils and macrophages, impaired neutrophil chemotaxis, and impaired generation of antigen-specific T cell responses[17-21], all of which could account for more rapid growth of *M. tuberculosis* early in the course of infection. However, few studies have emphasized the mechanisms that increase susceptibility to pathogen-specific diseases where diabetes has a demonstrated impact on the manifestation, progression, severity and treatment of the disease, as is the case with tuberculosis.

Among the most important stages of the response during TB in the mouse model is the transition from innate to adaptive immunity. It is well known that a Th1 T cell response is critical for limiting progressive inflammation because it greatly enhances bactericidal capacity of *M. tuberculosis* infected macrophages, and corresponds with the stage of disease progression where bacterial growth is controlled[22-24]. In the absence of IFNγ, the signature of a Th1 response, bacterial growth continues and severe and fatal inflammation ensues[25]. A requirement for timely development of a cell-mediated immune response is the transport of viable bacteria from the lung to the draining lymph node by dendritic cells for antigen presentation[26]. The expedited transport of *M. tuberculosis* to the lymph node has consistently led to the development of earlier pulmonary cell-mediated immunity and better control of bacterial growth in animal models[27-29]. Therefore, it is reasonable to expect that any impairment in the development of an antigen-specific response would lead to higher bacterial growth. Such results have been demonstrated in the mouse model of diabetes, which develops a delayed adaptive immune response[30]. However, whether this also occurs in the comorbidity with the guinea pig model of type 2 diabetes is unknown. In this study, we aimed to identify whether altered early innate responses and transition to adaptive immunity account for rapid bacterial

growth and severe inflammation characteristic of type 2 diabetic guinea pigs later in the course of *M. tuberculosis* infection.

Materials and Methods.

Induction of type 2 diabetes in guinea pigs.

Outbred Dunkin-Hartley guinea pigs, weighing between 250-350 g, were obtained from Charles River Laboratories (Wilmington, MA) and maintained in individual housing. Guinea pigs were divided into two groups, either non-diabetic controls or those that would receive treatments to induce type 2 diabetes. The induction of type 2 diabetes was performed as previously described[8]. Briefly, 11 weeks prior to infection with *Mycobacterium tuberculosis,* guinea pigs were fed a custom-formulated high fat, high carbohydrate (HFHC) diet (Dyets Inc., Bethlehem, PA) *ad libitum* to induce impaired glucose tolerance. After 8 weeks of HFHC diet and confirmation of glucose intolerance by a 2g/kg oral glucose tolerance test, type 2 diabetes was induced with a single 200 mg/kg subcutaneous injection of anomer-equilibrated streptozotocin (STZ)[31], approximately 20 minutes after intramuscular treatment with 0.5 mg/kg of yohimbine[32]. Diabetic glucose intolerance was confirmed by oral glucose tolerance at 14 and 21 days post-STZ treatment.

Infection with *Mycobacterium tuberculosis*.

An aliquot of the H37Rv strain of M tuberculosis (TMC102, Trudeau Institute, Saranac Lake, NY), grown to log phase in Proskauer Beck medium, was diluted to 1 x 10^6 CFU/ml for delivery to guinea pigs by low dose aerosol exposure using the Madison chamber aerosol generation device calibrated to deliver approximately 20 bacilli per guinea pig. The course of infection was evaluated in non-diabetic and diabetic guinea pigs at the predetermined endpoints of days 10 and 19 of infection (n=4 per group, per time point). Prior to euthanasia, blood was

collected under isoflurane anesthesia by venipuncture of the cranial vena cava accessed by intrathoracic collection at the junction of the manubrium and first rib. Euthanasia was performed by anesthetic induction with ketamine (40 mg) and diazepam (1 mg) prior to intraperitoneal injection of an overdose of sodium pentobarbital (1.5ml/kg) and followed immediately by necropsy and tissue collection.

Histopathology and determination of lesion burden.

Tissues collected at day 10 and 19 of infection were fixed in 4% buffered paraformaldehyde and 5 μm sections of paraffin embedded tissues were stained with hematoxylin and eosin for histopathologic evaluation using routine methods. Morphometric analysis was performed using a Nikon 80i Eclipse microscope and StereoInvestigator software, version 10.02 (MBF bioscience, Williston, VT) with tissue area and lesion area estimated using the area fraction fractionator method and expressed as a percent ratio of lesion to total tissue area, as previously described[33, 34].

Quantification of tissue bacterial burden.

Lung, spleen, and liver were homogenized in 1 ml of PBS, plated in serial dilutions on nutrient 7H11 agar and incubated at 37°C for 3-6 weeks. Colony forming units (CFUs) were log transformed and expressed as CFUs/g of tissue.

RNA isolation and relative gene expression by quantitative RT-PCR.

The pulmonary circulation was perfused with RNasin RNase inhibitor (Promega, Madison, WI) (40 units/ml) in Hank's balanced salt solution (HBSS) at the time of necropsy. The right cranial lung lobe and head of the spleen at days 10 and 19, and one third of the peribronichial lymphnodes at day 19, were collected for RNA isolation and homogenized in RNA Later solution (Ambion). RNA isolation from lymph node at day 19 was not possible due to size

and consequently, all tissue was used for bacterial culture. Preserved lung and spleen tissues were incubated at 4°C overnight then frozen at -80°C until processed for RNA isolation. RNA isolation with Trizol reagent (Life Technologies, Grand Island, NY) was performed as instructed by the manufacturer followed by treatment with 10 units of DNaseI and recovery with an additional phenol/chloroform separation and sodium acetate-ethanol precipitation. The early immune response was evaluated by relative gene expression of selected key cytokines, chemokines and receptors. Following cDNA synthesis, quantitative RT-PCR was performed with SYBR green detection (BioRad, Hercules, CA) using the BioRad CFX-96 real time thermal cycler per manufacturer's instructions with each reaction containing 0.2 μM of each primer and 50 ng of cDNA template. Under these reaction conditions, primer design was optimized for equivalent amplification efficiencies. Gene expression was normalized to two reference genes, hypoxanthine-guanine phosphoribosyltransferase (HPRT) and GAPDH, both of which were validated for consistent expression under these experimental conditions. The HPRT, GAPDH, IL-12p40, IFNγ, TNFα, IL-1β, MCP-1, IL-10, IL-23 and IL-17A primer sequences used in this study are previously published[8, 35-37]. IL-12p35, IL-6, CCR7, CCL19 and CCL21 primers were designed using transcripts from the EMBL Ensembl annotated genome browser[28] for the guinea pig with the aid of Primer3 primer design software[29].

The primer sequences are as follows: CCR7 (ENSCPOT00000012440) Forward 5'-TGCTGACCCTTCCCTTCTGG, Reverse 5'-CATGCCGCTGAAGAAGCTCA; IL-12p35 (ENSCPOT00000000016) Forward 5'-CTGAGGGCGGTGAACAGTGA, Reverse 5'-GGCTTTCACGGTGCTGGTTT; IL-6 (ENSCPOT00000002030) Forward 5'-TCCAGATGCCACCCTGCTGG, Reverse 5'-TCTTCAAGGCGCTGAAGGACGA; CCL19 (ENSCPOT00000002661) Forward 5'-CCCTCAGCCTGCTGGTTCTC, Reverse 5'-TCACGATGTTCCCAGGGATG; CCL21 (ENSCPOT00000005489) Forward 5'-CCCTCTGCATCACCCAGACA, Reverse 5'-CTGCGGACGACCTTGTAGGG. RNA isolates

without reverse transcription were amplified for each tissue isolate to ensure a lack of contaminating genomic DNA and all assays performed included no-template controls. Data was expressed as normalized fold expression on a $\log_2$ scale.

IFNγ release assay by relative gene expression.

Prior to euthanasia on days 10 and 19 of infection, 1 ml of peripheral blood was collected in EDTA anticoagulant from non-diabetic and diabetic guinea pigs. Peripheral blood mononuclear cells were isolated from whole blood using Polymorph Prep (Cosmo Bio, Carlsbad, CA), which yields a population of lymphocytes and monocytes of greater than 95% purity from guinea pig blood based on cytologic evaluation (data not shown). The collected whole blood was layered on top of 1.5 ml of Polymorph Prep and centrifuged at 400 x g for 30 minutes at room temperature to separate PBMCs from granulocytes and erythrocytes. PBMCs were isolated and washed in 5 ml of Hank's Balanced Salt Solution, then plated at a cell density of 100,000 cells per well of a 48-well tissue culture plate in RPMI-1640 with 5% fetal calf serum. Cells from each animal were stimulated with a combination of immunodominant antigens ESAT-6 and CFP-10, concanavalin A as a non-specific mitogen, or ovalbumin as a non-specific antigen and negative control. Cells were cultured under these conditions for 24 hours then RNA isolated using methods outlined above, cDNA synthesized, and the relative expression of IFNγ transcripts measured by qRT-PCR. All data was normalized to the IFNγ expression levels in ovalbumin negative controls.

5.3 Results

The rate of bacterial growth is not altered by type 2 diabetes, but dissemination from the lung is delayed.

