Functional and genetic dissection of anti-mycobacterial immunity

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Abstract

Tuberculosis (TB) is a major global health problem with an estimated 2 billion people worldwide infected with *M. tuberculosis*, the causative agent of TB, and 9.2 million new cases of active TB disease reported in 2008. Despite the enormity of the problem, little is known about the anti-mycobacterial immune responses used to detect TB infection. The aim of my studies was to investigate how host factors impact on anti-mycobacterial immune responses and to evaluate if these responses can provide clues about host susceptibility to infection and progression to disease. Healthy nuclear families were enrolled from a hyper-endemic TB region of Cape Town, South Africa. We measured *in vivo* tuberculin skin test (TST) responses, and used *in vitro* whole blood assays to determine antigen-specific IFNγ cytokine release as well as the frequency of antigen-specific $IFN\gamma^+ CD4^+$ and $IFN\gamma^+ CD8^+$ cells. We showed that the *in vivo* TST and *in vitro* IFNγ release assays, two assays used to detect *M. tuberculosis* infection, measure non-redundant pathways of anti-mycobacterial immunity. We observed a significant impact of age, but not sex, on all the immune phenotypes measured. We calculated the heritability of the immune phenotypes and observed high estimates. We performed the first genome-scan of the TST and identified a major locus on 11p14 ($p = 1.5x10^{-5}$) impacting on TST positivity (i.e. T-cell independent resistance to *M. tuberculosis*) and a second major locus on 5p15 ($p < 10^{-5}$) controlling the extent of the TST response. The results of the genome-scan suggested that host genetics confounds the use of TST in detection of TB infection and critically, modulates the protective immune response to *M. tuberculosis* infection. Finally, we provided evidence for the functional impact of *NRAMP1* TB disease risk alleles on protein function. Together, the studies demonstrated the importance of host factors, in particular host genetic factors, for immunity to TB. Study of these host factors will provide valuable mechanistic insight into the responses underlying immuno-diagnosis and disease pathogenesis of TB.

Résumé

La tuberculose reste un problème majeur de santé publique à travers le monde. On estime qu'environ 2 milliards de personnes dans le monde sont infectées par la bactérie *M. tuberculosis*, l'agent causal de la tuberculose, et on a rapporté 9,2 millions de nouveaux cas de tuberculose évolutive en 2008. Malgré l'énormité du problème, on en sait peu sur les réponses immunitaires anti-mycobactériennes utilisées pour dépister l'infection tuberculeuse. L'objectif de mon travail était de rechercher l'impact des facteurs de l'hôte sur les réponses immunitaires anti-mycobactériennes et de déterminer comment ces réponses peuvent fournir des indices sur la susceptibilité de l'hôte à développer une infection tuberculeuse et sa progression en maladie. Nous avons recruté des familles nucléaires en bonne santé vivant dans une zone hyperendémique tuberculeuse du Cap en Afrique du Sud. Nous avons interprété les réponses de l'intradermo-réaction (IDR) à la tuberculine *in vivo* et avons réalisé des essais immunologiques sanguins *in vitro* pour mesurer la production spécifique de cytokines IFNγ vis-à-vis d'antigènes ainsi que la fréquence de production spécifique de cellules $IFN\gamma^+$ $CD4^+$et $IFN\gamma^+$ $CD8^+$ vis-à-vis d'antigènes. Nous avons démontré que l'IDR à la tuberculine *in vivo* et les essais sanguins *in vitro*, deux essais servant au dépistage de l'infection tuberculeuse, mesurent les réponses immunitaires anti-mycobactériennes sans redondance. Nous avons observé que l'âge a un effet significatif, mais non le sexe, sur les essais immunitaires. Nous avons évalué l'héritabilité des phénotypes immunologiques et observé des taux élevés d'héritabilité. Nous avons effectué pour la première fois un balayage du génome à partir de l'IDR à la tuberculine et avons identifié un locus majeur sur 11p14 ($p = 1.5x10^{-5}$) ayant un impact sur la positivation de l'IDR à la tuberculine (i.e. résistance indépendante des cellules T à *M. tuberculosis*) et un deuxième locus majeur sur 5p15 ($p < 10^{-5}$) celui influençant la réponse quantitative à l'IDR à la tuberculine. Les résultats du balayage du génome suggèrent que les facteurs génétiques de l'hôte faussent les résultats de l'IDR à la tuberculine dans le dépistage de l'infection tuberculeuse et d'une manière critique contrôlent

la réponse protectrice immunitaire à l'infection par *M. tuberculosis*. En dernier lieu, nous avons fourni des éléments de preuve à l'effet que les allèles à risque de TB du gène *NRAMP1* ont un impact fonctionnel sur la fonction protéique. Dans l'ensemble, l'étude a démontré l'importance des facteurs de l'hôte, tout particulièrement les facteurs génétiques de l'hôte, vis-à-vis une réponse immunitaire à la tuberculose. L'étude des ces facteurs de l'hôte sera d'une aide précieuse dans la connaissance des mécanismes régissant le diagnostique des réponses immunitaires et la pathogénie de la tuberculose.

Preface

The work described in Chapter 1 and 6 of this thesis has been published as follows:

Chapter 1:
Gallant CJ, Di Pietrantonio T, E Schurr. 2003. Host genetics of tuberculosis susceptibility. McGill J Med 7(1): 113-125.

Gallant CJ, Di Pietrantonio T, Schurr E. 2008. Genetics of susceptibility to tuberculosis. S.K. Sharma, ed.. In Tuberculosis, 2nd Edition. India.

Chapter 6:
Gallant CJ, Malik S, Jabado N, Cellier M, Simkin L, Finlay BB, Graviss EA, Gros P, Musser JM, Schurr E. 2007. Reduced *in vitro* functional activity of human *NRAMP1* (*SLC11A1*) allele that predisposes to increased risk of paediatric tuberculosis disease. Genes & Immunity 8(8): 691-8.

In addition, at the time of thesis submission Chapters 2 to 5 had been submitted for publication.

Contribution of Co-Authors

The work described in Chapters 1 to 6 has been performed with the collaboration of the co-authors as follows:

Chapter 1: Research done for two published reviews on genetic susceptibility to tuberculosis contributed towards the writing of the *Introduction and Literature Review*. The reviews were divided into two sections: genetic susceptibility to tuberculosis in human and in mice. I wrote the section related to humans while T.D.P. wrote the section related to mice.

Chapter 2, 3 and 4: A.C. contributed to the data analysis, statistics and writing of the manuscripts; L.S. worked on database development and management; G.F.B. performed ELISA experiments and contributed edits to the manuscripts; K.S. performed ELISA experiments; J.H. performed FACS experiments and contributed edits to the manuscripts; T.M.D. provided GLP quality ESAT-6 and contributed edits to the manuscripts; W.A.H. and B.E. advised on and supervised the FACS experiments; N.B. contributed to field work and clinical data collection; J-P.J. contributed to the statistical analysis; P.V.H. contributed to the field work; L.A. contributed to the statistical analysis and edits on the manuscripts; A.A. supervised the statistical analysis and contributed to the writing of the manuscripts; E.G.H. supervised the collection of phenotypes and field work and contributed edits to the manuscripts.

Chapter 5: Contributions as in Chapters 2 to 4 plus J-L.C. contributed to the writing of the manuscript; A.B-A. organized the genotyping for the genome-wide linkage scan.

Chapter 6: S.M. genotyped the *NRAMP1* variants in the paediatric tuberculosis patients; N.J. advised on the development of the assay; M.C. provided the U937+*NRAMP1* transformed cell line; L.S. worked on the database development and management; B.F. contributed the GFP-*Salmonella;* E.A.G. and J.M. enrolled the paediatric patients in Houston, Texas; P.G. advised on the development of the assay.

Erwin Schurr provided expert supervision throughout all the studies.

Table of Contents

Abbreviations

ARI: annual risk of infection

BCG: *M. bovis* bacille Calmette Guérin

CFP-10: culture filtrate protein-10

CNV: copy number variation

DTH: delayed-type hypersensitivity

ELISA: enzyme-linked immunosorbent assay

ELISPOT: enzyme-linked immunospot

ESAT-6: early-secreted antigenic target-6

IBD: identical-by-descent

IGRA: interferon-gamma release assay

IFN-γ: interferon-gamma

LD: linkage disequilibrium

LOD: logarithm of the odds

M6PR: mannose 6-phosphate receptor

MDM: monocyte-derived macrophage

MHC: major histocompatibility complex

MLB: maximum-likelihood binomial

MTBC: *M. tuberculosis* complex

NRAMP1: natural resistance-associated macrophage protein-1

NTM: nontuberculous mycobacteria

PMA: phorbol 12-myristate 13-acetate

PPD: purified protein derivative

QTL: quantitative trait locus

TST: tuberculin skin test

SCV: *Salmonella*-containing vacuole

SNP: single nucleotide polymorphism

TNFα: tumor necrosis factor alpha

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Objectives of the Present Work

Little is known about the baseline immune reactivity profiles of high tuberculosis incidence populations. The study of these profiles may contribute to the identification of host factors that control resistance to infection by *M. tuberculosis* and progression of infection to clinical tuberculosis disease. At present, there is a lack of studies aimed at identifying the genetic factors that influence resistance to infection. Likewise, while a number of studies have succeeded in identifying genetic risk factors for advancement from infection to tuberculosis disease, they have largely failed to identify which biological phenotypes are modulated by these genetic risk variants. Therefore, the overall objective of my thesis is to systematically describe key aspects of anti-mycobacterial immunity in a highly tuberculosis endemic area and to map the major genetic factors that impact on this immunity. In addition, I aim to associate a known biological function to well replicated tuberculosis progression susceptibility variants of the *NRAMP1* gene.

Specifically, I propose:

a. To determine how key quantitative anti-mycobacterial immune responses measured in young individuals living in a hyper-endemic tuberculosis setting relate and how they are impacted by age and sex;

b. To estimate the heritability of anti-mycobacterial immune responses both as univariate and multivariate traits;

c. To identify the major genetic factors that impact on tuberculin skin test reactivity either as a binary trait (TST = 0 mm vs. TST >0 mm) or as a quantitative trait by performing a genome-wide linkage scan; and

d. To test the impact of specific risk alleles on the function of the well established tuberculosis gene, *NRAMP1,* by means of a newly developed biological assay.

Chapter 1
Introduction and Literature Review

1.1. A BRIEF HISTORY OF TUBERCULOSIS

Tuberculosis (TB) is an ancient disease with a long history of co-evolution with humans and a wide geographic distribution. *Mycobacterium tuberculosis*, the primary causative agent of TB disease in humans, is a member of the *M. tuberculosis* complex (MTBC), a group of pathogens with high DNA sequence similarity but different host tropism, epidemiology and pathogenicity[1,2]. Ancient MTBC DNA has been isolated in different parts of the globe, including from 5000-year-old samples from Egypt[3] and pre-Columbian mummies from Ecuador[4]. Descriptions of symptoms related to TB are found in early Hindu texts (~2000 B.C.) and in the writings of the ancient Greek physician Hippocrates (~ 400 B.C.)[5]. Ancient theories on the origins of TB disease vary wildly, but include references to both a heritable and infectious origin[5].

The first modern and explicit observation of an infectious origin of TB disease was made in the early 18th century, over a hundred years before the establishment of the germ theory. In 1722, a English physician named Benjamin Marten stated that "animalculae..or wonderfully minute living creatures..fretting or gnawing the Vessels of the Stomach, Lungs, Liver" were the causes of disease[5]. Marten's contemporaries dismissed the theory as they could not reconcile how "animalculae" could cause the diverse pathology – tubercles, cavities and ulcers – observed in TB patients. In addition, given that the prevalence of TB disease was very high in large European cities, many individuals were exposed their entire life without falling ill and it was thought that only those with "natural" susceptibility became afflicted. Thus, a theory of heredity rather than infection remained the predominant view at that time.

As the germ theory of disease gained widespread acceptance, Robert Koch, building on experiments of his predecessors and contemporaries[6], demonstrated that TB is an infectious disease and that the bacillus was a constant presence in the tuberculous lesions of men and animals[7]. He cultivated TB bacilli in culture, and showed that the organism could cause disease when introduced into a healthy host. These experiments, together with the requirement that bacilli must be re-isolated from the inoculated and diseased host animal and shown to be identical to the original causative agent, became the basis of Koch's postulates designed to show causality between a microbe and a disease.

Today, the once mutually exclusive theories of an infectious versus hereditary origin of TB disease have been reconciled. It is firmly established that TB disease has both an infectious and hereditary origin – exposure and infection with the causal factor (i.e. infectious agent) is necessary but often not sufficient to cause disease. The interplay of additional risk factors, both genetic and environmental, is necessary for infection and progression to disease.

1.2. GLOBAL TUBERCULOSIS DISEASE BURDEN

In 2008, the World Health Organization reported that the global TB incidence rate and prevalence continue to increase. It was estimated that 2 billion people worldwide were infected with *M. tuberculosis*, and that there were an estimated 9.2 million new and 14.4 million active TB cases[8]. TB disease is a particular major health problem in the regions of Africa, South-East Asia and Western Pacific which in 2006 accounted for 83%

of the total TB case notifications[8]. In addition, the rise of the often deadly combination of TB-HIV co-infection and multi- and extensively drug-resistant strains of *M. tuberculosis* threaten to amplify the global TB disease burden[9-11]. TB disease in children is often overlooked despite estimates that they represent approximately 11% of the global cases[12] and as high as 14 to 39% of the case load in high burden countries such as South Africa[8,12-14].

1.3. NATURAL HISTORY OF TUBERCULOSIS DISEASE

TB is a chronic infectious disease. Knowledge of TB pathogenesis has largely been derived from animal models and is classically described as a divergent multi-stage process[15,16]. Upon exposure and inhalation of air-borne tubercle bacilli into the lung, the bacilli are ingested by phagocytes – alveolar macrophages and dendritic cells – and are either killed or grow to a limited extent intracellularly. If the infection is not immediately cleared at this stage, the bacilli multiply, spread and activate T cells in the lung-draining lymph node[17-19]. Two to six weeks following infection, cell-mediated immunity develops and contains the localized, granulomatous lesions, killing most, but not necessarily all of the bacilli. If the cell-mediated immune response is not effective, the primary infection will progress to active disease (primary TB disease). In young children and immunocompromised persons, the pathogen can disseminate and forms small miliary lesions, disease in distinct organs or life-threatening meningitis[20-22]. In the majority of cases, the primary infection is controlled but not cleared and the bacteria remain dormant intracellularly and within granulomas (latent TB infection)[23]. A small fraction of latently infected individuals will develop active clinical TB over the course of their lifetime under

conditions of failed immune surveillance (reactivation TB disease)[24,25]. In general, *M. tuberculosis* has a strong affinity for the lungs and the majority of infections result in pulmonary disease. Once an infected individual converts to active disease, cavitary lesions develop and the mycobacteria proliferate. If the cavity expands into the alveoli, the patient becomes infectious and spreads the bacilli by speaking, coughing and sneezing[25].

Infection with mycobacteria other than *M. tuberculosis* has been shown to cause disease in humans. *M. leprae* is the causative agent of leprosy, a disease that occurs primarily in the skin and peripheral nerves and commonly causes sensorial loss, physical deformities and permanent nerve damage[26]. *M. africanum,* a member of the MTBC, primarily infects humans and causes disease as severe as infection with *M. tuberculosis*[27-29]. Interestingly, studies have demonstrated that *M. africanum* is equally transmitted but progression to disease occurs at a lower rate than infection *M. tuberculosis*[28] suggesting distinct host-pathogen interactions at different stages of disease. *M. bovis,* also within the MTBC, is a common pathogen of cattle and is spread to humans through infectious milk. *M. bovis* infection in humans is primarily associated with lymphatic or pulmonary TB disease[30,31]. Nontuberculous mycobacteria (NTM) is a broad term used to define the group of mycobacteria outside of the MTBC. NTM are often ubiquitous in the environment and can cause pathology in both immuno-competent and immuno-compromised hosts[32,33]. For example, the *M. avium* complex is responsible for pulmonary disease in otherwise healthy elderly men and women and severe disseminated disease in AIDS patients[34-36].

1.4. EPIDEMIOLOGY OF TUBERCULOSIS INFECTION AND DISEASE

In order for an individual to develop TB disease, two events need to occur: (1) exposure and infection with *M. tuberculosis,* and (2) infection needs to progress to active disease. The occurrence of these events depends on a number of factors which modulate the risk of exposure to the bacilli, the risk of infection and the risk of developing TB disease. The known risk factors will be explored below.

1.4.1. Risk of exposure and infection

Overall, the risk of TB exposure and infection depends on the presence of incident cases. At the population level, the risk of being infected is classically estimated by the annual risk of infection (ARI) which measures the incidence of infection in a given population[37]. The ARI is reported to be approximately 1-2 % in TB endemic developing countries and 0.1-1% in developed countries[38]. In the suburbs of Ravensmead and Uitsig, Cape Town, South Africa, the site for many of the studies included in this thesis, the ARI is estimated at 4%. Therefore, the risk of exposure and infection is very high in this community (unpublished data).

Among persons exposed to an infectious TB case, the risk of infection is modulated by a number of factors including: (1) the infectivity and duration of infectiousness of the source case, and (2) the degree of exposure to the case. Social factors, community TB prevalence and age determine where exposure is most likely to occur. Close contacts of TB patients, in particular young children living with an infectious caretaker (e.g. parent or grandparent), are at particular risk of exposure due to

close and on-going interactions[39-42]. In areas of poverty, poor housing and overcrowding, and in TB populations with a high ARI and thus a high rate of transmission, the risk of exposure from sources outside the household may be higher and more important than in areas with low incidence[13,22,40,42-46].

Following transmission, establishment of infection depends on both bacterial (e.g. strain or species diversity)[28,47-51] and host (e.g. innate immunity) factors[52-56,41,53,57-60]. For example, there exists a significant variability in inter-individual responses to *M. tuberculosis* exposure. In highly-endemic regions, epidemiological studies show a consistent pattern of approximately 20% of individuals who remain negative in diagnostic tests to measure TB infection throughout their lifetime despite repeated exposure to the bacteria[41,61,62]. It is unlikely that this subgroup all have an intrinsic impairment of delayed-type hypersensitivity or acquired T-cell immunity, and it is possible that a significant fraction of non-responders represent individuals that are naturally resistant to TB infection[63]. At present, few studies have investigated the initial encounter between the host and pathogen, and therefore little is known about the exact host mechanisms modulating resistance to infection.

1.4.2. Risk of disease

Following exposure and infection, progression to TB disease is the exception rather than the rule for the majority of individuals. Although there is a lack of controlled studies, it is estimated that 90% of infected individuals have effective and lasting anti-TB immunity and remain asymptomatic throughout their lifetime. Approximately 5% of individuals will progress to active disease within two years of infection (primary disease) and an

additional 5% of infected individuals will have TB disease within their lifetime (reactivation disease)[64,65]. If latently TB infected individuals become infected with HIV, the annual risk of progression is significantly higher ranging from 5 to 15%[66,67].

In children as in adults, many cases of primary TB infection are asymptomatic and self-healing[68]. However, reviews of childhood TB studies from the pre-chemotherapy era showed that most disease in children occurred in the first year following infection[22,68]. Therefore, childhood disease primarily reflects recent primary infection rather than reactivation disease. The risk of disease is greatest for children under the age of 2 years and for children greater than the age of 10 years, and lowest in children between 5 and 10 years of age[69]. Infected children who do not progress to primary disease become reservoirs of infection and disease upon reactivation later in life[70].

Primary infection in children less than 2 years of age, and to a lesser extent in children less than 5 years of age, often progresses to severe disseminated disease, including TB meningitis or miliary TB disease, due to early uncontrolled haematogenous spread of the mycobacteria[20,21,68,71]. In contrast, infection after 10 years of age more commonly progresses to an adult-type pulmonary disease[68]. These two major clinical forms of TB disease – severe disseminated and pulmonary disease – correspond to two age-dependent peaks of disease incidence[72], suggesting that they are the result of yet undefined differences in immunologic and genetic control[63,70].

In addition to an age-dependent risk of disease, a number of risk factors of varying impact have been identified. HIV infection is the most important known risk factor for

progression to active disease or reactivation of latent infection[9,73]. Other risk factors include sex - reflected in difference in disease notification rates between males and females primarily after 15 years of age[41,74], variability in bacterial strain factors[28,75,76], nutritional status[77] including the level of vitamin D[78-80], smoking[81,82], use of immunosuppressive drugs[83-85], renal failure[86] and diabetes mellitus[87]. Only a small number of studies have identified and replicated major genetic risk factors impacting on progression from infection to disease, including rare deleterious mutations in genes in the IL-12/IFN-γ cytokine pathway[88], and common variants in the natural resistance-associated macrophage protein 1 (*NRAMP1)*[89,90] and monocyte chemoattractant protein 1) (MCP1)[91,92]. Overall, despite the known risk factors described above, for the majority of TB infected individuals we have yet to identify the exact triggers that cause progression to active disease and therefore cannot predict disease risk with any great accuracy.

1.5. DETECTION OF TUBERCULOSIS INFECTION

The purpose of screening for *M. tuberculosis* infection include to diagnose latent TB infection as part of contact screening and epidemiological surveys, and in combination with other tests, to support or refute a diagnosis of active TB disease[93]. At present, there are two different ways to diagnose infection – the tuberculin skin test (TST), which until recently was the only method but remains largely the imperfect reference diagnostic, and the newly developed interferon-gamma release assays (IGRAs). Both assays, unfortunately, suffer from variable diagnostic sensitivity and specificity.

Details of the TST and IGRA and the known factors affecting their ability to reliability detect *M. tuberculosis* infection are described below.

1.5.1. Tuberculin skin test

The TST was first introduced in principal in 1890 by Robert Koch who showed that patients with and without TB diseases had different hypersensitivity responses to injection of tuberculin – extracts prepared from heat-killed tubercle bacilli[5,94,95]. Although Koch first believed that he had discovered a treatment for TB, he and others later recognized the potential of tuberculin as a diagnostic tool. Koch's observation led to the development of a standardized *M. tuberculosis* purified protein derivative (PPD) and the practical application of a TST in the 1930s[94-96].

1.5.1.1. Immunologic basis for tuberculin skin test reactivity

The TST measures cell-mediated immunity in the form of a classic delayed-type hypersensitivity response to mycobacterial antigens. Following intradermal injection of PPD, antigen-presenting cells (e.g. Langerhans cells)[97] present mycobacterial antigens to local T cells inducing the secretion of cytokines and chemokines and an influx of monocytes, macrophages and lymphocytes at the site of antigen exposure[98-102]. The expression of lymphokines by sensitized T cells induces induration through local vasodilation, edema, fibrin deposition and recruitment of other inflammatory cells to the area[103,104]. The TST reaction begins 5 to 6 h after injection, causes maximal induration at 48 to 72 h and subsides over a period of days[53]. Of note, no reaction will occur if an individual is infected but the bacilli are killed without the involvement of cell-mediated immunity[105].

1.5.1.2. Test administration and reading

The TST is applied primarily by the Mantoux method developed by the French physician Charles Mantoux[106]. In the Mantoux skin test, 0.1 ml of *M. tuberculosis* PPD solution (5 tuberculin units (T.U.) of PPD-S or 2 T.U. of PPD-23) is injected intradermally in the volar or dorsal surface of the forearm. The injection is made using a one-quarter- to one-half-inch, 27-gauge needle and a tuberculin syringe[53]. The test is read 48 to 72 h after injection by measuring in millimetres the diameter of the cutaneous response – the induration. There are two methods to measure the induration size. The first method uses palpation to delineate the borders of the induration. The second method uses a ball-point pen by moving the pen from the lateral edge to the centre of the reaction area and marking the border where resistance is felt. After delineating the border, a ruler is used to measure the largest diameter. Use of the ball-point pen method is suggested to decrease inter-observer variability[107-109] and increase sensitivity[110].

1.5.1.3. Interpretation of tuberculin skin test reactions

1.5.1.3.1. Sensitivity and false negatives

Sensitivity of the TST is influenced by false negatives which can be caused by a number of factors. Among TB patients, the rate of false negatives can range from 10-25% of cases although it is not known why they present with TST anergy[111-115]. False negative TST results can also occur due to infection with bacterial or viral disease including HIV[116,117], malnutrition[118], use of immunosuppressive drugs[53], disease affecting lymphoid organs, chronic renal failure, and very young or old age[53,114,119,120].

1.5.1.3.2. Specificity and false positives

The *M. tuberculosis* PPD used for the TST is a crude mixture of antigens that are also present in the TB vaccine *M. bovis* bacille Calmette Guérin (BCG) and in several NTM[121,122]. Cross-reactivity due to infection with NTM or BCG vaccination may make it difficult to distinguish whether sensitization to PPD was by BCG vaccination[123,124], NTM exposure[123] or *M. tuberculosis* infection, and therefore affect the specificity of the test. In populations with low-frequency of NTM, there is a clear separation of TST non-responders and responders[125]. Conversely, in populations with moderate to high frequency of NTM exposure, the separation is less clear although cross-reactivity of antigens primarily affects the specificity of the test in the low response range (i.e. less than 10 mm)[123,126]. BCG-induced TST sensitivity also often ranges below 10 mm induration and wanes after vaccination[124,127], especially when BCG is given at birth[124,128].

1.5.1.3.3. Recommendations for determining a positive test

In order to increase the likelihood of identifying individuals truly infected with *M. tuberculosis* and at high risk of developing TB disease, the American Thoracic Society developed recommendations to interpret TST results based on risk of exposure and disease[53]. The results of the TST are interpreted as binary, with a positive test indicating infection. A cut-off of ≥ 5 mm is recommended for individuals at very high risk of developing TB, including those in close contact with an active TB case or those that are immuno-suppressed. A cut-off of ≥ 10 mm is recommended for persons with normal or mildly impaired immunity and a high likelihood of being infected with *M. tuberculosis*. This cut-off is also applied to test results in children less than 4 years of age or infants,

children, and adolescents exposed to high-risk adult cases. Finally, a high cut-off of ≥ 15 mm is used in persons who have no risk factors for TB[53].

Although the recommended thresholds to determine TST positivity were determined for logistic ease and to minimize the rate of false negatives and false positives, exposure history of the population as well as the individual clinical context should be considered when interpreting the result of the TST[53,124]. Likewise, the prevalence of TB infection in the population determines the likelihood that a positive TST truly represents infection, with high prevalence associated with a high positive predictive value[53].

1.5.1.4. Tuberculin skin test characteristics

Despite the limitations of the TST in regards to sensitivity and specificity, it is still widely used globally. A positive TST remains an important indicator for therapy among TB case contacts[129,130], and under certain conditions it is associated with progression to disease[64,131,132]. In addition, treatment of latent TB based on positive TST results reduces the risk of active disease significantly[53,133-135]. However, the results of a single TST cannot distinguish if the infection is a result of recent exposure where symptoms have not yet developed or from a long-term infection where the host successfully contained the pathogen[136]. In addition, the relationship between the magnitude of the TST response and disease risk, and whether the cellular targets of the delayed-type hypersensitivity reaction and those to *M. tuberculosis* are the same, remain unclear[64,98,131,137,138].

1.5.2. Interferon-gamma release assays

For nearly a century, the TST was the only test available to detect TB infection. However, a new class of tests – IGRAs – have recently been developed and proposed as an alternative to the TST. Two IGRAs are now commercially available - the QuantiFERON-TB Gold and the T-SPOT.TB. Their use is increasing despite a number of unanswered questions concerning the interpretation of assay results and IGRA performance in high-risk populations[139].

1.5.2.1. Immunologic basis for interferon-gamma release assays

The IGRAs test for *in vitro* IFNγ production by sensitized T cells in peripheral blood after 16 to 24 h incubation with specific mycobacterial antigens. IFNγ production is measured by enzyme-linked immunosorbent assay (ELISA) or enzyme-linked immunospot (ELISPOT).

1.5.2.2. Interpretation of interferon-gamma release assays

One of the major barriers to developing new diagnostics to detect *M. tuberculosis* infection is the absence of a diagnostic gold standard. Extensive longitudinal studies with the TST have allowed clinicians to make informed interpretations of the results, but as discussed, it is still an imperfect tool to detect infection. Without an alternative method to confirm infection, sensitivity and specificity of IGRAs cannot be directly estimated. Specificity of IGRAs are evaluated in healthy populations with low TB endemicity where the proportion of individuals without active TB disease who are negative by the assay are calculated. Sensitivity of IGRAs are estimated from patients with active TB disease (as a

surrogate for latent infection) and from persons in contact with patients with active TB disease – with contact categorized in gradients of exposure[140]. In addition, a number of studies measure the concordance of IGRA and TST results[62,140]. These estimates of specificity, sensitivity and concordance are problematic for a number of reasons. Most importantly, patients with active TB disease display a reduction of cell mediated responses and therefore do not represent the best reference population[112-114]. In addition, we know very little about how the immune responses underlying the TST and IGRAs relate. However, despite the problems with evaluating IGRAs, the new class of assays appear to address some of the disadvantages of the TST related to specificity.

