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Maziar Divangahi *Editor*

The New Paradigm of Immunity to Tuberculosis

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The New Paradigm of Immunity to Tuberculosis

Editor
Maziar Divangahi
McGill University
Montreal, QC
Canada

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Epidemiology of Tuberculosis Immunology

G. J. Fox and D. Menzies

Abstract Immunological impairment plays a major role in the epidemiology of TB. Globally, the most common causes of immunological impairment are malnutrition, diabetes, HIV/AIDS, aging, and smoking. With the notable exception of HIV, each factor leads to relatively mild immunological impairment in individuals. However, as these conditions affect a significant proportion of the population, they contribute substantially to the incidence of TB at a global scale. Understanding immunological impairment is central to understanding the global TB pandemic, and vital to the development of effective disease control strategies.

Keywords Prevalence · Association · Impact · *Mycobacterium tuberculosis* · Malnutrition · Vitamin deficiency · Diabetes · HIV · Aging · Immunosenescence · Smoking · Alcohol use · Chronic kidney disease · Chronic obstructive pulmonary disease · Rheumatoid arthritis

1 Introduction

1.1 Global Trends in Tuberculosis Epidemiology

Tuberculosis (TB) is an airborne bacterial infection that causes disease in 9.4 million people a year worldwide, most of whom live in low- and middle-income

G. J. Fox
Woolcock Institute of Medical Research,
University of Sydney, 431 Glebe Point Road, Glebe, NSW 2037, Australia

D. Menzies (✉)
Respiratory Division, Montreal Chest Institute, MUHC and McGill University,
Room K1.24, 3650 St. Urbain St., Montreal, PQ H2X 2P4, Canada
e-mail: Dick.Menzies@McGill.ca

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countries [1]. The disease causes 1.7 million deaths each year, of which 20 % are estimated to occur in people living with HIV (PLHIV) [1]. The global prevalence of disease has begun to fall slowly since peaking in 2003 [2, 3], however TB control programs are yet to make a measurable impact upon the burden of disease in most regions of the world [4].

The epidemiology of TB is driven by the natural history of the disease and the susceptibility of the at-risk population. About one-third of the world's population has been infected with TB and has latent tuberculosis infection (LTBI) [3, 5].

1.2 The Role of Immunological Factors in Global Tuberculosis Epidemiology

Most exposed individuals are able to mount a sufficient immune response to contain or eliminate the bacteria, and therefore do not develop disease [5].

About 10 % of individuals with latent infection will progress to active TB during their lifetimes [5–7]. The greatest risk of progression is within the initial 2–5 years after infection, however disease reactivation may occur at any time. Individual susceptibility to progressing from latent infection to active disease varies considerably, and is strongly affected by immunological factors [7–10].

A recent study of TB rates in 134 countries identified a number of biological risk factors that were associated with higher rates of TB [4]. These risk factors included undernourishment, HIV infection, diabetes, and tobacco smoking—all of which are known to increase immune susceptibility. This study demonstrated that the impacts of immunological impairment are evident at both an individual and a population level.

Similarly, indicators of improving general health of the whole population have been associated with declining TB incidence. A recent study of national data from 165 countries, from 1990 to 2005, found that increasing life expectancy and improving vaccination rates were associated with declining TB incidence [11]. This suggests that improved immunological function, likely to be associated with improved general health, has an impact upon TB at a population level.

The epidemiological impact of conditions that impair immunity depends both upon the prevalence of the condition and the severity of immunological impairment that it causes. For example, the immunological impairment due to HIV infection confers a risk of developing TB of over 20 times than that of the general population [12, 13]. Consequently, HIV has had an important impact upon the TB epidemic, with an estimated 12 % (95 % CI 8–15 %) of TB-related deaths attributable to HIV infection [14]. In contrast, the relative risks of TB conferred by diabetes [9] and smoking [15] are less than five times that of healthy control subjects. However, although these conditions may not confer as high a risk of disease, they are much more common, and therefore also have an important impact upon TB epidemiology.

Together, immunological risk factors are major drivers of the ongoing global TB pandemic. Malnutrition affects around 1 billion people [16], diabetes affects

200 million people, alcohol abuse is highly prevalent in many populations, and there are 1.45 billion smokers worldwide [17]. The impact, or contribution that these risk factors make to overall incidence of TB in a population, termed the population attributable fraction (PAF), increases as the prevalence of these risk factors increases in the population.

This chapter will examine the relationship between TB and key risk factors that increase immunological susceptibility. It will examine the evidence that common causes of immunological impairment such as malnutrition, diabetes, aging, smoking, alcohol, chronic disease, and HIV increase the risk of TB. The complex interaction between TB and host immune function must be better understood if the TB pandemic is to be tackled effectively.

1.3 Sources of Evidence for the Impact of Immunologic Disease Upon Tuberculosis

The evidence for an association between immunologic disease and TB ranges from small clinical case series to large studies of entire populations. Even if a given risk factor is biologically plausible, and has been shown to be associated with TB, it is often quite difficult to prove a causal relationship definitively. In particular, it is notoriously difficult to adjust for confounding factors that coincide with the risk factors being studied. In contrast to laboratory studies, epidemiological studies occur in a complex environment where many variables are impossible or difficult to control or measure. For example, low socioeconomic status is often co-located with other environmental risk factors such as crowded housing, poor nutrition, limited access to health care, and increased tobacco and alcohol use.

Evidence for the impact of immunological factors upon TB can be derived from a variety of study designs [18]. The randomized-controlled trial design provides the best level of evidence for an association and best controls for confounding factors. However, it is not possible to randomly allocate exposures that will cause immunological impairment.

Observational studies are more feasible, ethically and morally acceptable, and are commonly used to test for associations in epidemiological studies. However, evidence derived from observational studies is limited, as the exposures in these studies are not experimentally assigned. Consequently, observational studies provide weaker evidence of a true association because they cannot avoid the influence of confounding factors upon the study outcome. It can be very challenging to dissect the contribution of each of the biological factors from the background social determinants of disease [19]. Cohort studies have the advantage of ensuring that a risk factor (such as alcohol consumption or diabetes) is measured prior to the time that the outcome occurs, which somewhat overcomes recall and selection bias.

Case-control studies have a number of limitations. In particular, these studies face difficulty in selecting a control group that is comparable with the group with

disease. This can lead to selection bias or confounding. Cross-sectional studies (also called *prevalence studies*) are unable to show a temporal association between the risk factor and outcome, or properly control for confounders.

Given the weaknesses inherent in the designs of most published studies, it is important to draw evidence about a particular risk factor from a variety of studies in a range of settings, before concluding that there is actually a causal relationship. Each study must be assessed for its intrinsic methodological weaknesses and interpreted with caution. Consequently, systematic reviews and meta-analyses [9, 20–26] can provide a very useful indication of overall trends. However, even these studies must be interpreted with caution as their conclusions are prone to publication bias, and may not necessarily apply to all settings.

1.4 Key Epidemiological Terms

This chapter applies a number of key terms that are important in interpretation of the epidemiological evidence.

Prevalence refers to the number of individuals in a population with disease at a specific point in time.

An *association* occurs when an environmental exposure or host characteristic is statistically correlated with a disease or health outcome. Not all associations are causal. Even if there is an association between an exposure and an outcome, it may not be possible to conclude that one event caused the other. In fact, there may be a third independent factor common to both exposure and outcome that explains the association, or the direction of causation may be the reverse (i.e. what is assumed to be the outcome, is actually the exposure).

Risk is the probability that an event (such as a disease) will occur following a particular exposure. In this chapter, a *risk factor* is an exposure that is statistically related to a particular disease outcome [27].

An *impact* is an outcome that is caused by exposure to a risk factor, such as "*the impact* of HIV on tuberculosis disease or mortality".

In the subsequent sections, we have included a wide variety of epidemiological evidence that examines the association between TB and key risk factors related to immunological impairment. Where evidence is available, we have examined the impact of these risk factors on TB infection, development of disease and mortality.

1.5 Immunological Impairment and the Natural History of Tuberculosis

An individual whose immune function is impaired will only develop TB in the presence of the *Mycobacterium tuberculosis* bacillus. Therefore, a person's risk of

developing active disease is not only influenced by the degree of immune impairment of the person but also depends upon exposure to TB and the biology of the bacterium.

After exposure to airborne droplets containing *M. tuberculosis*, an individual may become infected. He or she may either rapidly progress to TB disease after a short incubation period (so-called ‘primary progressive disease’), may develop ‘latent tuberculosis infection’ (LTBI) or may eradicate the organism. In individuals with longstanding LTBI, lasting 6 months or more, TB may undergo subsequent reactivation and progress to active disease [28]. Hence, there is a dynamic interplay between the determinants of immunological impairment and the stage of the infection in each individual.

A condition impairing immunity in an individual may increase the risk of initial infection and primary progression after recent exposure, or it may increase the risk of subsequent reactivation of LTBI, or both. The condition may also affect the risk of mortality compared to an individual with normal immunity.

In this chapter, we explore the evidence for associations between common causes of immunological impairment and TB.

1.6 Methods of this Review

A literature review was undertaken to identify risk factors associated with TB. We first identified the main factors known to be associated with immunological susceptibility to TB at a population level in multiple countries, based on recent reviews of epidemiologic risk factors for TB [19]. We also conducted a search using PubMed for reviews of immunity and TB. We performed a series of searches on PubMed, combining “tuberculosis” with keywords for the identified risk factors including nutritional status, diabetes, smoking, chronic disease, alcohol, aging, and immunosuppressive medications. Based on these searches, we obtained recent systematic reviews for each topic [27, 29–36] and the full text of other relevant primary studies. We also identified information about the epidemiology of each risk factor from reports by the World Health Organization (WHO) and the United Nations.

2 Specific Immunologic Factors Associated with Tuberculosis

2.1 Malnutrition

2.1.1 The Global Burden of Malnutrition

Malnutrition, or the lack of nutritional elements necessary for human health, is common in populations with a high TB prevalence [19]. According to United

Nations data, an estimated 925 million people were undernourished in 2010, comprising almost 16 % of the population of low- and middle-income countries [16]. There have been dramatic improvements in food supply and per capita food availability over recent decades in many regions of the world [37]. However, these gains have not been uniform. Substantial improvements in nutrition have been made in India and China, while improvements have been much less in regions such as sub-Saharan Africa. Children are at a particularly high risk of the effects of malnutrition, not only due to protein and energy insufficiency but also as a result of limited access to essential micronutrients.

Micronutrient deficiency, also called "hidden hunger", is extremely common in resource-limited settings. Globally, it is estimated that iron deficiency anemia affects more than 1.2 billion people, Vitamin A deficiency affects between 100 and 140 million children and that almost 1 billion people are at risk of iodine deficiency [37]. These nutritional deficiencies are often closely linked to poverty, inadequate access to food, poor sanitation, and water supply—each of which have an impact upon the health of these populations.

2.1.2 The Mechanism for Malnutrition and Susceptibility to Tuberculosis

Nutrition, immunity, and infection are known to interact in complex and dynamic ways. Infection is both a cause and a perpetuating factor in protein energy malnutrition within a population, by reducing productivity, increasing socioeconomic and political stability, and impairing productive capacity of the society (Fig. 1) [38].

An association between malnutrition and TB is certainly biologically plausible. In experimental mice models of TB, protein calorie malnutrition has been shown to impair biological mechanisms in TB control including production of TNF, iNOS, and interferon-gamma. This deficiency could be reversed by restoring protein nutrition [39, 40]. Other experimental evidence also supports the association [40]. However, epidemiological studies are required to assess the relevance of these models to the human setting.

2.1.3 Malnutrition and the Risk of Tuberculosis

Tuberculosis and malnutrition have long been understood as being closely linked [41], as both are the consequence of poverty, economic instability, and food insecurity. However, until recently it has been surprisingly difficult to prove an association among malnutrition, immunological impairment, and TB at a population level [40].

We found little evidence of an association between malnutrition and susceptibility to latent infection. However, a range of epidemiologic studies have shown an association between malnutrition and active disease [38, 40, 42–45]. The main

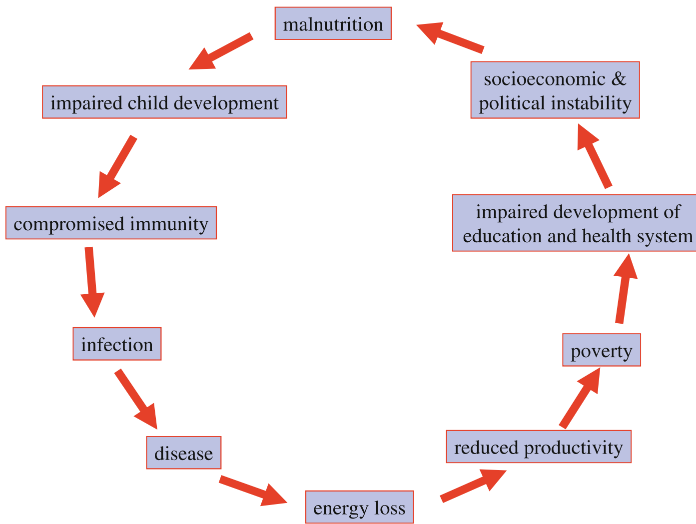

Figure 1. Protein Energy Malnutrition Increases Prevalence of Infection, Leading to Energy Loss for the Individual
On the community level, this burden reduces productivity, including food production, and perpetuates the relentless spiral of further malnutrition, infection, disease, poverty, and socioeconomic and political instability.

Fig. 1 The cycle of malnutrition, infection, disease, and poverty associated with tuberculosis in low-income settings [38]

limiting factor in most studies is the difficulty adjusting for independent factors related to poverty that may confound the association, such as crowded housing, smoking, or limited access to health care. This area of research is particularly challenging because the causal link may operate in either direction—it may actually be the TB itself that causes reduced body mass, muscle bulk, and biochemical parameters. We can obtain some fairly weak evidence from a number of ecological, cross-sectional, and case-control studies.

Ecological studies provide an insight to the association between nutritional deficiency and TB. An historical report from Denmark during the First World War described TB rates reducing dramatically once food supplies were restored to the population, in comparison to surrounding countries where shortages persisted [40]. During both the First and Second World Wars, there were increases in TB incidence in many European countries [46, 47]. Populations that maintained reasonable nutritional status tended to avoid the increase, while in the Netherlands a rise in TB coincided with periods of severe famine during the later years of the war [47]. Although such observations are suggestive of a correlation between malnutrition and TB at a population level, there are other potential confounders such as health system disarray that may also explain the phenomenon.

A study from Trondheim Naval School in Norway found that improvements to housing did not change TB incidence, but subsequent reductions in TB incidence

occurred with the supplementation in the diet of margarine, cod liver oil, whole wheat bread, fresh fruits and vegetables, and milk [48]. McKeown's analysis of TB mortality in England from 1770 to 1900 elaborated on the alternate hypotheses for explanation of reducing rates, and concluded that diet was highly likely to be responsible [48].

The beneficial impact of social interventions, including improved nutrition, was evident in the English village of Papworth during the period 1918–1943. Tuberculosis patients and their families were brought into the village and received adequate wages, improved nutrition and housing. Child contacts of TB patients born within the village, and receiving adequate nutrition, had a significantly lower risk of TB than contacts born outside this environment. The study provides an interesting example of how social and nutritional improvements correlate with reductions in TB [49].

Several published case series patients show an increase in TB risk associated with the nutritional deficiency associated with intestinal bypass [50, 51]. A number of cross-sectional studies have also shown that low body mass index (BMI) is associated with TB [43, 52]. A recent systematic review of six studies found an inverse log-linear relationship between TB incidence and BMI. The study showed a reduction of TB incidence of 13.8 % for each unit increase in BMI [52].

There are surprisingly few cohort studies available. This study design is relevant to establishing the direction of causation, by assessing the nutritional status of patients before they develop disease. One cohort study of 823,000 US navy recruits showed that TB occurred three times more commonly in young men who were 10 % or more below their ideal body weight, compared to those who were 10 % above it [53]. However, despite the large sample size, low socioeconomic status, and smoking may have been independently associated with susceptibility to TB in this cohort.

Objective measures of underweight have also been associated with TB. One study in Tanzania found that low levels of low subcutaneous fat and low hand grip strength were associated with TB [43]. Other studies have found that low BMI and mid arm circumference [54] were associated with TB in patients compared to a control group.

2.1.4 Vitamin Deficiency and Tuberculosis

In addition to low weight and energy malnutrition, reduced micronutrients (particularly vitamins A, C, E, zinc and selenium) have been associated with impaired immune response. Micronutrients are nutrients that are essential in the diet in small quantities, to enable a range of normal physiological functions. They include minerals, vitamins, and other organic compounds. A number of studies have studied the association between micronutrient deficiency and TB, but often it is difficult to determine whether such deficiencies are the cause or effect of TB [24, 55–57].

(a) Vitamin D and TB

Vitamin D has a range of important physiological functions, including a role in infectious immunity [58]. Vitamin D levels in individuals commonly fluctuate

annually, and are particularly low during the winter months in North American and European countries due to reduced sun exposure. This observation, and finding of an association between low vitamin D levels and TB [24], has led to the hypothesis that Vitamin D deficiency contributes to TB susceptibility. Vitamin D deficiency has been offered as a possible explanation for reactivation of TB among migrants from sunshine-rich tropical countries to Northern Europe and North America [24]. However, although peaks of TB coincide with low Vitamin D levels, this finding is not seen consistently [59].

A meta-analysis of seven studies evaluating the relationship between Vitamin D and TB showed a significantly lower levels of the vitamin in TB patients compared to controls (with an effect size of 0.68 (95 % CI 0.43–0.93). However, the included studies did not measure nutritional status prior to disease onset and so the direction of causation is not certain [24].

Hence, while both environmental [59] and genetic [60] factors influence the way that Vitamin D affects immunity, the association between Vitamin D and TB still remains unclear [58].

(b) Vitamin C and TB

Vitamin C, or ascorbic acid, has a number of essential metabolic functions including in cell-mediated immunity [61]. A large cohort study showed a correlation between low levels of Vitamin C and TB, however this became non-significant when adjusted for nondietary factors [55]. A number of other studies have also established associations between Vitamin C and TB [62].

(c) Vitamin A and TB

Vitamin A is an essential dietary vitamin that is important for vision and epithelial cell function and has a number of important roles in the immune system [61]. A small case-control study in India found lower Vitamin A levels in TB patients than in healthy controls. However, retinol binding may independently be lowered by acute illness [63, 64].

Other studies from Africa have found low Vitamin A levels in patients with TB and HIV co-infection [65, 66]. There is also evidence that HIV infection may also contribute to low Vitamin A levels. In one South African study, 90 % of TB patients had low levels of the vitamin [67], and another study found that a 64 % of HIV-positive blood donors also had low Vitamin A levels. Consequently, there is not yet clear evidence for a relationship between Vitamin A and TB susceptibility.

(d) Evidence for vitamin supplementation in TB

A 2008 Cochrane study examined the role of vitamin and micronutrient supplementation in TB [68]. The authors identified no evidence that supplements affected sputum conversion or cure rates, however there was a benefit for high energy supplements, multivitamins, and Vitamin A plus zinc in achieving significant weight gain compared to placebo. Additional recent trials have failed to demonstrate benefit [69], and other trials are currently underway to address the role of supplements in enhancing immune function in TB patients.

In a recent randomized controlled trial [56], TB treatment outcomes were unchanged by Vitamin D supplementation. Unfortunately, this study was underpowered and both arms had excellent outcomes. Furthermore, it does not address the relationship between Vitamin D deficiency and reactivation of latent TB infection.

Hence, while there are some evidences of an association between TB and micronutrient deficiency, further research is required to investigate whether there is a causal relationship between deficiency of specific micronutrients and TB susceptibility.

2.1.5 Malnutrition and Tuberculosis Mortality

Malnutrition is also associated with increased mortality due to TB. The link between TB mortality and nutritional factors was addressed in a recent systematic review [26], which showed evidence that TB outcomes were worse for underweight and malnourished patients.

A retrospective cohort study from the United States showed the odds of death to be 3.2 (95 % CI 2.1–4.9) times higher in malnourished patients [44]. Another study from Australia also found that patients with malnutrition had 3.2 times the odds of death (95 % CI 1.0–9.9) [70].

Another study in Malawi demonstrated that severe malnutrition was associated with death during the initial 4 weeks of treatment, and that risk of mortality was greater at lower body mass indices (a BMI of less than 16 had an odds ratio of 2.2 (95 % CI 1.3–3.8) [71]. Other studies in the United States [44] and Latvia [42] have also found an association.

A number of epidemiologic studies have shown a correlation between biochemical parameters, such as serum albumin and TB mortality [26]. However, as discussed previously, the temporal association is unclear. It is conceivable that such measures of malnutrition actually reflect the consequences of delayed diagnosis. Therefore, more advanced malnutrition may merely reflect prolonged disease and hence predict an understandably higher mortality rate.

2.1.6 The Impact of Malnutrition Upon Tuberculosis at a Population Level

According to a United States Surgeon General report “malnutrition may account for a greater population attributable risk of TB than HIV infection, and certainly a much more correctable one” [72].

Since a very large number of people worldwide suffer from some degree of malnutrition, modest reductions in malnutrition at a population level may translate to a substantial impact on TB incidence. However, there remains little direct evidence showing a benefit from nutritional supplementation upon TB incidence within a whole population. Ecological observations, such those described above from Europe during the Second World War, suggest that improving nutrition at a

population level may reduce TB incidence fairly rapidly [47]. Quasi-experimental studies such as the Papworth experiment also suggest a role for a comprehensive social and nutritional support to reduce the progression to disease [49].

The Millennium Villages program that is currently underway in Africa represents a modern equivalent that applies comprehensive social, nutritional, and health care interventions to improve health in whole communities [73]. Such strategies offer an excellent opportunity to evaluate the relationship between nutrition and TB in settings where marked socioeconomic improvements are achieved.

2.2 Diabetes and Tuberculosis

2.2.1 The Global Burden of Diabetes

Diabetes is an increasingly common condition globally, with a recent review of data from 199 countries showing a prevalence of 9.8 % among adults aged 25 years or over [74]. There were 171 million prevalent cases worldwide in 2000, and this number is projected to rise to 366 million by 2030 [75]. In high-income countries, diabetes prevalence has increased substantially over recent decades, and now 23.1 % of Americans aged over 60 years have diabetes. In Europe, noncommunicable diseases (particularly diabetes and alcohol) are now thought to be more significant causes of immunological impairment in TB patients than HIV [76].

Diabetes is also becoming more common in low- and middle-income countries, as these countries face the so-called "double-burden" of both communicable and noncommunicable disease [77].

2.2.2 Diabetes and Risk of Tuberculosis

Two recent systematic reviews of TB and diabetes have shown consistent evidence of an association [9, 78]. One such review included 13 studies, of which three were cohort studies and 10 were case control studies. The pooled relative risk of TB in the cohort studies was 3.1 (95 % CI 2.3–4.3) across these studies. This effect was also seen in the included case-control studies [9].

The second systematic review identified nine studies that compared the prevalence of TB in diabetics to that in nondiabetics, and found all studies showed an increased prevalence among diabetics. This study did not attempt to provide a summary statistic due to heterogeneity in study designs and research methods [78]. The included studies that quantified the prevalence of TB were from South Korea (OR 3.5 (95 % CI 3.0–4.0)), India (OR 2.4 (95 % CI 1.2–5.1)), Russia (OR 2.7 (95 % CI 1.1–6.5)), the United Kingdom (3.8 (95 % CI 2.3–6.1)), two studies from Indonesia (OR 4.2 (95 % CI 1.2–11.7) and OR 4.7 (95 % CI 2.7–8.1)), Mexico (6.8 (95 % CI 5.7–8.2)) and two studies from the United States of America (OR 2.95 (95 % CI 2.6–3.3) and 1.82 (95 % CI 1.6–2.1)).

A Pakistani cohort study found TB prevalence in diabetic patients was 10 times higher than in nondiabetic patients [79]. In Tanzania, a case-control study found diabetes was four times as frequent and impaired glucose tolerance was twice as frequent in diabetics than in controls [80]. A South Korean longitudinal study among 800,000 civil servants found 5.2 (95 % CI 3.8–7.0) times more microbiologically confirmed cases in diabetics [81], and 3.5 (95 % CI 3.0–4.0) times more among all diabetics. However, in this study people with no diabetic history were not tested for diabetes.

A large prospective cohort from Canada, comprising over 500,000 people, found the diabetic population had a relative risk of 1.2 (95 % CI 1.1–1.4) compared to nondiabetics [82].

It is important to recognize that the prevalence of latent TB infection in a population will influence the absolute risk of a diabetic patient developing TB. In countries with a low prevalence of TB, such as Canada, diabetic patients are very unlikely to be exposed to infectious organism. Hence, although they may be more susceptible, in the absence of infection this susceptibility will usually not result in TB.

By contrast, in countries with a high prevalence of latent TB infection, patients with diabetics are more likely to be infected. Consequently, patients with diabetes have a much greater likelihood of progressing to TB than those in low-prevalence settings. This observation explains why studies conducted in countries with low incidence of TB require much larger sample sizes to detect an effect due to diabetes, and the overall effect size appears small.

2.2.3 Does the Adequacy of Diabetic Control Affect Tuberculosis Susceptibility?

A small number of studies show that poorly controlled diabetes is more likely to be associated with TB.

In a Hong Kong study of 42,116 diabetics aged 65 or above with a glycosylated hemoglobin (HbA1c) of seven or more (poorer diabetes control) had 3.1 (95 % CI 1.6–5.9) times the odds of developing culture confirmed pulmonary (but not extrapulmonary) TB compared to the group of diabetics with better control [83].

A prospective cohort study of diabetes in Tanzania found that 8.8 % of patients with type 1 diabetes developed pulmonary TB, compared to 2.2 % of patients with type 2 diabetes that did not require insulin [84]. In this study, nutritional status may have been a confounding factor as patients with type 2 diabetes had a higher mean BMI than type 1 diabetics, and may have been protected against TB due to better nourishment.

The relationship between BMI and diabetes is complex. In some settings such as India, the gains made by improvements in nutrition may be offset by rising levels of diabetes [85].

2.2.4 Diabetes and Treatment Outcomes of Tuberculosis

Evidence from two cohort studies, one from India [86] and one from the United States found that the time to sputum conversion was not significantly different in diabetic patients with active TB [87].

Most studies examining mortality among TB patients found significantly increased mortality in diabetics. One study from the United States found all-cause mortality was 4.7 (95 % CI 1.9–12.5) times greater in diabetics when adjusted for age [88]. A second retrospective cohort study from Baltimore found the odds of death were 6.5 (95 % CI 1.1–38.0) times higher in patients with diabetes (1.1–38.0), when adjusted for HIV, age, weight, and foreign birth [87]. Another small cohort study found that diabetes was associated with a mortality of 3.8 (95 % CI 1.4–10.3) [89] times that of nondiabetics. In contrast, a retrospective cohort study from St Louis found no association with mortality [44].

Consequently, it appears likely that diabetes, particularly if it is poorly controlled, does increase the mortality of patients with TB.

There is conflicting evidence about the impact that diabetes has upon treatment failure rates, as distinct from overall mortality. The aforementioned study from Baltimore found no significant difference between treatment failure in patients with and without diabetes [87].

A case-control study from Egypt did find diabetes to be a risk factor for treatment failure among those who were compliant with treatment (OR 10.08, 95 % CI 2.5–41.3), although the sample was small and drug resistance patterns were not reported [90].

A study from Saudi Arabia found no difference in treatment outcomes, but found that diabetics had lower rates of multi-drug-resistant (MDR) disease and higher initial sputum load of acid-fast bacilli and higher 3-month conversion rates than nondiabetics [91]. Interestingly, in this study the mean time for sputum conversion was longer in diabetics, even though they had lower rates of MDR disease. By contrast, another from the United States actually found higher rates of MDR in among diabetic patients with TB [92] (adjusted OR 5.3, 95 % CI 1.9–14.6)). It is difficult to be certain about the reason for this finding, particularly since the odds actually increased when failure rates were adjusted for participation in observed therapy (OR 8.6 (95 % CI 3.1–23.6)), meaning that primary resistance was unlikely to have caused the MDR TB. One possible explanation is that diabetic patients were more likely to utilize hospital services, and that increased nosocomial transmission may drive the higher prevalence in these patients, particularly given the high rates of MDR in New York City at the time.

The evidence for the impact of diabetes upon the clinical presentation of TB is mixed. Radiological studies give varying evidence about the lobar distribution of TB among diabetics. Some studies have showed more lower lobe involvement in TB with diabetes [83], one study found more cavitatory disease [93], while another found the pattern of disease diagnosed radiologically among diabetics to be similar

to that of nondiabetics [94]. The difference in radiologic signs between diabetics and nondiabetics is probably overstated [36]. Two studies identified in a recent systematic review found that the risk of diabetes was highest risk in the young, and declined with older age [78]. These findings may be specific to the populations in which they were studied, and further research is warranted to further explore the clinical presentation of TB among diabetics.

2.2.5 The Impact of Diabetes Upon Tuberculosis at a Population Level

Not only is there is strong evidence for an association between TB and diabetes in individuals, but there are also clear public health implications behind increasing rates of diabetes worldwide [78]. Particularly in populous countries such as India, with a large proportion of both the world's TB and diabetes, the interplay between the two is likely to continue to undermine efforts at TB control [95].

2.3 HIV and Tuberculosis

2.3.1 The Global Burden of HIV

There were 33.3 million PLHIV and 2.6 million new cases in 2009 [96]. Despite the rapid rise in HIV incidence in the 1980s and 1990s, the number of new cases peaked globally in 1999. Incidence has been falling due to a variety of factors including preventive efforts, behavioral change, and better access to treatment [96]. Despite improvements in most countries, there were still seven countries where incidence increased by more than 25 % from 2001 to 2009 [96]. HIV prevalence varies considerably between and within countries, ranging from 0.1 % of adults aged 15–49 years in East Asia to 5.0 % in sub-Saharan Africa [96]. The latter region is home to 68 % of the total number of people living with the virus, where HIV infects more women than men.

Globally, the majority of people who acquire human immunodeficiency virus (HIV) do so through unprotected heterosexual intercourse. Consequently, in settings with a 'generalized' epidemic (HIV prevalence is 1 % or more of the general population), women and men are often affected to a similar degree [97]. In 'concentrated' epidemics, the prevalence is less that 1 % in the general population but over 5 % in specific at-risk populations [97] such as injecting drug users. Consequently, the results of HIV-related immunological impairment predominantly affect those groups.

If untreated, affected individuals are at high risk of acquiring opportunistic infections and AIDS-related illnesses and suffering increased mortality. Approximately, 1.8 million people died due to HIV/AIDS in 2009. As with the global

trend in the disease, HIV-related mortality has also begun to decline, particularly among children [96].

2.3.2 The Mechanism for HIV Infection and Susceptibility to Tuberculosis

People living with HIV (PLHIV) are highly susceptible to TB. This may be either due to the rapid progression of new TB infection or re-infection, or due to the reactivation of pre-existing latent TB [98]. Not only does HIV affect TB, by causing profound immunological impairment, but TB disease may also accelerate the progress of HIV [99]. Conversely, treating TB improves HIV control [23]. Therefore, the epidemiologic impacts of both pathogens are closely related.

2.3.3 HIV and Progression to Tuberculosis Disease

There is very strong evidence for an association between HIV and TB, which has been evident since the beginning of the HIV epidemic in the early 1980s [100].

The risk of TB among PLHIV is significantly higher than the general population, although the magnitude of the risk varies depending upon the background population prevalence of both HIV and TB. A WHO report of directly measured data from 64 countries found that HIV-positive people are over 20 times more likely than HIV-negative people to develop TB in settings with a generalized HIV epidemic. In countries with a low prevalence of HIV, the risk was 26–37 times that of HIV-negative individuals [101].

Another analysis found that in countries with a generalized HIV epidemic, the relative incidence of TB among PLHIV was 20.6 (95 % CI 15.4–27.5) compared to uninfected individuals. In settings with an HIV prevalence of less than 5 %, risk of TB was 36.7 (95 % CI 11.6–116) times that of the HIV uninfected population [12].

As for all HIV-related opportunistic infections, the prevalence of TB increases at lower CD4 T cell counts [31].

The high degree of susceptibility was highlighted in a cluster outbreak study from the United States, where 37 % of PLHIV developed active TB compared to no cases among the uninfected group [98]. A recent meta-analysis of molecular epidemiologic studies showed HIV to be significantly associated with clustering of disease (adjusted OR 0.13 (95 % CI −0.19 to 0.44)) [20]. However, molecular epidemiology studies are likely to be biased toward detecting an association between TB and HIV, because clustering is based upon recent transmission. The inclusion of a case in a cluster usually requires a diagnosis within 2 years of the initial index case. Consequently, as there is more rapid progression to disease in HIV-infected patients, it is likely that they will be over-represented. An additional source of possible bias is that outbreak investigations often begin within health care institutions, where HIV is more likely to occur. The alternative explanations for the markedly higher rates of disease in clusters, that HIV-infected patients are

more likely to be infected or that they are more likely to transmit disease, are both unlikely. More likely, HIV is independently related to the identification of the sorts of patients included in an outbreak investigation.

The risk of acquiring TB increases after the patient undergoes HIV seroconversion and doubles within the first year of HIV infection [102]. In advanced AIDS, the risk of progression from latent infection to active disease increases significantly compared with that of a person who is HIV negative [100] HIV is also a predisposing factor for developing MDR-TB [103].

2.3.4 HIV and Susceptibility to LTBI

While HIV is clearly associated with progression from LTBI to active disease, there is little data to determine whether HIV infection initially increases susceptibility to latent TB infection. A meta-analysis of 168 studies of contact investigation found the prevalence of LTBI in HIV-infected individuals was similar to the prevalence of LTBI in all contacts. However, there was considerable heterogeneity in the outcomes of included studies, in part due to variations in study design and different epidemiological settings in which the studies were conducted [104].

2.3.5 Mortality of HIV/Tuberculosis Co-infection

HIV infection also appears to increase the risk of mortality due to TB. Of the estimated 1.8 million deaths each year among PLHIV worldwide, 0.4 million deaths (22 %) occurred among incident TB cases with HIV [1].

All-cause mortality in a cohort of 239 TB patients treated in Zambia found the mortality among HIV-positive patients to be five times that of an HIV-negative patient over 24 months following diagnosis with TB [105]. A total of 42 out of the 47 patients in this study who died were HIV positive [105].

Autopsy studies from a West African city found that TB was present in 54 % of people dying with AIDS [106].

2.3.6 The Impact of HIV Upon Tuberculosis at a Population Level

HIV can have a significant effect upon the incidence of TB at a population level. One study of population-wide data from 134 countries showed that the annual increase in TB incidence in Latin American and the Caribbean countries positively correlates with HIV rates in those countries [4]. The impact of HIV was a major driving force behind the sharp increases in TB incidence in sub-Saharan Africa in the 1980s and 1990s [33]. Indeed, global TB incidence would have fallen substantially between 1990 and 2000 if there had been no concomitant HIV epidemic. [14, 33].

Although the global incidence of HIV has peaked [96] sustained access to effective therapy and health care capacity remain major barriers to global HIV

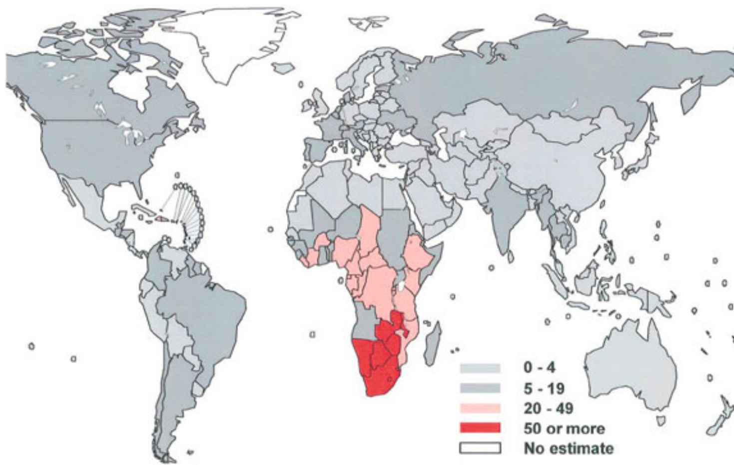

Figure 2. Estimated HIV infection prevalence (%) among new adult patients with tuberculosis (15–49 years of age). Reprinted from the World Health Organization [7]. The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city, or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines denote approximate borders on which there may not yet be full agreement.

Fig. 2 Estimated HIV infection prevalence among new adult patients with tuberculosis [33]

control. Consequently, the impact of immunological impairment due to HIV will remain a major factor in global TB epidemiology this century (Fig. 2).

2.4 Aging and Tuberculosis

2.4.1 Global Trends in Aging

The world population is aging at a scale unprecedented in human history, with the older population (aged 60 or over) increasing at 1.9 % per annum compared to 1.2 % growth of the overall population. The median age of the world's population is currently 26 years, but by 2050 the median age will increase to 36 years [107, 108]. By then, people aged 60 and above will comprise 21 % of the world's population, and exceed the number of people aged younger than 15 years [107]. These trends will have profound implications for the patterns of disease in these populations.

2.4.2 The Impact of Age Upon Immune Function

Immune function varies dynamically with age. At both extremes, TB susceptibility is increased, and these changes are accompanied by differences in presentation of the disease [109].

Early pre-antibiotic studies show the risk of children developing TB was greatest in children up to 4 years of age, and it then slowly declined up to 10 years of age [8]. Following primary infection, the mortality of childhood TB is highest in infancy, subsequently declining to 1 % between 1 and 4 years of age, before rising to 2 % from 15 to 25 years of age. This reflects the changing immunological function over the early life span [8], as well as socioeconomic and nutritional factors.

Age-related decline in immune function is known as immunosenescence. The predominant changes in immune function with aging are progressive decline in cell-mediated immunity, including both defects in T and B cells as well as innate immune function [110]. These findings have also been shown in mice, where T cells undergo age-associated functional changes [111]. In addition to direct immunological effects of aging, malnutrition also contributes significantly to the increase in infectious diseases in the elderly. The impacts of age-related chronic diseases also have an impact on immunological susceptibility to TB [112], and are addressed in detail in other sections of this chapter.

2.4.3 The Impact of Aging Upon the Epidemiology of Tuberculosis

Population aging clearly has differing impacts upon TB epidemiology depending upon the demographic factors and existing burden of disease affecting each country.

In high-income countries, there has been a steady decrease in TB over at least the past 50 years [113]. However, the elderly population has experienced a relatively slower rate of decline in incidence. This is largely because the elderly populations, having been exposed to *M. tuberculosis* in their youth, experience reactivation of the disease in later life [114].

In the United States, the incidence of TB increases steadily with age albeit from a relatively low absolute rate. The same trend has been seen in Japan, which has decreased its TB notification rate from 303 per 100,000 population in 1962 to 22 per 100,000 by 2005 [115].

In low- and middle-income countries, the age distribution of TB differs dramatically from high-income settings. In African countries, the age-specific case-notification rate is in the age group 25–44, while in the USA it is highest in the population above 65 years of age [115].

The clinical presentation of TB appears to be similar across different age groups. A meta-analysis of 12 studies assessed the effects of aging on pulmonary TB and found that there were no significant differences between patients aged over 60 years and patients who were younger, with respect to most clinical, radiological, and biochemical parameters [35]. The analysis showed slightly higher rates of sputum smear positivity, and reduced lung cavitation as well as lower albumin levels. In some studies, extrapulmonary disease has been reported to be more common among the elderly [30]. Elderly patients are also more likely to suffer cardiovascular disorders, diabetes, chronic obstructive pulmonary disease (COPD), and malignancy, each of which may confer susceptibility to TB [115].

The effects of aging on the epidemiology of TB are therefore complex. As the overall rate of TB in a population is decreasing, the rate of decline is likely to be slower in the elderly.

2.4.4 The Impact of Aging Upon Tuberculosis Infection, Reactivation and Mortality

The prevalence of LTBI increases as a person ages, due to the increased lifetime risk of exposure to *M. tuberculosis*. In high-incidence countries, the prevalence of latent infection steadily rises throughout the life of most individuals, so that by the age of 30, the majority of people will have been infected [113]. Although some go on to develop disease immediately, a proportion will retain the infection for years. *M. tuberculosis* may then reactivate as the person ages [109].

Hence, the risk of disease in the elderly is both related to the cumulative lifetime risk as well as the risk of transmission in the past decades [115]. Unless there are high rates of ongoing transmission, the disease is much more likely to represent reactivation than progression following recent infection.

2.4.5 Malnutrition in the Elderly

While immunological impairment in the elderly may be attributed to immunosenescence, malnutrition is in fact an important contributor to the increase in infectious diseases [116], 5–10 % of community dwelling individuals and up to 60 % of hospitalized elderly suffer from protein, energy, and micronutrient deficiency.

2.4.6 The Impact of Aging on Global Tuberculosis Incidence

Aging is affecting the global TB pandemic in different ways depending upon demographic factors within each country. In high-income countries, the elderly comprise an increasing proportion of all TB patients. This population is also often more likely to be infected with TB due to past exposure. Consequently, in Japan the prevalence of latent TB infection among those over 70 years is as high as 70 %, but among those aged 20 years it is only 1 %. The elderly are therefore both at risk due to the immune impairment of aging as well as their higher likelihood of past exposure [115].

In contrast, the role that aging has in TB is much less in countries with younger populations. In Africa, the highest incidence is in 15–24 years old and elderly people comprise a relatively small proportion of all cases [115]. However, as populations of countries such as India and China age, the contribution of immune impairment among the elderly to the population incidence of disease will also steadily increase.

2.5 Smoking, Indoor Air Pollution, and Tuberculosis

2.5.1 The Global Burden of Smoking and Indoor Air Pollution

Worldwide there are an estimated 1.45 billion smokers, with rates of smoking among males five times higher than among females [17]. Per capita cigarette consumption is continuing to increase in low- and middle-income nations, while it has steadily declined in high-income countries. Today, the vast majority of smokers live in low- and middle-income countries. China, where 52.9 % of men and 2.4 % of women smoke [117], is now home to 301 million current smokers. This has significant implications for the health of the whole population.

In most low- and middle-income countries, the majority of the population uses heavily polluting combustion heaters to heat and cook, burning wood, dung, and crop residues [118]. These materials are typically incompletely combusted, and associated with considerable indoor air pollution. Children and women are particularly exposed to high levels of indoor air pollution, and biofuel use is particularly associated with poverty [118]. Although its use is declining in many countries, the slow progress in economic development in many countries makes it likely that biofuel use will remain an important contributor to morbidity among the poor.

2.5.2 The Impacts of Smoke Exposure Upon Immune Function

Chronic exposure to both tobacco smoke and other indoor air pollutants may directly impair immune function—by impairing normal clearance of secretions [119], and therefore impair the initial clearance of bacteria. Smoke exposure may also impair alveolar macrophage function [120]. Cigarette smoke contains more than 4,500 compounds, many of which are immunosuppressive, including nicotine. Therefore, it is biologically plausible that smoking impairs the host immune response to TB [120]. For example, components of tobacco such as nicotine may decrease TNF production by macrophages. Consequently, it is conceivable that smoking will impact upon the susceptibility of individuals and populations to TB.

2.5.3 Smoking and Susceptibility to LTBI

Tobacco smoking has been clearly recognized to increase the risk of latent TB infection in smokers [22]. A meta-analysis in 2007 identified 38 papers relating to the effects upon TB susceptibility by tobacco. Data from six included studies showed that the odds of having a tuberculin skin test (TST) result of 5 mm or more was 2.1 (95 % CI 1.5–2.8) times higher in smokers, and the odds of having a TST of 10 mm or more was 1.8 (95 % CI 1.5–2.2) [22]. When adjusted for alcohol, the odds ratio was still 1.8 (95 % CI 1.4–2.2). Interestingly, the significant difference in infection rates was found only among cross-sectional studies but not case-control studies.

Another study in nursing homes showed that subjects were more likely to have latent TB infection if they were than smokers than if they were nonsmokers (OR 1.6) [121]. The study found that Heaf test positivity directly related to pack years of smoking. Similarly, migrant workers in California showed increased prevalence in former smokers than nonsmokers (OR 3.1 (95 % CI 1.2–8.9)) [122].

2.5.4 Associations Between Smoking and Tuberculosis

There is considerable evidence that smoking is associated with TB disease. An early study compared smoking habits in 1,200 TB patients to 979 controls from non-TB inpatient and outpatient settings. They concluded there is a "direct association between smoking habits and respiratory tuberculosis" [123].

A meta-analysis of 23 studies addressing the effect of smoking upon TB from 12 countries showed a significantly higher risk of active TB among smokers compared to nonsmokers for both pulmonary TB and all TB [22]. This meta-analysis was limited by the considerable heterogeneity associated with inter-study variation.

A separate review of seven studies showed an association between TB and smoking. Four of these studies showed a dose–response effect, where higher tobacco consumption was associated with a higher risk of active disease [124].

A cross-sectional study from Shanghai that compared heavy smokers to non-smokers found the odds of smokers developing TB were 2.2 (95 % CI 1.3–2.6) times higher than nonsmokers, after adjustment for possible confounders including age, sex, and contact with TB patients. This study found no association for patients reporting mild or moderate levels of smoking [125].

The amount smoked also appears to influence the risk of TB. A study from the United States found that smoking conferred a TB risk of 30–50 % higher than the risk for nonsmokers [126]. The largest risk was for those smoking more than 30 years. Another study showed that in a cross-sectional study of 76,589 volunteers that there was a gradient of increase in TB rate for increasing amount smoked in both men and women [127].

Smoking has also been shown to increase the risk of relapse after successful treatment for TB [128].

2.5.5 Smoking and Tuberculosis Mortality

There is mixed evidence about the association between smoking and TB mortality. Five studies have looked at TB mortality related to smoking. There was substantial heterogeneity in the findings form different settings, however among patients with pulmonary TB mortality among smokers was twice that of nonsmokers [22]. Other studies have found no association between TB deaths and smoking [26].

2.5.6 Indoor Air Pollution and Tuberculosis

There is currently mixed evidence about the impact of indoor air pollution on the risk of developing TB. One systematic review of the available literature identified six studies, of which only two adjusted for cigarette smoking [34]. Many of these studies did not adequately quantify the exposure. The results from three studies reached statistical significance, showing odds of TB to be increased by 2.5 (95 % CI 1.1–6.0), 2.6 (95 % CI 2.0–3.4), and 2.2 (95 % CI 1.1–4.2) times, respectively. Another more recent case-control study in China found no significant association [129]. A challenge in these studies is quantifying the true long-term exposure to biofuels.

Consequently, there is still insufficient evidence to confirm the biologically plausible association between indoor air pollution and TB. Further studies are required to elucidate the issue, and to quantify the necessary threshold of exposure.

2.5.7 Passive Smoking and Tuberculosis

Passive smoking has also been shown to be a risk factor for TB. An unmatched case-control study from Spain compared 93 child contacts of TB patients who developed disease to 95 contacts who did not develop disease [130]. The study showed passive smoking is a risk factor for TB (OR 5.4 (2.4–11.9, $p < 0.01$)), particularly in children aged less than 10 years. It also found a dose response association between risk of acquiring active TB and amount smoked in the household. This study adjusted for socioeconomic status of the parents and age of the children, and still showed an association. The relationship was only significant for >20 cigarettes per day consumed in the household.

2.5.8 The Impact of Smoking and Indoor Air Pollution Upon TB at a Population Level

At a population level, exposure to cigarette smoke and other airborne pollutants have an important effect upon prevalence of TB [131]. Modeling in one study suggested that complete cessation of smoking would reduce the projected incidence of TB in China by between 14 and 52 % [132]. At a population level, cigarette smoking is likely to be a key reason why men are more likely to develop TB than women. In the south of Vietnam, where males are more than four times more likely to develop TB than women [133], current smoking rates are 67.8 % in men, and only 1.1 % in women [134]. It is very likely that this striking difference in smoking rates explains some of the difference in TB prevalence. Clearly smoking cessation is an important health care priority for many reasons, including its impact upon TB.

2.6 Alcohol and Tuberculosis

2.6.1 The Global Burden of Alcohol Use

Alcohol is one of the most commonly abused substances worldwide. The annual per capita consumption in 2005 was 6.13 liters of pure alcohol per person aged 15 years and older, of which 28.6 % was homemade [135]. According to a recent WHO report on global alcohol status, there is significant variation in consumption of alcohol, with the highest levels found in high-income countries, medium levels of consumption found in southern Africa, North and South America and lower consumption found in North Africa, southern Asia, and the Eastern Mediterranean [135]. More than 45 % of alcohol is consumed in the form of spirits and 36 % is consumed as beer. Global estimates suggest that consumption is stable in most regions, but there has been an increase in Africa and South-East Asia.

2.6.2 The Impact of Alcohol on Immune Function

Alcohol is a common cause of immunological impairment in both developed and developing countries. In addition to an association with smoking, alcohol abuse is often associated with malnutrition. All of these factors confer a strong biological explanation for immunological susceptibility to TB. The mechanisms by which alcohol impairs immune function have been well described [136].

2.6.3 The Impact of Alcohol on Tuberculosis Risk

There is now a substantial body of literature that shows a strong association between alcohol and TB, independent of smoking. A recent meta-analysis on the risk of TB with alcohol use disorders found a pooled relative risk of 2.9 (95 % CI 1.9–4.6) [25]. The meta-analysis combined the effect of three cohort studies and eight case-control studies including patients that consumed 40 g of alcohol or more daily. Another meta-analysis of molecular epidemiology studies showed alcohol abuse was associated with increased clustering of TB cases, suggesting increased risk of transmission in this setting [20].

There is evidence that heavy alcohol use will increase susceptibility to active disease TB and reactivation of latent infection as well as other respiratory infections [25]. A number of studies also show an association between alcohol use, earlier TB relapse and more destructive forms of TB [25]. Heavy drinkers also have higher rates of MDR-TB and treatment default than nondrinkers [25].

Alcohol use may also be associated with higher rates of mortality in TB, although there have been mixed findings. A systematic review found evidence of an association between TB-related death and alcohol in Russia and Brazil, however no association was found in another Indian study [26]. Another study from the

United States found that the people with heavy alcohol consumption had twice the rate of TB even after adjusting for smoking (OR 2.0, 95 % CI 1.1–3.7) [126].

2.6.4 The Population Impact of Immunological Impairment Due to Alcohol on Tuberculosis

Alcohol is likely to have a marked impact on TB epidemiology globally, despite the inherent difficulty in quantifying alcohol consumption in individuals or at a population level. One systematic review estimated that 10 % of TB globally could be attributed to alcohol [25]. As alcohol consumption varies considerably between populations, also does its impact upon TB.

Heavy alcohol consumption is likely to be one of the reasons that explain why males are more likely to develop TB than females [137]. However, it is difficult to disentangle the confounding factors that may cause increased risk of TB, particularly smoking, malnutrition, and poverty.

2.7 Chronic Disease and Tuberculosis

2.7.1 The Epidemiology of Chronic Disease

Chronic diseases, such as heart disease, diabetes, COPD, and cancer, are now the leading causes of mortality worldwide [138]. In addition to being the major cause of illness in high-income countries, chronic illness is responsible for an increasing proportion of morbidity in low- and middle-income settings [139]. Many of these diseases are related to lifestyle changes that have accompanied increases in per capita income, including smoking, lack of physical exercise, changes in diet, and use of alcohol. There is a vast literature on the epidemiology of different chronic diseases, including a recent global report by the WHO [138].

2.7.2 The Impact of Chronic Illness Upon Tuberculosis Epidemiology

As societies in low- and middle-income countries become wealthier, they tend to undergo an epidemiologic transition from primarily infectious disease to higher rates of chronic disease [77]. Paradoxically, even if TB rates are falling as overall health standards rise, those individuals with chronic illness may become more susceptible.

The relationship between chronic disease and TB is well documented. Higher rates of TB have been shown in patients with chronic kidney disease, COPD, and other chronic diseases in comparison to the unaffected population [36].

Chronic kidney disease is becoming increasingly common in many societies, and a number of studies have identified it as a risk factor for TB. For example, one

study in Mexico found the risk of diabetes in chronic renal failure to be three times than that of the general population (OR 3.09, and 95 % CI 1.9–5.1) [140]. Chronic renal failure has been shown to be associated with increased mortality due to TB (OR 8.1 (3.4–19.8)) [88]. A retrospective cohort study from the USA, showed that mortality in culture positive TB was seven times more common in patients receiving dialysis for chronic renal failure (95 % CI 3.7–13.3) [44]. A separate retrospective cohort study in China supports this finding [141].

COPD and TB share a complex relationship. Smoking is a common risk factor for both, and COPD is recognized as a long term complication of TB [142]. There is limited published literature about the impact of COPD upon TB. A number of studies have shown COPD to be one of the most common comorbidities among TB patients, particularly in elderly patients with TB [142]. The diagnosis of COPD has been shown in a number of small studies to be more common in patients with isoniazid resistance and more commonly associated with mortality. However, further research is required to clearly establish the discrete role of COPD separate from smoking and socioeconomic status.

Diabetes has been shown to be associated with TB, and is described in detail in the previous section. Various forms of cancer have been associated with TB in a number of studies [89].

A recent review of TB and rheumatoid arthritis highlighted a possible association between the two diseases, but noted substantial variation in findings between studies in different settings [108]. Studies from Spain and Asia found an association, however other studies from Peru and the United States found no difference in TB prevalence among patients with rheumatoid arthritis than the general population.

Over recent years, the increased interest in TB among rheumatoid arthritis has arisen primarily due to new immunosuppressive agents, particularly TNF inhibitors [108]. These drugs are associated with significantly increased susceptibility to TB for individual patients, by severely impairing innate immune function. However, on a global scale only a very small number of people take these medications. Consequently, the global PAF of TB due to biologic agents for autoimmune disease is very small.

Over coming decades, as the populations of countries with high rates of TB such as China, Russia, and India become older, these chronic illnesses will become increasingly important in the epidemiology of TB.

3 Conclusion

Over recent decades, we have better come to appreciate that the epidemiology of TB is not just driven by exposure to bacteria, but that the host susceptibility is of crucial significance. Globally, the most common factors impacting upon immune function include malnutrition, diabetes, smoking, alcohol, HIV, and aging. While most of these factors cause only mild immunological impairment at an individual

level, their cumulative effect on TB incidence in a population is likely to be considerable. It is essential that policy makers, researchers, and clinicians improve their understanding of these immunological factors, and develop better strategies to combat them, if the global pandemic of TB is to be combated effectively.

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Host–Pathogen Specificity in Tuberculosis

Tania Di Pietrantonio and Erwin Schurr

Abstract The host response to mycobacterial infection including tuberculosis depends on genetically controlled host and bacterial factors and their interaction. A largely unknown aspect of this interaction is whether disease results from an additive and independent effect of host and pathogen or from specific host–pathogen combinations. The preferential association of specific mycobacterial strains with specific ethnic groups provided tentative evidence in favor of host–pathogen specificity in tuberculosis and is consistent with the hypothesis of host–mycobacterial co-adaptation. Substantial evidence for specificity has now been provided by animal models and human case–control association studies. These studies indicate that differences in the host response to infection are at least in part due to specific combinations of host genetic factors and genetic and phenotypic characteristics of the infecting mycobacterial strain.

Keywords *Mycobacterium tuberculosis* · Host–pathogen specificity · BCG infection · ANOVA · RC strains · Chemokine and chemokine-related genes · Toll-like receptors · Pathogen-associated molecular patterns · Meningeal tuberculosis · Phagosome maturation · Autophagy · Mannose-binding lectin (MBL)

T. Di Pietrantonio · E. Schurr (✉)
McGill University Health Centre McGill Centre for the Study of Host Resistance,
Departments of Medicine and Human Genetics, 1650 Cedar Avenue, Room L11.521,
Montreal H3G 1A4 QC, Canada
e-mail: erwin.schurr@mcgill.ca

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1 Introduction

The host response to *Mycobacterium tuberculosis* is highly variable. Among individuals exposed to *M. tuberculosis*, approximately 30–50 % become infected during an outbreak and only about 10 % of those infected progress to clinical disease [1]. Several lines of evidence support the importance of host genetic factors in tuberculosis susceptibility. These include the geographic and ethnic clustering of tuberculosis cases [2, 3], increased concordance rates of tuberculosis in monozygotic compared to dizygotic twins [4, 5], and documented cases of Mendelian predisposition to disease [6, 7]. Genetic variants have been identified as risk factors for tuberculosis through genome-wide linkage [8–14] and association studies [15, 16] as well as candidate gene studies [17]. Although these studies have greatly increased our insight into the genetic basis of tuberculosis susceptibility, the results have been somewhat inconsistent. With candidate gene studies, the identified effects have generally been weak and poorly reproducible. Similarly, there is minimum overlap between susceptibility regions identified through genome-wide studies. Non-reproducibility has been attributed primarily to population genetic factors, differences in phenotype definition, and genetic diversity among *M. tuberculosis* strains [17, 18].

Increasing evidence suggests genetic differences among *M. tuberculosis* strains may have important phenotypic consequences. For example, *M. tuberculosis* strains differ in their virulence and immunogenicity in animal models [19–22]. In humans, *M. tuberculosis* isolates have been associated with specific clinical phenotypes [23]. An increasing number of studies also indicate that specific strains of *M. tuberculosis* are more prevalent in certain geographical areas [24]. Six geographically constrained lineages have been identified using isolates from San Francisco [25] and replicated independently using a Montreal cohort [26]. These include the East-Asian, Euro-American, Indo-Oceanic, East-African-Indian, and West-African-1 and -2 lineages. East-Asian strains include, but are not confined to, the Beijing family of strains [25]. Strains of the Euro-American lineage predominate in Europe and the Americas but are also found in regions of Africa and the Middle East [25]. The East-Asian, Euro-American, Indo-Oceanic, and East-African-Indian groups are sub-lineages of *M. tuberculosis* sensu stricto, whereas the West African lineages 1 and 2 are traditionally referred to as *M. africanum* [27]. These geographical associations are highly stable [28] and were shaped by human migration and demography [29], suggesting *M. tuberculosis* strains are preferentially transmitting and causing disease among specific ethnic groups. They may also reflect co-adaptation of *M. tuberculosis* with its human host.

Together, these observations raise the biological question if host and mycobacterial factors are acting additively and independently to cause disease or if specific combinations of host and pathogen are associated with increased risk of tuberculosis. If host and pathogen factors contribute independently to disease, hosts would display a range of phenotypes from highly resistant to highly susceptible regardless of the infecting strain and mycobacteria would vary from

highly to mildly virulent across all hosts. Only hosts that are intrinsically susceptible to *M. tuberculosis* or those exposed to highly virulent strains of *M. tuberculosis* would present with disease. Alternatively, if disease results from specific combinations of host and pathogen, specific strains of *M. tuberculosis* would only cause disease in certain hosts while hosts would only be susceptible to particular strains of *M. tuberculosis*. In the latter instance, the concepts of "virulence" or "susceptibility" would only hold true when jointly considering host and *M. tuberculosis* strain. Understanding the dynamics and specificity of host–pathogen associations in tuberculosis pathogenesis is imperative for the development of effective tuberculosis control tools. This chapter reviews the current evidence supporting host–pathogen specificity in increased tuberculosis risk.

2 Mycobacterial Strain Specificity in Host Response Phenotypes

Inbred mice display a switch in host susceptibility/resistance to virulent *M. tuberculosis* and attenuated *M. bovis* Bacille Calmette-Guérin (BCG). Mouse strains, which are classically considered resistant to BCG infection, are susceptible to infection with *M. tuberculosis* and vice versa [30, 31]. Susceptibility of the mouse to tuberculosis is defined as early death caused by progressive lung disease and/or uncontrolled bacterial replication. Unlike *M. tuberculosis,* BCG produces a non-lethal, self-limiting infection. Susceptibility to BCG is assessed by the extent of bacterial replication in the organs of the mononuclear phagocyte system within the first 3 weeks of infection. The A/J and C57BL/6J mouse strains represent polar ends of this operationally defined susceptibility/resistance spectrum. Death following infection with *M. tuberculosis* in the susceptible A/J strain results from a progressive interstitial pneumonitis characterized by diffuse cellular infiltration, widespread tissue necrosis, and bacterial dissemination. *M. tuberculosis* infection is also lethal in the resistant C57BL/6J strain, although these mice survive for prolonged periods due to the development of functional lung granulomas and corresponding bacterial containment [Di Pietrantonio et al., unpublished data, [32–34]. Host susceptibility is reversed in response to infection with either the Montreal or Pasteur substrains of BCG: *M. tuberculosis*-resistant C57BL/6J mice are permissive to BCG replication within the reticuloendothelial organs, whereas the *M. tuberculosis*-susceptible A/J strain is resistant [35, 36]. The shift in host susceptibility in response to attenuated and virulent mycobacteria provides strong support for pathogen-specific susceptibility in infections caused by closely related mycobacterial species.

To provide formal and direct evidence for specificity in the host–pathogen crosstalk, biological and mechanistic phenotypes were investigated in A/J and C57BL/6 J mice following intravenous infection with *M. tuberculosis* or the Russia, Japan, and Pasteur substrains of BCG [37]. Biological phenotypes are markers of host susceptibility and resistance, whereas mechanistic phenotypes

describe the mechanisms underlying susceptibility and resistance traits. Host phenotypes also provide a measure of bacterial virulence, a property that is investigated in terms of changes induced by a panel of bacterial strains in genetically identical hosts. The individual and joint contribution of the host and mycobacteria on phenotype expression can be assessed using three-way analysis of variance (ANOVA). With ANOVA, the significance of each variable (here host, bacteria, and time) and the interaction between each pair of variables is determined by independent F tests. Evidence for host–pathogen specificity is provided by the host $\times$ bacteria interaction term [38].