Bacterial loads were assessed from lung, lymph node, spleen and liver on days 10 and 19 of infection. Bacterial growth was similar in both diabetic and non-diabetic lung at day 10 of infection and as expected, no extrapulmonary dissemination to spleen or liver was evident at day 10 of infection (Figure 5.1). However, the frequency of cultivatable bacilli did differ in the lymph node early in infection on day 10. In non-diabetic guinea pigs, 3 out of 4 had viable bacilli in the lymph node ranging from 2 to above 3 $\log_{10}$ CFU per gram of tissue. In contrast, only 1 out 4 type 2 diabetic guinea pigs contained viable bacilli in the lymph node on day 10 of infection. The one remaining diabetic guinea pig had a lymph node bacterial load consistent with non-diabetic guinea pigs (Figure 5.2). By day 19 of infection, extrapulmonary dissemination and bacterial loads in the lung in diabetic guinea pigs was not statistically different from non-diabetics (Figure 5.1), including similar bacterial load in the lung-draining lymph node (Figure 5.2). As a result, the average rate of growth between days 10 and 19 of infection in non-diabetic and type 2 diabetic guinea pigs was similar, although minimally higher in type 2 diabetic guinea pigs at both time points evaluated.

Development of an antigen-specific IFNγ response is delayed in type 2 diabetic guinea pigs.

At day 10 of infection, there was no detectable induction of IFNγ by the immunodominant antigens ESAT-6 or CFP-10 when compared to non-specific ovalbumin controls (Figure 5.3). However, non-specific expression of IFNγ was induced by the mitogen concanavalin A,

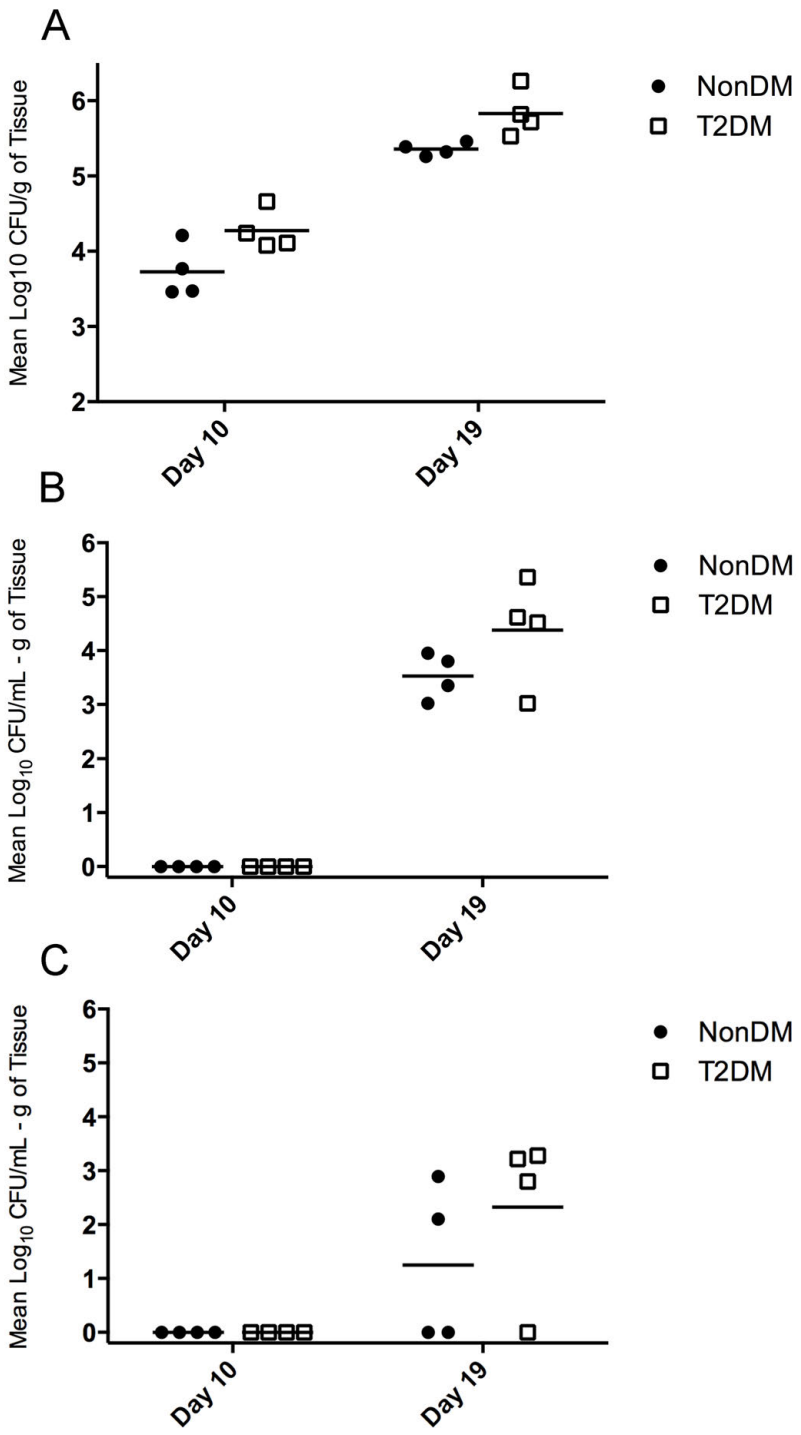

Figure 5.1. Pulmonary and extrapulmonary bacterial burdens do not differ early in *M. tuberculosis* infection. Bacterial burdens in the lung **(A)** on day 10 and 19 of infection are minimally higher in type 2 diabetic guinea pigs but no significant change in the rate of *M. tuberculosis* growth can be demonstrated between these two infection time points. As would be expected, no bacteria were cultured from spleen **(B)** or liver **(C)** on day 10 of infection, which precedes the stage of extrapulmonary dissemination. By day 19 of infection, extrapulmonary burdens in spleen and liver are similar although minimally higher in type 2 diabetic guinea pigs. (n=4 per group)

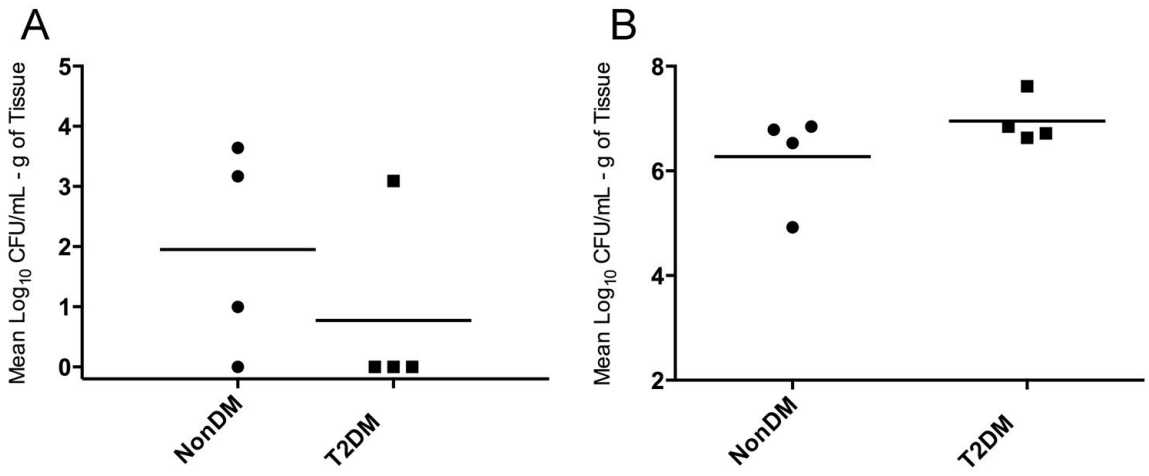

Figure 5.2. Arrival of viable bacteria to the lung draining lymph node is delayed in type 2 diabetic guinea pigs. (A) At day 10 of infection, a time when bacterial transport to the lymph node is expected, three of four non-diabetic guinea pigs had cultivatable organisms in the lung draining lymph node. In contrast, bacilli were cultured from the lymph node of only one of four diabetic guinea pigs. **(B)** By day 19, lymph node bacterial burdens were similarly elevated in both non-diabetic and diabetic guinea pigs. (n=4 per group)