1.5.2.2.1. Sensitivity and false negatives

Studies of IGRA performance have reported variable sensitivity of the test to detect TB infection[62,140]. In studies comparing T-SPOT.TB (ELISPOT-based) and QuantiFERON (ELISA-based) assays, differences have been observed in diagnostic sensitivity to detect TB infection in children compared to adults[141,142], between sexes[143,144], and across tests in young individuals[119,145]. Very few studies have investigated the performance of IGRAs in high-risk populations[62,93,119,140,141,146], although there is evidence that IGRAs show low sensitivity in infants, in individuals with HIV-co-infection or in those suffering from malnutrition[119,147-149].

1.5.2.2.2. Specificity and false positives

In contrast to the variable results reported on IGRA sensitivity, many studies report high specificity to detect *M. tuberculosis* infection, including in BCG-vaccinated and TB-endemic populations[62,140,150]. Commercial IGRAs employ the antigens early-secreted antigenic target-6 (ESAT-6), culture filtrate protein-10 (CFP-10) and TB7.7. ESAT-6 and CFP-10 are produced by a small number of mycobacterial species (including pathogenic *M. africanum*, *M. bovis*, *M. kansaii*, *M. marinum*, *M.szulgai and M. leprae*)[151-153] but not BCG vaccines[154] and most environmental mycobacteria[155] and are therefore more specific than the relatively crude mix of antigens in PPD.

1.5.2.3. Interferon-gamma release assay test characteristics

Unlike the TST, IGRAs do not benefit from extensive use in order to aid interpretation of assay results. Little is known about the IGRAs' ability to distinguish individuals with recent versus remote infection and those with latent infection at high risk of developing active TB disease[62,93,136,144]. One study, however, concluded that the IGRA test was at least as sensitive as the TST for identifying TB contacts at high risk for progression to active TB[156]. In addition, IGRAs were shown to be at least as or more sensitive that the TST to gradients of TB exposure[142,156,157].

Differences in IGRA response between short-term (24 h to 3 days) vs. long-term (6 to 7 days) incubation times exist[158,159]. It has been hypothesized but not yet shown that short incubation assays detect responses of activated effector T cells that have recently encountered antigens *in vivo* (i.e. recent exposure and infection), and can therefore

rapidly release IFNγ when stimulated *in vitro*. In comparison, long-term incubation assays may stimulate central memory T cells - cells that are associated with protective immunity[160,161] and persist after clearance of infection or during latency[158,159,162]. Finally, it is not known why a consistent discordance between TST and IGRA results occurs across populations, although it is hypothesized that the assays measure different components of the cellular immune response[93,140,163].

1.6. TUBERCULOSIS VACCINATION

The family of BCG strains are the only vaccines currently available for TB. BCG was first used to immunize humans in 1921 following serial *in vitro* passages of a virulent *M. bovis* isolate for 13 years in the lab of French scientists Albert Calmette and Camille Guérin leading to its attenuation. Subsequent passages of the attenuated isolate in different labs around the world created BCG daughter strains that are characterized by genomic and phenotypic differences[164,165]. Four strains, the Pasteur strain 1173 P2, the Danish strain 1331, the Glaxo strain 1077, and the Tokyo strain 172, account for approximately 90% of BCG vaccinations globally[166]. Difference in efficacy between strains has not been shown and there is no global consensus as to which strain is most optimal.

Today, BCG vaccines are administered to approximately 100 million children each year. The WHO guidelines recommend administration of BCG as soon as possible after birth to all infants in countries with high TB prevalence. In industrialized countries

where the incidence of disease is very low, vaccination strategies include targeting only defined high–risks groups[166].

The greatest effect of BCG vaccination is the protection against severe disseminated disease in young children. It seems to not prevent primary infection or reactivation of latent pulmonary infection, the principal source of bacillary spread in the community[166-169]. One meta-analysis estimated the overall protective efficacy to be 50% for all forms of TB disease with studies reporting estimates from 0% to 80%[167]. The range of protective efficacy for disseminated disease and TB meningitis is reported to be 47-98%[169].

In order to effectively reduce the burden of TB disease, new vaccines are urgently needed. Given the evidence of protection against severe forms of childhood TB disease, many vaccines currently in development are working to either provide better and longer-term protection than BCG, or boost pre-existing BCG-derived immunity[170]. In addition, researchers are working to develop vaccines to prevent infection in immunologically-naive individuals, to prevent reactivation of latent infection and to that take into account the effects of co-HIV-TB infection. At present, the major obstacle to vaccine development is the limited understanding of the biology of TB infection and disease, and as a result, a lack of well defined correlates of protective immunity[171].

1.7. IMMUNITY TO TUBERCULOSIS

Dissection of the molecular and cellular mechanisms related to immunity to *M. tuberculosis* disease has primarily been performed using murine and rabbit experimental disease models. *In vitro* experiments with human cells, new models such as the zebrafish, the effect of rare human mutations and observation of post-mortem pathology in *M. tuberculosis* infected humans have also contributed to our understanding of anti-TB immune responses. Despite the existing knowledge, considerable gaps exists in our understanding of the key immune factors influencing susceptibility to infection and progression to both primary and reactivation disease. A review of some of the more established aspects of TB immunity is presented below.

1.7.1. Overview

Upon inhalation of aerosolized TB bacilli, the pathogen encounters and is engulfed by alveolar macrophages and interstitial dendritic cells[172,173]. Infected macrophages contain the bacteria and both macrophages and dendritic cells induce a localized pro-inflammatory response, including the secretion of cytokines (e.g. IL-12, IL-18, TNFα)[174-176] and chemokines (e.g. CXCL8, CCL2, CCL5)[175,177-179] which drives successive waves of recruitment of neutrophils, natural killer (NK) cells, $CD4^+$ T cells and $CD8^+$ T cells. Each wave of recruitment produces a different combination of chemokines and cytokines to amplify the immune response[178,180-182]. Unconventional T cells, $\gamma\delta$ and CD1-restricted T cells are activated during *M. tuberculosis* infection but their role in anti-mycobacterial immunity and protection is not completely understood[183-

[185]. IL-12 induces migration of activated dendritic cells to the draining lymph node which is required for the activation of immunologically-naive and antigen-specific T cells[186,187]. Activated T cells, by way of recirculation and tissue surveillance, recognize *M. tuberculosis* infected macrophages and DCs in lung lesions. Secretion of IFNγ by antigen-specific $CD4^+$ and $CD8^+$ T cells activate infected macrophages leading to the initiation of microbicial effector mechanisms including phagosome maturation[188-190], autophagy[191], the production of anti-microbial peptides[79,192,193] and in turn to IFNγ secretion[194,195].

A hallmark of pulmonary TB disease is the formation of granulomas in the lung to contain the bacteria. The mature granuloma is composed primarily of infected macrophages surrounded by foamy macrophages, giant cells, lymphocytic infiltrate and a fibrous "cuff" of collagen and other extracellular matrix at the periphery[182,196,197]. The involvement of TNFα in granuloma formation is discussed in Section 1.7.3.2. If the granuloma is capable of inhibiting bacterial spread and proliferation, the infection is contained but not eradicated and the individual remains asymptomatic and non-infectious. It is hypothesized that latency is maintained throughout life unless host immunity or the signals maintaining the granuloma structure are disrupted or wane, leading to granuloma decay, rupture and spread of infectious bacilli in the host's airways[197,198].

1.7.2. Innate Immunity

A number of recent studies support an important independent role of innate immunity in modulating protection in the early stages of TB infection and in initiating

and instructing the adaptive immune response. It is therefore hypothesized that the inability to resolve infection is due to an intrinsic deficiency in innate immunity and macrophage function[54,55,199,200]. The population of individuals who remain TST and IGRA negative despite repeated exposure to *M. tuberculosis* most likely represent a subset of individuals resistant to infection. And as this population shows no detectable TB-specific T cell memory, it is implied that resistance is mediated by innate immunity. Therefore, a strategy to reduce the incidence of infection would be to learn the characteristics of the natural resistance immune response and develop a vaccine that mimics it or a treatment that repairs deficiencies[171]. Characterization of natural resistance could be done through large, prospective, population-based studies in TB endemic regions in order to understand the distribution of key immune responses (i.e. do a subset of individuals with a specific immune profile remain resistant to infection or progress to disease) and through genetic studies to identify the factors that modulate protection. Experiments described in this thesis directly contribute to this important goal.

1.7.3. Key effector cytokines and cells

1.7.3.1. IL-12/IFN-γ cytokine pathway

Mice with a targeted *Ifng* or *Il12p40* gene deletion exhibit extreme susceptibility to TB disease[201,202]. In addition, genetic disruption of the ifn-γ receptor-1 (*Ifngr1*)[203] or ifnγ-response genes including signal transducer and activator of transcription 1 (*Stat1*)[203,204], the nuclear factorκBp50 (*Nfkb1*)[205], the interferon regulatory factor-1 (*Irf1*)[206] and the interferon inducible protein Lrg-47 (*Ifi1*)[207] also produce severe pathological conditions characterized by early death following *M. tuberculosis* infection.

More importantly, in humans with lack or partial deficiency of proteins in the IL-12/IFN-γ cytokine pathway are hyper-susceptible to mycobacterial infection (discussed in more detail in Section 1.9.3.1). IL-12 is secreted by hematopoietic phagocytic cells and dendritic cells following phagocytosis of *M. tuberculosis*[174,176]. It drives the development of a Th1 cell-mediated immune response including the production of IFN-γ by $CD4^+$ T cells[208], $CD8^+$ T cells[209,210] and natural killer cells[211]. In return, IFN-γ secretion stimulates a mycobactericidal response in macrophages necessary to control bacterial growth[203,212-214]. Together, the evidence from murine and humans studies demonstrates that the IL-12-IFN-γ pathway is essential for protection against TB disease.

1.7.3.2. TNFα

In the murine model of TB infection, animals deficient of TNFα or the TNFα receptor failed to control a primary challenge with *M. tuberculosis* and show impaired granuloma formation[215-217]. In humans, patients receiving TNFα-neutralizing drugs to treat immune and inflammatory conditions are at increased risk of reactivation of latent TB infection[83,218,219]. TNFα is produced by variety of immune cells during TB infection, including macrophages[220], dendritic cells[176] and T cells[221]. It has pleiotropic effects in inflammation that affect cell activation, migration and apoptosis[222,223]. Synergy between TNFα and IFN-γ mediates killing of intracellular pathogens[214,224,225] largely through activation of macrophage antimicrobial functions[214].

Recent research in the zebrafish model of TB infection suggests an important role for TNFα in innate immunity and the control *M. tuberculosis* infection. In young,

optically transparent zebrafish infected with the pathogenic *M. marinum* before the development of an adaptive immune system, TNFα was produced within 24 hours of infection and TNFα signalling mediated early protection by controlling bacterial growth within macrophages. In the absence of TNFα signalling, a granuloma formed but considerable necrotic death occurred and the granuloma rapidly disintegrated. Thus, independent of adaptive immunity, TNFα in this system was not required for TB granuloma formation, but for the maintenance and integrity of the structure by restricting bacterial growth, preventing macrophage necrosis and bacterial spread[200].

1.7.3.3. $CD4^+$ T cells

Evidence of the importance of $CD4^+$ T cells for protection against TB disease progression comes from both murine and human studies. Mice deficient of the surface molecules CD4 or MHC class II[226,227] or depleted of $CD4^+$ T cells[228] cannot control *M. tuberculosis* replication. In addition, it has been shown that adoptive transfer of *M. tuberculosis*-specific $CD4^+$ T cells can protect previously naïve mice against infection challenge with *M. tuberculosis*[229]. In humans, $CD4^+$ T cells are a major source of cell-mediated IFN-γ production and have been shown to be important in granuloma formation and function[230]. In addition, TB disease in AIDS patients have demonstrated the importance of $CD4^+$ T cells in controlling dissemination of the bacilli. If TB disease develops in a HIV-infected person early on in the course of HIV infection, pulmonary TB disease is the most common outcome. However, with progression of immuno-suppression (i.e. decreasing $CD4^+$ cell count), TB disease often presents as

extrapulmonary – lymphatic, pleural, peritoneal or meningeal. In patients with very low $CD4^+$ cell counts, disseminated TB disease is common[231].

$CD4^+$ T cells produce cytokines in response to antigens presented by MHC class II molecules. They can acquire phenotypes that are associated with distinct profiles of cytokine expression after antigenic stimulation[232]. Th1 cells are induced by IL-12 and produce primarily IFN-γ, IL-2 and TNFα[233]. Th2 cells are induced by IL-4 and produce high amounts of this cytokine and others, including IL-5, IL-10 and IL-13, which are important for an antibody and allergic-mediated responses. Finally, Th17 cells are induced by TGF-B and IL-6, sustained by IL-23 and produce high concentrations of IL-17[234]. Although there is a large body of evidence supporting an important role of Th1-mediated cytokines, evidence of the contribution of Th2[235,236] and Th17 responses[237,238] to anti-TB immunity is conflicting and less established.

1.7.3.4. $CD8^+$ T Cells

$CD8^+$ T cells recognize peptide antigens retrieved in the cytosol and presented via MHC class I molecules. Even though *M. tuberculosis* primarily resides in vacuoles within phagocytes, recent research demonstrated that *M. tuberculosis* antigens are processed and presented via MHC class I molecules and are recognized by $CD8^+$ T cells[239]. In the mouse, $CD8^+$ T cells are required for optimum host defense following *M. tuberculosis* infection[239,240], however their effector mechanisms are not fully understood. In humans, whereas the impact of HIV infection on $CD4^+$ T cells have provided evidence of their important role, the direct impact of $CD8^+$ T cells during *M. tuberculosis* infection

has been more difficult to determine. Mycobacterial-specific $CD8^+$ T cells have been isolated from BCG-vaccinated subjects[221,241], individuals with latent and active TB disease[242,243 210] and have shown to contribute significantly to granulomas[244]. It is largely thought that $CD8^+$ T cells contribute to the control of *M. tuberculosis* infection by mediating specific effector functions including IFNγ production[161], lysis of infected host cells[210,243] and direct killing of mycobacteria[243].

1.8. GENETIC DISSECTION OF HUMAN DISEASE

Genetic dissection of human disease has shed new light on disease pathogenesis and has helped identify novel factors modulating resistance and susceptibility[245,246]. For infectious diseases in particular, genetic studies have contributed greatly to our understanding of immunity to infection[247-249] and have complemented and expanded the knowledge gained from developmental, cellular and molecular immunology[249,250]. In the following sections, the basic concepts underlying the genetic dissection of human disease will be briefly introduced, as well as an overview of our understanding of human genetic susceptibility to TB.

1.8.1. Genetic architecture of human disease

The architecture of human disease can currently be explained by a wide spectrum of genetic effects. Genetic factors underlying disease range from low to highly penetrant, and from very rare to common in frequency[245]. Rare, Mendelian diseases are characterized by intermediate to highly penetrant but very rare disease alleles. Complex

traits, which may be common in the population, can be influenced by a number of environmental and genetic factors, and potentially by interactions between them. The range of genetic factors underlying complex diseases is incompletely understood, however there is evidence that both common, modest-risk variants[251,252] and rare, moderate to high risk variants[253-255] contribute to disease[256,257].

1.8.1.1. Human genetic variation

With the completion of the human genome sequencing and the International HapMap Project, we have a better understanding of the characteristics and extent of DNA sequence variation in humans[258-260]. The increase in the number of informative genetic markers in the human genome has been instrumental in expanding genetic mapping efforts and expanding the range of genetic effect that can be detected. Genetic variation at the level of the sequence can be classified in different ways – by the physical nature of the sequence variation, by the effect on transcript levels and protein formation, and by the associated susceptibility to a disease. Historically, the two most important classes of variation include microsatellites and single nucleotide polymorphisms (SNPs). However, recent studies of structural variation has revealed that this class contributes significantly to genetic variation, accounting for at least 20% of all genetic variants in humans[261].

Microsatellites are characterized by sequence repeats (e.g. CACACA), are typically not found in coding regions, are highly variable (i.e. vary at the number of sequence repeats) with a high degree of heterozygosity at any given locus. Given that microsatellites are highly polymorphic and found throughout the genome, they have been used widely in linkage analysis studies.

SNPs represent variation at the level of a single nucleotide and are largely biallelic (e.g. substitution of C for A). They are abundant in the genome[260], are located in exons, introns, promoters, enhancers and intergenic regions[251], and are more stable than other types of polymorphism[262,263]. The alleles of SNPs located in the same genomic interval are often correlated with one another. This correlation structure, or linkage disequilibrium, varies in a complex and unpredictable manner across the genome and between different populations[264]. The International HapMap Project has identified groups of highly correlated SNPs that are inherited together (linkage disequilibrium bins), and SNPs that tag these bins (tag SNPs) across different continental population groups. The use of tag SNPs have facilitated association-based mapping studies by reducing the number of markers that need to be assayed in order to capture common genetic variation in a given region. Given the advantages of their use, SNPs are now preferentially used over microsatellites for most genetic studies[265].

Structural variants – such as large deletions, duplications and inversions ranging from tens to hundreds of kilobases in size – were previously thought to be pathogenic and associated with rare developmental disorders. Recently, a number of groups have showed that there is a high frequency of these copy number variants (CNVs) segregating in the general population[259,266,267]. It is not yet fully understood how CNVs contribute to disease susceptibility although recent studies have demonstrated an effect of CNVs on gene expression[268,269] and complex disease susceptibility[270-272].

1.8.2. Methods for the genetic dissection of complex traits

A common framework to identify the genetic control elements underlying complex diseases begins with determining the strength of evidence of a genetic effect by studying familial aggregation (e.g. familial relative risk or twin studies) followed by establishing whether the observed aggregation is consistent with a genetic effect (e.g. calculating heritability estimates)[273]. Segregation analysis in families, although not very common, can determine if there is evidence of a major gene effect and whether to proceed with genome-wide linkage scans[274]. Alternatively, genome-wide association scans have shown increased power over linkage studies to detect weak to moderate gene effects caused by relatively common polymorphisms[257]. Both gene-mapping methods allow the cloning of genes responsible for a phenotype without *a priori* knowledge of their location in the genome or their function. Fine mapping is often used to narrow down the genetic region of significant linkage, which includes testing by association analysis candidate genes within the linkage interval[275]. Finally, functional studies are necessary to determine if the associated genetic variant is causal and to determine its role in the molecular and physiological process related to the studied trait[276].

The concepts of linkage, association and functional validation will be reviewed next generally, whereas all concepts underlying the genetic dissection of complex diseases will be reviewed in the next section in the context of TB disease.

1.8.2.1. Linkage studies

Linkage is a powerful method to identify genetic variants with strong genetic effect and penetrance. It has been a very fruitful method to identify disease genes for

traits which exhibit classic Mendelian recessive or dominant inheritance attributable to a single gene locus[277,278] and to a lesser extent for complex traits that display familial clustering and prior evidence of major gene effects[279-284].

1.8.2.1.1. Basic concepts in linkage analysis

There are two approaches in linkage analysis – parametric and non-parametric. In parametric linkage analysis, *a priori* knowledge of the genetic model underlying the trait is necessary and parameters such as the mode of inheritance (e.g. dominant vs. recessive), penetrance and disease allele frequencies must be specified. The parameter estimates can be calculated from population based studies, including segregation analysis[274]. The power of parametric linkage analysis lies in the correct specification of the genetic model. For complex traits, which often present with no clear pattern of inheritance and incomplete penetrance, and are the result of both genetic and environmental factors, it is difficult or next to impossible to estimate the parameters with a high degree of certainty. Therefore, if the genetic model cannot be specified, the alternative is to perform non-parametric (or model-free) linkage analysis.

In non-parametric linkage analysis, no assumption is made regarding the genetic mode of disease transmission, and linkage analysis is based on the relationship between phenotypic similarities and the proportion of allele sharing identical-by-descent (IBD) for pairs of relatives - usually sibpairs. Two loci alleles which are copies of a common ancestral locus are said to be IBD. At any locus, according to the null hypothesis of no linkage, the probability of sibs sharing zero, one or two alleles IBD is 0.25, 0.5 and 0.25

respectively. Evidence of linkage is present if the affected sibs share significantly more alleles IBD than expected by chance.

Several model-free methods have been developed for the analysis of binary[285-288] and quantitative traits[289-292]. The method used for analysis in our studies is the maximum likelihood binomial (MLB)[288,293]. Unlike other methods, the MLB does not require the decomposition of sibships of size greater than two into their constitutive sibpairs[294]. This is advantageous because sibships of large size contain more linkage information than independent pairs[295,296] and it avoids the problem of non-interdependence of artificially extracted pairs. The test of linkage is a maximum-likelihood ratio test that compares the likelihoods under the null hypothesis of no linkage (i.e. parental allele transmission is independent of the offspring phenotype) and the hypothesis of linkage (i.e. siblings whose phenotypes are more similar receive the same parental allele more often). When compared to other methods, the MLB showed consistent results in terms of type I errors with various sample sizes and provided equivalent power to detect linkage[288].

The classic sib-pair linkage analysis for quantitative traits was developed by Haseman and Elston in 1972. Their method regresses the square difference in the siblings' traits values on the expected proportion of alleles shared IBD – two siblings that share more alleles IBD would be expected to have more similar trait values if the marker is linked to a locus influencing the trait. The Haseman and Elston method assumes a normally distributed trait which is likely to be false in a number of cases[297,298]. The MLB-Q, an extension of the MLB method, extends the capability of the original approach from binary to quantitative traits. It makes no assumptions about the distribution of the

quantitative phenotype under study which is important for a non-randomly selected sample set where the hypothesis of normality is violated by definition[289].

The MLB method has successfully been used to identify major susceptibility loci for leprosy[299], TB[283], the quantitative granulomatous Mitsuda reaction[300], and was also used for the linkage analysis carried out as part of this thesis.

1.8.2.1.2. Statistical evidence and interpretation

Evidence of genetic linkage is often reported as likelihood of odds (LOD) scores which are based on likelihood ratios. Classically, in parametric linkage analysis of single-gene disorders, a LOD score of 3 (observed data is 10^3-fold more likely to arise under a specified hypothesis of linkage than under the null hypothesis of independent assortment)[277] was considered a significant linkage result. The relatively low stringency of the threshold (genome-wide significance level of 9%) was sufficient given the limited number of genetic markers used in early linkage studies and low estimates of the prior probability of detecting linkage by chance[301-303]. However, with the use of an increasing number of genome-wide markers, a significant LOD score of 3 in complex trait mapping is more contentious[277]. Lander and Kruglyak, assuming a dense map of fully informative markers, derived the threshold required for a LOD score or corresponding *p*-value to achieve genome-wide significance of 5%[277]. For parametric linkage analysis, a LOD score of 3.3 or *P*-value 5 X 10^{-5} was determined to be significant. For affected sib-pair analysis, genome-wide significance is achieved at a pointwise *P*-value of 2.2 x 10^{-5} or a LOD score of 3.6[277], although the significance threshold can vary depending on the exact method used[304]. In order to harmonize reports of linkage, Lander and Kruglyak

recommended that researchers follow a standardized classification of linkage results: suggestive linkage (statistical evidence that would be expected to occur one time by chance in the genome) and significant linkage (expected to occur 0.05 times in a genome scan by chance)[277]. An alternative to Lander and Kruglyak recommended stringent thresholds is to determine empirical level of genome wide significance using simulations using features of a particular study (e.g. density of markers, family structure, patterns of missing data)[305].

1.8.2.2. Association studies

Association analysis, in contrast to linkage analysis, has high power to identify common variants exerting moderate to weak genetic effect[306,307]. It can be applied to study candidate genes identified from linkage hits in whole-genome linkage scans[308], from studies of animal models *in vivo*[309], from comparison with human inherited disorders with a related clinical phenotype[310], or from the known physiology of the studied trait[80]. Most recently, association analysis has been applied to whole-genome scans with success in identifying common, modest-risk variants for complex traits such as inflammatory bowel disease[246]. Finally, association analysis is a common method used in fine mapping in order to narrow down relatively large genomic regions originally identified by linkage analysis[264].

1.8.2.2.1. Basic concepts in association analysis

In association studies, the aim is to determine if an excess or deficit of marker alleles occurs among cases (or affected individuals) when compared with a control (or unaffected) group. Association can be tested using a population- or family-based design.

Population-based sample designs are based on the comparison of unrelated cases and controls. An allele is associated with the studied trait if it occurs at a significantly different frequency in the cases vs. the controls. Statistical analysis of population-based association includes a χ^2 test of association, or regression analysis (logistic for binary traits, linear for quantitative traits)[311]. In population-based design, careful selection of the population sample (i.e. matched cases and controls) is required in order to avoid spurious association due to population stratification. Population stratification refers to the differences in allele frequencies between cases and controls due to systematic differences in ancestry rather than association of genes with disease.

In family-based designs, the most common test – the transmission disequilibrium test (TDT) - measures whether an allele is significantly over- or under-transmitted from heterozygous parents to affected progeny[312,313]. Under independent assortment, we expect the allele to be transmitted with a probability of 0.5. If the allele is associated with the trait, a distortion of the expected random transmission is observed and the cause of the association is linkage between the marker and the phenotype. A great advantage of the TDT is that it is robust against population stratification[313,314]. The TDT can also be extended for use with quantitative traits and designs with multiple sibs.

1.8.2.2.2. Statistical evidence and interpretation

Positive, significant association between marker alleles and a trait can occur for three main reasons: 1) the marker allele has a causal role; 2) the marker allele has no causal role but is associated with the causative allele via linkage disequilibrium; and 3)

spurious association due to underlying population stratification or admixture[315,316]. A large number of association studies either fail to find an association or fail to validate studies that found a significant result[317,318]. The lack of reproducibility is often ascribed to small sample sizes with inadequate power, biological and phenotypic complexity and population-specific differences[254,306,318]. It is argued that positive association should be considered tentative until validation in at least one independent population and reserved for findings involving the same allele or haplotype, and the same phenotype, genetic model and direction of association as the original signal. In addition, studies should be adequately powered to detect significant association (i.e. large samples sizes) and use appropriately corrected *P*-values to determine significance[319,320].

1.8.2.3. Functional validation and translation

In gene mapping studies, it is important to realize that statistical significance does not imply causality. Once putative causal variants are identified, functional validation is necessary to determine if the variant is causal and to determine its role in the molecular and physiological processes related to the studied trait. Validation assays need to be developed according to the biological function of the implicated gene or region. The assays can entail, for example, expression analysis or measuring the effect of the variant on a specific mechanism (e.g. apoptosis, bacterial growth or receptor signalling). Upon validation, the knowledge acquired through the genetic research – either mechanistic insights or disease risk prediction – can hopefully be translated to advances in clinical care[257]. Functional validation for genetic studies in complex diseases has not been straightforward. Associated variants are often common and found in non-coding or intergenic regions thereby providing few clues of their impact[261]. As an example,

genome scans have robustly identified 11 susceptibility genes and loci for inflammatory bowel disease[246]. However, identification of the causal variants and the biological impact of these variants have only been identified for two genes – *NOD2*[321] and *IRGM*[269].

1.9. GENETIC SUSCEPTIBILITY TO TUBERCULOSIS

1.9.1. Population variability in susceptibility to tuberculosis

There is significant historical evidence demonstrating the importance of host genetic factors in susceptibility to TB. It is thought that the variable patterns of TB incidence reflect in part a population's history of exposure to *M. tuberculosis*. Infectious diseases, such as TB, that entail a high morbidity and mortality in early life are expected to select for genetic variants that confer resistance. Consequently, populations with a long history of exposure to *M. tuberculosis*, such as Europeans, compared with populations only recently exposed, such as North American Natives and sub-Saharan Africans, show greater resistance to TB[322]. Two historical events illustrate both population differences and inter-individual variability in TB susceptibility.

The accidental inoculation of infants with a mix of virulent *M. tuberculosis* instead of the live attenuated vaccine strain *M. bovis* BCG in Lübeck, Germany, in 1929 provided inadvertent verification that human individual variation exists in susceptibility to TB following uniform infectious exposure. Of the 251 immunologically-naive infants administered virulent *M. tuberculosis*, 77 died with proven TB within 1 year of infection. Of the 174 children who overcame the infection, 126 showed clear radiological signs of TB infection[323]. In comparison to the high survival rate of the German infants, North

American Natives were devastated by TB upon initial exposure and TB death rates among American Natives during the late 19th century were the highest ever recorded[322].