In a proof-of-principle study, pulmonary bacterial counts (CFU) and transcript levels of three cytokines (*Ifng*, *Il12b*, and *Il4*) were measured at 1, 3, and 6 weeks post-infection as biological and mechanistic phenotypes, respectively. The bacterial load is an important biomarker of host resistance and susceptibility to infection and is a useful parameter to assess differences in virulence among mycobacterial strains. In the lung, *M. tuberculosis* had the highest bacillary counts in both A/J and C57BL/6J mice when compared to the three BCG strains. Among the BCG strains, the pulmonary counts of BCG Russia increased progressively over time while BCG Pasteur remained consistently low and BCG Japan was unrecoverable in both mouse strains. When the impact of the mycobacterial strain on the pulmonary counts was assessed using three-way ANOVA, significant bacterial-related effects were detected independent of the host and time [$F(2, 30.9) = 118.3$; $P < 0.001$]. For the host component, a significant contribution of the mouse strain was observed independent of the bacteria and time [$F(1, 34.3) = 18.6$; $P < 0.001$]. The effect of time was also significant [$F(2, 23) = 49.2$; $P < 0.001$]. Importantly, significant interaction effects were observed between the bacteria and mouse strain [$F(2, 30.9) = 5.5$; $P < 0.01$], providing significant evidence of host–pathogen specificity in pulmonary bacterial replication. For the mechanistic phenotypes, host–pathogen specificity was detected in the transcriptional induction of *Ifng* and *Il12b* but not *Il4*. A significant contribution of host genetic background on phenotype expression was observed for *Il12b* transcription only, whereas an effect of the mycobacterial strain was observed across all mechanistic phenotypes. Pulmonary expression profiles of chemokine and chemokine-related genes further indicated that variation across mycobacterial strains had a larger impact than host genetic variation. While differences in the transcriptional response between A/J and C57BL/6J mice were modest, a large subset of immune genes was differentially regulated across mycobacteria. Importantly, mycobacterial-related effects were more prominent in the A/J mouse strain, indicating that the host genetic background modulated the impact of the mycobacterial strain on these responses. These results suggested that the mycobacterial strain had a greater effect on the studied phenotypes compared to the host genetic background in this experimental setting. However, it is important to realize that the genetic similarity among the host strains was comparatively higher relative to the mycobacteria, which included two different species. In situations involving outbred hosts, both the impact of the host and the significance of host–pathogen effects are expected to be substantially stronger [37].

3 Mycobacterial Strain Specificity in the Genetic Control of Infection and Disease

Mouse genetic studies have generally focused on one mycobacterial strain to map and identify the host genetic factors that control infection and the progression of infection. Using this approach, differential susceptibility of inbred mouse strains to BCG infection was shown to be largely under the control of the natural resistance-associated macrophage protein 1 (*Nramp1,* alias solute carrier family 11 member 1, *Slc11a1*) gene [35], whereas the host genetic control of *M. tuberculosis* infection was found to be multigenic [39–43]. The shift in genetic control from simple to complex across mycobacteria of varying virulence strongly suggested that pathogen genome variability impacted on the host genetic response to infection. This raised the question of if and to what extent different strains of the same mycobacterial species were under different host genetic control, a necessary consequence of specificity in host–pathogen interactions. To test for strain-specific genetic control of susceptibility, a comparative genetic analysis was performed in the A/J- and C57BL/6J-derived recombinant congenic mouse (RC) strains following infection with BCG Russia or BCG Pasteur. A unique feature of RC strains is that host response phenotypes are estimated with greater accuracy due to repeat measurements in genetically identical animals. Linkage genome scans can then be conducted with high resolution using the relatively limited number of RC strains within the panel. Employing eight parallel genome scans, bacillary counts in the lung and spleen were shown to be under the control of shared as well as tissue- and BCG-specific susceptibility loci. A locus indistinguishable from *Nramp1* on chromosome 1 impacted on both BCG Pasteur and BCG Russia infection in a spleen-specific manner. Loci influencing the counts of BCG Russia but not BCG Pasteur were identified on chromosome 13 for the spleen and on chromosome 11 for the lung and spleen [44]. The observation that only a minority of genetic control elements was shared among closely related strains of BCG and across tissues in the mouse indicated that the genetic control of BCG infection was sensitive to the infecting strain and to phenotype definition (here, bacillary counts in the lung versus the spleen). Another important aspect of phenotype definition is the stage of the infectious process. Clearly, more advanced stages are under different genetic control as compared to the initial stages of infection [44]. It is likely that the same disease stage specificity of the genetic control also applies to tuberculosis and contributes to the difficulty in identifying strong genetic risk factors of susceptibility.

At present, five human genetic studies have incorporated the *M. tuberculosis* genotypes of their sample population into the analysis of genetically controlled tuberculosis susceptibility. All five studies found an interaction between a human genetic variant and the mycobacterial lineage known to be associated with the corresponding geographic region and human population.

3.1 Toll-Like Receptor 2 (TLR2)

Toll-like receptors (TLRs) are signaling molecules involved in innate immunity. TLRs detect pathogen-associated molecular patterns (PAMPs) on bacterial molecules. Binding of the PAMP ligand to the extracellular domain of the TLR initiates a signaling cascade through the Toll/IL-1 receptor domain, resulting in the expression of pro-inflammatory molecules including cytokines and chemokines. Among the TLRs, TLR2 recognizes lipoprotein/lipopeptides which are expressed by all bacteria including mycobacteria [45].

In a population-based study from Vietnam, the *TLR2* T597C polymorphism was shown to be associated with increased risk of developing tuberculosis. The association was strongest in cases caused by the East Asian/Beijing isolates [P = 0.004, odds ratio (OR) 1.57 (95 % confidence interval (CI) 1.45–2.15)], but not significant in cases caused by the Indo-Oceanic and Euro-American isolates, although heterogeneity testing was not reported. This suggests that the *TLR2* T597C polymorphism or a polymorphism in linkage disequilibrium with T597C specifically affects the interaction of the East Asian/Beijing strains with TLR2. The association between disease caused by the East Asian/Beijing genotype and *TLR2* T597C increased when the clinical phenotype was considered: the OR for meningeal tuberculosis caused by East Asian/Beijing isolates was 1.91 [P = 0.001, 95 % CI 1.28–2.86]. By contrast, the Euro-American lineage was associated with pulmonary rather than meningeal tuberculosis: the OR for meningeal tuberculosis caused by Euro-American strains was 0.395 [P = 0.009, 95 % CI 0.193–0.806] [23]. The increased propensity of East Asian/Bejing strains to cause meningeal tuberculosis may be linked to production of a phenolic glycolipid (PGL-tb) molecule. Experiments involving animal models have shown that PGL-tb specifically inhibits the immune response and promotes dissemination from the lungs [22, 46]. Euro-American strains lack expression of PGL-tb due to a seven-base pair deletion in the polyketide synthase (*pks1/15*) gene [25, 47] and may conversely cause less extra-pulmonary disease.

3.2 Solute Carrier Family 11 Member 1 (SLC11A1) Natural Resistance-Associated Macrophage Protein 1 (NRAMP1)

In inbred mice, allelic variation in *Nramp1* (also known as *Slc11a1*) is strictly correlated with susceptibility to low dose BCG infection [48]. During BCG infection, Nramp1 recruitment to the membrane of BCG-containing phagosomes abrogates the ability of BCG to block phagosome-lysosome fusion, causing increased vacuolar acidification and decreased intracellular replication [49]. In humans, NRAMP1 (SLC11A1) was shown to promote phagosome maturation in a monocytic cell line [50].

The contribution of *SLC11A1* variants to *M. tuberculosis* genotype-specific susceptibility was studied in an Indonesian population sample. The GG genotype of the non-synonymous exonic variant D543 N (G1703A) was significantly associated with increased risk of tuberculosis caused by Beijing strains [$P = 0.05$, OR 2.15 (95 % CI 1.25–3.70)]. The homozygous insertion genotype for the TGTG insertion/deletion polymorphism in the 3′ untranslated region (3′ UTR) (1729 + 55 ins/del4) was also associated with tuberculosis caused by strains of the Beijing genotype [$P < 0.001$, OR 2.40 (95 % CI 1.19–4.83)] [51]. The increased risk of disease caused by Beijing strains in carriers of these *SLC11A1* variants suggests strains of this lineage may be more resistant to SLC11A1-mediated protection.

3.3 Immunity-Related GTPase Protein Family, M Gene (IRGM)

The immunity-related GTPase M (IRGM) participates in the host defense against *M. tuberculosis* by inducing autophagy. During autophagy, cytosolic components such as organelles are sequestered in an autophagosome and become degraded following fusion with a lysosome [52]. *M. tuberculosis* is accessible to autophagy either through the phagosome or directly from the cytoplasm following translocation from the phagosome [53]. Formation of the autophagosome involves light chain 3 (LC3), which is activated by IRGM [54].

The *IRGM* -261TT genotype was associated with increased protection to tuberculosis in a population-based study from Ghana. The protective effect applied to tuberculosis caused by *M. tuberculosis* sensu stricto [$P_{corrected} = 0.0045$, OR 0.66 (95 % CI 0.52–0.84)] but not to *M. africanum* or *M. bovis*. Stratification by *M. tuberculosis* lineage further revealed that protection was exclusive to the Euro-American subgroup [$P_{corrected} = 0.0019$, OR 0.63 (95 % CI 0.49–0.81)] and did not apply to the East-African-Indian, East-Asian/Beijing, or Indo-Oceanic/Delhi genotypes [55]. The increased vulnerability of the Euro-American strains to IRGM-triggered innate immune mechanisms including autophagy may be related to absence of PGL-tb.

3.4 Arachidonate 5-Lipoxygenase (ALOX5)

Arachidonate 5-lipoxygenase (also known as 5-lipoxygenase or 5-LO) is an enzyme involved in the biosynthesis of leukotrienes (LT) and lipoxins (LX) from arachidonic acid. Among the different classes of LTs, LTB_4 is a pro-inflammatory mediator which attracts and stimulates leukocytes, resulting in increased production of IFN-γ and IL-12 [56, 57]. LXs antagonize LTB_4 activity by suppressing

leukocyte effector functions, downregulating IL-12 production [56] and enhancing IL-4 secretion [58].

The non-synonymous exonic variant g.760G/A (Glu254Lys) of *ALOX5* was shown to be associated with increased risk of developing tuberculosis in a case–control sample from Ghana, West Africa. Stratification by phylogenetic mycobacterial clades revealed that the association was strongest for tuberculosis caused by the *M. africanum* West African-2 genotype [$P_{corrected} = 0.024$, OR 1.70; (95 % CI 1.2–2.6)] or for the West African-2 and *M. bovis* strains combined [$P_{c12} = 0.006$, OR 1.85 (95 % CI 1.27–2.65)] [59]. The implications of these findings are two-fold. First, they suggest that *M. africanum* and *M. bovis* may differentially activate 5-lipoxygenase compared to *M. tuberculosis*. Secondly, the association between the West African-2 lineage which is virtually unique to Africa and the g.760G/A polymorphism which has a higher frequency in African relative to European and Euro-American populations is consistent with the hypothesis of co-evolution.

3.5 Mannose Binding Lectin 2 (MBL2)

Mannose-binding lectin (MBL) is an innate immune effector. It binds carbohydrate structures on the surface of a wide variety of pathogens and promotes phagocytosis either directly by acting as an opsonin or indirectly by activation of classical complement pathway [60].

The impact of genetic *MBL2* variants on susceptibility to pulmonary tuberculosis was investigated in a Ghanian study population. Among the three structural variants tested in exon 1, the G57E polymorphism at position 57 was protective against tuberculosis caused by *M. africanum* or *M. bovis* [$P_{nominal} = 0.008$, OR 0.60 (95 % CI 0.4–0.9)] but not by *M. tuberculosis* sensu stricto. A haplotype of three polymorphisms of the *MBL2* promoter at positions −550 (alleles H and L), −221 (alleles Y and X) and +4 (alleles P and Q) together with the variant at position 57 of exon 1 (alleles A and C) revealed that the LYQC combination was protective against *M. africanum*/*M. bovis* [$P_{corrected} = 0.007$] but not *M. tuberculosis* [61]. The LYQC haplotype is almost exclusive to sub-Saharan Africa and occurs there at a high relative frequency, suggesting it may have been selected for because of its protective effect against *M. africanum* and *M. bovis*.

Given the presumed function of MBL, it is tempting to speculate that *M. tuberculosis* differs from *M africanum* and *M. bovis* in the expression of cell surface structures which may be relevant to MBL binding. For example, the TBd1 region is absent in all *M. tuberculosis* sensu stricto strains but not in *M. africanum*/*M. bovis*. This region contains genes encoding membrane proteins which may be involved in lipid transport and may impact on cell wall composition [61].

4 Conclusion

There is now clear evidence from experiments in both animal models and human case–control samples for pathogen specificity in the genetic control of tuberculosis susceptibility. These findings are not unexpected since the mechanisms of pathogenesis in animal models and cell explants differ among closely related strains and species of mycobacteria. Differences in pathogenesis open entry points for variable host genetic control of mycobacterial infection and disease. While the presence of host–mycobacteria specificity is indisputable, the genetic effects detected are of moderate size. Whether this reflects a true minor contribution to disease risk of such host–mycobacteria specific interactions or the lack of high-resolution genetic characterization of host and pathogen will be the object of further investigation.

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Genetic Determinants of Susceptibility to Mycobacterial Infections: IRF8, A New Kid on the Block

S. Salem and P. Gros

Abstract Genetic and population studies suggest that onset, progression and ultimate outcome of infection with Mycobacteria, including the agent of tuberculosis *Mycobacterium tuberculosis*, are strongly influenced by genetic factors. Family-based and case-control linkage and association studies have suggested a complex genetic component for susceptibility to tuberculosis. On the other hand, patients with inborn errors in the IL12/IFNγ circuit may develop disseminated mycobacterial infections following perinatal BCG vaccination. The study of such MSMD (Mendelian Susceptibility to Mycobacterial Diseases) patients has provided much insight into innate and acquired immune defenses against mycobacteria. Parallel genetic analyses in mouse models of mycobacterial infections have also indicated complex genetic control, and have provided candidate genes for parallel testing in humans. Recently, mutations in human *IRF8* were discovered and shown to cause two distinct forms of a novel primary immunodeficiency and associated susceptibility to mycobacteria. Autosomal recessive IRF8 deficiency is caused by mutation K108E and associated with severe disease with complete depletion of monocytes and dendritic cells. Mutation T80A causes autosomal dominant IRF8 deficiency and a milder form of the disease with selective loss of a subset of dendritic cells. These findings have established that IRF8 is required for ontogeny of the myeloid lineage and for host response to mycobacteria. The ongoing study of the IRF8 transcriptome has shown promise for the identification of IRF8 dependent pathways that play a critical role in host defense against mycobacteria in particular, and against intracellular pathogens in general.

S. Salem · P. Gros (✉)
Department of Biochemistry, Complex Traits Group, McGill University,
3649 Drummond St., room 366, Montreal, QC H3G-1Y6, Canada
e-mail: Philippe.gros@mcgill.ca

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1 Pathogenesis of Infection with *Mycobacterium tuberculosis*

Mycobacteria form a group of important human pathogens. *Mycobacterium tuberculosis* (MTB) is the causative agent of human tuberculosis (TB). It is estimated that as much as one-third of the world's population is or has been infected with MTB, hence representing an enormous reservoir for latent infection and possible transmission. Of these individuals, a subset progresses from infection to disease, resulting in an estimated 8.9 million new cases and 1.7 million deaths per year worldwide [1]. The TB problem is exacerbated by the emergence of highly drug resistant variants [2] and the increased susceptibility of AIDS patients [3]. MTB is not the only human pathogenic mycobacteria and infection with *Mycobacterium leprae* causes leprosy, a disease that is still prevalent in several countries, with about 250,000 new cases recorded in 2006 [4]. Leprosy presents with a broad pathological spectrum, from the localized tuberculoid paucibacillary form to the more disseminated lepromatous multibacillary form. On the other hand, an additional group of mycobacterial species represent opportunistic environmental pathogens that infect hosts with a local or systemic immunodeficiency (e.g. *Mycobacterium abscessus* lung infections in patients with cystic fibrosis, *Mycobacterium avium* bacteremia in patients with AIDS). Finally, disseminated infections with weakly virulent mycobacterial species, such as *Mycobacterium bovis* BCG vaccines, *Mycobacterium marinum* and *M. avium*, can also appear in otherwise healthy individuals, a rare syndrome known as Mendelian Susceptibility to Mycobacterial Diseases (MSMD) [5].

The pathogenesis of infection with virulent Mycobacteria such as MTB can be summarized as follows. Virulent MTB enters the lung via the aerosol route and it is promptly engulfed by resident alveolar macrophages and dendritic cells. Infected dendritic cells migrate to peripheral lymph nodes where they present mycobacterial antigens to $CD4^+$ and $CD8^+$ T lymphocytes. Primed T cells migrate to the lungs to initiate the formation of protective granulomas; Infected macrophages and in particular dendritic cells secrete the key cytokines IL-12 and IL-23 to activate T lymphocyte response (early Th1 polarization); $CD4^+$ and $CD8^+$ T cells recognize bacterial antigens presented by phagocytes in association with Class II and Class I MHC molecules, respectively, and secrete INF-γ and TNF-α causing macrophage activation. Activated macrophages produce bacteriostatic and bactericidal species (reactive oxygen radical, nitric oxide, iron chelators) but also

display increased maturation of MTB phagosomes including increased acidification, and enhanced delivery of lysosomal enzymes to the phagosomal space. T cells also directly produce perforin and granulysin that contribute to microbicidal activity [6]. Despite this impressive immune response, infected hosts usually fail to clear the infection and become asymptomatic carriers, with a 5–10 % lifetime risk of progressing to active disease [7].

One of the most mysterious aspects of pathogenesis is the ability of virulent mycobacteria to survive in fully bactericidal macrophages. Like other intracellular pathogens, MTB has developed unique strategies to avoid/override macrophage defenses, and studies of such mechanisms have shed new light on molecules and pathways that macrophages use to destroy invading microbes [8–10]. It has been known for 40 years that virulent MTB resides in a phagosome that fails to mature, including incomplete acidification (decreased recruitment of vacuolar H^+/ATP-ase), inhibition of fusion to Rab7 and LAMP-1 positive late endosomes and lysosomes, and retention of early endosome markers and coronin-1 (TACO), a process influenced by lipids from the mycobacterial cell wall. In addition, virulent MTB (but not avirulent mycobacteria) has been shown to inhibit early apoptotic cell death in macrophages while inducing sustained membrane damage and necrotic cell death in these cells [11]. This process involves MTB-driven proteolytic cleavage of the amino terminus of annexin 1, preventing annexin 1 deposition and assembly of the apoptotic envelop. Predominant necrotic cell death results in reduced mycobacterial antigen presentation by macrophages and dendritic cells [11]. In addition, it has been shown that experimental stimulation of autophagy can reduce viability and restrict replication of intracellular MTB [12, 13]. Furthermore, in a recent genome-wide RNAi screen in MTB-infected THP1 human macrophages, autophagy has also been identified as a key defense pathway in macrophages, which is modulated during MTB infection [14]. Recent studies have also pointed at a critical role of lipoxin LXA4 and prostaglandin PGE2-dependent inflammation in regulating MTB replication in macrophages in vitro and TB susceptibility in vivo [15–17]. Finally, it has also been observed that some pathogenic mycobacteria can escape the phagosome and replicate in the cytosol of macrophages [18].

The host defense mechanisms against MTB, and in particular the host innate and adaptive immune defenses that MTB defeats to persist for years in infected cells and tissues still remains poorly understood. Such mechanisms can manifest themselves as genetic determinants of innate resistance or susceptibility to infections in human populations [19–23], and in corresponding animal models [22, 24, 25]. Association studies with candidate genes and linkage analysis by whole genome scanning have shown that susceptibility to MTB in humans is multifactorial and genetically heterogeneous [7–22]. In addition, the host genetic component is further modified by complex microbial pathogenicity determinants that ultimately reveal host susceptibility [26]. Therefore, a genetic approach to susceptibility to infections is difficult in humans. Such complex traits can be dissected in the laboratory mouse using well defined but phenotypically distinct inbred or mutant mouse stocks, where host genetic factors and pathogen-related effects can be controlled [25, 27]. The mouse

has proven extremely useful to study several aspects of host response to TB including pathogenesis, innate and acquired immune responses, and predisposing genetic effects. The intracellular survival strategy of MTB in macrophages is similar in mouse and man (inhibition of phagosome maturation, inhibition of apoptosis) [8, 11, 13, 28–32]. In addition, innate and acquired immune responses to MTB in mice resemble those in humans, including the protective roles of $CD4^+$ T cells, IFN-γ, TNF-α, and IL-12 [6]. The importance of these molecules, initially demonstrated in knock-out mice [6, 33, 34] (Table 1), was validated by the discovery that humans bearing mutations in these genes show hypersensitivity to mycobacterial infections (pediatric TB, susceptibility to BCG vaccines) [5]. Likewise, the relevance of genes such as *Nramp1,* and *sst1* that control susceptibility to mycobacteria in inbred mouse strains, was validated in humans from endemic areas of mycobacterial diseases [22].

We will herein review recent literature on the parallel genetic analyses of susceptibility to mycobacterial infections in humans and mice, with an emphasis on genetic effects that are common to both species, and that were discovered by forward genetic approaches.

2 Genetic Factors and Susceptibility to Infection with Weakly Virulent Mycobacteria

Live BCG vaccines can cause severe clinical disease in children with primary immunodeficiencies, a condition that has been termed BCG-osis [35]. MSMD (MIM 209950) [36] is a broader syndrome defined by the development of severe clinical conditions upon infection with weakly virulent mycobacterial species, such as BCG vaccines and nontuberculous, environmental mycobacteria, in otherwise healthy individuals [35, 37–39]. Patients with MSMD are also susceptible to the more virulent MTB [40–45]. Nearly half the cases of MSMD show severe disease caused by nontyphoidal and, to a lesser extent, typhoidal *Salmonella* serotypes [37, 46, 47]. Although associated with a narrow range of infections, "idiopathic" BCG-osis and infection with environmental mycobacteria were often diagnosed in consanguineous and/or multiplex kindreds, strongly suggesting a Mendelian inheritance. Over the past 15 years, Casanova and colleagues have used a candidate gene approach to elucidate the genetic etiology of MSMD in a cohort of 450 such patients recruited all over the world. They have identified in certain of these patients mutations in IL-12p40 (*IL12B*) [48], and its receptor (*IL12RB1*) [45, 48–51], the receptor for interferon γ (IFNRG1; IFNRG2) [43, 52–54], the IFNRG signaling partner STAT1 [55–57], and the NFκB regulator I$\kappa\kappa\gamma$ (NEMO) [22, 39, 58]. In all cases, the immunodeficiency associated with these mutations is inherited as autosomal recessive with partial or complete loss-of-function alleles. These studies have clearly highlighted the critical role of the IL-12/IFNγ circuit in macrophage priming and T cell function (including Th1 polarization) in early host defenses against mycobacterial infections. Recently,

Table 1 Response of mouse mutants to Mycobacterium tuberculosis

Mouse Strain	Mycobacterial Strain	Infection	Phenotype	Anti/Pro-inflammatory Mediators Production in response to M.tb infection	Ref
Beta 2-microglobulin (B2m)	Virulent M. tuberculosis Erdman Strain	I.v (10^6)	Rapid Death Uncontrolled Mtb growth	Normal production	182
Caspase Recruitment Domain containing protein 9 (Card-9)	M.tuberculosis H37Rv	Aerosol (450)	Rapid death High Mtb load in lungs Uncontrolled Mtb growth Increased apoptosis in lungs	Lower production of TNFα, IL-6, CCL5, IL-12	183
Caspase Recruitment Domain 15 / Toll-like Receptor 2 (Card15/Tlr2)	M.tuberculosis H37Rv	Aerosol (100)	Normal Mtb load in lungs	Normal production	184
Chemokine (C-C motif) Receptor 2 (Ccr2)	M.tuberculosis H37Rv	I.v (3.3–8.0x10^5)	Rapid Death High Mtb load in lungs Uncontrolled Mtb growth in lungs	Lower early IFN-γ production	185
Cluster of Differentiation 4 (Cd4)	M.tuberculosis H37Rv	Aerosol (100)	Altered granuloma formation Defect in late containment of Mtb growth	Normal IFN-γ production	186
Cluster of Differentiation 8 alpha (CD8α)	Virulent M. tuberculosis Erdman Strain	I.v (10^5, 10^6)	Decreased survival Uncontrolled Mtb growth	N/A	187
Cluster of Differentiation 36 (Cd36)	M.tuberculosis H37Rv	MOI (10:1)	Reduced Mtb load	Normal production	188
Class II, major histocompatibility complex Transactivator (Ciita)	Virulent M. tuberculosis Erdman Strain	Aerosol (100)	Decreased survival High Mtb load Uncontrolled Mtb growth	Lower IFN-γ production	189

(continued)

Table 1 (continued)

Mouse Strain	Mycobacterial Strain	Infection	Phenotype	Anti/Pro-inflammatory Mediators Production in response to M.tb infection	Ref
Complement Receptor 3 (Cr3)	Virulent M. tuberculosis Erdman Strain	I.v ($2x10^5$)	Normal survival	Normal IL-12, IFN-γ production	190
C-type lectin domain family 7 member A (Clec7a)	M.tuberculosis H37Rv	Aerosol (100)	Reduced Mtb load in lungs Normal survival	Normal production	191
CXC Chemokine Receptor 3 (Cxcr3)	M.tuberculosis H37Rv	Aerosol (10–100)	Normal survival Normal Mtb load	N/A	192
DNAX Activation Protein 10 (Dap10)	M.tuberculosis H37Rv	Aerosol (100)	Normal survival Normal Mtb load in lungs	Normal production	193
Fragment Crystallizable gamma Chain (Fcγ)	Virulent M.tuberculosis	Aerosol (150)	Decreased survival High Mtb load in lungs and spleen	Higher IL-10 production	194
Fragment Crystallizable gamma Receptor II B (FcγRiib)	Erdman Strain		Reduced Mtb load	Higher IFN-γ, IL-12p40 production	
Granzyme b (Go)	Virulent M.tuberculosis Erdman Strain	Aerosol (100)	Control of Mtb growth	Normal IFN-γ production	195
Guanylate binding protein 1 (Gbp1)	M.bovis BCG	I.v (10^5)	Uncontrolled growth Normal granuloma formation	Normal IFN-γ production, NO release	196
Ig mu Chain C region (Ighm)	M.tuberculosis H37Rv	Aerosol (100)	Control of Mtb growth Normal Mtb load	Normal IFN-γ production	197
Immunity Related GTPase A6 (Irga6)	M.tuberculosis H37Rv	Aerosol (100)	Normal Mtb load	N/A	198

(continued)

Table 1 (continued)

Mouse Strain	Mycobacterial Strain	Infection	Phenotype	Anti/Pro-inflammatory Mediators Production in response to M.tb infection	Ref
Immunity Related GTPase D (Irgd)	Virulent M. tuberculosis Erdman Strain	Aerosol (5×10^2) I.v (10^5)	Control of Mtb growth Normal Mtb load	N/A	91
Immunity Related GTPase M1 (Irgm1)	Virulent M. tuberculosis Erdman Strain	Aerosol (5×10^2) I.v (10^5)	Decreased survival Uncontrolled Mtb growth	Normal IFN-γ, TNFα, NOS2 production	
Immunity Related GTPase M3 (Irgm3)	Virulent M. tuberculosis Erdman Strain	Aerosol (5×10^2) I.v (10^5)	Control of Mtb growth Normal Mtb load	N/A	
Inducible Nitric Oxide Synthase (Nos2)	Virulent M. tuberculosis Erdman Strain	I.v (10^5)	Decreased survival Uncontrolled Mtb growth	Higher production of IFN-γ, TNFα No production of NO	199
	Virulent M. tuberculosis Erdman Strain	Aerosol (50)	Decreased survival Uncontrolled Mtb growth	Lower RNI production	200
Integrin Alpha L (Itgal)	Virulent M. tuberculosis Erdman Strain	Aerosol (7.2×10^1)	Decreased survival Uncontrolled Mtb growth High Mtb load Altered granuloma formation	Lower IFN-γ production	201
Integrin Beta-2 (Itgb2)	Virulent M. tuberculosis Erdman Strain	Aerosol (2.4310^2)	Decreased survival	N/A	
Interferon-gamma (Ifnγ)	Virulent M. tuberculosis Erdman Strain	I.v (10^6)	Decreased survival Uncontrolled Mtb growth	No production of ROI	202
	Virulent M. tuberculosis Erdman Strain	I.v (10^5) Aerosol (5×10^1)	High Mtb load Uncontrolled Mtb growth	N/A	33
Interferon-gamma Receptor (IfnγR)	M.bovis BCG	I.v (10^7)	Decreased survival Altered granuloma formation in liver	Lower TNFα production	203

(continued)

Table 1 (continued)

Mouse Strain	Mycobacterial Strain	Infection	Phenotype	Anti/Pro-inflammatory Mediators Production in response to M.tb infection	Ref
Interferon Regulatory Factor 1 (Irf1)	Virulent M. tuberculosis	Aerosol (10^6)	Rapid death	Normal IFN-γ, TNFα production	166
			High Mtb load	Lower NOS2, IFNα production	
	Kurono Strain		Dissemination	Higher IL-6, IL-18 production	
Interferon Regulatory Factor 8 [R294C] (Irf8)	M.tuberculosis	I.v (10^4)	Rapid death Uncontrolled Mtb growth	No production of IL-12p40, IFN-γ	132
		Aerosol (100)	Uncontrolled Mtb growth		
	H37Rv		Dissemination		
Interleukin 1 alpha/beta (Il1α/β)	M.tuberculosis	Aerosol (10^6)	Normal survival Normal Mtb. Load	Normal production	204
	H37Rv		Larger granulomas		
Interleukin -1 beta (Il1β)	M.tuberculosis H37Rv	Aerosol (100)	Decreased survival	Higher TNFα, IFN-γ, IL-1α production	205
			High Mtb load	Lower IL-12p40 production	
Interleukin 1 Receptor (Il1R)	M.tuberculosis H37Rv	I.n ($2x10^2$)	High Mtb load Uncontrolled Mtb growth Altered granuloma formation	Lower IL-12p40 production	206
Interleukin 4 (Il4)	M.tuberculosis H37Rv	Aerosol (100)	Normal defense against Mtb	N/A	207
Interleukin 6 (Il6)	M.tuberculosis	I.v (10^6)	Decreased survival	Lower IFN-γ, IL-12 production	208
	H37Rv		Uncontrolled Mtb growth	Higher IL-4, TNFα production	

(continued)

Table 1 (continued)

Mouse Strain	Mycobacterial Strain	Infection	Phenotype	Anti/Pro-inflammatory Mediators Production in response to M.tb infection	Ref
Interleukin 10 (Il10)	M.tuberculosis H37Rv	Aerosol (100)	Normal defense against Mtb	N/A	207
Interleukin 12 subunit a (Il12-p35)	Virulent M. tuberculosis Erdman Strain	Aerosol (50)	Uncontrolled Mtb growth Dissemination	Normal production of IL-23p19	209
Interleukin 12 subunit b (Il12-p40)	Virulent M. tuberculosis Erdman Strain	I.v (10^5)	Decreased survival Uncontrolled Mtb growth	Lower IFN-γ, TNFα production	34
Interleukin 17 A (Il17a)	M.tuberculosis H37Rv	I.t (10^3)	High Mtb load Altered granuloma formation	N/A	210
Interleukin 18 (Il18)	Virulent M. tuberculosis Kurono Strain	Aerosol (10^5,10^6)	Normal survival Larger granulomas	Lower IFN-γ production	211
	M.tuberculosis H37Rv	Aerosol (100)	Decreased survival High Mtb load	Lower IFN-γ production	212
Interleukin 18 Receptor (Il18R)	M.tuberculosis H37Rv	I.n (2×10^2)	Normal defense against Mtb	Normal production	206
Interleukin 23 subunit p19 (Il23-p19)	M.tuberculosis H37Rv	Aerosol (100)	Normal Mtb load	Normal IFN-γ, NOS2 production Lower IL-17 production	213
Interleukin 27 Receptor (Il27R)	M.tuberculosis H37Rv	Aerosol (100)	Reduced Mtb load in lung	Higher TNFα, IL-12p40, IFN-γ production	214
Intracellular Adhesion Molecule 1 (Icam1)	Virulent M.tuberculosis Erdman Strain	Aerosol (100)	Decreased survival Defective granuloma formation	N/A	215
Intracellular Adhesion Molcule 1 [del Exon 5] (Icam1)	Virulent M.tuberculosis Erdman Strain	Aerosol (100)	Normal survival Normal Mtb. load	Normal production of IFN-γ, IL-12p40	216

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Table 1 (continued)

Mouse Strain	Mycobacterial Strain	Infection	Phenotype	Anti/Pro-inflammatory Mediators Production in response to M.tb infection	Ref
Monocyte Chemoattractant Protein 1 (Mcp1)	M.tuberculosis H37Rv	I.v (10^5)	Control of Mtb growth	N/A	217
Multidrug Resistance Protein 1 (Mrp1)	M.tuberculosis	I.n (10^5)	Defective early granuloma formation		
	H37Rv		Normal survival at late stage	Lower early IFN-γ production	218
Myeloid Differentiation primary response 88 (MyD88)	Virulent M. tuberculosis Kurono Strain	Aerosol (10^6)	Normal defense against Mtb	Higher IFN-γ, TNFα, IL-12 production Slightly decreased production of NOS2, IL-18	219
	M.tuberculosis H37Rv	I.n (20–50)	Decreased survival High Mtb load in liver	Lower IL-12p40, IFN-γ, TNFα, NOS2 production	220
NACHT, LRR and PYD containing Protein 3 (Nalp3)	M.tuberculosis H37Rv	Aerosol (10^2, 10^3)	Normal Mtb load in lungs	Normal production of IL-12p40, IL-1α	221
Natural Resistance-Asociated Macrophage Protein 1 (Nramp1)	M.tuberculosis	I.v (10^4)	Normal control of Mtb growth Normal survival	Normal production	222
	H37Rv	Aerosol (2×10^2)	Normal survival	Normal production	
	M.bovis BCG	I.v (2×10^4)	Uncontrolled BCG growth	N/A	62
Nuclear Factor kB 1 (Nfκb1p50)	Virulent M. tuberculosis	Aerosol (10^6)	Decreased survival	Lower IFN-γ, TNFα, IL-12, NOS2 production	223
	Kurono Strain		High Mtb load	Higher IL-6 production	

(continued)

Table 1 (continued)

Mouse Strain	Mycobacterial Strain	Infection	Phenotype	Anti/Pro-inflammatory Mediators Production in response to M.tb infection	Ref
Nucleotide-binding Oligomerization Domain containing 2 / Caspase Recruitment Domain 15 (Nod2/ Card15)	M.tuberculosis H37Rv	Aerosol ($35\text{-}1.5\times10^3$)	Normal Mtb load in lungs	Normal production	184
	M.tuberculosis	Aerosol (100)	Normal Mtb load in lungs	Lower IFN-γ, TNFα, NO production	224
	H37Rv		Defective Adaptive Immunity	Normal IL-12p40, IL-10 production	
Perforin (Po)	Virulent M.tuberculosis Erdman Strain	Aerosol (100)	Control of Mtb growth	Normal IFN-γ production	195
Phagocyte Oxidase p47 subunit (p47phox)	Virulent M.tuberculosis	Aerosol (100)	Normal survival High Mtb load in early response	Increased IFN-γ response	225
	Erdman Strain		Controlled Mtb load		
Programmed Death 1 (Pd1)	M.tuberculosis H37Rv	Aerosol (50–320)	Rapid death High Mtb load in lungs	Higher TNFα, IL-6, IL-17, IFN-γ, IL-12 production	226
Prostaglandin E Synthase (Pges)	M.tuberculosis H37Rv	Aerosol (100)	High Mtb load in lungs	N/A	15
Signal Transducer and Activator of Transcription 1 (Stat1)	Virulent M. tuberculosis Kurono Strain	Aerosol (10^6)	Decreased survival High Mtb load	Lower IL-12p40, IFN-γ, TNFα, IL-18, NOS2 production	227
Surfactant Associated Protein A (SpA)	Virulent M.tuberculosis	Aerosol (100)	Normal survival Normal Mtb load in lungs	Normal IFN-γ production	228
	Erdman Strain	Aerosol (10^3)	High Mtb load in lungs		
Surfactant Associated Protein D (SpD)	Virulent M.tuberculosis	Aerosol (100)	Normal survival Normal Mtb load in lungs	Normal IFN-γ production	
	Erdman Strain	Aerosol (10^3)	High Mtb load in lungs		

(continued)

Table 1 (continued)

Mouse Strain	Mycobacterial Strain	Infection	Phenotype	Anti/Pro-inflammatory Mediators Production in response to M.tb infection	Ref
T cell Receptor alpha/beta (TcRα/β)	M.tuberculosis H37Rv	Aerosol (100)	Decreased survival Uncontrolled Mtb growth	Lower IFN-γ, NOS2 production	229
T cell Receptor beta (TcRβ)	M.tuberculosis H37Rv	I.v (1×10^5, 5×10^6)	Decreased survival Uncontrolled Mtb growth	Lower IFN-γ production	230
T cell Receptor gamma/delta (TcRγ/δ)	M.tuberculosis H37Rv	Aerosol (100)	Control of Mtb growth	Normal production	229
Toll-Like Receptor 2 (Tlr2)	Virulent M. tuberculosis Kurono Strain	Aerosol (10^6)	Normal defense against Mtb	Lower NOS2, TNFα, TGFβ, IL-1β, IL-2 production Higher IL-4, IL-6 production	231
Toll-like Receptor 2/9 (Tlr2/9)	M.tuberculosis H37Rv	Aerosol (50–100)	Decreased survival High Mtb load in lungs	Lower IFN-γ, IL-13 production	232
Toll-like Receptor 2/4/9 (Tlr2/4/9)	M.tuberculosis H37Rv	Aerosol (100)	Control of Mtb growth	Normal TNFα, IL12/23p40, IFN-γ, NOS2 production	233
Toll-Like Receptor 4 [P712H] (Tlr4)	M.tuberculosis H37Rv	I.n (10^5)	Decreased survival High Mtb load	Higher IFN-γ production Lower IL-4 production	234
Toll-Like Receptor 6 (Tlr6)	Virulent M. tuberculosis Kurono Strain	Aerosol (10^6)	Normal defense against Mtb	Lower NOS2, TNFα, TGFβ, IL-1β, IL-2 production Higher IL-4, IL-6 production	231

(continued)

Table 1 (continued)

Mouse Strain	Mycobacterial Strain	Infection	Phenotype	Anti/Pro-inflammatory Mediators Production in response to M.tb infection	Ref
Toll-like Receptor 9 (Tlr9)	M.tuberculosis	Aerosol (50–100)	Normal survival	Lower IFN-γ, IL-12 production	232
			Normal Mtb load in lungs		
		Aerosol (5×10^2)	Decreased survival		
	H37Rv		High Mtb load in lungs		
Transporter Associated With Antigen rocessing 1 (Tap1)	Virulent M. tuberculosis	I.v (10^6)	Decreased survival	N/A	235
			Uncontrolled Mtb growth		
	Erdman Strain		Hepatosplenomegaly		
Tumor Necrosis Factor (Tnfα)	M.tuberculosis	Aerosol (100)	Decreased survival	Normal RNI production	236
			High Mtb load	Normal IFN-γ production	
	H37Rv		Dissemination		
			Altered granuloma formation		
Tumor Necrosis Factor Receptor 1 TnfαR1)	M.bovis	I.v (10^7)	Decreased Survival	N/A	237
			High BCG load		
			Altered granuloma formation		
Tumor Necrosis Factor Receptor 2 (TnfαR2)	BCG		Normal defense	N/A	
5-lipoxygenase (5-Lo)	M.tuberculosis	I.n (50–300)	Enhanced survival	Higher IL-12p40, IFN-γ production	238
			Reduction in Mtb load	Enhanced NOS2 production	
	H37Rv		Decreased lung inflammation/ necrosis		

germline mutations in the *CYBB* gene encoding the gp91phox subunit of NADPH oxidase, which selectively impair the activity of this enzyme and associated respiratory burst in monocyte-derived macrophages, were identified in MSMD patient, thereby highlighting the role of this pathway in defenses against mycobacteria [59]. Finally, the genetic etiology of MSMD in about half of these patients remain unknown.

In mice, susceptibility to infection with several *Mycobacterium* species, *Salmonella* (*S. typhimurium*) and *Leishmania donovani*, as measured by microbial replication in spleen and liver in vivo and in explanted macrophages in vitro, is controlled by the *Bcg/Ity/Lsh* locus [60]. The *Nramp1* gene (*N*atural *R*esistance *A*ssociated *M*acrophage *P*rotein; *Slc11a1*) [61, 62] was identified as the gene responsible for the *Bcg/Ity/Lsh* effect. *Nramp1/Slc11a1* codes for a membrane protein present in lysosomes of macrophages, and in gelatinous granules of neutrophils which are rapidly recruited to the membrane of microbe-containing phagosomes [63]. *Nramp1/Slc11a1* acts as a pH-dependent efflux pump for Fe^{2+} and Mn^{2+} ions at the phagosomal membrane to inhibit intraphagosomal bacterial replication [64]. This results in increased acidification and enhanced recruitment of lysosomal enzymes to *Nramp1*-positive mycobacterial phagosomes, and increased recruitment of newly formed endosomes and late endosomal markers (manose-6-phosphate receptor) to *Salmonella*-containing vacuoles [65, 66]. Susceptibility to infections in Bcg^{s} mouse strains is caused by a G169D mutation in predicted TM4 of *Nramp1*/*Slc11a1* that interferes with protein folding and targeting, resulting in absence of mature functional protein in macrophages from these strains [60].

Additional genetic studies in mice have suggested the presence of a number of additional genetic effects that can modulate *Nramp1/Slc11a1*-determined susceptibility to infection with mycobacteria and other intracellular pathogens. For example, a locus on chromosome 17 (near the MHC) has been shown to modify the *Nramp1* effect in mouse crosses where one of the strains used is wild-derived *Mus spretus* [67]. Likewise, two loci on chromosomes 1 and 11, and designated *Ity2* and *Ity3*, were found to control survival to *Salmonella* infection in an *Nramp1* independent fashion in F2 mice derived between C57BL/6J and the genetically resistant ($Nramp1^{G169}$), but phenotypically highly susceptible wild-derived strain *Mus molossinus* (MOLF/Ei) [68]. Loss-of-function mutations at other loci, such as the erythrocyte pyruvate kinase (pklr) also cause susceptibility to *Salmonella*, despite presence of resistance allele at *Nramp1* [69]. A similar situation is observed in the mouse strain BXH2 which is highly susceptible to mycobacterial infections as result of a mutation in IRF8, a transcription factor which is essential for many aspects of host defenses against infections including transcriptional activation of *Nramp1* [70, 71].

3 Genetic Factors and Susceptibility to Virulent *Mycobacterium tuberculosis*

Genetic factors have long been thought to play a role in onset, progression, and ultimate outcome of infection with *M. tuberculosis* [22], and in fact reported familial clustering of TB cases led to the belief in the eighteenth and the nineteenth century that TB was a hereditary disease [72]. Epidemiological data pointing to sex, and racial differences in susceptibility, as well as geographical distribution and familial aggregation of disease suggested a genetic component to TB susceptibility [73–75]. In addition, population studies in endemic areas of disease and during first contact epidemics [76, 77], together with studies in twins [78], have established a clear but complex genetic component of susceptibility to TB in humans. This genetic component has been studied for TB by association studies with candidate genes, and by genome-wide linkage and association studies. While we will only review literature addressing the role of genetic factors in susceptibility to TB, susceptibility to leprosy has also been shown to be under genetic control and the reader is referred to recent reviews on this topic [79, 80], which will not be systematically covered herein.

Considering the key role of the *Nramp1/Slc11a1* gene in susceptibility of mice to mycobacterial infections (see above), polymorphic variants at or near *Nramp1* were identified and were found to show a strong association with onset of TB in endemic areas of the Gambia [81] and South-East Asia [82], and in linkage studies of a large aboriginal population of Northern Canada [83]. *Nramp1* alleles have been shown to represent a major risk factor for pediatric TB in 184 ethnically diverse families from the greater Houston area [84]. Finally, *Nramp1* alleles have also been linked to susceptibility per se [85] and response to [86] leprosy (*M. leprae*) in South-East Asia, and Buruli ulcer [87]. Importantly, it has been shown that monocytes derived from individuals bearing *Nramp1* alleles associated with TB susceptibility in field studies, display reduced functional activity of the *Nramp1* protein as measured by extent of maturation of *Salmonella*-containing vacuoles (recruitment of M6PR) formed in these cells [88]. Together, these studies have demonstrated the critical role of the *Nramp1/Slc11a1* protein in macrophage function and in defenses against mycobacterial infections in mouse and man.

In addition to the validated *Nramp1* effect, a large number of common polymorphisms providing moderate individual risks for complex genetic susceptibility to TB have been detected in humans through association studies with other candidate genes. These include the *SP110* gene (homolog of mouse *Ipr1* which alterations cause susceptibility to pulmonary TB in mice) [32, 89], and the human IRGM1 gene [90] that codes for an interferon-inducible and phagosomal membrane associated GTPase which deletion causes susceptibility to TB in mice [91, 92]. Other TB susceptibility associated polymorphic variants reported include the HLA class II DR2 and DQB1 [93, 94], Vitamin D receptor, mannose-binding protein [95, 96], CD20 (DC-SIGN), CD209 [97, 98], the chemokine MCP-1 [99], the toll-like receptor Tlr1 [100], the lysosomal cysteine proteinase cathepsin Z

[101–103], and the melanocortin receptor *MC3R* gene promotor [101], and the ATP receptor *P2X7* [104]. Polymorphic variants in prostaglandin-associated genes such as leukotriene A4 hydrolase [17] and 5-lipoxygenase [105] have also been associated with susceptibility to TB. A convincing effect of a functional polymorphism in the NFkB binding site in the *IFNγ* gene (+874 A/T, with the A allele predisposing to TB) on TB susceptibility has been validated in several populations [106, 107].

In addition, a whole genome scan in affected sib-pairs from The Gambia and South Africa has identified suggestive linkages (LOD $\sim$ 2) on Chr. 15q and Xq [108]. A major locus on chromosome 8q12-q13 and that predisposes to adult pulmonary TB with a dominant mode of inheritance (LOD 3.6) was identified by a genome-wide screen in a Moroccan population [109]. Additional genome-wide linkage analyses [110], followed by association studies with candidate gene subsets [111] identified chromosome 5q31 as a suggestive linkage (LOD 2.29) contributing to TB susceptibility in that population. The genes responsible for the effects detected in these genome-wide surveys remain unidentified. Finally, a genome-wide association studies conducted in over 11,000 individuals (discovery and replication cohorts) from Ghana and the Gambia identified a region on chromosome 18q11.2 (rs4331426, located between the *CTAGE1* and the *RBBP8* genes) that shows association with TB in these African populations (combined $P = 6.8 \times 10^{-9}$, odds ratio = 1.19, 95 % CI = 1.13–1.27) [112]. Together, these genetic studies in humans point to a complex genetic determinant of TB susceptibility in human sporadic cases, with independent and population-specific modest genetic effects.

Parallel studies have also pointed to a complex genetic determinant of susceptibility to pulmonary TB in mice [22]. Inbred strains have been classified as highly susceptible (I/St, CBA, C3H, DBA/2, 129SvJ, A/Sn) or highly resistant (C57BL/6J, BALB/c) to intravenous or aerosol challenge with virulent human *M. tuberculosis*[113, 114]. As with humans, differential susceptibility to*M. tuberculosis* in mice is genetically complex, and phenotypically expressed as different rates of pulmonary microbial replication, distinct histopathology, robustness of inflammatory response in situ, and survival time. The high susceptibility of C3HeB/FeJ mice to pulmonary TB was shown to be controlled by a complex locus on Chr. 1 designated super-susceptibility TB (*sst1*) [115], and has been shown to be caused by rearrangement in the Intracellular pathogen resistance 1 (*Ipr1*) gene [32, 116] that codes for a protein (Ifi75; interferon-induced protein 75) regulating apoptotic response of macrophage following phagocytosis of *M. tuberculosis* [117]. The *sst1* locus also affects resistance of macrophages to infection with *Listeria monocytogenes* [32]. Studies in *sst1* congenic and subcongenic lines have indicated that the determining effect of the *sst1* gene is further modulated by four additional loci mapping to chromosomes 7 (central; LOD 4.8), 12 (distal; LOD 6.6), 15 (distal; LOD 4.6), and 17 (proximal; LOD 5.5) that influence survival to infection [118]. The genes responsible for these effects remain to be identified. In addition, the study of differential susceptibility of strain pairs I/St (susceptible):A/Sn (resistant) to pulmonary TB [119] has led to the mapping of the *Tbs1* and *Tbs2*

loci (TB Severity) that affect, in a gender-specific fashion, body weight loss following infection with high doses of *M. tuberculosis* [120, 121]. In females, significant linkages were identified on proximal Chr. 9 (*Tbs2*; LOD 6.7), and distal Chr. 3 (*Tbs1*; LOD 3.9) with additional suggestive linkages detected on proximal Chr. 8 (LOD 3.0), and Chr. 17 (LOD 2.9); In males, only suggestive linkages were detected on proximal Chr. 5 (LOD 3), and distal Chr. 10 (LOD 2.3). A new coding mutation in the TNFα leader sequence in TB-sensitive I/St mice that causes higher secretion levels of soluble TNFα may represent the Chr. 17 contribution to pathophysiology in I/St mice [122].

DBA/2 (D2) mice are susceptible to pulmonary TB, while C57BL/6 are resistant, as measured by extent of pulmonary bacterial replication, lung pathology, and survival time [123]. Enhanced bacillary replication in D2 is lung specific as D2 mice can, alike B6, stabilize infection in liver and spleen [124]. Transcript profiling studies suggest that early death in D2 mice is caused by an altered tissue remodeling response, leading to lung fibrosis (elevated expression of Fn1, Sparc, Col1a1, Col1a2, Col2a1, Col3a1, Col4a1, Col4a2, Mmp-2, Timp1, and Arg1) [125]. Genome scans in informative [B6 X D2]F2 mice using extent of pulmonary bacterial replication and overall survival time detected four loci contributing to phenotypic variance in this cross: on distal Chr.1 [*Trl-1;* LOD = 4.80], on proximal Chr.3 [*Trl-2*; LOD = 3.93], on proximal Chr.7 [*Trl-3*; LOD = 4.66] and on central Chr. 19 [*Trl-4;* LOD = 5.6]. For all loci, B6-derived resistance alleles are inherited in a co-dominant fashion, as expected [123, 124]. Studies in congenic mice showed that the protective effect of *Trl3* does not involve modulation of timing or magnitude of Th1 response in the lung, as investigated by measuring the number of antigen-specific, IFN-γ producing $CD4^+$ and $CD8^+$ T cells. Rather, *Trl3* appears to affect the intrinsic ability of activated macrophages to restrict intracellular mycobacterial replication. Microarray experiments identified a number of positional candidate genes in the *Trl3* interval on Chr.7, which level of expression either prior to infection or in response to *M. tuberculosis* infection is differentially regulated in a parental haplotype dependent fashion [71].

4 IRF8 and Susceptibility to Mycobacterial Infections in Mouse

While searching for genetic modifiers of resistance to intracellular infection afforded by *Nramp1*, we observed that the BXH2 mouse strain is susceptible to *M. bovis* (BCG) infection despite presence of resistance alleles at *Nramp1* (*$Nramp1^{G169}$*) [126]. Susceptibility in BXH2 is associated with uncontrolled replication of BCG early and late during infection, with absence of granulomas formation in spleen and liver [70]. In subsequent studies, we found that BXH2 mice are also susceptible to infection with other microbial pathogens including *S. typhimurium* [70], *Legionella pneumophila* [140], as well as with the malarial

parasite *Plasmodium chabaudi* AS [70]. BXH2 mice suffer from a preneoplastic syndrome with important infiltration of neutrophil precursors in spleen, liver, and bone marrow; BXH2 succumb a <1 year of age of monoclonal leukemias produced by insertional mutagenesis with a replication-competent B-ecotropic retrovirus, which is produced by recombination between two replication-incompetent integrated viral copies (*Emv1*, *Emv2*) [127]. BXH strains are recombinant inbred strains derived from C57BL/6J and C3H/HeJ, and although other BXH strains harbor both *Emv1* and *Emv2*, BXH2 is the only that produces high titer of recombination competent virus. Susceptibility to BCG, granulocytic hyperplasia, and hepatosplenomegaly in BXH2 map to the same locus on chromosome 8 (originally mapped as the *Myls* locus), that we identified by positional cloning as a partial loss-of-function in the IRF family member, IRF8 (IRF8^{R294C}) [127, 128]. BXH2 splenocytes do not produce IL-12p40 in response to IFNγ/CpG stimulation in vitro. Independently, it was observed that the proximal promoter of *Nramp1* contains an ISRE that binds IRF8; IRF8 and Miz1 were found to be required for IFNγ/LPS activation of *Nramp1* transcription in macrophages, and ablation of IRF8 in these cells reduces *Nramp1* expression and causes susceptibility to *M. bovis* (BCG) in vitro [129–131]. As expected, BXH2 mice are supersusceptible to infection with virulent MTB and develop acute and rapidly lethal pulmonary TB [132]. BXH2 mice develop a fulminant infection following either intravenous or aerosol infection, and characterized by (a) uncontrolled microbial replication (detected within 10 days postinfection), (b) impaired granuloma formation and rapid dissemination from the lung to spleen and liver, (c) extensive tissue necrosis in infected organs, and (d) early death (20 days, compared to 160 days for C3H and >250 days for B6 controls). As opposed to C3H and B6 controls, BXH2 mice show a selective and almost total depletion of CD8α^+ dendritic cells (DCs) whereas other DC subsets such as plamcytoid DCs (pDCs) are normal [133]. CD8α^+ DCs are the main producers of IL-12 and direct differentiation of T helper cells into the Th1 lineage [134]. BXH2 mice show severely depressed numbers and impaired priming of CD4$^+$ and CD8$^+$ IFNγ producing T cells in the lungs, with no detectable serum IFNγ and IL12p70 [132]. These results highlight the critical role of IRF-8 in defenses against mycobacterial infections.

IRF8 is one of the nine members of family of *I*nterferon *R*egulatory *F*actor. IRF8 has a DNA-binding domain of the helix-turn-helix type that binds to ISRE sites (*I*nterferon *S*timulated *R*esponse *E*lements; core sequence 5′AANNGAAA3′) present in proximal promoters of type II IFN-regulated genes. IRF8 is expressed in myeloid cells (macrophages, DCs), and upon stimulation with IFNγ, LPS, and PAMPs (pathogen-associated molecular patterns), it binds to ISREs in association with other members of the IRF (e.g. IRF1), or ETS (e.g. PU.1) families, to activate or repress gene expression [135]. IRF8 plays an important role in several aspects of myeloid cells. First, IRF8 is required for expression of intrinsic macrophage antimicrobial defenses, through activation (in response to IFNγ/PAMP) of effector targets essential for destruction of intracellular pathogens including *Nramp1* [129–131], iNOS [141], gp91^{phox} [136], p67^{phox} [135], and key surface receptors such as FcgR1 [137], *TLR4* [138] and *TLR9* [139]. Thus, IRF8-deficient macrophages have

weakened anti-microbial defenses and are susceptible to ex vivo infection with *M. bovis*, *S. typhimurium*, and *L. pneumophila* [131, 140]. IRF8 is also required for Th1 polarization of early immune response [IL12p40/IFNγ loop] [138, 141]. IRF8 binds to the promoter regions and activates expression of IL12p40 [142–145], IL12p35 [146], and IL-18 [147] genes in response to IFNγ; IRF8$^{-/-}$ mice do not produce IL12p40, lack Th1 polarization (absence of antigen-specific CD4^{+}, IFNγ producing T cells), and are susceptible to in vivo infection with intracellular pathogens including *Leishmania major* [142], *L. monocytogenes* [148], *Yersinia enterocolitica* [149], *Toxoplasma gondii* [150], and *Brucella abortus* [151]. In addition, IRF8 physically interacts with RORγt and was recently shown to act as a transcriptional inhibitor to negatively regulate TH17 cell differentiation and associated immune response [152]. IRF8 drives differentiation of myeloid progenitors toward macrophages, while positively regulating apoptosis of the granulocytic lineage [153–156]. IRF8-deficient mice show a profound defect in dendritic cells, as they lack both CD11c^{+}CD8α^{+} DCs and pDCs [133, 157, 158]. In addition, the small number of CD11c^{+}CD8α^{+} and CD8α^{-} DCs present in these mice fail to up-regulate co-stimulatory molecules and to produce IL12p40 in response to microbial products [159, 160].

IRF1 is a key heterodimerization partner of IRF8 [135]. IRF8 and IRF1 synergize in the IFNγ-dependent activation of intrinsic macrophage anti-microbial defenses (iNOS, gp91^{phox}, p67^{phox}, caspase-1, Cox2), of inflammatory cytokines activating early immune response (IL12-p40, IL18, RANTES, TNFα, IL27-p28) [161–164], and of genes affecting maturation of myeloid (DCs) and lymphoid (NK, CD8^{+} T cells) cells [135, 165]. IRF1$^{-/-}$ mice are also susceptible to certain infections, including *M. tuberculosis* [166]. IRF8 physically interacts with PU.1 to activate transcription of the MHC II transactivator, CIITA, thereby up-regulating expression of MHC class II genes and enhancing antigen presentation [167]. IRF8 is also involved in bone metabolism and prevents pathological bone resorption or erosion, which can occur during microbial infections, through direct transcriptional inhibition of NFATc1 [168]. IRF8 is therefore a negative regulator of osteoclastogenesis and a direct target for bone homeostasis genes (BLIMP1) [169]. Finally, IRF8 is involved in acquired immunity by regulating differentiation, commitment, and development of B cells [170], including restricting the size of marginal zone and follicular B cells [171].

5 IRF8 Deficiency in Humans

Recently, mutations in IRF8 have been identified in humans and have been shown to cause mild or severe forms of a novel type of immunodeficiency of the myeloid compartment and that particularly target dendritic cells [172]. Autosomal recessive IRF8 deficiency was identified in a 10-week-old infant presenting with disseminated BCG disease (following vaccination), cachexia, and oral candidiasis.

Hematological analysis showed severe depletion of the CD3/19/56^{-} (lin^{-}) HLA-DR^{+} cellular compartment, with total absence of both CD14^{+} and CD16^{+} monocytes, and with very high CD34^{+} neutrophil count. The patient's blood was also devoid of dendritic cells (DCs), whether CD11c^{+} myeloid (CD1c^{+} or CD141^{+}) or CD123^{+} plasmacytoid; CD1α^{+} and CD14^{+} dermal DCs were also very low, while epidermal Langerhans cells were present in normal numbers as were circulating B and NK cells. The patient's bone marrow and lymph nodes also showed granulocytic hyperplasia. Studies with peripheral blood mononuclear cells showed a severe defect in production of IL12 and IFN-γ in response to BCG, phytohaemagglutinin or LPS. The patient was found to be homozygote for a lysine-to-glutamic acid substitution at position 108 (K108E) in the DNA-binding domain of IRF8, with both parents being heterozygotes. K108R was patient-specific and affected a residue that is invariant in IRF8 orthologs and highly conserved in the IRF family. K108 makes a critical hydrogen bond with the sugar backbone of DNA, and its replacement by a negatively charged glutaminic acid abrogates this interaction. Functional studies showed that the K108E variant has severely reduced DNA-binding characteristics, and is transcriptionally inactive for known IRF8 targets such as the *IL-12p40* and the *NOS2* gene promoters. This patient was, therefore, found to be homozygote for a loss-of-function mutation in *IRF8*.

IRF8 was also sequenced in a group of 454 patients suffering from MSMD, and of which the genetic etiology was unknown [171]. Two unrelated individuals from Chile and Brazil, and suffering from recurrent episodes of disseminated BCG disease, were each found to carry the same de novo heterozygote mutation, causing a threonine-to-alanine substitution at position 80 (T80A) of IRF8. T80 is a strictly conserved residue across IRF8 orthologs and paralogs genes and maps to the major DNA binding helix of IRF8 that inserts into the major groove of double-stranded DNA. T80A showed a strong reduction in both DNA binding activity and in the transactivation of *IL12B* and *NOS2* promotors. In addition, the T80A variant was shown to behave as a dominant negative isoform, and was able to suppress the transactivation potential of wild type IRF8, when tested together in the same assay. It was concluded that the T80A mutation causes autosomal-dominant MSMD. Phenotypically, the two T80A patients showed the same subtle defect in DC populations: within the CD11c^{+} myeloid DC subset, normally divided into minor CD141^{+} and major CD1c^{+} subsets, there was marked loss of CD1c^{+}DCs, while the CD141^{+} subset and total number of CD11c^{+}DCs remained intact. Peripheral blood mononuclear cells harboring IRF8^{T80A} produced one-third of the amount of IL-12 produced by control cells suggesting that depletion of IL-12-producing CD1c^{+} DCs contributes to susceptibility to mycobacterial disease in these individuals.