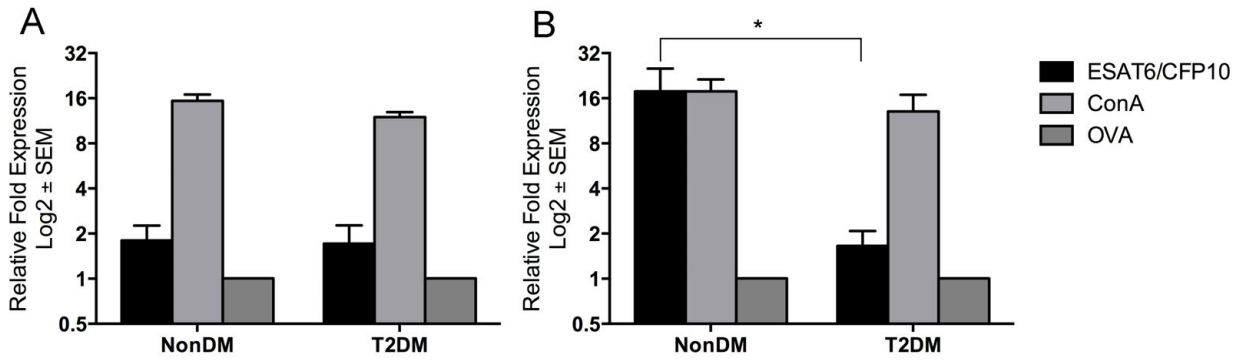

Figure 5.3. Development of antigen-specific cell-mediated immunity is delayed in type 2 diabetic guinea pigs. IFNγ expression was measured in PBMCs in response to specific antigen stimulation, non-specific mitogen stimulation, and in the presence of a non-specific antigen **(A)** On day 10 of infection, prior to onset of adaptive immunity, there is no antigen specific induction of IFNγ in either non-diabetic or diabetic guinea pigs. However, mitogen stimulated induction was equivalent in both groups. (n=4 per group) **(B)** By day 19 of infection, inducible IFNγ production by ESAT-6 and CFP-10 antigens is evident in non-diabetic guinea pigs while diabetic guinea pigs remain unresponsive. Similar to day 10, mitogen stimulation is equivalent in both groups. (n=4 per group) All gene expression data is normalized to ovalbumin as a non-specific antigenic stimulus. *P<0.05

the response to which was similar between both non-diabetic and type 2 diabetic guinea pigs. However, by day 19 of infection, the production of antigen inducible IFNγ was detected in cultured PBMCs of non-diabetic guinea pigs (Figure 5.3). In contrast, type 2 diabetic guinea pigs did not demonstrate any significant inducible IFNγ expression even at day 19 of infection, which was significantly different from the induction present in non-diabetic guinea pigs. However, mitogen stimulated IFNγ expression, induced by concanavalin A was similar between both diabetic and non-diabetic guinea pigs on day 19 of infection. The ESAT-6/CFP-10 inducible IFNγ expression in the non-diabetic guinea pigs correlated with an elevation in IFNγ in the lungs overall when compared to type 2 diabetic guinea pigs. Similar elevation in total IFNγ was also present in the lymph node of non-diabetic guinea pigs on day 19 of infection compared to lymph nodes of type 2 diabetics (Figure 5.4).

Disease severity does not differ early in disease but lymphatic lesion development is impaired in diabetic guinea pigs.

In non-diabetic and type 2 diabetic guinea pigs at day 10 of infection, there was minimal detectable lesion within the pulmonary parenchyma. These areas consisted of infrequent and small aggregates of macrophages with rare neutrophils. These lesions were present in 2 of 4 guinea pigs in both non-diabetic and type 2 diabetic groups. However, significantly different lesion manifestations were observed in the peribronchial tissue where aggregates of macrophage type cells surrounded, invaded and filled peribronchial lymphatic vessels. These changes were present in all four of the non-diabetic guinea pigs with multiple sites in each lung section. In contrast, only one of four type 2 diabetic guinea pigs displayed this lymphatic lesion, which consisted of a single small aggregate of cells (Figure 5.5). Interestingly, this single animal was also the only diabetic guinea pig to contain cultivatable bacilli in the lung draining lymph node.

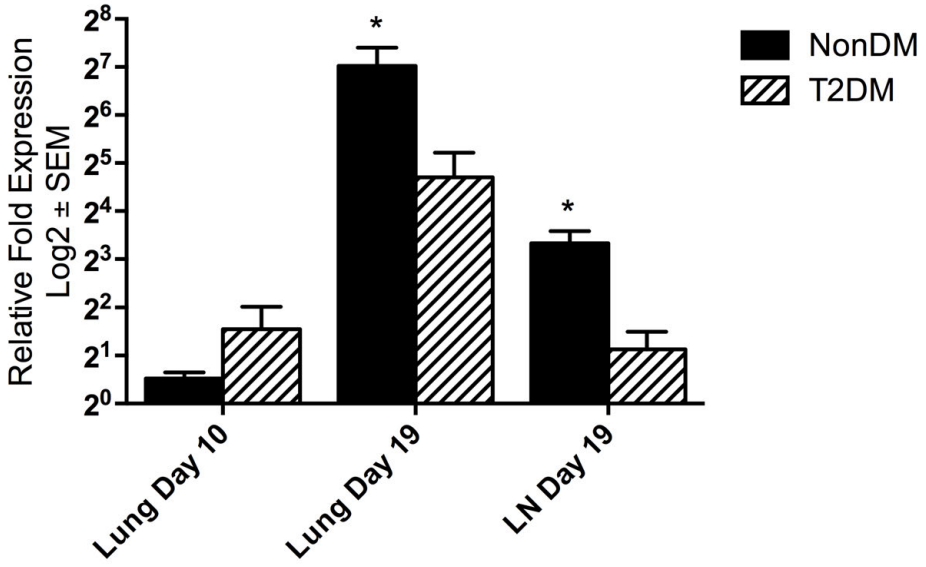

Figure 5.4. Expression of IFNγ is reduced in the lung and draining lymph nodes of type 2 diabetic guinea pigs. Total expression of IFNγ was measured in the lung on day 10 and 19 of infection and draining lymph nodes on day 19 of infection. IFNγ expression is reduced in diabetic guinea pigs on day 19 in both lung and lymph node, compared to non-diabetic guinea pigs. (n=4 per group) *P<0.05

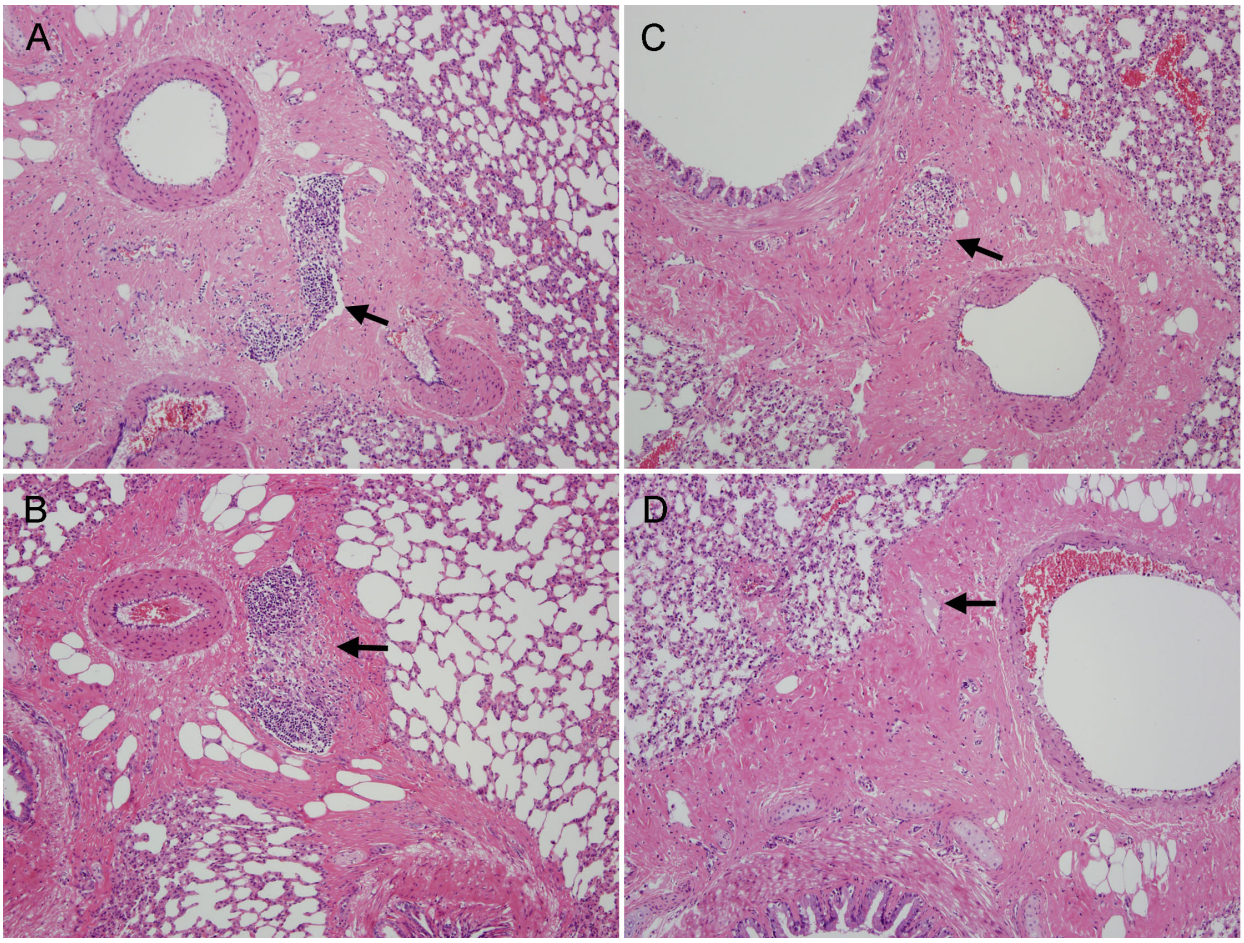

Figure 5.5. Early development of pulmonary lymphangitis is greatly reduced in type 2 diabetic guinea pigs. Images from day 10 of infection are representative of pulmonary pathology present in the non-diabetic and type 2 diabetic guinea pigs. **(A, B)** Non-diabetic lungs have consistent and larger inflammatory lesions surrounding and involving the peribronchial lymphatic vessels (arrow). **(C)** In contrast, type 2 diabetic guinea pigs, in general, lack this lesion (arrow) or **(D)** in one animal, displayed a single small focus of lymphatic inflammation (arrow).