1.9.2. Familial aggregation and heritability

The study of familial aggregation has helped dissect the relative contribution of genes and environment to disease and quantitative traits. In two studies, concordance of TB disease was significantly higher among monozygotic twins than dizygotic twins[324,325], indicating a strong genetic component for host susceptibility to TB. Complex segregation analysis is a method to evaluate the transmission of a trait within a pedigree, the genetic effect and the magnitude of the genetic sources of variation in the trait[274]. Analysis of multicase TB families from Brazil found evidence of an oligogenic model of inheritance that includes major gene effects[326].

While the contribution of genetic factors to TB susceptibility has been extensively studied, the effect of genetics on anti-mycobacterial and anti-TB immune responses is less well known. Heritability analysis estimates the proportion of phenotypic variability in a population attributable to genetic variation among individuals. Such analysis has been performed for a number of immune responses related to recall responses to mycobacterial antigens. Studies of twins in a TB endemic region of West Africa observed a strong contribution of host genetic factors to PPD-triggered IFN-γ response (range 39% - 41%)[327-329]. In an East-African population, household contacts of smear-positive pulmonary TB cases showed high heritability (68%) of TNFα production in response to *M. tuberculosis* culture filtrate[330]. In addition, complex segregation analysis

reported evidence of a major gene controlling TNFα production in a population of TB disease patients and their contacts living in a TB endemic region[331].

Heritability for delayed-type hypersensitivity to PPD measured by TST has been estimated to be 28% and 71% in two studies of twins from Chile and The Gambia respectively[327,332]. Although the difference is striking, it should be noted that heritability is a population-specific parameter as both the extent of genetic and environmental variance vary across populations[273]. Therefore, the different heritability estimates should be interpreted in the context of the study design and the populations studied. The lower estimate was determined in a small sample of young (n = 35, age < 3 years) monozygotic and dizygotic twins living in a region of low TB incidence. Skin reactivity was measured in response to BCG vaccination at birth and not TB exposure. In contrast, the second study investigated TST reactivity in a context of high TB exposure and in a significantly larger sample of older twins (n = 255, age > 12 years)[327]. These results suggest that for TST reactivity and perhaps for anti-mycobacterial immunity generally, genetic variation has a greater impact in areas of high exposure. Finally, the heritability estimates calculated for TB-related quantitative phenotypes are in-line with those estimated for other infectious diseases, including whipworm (*Trichuris trichuria*) egg burden (28-36%)[333], total serum IgE (60%) and infection burden (31%) in response to *Shistosoma mansoni*[334].

Collectively, these studies demonstrate that infectious-disease related intermediate phenotypes are highly heritable and consistently show the contribution of a strong genetic component to TB and anti-mycobacterial immunity.

1.9.3. Spectrum of genetic effects

Abel and Casanova[52,249,335] describe the genetic control of TB as a continuous spectrum, with simple Mendelian disease at one end, complex polygenic disease predisposition at the other end, and intermediate major susceptibility genes providing the link. High-risk alleles are rare in a population and are thought to explain very little of the overall disease prevalence. However, observations that children with rare mutations leading to deficiency in IL-12Rβ1 and IFN-γR1 developed severe disseminated TB disease raise the possibility that a proportion of paediatric cases with severe TB disease have a Mendelian disposition to disease[52,63,336]. There is evidence for major-gene control of susceptibility in certain populations[92,283] or in well-defined epidemiologic settings where gene-environment interactions can be modelled[89,90]. It is unknown if major gene effects are rare occurrences limited to specific epidemiological situations or go undetected due to the inherent difficulties in capturing important gene-environment interactions in complex diseases such as TB. Numerous common genetic variants contributing moderately to TB susceptibility have been identified but their functional relevance and impact at the population level remain elusive[337-344].

1.9.3.1. Mendelian susceptibility to mycobacterial disease

The lack or partial deficiency of proteins in the IL-12/IFN-γ axis can lead to a rare syndrome called Mendelian susceptibility to mycobacterial disease . Individuals with the syndrome are highly susceptible to poorly virulent mycobacteria such as NTM and BCG. With the exception of salmonellosis occurring in fewer than half the syndrome cases, disease caused by other microorganisms is very rare[52]. Several patients with the syndrome have been diagnosed with clinical TB but it is unclear to what extent the mutations are important in *M. tuberculosis* infection or disease progression[52,310,345-348].

Five genes have been found to be mutated in patients with the syndrome. Mutations in the interferon gamma receptor 1 (*IFNGR1*) and interferon gamma receptor 2 (*IFNGR2*), which encode the two IFN-γ receptor chains, and signal transducer and activator of transcription 1 (*STAT1*), an essential signalling component, result in impaired cellular responses to IFNγ. Mutations in the interleukin 12 subunit p40 (*IL12B*) and interleukin 12 receptor beta-1 subunit (*IL12RB1*) result in impaired IFN-γ production. The mutations can be classified into three classes of alleles: recessive non-functional; recessive, partially-functional; and dominant-negative partially-functional[52]. The classes of alleles correspond to distinct clinical, immunological and histopathological outcomes[349].

Although the phenotype-genotype relationship for IFN-γ-mediated immunity against mycobacterial disease is relatively clear, the importance of genetic variation in the IL-12/IFN-γ cytokine pathway at the population level is less well understood[249]. The

extent of cases of rare Mendelian mutations responsible for cases of TB in areas of endemicity is unknown. However, a number of studies have found a modest association between TB disease and common genetic variation within the IL-12/IFN-γ pathway[350-353,337,351,352,354-361]. These results suggest a possible role for gene-gene interactions, although common individual gene variants exert only a modest effect, the summed impact across variants on the pathway may be strong[362]. Overall, additional studies are necessary to determine the contribution of rare to common genetic variation in the IL-12/IFN-γ cytokine pathway to TB disease susceptibility.

1.9.3.2. Tuberculosis candidate genes

A number of genes have repeatedly been associated with TB susceptibility, including the natural resistance-associated macrophage protein 1 (*NRAMP1)*, vitamin D receptor (*VDR)* and MHC class II loci. Evidence supporting their role as TB susceptibility genes will be reviewed.

1.9.3.2.1. *NRAMP1*

Nramp1 is expressed at the phagosomal membrane in professional phagocytes and functions as a pH-dependent divalent cation pump[363-365]. The murine *Nramp1* orthologue confers susceptibility to specific *Salmonella*, *Leishmania* and *Mycobacterium* species[366]. In humans, *NRAMP1* has been tested in numerous association studies. Initially, *NRAMP1* variants were found to be associated with TB susceptibility in a West African population. Individuals with TB were four times more likely to have a disease-associated *NRAMP1* genotype combination than healthy controls[367]. These observations

have been replicated in a number of studies of patients from China, Japan, Korea, Malawi, Guinea-Conakry, Cambodia, U.S.A., and East-Africa[368-372]. The independent replication of *NRAMP1* association with TB across different populations makes *NRAMP1* the most solidly established TB susceptibility gene. Nevertheless, several studies failed to detect an *NRAMP1* - TB association[373-375] providing evidence for genetic heterogeneity in TB susceptibility.

The modest genetic impact of *NRAMP1* alleles on susceptibility has been interpreted to suggest that the gene accounts for only a small proportion of the total genetic contribution to TB susceptibility[326,376]. However, an alternative explanation is provided by a genetic study of TB susceptibility in an Aboriginal Canadian community. In this study, it was possible to detect a very strong genetic effect (relative risk of 10) of the *NRAMP1* region on TB[89]. Of note, this strong genetic effect was only detected when exposure history was included into the analysis. The importance of gene-environment interactions was further exemplified in a study of paediatric TB where the association between *NRAMP1* variants and paediatric TB was stronger in simplex vs. multiplex families suggesting interplay between genetic control and exposure intensity[90]. Despite substantial genetic evidence implicating *NRAMP1* in TB susceptibility, only one study has shown a relationship between *NRAMP1* genetic variants and protein function[377]. However, how variation in protein function is associated with increased TB susceptibility remains to be established.

1.9.3.2.2. *VDR*

Before the development of anti-mycobacterial drugs, vitamin D was prescribed to TB patients in the form of cod-liver oil and sunlight exposure and was administered with some success to treat TB[378]. It has since been discovered that the biologically active form of vitamin D, 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), interacting with the vitamin D receptor (VDR), acts as an important immunomodulatory molecule[379]. *In vitro*, $1,25(OH)_2D_3$ activates antimicrobial pathways[79,380,381] and restricts the growth of *M. tuberculosis* in human macrophages[190,213,382]. In addition, results from epidemiologic studies point to a link between vitamin D deficiency and a higher risk of TB[383,384]. This is demonstrated by seasonal variation of TB incidence, lower vitamin D serum levels in untreated TB patients, and a higher incidence of TB in individuals with relatively low serum vitamin D levels[385].

Given that vitamin D exerts its effects via the VDR, and that the receptor is present on monocytes and on T and B lymphocytes[386], several studies have investigated the role of VDR genetic variants in TB susceptibility with conflicting results[368,371,387-389]. Studies that took into account vitamin D serum levels or treatment outcome were more successful in detecting a significant genetic effect. A small case-control study in a Peruvian population found no association with pulmonary TB however specific VDR polymorphisms were associated with the time to sputum culture and auramine stain conversion during treatment[390]. A case-control association study of Gujarati Asians living in West London, England, investigated the interaction between VDR genotype and serum vitamin D concentrations. The study failed to show a significant association between *VDR* genotype and increased risk of TB. However, a strong association was

observed between vitamin D deficiency and TB. In addition, the study was able to detect evidence for an interaction between genotype (either TaqI TT/Tt or FokI ff), deficient or undetectable vitamin D serum levels and susceptibility to TB[80].

Collectively, the epidemiological and genetic studies demonstrate that a gene-environment interaction – vitamin D levels and VDR variants – play an important role in host defence against *M. tuberculosis,* and that VDR variants on their own may not be important TB susceptibility factors[391].

1.9.3.2.3. MHC Class II

The highly polymorphic major histocompatibility complex (MHC) class II loci encode molecules responsible for antigen presentation to $CD4^+$ T cells. Reports of association between class II loci and TB susceptibility have been reported across populations. Associations have been found with HLA-DR2 or HLA-DQB1 alleles in Northern India[392], Indonesia[393], Mexico[394], South Africa[395] and Cambodia[396]. A recent study demonstrated the functional impact of the HLA-DQB1*503 allele shown to be previously associated with TB susceptibility[397]. They showed that the presence of an aspartic acid at codon 57 – HLA-DQB57-Asp - impacted on the protein's binding affinity for *M. tuberculosis* immunogenic protein ESAT-6 and on the subsequent activation of $CD4^+$ T cells. In addition, they showed a significant association between homozygosity for alleles encoding HLA-DQB57-Asp and susceptibility to TB disease[397]. These results suggest that the impact of HLA class II allele variation on anti-mycobacterial immunity is important to consider in the interpretation of immune-based diagnostics (e.g. ESAT-6-

specific activation of T cells) and to identify individuals at particular risk of progressing to TB disease.

1.9.3.3. Whole-genome linkage studies

Whole-genome scans have been used to identify susceptibility loci for several human infectious diseases, including schistosomiasis[280], visceral leishmaniasis[398], malaria [399], leprosy[279], and TB. The best evidence to date of a major risk locus for pulmonary TB diseases is from a genome scan in multiplex Moroccan families which identified a major locus on chromosome 8p (LOD = 3.9)[283]. Of note, the authors showed that linkage was strongest in the subset of families in which one parent was also affected by TB compared with families without affected parents, supporting a dominant mode of inheritance of the susceptibility locus[283]. A number of other studies have found evidence for suggestive linkage with TB disease and tentatively identified candidate genes via positional cloning. One study was performed in 92 sib-pairs with TB from Gambia and South Africa. Weak evidence for linkage was detected on chromosome regions 15q and Xq[400]. Expectations that novel loci had been identified were not borne out in follow-up association studies of both regions[401,402]. A genome scan for TB in a Brazilian population found three regions with suggestive evidence for linkage: 10q26, 11q12 and 20p12[403]. A linkage study of TB affected sibling-pairs from South Africa and Malawi identified two loci with suggestive linkage on chromosome regions 6p21-q23 and 20q13[404]. Association testing was done in an independent population from West Africa and positive association was detected with two genes on chromosome 20q13: the *melanocortin 3 receptor* (*MC3R*), a member of a family of proteins involved in obesity and weight control, and *cathepsin Z* (*CTSZ*), a member of the cathepsin protease family[404]. Linkage at 20q13, although not attaining

significance, was also detected in a scan of TB patients from Uganda[405]. Finally, a genome-scan in a Thai population of multiplex families detected evidence of suggestive linkage with TB on chromosome region 5q23.2-31.3. Interestingly, in an ordered subset analysis using minimum age at onset of TB as the covariate, they identified two other chromosomes with suggestive linkage on regions 17p13 and 20p13, implying different disease mechanisms in young versus older patients[406]. Across studies, only the chromosome region 20q13 has been replicated across populations, although the functional impact of the two candidate susceptibility genes in this region – *CTSZ* and *MC3R* – has not been identified.

Only one whole-genome linkage scan published to date has studied resistance to TB infection[405]. Resistance to infection was defined as a negative TST on 2 or more occasions over 2 years of observation of an individual living with a TB index case. Suggestive linkage was observed on chromosome regions 2q21-24 and 5p13-5q22[405]. HIV positive individuals were included in the group defined as resistant to infection which is problematic considering HIV infection is known to cause TST anergy[116,117]. When the HIV positive individuals were removed from the analysis, suggestive linkage remained only on chromosome region 2q21-24[405].

A number of linkage scans of quantitative traits related to immunity have been performed including IgE levels in asthma[407], variation in T and B cell subpopulations in healthy persons[408] and the Mitsuda reaction, a delayed granulomatous skin reaction in response to heat killed *M. leprae*[300]. All the studies identified significantly linked QTLs. To date, only one whole-genome linkage scan has studied a TB-related quantitative

phenotype – TNFα production in response to *M. tuberculosis* culture filtrate[405]. The analysis was done in a heterogeneous group of individuals including persons both HIV positive and negative, and with confirmed TB disease, latently infected or with no signs of TB infection (TST negative). No significant or suggestive linkage peaks were identified in this study. It is possible, however, that any genetic impact was masked by the significant variation in cytokine production introduced by differences in infection or disease status across subjects included in the study[405].

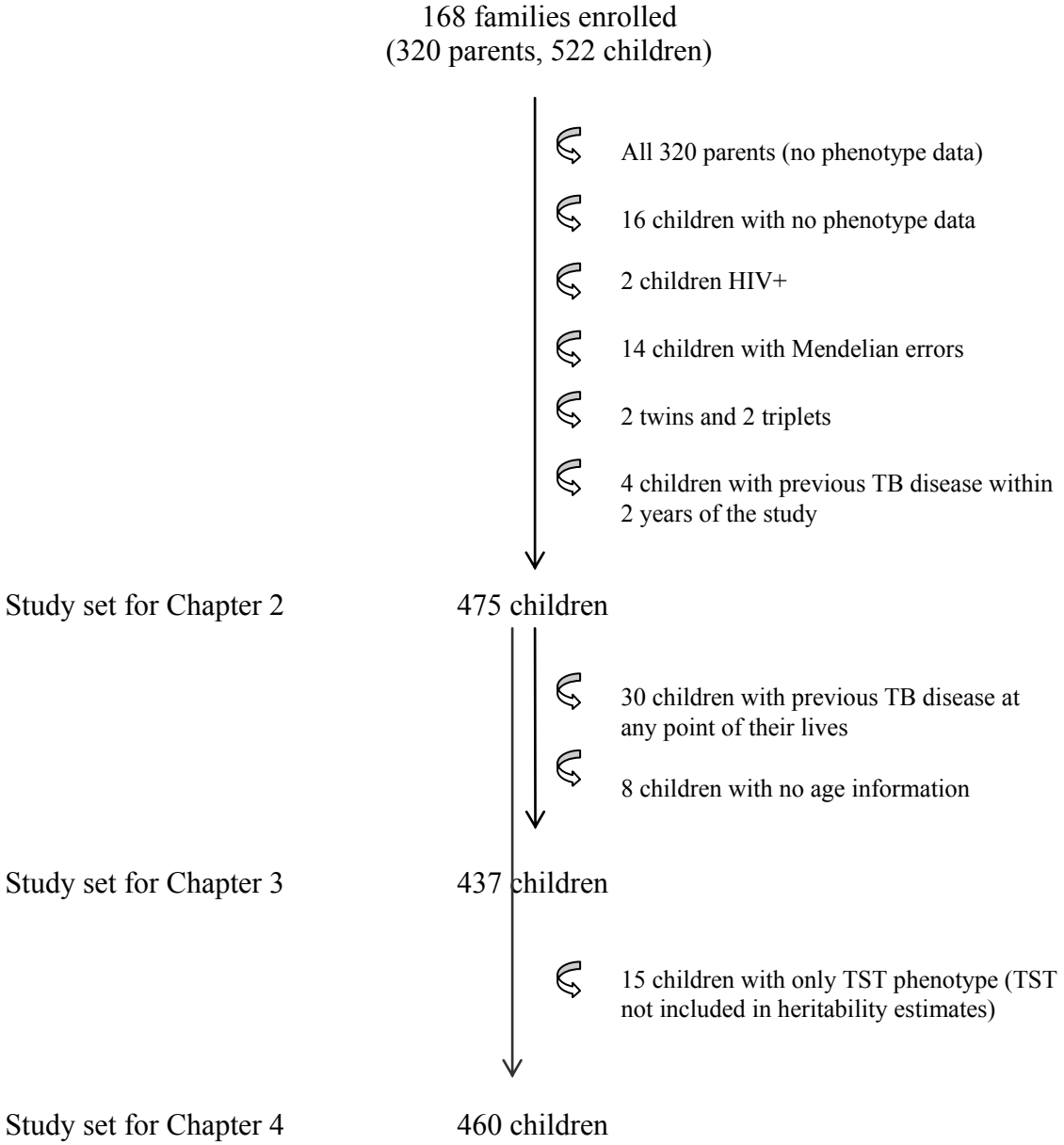
Overall, little is known about how genetic variation affects key anti-mycobacterial immune responses and susceptibility to TB infection. Greater understanding of the genetic impact on immunity is critical in order to inform TB diagnostics, vaccine development and help identify the immune pathways modulating TB susceptibility to infection and disease.

Chapter 2
Quantitative analysis reveals low redundancy between *in vitro* and *in vivo* measures of anti-mycobacterial immunity in children and adolescents

Chapter 2 describes and comparatively analyzes the immune-phenotypes measured in the children enrolled from a hyper-endemic TB area of Cape Town, South Africa. The results of this study helped inform our approach and analysis for the work presented in Chapters 3 to 5.

The following page provides information on which Cape Town individuals were included in which analysis described in Chapters 2 to 5.

Flow chart of children and parents enrolled and included in the studies described in Chapters 2,3 and 4.



The children included in the genome-wide linkage study described in Chapter 5 are a sub-set of children studied in Chapter 2 with available TST and genotype data plus the children's parents with genotype data (see Results section in Chapter 5 for more information). The children enrolled for the study described in Chapter 6 are from a different population (see Chapter 6 for more details).

Quantitative analysis reveals low redundancy between *in vitro* and *in vivo* measures of anti-mycobacterial immunity in children and adolescents

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Abstract

Background: While many studies have compared *in vitro* tuberculosis diagnostic tests with the venerable tuberculin skin test (TST), there is little understanding of the quantitative relationship between critical measures of anti-mycobacterial immunity used to detect tuberculosis infection. We therefore decided to determine the degree of redundancy between quantitative read-outs of *in vivo* and *in vitro* assays of anti-mycobacterial immunity.

Methods: We enrolled 475 healthy HIV-negative children and young adults living in a hyper-endemic area of tuberculosis. We measured *in vivo* TST responses, and used *in vitro* whole blood assays to determine the antigen-specific IFNγ cytokine release as well as the frequency of antigen-specific $IFN\gamma^+$ $CD4^+$ and $IFN\gamma^+$ $CD8^+$ cells.

Results: *In vivo* TST responses segregated into two well separated groups with either no measurable response (TST induration < 5mm; n = 164) or a normally distributed group with TST indurations ≥ 5 mm with peak at 15 mm (n = 260). *In vitro* assays provided a less pronounced separation of responders and non-responders. Correlation analysis of responses among persons with TST ≥ 5 mm demonstrated that extent of TST response was poorly correlated with IFNγ release (coefficients of correlation ρ = 0.17-0.22) and frequency of $IFN\gamma^+$ $CD4^+/CD8^+$ cells (ρ = 0.05-0.17) across three stimulating antigens (BCG, PPD, and ESAT-6).

Conclusions: *In vivo* and *in vitro* assays are non-redundant, complementary measures of anti-mycobacterial immunity.

Introduction

In 2008, the World Health Organization (WHO) reported that two billion people worldwide were infected with *Mycobacterium tuberculosis*, the bacterium causing tuberculosis (TB), with an estimated 14.4 million active TB cases[1]. Out of 22 high burden countries, South Africa has the highest TB incidence rate at 940/100,000, with children constituting up to 39% of this case load[1,2]. Research on TB in children is complicated by several factors, including difficulty in establishing a definitive diagnosis of infection and disease[3,4]. Several organizations have prioritized the standardization of definitions for immune response profiles in childhood TB in highly endemic populations[5,6]. Besides facilitating vaccine studies, such experiments are required to derive reliable immunodiagnostics that indicate both latent infection with *M. tuberculosi*s and the risk that such latent infection will advance to active tuberculosis.

For most of the last century, the tuberculin skin test (Mantoux; TST) has been employed to detect tuberculosis infection. The test measures induration of the skin (mm) 48-72 hrs following intradermal inoculation of *M. tuberculosis* purified protein derivative (PPD) and represents a delayed-type hypersensitivity (DTH) response to PPD. Although it has the disadvantage of reduced specificity, especially in the low mm reaction range[7], there is a large body of longitudinal data that can be used to aid in the interpretation of TST reactivity[8]. Due to the known disadvantages of the TST, alternative methods for immunodetection of *M. tuberculosis* infection have been developed that are based on *in vitro* T-cell measures of anti-mycobacterial immunity[9]. Since the results of such tests are used to inform clinical and public health decisions, it is common practice to report test results as either “positive” or “negative” employing cut-off points that aim to minimize

and balance the risk of false positive and false negative results. Hence, the concordance of positive and negative results from *in vivo* and *in vitro* assays is of great practical importance and has been the focus of numerous studies[9]. However, there is profound disagreement on what discordant results represent[10] and few studies center on the immune responses these tests measure.

While the use of cut-off points for assay results is justified from an operational perspective, it is important to remember that such cut-off points have little bearing on the understanding of the biology of the underlying immune responses. Indeed, a great deal of basic biological information is lost by dichotomizing what are naturally continuous responses. We decided to study the biology of the *in vitro* T-cell assays and the *in vivo* TST by analyzing comparatively the quantitative assay responsiveness of a young population living in a tuberculosis endemic area of the Western Cape, South Africa. For the *in vitro* assays, we stimulated whole blood separately with three antigens: *M. bovis* bacillus Calmette-Guérin (BCG) and *M. tuberculosis* PPD, two antigens that measure exposure to mycobacteria, and early-secreted antigenic target-6 (ESAT-6), a RD1-antigen primarily produced by *M. tuberculosis* and a minority of other mycobacterial species but not BCG vaccines[11]. Following exposure of whole blood to antigen, the production of IFNγ, was measured in assay supernatants. In addition, the frequency of $IFN\gamma^{+}CD4^{+}$ and $CD8^{+}$ cells in response to BCG and *M. tuberculosis* PPD was measured in whole blood. Increasing evidence supports a role for both cell types in protective anti-tuberculosis immunity[12,13]. We show that there is low redundancy between *in vitro* and *in vivo*

measurements, which strongly suggests that independent aspects of anti-mycobacterial immunity are being measured by these *in vitro* and *in vivo* tests.

Materials and Methods

Study site & subjects

Study subjects were enrolled from Ravensmead and Uitsig, two suburbs of Cape Town with a very high incidence of tuberculosis disease. There was no requirement for subjects to be household contacts of TB cases. However, this area has a notification rate of TB (all cases) of 761/100,000 and of new bacteriologically confirmed cases of 313/100,000[14]. BCG vaccination is routine in the study area. Informed consent was obtained for all study participants. The study was approved by the Institutional Review Boards of Stellenbosch University, Tygerberg, South Africa; the University of Cape Town, Cape Town, South Africa; and the Research Ethics Board at the Research Institute of the McGill University Health Centre, Montreal, Canada.

Enrolment strategy

The enrolment strategy was to target large households with priority being given to the largest households to allow later reconstruction of nuclear families. In addition, if TST reactivity was known at time of enrolment households that contained both strongly TST positive and TST negative subjects were prioritized for enrolment. The reason for this enrolment strategy is the suitability of the collected phenotype information for subsequent human genetics analyses. Subjects who had had clinical tuberculosis disease in the two years preceding the study were excluded. Individuals who were HIV positive,

pregnant or using immuno-modulatory chemotherapy were also excluded at the time of enrolment. BCG vaccination at birth is routine in the study area.

Blood collection

A maximum of 10 ml of blood was drawn in heparinized tubes for the immune assays. Particular effort was taken to ensure that the whole blood intracellular IFNγ assay samples were maintained at 37^{o}C in a portable incubator during transport and that all bloods arrived to be processed in the laboratory within 1 hour of being drawn from the subject.

Tuberculin skin test

Tuberculin skin tests were carried out using the Mantoux method with *M. tuberculosis* PPD batch RT23 (2 T.U., Statens Serum Institut, Copenhagen, Denmark). TST reactivity was read between 48-72 h after the skin test was performed using a set of calipers calibrated to the nearest 0.5 mm. The TST was usually performed immediately after drawing blood for the other immune assays.

Measurement of IFNγ production

Heparinised whole blood was diluted 1 in 10 with serum-free medium (RPMI, Gibco, Carlsbad, California, USA) and plated in 96-well round bottomed tissue culture plates (Nunc, Amersham, Uppsala, Sweden) at 100 µl/well. Cells were stimulated in quadruplicate with antigen, antigen plus cytokine, mitogen, or with serum-free medium, giving a final volume of 200 µl/well. Live BCG (Danish 1331 strain, Statens Serum

Institut) was added at a MOI of 100:1, either alone or together with IFNγ at a concentration of 0.25 μg/ml (Human IFNγ, Sigma-Aldrich, St-Louis, Missouri, USA). The antigens *M. tuberculosis* PPD (Statens Serum Institut) and early secreted antigen target -6 (ESAT-6; Statens Serum Institut) were used at a final concentration of 5 μg/ml and 20 μg/ml, respectively. The mitogen phytohemaglutinin (PHA; Sigma-Aldrich) was used as a positive control (final concentration of 5 μg/ml), and cultures incubated without stimulation in serum-free medium provided the negative control. Cell cultures were incubated at 37^{o}C with 5% CO_2. Supernatants were harvested after 1 day, 3 days or 7 days incubation and stored at -80^{o}C prior to ELISA.

IFNγ after 3 and 7 days was measured in quadruplicate samples diluted 1:2 by ELISA in DuoSet ELISA system plates (Human IFNγ, R&D Systems, Minneapolis, Minnesota, USA). Recombinant IFNγ provided by the DuoSet ELISA system was used as standard curves at a range of 15 - 2000 pg/ml and 31- 4000 pg/ml, respectively. A standard curve was included on each ELISA plate. The ELISA was developed using streptavidin-HRP and read at 450 nm using the Benchmark Microplate Reader (Biorad, Hercules, California, USA). Data collection and analysis were done via the Microplate Manager Software (Version 5.2, Biorad). A cubic spline curve fit was used for the standard curve. Data outside the standard curve were extrapolated. The limit of detection for IFNγ assays was 31 pg/ml, respectively.

Whole blood intracellular IFNγ assay

As previously described[15], heparinized whole blood was immediately incubated at 37^0C with *M. tuberculosis* PPD (50 μg per ml of blood; Statens Serum Institut) or BCG SSI Danish (1.2 x 10^6 organisms per ml of blood; Statens Serum Institut). Streptococcal enterotoxin B (SEB; 10 μg per blood; Sigma-Aldrich) was used as a positive control and medium alone (nil antigen) as a negative control. Co-stimulatory antibodies anti-CD28 (BD Biosciences, San Jose, California, USA) and anti-CD49d (BD Biosciences) were added to each tube at 1 μg/ml blood. After 7 hours of incubation, Brefeldin A (Sigma Aldrich) was added at 10 μg/ml to each sample to capture cytokines intracellularly. After a total of 12 h incubation, 2 mM EDTA was added, red blood cells lysed and white blood cells fixed with FACS Lysing Solution (BD Biosciences). Cells were subsequently cryopreserved at -70°C in 10% DMSO/20%FCS in RPMI.