Together, these findings established that in humans, IRF8 plays a critical role in the ontogeny of the myeloid lineage, in general, and of dendritic cells in particular. They also demonstrate that IRF8 is required for defenses against mycobacterial infections.

6 Transcriptional Targets of IRF8 in Myeloid Cells

What are the transcriptional targets of IRF8 in myeloid cells that are important for intrinsic defenses of macrophages against intracellular pathogens such as mycobacteria? Several published reports have used transcriptional profiling with microarrays to get insight into the IRF8 transcriptome in a number of different cell lines [157, 173, 174]. These studies compared RNA preparations from cell types obtained from either wild type or IRF8 mutant mice, or from cell types in which IRF8 had been silenced using inhibitory RNA (RNAi) strategies, and obtained either with or without prior treatment with IFNγ or other known inducers of IRF8. More recently, two reports have used a combination of expression profiling and chromatin immunoprecipitation on promotor arrays (ChIP-chip) to identify direct transcriptional targets of IRF8 in myeloid cells.

In a first report [175], gene expression profiles of siRNA knock-down samples and genome-wide binding locations by ChIP-chip were studied in the THP-1 myelomonocytic human cell line. This analysis (overlap between the two datasets) identified 84 genes as direct transcriptional targets of IRF8 in these cells. The authors also used the same approach to identify 53 direct targets of the IRF8 transactivation partner PU.1 (also known as SPI1) in these cells. The IRF8 and PU.1 overlap was found to consist of 19 genes, which included key molecules of IFN signaling such as OAS1 and IRF9 [175].

In a second study, Marquis [176] used bone marrow macrophages from [BXH2 and BALB/c] F2 mice to identify IRF8 targets that would segregate as stable expression quantitative trait loci (eQTLs) in groups of F2 mice of such mixed genetic background but fixed for homozygosity for wild type (BALB/c) or mutant IRF8 variants (BXH2). This analysis led to the identification of IRF8-dependent eQTLs (basal, and inducible), including a set of 368 differentially expressed genes in response to treatment of macrophages with IFNγ and CpG. ChIP-chip experiments in J774 macrophages stimulated with IFNγ and CpG identified 319 IRF8 binding sites, corresponding to 333 different genes. Examination of the binding site sequences produced the consensus IRF8 DNA binding motif G/AAGAAnTGAAA, and ~50 % of these sites were found to contain a PU.1 binding motif (GAGGAA) with co-localization of the two sites at the binding peak. An overlap of 29 genes was detected between IRF8 binding sites (ChIP/chip) and IRF8 regulated transcripts (detected as eQTLs in response to IFNγ/CpG) and ChIP/chip, while nearly 2/3 of the IRF8 binding sites ($n = 213$) were associated with genes that show regulated expression in the lungs of *M. tuberculosis*-infected mice 30 days following aerosol infection. Gene Ontology analysis for genes exhibiting an IRF8 binding peak detected by ChIP-chip revealed a strong enrichment for the "immune response" category (29 genes) [177]. This list includes several genes encoding proteins involved in recognition, processing, and presentation of antigens by antigen-presenting cells (dendritic cells, macrophages), including members of the Toll-like receptors family that play a crucial role in recognition of pathogen-associated molecular signatures, including *Tlr4* (interaction with LPS from

Gram-negative bacteria), *Tlr9* (unmethylated CpG containing DNA), and *Tlr13* (vesicular stomatitis virus). This list also comprised an additional subgroup of several genes that play a key role in antigen processing and presentation in dendritic cells and macrophages, including Class I and Class II MHC (major histocompatibility complex) molecules, as well as proteases, membrane transporters, and structural proteins involved in generation, transport, and loading of antigenic peptides onto Class I or Class II molecules. Indeed, a strong enrichment of IRF8 binding sites was noted in the MHC on chromosome 17. A third subgroup contained nucleotide binding proteins, several of which corresponding to members of the IFN-inducible GTPase superfamily, including the Gbp (guanylate binding proteins; Gbp2, Gbp3, Gbp6), Mx (Mx2), and p47 (immunity-related GTPases; IRGM1/Lrg-47, Ifi47/Irg-47, lgtp/Irgm3) families which are involved in early innate immune response to intracellular infection in many cell types [178–180]. Some of these proteins have been found associated with the membrane of microbe-containing phagosomes formed in macrophages, and shown to play an important role in maturation to bactericidal phagolysosomes. Mutations in one of them, Lrg47/IRGM1, cause susceptibility to mycobacterial infections in mice [91, 92], and polymorphic variants in their human counterpart have been found associated with susceptibility to TB [90].

Finally, a comparison of direct IRF8 targets identified in THP-1 cells [175, 176] identified a 43 % overlap in the list of IRF8 binding peaks (ChIP-chip), and a 21 % overlap with the list of genes regulated by IFNγ/CpG in an IRF8-dependent fashion, and 70 % overlap with the list of genes differentially regulated during pulmonary TB in vivo [176]. Together, these studies emphasize the predominant role of IRF8 in myeloid cell functions and in host defenses against infection with intracellular pathogens.

7 Conclusion

The parallel genetic analysis of susceptibility to infection with avirulent (BCG and environmental mycobacteria) and virulent (*M. tuberculosis*) strains of mycobacteria in mice and humans has been remarkably successful in identifying key genes, proteins, and biochemical pathways that play critical roles in innate and adaptive immune responses to this group of intracellular pathogens. In particular, they have shed considerable light on the intrinsic mycobactericidal defenses of phagocytes, the first line of defenses against these microbes, as well as the all important IL12-IFNγ amplification loop that activates protective T-cell-mediated immune responses that ultimately contain the infection and prevents clinical disease. More recent studies have discovered a major role for IRF8 in ontogeny of myeloid cells, in particular the function of dendritic cells and macrophages. This has been demonstrated both in mouse mutant lines, as well as in humans suffering from autosomal recessive, and autosomal dominant forms IRF8 deficiency.

The characterization of transcriptional targets of IRF8 has provided an additional rich source of candidate genes at the center of macrophage defenses against infection with intracellular pathogens.

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Evolution of *Mycobacterium tuberculosis*

Marcel A. Behr

Abstract Genomic studies have provided a refined understanding of the genetic diversity within the Mycobacterium genus, and more specifically within *Mycobacterium tuberculosis*. These results have informed a new perspective on the macro- and micro-evolution of the tubercle bacillus. In the first step, a *M. kansasii*-like opportunistic pathogen acquired new genes, through horizontal gene transfer, that enabled it to better exploit an intracellular niche and ultimately evolve into a professional pathogen. In the second step, different subspecies and strains of the *M. tuberculosis* complex emerged through mutation and deletion of unnecessary DNA. Understanding the differences between *M. tuberculosis* and related less pathogenic mycobacteria is expected to reveal key bacterial virulence mechanisms and provide opportunities to understand host resistance to mycobacterial infection. Understanding differences within the *M. tuberculosis* complex and the evolutionary forces shaping these differences is important for investigating the basis of its success as both a symbiont and a pathogen.

Keywords Tuberculosis · *Mycobacterium tuberculosis* · Genomic deletions · Single nucleotide polymorphisms (SNPs) · Horizontal gene transfer · Mycobacterium genus · Non-tuberculous mycobacteria (NTM) · Bacille de Calmette et Guérin (BCG) vaccine · Evolution · Single nucleotide polymorphism (SNP) · Immune response · *M. tuberculosis* complex

Abbreviations

TB	Tuberculosis
BCG	Bacille de Calmette et Guérin

M. A. Behr (✉)
McGill University Health Centre, Montreal General Hospital,
1650 Cedar Avenue, Room A5.156, Montreal H3G 1A4, Canada
e-mail: marcel.behr@mcgill.ca

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NTM Non-tuberculous mycobacteria
RD Region of difference, representing a locus of the bacterial genome that is absent from a closely related species, as in RD1, RD2, etc.
SNP Single nucleotide polymorphism

1 Overview and Definitions

A key challenge for the post-genomic era has been uncovering the genomic basis of bacterial pathogenicity, and in the specific case of tuberculosis (TB), the genomic basis for the capacity of this organism to continue to infect and cause disease despite the best efforts of host immunity. To this end, the introduction of a variety of genetic approaches, built conceptually upon complete genome sequence analysis [1], has enabled the TB research community to pose anew a set of questions that had been largely defined in the early twentieth century. Why is *M. tuberculosis* more virulent than the BCG vaccine? Why is *M. tuberculosis* more virulent than non-tuberculous mycobacteria? How closely related are *M. tuberculosis* strains and what is the nature of variability between such strains?

The goal of this chapter will be to present selected lessons regarding the evolution of *M. tuberculosis* that may be useful for contemplation in a book that largely deals with the immune response to this pathogen. Following a definition of the organisms that may cause TB or TB-like disease, I will discuss the current understanding of mycobacterial evolution, in order to situate *M. tuberculosis* within its genus. In the following section, I will discuss genetic studies looking within the *M. tuberculosis* complex, focussing on differences within *M. tuberculosis* sensu stricto. Finally, I will provide personal reflections on how these data might instruct our expectations and our investigations of the host immune response to *M. tuberculosis*.

Throughout the chapter, I will use the term *M. tuberculosis* complex to refer to related organisms that cause TB in their respective hosts (e.g. *M. bovis*, *M. caprae* and *M. pinnipedii*) reserving *M. tuberculosis* for the human pathogen. To distinguish the bovine pathogen (*M. bovis*) from the vaccine (*M. bovis* BCG), I will refer to virulent *M. bovis* and BCG vaccine. I will retain the classic name for human isolates from Western Africa, which is *M. africanum*, recognizing that certain groups consider these to comprise two lineages of human-adapted *M. tuberculosis* [2]. Regarding the terms pathogen and symbiont, I will use both of these terms to describe *M. tuberculosis*, as the former is a medical term (causes pathology) while the latter is a biological term (lives together with a divergent organism) [3]. Since *M. tuberculosis* causes disease in humans, it is clearly a pathogen; since *M. tuberculosis* spreads among people with no extra-human reservoir, it meets the definition of a human symbiont.

2 The Mycobacterium Genus

The availability of sequence-based modalities to define bacterial variants has led to an explosion in non-tuberculosis mycobacterial (NTM) species; at last count, there are ~150 species in this genus (http://www.bacterio.cict.fr/m/mycobacterium.html). This list is probably growing for two reasons: (1) the discovery of organisms in obscure habitats that had not previously been subject to mycobacteriologic study and (2) the redefinition of known organisms that were previously considered to be variants of other species. Classically, the genus has been divided into the rapid-growing organisms (colonies seen within a week) and the slow-growing organisms (see Fig. 1) for a phylogenetic representation of these species based on sequence analysis of organisms with complete genome data [4]. The group of rapid-growing organisms has only one important human pathogen (*M. abscessus*) whereas the slow-growing group of mycobacteria includes the *M. tuberculosis* complex and the following species associated with disease in humans: *M. avium*, *M. intracellulare*,

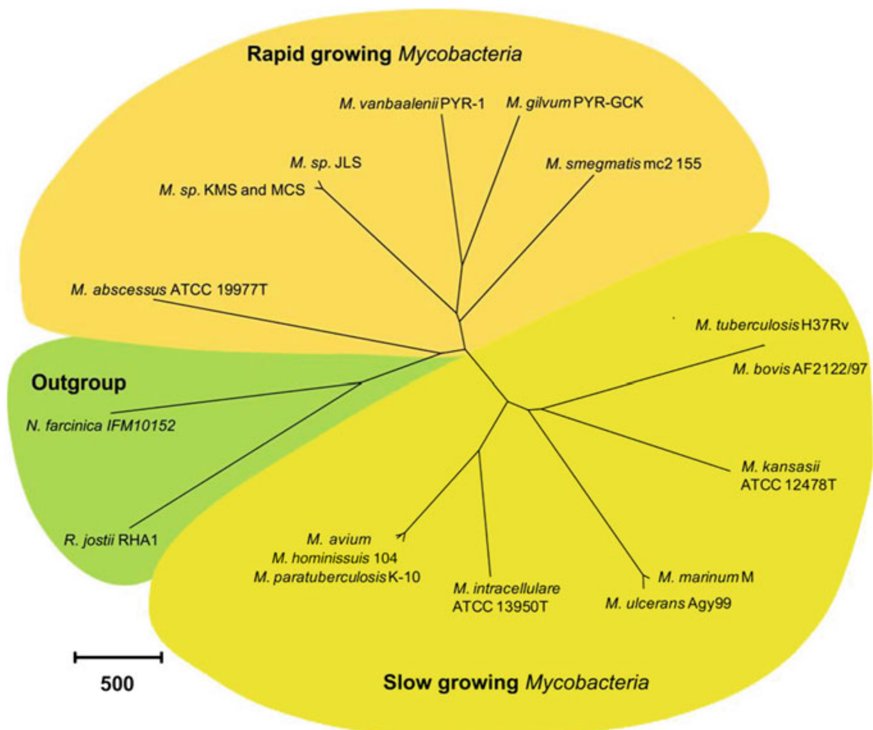

Fig. 1 A phylogenetic representation of the mycobacterium genus, based on concatenates from 20 compete genes in all mycobacterial genomes that have been sequenced to date. Nocardia and Rhodococcus are presented as out-groups. The distinction between rapid-growing mycocbacteria and slow-growing mycobacteria is highlighted through coloring [4]

M. leprae, *M. marinum*, *M. ulcerans* and *M. kansasii*. It remains to be determined whether there is increased pathogenicity associated with slow-growth, and if so, how a reduction in the replication rate would present an advantage to the bacterium in terms of either generating a productive infection and/or inducing clinical disease in eukaryotic hosts.

The slow-growing NTM associated with human disease do not transmit from person-to-person, and so they are effectively environmental organisms that are accidental pathogens if introduced into the appropriate host. In some cases, the host defect associated with disease is well documented (e.g. disseminated *M. avium* disease in a person with AIDS), whereas in other cases, the specific host defect is presently unknown (e.g. pulmonary *M. avium—intracellulare* infection in someone who is otherwise healthy). Of note for a book on immunity to TB, NTM potentially offer two perspectives: (1) outlier organisms that may teach us about the evolution of mycobacterial virulence, or (2) low-pathogenicity organisms that might be particularly instructive in understanding host susceptibility. The second perspective suggests that compared to patients with TB, individuals suffering from NTM disease must have a greater host defect, as their organism is not adapted to living in the face of the human immune system. To some extent this prediction is supported by the published literature, as a number of pathways critical for mycobacterial resistance have been mapped by the study of patients with disseminated disease due to environmental mycobacteria [5]. The search for human genetic determinants of *M. tuberculosis* susceptibility is ongoing and will be covered in Chap. 10.

Mycobacteria evolve by both vertical inheritance of core genes (shared across species) and also show evidence of horizontal gene transfer (HGT), in the form of plasmids or chromosomal DNA (that present as species–species or subspecies-specific elements). These two modalities of bacterial evolution have been documented in the case of the *M. marinum*/*M. ulcerans* complex and the *M. avium—intracellulare* complex, leading to a bi-phasic model of mycobacterial evolution, in which there is acquisition of novel genetic material by a particular organism is then followed by deletion of non-essential elements [6]. Recently, this model was extended to describe the genesis of *M. tuberculosis* from a related NTM species, revealing that after its common ancestor with a *M. kansasii*-like organism, the ancestral MTC organism acquired at least 55 new genes via horizontal gene transfer (HGT) [4]. This finding confirmed an analysis using a different but complementary approach, also presenting evidence of HGT in *M. tuberculosis* [7]. This process apparently continued beyond the common ancestor of *M. tuberculosis* and *M. canetii*, as the former organisms contain certain genes lacking in the closely related *M. canetii* species [8]. After the establishment of *M. tuberculosis* sensu stricto, there is no evidence of further HGT specific to distinct lineages of the organism. The absence of ongoing HGT is presumably the consequence of living in an intracellular niche where there is limited opportunity to acquire foreign DNA. From the bacterial vantage, a key research priority is to define the extent to which these genes acquired during the emergence of the *M. tuberculosis* complex

contributed to the unique pathogenicity of *M. tuberculosis*, either through targeted gene disruption studies in *M. tuberculosis* or via heterologous complementation of putative virulence genes into less virulent organisms such as *M. kansasii*.

3 *Mycobacterium tuberculosis* Complex Evolution

After the completion of the *M. tuberculosis* H37Rv genome, comparative genomic studies using BAC libraries and DNA microarrays detected large sequence polymorphisms that distinguished the interrogated organisms from the referent; since the comparisons were unidirectional, there was a bias towards finding genomic deletions in a particular subspecies or strains [9, 10]. When the presence of such regions was assessed across collections of clinical isolates, it was seen that some genomic regions (known as "regions of difference" or RDs) were deleted across large groups of organisms (e.g. RD9 is absent from *M. africanum* and the veterinary variants [11], while others were specific to highly specific lineages (e.g. RD1 is absent from BCG vaccines [12]. Because *M. tuberculosis* complex exhibits no ongoing HGT [13], these deletions were predicted to represent unique evolutionary events that are highly unlikely to revert. Hence these deletions were used to derive first-generation phylogenies of *M. tuberculosis* complex organisms wherein *M. tuberculosis* was situated in a more ancestral position, with a larger genome, while *M. bovis* was situated in a more derived position, with a smaller genome, serving as the parental strain for BCG vaccines [14, 15]. At the same time, the availability of spacer oligotyping, or spoligotyping, as a DNA fingerprinting tool, allowed investigators to interrogate collections of organisms and determine that the number of spacers at the direct repeat locus also presented a pattern of deletions, again placing *M. tuberculosis* before *M. bovis* before BCG [16]. Together, these modalities suggested that the traditional subspecies names were robust and that different strains and lineages of the *M. tuberculosis* complex had distinct genomic identities [17].

In recent years, these kinds of genomic comparisons have expanded from *ad hoc* study of the strain of interest [18–20] towards a more comprehensive assessment of genetic variability through study of large, representative collections of strains from around the world [21, 22]. These studies have employed GeneChip-based interrogation for genomic deletions, SNP-based analysis, spoligotyping, multi-locus sequence analysis, and most recently whole genome sequence analysis [23]. Reassuringly, the overall genetic architecture of the *M. tuberculosis* complex has remained consistent across these different analyses, suggesting again that the subspecies classifications are robust. Furthermore, it was observed that Africa is the only continent which harbors all six main lineages of human-adapted *M. tuberculosis* complex. Given that the greatest human genetic diversity is also found on that continent, Hershberg and colleagues proposed a scenario for the evolution and global spread of human tuberculosis, whereby *M. tuberculosis* originated in Africa and accompanied the ancient Out-of-Africa migrations of

modern humans which occurred about 50,000 years ago [24] (see Fig. 2). Together with ancient DNA evidence indicating that humans have been infected with *M. tuberculosis* for at least 9,000 years [25], the global phylogeography supports the hypothesis that humans have lived with *M. tuberculosis* for millennia, a period during which there has been ample opportunity for each organism to shape the evolution of the other.

From this evolutionary paradigm, a number of immunologic propositions emerge. First, in order for the organism to infect and cause transmissible disease, there is a selective advantage for the organism not to kill its host. In the absence of antibiotics, TB meningitis is lethal for the host; whether treated or not, TB meningitis is dead-end for the pathogen. The most adaptive outcome for the organism will occur if it induces a chronic, low-grade disease with long-term expectoration and no life-threatening pathology. Indeed, while clinicians tend to remark at the size of a TB-associated cavity, it can also be noted that during active TB, most humans are ambulatory and do not require respiratory support, as disease is typically confined to one lobe of one lung. Second, if *M. tuberculosis* has lived with humans for millennia, strains circulating today (and hence subject to genetic analysis) by definition represent the successful strains. It can be argued that there is

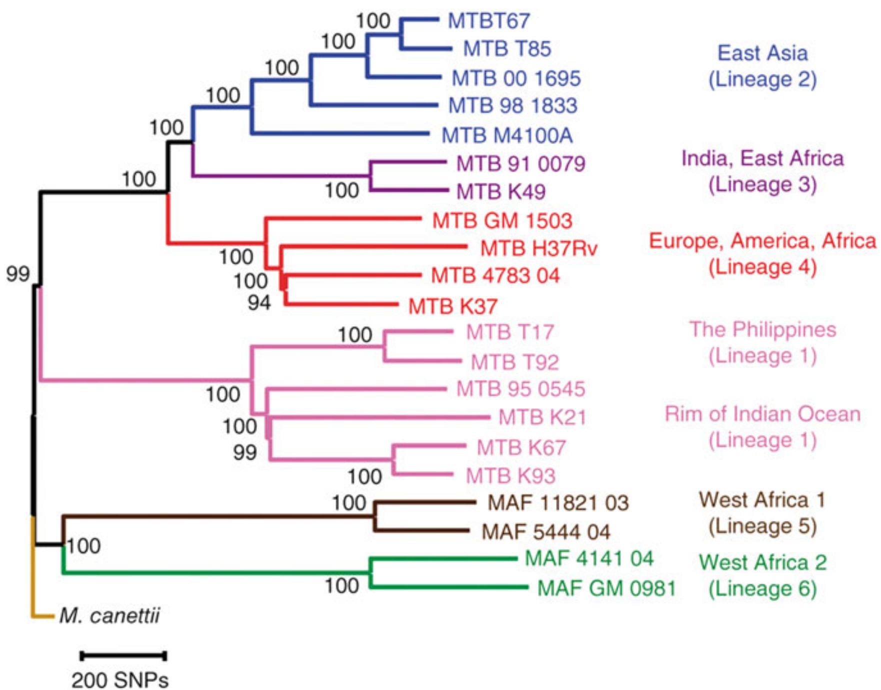

Fig. 2 The global phylogeny of *Mycobacterium tuberculosis* complex based on complete sequence analysis of 23 strains representing global *M. tuberculosis* diversity. The tree is rooted with *M. canettii*, the closest known out-group and branches are colored according to the six main phylogeographic lineages of MTC [23, 29]

unlikely to be a massive difference in virulence between one contemporary strain and the other for at least two reasons. From the vantage of competition, extremely successful strains should be dominant wherever they have been introduced while unsuccessful strains should no longer be with us. Yet, while the Beijing strain appears to dominate in certain settings [26], there is no evidence that this strain behaves unusually when introduced into Montreal [27] or Switzerland [28]. Instead, when looking at specific clinical outcomes in over 1,000 patients with TB diagnosed and treated in a setting with uniform access to health care, there is no evidence that any one strain is associated with more outbreaks, more severe disease or worse treatment outcomes; the only statistically significant associations were with less severe disease, suggesting that if anything, some strains of *M. tuberculosis* are evolving towards a more indolent disease [27]. From the vantage of the disease process, a more invasive strain would be lethal to its host (reducing transmission) and a less virulent strain would have a decreased capacity to generate inflammatory pathology (again reducing transmission). In order to successfully exit a human host, the strain of *M. tuberculosis* must be carefully calibrated to cause just enough disease, but not too much.

Additionally, when assessing strains currently circulating in human populations, it can be inferred that whatever strategies have been developed and deployed by the host with the aim of eliminating *M. tuberculosis*, these have by definition failed. This indicates that for each human immune effector that is discovered using reductive experimental approaches there is likely to be a bacterial counter-effector in *M. tuberculosis* that successfully subverts or neutralizes the effort. From this, one can draw the sobering inference that immune parameters observed during active TB need not represent protective responses; they may instead only be host reactions to a successful infection. While these are largely theoretical considerations, an inspection of the genetic record for *M. tuberculosis* provides some support for these notions. An analysis of 23 whole-genome sequences representative of the global diversity of *M. tuberculosis* found that among the most invariant genes of the bacteria are those coding for antigenic proteins [29]. In stark contrast to various pathogenic viruses, protozoa, and bacteria, where immune escape is achieved through the accumulation of antigenic variation, the authors found that T cell antigens are as conserved as essential genes, and that within the gene, the specific antigen regions known to interact with human T cells (i.e. the T cell epitopes) was the most highly conserved. This study supports the view that *M. tuberculosis* does not evade our immune system, but instead depends on it to generate the pathology required for transmissible infection. Indeed, while there are intuitive benefits to immune-evasion for persistence in the host, it is difficult to envision how immune-evasion can ultimately offer an exit strategy for the organism. Indirect support for the importance of immune-recognition is provided by the natural experiment of HIV/AIDS; with an impaired host immune response, there is an increased risk of disease but HIV-infected TB patients are generally less contagious [30].

4 Pending Questions and Concluding Thoughts

The availability of genome-level data has permitted a number of fields of research to revisit old dogma, challenge assumptions about the biology of the disease, and uncover new targets for post-genomic approaches to addressing that particular process. This is clearly the case for TB. Over 100 years ago it was known that *M. tuberculosis* is less virulent than *M. bovis* for rabbits and cows, but more likely to cause human disease. For a number of reasons it was subsequently assumed that the human pathogen had evolved from the bovine pathogen, which set up a model whereby *M. bovis* may have had a larger genome, with greater flexibility to cause disease in a wider range of hosts, including the ability to produce antigenic proteins such as MPB70 and MPB83 that are not detected in culture filtrates of *M. tuberculosis*. Now we know that this scenario was wrong. *M. bovis* has a smaller genome, the reason for its ability to cause disease in a large number of hosts is still unknown and the production of copious amounts of these antigenic proteins is not because *M. tuberculosis* lacks the necessary genes, but rather because *M. bovis* has an anti-sigma factor mutation resulting in constitutive expression of genes in the SigK regulon [31]. This anecdote serves to remind us of the greatest value of genome-level interrogation, which is the elimination of pre-experimental biases that may be present when doing directional and sequential research. So what are the key questions about MTC evolution in 2012?

At the fundamental level, we still do not know why *M. tuberculosis* spreads so efficiently between people, unlike non-tuberculous mycobacteria, and unlike other members of the *M. tuberculosis* complex. There have been anecdotes of a perfect storm, where a patient with severe NTM lung disease transmits their organism to a highly susceptible host, but clearly this is not the norm [32]. Even transmission of virulent *M. bovis* between humans remains remarkable and publishable [33]. An understanding of why transmission is more or less successful, coupled with an associated intervention, could serve to convert TB from a public health problem (epidemic disease) into a clinical problem (infectious disease). One can envision two approaches to resolving this question: starting at with *M. tuberculosis* sensu stricto and working outwards, or starting at the extremes and working inwards. In the former approach, one can analyze sequence variability among closely related strains to determine the nature of genetic change, e.g., among sub-strains of the Beijing lineage of *M. tuberculosis*. Such an analysis will reveal the smallest number of SNPs but may point to key regions in the genome tolerating variability, such as drug-resistance genes for which variability in such comparisons is now well documented [34]. The contrasting approach starts with the premise that genes present in *M. tuberculosis* that are absent from *M. kansasii*, *M. marinum* and other non-tuberculous mycobacteria may hold the key to the capacity of *M. tuberculosis* to efficiently cause disease and spread between humans. Here, the challenge is not so much to detect differences, but rather to sort through a much longer list of differences to generate candidates for experimental assessment in the laboratory. Notably, among the genes conserved between *M. tuberculosis* and *M. kansasii* are

a number of well-established virulence genes, including the ESX-1 locus; this argues that while this virulence system may be necessary for full virulence, it is clearly not sufficient.

Ultimately, the driving goal of TB research is to develop the tools needed to combat the epidemic. Conceptually, this occurs in clusters of researchers interested in better diagnosis, better treatment or a better vaccine against TB; likely, synergy will be achieved if these advances are applied in concert. Key questions in coming years include the importance of pathogen variability in each of these programs of research, from conception to product development to validation in the field [35]. Will new immunodiagnostic tests based on *M. tuberculosis*-specific antigens detect all cases of TB infection, or are there strains that will elude detection? Will these tests be confounded by infection with NTM, and is this a problem or an opportunity? Do drug treatments produce a predictable rate of antibiotic resistance, across strains, or is there reason to believe that certain lineages have a genetically programmed propensity for antibiotic tolerance and/or selection of antibiotic resistance? Do new vaccines provide protection against all strains of *M. tuberculosis*, or just laboratory strains that are used for convenience because they lend themselves to reproducible experimental infections? For all of these questions, an evolutionary understanding of *M. tuberculosis* can inform parameters that do matter, conceptually and empirically, or do not matter. The proximal challenge will be to determine which of these features of the disease are and are not under the influence of bacterial variability, to design the fundamental experiments and evaluate the new tools appropriately.

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Glossary

Mycobacterium tuberculosis complex: A bacterial species that comprises *M. tuberculosis* sensu *stricto* *M. bovis* and other related agents of tuberculosis in their respective mammalian hosts

Bacille de Calmette et Guérin (BCG) vaccine: A family of attenuated strains of *M. bovis* originally derived by serial passage of virulent *M. bovis* in the laboratory between 1908 and 1921. Named for the scientists who developed this vaccine

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Mycobacterium tuberculosis Genes Involved in Regulation of Host Cell Death

Volker Briken

Abstract The topic of host cell death response upon *Mycobacterium tuberculosis* (Mtb) infection has been a controversial one [1]. Recent findings demonstrate that one of the important confounding factors was most likely the fact that while Mtb inhibits host cell apoptosis induction early during the infection it clearly induces a necrotic form of cell death during later infection stages [2, 3]. This bi-phasic intracellular lifestyle in regard to host cell death manipulation is emerging as a common theme shared with other facultative and obligate intracellular bacterial pathogens such as *Chlamydia* and *Legionella* [4–6]. Accordingly, the list of discovered bacterial proteins involved in host cell apoptosis inhibition is growing [7, 8]. At the same time it is clearly beneficial for the resistance of the host to overcome the bacterial apoptosis block during the early stage of the infection [9–11]. Hence, host cell components have evolved to recognize intracellular pathogens and mediate host cell apoptosis induction if necessary [12]. There have been several reviews on the various aspects of the host cell death response upon Mtb infection [1, 3, 13–15]. Thus in this chapter I will focus on the pathogen side of the equation and describe the tremendous progress that has been made in the identification and characterization of Mtb genes involved in manipulation of host cell death pathways.

Keywords Apoptosis · Pyroptosis · Necrosis · Mycobacterium · Tuberculosis · Cell death

V. Briken (✉)
Department of Cell Biology and Molecular Genetics, University of Maryland, Microbiology Building 231, Room 2201, College Park, MD 20742, USA
e-mail: vbriken@umd.edu

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1 Introduction

The topic of host cell death response upon *Mycobacterium tuberculosis* (Mtb) infection has been a controversial one [1]. Recent findings demonstrate that one of the important confounding factors was most likely the fact that while Mtb inhibits host cell apoptosis induction early during the infection it clearly induces a necrotic form of cell death during later infection stages [2, 3]. This bi-phasic intracellular lifestyle in regard to host cell death manipulation is emerging as a common theme shared with other facultative and obligate intracellular bacterial pathogens such as *Chlamydia* and *Legionella* [4–6]. Accordingly, the list of discovered bacterial proteins involved in host cell apoptosis inhibition is growing [7, 8]. At the same time it is clearly beneficial for the resistance of the host to overcome the bacterial apoptosis block during the early stage of the infection [9–11]. Hence, host cell components have evolved to recognize intracellular pathogens and mediate host cell apoptosis induction if necessary [12]. There have been several reviews on the various aspects of the host cell death response upon Mtb infection [1, 3, 13–15]. Thus in this chapter I will focus on the pathogen side of the equation and describe the tremendous progress that has been made in the identification and characterization of Mtb genes involved in manipulation of host cell death pathways.

2 Genes Involved in Inhibition of Host Cell Death

2.1 Superoxide Dismutase A

The first evidence for a gene of Mtb with anti-apoptotic function was provided by the analysis of lungs of mice infected with either Mtb or a strain with reduced superoxide dismutase A (SodA) expression using an anti-sense approach [16]. The SodA-knock-down strain was attenuated in mice and induced more apoptosis in lung cells [16]. These findings could be confirmed and extended in a more recent study which used an Mtb mutant deficient in the SecA2 secretion system that induced increased caspase-dependent apoptosis in macrophages. The pro-apoptotic phenotype of the *secA2* mutant was caused by deficient export of SodA since episomal expression of SodA with a signal peptide, targeting it to the SecA1 secretion system for export, could rescue the *secA2* mutant phenotype [17]. Interestingly, the overexpression of SodA in the vaccine strain BCG resulted in a reduction of macrophage apoptosis induction and a reduced protective capacity of the vaccine against challenge with Mtb in the mouse and guinea pig models [18].

2.2 nuoG *(NDH-1) and* eis

The first conclusive report that the inhibition of host cell apoptosis is important for the virulence of Mtb was provided by the analysis of the pro-apoptotic mutant of Mtb deficient in the *nuoG* gene [19]. In this study a "gain-of-function" genetic screen was used to identify three genomic regions in Mtb that contain anti-apoptotic genes and *nuoG* was identified as an anti-apoptotic gene in one of these regions [19]. The *nuoG* gene is part of a 14 gene operon coding for the type I NADH dehydrogenase, NDH-1. Subsequent investigations discovered that a functional NDH-1 is important for neutralizing reactive oxygen species (ROS) created by the macrophage phagocyte oxidase (NOX2) [20]. In *nuoG* mutant infected macrophages ROS accumulated in the phagosome as late as 24 h after infection but not in NOX2-deficient macrophages. The nuoG mutant did not induce apoptosis in the NOX2 knockout macrophages demonstrating the causal effect of increased ROS on host cell apoptosis. Phagosomal ROS mediated host cell apoptosis induction via the extrinsic apoptosis pathway was dependent upon TNF receptor signaling and caspase-8 activation [20]. The enhanced intracellular survival (*eis*) gene of Mtb is also important for suppression of ROS increases in the host cell but in this case the *eis* mutant induced ROS generation not via NOX2 but also via host cell mitochondria [21]. Another important difference of this mutant with the *nuoG* mutant phenotype is that the *eis* mutant induces caspase-independent cell death and autophagy. Eis is a member of the GCN5-related family of N-acetyltransferases and the acetyltransferase activity of Eis is necessary for its anti-apoptotic activity [21]. Eis is a secreted protein and thus it is conceivable that it reaches the host cell cytosol to acetylate and thus inactivate target proteins in a manner similar to the *Yersinia* spp. secreted effector YopJ [21, 22].

2.3 Protein Kinase E

It is of interest to note that neither the *nuoG* nor the *eis* mutant-induced apoptosis depends on the phagosomal generation of reactive nitrate intermediates (RNI) that are derived from nitric oxide produced by the iNOS enzyme. Instead, nitric oxide induces transcription of protein kinase E (*pknE*) and in the absence of PknE, Mtb causes more host cell apoptosis early during infections and less necrosis during the later stage of the infection [23]. A detailed analysis of the host transcriptome upon infection with the *pknE* mutant demonstrated an increased expression of pro-apoptotic BCL-2 family members *bax* and *bid* genes and suppressed expression of anti-apoptotic *mcl-1* among a list of genes involved in apoptosis regulation, co-stimulation, pro- and anti-inflammatory cytokines [24]. It is still unclear which of these host genes are of functional importance for the phenotype of the *pknE* mutant, neither is the target of pknE known. Nevertheless, a model emerges in which Mtb has evolved to sense the important host phagosomal defense responses

in ROS and RNI and developed multiple pathways to evade their detrimental effects. Furthermore, besides their involvement in direct bactericidal activity, the prolonged production and accumulation of ROS and RNI leads to host cell apoptosis induction. This could be regarded as a last resort of the macrophage defending against intracellular pathogens that have adapted to the hostile macrophage environment.

2.4 Rv3364c *and* Rv3654c

Several anti-apoptotic Mtb genes have recently been discovered via a "loss-of-function" genetic screen [25, 26]. The first report describes the importance of a 7-gene operon (*Rv3654c*-*Rv3660c*) for Mtb-mediate apoptosis inhibition. The operon contains four genes with homology to type IV pili. It could be determined that Rv3654c is secreted into the host cell cytosol and binds to protein-associated splicing factor (PSF) which inhibits splicing of caspase-8 pre-mRNA and thus downregulates caspase-8 protein levels in macrophages [26]. Consistently, knock-down of host cell PSF via siRNA abrogates the pro-apoptosis phenotype of the *Rv3654c* mutant [26]. The second finding reported from this genetic screen describes another operon (*Rv3361c*-*Rv3365c*) but this operon was important for inhibition of host cell pyroptosis [25]. Pyroptosis is defined as a form of apoptosis that depends upon inflammasome activation and in particular the activation of the caspase-1 [12]. Interestingly, the Rv3364c protein can enter the host cell cytosol, bind to, and inhibit the protease cathepsin G which leads to less apoptosis induction. Furthermore, a reduction of cathepsin G activity also reduces activation of caspase-1 which further

Table 1 Mtb genes important for apoptosis inhibition

Gene	Protein function	How it inhibits apoptosis	Ref.
sodA	Superoxide dismutase	Neutralizes phagosomal ROS?	[16–18]
nuoG	Electron acceptor; part of type I NADH dehydrogenase	Neutralizes and/or inhibits production of phagosomal ROS	[19, 20]
pknE	Serine/threonine protein kinase	Suppresses nitric oxide stress induced apoptosis	[23, 24]
Rv3364c	Roadblock/LC7 family like protein	Binds to host cell cathepsin G which leads to suppression of pyroptosis	[25]
eis	GCN5-related family of N-acetyltransferases	Mediates ROS suppression via N-acetyltransferase domain; inhibits autophagy and caspase-independent cell death	[21]
Rv3654c	Part of 7-gene operon (type IV pili like)	Binds and cleaves host cell protein-associated splicing factor (PSF) leading to less caspase-8 in host cell	[26]

cements the importance of cathepsin G in pyroptosis induction and suggests a role for cathepsin G in caspase-1 activation [25] (Table 1).

3 Genes Involved in Induction of Host Cell Death

3.1 esxA (esat-6)

After replication and inhibition of host cell apoptosis Mtb escapes the phagosome [27, 28] and induces a form of necrotic cell death [2, 3]. It is unclear which genes of Mtb are important for triggering this necrotic death response but it seems likely that active bacterial manipulation is required [3, 29]. Mycobacteria contain five type VII secretion systems (ESX1-ESX5) [30, 31]. The ESX-1 secretion system is clearly important for escape of Mtb and *M. marinum* (Mm) out of the phagosome into the host cell cytosol [27, 28, 32, 33]. It is also well established that Mtb and Mm mutants deficient in functional ESX-1 induce less host cell necrosis and reduced inflammasome activation [32–36]. In general, the problem with the analysis of the ESX-1 system is that the deletion of any of its known components inhibits the whole system and hence it is difficult to demonstrate which proteins of the system are the secreted effectors and which are part of the secretion machinery. The esxA (ESAT-6) component of ESX-1 is able to form dimers which can insert into lipid bilayers and form pores [32, 34]. This could explain the importance of ESX-1 for phagosomal escape and it also makes the ESX-1 system a candidate for the induction of necrosis-type cell death induction during the later stage of the infection [37, 38]. Alternatively, the recognition of ESX-1 effectors by host cell the NLRP3 inflammasome induces pyronecrosis in human macrophages [39]. It remains to be seen if these findings can be linked to the previously described exit pathway involving host cell eicosanoide manipulation [3]. It will be experimentally challenging to prove the role of ESX-1 in host cell exit since ESX-1 mutants do not escape from the phagosome and hence do not enter the normal later stage of the replication cycle. Nevertheless, the dual role of the ESX-1 system for two important functions in the intracellular life cycle of Mtb would also explain the severe attenuation of mycobacteria without this secretion system [34, 40–42].

Interestingly, the *esxA* mutant induces less host cell apoptosis compared to wild-type Mtb early during the infection when Mtb inhibits phagosome maturation and replicates in the phagosome [38, 43]. This suggests that host cell proteins are able to recognize ESX-1 secreted effectors to induce apoptosis. Interestingly, purified EsxA can bind to and induce signaling of the TLR-2 which leads to inhibition of signaling response of TLR-4, TLR-7, and TLR-9 [44]. These studies were performed using purified ligands and hence it remains to be seen how all of these signals integrate during an infection with live Mtb. It would not be surprising to find that human macrophages have evolved to target one or more of the components of Mtb essential for intracellular survival during the early stage of the

infection to induce host cell apoptosis. To that effect, genetic variability in the capacity to mediate an apoptotic response is associated with differences in host susceptibility to mycobacterial infections [9–11].

3.2 ESX-5 Locus and PE_PGRS33

The ESX-5 type VII secretion system is a major pathway for the export of PE and PPE domain containing family of proteins [45, 46]. The ESX-5 deficient Mtb and Mm induce less inflammasome activation and host cell necrosis [36]. The host cell necrosis induction is mediated via lysosomal rupture and depends upon the cathepsin B protease. Interestingly, the ESX-5 system is not required for phagosomal escape and thus a model was proposed where ESX-1 is important for phagosomal escape and ESX-5 for necrosis induction and consequently the exit of Mtb out of the host macrophage [36]. The PE family protein PE_PGRS33 has pro-apoptotic activity via interaction with Toll-like receptor 2 when purified protein was added to various cell types [47]. It remains to be shown if this protein has any activity when analyzed in the context of live mycobacteria and if it could be an ESX-5 secreted effector involved in host cell death induction.

3.3 Heparin-Binding Hemagglutinin

The Heparin-binding hemagglutinin (HBHA) is a 28 kDA protein found in the cell wall and culture filtrate of Mtb. It facilitates binding of Mtb to nonphagocytic epithelial cells and is important for extrapulmonary dissemination of Mtb [48, 49]. Interestingly, in macrophages HBHA is targeted to host cell mitochondria after infection to induce apoptotic cell death [50]. In this study, BMDMs were infected with either Mtb deficient in HBHA expression which led to less apoptosis induction or with Msme ectopically expressing Mtb-HBHA which led to an increase in apoptosis. The apoptosis induction is dependent on the activation of pro-apoptotic host cell protein Bax and on an increase in mitochondrial ROS. A specific role of this apoptosis induction for Mtb pathogenesis remains to be determined but it seems unlikely that this mechanism is involved in the exit of Mtb from macrophages because it does not induce cell membrane rupture [50].

3.4 OppABCD

Methylglyoxal (MG) and advanced glycation end products (AGE) may cause apoptosis and IL-1β, IL-6 and TNF cytokine secretion [51–53]. Interestingly, both of these products accumulate in host cells upon Mtb infection [54]. The oligopeptide transporter OppABCD of Mtb is able to bind glutathione and import it into

Table 2 Mtb genes important for apoptosis/necrosis induction

Gene	Proposed function	How it induces apoptosis/necrosis	Ref.
esxA (esat-6)	Component of ESX-1 type VII secretion system	Forms homodimers to induce pores in host cell membranes	[32, 34, 43]
hbha	Adhesion to lung epithelial cells	Localizes to host cell mitochondria to induce Bax activation, loss of MOMP and cytochrome C release	[50]
ESX-5 locus	Type VII secretion system	Necrotic cell death dependent upon cathepsin B	[36]
oppA-D	Oligopeptide transporter	OppA binds to host cell glutathione, leads to increase in host cell TNF production	[53]

the bacterial cytosol [53]. The decreased glutathione levels in the host cell lead to an accumulation of MG and AGE in cells infected with wild-type Mtb but not in cells infected with an *oppD* mutant [53]. These findings thus provide a molecular mechanism for the Mtb-mediated manipulation of host cell glutathione levels and its effect on cell apoptosis and cytokine response. The role for this regulation in pathogenicity of Mtb during in vivo infections remains to be determined. In view of the in vitro data, one could speculate that an *oppA-D* deficient mutant will be hypervirulent since it induces less apoptosis and cytokine secretion (Table 2).

4 Conclusion

The manipulation of host cell death pathways by Mtb is complex but can be divided into at least two phases: an early anti-apoptotic and a later pro-necrotic phase. The analysis and characterization of the genes involved in the pro-necrotic phase will be complicated by the fact that the host cell also aims to undergo apoptosis and hence recognizes mycobacterial components for cell death induction. Consequently, in future studies it will be important to differentiate between mycobacterial genes important for the regulated exit and genes encoded for components that are recognized by the host cell for immune defense purposes. The execution of "loss-"and "gain-of-function" genetic screens has been successful in identifying Mtb genes dedicated to host cell apoptosis inhibition during the early phase of infection. These approaches revealed that the Mtb genome encodes multiple effectors dedicated to inhibiting the extrinsic pathway of caspase-dependent apoptosis, the caspase-1-dependent pathway of pyroptosis, and caspase-independent, autophagy-associated apoptosis.

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Dying to Live: How the Death Modality of the Infected Macrophage Modulates Immunity to Tuberculosis

Maziar Divangahi, Samuel M. Behar and Heinz Remold

Abstract Virulent *Mycobacterium tuberculosis* (*Mtb*) inhibits apoptosis and triggers necrosis of host macrophages to evade innate delay in the initiation of adaptive immunity. Necrosis is a mechanism used by bacteria to exit macrophage, evade the host defenses, and disseminate while apoptosis is associated with diminished pathogen viability. We have recently demonstrated that eicosanoids regulate cell death program of either human or murine macrophages infected with *Mtb*. We have defined prostaglandin E_2 (PGE_2) as a pro-apoptotic host lipid mediator which protects against necrosis. In contrast, lipoxin A_4 (LXA_4) is a pro-necrotic lipid mediator which suppresses PGE_2 synthesis, resulting in mitochondrial damage and inhibition of plasma membrane repair mechanisms; this ultimately leads to the induction of necrosis. Thus, the balance between PGE_2 and LXA_4 determines whether *Mtb*-infected macrophages undergo apoptosis or necrosis and this balance determines the outcome of infection.

Keywords *Mycobacterium tuberculosis* (*Mtb*) · Macrophages · Necrosis · Apoptosis · B-cell lymphoma 2 (Bcl-2) · Bcl-2 associated X Protein (BAX) · Bcl-2 homologous antagonist killer (BAK) · BH3 interacting domain (BID) ·

M. Divangahi (✉)
Meakins-Christie Laboratories, Department of Microbiology and Immunology,
Department of Medicine, McGill University Health Centre,
Montreal, QC H2X 2P2, Canada
e-mail: maziar.divangahi@mcgill.ca

S. M. Behar (✉) · H. Remold (✉)
Division of Rheumatology, Immunology, and Allergy, Department of Medicine,
Brigham and Women's Hospital, Harvard Medical School,
Boston, MA 02115, Canada
e-mail: sbehar@rics.bwh.harvard.edu

H. Remold
e-mail: hremold@rics.bwh.harvard.edu

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Extrinsic pathway · FLICE-inhibitory protein (FLIPS) · Intrinsic pathway · Mitochondrial outer membrane permeabilization (MOMP) · Mitochondrial permeability transition (MPT) · Cell death program · Lipoxins (LX) · Prostaglandins (PG) · Eicosanoids · Plasma membrane microdisruptions · Mycobacterial antigens · BCG vaccine · T cell response

1 Introduction

Mycobacterium tuberculosis (*Mtb*) is an extremely successful bacterium that is transmitted person-to-person by the aerosol route. The World Health Organization (WHO) has estimated that more than 2 billion persons are latently infected with *Mtb*. From this large reservoir of asymptomatic infected people emerges 8–10 million cases of active TB each year resulting in the deaths of nearly 1.7 million people each year [1]. The increased incidence of TB has been attributed to three major factors: the HIV pandemic, the emergence of multidrug-resistant strains of *Mtb*, and the failure of the major vaccine, BCG, to prevent pulmonary tuberculosis [2–4].

The success of this pathogen is closely linked to its ability to alter the intracellular environment of the alveolar macrophage. When inhaled, *Mtb* enters the lower respiratory tract and reaches distal alveoli where initially infects alveolar macrophages. Although macrophages excel at phagocytizing and destroying biological particles including dead cells and bacteria, *Mtb* has adapted to the harsh intracellular environment, which allows it to survive and replicate within these phagocytic cells. By subverting or avoiding critical components of macrophage immunity including phagolysosomal fusion, microbicidal effectors, and as will be discussed in this chapter, cell death pathways, *Mtb* evades both innate and adaptive immune responses. Therefore, delineating how *Mtb* and macrophages interact is fundamental to understand immunity to *Mtb*.

Manipulation of macrophage death pathways is one mechanism that allows *Mtb* to evade host defenses. Three major outcomes are observed following productive *Mtb* infection of human or murine macrophages in vitro: (a) necrosis, a form of death characterized by plasma membrane disruption; (b) apoptosis, a form of death in which the plasma membrane integrity is preserved; and (c) survival of the infected macrophages. Characterization of these different phenotypes is challenging because of the asynchronous nature of intracellular infection and heterogeneity among the bacteria and macrophages. Other factors such as the percentage of infected macrophages and variation in the number of bacteria internalized by each macrophage can affect the kinetics of cell death when studied in vitro. Nevertheless, a spectrum of all three phenotypes can be observed following infection of normal macrophages with virulent *Mtb*. In general, highly virulent *Mtb* strains predominantly induce necrosis [5]. The concept that virulent *Mtb* induce necrosis in part by actively inhibiting macrophage apoptosis [6], has gained additional support by the identification of bacterial mutants that induce apoptosis

instead of necrosis [7, 8]. The different cellular fates of *Mtb* infected macrophages are of great interest as the death modality influences the outcome of infection. In particular, apoptotic death reduces the viability of different mycobacterial species [9, 10] including *Mtb* [11, 12, 13]. Here, we discuss the cellular mechanisms that regulate the death modality of *Mtb*-infected macrophages and lead to important functional consequences on immunity to *Mtb*.

2 Macrophage Apoptosis is a Host Defense Mechanism Against *Mtb*

The discovery that many attenuated strains of mycobacteria induce more apoptosis than their wild-type counterparts supports the hypothesis that virulent mycobacteria inhibit macrophage apoptosis. Indeed, there exists a reciprocal relationship between virulence and apoptosis. As such, *Mtb* infection predominantly results in necrosis, while attenuated mutant strains including BCG and H37Ra primarily induce apoptosis. Now, investigators are identifying single gene mutations in *Mtb* that shift the balance from necrosis to apoptosis [7, 8]. Although it is not yet clear whether virulent *Mtb* block the triggering of apoptosis or inhibit downstream events that give rise to the typical cellular changes associated with apoptosis, it can be argued that by inducing necrosis, *Mtb* evades host defenses and provides a pathway for its exit from the infected cell and its dissemination. Detailed analysis of "necrosis" reveals to be heterogeneous and certain subtypes have been defined that have unique cellular triggers and molecular mechanisms. For instance, pyroptosis and necroptosis are forms of necrosis that are dependent on caspase 1 and receptor-interacting proteins 1 and 3 (RIP-1/3), respectively [14–16]. Thus, the idea that necrosis is a passive, accidental, and unregulated form of cell death is an old dogma that needs to be revisited. In fact, how *Mtb* induces necrosis is a question that remains unanswered.

In contrast to necrosis, the past decades have made tremendous progress in unraveling the signaling pathways that lead to initiation of apoptosis. Hallmarks of apoptosis include the segmentation of DNA [17], exposure of phosphatidylserine on the outer leaflet of the plasma membrane, and finally, packaging of cellular components into membrane-bound blebs [18, 19]. During apoptosis the dying cell produces 'find me' and 'eat me' signals that aid its rapid clearance by phagocytes through the process of efferocytosis [13, 20].

Apoptosis is initiated by two major pathways

(1) *The extrinsic pathway:* The induction of apoptosis by attenuated *Mtb* in human monocyte-derived macrophages is mediated by the executioner caspases 3 and 7 and requires two distinct signals: one is a lipid and the other is a protein [21, 22] the protein-dependent signal can be reconstituted by TNF. Indeed, the induction of apoptosis in macrophages by *Mtb* requires the action of TNF to

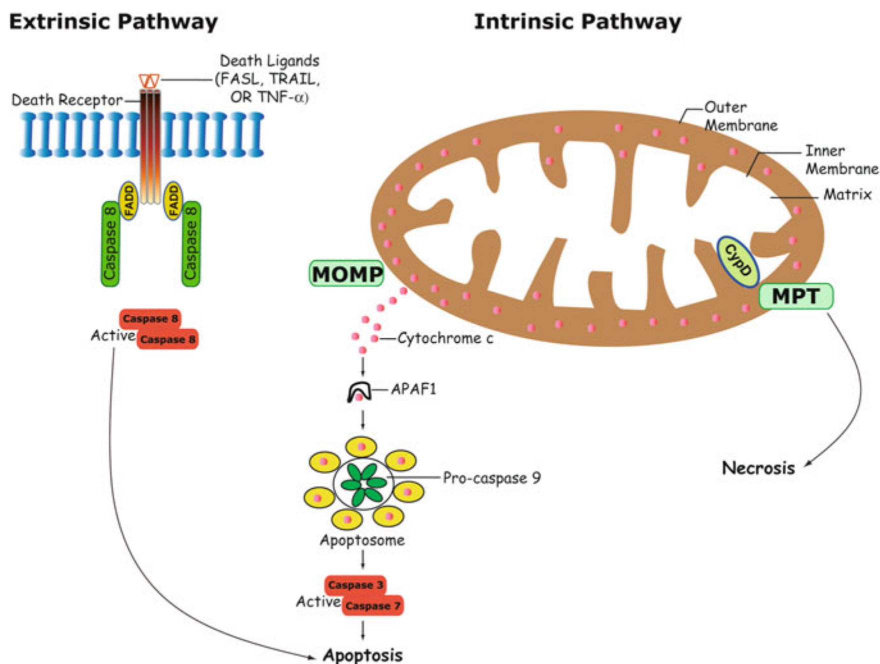

Fig. 1 Two major pathways leading to macrophage death following *M. tuberculosis* infection. Some macrophages death programs are triggered by the extrinsic pathway (surface receptor mediated): Ligation of death receptor (e.g., Fas or TNF-α receptors) is followed by activation of caspase 8 which leads to apoptosis. Alternatively, the intrinsic pathway (mitochondria-mediated) is activated: Permeabilization of mitochondria outer membrane potential (MOMP) leads to the release of apoptotic mediators such as cytochrome c from the mitochondrial intermembrane space into the cytosol leading to formation of apoptosome complex and activation of caspases (9, 3, and 7) which in turn induce apoptosis. However, during necrosis mitochondrial permeability transition (MPT) causes mitochondrial inner membrane perturbation (MIMP), collapse of the membrane potential, uncoupling of the respiratory chain, and overproduction of reactive oxygen species (ROS). Cyclophilin D (CypD) is a mitochondrial protein which is involved in MPT and necrosis

activate the extrinsic death receptor-dependent pathway (Fig. 1). Although both bacterial strains produce comparable amounts of TNF [23], the avirulent strain H37Ra much more potently induces apoptosis than the virulent H37Rv strain. One potential explanation for this observation is that soluble TNFR-2 is shed by macrophages infected with virulent *Mtb*, and neutralizes TNF, resulting in a "TNF-poor" microenvironment [24]. This model is also consistent with data that caspase 8 activation, which is an essential and early step in the induction of apoptosis by the extrinsic pathway, is inhibited in H37Rv-infected macrophages (Remold, unpublished observation). Recently, some of the early components of the extrinsic apoptotic pathway activated by *Mtb* in the murine macrophage cell line RAW 264 have been identified [25]. In *Mtb*-infected cells, TNF production induces reactive oxygen species

(ROS)-dependent activation of apoptosis signal-regulating kinase (ASK1; A002816), a member of the mitogen-activated protein kinase family causing $FLIP_s$ phosphorylation. Phosphorylated $FLIP_s$ interacts with the E3 ubiquitin ligase c-CYBL facilitating proteasomal $FLIP_s$ degradation involving the tyrosine kinase c-Abl. $FLIP_s$ degradation then enables activation of caspase 8 leading to caspase 3/7 activation and apoptosis.

(2) *The intrinsic pathway:* Induction of apoptosis in vertebrate cells most commonly proceeds through the intrinsic apoptotic pathway, which is functionally defined by mitochondrial outer membrane permeabilization (MOMP) [26]. MOMP is a central event that can lead to apoptosis as it results in release of apoptotic mediators, including cytochrome c, Smac-DIABLO, AIF, and other factors from the mitochondrial inter-membrane space and ultimately results in the activation of caspases 9, 3, and 7 (Fig. 1). Although these events usually occur independently of other changes in the mitochondria, they can also be associated with the opening of the mitochondrial inner membrane pore (PT pore), which leads to mitochondrial permeability transition (MPT), loss of the mitochondrial inter-membrane potential ($\Delta\psi_m$), and necrosis. We found that *Mtb* infection, whether virulent or avirulent, induces such changes in the mitochondrial membranes and that these changes are the key events that determine the death modality of infected macrophages.

3 Mitochondrial Damage and Macrophage Death

Considering mitochondria as a key player in regulation of cell death program, the different combinations of MOMP and mitochondrial permeability transition (MPT, the opening of a pore in the inner mitochondrial membrane) in model experimental systems, and their effect on the cellular outcome are reviewed below. Thus the changes in MOMP and MPT induced by either virulent or avirulent *Mtb* infection will be discussed in the context of these scenarios.

In Scenario I, MPT causes the mitochondria to become leaky to water, which results in swelling, dysfunction, and eventually necrosis [26]. Irreversible MPT can lead to outer mitochondrial membrane damage, which manifests itself as MOMP, in this case a by-product of MPT. This scenario emerges when hepatocytes under oxidative stress or due to other toxic treatment undergo both necrosis and apoptosis [27].

However, in Scenario II, MOMP and apoptosis can occur independently of MPT. This is the case when MOMP is induced by members of the Bcl-2 family of apoptosis-inducing proteins, which do not affect the mitochondrial inner membrane [28]. Specifically, processing of the Bcl-2 protein by BID leads to activation of the pro-apoptotic Bcl-2 family proteins, BAX and BAK, causing MOMP and translocation of pro-apoptotic factors including cytochrome c into the cytosol, activation of caspase 9, and eventually caspase 3. This process neither induces nor requires MPT [29].

In Scenario III, effector molecules capable of damaging the mitochondrial inner membrane gain access to the mitochondrial inter-membrane space if the mitochondrial outer membrane is permeable. This seems to be the mechanism by which Ndufs1, the 30 kDa subunit of mitochondrial complex 1 of the electron transport chain, is damaged by caspase 3 [30]. Caspase 3 is thought to access the mitochondrial inter-membrane space via pores generated in the mitochondrial outer membrane, which allow pro-apoptotic factors including cytochrome c to escape into the cytosol [29]. Damage of Ndufs1 disrupts the electron transport chain in the inner membrane leading to ROS accumulation and necrosis.

Finally, MPT can also occur independently of MOMP (Scenario IV). This is thought to be how granzyme A damages components of the mitochondrial inner membrane [31]. Hsp70 and Hsp90 are candidate molecules that serve as cytosolic chaperones for granzyme A and allow the protease to enter the mitochondrial inter-membrane space without damaging the mitochondrial outer membrane leading to cleavage of Ndufs3 [31].

In macrophages infected with attenuated *Mtb*, apoptosis is associated with MOMP yet MPT is not induced, as described in Scenario II [29]. Inhibition of MOMP diminishes only apoptosis, but does not affect MPT [5]. Silencing of the gene for the pro-apoptotic Bcl-2 protein BAX, which is required for the release of cytochrome c and AIF from the mitochondrial inter-membrane space, abrogates *Mtb*-induced apoptosis, but does not affect MPT or necrosis [5]. In contrast, virulent *Mtb* induce both MOMP and MPT leading to irreversible mitochondrial swelling and necrosis [5]. MPT can be inhibited by cyclosporin A (which selectively blocks the function of cyclophilin D in the mitochondrial inner membrane), has a requirement for mitochondrial Ca^{++} loading and is independent of Bcl-2 family member-induced apoptosis [32]. Inhibition of MPT vis Cyclophilin D, downregulates only necrosis, but does not affect the degree of MOMP or apoptosis [33, 34]. It is not clear at present whether in *Mtb* infected macrophages MPT is dependent on opening of pores in the mitochondrial outer membrane (MOMP—Scenario III) or whether toxic molecular species enter the mitochondrial inter membrane space via chaperones (Scenario IV). The different mechanisms induced by virulent and avirulent *Mtb* indicate that in *Mtb*-infected macrophages MOMP and MPT are independent phenomena; virulent *Mtb* are unique in their induction of MPT that leads to the destruction of the mitochondrial outer membrane causing secondary cytochrome c release (see Scenario I) [5]. In summary, induction of apoptosis or necrosis in *Mtb*-infected macrophages depends on highly specific mechanisms leading to different types of mitochondrial membrane perturbation. Attenuated and virulent *Mtb* alike cause transient MOMP characterized by cytochrome c release from the mitochondrial inter-membrane space, which requires BAX. In contrast, only virulent H37Rv causes MPT.

While cell death is a tightly regulated process, the host–pathogen interaction adds several layers of complexity. How the death modality of infected cells affects the outcome of infection, particularly during different clinical states in people (e.g., latency versus disease), remains a pertinent question. Here, other investigators working on the genetics of susceptibility to *Mtb* provide an important

perspective. Gene expression profiling finds that several genes related to apoptosis are expressed less in active TB patients than in latently infected people, suggesting that decreased apoptotic activity is associated with the reactivation of latent infection [35]. Using a more targeted approach, Abebe et al. found that patients with active TB in Ethiopia had elevated expression of genes associated with the extrinsic apoptosis pathway including TNF, Fas, FasL, and caspase 8. However, the expression of FLIP, an intrinsic inhibitor of caspase 8 was also significantly elevated [36]. Although the upregulation of TNF, Fas/FasL, and caspase 8 may be the signature of an immune response capable of inducing apoptosis in infected cells, the authors propose a model in which *Mtb* inhibits the extrinsic apoptosis pathway by upregulating FLIP to evade an apoptotic death. Finally, the eicosanoid biosynthetic pathways, which regulate the death modality of infected human and murine macrophages, have now been identified as important genetic loci that regulate susceptibility to tuberculosis and leprosy in people [37–39]. While the genetic and functional data require greater scrutiny and functional correlation, they independently provide scientific motivation to better understand how death is regulated in *Mtb*-infected macrophages.