By day 19 of infection, early granuloma structures were present with central areas of neutrophil infiltration and loosely arranged peripheral walls of macrophages. This lesion morphology was present in both diabetic and non-diabetic lung. Lesion area was measured in the lung of both diabetic and non-diabetic guinea pigs. Although 2 of 4 diabetic guinea pigs displayed higher total lesion area, no significant differences were present between non-diabetic and diabetic groups (Figure 5.6).

Type 2 diabetic guinea pigs have reduced expression of critical chemokines and receptors.

Because lymphatic lesions were slow or failed to form in the type 2 diabetic guinea pigs at day 10 of infection, we investigated the chemokine signals known to be critical for migration of dendritic cells. CCR7 chemokine receptor transcripts were measured in lung and lymph node as well as the corresponding chemokine signals, CCL19 and CCL21. In the lymph node at day 19 of infection, relative expression of CCL19 and CCL21 were similar with slightly higher, but not statistically significant, expression in the type 2 diabetic guinea pigs (Figure 5.7). Although less biologically relevant, the CCR7 receptor expression was also measured in the lymph node and again, no significant differences were identified between non-diabetic and type 2 diabetic guinea pigs. However, in contrast, the relative expression of the CCR7 receptor was reduced in the lungs of type 2 diabetic guinea pigs early at day 10, which persisted with a more significant reduction by day 19 of infection (Figure 5.8). An additional chemokine signal measured responsible for recruitment of inflammatory macrophages was MCP-1 (CCL2). Although no significant differences were identified in the lung at day 10 of infection, by day 19, the expression of MCP-1 was significantly reduced in diabetic guinea pigs.

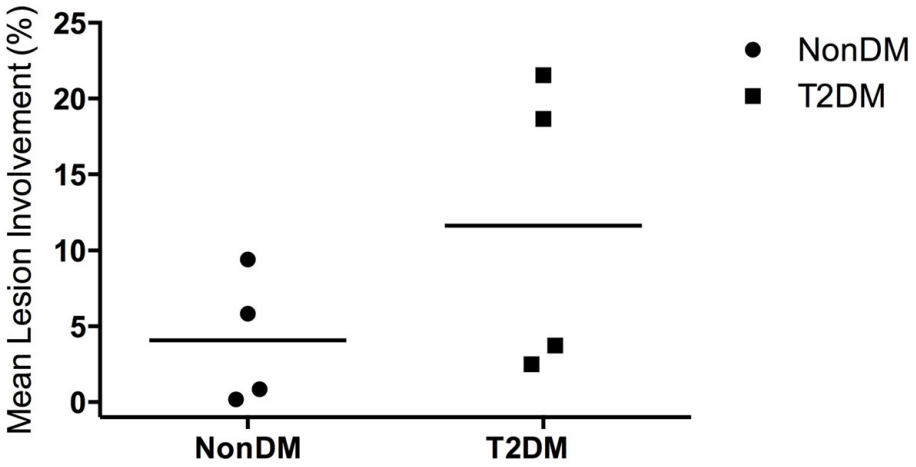

Figure 5.6. Severity of pulmonary disease does not differ between diabetic and non-diabetic guinea pigs. Disease severity was measured by proportional lesion area based on total pulmonary TB lesions as a fraction of total lung area. Although two diabetic guinea pigs display higher lesion burden in the lung, overall there is no significant difference between non-diabetic and diabetic guinea pigs. (n=4 per group)

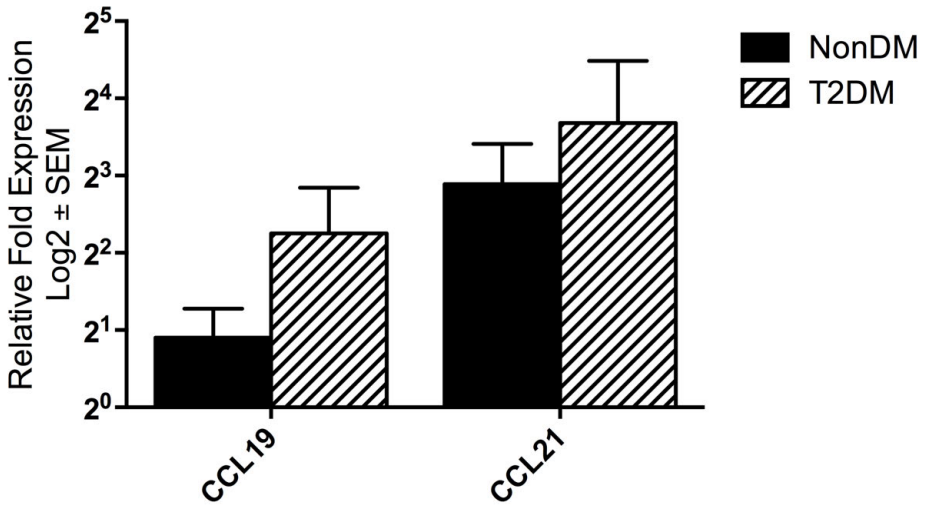

Figure 5.7. Expression of chemokines for dendritic cell migration is not altered in lymph node of type 2 diabetic guinea pigs. The chemokines CCL19 and CCL21, responsible for recruitment of activated dendritic cells, were measured on day 19 of infection in the lung draining lymph nodes. There is no significant difference in the expression of either chemokine between non-diabetic and diabetic guinea pigs. (n=4 per group)

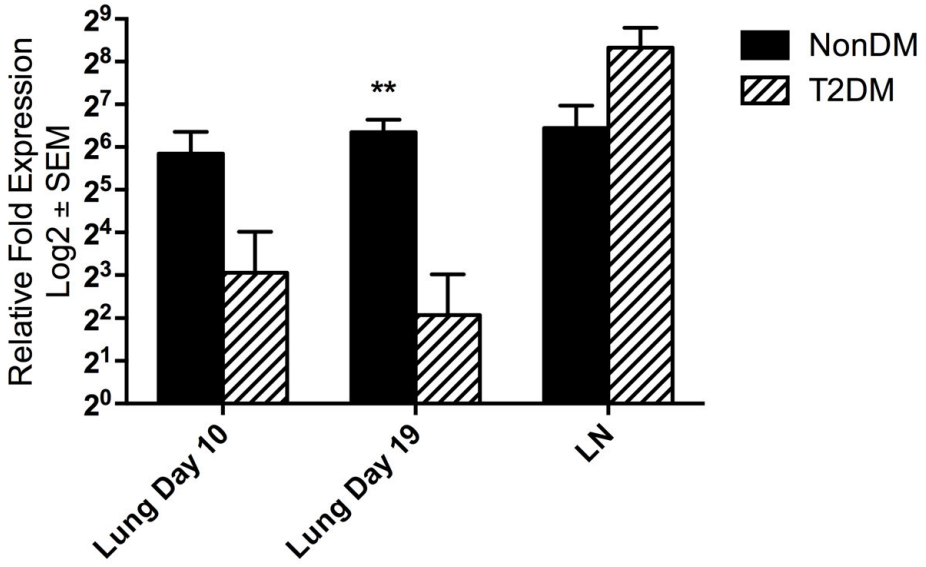

Figure 5.8. The expression of dendritic cell chemokine receptor, CCR7, is reduced in the lung of type 2 diabetic guinea pigs. The chemokine receptor CCR7, required for migration of dendritic cells to the draining lymph node for antigen presentation, is reduced on day 10 of infection in diabetic lung and more significantly reduced by in diabetic guinea pigs by day 19 of infection. There is no significant difference in CCR7 expression in the lymph node on day 19 of infection. (n=4 per group) **P<0.01

The early innate pulmonary cytokine profile is shifted in type 2 diabetic guinea pigs.

Cytokines that mediate a pro-inflammatory response, or the transition from innate to Th1-biased cell mediated immunity were evaluated. At day 10 of infection, few significant differences were identified between non-diabetic and type 2 diabetic guinea pigs (Figure 5.9). At day 10 of infection, IL-8 expression and IL-1β expression were both elevated in type 2 diabetic guinea pigs at 6.4- and 6.1-fold higher relative expression, respectively. No significant differences were noted at day 10 of infection in TNFα, IL-6, IFNγ, IL-12p40, IL-12p35, IL-23 or IL-17. In contrast, by day 19 of infection, there were multiple significant differences in the innate cytokine expression profile of type 2 diabetic guinea pigs (Figure 5.10). Although no significant changes were identified in the expression of the heterodimeric cytokine IL-12, as indicated by equivalent IL-12p40 and IL-12p35 expression, the expression of IL-23 (IL-12p19) was 52.3-fold lower in diabetic guinea pigs, which corresponded with a 40.2-fold reduction in expression of its induced cytokine, IL-17. Also reduced in type 2 diabetic guinea pigs was the relative expression of TNFα by 3.1-fold (P=0.11). In contrast, the cytokines IL-6 and IL-8 (P=0.1) were elevated in type 2 diabetic guinea pigs by 18.2- and 8.4-fold, respectively.

Discussion.