Cryopreserved cells were thawed, centrifuged and the supernatant decanted. Cells were permeabilized (0.1% saponin in 0.1% BSA in PBS) for 10 mins at room temperature and incubated with fluorescent-conjugated antibodies for 30 min at RT. Cytokine profiles of BCG- and PPD- specific cells were examined using the following conjugated antibodies: anti-CD4 APC, anti-CD8 FITC and anti-IFNγ PE (all from BD Biosciences). Cells were fixed with 1% para-formaldehyde (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) in PBS. The frequency of $IFN\gamma^+$ $CD4^+$, $CD8^+$ and total small, non-granular lymphocytes was measured on a 4-colour FACSCalibur flow cytometer (BD Biosciences). Flow cytometric acquisition was completed on a 4-colour FACSCalibur (BD Biosciences). The gating strategy was as follows: first $CD4^+$, $CD8^+$ and total

lymphocytes were selected, then $CD4^+$ vs side scatter, $CD8^+$ vs side scatter and forward scatter vs side scatter, respectively, were selected. After autofluorescing cells were excluded, the frequency of $IFN\gamma^+$ cells as selected was measured against forward scatter for each population. Hence, we cannot exclude the presence of a small number of $CD8^+$ NK cells among the $CD8^+$ T cell population. For antigen stimulated samples, the frequency of $IFN\gamma^+$ cells in the corresponding antigen-free control was subtracted before analysis.

Statistical analysis

For soluble cytokine ELISAs, the cut-off for a positive IFNγ response was set at twice the limit of detection of the respective ELISA assay. For the proportion of antigen-specific $IFN\gamma^+$ cells, the cut-off for a positive IFNγ response was defined to be the 90% threshold of the non-stimulated distribution for $CD4^+$ cells and $CD8^+$ cells.

The cytokine concentrations are presented as the mean of quadruplicate samples minus the negative control in pg/ml. No sample showed a lack of (< 62 pg/ml) PHA response on day 1 and only 0.7% were PHA negative on day 3 and 7. There was no overlap of individuals with a negative PHA response on day 3 and day 7. For the negative control, only 1.9% and 3% had a positive (> 62 pg/ml) IFNγ response on day 3 and 7 respectively. Five individuals showed positive IFNγ production in the absence of antigen on both day 3 and 7. For $CD4^+$ cells and $CD8^+$ cells, the lower 5% threshold of the antigen-specific or positive control IFNγ distribution for each cell subset was determined. We decided to exclude an individual's $CD4^+$ cell and $CD8^+$ cell frequency

data if both their positive control and antigen-specific IFNγ responses were below the 5% threshold. This strategy resulted in the exclusion of data from two individuals.

Two quality control steps were taken to evaluate the ELISA data. First, any replicate outside the mean ± 2SD would be considered an outlier and excluded. Second, to distinguish if non-responses were due to experimental error, the lower 5% threshold of the positive control distribution and the lower 5% threshold of the antigen specific distributions for day 1, 3 and 7 were calculated for both cytokines. We decided to exclude an individual's ELISA data if their positive control on each of day 1, day 3 and day 7 was lower than the respective threshold of 5% of the positive control distribution, and all antigen specific responses were below their respective 5% thresholds. When these two steps were taken, no ELISA data was required to be excluded from the analysis.

In comparisons between TST and IFNγ production, only those persons were considered where TST was read within 2 weeks after cytokine assays. In order to account for familial dependencies between the individuals, correlations between quantitative immune phenotypes were estimated using BIvariate Estimating Equations (BIEE) as proposed by Tregouet et al.[16].

Results

Description of study population

A total of 475 healthy individuals belonging to 155 families with at least one immune phenotype assayed were included in the study. Results were available for TST

responses in 426 (89.7%) individuals, for whole blood IFNγ release assays in 464 (97.7%) individuals, and for whole blood intracellular IFNγ assays in 412 (86.7%) individuals. The proportion of males (50.3%) and females (49.7%) was approximately equal and mean age of the subjects was 13.9 years (SD=6.1 years). Thirty individuals (6.3%), 18 female and 12 male, with a median age of 13.8 years (SD = 5.8 years, had previous tuberculosis disease at least two years before their enrolment in the study.

TST skin reactivity

The distribution of TST skin responses is shown in Figure 1. The distribution is bimodal with a first peak centered on 0 mm and a second peak centered on 15 mm. The median induration was 11.8 mm (range, 0 – 43 mm). One hundred and sixty-two individuals (38%) had no measurable induration while two had a TST between 0-5 mm (Figure 1). Of the remaining 262 subjects, 260 (99.2%), 232 (88.5%) and 144 (55.0%) had indurations greater than 5 mm, 10 mm and 15 mm, respectively (Figure 1). This distribution of TST skin reactivity differed slightly in comparison to the distributions in other endemic regions, since we did not observe many responses in the >0 mm to 5 mm range[17,18]. Reactions in the latter range are usually attributed to sensitization by cross-reacting antigens. By contrast, in our study population there was a clear dichotomy between a sizeable minority of subjects (n = 38%) with no detectable skin test reactivity (TST induration size < 5mm) and a majority with varying degrees of skin test reactivity (TST induration size ≥ 5 mm; Figure 1). Skin test reactivity is usually taken as a proxy for cell-mediated immunity.

IFNγ release assays

In a second set of assays, whole diluted blood was stimulated with live BCG, *M. tuberculosis* PPD or ESAT-6 and the production of IFNγ was measured after 3 or 7 days incubation (Figure 2). The distribution of IFNγ production was largely bimodal with two groups of responders and non-responders. In addition, there was a group of intermediate responders (e.g. PPD, day 3: 63-500 pg/ml; PPD, day 7: 63-1000 pg/ml) that may belong to the tail ends of the distributions of non-responders or positive responders (Figure 2). On day 3, 31.0%, 30.8% and 63.4% of individuals (Figure 2A) and on day 7, 15.5%, 17.9% and 55.8% of individuals (Figure 2B) displayed no measurable IFNγ response (< 62 pg/ml) to BCG, PPD and ESAT-6, respectively. Overall, 20.5% of individuals showed absence of response to all 3 antigens on day 3. This proportion of mycobacterial antigen non-responders dropped to 11.1% of individuals on day 7. Among the 3 antigens, PPD was the strongest inducer of IFNγ secretion. The number of non-responders to BCG and PPD was approximately equal and substantially lower than the number of non-responders to ESAT-6.

There was a strong correlation between extent of antigen-specific IFNγ secretion on day 3 and day 7 for all 3 antigens (Figure 2C). However, a substantial number of individuals, i.e. 79 (17.0%) for BCG, 65 (14.0%) for PPD and 47 (10.1%) for ESAT-6, converted from undetectable responses (< 62 pg/ml) to positive responses (> 62 pg/ml) between day 3 and day 7. A number of studies have highlighted similar differences in mycobacterial-specific responses between short-term and long-term incubations and have suggested that the differences may reflect the response of different immune cell subsets

invoked by recent versus remote infections, i.e. effector vs. memory cells[19,20]. Since an incubation period of 7 days appeared to provide a better separation of responders and non-responders, in further analysis, only day 7 was considered.

Frequencies of antigen-specific IFNγ$^+$ CD4$^+$ and CD8$^+$ cells

To determine the contribution of CD4$^+$ and CD8$^+$ cells and total blood lymphocytes to antigen-specific IFNγ responses, we measured the absolute count and determined the frequency of BCG and PPD-specific IFNγ-producing lymphocytes in diluted whole blood after 12 h co-incubation with antigen. Since absolute counts were very strongly correlated with frequencies ($\rho > 0.78$; data not shown), we present results for frequencies only. The distribution of antigen-specific IFNγ$^+$ cells in blood samples is shown in Figure 3. The majority of blood assays detected a positive response in the induction of BCG- (85.2%) and PPD-specific (73.4%) CD4$^+$IFNγ$^+$ T cells (Figure 3A). In comparison, fewer blood samples revealed positive induction of BCG-specific (64.3%) or PPD-specific (27.6%) CD8$^+$ IFNγ$^+$ cells (Figure 3B). For both CD4$^+$ and CD8$^+$ IFNγ$^+$ cells, BCG was a better inducer than PPD, which was unexpected given the higher induction of IFNγ secretion by PPD relative to BCG (Figure 2A, B). When we summed the frequency of CD4$^+$ and CD8$^+$IFNγ$^+$ cells and compared this proportion to the total frequency of lymphocytes positive for IFNγ, there was no significant difference to the overall distribution of IFNγ positive cells, suggesting that CD4$^+$ and CD8$^+$ cells were the major contributors to IFNγ production in the ELISA-based assays (Figure 3C).

Comparison of TST reactivity with cytokine release

Across all measured induration sizes, TST responses were moderately correlated with IFNγ production triggered by BCG ($\rho = 0.49$), PPD ($\rho = 0.62$) and ESAT-6 ($\rho = 0.39$; Figure 4). It is evident from Figure 4 that persons who tested TST "negative" (< 5 mm) contributed disproportionally to the overall correlations. However, individuals who were never sensitized by prior mycobacterial antigen exposure and who can be considered as immunologically naive constitute an unknown proportion of those TST negatives and inflate assay correlation. If correlations are done by restricting the analysis to TST > 5mm, the correlation between TST and IFNγ release is vastly diminished (BCG: $\rho = 0.17$; PPD: $\rho = 0.22$; ESAT-6: $\rho = 0.11$; Figure 4). The latter observations strongly suggest that different aspects of immune reactivity are being probed by *in vivo* TST responses and *in vitro* IFNγ release assays.

While the focus of our analysis was on the quantitative comparison of responses, we also computed the proportions of responders (IFNγ > 62 pg/ml) and non-responders (IFNγ < 62 pg/ml) according to TST status. We found that of the 100 persons who had a TST < 5mm, 74% (74/100), 65% (65/100) and 7% (7/100) had positive (> 62 pg/ml) IFNγ production in response to BCG, PPD, and ESAT-6, respectively. An excess of positive interferon responses among TST negative persons was also observed by previous studies[21,22]. Inversely, using TST >5mm as cut-off for positivity, we observed a very high number of IFNγ >62 pg/ml responders: BCG = 92.7% (114/123), PPD = 95.1%, (117/123), and ESAT-6 = 69.1% (85/123). When we used TST induration > 10 mm as cut-off for a positive response – the standard for South Africa – again the majority of

persons classified as TST positive were IFNγ positive responders across all three antigens: BCG, 92.1% (105/114); PPD, 94.7% (108/114); ESAT-6, 70.2% (80/114). Together with the TST distribution shown in Figure 1, these data may be used to argue that the cut-off for TST positivity in Cape Town should be moved to 5 mm from the current 10 mm. More importantly, these results show that both *in vitro* and *in vivo* tests are valid assays for complementary pathways of anti-mycobacterial immunity.

Comparison of TST induration size with frequency of antigen-specific IFNγ^+ cells

For all subjects, we analyzed the correlation between TST responses and the frequency of antigen-specific $CD4^+$ $IFN\gamma^+$ T cells (BCG, $\rho = 0.43$; PPD, $\rho = 0.47$), and that of $CD8^+$ $IFN\gamma^+$ cells (BCG, $\rho = 0.13$; PPD, $\rho = 0.29$). However, as for the cytokine release assays, TST negative persons inflated the overall assay correlations (Figure 5). When the analysis was focused on persons with TST >5mm, the correlations between TST and the frequency of antigen-specific $CD4^+$ $IFN\gamma^+$ cells (BCG, $\rho = 0.05$; PPD, $\rho = 0.17$) and antigen-specific $CD8^+$ $IFN\gamma^+$ cells (BCG, $\rho = -0.17$; PPD, $\rho = -0.09$) were very weak (Figure 5).

Among persons with a negative TST response (< 5 mm), for BCG antigen 65% (59/91) and for PPD antigen 28% (25/90) were classified as $CD4^+$ $IFN\gamma^+$ positive responders. Likewise, of the TST < 5 mm subjects, 47% (43/91) and 7% (6/90) were classified as $CD8^+$ $IFN\gamma^+$ positive responders for BCG and PPD, respectively. The consistently higher proportion of BCG positive responders in both ELISA and FACS assays may suggest an effect of previous BCG vaccination in *in vitro* assays that is not

seen in the TST *in vivo* assay (Figure 5). Alternatively, it may reflect an immunostimulatory effect of BCG *in vitro*. Interestingly, the relatively higher ratio of CD8 cells responding to BCG than PPD (Figure 3A, 3B) implies that while more cells responded, many of these were not strong producers of IFNγ[23].

Discussion

With increasing use of *in vitro* tests for TB diagnosis in clinical practice, the question of how results should be interpreted is increasingly important. The debate is particularly acute with regard to how to handle discordant results between *in vitro* assays and the TST. While TST has decades of studies that can guide interpretation of the result, it is not clear how much – if at all – these same conclusions can be applied to *in vitro* assays. The aim of the present study was therefore to perform a quantitative comparison of key *in vitro* assays of anti-mycobacterial immunity with *in vivo* TST response in healthy children and young adults. Hence, the focus of our study was on a better understanding of the biology underlying these assays rather than on refining and extending the practically important use of cut-off point defined "positive" and "negative" responders. We observed that *in vitro* antigen-triggered IFNγ release in immuno-sensitized persons correlated very poorly with TST responses. Since the *in vitro* T-cell responses showed low redundancy with *in vivo* TST reactivity, we concluded that *in vitro* and *in vivo* assays measured different pathways of anti-mycobacterial immunity. More specifically, since T-cell assays are geared towards IFNγ-mediated immunity, it is likely that this aspect of the acquired anti-mycobacterial immune response is only part of the

TST response. This conclusion is consistent with our current understanding of the Mantoux reaction and anti-mycobacterial immunity. The TST is a typical DTH reaction that involves immune cell influx and local T- cell proliferation in the skin[24]. There is probably an important role of the local tissue in the development of the response that cannot be easily captured by whole blood assays[24]. Neither TST reactivity nor IFN-γ production alone have proven effective correlates of protective immunity in tuberculosis, although IFNγ is an important effector molecule of the anti-mycobacterial host response[25,26]. Support for our results is also provided by recent studies in HIV-infected persons where the extent of TST is independent of the number of circulating $CD4^+$ cells[27]. Finally, our conclusions are consistent with the observation that children defective in their IL-12/IFNγ circuit displayed unimpaired TST reactivity[28] but showed increased susceptibility to mycobacterial diseases, including tuberculosis[29].

In our study, we employed PPD antigen in both in-house *in vitro* assays and TST, thus avoiding variable antigen specificity as a potential confounder for assay comparisons. Overall, we observed a medium strength correlation between *in vitro* assays and TST ($\rho \sim 0.5$). However, on excluding the group of persons with TST <5 mm, the correlation between TST and IGRA, whatever the antigen, was strikingly poor ($\rho \sim 0.2$). This observation highlighted the strong impact on assay correlations of (presumably immunologically naive) persons that were non-responders in TST assays. While immunologically naive persons are important from a public health perspective, for our purpose of comparing the extent of reactivity, they add little information. In agreement with our findings, a study of TST positive subjects failed to observe a significant

correlation between TST induration and extent of IFNγ responses[30]. In an earlier study, Converse et al., (1997) observed a Spearman coefficient of $\rho = 0.45$ for TST induration and IFNγ responses and a similar Spearman correlation coefficient ($\rho = 0.6$) was also reported from a New Zealand study[31]. However, both studies included double negatives in the calculation of correlation coefficients and it seems plausible that correlation of responses for TST positives only would be significantly lower A large study of healthy non-vaccinated persons in Malawi reported a significant increase of median IFNγ responses for 5 selected sizes of TST in a 6 day blood incubation assay[32]. How this observation relates to our own results is difficult to know. In our own data, we see a clear effect of TST size on IFNγ release when data are grouped in three TST sizes (< 5 mm; 5-15 mm, > 15 mm; $P < 0.0001$). This effect disappears when the < 5 mm group is omitted from analysis ($P > 0.1$). Hence, it is possible that inclusion of TST < 5 mm in the Malawi study underlies the significance of the observation.

In summary, we show that *in vitro* IFNγ release assays and TST are non-redundant measures of anti-mycobacterial immunity in young persons from a highly TB endemic area. Consequently, the induction of IFNγ immunity and TST may depend differently on host characteristics, including sex and age, exposure setting to *M. tuberculosis*, and the nature and frequency of other non-tuberculous mycobacteria. Together, these factors may explain why assay concordance can vary substantially from study to study. Our findings strongly suggest that *in vitro* and *in vivo* assays of anti-mycobacterial immunity are complementary rather than competing measures of anti-mycobacterial immunity.

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Figure legends

Figure 1: TST skin response distribution from children and young adults living in a highly tuberculosis endemic community

The distribution of TST responses among all individuals tested (n = 426). The size of the TST response, measured in millimetres (mm), is plotted against the number of individuals with a given response.

Figure 2: IFNγ production in response to BCG, PPD and ESAT-6

The concentration of IFNγ in assay supernatants in response to BCG, PPD and ESAT-6 was measured by ELISA after **(A)** 3 days and **(B)** 7 days co-incubation with antigen and grouped into bins where each subsequent bin doubles in concentration range. Positive responders were defined as having an IFNγ concentration > 62 pg/ml, double the minimum detectable limit of the assay. **(C)** BIEE correlations were calculated between the extent of IFNγ production after 3 and 7 days co-incubation with antigen.

Figure 3: Distribution of the proportion (%) of antigen-specific IFNγ-producing blood lymphocytes

The frequency of BCG- and PPD-specific $CD4^+$ **(A)** and $CD8^+$ **(B)** cells and total lymphocytes **(C)** producing IFNγ was measured after 12 h co-incubation with antigen. In panel **(C)**, the sum frequency of $CD4^+$ $IFN\gamma^+$ and $CD8^+$ $IFN\gamma^+$ cells is displayed beside the frequency of total lymphocytes for comparison. The negative (NIL) and positive (SEB) control distribution for each cell subset is also shown. The red horizontal bar corresponds to the median. The cut-off for a positive response was set at the 90% threshold of the no

antigen (NIL) control distribution and is shown by a solid orange horizontal bar in each graph.

Figure 4: Correlation of BCG, PPD and ESAT-6 specific IFNγ-production with extent of TST response. Extent of TST induration is plotted against the quantitative IFNγ release as detected in assay supernatants. PPD, BCG and ESAT-6 were used to stimulate whole blood cultures for 7 days. Across all antigens there is an excess of individuals with zero reactivity for both measures, represented by the grey circles. The size of the circle is relative to the number of double negatives (BCG n = 15; PPD n = 26; ESAT-6 n = 81). Orange circles identify persons with TST < 5mm and IFNγ >0. Blue circles identify individuals with TST > 5mm that were used for calculation of correlation coefficients.

Figure 5: Correlation of the proportion of antigen-specific $IFN\gamma^+CD4^+$ and $IFN\gamma^+CD8^+$ with extent of TST response. Extent of TST induration plotted against the frequency of $IFN\gamma^+CD4^+$ and $IFN\gamma^+CD8^+$ cells. Whole blood assays were stimulated with BCG or PPD as antigen. The grey circles indicate counts of persons with no detectable reactivity for both measures (BCG CD4 n = 7; BCG CD8 n = 11; PPD CD4 n = 7; PPD CD8 n = 33). Orange circles indicate persons with TST <5mm and $IFN\gamma^+$ cell frequencies > 0. Blue circles identify individuals with TST > 5mm that were used for calculation of correlation coefficients.

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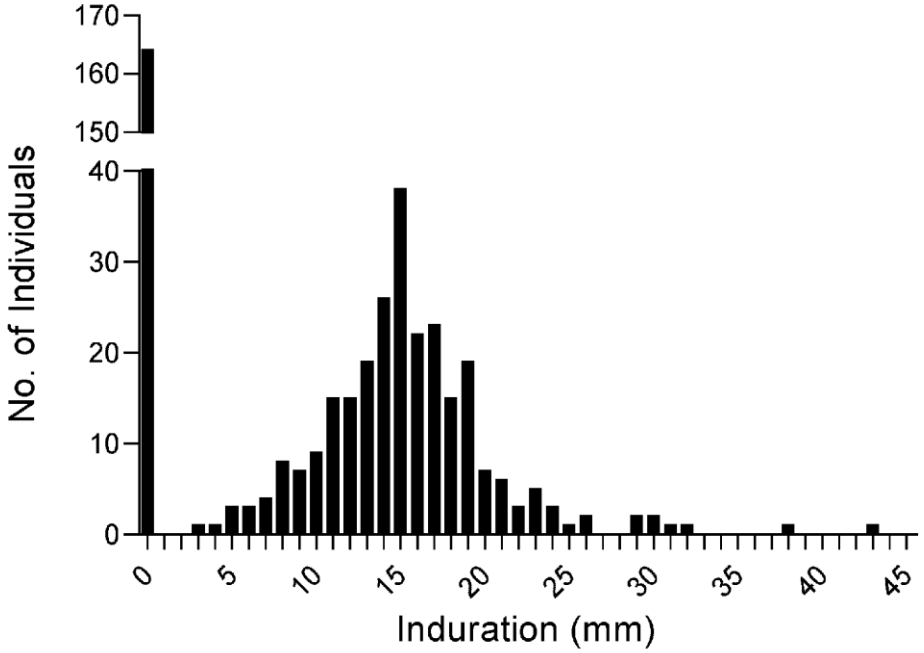
No. of Individuals
170
160
150
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
Induration (mm)

Figure 1

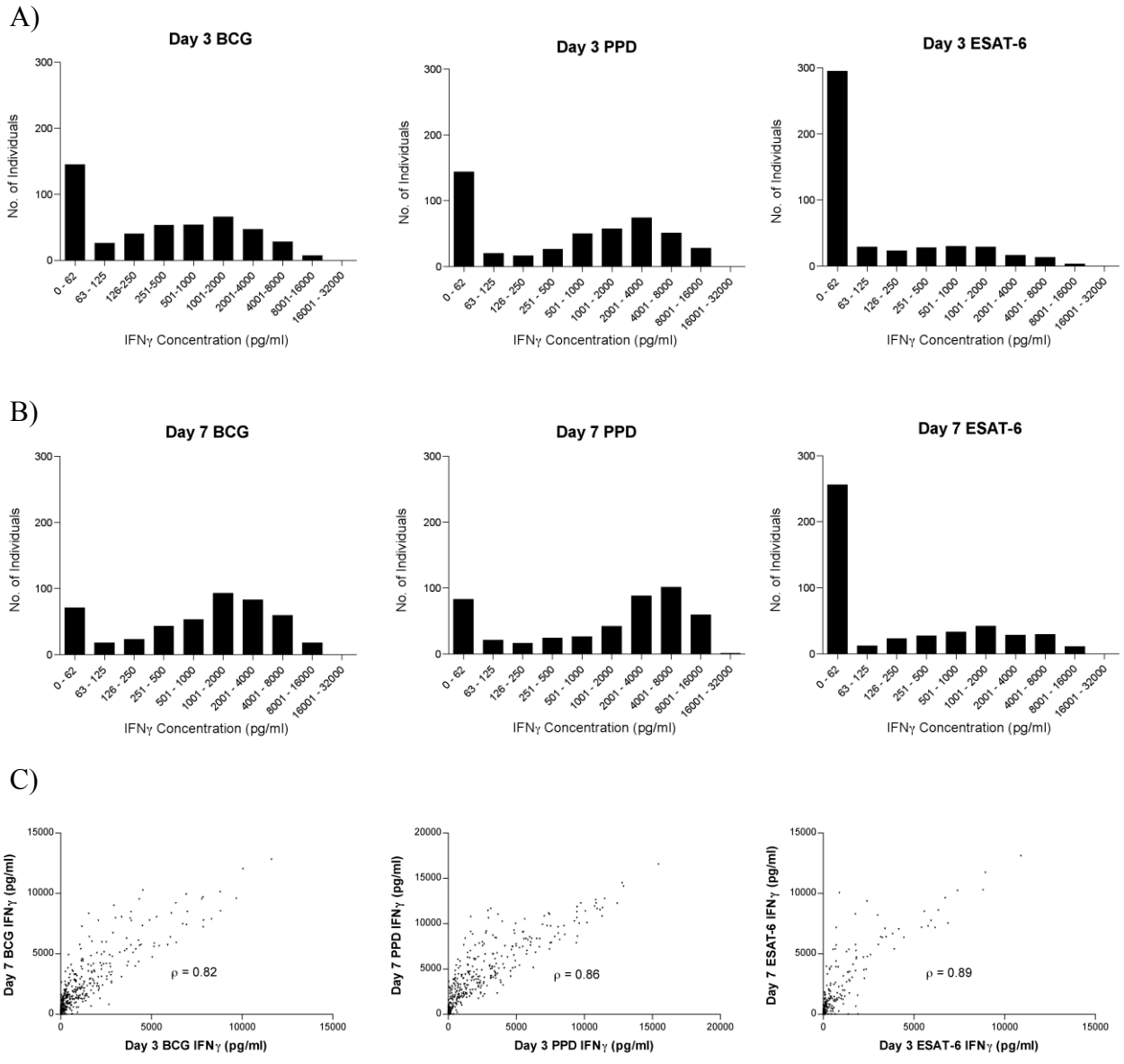
A)
Day 3 BCG
Day 3 PPD
Day 3 ESAT-6
No. of Individuals
IFNγ Concentration (pg/ml)
0 - 62
63 - 125
126 - 250
251 - 500
501 - 1000
1001 - 2000
2001 - 4000
4001 - 8000
8001 - 16000
16001 - 32000
B)
Day 7 BCG
Day 7 PPD
Day 7 ESAT-6
C)
Day 7 BCG IFNγ (pg/ml)
Day 3 BCG IFNγ (pg/ml)
ρ = 0.82
Day 7 PPD IFNγ (pg/ml)
Day 3 PPD IFNγ (pg/ml)
ρ = 0.86
Day 7 ESAT-6 IFNγ (pg/ml)
Day 3 ESAT-6 IFNγ (pg/ml)
ρ = 0.89

Figure 2

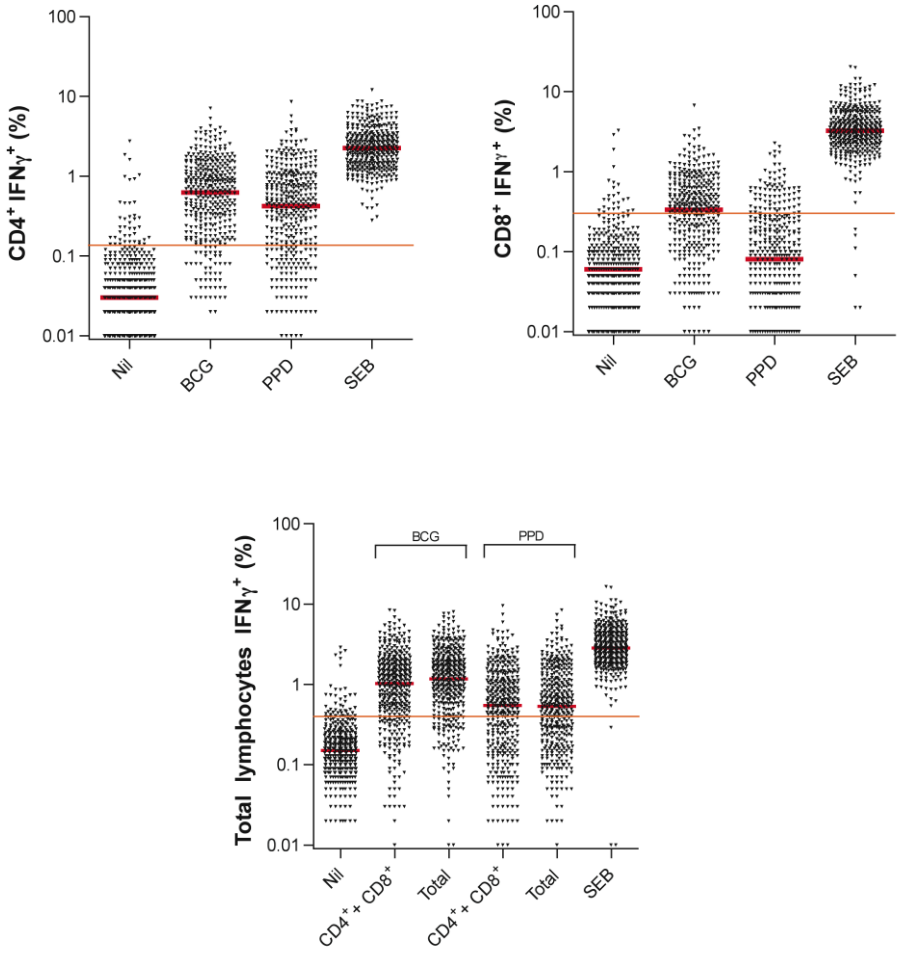
CD4+ IFNγ+ (%)
100
10
1
0.1
0.01
Nil
BCG
PPD
SEB
CD8+ IFNγ+ (%)
100
10
1
0.1
0.01
Nil
BCG
PPD
SEB
Total lymphocytes IFNγ+ (%)
100
10
1
0.1
0.01
BCG
PPD
Nil
CD4+ + CD8+
Total
CD4+ + CD8+
Total
SEB