4 Host Lipid Mediators Modulate *Mtb*-Infected Macrophage Death Modality

As bacterial factors can affect the death modality, host factors also determine whether an infected cell undergoes apoptosis or necrosis. In particular, the eicosanoids appear to be critical regulators of apoptosis following *Mtb* infection [11, 12, 40]. *Mtb* induces apoptosis and triggers concomitant antimycobacterial activity of human macrophages based on the activity of cytosolic phospholipase A_2-γ (cPLA_2-γ), a group IV cytosolic PLA_2, which catalyzes the release of arachidonic acid from the *sn-2* position of membrane phospholipids [22]. Arachidonic acid and its diverse products regulate death in several cell types [41]. For example, arachidonic acid products are second messengers in TNF-induced apoptosis [42], and oxygen radicals, which are produced during lipoxygenation of arachidonic acid, induce ROS production, which can induce cell death [43]. Arachidonic acid also activates sphingomyelinase leading to ceramide production and apoptosis [44]. Which of these mechanisms are important in vivo is not clear [45].

An interesting area of research focuses on the role of the eicosanoids prostaglandin E_2 (PGE_2) and lipoxin A_4 (LXA_4) in regulating programmed cell death of macrophages [11, 12, 40]. The cyclooxygenases COX1 and COX2 convert arachidonic acid into the central intermediate PGH_2 [46], which is converted by specific synthases into diverse prostanoids [47]. Interaction of these prostanoid species, which includes the prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane, with an array of specific prostanoid receptors affects many cellular pathways. In the case of PGE_2, interaction with one of four receptors, EP1, EP2,

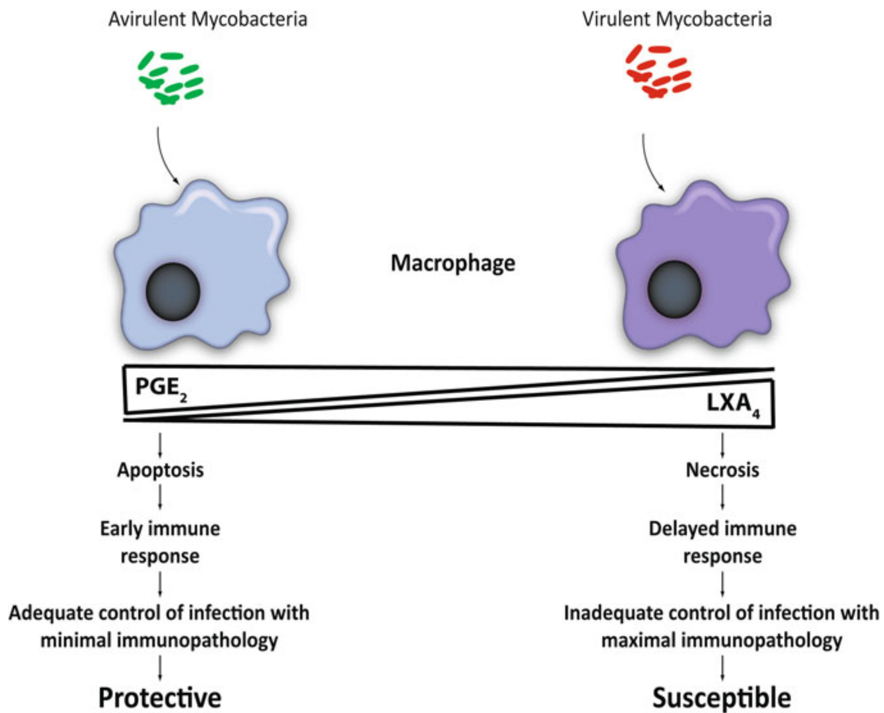

Fig. 2 Virulent mycobacteria tip the balance between PGE_2 and LXA_4 production in macrophages. Infection with virulent *Mtb* induces LXA_4, which inhibits the production of COX-2 dependent PGE_2. In the absence of PGE_2 mitochondria are damaged and membrane microdisruptions remain unrepaired triggering macrophage necrosis. We hypothesize that bacterial inhibition of prostaglandin production is an immune evasion strategy that allows *Mtb* to avoid the consequences of apoptosis, which leads to early immune response

EP3, and EP4 triggers intracellular pathways that either promote or inhibit inflammation [48]. Importantly, the functional outcome of PGE_2 signaling is largely determined by its interaction with its specific receptors [48]. For example, EP1 mediates the elevation of intracellular Ca^{++}. By contrast, EP2, which is involved in joint inflammation and neutrophil recruitment, and EP4, which induces cell migration in tumor invasion, both lead to an increase in intracellular cAMP levels. EP2 signaling results in PKA activation and triggering EP4 activates adenylate cyclase and phosphatidylinositol 3 kinase. Triggering EP3 decreases cAMP concentrations and is known to mediate fever and angiogenesis [48].

D'Avila et al. find that lipid bodies form at distinct cytoplasmic sites following infection of murine macrophages with the attenuated *M. bovis* strain BCG. These lipid bodies are the site of COX2 activity and PGE_2 generation [49]. Indeed, PGE_2 production has been a consistent finding following BCG infection of mice and macrophages [50]. We find that macrophages infected with attenuated *Mtb* also activate the PGE_2 production, which prevents necrosis and leads instead to an

apoptotic death (Fig. 2) [12]. In contrast, virulent *Mtb* strains, such as H37Rv or Erdman, only minimally induce the production of PGE_2 by macrophages [12]. This raises the possibility that virulent *Mtb* actively inhibits PGE_2 production. Thus, an important strategy that *Mtb* exploits to avoid death by apoptosis is the subversion of host eicosanoid biosynthetic pathways [11, 12].

Lipoxins are also generated from arachidonic acid but require the action of different enzymes including 5- and 15-lipoxygenases [51]. Lipoxins are anti-inflammatory and modulate chemokine and cytokine expression, monocyte trafficking and efferocytosis (phagocytosis of apoptotic cells) [52]. In contrast to attenuated strains, virulent *Mtb* induces LXA_4 production, which inhibits cyclo-oxygenase-2 production effectively shutting down PGE_2 biosynthesis, and provides an explanation for how *Mtb* inhibits PGE_2 production [11, 12]. In a PGE_2-poor microenvironment, the macrophage cannot prevent mitochondrial damage nor enable repair of plasma membrane disruptions effectively [5, 11, 12]. Both processes are required to prevent macrophage necrosis and induce apoptotic cell death [11, 12]. Virulent *Mtb* in pre-necrotic macrophages continues to replicate and once the cells are lysed, propagate the infection by spreading to uninfected macrophages. Thus, the balance of PGE_2 and LXA_4 production by the infected macrophage regulates the relative amount of apoptosis and necrosis following *Mtb* infection and has important functional consequences for innate control of intracellular *Mtb* infection.

Induction of LXA_4 by virulent *Mtb* inhibits PGE_2 production and triggers mitochondrial permeability transition (MPT) leading to irreversible mitochondrial damage [12]. By triggering LXA_4 production in the host macrophage virulent *Mtb* inhibits prostanoid production by blocking COX2 mRNA accumulation. By contrast, attenuated *Mtb* induce only minimal amounts of LXA_4 and cause instead production of substantial amounts of PGE_2. We found that when macrophage are infected with attenuated *Mtb*, PGE_2 actively suppresses mitochondrial inner membrane perturbation, which is the outcome in an infection with virulent *Mtb* [12]. Therefore, infection with virulent H37Rv, a PGE_2 non-inducer, causes MPT, which is suppressed by reconstitution with PGE_2.

Our model that lipoxin production by *Mtb*-infected macrophages is associated with increased bacterial replication and greater virulence is strengthened by the recent genetic analysis of zebrafish susceptibility to *M. marinum* [53]. Multiple mutant classes with different innate susceptibilities to *M. marinum* were identified by Tobin et al. [53]. A hypersusceptible zebrafish mutant was found to map to the LTA4H locus, which encodes leukotriene A_4 hydrolase (LTA4H), an enzyme that is required for the final step of leukotriene B_4 (LTB_4) synthesis. While LTA4H deficiency results in the loss of LTB_4 production, addition of LTB_4 did not complement the genetic defect nor increase host resistance. In the absence of LTA4H, its substrate, LTA_4, accumulates and can lead to redirected eicosanoid synthesis and increase lipoxin synthesis. Therefore, Tobin et al. hypothesize that the increased susceptibility of the zebrafish LTA4H mutant is due to an increase in lipoxin production. The same study presents human genetic data that polymorphisms in the

LTA4H gene are associated with susceptibility to pulmonary and meningeal tuberculosis [39, 53]. Thus, from fish to man, eicosanoids appear to play an unexpected role in susceptibility to tuberculosis.

5 Blocking Plasma Membrane Repair

The ESAT-6 secretion system 1 (ESX-1), a specialized Type VII secretion system, is required for the secretion of certain virulence factors including the immunodominant antigens early secreted antigen 6 kilodaltons (ESAT6) and culture filtrate protein 10 (CFP10). Although known to contribute to bacterial virulence, why ESX-1 is required for bacterial survival in the host is unknown. Some data indicate that ESAT6 damages host cell membranes [54, 55]. We hypothesized that disruption of the plasma membrane by *Mtb* is one mechanism that induces necrosis of the macrophage. Interaction of mycobacteria with the host macrophage results in plasma membrane microdisruptions. Microdisruptions induced by attenuated *Mtb* are rapidly resealed by plasma membrane repair mechanisms that include recruitment of lysosomal and Golgi apparatus-derived vesicles to the macrophage surface lesions [11, 56, 57]. Lysosomal or Golgi membrane recruitment to the plasma membrane can be assessed by measuring LAMP1 or mannosidase II translocation to the macrophage surface [58, 59]. Active membrane repair prevents necrosis and is required for induction of apoptosis. By contrast, if resealing of the plasma membrane microdisruptions inflicted by the bacteria is inhibited, as is the case with virulent *Mtb* infection and necrosis ensues.

Ca^{++} sensors are of crucial importance for the recruitment of both lysosomal and Golgi vesicles to the membrane lesions. Gene silencing of the lysosomal Ca^{++} sensor synaptotagmin 7 (SYT7) impairs the recruitment of lysosomal, but not Golgi membranes to the cell surface [11, 60]. The recruitment of Golgi-derived vesicles to the cell surface, which occurs independently of lysosomal vesicle recruitment, requires the expression of neuronal calcium sensor 1 (NCS-1), a Ca^{++} sensor that is particularly abundant in the Golgi [11, 61]. Silencing NCS-1 gene expression, or the use of brefeldin A, a Golgi-specific transport inhibitor, both inhibit translocation of Golgi membranes. These data show that both lysosomal and Golgi membranes are involved in plasma membrane repair and are recruited independently to plasma membrane lesions of infected macrophage.

Plasma membrane resealing is cAMP dependent [62], and addition of forskolin, an activator of adenylate cyclase, results in greater translocation of lysosomal membranes to the cell surface [11]. The protective effect of PGE_2 on mitochondrial stability is mediated through the PGE_2 receptor EP2 [12] and binding of PGE_2 to either EP2 or EP4 causes increased cAMP accumulation [63]. Consistent with this, PGE_2 treatment of human macrophages infected with virulent H37Rv reconstitutes repair mediated by lysosomal membranes. By contrast, PGE_2 does not affect Golgi mediated repair [11]. Although the protective effects of PGE_2 on mitochondria require the EP2 receptor, PGE_2-dependent lysosomal membrane

translocation requires PI3 K activation, which indicates that signaling through EP4 is involved [11]. These findings have important functional consequences for control of intracellular mycobacterial replication. First, *Alox-5*$^{-/-}$ mice (unable to produce LXA_4 and other *Alox-5*-dependent products) survive longer than wild-type (WT) control mice after low dose aerosol infection with virulent *Mtb* [64]. Conversely, *Ptges*$^{-/-}$ (unable to produce PGE_2) mice succumb earlier than WT mice (unpublished observation: Divangahi, Behar, and Remold). However, as many cell types produce eicosanoids, these results do not provide information about the role of eicosanoids during innate immunity. In experiments using macrophages from *Ptges*$^{-/-}$ and *Alox-5*$^{-/-}$ mice, we found that *Ptges*$^{-/-}$ macrophages were unable to control intracellular *Mtb* infection, while *Alox5*$^{-/-}$ macrophages limited *Mtb* replication better than WT macrophages [11]. This phenotype is replicated in vivo when *Mtb* infected *Ptges*$^{-/-}$, *Alox5*$^{-/-}$ and WT macrophages were adoptively transferred into the lungs of V(D)J recombination-activating protein 1-deficient (*Rag*$^{-/-}$) recipient mice. Recipient mice that received infected *Alox5*$^{-/-}$ macrophages had a substantially lower mycobacterial lung burden than recipients that received infected *Ptges*$^{-/-}$ or WT macrophages. Since *Rag1*$^{-/-}$ mice lack B and T cells, the greater capacity of *Rag1*$^{-/-}$ mice to control pulmonary infection following transfer of *Mtb* infected *Alox5*$^{-/-}$ macrophages must be attributed to an intrinsic property of *Alox5*$^{-/-}$ macrophages or a unique interaction between *Alox5*$^{-/-}$ macrophages and the innate immune system [11].

One conceivable explanation for the role of PGE_2 in fostering membrane repair is that PGE_2 is required for the generation of SYT7, the lysosomal Ca^{++} sensor essential for plasma membrane repair. Virulent *Mtb* stimulate LXA_4 production in macrophages, which inhibits PGE_2 production by down regulation of COX2 mRNA accumulation [12]. Indeed we find that in contrast to LAMP1 expression, SYT7 transcription is specifically induced by PGE_2. Likewise, *Alox5*$^{-/-}$ macrophages infected with virulent *Mtb* express more SYT7 than WT or *Ptges*$^{-/-}$ macrophages [11]. Although it is not known how PGE_2 modulates SYT7 expression, collectively these data indicate that PGE_2 is an essential mediator of SYT7 expression and is therefore of critical importance for the prevention of necrosis and induction of apoptosis. Cumulatively, these studies show that the balance of PGE_2 and LXA_4 production by infected macrophages affects the outcome of infection in the microenvironment of the lung (Fig. 2).

6 The Fate of *Mtb*-Infected Macrophages Determines Cross-Presentation of Mycobacterial Antigens

As elegantly discussed in Chap. 8 by Dr. Behar, an alternate possibility is that phagocytosis of dying infected macrophages leads to acquisition of bacterial antigens by DC, as has been shown for influenza and Listeria [65, 66]. The relevance of these processes to mycobacterial antigen presentation was first

investigated by Schaible et al. [67]. Extracellular vesicles derived from infected DC and macrophages were identified that were free of viable bacteria but contained mycobacterial lipids and proteins. The origin of these vesicles was not entirely clear, but they appear to be apoptotic blebs or possibly exosomes. While infected macrophages were not efficient to directly stimulate CD8+ T cells, their co-culture with uninfected DC led to the transfer of mycobacterial antigens to DC, which became competent to cross-present the antigens to CD8+ T cells. Presentation was TAP-1-dependent and required an intact class I MHC pathway. Thus, the antigenic cargo contained in these vesicles could be cross-presented by DC to CD8+ T cells. As these studies were done with previously activated T cells, the observed T cell activation was not true cross-priming but would be more accurately categorized as cross-presentation. Nevertheless, the uptake of antigen-containing vesicles by DC provides a mechanism by which uninfected DC can acquire *Mtb* antigens and prime naïve T cells.

Winau et al. used similar vesicles purified from BCG-infected murine macrophages to immunize mice [68]. Again, the purified apoptotic bodies contained bacterial antigens but no bacteria. CD8+ T cell priming was observed and required an intact class I MHC pathway. Successful T cell priming was associated with DC homing to the tissue sites where the purified vesicles were injected. Interestingly, initiation of the endosomal processing pathway abrogated CD8+ T cell priming—a feature that may be unique to cross-presentation of class I MHC-restricted peptides. The generation of CD8+ T cell responses in naïve mice indicates that true cross-priming occurred. Remarkably, not only did a CD8+ T cell response develop, but also vaccination with the vesicles generated immunity that protected mice against challenge with virulent *Mtb*.

The studies by Schaible and Winau provide the foundation for the "Detour Model" as proposed by Kaufmann [69]. They convincingly show that the mycobacterial antigens contained in purified vesicles are taken up by both human and murine DC and enter the class I MHC pathway. However, these studies fall short of demonstrating whether apoptosis of infected macrophage is required for the transfer of antigens to DC and whether this process occurs in vivo indicating physiological significance. Additionally, the apoptotic vesicles used in the studies by Winau et al. and Schaible et al. were derived from BCG-infected macrophages [68, 69] and it is not clear whether infection of macrophage with wild-type virulent *Mtb* would lead to apoptosis and enhanced T cell immunity. Finally, while immunization with purified vesicles cross-primes antigen-specific T cells, it is uncertain whether the generation of vesicles from infected macrophages is required for CD8+ T cell priming in vivo. The finding that *Mtb* infected DC traffic from the lung to the regional LN with kinetics mirroring T cell priming could be consistent with *Mtb*-infected DC directly priming *Mtb*-specific T cells and could indicate the existence of a priming pathway independent of the "Detour Pathway" [69]. Thus, the role of apoptosis and cross-priming in the generation of adaptive immunity during virulent *Mtb* infection remained an important unanswered

question. To confirm the existence of these pathways and to begin to elucidate their relevance, a better understanding of the host factors regulating cell death during *Mtb* infection was required.

7 The Role of Eicosanoids in Apoptosis-Mediated Cross-Presentation

Eicosanoids have been identified as important host lipid mediators that regulate inflammation and susceptibility following mycobacterial infection. One effect of eicosanoids is the regulation of cell death in both human and murine macrophages infected with *Mtb* [11, 12]. As discussed above, prostanoids such as the host lipid mediator PGE_2 induce plasma membrane repair and prevent mitochondrial damage; together these events protect infected macrophages against necrosis and instead promote apoptosis. Importantly, products of 5-lipoxygenase including LXA_4 are produced by macrophages after infection with virulent *Mtb*. LXA_4 inhibits COX-2 activity, which shuts down prostaglandin synthesis. As predicted, macrophages from mice that lack 5-lipoxygenase, produce prostaglandins even after infection with virulent *Mtb* and undergo more apoptosis than necrosis. Interestingly, *Alox5*$^{-/-}$ mice are more resistant to *Mtb*. Studies from Bafica et al. found that a more pronounced Th1 cytokine response is detected in the lungs of infected *Alox5*$^{-/-}$ mice compared to WT controls mice [64].

In order to determine whether apoptotic macrophages contribute to adaptive immunity, we established a novel adoptive transfer model in which macrophages from wild-type or knockout mice were infected in vitro with *Mtb* and then transferred by intra-tracheal instillation into normal recipient mice. This strategy was used to determine whether the macrophage genotype influences the T cell response and control of infection [40]. By using knockout macrophages that are prone to undergo either apoptosis (e.g., *Alox5*$^{-/-}$) or necrosis (e.g., *Ptges*$^{-/-}$) following infection, we determined how these two different cellular fates alter the course of infection in vivo. One advantage of this adoptive transfer infection model is that the development of tuberculosis occurs in a developmentally normal host with an intact immune system, which avoids the pitfalls of studying *Mtb* infection in knockout mice in which the genetic lesion affects multiple cell types and physiological processes.

We used the CD8+ T cell response to TB10.4, a mycobacterial antigen that elicits an immunodominant response following low dose aerosol *Mtb* infection, to track the CD8+ T cell response following intra-tracheal transfer of *Mtb*-infected macrophages [40]. An earlier TB10.4-specific CD8+ T cell response was detected both in the draining pulmonary LN and in the lung following transfer of pro-apoptotic macrophages compared to wild-type macrophages. Importantly, the cellular fate of the infected macrophages was crucial; pre-treatment of the pro-apoptotic macrophages with inhibitors of caspase 8 and caspase 9, which

prevented apoptosis of the infected macrophages, abrogated the enhancement of the CD8+ T cell response [40].

To determine how the *Mtb*-infected macrophages enhanced the CD8+ T cell response, the infected macrophage adoptive transfer model was adapted for use with OT-I TCR-transgenic mice (carry a transgenic CD8 T cell receptor (TCR) for the MHC class I-restricted $OVA_{257-264}$ peptide), so early events in T cell priming could be easily assessed. Similar to the intra-tracheal adoptive transfer of *Mtb*-infected macrophages, OT-I CD8+ T cell priming was detected earlier after the transfer of *Mtb*-infected OVA-pulsed *Alox5*$^{-/-}$ macrophages compared to wild-type macrophages. Importantly, the infected macrophages did not directly activate CD8+ T cells; instead, T cell priming required endogenous DC, since DC depletion abrogated OT-I CD8+ T cell expansion. Similar to the results of Winau et al. [68], CD8+ T cell priming required TAP-1 and an intact class I MHC pathway. These experiments show that CD8+ T cell priming requires cross-presentation of antigen acquired by DC from apoptotic *Mtb*-infected macrophage via the detour pathway. In addition, after the transfer of *Mtb*-infected pro-apoptotic *Alox5*$^{-/-}$ macrophages, not only was there an earlier and more robust *Mtb*-specific CD8+ T cell response, but the CD4+ T cell response to ESAT6 and Ag85B was also enhanced [40]. This may not be too surprising if DC phagocytosis of apoptotic vesicles transfers their cargo of *Mtb* antigens to the endocytic system, which intersects with the MHC II processing pathway. However, the mechanisms that govern this potential transfer have yet to be elucidated. Thus, while apoptosis has been directly linked to increased CD8+ T cell responses via cross-presentation, it also enhances class II MHC-restricted antigen presentation. This has important implications for the finding that vaccination with apoptosis-inducing bacterial vaccines or apoptotic vesicles induces protection against virulent *Mtb*: namely, the protective immunity elicited may be due to a combination of *Mtb*-specific CD4+ and CD8+ T cells.

Moreover, the pro-apoptotic mutants of *Mtb* prime a greater T cell response and enhance host control of infection [7]. This has generated considerable interest in whether pro-apoptotic mutants of *Mtb* could be used as a vaccine strategy. For example, vaccination with attenuated BCG or *Mtb* that induce greater macrophage apoptosis or with purified apoptotic bodies [68] may stimulate an enhanced T cell response. Collectively, these studies have provided important evidence that during pulmonary *Mtb* infection apoptosis of infected macrophages: (1) leads to innate control of early bacterial growth; and (2) acts as a reservoir of antigen that facilitates initiation of acquired T cell immunity via cross-priming by DC.

8 Conclusions

The finding that macrophages infected with virulent *Mtb* undergo necrosis while macrophages infected with attenuated mutant strains of *Mtb* undergo apoptosis, suggests that wild-type *Mtb* actively inhibits apoptosis. This forms the foundation

for the concept that apoptosis is an innate macrophage defense mechanism. Apoptosis is associated with a reduction in the viability of intracellular *Mtb* and provides an important link to the establishment of T cell immunity. Investigation of the interaction between *Mtb* and macrophages finds that three distinct mechanisms contribute to macrophages necrosis. First, *Mtb* inhibits plasma membrane repair. Second, virulent *Mtb* causes inner mitochondrial membrane damage. Third, *Mtb* inhibits generation of the apoptotic cellular envelope. These three effects predispose the infected macrophages to necrosis. In part, these events occur because virulent *Mtb* inhibits the production of PGE_2, a prostaglandin that is important for stimulation of membrane repair and protection of the mitochondrion. However, it is also important to note that some investigators have found that PGE_2 can impair immunity to other bacterial infections [70, 71] or Influenza viral infection (Divangahi, unpublished observation). Thus, how virulent *Mtb* subvert eicosanoid biosynthesis to alter the death modality of macrophages to foil both innate and adaptive immunity is an important area for future investigation. Given our capacity to manipulate eicosanoid-pathways, a better understanding of how their regulation is altered by mycobacteria may lead to novel approach to intervene therapeutically as well as to develop immunomodulatory strategies that can enhance vaccine efficacy.

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Cytokines in the Balance of Protection and Pathology During Mycobacterial Infections

Egídio Torrado and Andrea M. Cooper

Abstract The outcome of natural infections with pathogenic mycobacteria can range from early asymptomatic clearance through latent infection to clinical disease. Different host and pathogen-specific factors have been implicated in determining the outcome of these infections; however, it is clear that the interaction of mycobacteria with the innate and acquired components of the immune system plays a central role. Specifically, the recognition of mycobacterial components by innate immune cells through different pathogen recognition receptors (PPRs) induces a cytokine response that can promote early control of the infection. In fact, in the majority of individuals that come into contact with mycobacteria, this response is enough to control the infection. Among PRRs, Toll-like receptors (TLRs), Nucleotide Oligomerization Domain (NOD)-like receptors, and C-type lectins have all been implicated in recognition of mycobacteria and in the initiation of the cytokine response. Defining the mechanisms by which distinct mycobacterial components and their receptors stimulate the immune response is an area of intense research.

Keywords Cytokines · Innate cytokine response · *Mycobacterium tuberculosis* · IFN-producing T cells · Mycobacterial infection · T cell response · Macrophages · Cell death · Granulocytes · Tumor necrosis factor · Eicosanoids · CD4 T cells · Cell survival · Phagocytes · Pulmonary fibrosis

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E. Torrado · A. M. Cooper (✉)
Trudeau Institute, Inc, 154 Algonquin Ave., Saranac Lake, NY 12983, USA
e-mail: acooper@trudeauinstitute.org

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1 Introduction

The outcome of natural infections with pathogenic mycobacteria can range from early asymptomatic clearance through latent infection to clinical disease. Different host and pathogen-specific factors have been implicated in determining the outcome of these infections; however, it is clear that the interaction of mycobacteria with the innate and acquired components of the immune system plays a central role. Specifically, the recognition of mycobacterial components by innate immune cells through different pathogen recognition receptors (PPRs) induces a cytokine response that can promote early control of the infection. In fact, in the majority of individuals that come into contact with mycobacteria, this response is enough to control the infection. Among PRRs, Toll-like receptors (TLRs), Nucleotide Oligomerization Domain (NOD)-like receptors, and C-type lectins have all been implicated in recognition of mycobacteria and in the initiation of the cytokine response. Defining the mechanisms by which distinct mycobacterial components and their receptors stimulate the immune response is an area of intense research.

The innate cytokine response is critical to determining the subsequent acquired immune response, which is essential to pathogen control once the infection is established. It is thought that T helper (Th)1 cells, characterized by the secretion of Interferon (IFN)γ, are key in the control of mycobacterial infections. However, the magnitude of the Th1 response does not always correlate with bacterial clearance or increased resistance. Th17 cells and regulatory T cells (Tregs) are also induced upon infection; however, their role in the protective immune response is still under investigation. Furthermore, the chronic nature of mycobacterial infection results in constant activation of the immune response, which eventually leads to the development of granulomatous structures that allow both containment and transmission of the disease. While we do not fully understand the mechanisms that underlie the generation of the granuloma, cytokines are known to play a central role in the initiation and maintenance of these structures and thus have a critical impact on both the generation of protective immunity and the development of pathological consequences. Therefore, it is important that we define the role and mechanisms of action of different cytokines at different stages of mycobacterial infection. This knowledge will improve our understanding of how host resistance is induced, maintained, and regulated, and provide a basis for potential prophylactic and therapeutic interventions.

In this chapter we will provide an overview of the roles of the major cytokine families that are produced during the innate and acquired immune response to mycobacteria, in particular, to *Mycobacterium tuberculosis*. The modes of action of these cytokines and their impact on the control of infection and development of pathological consequences will also be discussed.

2 The IL-12 Family: Initiators and Regulators of Acquired Immunity

The IL-12 family of cytokines comprises 4 heterodimeric members: IL-12p70, IL-23, IL-27 and the newest member of the family, IL-35. These cytokines share homology at the subunit, receptor, and signaling levels and play distinct roles in the generation and maintenance of acquired immune responses to mycobacteria. Both IL-12p70 and IL-23 share the IL-12p40 subunit that is covalently bound to IL-12p35 to form IL-12p70 or to IL-23p19 to form IL-23. On the other hand, IL-27 is formed by the association of Epstein-Barr Virus-Induced gene 3 (EBI3) and the subunit IL-27p28. While EBI3 resembles IL-12p40 and IL-27p28 is related to IL-12p35, EBI3 and IL-27p28 are not covalently bound and they can be secreted by different cells to heterodimerize in the extracellular compartment. This difference can have important implications in the biological activity of the single subunits as it was recently shown that IL-27p28, when not bound to EBI3, can act as an antagonist for IL-6 signaling [1]. Finally, IL-35 is formed by the association of IL-12p35 and EBI3 subunits. This cytokine is the most recent member of the IL-12 family and its function is still under scrutiny. However, recent data point to an immunoregulatory role of IL-35, as regulatory T cells (Tregs) seem to be an important source of this cytokine [2].

2.1 The IL-12 Family in the Initiation of Acquired Immunity to Mycobacteria

Upon infection with *M. tuberculosis*, bacilli are deposited in the lower airways where they are thought to be initially phagocytosed by resident myeloid populations, including dendritic cells (DCs) and macrophages. In the mouse aerosol model of infection, *M. tuberculosis* grows in an unrestricted manner for the first 20 days of infection, followed by growth arrest corresponding to the accumulation of IFNγ-producing T cells in the lung [3–5]. Recent data suggest that the T cell response is initiated in the draining lymph node (dLN) of the lung between days 7 and 10 post-infection [6–8]. Since the lung is the main site of infection, it is likely that the DCs that pick up bacteria or antigen migrate from the lung to the dLN where they present antigen and initiate the T cell response (Fig. 1) [9]. In the dLN, both IFNγ- and IL-17-producing T cells are induced and these cells then migrate to the lung where they exert effector function.

IL-12p70 is the critical factor that drives the generation of IFNγ-producing T cells that are thought to be essential for bacterial control [10]. Indeed, deficiencies in the IL-12 or IFNγ signaling pathways have been associated with human susceptibility to tuberculosis [11]. Similarly, in the mouse model, the absence of IL-12p35 (and therefore IL-12p70) is associated with increased susceptibility to infection, corresponding with a significant reduction in the number of antigen-specific, IFNγ-producing T cells in the lung [12]. However, these mice still have an adaptive

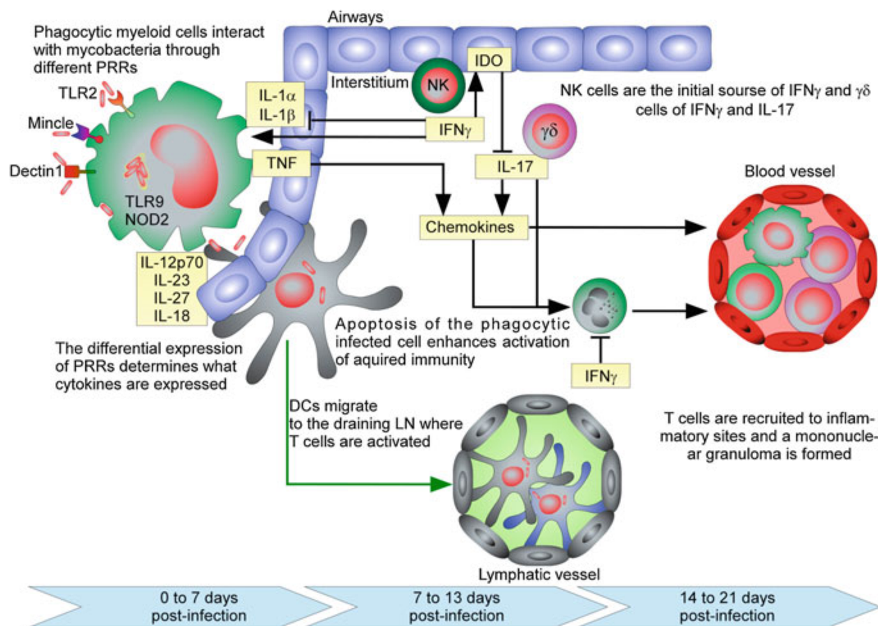

Fig. 1 Cytokines act during the initiation of the immune response and after *M. tuberculosis* infection has been established. At very early stages of infection (0–7 days in the mouse model) the interaction of mycobacteria or mycobacterial products with myeloid phagocytic cells through distinct PRRs induces the expression of innate cytokines. TNF and other inflammatory cytokines promote the expression of chemokines that recruit inflammatory cells to the infection foci. Between days 7 and 13 of infection, after DCs migrate to the draining lymph node and T cells are activated. At the infection site, innate source of IL-17 and IFNγ counter-regulate each other. While IL-17 recruits neutrophils, IFNγ regulates the response of the interstitium to promote a regulatory environment, possibly dependent upon induction of IDO. From day 14 onwards the inflammation generated at the site of initial infection attracts newly activated T cells that, upon accumulation restrain bacterial growth in IFNγ- and TNF-dependent and independent ways

IFNγ response, which is virtually nonexistent in mice that lack both IL-12p35 and IL-23p19 (IL-12p70 and IL-23 double-deficient mice) or IL-12p40 [12, 13] suggesting that, IL-23 can compensate for the absence of IL-12p70 and induce IFNγ-producing T cells. This response is not potent enough to maintain long-term control of the infection, supporting an important role of IL-12p70 in maintenance of long-term Th1 responses. The main role for IL-23 during mycobacterial infection is the maintenance of the IL-17 response [14]. Indeed, mice deficient in IL-23p19 are unable to maintain IL-17-producing cells or IL-17 mRNA expression in the lung throughout infection [13]. However, there is still an IL-17 response in the absence of IL-23p19 in the dLN suggesting that, as in other models of disease, IL-23 is not required to initiate the Th17 response but it is critical to sustain it [15, 16].

Unlike IL-12p70 and IL-23, IL-27 appears to play mostly a regulatory role during mycobacterial infections. IL-27 was originally described as a Th1 differentiating

factor in vitro [17]; however, in vivo, in distinct infection models, including the *M. tuberculosis* aerosol infection model, the absence of IL-27 activity does not significantly compromise the generation of protective IFNγ-producing cells [18–21]. In contrast, during *M. tuberculosis* infections in the absence of IL-27 signaling, T cells express less IFNγ on a per cell basis [18], suggesting that, IL-27 may be critical to maximize IFNγ production by these cells. Interestingly, mice deficient in IL-27 signaling are more resistant to *M. tuberculosis* infection [18, 19]. Since IL-27 has been shown to inhibit Th17 differentiation [22] and to induce IL-10 production from activated T cells [23, 24], it is likely that, during *M. tuberculosis* infections, IL-27 function is to suppress excessive T cell activation. Indeed, although bacterial burdens are reduced in mice that lack IL-27 signaling, these mice also display greater inflammatory responses and reduced survival when compared to wild-type mice [19]. It will be important to further dissect the mechanisms whereby IL-27 activity reduces protection against *M. tuberculosis* as this may be a pathway induced to tolerate the pathogen in order to protect the infected organ from immunopathological consequences.

2.2 *Impact of IL-12-Related Cytokines in the Chronic T Cell Response*

Chronic exposure to IL-12 and IL-23 in the site of infection can have an important impact in the T cell response, specifically in circumstances where these cytokines are overexpressed. Indeed, it has long been known that repeated exposure to high level of antigens in *M. tuberculosis*-infected hosts can lead to exacerbated inflammatory responses, known as the Koch Phenomenon. Recently, it was shown that the local expression of IL-23 is important for this response, as this cytokine promotes further expansion of the ongoing IL-17 response and shifts the chemokine profile resulting in enhanced granulocytic inflammation without impacting the IFNγ protective response [25]. In line with these data, it was also recently shown that IFNγ signaling by lung stromal cells is critical to regulate IL-17-dependent immunopathology during tuberculosis in mice [26]. Indeed, stromal cells that lack the ability to signal IFNγ have impaired expression of the enzyme indoleamine 2,3-dioxygenase (IDO) and a reduced accumulation of by-products of the tryptophan catabolism [26]. These products were shown to reduce the immunopathological consequences caused by the *M. tuberculosis* infection, by restraining the IL-23-dependent IL-17 response [26]. These data show that IL-23 plays an important role in the maintenance of the IL-17 response at the site of infection and that elevated expression of IL-23 further expands the ongoing IL-17 response, causing extensive recruitment of neutrophils with important pathological consequences. IFNγ appears to play a central role in the regulation of these responses, both directly in the differentiation of IL-17-producing cells [27] and indirectly by inducing IDO activation by non-hematopoietic cells (Fig. 2) [26].

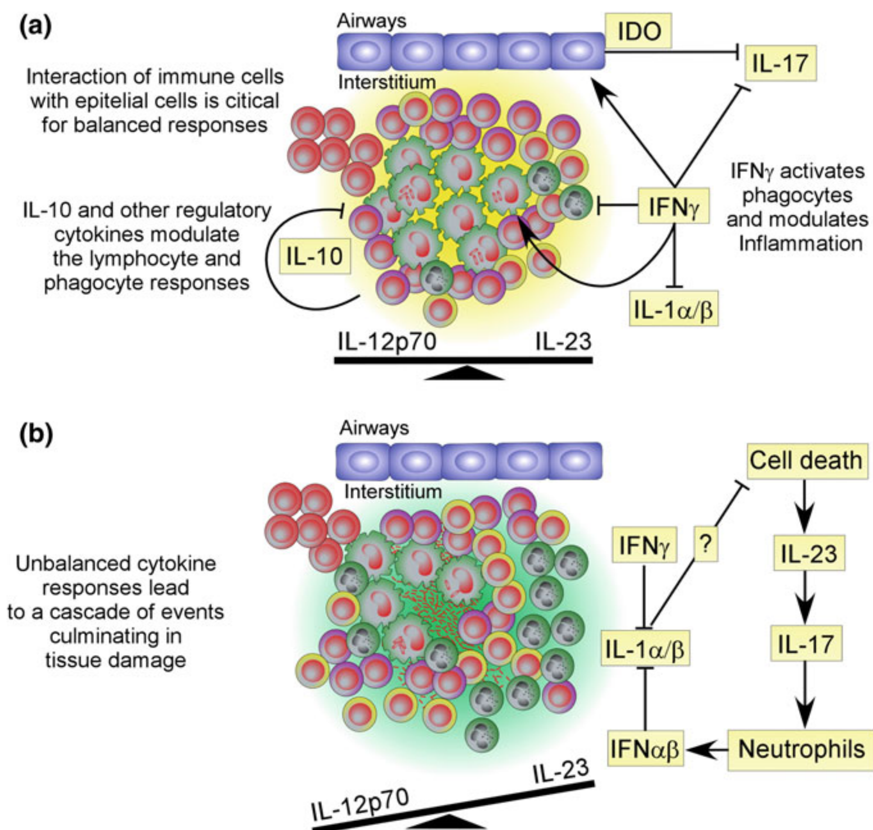

Fig. 2 Balanced cytokine responses during chronic mycobacterial infection limits tissue damage. **a** During chronic infection, IFNγ activates phagocytic cells and modulates the inflammatory environment by regulating IL-17 and IL-1 from inflammatory macrophages while IFNα/β regulates IL-1 in a more general manner. IL-10 produced by highly activated T cells and Tregs limits lymphocyte and phagocyte responses. **b** When the cytokine balance is shifted or excessive cell death occurs (absence of IL-1 or high antigen availability), elevated IL-23 expression can enhance ongoing IL-17 responses, culminating in excessive the neutrophil recruitment and tissue damage

Overall, these data show that the relative balance of each IL-12 family member during T cell priming and at the site of infection can have important implications for disease control and pathological consequences (Fig. 2). Understanding how these cytokines are regulated during infection will have important implications in our ability to modulate the immune response to promote bacterial control with minimal pathological consequences.

3 The IL-1 Cytokine Family: Mediators and Regulators of Inflammation

The IL-1 family of cytokines comprises 11 members of which IL-1α, IL-1β, IL-18, and IL-33 have been studied during mycobacterial infections. Recent data support an important role for both IL-1α and IL-1β during *M. tuberculosis* infections, whereas the role of IL-18 is still under investigation. On the other hand, IL-33 is a Th2-related cytokine with little impact in the immune response to mycobacteria as demonstrated by the similar inflammatory response and bacterial burdens of mice deficient in the IL-33 receptor chain *st2* and wild-type mice [28].

3.1 Regulation of IL-1β Production During Mycobacterial Infection

IL-1β signaling is mediated through the adaptor molecule MyD88, shared by most TLRs. Interestingly, the first observations regarding the high susceptibility of MyD88-deficient mice to mycobacteria were interpreted as a requirement for TLR signaling [29, 30]. However, mice deficient in different TLRs were not as susceptible to mycobacterial infection as MyD88 deficient mice. Recently, the susceptibility of IL-1β and IL-1R-deficient mice was shown to be indistinguishable from that of MyD88-deficient mice, suggesting that, the signals conveyed by MyD88 that required for host survival during tuberculosis are from the IL-1R [31, 32].

Mycobacterium tuberculosis is a strong inducer of both IL-1α and IL-1β at the site of infection [31, 32]. Unlike IL-1α, IL-1β is produced in the form of a pro-cytokine, i.e., in a non-active form. In order to become active, a multi-protein complex known as the inflammasome is required to trigger the activation of caspase-1, the enzyme that converts pro-IL-1β into mature IL-1β [33]. In vitro, macrophages produce mature IL-1β through the NOD-like receptor family, pyrin domain containing 3 (NLRP3)-inflammasome-mediated caspase-1 activation [34–37]. Interestingly, the ESAT-6 secretion system 1 (ESX-1), a system that mediates the secretion of virulence factors encoded by the region of difference 1 (RD1), is required for mature IL-1β secretion [34, 37]. Accordingly, macrophages and DCs that are deficient in the apoptosis-associated spek-like protein containing a caspase recruitment domain (ASC), a critical component of the NLRP3 inflammasome, are unable to secrete IL1β upon stimulation with *M. tuberculosis* in vitro [31, 34, 35, 37]. However, mice deficient in caspase 1, ASC or NLRP3 do not show the same susceptibility to *M. tuberculosis* as IL-1β or IL-1R-deficient mice [31, 35] suggesting that, there are alternative pathways for IL-1β cleavage in vivo.

Recent data show that the recognition of some species of mycobacteria though Dectin 1 triggers the activation of a non-canonical caspase-8-dependent inflammasome resulting in the processing of pro-IL-1β [38]. Indeed, the release of IL-1β

by in vitro cultured DCs in response to *Mycobacterium leprae* was completely dependent on this pathway, whereas *M. tuberculosis* stimulated the processing of pro-IL-1β via the canonical caspase-1 pathway [38]. It is likely that, in vivo, *M. tuberculosis* induces the cleavage of pro-IL-1β through an inflammasome-independent, or caspase-1- and caspase-8-independent mechanism. Candidate cleavage enzymes include other caspases, chymases, cathepsins, and elastases [39]. Recent data suggest that inflammatory monocytes/macrophages and DCs are the major sources of both IL-1α and IL-1β in the lungs of *M. tuberculosis* infected mice [32]. It will be important to determine the mechanisms that lead to the cleavage of pro-IL-1β by these populations in vivo and the effector function of these cytokines in the immune response to mycobacteria.

3.2 The Role of IL-1α and IL-1β in Mycobacterial Control

Studies conducted in gene-deficient mice clearly support a critical role for IL-1α and IL-1β in the immunity to mycobacteria. However, the mechanism by which these cytokines impact bacterial control and ensure host survival is still not completely understood. It is clear that the high susceptibility of mice deficient in IL-1α, IL-1β or IL-1R to *M. tuberculosis* is not associated with impaired Th1 responses [31]. IL-1β was previously implicated as a cofactor for the generation of Th17 cells, and it is likely that this is also the case during mycobacterial infections. Indeed, it was shown that *M. tuberculosis*-induced IL-17 response by human cells was strongly dependent on IL-1β signaling [40]. In this system, TLR4 and dectin 1 were the main receptors responsible for mediating IL-17 production [40]. Whether or not IL-1β is critical for Th17 generation in vivo, these data do not explain the high susceptibility of IL-1β-deficient mice to *M. tuberculosis,* as the absence of IL-17 does not significantly impact control of infection [41]. On the other hand, extensive granuloma necrosis is a hallmark of *M. tuberculosis* infection in IL-1β and IL-1R-deficient mice [31], and it is possible that in vivo, IL-1 activity regulates host resistance by modulating cell death (Fig. 2b).

As mentioned above, IL-1α does not need proteolytic cleavage and can function as a nuclear transcription factor. In viral models, it has been suggested that the constitutive expression of IL-1α is critical for the antiviral effects of IFNγ [42]. During tuberculosis, IFNγ is critical to induce the expression of *nos2* to produce nitric oxide and restrain bacterial growth (discussed below). As mice deficient in IL-1α, IL-1β or IL-1R have equivalent expression of effector molecules important to control *M. tuberculosis* as wild-type mice [31, 32], it is likely that the IL-1 cytokines act in concert with other inflammatory mediators to regulate inflammation and induce bacterial growth arrest by an, as yet, undefined mechanism.

Finally, the role of IL-1 signaling in human disease is supported by different studies suggesting associations of polymorphisms in the *Il1* or *Il1r1* genes with susceptibility to tuberculosis [43, 44]. Therefore, it is important that we continue investigating the role of IL-1 during mycobacterial infections. Specifically, if IL-1

is as central in controlling *M. tuberculosis* in humans as it is in mice, blockade of IL-1 may have important implications in tuberculosis progression or reactivation.

3.3 The Role of IL-18

IL-18 like IL-1β is produced as a pro-cytokine that was originally identified as a Th1 differentiating factor [45]. Early reports in the mouse model of tuberculosis confirmed the role for IL-18 in the IFNγ response, as mice deficient in IL-18 had lower IFNγ responses when compared to wild-type mice [46, 47]. Despite this, the control of *M. tuberculosis* bacterial burdens was only modestly impaired in the absence of this cytokine [46, 47].

On the other hand, in a more recent study it was shown that mice deficient in IL-18 were extremely and acutely susceptible to aerosol infection with *M. tuberculosis*, to a similar extent as MyD88 and IL-1β-deficient mice [48]. As shown in the earlier studies, there was a reduced IFNγ response in the absence of IL-18, corresponding to a reduced expression of effector genes downstream IFNγ, and an elevated accumulation of granulocytes [48]. It is intriguing, however, that mice deficient in the IL-18 receptor were not more susceptible to *M. tuberculosis* [48]. The discrepancy in terms of susceptibility in the absence of IL-18 versus its receptor suggests redundancy in receptor usage. However, this hypothesis requires experimental investigation.

While these data suggest a potentially important role for IL-18 in the control of *M. tuberculosis*, it is possible that the different susceptibility observed in different studies is related with the dose of infection and/or virulence of the *M. tuberculosis* strain. It will be important to determine the factors behind these differences, as IL-18 may have a central, not yet identified role in the protective immune response to *M. tuberculosis*.

4 Tumor Necrosis Factor: From Phagocyte Activation to Granuloma Formation

The potential for using TNF blocking agents to treat inflammatory diseases, such as rheumatoid arthritis, has reinvigorated the interest in the role of TNF during intracellular infections, as in some cases TNF blockade caused reactivation of tuberculosis [49]. Indeed, mice deficient in this cytokine, together with mice deficient in IFNγ or IL-12p40 are the most susceptible to *M. tuberculosis* infection. The most striking characteristic of TNF deficiency is poor phagocyte activation; however, the mechanisms underlying the activity of TNF during mycobacterial infections are not restricted to phagocyte activation, but also to a deficiency in chemokine expression with important implications in granuloma organization [50].

The complex role of TNF during mycobacterial infections may, in part, be associated with the different forms of the cytokine and the receptors it engages.

Indeed, TNF is produced primarily as a type II transmembrane protein that can be cleaved by the metalloprotease TNF alpha converting enzyme to become soluble TNF. Both soluble and transmembrane TNF bind to the TNFRI, whereas the TNFRII can only be fully activated by transmembrane TNF. Both receptors can transduce pro-inflammatory and anti-apoptotic signals by activating the NF-κB pathway and the mitogen-activated protein kinase. However, TNFRI can also transduce apoptotic and anti-inflammatory signals by recruiting the Fas-associated death domain and caspase 8.

It has been shown that mice that are deficient in the soluble form of TNF can control acute infections by *M. tuberculosis*; however, long-term control requires the full complement of TNF activity [51]. TNF-deficient mice are also more susceptible and succumb earlier to *Mycobacterium avium* infections; however, the reduced survival of these mice is associated with an exacerbated immune response that is characterized by an elevated accumulation of IFNγ-producing T cells and disintegration of the granuloma [52]. It will be important to determine whether this is caused by absence of TNF signaling in the myeloid population, or directly on the T cells, as it is likely that TNF also acts on the T cells in order to regulate their function and immunopathological potential.

Eicosanoids have been shown to have an impact in the TNF pathway, and therefore in the control of infection. A recent study shows that mutations in the *lta4h* locus encoding leukotriene A_4 hydrolase, which catalyzes the final step in the synthesis of leukotriene B_4, were extremely susceptible to mycobacterial infection, caused by a redirection of eicosanoid substrates to anti-inflammatory lipoxins [53]. The resultant anti-inflammatory state permits increased mycobacterial proliferation by limiting the production of TNF [53]. These data highlight the importance of innate-derived TNF in the control of mycobacterial infections in the absence of acquired immunity.

5 The IFN Cytokine Family: Effectors and Regulators of Acquired Immunity

IFNs have long been recognized as important mediators of immunity to mycobacteria. Of extreme importance is the type II IFN, IFNγ, which is essential to activation of phagocytic cells to kill mycobacteria. However, the role of IFNγ during mycobacterial infections is not as straightforward as it appears to be.

5.1 CD4 T Cells as the Main Cellular Source of IFNγ

Antigen-specific CD4 T cells are thought to be the most important source of IFNγ in vivo. Indeed, upon aerosol infection with *M. tuberculosis* bacterial control correlates with the accumulation of IFNγ-producing CD4 T cells into the lung [5]. However, there is still no experimental proof that IFNγ production by CD4 T cells,

is required to control bacterial proliferation [54]. Recent data suggest that, CD4 T cells unable to secrete IFNγ are equally capable of inducing bacterial control as wild-type CD4 T cells [55]. In fact, antigen-specific CD4 T cells within the lungs of *M. tuberculosis* infected mice produce very low amounts of IFNγ, even at the peak of the response [56, 57]; yet, bacterial control is maintained for long periods of time. It is likely that the cause for this apparent inability of the antigen-specific cells to secrete IFNγ is a very low level of cognate antigen. Indeed, it has been shown that the frequency of IFNγ-producing CD4 T cells correlates with the expression of the cognate antigen by *M. tuberculosis*, and that delivery of the cognate peptide results in greatly increased frequency of IFNγ production [56]. The location of the T cells in the infection site is also important to induce bacterial control. During mycobacterial infection, it was recently shown that both antigen-specific and nonspecific cells migrate very rapidly through the granuloma, with very few cells showing migration arrest, a hallmark of antigen recognition and presentation [57]. In combination with the observation that antigen-specific cells express low levels of cytokine in real time, these data suggest that antigen availability at the infection site is limited and this limits migration arrest and cytokine production [57].

These data suggest that the classical mechanism in which T cell-derived IFNγ activates the macrophage to restrain *M. tuberculosis* growth may not provide the full mechanism of control. There is also the potential for T cells to mediate their effector function independently of cytokine-secretion. As our ability to measure effector function is limited by measuring bacterial arrest, we cannot exclude the possibility that the appropriate effector functions are being expressed, and this does not require migration arrest or cytokine production.

5.2 The Impact of IFNγ in Cell Survival in the Inflammatory Environment

If T cells are capable of limiting bacterial growth, even when they are unable to secrete IFNγ, why is that both mice and humans with deficiencies in the IFNγ signaling pathway are so susceptible to tuberculosis? The hallmark of *M. tuberculosis* infection in IFNγ-deficient mice is the accumulation of polymorphonuclear granulocytes in the infection site [58]. IFNγ is known to limit the IL-17 response during mycobacterial infections [26, 27] and thus IFNγ also inhibits the inflammatory programs initiated by IL-17 that culminate in neutrophil influx to the infected lungs [59]. In a recent study, IFNγ was also shown to have a direct and negative impact in the survival of neutrophils in the infected lung [59]. Also, in *M. avium* and *M. leprae* infected IFNγ-deficient mice show mild increases in bacterial burdens, but have robust granulocyte recruitment to the infection site [60]. As neutrophil accumulations are associated with a poor disease outcome, these data suggest that neutrophilic lesions during tuberculosis are probably caused by impaired IFNγ responses or IFNγ signaling and predispose to poor bacterial control.

The impact of IFNγ in cell survival is not restricted to neutrophils. Indeed, IFNγ has also an important impact in the survival of CD4 T cells in the inflammatory environment. This is demonstrated in the mouse model of *M. avium* infections, known to induce strong lymphocyte depletion during the chronic stages of infection which is mediated by IFNγ [61]. However, it is still not clear whether these effects occur directly in the T cell or indirectly, via the induction of inflammatory mediators that are detrimental for the survival of T cells in the inflammatory environment.

While there has long been appreciation for IFNγ as a regulator of the inflammatory response during mycobacterial infection [60] this appreciation has increased dramatically with the recent papers demonstrating the mechanisms whereby IFN mediates these anti-inflammatory effects (Fig. 2). Keeping in mind both the antibacterial and the anti-inflammatory roles of IFNγ when considering interventions with this chronic inflammatory disease will be critical to successful outcomes.

5.3 The Immunoregulatory Properties of Type I IFNs

Contrary to IFNγ, type I IFNs appear to have a largely detrimental role during mycobacterial infections. Indeed, type I IFN receptor-deficient mice are more resistant to *M. tuberculosis* infection and display significantly reduced bacterial burdens during the chronic stage of infection when compared to wild-type animals [62]. Interestingly, the virulence of *M. tuberculosis* clinical isolates has been correlated with the induction of type I IFN, which was associated with impaired Th1 responses [63]. Furthermore, macrophages express type I IFN-associated genes and IFNβ in response to virulent *M. tuberculosis* but not to a less virulent strain with an inactive ESX-1 secretion system [64]. This response was found to be independent of the TLR adaptor TRIF and the adaptors for NOD1 and NOD2, but dependent on the activity of the TANK-binding kinase 1 [64], which are also necessary for type I IFN induction by *Listeria monocytogenes* [65].

In line with the detrimental role of type I IFN, it was recently shown that *M. tuberculosis*-infected mice treated with the type I IFN-inducer poly-nosinic-polycytidylic acid have exacerbated lung pathology and bacterial burdens [62]. The elevated susceptibility was not associated with impaired T cell responses, but with the accumulation of a myeloid population that was permissive to mycobacterial growth when compared to the same population isolated from non-treated mice [62]. Also recently, type I IFNs were shown to be strong inhibitors of IL-1α and IL-1β production by macrophages and DCs in the lungs of *M. tuberculosis* infected mice (Fig. 2) [32]. This inhibition was shown to occur directly in the IL-1α/β producing cell as, in the same environment, IL-1 α/β expression by *Ifnar1*-deficient cells was not affected [32]. As discussed above, IL-1 α and IL-1β cytokines are strong inflammatory mediators and thus it is likely that this is a negative feedback mechanism that prevents extensive generation of pathological consequences during infection.

Type I IFNs have also been suggested as important factors in determining the outcome of human tuberculosis. Indeed, a recent study shows that most patients with active tuberculosis display an expression signature associated with type I IFN genes in neutrophils [66]. This same profile is also present in some of the asymptomatic patients, suggesting that these are at higher risk to develop active tuberculosis [66].

Overall, the IFN family of cytokines appears to be critical to the outcome of mycobacterial infection with roles in containment of bacterial growth as well as regulation of immunopathological consequences (Fig. 2).

5.4 IL-17 and TH17-Related Cytokines

Early studies clearly established IL-17 as a critical cytokine in the protective immune response to rapidly growing extracellular pathogens with the protective response mediated by rapid neutrophil recruitment and tissue repair [67–69]. On the other hand, in infections caused by intracellular pathogens the role of IL-17 is not as clear. In some infection models, IL-17 appears to play a mostly protective role, however, not as dramatically as observed for extracellular pathogens. For instance, during *L. monocytogenes* infections, mice have increased bacterial burdens and defective granuloma generation in the absence of IL-17 [70]. As for mycobacteria, the data are equivocal as to whether IL-17 is required to control infection. In the low dose aerosol infection model, IL-17 is not required [41] although following slightly higher intratracheal infection with *M. tuberculosis* or BCG [71], IL-17 does have a protective role. This apparent discrepancy suggests that the circumstances of infection are critical for defining the role of IL-17. A possible mechanism for this impact may be that neutrophils have recently been implicated in limiting the early activation of acquired immunity to *M. tuberculosis* [72] suggesting that the level of IL-17, and potentially neutrophil recruitment, very early after infection may impact the ability of the bacteria to limit the initiation of immunity (Fig. 1).

As IL-17 acts mostly by inducing inflammatory programs associated with neutrophil recruitment, it is possible that in the low dose aerosol infection model IL-17 acts to maintain granuloma integrity independently of the protective immune response. Indeed, depletion of neutrophils later in *M. tuberculosis* infection has been shown to delay granuloma formation with little impact on bacterial burden [73]. Neutrophil-mediated regulation of granuloma formation has been shown to be mediated by CXCR3-ligating chemokines, specifically CXCL9 [73]. Indeed, neutrophils are an important source of this chemokine early after infection, and antibody blockade of CXCL9 results in defective granuloma formation [73]. It is thought that neutrophils and macrophages can co-operate to limit mycobacterial survival [74] and this may be via macrophage phagocytosis of apoptotic neutrophils [72]; although it appears that virulent mycobacteria may limit this process [72].

Further complexity in the role of IL-17 during mycobacterial infections may be associated with the Th17 related cytokine IL-22. In recent studies, it was shown that IL-22-deficient mice or IL-22 neutralization did not have a significant impact in the ability of mice to control infection [75, 76]. However, IL-22 can activate inflammatory programs similar to those activated by IL-17. Furthermore, in vitro and in vivo generation of Th17 cells can lead to the development of cell expressing only IL-17, IL-22 and cells expressing both cytokines [77]. This is important because, in the infected tissue, IL-17 and IL-22 may be secreted and act independently of each other and have redundant roles. Indeed, in healthy humans exposed to *M. tuberculosis*, IL-22-expressing CD4 T cells were reported to be distinct form Th17 and Th1 cells [78]. It will be important to address the redundancy of IL-17 and IL-22 during infection as they can be produced and act independently of each other.

5.5 IL-10 and Other Immunosuppressive Cytokines

As discussed above, the chronic nature of mycobacterial infections requires constant activation of the immune system in order to maintain control over bacterial proliferation. At the same time, it is important to control the inflammatory response to ensure survival of the host. In this respect, IL-10 may be important in the protective immune response to mycobacteria on two fronts (Fig. 2). On one hand, IL-10 has been shown to modulate the activity of phagocytes in the lung by negatively impacting their ability to secrete TNF and IL-12p40 [79, 80] and by blocking the maturation of the phagosome [81]. Indeed, mice strains that express high levels of IL-10 upon infection, such as the CBA, are naturally more susceptible to tuberculosis and when IL-10 activity is neutralized, these mice are better able to control *M. tuberculosis* [80, 82]. Recent data also suggest that IL-10 can have a negative impact in the recruitment of T cells into the lung by inhibiting the expression of T cell recruiting chemokines [82]. IL-10 may also act to prevent strong activation of T cells thereby ensuring their survival and possibly limiting immunopathological consequences. Interestingly, IL-27 has been shown to be a critical factor in induction of IL-10 by activated T cells [23, 24]. The role of IL-27 in inducing IL-10 is yet to be demonstrated during tuberculosis but as discussed before, mice deficient in IL-27 signaling are more resistant to infection, at the cost of greater inflammatory responses [18, 19].

Mycobacteria are strong inducers of Th1 immunity, but it is still not clear whether Th2 responses or Th2 derived cytokines can have a negative impact in the control of infection. In the mouse model of tuberculosis, IL-4 is very low or undetectable in the site of infection. In humans however, IL-4 can be detected in some lesions [83]. It is also relevant to understand the impact of Th2 responses in the context of mycobacterial infections, even when these responses are not

directed to mycobacterial antigens. In this respect, it was recently shown that pre-exposure of mice to the intestinal helminth *Nippostrongylus brasiliensis* is detrimental for the control of a subsequent aerosol infection with *M. tuberculosis* [84]. Indeed, the Th2 response induced by *N. brasiliensis* skewed macrophage activation to the alternative state, without affecting the protective T cell response [84]. Alternatively activated macrophages express high levels of the arginine hydrolytic enzyme arginase 1, which competes with iNOS for the same substrate, arginine, and impairs the production of nitric oxide [85]. In the above discussed co-infection model, arginase I was induced by IL-4 [84]; however, it has been shown that the expression of this enzyme can be induced in mycobacterial infections in a TLR-dependent way [85] or in settings where IL-10 expression is high [86].

While we generally associate immunopathology during tuberculosis to Th1 and Th17 responses, Th2 responses and Th2-derived cytokines are in fact the major cause of pulmonary fibrosis in different diseases, such as systemic sclerosis, idiopathic pulmonary fibrosis, and radiation induced pulmonary fibrosis and chronic lung allograft rejection [83]. We cannot discard the hypothesis that fibrosis during tuberculosis may be, at least in part, dependent on Th2 cytokines.