Previously we had investigated the course of TB progression and immune response in diabetic guinea pigs at day 30 of infection, a time when the cell-mediated immune response was strongly evident based on high IFNγ production, presence of activated $CD45^{high}$ CD4 and CD8 T cells, and a distinguishable population of activated $MHCII^{high}$ macrophages[8]. At this time in the course of infection, diabetic guinea pigs responded with what typically would be interpreted as an appropriate cell-mediated response with Th1 skewed IFNγ-expression and a higher proportion of macrophage activation in the presence of high IFNγ. Despite this presumably

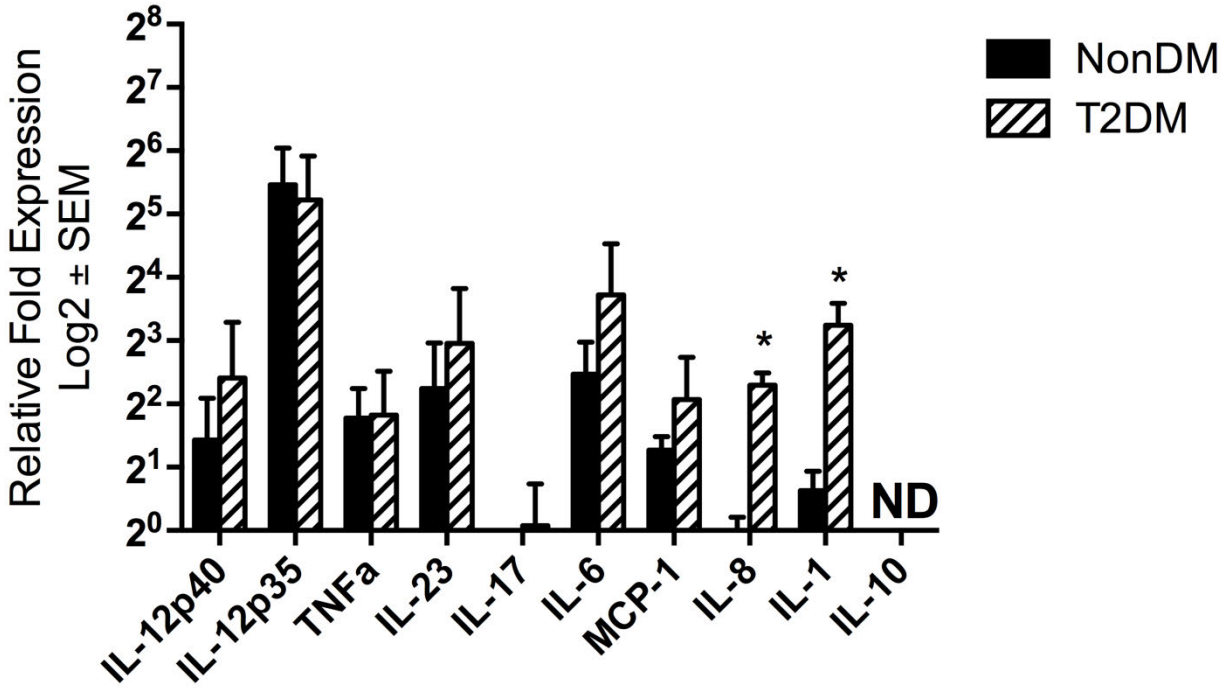

Figure 5.9. Cytokine profiles at day 10 of infection are indicative of a pro-inflammatory response in the lung of diabetic guinea pigs. Select cytokines that function in macrophage activation and bactericidal activity or promotion of a Th1 skewed cell-mediated response as well as chemokines for neutrophil and macrophage recruitment was measured by relative gene expression qRT-PCR. Alterations in early innate cytokine expression on day 10 of infection are limited to elevations in pro-inflammatory cytokine IL-1β and chemokine IL-8 in diabetic guinea pigs. (n=4 per group) *P<0.05.

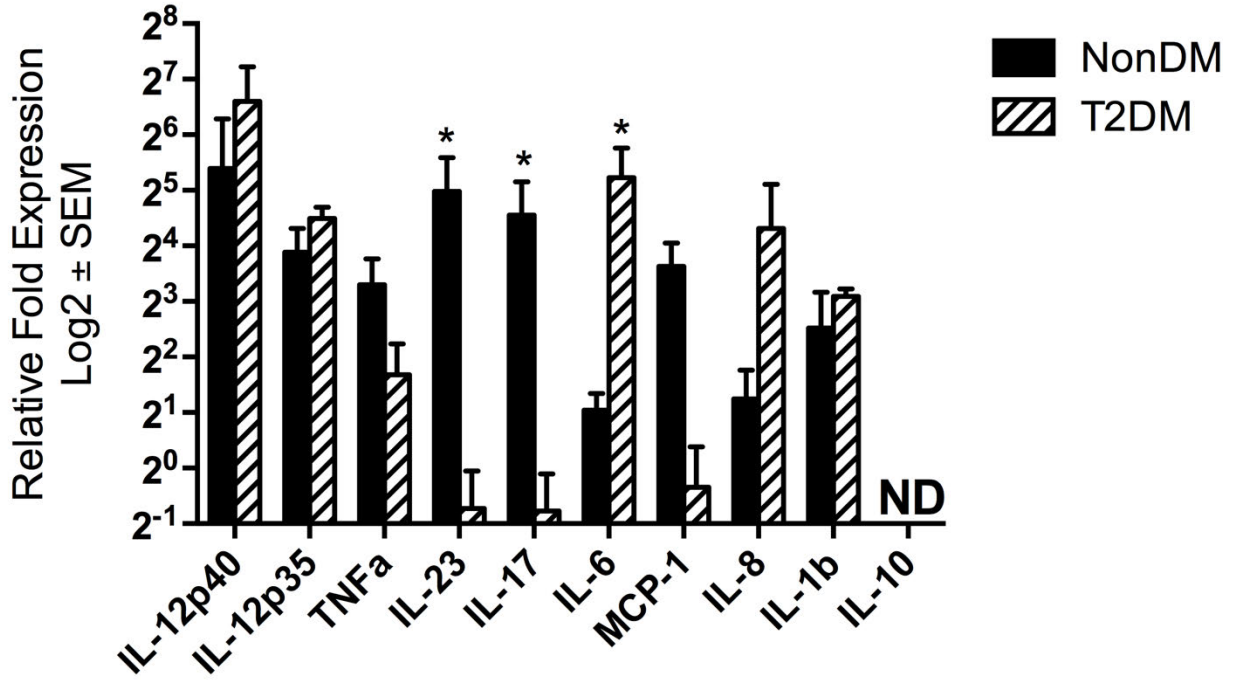

Figure 5.10. The inflammatory profile in the lung of diabetic guinea pigs at day 19 of infection is dominated by atypical cytokines. The same activating and Th1 cytokines were measured on day 19 of infection in lung. Expression of IL-6 and IL-8 (P=0.1) are elevated in diabetic guinea pigs while TNFα (P=0.11), MCP-1 (P=0.1), IL-23 and IL-17 are all reduced in diabetic guinea pigs compared to non-diabetics. (n=4 per group) *P<0.05

favorable response, diabetic guinea pigs were unable to control the bacterial growth and in contrast, to non-diabetic guinea pigs, manifested with a pro-inflammatory response involving heightened innate cytokine expression and damaging suppurative inflammation. However, it was not apparent whether this severe manifestation of TB in diabetic guinea pigs occurred only after onset of adaptive immunity or if such a response was consistent throughout infection in diabetic guinea pigs; or what factors may have contributed to such high bacterial growth. In this study, we targeted day 10 and day 19 of infection as predetermined endpoints to evaluate disease progression because this spans the initiation of transitional innate and adaptive immunity in the lymph node and the earliest onset of antigen-specific cell-mediated immunity in the lung in guinea pigs, inferred from previous histopathological and immnunohistochemical analyses in the guinea pig and immunological investigations in mouse models[38-41].

The induction of a Th1 dominated cell-mediated immune response is a critical factor in the progression of TB disease because it both limits bacterial growth and progressive inflammation. It is well documented that disease progression is limited after the onset of IFNγ production in *M. tuberculosis* infection and recently an anti-inflammatory function of IFNγ has been attributed to limiting the neutrophilic inflammatory response[42]. Additionally, IFNγ generously promotes the production of nitric oxide in infected macrophages and greatly enhances their bactericidal capacity[43]. Therefore, with an assumed protective function of this key cytokine, it was surprising to identify more severe TB disease in the face of high IFNγ expression in diabetic guinea pigs and we hypothesized that the response may be exacerbated by the significantly higher bacterial burdens present in diabetic guinea pigs.

Among the most critical factors in the establishment of a cell-mediated immune response during *M. tuberculosis* infection is the timing of this response. In any circumstance, the earlier generation of cell-mediated immunity, either through BCG-induced memory response after immunization[44], or through the use of mutated and less virulent *M. tuberculosis* strains, has

resulted in earlier pulmonary cell-mediated immunity and consequently, better control of bacterial growth[27, 45, 46]. Consistent with previous data in the mouse model, we have identified a significant delay in the onset of antigen-specific cell-mediated immunity in diabetic guinea pigs measured based on IFNγ transcription in response to stimulation with ESAT-6 and CFP-10, two immunodominant antigens of *M. tuberculosis*, which serve as the basis of human IFNγ release diagnostic assays[30, 47]. However, in contrast to previous studies in human patients, PBMCs of diabetic guinea pigs did not display reduced non-specific IFNγ production in response to mitogen stimulation[48]. This delayed response may represent a fundamental underlying mechanism of diabetic susceptibility to TB by allowing bacterial growth to continue unopposed until finally, when pulmonary adaptive immunity arrives, effector T cells must respond to a very high antigen load.