Figure 3

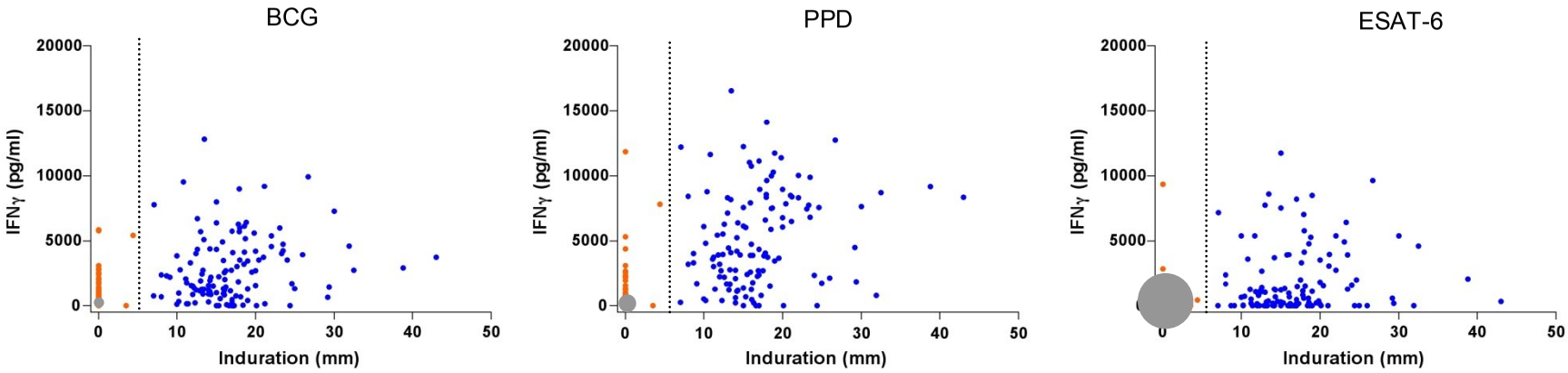
BCG
PPD
ESAT-6
IFNγ (pg/ml)
Induration (mm)
20000
15000
10000
5000
0
0
10
20
30
40
50

Figure 4

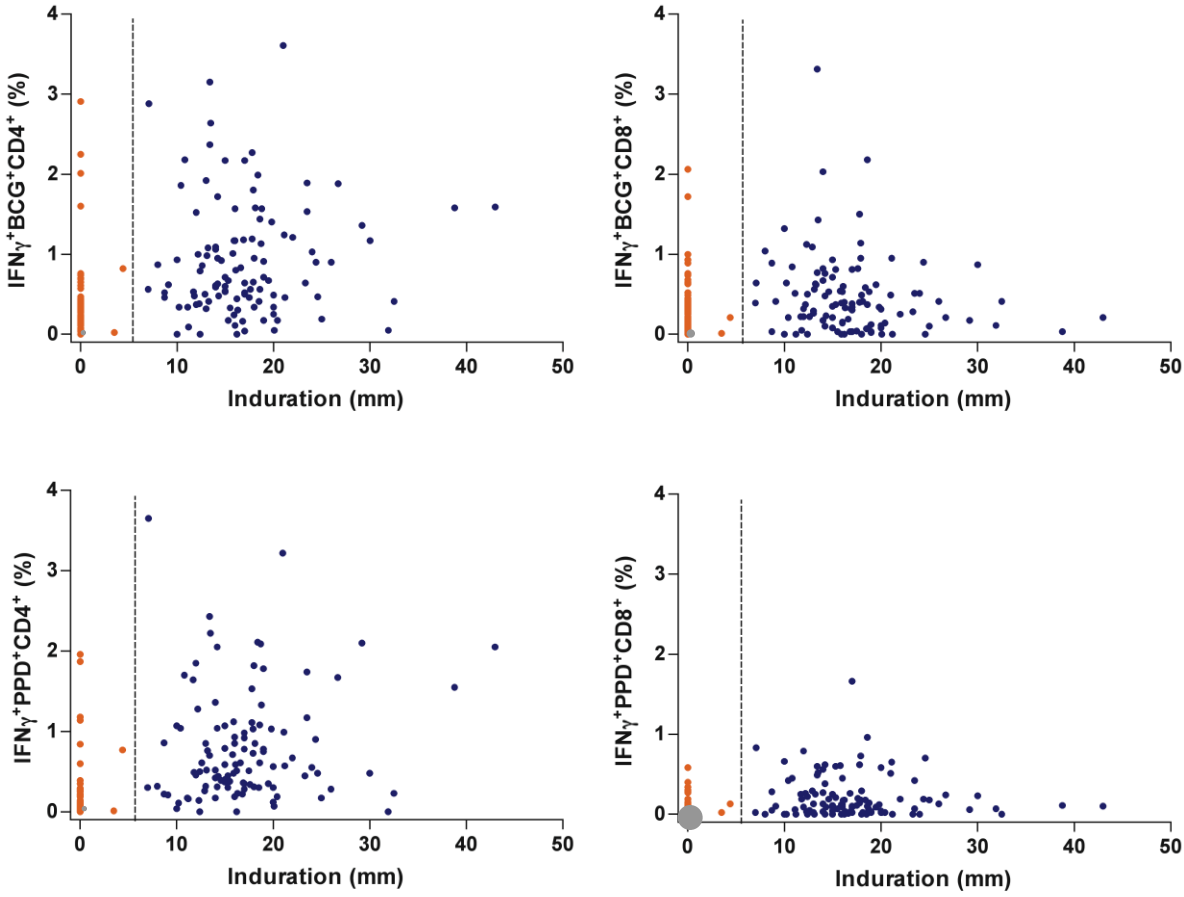
IFNγ+BCG+CD4+ (%)
IFNγ+BCG+CD8+ (%)
IFNγ+PPD+CD4+ (%)
IFNγ+PPD+CD8+ (%)
Induration (mm)
0
1
2
3
4
0
10
20
30
40
50

Figure 5

Chapter 3
The impact of age and sex on anti-mycobacterial immunity of children and adolescents in an area of high tuberculosis incidence

Chapter 3 presents the study where we evaluated the impact of age and sex on the immune-phenotypes presented in Chapter 2. The age-related results informed the analysis for the TST linkage scan presented in Chapter 5.

The impact of age and sex on anti-mycobacterial immunity of children and adolescents in an area of high tuberculosis incidence

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EGH and ES share senior authorship

Abstract

Background: The concordance between the tuberculin skin test (TST) and IFNγ T cell assays is understudied among children and adolescents. Likewise, the extent of immune reactivity measured by assays is usually not analysed.

Objectives: To determine the impact of age and sex on assay positivity and on extent of reactivity for both TST and T-cell assays in young persons in an area of high TB transmission.

Results: Age had a strong impact on assay positivity for all seven tested immune phenotypes ($P < 0.0001$). Among positive responders, the extent of PPD-triggered IFNγ release and frequency of PPD-specific $IFN\gamma^{+}CD4^{+}$ cells were sensitive to age ($P < 0.01$). By contrast, the extent of TST induration, ESAT-6-triggered IFNγ release and frequency of PPD-specific $IFN\gamma^{+}CD8^{+}$ cells were independent of age ($P > 0.05$). Sex had no significant impact on any phenotype measured ($P > 0.05$). The high proportion of positive responders in the]0-10] age group observed with long-term whole blood assays, but not with three day assays and TST, suggests that long-term whole blood assays are confounded by BCG vaccination.

Conclusion: There is a significant impact of age, but not sex, on different assays of immune reactivity in this high TB transmission setting.

Keywords: TST, IFNγ Release Assays, Tuberculosis, Covariates

Introduction

The most widely used assay for detection of latent infection with *Mycobacterium tuberculosis* is the tuberculin skin test (TST)[1]. The most commonly-used form of TST, the Mantoux test, measures the size of induration 48 to 72 hours post intradermal injection of standardized purified protein derivative (PPD) obtained from cultures of *M. tuberculosis*. The TST can be confounded by cross-reactivity with non-*M. tuberculosis* antigens (false positives)[2,3] and is also prone to an increased rate of false negative results among immmune-compromised persons[4,5]. To overcome the problem of TST limited specificity, alternative assays have been developed that employ antigens (e.g. ESAT-6) with increased specificity for *M. tuberculosis*[6]. These newer assays are *in vitro* tests that either determine the frequency of lymphocytes secreting IFNγ or measure the amount of IFNγ secreted following co-incubation of whole blood with *M. tuberculosis* antigens.

An increasing number of studies have sought to establish the concordance among these tests by using empirically and operationally derived cut-off points for assay "positivity" in different populations[6,7]. The combined results from these studies indicate unexplained variance in agreement between assay positivity. Although very few studies have systematically compared the impact of age and sex, especially among children and adolescents, on concordance rates of test assays, initial results suggest that age influences assay results[8-11]. Another aspect that warrants closer study is the immune basis for the discordance between the TST *in vivo* assay and the *in vitro* based IFNγ assays. Similarly, while cut-off points are important tools for clinical practice and public health,

enforced qualitative end points can miss valuable biological and physiological information about the nature and relationship of different measures of immune reactivity.

In this study, we investigated the comparative biology of the *in vitro* and *in vivo* assays of anti-mycobacterial immunity by comparing the quantitative extent of immune reactivity, employing the same antigens across assays rather than focusing exclusively on qualitative assay positivity/negativity (see Chapter 2). The study population comprised children and adolescents from an area of high tuberculosis transmission in South Africa. We describe the impact of age and sex on assay positivity and extent of reactivity in this age group. We observed an important impact of age on both positivity and extent of reactivity. By contrast, sex did not impact on any of the measured phenotypes.

Materials and Methods

Study design and subjects

Healthy study subjects were recruited from Ravensmead and Uitsig, two suburbs of Cape Town, South Africa, with a very high incidence of tuberculosis disease. The notification rate of tuberculosis (all cases) in the area is 761/100,000 and of new bacteriologically confirmed cases is 313/100,000[12]. BCG vaccination at birth has been mandatory in the study area since 1975. The enrolment strategy was to target large households with healthy children over one year of age and young adults. Priority was given to the largest households to allow later reconstruction of nuclear families. This family-based enrolment strategy allows a subsequent genetic study of the established immune-reactivity phenotypes. Individuals who were HIV positive, pregnant or using

immuno-modulatory drugs were excluded at the time of enrolment, and subjects who had had clinical tuberculosis disease were also excluded from this study. Informed consent was obtained for all study participants. The study was approved by the Institutional Review Board of Stellenbosch University, Tygerberg, South Africa; Cape Town University, Cape Town, South Africa; and the Research Ethics Committee at the Research Institute of the McGill University Health Centre, Montreal, Canada.

Immune phenotypes

TSTs were carried out using the Mantoux method with *M. tuberculosis* PPD batch RT23 (2 T.U.). TST reactivity was measured 48-72 h after the skin test was performed. Before the TST was performed, blood was drawn for a series of *in vitro* whole blood assays. Production of IFNγ in response to live BCG, *M. tuberculosis* purified protein derivative (PPD), or *M. tuberculosis* early secreted antigen target-6 (ESAT-6) was measured after 3 and 7 days of incubation with the antigens. IFNγ production in assay supernatants was measured using ELISA. The frequency of BCG- or PPD-specific $CD4^+$ $IFN\gamma^+$ cells, and of $CD8^+$ $IFN\gamma^+$ cells was measured using flow cytometry. Details of the assays are described elsewhere (see Chapter 2).

Statistical Analysis

The impact of sex (male/female) and age (coded in 4 classes:]0-10],]10-15],]15-20] and >20 years) was evaluated on the positive quantitative responses only and on the proportion of positive responders. Positive TST reactivity was defined as ≥ 5 mm, positive IFNγ production as twice the detectable limit of the ELISA assay (> 62 pg/ml), and the frequency of $IFN\gamma^+$ cells as the 90% threshold of the negative control distribution

($CD4^+$ cells > 0.13%; $CD8^+$ cells > 0.2%). To account for familial dependencies between the individuals, univariate and multivariate regression analyses were performed using generalized estimating equation (GEE), as implemented in the GENMOD procedure of the SAS software version 9.1 (SAS Institute, Cary, NC). An identity or logit link function was used for the analysis of quantitative and qualitative phenotypes, respectively.

Results

Description of assays and study population

In the present analysis, we examined seven immune-phenotypes (Table I) that represent two types of immune function: the *in vivo* TST, and *in vitro* T-cell assays. To investigate T-cell based immunity, we employed two types of assays: (i) IFNγ release assays where the amount of IFNγ is determined by ELISA in the supernatant of whole blood cultures and (ii) intracellular IFNγ staining assays where the proportion of IFNγ positive $CD4^+$ and $CD8^+$ cells in whole blood cultures is determined by FACS. In all tests PPD was used as the stimulating antigen, but for whole blood ELISA tests we also used ESAT-6. The time interval between antigen stimulation and subsequent phenotype measurement varied from one day for whole blood FACS assays to seven days for long-term ELISA IFNγ assays (Table I). The objective of our analysis was to determine the impact of age and sex on the above phenotypes both as a qualitative measure of test positivity (Figure 1) and as a quantitative measure of immune responsiveness (Figure 2). Immune-phenotypes were determined for a total of 437 healthy individuals belonging to 155 families. The mean age of subjects was 13.9 years (SD 6.1). The proportions of

males (51.3%) and females (48.7%) were similar (Table II). Not all individuals were phenotyped for all tests; the number of available outcomes is listed in Table I.

Prevalence of positive assay responders

Negative and positive responders have been defined in the Materials and Methods section. The non-age stratified distributions of immune reactivities, including positive and negative responders, have been described previously (see Chapter 2). In Figure 1A, the proportions of positive responders across all age classes are shown for the TST and the whole blood ELISA assays. Increased prevalence of positivity with increasing age is highly significant ($P < 0.0001$) whatever the age coding scheme (i.e. four classes or quantitative) and the phenotype under study. The proportion of persons with day 3 IFNγ release >62 pg/ml in response to PPD stimulation was nearly identical to the proportion of persons with TST > 5 mm across all age groups (Figure 1A). The proportions of positive responders for PPD-triggered IFNγ release at day 7 were consistently higher than for TST and day 3 responses. This effect was particularly significant for the]0-10] years age class where the proportion of day 7 positives was nearly double that of TST and day 3 positives (Figure 1A). This effect is unlikely to be a sampling error since the same effect was also seen when BCG was used as antigen (data not shown). When ESAT-6 was used as antigen, the proportions of positive responders were approximately half of those observed for TST and for day 3 PPD, except for subjects older than 20 years, where the proportions of positive responders were approximately one quarter of those observed for TST and for day 3 PPD (Figure 1A). The proportions of subjects testing positive for PPD-specific $IFN\gamma^+$ $CD4^+$ cells were nearly identical to the proportion of TST positive

subjects across all age groups (Figure 1A). By contrast, the age distribution of subjects testing positive for the presence of IFNγ^+ CD8$^+$ cells was similar to that observed for ESAT-6-triggered IFNγ secretion (Figure 1A).

There was no significant impact of sex on assay positivity, either in univariate or multivariate analysis. The non-age stratified proportions of positive responders for each assay are shown in Figure 1B. We also investigated whether there was an impact of sex in the younger (< 15 years) or older (> 15 years) age groups and again found no effect (data not shown).

Extent of immune reactions

For each phenotype, we established the quantitative distributions of positive responders and then evaluated the impact of age and gender on extent of reactivity. In Figure 2, the extent of immune reactivity across 4 age classes for all immune assays is shown. The age-dependent pattern of immune reactivity segregated into two groups, those that did show a significant overall impact of age and those that did not (Figure 2). Specifically, there was no significant age-effect (P = 0.09) on extent of TST reactivity (Figure 2A) or the frequency of PPD-specific IFNγ^+ CD8$^+$ cells (P = 0.82; Figure 2B). Similarly, there was no significant (P = 0.10; day 3) or borderline significant effect of age (P = 0.03; day 7) on extent of ESAT-6-triggered IFNγ secretion (Figure 2C). In contrast to TST and ESAT-6-triggered IFNγ release, there was a pronounced increase of PPD-triggered IFNγ secretion with increasing age (Figure 2D) and this increase was highly significant at both day 3 and day 7 (P < 0.0001). Similarly, we observed significantly

higher proportions of PPD-specific $CD4^+$ $IFN\gamma^+$ cells with increasing age (P = 0.002; Figure 2B). We also studied the effect of sex on extent of immune reactivity. As observed for the proportion of positive responders, sex had no significant impact on immune reactivity, independent of the immune phenotype analysed, in either univariate or multivariate analyses. The male and female specific mean reactivities are shown next to the age-class means for each assay (Figure 2).

Discussion

Persons latently infected with *M. tuberculosis* are central to the spread of *M. tuberculosis* in exposed populations. Consequently, the reliable identification and subsequent treatment of latent tuberculosis are important tools of tuberculosis control. The standard assay for detection of latently-infected persons is the TST[4]. Depending on the epidemiologic setting, TST may show poor specificity in the low reaction range[2,13,14]. For this reason, newer and more specific assays based on anti-mycobacterial T–cell immunity have been developed[6]. Both TST and the newer assays have been compared in numerous studies for both specificity and sensitivity in the detection of latently infected persons employing operationally defined cut-off points of assay positivity. However, little effort has been dedicated to studying the population biology of the corresponding immune responses in a comparative fashion.

In the present analysis we observed that the proportion of positives in all phenotypes analysed was highly dependent on age ($P < 0.001$). This strong age effect

that presumably reflects age-dependent cumulative exposure is seen in many studies of TST and thus far only a few IFNγ release assays[15-21]. Here we also showed a strong age effect for frequency of PPD-specific $IFN\gamma^+$ $CD4^+$ and $IFN\gamma^+$ $CD8^+$ cells. In addition, the proportions of positive responders were very similar across four age groups for TST, three day IFNγ release assays and 24 hrs $CD4^+$ $IFN\gamma^+$ staining assays which employed the same PPD antigen. The nearly identical proportions of positive responders across three independent measures (TST, IFNγ release, IFNγ cytokine staining) is likely a reflection of the young age of the study group where all positive responders must have been recently infected[22]. In IFNγ release assays employing ESAT-6 as antigen, the proportion of positive responders was substantially lower. This effect may reflect the known reduced IFNγ release assay sensitivity when using ESAT-6 as sole antigen[23,24].

Of interest was the comparison of day 3 and day 7 PPD-stimulated IFNγ release assays. In general, day 7 assays measure the effect of memory cells and detect a higher number of positive responders[25,26]. We observed a striking increase in the proportion of positive responders from 42% to 70% in the day 7 PPD assay in the]0-10] years age group that was not seen in the day 7 ESAT-6]0-10] years group. It seems likely that this increase is caused by prior BCG vaccination immunity, which will be missed by ESAT-6 since this antigen is missing from BCG[27,28]. That long-term PPD co-culture assays are confounded in children by BCG vaccination is supported by the observation that when BCG was employed as antigen, 76% of children in the]0-10] years group tested positive in a 7 day assay as compared to 50% in a 3 day assay (data not shown). If the excess of day 7 positive responders is due to BCG vaccination, this would support the suggestion,

that if BCG is given at birth, as is done in South Africa since 1975, BCG immunity wanes in children older than 10 years[3,7] . Finally, the above interpretation would also imply that BCG vaccination in the Western Cape does not confound TST testing although the number of children less than 2 years old was small in our study. A similar conclusion was also reached by Mahomed et al (2005)[29] in a cross-sectional study of healthy adults in South Africa, as well as in Taiwan[20] and India[17,30]. If this conclusion is generally correct, it would support the idea that loss of TST specificity is mainly due to non-BCG cross reactivities, most likely with environmental mycobacteria[31].

When we compared extent of immune reactivity across age groups we noticed distinct patterns of reactivity. The greatest impact of age was on PPD-triggered IFNγ release and on the proportion of $CD4^+$ $IFN\gamma^+$ cells; age strongly correlated with extent of reactivity. By contrast, TST- and ESAT-6-triggered IFNγ release displayed a pattern where only certain age groups showed an increase in reactivity: the older than 20 years age group for TST and the]10-15] years group for ESAT-6. The differences in age-dependency indicate that different biological processes underlie these assays. In our study we have chosen very stringent cut-off points that are defined by the technical limits for detecting a response and this approach provided consistent results across assays. Our interpretation is that each assay is a good indicator of a particular aspect of anti-mycobacterial immunity. Consequently, concordance among assays will be best when cross reactivities can be excluded and cut-off points can be moved to the limits of technical detectability. At least for TST, cross-reactivities are known to impact predominantly on the low end of the TST distribution[2,13,14,18]. As cut-off points are

shifted upward to minimize the risk of false positive results, age will have different impact for different assays and discordant results will be observed[6,32-34].

An intriguing aspect of our analysis was the absence of a significant impact of sex on any of the studied phenotypes. From this we conclude that susceptibility to infection is independent of sex in the age groups studied. In similar studies in Malawi, a sex effect on the proportions of positive responders in interferon gamma release assays and TST has been observed[15,16]. Sex-specific differences for both assays were found to be age-dependent and became most evident in the older than 20 years age group. In our study, only 51 subjects fell into this age class and we might have lacked power to detect a small impact of sex on positivity. Similarly, a higher proportion of male positives responders in QuantiFERON assays was observed for an adult South African population[29]. Whether such differences reflect biological sex differences or gender-specific differences in high risk exposure behaviour is difficult to know[16,35]. By contrast, the interpretation of our data seems more straightforward. Considering the absence of a sex effect on both binary (positive/negative) and quantitative responsiveness of three independent measures of anti-mycobacterial immunity, we conclude that in the high incidence tuberculosis setting of the Western Cape sex is not a risk factor for infection with *M. tuberculosis* among young persons.

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Legends to Figures

Figure 1: Impact of age and sex on the proportions of positive responders in seven assays of anti-mycobacterial immunity. A) The proportion of positive responders is plotted across four age classes for TST —●—, D3PPD ···□··· (green), D3ESAT-6 ··●··, D7PPD ···▲··· (green), D7ESAT-6 –■–, PPDCD4 —✱— (red), PPDCD8 —○—. For sake of clarity, error bars are omitted. B) Overall proportions of positive responders among males (M) and females (F) for seven immune assays (+/- standard error of the proportion). TST: tuberculin skin test; D3PPD: IFNγ release after 3 days of co-incubation with PPD antigen, D3ESAT-6: IFNγ release after 3 days of co-incubation with ESAT-6 antigen, D7PPD: IFNγ release after 7 days of co-incubation with PPD antigen, D7ESAT-6: IFNγ release after 7 days of co-incubation with ESAT-6 antigen, PPDCD4: $CD4^+$ cells showing intracellular IFNγ stain after 1 day of co-incubation with PPD antigen, PPDCD8: $CD8^+$ cells showing intracellular IFNγ stain after 1 day of co-incubation with PPD antigen.

Figure 2: Quantitative immune reactivity among positive responders for seven anti-mycobacterial immune assays across age and sex. The mean values +/- standard error of the mean are given for all data points. A) TST induration size across 4 four age classes and sex. B) Percentage of IFNγ positive staining $CD4^+$ cells ···■··· and $CD8^+$ cells —◆— across age and sex following co-incubation with PPD antigen. C) Release of IFNγ in pg/ml following co-incubation with ESAT-6 antigen for 3 days —◆— or 7 days ···■···. D) Release of IFNγ in pg/ml following co-incubation with PPD antigen for 3 days —◆— or 7 days ···■···. Error bars indicate standard error of the mean. Abbreviations as in Figure 1.

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Table I: Summary of phenotypes studied

Test	Type	Antigen	Measurement	Time	Number of outcomes
Tuberculin skin test	*in vivo*	PPD	skin induration	2 days	401
Whole blood ELISA	*in vitro*	PPD	release of IFNγ into supernatant	3 days	435
Whole blood ELISA	*in vitro*	ESAT-6	release of IFNγ into supernatant	3 days	435
Whole blood ELISA	*in vitro*	PPD	release of IFNγ into supernatant	7 days	430
Whole blood ELISA	*in vitro*	ESAT-6	release of IFNγ into supernatant	7 days	430
Whole blood FACS	*in vitro*	PPD	proportion of $IFN\gamma^{+}CD4^{+}$ cells	1 day	385
Whole blood FACS	*in vitro*	PPD	proportion of $IFN\gamma^{+}CD8^{+}$ cells	1 day	385

Table II: Participant characteristics (N = 437)

Characteristic	Number of participants (%)
*Age in years**	
]0-10]	124 (28.4)
]11-15]	149 (34.1)
]16-20]	110 (25.2)
]20-41]	51 (11.7)
Sex	
Male	224 (51.3)
Female	213 (48.7)

* Age unknown for 3 individuals (0.6%)

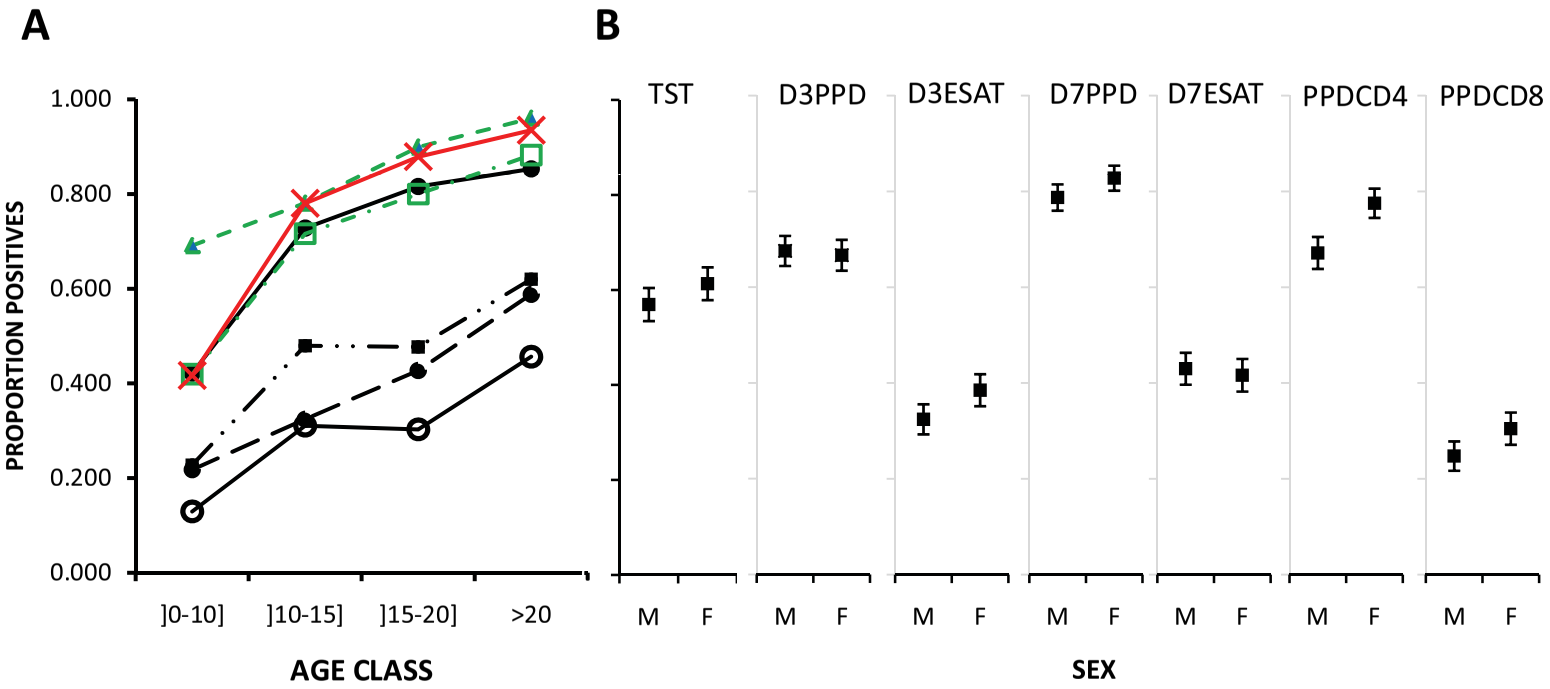
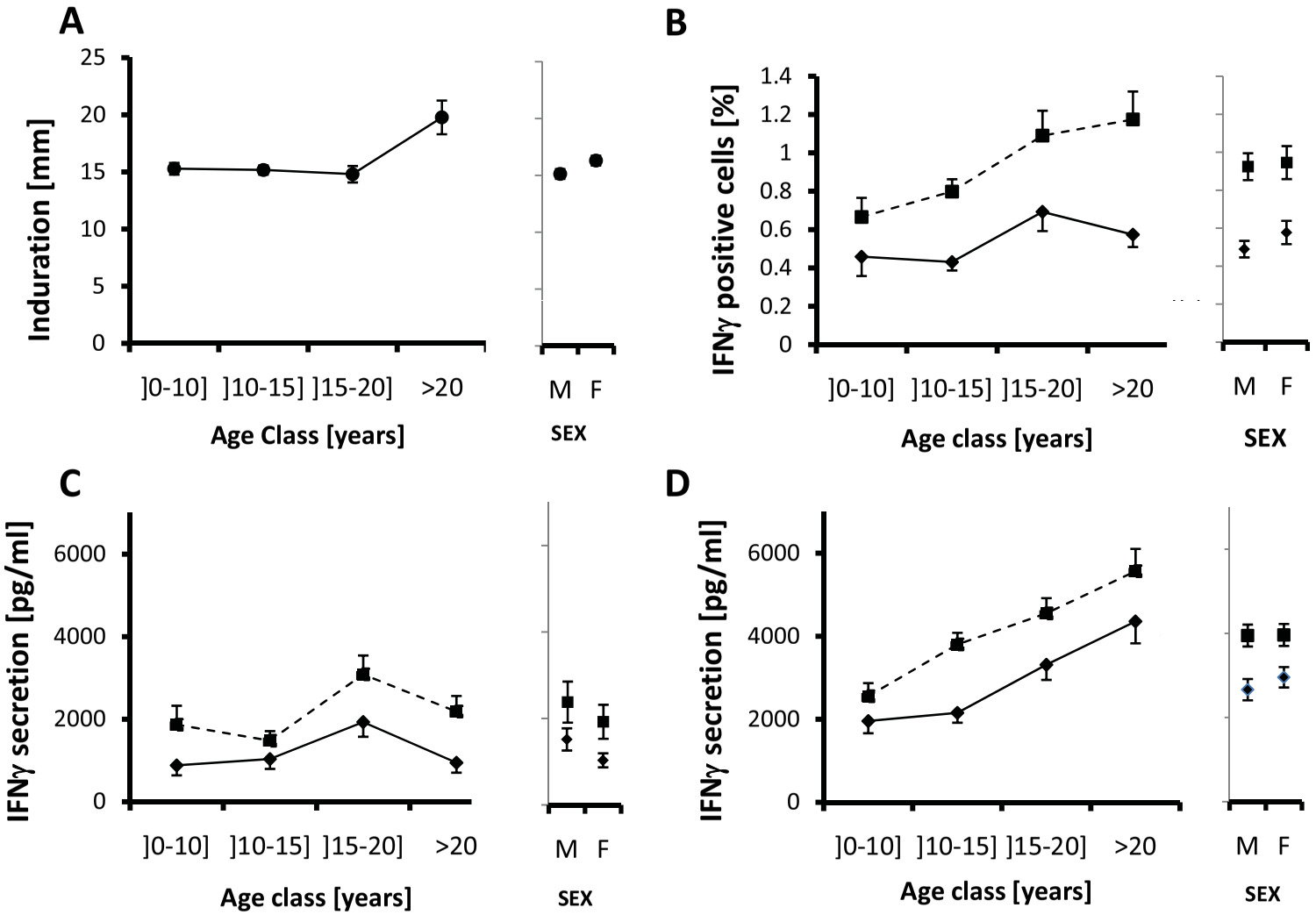