Overall, immunosuppressive cytokines such as IL-10 seems to be mostly detrimental at early stages of infection, but may be required to control long-term inflammatory responses. In addition, Th2-derived cytokines may have a negative impact in high incidence areas where infections by helminths can skew macrophage activation to the alternative state.

6 Conclusions

Mycobacterial infections are of enormous clinical importance. It is clear that the cytokine response induced by mycobacteria have a critical impact in the development of disease both by limiting bacterial growth and by regulating inflammation. Only by determining the pathways by which specific cytokines modulate the immune response to infection and by defining the specific cell types that produce each cytokine will we be able to effectively intervene. It is also important that we consider the interaction between different cell populations and how these interactions impact the cytokine balance and the inflammatory environment. These interactions are not restricted to immune cells, as recent data show an important role of non-hematopoietic cells in modulating the inflammatory environment. It is therefore important that we understand how each cytokine acts during the initiation of the immune response and after infection has been established and disease is ongoing. This will allow us to generate better preventive and prophylactic strategies that enforce balanced immune responses and allow containment of infection with minimal pathological consequences.

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Antigen-Specific CD8$^+$ T Cells and Protective Immunity to Tuberculosis

Samuel M. Behar

Abstract The continuing HIV/AIDS epidemic and the spread of multi-drug resistant *Mycobacterium tuberculosis* has led to the perpetuation of the worldwide tuberculosis epidemic. While *M. bovis* BCG is widely used as a vaccine, it lacks efficacy in preventing pulmonary tuberculosis in adults [1]. To combat this ongoing scourge, vaccine development for tuberculosis is a global priority. Most infected individuals develop long-lived protective immunity, which controls and contains *M. tuberculosis* in a T cell-dependent manner. An effective T cells response determines whether the infection resolves or develops into clinically evident disease. Consequently, there is great interest in determining which T cells subsets mediate anti-mycobacterial immunity, delineating their effector functions, and evaluating whether vaccination can elicit these T cells subsets and induce protective immunity. CD4$^+$ T cells are critical for resistance to *M. tuberculosis* in both humans and rodent models. CD4$^+$ T cells are required to control the initial infection as well as to prevent recrudescence in both humans and mice [2]. While it is generally accepted that class II MHC-restricted CD4$^+$ T cells are essential for immunity to tuberculosis, *M. tuberculosis* infection elicits CD8$^+$ T cells responses in both people and in experimental animals. CD8$^+$ T cells are also recruited to the lung during *M. tuberculosis* infection and are found in the granulomas of infected people. Thus, how CD8$^+$ T cells contribute to overall immunity to tuberculosis and whether antigens recognized by CD8$^+$ T cells would enhance the efficacy of vaccine strategies continue to be important questions.

S. M. Behar (✉)
Division of Rheumatology Immunology, and Allergy, Brigham and Women's Hospital,
Smith Building, Room 516 One Jimmy Fund Way, Boston, MA 02115, USA
e-mail: sbehar@rics.bwh.harvard.edu

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Abbreviations

-/-	Genetically deficient (i.e., “knockout”)
APC	Antigen presenting cell
DC	Dendritic cell
CFU	Colony forming unit
IFN	Interferon
IL	Interleukin
LN	Lymph node
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MNC	Mononuclear cells
PLN	Pulmonary LN
TCR	T cells receptor
TNF	Tumor necrosis factor
WT	Wild-type

1 Introduction

The continuing HIV/AIDS epidemic and the spread of multi-drug resistant *Mycobacterium tuberculosis* has led to the perpetuation of the worldwide tuberculosis epidemic. While *M. bovis* BCG is widely used as a vaccine, it lacks efficacy in preventing pulmonary tuberculosis in adults [1]. To combat this ongoing scourge, vaccine development for tuberculosis is a global priority. Most infected individuals develop long-lived protective immunity, which controls and contains *M. tuberculosis* in a T cell-dependent manner. An effective T cells response determines whether the infection resolves or develops into clinically evident disease. Consequently, there is great interest in determining which T cells subsets mediate anti-mycobacterial immunity, delineating their effector functions, and evaluating whether vaccination can elicit these T cells subsets and induce protective immunity. $CD4^+$ T cells are critical for resistance to *M. tuberculosis* in both humans and rodent models. $CD4^+$ T cells are required to control the initial infection as well as to prevent recrudescence in both humans and mice [2]. While it is generally accepted that class II MHC-restricted $CD4^+$ T cells are essential for immunity to tuberculosis, *M. tuberculosis* infection elicits $CD8^+$ T cells responses in both people and in experimental animals. $CD8^+$ T cells are also recruited to the lung during *M. tuberculosis* infection and are found in the granulomas of infected people. Thus, how $CD8^+$ T cells contribute to overall immunity to tuberculosis and whether antigens recognized by $CD8^+$ T cells would enhance the efficacy of vaccine strategies continue to be important questions.

2 Do $CD8^+$ T Cells Contribute to Immunity Against Tuberculosis?

In 1992, Flynn and colleagues showed that mice lacking β2-microglobulin (β2m) succumb rapidly following IV infection [3]. Because β2m is required for assembly and trafficking of the class I heavy chain, no class I MHC is expressed on the cell surface in the absence of β2m. Consequently, class I MHC-restricted $CD8^+$ T cells fail to be positively selected during thymic development, leading to a developmental deficiency of $CD8^+$ T cells. These data were the first truly convincing evidence that $CD8^+$ T cells were required for optimum resistance to tuberculosis. However, this interpretation was confounded by the fact that other β2m-associated proteins exist and some function as antigen presenting molecules including CD1, H2-M3, and class Ib MHC proteins [4, 5]. Subsequently, a comparison of transporter associated with antigen processing (TAP)-1 knockout (-/-) mice (defective in class I MHC processing) and $CD1d^{-/-}$ mice, demonstrated a requirement for an intact class I MHC antigen processing pathway, and confirmed the requirement for $CD8^+$ T cells in resistance to *M. tuberculosis* [6]. Mice with disruptions in the β2m or TAP1 genes are unable to control *M. tuberculosis* replication in the lung and die prematurely compared to normal mice following infection via the intravenous or aerosol route [3, 6, 7]. The increased susceptibility of $CD8^{-/-}$ mice and the class I MHC heavy chain knockout ($K^bD^{b-/-}$) further corroborated the requirement for $CD8^+$ T cells following primary infection [8, 9]. In addition to these genetic models, a variety of other experimental approaches confirm that $CD8^+$ T cells mediate protection against tuberculosis [reviewed in [10]]. These include $CD8^+$ T cells deletion, adoptive transfer of $CD8^+$ T cells, and vaccination to elicit $CD8^+$ T cells, all which show that $CD8^+$ T cells are required for optimal immunity against virulent *M. tuberculosis*. Thus, antibody-mediated depletion of $CD8^+$ T cells in vivo increases host susceptibility, while adoptive transfer of purified $CD8^+$ T cells enhances host resistance [11–13]. While the above-cited studies using the mouse model show that $CD8^+$ T cells play a crucial role in immunity to *M. tuberculosis*, the relative contribution of $CD8^+$ T cells still needs to be more clearly defined. For example, it is not known when during the natural history of tuberculosis $CD8^+$ T cells are important, how $CD8^+$ T cells mediate their protective effect, and whether $CD8^+$ T cells perform any unique effector functions. The possibility that $CD8^+$ T cells adversely affect host immunity to tuberculosis and worsen disease under some circumstances has not been ruled out.

Furthermore, several misconceptions exist concerning the role of $CD8^+$ T cells during infection. For example, $CD4^+$ T cells are perceived to be more important than $CD8^+$ T cells in host immunity. This is based in part on studies showing that mice lacking $CD4^+$ T cells are more susceptible than mice lacking $CD8^+$ T cells [11]. However, it is now understood that $CD4^+$ T cells are required for the development of $CD8^+$ memory T cells. Therefore, mice lacking class II MHC are more susceptible to tuberculosis because they lack $CD4^+$ T cells, but also because they mount a suboptimal $CD8^+$ T cell response. On the other hand, the ability of

DNA vaccination to prolong survival and decrease bacterial load after aerosol infection in $CD4^{-/-}$ mice supports a protective role for class I MHC-restricted $CD8^+$ T cells [14, 15]. However, these data need to be re-examined. It has been known for some time that $CD4^{-/-}$ mice have class II MHC-restricted T cells [16]. Now there is evidence that these class II MHC-restricted T cells make up a large fraction of the residual T cells. Furthermore, many of these class II MHC-restricted T cells express CD8 [17]. Thus, depletion of $CD8^+$ T cells in the $CD4^{-/-}$ model does not prove that class I MHC-restricted T cells mediate immunity following vaccination and $CD4^{-/-}$ mice are unlikely to reveal the full potential of $CD8^+$ T cells to protect mice from tuberculosis. Finally, class I MHC has other functions distinct from antigen presentation such as acting as an inhibitory ligand for NK cell receptors [18]. Thus, the exact contribution of $CD8^+$ T cells in host defense against *M. tuberculosis* remains to be delineated.

Perhaps the most important issue is whether $CD8^+$ T cells mediate immunity against *M. tuberculosis* in people. Although at this time, we cannot definitively answer this question, data that $CD8^+$ T cells are crucial for immunity to *M. tuberculosis* in non-human primates [19] and cattle [20, 21] bolster the argument that $CD8^+$ T cells are likely to be relevant to mycobacterial infection in general. The results of ongoing vaccination studies have the greatest potential to determine their true relevance in people. However, there is abundant circumstantial data that infected people generate $CD8^+$ T cells and those $CD8^+$ T cells express effector functions that can suppress bacterial growth [22–24]. The study of human $CD8^+$ T cells has also identified T cells distinct from class Ia MHC-restricted $CD8^+$ T cells—such as $\gamma\delta$-TCR^+ T cells, CD1-restricted T cells, and MAIT cells—which also are activated and specifically recognize antigens produced by *M. tuberculosis*. These additional subsets of $CD8^+$ T cells have been reviewed elsewhere [25–27].

3 Which Mycobacterial Antigens are Recognized by $CD8^+$ T Cells?

While $CD4^+$ T cells primarily recognize antigens that enter the endocytic pathway and are presented by class II MHC, most $CD8^+$ T cells recognize short peptides of 8–10 amino acids derived from cytosolic proteins that are presented by class I MHC molecules. Under certain conditions, class I MHC can present antigens that enter the endocytic pathway, a process known as cross-presentation. Both self and foreign proteins in the cytosol are cleaved by the proteosome and the resulting peptides are translocated from the cytosol into the endoplasmic reticulum (ER) by the TAP1/TAP2 heterodimer. Once in the ER, the peptides assemble with the class I MHC heavy chain and β2m to form a trimeric complex, which is then transported to the cell surface. The sampling of the cytosol by class I MHC explains why $CD8^+$ T cells are critical for host resistance to viral infections and certain intracellular bacterial infections. One impediment to understanding the role of class I MHC-restricted

CD8+ T cells in immunity to tuberculosis is the paucity of mycobacterial antigens that are known to be recognized by CD8+ T cells. Following macrophage infection, *M. tuberculosis* survives and replicates in the phagosome. Just how bacterial antigens traffic from the phagosome to the cytoplasm where they can enter the class I MHC processing pathway is a matter of controversy and several mechanisms have been proposed [28, 29]. Ultimately, mycobacterial antigens do enter the class I MHC pathway, since class I MHC-restricted CD8+ T cells are elicited by infection in both people and experimental animals.

Secreted *M. tuberculosis* protein antigens have been extensively studied in part because they are targets of T cell-mediated immunity [30]. Antigens such as Antigen 85 (Ag85), early secretory antigen target-6 (ESAT6) (esxA; Rv3875), culture filtrate protein-10 (CFP10) (esxB; Rv3874), and others elicit strong CD4+ T cells responses in both mice and humans [31, 32]. In contrast, fewer *M. tuberculosis* antigen epitopes have been identified that are recognized by CD8+ T cells. In 2000, Lewinsohn et al. cloned CD8+ T cells from a PPD+ individual using autologous *M. tuberculosis* infected DC [24]. Four of the CD8+ T cells clones were class I MHC-restricted and two recognized distinct peptide epitopes derived from the CFP10 protein [8]. These data are significant for several reasons. First, it demonstrates that CFP10 has access to the class I MHC processing pathway and shows that CFP10-specific CD8+ T cells are primed following infection. Second, CFP10-specific CD8+ T cells clones recognize infected cells, which indicates their potential to act as effector cells [8, 33]. Finally, CFP10-specific CD8+ T cells were detected at frequencies as high as 1/700 total CD8+ T cells, suggesting that CFP10 can be an immunodominant antigen in people. A study by Shams et al. defined a 15mer peptide ($CFP10_{71-85}$), which contains at least two distinct epitopes, each one that can be presented by class I and II MHC to both CD8+ and CD4+ T cells, respectively [33]. The promiscuity of these epitopes for different alleles of class I and class II MHC proteins may explain why nearly all of people with latent *M. tuberculosis* infection recognize CFP10.

CFP10 is encoded within the RD1 locus of the *M. tuberculosis* genome, a region of DNA that is present in *M. tuberculosis* and pathogenic *M. bovis* strains, but is deleted from all BCG strains [34–39]. RD1 appears to be the original deletion that resulted in the attenuation of *M. bovis* BCG, the widely used vaccine strain. Through targeted mutation, it is now confirmed that the RD1 locus is critical for the virulence of *M. tuberculosis*. The RD1 locus encodes several proteins that by definition are implicated in virulence. Two of the proteins encoded within the RD1 locus are CFP10 and ESAT6, which are secreted as a heterodimer [40]. The *cfp10* gene is in the same operon as the *esat6* gene, and the two genes share 40 % sequence homology and belong to the ESAT6 family of small proteins [36, 39, 41]. In addition to RD1, *M. tuberculosis* H37Rv has 11 distinct loci that encode 23 ESAT6-related proteins (reviewed in [42]). This extended family of ESAT6-related proteins has been given the name EsxA through EsxW, with the best characterized ESAT6 and CFP10 proteins named EsxA and EsxB. Other proteins in the RD1 locus, adjacent to ESAT6 and CFP10, are required for the

secretion of the CFP10 and ESAT6, and together, the locus appears to encode a specialized secretory apparatus, which is referred to as ESX-1.

Just as CFP10 elicits antigen-specific $CD8^+$ T cells following *M. tuberculosis* infection, specific epitopes within the ESAT6 protein are recognized by $CD8^+$ T cells obtained from the blood of infected individuals. Lalvani et al. identified two class I MHC-restricted epitopes from the ESAT6 protein in an individual with active tuberculosis [43]. In a second study, $CD8^+$ T cells specific for ESAT6 were detected in two unrelated individuals; one a PPD^+ household contact and a second with untreated healed tuberculosis [44]. In both cases the $CD8^+$ T cells recognized $ESAT6_{21–29}$ presented by HLA-A68.02 and these T cells were present at a frequency of up to 1/2500 peripheral blood lymphocytes. The frequency of T cells recognizing $ESAT6_{21–29}$ was similar to the frequency of T cells specific for PPD. Furthermore, the frequency of $ESAT6_{21–29}$-specific $CD8^+$ T cells was stable during 21 months of follow-up. Pathan et al. argue that the persistent high frequency of these T cells suggest that ESAT6-specific $CD8^+$ T cells have a role in protection against tuberculosis [44].

These observations prompted my own lab to ascertain whether members of the ESAT6-related family of proteins are targets of the $CD8^+$ T cells response in the mouse model. We identified two epitopes from the CFP10 protein: $CFP10_{11–25}$ and $CFP10_{32–39}$, which are recognized by $CD4^+$ and $CD8^+$ T cells, respectively. The $CFP10_{32–39}$-specific $CD8^+$ T cells were H-2 K^k-restricted and were elicited following infection with virulent *M. tuberculosis* (Erdman and H37Rv) but not H37Ra or BCG. $CFP10_{32–39}$-loaded K^k-tetramers identified CFP10-specific $CD8^+$ T cells in the lung, spleen and LN. Surprisingly, nearly 30 % of pulmonary $CD8^+$ T cells recognize $CFP10_{32–39}$, demonstrating the CFP10 is an immunodominant antigen in mice of the H-2 ^{k} haplotype [45]. We also studied other family members including TB10.3 (esxR; Rv3019c) and TB10.4 (esxH; Rv0288) [46–48]. These two proteins both contain an identical epitope ($TB10.3/10.4_{20–28}$), which is recognized by H-2 K^d-restricted $CD8^+$ T cells following respiratory *M. tuberculosis* infection [45, 49]. As many as 30–40 % of the $CD8^+$ T cells in the lungs of BALB/c mice recognize this epitope and these $CD8^+$ T cells have cytolytic activity both in vitro and in vivo [45, 50]. The identification of immunodominant epitopes such as $CFP10_{32–39}$ and $TB10.3/10.4_{20–28}$ that are recognized by $CD8^+$ T cells following infection is critical for studies elucidating the key elements of protective immunity. In contrast to $CD8^+$ T cells that recognize subdominant epitopes, the high frequency of $CD8^+$ T cells that are specific for dominant epitopes can facilitate studies on the activation, differentiation, trafficking, and effector functions of these T cells.

There now exist five well-characterized epitopes that are recognized by murine $CD8^+$ T cells elicited by infection. Three of these, $TB10_{4–11}$, $TB10_{20–28}$, and $CFP10_{32–29}$ are immunodominant in H-2^b, 2^d, or 2^k mice, respectively, and represent 20–50 % of the $CD8^+$ T cells in the lungs of chronically infected mice [45, 50]. The other two (32C, EspA) account for 1–10 % of $CD8^+$ T cells [51–53]. It is quite remarkable that a number of ESAT6-related proteins including ESAT6 (EsxA), CFP10 (EsxB), and TB10.3/10.4 (EsxR/H) generate $CD8^+$ T cells responses in infected people and mice. Why this group of proteins should be so

frequently recognized by T cells is unknown. However, as they are all small secreted proteins, they may efficiently enter the class I and class II MHC antigen processing pathways (see below).

4 What is the Basis for the Extreme Immunodominance of Certain Mycobacterial Antigens Recognized by CD8$^+$ T Cells Following *M. tuberculosis* Infection?

During infection, pulmonary CD8$^+$ T cells responses are dominated by large expansions of T cells that recognize a limited number of antigens. While the identity of the immunodominant bacterial epitopes varies from person to person, people infected with *M. tuberculosis* also mount CD8$^+$ T cells responses that preferentially recognize a limited number of bacterial epitopes [22]. The person-to-person variation in which epitopes are immunodominant presumably reflects the HLA diversity of people. Why there should be such extreme immunodominance in a complex microorganism with ~4,000 genes is unknown. Some possibilities include the kinetics of the response (do early responses become immunodominant?), the amount of antigen produced by the bacteria (do abundant proteins drive immunodominance), or T cells recognizing immunodominant epitopes are overrepresented in the naive T cells repertoire. An important question is whether the immunodominance of certain antigens is beneficial or detrimental to the host. Investigators studying the CD8$^+$ T cells response to HIV debate a similar issue [54]. Some believe that a more diverse T cells response is associated with better host protection. Based on paradigms established studying other pathogens, immunodominant antigens could serve as decoys that focus the host response on antigens that do not lead to protective immunity. Such questions have been difficult to address to date, since bacterial genes that are required for bacterial virulence encode nearly all of the epitopes that have been defined (including TB10.4 and CFP10).

Antigen-specific CD8$^+$ T cells responses to three different proteins have been intensively studied in the mouse model: TB10.4, CFP10, and EspA (Rv3616c). Both CFP10 and TB10 contain epitopes that are recognized by CD4$^+$ and CD8$^+$ T cells. This facilitates comparison of the kinetics of the CD4$^+$ and CD8$^+$ T cells responses to a single protein following infection. The CD4$^+$ and CD8$^+$ T cells response to CFP10 and to TB10.4 develop with nearly identical kinetics [45]. These responses develop early during infection and the increase in CFP10- and TB10-specific CD4$^+$ and CD8$^+$ T cells closely parallel the pulmonary bacterial burden. While both CD4$^+$ and CD8$^+$ T cells responses are detected within 2 weeks of infection, the CD4$^+$ T cells response generally peaks slightly earlier (4–5 weeks post infection) than the CD8$^+$ T cells response (5–8 weeks). The frequency of antigen-specific T cells in the spleen is substantially lower although the relative hierarchy of immunodominance is similar.

We considered whether the immunodominance of these epitopes is related to their initial precursor frequency in the naïve T cells repertoire. Immunomagnetic selection of tetramer bound T cells followed by dual-color tetramer staining and analysis by flow cytometry can sensitively and specifically enumerate naïve antigen-specific T cells [55, 56]. We determined the precursor frequency of naïve $CD8^+$ T cells that recognize the immunodominant epitope TB10 as well as a less dominant epitope, EspA, in naïve BALB/c mice. For comparison, we also determined the frequency of $F2_{26-34}$ a well-characterized vaccinia epitope. Relative to the frequency of $F2_{26-34}$-specific naïve $CD8^+$ T cells in uninfected mice ($\sim$1:16,000), TB10.4 ($\sim$1:416,000) and EspA ($\sim$1:778,000)-specific naïve $CD8^+$ T cells were 25–50-fold less abundant. In addition, the frequency of naïve TB10.4-specific $CD8^+$ T cells was consistently 1.9-fold greater than EspA-specific $CD8^+$ T cells; however, we do not believe that this two-fold difference is sufficient to explain the observed dominance of the TB10.4-specific response following *M. tuberculosis* infection. While the precursor frequency did not explain the extreme immunodominance of TB10-specific $CD8^+$ T cells response, these data allow one to assess the magnitude of the expansion of antigen-specific T cells following infection. Assuming that the entire repertoire of a naïve mouse has $\sim$50 TB10-specific $CD8^+$ T cells, the detection of 500,000 TB10-specific $CD8^+$ T cells in the lung at the height of the response to tuberculosis represents at least a 10,000-fold expansion.

5 Cross-Priming of $CD8^+$ T Cells

The process by which a naïve T cells becomes activated, which allows the T cells to leave the lymph node, proliferate, and express different effector functions, is known as T cells priming. Conventional wisdom is that T cells priming occurs in the draining lymph node and the dendritic cell (DC) is the antigen-presenting cell (APC) that most efficiently is able to present microbial antigens and activate T cells. As described above, the sequence of events that lead to activation of $CD4^+$ T cells is reasonably well understood. DC, which survey different tissues, will become activated when they encounter microbial products or become infected. DC activation allows the cell to migrate from the peripheral tissue to the draining LN where they can interact with naïve T cells. Because most such encounters with bacteria or bacterial products lead to acquisition of the microbial antigens through phagocytosis or macropinocytosis, the antigen enters the endocytic pathway, which intersects with the class II MHC presentation pathway. For example, viable *M. tuberculosis* survives in intracellular compartments including the phagosome. One can easily imagine how its secreted antigens are selectively presented via the class II MHC pathway to $CD4^+$ T cells. It is considerably less clear how antigens from *M. tuberculosis* generate $CD8^+$ T cell responses. How bacterial antigens traffic from the phagosome to the cytoplasm where they can enter the class I MHC processing pathway is a matter of controversy.

How do DC acquire mycobacterial antigens? Following aerosol infection, the dogma is that *M. tuberculosis* preferentially infects alveolar macrophages. This view is beginning to change as we recognize (at least in the mouse model) that numerous types of myeloid cells are infected soon after infection. In addition to macrophages, there is good evidence that neutrophils and DC can both be directly infected [57–59]. Furthermore, although there is evidence that immunity can be initiated in the lung [60], most investigators have found that immunity to tuberculosis is initiated in the local draining LNs of the lung [57, 59, 61, 62]. Since the DC is specialized for migrating from tissue beds to the lymph node, this is strong circumstantial evidence that acquisition of mycobacterial antigens by DC is required for T cell priming. It should be noted that we are beginning to realize that other cell types such as neutrophils can also become infected and traffic to the LN where they can prime T cells although the physiological relevance of this process is still be worked out [58]. We have previously shown that DC are required to generate CD4$^+$ T cells immunity following *M. tuberculosis* infection. By taking advantage of the CD11c/DTR Tg mouse model, which allows transient ablation of DC, we demonstrated that DC are required to prime ESAT6-specific CD4$^+$ T cells. In addition, deletion of CD11c$^+$ cells and the subsequent delay in adaptive immunity impairs control of *M. tuberculosis* replication. While these experiments show a critical role for DC in the initiation of adaptive immunity to *M. tuberculosis*, they do not shed light on how DC acquire mycobacterial antigen, which can occur by two different processes. Although we have known for some time that both human and murine DC can be infected experimentally in vitro, there is now good evidence that DC are directly infected in vivo. Nevertheless, it has been difficult to ascertain whether the DC that are directly infected are the same DC that traffic to the draining LN and prime T cells. A second possibility is that DC acquire mycobacterial antigen indirectly. Immature tissue DC can phagocytose dead bacteria, bacteria products, and apoptotic vesicles that contain mycobacterial antigens.

Although *M. tuberculosis* can grow extracellularly, it is observed to be mostly intracellular except during active disease when it can reach great numbers at the center of caseous granulomas. It is unlikely that its antigens would be found in the extracellular space in the absence of cell death. Thus, it should come as no surprise that the cell death modality of *M. tuberculosis* infected macrophages influences innate and adaptive immunity. During *M. tuberculosis* infection, cellular necrosis and loss of plasma membrane integrity occurs, which among other things allows the bacteria to infect other cells [63–66]. Apoptosis of *M. tuberculosis* infected cells enhances innate control of intracellular bacterial replication as well as augments T cell-mediated immunity. Previous studies delineated the importance of apoptosis in cross-presentation of *M. tuberculosis* antigens by human DC in vitro or by apoptotic vesicles purified from BCG infected macrophages in vivo [67, 68]. We were particularly interested in how the death modality of host macrophages infected with live *M. tuberculosis* in vivo affected initiation of T cells immunity. To explicitly study this question, we established a novel adoptive transfer model in which macrophages from wild-type or knockout mice are infected in vitro with *M. tuberculosis* and then transferred into normal recipient mice. This strategy allows

one to determine how the macrophage genotype influences T cells immunity and control of infection. Transfer of *M. tuberculosis* infected pro-apoptotic macrophages enhanced T cells immunity and led to better protection than observed after transfer of infected pro-necrotic or wild-type macrophages [69].The enhanced antigen-specific $CD8^+$ T cells response requires presentation of antigen derived from apoptotic macrophages by the Detour pathway [67, 68, 70], as defined by TAP-1-dependent and class I MHC-restricted cross-presentation by DC [28, 67, 69]. Enhanced T cell-mediated immunity was not restricted to $CD8^+$ T cells and we observed that *M. tuberculosis*-specific $CD4^+$ responses were also increased after transfer of infected pro-apoptotic macrophages. Thus, while apoptosis has been directly linked to increased $CD8^+$ T cells responses via cross-presentation, it also enhances class II MHC-restricted antigen presentation. Presumably, DC phagocytosis of apoptotic vesicles delivers their cargo of *M. tuberculosis* antigens to the endocytic system, which intersects with the MHC II processing pathway, ultimately increasing priming of $CD4^+$ T cells. We speculate that the pro-inflammatory function of apoptosis during infection, compared to its anti-inflammatory propensity during physiological cell death, may be a consequence of TLR ligands and other microbe-derived signals that are contained in apoptotic vesicles that activate DC upon their acquisition.

Why are secreted antigens preferentially presented to $CD8^+$ T cells? Although the number of antigens that have been discovered to be recognized by $CD8^+$ T cells elicited by *M. tuberculosis* is still relatively small, it is striking that proteins secreted by *M. tuberculosis* are overrepresented [10, 22, 71, 72]. Secreted *M. tuberculosis* protein antigens have been extensively studied as targets of T cell-mediated immunity in part because they are easy to obtain in purified form. These include antigens such as Ag85, ESAT6, and CFP10, which elicit strong $CD4^+$ T cells responses in both mice and humans. Another feature is that most of the antigens are small MW proteins. In a limited number of cases, it has been shown that protein secretion is required for priming of $CD8^+$ T cells during infection. The best characterized is the response to CFP10. As discussed above, CFP10 is secreted by the ESX-1 type 7-secretion system, and bacterial mutants that have defects in this secretion apparatus produce ESAT6 and CFP10 but do not secrete them. If *M. tuberculosis* does not secrete CFP10, the host does not generate a CFP10-specific $CD8^+$ T cells response following in vivo infection [73]. As the ESX-1 locus is required for bacterial virulence, another interpretation of these data is that the capacity of the bacteria to grow and induce inflammation modulates the host $CD8^+$ T cells response. To address this issue, we evaluated an interesting point mutant of EspA (Rv3616). The protein EspA is not encoded in the ESX-1 locus, but is required for the function of the ESX-1 complex [74]. The point mutant secretes CFP10, ESAT6, and EspA, but no longer assembles the ESX-1 complex, and consequently, is attenuated [53]. Following in vivo infection with this mutant, a CFP10-specific $CD8^+$ T cell response is generated, showing that virulence and immunogenicity of the ESX-1 locus can be dissociated [53]. These data are consistent with the hypothesis that secretion is a prerequisite for T cells priming and occurs independently of the virulence of the bacteria.

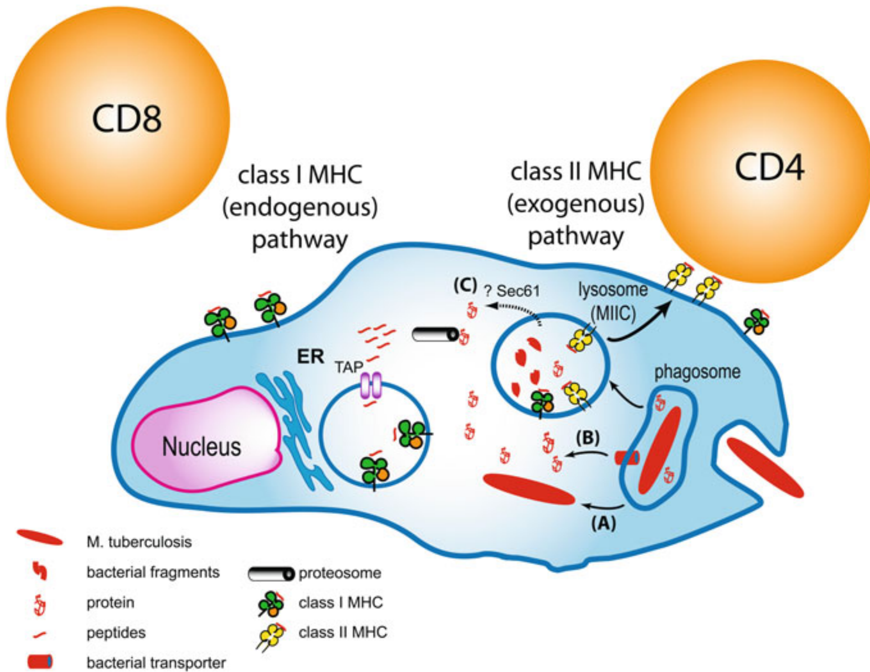

Fig. 1 Antigen presentation pathways in infected APC. Mycobacterial peptides can be loaded onto class II MHC within the endocytic pathway. How mycobacterial peptides enter the class I MHC pathway is unknown. One possibility is that (**a**) the bacterium can translocate from the phagosome and enter the cytosol. Its secreted products could then be processed by the proteasome and the peptide products transported into the ER by the TAP proteins. Alternately, a bacterial secretion system, such as ESX-1, might actively transport secreted proteins across the phagosomal membrane (**b**). Another possibility is that host machinery, such as sec61, which performs retrograde translocation of proteins, could transport bacterial proteins into the cytosol (C)

Once the mycobacterial antigens are in the endosomal compartment of DC, how do they enter the class I MHC processing pathway? The propensity of secreted mycobacterial antigens to be recognized by CD8+ T cells may provide some clues as to how such antigens enter the class I MHC processing pathway (Fig. 1). Our thinking has changed from the idea that *M. tuberculosis* is a pure phagosomal pathogen to the realization that under certain circumstances, either the bacteria or bacterial products can gain access to the cytosol. For example, van der Waal et al. showed that virulent but not avirulent *M. tuberculosis* can translocate from the phagosome into the cytosol [75]. This paradigm-shifting observation potentially solves the problem of how mycobacterial antigens enter the class I MHC processing pathway. If the bacteria can survive in the cytosol, there is no need for cross-presentation. The class I MHC pathway could directly sample bacterial proteins secreted into the cytosol. Despite the attractiveness of this model, there are some problems with this idea. First, the kinetics of the class I MHC antigen presentation is rapid and bacterial antigens are presented to CD8+ T

cells before one can detect the first evidence of escape [76]. Second, it does not explain how the CD8$^+$ T cells responses elicited by less virulent bacteria, which do not escape, are generated. For example, although bacteria with mutations in ESX-1 escape and do not generate CFP10-specific CD8$^+$ T cells responses, they do generate CD8$^+$ T cells responses to other antigens that are secreted by mechanisms that are independent of the ESX-1 type VII secretion system. An alternate view of bacterial translocation into the cytosol is that breakdown of the phagosomal membrane is associated with necrosis, and the appearance of cytosolic bacteria is a transient state before cellular necrosis occurs, allowing the bacteria to disseminate [77]. The significance of cytosolic bacteria for generating CD8$^+$ T cells responses remains to be determined.

Short of bacterial translocation, there is increasing evidence that *M. tuberculosis* has the capacity to damage host cells membranes and it is likely that this includes damage to the phagosomal membrane. Damage of the phagosomal membrane would allow bacterial products to leak out of the phagosome and into the cytosol. Alternately, active transport by a secreted bacterial transport apparatus (similar to the ESX-1 type VII secretion system) could translocate bacterial proteins across the phagosomal membrane. Both of these scenarios would introduce mycobacterial antigens into the cytosol of the infected cell where they could be sampled by the class I MHC processing pathway.

Finally, the finding that uninfected DC that take up apoptotic infected cells cross-present mycobacterial antigens, leading to cross-priming of CD8$^+$ T cells, indicates that phagosomal antigens in DC have the capacity to enter the class I MHC processing pathway whether or not there are viable bacteria present [28, 67–70]. The bias towards presentation of secreted proteins could arise if soluble proteins are preferentially packaged into apoptotic blebs. How the antigens get out of the apoptotic vesicles, which are taken up by efferocytosis, and into the cytosol, still remains a formidable problem to solve. There must be host mechanisms that facilitate sampling of antigens from endocytic compartments. One possible mechanism involves the host protein Sec61, which normally mediates retrograde protein translocation as a way to transport misfolded proteins back into the cytosol where they can be targeted for degradation. Proteins secreted by *M. tuberculosis* into the phagosomal compartment might be translocated by Sec61 across the phagosomal membrane into the cytosol where they could enter the class I MHC processing pathway [78].

6 Effector Functions of CD8$^+$ T Cells During *M. tuberculosis* Infection

T cells immunity is crucial for controlling *M. tuberculosis* infection and both CD4$^+$ and CD8$^+$ T cells play important roles in mediating host protection. The dominant paradigm of T cells immunity to tuberculosis postulates that interferon-γ

(IFNγ) plays a central role in host defense. This model is supported by the finding that IFNγ is essential for immunity in both humans and in animal models [79–85]. T cells are the main producers of IFNγ and IFNγ stimulates antibacterial activity in *M. tuberculosis* infected macrophages. This is the basis for the (oversimplified) schema that the IFNγ production by T cells activates microbicidal activity in macrophages, which kills, or at least suppresses, the growth of *M. tuberculosis*. However, more and more data show that IFNγ has other important roles during *M. tuberculosis* infection in addition to direct activation of macrophages. It serves an important immunoregulatory role and has anti-inflammatory properties that reduces immunopathology. Conversely, T cells can mediate antimicrobial immunity independent of IFNγ. Finally, and relevant to CD8$^+$ T cells, while IFNγ production by CD8$^+$ T cells can suppress bacterial growth, it is not sufficient to replace IFNγ production by CD4$^+$ T cells. This indicates that CD4$^+$ and CD8$^+$ T cells mediate fundamentally different functions during infection.

There are several unique roles for CD8$^+$ T cells during the immune response to tuberculosis. Although CD8$^+$ T cells appear to be less efficient at suppressing bacterial growth compared to CD4$^+$ T cells, this is a complicated comparison since some of the functions expressed by CD8$^+$ T cells are dependent on CD4$^+$ T cells help [86, 87]. Therefore, in the absence of CD4$^+$ T cells, CD8$^+$ T cells may not function optimally. There is also the possibility that CD8$^+$ T cells do not functional optimally during *M. tuberculosis* infection because chronic infection impairs T cells immunity. T cells immunity may be suboptimal because chronic inflammation leads to the development of T cells exhaustion or anergy. Alternately, *M. tuberculosis* may actively evade T cells immunity by impairing antigen presentation. There is great interest in determining whether certain vaccination strategies could enhance immunity to tuberculosis, not only by increasing the priming and expansion of *M. tuberculosis*-specific CD8$^+$ T cells, but also by increasing the expression of functions that contribute to host resistance. Designing such strategies could be done more rationally if we understood the mechanisms that CD8$^+$ T cells use to inhibit bacterial growth.

While it is unknown how CD8$^+$ T cells mediate protection against tuberculosis, there are several unique functions that CD8$^+$ T cells have. CD8$^+$ T cells are more likely to recognize infected cells [88], while CD4$^+$ T cells can recognize infected cells or cells that have phagocytosed dead bacteria or their antigens. CD8$^+$ T cells can recognize class II MHC negative cells, and while the relevance of such cells is hypothetical, class II MHC expressed by infected macrophages is resistant to upregulation by IFNγ [89, 90], which may allow the bacteria to "hide" from CD4$^+$ T cells; however, class I MHC is not altered under these conditions. This idea is supported by a unique role for CD8$^+$ T cells in controlling bacterial burden during latency, since depletion of CD8$^+$ T cells, but not CD4$^+$, leads to an increase in bacterial replication [91]. Cytokine production (IFNγ, TNF) and cell-mediated cytotoxicity (CTL activity) are the two functions most commonly described for CD8$^+$ T cells. We will now review what is known about these two functions of CD8$^+$ T cells during *M. tuberculosis* infection.

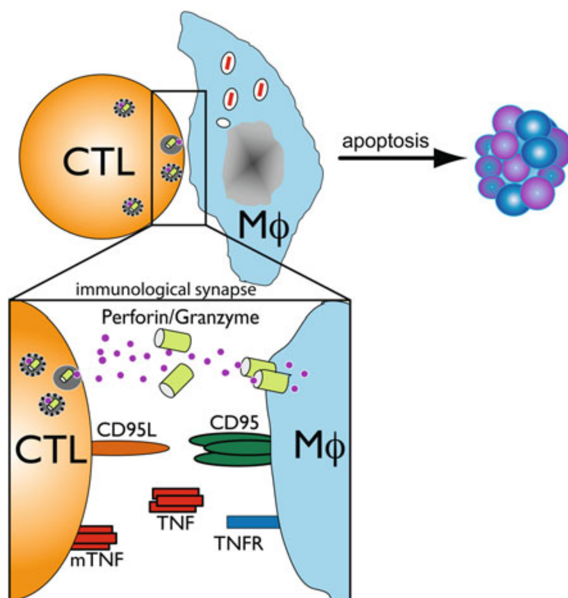

Fig. 2 Mechanisms of target cell lysis by CD8+ T cells

Cytokine production. Activated CD8+ T cells produce type 1 cytokines including IFNγ and TNF, which activate macrophages to produce nitrogen and oxygen radicals and LRG47 [92–94]. IFNγ production by CD8+ T cells can potentially be protective. Tascon et al. showed that the ability of adoptively transferred naïve CD8+ T cells to protect T cell-deficient recipients required IFNγ [95]. However, it is clear from others that CD8+ T cells, even ones producing IFNγ and inducing NO, cannot replace the protection mediated by CD4+ T cells [14]. Thus, the relative importance of IFNγ secretion by CD8+ T cells in hosts with intact immunity is unknown.

CTL activity. We find that CD8+ T cells elicited by *M. tuberculosis* infection are cytolytic in vivo [13, 45]. TB10-specific CTL activity is detected within 4 weeks of *M. tuberculosis* infection and persists for at least 9 months. *M. tuberculosis*-specific CD8+ T cells lacking perforin have reduced cytolytic activity in vivo, and in the absence of perforin, the residual cytolytic activity is FasL (CD95L) and TNFR-dependent (Fig. 2) [13]. In the lung, disruption of both perforin and FasL eliminates target cell lysis. The importance of CTL activity in mycobacterial immunity remains unknown. Although murine and human *M. tuberculosis*-specific CD8+ cytolytic T cell lines have been established in vitro, it has been difficult to demonstrate cytolytic activity of freshly isolated CD8+ T cells against infected macrophages [43, 96–99]. Following in vitro stimulation of pulmonary CD8+ T cells with *M. tuberculosis* infected DC, CD8+ T cells acquire the ability to lyse infected macrophages in vitro, and the lysis is dependent upon granule exocytosis [100].

Protection. Importantly, immune CD8+ T cells isolated from WT, but not perforin-deficient mice, transfer protection against *M. tuberculosis* infection to recipient mice [13]. Thus, although *M. tuberculosis*-specific CD8+ T cells use several cytolytic pathways in a hierarchical and compensatory manner, adoptively transferred *M. tuberculosis*-*specific CD8+ T cells require perforin to protect animals from M. tuberculosis infection*. Human CD8+ T cells also require perforin to restrict intracellular *M. tuberculosis* growth, indicating use of a granule-dependent mechanism [101, 102]. Human CD8+ T cells, which express the antimicrobial peptide granulysin in their cytotoxic granules, also require perforin to restrict intracellular *M. tuberculosis* growth [101, 102]. For human CD8+ T cells, granulysin is an important granule constituent [101]; the critical effector molecules for murine CD8+ T cells are unknown.

Genetic models. Mice lacking CD8+ T cells (i.e., *β*2m, TAP1, and CD8 knockout mice) die more rapidly than wild-type mice lacking either CD95 or perforin [7, 9, 103, 104]. This suggests that these two major cytotoxic pathways may be functionally redundant in tuberculosis, as for certain viruses [105–107], or may be compensated by another (undefined) mechanism. On the other hand, none of these molecules are specific for CD8+ T cells and most are expressed by NK cells and some CD4+ T cells. Further complicating the interpretation of these data is the finding that both CD95 and perforin-deficient mice have increased Th1-type cytokine production that may confound a clear interpretation of the knockout results. These results highlight the role of CD95 and perforin in lymphocyte homeostasis and immunoregulation [108]. Despite the imperfect nature of these models, mice lacking perforin, generally succumb to *M. tuberculosis* late during infection, which suggests that CTL are more important during chronic [7] or even latent [91] infection. CD8+ T cells may be particularly important in controlling bacterial replication during latency, since depletion of CD8+ T cells, but not CD4+, leads to an increase in bacterial burden [91].

Why would killing infected macrophages be beneficial? Lysis of *M. tuberculosis* infected macrophages could be beneficial if the released bacteria are subsequently taken up by activated macrophages that can mediate bacterial killing. However, most killing mechanisms used by CD8+ T cells induce target cell apoptosis, which is known to reduce intracellular bacterial viability. Furthermore, apoptosis induced by CD95 ligation on infected macrophages leads to reduced viability of *M. tuberculosis* and supports a possible role for the CD95/CD95L pathway in the host response [109]. In addition, CD8+ T cells have unique effector mechanisms that can kill intracellular bacteria [102, 110]. For example, human CD8+ T cells express granulysin in their cytotoxic granules that can directly kill intracellular *M. tuberculosis* [111, 112]. Finally, apoptotic infected cells are engulfed by uninfected macrophages, which are able to more efficiently target the efferosome containing bacteria to the lysosome where the bacteria are destroyed [113].

Are there different subsets of effector CD8+ T cells during the host response to infection? CD4+ T cells have been divided into different subsets based on their effector functions (Th1, Th2, Th17, Treg, etc.). CD8+ T cells with different functions have been identified (Tc1, Tc2), although it is not clear whether these represent

stable phenotypes. In addition, dramatic heterogeneity is observed during antigen-specific $CD8^+$ T cell responses. Effector molecules such as IFNγ, perforin, and granzyme A, B, and C, are heterogeneously expressed at the single cell level by $CD8^+$ T cells [114]. The early gene expression of these molecules appears stochastic, although RNA expression does not always correlate with protein expression [114]. During the acute phase of influenza infection, an infection that requires CTL to clear virus, considerable heterogeneity is evident among antigen-specific $CD8^+$ T cells [115]. In particular, the regulation of $CD8^+$ T cell cytotoxic activity is complex. Only a minority of responding $CD8^+$ T cells expressed both perforin and granzymes. The co-expression of perforin and granzymes is thought to be important for target cell killing by the granule-exocytosis pathway. If the expression of granule contents is uncoordinated, $CD8^+$ T cells may be "shooting blanks" [116]. The basis for the heterogeneity among $CD8^+$ T cells is not understood nor whether it affects host resistance to infection. Even though CTL activity is detected early during in vivo *M. tuberculosis* infection [13, 45], it is unknown what percentage of the antigen-specific $CD8^+$ T cells are competent CTL that restrict bacterial replication—it could be a small population early during infection. This may be particularly true if multiple pathways (cytotoxic granules, fas/fasL, TNF/TNFR) lead to target cell cytolysis but only a unique combination of effector molecules lead to *M. tuberculosis* killing (perforin + granzymes). In other words, the CTL activity may overestimate the "protective" capacity of $CD8^+$ T cells.

Heterogeneity of T cells during *M. tuberculosis* infection. Heterogeneity is observed for $CD8^+$ T cells recruited to the lung during *M. tuberculosis* infection. Joanne Flynn's lab reported distinct populations of IFNγ-producing and cytolytic $CD8^+$ T cells during persistent infection (as determined by CD107 expression, a measure of cytotoxic degranulation) [117]. Our enumeration of antigen-specific $CD8^+$ T cells from *M. tuberculosis* infected mice finds a discrepancy depending on whether we use tetramers (which quantify $CD8^+$ T cells independently of function) or an elispot (which enumerates cells based on their secretion of IFNγ). We find that only 10–15 % of TB10-specific $CD8^+$ T cells produce IFNγ [50, 52]. Thus, a substantial fraction of TB10-specific $CD8^+$ T cells do not produce IFNγ but may express other functions. The polarization of $CD8^+$ T cells during *M. tuberculosis* infection raises the question of whether these two functional subsets represent the *emergence of two distinct* $CD8^+$ T cells populations or a *loss of function* by polyfunctional T cells and progressive T cell exhaustion.

7 Conclusions

Despite the worldwide application of BCG-vaccination and other anti-*M. tuberculosis* interventions, *M. tuberculosis* remains one of the most successful human pathogens. Eight to ten million new cases of active tuberculosis occur each year due in large part to the large reservoir of asymptomatic people chronically infected with *M. tuberculosis*. Estimates suggest that up to a third of the world's population

is latently infected with *M. tuberculosis* indicating *M. tuberculosis* is able to persist long term in humans. Understanding the role of $CD8^+$ T cells in host immunity is required to design the best vaccine. Such an understanding is particularly important in the setting of HIV coinfection as HIV infects and kills $CD4^+$ T cells, crippling one arm of T cells immunity against TB. Whether vaccine strategies can be developed to elicit $CD8^+$ T cells that provide protection against tuberculosis remains an important question. Consequently, both commercial and academic ventures are pursuing vaccine strategies that stimulate $CD8^+$ T cells responses. To activate $CD8^+$ T cells, mycobacterial antigens need to enter and be processed by the MHC class I pathway, which can be targeted by DNA vaccines, recombinant viruses, and live attenuated mycobacteria. Evaluating whether or not such strategies are successful in activating $CD8^+$ T cells has been hampered because few mycobacterial antigens have been identified that are presented by class I MHC. Thus, even basic questions concerning the function of $CD8^+$ T cells during *M. tuberculosis* infection remain unanswered. These include uncertainty concerning when during infection $CD8^+$ T cells are most important, whether $CD8^+$ T cells elicited by vaccination mediate protection, whether $CD8^+$ T cells perform any unique effector functions, and how $CD8^+$ T cells mediate their protective effect. The possibility exists that $CD8^+$ T cells effector molecules may correlate with protection. Therefore, the precise contribution of $CD8^+$ T cells in host defense against *M. tuberculosis* requires elucidation. A better understanding of the effector functions used by $CD8^+$ T cells to restrict intracellular bacterial growth would help in the development of new vaccines.

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Foxp3^{+} Regulatory T Cells in Tuberculosis

Ryan P. Larson, Shahin Shafiani and Kevin B. Urdahl

Abstract The immune response to *Mycobacterium tuberculosis* (Mtb) must be tightly regulated to mount a sufficient response to limit bacterial growth and dissemination while avoiding excessive inflammation that could damage host tissues. A wide variety of cell types, cell surface molecules, and cytokines are likely to contribute to this regulation, but recent studies have revealed that a subset of CD4 T cells expressing the transcription factor Foxp3, called regulatory T (reg) cells, play a critical role [1–3]. Although the first reports of T reg cells in tuberculosis (TB) occurred only recently (i.e., 2006) [4, 5], we have already gained many insights into their activity during TB. While it is likely that T reg cells do play some beneficial roles by preventing inflammation-mediated damage to host tissues during TB, this aspect of their function has not been well studied to date. What is clear, however, is that during the initial T cell response to Mtb infection, Mtb induces the expansions of T reg cells that delay the onset of adaptive immunity, suggesting that Mtb has hijacked T reg cell-mediated immune suppression to allow it to replicate unabated in the lung until T cells finally arrive [6]. In this chapter, we will first provide an overview of the delayed T cell response to Mtb and a brief introduction to regulatory T cells. We will then review what is known about T reg cells from observations in human populations, discuss mechanistic insights revealed in the mouse model, and speculate about the relevance of this understanding for future efforts to prevent and treat TB.

R. P. Larson · S. Shafiani · K. B. Urdahl (✉)
Tuberculosis Program, Seattle Biomedical Research Institute, 307 Westlake Avenue North, Suite 500, Seattle, WA 98109, USA
e-mail: kevin.urdahl@seattlebiomed.org

R. P. Larson · K. B. Urdahl
Department of Immunology, University of Washington, Seattle, WA 98195, USA

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1 The Delayed T Cell Response to TB

The adaptive immune response to Mtb is significantly delayed compared to responses to other pathogens. Studies in humans recently exposed to Mtb found that those infected did not become tuberculin test positive until 6 weeks after exposure [7, 8]. Similarly, mice infected with a low dose of aerosolized Mtb also exhibit a delayed T cell response, peaking at ~30 days post-infection, whereas responses to acute infections (e.g., *Listeria monocytogenes*, lymphocytic choriomeningitis virus, and influenza virus) typically peak between days 7–10 post-infection [9]. Because accelerated T cell responses are associated with early control of Mtb replication in the lung and improved long-term outcomes, a primary goal of vaccination should be to achieve a rapid T cell response in the lung. Thus, understanding the mechanisms underlying this delay is critical, and the mouse provides an ideal model to dissect the contributing factors.

Mouse studies, to date, have shown that initiation of adaptive immunity is delayed at multiple steps, from Mtb-infected DC arrival in lung draining lymph nodes (LDLN) to effector T cell expansion, and subsequent migration to infected lungs [10–14]. Initiation of the effector T cell response requires Mtb-infected DCs to migrate from the lung to the LDLN, which does not occur until between days 9 and 11 after infection [11, 12]. Recently, this delayed migration has been attributed to the ability of virulent Mtb to prevent apoptosis of infected macrophages and neutrophils [15, 16], thus delaying DC-mediated uptake of Mtb-containing apoptotic bodies and subsequent egress from the lung to the LDLN [17, 18]. Alterations in Mtb or host factors that promote apoptosis of Mtb-infected macrophages and neutrophils result in enhanced adaptive immunity to Mtb. For example, mice infected with apoptosis-inducing mutant strains of Mtb, or mice deficient in eicosanoid biosynthetic pathways that prevent apoptosis, exhibit enhanced effector CD8 T cell generation due to increased cross-presentation of Mtb-associated antigen by dendritic cells [18, 19]. Thus, manipulation of phagocyte cell death pathways is a critical factor mediating the initial delay in T cell priming. Even after Mtb is delivered to the LDLN, the kinetics of effector T cell expansion and migration to the lungs is also slowed[6]. We now understand that T reg cells play an important role in mediating this second delay, and the evidence for this involvement will be reviewed in this chapter.

2 Regulatory T Cells

T reg cells play a critical role in preventing autoimmune disease, and it is this role that led to their discovery. Thymectomized neonatal mice were observed to develop multi-organ autoimmune disease that was prevented by transferred T cells from adult mice [20, 21]. These studies led to the hypothesis that adult mice contained a subset of T cells with immunosuppressive properties that first emerged from the murine thymus in the first week of life and were required to prevent widespread autoimmunity. Subsequent studies revealed that this subset could be identified, at least to some degree, by their high level expression of IL-2Rα (CD25), and more specifically by the transcription factor Foxp3 [22–25]. The relevance of T reg cells to human disease was clearly demonstrated by the discovery that individuals lacking functional regulatory T cells, due to mutations in their *foxp3* locus suffered from widespread autoimmunity, termed immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), a lethal disease without bone marrow transplantation [26]. The role of Foxp3 in driving the suppressive program in T reg cells was confirmed by the finding that mice deficient in Foxp3 have similar lethal autoimmune disease as IPEX patients [25, 27]. Although Foxp3-negative T cell subsets with regulatory properties also have been described (e.g., type 1 regulatory T cells that produce IL-10) and undoubtedly can function to dampen immune responses during TB, this review focuses solely on those CD4 T cells that express Foxp3.

Foxp3^{+} T reg cells can be separated into two subsets; natural T reg cells that develop in the thymus as a consequence of relatively high affinity interactions with self-antigens, and induced T reg cells that arise when Foxp3 is upregulated by conventional CD4 T cells in the periphery, a process promoted by chronic antigen exposure, limited costimulation, or the presence of the immunoregulatory cytokine TGF-β [28]. The immunosuppressive function of both natural and induced T reg cells is dependent on signals transmitted through their antigen-specific T cell receptors (TCRs). Because of their distinct developmental pathways, natural and induced T reg cells have nonoverlapping specificities, and as a result can each play complementary roles in mediating immune suppression [29, 30]. It has become clear that Foxp3 drives a suppressive program in T reg cells that results in a multitude of suppressive mechanisms, many of which are redundant, confounding attempts to pinpoint a specific pathway mediating immunosuppression. Natural and induced T reg cells share many of these molecular mechanisms of suppression, including cell surface molecules such as CTLA-4, LAG, and NRP1 that mediate contact-dependent inhibition and soluble molecules such as IL-10 and TGF-β [31].

3 Regulatory T Cells in Infection

The discovery that T reg cells, in addition to preventing autoimmunity, could also dampen immune responses to microbial pathogens was first made in mice infected with *Leishmania major*. Depletion of T reg cells during chronic infection, was shown to result in enhanced effector T cell function and rapid pathogen clearance [32]. Subsequent studies showed that the T reg cells mediating this activity proliferated specifically in response to *Leishmania*-infected dendritic cells, suggesting they recognized a pathogen-derived antigen [33]. T reg cell responses have now been implicated in a wide range of bacterial, viral, and parasitic pathogens, especially those that establish chronic infections [34]. During chronic infection T reg cells may serve to prevent tissue damage due to excessive inflammation, but this immune suppression can also prevent microbial eradication and facilitate chronic infection [32, 34]. Because TB is arguably the most important chronic infection in terms of human morbidity and mortality, the activity of T reg cells during human TB disease has been an active area of investigation over the last several years.

4 Regulatory T Cells in Human Tuberculosis

The portal of human infection is the lung, which occurs when aerosolized droplets containing Mtb produced by the cough or sneeze of an individual with active TB, is inhaled. In the first few weeks after such an infection occurs, T reg cells seem to be recruited to the lung nonspecifically. This finding was first suggested by the surprising observation that individuals recently exposed to Mtb had reduced numbers of T reg cells in their peripheral blood [35]. The whereabouts of these T reg cells was subsequently demonstrated in the macaque model; macaques likewise had low numbers of T reg cells in their blood during early infection, but exhibited increased T reg cell numbers at the site of infection in the lung [36]. The rapid recruitment of T reg cells to the lung can likely be explained by the fact that T reg cells, even in steady-state conditions, display an activated phenotype, including the expression of cell surface molecules and chemokine receptors that facilitate migration into inflamed tissues [37].

4.1 Correlation Between T Reg Cell Frequencies and TB Severity

During later stages of infection there seems to be a direct correlation between the severity of the infection or the bacterial burden, and T reg cell numbers. In most individuals the immune system successfully curtails Mtb replication, resulting in

latent or asymptomatic infection with a low bacterial burden, and T reg cell numbers return to normal or slightly elevated levels [38, 39]. However, in the 5–10 % of individuals that develop active TB with a high bacterial burden, increased numbers of T reg cells are observed at sites of infection (lung and pleural sac) and also in the blood [4, 5, 35, 40]. Histological analysis has further shown that cavitating granulomas, those possessing the highest bacterial burden, contain significantly more T reg cells than other granulomas [41]. Moreover, individuals with miliary TB, the most severe form of disseminated TB, have elevated T reg cell: effector T cell ratios compared to those with localized pulmonary TB [42]. Despite these correlations, it is not clear whether T reg cells are the cause of severe forms of TB, or simply the effect of higher levels of inflammation associated with greater bacterial burdens. Likewise, the degree to which T reg cell function is harmful versus beneficial to the host during active TB disease is also unknown.

4.2 What is Driving T Reg Cell Proliferation?

It is clear that T reg cells undergo massive expansion during human TB, but these studies raise many questions. T reg cell proliferation requires signals transmitted through their antigen-specific TCR [43], but whether the antigens recognized are derived from Mtb, or are self-antigens, perhaps those induced by inflammation, is not known. In addition to TCR-mediated signals, other costimulatory signals are likely to be involved. For example, Mtb infection induces increased levels of PD-L1 expression on dendritic cells, and interactions between PD-L1 and PD-1 expressed by T reg cells may play a role in their expansion [44]. Another question from these human studies is whether the expanded population of T reg cells arise from the pre-existing pool of T reg cells that originated in the thymus (i.e., natural T reg cells) and/or whether they arise from conventional CD4 T cells that are induced to express Foxp3 by the inflammatory milieu of TB (i.e., induced T reg cells). Answers to these questions are needed to determine whether targeting T reg cell activity is a feasible strategy to prevent or treat TB, and if so, to help inform approaches to do so.

4.3 What is the Functional Role of T Reg Cells During TB?

Efforts to elucidate the role of T reg cells in human TB have largely relied on in vitro assays assessing their ability to suppress T cell function. Depletion of T reg cells from in vitro cultures of peripheral blood T cells results in increased IFN-γ production and proliferation by effector T cells stimulated in a polyclonal or antigen-specific manner, demonstrating their suppressive function [40]. Immunosuppression in in vitro assays, however, is not specific to TB and sheds little light on the activity of T reg cells in vivo. In fact, even T reg cells from uninfected

individuals, or from individuals with other diseases, show a similar ability to suppress T cell function [34, 45]. Further studies have demonstrated that T reg cells can suppress T cell responses to some Mtb antigens (e.g., heparin-binding hemagglutinin) but not other Mtb antigens (e.g., ESAT-6), suggesting that T reg cells may be specific for some, but not all epitopes [46]. However, other possible explanations for this selective suppression exist, such as differences in the relative affinities of each set of T cells for their cognate antigen. Thus, the definitive identification of Mtb-specific T reg cells in humans will require the use of MHC class II tetramers. Overall, human studies have revealed interesting correlations that suggest that T reg cells may play a critical role in disease pathogenesis, but evidence proving causality has been unattainable due to inherent limitations of human studies. Insights into the role of T reg cells in vivo during TB have come from the mouse model, a tractable animal model in which T reg cell numbers and function can be readily manipulated.

5 Regulatory T Cells in the Mouse Model of Tuberculosis

Regulatory T cells were not only discovered in the mouse, but most of our understanding of the in vivo functional activities of T reg cells during both homeostasis and disease has also come from mouse studies [28]. The mouse is also the most widely used animal model for studying tuberculosis, and has proven to reflect the immunobiology of human TB in many important ways. Both mice and humans exhibit a dramatically delayed T cell response after low dose aerosol infection, and the ultimate arrival of IFN-γ-producing CD4 T cells in the lung is critical for protective immunity [47]. The utility of the mouse to study T reg cell biology and TB stems from the tractable nature of model and the wealth of available reagents to monitor and manipulate various aspects of the immune response in vivo. These reagents include, but are not limited to, a wide array of antibodies to monitor and deplete specific cell types, inbred and congenic strains of mice, and genetically engineered mice (e.g., transgenic mice containing T cells with a single specificity, reporter mice in which T reg cells express fluorescent proteins, and mice with conditional knockouts of specific genes of interest in Foxp3^{+} T cells). Using these tools, researchers have begun to gain insight into the function of T reg cells during TB.