The potential involvement of delayed cell-mediated immunity in impaired control of bacterial growth in diabetic guinea pigs is further supported by the observation that pulmonary bacterial growth in diabetic guinea pigs did not differ significantly from non-diabetic controls out to day 19 of infection, indicating no difference in the rate of growth between the groups. Additionally, by day 19 of infection, bacterial growth in non-diabetic had reached a level similar to what was also observed previously at day 30 of infection. This suggests that while bacterial growth is controlled after onset of cell-mediated immunity in non-diabetics, the growth continues in diabetic guinea pigs in the absence of T cell help. However, an additional consideration for bacterial growth in diabetics is the excess availability of both lipid and carbohydrate nutrients. Previously, we had shown that exogenous dietary supplementation with sucrose increases bacterial growth in the guinea pig model of TB, but the impact of this on immune function is unknown.

This delayed adaptive response in diabetic guinea pigs was evident at day 19 of infection, a point in the course of infection where pulmonary cell-mediated immunity is being

initiated and suggests that the development of this response is fundamentally delayed at the level of antigen presentation in the lymph node. This interpretation is supported by the fact that IFNγ production was lower at day 19 of infection not only in the lung of diabetic guinea pigs, but also in lung-draining lymph node. In contrast, non-diabetic guinea pigs demonstrated higher IFNγ production in both sites, which correlated with an antigen-inducible response in PBMCs stimulated *in vitro*.

Lymphatic pathology, characterized by lymphangitis due to infiltration with macrophage-type cells, is one of the earliest characteristic lesions to form in the guinea pig model of TB and the inflammatory involvement of the lymph node is also well-documented in human TB, particularly in children[38, 49]. This highlights the importance of cell migration to lymphatics and subsequent development of lymphadenitis in this model species, which closely reflects the lesion morphology and progression in human TB. In this study, we observed a generalized lack of early pulmonary lymphangitis in diabetic guinea pigs on day 10 of infection, which suggests impaired migration of antigen presenting cells to the draining lymph node; however, this finding would be better supported by additional phenotypic identification of the cells and a larger sample size to account for low sensitivity of the histopathological approach to tissue sectioning. However, impaired migration is further supported by the observation that cultivatable bacilli in the lung draining lymph node were isolated from only 25% of diabetic guinea pigs on day 10 of infection, in contrast to 75% of non-diabetics. Previously, it has been shown that transport of viable bacilli to the lymph node by pulmonary dendritic cells is required for the timely establishment of an adaptive immune response to TB[41, 50], and our data suggests that impaired migration of antigen presenting cells may underlie the delayed development of adaptive immunity in diabetic guinea pigs.

We have identified several potential factors in this study that may contribute to impaired antigen presenting cell migration and delayed antigen-specific Th1 immunity. Perhaps the most

important, and a potential driving force in the diabetic susceptibility to TB, is the early increased expression of IL-6 in the lungs of diabetic guinea pigs. IL-6 is a pleiotropic cytokine whose role in the response to *M. tuberculosis* infection is not well understood, but has a documented role in the inflammatory pathogenesis of type 2 diabetes[51-53]. Many cell types, beyond immune cells, have the capacity to produce IL-6, including pulmonary epithelium[54]. The production of IL-6 is not limited to immune cells and in a state of insulin resistance, the majority of IL-6 is produced by adipose, liver, and skeletal muscle. The induction of IL-6 is dispensable for control of *M. tuberculosis* growth and antigen specific immunity, but its absence leads to progressive inflammation early in disease[55-57]. However, when present in high concentrations, IL-6 impairs the induction of a productive Th1 response[58]. More recently, a reduced Th1 effector response has been demonstrated in SOCS3 deletion mice lacking the primary suppressor of IL-6 signaling, wherein it was hypothesized that persistently high IL-6 impairs IL-12 production and therefore generation of Th1 biased immunity from the critical IL-12-IFNγ axis[59]. A reduced production of IL-12 has also been demonstrated during *in vitro M. tuberculosis* infection of PBMCs from type 2 diabetic human patients, linked to the oxidative stress of diabetes and lower concentrations of intracellular reduced glutathione (GSH)[60]. Although IL-12 is required for dendritic cell migration and T cell priming[61], our data does not reflect the conclusions of these two studies because IL-12, measured as relative expression of both IL-12p35 and IL-12p40 components of the cytokine, did not differ between non-diabetic and diabetic guinea pigs.

Equivalent expression of IL-12 and the observation of delayed arrival of *M. tuberculosis* bacilli at the regional lymph nodes led us to investigate other potential contributions to a delayed onset of cell-mediated immunity. Chemokines play an important role in the transition from innate to adaptive immunity during *M. tuberculosis* infection and because of the lack of lymphatic lesions in diabetic guinea pigs, we investigated the expression of chemokines and their receptor implicated in dendritic cell migration to the lymph node during infection[62]. CCL19

and CCL21 are chemokines produced in the lymph node and provide a gradient for the migration of activated dendritic cells expressing the chemokine receptor, CCR7[62, 63]. In this study, we show that the expression of CCL19 and CCL21 in lung-draining lymph nodes is not impaired in diabetic guinea pigs and actually is slightly elevated, suggesting that the migratory signal is not involved in delayed delivery of viable bacilli to the lymph node. However, we observed a significant reduction in the CCR7 receptor in the lung on both days 10 and 19 of infection, which may have an impact on the migratory responsiveness of dendritic cells to CCL19 and CCL21. It should be noted however, that CCR7 is also expressed on lymphocytes and this reduction may not be entirely reflective of the dendritic cell population. Previously, suppressive effects of IL-6 on dendritic cell function have been demonstrated by impairing migratory capacity through reduction of surface CCR7 expression[64]. Our data of high pulmonary IL-6 in the presence of low CCR7 expression are consistent with the findings of this study and suggest that diabetes-induced IL-6 may play a central role in the delayed establishment of Th1 cell-mediated immunity in diabetic guinea pigs.

Other factors may also be involved in the early diabetic response to infection. TNFα has been shown to make a large contribution to the overall early innate response to *M. tuberculosis* infection[65]. In the absence of TNFα, early bacterial control is impaired and Th1 immunity is reduced. Additionally, TNFα contributes significantly to the fate of macrophages containing intracellular bacilli[66]. When TNFα is blocked or induced in excessive concentrations, the induction of apoptosis associated with TNFα is reduced[67]. Apoptosis is a critical mediator in the development of cell-mediated immunity by increasing the acquisition of bacilli and subsequent activation of pulmonary dendritic cells[45]. We have identified here that TNFα expression is reduced in diabetic guinea pigs early in infection. Interestingly, high IL-6 is known to reduce TNFα expression[68-71], further implicating the role of IL-6 in diabetic susceptibility to TB.

In stark contrast to our previous evidence of high IL-17 in diabetic guinea pigs at day 30 of infection, which may support a damaging neutrophilic inflammatory response[72], we show here that early in infection, the opposite is true of diabetic guinea pigs where expression of both IL-17 and its inducing cytokine, IL-23 are markedly reduced in diabetic guinea pigs. The role of this amidst the other immunological alterations identified early in the course of diabetic infection is uncertain. IL-23 promotes early IL-17 production by γδT cells early during *M. tuberculosis* infection and recruits neutrophils to the site of infection, a cell type also shown to be important in the development of adaptive immunity[46, 73]. However, in mice, the adaptive response develops adequately in the absence of IL-12p19, the component defining the IL-23 heterodimeric cytokine, as long as IL-12p70 is present[44, 74]. Considering these studies, our data showing that expression of IL-12p70 components was unaltered questions the importance of low IL-23 in the pathogenesis of tuberculosis-diabetes comorbidity in our guinea pig model.

Although this study is limited by low sample size, we have identified some important alterations in early immune function with statistical differences. From this study, it is apparent that a delayed cell-mediated immune response may represent a fundamental pathway leading to more severe TB disease in diabetic guinea pigs. We have learned that bacterial growth does not occur earlier or more rapidly in diabetic guinea pigs but rather, bacterial burdens reach higher levels before they can be controlled, which may be a direct result of delayed cell-mediated immunity. It is anticipated that the severe TB disease in diabetics is in response to this high bacterial growth, but determining the role of antigen load requires further investigation. In order to improve the timing of the cell-mediated response that occurs due to diabetes, a better understanding of the underlying mechanisms for this delay will be necessary. We have identified factors that could contribute to delayed immunity in diabetic guinea pigs, all of which have been previously linked to the over production of IL-6. Therefore, further investigation of the role for this cytokine in more severe TB disease in diabetic guinea pigs will help to identify novel

pathways and therapeutic targets to reduce the burden of TB disease in patients with diabetes. In conclusion, we have demonstrated that severe disease and damaging inflammation is not a continual process in diabetic guinea pigs but instead, occurs after a delayed onset of adaptive immunity potentially in response to high bacterial load.

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CHAPTER 6

Final Discussion and Future Directions

Less than 10% of individuals exposed to *M. tuberculosis* will develop active TB disease. The active form of TB, whether developing upon exposure or as reactivation from the latent form of *M. tuberculosis* infection, is often associated with a factor of susceptibility intrinsic to the host or from an acquired risk factor[1]. The previous epidemic and ongoing association of HIV with *M. tuberculosis* infection highlights the impact that a particular risk factor can have on TB incidence[2]. In the 1990's HIV-associated TB had nearly doubled the incidence of TB in some areas of subsaharan Africa where HIV was most prevalent, and during this time, TB emerged as the most important opportunistic infection and cause of death in HIV patients[3-5]. In hindsight, more controlled and dedicated efforts to diagnose and treat HIV-TB comorbid individuals may have limited the impact of this epidemic on the overall control of TB[2].