A
B
1.000
0.800
0.600
0.400
0.200
0.000
PROPORTION POSITIVES
]0-10]
]10-15]
]15-20]
>20
AGE CLASS
TST
D3PPD
D3ESAT
D7PPD
D7ESAT
PPDCD4
PPDCD8
M
F
SEX

Figure 1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Induration [mm]
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]0-10]
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>20
Age Class [years]
M
F
SEX
B
IFNγ positive cells [%]
1.4
1.2
1
0.8
0.6
0.4
0.2
0
]0-10]
]10-15]
]15-20]
>20
Age class [years]
M
F
SEX
C
IFNγ secretion [pg/ml]
6000
4000
2000
0
]0-10]
]10-15]
]15-20]
>20
Age class [years]
M
F
SEX
D
IFNγ secretion [pg/ml]
6000
4000
2000
0
]0-10]
]10-15]
]15-20]
>20
Age class [years]
M
F
SEX

Figure 2

Chapter 4
High heritability of anti-mycobacterial immunity in a hyper-endemic area for tuberculosis disease

As a prelude to linkage studies, Chapter 4 presents the heritability estimates of the quantitative immune-phenotypes presented in Chapter 2. Of note, the heritability for TST reactivity was not calculated as siblings were preferentially enrolled based on discordant TST results. Also, in contrast to Chapter 2,3 and 5, the immunephenotypes from the Cape Town population were log transformed before heritability analysis as the log phenotypes were more normally distributed.

A more detailed discussion of the concept of heritability, its interpretation and examples of estimates for other quantitative traits can be found in the Introduction (p. 42-43).

High heritability of anti-mycobacterial immunity in a hyper-endemic area for tuberculosis disease

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Abstract

Human anti-mycobacterial immunity is a critical component of tuberculosis (TB) pathogenesis that is often used to infer presence of TB infection. We report high heritability (30 - 74%) for *in vitro* secretion of TNFα and IFNγ and the frequency of antigen-specific $IFN\gamma^{+}CD4^{+}$ and $IFN\gamma^{+}CD8^{+}$ cells in whole blood in response to mycobacterial challenge. In principal component analysis, the first three components explain 75% of the overall variance consistent with the effect of pleiotropic regulatory genes of human anti-mycobacterial immunity. These results directly demonstrate the pivotal role of host genetics in quantitative measures of anti-mycobacterial immunity and suggest that immune-diagnosis of TB infection may be confounded by host genetics.

Introduction

Specific immune assays are often used to estimate the risk of an individual being infected by *Mycobacterium tuberculosis*[1]. The underlying assumption is that in immuno-competent individuals, reactivity in these assays is largely independent of intrinsic host factors and that therefore universal thresholds can be used to detect infection by *M. tuberculosis*. To test this assumption, we estimated the impact of host genetic factors on read-outs of immune assays by means of heritability analysis. In a sample of 475 healthy individuals belonging to 155 families from a tuberculosis (TB) hyper-endemic suburb of Cape Town, South Africa[2,3], we investigated the *in vitro* secretion of TNFα and IFNγ as well as the frequency of $IFN\gamma^{+}CD4^{+}$ and $IFN\gamma^{+}CD8^{+}$ cells in whole blood in response to BCG, PPD and ESAT6 antigens.

Previous studies have reported high levels of heritability in endemic areas for immune assay read-outs[4-6]. These estimates ranged from 71% for the tuberculin skin test[4], 20%-40% for antigen specific IFN-γ production[4,6], and 68% for TNFα production[6]. Further segregation analysis of TNFα production in a TB-endemic community in Uganda supported the presence of a major gene that explained one-third of TNFα-production variance[7]. In our own studies we have recently identified two major genes that impact on intensity of tuberculin skin test reactivity (Chapter 5). These observations provide the proof-of-principle that high heritability is a valid surrogate for host genetic factors with an important role in human anti-mycobacterial immunity.

Our analysis expanded previous results and showed for the first time a very strong impact of host genetic factors on the frequency of BCG- and PPD-specific $IFN\gamma^+CD4^+$ and $IFN\gamma^+CD8^+$ blood cells. We also demonstrated the presence of shared, strong genetic control elements for IFNγ and TNFα release as well as the frequency of $IFN\gamma^+CD4^+$ and $IFN\gamma^+CD8^+$ cells, an observation that points to the action of pleiotropic immune response genes. Since we showed that the host genetic background is an important determinant of inter-individual variability of anti-mycobacterial immunity, our results represent a critical contribution towards the dissection of TB pathogenesis. Equally important, a large impact of the host genetic background on two immune pathways exploited in the detection of tuberculosis indicated a possible confounding of test results by genetic host factors.

Materials and methods

Study design and subjects

Healthy study subjects were recruited from Ravensmead and Uitsig, two suburbs of Cape Town, South Africa with a very high incidence of TB disease. The notification rate of TB (all cases) in the area is 761/100,000 and of new bacteriologically confirmed cases it is 313/100,000[3]. BCG vaccination at birth has been routine in the study area since 1975 and HIV rates are below 4% in the pediatric population[3]. The enrolment strategy was to target large households with healthy children over 1 year of age and young adults. Individuals who were HIV positive, pregnant or using immuno-modulatory drugs were excluded at the time of enrolment. Subjects who had had clinical TB disease

within 2 years of the study were excluded from the analysis. Informed consent was obtained for all study participants. The study was approved by the Institutional Review Boards of Stellenbosch University, Tygerberg, South Africa; Cape Town University, Cape Town, South Africa; and the Research Ethics Committee at the Research Institute of the McGill University Health Centre, Montreal, Canada.

Whole blood ELISA assay and frequencies of antigen-specific IFNγ^+ T cells

Blood was drawn for a series of *in vitro* whole blood assays. Production of TNFα was measured after 1 day of incubation with live BCG (D1BCG), or BCG plus IFNγ (D1BCG+γ). Production of IFNγ in response to live BCG, *M. tuberculosis* PPD, or *M. tuberculosis* ESAT-6 was measured after 7 days of incubation with the antigens (D7BCG/PPD/ESAT). Cultures incubated without stimulation in serum-free medium were used as negative controls. TNFα and IFNγ production in assay supernatants were measured using ELISA. The frequencies of BCG- or PPD-specific $CD4^+IFN\gamma^+$ cells (BCG- PPD-CD4) and $CD8^+IFN\gamma^+$ cells (BCG-PPD-CD8) were measured using flow cytometry. Details of the assays are described elsewhere (see Chapter 2).

Statistical analysis

Analyses were performed on log-transformed phenotypes. The negative control value after log-transformation was subtracted from the log-transformed stimulated values. For TNFα and IFNγ assays, where cells were stimulated with antigens in quadruplicate, we used the mean of the log-transformed data.

Prior to further analysis, log-transformed phenotypes were adjusted systematically for age, previous TB disease and sex by use of multivariate linear regression, as implemented in the PROC GLM of the SAS software v9.1 (SAS institute, Cary, NC), to remove confounding environmental effects. For each phenotype, the best model for the age effect was determined among a set of multivariate fractional polynomials models (FP) as proposed by Royston et al.[8] and implemented in the SAS-MACRO *%mfp8* [http://www.imbi.uni-freiburg.de/biom/mfp/].

Principal component analysis was conducted on the adjusted log-transformed phenotypes using the PROC PRINCOMP of the SAS software. Such analysis consisted of converting a given number of correlated quantitative phenotypes into fewer uncorrelated traits defined as the linear combinations of the original phenotypes that maximize the overall variance of the data. The 3 first principal components (PC) with an eigenvector greater than one, explaining 78% of the variance, were retained for heritability analysis. The first PC accounts for the maximal amount of total variance in the observed phenotypes while the second and thirds components account for a maximal amount of variance in the data set not accounted for by the first component and second components respectively. The eigenvalues represent the amount of variance that is accounted for by each component with the total variance equal to the number of observed quantitative phenotypes included in the analysis (n = 9).

Familial correlation coefficients were estimated by use of the class D regressive model developed by Bonney[9] and implemented in the SEGREG routine of the S.A.G.E. As the parental phenotypes were not available, only the sib-sib correlation coefficient

(ρ_{SS}) was estimated and the heritability approximated under the hypothesis of a polygenic model as 2 $\rho_{SS,}$. For each phenotype, a null model (without correlation) and a model with sib-sib correlation were fitted. The statistical test is a likelihood ratio test between the two models and is distributed, under the null hypothesis H_0 of no sib-sib correlation, as a χ^2 with 1 degree of freedom.

Results and discussion

At least one immune phenotype was available for each of the 460 children and young adults, distributed in 148 extended pedigrees (45, 57, 36, 7, 1 and 2 families with 2, 3, 4, 5, 6 and 7 sibs respectively). The median range age was 14 (range 1-41) years and the sex ratio was 1.05. A total of 29 individuals had had clinical TB disease at least 2 years prior to their enrolment in the study. The heritability estimates of the log-transformed phenotypes are summarized in Figure 1. Overall, heritability estimates were high and strongly suggested that host genetic factors were involved in the regulation of the anti-mycobacterial immune response. For TNFα production triggered by co-incubation with BCG alone or BCG plus IFNγ, heritability was found to be 30% and 39%, respectively (Figure 1). Those results further support a strong genetic control of TNFα production in response to mycobacterial antigens. A study in Uganda had described a major gene effect on mycobacterial antigen triggered TNFα production[7] and the corresponding heritability was estimated at 68%[6]. The results obtained in Uganda and in the Cape Town sample identify TNFα production as excellent quantitative phenotype for further genetic study. Indeed, a first genome-wide linkage analysis conducted in the Ugandan sample reported suggestive linkage of a locus impacting on TNFα production

on chromosome region 21q22[10]. The latter result provided direct experimental support to the notion that high heritability estimates are a good indicator of the possibility of identifying genes underlying trait heritability.

Heritability of IFNγ production was estimated from 43% to 58% ($p<10^{-3}$), depending on the nature of the stimulating antigen (Figure 1). Interestingly, ESAT6 triggered IFNγ secretion showed the highest level of heritability (58%) while using BCG as stimulating antigen produced lower heritability estimates (43%). It is reasonable to expect lower heritability for more complex antigens and higher heritability for less complex ones. Generally, the estimates for heritability of IFNγ secretion tended to be higher in our study than those previously observed. Heritability of IFNγ production following stimulation by *M. tuberculosis* culture filtrate in Uganda was estimated from 17% and 30%, depending on the analytical strategy used[6]. In a twin study in The Gambia, heritability of IFNγ production triggered by PPD was found to be 38% but with a large 95% confidence interval [0-68][4]. In our study, the heritability estimates for the frequency of antigen-specific $IFN\gamma^+$ T cells ranged from 53% to 74%. The highest estimate was 74% for BCG-specific $IFN\gamma^+CD8^+$ cells, while remaining estimates were in the 50%-60% heritability range (Figure 1) This is the first report of heritability analysis for the frequency of antigen-specific $IFN\gamma^+CD4^+$ and $^+CD8^+$ cells, two cell types with mounting evidence for a role in protective anti-tuberculosis immunity[11,12]. The observation that both IFNγ secretion and frequency of antigen-specific $IFN\gamma^+CD4^+$ and $IFN\gamma^+CD8^+$ cells are strongly impacted by the host genetic background may have important practical implications since similar immune assays are used for TB detection.

The different assay read-outs in our study are all measures of anti-mycobacterial immunity and are therefore correlated to some degree. The most popular approach to reduce data dimensionality in correlated data sets is principal component analysis (PCA; see M&M). The first three components had eigenvalues greater than 1 and together explained 75% of the total variability (Table 1). The first principal component (PC1) which explained 41% of the overall variance reflects with approximate equal weights all aspects of IFNγ production. Heritability of PC1 was 60% consistent with a similar heritability for the normalized marginal IFNγ phenotypes. PC2 mainly corresponded to TNFα production-related phenotypes. Heritability PC2 was estimated at 37% again in good agreement with the estimates of the marginal TNFα phenotypes. PC3 was mainly related to IFNγ phenotypes and gave special weight to the frequency of $IFN\gamma^+$ cells (negative coefficients). Heritability of PC3 was estimated at 59% (Table 1).

We used sib-sib correlation of phenotypes to estimate the heritability of the underlying trait. In this approach, any shared environmental components within a family may induce non genetic familial correlation that might inflate heritability estimates. In our study, by far the most important environmental factor was exposure to and infection by *M. tuberculosis*. A good correlate of infection is transmission of TB. It has been demonstrated that TB transmission in our study area occurs mainly outside the household[2]. This argues against inflation of heritability estimates by household specific exposure factors. Likewise, we failed to observe an influence of neighbourhood tuberculosis incidence on any of the immune phenotypes (data not shown) which is

further evidence that exposure and infection happen at the level of the community and not at the level of the household or neighbourhood.

The genetic component that underlies the very significant heritability estimates of the univariate and multivariate phenotypes is currently unknown. However, the dominant impact of genetics on immune assays suggested that at least some of the genes underlying the high heritability of phenotypes can be identified by, for example, positional cloning approaches. Of particular interest in this context is the observation that multivariate phenotypes display heritability estimates of very similar magnitude as univariate traits. Hence, the first components of the multivariate analysis can now be used as the phenotypes of interest for linkage analysis[13]. There is a clear gain of power with PC techniques over univariate analysis when the traits are influenced by a QTL in a manner consistent with the linear combination in the PC[14]. What does this mean in molecular terms? The most parsimonious explanation is the existence of immune response regulatory genes with pleiotropic effects on different immune pathways. The molecular identification of trait specific and pleiotropic immune regulator genes will provide much needed insight into host genetic control of anti-mycobacterial immunity.

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Figure legends

Figure 1: Heritability estimates of quantitative anti-mycobacterial immunity.

Level of heritability is indicated on the top left side of the graph. The bars represent heritability estimates of the adjusted log-transformed phenotypes. Asterisks indicate the level of significance of a genetic component for phenotype expression. D1BCG: TNFα production following stimulation by BCG antigen; D1BCG+γ: TNFα production following stimulation by BCG antigen plus IFNγ ; D7BCG: IFNγ production following stimulation by BCG; D7PPD: IFNγ production following stimulation by PPD; D7ESAT: IFNγ production following stimulation by ESAT-6; BCGCD4: frequency of $IFN\gamma^{+}CD4^{+}$ cells after stimulation by BCG; PPDCD4 : frequency of $IFN\gamma^{+}CD4^{+}$ cells after stimulation by PPD; BCGCD8 : frequency of $IFN\gamma^{+}CD8^{+}$ cells after stimulation by BCG; PPDCD8 : frequency of $IFN\gamma^{+}CD8^{+}$ cells after stimulation by PPD.

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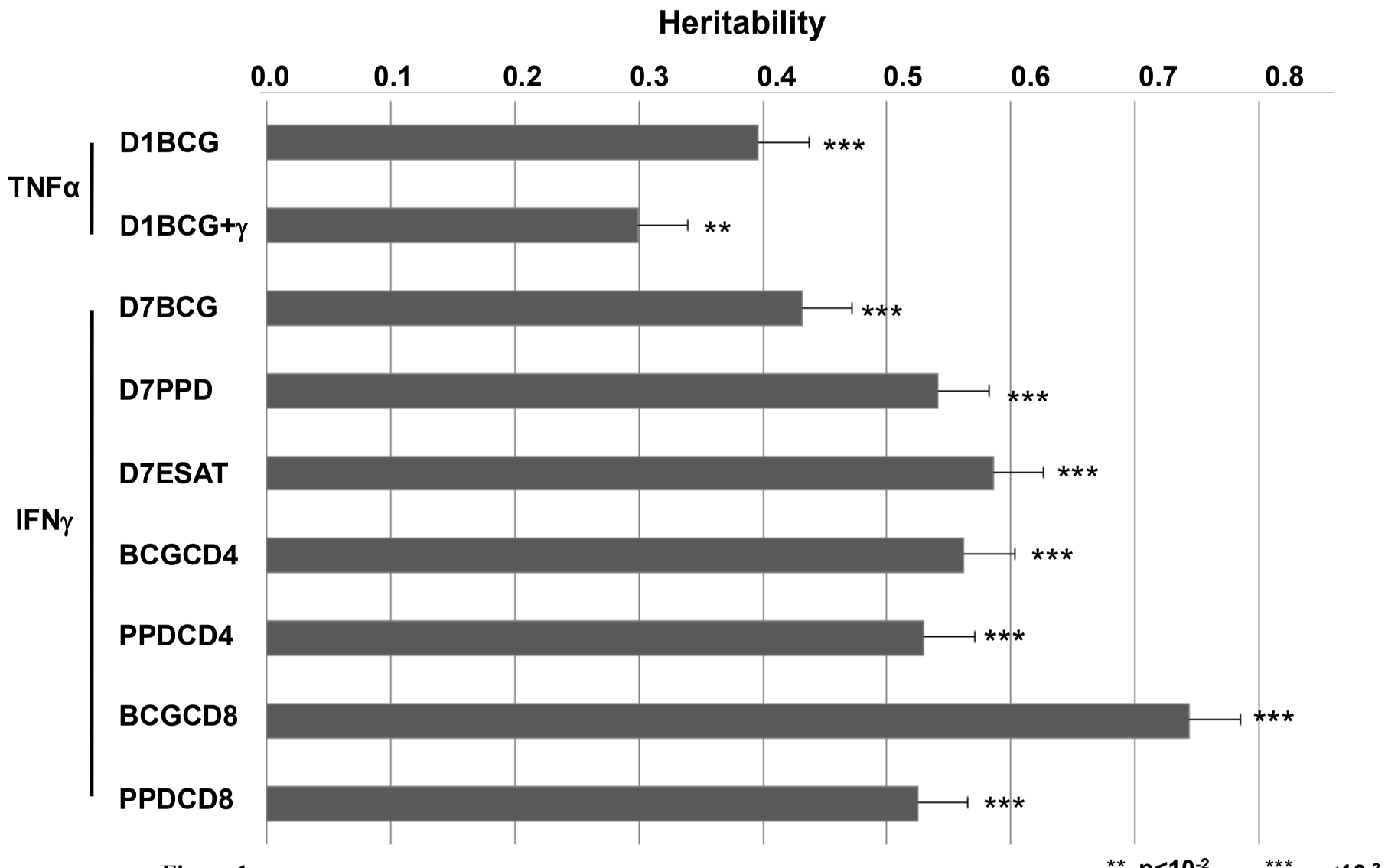
Table 1. Description and heritability (h^2) estimates of the first three principal components of the PCA for adjusted and log-transformed phenotypes

	Phenotypes												
	D1BCG*	**D1BCG+γ**	**D7BCG**	**D7PPD**	**D7ESAT**	**BCGCD4**	**PPDCD4**	**BCGCD8**	**PPDCD8**	**Eigenvalue**	**Cumulative proportion variance**	**h^2**	**p-value**
PC1**	0.06	0.06	0.2	0.2	0.15	0.23	0.23	0.17	0.19	3.65	0.41	0.6	$6x10^{-8}$
PC2	0.5	0.5	-0.05	-0.07	-0.08	-0.04	-0.05	0.003	-0.02	1.9	0.62	0.37	$5x10^{-4}$
PC3	0.05	0.06	0.34	0.38	0.37	-0.21	-0.14	-0.35	-0.33	1.44	0.78	0.59	$1x10^{-7}$

* Abbreviations as in Figure 1

** Score coefficients of marginal phenotypes are given for standardized PC


Heritability
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
TNFα
IFNγ
D1BCG
D1BCG+γ
D7BCG
D7PPD
D7ESAT
BCGCD4
PPDCD4
BCGCD8
PPDCD8
***
**
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***
***
***
***
***
** p<10-2
*** p<10-3

Figure 1

Chapter 5
Two loci sequentially control tuberculin skin test reactivity in an area hyper-endemic for tuberculosis

Chapter 5 presents the results of the genome-wide linkage scan of the quantitative immune-phenotype TST reactivity. The subjects in this study include the children described in Chapters 2-4 as well as their parents.

Two loci sequentially control tuberculin skin test reactivity in an area hyper-endemic for tuberculosis

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Abstract

Approximately 20% of exposed persons appear to be naturally resistant to infection by *M. tuberculosis*[1] as estimated by means of the tuberculin skin test (TST). However, the molecular basis of TST reactivity is not known. We report here on a genome-wide search for loci impacting on TST reactivity defined either as a binary (i.e. zero vs. non zero, TST-BIN) or a quantitative (i.e. TST in mm, TST-QTL) trait in a panel of 128 families including 350 siblings from an area hyper-endemic for tuberculosis (TB). We detected a major locus (*TST1*) on chromosomal region 11p14 ($P = 1.5x10^{-5}$) that controlled TST-BIN, i.e. T-cell-independent resistance to *M. tuberculosis*. We also detected a second major locus (*TST2*), on chromosomal region 5p15 ($P < 10^{-5}$) that controls TST-QTL, i.e. the intensity of T-cell-mediated delayed type hypersensitivity (DTH) to tuberculin. Refined genetic analysis demonstrated that these two loci control TST reactivity in a sequential manner. Our results pave the way for the understanding of the molecular mechanisms involved in resistance to *M. tuberculosis* infection in endemic areas (*TST1*), and for the identification of a critical regulator of T-cell dependent DTH to tuberculin (*TST2*).

Introduction

Only an estimated 10% of individuals infected with *M. tuberculosis* develop clinical TB, whether primary TB, typically an acute systemic disease of children, or reactivation TB, typically a chronic pulmonary disease of adults[2]. It has long been suspected that inter-individual variability in progression from infection to clinical TB disease is under tight genetic control[3]. Genetic epidemiological evidence includes the large inter-population variability in both incidence and severity of natural TB disease, an equally remarkable inter-individual variability following accidental inoculation of babies with *M. tuberculosis,* a higher incidence risk of the disease in first-degree relatives of TB cases as compared to first degree-relatives of unaffected individuals, and a higher concordance rate of TB among identical than fraternal twins[4]. Studies of experimental infection in animal models, in the mouse in particular, have first provided a molecular basis to genetic predisposition to TB [reviewed in Schurr and Kramnik[5]]. Subsequent human molecular genetic studies have identified candidate genetic risk factors for pulmonary TB in adults [reviewed in Schurr and Kramnik[5]], and have documented Mendelian predisposition to disseminated TB in a few children, IL-12Rβ1 deficiency in particular[6]. Taken together, these studies have provided the long-awaited molecular proof-of-principle for the contribution of human genetic factors to TB susceptibility.

There is also inter-individual variability at the earlier, initial step of the infectious process, as approximately 20% of long-exposed persons appear to be naturally resistant to infection by *M. tuberculosis*[1]. This estimate is based on the detection of *M. tuberculosis* infected and non-infected persons by means of the tuberculin skin test (TST, Mantoux).

This assay measures induration of the skin following intradermal inoculation of *M. tuberculosis* purified protein derivative (PPD). The test triggers a classic T cell-mediated, delayed type hypersensitivity (DTH) reaction against mycobacterial antigens[7]. A lack of TST reactivity is suggestive of a T cell-independent resistance to mycobacteria, *M. tuberculosis* in particular in hyper-endemic areas. Interestingly, several genetic epidemiological studies in endemic areas have reported high levels of heritability for TST, considered either as a binary or quantitative trait, following *M. tuberculosis* exposure[8,9]. For example, there is 92% heritability for quantitative TST in household children exposed to an adult TB case in Chile[8]. These observations suggested an important contribution of host genetic factors to resistance to *M. tuberculosis* infection, and to the immune reactions underlying TST intensity.

However, surprisingly few studies have aimed to dissect the underlying genetic variants. A candidate gene study failed to identify significant association between quantitative TST and interleukin(IL)-1 receptor antagonist[10] ; this gene does not appear to be critically involved in TST reactivity. A genome-wide linkage study in an endemic area reported suggestive linkage of persistently low TST reactivity with chromosome regions 2q21-2q24 and 5p13-5q22[11]. The absence of significant linkage peaks in the latter study might be explained by the threshold-dependent categorization of the TST phenotype (no infection / infection), and by the variable BCG vaccination and/or HIV infection status of the subjects enrolled. Better controlled studies exploiting the full range of TST reactivity are thus needed, since there is considerably more information for linkage analysis in quantitative variation than there is in any binary trait[12].

To address this question, we have collected over the last five years a unique population sample of 128 large nuclear families from a hyper-endemic suburb of Cape Town, South Africa, with an estimated TST-based annual risk of infection as high as 4% despite low rates of HIV infection[13]. Out of 22 hyper-endemic countries, South Africa has the highest TB annual incidence rate at 940/100,000 with children constituting up to 39% of this case load[14]. The Western Cape offers the advantage that tuberculosis detection by TST is not confounded by cross-reactivities to environmental mycobacteria and BCG vaccination as there are virtually no results in the low reading range (0-5mm) (ref 15 and unpublished data). Consequently, the Western Cape is uniquely suited for a genetic study of immune reactivity against *M. tuberculosis* antigens, as measured by TST. To decipher the molecular basis of T cell independent resistance to *M. tuberculosis* in endemic areas and the molecular basis of T cell dependent TST intensity, we took advantage of the Western Cape epidemiologic setting and undertook the first genome-wide linkage scan for both binary (zero vs. non zero) and quantitative TST reactivities.

Material and methods

Subjects and families

Nuclear families (i.e. parents and offspring) with at least two children were enrolled from Ravensmead and Uitsig, two suburbs of Cape Town, South Africa, that are hyper-endemic for TB. All individuals of the sample belong to the Cape Coloured ethnic group. There was no requirement for subjects to be household contacts of TB cases. However, this area has a notification rate of TB (all cases) of 761/100,000 and of new

bacteriologically confirmed cases of 313/100,000[13] suggestive of a high level of exposure to *M. tuberculosis*. The enrolment strategy was to target large households to allow later reconstruction of nuclear families. In addition, if TST reactivity was known at time of enrolment, households that contained both strongly TST positive and TST negative subjects were prioritized for enrolment as this sampling strategy has been shown to be the most powerful for linkage analysis.