As in human TB, low dose aerosol infection of mice with Mtb leads to T reg cell proliferation, and increased numbers of activated T reg cells in the lung and other sites of infection. Early attempts at determining a functional role for T reg cells during Mtb centered on depleting T reg cells using anti-CD25 antibodies. Mice treated with anti-CD25 antibodies and subsequently infected with Mtb exhibited slightly enhanced Th1 effector cell proliferation and cytokine production, but no change in the lung bacterial burden [3]. Although this treatment was

intended to deplete T reg cells, many T reg cells express minimal levels of CD25 and others simply transiently down-modulate the molecule in response to anti-CD25 exposure [48, 49]. Thus, the inefficient elimination of T reg cells using anti-CD25 probably explains why two groups obtained greater effects when approaches resulting in more complete elimination of T reg cells were employed. Kursar et al. transferred CD4^{+}CD25-negative T cells (effectors) with or without CD4^{+}CD25^{+} T cells (T reg cells) into T cell-deficient mice and subsequently infected them with Mtb [1]. They found decreased bacterial loads in mice that received effectors alone compared to mice that also received T reg cells, suggesting that CD25^{+} T reg cells could inhibit the protective immune response to Mtb. Because effector T cells can also express CD25, we employed a strategy to eliminate all Foxp3^{+} T reg cells regardless of their expression of CD25. Mixed bone marrow chimeras were generated using Foxp3 sufficient and deficient bone marrow and antibodies against a congenic marker (Thy1.1) were used to deplete all T cells capable of expressing Foxp3. Mice depleted of T regs in this manner and subsequently infected with Mtb were shown to exhibit enhanced bacterial clearance [2], again supporting the idea that T reg cells could suppress protective immune responses. A major caveat of these experiments that confounded their interpretation was that mice rigorously depleted of T reg cells displayed multi-organ autoimmunity. Thus, while Mtb infection is better controlled in the setting of such dysregulation, it was not necessarily clear whether this indicated that T reg cells were capable of specifically suppressing immunity against Mtb during steady-state conditions, or whether they simply prevented the autoimmune inflammation from occurring. At a minimum, however, these results indicated that T reg cells had the potential to suppress immune responses that were capable of controlling Mtb.

5.1 Do Mtb-Specific T Reg Cells Arise from Effector T Cells?

In addition to being a caveat that confounds interpretation of the experimental results, the autoimmunity induced by T reg cell elimination precludes such an approach from being a practical strategy to prevent or treat TB. However, if the T reg cells that inhibited protective immunity were found to be a subset of pathogen-specific cells, it may be possible to devise interventions targeting these cells without also causing adverse effects. One way that Mtb-specific T reg cells could arise is if Mtb-specific effector CD4 T cells were induced to express Foxp3 when stimulated during the course of TB. It is now clear that induced T reg cells specific for antigens derived from commensal microbiota arise in the lymphoid compartment of the gastrointestinal tract during homeostatic conditions [29, 50, 51]. Whether this occurs during the more inflammatory milieu of a pathogenic infection remains unclear and our group has found no evidence for this phenomenon during murine TB. Naïve Foxp3-negative CD4 TCR transgenic T cells specific for

an Mtb antigen (Ag85B$_{240\text{-}254:}$I-A^{b}) transferred into Mtb-infected mice undergo robust expansion and accumulate at sites of infection including the lung, LDLN, and spleen, but expression of Foxp3 was not observed at any timepoint from early to late infection. In case the fate of Ag85B-specific T cells did not reflect the fate of endogenous CD4 T cells, we repeated these experiments by transferring a polyclonal population of Foxp3-negative CD4 T cells into Mtb-infected mice, but we observed similar results—induction of Foxp3 was not observed in any of the transferred cells [6]. Thus, responding T reg cells in murine TB do not arise from the induction of Foxp3 in conventional CD4 effector T cells, but expand from a pre-existing natural T reg cell population.

5.2 Do Proliferating Natural T Reg Cells Contain a Population of Mtb-Specific Cells?

A second possible way that pathogen-specific T reg cells could arise is through clonal expansion of antigen-specific cells from within the natural T reg cell population, as has previously been suggested to occur during chronic Leishmania infection [33]. Although natural T reg cells clearly proliferate during TB [2, 6], it initially was unclear whether they were responding to Mtb antigens or self-antigens. The latter possibility could occur either because Mtb-associated inflammation induces the expression of new self-antigens, or because the altered cytokine milieu lowers the activation threshold of T reg cells, causing them to proliferate in response to self-antigens that are always expressed. To begin to address these questions we examined T reg cells at multiple early time points to determine when they start to proliferate. Surprisingly, despite increasing levels of inflammation in the lung during the first 2 weeks of infection, T reg cells did not begin to proliferate until after day 15, and then did so in the LDLN [6]. Thus, like effector T cell responses, T reg cell responses are also delayed and do not occur until after DCs have transported bacteria to the LDLN. These results provided the first suggestion that T reg cells responding to Mtb may be pathogen-specific, but they could also be explained if self-reactive T reg cells required IL-2 or other factors derived from activated effector T cells to support their proliferation. To address this question we used Mtb-specific T reg cells from TCR transgenic mice to determine the requirements for T reg cell proliferation during TB. We showed that Mtb-specific T reg cells proliferated robustly in Mtb-infected mice, whereas T reg cells specific for irrelevant antigens did not proliferate at all [6]. Therefore, most T reg cells do not have sufficient self-reactivity to drive proliferation even during the inflammatory milieu of TB. Instead, Mtb-specific T reg cells respond preferentially, supporting the idea that the majority of T reg cells responding to early Mtb infection are indeed pathogen-specific.

5.3 Mtb-Specific T Cells Restrict Effector T Cell Priming

To determine the role of Mtb-specific T reg cells during early Mtb-infection, we transferred Mtb-specific T reg cells from TCR transgenic mice into recently infected mice. Surprisingly, even small numbers of Mtb-specific T reg cells (i.e., 50–75 antigen-specific T reg cells in the LDLN) restricted priming of effector T cells in the LN and delayed their subsequent migration to the lung. The end result was a prolonged period of unrestricted bacterial replication and a higher lung bacterial burden (Fig. 1). The ability of only a few Mtb-specific T reg cells to mediate such a potent effect suggests that their impact may be amplified through DCs, the central antigen-presenting cells driving T cell priming in the LN. Indeed, previous in vitro studies have shown that interactions between activated T reg cells and DCs can lead to downregulation of CD80/CD86 and impair the ability of DCs to provide costimulatory signals that are essential for priming naïve T cells [52, 53]. Future studies are needed to address whether this mechanism occurs in vivo during TB, or alternatively, if T reg cells restrict the expansion of effector T cells directly. Regardless of the mechanism, these results support the idea that T reg cells are critical facilitators of the notoriously slow T cell response during TB. Because allowing effector T cells

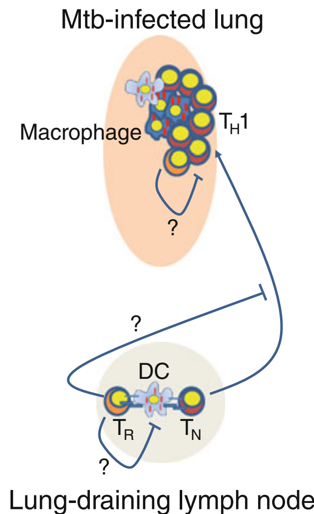

Fig. 1 Pathogen-specific regulatory T cells delay the priming of Mtb-specific effector T cells in the LDLN, resulting in prolonged bacterial replication and a higher bacterial burden in the lung. Small numbers of Mtb-specific T reg cells in the LDLN have a potent ability to restrict the priming of both CD4 and CD8 effector T cells by mechanisms that have yet to be described. This results in delayed arrival of effector T cells in the lung, prolonged bacterial replication, and a higher bacterial burden. T reg cells are also present in the lungs, including within granulomas, but the function of T reg cells in the lung have not yet been defined

to recognize Mtb in the infected lung as quickly as possible is one of the primary goals of TB vaccination, devising strategies to effectively circumvent this T reg cell-mediated delay in a lasting manner should be actively sought.

5.4 Specialization of the T Reg Cell Response During TB

Although targeting pathogen-specific cells may be one way to design T reg cell manipulations that lead to enhanced Mtb eradication without deleterious consequences to the host, an alternative strategy may be to target specific immunosuppressive pathways utilized by T reg cells to restrict immunity to Mtb that are relatively unimportant for the prevention of autoimmune responses. This possibility has been raised by the recent finding that T reg cells differentiate during TB and become specially equipped to function in the extreme Th1 inflammatory milieu of Mtb infection [37]. This specialization is driven by T reg cell expression of T-bet, a transcription factor that was previously thought to define the Th1 lineage and facilitate IFN-γ production. T reg cells in TB that co-express Foxp3 and T-bet do not produce IFN- γ, but do upregulate CXCR3, a chemokine receptor that enables these T reg cells to migrate to sites of Th1-mediated inflammation and inhibit immune responses at these sites. It has also been shown that T-bet expression renders T reg cells better able to survive and proliferate during TB and also in other scenarios of Th1-dominated inflammation [54]. Further studies are needed to characterize the global transcriptional profile promoted by T-bet expression in T reg cells, but the fact that this specialization occurs makes it plausible that a relatively specific mechanism of inhibition that restricts Mtb-specific immune responses, but plays a lesser role in preventing self-reactivity, might be discovered.

6 Next Questions and Potential Implications

As summarized in Table 1, many insights into the activity of T reg cells during TB have been gained in recent years, but many questions remain. The question of the specificity of T reg cells during infection is not unique to TB, but is a largely unanswered question in the fields of immunology and infectious disease research in general [34]. In tuberculosis, however, the answer to this question has enormous practical implications because of the high morbidity and mortality of TB globally, the poor efficacy of the current vaccine, and the rising incidence of Mtb strains that are resistant to currently available antibiotics. T reg cell-mediated immunosuppression may represent a roadblock hindering the development of effective vaccines and therapeutics targeting the host response. Thus, understanding the specificity of T reg cells in TB may provide new avenues to overcome these barriers and achieve new ways to prevent and treat TB.

Table 1 Observations made in Mtb-infected humans have led to advances in our knowledge of T reg function during TB through mechanisticstudies in animal models

Observations from Humans	Questions raised	Mechanisms defined in animal models
Recently infected individuals havereduced T reg cell numbers in theirblood	What happens to the T reg cells that were in the blood prior toinfection?	Recently infected macaques have reduced Treg cell numbers in their blood and increasednumbers in the Mtb-infected lung
During active Mtb infection T reg cellnumbers are increased in the bloodand sites of infection	What is the origin of theexpanded T reg cell population?	InMtb-infected mice, expansion occurs from apreexisting T reg cell population, suggestingthey are natural or thymic in origin
	What is the antigen specificityof the expanded T reg cells?	Mtb antigen-specific T reg cells preferentiallyexpand in Mtb-infected mice while polyclonalT reg cells or T reg cells with an irrelevantspecificity undergo minimal expansion
T reg cells from Mtb-infected patientssuppress Mtb antigen-specific T cellresponses *in vitro*	Do T reg cells suppress theimmune response to Mtb *invivo*?	Transfer of small numbers of Mtb antigenspecificT reg cells into previously Mtbinfectedmice restricts effector T cell primingand migration to the lung, allowing forbacterial replication and higher bacterialburden in the lung
Individuals with severe forms of TBhave higher T reg cell to effector T cellratios at sites of infection thanindividuals with milder disease	Do T reg cells hamper theimmune response to Mtband/or helpful in preventingdamage to the host?	Depletion of all T reg cells early during Mtbinfection leads to enhanced bacterialclearance in Mtb-infected mice

Most research to date has focused on the role of T reg cells during the first few weeks of infection, and indeed, understanding T reg cell activity during this period may have important implications for strategies that seek to prevent active or severe forms of TB from occurring. If the immune pathways that promote antigen-specific T reg cell expansion during early TB could be identified, pharmacologic blockade of these pathways in recently exposed individuals may accelerate protective T cell responses, establish earlier control of Mtb replication in the lung, and ultimately, improve infection outcomes. These insights regarding early infection, however, may be of little benefit for individuals who already have active TB. For this we need to understand the function of T reg cells during chronic stages of infection and currently this subject is almost completely unexplored.

Although mouse studies strongly support the notion that T reg cells responding during TB are pathogen-specific, there are still many important questions to address. What Mtb epitopes are recognized? Are they the same epitopes recognized by effector T cells, completely different, or partially overlapping? Because T reg cells and conventional CD4 T cells are selected in the thymus via different affinity interactions with self-antigens [55], it is a distinct possibility that the foreign epitopes they cross-react with will not be identical. If T reg cell epitopes are different from effector T cell epitopes, identifying these epitopes will be a challenging enterprise. T reg cells do not produce effector cytokines, such as IFN-γ, that are typically used in screening antigenic libraries to discover effector T cell epitopes. Thus, new technologies will need to be developed for T reg cell epitope discovery. Ultimately, once epitopes are identified, MHC class II tetramer studies will be needed to monitor the activity of Mtb-specific T reg cells throughout the course of infection in both mice and humans.

The finding that T reg cells responding during TB are likely to be Mtb-specific and arise from the pre-existing T reg cell population has many important implications. For example, vaccines or environmental exposures that increase the precursor frequency of antigen-specific T reg cells may restrict an individual's protective immune response to TB. It also seems possible that some individuals may possess genetic polymorphisms that increase their propensity to expand or induce Mtb-specific T reg cells. Would such individuals have an increased likelihood to develop active or severe forms of TB? The attenuated vaccine strain, BCG, is closely related to Mtb and shares many common antigens [56]. Future studies are needed to determine whether BCG immunization induces the expansion of antigen-specific T reg cells, and if so, whether they are long-lived and can mount a recall response to Mtb infection. Likewise, nontuberculous mycobacteria that also share many antigens with Mtb are relatively ubiquitous in the environment, particularly in soil and water [57]. Can exposure to nontuberculous mycobacteria, especially in the tolerogenic environment of the gut, cause the expansion of T reg cell populations with the potential to restrict immunity to Mtb?

Several epidemiologic studies have suggested that the BCG vaccine has limited efficacy in populations with high levels of exposure to nontuberculous mycobacteria in the environment, but the reasons for this negative association are unknown [56, 58–60]. One possibility that should be explored is whether exposure to

environmental mycobacteria can expand antigen-specific T reg cell populations that limit immunity induced by BCG and/or restrict responses to subsequent Mtb infection. Two murine studies have shown that inactivation of T reg cells prior to immunization enhances BCG-induced T cell responses, but only marginally improves, or does not improve, protection against Mtb [61, 62]. These studies, however, were performed in mice reared in relatively sterile environments that most likely would not have been exposed to nontuberculous mycobacteria. Different results may have been obtained if the mice had expanded populations of antigen-specific T reg cells from prior exposures, and experimental models need to be developed so these possibilities can be tested. Despite the central importance of IFN-γ-producing T cells in immunity to Mtb, multiple studies have shown that their frequency in immunized individuals correlates poorly with the degree of protection conferred [63–66]. Rather than the magnitude of the Th1 response, it seems possible that an individual's overall level of immune protection may depend more on their relative ratio of Mtb-specific protective versus suppressive T cells. The presence and abundance of Mtb-specific T reg cells and other T cell subsets with immunosuppressive properties are not being measured by the current approaches used to assess immunity against Mtb. As more and more vaccine candidates are beginning to be tested in human populations, there is an urgent need to identify biomarkers that can predict an individual's degree of protection. The development of new assays that could identify Mtb-specific T cells that do not produce IFN-γ, but instead inhibit immune responses that might otherwise be protective, may help us achieve this goal.

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CD1a, CD1b, and CD1c in Immunity Against Mycobacteria

Ildiko Van Rhijn, Dalam Ly and D. Branch Moody

Abstract The CD1 system is composed of five types of human CD1 proteins, CD1a, CD1b, CD1c, CD1d, and CD1e, and their mammalian orthologs. Each type of CD1 protein has a distinct antigen binding groove and shows differing patterns of expression within cells and in different tissues. Here we review the molecular mechanisms by which CD1a, CD1b, and CD1c capture distinct classes of self- and mycobacterial antigens. We discuss how CD1-restricted T cells participate in the immune response, emphasizing new evidence for mycobacterial recognition in vivo in human and non-human models.

1 Introduction

The discoverers of the CD1 locus originally separated CD1 proteins into group 1 (CD1a, CD1b, and CD1c) and group 2 (CD1d), based on amino acid sequence homology [1]. The cellular expression pattern of CD1a, CD1b, CD1c, and CD1d also supports grouping into group 1 and group 2 CD1 because the expression of group 1 CD1 molecules is limited to CD4 and CD8 double-positive thymocytes and professional antigen presenting cells, while group 2 CD1 has a more extensive

I. Van Rhijn (✉) · D. Ly · D. B. Moody (✉)
Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA
e-mail: i.vanrhijn@uu.nl

D. B. Moody
e-mail: bmoody@rics.bwh.harvard.edu

I. Van Rhijn
Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

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distribution pattern that also includes non-hematopoietic cells. Group 1 proteins are more likely to be constitutively expressed on cells, and recent studies show inverse expression of group 1 and group 2 protein surface density when encountering bacterial stimuli [2].

Published research on CD1 function strongly emphasizes the role of NKT cells in mice, which are defined by the expression of a nearly invariant T cell receptor (TCR) and recognition of CD1d. Through germline deletion of murine CD1d or the invariant TCR, as well as the use of CD1d tetramers, invariant NKT cells have been shown to function in mycobacterial, bacterial, fungal and protozoal infections, as well as mouse models of cancer, atherosclerosis, diabetes, multiple sclerosis, and fat maintenance [3, 4]. Much of this research has emphasized the invariant nature of CD1d-restricted TCRs and a stereotyped function relating to rapid secretion of cytokines, especially Interferon-γ. However, invariant NKT cells are only part of the CD1d system, and CD1d-restricted responses are in turn only part of the much larger system of CD1-reactive T cells that respond to human CD1a, CD1b, and CD1c, as well as their mammalian orthologs. Antigen presentation by CD1a, CD1b, and CD1c is recently gaining a broader interest [5]. Because mice lack group 1 CD1 proteins, studies of CD1a, CD1b and CD1c use human T cells in vitro or ex vivo, non-murine animal models, or human CD1 proteins expressed in mice, and they are now revealing the functions of T cells that normally respond to CD1a, CD1b, and CD1c. It has become clear that many of the most well known features of invariant NKT cells, including strict TCR conservation, liver homing, α-galactosylceramide specificity, and an Interferon-γ dominated immunophenotype, are not broadly shared among the larger CD1-restricted repertoire.

Here we emphasize that the group 1 CD1 versus group 2 CD1 classification lumps CD1a, CD1b, and CD1c together in ways that obscure the distinctiveness of the individual group 1 CD1 isoforms, each of which has different patterns of expression and mechanism of antigen presentation. Here we review the data on the separate functions of the CD1a, CD1b, and CD1c isoforms and describe how different or similar they are, with an emphasis on their roles in recognizing foreign, mycobacterial antigens during infection. Further, we review CD1 genes and function among non-human mammals. Both functional and evolutionary data support the idea that CD1a, CD1b, and CD1c are remarkably different from each other.

2 Cellular Expression and Intracellular Trafficking of CD1

Human CD1a, CD1b, and CD1c vary in size of their antigen binding grooves, patterns of expression across different tissues and cell types [6], and subcellular trafficking patterns, leading to presentation of different types of antigens presented in different tissues. Whereas CD1d is abundantly expressed in the liver and gastrointestinal tract, CD1a proteins are expressed in the skin at high density on the surface of Langerhans cells. Accordingly, CD1d-restricted invariant NKT cells home to and accumulate to large numbers in the liver, whereas CD1a autoreactive T cells home to

the skin [7]. CD1b expression in the periphery is limited to the surface of dendritic cells and its inducible appearance on monocytes, and under those conditions CD1a and CD1c are also expressed. In addition, CD1c is expressed on marginal zone B cells in lymph nodes and peripheral blood. These clearly different, inducible, or constitutive patterns of each type of CD1 protein represent the first line of evidence that each CD1 gene likely plays a distinct role in immune response.

The subcellular trafficking of each type of CD1 molecule to differing steady-state distribution among endocytic compartments is mainly determined by motifs in the CD1 cytoplasmic tail, which bind to adaptor protein complexes [8–10]. Whereas CD1a lacks AP binding sequences and is found on the cell surface and sorting endosomes, human CD1c and CD1d bind to AP2, which mediates recycling and antigen capture in endosomes. Mouse CD1d and human CD1b bind both AP2 and AP3, driving their trafficking and steady-state accumulation in late endosomes and lysosomes. Therefore, human and mouse CD1 proteins differ and can be ranked as to the extent of penetration into the endosomal compartment with huCD1a < huCD1c < huCD1d < huCD1b and muCD1d. These differing trafficking patterns influence their function in T cell activation because endocytic compartments differ in their antigenic content. Also, the presence of endosomal lipid transfer proteins and low pH promote antigen loading onto CD1, yet highly acidic compartments can degrade some antigens so that they are not recognized [11–16]. These data provide clear evidence that each type of CD1 protein differs with regard to the type of cell on which it is expressed, and the cellular subcompartment to which it most strongly localizes.

3 Antigen Presentation by CD1a, CD1b, and CD1c

Complementing another review on CD1d in this same edition, this review focuses on the group 1 CD1 proteins, CD1a, CD1b, and CD1c. Even though CD1a, CD1b, and CD1c are able to present mammalian self-lipids and lipids from several types of bacteria, mycobacterial lipids are the focus of this section. Here we review mycobacterial antigens, which were the first described antigens for the CD1 system [17] and for which the most information is available, including ex vivo studies of human patients. The molecular interactions of each type of CD1 groove with differing antigens are considered separately.

3.1 CD1a

Of all the human group 1 CD1 molecules, CD1a has the smallest antigen binding groove ($\sim$1,350 Å^3) and apparently the least stringent conditions for loading antigens [18, 19]. Upon *de novo* synthesis, all CD1 molecules appear on the cell surface and subsequently undergo multiple rounds of internalization via the

Fig. 1 *Sources of CD1a-, CD1b-, and CD1c-presented antigens*. Group 1 CD1-expressing antigen presenting cells internalize mycobacterial lipids via the endocytic route in the form of bacteria or bacterial debris, debris of infected cells, as free lipids, or lipid-protein complexes. Lipids are loaded into the CD1 molecules in the early or late endocytic compartments to which CD1 isoforms differentially traffic. The structures of representative mycobacterial antigens that are presented are shown. R is glucose in glucose monomycolate, glycerol in glycerol monomycolate, or hydrogen in mycolic acid

endocytic route. In contrast to all other human CD1 proteins, human CD1a lacks discernable AP binding motifs and only cycles through early endosomes, bypassing late endosomes, before returning to the cell surface (Fig. 1) [14, 20]. CD1a may bind its antigens in the endoplasmic reticulum in the case of endogenous phospholipids, but also egresses to the cell surface and recycles through early endosomes, where it has access to exogenously added antigens and serum lipids. However, CD1a is not known to have requirements for low pH to capture antigens, and there is no clear evidence that early endosomes provide antigen loading factors, like activated saposins, which are preferentially located and activated in late endosomes and lysosomes. As contrasted with other CD1 proteins, which have mechanisms for preferential antigen capture in endosomes, these findings suggest that CD1a may have the most promiscuous mechanisms for binding endogenous lipids or exogenous lipids encountered at or near the cell surface.

The potential of CD1a to contribute to anti-mycobacterial immunity is demonstrated by its ability to present dideoxymycobactin (Fig. 1), a precursor molecule in the biosynthesis of mycobactin [21, 22]. The mycobactin siderophores are

essential in mycobacterial iron acquisition and survival of the bacterium in vivo [23, 24]. Mycobactins and related molecules have peptidic backbones that are synthesized by non-ribosomal protein synthases. The crystal structure of CD1a bound to a mycobactin shows that the acyl chain is bound deeply within the A' pocket in the antigen binding groove of the CD1a molecule, and the peptidic part is folded into a U-shaped conformation, so that it descends into the C' pocket with its' termini near the predicted plane of TCR contact. In contrast to the extended conformation of MHC I-presented peptides, for which the center of the peptide provides the key surface for TCRs, the structure of CD1a-dideoxymycobactin complexes predicts that only the termini of peptides are presented to the TCR [25]. However, unlike for CD1b- and CD1c-restricted anti-mycobacterial responses, which have been directly observed among polyclonal T cells ex vivo, published evidence for dideoxymycobactin recognition is limited to detailed studies of one clone so that broader studies of polyclonal responses ex vivo are needed.

CD1a also activates T cells in the absence of exogenous antigen, suggesting that self-antigens for CD1a exist [7, 26–29]. The antigens recognized by these clones are unknown, but most likely represent cellular lipids. Using purified or commercially available lipids to prime human T cells ex vivo, it is possible to detect responses against molecules like gangliosides and sulfatides [30] and unknown ligands possibly originating from serum [18]. The very high expression of CD1a by Langerhans cells and the recognition of CD1a in the absence of exogenously added antigen by T cell clones and polyclonal T cell populations with skin homing phenotype strongly suggests that there is a relevant physiologic function of CD1a in the skin, independent from a role in antimycobacterial immunity, possibly related to skin homeostasis [7]. For CD1d-restricted NKT cells the phenomenon of self-reactivity and reactivity to exogenously added foreign antigens is well described, including the physiologic relevance of this dual reactivity [31]. It seems that CD1a represents a comparable situation in that foreign bacterial ligands certainly exist and are capable of stimulating the immune system in a CD1a-dependent manner, but self-antigens also importantly contribute to its normal function.

3.2 CD1b

Among all CD1 isoforms, the size and shape of the CD1b groove stands out as having the largest volume ($\sim$2.200 Å^3). The CD1b groove is nearly twice as large as the CD1a groove, and compared to other CD1 isoforms, CD1b binds correspondingly larger lipids, including mycolyl lipids and sulfoglycolipids that are up to C80 in length [32–37]. Because the volume of the groove is estimated to accommodate optimally C76 [38] and the fact that mycobacterial mycolyl lipids are amongst the longest hydrocarbon chains known in biology, CD1b likely plays a biological role in presenting particularly large foreign lipids. The crystal structure of human CD1b bound to glucose monomycolate shows that the very long

meromycolate chain is twisted through the combined length of the A', T', and F' pockets, which are oriented end to end, creating a superchannel in the CD1b molecule. The shorter α-branch lies in the C' pocket [32]. The large volume of the CD1b groove may represent an evolved adaptation by which CD1b particularly focuses on presentation of foreign lipids. Supporting this speculation, CD1b autoreactive T cells are rarely observed among studies of autoreactive T cell populations or clones [7, 26]. Also, ~C76–C80 lipids that are commonly found in mycobacteria are generally absent in mammalian cells and were not detected in studies that broadly measured cellular CD1b ligands in eluents [39].

Considering all antigens known for all CD1 isoforms, CD1b shows the greatest flexibility for capture of lipids of different size, ranging from C12 to C80, including glucose monomycolate [37], glycerol monomycolate [36], free mycolic acid [33], diacylated sulfoglycolipids [40] and phosphatidylinositol mannosides (Fig. 1) [41, 42]. Long chain self-lipids in the range of C80 are not yet known and may not exist, begging the question of how smaller ~C40 self-lipids can bind to CD1b.

This question has been recently solved by showing that deeply seated scaffold lipids and antigenic lipids simultaneously bind to CD1b, so that their combined length fills the complete antigen binding groove of CD1b [34, 39, 43]. Crystallographic and functional studies now show that small self-antigens, like phosphatidyl ethanolamines with approximately ~C40 alkyl chains, are seated in the 'upper' region of the groove so that their head groups protrude for TCR contact. These antigens bind simultaneously with ~C32–40 scaffold lipids. Scaffold lipids derive their name from their deep seating within the 'bottom' of the groove, where provide upward support to position antigens near the A' portal for TCR contact from the 'bottom' of the groove to support antigens. Scaffold lipids were originally suggested to exist based on co-crystallization of detergents in the groove [43], and naturally occurring spacer lipids have been recently identified as diacylglycerols and deoxyceramides [39, 43, 44]. It has been known for more than a decade that CD1b can present glucose mycolate antigens with either C80 or C32 tails, and the latter finding is likely explained by binding a diacylglycerol spacer beneath the short chain mycolic acid [37, 39].

Thus, CD1b has evolved specific mechanisms for sampling antigens greatly vary in length. A general structural formula of mycolyl lipids is provided in Fig. 1. CD1b also presents big non-mycolyl lipids, such as mycobacterial diacylated sulfoglycolipids (Fig. 1) and phosphatidylinositol mannosides, which have both been reported to bind CD1b and activate T cell clones [35, 40, 41]. Last, CD1b is known to present the ganglioside self-lipids GM1, GalNac GD1a, GD1b, and GQ1b [45].

For the presentation of long chain glucose monomycolate and mycolic acid, as well as diacylated sulfoglycolipid, trafficking of CD1b to low pH lysosomal compartments is necessary for antigen loading (Fig. 1) [13, 17, 40, 41, 46]. The low pH causes release of interdomain tethers that are present at neutral pH and normally connect the α1 helix across to the α2 helix and to a loop located at the end of the groove. Acidic pH at levels found in lysosomes protonates negatively charged residues that from ionic bonds within tethers, so interdomain contacts located immediately adjacent to the antigen entry portal are dissolved, providing

greater access to the groove, as well as unloading of prebound self-lipids [47]. Because antigens with small alkyl chains would be less affected by this proposed dilation of the antigen entry portal, this mechanism might explain how smaller self-antigens are initially captured in the secretory pathway at neutral pH, whereas larger mycobacterial antigens strictly require low pH found in endosomes.

In addition, late endosomes and lysosomes are likely enriched for exogenous or foreign antigens by the action of the mannose receptor, DC-SIGN, Langerin, LDL, or scavenger receptor-mediated endocytosis of bacteria and bacterial debris [48–52]. CD1b expressing cells that are infected with mycobacteria present lipids to T cells, but whether antigens can gain direct access from the bacterium that resides in the cytoplasm or phagolysosome to the lysosome where CD1b loads its antigens, or whether antigens need to be shed and endocytosed has not yet been solved. Supporting the notion that CD1b is of specific relevance to the immune responses against antigens with long alkyl chains, CD1b-mediated T cell responses have not only been detected among T cell clones, but also polyclonal CD1b-restricted T cell responses from infected humans and bovines against mycolic acid, glucose monomycolate, glycerol monomycolate, and sulfoglycolipid have been described by independent research groups and will be discussed in more detail in Sect. 4 [36, 40, 53–56].

3.3 CD1c

CD1c shows interaction with both $\alpha\beta$ or $\gamma\delta$ T cells [28, 57]. CD1c molecules broadly survey the endocytic compartments, trafficking into early and late endosomes, an observation suggesting CD1c may have a larger antigen sampling pool than other isoforms [58, 59]. A role for CD1c in mediating T cell responses against *M. tuberculosis* was first demonstrated by the successful derivation of human T cell clones recognizing mycobacterial lipid extracts [60, 61]. Several CD1c-restricted T cell lines have been successfully derived against mycobacterial lipid extracts and patients infected with *M. tuberculosis* have CD1c-restricted immune responses [61].

Identification of the antigenic compounds that are recognized by these T cell clones revealed that CD1c presents structurally related branched chain lipids to clones and polyclonal T cells, the first description of which defined a new class of mycobacterial lipid, mycoketides (Fig. 1). Using the CD1c-restricted T cell line CD8-1, mannosyl phosphomycoketide was identified from mycobacterial lipid extracts. Mycoketides were found in lipid extracts of various medically important mycobacterial species, including *M. avium*, *M. bovis* BCG, and *M. tuberculosis* [61, 62]. The methyl branching pattern of mycoketides is important in positioning the lipid within CD1c for optimal T cell activation. More specifically, comparisons of synthetic mycoketide analogs demonstrate that optimal T cell activation occur with all five methyl branches with S-stereocenters [63]. In addition, T cell clones can show specificity for the presence and linkage of carbohydrate moieties within dolichyl or mycoketide phosphoglycolipids [61, 63].

The crystal structure of CD1c in complex with all-S mannosyl-phosphomycoketide reveals the second largest binding groove amongst all CD1 isoforms, at 1780 Å^3. CD1c has two hydrophobic binding pockets, called A' and F'. The CD1c A' pocket has a toroidal shape, which is large enough to accommodate the bulky alkyl chain of mycoketides and binds the branched lipid with a hand in glove fit. The complex also highlighted the importance of the all-S configuration of methyl branches, which allows for the favorable clockwise rotation of the lipid, an orientation that may not occur with stereorandom methyl branching [64]. Thus the lipid tail helps to orient interactions within the CD1c A' pocket to position the glycan headgroup for recognition by T cell. The short length (C30–36), saturation, and methyl branching of mycoketides differ from that seen in mammalian isoprenoid structures and may represent a pathogen-associated pattern that specifically binds to CD1c and supports recognition. Although less studied than CD1b-restricted T cell responses, CD1c-restricted T cells recognizing synthetic mycoketide homologs, mannosyl-phosphodolichols, have been observed to be expanded in number in the blood of human *M. tuberculosis* infected patients as compared to uninfected controls, providing evidence for an in vivo immune response to this phospholipid antigen during natural human infections [61].

4 Function of Group 1 CD1 In Vivo

The in vitro generated data discussed above suggest a possible function of group 1 CD1 in mycobacterial infection by providing proof of principle that mycobacterial lipids can be recognized by T cells and by identifying many such examples through the study of T cell clones. New data for presentation of mycobacterial lipids to polyclonal T cells in vivo or ex vivo provide direct evidence for a function of group 1 CD1 during mycobacterial infection in animal models and humans.

Guinea pigs (*Cavia porcellus*), a species in which group 1 CD1 is represented by multiple CD1b and CD1c orthologs, have been used in immunization experiments with *M. bovis* Bacille Calmette-Guérin (BCG) or with partially purified lipid extracts of *M. tuberculosis,* and were shown to develop strong proliferative and cytolytic T cell responses restricted by CD1b and CD1c [65, 66]. Guinea pigs are naturally highly sensitive to infection with *M. tuberculosis*, but immunization with *M. tuberculosis* lipids resulted in significantly smaller lung lesion size upon aerosol challenge with *M. tuberculosis* than vehicle only immunized animals. The level of protection was comparable to BCG vaccinated animals [67].

Cattle (*Bos taurus*) express multiple CD1a and CD1b proteins, but lack a gene for CD1c. Cattle are sensitive to natural infection with *M. bovis*, causing bovine tuberculosis that shares many clinical and pathological characteristics with human tuberculosis. Also, *M. avium paratuberculosis* causes bovine paratuberculosis, also known as Johne's disease. Polyclonal T cells of naturally infected animals have been stimulated with lipid antigens, and despite considerable overlap in lipid content, lipid extracts from *M. bovis* and *M. avium paratuberculosis* could

specifically stimulate T cells from cattle infected with *M. bovis* and *M. avium paratuberculosis* respectively, with very little crossreactivity [56]. The immunodominant lipid in *M. avium paratuberculosis* is glucose monomycolate [56]. In uninfected animals, immunization experiments with pure glucose monomycolate demonstrated that a CD1b-restricted T cell response against glucose monomycolate was generated and could be detected until 4 months after the last immunization, but no antibody response was induced [68].

Working with guinea pigs, cattle, or any other animal species that naturally expresses group 1 CD1 is much more cumbersome than working with mice. To create the possibility to study group 1 CD1 mediated immunity in mice, a mouse that is transgenic for the human group 1 CD1 molecules has been created [69]. The expression pattern of the individual human CD1 proteins in this mouse reflects the pattern in humans. This mouse gave rise to multiple T cell lines that were reactive to human CD1 proteins in the absence of exogenous antigens. Upon immunization with *M. tuberculosis* lipids or infection with *M. tuberculosis*, T cells that recognize *M. tuberculosis* lipids presented by human CD1 proteins could also be detected. An alternative way to create mouse models for human CD1 proteins is the humanized SCID mouse [70]. An important difference between the human CD1 transgenic mice and the humanized SCID mice is that the latter also has a human T cell compartment, while the human group 1 CD1 transgenic mouse uses its endogenous murine TCR gene segments.

Working with T cells from *M. tuberculosis* exposed human subjects provides the most physiologically relevant data on function of group 1 CD1 but in the past was mainly limited to studies of human clones in vitro. However analysis of fresh polyclonal T cells is feasible and is becoming more widespread with the recent invention of new tools. One approach is to stimulate ex vivo human T cells with group 1 CD1-expressing antigen presenting cells and *M. tuberculosis* lipids followed by detection of polyclonal T cell proliferation or the release of Interferon-γ directly. This approach has been taken to demonstrate the recognition of CD1c-restricted T cells recognizing phosphomycoketides selectively expanded in human *M. tuberculosis* infected patients, providing evidence for an in vivo immune response [61]. Also, CD1b-restricted responses against glycerol monomycolate were detected ex vivo in latent tuberculosis patients and BCG vaccinated individuals, but not in healthy controls or patients with active tuberculosis [36]. Glucose monomycolate was recognized by ex vivo T cells from latent tuberculosis patients, but not by T cells from healthy donors, patients with active tuberculosis, or BCG vaccinated individuals [55]. CD1b-restricted responses against mycobacterial sulphoglycolipid have been demonstrated in both latent and active *M. tuberculosis* infected patients, but not in healthy controls [40]. Finally, responses to mycolic acid were detected in active tuberculosis patients but not in healthy controls. During treatment, the mycolic acid-specific T cell population contracted and became mostly undetectable at 6 months after initiation of treatment, but T cells specific for the mycobacterial proteins ESAT-6 and CFP10 were still detectable [54].

An alternative approach to demonstrate group 1 CD1 T cell responses during mycobacterial infections in human subjects is the use of tetramers made from group 1 CD1 proteins. Two advantages of using tetramers is that CD1-restricted,

antigen specific T cells can be visualized directly ex vivo without stimulation, and antigen specific cells can be physically captured in a live state after flow cytometric sorting. Tetramer studies of human group 1 reactive T cells were first reported last year using a glucose monomycolate-loaded CD1b tetramer. A small CD4+ T cell population was clearly detectable in tuberculosis patients, while no such population could be detected using unloaded CD1b tetramer. Sorting of this population confirmed the CD1b restriction and antigen specificity of these cells in an Interferon-γ ELISPOT assay and provides new avenues of research into TCR diversity and natural effector functions [53].

5 Characteristics of Group1 CD1-Restricted T Cells

The different mammalian group 1 CD1 genes and the chemical diversity of the antigens that can be presented by the group 1 CD1 proteins can only exert an effect on the course of an infectious disease, if there are also group 1 CD1 responsive immune cells. The evidence that T cells respond to antigen presentation by CD1 is very strong, but interactions of CD1 with a receptors other than the TCR were recently demonstrated in reports that showed that Ig-like transcript 4 (ILT4) expressed on monocytes can bind CD1c and CD1d [71, 72]. Binding of molecules other than TCR has also been demonstrated for classical MHC class I molecules and is by no means inconsistent with the activation of T cells as the main function of these molecules.

Very low precursor frequency MHC class I- and II-restricted T cells expand upon primary exposure to their cognate antigen, and this forms the basis of immunological memory. Therefore it was a surprise that invariant NKT cells, a subset of T cells that are restricted by CD1d, exist as a population with a very high precursor frequency with an activated phenotype. Immunization with their cognate antigen does not durably change their precursor frequency and phenotype. It is unknown whether CD1a-, CD1b-, and CD1c-restricted T cells behave like MHC class I- and class II-restricted cells, or rather like NKT cells. Pathogen-specific CD1a-, CD1b-, and CD1c-restricted T cell responses are virtually undetectable in blood in naïve individuals, and they appear upon priming with antigen either by infection in vivo with CD1-presented antigen-producing pathogens or by experimental immunization with CD1-presented antigens. These responses can be detected several months after the initial exposure with antigen or immunization, but it is unknown how long they persist in the absence of antigen. Thus, the question whether group 1 CD1-restricted T cells generate years-long durable memory responses like conventional MHC-restricted T cells has not yet been resolved.

Double-negative (CD4-CD8-) cells were among the first CD1a-, CD1b-, and CD1c-restricted T cells that were described [27]. This observation is likely attributable to the fact that early studies of CD1 specifically deleted CD4+ cells from cultures to reduce alloreactivity rather than the lack of naturally occurring CD4 cells that recognize group 1 CD1 proteins. More recent studies have detected many CD4+ CD1b-restricted clones, and T cells sorted with CD1b tetramers bound to glucose monomycolate are predominantly or exclusively CD4 positive [26, 36, 53, 73, 74].

The early in vitro studies on reactivity of CD1a-, CD1b-, and CD1c-restricted T cell clones and also polyclonal T cell responses have emphasized assays that determine cell proliferation, cytotoxicity, or Interferon-γ release, which are anti-mycobacterial effector mechanisms. Indeed, most known mycobacterial lipid-specific T cells can efficiently kill infected cells, which is a phenotype that suggests that these cells might contribute to immunity against mycobacteria [27, 46, 73, 75]. Interestingly, the release of granulysin, a mycobactericidal peptide, in cytolytic granules been described and has been shown to be able to kill *M. tuberculosis* bacteria [76]. However, T cell clones may show functional drift during in vitro culture. Therefore, candidate effector functions previously identified in clones must now be tested using new methods to survey fresh polyclonal T cells.

A conceptual problem with a primary cytotoxic and mycobactericidal role is that CD1a, CD1b, and CD1c are not expressed on macrophages, which form the bulk of the infected cells during tuberculosis. Also, arguing against a mainly cytotoxic and mycobactericidal function in vivo are the low percentages of circulating CD1b-restricted cells that have been detected compared to the percentages of cytotoxic MHC class I-restricted T cells during acute viral infection [53, 54]. The relatively low absolute precursor frequency in the blood raises the question as to whether these cells might migrate from the blood toward infected sites. CD1-restricted T cells might have helper functions by which the effect of a small number of antigen-specific T cells might be amplified through other effector cells, a concept that is well established for NKT cells.

It must be noted that our view of the T cell response is determined by the choice of the T cell assays. Because release of Interferon-γ is thought to be beneficial to the host during mycobacterial infection, and sensitive reagents for its detection have been widely available, it may have been the preferred readout for T cell function for convenience sake rather than the dominant natural effector function of cells in vivo. Whether CD1a-, CD1b-, and CD1c-restricted T cells perform other tasks in vivo, including regulatory, Th1, Th2, Th17-like helper-like functions, or have completely novel functions, remains to be established. These questions could be addressed with more unbiased and descriptive techniques like gene expression profiling. The recently described technology of detecting CD1-restricted T cells using antigen-loaded tetramers should help in the elucidation of the function of these cells because the Th1 biasing effects of in vitro culture can be avoided and diverse effector functions might be detected.

Another piece of information about a T cell subset lies in the TCR itself. The available TCR sequences of T cells that recognize CD1a-, CD1b-, and CD1c-presented antigens show diverse variable region usage, incorporate non-template encoded amino acids, and otherwise do not contain any features that stand out that distinguish these TCRs from the TCRs of conventional peptide specific MHC class I- or II-restricted T cells. Due to the limited number of available TCR sequences from group 1 CD1-restricted T cell clones with known antigen specificity, little is known about whether subpopulations with invariant characteristics exist. With the advance of tetramer technology and discovery of novel group 1 CD1-presented antigens, more TCR sequences will become available and invariant group 1

CD1-restricted T cells may be identified. However, the possibilities of diverse and invariant group 1 CD1-restricted are not mutually exclusive. In fact, CD1d presents lipid antigens to invariant NKT cells, but also to T cells that do not fit the criteria of invariant T cells [77–79].

6 Concluding Remarks

The CD1 family of antigen presenting molecules is a varied group of molecules that is unevenly spread among mammalian species. The functions of the CD1a, CD1b, and CD1c isoforms might be as different from each other as from CD1d. Although all the CD1 isoforms can present mycobacterial lipids to T cell clones, data suggest that for CD1a self-antigen reactive T cells are very common. In contrast, CD1b has most convincingly and repeatedly shown to present mycobacterial antigens in physiologically relevant settings. It is the right time now to to focus on animal models and study of fresh human T cells in the ex vivo setting to expand the knowledge of group 1 CD1-restricted T cells, bringing it to a level that equals our extensive knowledge of classical MHC- and CD1d-restricted T cells, and prepare the road for application of lipids in vaccines against tuberculosis.

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CD1d and Natural Killer T Cells in Immunity to *Mycobacterium tuberculosis*

Pooja Arora, Erin L. Foster and Steven A. Porcelli

Abstract The critical role of peptide antigen-specific T cells in controlling mycobacterial infections is well documented in natural resistance and vaccine-induced immunity against *Mycobacterium tuberculosis*. However, many other populations of leukocytes contribute to innate and adaptive immunity against mycobacteria. Among these, non-conventional T cells recognizing lipid antigens presented by the CD1 antigen presentation system have attracted particular interest. In this chapter, we review the basic immunobiology and potential anti-mycobacterial properties of a subset of CD1-restricted T cells that have come to be known as Natural Killer T cells. This group of lipid reactive T cells is notable for its high level of conservation between humans and mice, thus enabling a wide range of highly informative studies in mouse models. As reviewed below, NKT cells appear to have subtle but potentially significant activities in the host response to mycobacteria. Importantly, they also provide a framework for investigations into other types of lipid antigen-specific T cells that may be more abundant in larger mammals such as humans.

Keywords *Mycobacterium tuberculosis* · Natural killer T (NKT) cells · Mammalian major histocompatibility complex (MHC) class I proteins · *CD1D1* genes · T cell antigen receptors · Cortical thymocytes · CD1d expression · Macrophages · Apolipoprotein E (ApoE) · Saposin family (saposins A–D) · Microsomal triglyceride transfer protein (MTP) · Niemann-pick type C2 (NPC2) protein · Invariant NKT (iNKT) cells · Glycolipids · *Mycobacterium bovis* BCG

P. Arora · E. L. Foster · S. A. Porcelli (✉)
Department of Microbiology and Immunology, Albert Einstein College of Medicine,
1300 Morris Park Avenue, Bronx, NY 10461, USA
e-mail: steven.porcelli@einstein.yu.edu

S. A. Porcelli
Department of Medicine, Albert Einstein College of Medicine,
1300 Morris Park Avenue, Bronx, NY 10461, USA

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1 Introduction

The critical role of peptide antigen-specific T cells in controlling mycobacterial infections is well documented in natural resistance and vaccine-induced immunity against *Mycobacterium tuberculosis*. However, many other populations of leukocytes contribute to innate and adaptive immunity against mycobacteria. Among these, non-conventional T cells recognizing lipid antigens presented by the CD1 antigen presentation system have attracted particular interest. In this chapter, we review the basic immunobiology and potential antimycobacterial properties of a subset of CD1-restricted T cells that have come to be known as Natural Killer T cells. This group of lipid reactive T cells is notable for its high level of conservation between humans and mice, thus enabling a wide range of highly informative studies in mouse models. As reviewed below, NKT cells appear to have subtle but potentially significant activities in the host response to mycobacteria. Importantly, they also provide a framework for investigations into other types of lipid antigen-specific T cells that may be more abundant in larger mammals such as humans.

2 CD1 and Lipid Antigen Presentation

2.1 The CD1 Protein Family

One of the first monoclonal antibodies produced against human leukocytes provided the basis for defining CD1 as the first of many cluster of differentiation (CD) designations for the classification of cell surface antigens [1]. Subsequent work on other related monoclonal antibodies and from molecular cloning revealed that CD1 in humans is actually a family of five different proteins, now known as the CD1 isoforms designated CD1a, -b, -c, -d, and -e [2, 3]. All of these proteins show homology to the transmembrane heavy chain of mammalian major histocompatibility complex (MHC) class I proteins, and like MHC class I proteins they associate noncovalently with β2-microglobulin. However, unlike MHC I proteins, CD1 proteins are strikingly nonpolymorphic. It has become customary to classify known CD1 proteins into three separate groups, based on levels of sequence homology, patterns of expression, and differences in functions. In this classification, CD1a, -b, and -c comprise group 1 CD1 molecules, while CD1d is designated as group 2 and CD1e as group 3 [4]. Group 2 appears to be the most highly conserved among different mammals, and is the only CD1 protein shared between humans and rodents such as mice and rats, which have been found to entirely lack groups 1 and 3 CD1 proteins [5]. In mice, gene duplication has created two highly homologous murine *CD1D* genes ($\sim$95 % sequence identity), although in most mouse strains only the *CD1D1* gene is functional while the *CD1D2* gene is either an untranslated pseudogene or has relatively little immunological function [6, 7]. The high level of conservation of CD1d most likely reflects a strong evolutionary

pressure that is directly related to the unique role of this protein in the development and function of an important class of innate-like T lymphocytes, now known almost universally as CD1d-restricted natural killer T cells (NKT cells).

3 Structure and Expression of CD1d

The CD1d protein shows its highest sequence homology with MHC class I proteins in the $\alpha1$ and $\alpha2$ domains, which adopt the typical peptide antigen binding superdomain found in both MHC class I and II proteins. Supporting the $\alpha1$ and $\alpha2$ domains is the immunoglobulin-like $\alpha3$ domain that forms the majority of the noncovalent bonds with $\beta2$-microglobulin. The most striking differences between CD1d and the MHC encoded proteins are in the structure of the ligand-binding groove. This is a shallow cavity in the case of both MHC class I and II, whereas in CD1d, as in all CD1 proteins, this cavity is much deeper and has a notably nonpolar or hydrophobic surface. In CD1d, this cavity is bifurcated, resulting in the formation of two hydrophobic ligand binding sites that have been designated as the A' and F' pockets. These structural features are now well recognized as the basis by which CD1d, like other CD1 molecules, binds a range of lipid antigens and presents these to specific T cells. The size and shape of the two hydrophobic pockets of the CD1d ligand-binding groove explain the unifying characteristics of lipids presented by CD1d, which in most cases are glycolipids with two alkyl tails that are covalently anchored to a polar head group. The unique structure of CD1d allows the lipid chains to be sequestered within the protein, while the polar head group protrudes at the surface of the protein where it can form direct contacts with T cell antigen receptors.

In addition to its expression as a cell surface glycoprotein, CD1d traffics rapidly between the cell surface and intracellular compartments of the endocytic system. In fact, in most CD1d expressing cells, a majority of the CD1d protein is retained in late endosomal and lysosomal compartments, giving a steady-state distribution similar to that of MHC class II molecules. The newly synthesized CD1d arrives on the cell surface with kinetics very similar to MHC class I, suggesting a direct route for the transport of mature, glycosylated CD1d from the endoplasmic reticulum to the cell surface. At least a fraction of this newly synthesized CD1d is associated with the MHC class II invariant chain, and following delivery to the plasma membrane is rapidly internalized to endosomal and lysosomal compartments [8, 9]. CD1d also has an intrinsic tyrosine-based targeting signal in its short cytoplasmic tail that plays a major role in controlling its internalization and trafficking to endosomal compartments [10–12]. The broad intracellular localization of CD1d may be important in facilitating its ability to sample a wide spectrum of antigens at multiple locations of the endocytic network.

CD1d is expressed constitutively at least at low levels on almost all types of hematopoietic cells in both humans and mice [13, 14]. Functionally relevant levels of murine CD1d are expressed on B cells, with the highest levels on splenic

marginal zone B cells, as well as on phagocytic and specialized antigen presenting cells including macrophages, monocytes and dendritic cells [15]. Like all CD1 proteins, CD1d expression is high on immature cortical thymocytes, and is downregulated during the thymic maturation process so that it is only minimally retained on mature T cells. Expression on cortical thymocytes has been shown to be necessary for positive selection of CD1d-dependent NKT cells [16]. CD1d has also been reported to be transiently upregulated on some types of cells, such as T cells undergoing activation, on intestinal epithelial cells in response to damage and stress, and on keratinocytes in certain types of inflammatory skin disorders [17–19].

A number of pathogens have been shown to influence CD1d expression. Several viruses including vaccinia, herpes simplex, hepatitis, and lymphocytic choriomeningitis viruses decrease CD1d expression in myeloid cells, possibly as a component of an immune evasion strategy [20–22]. In contrast, there are many reports demonstrating increased CD1d expression following bacterial infections or exposure to bacterial products. For example, exposure of mouse dendritic cells to LPS derived from *Salmonella typhimurium* or *E. coli* increased CD1d expression [23], and infection with *Mycobacterium tuberculosis* or exposure to lipid extracts of that bacterium have been shown to upregulate CD1d on bone marrow-derived macrophages [24]. In addition, inflammatory cytokines like interferon-β have also been shown to augment CD1d levels on infected dendritic cells [24, 25]. All of these studies were performed with cells cultured in vitro, leaving open the question of physiological relevance. However, a study of *Listeria monocytogenes* infection in mice showed increased CD1d expression both in vitro and in vivo [26], suggesting that induction of higher levels of CD1d may be a relevant feature of immune responses to at least some intracellular bacterial pathogens.

4 Lipid Antigen Loading of CD1d

Lipid molecules are water insoluble and exist naturally within lipid-protein complexes or as principal components of cellular membranes in mammalian systems. Several studies have revealed that newly synthesized CD1d is already bound to endogenous, host-derived lipids, suggesting that these lipids must be exchanged with foreign lipids for antigen presentation to occur [27, 28]. Lipid antigen presentation may therefore be regulated by endogenous host lipids, and by the differential expression of lipid binding and lipid exchange proteins or their localization within cells (Fig. 1).

A number of specific proteins have been implicated in this process. The very low-density lipoprotein associated apolipoprotein E (ApoE) has been demonstrated to be important for the uptake of foreign glycolipid antigens by antigen presenting cells, and their subsequent association with CD1d [29, 30]. In addition, lipid exchange is accomplished by members of the saposin family (saposins A–D), which are lipid transfer proteins generated by cleavage of prosaposin within the endosomal compartment [27, 31–33]. Several other intracellular proteins have

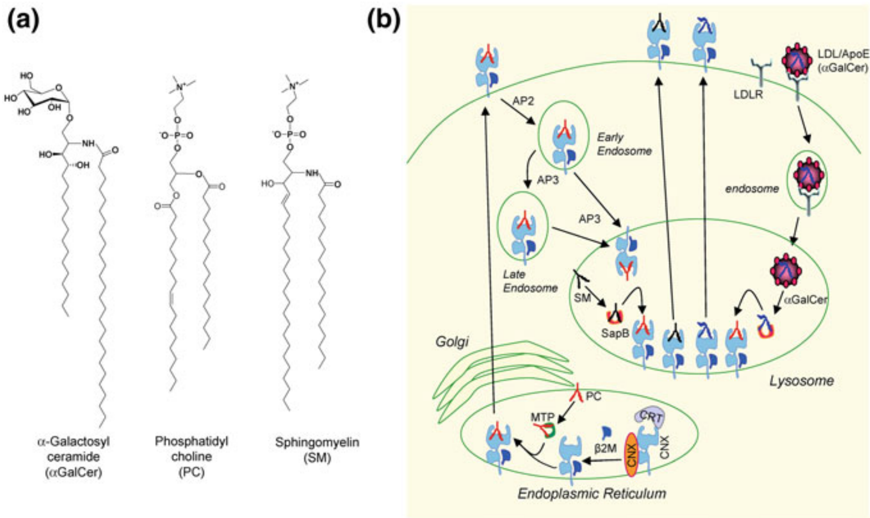

Fig. 1 *Synthesis, trafficking and antigen loading pathways of mouse CD1d molecules*. **a** Lipid ligands involved in formation of CD1d-lipid complexes. Structures of phosphatidylcholine (PC) and sphingomyelin (SM), two cellular lipids involved in formation of CD1d-lipid complexes, are shown. The synthetic CD1d binding glycolipid ligand α-galactosylceramide (αGalCer) is also shown as an example of a CD1d binding lipid acquired by lipid exchange in the lysosome. **b** Newly synthesized CD1d heavy chain associates with chaperone proteins including calnexin (CNX) and calreticulin (CRT) in the endoplasmic reticulum. After glycosylation and disulfide bond formation, the CD1d heavy chain (*light blue*) is released from these chaperons and associates with β2-microglobulin (β2M) to form the mature CD1d protein. In the ER lumen, microsomal triglyceride transfer protein (MTP) loads abundant phospholipids like phospatidylcholine (PC) onto CD1d. These CD1d-PC complexes traffic to the cell surface via the trans-Golgi network. From the cell surface, CD1d-PC complexes are rapidly internalized by a clathrin mediated endocytosis step requiring adapter protein complexes AP-2 and AP-3, which eventually delivers them to late endosomal and lysosomal compartments. Lysosomal lipid exchange proteins like saposin B (SapB) promote the exchange of ER-derived phospholipids with other intracellular lipids, predominantly sphingomyelin (SM). Transport of exogenous lipids like αGalCer is mediated by low density lipoprotein (LDL/ApoE) and LDL receptor (LDLR) into the endosomal compartment where lipid exchange can take place. Finally, the CD1d-αGalCer complexes are transported to the cell surface where they can be presented to iNKT cells

been shown to modulate the binding and presentation of lipid antigens by CD1d. For example, the microsomal triglyceride transfer protein (MTP), which resides largely in the endoplasmic reticulum, appears to have a major role in regulating CD1d protein biosynthesis and function [34]. Deletion of MTP resulted in reduced expression of CD1d on the cell surface with increased accumulation in the endoplasmic reticulum [34], and silencing of MTP in dendritic cells impaired their ability to present exogenous lipid antigens [35]. In vitro studies with purified MTP protein showed that this protein can directly transfer lipid antigen to recombinant CD1d proteins, suggesting that MTP may regulate lipid antigen presentation both by controlling CD1d biosynthesis and also through effects on lipid exchange.

Another potentially important protein in this process is the Niemann-Pick type C2 (NPC2) protein, a lysosomal lipid transfer protein that has been reported to facilitate the loading of lipids onto CD1d proteins. Mice deficient in NPC2 show defects in lipid antigen presentation by CD1d [36]. Recently, a serum protein known as fatty acid amide hydrolase (FAAH) has also been shown to contribute to exogenous glycolipid uptake from serum by antigen presenting cells to facilitate presentation by CD1d [37].

5 T Cell Populations Restricted by CD1d

Natural killer T (NKT) cells are a subset of T cells that were initially defined mainly by their coexpression of CD3-associated T cell receptors (TCRs) and several NK cell associated receptors, especially the cell surface lectin NK1.1 [38–41]. Subsequent investigations and efforts at creating a precise nomenclature have led to a current consensus that $CD3^+$ $NK1.1^+$ T cells fall into three distinct groups, based mainly on their TCR structures, their dependence on CD1d, and the nature of the antigens they recognize. Under the currently accepted system of nomenclature, these groups are called Type I, II, and III NKT cells, and each has distinct functional characteristics (Fig. 2).

Type I NKT cells are the most well studied of the different NKT cell subsets, and are the main focus of this chapter. These cells are often referred to as invariant NKT (iNKT) cells, because they express an invariant TCRα chain that arises by the precise joining of one particular Vα gene segment (Vα14 in mouse, and the highly homologous Vα24 in human) with one particular Jα gene segment (Jα18 in both mouse and human, which was formerly known as Jα281 in mouse) [42–44].

- **Type I NKT Cells**
  aka invariant NKT or "iNKT cells"
  - CD1d-restricted
  - invariant TCRα chain,
  - limited TCRβ variability
  - Lipid and glycolipid reactive (αGalCer)
- **Type II NKT Cells**
  aka non-invariant or diverse NKT cells
  - CD1d-restricted
  - Diverse TCRs
  - Lipid and glycolipid reactive (not αGalCer)
- **Type III or "NKT-like" Cells**
  - Not CD1d-restricted
  - Diverse TCRs
  - Reactivities poorly defined

Fig. 2 Classification of NKT cell subsets

This invariant TCRα chain is paired with TCRβ chains that have some diversity, but are also quite limited in their variability compared to the TCRβ chains of conventional MHC-restricted T cells [45–48]. Mice in which the gene encoding Jα18 has been deleted are essentially devoid of iNKT cells [49]. A subtle exception to the foregoing rules has recently been noted with the discovery of a second, relatively small population of mouse NKT cells that express a different invariant TCRα chain, but in other respects appear very similar to the classic iNKT cells [50]. iNKT cells recognize a range of lipid antigens presented by CD1d molecules on antigen-presenting cells, and all of these cells are strongly reactive to the synthetic model antigen α-galactosylceramide (αGalCer) [51]. Other lipids recognized by iNKT cells can be derived from endogenous or exogenous sources, including microbial cell walls [32, 52–54].

Type II NKT cells are also CD1d-restricted T cells and also appear to be predominantly or exclusively responsive to lipid antigens. However, in contrast to type I NKT cells, type II NKT cells express highly diverse TCRs and recognize a different range of lipid antigens that in most cases does not include αGalCer [55]. Type II NKT cells are believed to circulate with an activated or memory phenotype similar to type I NKT cells, and are absolutely dependent on CD1d expression in the thymus for their selection. Thus, whereas mice with a deletion of the Jα18 gene lack type I NKT cells, these mice retain type II NKT cells. In contrast, knockout mice with homozygous deletion of their *CD1D* genes lack both type I and type II NKT cells [56]. Much less is known about type II NKT cells in general, largely because reagents to unequivocally identify and isolate them are currently lacking, and animal models in which they are selectively deleted are not yet available.

Type III NKT cells comprise a third category of $CD3^+$ T cells coexpressing NK1.1 or other NK cell-associated receptors. These cells are not dependent on CD1d for their development or function, have diverse TCRs, and may be predominantly a heterogeneous group of cells that are mainly or exclusively MHC restricted [57]. They also sometimes display markers associated with na T cells, unlike type I and type II NKT cells. It is believed that some or many of these type III NKT cells have innate-like qualities that link them functionally to type I NKT cells, although their functions and phenotype are still largely unknown.