A primary contributing factor to this comorbidity was the convergence of the HIV epidemic on countries in Africa already having high endemic prevalence of TB. Now, TB endemic countries are faced with a growing epidemic of non-communicable diseases, perhaps the most important of which is diabetes, a known risk factor for TB[6]. Although at the individual level, diabetes has a lesser impact than that of HIV, there is a much larger and rapidly growing diabetic population, which creates a population attributable risk for TB that is approximately equivalent to HIV[7]. Reflecting on lessons learned from the HIV-TB epidemic, a collaborative framework has already been established recognizing the importance of early detection and treatment in diabetes-TB comorbidity and has defined a research agenda to reduce the impact of diabetes on TB control[8, 9].

However, the mechanisms by which diabetes predisposes to the development of active TB still remain poorly defined. A better understanding of this pathogenesis will contribute

greatly to the prioritized agenda to identify the most successful approaches for diagnosing and treating TB in the face of diabetes and *vice versa*. However, investigations using animal models to gain further insight into TB pathogenesis, efficacy of new treatment strategies and evaluation of preventive strategies are infrequently modeled in the context of TB risk factors, including diabetes, which we have shown in these studies can have a significant impact on the response to infection with *M. tuberculosis*[10].

In the previous chapters, we have identified a number of important conclusions, which have led to the development of novel hypotheses, a theoretical model for how diabetes leads to more severe TB disease, as well as new therapeutic targets to improve the course of TB and response to treatment, both in diabetes-TB comorbidity and in the absence of diabetes as a risk factor.

The model chosen to study any disease, including tuberculosis, should be carefully selected to have the greatest translational potential for application to human disease. Unfortunately, an exact replicate of human TB disease is not provided by any animal species, and all have their advantages and disadvantages[11]. Although the non-human primate is arguably the most reflective animal model of TB, the use of this species in TB studies is cumbersome and expensive. In our work, the guinea pig model was chosen because this model closely reflects the pathology and progression of active TB in humans and has been relied upon with frequency for the evaluation of new treatment and preventive strategies. During these studies, we developed the first model of type 2 diabetes in the guinea pig, which develops diet-induced glucose intolerance and reduced β-cell mass based on a design to closely mimic the pathogenesis of insulin resistance and subsequent type 2 diabetes in humans.

Evaluating this model in the context of type 2 diabetes and TB comorbidity, we learned that type 2 diabetic guinea pigs infected with *M. tuberculosis* develop many of the reported clinical features of TB in diabetic human patients including high bacterial burdens, more numerous and severe TB lesions, and a dysregulated immune response involving higher innate

and type 1 cytokines[10, 12-14]. Using this model, we have shown that despite strong Th1 cell-mediated immunity, which is known to be critical for the control of TB, diabetic guinea pigs infected with *M. tuberculosis* develop a high lung and extrapulmonary bacterial burden accompanied by an unfavorable inflammatory response predominated by neutrophilic infiltration[10]. These data indicate that in diabetic guinea pigs, early immune responses are unable to contain bacterial growth, which leads to exacerbated immunopathology.

The observation that diabetic guinea pigs developed more severe disease in the presence of a bacterial burden nearly 100-fold higher than non-diabetic guinea pigs led us to investigate the early immune factors that might allow for such exuberant bacterial growth. We had originally hypothesized that an exacerbated immune response would be present from early on in the course of infection, rationalized based on the robust inflammatory response identified in our type 2 diabetic guinea pig model and also reported in the human comorbidity[13, 14]. However, we have now linked the severe TB disease manifested in diabetic guinea pigs to generally hyporesponsive innate immunity early in the course of disease, highlighted by reduced involvement of the pulmonary lymphatic system and draining lymph nodes, which is the site of antigen presentation and development of the cell-mediated immune response[15, 16]. Furthermore, diabetic guinea pigs had delayed development of antigen-specific cell-mediated immunity, the timing of which is critical for the effective control of bacterial growth in the lung and extrapulmonary sites because the production of IFNγ by antigen-specific T cells is generally accepted to enhance the bactericidal capacity of infected macrophages and limit progressive inflammation[17-20].

From these conclusions we have generated a hypothetical model of the pathogenesis of tuberculosis in diabetic guinea pigs, depicted in the following figure, that ultimately leads to more severe TB disease:

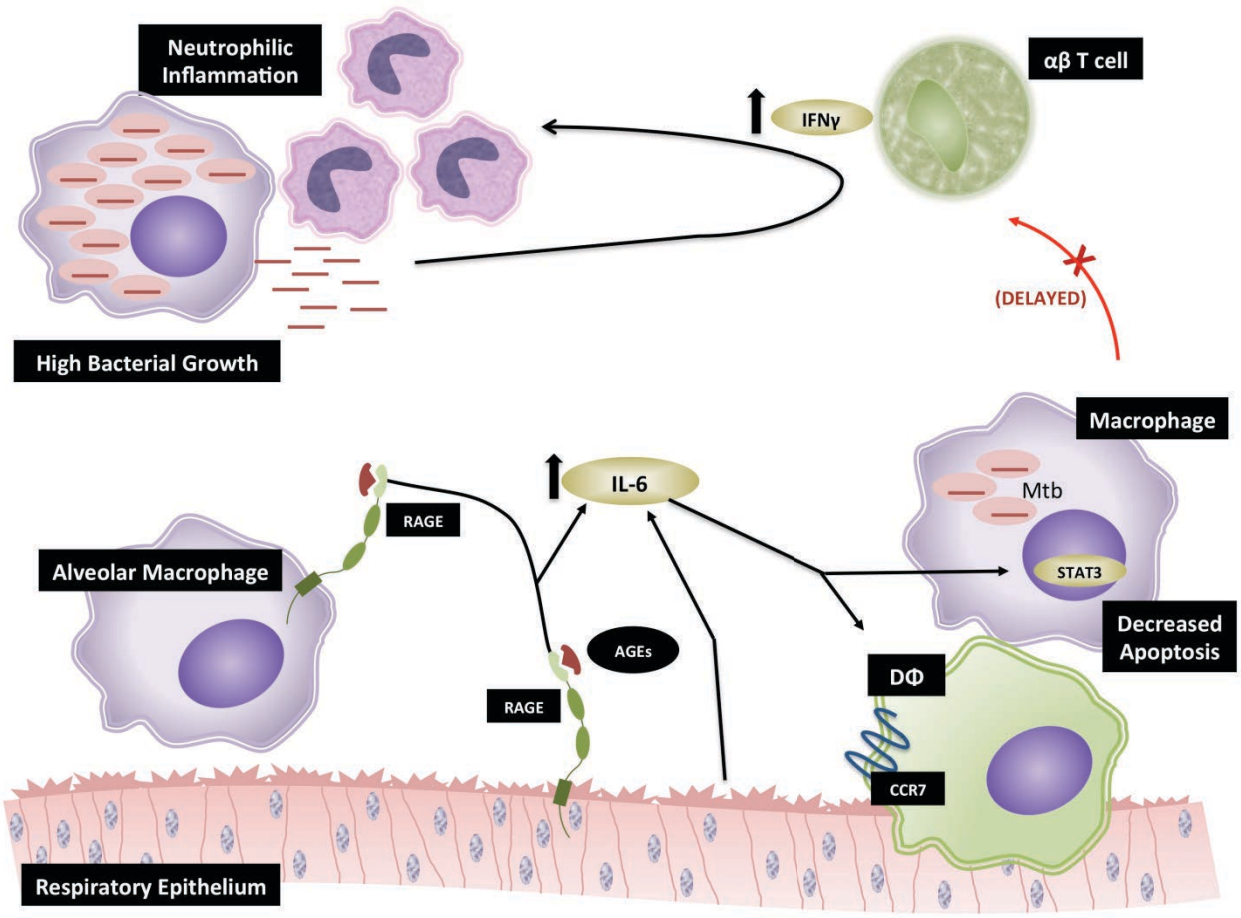

Figure 6.1. Proposed pathway leading to delayed cell-mediated immunity and more severe TB disease in diabetic guinea pigs. IL-6 is a proposed central mediator, which may be the result of AGE-induced RAGE signaling on respiratory epithelium and macrophages. IL-6 serves as a survival signal to macrophages by activation of STAT3, leading to decreased apoptosis, and also impairs dendritic cell migration by reducing CCR7 expression. In turn, a delayed cell-mediated response occurs because of its dependence on apoptosis and bacterial transport to the lymph node for timely initiation. This delay allows for substantial bacterial growth in the lung such that, upon encounter by a Th1 response, the severity of inflammation is exacerbated.