Subjects who had had clinical TB disease in the two years preceding the study were excluded. Individuals who were HIV positive, pregnant or using immuno-modulatory chemotherapy were also excluded at the time of enrolment. BCG vaccination at birth is routine in the study area and was therefore not a confounding factor in our study, i.e. because all individuals are vaccinated at the same age there is no differential impact on the TST, if any (several studies have shown that the impact of BCG at birth on the TST vanishes rapidly[25]). The study was approved by the Institutional Review Board of Stellenbosch University, Tygerberg, South Africa; Cape Town University, Cape Town, South Africa; and the Research Ethics Board at the Research Institute of the McGill University Health Centre, Montreal, Canada.

Phenotype and Covariates

Tuberculin skin tests were carried out by specially trained health care providers using the Mantoux method with *M. tuberculosis* PPD batch RT23 (2 T.U., Statens Serum Institut, Copenhagen, Denmark). TST reactivity was read between 48-72 h after the skin test was performed and the diameter of induration was measured in millimetres using a set of calipers calibrated to the nearest 0.5 mm. Two phenotypic definitions were used

(Figure 1). First, we dichotomized the TST distribution using zero mm as the threshold to study the TST positivity (TST-BIN). Second, we analysed the extent of the TST reactivity as a quantitative trait (TST-QTL) by Tobit regression.

Prior to linkage analysis, TST-BIN and TST-QTL phenotypes were all adjusted on previous clinical TB (at least two years preceding the study), sex (male, female) and age (in years). Pearson and Tobit residuals were used for linkage analysis of TST-BIN and TST-QTL, respectively. TST-BIN was adjusted by means of logistic regression (Figure 1.C), as implemented in the PROC LOGISTIC of the SAS software v9.1 (SAS institute, Cary, NC). TST-QTL was adjusted by means of the Tobit censored regression as implemented in the PROC QLIM of the SAS software v9.1 (SAS institute, Cary, NC), with the censoring threshold fixed at zero (Figure 1.D). For each phenotype, the best fitting model for the age effect was determined among a set of multivariate fractional polynomials models (FP) as proposed in Royston et al [26]. First (FP1) and second (FP2) degree fractional polynomial models were fitted with power p for FP1 and p and q for FP2 chosen from (-2, -1, -0.5, 0, 0.5, 1, 2, 3), 0 denoting log transformation. Among the 44 possible combinations, the model providing the smaller Akaïke Information Criteria was selected. A simple FP1 model was retained with power p=0.5 for TST-BIN wheras a FP2 model was retained for TST-QTL (power p=1 and q=2).

Genotyping

High density genotyping was performed at the 'Centre National de Génotypage' (CNG), Paris, France, with the Illumina® linkage IVb Panel, containing more than 6,000 single nucleotide polymorphisms (SNPs). Eleven non polymorphic SNPs and 79 SNPs with a call rate lower than 80% were excluded. None of the remaining SNPs showed departure from Hardy-Weinberg equilibrium among the founders at the 0.001 level. Pair-wise linkage disequilibrium (LD) analysis between adjacent SNPs was performed using the Haploview software[27] in the 186 parents of our sample. In our sample, pairwise LD between adjacent SNPs was very weak with most of SNPs-pairs having r^2 less than 0.1 (mean pair-wise $r^2 = 0.07$).

Internal population structure analysis

As suggested by Thompson et al[28], we checked for population substructure in order to minimize genetic heterogeneity of the sample prior to linkage analysis. We performed a principal component analysis (PCA) of the 5567 autosomal SNPs in the 186 genotyped founders of our sample as implemented in the SMARTPCA software[29]. The principle of such a method is to determine the major axes of genetic variation in the sample and to output each individual's coordinates along axes of variations, without formally clustering individuals into discrete population. No population substructure was found in our data but we identified five outliers distributed in four families that were excluded from the analysis (data not shown).

Linkage analysis

We performed quantitative model-free multipoint linkage analysis on the Pearson residuals of the TST-BIN logistic regression and on the residuals of the TST-QTL censored Tobit regression. As residuals were not normally distributed and pedigrees were extended, we used the maximum-likelihood binomial (MLB) method extended to quantitative trait linkage analysis (MLB-QTL v.3.0, available upon request)[30] that has been implemented in an extension of the GENEHUNTER program.

This approach considers the sibship as a whole and does not make any assumption about the distribution of the phenotype. The idea of the MLB method is to introduce an individual latent binary variable, which captures the linkage information between the observed quantitative trait and the marker. The probability that the latent variable for an individual takes the value zero or one depends on the quantitative trait value of the individual and on a link function, which can be parametric (like the standard cumulative normal distribution) or empiric (derived from the observed phenotypic distribution).

The test of linkage is a maximum-likelihood ratio test that compares the likelihoods under the null hypothesis of no linkage H_0 and the hypothesis of linkage. The test statistic is asymptotically distributed as a 50:50 mixture of χ^2 distribution with 0 and 1 degree of freedom and can be expressed as a classical LOD-score. Here, we used an empirical link function based on the deciles of the residuals distribution as suggested in Alcais and Abel[30]. Confidence intervals (CIs) for the location of QTL underlying the linkage peaks were calculated by use of the support interval method in which a 1-LOD

interval corresponds to a 90% CI[31]. A LOD-score greater or equal to 3.6 ($P \leq 2 \times 10^{-5}$) and 2.2 ($P \leq 7 \times 10^{-4}$) were considered as significant and suggestive of linkage, respectively, as proposed in Lander and Kruglyak[16]. Analysis of chromosome X is not implemented in the MLB method and was therefore not analysed.

Results

The distribution of TST is bimodal in the individuals studied

We studied 128 informative families (including 186 parents and 350 children) comprising two to six children with available TST phenotypes and genome-wide SNP genotypes (Table 1). TST reactivity of the 350 children is shown in Figure 1A. Reactivity extended from 0 mm to 43 mm with a median size of 11.15 mm. Median age (range in years) at the time of TST was 8 years (1 - 35) and the sex-ratio was 1. As expected due to the cumulative exposure to *M. tuberculosis* in this high incidence area, age had an important impact on TST positivity (Figure 1B). Closer inspection of the extent of TST reactivity showed a clear bimodal distribution with 140 individuals (40%) having a value of zero and 210 (60%) having values that approximately followed a normal distribution centred around 16 mm. Only two children had a TST between 1 and 5 mm. This distribution is strongly suggestive of a gene or group of genes impacting on TST positivity *per se* and a different gene or set of genes controlling the extent of TST reactivity. To test this hypothesis, we genotyped ~6,000 SNPs in the 536 individuals (128 nuclear families) of the sample and we performed two complementary linkage analyses. The first analysis focused on the binary phenotype 'positivity *per se*', i.e. TST=0 vs. TST>0 (TST-BIN). The second analysis focused on the quantitative

phenotype 'extent of TST reactivity' (TST-QTL) with a particular emphasis on individuals with a TST>0. Prior to linkage analysis, the two phenotypes were adjusted on age, gender and previous clinical TB in order to remove as many environmental or non specific genetic sources of TST variation as possible. Subsequent linkage analyses were therefore performed on the resulting adjusted residuals shown in Figure 1C (TST-BIN) and Figure 1D (TST-QTL).

A major locus for TST positivity per se maps to chromosome region 11p14

We first searched for genetic factors controlling TST positive response *per se*. Results of the TST-BIN linkage analysis are shown in Figure 2. Information content (IC) was very high across all autosomes with mean genome-wide information of 94.4% (from 80.6% to 98.7%). This IC is excellent and corresponds to the level only accomplished at the fine-mapping stage in microsatellites-based genome scans. Linkage analysis of TST-BIN identified a significant linkage signal on chromosomal region 11p14 (LOD score = 3.81) at chromosomal position 26.37 Mb (IC = 91.15%, $P = 1.4x10^{-5}$; Figure 2). This level of statistical support exceeds the stringent threshold of significance (LOD score = 3.6) for genome-wide linkage (GWL) scans[16]. The 1-LOD confidence interval for the location of the major locus (corresponding to the 90% confidence interval for the location of the QTL underlying the linkage peak) spanned from 22.35 - 28.82 Mb (Figure 2, bottom). A suggestive linkage signal was also observed on chromosomal region 5p15 (LOD-score = 2.39, $P = 0.0005$, IC = 90.5%) at the same position as the TST-QTL locus (see below). In addition, five weaker linkage peaks with $p<0.01$ were observed on chromosomal regions 3p24 (LOD-score = 1.40, $P = 0.0056$, IC = 96.8%), 4q28 (LOD-

score = 1.57, *P* = 0.0036, IC = 94.7%), 15q26 (LOD-score = 1.20, *P* = 0.0094, IC = 93.4%) ,19q13 (LOD-score = 1.83, *P* = 0.0018, IC = 92.4%) and 20p13 (LOD-score= 1.23, *P* = 0.0087, IC = 93.9%; Figure 2, top). As a substantial proportion of TST=0 persons are most likely resistant to infection with *M. tuberculosis* (see discussion), these data therefore point toward the identification of one major locus (*TST1*) controlling human resistance to *M. tuberculosis*.

A major locus for TST intensity maps to chromosome region 5p15

Results of the TST-QTL linkage analysis are shown in Figure 3. A highly significant linkage signal was observed on chromosomal region 5p15 with a multipoint LOD-score of 4.00 ($p < 10^{-5}$) at position 2.70 Mb. Again, this is substantially above the commonly accepted threshold for significance in GWL studies[16]. The 1-LOD confidence interval for location of the quantitative trait locus (QTL) was small, spanning ~2 Mb from 1.39 Mb – 3.23 Mb (Figure 3, bottom). In addition to this major locus, four weaker linkage peaks with *P* <0.01 (i.e. LOD score > 1.17) were observed in chromosomal regions 11p14 (LOD-score = 1.47, *P* = 0.0046, IC= 93.1%), at the same position as the TST-BIN locus, 10p15 (LOD-score = 1.76, p = 0.002, IC= 93.3%), 13q21 (LOD-score = 1.51, *P* = 0.0042, IC=96%) and 22q11 (LOD-score = 1.34, *P* = 0.0065, IC= 97%). It has been shown that linkage analysis using a dense SNP panel in familial samples with missing parents could inflate the type I error rate in regions where SNPs display high level of LD[17]. Therefore, although the LD was very weak at the genome-wide level and between markers underlying the two different linkage peaks (mean pair-wise $r^2 = 0.07$), we removed the SNP with the lowest minor allele frequency for each pair of SNPs with an $r^2 > 0.1$. This had no impact on the LOD-scores or the IC (data not shown) ruling out

the possibility of *TST1* and *TST2* being false positive linkage signals due to LD patterns. Therefore, these results support the hypothesis of a second major locus (*TST2*) on chromosomal region 5p15 controlling the intensity of TST reactivity as a quantitative trait, i.e. the intensity of T cell-mediated DTH to tuberculin.

Genetic control of TST reactivity is sequential

An interesting observation is the mirror effect between the two previous analyses with a suggestive linkage signal on chromosomal region 5p15 in the analysis of TST-BIN and on chromosomal region 11p14 in the analysis of TST-QTL. This is suggestive of some level of redundancy between the two phenotypes. The most straightforward explanation for this redundancy is the presence of individuals with a TST equal to zero mm in both analyses since these are coded as zero in both analytical approaches. By contrast, individuals with TST > 0 mm will be coded as 1 in TST-BIN whereas they will be coded at the extent of their TST in TST-QTL. Indeed, the correlation between the logistic (TST-BIN) and the Tobit (TST-QTL) residuals among individuals with TST=0mm was >0.95. If the hypothesis of a two-step control of TST is valid (i.e. *TST1* is impacting on positivity *per se* and *TST2* is impacting on the extent of TST reactivity) then restricting the TST-QTL analysis to positive individuals only should have a limited impact on the 5p15 linkage peak (*TST2*) but a much more dramatic impact on the 11p14 peak (*TST1*). Therefore, we performed a linkage analysis of TST-QTL among individuals with TST >0mm only. Despite a dramatic reduction in family numbers (the sample size was reduced to 68 families comprising 164 children) we still found evidence of linkage at chromosomal region 5p15 (LOD-score=2.17; $P < 8.10^{-4}$). By contrast, the linkage signal on 11p14 totally disappeared (LOD-score=0.14; $P = 0.21$). These results are in strong

support of a sequential genetic control of TST reactivity, with *TST1* controlling TST reactivity as a binary trait and, presumably, T-cell-independent innate resistance to *M. tuberculosis,* and *TST2* controlling the intensity of TST reactivity as a quantitative trait and, presumably, the intensity of T cell-mediated DTH to tuberculin.

Discussion

We report here that negative TST reactivity (zero induration) has a major human genetic basis, with a locus *TST1* that maps to chromosome region 11p14. As the risk of developing TB for persons with *M. tuberculosis* exposure but TST = 0 mm was previously shown to be extremely small, such persons are most likely not infected with *M. tuberculosis*[18]. Persons who experience continued high exposure to *M. tuberculosis* and display persistent lack of T-cell sensitization, are much more likely to be naturally resistant to infection with *M. tuberculosis* than intrinsically deficient in mounting a DTH response. An estimate of infection resistant persons can be obtained from countries where TB is highly endemic. In such conditions, where exposure to the tubercle bacillus is intense and sustained, approximately 20% of individuals remain TST negative[1]. In our families, it is not possible to distinguish between individuals who have never been exposed to *M. tuberculosis* and those who have been exposed but are naturally resistant to infection. However, since the lack of exposure is difficult to reconcile with a genetic component, the most parsimonious explanation of our data is that the *TST1* locus reflects T-cell independent resistance to *M. tuberculosis* infection. Consequently, a substantial fraction of TST = 0 mm persons in the study families must have been exposed to *M. tuberculosis* but due to very effective T-cell independent responses those persons were

resistant to *M. tuberculosis* before T cell sensitization could occur. Our findings make it likely that being spared from infection with *M. tuberculosis* is not simply a matter of lack of exposure but reflects genetically controlled profound differences of T-cell independent resistance among exposed persons. The targeted strengthening of T-cell independent resistance loci in infection susceptible persons offers an attractive avenue of protection from TB disease, especially in persons with deteriorated acquired immunity who are at high risk of progression from *M. tuberculosis* infection to clinical TB disease. The identification of the *TST1* locus will follow the strategy successfully applied in leprosy[19,20].

Our study also provides strong evidence that a major QTL (*TST2*) mapping to chromosome region 5p15 is involved in the control of TST reactivity in families living in a hyper-endemic region for TB. Consistent with our results, the same region showed some evidence of linkage ($p<0.05$) with persistently low TST reactivity in a familial sample from Uganda[11]. *TST2* is the first non-MHC locus impacting on intensity of T-cell mediated DTH to tuberculin. This identifies *TST2* as important immune modulator and possible confounder in the numerous studies that have aimed at correlating extent of TST reactivity with risk for future clinical TB disease. While not all studies agreed, overall there is significant positive correlation between size of TST and risk of subsequent TB disease[21]. However, there is a large variation in the strength of this association. To what extent results become more uniform and associations possibly stronger when the impact of *TST2* on TST induration size is considered will be an important aspect for future studies A more direct link of *TST2* with risk of disease is perhaps provided by the repeated observation of the 5p15 region as location of a sarcoidosis susceptibility locus[22].

Sarcoidosis is characterized by the immune paradox of extensive local inflammation (granuloma, cytokine secretion) associated with suppression of the immune response to tuberculin[23]. Multiple studies have investigated the association between mycobacterial antigens and sarcoidosis but no clear consensus has emerged[22]. Our results suggest that human genetics could serendipitously connect the mechanisms governing sarcoidosis and T-cell dependent DTH to mycobacteria. To the best of our knowledge, no other DTH response regulator has previously been mapped to the 5p15 (or the 11p14) chromosomal regions suggesting that *TST2* immune regulation involves an element of specific antigen recognition. Following the successful strategy used for the identification of *PARK2/PACRG* and *LTA* polymorphisms conferring susceptibility to leprosy[19,20], we are currently carrying out experiments to identify the gene variants controlling extent of TST reactivity.

The gene regions previously reported to play a role in clinical TB[5], including the only major TB susceptibility locus on chromosome 8p13[24], do not overlap with the two strongest chromosomal regions identified in this study. For the *TST1* locus on chromosome 5p15 this is not surprising since genetic studies are generally done on TST positive (i.e. infected) subjects which precludes identification of infection resistance genes. For the *TST2* region on chromosome 11p14 the lack of overlap with TB susceptibility genes is more surprising. However, since *TST2* has a strong impact on TST induration size, we can predict that even after adjustment on *TST2* alleles the correlation between TST reactivity and risk of subsequent TB will remain modest. HLA class II genes are validated TB susceptibility loci and MHC alleles are known from the mouse model to have a strong impact on DTH. These observations made the chromosome 6

HLA region a prime candidate for loci impacting on TST induration size. Yet, we failed to find any evidence for a role of HLA genes on TST. That the strongest genetic effect on tuberculin reactivity is caused by a non-HLA locus directly leads to the question as to whether this or other non-HLA loci will be modulators of vaccine–induced anti-mycobacterial protective immunity. While the *TST1* and *TST2* loci do not appear to substantially impact on progression from infection to TB disease, the two loci which control T-cell independent resistance to infection with *M. tuberculosis* (*TST1*) and T-cell dependent intensity of tuberculin reactivity (*TST2*) represent an important expansion in our understanding of TB immunity.

The TST is the most widely used assay to identify persons infected with *M. tuberculosis*. It is common practice that results of TST testing are reported as "positive" or "negative" for infection with *M. tuberculosis*. Cut-off points are chosen according to the epidemiological risk of infection and are an operationally justified approach to balance the risk of false positive and false negative results. Considering the strong impact of the *TST2* locus on extent of TST reactivity it is clear that the classification of positive and negative responders is confounded by this locus. Since *TST2* impacts on TST extent over the entire range of TST values, the use of binary (+/-) data for TST assays will be confounded irrespective of the specific cut-off point chosen. Results of TST assays are still the corner stone for the initiation of treatment for latent TB infection and a main public health TB surveillance tool. To our knowledge this is the first example on how host genetics confounds clinical decision making in an infectious disease and impacts on public health surveillance of a major infectious threat. Moreover, our results suggest that although more *M. tuberculosis*-specific, T cell based diagnostic procedures, such as

ESAT6/CFP10-trigered IFN-γ production by T cells in vitro, may also be under tight genetic control. 'Negativity' of any T cell assay may be confounded by *TST2* or other genetic loci. In any event, as a next generation of anti-microbial drugs may be based on immune-modulation of host responses, the proof-of-concept observation that *TST2* has a strong impact on anti-mycobacterial immunity may have far reaching implications not only for TB vaccine efficacy but also for drug development.

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Figure legends

Figure 1: Distribution of TST according to age before and after adjustment on relevant covariates

(A) Distribution of TST values among the 350 children used for the linkage analysis. A total of 140 subjects had no measurable reaction (red bar) and 210 subjects had TST induration > 0 mm (black bars).

(B) Distribution of TST values among the 350 children according to age in years (same color-coding as in A). Note that the red dots reflect variable numbers of subjects with TST=0. Overall, 3, 2, 6, 2, 5, 22, 10, 20,17, 11, 11, 13, 6, 2, 2 and 3 subjects had a TST = 0 at the age of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 19 and 20 years, respectively.

(C) Distribution of the Pearson residuals obtained by logistic regression of TST-BIN on age, sex and previous TB according to age in years. Color-coding indicates those subjects with TST=0 (red) or TST>0 (black). As detailed in panel B, red dots, i.e. subjects with TST=0, usually represent multiple individuals. The two outliers in red correspond to two subjects with previous TB and TST = 0.

(D) Distribution of the residuals obtained by Tobit regression of TST-QTL on age, sex and previous TB according to age in years. Color-coding indicates those subjects with TST=0 (red) or TST>0 (black). As detailed in panel B, red dots, i.e. subjects with TST=0, usually represent multiple persons. The two outliers in red correspond to two subjects with previous TB and TST = 0.

Figure 2: Genome-wide model-free linkage analysis of TST-BIN

(A) Multipoint LOD-score (black line; left Y-axis) and information content (red line; right Y-axis) are plotted for the 22 autosomes.

(B) Expanded view of the region with the highest LOD-score on chromosome 11. The multipoint LOD score (black line), information content at marker positions (red line) and 90% confidence interval for the location of the quantitative trait locus (horizontal arrow and dotted vertical lines) are given. Left and right Y axes indicate LOD score and information content, respectively. Chromosomal positions are given in megabases (Mb).

Figure 3: Genome-wide model-free linkage analysis of TST-QTL

(A) Multipoint LOD-score (black line; left Y-axis) and information content (red line; right Y-axis) are plotted for the 22 autosomes.

(B) Expanded view of the region with the highest LOD-score on chromosome 5. The multipoint LOD score (black line), information content at marker positions (red line) and 90% confidence interval for the location of the quantitative trait locus (horizontal arrow and dotted vertical lines) are given. Left and right Y axes indicate LOD score and information content, respectively. Chromosomal positions are given in megabases (Mb).

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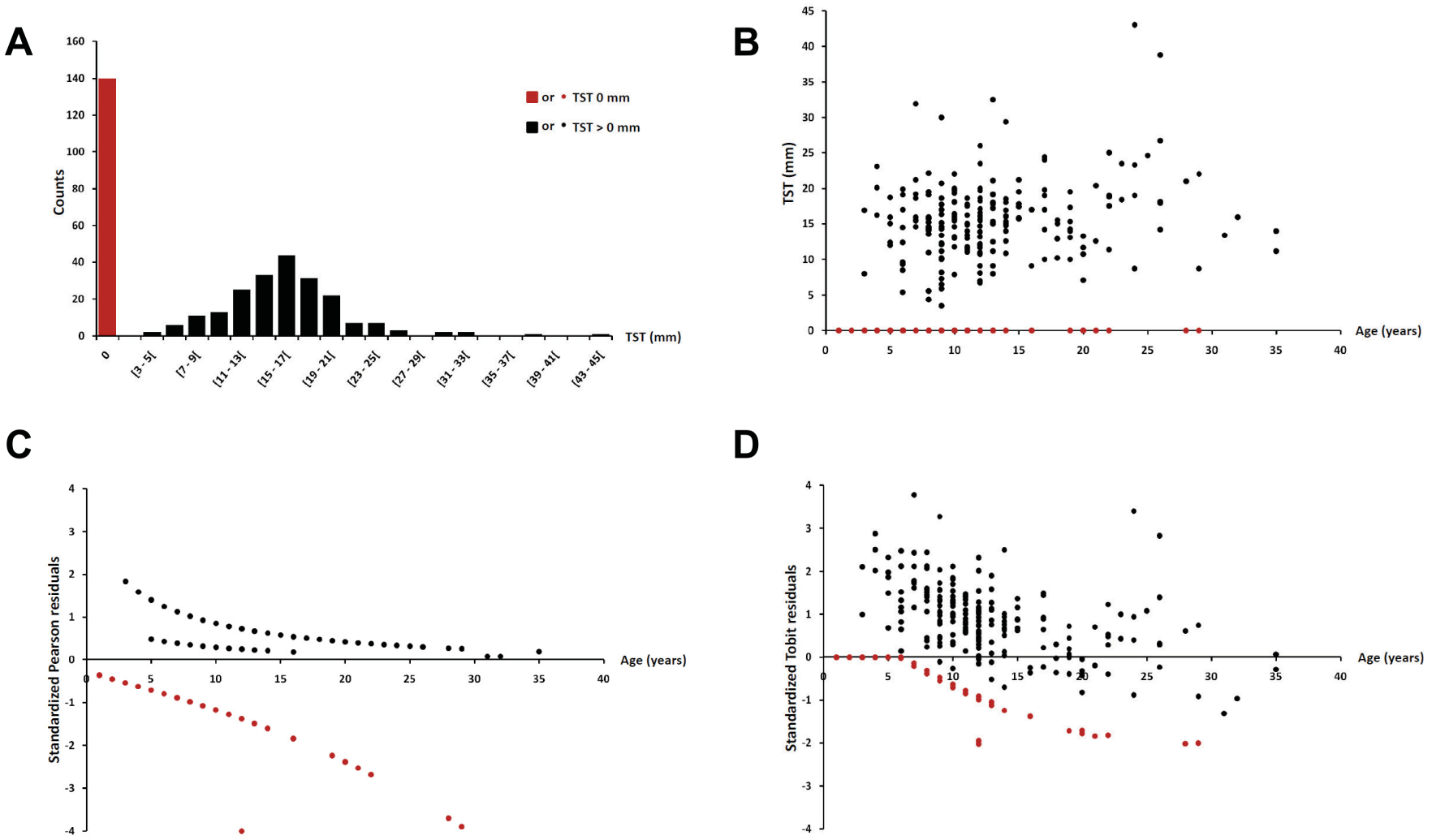
Table 1: Distribution of families according to the number of genotyped siblings with available TST data and the number of parents genotyped

Number of parents genotyped	Number of sibs per family					
	2	**3**	**4**	**5**	**6**	**Total**
0	2	2	0	0	0	**4**
1	33	23	5	1	0	**62**
2	22	29	7	3	1	**62**
Total	**57**	**54**	**12**	**4**	**1**	**128**


A
Counts
TST (mm)
or TST 0 mm
or TST > 0 mm
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[3 - 5[
[7 - 9[
[11 - 13[
[15 - 17[
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[23 - 25[
[27 - 29[
[31 - 33[
[35 - 37[
[39 - 41[
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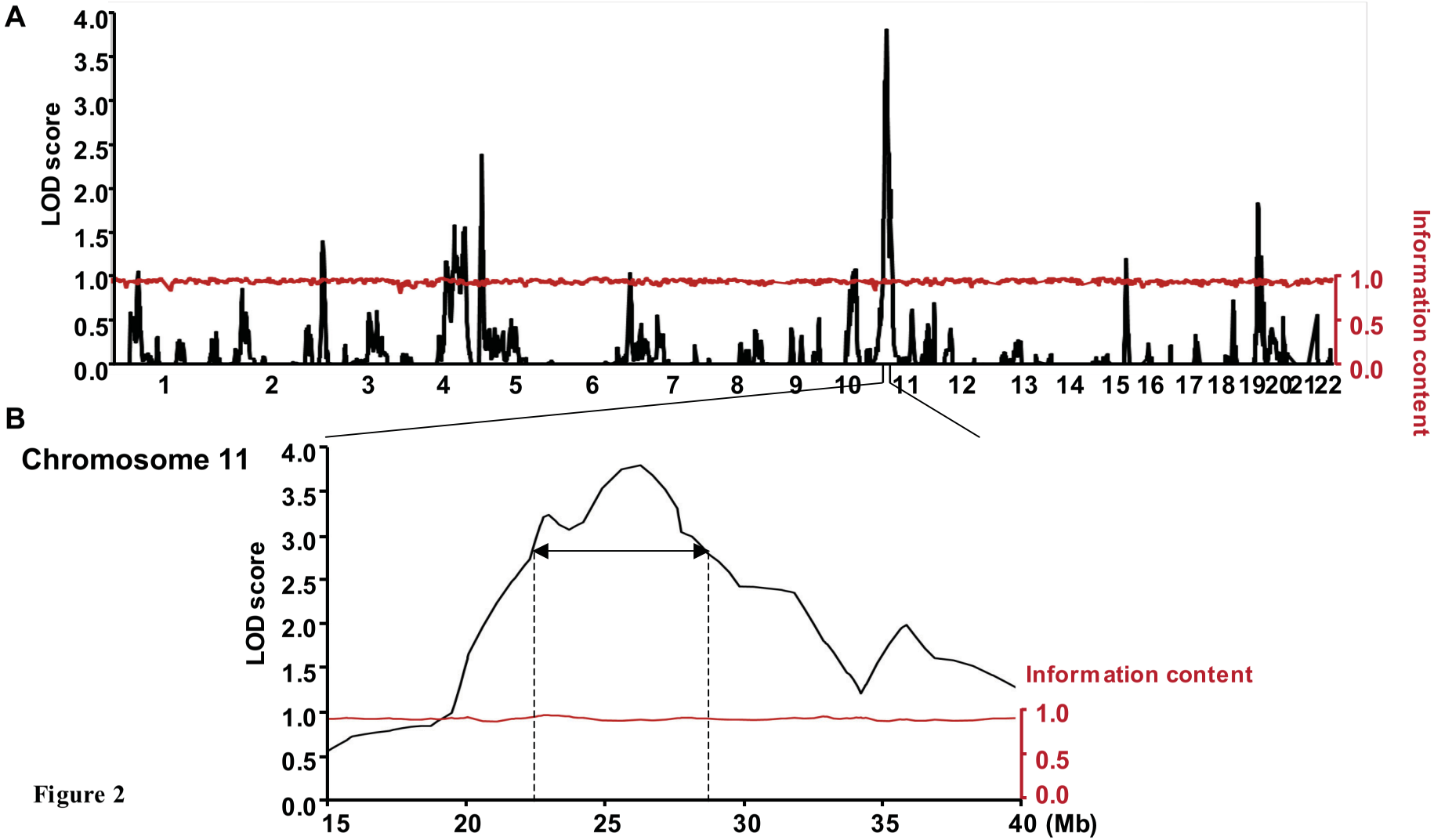
Age (years)
C
Standardized Pearson residuals
Age (years)
D
Standardized Tobit residuals
Age (years)