6 Development, Frequency, and Function of iNKT Cells

The development of iNKT cells involves thymic selection from a pool of immature thymocytes in the $CD4^+CD8^+$ heat-stable antigen expressing (HSA) population [58]. Several important features distinguish the intrathymic development and selection pathway for iNKT cells from that of conventional MHC-restricted T cells. One unique feature is the specific requirement for recognition of CD1d expressed by cortical thymocytes for positive selection of iNKT cells, which contrasts strongly with the positive selection of most conventional T cells by recognition of MHC class I or II molecules expressed on thymic epithelial cells [16, 56]. Alternately,

transgenic overexpression of CD1d in thymic dendritic cells leads to negative selection and the arrested development of iNKT cells [16]. The intrathymic positive selection of iNKT cells is also unusual in its absolute dependence on homotypic interactions between members of the SLAM family of surface proteins, in particular Slamf1 and Slamf6, and signals generated through their recruitment of the adaptor SLAM-associated protein (SAP) [59]. This process is highly dependent on the tyrosine kinase Fyn, as Fyn-deficient mice have a marked reduction in the number of iNKT cells while the numbers of CD1d-independent NKT cells and conventional T cells are not altered [57]. Developing iNKT cells upregulate NK1.1 and activation-associated markers such as CD69 as they develop in the thymus, and the acquisition of their unusual phenotypic and functional program is driven in large part by their expression of a key transcription factor known as promyelocytic leukemia zinc finger (PLZF) [60–62]. Nearly all developing iNKT cells extinguish the expression of CD8 early in their development as a result of expression of the transcription factor Th-POK, and a large fraction also downmodulate CD4 [63]. Thus, mature iNKT cells are either $CD4^+$ or $CD4^-CD8^-$ (double negative, or DN) [58].

After leaving the thymus, iNKT cells localize to many tissues and are found predominantly in the liver, spleen, and peripheral blood of healthy humans and mice, with lower numbers in the bone marrow and lymph nodes [38, 41, 64–66]. There is a wide range in the number of iNKT cells in the blood of healthy humans, reported to be as low as 0.003 % and as high as 1.78 % of total circulating T cells, with a mean value usually in the range of 0.1 % in healthy Caucasians with no significant differences observed between males and females or children and adults [67, 68]. However, the number of iNKT cells in peripheral blood, as well as their proliferative ability, decreases with age in healthy elderly humans, as documented with other T cell subsets [69]. In contrast to peripheral blood, iNKT cells are highly enriched in the liver, where they localize predominantly within the microvasculature of the hepatic sinusoids under normal, steady state conditions [70]. In mice, iNKT cells have consistently been found to comprise 20 % or more of total hepatic $CD3^+$ T cells. In humans, this percentage is lower, but still shows a substantial enrichment [71, 72]. Liver iNKT cells have been observed to decrease in mice as they age, and this appears also to be the case for humans [73, 74].

A remarkably broad range of functions characterizes iNKT cells, and these overlap with the functions of multiple different innate-like and adaptive immune effector cells [75]. Much emphasis has been given to the finding that iNKT cells secrete a large number of different cytokines when activated, including those produced by classical Th1 T cells (interferon-γ and TNF) as well as those that are characteristic of Th2 responses (IL-4, IL-5 and IL-13). iNKT cells also possess significant cytotoxic effector activities [76, 77], and can serve as helper cells for antibody responses [78]. The extraordinary diversity of iNKT cell functions undoubtedly explains their participation at some level in many types of immune responses, including those directed at virtually all classes of microbial pathogens [79, 80].

7 Antigens Recognized by iNKT Cells

All clearly confirmed examples of iNKT cell antigens are lipids, and in most cases they are glycolipids derived from natural or synthetic sources. The specific glycolipid ligand that has been used most extensively to investigate as well as therapeutically manipulate iNKT cell responses is a synthetic glycolipid known as KN7000, which is a form of α-galactosyl ceramide (αGalCer) containing a saturated C26 fatty acyl chain and a C18 phytosphingosine base (Fig. 1) [81]. This glycolipid, which is a derivative of a similar natural glycolipid that was identified as an immunostimulatory compound with anti-cancer activity in extracts from the marine sponge *Agelas mauritianus*, is well known for its ability to rapidly and potently stimulate a characteristic cascade of iNKT cell dependent cytokine responses in mice [82, 83]. In addition to the initial bursts of iNKT cell derived IL-4 and IFNγ stimulated by αGalCer administration, this glycolipid also induces the secondary activation of DCs, NK cells and other leukocytes through a process that is dependent on the initial iNKT cell response [84–86].

Several variants of αGalCer have been synthesized that induce a selective IL-4 or IFNγ response, and are therefore described as Th2- or Th1-biasing agonists [87–89]. Detailed analysis of αGalCer presentation has revealed that Th2-biasing agonists load CD1d directly at the cell surface, and the CD1d-glycolipid complexes formed in this way are excluded from cholesterol rich lipid raft microdomains in the plasma membrane. In contrast, Th1-biasing agonists load CD1d in endosomal compartments and are presented predominantly by CD1d that are localized plasma membrane lipid raft microdomains [27, 87]. Interestingly, protective effects of iNKT cells in infectious models involving intracellular pathogens are strongly correlated with IFNγ secretion and secondary activation of NK cells, which appear to be driven by lipid raft localized CD1d-antigen complexes. In this regard, it is intriguing that many microbes such as mycobacterial species secrete cell wall lipids that are selectively incorporated into the lipid raft microdomains of infected macrophages [90] and may indirectly modulate antigen presentation to iNKT activation to subvert this component of host protective immunity.

In addition to the extensive work on synthetic glycolipid antigens, iNKT cells have also been shown in many recent studies to recognize a range of natural self and foreign microbial lipids and glycolipids [88] (Fig. 3). Approaches relying mainly on an analysis of which cellular lipids are bound to CD1d extracted from cells initially were reported to show that glycosylphosphatidylinositols were the major bound self-lipid ligands [91]. However, subsequent refinements of this approach have shown that the majority of the naturally bound cellular lipids associated with CD1d are actually phosphatidylcholine (PC) and sphingomyelin moieties. In addition, studies relying mainly on analysis of iNKT cell recognition have identified different cellular lipids as the predominant potential self-antigens that are presented by CD1d to this T cell population. Early studies using this approach revealed that purified cellular phospholipids including phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) could activate some

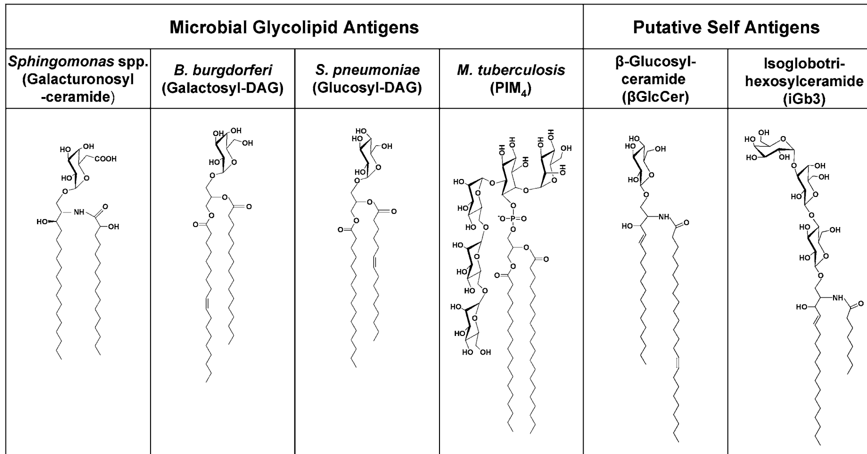

Fig. 3 Structures of representative microbial and putative self glycolipid antigens recognized by iNKT cells

but not all iNKT cell hybridomas to varying degrees, although usually weakly compared to the strong synthetic agonist αGalCer [92]. Another set of studies identified lysophosphatidylcholine, a lipid moiety generated by lipid-dependent signaling pathways, as self-lipids capable of stimulating iNKT cells [93].

Cellular glycosphingolipids, which are the self-lipids most closely resembling the synthetic antigen KRN7000 with the important difference that their proximal sugars almost invariably contain a *β*-anomeric linkage, have also been implicated as important self antigens for iNKT cells. Thus, the lack of iNKT cell stimulation by cells mutant in *β*-glucosylceramide synthase suggested lysosomal glycosphingolipids as candidate natural ligands. An initial candidate was identified as isoglobotrihexosylceramide (iGb3), which was proposed to be the major ligand recognized during CD1d-dependent thymic selection of iNKT cells [32], although this conclusion has been questioned by several subsequent studies [94, 95]. Recently studies have described particular forms of *β*-glucosylceramide as stimulatory self-lipids for iNKT cells [96], and this lipid which is known to be present in thymus tissue has also been proposed as a major candidate ligand involved in positive selection of iNKT cells.

In addition to the synthetic and self-lipid antigens discussed above, multiple studies have identified iNKT cell stimulating lipids as natural components of various microbes, including some that are well known as causative agents of disease in mammals. One of the first studies to seek such microbial iNKT cell antigens used lipids derived from *M. bovis* BCG to assess CD1d binding and iNKT cell recognition. These initial experiments identified a glycolipid fraction enriched in phosphatidylinositol mannosides (PIMs) as potential CD1d-presented iNKT cell antigens, with a tetramannosylated form (PIM_4) having the greatest apparent activity (Fig. 3) [97]. Although published in 2004, these findings still await confirmation and

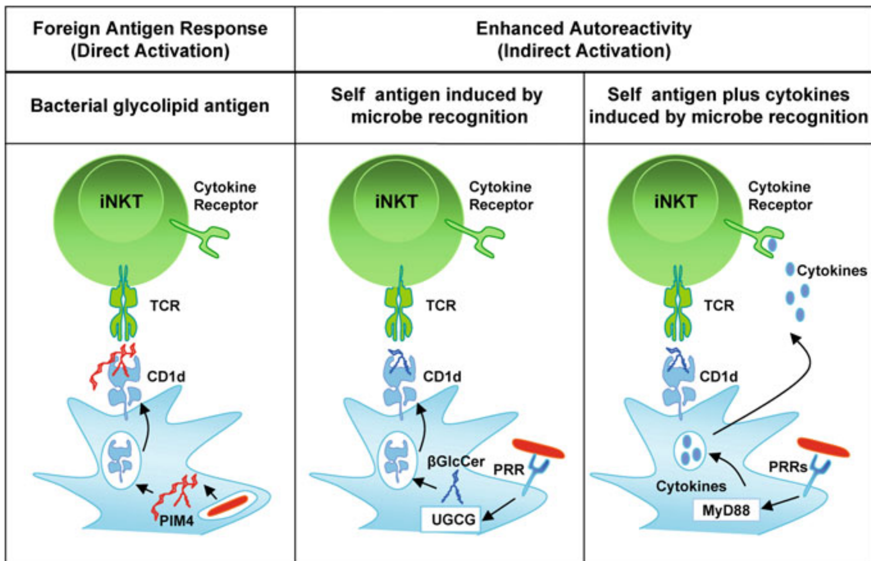

Fig. 4 *Direct and indirect pathways of iNKT cell activation*. Microbial pathogens carry CD1d restricted glycolipid antigens that can directly stimulate iNKT cells, such as the PIM_4 glycolipid of mycobacteria. Recognition of foreign glycolipid antigens by the TCR of iNKT cells results in direct activation (*left panel*). However, iNKT cell activation can also occur even when foreign microbial lipid antigens are absent or present in limiting amounts. This occurs through one or more indirect activation pathways. In one of these pathways, simulation of pattern recognition receptors (PRRs) by microbial products generates signals that increase the levels of *UDP-glucose:ceramide glucosyltransferase (UGCG), that can increase the levels of self* antigens like *β*-glucosylceramide (*β*GlcCer) (*center panel*). In other cases, innate recognition of microbes by pattern recognition receptors (PRRs) induces secretion of inflammatory cytokines like IL-12 and IL-18 via MyD88-dependent signaling that can stimulate iNKT cells primarily through cytokine receptors either with or without cognate antigen recognition through their TCRs (*right panel*)

further investigation with respect to their potential relevance to mycobacterial infections. However, studies on a number of other microbes have provided more convincing evidence of relevant CD1d-presented lipid antigens in bacteria. Striking examples are the diacylglycerol type glycolipids of *Borrelia burgdorferi* and *Streptococcus pneumoniae*, and the glycosphingolipids present in the cell walls of bacteria belonging to the genera *Sphingomonas* and *Novosphingobium* (Fig. 4). These glycolipids bind strongly to CD1d, and are capable of engaging the TCRs of iNKT cells to activate their immunological functions [54, 98–100]. Isolated studies have also identified potential antigenic lipids with iNKT cell stimulating capacity in protozoal parasites, including the surface glycophospholipids of *Leishmania* species [101] and the lipopeptidophosphoglycan of *Entamoeba histolytica* [102]. Evidence in mouse models of infection with these organisms supports the view that iNKT cell recognition of these glycolipid antigens may have an impact on the outcome of infections with the microbes that express them. However, other indirect mechanisms

of iNKT cell activation are also present in the setting of microbial infection, and there is still controversy concerning the extent to which recognition of specific microbial glycolipids plays a significant role in host immunity in vivo [96, 103].

8 Mechanisms of iNKT Cell Activation

Given the limited diversity of the TCRs of iNKT cells, their ability to participate in immune responses associated with a wide variety of microbial infections is surprising. This is particularly the case for infections caused by viruses, which would not be expected to harbor foreign lipid ligands of the type known to be presented by CD1d. This paradox has led to vigorous study of the structural basis for iNKT cell TCR interactions with cognate ligands, a process that forms the basis for direct TCR mediated activation of iNKT cells. In addition, a substantial amount of emphasis has been placed on defining indirect mechanisms of iNKT cell activation through mechanisms that are independent of foreign lipid antigen recognition (Fig. 4).

A remarkable array of X-ray crystallographic studies have been carried out to elaborate the unique flexibility of the CD1d presentation and iNKT cell TCR recognition process, providing a detailed molecular explanation for how such structurally diverse lipid ligands can lead to direct activation of iNKT cells. Structures of CD1d proteins have been obtained with a variety of different bound antigens, including microbial lipids from *Sphingomonas* and *Borrelia* species [54, 104] and *Streptococci* [98, 105], as well as candidate self-lipid antigens such as sulfatide [106] and iGb3 [107]. In addition, structures of ternary complexes of CD1d with bound synthetic glycolipid ligands and TCR have been solved for both human [108] and murine proteins [109]. These structures revealed significant differences from the conventional $\alpha\beta$ TCR binding to peptide/MHC complexes, as the iNKT cell TCR adopted an unusual docking mode and bound approximately parallel to the long axis of the ligand binding groove of the CD1d molecule. This is in marked distinction to the diagonal docking mode generally seen with conventional $\alpha\beta$ TCR/pMHC complexes. Comparison of ternary structures formed with CD1d presenting αGalCer, *Borrelia* and *Sphingomonas* glycolipids revealed that the binding mode was largely conserved among all the three structures [109]. Analysis of these structures strongly suggested that TCR binding can induce reorientation of the exposed polar head groups of glycolipid antigens, thus enabling the conserved TCR docking mode to recognize a broad range of antigens with reasonable affinity [110]. Comparison of ternary complexes formed with candidate self-glycolipids containing β-linked glycans reinforced this observation. The CD1d/lipid/TCR complexes formed with iGb3, β-galactosylceramide, and other glycosphingolipids with β-linked sugars all show flattening of the sugar moiety by the TCR to mimic the α-anomeric glycolipid pattern [106, 107, 111, 112]. The energy expended to induce the flattening of sugar head groups probably explains the low TCR affinity observed with these β-glycosyl lipid antigens compared to the much higher affinity for α-glycosyl lipid antigens like αGalCer.

Although the promiscuity of the iNKT cell TCR binding described above may contribute to the ability of iNKT cells to become activated in many types of infections, there is mounting evidence that indirect pathways of activation involving inflammatory cytokines may play a predominant role in many in vivo scenarios (Fig. 4). The first evidence for this alternate pathway of iNKT cell activation came from studies of in vitro stimulation of IFNγ secretion by human iNKT cell clones by monocyte-derived dendritic cells (DC) and heat killed bacteria [103]. Further characterization revealed that purified lipopolysaccharide (LPS) alone was sufficient to induce this response in a CD1d-dependent manner. Furthermore, iNKT cell activation by DCs infected with salmonella occurred rapidly and could be blocked by antibodies specific for either CD1d or IL-12. DCs deficient in MyD88 were incapable of inducing iNKT cell activation in response to LPS, suggesting that IL-12 induced by a TLR-4-dependent pathway is required for this process [53]. These findings are in contrast to αGalCer-induced iNKT cell activation, which is independent of MyD88 signaling. Since no exogenous glycolipid is required for this MyD88 dependent pathway of iNKT cell activation, it is believed that weak self-lipid antigen-mediated stimulation through the iNKT cell TCR is enhanced by IL-12 secreted by DCs in response to LPS. Such stimulatory self-ligands may be upregulated by conditions resulting from microbial infections or by TLR signaling. A leading candidate self-agonist that may be involved in such responses is β-glucosylceramide (βGlcCer), which has been shown to accumulate in DCs following TLR stimulation or in response to bacterial infection [96]. A possible mechanism is the increased activity in response to TLR signaling of UDP-glucose:ceramide glucosyltransferase (UGCG), an enzyme involved in the synthesis of membrane glycosphingolipids (Fig. 4) [113].

This indirect pathway of activation resulting from enhanced autoreactivity of iNKT cells by IL-12 has been found to induce secretion of IFNγ with little or no IL-4 secretion, and may thus be particularly effective for increasing innate host resistance to infection by intracellular pathogens. Consistent with this, studies have demonstrated that the indirect activation pathway is operational in mouse models of infection with *Mycobacterium bovis*, *Trypanosoma cruzi*, and *Listeria moncytogenes* [114–116]. Additional indirect pathways for iNKT cell activation have also been identified, such as one that is independent of CD1d presented self-antigens but requires IL-12 and IL-18 secreted by DCs in response to microbial stimulation [117].

9 The Role of iNKT Cells in Mycobacterial Infection

As powerful innate immune effectors and modulators of adaptive immune responses, iNKT cells have many functions that could potentially contribute significantly to immune responses against *M. tuberculosis* or other pathogenic mycobacteria. In particular, iNKT cells contain preformed IFNγ mRNA in their cytoplasm, and rapidly secrete this cytokine upon T cell receptor stimulation at levels that are up to 200-fold higher than na conventional CD4^{+} T cells [118, 119]. In addition, stimulation of iNKT

cells in vivo induces maturation of dendritic cells and facilitates their cross-presentation of soluble antigens, leading to increased priming of $CD8^+$ T cell responses [120]. This is relevant in the case of a mycobacterial infection for expanding antigen-specific $CD8^+$ T cells that participate in protective immunity [121]. However, despite these compelling reasons for considering an important role for iNKT cells in host protective immunity to mycobacteria, definitive data supporting this hypothesis are still lacking. The sections that follow summarize the available information addressing this point in mouse models and in human tuberculosis.

10 Insights from Mouse Models of Tuberculosis

Studies carried out in mice infected with live mycobacteria or injected with substances extracted from these bacteria have indicated the potential of iNKT cells to respond to *M. tuberculosis* and closely related pathogens. For example, 8 days after intravenous infection of mice with *M. bovis* BCG, there is a 6-fold expansion of iNKT cells in the lungs, with a subsequent decline by day 30 after infection [122]. These cells also decrease their surface NK1.1 expression and secrete IFNγ in the lung, consistent with their activation. Injection of mice with purified *M. tuberculosis* cell wall glycolipids such as trehalose-6,6′-dimycolate (TDM) and phosphatidylinositol mannoside (PIM) has also been associated with iNKT cell recruitment or activation [123, 124]. Other studies have suggested the importance of iNKT cells in the formation of granulomas in response to mycobacterial cell wall components [122, 125, 126]. Furthermore, in vitro experiments have shown the ability of iNKT cells to kill *M. tuberculosis*-infected macrophages and to concurrently mediate bactericidal effects [114].

Given the potential of iNKT cells to contribute to anti-mycobacterial responses, several groups have examined their importance for protective immunity in mycobacterial infection using knockout animals with specific gene deletions that eliminate iNKT cells (i.e., $CD1D^{-/-}$ or J$\alpha18^{-/-}$, both of which lack classic iNKT cell populations) [56, 127]. In initial experiments performed using CD1d-deficient knockout mice infected intravenously with 10^6 CFU of *M. tuberculosis*, there was no apparent significant difference in mortality rate whether iNKT cells were present or not [121]. A second study effectively confirmed these results, although higher bacterial counts were found in the lungs of CD1d-deficient animals at day 63 after intravenous infection with a lower dose of *M. tuberculosis* (10^5 CFU) [128]. However, this study also found ultimately no difference in survival or in lung bacterial burdens at later time points. Similar studies were also performed on Jα18 knockout mice, which specifically lack most iNKT cells. Rates of bacterial clearance, histologic findings, and mortality rates were similar in wild-type and Jα18 knockout mice, again indicating no major impact of iNKT cells on the progression or outcome of mycobacterial infection in mice [129, 130]. Although modest increases in lung CFUs were observed in one study following intratracheal infection with BCG [129], another study using intravenous BCG infection showed no significant difference in tissue CFUs in wild-type versus Jα18 knockout mice

[122]. A few studies have also been carried out using treatment of *M. tuberculosis* infected mice with anti-CD1d monoclonal antibodies as an alternative approach to assessing a role for iNKT cells during infection [131, 132]. While an effect on bacterial levels was observed in one case, these experiments are difficult to interpret because of the expression of CD1d on virtually all hematopoietic cells and the strong probability of nonspecific effects. Taken together, these studies argue against a major, nonredundant role for iNKT cells or other CD1d-restricted T cell populations in control of tuberculosis or other mycobacterial infections.

Another noteworthy observation is the depletion and anergy of iNKT cells, which has been revealed in studies of chronic infection with BCG in mice. Seven to nine days after intravenous BCG infection, the iNKT cell population contracts in the liver and spleen, most likely as a result of programmed cell death [133, 134]. In addition, the relatively few iNKT cells that persist after this contraction are unresponsive to T cell receptor stimulation by dendritic cells pulsed with αGalCer. This suggests that potentially protective functions of iNKT cells may be expressed only in the early acute phases of microbial infections, and thus further suggests that these cells may be of little importance in the natural course of prolonged chronic infections such as tuberculosis.

11 Studies of iNKT Cells in Human Tuberculosis

Most studies related to the role of iNKT cells in controlling *Mycobacterium tuberculosis* infection in humans are observational and characterize the percentage, numbers, or phenotype of iNKT cells in infected patients compared to healthy controls. Taken together, these studies do not give clear evidence for a requirement of iNKT cells in protection against pulmonary tuberculosis, although they do show changes in frequency and phenotype in association with *M. tuberculosis* infection. Interpretation of these studies in humans is complicated by the use of different methods to identify iNKT cells, some of which may not be sufficiently specific [135]. However, studies using reliable methods for quantitating iNKT cells have generally been consistent in finding a decrease in the circulating levels of these cells in human subjects with active tuberculosis compared with healthy uninfected controls [136–138]. These changes in overall iNKT cell levels were also associated with a phenotypic shift, resulting in an increased relative proportion of $CD4^+$ iNKT cells compared to $CD4^-CD8^-$ (double negative, or DN) iNKT cells. This alteration in circulating iNKT cell subsets during *M. tuberculosis* infection, which has also been observed in the mouse tuberculosis model [136], appears most likely to be due to a preferential egress of the DN iNKT cell subset out of the circulating pool and into infected tissues. This tissue localizing DN iNKT cell subset has been suggested by in vitro studies to produce a more proinflammatory cytokine pattern and to secrete the antimicrobial effector granulysin at higher levels than other iNKT cells, suggesting a potential protective role in antimycobacterial immunity [139].

12 Potential Therapeutic Opportunities

Although the available data argue against a major role for iNKT cells in the natural course of tuberculosis or other chronic mycobacterial infections, there remains a strong possibility that this T cell population can be manipulated to provide useful effects in the more acute stages of infection or in the setting of vaccination. Along these lines, several studies have shown that deliberate activation of iNKT cells using αGalCer in mouse tuberculosis models can enhance protective immunity and promote clearance of the bacilli, presumably through activation of bactericidal mechanisms. Thus, treatment of mice with αGalCer on days 1, 5, and 6 after infection with *M. tuberculosis* was shown to significantly increase survival and decrease bacterial burden through a mechanism that was dependent on CD1d expression [140]. Chronic administration of αGalCer to infected mice did not further increase survival or decrease bacterial burden, consistent with the development of iNKT cell anergy during chronic infection that has been demonstrated in studies using systemic BCG infection [134].

In a more detailed subsequent study of this approach, αGalCer was administered using several doses and treatment schedules before and after infection of mice with *M. tuberculosis* [141]. These experiments showed that intraperitoneal administration of a relatively high dose of αGalCer 1 day after infection gave significant enhancement of protective immunity, with treated mice showing increased duration of survival and decreased bacterial burden in their lungs. Similar findings were obtained in mice treated with intratracheal administration of a relatively low dose of αGalCer. Mice with established tuberculosis that had been infected 30 days previously also benefited from αGalCer treatment, with significantly enhanced survival, and αGalCer was effective as an adjunctive immunotherapy when added to isoniazid treatment. Together, these studies provide evidence that pharmacologic activation of iNKT cells during the early phases of tuberculosis can significantly strengthen natural antimycobacterial immunity, and may also retain at least some efficacy during later stages of disease or in combination with antimicrobial drug therapy.

Another approach to capturing the therapeutic potential of iNKT cells in tuberculosis has focused on the use of iNKT cell activators as vaccine adjuvants [142]. By directly incorporating αGalCer into live BCG organisms using a detergent-mediated uptake method, a modified vaccine was created that gave improved protection against challenge with virulent *M. tuberculosis* when compared to standard BCG. This effect was attributed to an increased priming of IFNγ-producing $CD8^+$ T cells specific for *M. tuberculosis* antigens, and was associated with reduced tissue CFUs, improved histology of infected lungs with decreased necrosis and PMN infiltration and increased lymphocyte recruitment. In addition, increased maturation of DCs was seen in animals immunized with αGalCer-modified BCG. These protective effects were dependent on the physical incorporation of the glycolipid into the bacilli, rather than simple co-administration, and also required the presence of functional iNKT cells and CD1d. These results strengthen the view that deliberate controlled activation of iNKT cells during vaccination against *M. tuberculosis* can have major beneficial effects in promoting protective recall responses against subsequent infection.

13 Summary and Conclusions

An impressive array of studies has contributed to our current understanding of iNKT cells as a prominent specialized T cell subset that participates in innate and adaptive immunity and in many aspects of immunoregulation. It is important to recognize that much of our knowledge on the potential functions of iNKT cells and other CD1d-restricted T cell populations come from studies of mice, which have a relatively large population of these T cells that may amplify their importance. Nevertheless, iNKT cells clearly show substantial conservation between mice and humans in many of their key features. Although there is currently no evidence to support the view that these cells play a major nonredundant role in tuberculosis or other mycobacterial infections, they do appear to participate in the immune response to this pathogen and may have subtle but important effects on the progression of disease. In addition, because of the impressive array of effector molecules produced by iNKT cells, their deliberate manipulation in the setting of vaccination or as an adjunct to therapy could have useful applications in strategies for prevention or cure of tuberculosis.

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The Role of B Cells and Humoral Immunity in *Mycobacterium tuberculosis* Infection

Lee Kozakiewicz, Jiayao Phuah, JoAnne Flynn and John Chan

Abstract Tuberculosis (TB) remains a serious threat to public health, causing 2 million deaths annually world-wide. The control of TB has been hindered by the requirement of long duration of treatment involving multiple chemotherapeutic agents, the increased susceptibility to *Mycobacterium tuberculosis* infection in the HIV-infected population, and the development of multi-drug resistant and extensively resistant strains of tubercle bacilli. An efficacious and cost-efficient way to control TB is the development of effective anti-TB vaccines. This measure requires thorough understanding of the immune response to *M. tuberculosis*. While the role of cell-mediated immunity in the development of protective immune response to the tubercle bacillus has been well established, the role of B cells in this process is not clearly understood. Emerging evidence suggests that B cells and humoral immunity can modulate the immune response to various intracellular pathogens, including *M. tuberculosis*. These lymphocytes form conspicuous aggregates in the lungs of tuberculous humans, non-human primates, and mice, which display features of germinal center B cells. In murine TB, it has been shown that B cells can regulate the level of granulomatous reaction, cytokine production, and the T cell response. This chapter discusses the potential mechanisms by which specific functions of B cells and humoral immunity can shape the immune

L. Kozakiewicz and J. Phuah contributed equally.

L. Kozakiewicz · J. Chan (✉)
Departments of Medicine and Microbiology and Immunology,
Albert Einstein College of Medicine, Jack and Pearl Resnick Campus,
1300 Morris Park Avenue, Forchheimer Building, Room 406,
Bronx, NY 10461, USA
e-mail: john.chan@einstein.yu.edu

J. Phuah · J. Flynn
Department of Microbiology and Molecular Genetics, Center for Vaccine Research,
University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

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response to intracellular pathogens in general, and to *M. tuberculosis* in particular. Knowledge of the B cell-mediated immune response to M. tuberculosis may lead to the design of novel strategies, including the development of effective vaccines, to better control TB.

Keywords *Myocobacterium tuberculosis* · Humoral immunity · B cells · Antibodies · T cell responses · Antigen presentation · Cytokines · Cytokine production · Fcγ receptors · Macrophages · Neutrophils · Granuloma · Immunopathology

1 Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is a major global health threat, resulting in over 2 million deaths each year [1]. *M. tuberculosis* is a remarkably successful pathogen due to its ability to modulate and to evade immune responses [2–4]. Cell-mediated immunity effectively regulates bacterial containment in granulomatous lesions in the lungs, usually without completely eradicating the bacteria, which persist in a latent state [5]. However, reactivation of TB can occur when the host immune system is compromised by various factors, such as HIV infection and the use of tumor necrosis factor (TNF) blockade therapy for a variety of inflammatory diseases [6–8]. The ability of *M. tuberculosis* to manipulate and evade immune responses presents a major challenge for the development of efficacious therapies and anti-TB vaccines [3, 4, 9–11]. Bacillus Calmette-Guèrin (BCG), an attenuated strain of *Mycobacterium bovis*, is the only anti-TB vaccine that is currently administered [12]. Although BCG protects adequately against pediatric TB meningitis, its protective effect for adult pulmonary TB, a most common form of the disease, is inconsistent at best [13–16]. A more thorough understanding of protective immunity and the ways by which *M. tuberculosis* manipulates these responses will aid in the control of TB [12, 17, 18].

It has been well established that cell-mediated immunity plays critical roles in defense against *M. tuberculosis* [3, 4, 11]; by contrast, B cells and antibodies generally have been considered unimportant in providing protection [19–21]. This notion has derived, at least in part, from inconsistent efficacy of anti-TB passive immune therapies tested in the late nineteenth century, which possibly could be due to the varied treatment protocols and reagents employed [20, 22]. In the late nineteenth century, the development of the concept of cell-mediated immune response based on Elie Metchnikoff starfish larvae observation as well as antibody-mediated immunity derived from Ehrlich's side-chain theory [23–25] set the stage for the subsequent emergence of the view that defense against intracellular and extracellular pathogens are mediated by cell-mediated and humoral immune responses, respectively [26, 27]. Guided by this concept of division of immunological labor, the role of humoral immune response in defense against *M. tuberculosis*, a prominent

intracellular pathogen, is generally thought of as insignificant [19, 28, 29]. However, accumulating experimental evidence derived from studying intracellular and extracellular pathogens suggest that the dichotomy of niche-based defense mechanisms is not absolute [19, 28, 29]. A more comprehensive unbiased approach to evaluate the contribution of both the cell-mediated immune response and B cells and humoral immunity to protection against pathogens regardless of their niche could further advance our knowledge of host defense that may eventually influence on the development of efficacious vaccines. The importance of this comprehensive approach is further reinforced by the advancement of our knowledge in immunology and vaccine development that highlights the significance of the interactions between innate and adaptive immunity, as well as those between various immune cells and subsets in the development of effective immune response against microbes [30]. This approach may be particularly important for pathogens, such as *M. tuberculosis*, for which consistently protective vaccines are still lacking.

2 Do B Cells and Humoral Immunity Contribute to Defense Against Intracellular Pathogens?

Based on the concept of division of labor by the cell-mediated and the humoral arm of the immune response in controlling pathogens, protection against intracellular microbes is generally thought to be mediated exclusively by cell-mediated immunity [28]. This has led to the use of highly T cell-centric strategies for the development of vaccines against intracellular pathogens including *M. tuberculosis* [31]. Complete exclusion of a role for B cell and humoral immune response in defense against microbes that gravitate to an intracellular locale is, however, problematic. Indeed, emerging evidence supports a role for B cells and the humoral response in protection and in shaping the immune response to pathogens whose life cycle requires an intracellular environment such as *Chlamydia trachomatis*, *Salmonella enterica*, *Leishmania major*, *Francisella tularensis*, *Plasmodium* spp., and *Ehrlichia chaffeensis* [32–38]. Interestingly, humoral immunity has been shown to contribute to protection against *E. chaffeensis*, a bacterium classified as an obligate intracellular pathogen [34]. This observation has led to the discovery of an extracellular phase in the life cycle of *E. chaffeensis* [34]. The Ehrlichia study suggests that even a brief extracellular sojourn may expose an obligate intracellular organism to antibody-mediated defense mechanisms operative in extracellular milieu. Indeed, it is likely that many intracellular pathogens exist in the extracellular space at some point in the infection cycle, making them vulnerable to the actions of antibodies [28]; and evidence exists that this notion is applicable to *M. tuberculosis* [39–41]. In the control of viruses, the quintessential class of obligatory intracellular pathogen, antibodies have been shown to play an important role in disease control and virion clearance from infected tissues involving mechanisms that are independent of neutralization resulting from direct interaction

of immunoglobulins with viral particles. For examples, binding of antibodies to membrane-associated viral antigens of infected cells have been shown to attenuate transcription and replication of the virus [42–44]. Additionally, immunoglobulins (e.g., certain anti-DNA [45] and anti-viral IgA antibodies [46, 47]) have been shown to be able to enter cells.

B cells can shape the immune response by modulating T cells via a number of mechanisms based on antigen presentation and the production of antibodies and cytokines [21, 48] (Fig. 1). B cells and humoral immunity contribute to the development of T cell memory [49–57] and vaccine-induced protection against a secondary challenge [21, 48] (two components critical to development of effective vaccines) with intracellular bacteria such as *Chlamydia* [58] and *Fransicella* [59]. Thus, infections with intracellular microbes where cell-mediated immunity is central to protection may also require humoral immunity for optimal clearance and vaccine efficacy. This dual requirement for both the cell-mediated and humoral

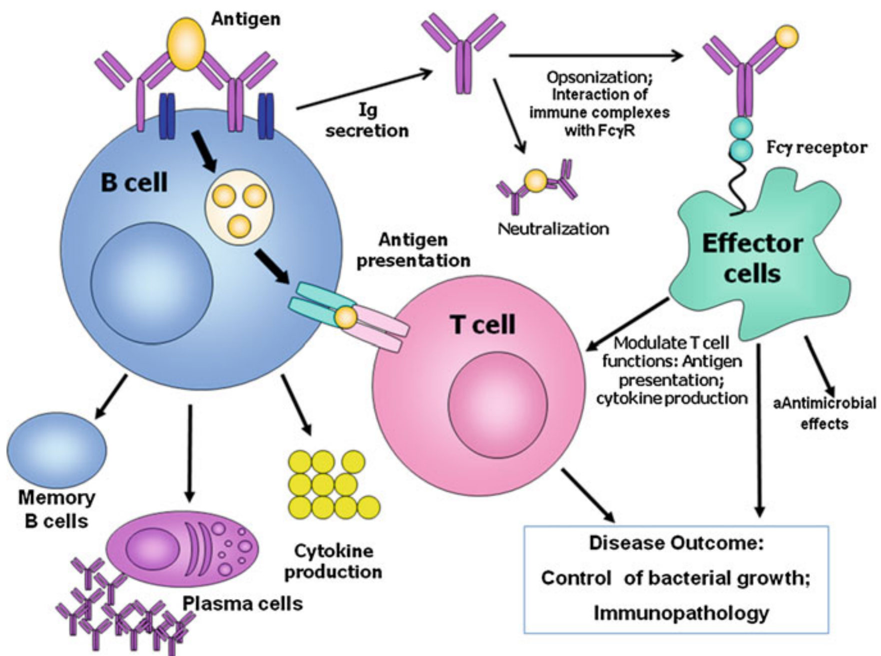

Fig. 1 *How do B cells modulate the immune responses to M. tuberculosis*. Production of *M. tuberculosis*-specific antibodies can mediate the formation of immune complex that can modulate the functions of effector cells such as dendritic cells and macrophages. It remains to be demonstrated whether specific neutralizing antibodies exist. B cells can serve as antigen presenting cells to influence T cell activation, polarization, and effector functions and the establishment of T cell memory. B cells can also modulate the functions of granulomatous immune cells. In concert, these antibody-dependent and independent functions of B cells play an important role in determining disease outcome in terms of the elimination of control of bacteria, as well as the development of immunopathology that could damage tissues and promote dissemination

immunity also applies to the development of optimal immune response to extracellular pathogens. For example, it has been reported that cellular immunity contributes to defense against *Streptococcus pneumoniae* [60] and T cells shapes the host response to *Escherichia coli* infection [61]; furthermore, the antigen-presenting attribute of B cells plays an important role in host defense against extracellular helminthes [57]. Together, these observations have provided evidence that, regardless of the preferred niche of the pathogens in the host, the immune response against invading microbes is shaped by the collaborative effects of cellular immunity and the B cell and humoral immunity. In the context of intracellular microbes such as *M. tuberculosis*, and particularly those for which efficacious vaccines are lacking, understanding how B cells regulate the immune response to the pathogens, and how these immune cells and antibody-dependent immunity interact with the cellular arm of the host response to mediate protective effectors will likely aid in the development of strategies to enhance anti-microbial immunity and vaccine efficacy.

3 B Cells Can Influence T Cell Responses

The interaction of T cells and B cells in response to an antigenic challenge has been well studied. These studies, however, have mostly focused on the characterization the mechanisms by which T cells provide help to B cells [62]. It has been firmly established that T cells play an important role in modulating the response of B cells to antigens, affecting biological functions as diverse as antibody production and cytokine secretion [62]. In contrast, the role of B cells in regulating T cell responses is less well-defined; and this is particularly the case for CD8+ T cells. This line of investigation has yielded conflicting results [48, 63], which are likely due, at least partially, to the complexity of the experimental systems employed, which use varied antigens and mouse models. For example, a much used model involves mice rendered deficient in B cells genetically, although non-B cell immunological aberrancy is known to exist in these strains [48]. The use of the B cell-depleting agent Rituximab in the treatment of a variety of human diseases have provided an excellent opportunity to study the role of B cells in shaping immune responses [64, 65]. These studies have provided compelling evidence that B cells regulate CD4+ T cell responses [48]. Although less well studied, accumulating evidence suggests a role for B cells in regulating CD8+ T cell responses including through antigen presentation [54, 66–68], even though, as in the case for CD4+ T cells, the results derived from these studies are not entirely congruent [69, 70].

Antigen presentation. Evidence that B cells and T cells cooperate in an immune response to induce the production of antibodies began to emerge in the late 1960s [71, 72]. Following up on this discovery, subsequent investigations revealed that this collaboration is mediated by the MHCII-restricted antigen presentation by antigen-experienced B cells to antigen-specific T cells [73, 74]. The T cells

provide help to the interacting B cells, leading to B cell activation, high-affinity antibody responses, the development of B cell memory and antibody-producing plasma cells [62] (Fig. 1). These studies established B cells as professional antigen presenting cells, thus revealing one of the many mechanisms by which these lymphocytes can influence T cell responses [48, 63]. Antigen-specific B cells are capable of presenting antigens to T cells with exceptionally high efficiency by capturing and internalizing antigens via surface immunoglobulins; these antigens are then processed and presented on the surface as peptide:MHC class II complexes [63, 73–75]. While It is generally believed that dendritic cells are the most efficient antigen presenting cell subset for priming na CD4+ T cells [76–78], ample evidence, derived from mouse models, supports a role for B cells as effective antigen presenting cells that participate in T cell priming [79–81]; and it has been reported that B cells have the ability to prime na CD4+ T cells in the absence of other competent antigen presenting cells [82]. A role for B cells in priming na T cells by virtue of their ability to present antigens is, however, not uniformly observed—this inconsistency suggests that the significance of this function of B cells in T cell priming likely depends on antigen types and specific immunological conditions [83]. The observation that B cells in lymph node follicles acquire injected soluble antigens within minutes post-inoculation strongly suggests the B cells have the ability to participate in early events of an immune response including antigen presentation and priming of T cells [63, 84, 85].

Vaccination strategies have exploited the potential antigen-presenting property of B cells for T cell activation [86, 87]: indeed, one such scheme has been shown to be effective in boosting BCG primed immunity against *M. tuberculosis* [88]. Data derived from a chronic virus infection model suggest that B cells can protect against disease reactivation through antigen presentation to T cells [89]. Activated B cells as antigen-presenting cells have been exploited to augment anti-tumor immunity [90]. The antigen-presenting property of activated B cells has been linked to the perpetuation of autoimmunity [91]; by contrast, resting B cells are noted for their ability to induce immune tolerance [92, 93]. Thus, it appears possible to target the antigen-presenting property of B cells to augment specific immune response as well as to suppress autoimmunity [94]. Accumulating evidence indicate that B cells regulate T cell proliferation early on in response to antigens, and affect the subsequent development of T cell memory responses [48, 63, 95]. In infectious diseases models, it has been observed that B cells are required for the development of memory T cell response to intracellular pathogens such as *F. tularensis* and *Listeria monocytogenes* [49, 54]. These observations underscore the importance of going beyond the concept of niche-based division of labor of cellular and humoral immunity in vaccine design to include strategies that target both arms of the immune response.

Priming of T cells to clonally expand requires two signals [96, 97]. The first is provided through the engagement of T cell receptor and with peptide:MHC complex of antigen presenting cells. The second signals derive from interaction of co-stimulatory molecules on the surface of T cells (such as CD28) and antigen presenting cells (such as the B7 family proteins) [98]. The two signals combine to

initiate the adaptive immune response. Absence of the second signal results in tolerance [97]. During the innate phase of the immune response, antigen presenting cells, destined to prime T cells to initiate the development of adaptive immunity, undergo maturation and upregulate the expression of surface co-stimulatory molecules [99]. By virtue of their ability to produce antibodies and cytokines, B cells can modulate the maturation process of antigen presenting cells, thereby regulating the ensuing adaptive immune response [100–105]. Natural antibodies can bind to and alter the activity of the costimulatory molecules B7 and CD40, thereby affecting the antigen presentation process [106, 107]. Finally, cytokines produced by B cells can polarize T cell responses [108]; for example, it has been shown that IL-10 produced by B cells in mice can promote a Th2 response [109].

Cytokine production. The composition of the immunological environment in which na CD4+ T cells interact with antigen presenting cells to clonally expand plays a critical role in determining the path of lineage development [110]. The cytokine milieu at the site of T cell-antigen presenting cell interaction strongly influences CD4+ T cell development. B cells produce a wide variety of cytokines either constitutively or in the presence of antigens, Toll-like receptor ligands, or T cells [108, 111–113]. Based on the pattern of cytokines they produce, B cells have been classified into different effector subsets: B effector-1 (Be1), B effector-2 (Be2), and regulatory B10 cells [48, 108, 113, 114]. During their initial interaction with T cells and antigen, B cells primed in a Th1 cytokine environment become an effector cell subset (Be1) that produces interferon (IFN)-γ and interleukin (IL)-12, as well as TNF, IL-10, and IL-6. B cells primed in the presence of Th2 cytokines produce IL-2, lymphotoxin, IL-4, and IL-13 and can secrete TNF, IL-10, and IL-6 as well [48, 108, 113–115]; these are designated the Be2 subset. Through differential cytokine production, these B cell effector subsets can influence the development of the T cell response. Thus, Be1 and Be2 cells can bias the differentiation of na CD4+ T cells into Th1 and Th2 effector T cells, respectively [57, 108]. Studies looking at cytokine production by human B cells have shown that B cells that produce IL-12 can stimulate a Th1 response in vitro [116], and, in contrast, B cells that produce IL-4 stimulate a Th2 response [117]. The ability of B cell-derived cytokines, notably IFNγ and IL-10 to regulate T cell differentiation provides a cross-regulatory link between B and T cells [108, 113, 115]. In a murine infectious disease model of *Heligomosomoides polygyrus*, a rodent intestinal parasite, it has been shown that B cells regulate both the humoral and cellular immune response to this nematode in multiple ways, including the production of antibodies, presentation of antigens, and the secretion of specific cytokines [57]. Cytokine-producing effector B cells are required for protection against *H. polygyrus*. Specifically, B cell-derived TNF is required for sustained antibody production and IL-2 is essential for Th2 cell expansion and differentiation [57]. In addition, the data revealed that specific functions of B cells might affect one particular phase of the immune response and not others [57]. This is a most comprehensive study designed to characterize the role of various effector functions of B cells modulating the development of the host response to a pathogen, providing compelling evidence to support a complex role for B cells during infection [57] (Fig. 1). We have recently observed that B cells immunomagnetically procured

from lungs of *M. tuberculosis*-infected mice produce a variety of cytokines (L. Kozakiewicz and J Chan, unpublished). The functions of these B cell-derived cytokines in TB remain to be evaluated.

It is clear from the above discussion that the ability of B cells to augment T cell immunity plays an important in the development of immune responses. Equally important is the ability of B cells to negatively affect T cell responses by a subset termed regulatory B cells [48, 114, 118]. Critical to this regulatory effect is the production of IL-10 and transforming growth factor (TGF)-β by this B cell subset [48, 114, 118]. These B cells downregulate T cell function either directly via IL-10 or TGF-β production or by augmentation of the regulatory T cell pathway [48, 114, 118]. Importantly, the regulatory B cells have been shown to play a role in the control of autoimmunity, inflammation, and cancer. Whether regulatory B cells modulate the development of immune responses to *M. tuberculosis* is currently unknown.

Fcγ receptor (FcγR) engagement. A most studied area of humoral immunity is perhaps the mechanisms by which antibodies regulate antigen-presentation through engagement of FcγR by antigen–antibody complexes [119]. The FcγR-immune complex engagement has been an area of active investigation for the development of vaccines against intracellular pathogens [58]. This interaction could be via engagement of the complex with stimulatory and/or inhibitory FcγRs, whose functions are determined by the presence of ITAM or ITIM motifs in the cytoplasmic domain of the receptor, respectively [120, 121]. Engagement of FcγRIIB, the sole inhibitory Fcγ receptor, negatively influences T cell activation by attenuating the process of dendritic cell maturation and subsequent antigen-presentation; interaction through the stimulatory FcγR's promotes both processes [101, 104]. The inhibitory property of FcγRIIB bestows upon this receptor a significant role in mediating peripheral T cell tolerance [122]. In murine models, blockade of FcγRIIB results in enhancement of T cell anti-tumor activity [100, 101, 104]. By contrast, the stimulatory FcγRs promote the development of Th1 or Th2 T cell response, the polarization direction being determined by the in situ inflammatory environment [123].

Due to the preferential engagement of specific immunoglobulin subclasses to FcγR [124], immunization protocols can be rationally designed to target stimulatory receptors to enhance cellular immunity against intracellular pathogens [58, 125]. Interestingly, the ITAM-containing FcγRIII exhibits immune suppressive effects in an IVIG model, suggesting that the ensuing inflammatory response upon engagement of FcγR is complex [126]. The availability of specific FcγR-deficient mouse strains has facilitated the evaluation of the importance of these receptors in various infectious disease models [127]. Disruption of the shared stimulatory Fcγ-chain results in suboptimal immune response to a variety of intracellular pathogens such as influenza virus, Leishmania species, *Plasmodium berghei*, and *S. enterica* [37, 128–132]. Passive immunization using IgG1 monoclonal antibodies against *Cryptococcus neoformans* requires functional stimulatory FcγR's [133]. These observations implicate the stimulatory FcγR in cellular defense against intracellular pathogens. We have recently shown that signaling through FcγRs can modulate immune responses to *M. tuberculosis* [134].

Together, these data suggest the possibility of enhancing efficacy of vaccines against intracellular pathogens by targeting specific FcγRs [58, 125]. Indeed, it has been proposed that recombinant Sindbis virus-based vectors engineered to target FcγR-bearing cells through expression of a bacterial component that bind the variable region of the kappa light chain, coupled with antibody-dependent infection enhancement, can be exploited to manipulate antigen-presenting cells for activation and immunization [135]. In mice, the *Plasmodium falciparum* merozoite surface protein MSP2 harbors a T cell epitope that can be exploited to preferentially induce isotype class-switching to IgG2b [116], a cytophilic immunoglobulin subclass with preferential affinity for stimulatory FcγRs [124].

4 B Cell Regulation of Effector Cells: The Influence on Macrophages

The distinct effector B cell subsets described earlier can polarize T cell development [48, 108, 111, 112]. Emerging evidence indicate that macrophages exist in distinct subsets with characteristic immunological functions [136, 137]. In mice, macrophages with the alternatively activated phenotype (M2) are conducive to persistence of certain pathogens [138] and contribute to the progression of tumor [139, 140]. The differentiation of macrophages into specific subsets can be modulated by B cells. For example, B1 cells have been shown to promote the polarization of macrophages into the M2 subset, with unique phenotypes characterized by upregulation of LPS-induced IL-10 production, downregulation of LPS-induced production of TNF, IL1β, and CCL3, and the expression of typical M2 markers including *Ym1* and *Fizz1* [141]. IL-10 plays a major role in promoting M2 polarization [141]. The significance of the B1 cell-mediated M2 polarization has been shown in a melanoma tumor model [141], providing evidence supporting the in vivo relevance of the ability of B cells to modulate macrophage functions. As macrophages are a major host cell for *M. tuberculosis*, and exist in close proximity to B cells in tuberculous granulomas [142], it is possible that B cells can affect the immune response to the tubercle bacillus by regulating macrophage functions.

The significance of the interaction by immune complex with FcγRs in modulating immune responses has been well established (discussed in previous section). One outcome of this interaction, as illustrated in a Leishmania infection model, is that ligation of FcγR with antibody-coated parasites leads to enhanced IL-10 and decreased IL-12 production by macrophages [143], thereby providing a cellular niche that allows leishmanial growth. This phenomenon is termed "antibody-dependent enhancement (ADE) of microbial infection" [144]. The ADE phenomenon, originally observed with viral pathogens, can be dependent on the nature of the immune complex [145]. In *M. tuberculosis* infection, mice infected with monoclonal antibodies-coated bacilli exhibited improved disease outcome relative to those infected with uncoated organisms [146]. Further, the FcγRIIB-deficient strain, compared to wild-type mice, displays enhanced ability to control

M. tuberculosis infection [134]. In addition, although immune complex engagement of activating FcγR has been reported to be a major mechanism underlying IL-10 enhancing ADE, we have observed that immune complex-treated *M. tuberculosis*-infected FcγRIIB KO macrophages produce enhanced IL-12p40 [134]. These data suggest that ADE may not be operative in vivo during *M. tuberculosis* infection. The precise mechanisms by which mycobacteria-IgG antibody complexes modulate disease outcome during *M. tuberculosis* infection in mice remain to be characterized. It is perhaps most appropriate to study such mechanisms in non-human primates, a species whose granulomas closely resemble the structure of that in humans, given the predicted relevance of the nature of the in situ conditions (the granuloma) in which IgG interacts with *M. tuberculosis* and/or its antigens (this issue will be discussed below). In sum, it is becoming clear that B cells can modulate macrophage functions through the production of antibodies and cytokines. In addition, B cells can indirectly influence macrophage biology through its ability to modulate T cell functions. Gaining insights into how B cells regulate macrophage functions in the course of *M. tuberculosis* infection should further illuminate the mechanisms underlying the immune responses to this pathogen.

5 Are Antibodies Effective in Defense Against *M. tuberculosis*?

Despite reports since the late nineteenth century that serum therapy can be effective against tuberculous infection, humoral immunity is generally considered insignificant in contributing to the immune response against the tubercle bacillus [20]. The latter notion derives from the inconsistent efficacy of passive immune therapy [20, 147], the discovery of effective anti-mycobacterial drug therapies in the mid-twentieth century [148], as well as the concept of the division of labor between humoral and cellular immunity in the control, respectively, of extracellular versus intracellular pathogens [19, 28, 29]. The history and the development of antibody-mediated immunity to *M. tuberculosis* have been discussed recently in excellent reviews [20, 149].

Accumulating evidence suggests a significant role for antibody-mediated response to intracellular pathogens [19, 21, 48]. Indeed, monoclonal antibodies specific for a number of mycobacterial components including arabinomannan, lipoarabinomannan, heparin-binding hemagglutinin and 16 kDa α-crystallin, have been shown to protect mice against *M. tuberculosis* to varying degrees [146, 150–153]. The protective effects of these antibodies manifest as either decreased in tissue mycobacterial loads or alteration of the inflammatory response [149]. Of note, serum therapy using polyclonal antibodies against *M. tuberculosis* is effective in protection against relapse of infection in SCID mice after treatment with anti-tuberculous drugs [154]. In addition, protective effects of IVIG in a mouse model of tuberculosis further suggest that humoral immune response contribute to the development of anti-tuberculous immunity in TB [155].

However, a vaccine comprising an *M. tuberculosis* arabinomannan–protein conjugate, while engendering an antibody response superior to that elicited by BCG in mice, was ineffective in improving survival of challenged animals [156]. These apparently discrepant results with regard to the significance of the humoral immune response in defense against *M. tuberculosis* can be due to multiple factors. First, the multifunctionality of B cells and humoral immunity predicts that analysis of this arm of the immune response is likely not straightforward. Second, the mouse may not be the most suitable species for these studies because of the dissimilarities of granulomatous response in this species and humans [157, 158], and because of the fact that anti-tuberculous responses in the mouse appears to be more robust than is needed for effective control of the infection [157]. This overly robust resistance of the mouse to *M. tuberculosis* could mask the significance of certain immunological factors that otherwise contribute substantially to defense against this pathogen. Demonstration of a correlation between antibody response and protection in human tuberculosis and in animal models should afford novel in vivo systems in which anti-TB vaccines that are based on B cells and humoral immunity can be effectively tested. Finally, a component of humoral immunity that has hardly been evaluated during tuberculous infection is the innate or natural antibody responses [159–161]. Given that complex lipids and polysaccharides constitute a major components of the *M. tuberculosis* cell envelope, the significance of T-independent antibody responses mediated by B1 and marginal zone B cells in defense against *M. tuberculosis* warrants examination [160–164].

Antibody-mediated immunity can shape the host response to pathogens in a number of ways (Fig. 1). These include antigen-specific neutralization, regulation of the inflammatory reaction through complement activation, FcγR cross-linking, release of microbial products due to direct anti-microbial activity, and impact on microbial gene expression upon binding of the organisms [19, 29]. Relevant to the local lung immune response during tuberculous infection, antibodies have been shown in a mycoplasma model to be able to modulate architectural changes in airway epithelium and vessels [165]. We have shown that adoptive transfer of B cells ameliorates the enhanced inflammatory response observed in B cell-deficient mice upon airborne challenge with virulent *M. tuberculosis* [166]. This B cell-mediated attenuation of exacerbated inflammatory response is associated with detectable levels of immunoglobulins in the recipient B cell-deficient mice but does not require the presence of B cell locally in the infected lungs [166], suggesting a role for immunoglobulin-mediated endocrine immune regulation during *M. tuberculosis* infection. It is thus apparent that antibodies, in addition to neutralizing and opsonizing microbes, can also be protective during microbial challenge by limiting inflammatory pathology [19, 29], the latter a well established function of immunoglobulins [167].

6 The Role of B Cells in the Development of the Immune Response to *M. tuberculosis*

Successful rational design of effective vaccines against *M. tuberculosis* has been hampered by the lack of definitive immunological correlates of protection, although strong evidence supports an important role for T cell-mediated responses in eliciting protective immunity [11]. As a result, TB vaccine development has focused predominantly on enhancing cellular immune responses against *M. tuberculosis* [12, 17]. A recent failed T cell vaccine trial against HIV should, however, caution against taking too narrow an approach in vaccine design [168]. Thus, it is possible that eliciting protective antibody responses may be required for successful immunization against *M. tuberculosis* [18]. Research effort directed at revealing how humoral immunity can be harnessed to enable protection against the tubercle bacillus is needed. Being that B cells are multifunctional, the mechanisms underlying how these lymphocytes modulate the immune response to *M. tuberculosis* are likely complex. For example, immunoglobulins, acting upon FcγRs, can influence the maturation process and functions of antigen-presenting cells, whose role in T cell activation and development has been well established [96, 97, 99]. B cells can conceivably shape anti-tuberculous immunity through direct effects of antibody on the pathogen, antigen-presentation, production of cytokines at the site of infection and by modulating intracellular killing mechanisms of leukocytes (Fig. 1).

B cell-deficient mice infected aerogenically with *M. tuberculosis*, compared to the parental wild-type C57BL/6 strain, display suboptimal defense against the pathogen (as assessed by tissue bacterial burden and mortality), as well as enhanced lung IL-10 expression, neutrophil infiltration, and inflammation [166]. These B cell-deficiency phenotypes are all reversible by adoptive transfer of B cells from *M. tuberculosis*-infected wild-type mice [166]. We have also shown that mice deficient in the inhibitory FcγRIIB are more resistant to *M. tuberculosis* compared to wildtype controls [134], with enhanced pulmonary Th1 responses, evidenced by increased IFNγ+ CD4+ T cells. Upon *M. tuberculosis* infection and immune complexes engagement, FcγRIIB−/− macrophages produced more p40 component of IL-12, a Th1-promoting cytokine. These data suggest that FcγRIIB signaling can dampen the Th1 response to *M. tuberculosis*, at least partially by attenuating IL-12 production, and that B cells can regulate CD4 Th1 response in acute TB through engagement of FcγRs by immune complexes. In contrast to the FcγRIIB−/− strain, mice lacking the common γ-chain of activating FcγRs are more susceptible to low dose *M. tuberculosis* infection with exacerbated immunopathology, increased mortality, and enhanced production of IL-10 [134]. These observations suggest that antibodies can significantly modulate host immune responses by mediating antigen–antibody complex engagement of FcγRs during *M. tuberculosis* infection. In addition, the data indicate that signaling through specific FcγRs can divergently affect disease outcome, suggesting that it is possible to enhance anti-mycobacterial immunity by targeting FcγRs.

In two different murine TB models involving mice with B cell deficiency, we and others have provided evidence that B cells can regulate IL-10 production in the lungs of *M. tuberculosis*- infected mice [166, 169]. We have also observed enhanced lung cell production of IL-10 in *M. tuberculosis*-infected mice deficient in the common γ chain of stimulatory FcγRs [134]. A wide variety of immune cells—B cells, dendritic cells, macrophages, and T cells—produce IL-10, whose anti-inflammatory functions have been well recognized [170]. The cellular source of this IL-10 increase remains to be determined. A feature of incompletely activated dendritic cells that have not undergone full maturation is IL-10 production [171]. Therefore, it is possible that B cells may indirectly influence the production of IL-10 by antigen-presenting cells through modulation of cellular activation via immune complex engagement of FcγRs. B cells can also activate or inhibit regulatory T cells, a significant cellular source of IL-10 [172, 173]. IL-10 has been reported to adversely affect disease outcome in murine TB [174]. It is possible that excess IL-10 production observed in the B cell- and γ chain-deficient mice can contribute to the inability of these strains to optimally control *M. tuberculosis* infection [134, 166]. Finally, this increased production of IL-10 may be a compensatory mechanism to counter the exacerbated immunopathology that develops in *M. tuberculosis*-infected B cell−/− and Fcγ-chain−/− mice [134, 166]. Much work needs to be done to characterize the mechanisms by which B cells and humoral immunity regulate the production of cytokines in general, and IL-10 in particular, at the site of tuberculous infection. The regulatory mechanisms are likely to be complex given the multiple immunological functions of B cells, which include the production of a wide variety of cytokines including IFN-γ [108], a critical anti-mycobacterial factor; and IL-10, which has been shown to attenuate resistance in murine TB [174].

As discussed in the previous sections, it is clear that the interaction of immune complexes with FcγRs plays an important role in immune regulation [120, 121]. Our FcγR knockout mouse studies suggest that the interaction of FcγRs with immune complexes during the course of *M. tuberculosis* infection can influence disease outcome [134]. The precise mechanisms by which this interaction modulates the infection in mice remain to be defined. Gallo et al. [175] has suggested a mechanism of regulation of macrophage FcγR signaling upon interaction with antibody–antigen immune complexes that might be pertinent during *M. tuberculosis* infection in humans or a species granuloma structure resembles that of humans. This mechanism proposes that macrophage FcγR signaling depends on the density of IgG within the immune complex. Immune complexes with high IgG densities promote anti-inflammatory responses, particularly the release of IL-10, while that of moderate densities tend to induce pro-inflammatory cytokine release by macrophages. The FcγR signaling pathway in a cell depends on cell surface receptor recruitment and cross-linking by the antigen–antibody immune complexes. The summation of FcγR members recruited and cross-linked is then translated as an inhibitory or activating signal to the cell. Whether the density of IgG within immune complexes can determine the recruitment of activating versus inhibitory class of receptors, a potential mechanism underlying the IgG density phenomenon, is presently unknown. The macrophage population is unique as these cells express both the

inhibitory and activating forms of FcγR, although there may be different ratios on the various types of differentiated macrophages that exist in the granuloma [176, 177]. Furthermore, the ratio of expression of inhibitory and activating FcγR can be influenced by the cytokine environment [177, 178]. Thus, macrophage behavior can become highly versatile depending on the composition of the immune complex.