It is recognized in both humans and mice that the onset of adaptive immunity is delayed normally in *M. tuberculosis* infection, even in the absence of known risk factors[15, 18, 21, 22]. This delay allows for bacteria to replicate to a high level, such that upon arrival of the cell-mediated response in the lung, the bacterial growth can only be controlled but not eliminated. We have demonstrated in these studies that cell-mediated immunity is further delayed under the influence of diabetes. We propose that this additional delay allows for continued growth of *M. tuberculosis* in the lung to an extent that exceeds the burden typical of a guinea pig without diabetes. This is further supported by the observation that the rate of bacterial growth is not accelerated in our type 2 diabetic guinea pig model compared to non-diabetic guinea pigs. Instead, we propose that bacterial growth is simply allowed to go unopposed for a longer period of time in diabetics because of prolonged absence of Th1-based IFNγ production.

Because plateaued growth of *M. tuberculosis* corresponds with the presence of antigen-specific Th1 immunity in the lungs[17], and diabetic guinea pigs did not develop severe disease until after Th1 immunity was evident in the lung, we suspect that this delay in adaptive immunity is a critical feature mediating the development of severe TB disease, which is driven by high antigen load. In support of this interpretation, these findings closely mimic immune reconstitution inflammatory syndrome (IRIS), where an injurious IFNγ response to excessive bacterial growth occurs upon restitution of immune function in end-stage HIV and *M. tuberculosis* co-infected individuals[23]. Individuals with TB-HIV IRIS develop severe and atypical neutrophilic inflammatory responses that are also manifested in the mouse model of IRIS and are very similar to the exacerbated neutrophilic response identified in the guinea pig model of type 2 diabetes[24].

The underlying mechanisms that lead to the severe disease associated with IRIS remain unknown but one fundamental requirement is the presence of antigen-specific T cells, indicating that this is an exacerbated innate response to T cell help[24], which we hypothesize also occurs in

response to high antigen load in diabetic guinea pigs. Previous research indicates that IFNγ may be involved in this process but these results contradict the normal regulatory and beneficial function assigned to this molecule in TB pathogenesis[25]. In future studies, it will be important to determine the role of CD4 T cells, and IFNγ itself, in the uncontrolled growth of bacteria in the lung and an exacerbated response to high antigen load. Conversely, it will also be important to determine if high bacterial growth in diabetic guinea pigs is what contributes to over-production of IFNγ and leads to severe and damaging inflammation.

It is known that insulin resistance is mediated by inflammation and as a result, type 2 diabetes can be classified as an inflammatory disease[26]. Consequently, it is possible that preexisting low-grade inflammation from type 2 diabetes may impact the response to infection. We have identified two potential pre-existing inflammatory factors that could have an early impact on the host response to *M. tuberculosis* infection; IL-6 and the interaction of advanced glycation end products (AGEs) with their receptor RAGE. The source of IL-6 in diabetes pathogenesis is not limited to inflammatory cells. Many cell types are capable of producing IL-6 and are involved in obesity-associated inflammation including adipocytes, hepatocytes, and skeletal myocytes[27, 28]. The RAGE receptor is over expressed in diabetes due to transcriptional activation by its own signaling pathway[29].

In diabetic guinea pigs, we have identified increased expression of the RAGE receptor in lung, which normally has the highest expression of all organs, and also increased pulmonary expression of IL-6, which may be epithelial derived[30, 31]. Linking these together, we have also confirmed that IL-6 is a major product of RAGE signaling in the presence of AGE ligands, using guinea pig macrophages. Since IL-6 is, (1) a major product of RAGE signaling[32]; (2) is known to impair a cell-mediated immune response in TB[33]; and (3) was shown to be significantly increased early in *M. tuberculosis* infection of diabetic guinea pigs, it will be important in future studies to identify the role that IL-6 may have in the delayed adaptive immune response of

diabetic guinea pigs. IL-6 is known to impair dendritic cell function by reducing CCR7 expression and inhibiting migration[34], data that is consistent with our observations made during early *M. tuberculosis* infection of diabetic guinea pigs. Additionally, IL-6 may promote survival of macrophages through STAT3 transcriptional activation. The STAT3 transcriptional factor is constitutively activated in type 2 diabetes and has previously been shown to inhibit macrophage apoptosis in obesity-associated inflammatory disease[35, 36]. Both dendritic cell migration and macrophage apoptosis are critical components in the transition from innate to adaptive immunity and the generation of a Th1 cell-mediated immune response[37], and will be investigated further in future studies.

In both diabetic and non-diabetic guinea pigs, we have shown that glucose intolerance and hyperglycemia exacerbates the progression of TB disease and leads to the formation of advanced glycation end products (AGEs)[38]. AGEs, a pathologic and unregulated byproduct of non-enzymatic glycation, function as pattern recognition molecules to promote a sustained pro-inflammatory host response by signaling through the receptor for AGEs (RAGE)[39, 40]. AGE-RAGE signaling and oxidative stress mediated by AGEs are fundamental pathogenic mechanisms directly linked to the high morbidity and mortality associated with the complications of uncontrolled diabetes[39-42]; however, the role of this interaction in diabetic susceptibility to infections, including *M. tuberculosis*, is unknown. The direct relationship between hyperglycemia and glycation in the pathogenesis of diabetic complications, the evidence that uncontrolled hyperglycemia is a greater risk factor for TB than individuals with controlled diabetes, and the presence of a dysregulated inflammatory response in diabetic, *M. tuberculosis* infected guinea pigs that accumulate AGEs, implicates AGE-RAGE signaling in the pathogenesis of diabetes-TB comorbidity. Studies investigating the role of AGEs in diabetes as well as RAGE in other infectious diseases have been performed previously using inhibitors of AGE formation as well as high-affinity RAGE inhibitors, and also through the therapeutic use of the decoy receptor for RAGE ligands known as soluble RAGE (sRAGE)[43-45]. These techniques

could be employed in future studies to evaluate the role of AGE-RAGE signaling in the development of severe TB disease in diabetic guinea pigs.

Up to this point, I have described a proposed pathogenesis for diabetic susceptibility to TB that revolves around immunological mechanisms. However, an alternative hypothesis that certainly deserves consideration is the contribution of host-derived nutrient availability to the bacterium. In these studies we identified that *M. tuberculosis* infection, by itself, is capable of inducing severe metabolic disturbances in both glucose and lipid metabolism, resembling insulin resistance and diabetes. As a result, we have established the guinea pig as an ideal animal model to study interventions on the TB-associated metabolic disturbances that mimic what had been reported in humans years ago[46, 47]. It is well known that TB disease results in a catabolic syndrome historically known as "consumption", leading to cachectic wasting of muscle and fat, a process that is likely a result of the metabolic disturbances that we identified in the guinea pig. Results from this study suggest that increased nutrient availability may significantly impact TB disease either at the host or bacterial level because exogenous feeding of sucrose enhanced bacterial growth and led to more severe TB pathology. Whether immune defects were present in these sucrose-fed, non-diabetic guinea pigs is unknown, but based on these results, a reasonable hypothesis to pursue is that excess nutrient availability, whether exogenous or host-derived, contributes to the growth and persistence of *M. tuberculosis* in the host.

It is anticipated that that the host metabolic derangements are an adaptive response of the host to chronic inflammation but that the *M. tuberculosis* bacterium benefits from host nutrient availability. At this point, the impact of excess host metabolites on immune function and host response to the infection is unknown. However, lipid sequestration and cholesterol accumulation within chronic granulomatous lesions of *M. tuberculosis* infected animals as well the frequent use of PET-CT imaging to identify experimental tuberculous lesions are indicative of altered metabolism in the host inflammatory response[48, 49]. Additionally, recent emphasis has been placed on metabolism of the organism itself and its utilization of host derived molecules.

Although the pool of carbon sources utilized by *M. tuberculosis* bacilli are not fully understood, those that are most readily used are fatty acids and cholesterol, which may be host derived and contribute to lipid accumulation within the bacteria that is characteristic of bacilli in a dormant or persistent state, as well as changes in cell wall lipid constituents[50-52]. Additionally, recent evidence suggests that bacilli may also use glucose *in vivo* to establish a persistent infection[53], which is likely to be readily available in a state of uncontrolled diabetes.

The hypothesis that derangement of host metabolism is a treatable response to infection has emerged from this study, where an improvement in glucose and lipid metabolism would be targeted to improve both the host response to infection and also the severity and progression of wasting associated with this disease. It would therefore be important to know the impact of limiting host-derived nutrients on the viability and metabolic state of TB organisms. The course of antimicrobial treatment for TB is exceedingly long and is complicated by adverse toxicity in human patients, especially in multidrug resistant TB cases, where treatment regimens may reach years in duration. Alternative and adjunctive therapies are greatly needed to reduce the length of treatment required with antimicrobials alone, and the implementation of metabolic interventions, by repurposing FDA-approved drugs for the treatment of type 2 diabetes, to target the metabolic disturbances associated with chronic inflammation of TB, may represent one such alternative approach.

The guinea pig model will serve as an excellent starting point to investigate these alternative treatment strategies. Ideally, first a better understanding of the degree of insulin resistance that occurs during TB and the pathogenesis leading to altered lipid metabolism should be identified. Using multiple therapeutic strategies to reduce glucose load, promote retention of adipose lipid content and reduce lipolysis, and interfere directly with the signaling mechanisms related to the pathogenesis of altered lipid metabolism in type 2 diabetes, represents an ideal approach to translating the manifestations of metabolic syndrome and type

2 diabetes to that of TB-related chronic inflammation. Based on the demonstrated alterations in glucose and lipid metabolism, we have a series of therapeutic targets that can be addressed to further clarify the pathogenesis of TB-associated metabolic disease and identify novel therapeutic targets.

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