The formation of immune complexes depends on the concentration of antigen and antibody. Conditions that favor the formation of complexes with high IgG densities can potentially direct macrophages to produce IL-10. Considering that *M. tuberculosis* bacilli are theoretically confined towards the center of a granuloma, antigen concentration would be the highest at the granuloma center and decrease towards the periphery (Fig. 2). Meanwhile, B cells and plasma cells are confined at the periphery meaning that antibody concentration should be highest at the lymphocytic cuff and lowest in the center (Fig. 3). Along this antibody–antigen gradient, we hypothesize that the immune complexes that form would have different in IgG densities and would signal the macrophage to behave differently. Thus, macrophages closest to the granuloma center would encounter immune complexes with lower IgG densities and can be predicted to be more pro-inflammatory than macrophages at the periphery, whose interaction with immune complexes with high IgG densities should result in the production of IL-10 (Fig. 3).

Given the highly stratified nature of the non-human primate and human granuloma, which is much different from the murine granuloma, B cells together with antibody production and immune complex formation may orchestrate targeted responses locally within specific regions of the granuloma (Figs. 2 and 3). Hence, antibody-mediated signaling would theoretically contribute towards control of *M. tuberculosis* by confining pro-inflammatory responses towards the center of the granuloma and thus increase bacterial killing. At the same time, bystander tissue damage at the peripheral areas of the granuloma is reduced as anti-inflammatory responses are dominant. Since control of *M. tuberculosis* relies on adequate

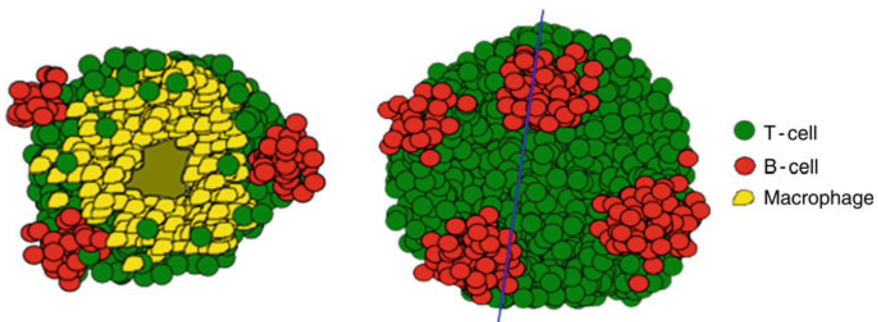

Fig. 2 A schematic representation of a granuloma showing how the B cell clusters (*red*) would be organized with respect to macrophages (*yellow*) and T-cells (*green*). The *blue line* denotes the cross-sectional area. B cell clusters would be located within the lymphocytic cuff on the surface of the granuloma analogous the *black spots* on a *white soccer ball*

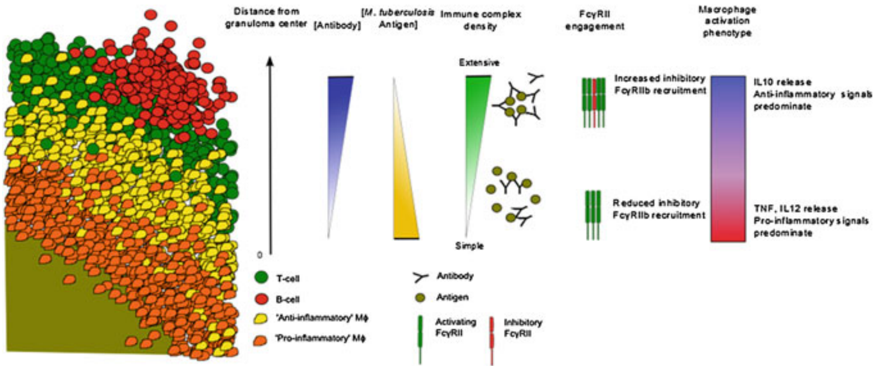

Fig. 3 The proposed mechanism of how antibody–antigen immune complexes can influence macrophage activation phenotypes with respect to distance from the granuloma center, antibody and antigen concentration, immune complex composition, and inhibitory receptor recruitment. A segment of a granuloma cross-section is used to illustrate how the proposed mechanism of immune complex interaction with macrophages would result in different modes of activation

balancing of pro- and anti-inflammatory responses within the granuloma, the proposed mechanism of how the humoral immune system influences macrophage function can possibly explain how such balance is achieved within the granuloma to realize disease resolution. Associated scenarios where inadequate control of disease occurs due to excess antibody responses or even treatment can potentially be addressed. The validity of the model requires a highly organized stratified granulomatous structure that exists in humans and non-human primates but not in the mouse. Verification of this model in non-human primates should underscore the importance of the choice of animal models in the study of TB.

An important aspect we would like to address before leaving this section is the effect of B cells and humoral immunity on the development of immunopathology in the course of *M. tuberculosis* infection. In the absence of B cells, mice with acute *M. tuberculosis* infection exhibit suboptimal anti-tuberculous immunity associated with exacerbated pulmonary pathology [166]. In a similar mouse model (albeit using a different strain of *M. tuberculosis*), it was observed that B cell-deficiency resulted in a delay in inflammatory progression during the chronic phase of tuberculous infection [179]. This paradox suggests that B cell functions during the course of TB are infection phase-specific: In acute infection, B cells are required for an optimal granulomatous response and effective immunity against *M. tuberculosis* aerosol infection; deficiency of these lymphocytes leads to dysregulation of granuloma formation and increased pulmonary inflammation is required to contain the growth of tubercle bacilli. In contrast, in the chronic phase of infection, the immunologically active B cell aggregates [54, 61, 62] (see below) likely play a role in promoting the perpetuation of effective local immunity so as to contain persistent bacilli and prevent disease reactivation. It is possible that a trade-off for the perpetuation of this local control of *M. tuberculosis* is the development of tissue-damaging immunopathology. As T cells exist within the B cell aggregates in the tuberculous lungs of

both human and mice ([142], P. Maglione and J. Chan, unpublished), the perpetuation of inflammation in chronic TB may occur in part through B cells acting as antigen-presenting cells. Thus, the inflammatory paradox observed in B cell-deficient mice may be due to the role of B cells shifting from optimizing host defense during acute challenge to perpetuating the potentially tissue-damaging chronic inflammatory response during persistent infection.

For those most severely affected by TB, morbidity and mortality of the infection is, at least in part, the result of a tissue-damaging host response [28, 180]: one with an excessive pathologic inflammatory reaction yet ultimately is ineffective in controlling the pathogen. Such an outcome may be due to an ineffective immune containment of the tubercle bacillus leading to excessive compensatory recruitment of leukocytes into the site of infection in the lungs. This is perhaps best exemplified in patients with reactivation TB. By mechanisms yet poorly defined, dormant bacilli reactivate to cause diseases that are associated with areas of intense pulmonary infiltrate [180]. In the active cases, neutrophils can be a dominant cell type in tuberculous pulmonary infiltrates [181]. The neutrophil is generally considered to be an innate immune cell that mediates early protection but can induce inflammatory damage in a variety of acute pulmonary diseases [182]. In mice, as in humans, susceptibility phenotype to *M. tuberculosis* is often associated an enhanced neutrophilic response [183]. Clearly, severe tissue-damaging host response can be observed in certain hosts in TB, however, the restricted Ghon complex pathology in humans indicate that successful containment of the tubercle bacillus needs not be associated with significant immunopathology. Emerging experimental results strongly suggest that B cells and humoral immunity may play a role in modulating the inflammatory response in TB [166, 179], and that this B cell-based modulation may be infection phase specific.

7 B Cells in Germinal Center-Like Structures in the Tuberculous Granuloma

B cells are a prominent component of the tuberculous granulomatous inflammation in the lungs of mice [142, 184], non-human primates (Y. Phuah and J. Flynn, unpublished) and human [142, 181], forming conspicuous aggregates. In the lungs of humans with TB, cellular proliferation is detected primarily in these B cell aggregates [181]. The B cell nodules are also characteristics of the progression of tuberculous granulomatous inflammation [142, 184, 185]. B cell nodules have been observed in many chronic inflammatory diseases such as multiple sclerosis and rheumatoid arthritis [186, 187]. Similar to TB, B cell clusters have been observed at the site of infection caused by a number of microbes such as influenza virus and *Helicobacter* [188, 189]. The ability of certain pathogens to promote expansion and inhibit apoptosis of B cells may contribute to the existence of aggregates of these lymphocytes during infection [190, 191].

The granulomas formed in *M. tuberculosis*-infected non-human primates are highly stratified, and represent the full range of granuloma types seen in humans [192, 193] (Figs. 2 and 3). The granuloma center generally contains the infected macrophages and can be cellular, infiltrated with neutrophils, necrotic with caseous cellular debris, or mineralized. Surrounding the granuloma center is a layer of epithelioid macrophages, which is in turn surrounded by a layer of lymphocytes interspersed with macrophages. Both T and B cells are found within the lymphocytic cuff of the granuloma but whereas the CD3+ T cells are generally homogenously spread throughout the cuff, the B cells are present in very discrete clusters (J. Phuah and J. Flynn, unpublished). These B cell clusters sit on the surface of the granuloma and are spaced away from other B clusters. A close analogy in the positioning of these B cell clusters would be like the black spots on a white soccer ball. Unlike T-cells, which can be seen infiltrating into the granuloma beyond the lymphocytic cuff, B cells are confined within the lymphocytic cuff (Fig. 2).

What are the effects of these ectopic B cell nodules on the local lung immune response in a tuberculous host? TB is a chronic condition that once established requires a long time to be successfully contained and resolved. It is possible that by forming these germinal centers in situ of the granuloma, antigen presentation and lymphocyte activation can occur with greater efficiency. The localization of cellular proliferation in the proximity of these B cell aggregates has led to the hypothesis that these structures function to perpetuate local host responses [181]. As discussed above, perpetuation of these local immune responses by the granulomatous B cell aggregates could contribute to the development of tissue-damaging immunopathology. T cells have been observed embedded within these B cell clusters [142], suggesting the possibility that antigen-presentation and B-cell maturation can occur in these aggregates. Indeed, experimental evidence suggests that the B-cell aggregates observed in tuberculous lungs represent tertiary lymphoid tissues [194], displaying cellular markers typical of germinal centers [166]. Our observation that B cell-deficient mice exhibit aberrant granulomatous reaction with exacerbated pulmonary pathology suggests that the B-cell aggregates may regulate the local lung immune response [166]. It has been reported that in the absence of secondary lymphoid organs, in situ lymphoid nodules can prime protective immunity in the lungs and memory responses against pulmonary influenza virus challenge [188, 195]. The significance of lymphoid neogenesis in the regulation of immune responses in TB remains to be defined.

8 Concluding Remarks

Only 10 % of those infected with *M. tuberculosis* develop disease; the remainder can apparently contain the infection without symptoms. Individuals in this latter group, generally thought to harbor dormant bacilli, run a 10 % lifetime risk of subsequent reactivation of the infection, often as a result of acquired immunodeficiency [11]. These epidemiological data attest to the tenacity of *M. tuberculosis*: this pathogen is

well-adapted to persist even in a host that can mount a disease-preventing immunity. This, together with the occurrence of exogenous reinfection in a previously infected host, suggests that effective preventive TB vaccines must elicit an immune response superior to that induced by natural infection [18]. This is a daunting task, and implies that rational design of effective vaccines should perhaps take a more comprehensive approach, going beyond the T cell-focused strategy to characterize the protective immunity in TB. In this respect, the vaccine effort may benefit from gaining insight into immune mechanisms that may not be immediately obvious in engendering major protection during natural infection, and how such pathways can be harnessed to optimize immunization protocols. One such pathway is the understudied B cell and humoral immunity. It is clear, as discussed in this review, that B cells and humoral immunity have a significant effect on the development of immune response against *M. tuberculosis*. How the B cell-mediated immunological pathways modulate the immune response to *M. tuberculosis* is just beginning to be understood and much remains to be learnt. For example, what are the mechanisms by which B cells regulate anti-mycobacterial T cell responses? What is the nature of the B cell memory that develops upon BCG vaccination or natural *M. tuberculosis* infection (are they one and the same?), and does it contribute to protection upon secondary challenge? Are there memory B cells that are specific for T-independent non-protein *M. tuberculosis* antigens (this is of relevance given the chemical composition of the mycobacterial cell envelop)? Are such antigens viable vaccine targets? What roles do natural antibodies play in defense against *M. tuberculosis* and can this pathway be a target for effective vaccines? Do protective antibodies against the tubercle bacillus exist and if so, how can they be targeted to develop more effective vaccines? What are the functions of the germinal center-like B cell clusters in the lungs of a chronically infected host—do they play a role in orchestrating local containment of *M. tuberculosis* or in the development of tissue-damaging immunopathology or both? If they do serve such functions, can B cells be manipulated to prevent reactivation and/or to ameliorate immunopathology (the latter an important means to prevent dissemination of infection)? Finally, are there B cell responses that adversely affect anti-TB immunity? Answers to these questions should illuminate the roles of B cells and humoral immune responses in TB. These will ultimately help develop strategies by which the humoral arm of immunity can be harnessed to optimize immune responses against *M. tuberculosis*. Given the global public burden of TB, it is not unreasonable to take a comprehensive approach to explore all possibilities that may lead to the development of novel TB control measures including vaccines. For decades, B cells have been relegated toward irrelevance in immune responses to *M. tuberculosis*, recent studies have provided evidence to suggest otherwise. Revisiting the role of B cells in anti-TB immunity may lead to better understanding of the mechanisms underlying the host response to *M. tuberculosis* infection.

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Looking Within the Zebrafish to Understand the Tuberculous Granuloma

Lalita Ramakrishnan

Abstract Tuberculosis is characterized by the formation of complex immune cell aggregates called granulomas, which for nearly a century have been viewed as critical host-beneficial structures to restrict bacterial growth and spread. A different view has now emerged from real-time visualization of granuloma formation and its consequences in the optically transparent and genetically tractable zebrafish larva. Pathogenic mycobacteria have developed mechanisms to use host granulomas for their expansion and dissemination, at least during the innate phases of infection. Host processes that are intended to be beneficial—death of infected macrophages and their subsequent phagocytosis by macrophages that are newly recruited to the growing granuloma—are harnessed by mycobacteria for their own benefit. Mycobacteria can also render the granuloma a safe-haven in the more advanced stages of infection. An understanding of the host and bacterial pathways involved in tuberculous granuloma formation may suggest new ways to combat mycobacterial infection.

Keywords Tubercles · Tuburculous granuloma · *Mycobacterium tuberculosis* · Macrophages · Necrosis · *Mycobacterium marinum* · Zebrafish · Tumor necrosis factor · *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine strain · Neutrophils · Cell death · Host matrix metalloproteinase 9 (MMP9) · Apoptosis · Mycobacteria

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L. Ramakrishnan (✉)
Department of Microbiology, Medicine and Immunology, University of Washington, Office K-443C HSC, 1959 NE Pacific Street, Box 357242Seattle, WA 98195, USA
e-mail: lalitar@uw.edu

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1 What is a Granuloma?

Tuberculosis is the most frequent cause of granulomas, which can form in response to a wide range of infectious and noninfectious stimuli that are not readily eradicated [1]. Granulomas, or tubercles, were first found associated with tuberculous lungs, hence the name “tuberculosis”. The basic definition of a granuloma is an organized aggregate of mature macrophages, which are characterized by an increase in cytoplasmic organelles and ruffled cell membranes suggestive of an increased microbicidal capacity [2–4]. However, the tuberculous granuloma can be much more complex. Its macrophages undergo several other distinct changes: they transform into so-called “epithelioid cells” due to the tightly interdigitated cell membranes linking them to adjacent cells; they can differentiate into lipid-rich foam cells; they can fuse into multinucleated giant cells; and they can die producing characteristic necrotic regions called caseum [5–13]. Frequently, it contains additional cell types, including neutrophils, dendritic cells (DCs), T and B cells, natural killer (NK) cells, fibroblasts, and extracellular matrix-producing cells [13]. The stages of tuberculous granuloma formation have been inferred from histological and radiological studies of humans and animal models. In humans, macrophages are thought to phagocytose inhaled *Mycobacterium tuberculosis,* transporting the bacteria across the alveolar epithelium and into the lung, the site of granuloma formation [14]. In mice aerosolized with *M. tuberculosis*, macrophages, neutrophils, and DCs are found soon after infection, suggesting the early participation of multiple phagocyte classes [15].

2 The Classical Model of the Tuberculous Granuloma as a Host-Protective Structure

The idea that the tuberculous granuloma is critical for host protection has its roots in two sets of observations. First, epidemiological and histological evidence together suggest that individuals who form granulomas in response to *M. tuberculosis* exposure can contain, or even eradicate, the infection without developing symptoms: autopsy studies of individuals who died from unrelated causes in the pre-chemotherapy era often revealed the presence of healed granulomas with no trace of viable bacteria, even upon guinea pig inoculation, an exquisitely sensitive assay [16–18]. However, since tuberculosis in immunocompetent individuals is also associated with well-formed granulomas, it is obvious that the granuloma frequently fails to eliminate infection. Thus, the classical model of the protective tuberculous granuloma attributes the latter findings to the granuloma “trying but failing”. This explanation is based on the inherent assumption that the granuloma is protective. An unbiased examination of the findings would suggest at least three equally plausible, alternative explanations: (1) granuloma formation is irrelevant to protection; (2) protection occurs despite granuloma formation; and (3)

granuloma formation is protective or not depending on context, for instance, extent, and cell types involved.

Second, multiple immunodeficiencies, e.g., those of tumor necrosis factor (TNF), IFNγ, IL-12, STAT4, and MyD88, result in hypersusceptibility to mycobacterial infection and the presence of disorganized granulomas [19–30]. In particular, the lack of granuloma structure associated with TNF deficiency has been used to marshal the protective granuloma argument, given TNF's role in inflammatory leukocyte migration [19, 31–37]. However, alternative explanations are plausible for these associations, and indeed have been found to be the case for TNF deficiency, which results in diminished macrophage microbicidal capacity and ultimately in granuloma destruction through necrotic lysis of the macrophages overladen with bacteria [38].

Together, these observations have been used to promote the notion that granuloma formation is critical for protection by "walling off" bacteria—a notion that has been presented as fact in the literature, including medical and immunology textbooks [39–44]. This chapter will explore the idea, based on experiments from the zebrafish model of tuberculosis, that the granuloma plays different, and often opposite roles in infection. This new understanding of the tuberculous granuloma, and its therapeutic implications, are beginning to be embraced [45].

3 The *Mycobacterium marinum*: Zebrafish Model of TB

Mycobacterium marinum, a close genetic relative of *M. tuberculosis,* is a natural pathogen of fish and other ectotherms (i.e. amphibians and reptiles), in which it produces a systemic tuberculosis-like disease [46]. Owing to its lower optimal growth temperature, *M. marinum* is also a well-known accidental pathogen of humans in which it produces a peripheral granulomatous infection (known as fish tank or acquarium tank granuloma) that is histologically similar to tuberculosis [47]. Among the natural *M. marinum* hosts developed for laboratory studies, leopard frogs develop lifelong asymptomatic infection with organized, non-necrotic granulomas [8, 48], while in contrast, zebrafish develop organized, necrotic granulomas associated with progressive disease [49]. The zebrafish is genetically tractable and, in early life, transparent, making it possible to dissect the contributions of host and bacterial determinants to granuloma formation and infection outcome in real-time. Known resistance factors in human TB such as TNF and lymphocytes, also mediate zebrafish resistance to TB; likewise *M. marinum* homologs of *M. tuberculosis* virulence determinants mediate virulence in the zebrafish [46]. Indeed, the zebrafish model led to the identification of eicosanoid mediators that modulate human inflammatory states, mycobacterial susceptibility and responsiveness to adjunctive therapies [50, 51].

4 Mature Tuberculous Granulomas Provide a Safe-Haven for Mycobacteria

The mature tuberculous granuloma is replete with adaptive immune cells [13], and we know that adaptive immunity is protective based on the susceptibility of HIV-infected humans and of *rag*- and CD_4 T cell-deficient mice [43–45, 52]. Yet the global burden of TB is sustained by transmission from immunocompetent humans with active granulomatous TB. In all animal models tested, granuloma maturation induces bacteriostasis but not eradication [13]. Thus, persistent infection and reinfection can occur [53–55] despite the concentration of presumably protective immune cells in the granuloma. In the same vein, the live attenuated vaccine strain (BCG) has proved largely ineffective despite multiple and varied attempts to improve its antigenicity [56]. This paradox has been explained by suggesting that newly infecting bacteria avoid existing granulomas with their concentration of effective host immune responses and instead establish infection in new areas [18, 57–59]. Additionally, mycobacteria present in the caseous centers were thought barricaded therein [14, 60]. However, work examining trafficking of *M. marinum* in frogs and zebrafish, or of *M. tuberculosis* in mice, with prior established infection revealed these ideas about physical barriers and compartments in the context of the TB granuloma to be overly simplistic. Superinfecting mycobacteria traffic rapidly into pre-existing mature granulomas, including the caseum, through *Mycobacterium*-directed, host phagocyte-mediated processes, and adapt quickly to persist long-term in them [61, 62]. Thus, established granulomas fail to eradicate even newly deposited mycobacteria that are “naïve” to the adaptive host responses concentrated therein.

Several explanations, albeit incomplete, are starting to emerge for why the mature granuloma fails. *M. marinum* rapidly induces the expression of a variety of granuloma-specific genes that might aid them in surviving host immune responses [63, 64]. Studies in the mouse model of TB attribute the failure of the granuloma to eradicate existing infection or prevent reinfection to a delay in initiation and activation of adaptive immunity that allows a prolonged period of exponential mycobacterial growth in the early innate phases of granuloma formation [65–68]. This of course brings up the question of why there is exponential bacterial growth in the early innate stage of infection, despite rapid phagocyte recruitment. The answers reveal yet another unsuspected and surprising role for the granuloma.

5 The Early Tuberculous Granuloma is a Dynamic Machine for Bacterial Expansion

In all animal models of tuberculosis tested—*M. tuberculosis* in mice, guinea pigs, and rabbits and *M. marinum* in leopard frogs and zebrafish—bacterial growth is rapid for the first 3 weeks of infection and plateaus coincident with the

development of adaptive immunity [48, 49, 69]. Because tuberculous granuloma formation was assumed to require adaptive immunity, granuloma formation was thought to be the critical event that curtailed bacterial expansion [69–71]. However, the ability to visualize the cellular events of granuloma formation at the whole-animal level in zebrafish larvae, in which adaptive immunity has yet to develop, revealed that bona fide granulomas comprising epithelioid cells form in the context of innate immunity alone [72]. Further work revealed that granuloma formation actually coincided with accelerated bacterial proliferation, a finding contrary to the dogma that enclosing bacteria within these structures curtails their growth [73]. Mycobacteria lacking a key virulence determinant, the ESX-1/RD1 (RD1) secretion system, produced attenuated infection in which the mutant bacteria grew normally within individual macrophages, which then failed to assemble into granulomas, or did so much more slowly than macrophages infected with wild-type bacteria [73]. These findings together support the surprising conclusion that granuloma formation is positively associated with bacterial expansion.

How does the early granuloma promote bacterial proliferation? Dynamic imaging studies in zebrafish larvae revealed that macrophages arriving at nascent granulomas continue to move rapidly within these structures until such time as they become heavily infected [72, 74]. These new recruits have morphological features of leukocytes actively undergoing chemotaxis in response to a chemokine gradient [74]. The macrophage chemotactic morphology and movement is dictated by the mycobacterial RD1 locus; in granulomas initiated by RD1-deficient mycobacteria, the few arriving macrophages are round and move slowly, and in this context the infection is not expanded [74]. The rapid and continuous migration of the newly arriving macrophages seen in the context of wild-type *M. marinum* infection allows them quick access to the numerous dying infected cells within the granuloma. Indeed, quantitation of phagocytosis of dying infected macrophages by new recruits revealed the mechanism by which granuloma formation promotes bacterial expansion; the bacterial contents of each dying cell are consumed on average by 2.3 new recruits, thereby increasing the number of infected cells. Within granulomas, macrophages move randomly, which suggests that they are migrating in response to a gradientless signal, until they are in the vicinity of a dead infected cell, when they acquire a distinct morphology and move towards that cell phagocytosing its bacterial contents or a part thereof [74]. These findings suggest two signals are operant, an RD1-dependent signal that recruits macrophages to, or retains them within, the forming granuloma and a second signal emanating from dying infected macrophages promotes their engulfment. These events suggest that pathogenic mycobacteria have accelerated the events of granuloma formation for their advantage. Based on the finding that RD1-deficient bacteria proliferate intracellularly, yet do not expand further, it is likely that the slower kinetics of macrophage recruitment, infected macrophage death and re-phagocytosis associated with that mutant represents what is optimal for the host.

Are these findings relevant to the situation in mature granulomas of immunocompetent adult animals? Three-dimensional time-lapse microscopy was performed on mouse liver granulomas 2–3 weeks after intravenous injection of the

attenuated vaccine strain *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), which lacks RD1 [75]. These granulomas consist of local and recruited Kupffer cells (liver-resident macrophages), and recruited monocyte-derived macrophages, as well as activated polyclonal T cells that arrive at the forming granulomas within days of infection. In contrast to the zebrafish observations, the myeloid cells are relatively static, although their membranes are in constant flux. The T cells are in constant motion throughout the granuloma, moving at similar speeds to macrophages in zebrafish granulomas [74]. The relative immotility of the various myeloid cells may be due to: (1) the absence of RD1, as would be predicted from the zebrafish studies; (2) the possibility that macrophages move more slowly during later adaptive phases of granuloma formation; or (3) intrinsic differences between fish and mouse macrophage behavior. In a subsequent study using virulent ($RD1^+$) *M. tuberculosis* in mice, with a limited visualization of macrophage motility, some arriving macrophages moved quickly whereas others moved slowly [76]. Since the bacteria were not simultaneously visualized, it is possible that the motile macrophages were uninfected whereas the immotile macrophages were infected and/or dying, similar to those the slowly moving infected macrophages in zebrafish granulomas [74]. Importantly, adult zebrafish and mice infected with attenuated RD1-deficient mycobacteria both have loose, poorly structured granulomas [49, 77], so this virulence determinant may direct bacterium-expanding macrophage motility even in later stages of granuloma formation and maturation.

The emerging picture of the granuloma is that of a highly dynamic accumulation of highly motile macrophages and T cells. This dynamic nature renders the granuloma a tool for bacterial proliferation by accelerating the normally host-beneficial processes of phagocyte apoptosis, chemotaxis, and efferocytosis to the point where they are detrimental to the host.

6 The Role of Cell Death in Determining the "Balance of Power" in the Tuberculous Granuloma

Bacterial exploitation of the granuloma requires host cell death, since newly arriving macrophages selectively phagocytose bacteria from dead or dying macrophages [74]. Mathematical modeling of early granuloma dynamics attributes 70–100 % of all bacterial proliferation to macrophage death and re-phagocytosis [74]. Furthermore, the zebrafish studies also showed that granuloma macrophages infected with virulent mycobacteria undergo bona fide apoptosis in that they exhibit the characteristic morphological hallmarks of nuclear collapse and fragmentation with the bacteria remaining encased within the apoptotic fragments [74]. These observations suggest a model in which migrating macrophages engulf infected apoptotic macrophages, with subsequent bacterial proliferation within the new recruits. The RD1 locus enhances the apoptosis of *M. marinum*-infected granuloma macrophages in the zebrafish [40, 73], similar to its action in *M. tuberculosis*- or *M. marinum*-infected cultured human and murine macrophages

[78, 79]. The RD1 secreted protein, ESAT-6, induces multiple cell death programs in cultured cells, and is a likely candidate for the induction of macrophage apoptosis in vivo [80–82]. (As will be discussed in the following section, ESAT-6 is also responsible for the recruitment of new macrophages to the growing granuloma, thus playing a dual role in granuloma expansion [73, 74]).

While the live imaging studies in the zebrafish suggest that *Mycobacterium*-induced apoptosis may enable bacterial proliferation during granuloma formation [74], a concurrent model based mainly on in vitro studies proposes that macrophage apoptosis is a host beneficial, bacterium-detrimental process [83–88]. What might account for this apparent discrepancy between the in vitro and in vivo findings? First, many of the in vitro studies rely on chemical agents to induce specific apoptotic death pathways, and these may override those induced by ESAT-6 [84, 85, 88, 89]. In support of this possibility, ATP-induced apoptosis kills intracellular mycobacteria whereas Fas ligand-induced apoptosis does not [90]. ESAT-6-induced apoptotic cell death in vivo may not be harmful to mycobacteria, similar to Fas ligand-induced apoptosis [90]. Alternatively or additionally, the rapid re-phagocytosis of the dead macrophages in the granuloma may nullify any bactericidal effects of apoptosis, thus rendering them essentially an artifact of a cultured cell system, where re-phagocytosis may not be as rapid and constant a feature. Recent work finds that phagocytosis of apoptotic [87] *M. tuberculosis*-infected macrophages in cell culture is bactericidal by placing the internalized bacteria in lysosomal compartments [87]. Again, while this dead cell phagocytosis, or efferocytosis, was found to occur in *M. tuberculosis*-infected macrophages mice, similar to what has been observed in the zebrafish [74], its effects on mycobacterial viability were studied only in vitro. Thus the detrimental effects of efferocytosis may reflect the activities of particular types, or activation states, of macrophages in vitro versus in vivo. For instance the thioglycollate-elicited peritoneal macrophages used in these experiments may have different microbicidal capacities from macrophages recruited to the granuloma. Indeed, there are many circumstances under which *M. tuberculosis* and *M. marinum* survive lysosomal residence [8, 46, 91, 92]. It is also possible that macrophage apoptosis per se is detrimental in vivo in that some bacteria are killed in the process of death and re-phagocytosis but that the overall effect of accelerating these processes is nevertheless beneficial for the bacteria. Finally, in addition to pro-apoptotic determinants like ESAT-6, mycobacteria also have virulence determinants that are demonstrated to have anti-apoptotic effects in tissue culture models [93–96]. Thus, it is possible that mycobacteria both enhance and inhibit apoptosis at different points in infection.

Another recent discovery relevant to the effects of macrophage apoptosis in vivo comes from the identification, in the zebrafish, of a protective role for neutrophils in the early granuloma [97]. It appears that mycobacteria evade direct neutrophil recruitment and phagocytosis, but that this evasion is then circumvented in the granuloma when macrophage death signals neutrophils to the structure. Like macrophages, these neutrophils also phagocytose the contents of the dying macrophages, and furthermore, a subpopulation of neutrophils kills their internalized

mycobacteria through oxidative mechanisms. In this manner, neutrophils are protective and their ability to kill the mycobacteria depends on infected macrophage apoptosis.

A long observed, yet poorly understood feature of the tuberculous granuloma is its necrotic regions famously known as caseum. Both necrosis and macrophage apoptosis have been observed in human tuberculous granulomas; their interrelationship is unclear but it appears that they can occur independently of each other [9, 98–100]. The consequences and importance of macrophage necrosis have been illuminated by studies in the zebrafish. First, an early study showed that in as much as macrophages can provide growth niches for mycobacteria, they are nevertheless growth restricting; macrophage-deficient larvae were found unable to both transport bacteria from peripheral sites of infection as well as unable to restrict infection [101]. Indeed, conditions rendering mycobacteria extracellular are associated with increased susceptibility to infection: conditions leading to both TNF deficiency and excess produce macrophage necrosis and hypersusceptibility in the zebrafish, probably by distinct mechanisms [38, 50, 51]. The relevance of these zebrafish findings is highlighted by the observation that modulations in eicosanoid pathways, which regulate TNF and are associated with a common human genetic variant, result in increased susceptibility to TB and leprosy in human populations [50, 51]. In addition, both mouse and zebrafish studies show that virulent mycobacteria induce the production of host lipoxins, anti-inflammatory eicosanoids that induce macrophage necrosis through TNF suppression [51, 102].

In summary, the zebrafish studies indicate that at least in the early granuloma, both apoptosis and necrosis promote bacterial proliferation. Necrosis seems to be more beneficial as intracellular bacterial proliferation through apoptotic death and rephagocytosis does not reach the level of extracellular growth [38, 101]. The induction of both apoptosis and necrosis can be linked to bacterial factors, and it is unclear if they represent mutually independent programs [38], alternative decisions in a common pathway [103, 104] or both. The RD1 determinants also promote the development of necrotic caseum in mature granulomas, and this could be through post-apoptotic necrosis or a mechanism distinct from its pro-apoptotic function [49, 73].

7 A Molecular Mechanism for the Dynamic Granuloma

The RD1 dependence of granuloma expansion also comes as a surprise [105]. The chemotactic effects of this bacterial determinant are mediated by its induction of host matrix metalloproteinase 9 (MMP9). MMP9 production and secretion occurs, not in the infected macrophages, but in the epithelial cells surrounding the growing granuloma, and promotes the recruitment of new macrophages to the granuloma. Specifically, it is ESAT-6 that appears to induce MMP9 independent of the presence of macrophages, suggesting a direct interaction with the epithelium. How

ESAT-6 comes in contact with epithelial cells is not yet clear, but it may be released from dead infected macrophages and/or from live ones through its pore-forming activity [81].

Why do mycobacteria induce MMP9 in epithelial cells in the context of tuberculous granulomas when macrophages can express MMP9 in many other inflammatory conditions [106]? One can speculate that the use of epithelial cells surrounding a single infected macrophage allows early amplification of MMP9 production. Alternatively, mycobacteria may simultaneously dampen macrophage inflammatory responses so as to aid their own survival [107–110], while inducing pro-inflammatory responses that include MMP9 production in epithelial cells so as to recruit macrophages for the purpose of niche expansion and dissemination. Consistent with this latter possibility, ESAT-6-mediated induction of epithelial cell *mmp9* is independent of pro-inflammatory MyD88 and TNF signaling, and probably involves a pathway distinct from pro-inflammatory responses [105]. In contrast, infection of zebrafish larvae with *Salmonella* spp. induces MMP9 production in a MyD88-dependent fashion [111].

The relevance of these findings in early zebrafish granulomas to mature tuberculous granulomas is supported by studies of mouse and human disease. *mmp9*-knockout mice infected with *M. tuberculosis* have decreased macrophage recruitment to the lungs, reduced granuloma formation, and decreased bacterial loads [112]. In human TB, MMP9 is induced in lung epithelia surrounding tuberculous granulomas, and increased MMP9 production in the cerebrospinal fluid is associated with worse outcomes in tuberculous meningitis [113–116]. The zebrafish model suggests that the role of MMP9 in promoting granuloma development may be the mechanistic basis for the human observations. The zebrafish studies may also explain the long-standing observation that although tuberculosis can affect most organs, tuberculosis of skeletal or cardiac muscle is extremely rare [12]. In the zebrafish, if a granuloma begins in muscle, the closest epithelium expressing MMP9 is at some distance from the infected macrophages [105], and perhaps this increased distance is the reason that granulomas forming in muscle generally fail to grow [117].

8 Granulomas Promote Dissemination of Infection

A major advantage of the zebrafish larval model is the ability to monitor infection serially throughout an individual animal. What this has revealed is that a primary granuloma can also seed new granulomas by the efflux of infected macrophages [74]. Animals beginning with a single infection focus will quickly advance to multiple foci throughout the body, implicating the granuloma as a primary means of disseminating infection. These observations, apart from discrediting the view of granulomas as static barriers, once again provide an explanation for the finding that dissemination occurs early in the course of human TB [58]. They also expand upon prior work in mammalian models, which suggested that mycobacteria

disseminate hematogeneously from the primary granuloma [57, 118]. The zebrafish work shows that dissemination occurs through infected macrophages departing granulomas, and that such spread may occur via blood vessels, as well as by transit through tissue parenchyma. Thus, phagocytes first transport bacteria to deeper tissues for the establishment of infection [15, 101, 119] and then again transport bacteria from primary to secondary granulomas. In this context it has been shown recently that inflammatory DCs rapidly traffic in and out of both acute and chronic mouse BCG granulomas [120]. The exiting DCs have a widespread dissemination pattern and seem to efficiently prime $CD4^{+}$ T cells. It is possible that macrophage egress represents yet another mycobacterial strategy to turn a host-beneficial process to its advantage.

The finding that the granuloma disseminates infected macrophages may be important even in the context of human tuberculosis treatment. Drug-tolerant mycobacteria are also expanded within and disseminated from the granuloma even during generally effective treatment of zebrafish [117]. This finding is very similar to radiological observations in humans where lesions containing genetically drug-sensitive bacteria appear and develop in new locations during tuberculosis treatment, even as most other lesions are regressing [120, 121].

9 Summary

For nearly a century, the tuberculous granuloma has been regarded as a fortress; largely as an impermeable barrier to bacterial proliferation and dissemination, save that its necrotic breakdown in the lung might favor bacterial growth and transmission to other hosts [122]. Work on the zebrafish presents a more nuanced and sophisticated view of the granuloma as a dynamic structure on the ever-changing frontline in the battle between host and pathogen. This new view of the granuloma's role in TB pathogenesis has important therapeutic implications. Modulation of host pathways, such as MMP9 induction, in such a way as to tip the balance of power towards the host, may be a particularly attractive approach to treat tuberculosis that is resistant to traditional antimicrobial agents. New technical developments for 96 well format larval husbandry, and automated fluorimetry to detect both larval viability and bacterial growth suggest that this model can be rendered into a rapid and inexpensive platform for the discovery of both bacterial and host targeting drugs [123].

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Immunization Strategies Against Pulmonary Tuberculosis: Considerations of T Cell Geography

Carly N. Horvath and Zhou Xing

Abstract Pulmonary tuberculosis (TB) remains a global health concern with an astounding 9 million new cases and 2 million deaths per year. This leading infectious cause of death remains highly prevalent with one third of the world's population latently infected with *Mycobacterium tuberculosis (M.tb)* despite routine vaccination against TB in endemic areas. The only approved TB vaccine is the Bacille Calmette-Guerin (BCG), which provides protection against childhood miliary tuberculosis and has been administered intradermally in humans for almost a century. While effective in preventing disseminated forms of TB, the BCG has variable efficacy in providing protection against pulmonary TB. Therefore, the BCG has been unable to control the instance of adult pulmonary TB which constitutes the global disease burden. Despite the fact that mechanisms underlying the lack of pulmonary protection provided by the BCG remain poorly understood, it remains the "Gold Standard" for vaccine-mediated protection against *M.tb* and will continue to be used for the foreseeable future. Therefore, continued effort has been placed on understanding the mechanisms behind the failure of BCG to provide sufficient protection against *M.tb* in the lung and to design new vaccines to be used in conjunction with the BCG as boost strategies to install protective immunity at the site of infection. Growing evidence supports that the route of immunization dictates the geographical location of TB-reactive T cells, and it is this distribution which predicts the protective outcome of such vaccine-elicited immunity. Such vaccines that are able to localize TB-reactive T cells to the lung and airway mucosa are thought to fill the "immunological gap" in the lung that is required for enhanced protection against *M.tb* infection. This chapter focuses on

C. N. Horvath · Z. Xing (✉)
MDCL-Rm.4012, Department of Pathology and Molecular Medicine,
McMaster Immunology Research Centre, McMaster University,
1280 Main Street West, Hamilton, ON L8S 4K1, Canada
e-mail: xingz@mcmaster.ca
URL: http://mirc.mcmaster.ca/xing

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the critical importance of T cell geography when designing new immunization strategies against pulmonary TB.

Keywords *Mycobacterium tuberculosis* (M.tb) · Bacille Calmette Guerin (BCG) vaccine · Effector T cells · T cell geography · Lung interstitium · Subunit vaccines · Genetic-based vaccines · Human type 5 adenovirus (Ad5-vectored) vaccines · Vesicular stomatitis virus-based vaccines · "Vaccineless" boost strategies · Chemokines · Viral-vectored TB vaccines

1 Introduction

Pulmonary tuberculosis (TB) is one of the leading infectious causes of morbidity and mortality, responsible for the deaths of approximately 2 million annually [1]. It is estimated that one-third of the world's population is currently latently infected with *Mycobacterium tuberculosis* (*M.tb*), of which 9 million individuals develop active disease each year [1]. The only TB vaccine, Bacille Calmette Guerin (BCG), is an attenuated strain of *Mycobacterium bovis* typically administered intradermally shortly after birth in TB endemic areas, and has been used in humans since the 1920s. However, while BCG is effective in protecting against severe forms of disseminated *M.tb* infections in children, it is largely ineffective in protecting against adult pulmonary TB of which constitutes the majority of the global burden of disease [2–8]. As such, a large proportion of individuals with active TB are BCG immunized, resulting in highly variable efficacy ranging from 0 to 80 %. The immunologic mechanisms underlying poor lung protection by BCG still remain poorly understood although it has been suggested that the variable efficacy of BCG can be attributed to a wide variety of factors including host genetics, the presence of environmental mycobacteria, and the type of memory T cells generated from the immunization [2, 4, 7]. However, despite its shortcoming, BCG is the "Gold Standard" for vaccine-mediated protection against *M.tb* infection, and BCG or an improved BCG vaccine will continue to be used in the foreseeable future. Thus, the continuing effort is required to better understand the immune mechanisms behind poor lung protection by parenteral BCG immunization and develop effective boost immunization strategies to fill up such "immunologic gap" for enhanced lung protection. Recent growing evidence has suggested that the route of immunization dictates the geographical distribution of TB-reactive T cells which determines the immune protective outcome in the lung following pulmonary *M.tb* infection. This chapter will focus on the important consideration of T cell geography in the design of immunization strategies against pulmonary TB.

2 T Cell Geography Following *M.tb* Infection in Non-Immunized and BCG-Immunized Hosts

It has now been widely accepted that one of the critical defects in the immune response following pulmonary *M.tb* infection is the delay in the initiation of T cell priming in the local draining lymph nodes [2]. This delay in T cell priming results in delayed recruitment of effector T cells to the airway lumen and lung interstitium, the principal site of infection. Thus, *M.tb* is permitted to increase at a logarithmic rate within the lungs of the infected host for approximately 20 days, creating a robust "foothold" before the arrival of antigen-specific effector T cells to the site of infection [2, 4, 9]. Concurrent with the mass arrival and abundance of effector T cells into the lungs approximately 18–20 days post-infection is the control of bacterial growth (a plateau) [2, 10].

Similar to their unimmunized counterparts, BCG vaccinated hosts also suffer delayed recruitment of antigen-specific effector T cells to the airway lumen and lung interstitium, albeit somewhat accelerated due to the peripheral presence of BCG-primed T cells poised for action. Given its intradermal route of administration, the BCG vaccine generates a population of T cells which reside in peripheral tissues. Although a moderate number of such T cells can also be found within the lung interstitium of BCG-immunized animals, several studies have demonstrated that these cells do not immediately undergo expansion upon *M.tb* infection [2, 10, 11] and there is still a lack of airway luminal T cells at least for up to 10 days [10]. Such a delay in effector T cell responses in the airway lumen of parenteral BCG hosts has been identified to be a critical immune mechanism for the lack of immune protection for the initial 14 days after *M.tb* challenge [10]. However, given the fact that parenteral BCG immunization does provide enhanced protection over unimmunized hosts around 4 weeks post-infection, BCG-primed T cells are functionally important. Increased effector T cell responses in the lung interstitium and appearance of airway luminal T cells are generally accelerated by approximately 5 days in parenteral BCG-immunized hosts challenged with *M.tb* when compared to the unimmunized controls [10]. Thus, antigen-specific T cells begin to increase within the lung interstitium and appear in the airway lumen around 12–14 days post-infection, which is associated with an earlier plateau of bacterial growth and a 1 log reduction in bacterial burden over unimmunized controls observed around 4 weeks post-infection [2, 10]. Therefore, in spite of a few days of acceleration in T cell responses both in the lung interstitium and airway luminal compartment of parenteral BCG-immunized hosts, there is a very significant "immunologic gap" (at least 10 days) present primarily within the airway lumen, which leaves the lung unprotected from *M.tb* in the early stage of infection. It is believed that this critical shortfall in T cell geography in parenteral BCG immunized hosts is one of the reasons behind the inability of BCG vaccination to effectively protect from pulmonary TB while it fares reasonably well in controlling disseminated forms of TB in children.

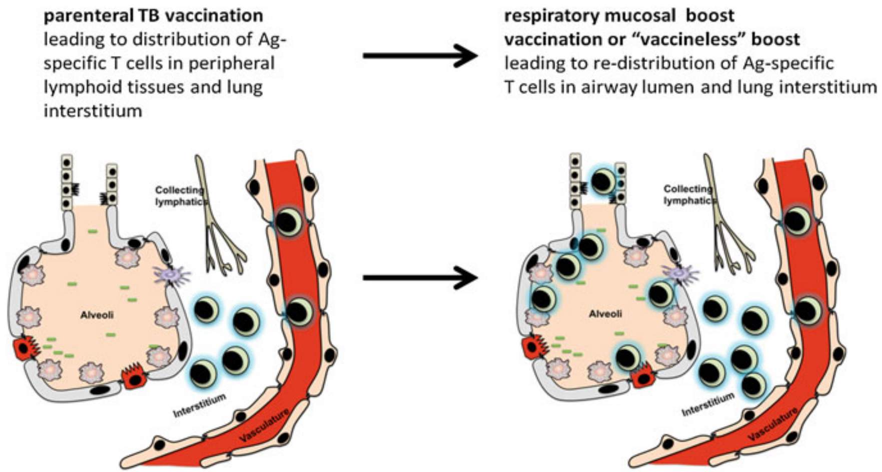

Fig. 1 Respiratory mucosal boost strategies used to install *M.tb* antigen-specific T cells on the surface of the respiratory mucosa for enhanced lung protection in parenterally TB immunized hosts

Thus, the emerging evidence establishes the importance of dividing the lung into the two major anatomic compartments for consideration of T geography and TB vaccination (Fig. 1): the lung interstitium present between the alveoli or between the airway and vasculature, and the mucosal surface of the lung identified as the airway lumen including both the conducting airway and alveoli [4, 5]. It is recognized that to date the majority of studies on pulmonary *M.tb* infection in naive or BCG-vaccinated host have focused on examining antigen-specific T cell responses in the whole lung without separately examining these cells in the lung interstitium and airway lumen [4]. Recent evidence gained from comparing parenteral and respiratory mucosal vaccination protocols has lent further support to the relevance of separately studying antigen-specific T cells both in the airway luminal and lung interstitial compartments [4] (Fig. 1).

3 Importance of T Cell Geography: Comparison of Primary Parenteral and Respiratory Mucosal Immunization Strategies

Immune control of *M.tb* in the lung following primary infection does not begin until the arrival of Ag-specific T cells in the lung following T cell priming in the local draining lymph nodes [2]. Although they bypass the initial priming stage in parenterally TB vaccinated hosts, peripherally located T cells still must enter the lung, particularly the airway lumen, in order to carry out their effector activities. While early vaccine studies primarily focused on the T cell distribution in the spleen and

lymph nodes [12–15], more recent studies have begun to examine T cell responses in both the lung interstitium and the spleen following parenteral TB immunization [16–19]. It is evident from such studies that parenteral immunization elicits robust T cell responses in the lung interstitium and peripheral lymphoid tissues such as the spleen, although the lung population varies with each vaccine candidate. However, when parenteral vaccination is compared head-to-head with mucosal administration with the same vaccine, it becomes apparent that robust T cell responses in the lung interstitium and spleen are not always correlated with protection against *M.tb* infection locally in the lung to the same extent as the presence of an airway luminal T cell population [4]. Thus, respiratory mucosal immunization fills up the "immunologic gap" associated with the parenteral immunization by installing abundant Ag-specific immune protective T cells on the respiratory mucosal surface and in the lung interstitium prior to pulmonary *M.tb* exposure.

Organism-based vaccines. While unlikely to be used as an intranasal vaccine in humans, BCG can be delivered parenterally, intranasally, or by aerosol. Intranasal immunization in mice was shown to provide better protection against pulmonary *M.tb* challenge than the subcutaneous route [20, 21]. Such improved protection was associated with more rapid T cell responses in response to mycobacterial challenge [20, 21]. While it is unknown whether mucosal administration of BCG resulted in a population of airway luminal T cells, there was an increase in the lung interstitial T cell population over that by parenteral administration [20]. The observation that respiratory mucosal delivery of BCG results in enhanced protection is not only limited to murine models, as aerosol-delivered BCG was also found to provide better protection than standard subcutaneous or intradermal BCG administration in guinea pigs [22].

Subunit vaccines. Due to the lack of immunogenic properties of purified or recombinant *M.tb* proteins alone, they require the addition of immune adjuvants as well as repeated deliveries in order to generate effective T cell responses [23, 24]. Nevertheless, several protein-based TB vaccines have been formulated and tested either via a parenteral or respiratory mucosal route. However, the head-to-head comparison studies are still lacking due to the fact that the immune adjuvant used for parenteral and respiratory mucosal deliveries is often different. One study [25] has compared the effect of subcutaneous with intranasal inoculation of a protein-based TB vaccine consisting of *M.tb* culture filtrate proteins and the adjuvant lipophilic quaternary ammonium salt, dimethyldioctadecylammonium bromide (DDA). It was found that the intranasal, but not subcutaneous, delivery resulted in elevated levels of antigen-specific T cell proliferation, IFN-γ, IL-12 in the cervical lymph nodes, and a greater induction of IgA in the nasal lavage [25]. Furthermore, the mice vaccinated intranasally exhibited enhanced protection in the lungs of *M.tb* challenged mice when compared to those immunized subcutaneously [25]. While this study did not fully characterize the T cell geography following the two vaccination routes, the results clearly support the advantage in vaccinating mucosally against pulmonary TB.

Ad5-vectored vaccines. Human type 5 adenovirus (Ad5)-based vector represents a robust *M.tb* antigen-expressing system due to its high level of transgene expression, excellent safety record in humans, high immunogenicity, and its

suitability for both parenteral and intranasal immunization [26, 27]. The first replication-defective Ad5-vectored TB vaccine engineered to express the *M.tb* Ag85A (AdAg85A) was evaluated both as a parenteral and mucosal vaccine candidate [16]. It was found that although intramuscular immunization with AdAg85A induced robust antigen-specific T cell responses in the spleen and lung interstitium, it failed to provide sufficient lung protection against *M.tb* challenge murine models [16]. It was further found that the peripherally distributed T cells activated by intramuscular AdAg85A immunization were not immunologically impaired as upon adoptively transferred into the airway lumen of na SCID mice, they were capable of potent immune protection [28]. On the other hand, respiratory mucosal administration of this vaccine elicited lower systemic levels of antigen-specific T cell responses, but much elevated T cell responses in the lung which correlated with superior protection following pulmonary mycobacterial infection, even surpassing that by parenteral BCG immunization [16]. Upon closer examination into the immune mechanisms, it was found that single respiratory mucosal immunization with AdAg85A elicited a significant antigen-specific CD8 T cell population in the airway lumen which was completely absent in the intramuscularly immunized group [28]. These T cells were of a long-lasting effector memory phenotype and were capable of self-renewing via continuing in situ proliferation in a specific Ag-dependent manner [29]. They remained immune-protective even in the absence of peripheral T cell supply [29] or in the absence of CD4 T cells [30]. These lines of evidence together indicate that following genetic-based TB immunization, the geographical distribution of antigen-specific T cells is closely associated with the route of immunization and ultimately the immune protective outcome against pulmonary *M.tb* challenge.

In addition to Ad5-based TB vaccines, other viral-vectored TB vaccines have also been developed and these include Ad35-based and vesicular stomatitis virus-based vaccines. However, following single intranasal immunization in murine models, these platforms were not as immune protective as the Ad5-based counterpart [31, 32].

4 Respiratory Mucosal Boost Immunization to Alter T Cell Geography in Parenteral BCG-Primed Hosts

The primary purpose of developing novel subunit- and genetic-based TB vaccines is to identify the candidate vaccines that can be used to boost protective immunity in the lung of parenteral BCG prime-immunized hosts [7]. As primary respiratory mucosal vaccination, by installing T cells on the surface of respiratory mucosa, consistently provides better protection against pulmonary *M.tb* exposure than parenteral vaccination [4], boost immunization via the respiratory tract shall most effectively enhance protective immunity in the lung of parenteral BCG prime-immunized hosts. Such respiratory mucosal boost immunization strategy enhances lung protection both by directly mobilizing the peripherally distributed pan-T cells

activated by parenteral BCG immunization into the lung and airway lumen and by activating the T cells specific to the *M.tb* antigen expressed by mucosally delivered vaccine and subsequently installing these cells in the lung.

Although most of the protein-based TB vaccine formulations are used parenterally, several studies have successfully used such vaccines intranasally to boost parenteral BCG immunization. Repeated intranasal deliveries of the fusion protein vaccine consisting of Ag85B-ESAT6 in LTK63 [33] or CTA1-DD/ISCOMs [34] induced potent T cell responses which served to boost the protection by parenteral BCG immunization. However, intranasal administration of an arabinomannan-tetanus toxoid conjugate (AM-TT) combined with a Eurocine adjuvant only enhanced protection in the spleen and not in the lungs of parenteral BCG-immunized mice [35]. Therefore, it appears that the efficacy of intranasal protein-based boost immunization in parenteral BCG hosts is dependent on the formulation of such boost vaccines.

The natural tropism of viral-vectored TB vaccines to the respiratory epithelium, their unique ability to derive robust transgenic *M.tb* Ag expression and their built-in immune adjuvant properties, make viral-vectored TB vaccines preferred candidates over protein- or plasmid DNA-based counterparts for respiratory mucosal boost immunization in parenteral BCG-immunized hosts [7, 26]. While the MVA-vectored TB vaccines when used as stand-alone vaccines do not induce potent T cell activation, when used for boosting parenteral BCG- or DNA plasmid-based prime immunization, these vectors are able to boost immune activation and protection against pulmonary *M.tb* challenge [12, 36]. Intranasal administration of MVAAg85A boosted BCG-primed T cell responses and protection to pulmonary *M.tb* infection [17], and is currently in clinical trials in South Africa based on an intramuscular immunization protocol. It remains to be seen whether this vaccine will also be amenable to respiratory mucosal immunization in humans [8].

The replication-defective Ad5-based vector, when delivered alone via the respiratory tract, has a record of proven safety and efficacy both in humans and animals [26, 27]. Thus, it makes an ideal respiratory boost vaccine candidate for parenteral BCG prime-immunized hosts. While respiratory mucosal delivery of AdAg85A alone provided a great level of protection following pulmonary *M.tb* challenge in mice, when delivered as a respiratory booster the immune protection in the lung was even further enhanced in parenteral BCG-immunized animals [37–39]. This was in contrast to the poorly enhanced lung protection by intramuscular boost immunization in the parenteral BCG hosts [37, 38]. This disparity between the respiratory and parenteral boosting was again attributed to the lack of Ag-specific airway luminal T cells by parenteral AdAg85A boosting despite its enhancing effects on lung interstitial and splenic T cells in parenteral BCG animals [4, 28]. In addition to murine models, the superior protection by respiratory mucosal boosting over the parenteral boosting with AdAg85A has also been demonstrated in parenteral BCG-immunized guinea pigs [40]. AdAg85A is currently under the evaluation in phase 1 clinical trials in both BCG-na and BCG-immunized healthy human volunteers in Canada but like all other TB vaccines currently in clinical trials, it is given parenterally. It is of importance to further

evaluate the effect of Ad5-based TB vaccine delivered via the respiratory tract in humans. This will allow us to appraise whether the pre-existence of anti-Ad5 immunity in most human populations may interfere with the safety and efficacy of Ad5-based TB vaccine. Apart from parenteral BCG priming and respiratory mucosal boosting strategies, a new immunization regimen involving simultaneous parenteral BCG and respiratory mucosal AdAg85A delivery has recently been investigated with promising results [41].

5 Respiratory Mucosal Manipulations of T Cell Geography Using "Vaccineless" Boost Strategies in Parenteral BCG-Primed Hosts

The "vaccineless" creation of airway luminal T cells in models of parenteral genetic-based immunization. The concept that respiratory mucosal boost immunization with various vaccine vectors, via installing T cells at the site of *M.tb* entry, provides the most robust protection in the lung, prompted the inquiry into whether the "vaccineless" boost strategies can be employed in place of boost vaccines. This concept is built in part on the surmise that early pulmonary *M.tb* exposure itself triggers insufficient innate immune responses within the airway luminal compartment, thus incapable of quickly mobilizing the peripherally spawned T cells by parenteral immunization into the airway lumen and lung interstitium [9]. Indeed, the concept of respiratory mucosal "vaccineless" boost strategy has been validated in murine models of viral- or plasmid DNA-based parenteral immunization. In this regard, intranasal delivery of CpG or the "empty" adenovirus elicited airway luminal T cells and restored the otherwise missing lung protection in intramuscularly AdAg85A-immunized mice [42]. However, such pro-inflammatory treatment does not deposit the specific *M.tb* antigens to the respiratory mucosa and thus can only confer a transient restoration of airway T cells and protection. On the other hand, intranasal delivery of soluble, nonadjuvanted Ag85 complex proteins creates a protracted restoration in either intramuscularly AdAg85A- or DNAAg85A-immunized animals [18, 42]. It was found that intranasal delivery of soluble mycobacterial antigens was sufficient to trigger the necessary responses of cytokines including TNF-α, MIP-1α, MCP-1, and IP-10 while keeping undesired tissue immunopathology to a minimum [18]. Thus, the blockade of either IP-10 or MIP-1α at the time of soluble antigen-delivery impaired the migration of peripherally primed antigen-specific T cells into the airway, associated with the loss of enhanced protection, indicating the essential role of chemokines for T cells to populate this lung compartment [18]. These data lend the support to using the "vaccineless" soluble, unadjuvanted *M.tb* culture filtrate proteins to elicit airway luminal T cells in parenteral BCG-immunized hosts.

The "vaccineless" creation of airway luminal T cells in parenteral BCG-immunized hosts. Parenteral BCG-immunized hosts fail to develop the airway luminal T cells in the initial 10–14 days after pulmonary *M.tb* exposure which is

responsible for the lack of lung protection in the first 2 weeks of time after *M.tb* challenge [10]. The arrival of T cells in the airway lumen ramps up to increased protection by 4 weeks post-challenge. Of note, in the same study it was observed that the T cells present within the lung interstitium were a poor correlate with lung protection [10]. These findings suggest that the "vaccineless" strategy capable of increasing airway luminal T cells would be expected to enhance lung protection by parenteral BCG immunization. Indeed, the intranasal delivery of soluble, unadjuvanted purified *M.tb* culture filtrate proteins artificially established a population of airway luminal T cells prior to *M.tb* infection in parenteral BCG-immunized mice. This airway luminal T cell population rapidly expanded upon pulmonary *M.tb* challenge whereas the lung interstitial T cell population remained unaltered [10]. Thus, the installation of airway luminal T cells prior to *M.tb* challenge in parenteral BCG-immunized hosts restored early lung protection that is otherwise missing in these animals [10]. Extrapolating from these findings, future research should focus on enhancing the speed at which T cell priming and migration to the airway luminal space occurs. Following primary *M.tb* infection it is essential to understand the role that the various immune molecules play in the recruitment of T cells both to the lung interstitium, and most importantly to the airway lumen. Such knowledge will provide further insights into the mechanisms of delayed or impaired T cell trafficking to the lung, and thus help design the strategies by which lung protection by parenteral BCG immunization can be enhanced.

Pros and cons of respiratory mucosal "vaccineless" boost strategies. The "vaccineless" purified *M.tb* antigen preparations (Ag85 complex or *M.tb* culture filtrate proteins) are not adjuvant-formulated, thus unable to prime T cells on their own. Compared to respiratory mucosal boost vaccination strategies, the "vaccineless" approach has several advantages. It is simple and cheap and potentially less pro-inflammatory, hence the safety for respiratory mucosal inoculation. Indeed, following intranasal inoculation of *M.tb* culture filtrate proteins, there was only a very mild inflammatory infiltrate seen around the bronchi [10, 18]. On the other hand, at least two repeated intranasal deliveries of *M.tb* antigen preparation are required to elicit robust airway luminal T cell responses in parenterally immunized animals [10, 18, 42]. In comparison, when a mucosal TB vaccine is used, a single administration suffices [16, 37]. It is also likely that vaccine-based strategies can most robustly create long-lasting memory T cells on the respiratory mucosal surface. Notwithstanding, the mucosal "vaccineless" boost strategy provides a new way to manipulate T cell geography for the benefit of enhancing local lung immune protection in parenterally immunized hosts.

6 Final Remarks

It is now widely accepted that parenteral BCG immunization fails to provide much needed mucosal protection against adult pulmonary TB. However, such parenteral immunization with BCG or an improved BCG vaccine is expected to continue in

human immunization programs. Emerging evidence suggests that the key to mend unsatisfactory lung protection by parenteral BCG is to increase immune protective T cells in the lung, particularly on the surface of the respiratory mucosa (Fig. 1). In this regard, there have been a few frontrunners including MVA- and adenovirus-based TB vaccines which are amenable to robust respiratory mucosal boost immunization in experimental models. These candidates have entered various phases of human trials but noticeably they are being given parenterally [43]. The challenge is to take the next step to evaluate the safety and efficacy of these vaccines following respiratory mucosal inoculation in humans. In order for this to happen, it is urgently needed to identify the reliable immunologic signatures in the peripheral blood that can be used to calibrate the levels of beneficial T cell responses occurring in the lung following respiratory mucosal immunization.

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