Figure 1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LOD score
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3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Information content
1.0
0.5
0.0
B
Chromosome 11
LOD score
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
15 20 25 30 35 40 (Mb)
Information content
1.0
0.5
0.0

Figure 2

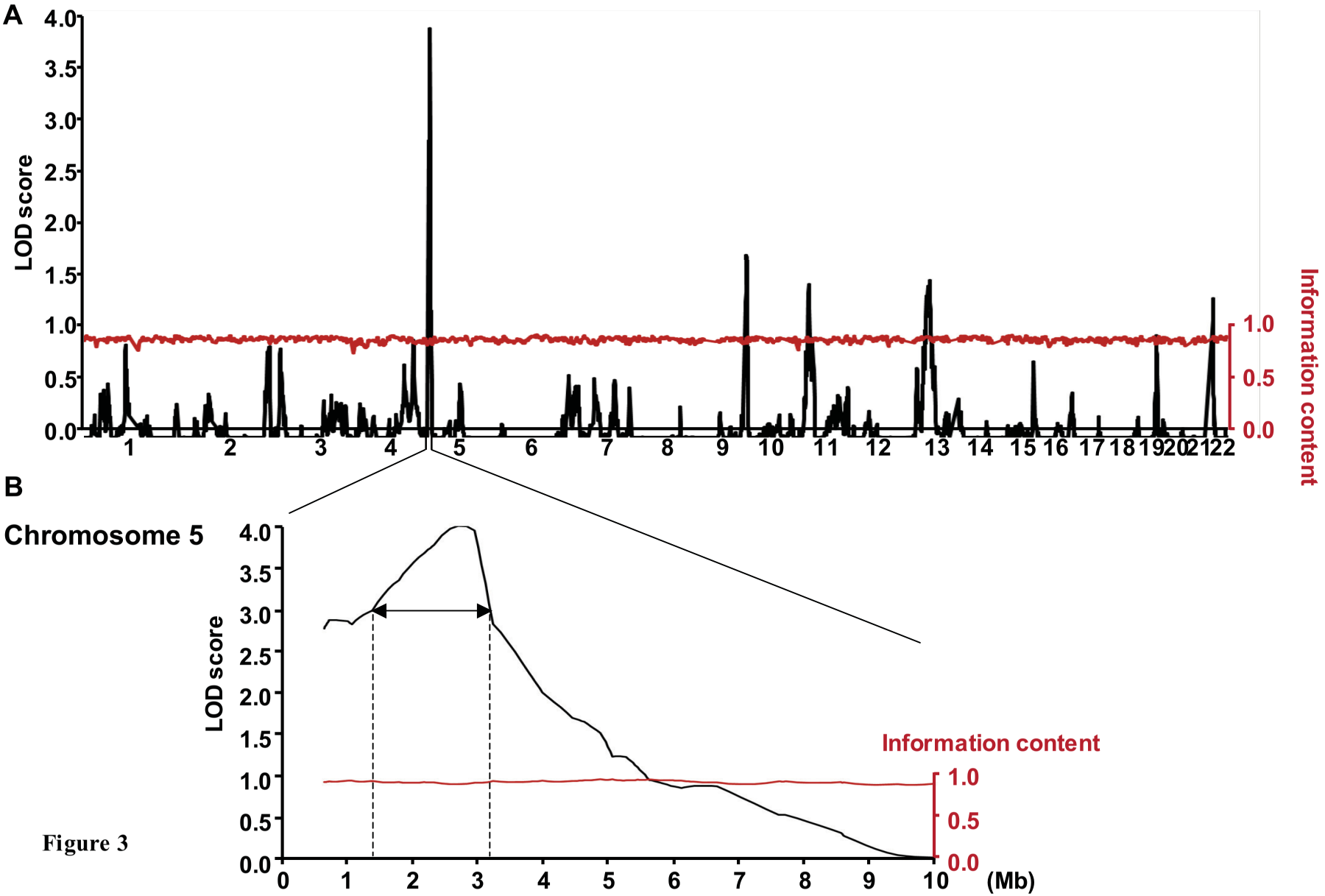
A
LOD score
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Information content
1.0
0.5
0.0
B
Chromosome 5
LOD score
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 (Mb)
Information content
1.0
0.5
0.0

Figure 3

Chapter 6
Reduced *in vitro* functional activity of human *NRAMP1* (*SLC11A1*) allele that predisposes to increased risk of pediatric tuberculosis disease

Chapter 6 presents the development and application of a functional assay to test the impact of specific risk alleles of *NRAMP1*, a well established tuberculosis susceptibility gene. The study was performed in a different group of subjects than described in Chapter 2-5. However, along with all the other studies presented in this thesis, it presents evidence for the importance of host factors for TB immunity.

The contents of this chapter is published in *Genes and Immunity* (2007) 8: 691-698. A copy of the published paper can be found in Appendix II.

Reduced *in vitro* functional activity of human *NRAMP1* (*SLC11A1*) allele that predisposes to increased risk of pediatric tuberculosis disease

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Keywords: tuberculosis, NRAMP1, functional assay, complex traits

Abstract

Polymorphic variants within the human natural resistance-associated macrophage protein-1 (*NRAMP1;* alias *SLC11A1*) gene have been shown to impact on susceptibility to tuberculosis in different human populations. In the mouse, Nramp1 is expressed at the macrophage phagosomal membrane and its activity can be assayed by the relative acquisition of mannose 6-phosphate receptor (M6PR) in *Salmonella*-containing vacuoles (SCV). Based on this M6PR recruitment assay, we have now developed an assay in primary human macrophages to test the function of human *NRAMP1* gene variants. First, we established that M6PR acquisition was significantly higher ($P = 0.002$) in human U-937 monocytic cell lines transfected with *NRAMP1* as compared to untransfected U-937 cells. Secondly, the M6PR assay was shown to be highly reproducible for NRAMP1 activity in monocyte-derived macrophages (MDM) from healthy volunteers. Finally, the assay was investigated in MDM from pediatric tuberculosis patients and significantly lower NRAMP1 activity was detected in MDM from individuals homozygous for the *NRAMP1*-274 high-risk allele (CC genotype) in comparison to heterozygous individuals (CT genotype; $P = 0.013$). The present study describes both an assay for human NRAMP1 functional activity and concomitant evidence for reduced NRAMP1 function in the common genetic variant shown to promote tuberculosis susceptibility in pediatric patients.

Introduction

According to recent figures, approximately 2 billion people worldwide are infected with *Mycobacterium tuberculosis*, the bacterium causing tuberculosis. The ongoing prevalence of tuberculosis results in nearly 9 million new tuberculosis disease cases and 2 million deaths each year[1]. In addition, there is an alarming rise in multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis*[2], which points to the urgent need to develop treatment strategies outside of the antibacterial spectrum. One avenue towards new therapeutic targets is based on identifying host genetic factors underlying susceptibility to infection.

Numerous genetic studies of tuberculosis disease have now been conducted, primarily in adults with pulmonary tuberculosis where the time lapse from infection with *M. tuberculosis* to clinically evident disease is unknown. Recently, we have focused on studies of primary tuberculosis disease (development of disease within 2 years of infection) on the assumption that early- and rapid-onset of tuberculosis disease may be less confounded by unmeasured environmental influences. In our studies, we have analyzed the human natural resistance-associated macrophage protein-1 gene (*NRAMP1,* alias *SLC11A1*) as a candidate gene, as several groups have identified an impact of *NRAMP1* on susceptibility to tuberculosis, as well as leprosy and inflammatory diseases[3-5]. We have identified common alleles of *NRAMP1* polymorphisms as major risk factors for primary tuberculosis in two exposure settings: among previously-unexposed adults in a tuberculosis disease outbreak in Northern Alberta, Canada[6], as well as among children in a low *M. tuberculosis* exposure setting in Houston, Texas[7]. Specifically, the *NRAMP1*-274C allele was identified as a major pediatric tuberculosis disease risk factor in families

with low exposure intensities to *M. tuberculosis*[7].

The *NRAMP1*-274C/T polymorphism corresponds to a silent nucleotide substitution in codon 66 (Phe) in exon 3 of the gene. In order to study the possible impact of the *NRAMP1*-274C allele on protein function, we have turned to the murine model of Nramp1 *in vitro* activity. Murine Nramp1 is primarily expressed at the phagosomal membrane of macrophages and functions as a pH-dependent divalent cation pump[8,9]. A single G169D mutation in murine Nramp1 leads to loss of a mature protein and causes increased susceptibility to intracellular pathogens, such as *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), *Salmonella typhimurium* and *Leishmania donovani*[10]. These pathogens avoid host killing by sequestration in phagosomes, albeit through different evasion mechanisms. Once *Salmonella* species invade their host cells, the bacteria are able to modify the composition and location of their phagosomal vacuoles in the cell to form an intracellular replication niche called the *Salmonella*-containing vacuole (SCV)[11-13]. The late-endosomal/lysosomal marker mannose-6-phosphate receptor (M6PR) is largely excluded from vacuoles containing live *Salmonella* bacteria[14,15]. However, there is greater recruitment of the M6PR to the SCV in *Nramp1*-expressing murine macrophages compared with non-expressing cells. Therefore, the influence of Nramp1 on recruitment provides a quantitative measure of Nramp1 activity[14].

In the present study, we have adapted the recruitment of M6PR to the SCV in human macrophages as a measure of NRAMP1 activity. Specifically, we have developed the SCV-M6PR assay in macrophages derived from blood as a method to study the impact on NRAMP1 function of known genetic variants. The results suggest that the

NRAMP1-274C allele may be linked with low innate macrophage function.

Material and Methods

Subjects: Pediatric tuberculosis patients were enrolled by the Baylor College of Medicine. Clinical criteria for diagnosis have previously been described[7]. Informed consent was obtained from all study participants. Upon agreement to participate, 8-10 ml blood was obtained from 10 patients and 5 healthy controls. Of the ten patient samples, one was lost during set-up of recruitment experiments. The study received ethical clearance from the Institutional Review Board at Baylor College of Medicine, Houston, TX, USA, and the Research Ethics Committee at the Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada.

NRAMP1 expression: RT-PCR and Real-Time PCR was performed to detect *NRAMP1* expression in U-937 and U-937+*NRAMP1* cells before and after stimulation with phorbol 12-myristate 13-acetate (PMA). RNA was isolated from the cells using a Qiagen (Mississauga, Canada) RNeasy Mini Kit and then 2 μg RNA was reversed transcribed into cDNA. RT-PCR was performed using specific NRAMP1 primers spanning intron 2 (forward: 5'gaagatccccatcccagac3'; reverse: 5cttccctagcccagctcct3') as well as GAPDH primers as a control. Real-Time PCR was performed on the same cDNA using a specific *NRAMP1* primer and TaqMan probe spanning intron 11 (Hs00184453_m1, TaqMan Gene Expression Assay, Applied Biosystems, California, USA). Expression of GAPDH, TBP and 18s genes were each tested to determine the best endogenous control

(Hs99999905_m1, Hs99999910_m1, Hs99999901_s1, respectively, TaqMan Gene Expression Assay, Applied Biosystems). All three performed equally well but only GAPDH was subsequently used as an endogenous control for the expression experiments.

M6PR and GFP-*Salmonella typhimurium* recruitment assay: Human U-937 and U-937 cells over-expressing a *NRAMP1*-c-Myc-tagged construct were seeded ($5x10^5$ cells/well) in a 12-well plate on glass coverslips. The *NRAMP1* construct was made with a pCB6 plasmid and a CMV-based promoter. Transfectant clones were verified for membrane protein expression via Western blot (not shown). The clone with the highest expression was chosen to be used in our study. U-937 cells carry the *NRAMP1*-274CC genotype. The transfected construct also carries a "C" at the nucleotide position 274. The cells were differentiated using 20 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Missouri, USA) for 48 hrs followed by a 24 hrs incubation without stimulation. The cells were grown in antibiotic-free RPMI 1640 supplemented with 10% fetal calf serum (inactivated, endotoxin tested), 2 mM L-glutamine and 1 mM sodium pyruvate (Invitrogen, Burlington, Canada). The cells were infected with *Salmonella enterica* serovar *Typhimurium* (*Salmonella)* containing a plasmid expressing the green fluorescent protein (GFP). The bacteria were grown in Luria-Bertani broth supplemented with tetracycline (12 μg/ml) overnight with shaking at 37 ^oC. The bacteria were subcultured at a 1:33 dilution for 3 hours at 37 ^oC to late log phase. After harvesting by centrifugation, the bacteria were washed with PBS and resuspended in Earle's Buffered Saline Solution (EBSS, pH 7.7; Invitrogen) to give an OD_{600} of approximately 0.2 before being added to the differentiated cells for 10 min at 37 ^oC, 5% CO_2 at a multiplicity of infection of

approximately 100:1. Subsequently, the cells were washed gently 3 times with PBS to remove non-internalized bacteria.

For experiments with MDM, a total of 8-10 ml of blood were used for each experiment. The blood was diluted 1:2 using RPMI 1640 with no supplements and layered over Ficoll-Paque (GE Healthcare, New Jersey, USA) and centrifuged for 30 min at 400 x g. The layer containing the mononuclear cells was removed. The cells were washed twice with RPMI for 10 min at 100 x g before being resuspended in RPMI with supplements. Mononuclear cells were seeded in 3-4 wells of a 12-well plate over glass coverslips. Macrophages were allowed to adhere overnight, washed once with both RPMI and PBS before being infected as described for U-937 cells such that a majority of infected cells phagocytosed 1-3 *Salmonella* bacteria.

Immediately following invasion, the cells were incubated in RPMI 1640 with supplements for 90 min at 37 ^{o}C, 5% CO_2 to allow for SCV maturation. Ice-cold blocking-buffer (5% normal goat serum in PBS) was added for 10 min. Extracellular *Salmonella* were detected using a rabbit anti-*Salmonella* antibody (1:300; Cortex Biochem, California, USA) for 15 min followed by a 20 min incubation with Alexa Fluor 350 labeled goat anti-rabbit secondary antibodies (1:500; Jackson ImmunoResearch, Pennsylvania, USA). Cells were fixed with 4% paraformaldehyde (PFA) for 45 min at room temperature, and blocked and permeabilized overnight with 5% normal goat serum plus 0.2% Triton X-100 in PBS. To label late-endosomes, cells were incubated the following day with a mouse anti-M6PR antibody (1:50; Affinity BioReagents, Colorado,

USA), mouse anti-LAMP1 antibody (1:300; Developmental Studies Hybridoma Bank, Iowa, USA), mouse anti-c-Myc (1:400; BD Biosciences, Mississauga, Canada) or rabbit anti-NRAMP1 (1:200) [16] for 1 hr followed by a Cy-3 conjugated goat anti-mouse or anti-rabbit secondary antibody for an additional 1 hr (1:500; Jackson ImmunoResearch).

Mounted glass coverslips were analyzed using conventional epifluorescent microscopy with a 63X oil objective. To quantify the level of recruitment between M6PR, LAMP1 or c-Myc-tagged NRAMP1 and GFP-expressing *Salmonella* bacteria, photos of the *Salmonella* (green), the M6PR or LAMP1 (red) and the extracellular-*Salmonella* (blue) were taken in the same plane and merged using the Northern Eclipse image software (Empix Imaging Inc., Mississauga, Canada). Extracellular bacteria were labeled to allow discrimination from phagocytosed bacteria. Only cells containing 1-3 bacteria were considered. At least 100 randomly chosen bacteria per slide were counted for each experiment.

***Salmonella* growth assay**: U-937 and U-937+*NRAMP1* cells were seeded (2-3 X 10^5 cells/well) in a 12-well plate. The cells were differentiated and infected as described for the M6PR and GFP-*Salmonella typhimurium* recruitment assay. Following infection, cells incubated for 24 h were maintained in RPMI with 100 μg/ml gentamicin for 2 h and then in 10 μg/ml gentamicin for the remaining time. At either 0 h or 24 h post-infection, cells were washed once with PBS and then lysed (1% Triton X-100/0.1% SDS in PBS) for 5 min at RT. The lysate was serially diluted and plated on LB agar plates. The CFUs were counted after incubating the plates for 24 h at 37 ^oC. Each time point was

performed in triplicate and each individual experiment was repeated six times. To accommodate experimental variation of baseline uptake of *Salmonella* by U-937 cells, *Salmonella* CFU at t = 0 h was subtracted in all experiments.

Statistical analysis: The difference in mean percent recruitment of *Salmonella* and the endocytic marker M6PR in U-937 cells was analyzed using a paired *t*-test. Recruitment values are shown as the mean ± standard error. The differences in *Salmonella* growth measured as LOG CFU 24 h – CFU 0h for U-937 and U-937+*NRAMP1* cells, and M6PR recruitment among MDM obtained from carriers of different *NRAMP1*-274C/T genotypes were analyzed using a two-tailed *t*-test. *P*-values < 0.05 were considered significant.

Results

Expression of *NRAMP1* in U-937 cells: The first series of experiments were undertaken in the human monocytic cell line U-937, both untransfected and stably expressing a *NRAMP1*-c-Myc-tagged construct (U-937+*NRAMP1*), to establish assay parameters. The level of *NRAMP1* mRNA expression in both cell types was determined before and after PMA treatment of U-937 cells, which is required for differentiation and adhesion of U-937 cells[17-19]. In wild-type U-937 cells there was no *NRAMP1* expression in PMA non-stimulated cells and very low expression after PMA stimulation. In contrast, there was strong *NRAMP1* expression in U-937 transfectants both before and after PMA stimulation as shown by PCR (Figure 1). The results of the mRNA expression studies were confirmed by immunolabeling with a *NRAMP1*-specific antibody. Employing immunofluorescence microscopy, wild-type U-937 expressed no or very low levels of

NRAMP1, while U-937+*NRAMP1* PMA stimulated cells expressed clearly detectable levels of c-Myc-NRAMP1 protein (Figure 2).

Recruitment of *Salmonella* with the late-endosomal marker LAMP1 and NRAMP1 in U-937 cells: In these experiments, we tested the intracellular localization of NRAMP1-c-Myc protein in the U-937 transfectants. U-937 cells were infected with *Salmonella typhimurium* and SCVs were identified by green fluorescent protein (GFP)-expressing *Salmonella*. NRAMP1-SCV recruitment was compared with the SCV marker lysosomal associated membrane protein-1 (LAMP1), a late-endosomal/lysosomal protein [14,20]. LAMP1 was immunolabelled with anti-LAMP1 and NRAMP1 was detected with an anti-c-Myc antibody since the use of NRAMP1 antibodies did not produce a signal of sufficient strength for recruitment experiments. *Salmonella* bacteria were verified to be internalized by immunolabeling extracellular bacteria with anti-LPS antibodies in the absence of host cell permeabilization. After immunolabeling the cells, we measured recruitment of LAMP1 and NRAMP1 to the GFP-expressing SCV in PMA-differentiated U-937 and U-937+*NRAMP1* cells (Figure 2). We found in four independent experiments that LAMP1 co-localized with 73.8% ±1.7% of SCV in U-937 cells and 72.9% ±1.7% in U-937+*NRAMP1* cells. To obtain an estimate of NRAMP1 recruitment to the SCV in U-937+*NRAMP1* cells, recruitment was quantified between the SCV and c-myc-tagged-*NRAMP1* in five independent experiments. The majority (70.9% ±2.7%) of SCVs in PMA stimulated U-937+*NRAMP1* cells show NRAMP1 recruitment (Figure 2). Nearly identical recruitment of LAMP1 to the SCV in U-937 and U-937+*NRAMP1* cells, and the efficient recruitment of NRAMP1 to the SCV suggest that over-expression of *NRAMP1*

does not cause a generalized de-regulation of endocytic vesicle trafficking.

Recruitment of *Salmonella* with the late-endosomal marker M6PR in U-937 cells: We assayed the number of phagocytosed GFP-expressing *Salmonella* that co-localize with M6PR in human PMA-differentiated wild-type U-937 and *NRAMP1* transfectants. Care was taken to analyze host cells that had phagocytosed only 1-3 *Salmonella* bacteria. Quantification of M6PR recruitment to SCV was done by counting at least 100 internalized bacteria per experiment in seven independent experiments. As in the LAMP1/NRAMP1 recruitment experiments, *Salmonella* bacteria were confirmed to be internalized by immunolabeling of extracellular bacteria with anti-LPS antibodies. Subsequently, U-937 wild type and *NRAMP1* transfectant cells were permeabilized and the proportion of SCV that had recruited M6PR was determined. A significantly higher percentage of recruitment between *Salmonella* and M6PR was reproducibly obtained in PMA differentiated U-937+*NRAMP1* cells (34.0% ± 2.9%) as compared to U-937 wild type cells (21.3% ± 2.42%, $P = 0.0002$) (Figure 3).

***Salmonella* growth assay in U-937 cells:** We next asked if there were differences in intracellular *Salmonella* growth in PMA-differentiated U-937+*NRAMP1* tranfectants and U-937 wildtype cells. In six independent experiments, PMA differentiated U-937 and U-937+*NRAMP1* cells were infected with *Salmonella* grown to late-log phase and CFUs were determined at 0 h and 24 h post-infection. The differences in *Salmonella* growth were measured as log CFU 24 h – 0 h for U-937 and U-937+*NRAMP1* cells. In U-937 cells, *Salmonella* consistently showed growth at 24 h post-infection. In comparison, U-937+*NRAMP1* cells either maintained their bacterial load (similar number of CFUs at 0 h

and 24 h post-infection) or showed bacterial clearance (P =0.0052; Figure 4). Taken together with the M6PR recruitment studies, these results demonstrated that both M6PR recruitment and *Salmonella* survival are modulated by the NRAMP1 protein.

Recruitment of *Salmonella* bacteria with the late-endosomal marker M6PR in monocyte-derived macrophages from healthy volunteers: In the next series of experiments, we tested if recruitment of M6PR to the SCV could also be detected in primary macrophage explants. We obtained monocyte-derived macrophages (MDM) from blood of healthy volunteers and normalized the number of cells per well before they were infected with GFP-expressing *Salmonella* bacteria at an approximate MOI 100:1. As in the U-937+*NRAMP1* transfectant system, a relatively weak immunofluorescence signal obtained with NRAMP1 antibodies precluded the determination of NRAMP1 recruitment. Recruitment of M6PR with the SCV was determined as for the U-937 cell experiments. We observed recruitment of M6PR to the SCV in MDM obtained from all five donors with experiments repeated at least twice for each donor. Overall, there was good inter-experimental reproducibility of M6PR recruitment by cells from the same donor suggesting that the observed differences in M6PR recruitment reflected intrinsic properties of the MDM (Table 1). Interestingly, recruitment efficiencies of M6PR to SCV in MDM from donors with the *NRAMP1*-274CC genotype did not overlap with those obtained in MDM from *NRAMP1*-274CT and *NRAMP1*-274TT donors.

Recruitment of *Salmonella* with the late-endosomal marker M6PR in monocyte-derived macrophages from pediatric tuberculosis patients: In a previous study, we had shown that among pediatric tuberculosis disease patients, *NRAMP1* was a strong risk

factor for tuberculosis disease. Specifically, we showed that the *NRAMP1* 274C allele predisposed to tuberculosis disease among simplex families with an odds ratio of 3.13 (95% confidence interval: 1.54 - 6.25). We therefore applied the M6PR assay to MDM from pediatric tuberculosis patients to test if risk alleles correlated with reduced functional NRAMP1 activity. We were able to obtain a second blood sample from 10 pediatric tuberculosis disease patients enrolled in the initial genetic study. In total, we successfully derived and infected MDM of 9 patients with GFP-*Salmonella* and determined the level of M6PR recruitment to the SCV for each individual.

The *NRAMP1* genotype for each patient was obtained only after M6PR/SCV recruitment had been determined for all samples. None of the patients carried a homozygous protective *NRAMP1*-274TT genotype. Hence, patients were assigned to two groups: those that were homozygous for the high-risk C allele (CC) and those that were heterozygous (TC). There was a significant difference in M6PR recruitment to the SCV between both groups ($P = 0.0129$; Figure 5). Individuals homozygous for the high-risk *NRAMP1* 274C allele recruited 29.0% ±1.0% while heterozygote 274C/T individuals on average showed significantly higher recruitment of 34.8% ±1.5%. Representative experiments of SCVs recruiting M6PR in MDM from the pediatric tuberculosis patients are shown in Figure 5. There was no discernible difference of genotype or M6PR recruitment between Black and Hispanic children; i.e. of the five *NRAMP1*-274CC homozygotes, two were Black and three were Hispanic and M6PR recruitment overlapped between cells obtained from children of both ethnic backgrounds.

Discussion

The human *NRAMP1* gene has been implicated with greater risk of tuberculosis disease by a number of studies and polymorphisms within *NRAMP1* have now been linked or associated with susceptibility to tuberculosis in at least 10 independent populations[4,6,7,21-27]. The focus of most studies was on susceptibility to smear-positive tuberculosis disease among adult populations. In order to dissect the genetic contribution to the onset of tuberculosis disease, we had previously analyzed the effect *NRAMP1* alleles had on risk of primary tuberculosis disease in a sample of pediatric patients from Houston, Texas. We identified the common *NRAMP1*-274C allele as major risk factor for early-onset pediatric tuberculosis[7]. Importantly, this finding replicated the results of the genetic analysis in an outbreak of primary tuberculosis among previously unexposed Canadian Aboriginals[6]. Considering that approximately half of all tuberculosis disease cases qualify as primary tuberculosis, a better understanding of NRAMP1 function is of critical importance.

The mechanism by which human *NRAMP1* disease associated variants impact on NRAMP1 activity, and how this altered activity is translated into increased disease risk is presently unknown. Murine Nramp1, found in the membranes of the late endosome/lysosome, influences phagosome maturation in both *Salmonella* and *Mycobacteria* infected cells[14,28]. However, the impact of Nramp1 on phagosome maturation is likely a secondary effect of its function as a metal transporter across the phagosomal membrane[9,29]. Here, we show that in analogy to murine Nramp1[14,29], NRAMP1 also impacts on intracellular vacuolar trafficking as revealed by differential recruitment of M6PR to SCVs at 90 min post-infection in PMA differentiated *NRAMP1*-

expressing or deficient U-937 cells. We used one time point to quantify recruitment as established in the murine model. In this model, a difference of recruitment efficiency of approximately 20% was detected between cell lines expressing (33%) or devoid of *Nramp1* (13%)[14]. It is possible that the smaller difference in recruitment between U-937 and U-937+*NRAMP1* cells was due to background NRAMP1 expression in PMA stimulated U-937 cells or other background differences between human and murine cells. Nevertheless, the results obtained in the human system were remarkably consistent with the mouse model and strongly support that human and murine NRAMP1 proteins impact on the same biological pathways in phagocytic cells. Importantly, these results also support that, like in murine macrophages, the extent of recruitment of M6PR to SCVs is a valid assay for quantitation of human *NRAMP1* functional activity.

The biological consequence of increased NRAMP1-dependent M6PR recruitment is not clear. It is likely that increased M6PR recruitment mirrors changes in phagosomal maturation that correct the ability of *Samonella* bacteria to subvert the normal degradative pathway of the phagosome to avoid killing by host cells. In addition, M6PR is an important mediator for the delivery of vacuoles containing lysosomal enzymes to the phagosome[30]. The observed difference in *Salmonella* bacterial growth between U-937 wild-type and *NRAMP1* transfectants supports the claim that altered vesicular trafficking, demonstrated by differential M6PR recruitment in this study, impacts on bacterial replication and survival. Further studies are required to fully elucidate the role of NRAMP1 in modulating bacterial replication by comparing intracellular growth of *Mycobacteria* and other pathogens[28].

Here, we provided direct evidence that primary explanted macrophages from carriers of high-risk *NRAMP1* genotypes displayed a phenotype of reduced NRAMP1 function as compared to carriers of an intermediate-risk genotype. Specifically, we detected significant differences of M6PR recruitment to SCVs in MDMs from pediatric tuberculosis patients homozygous for the high risk *NRAMP1*-274C allele as compared to heterozygous donors. These findings are in good agreement with the results of the genetic analysis done in this population and suggest that lower NRAMP1 activity is underlying the observed association of the *NRAMP1-274C* allele with risk of pediatric tuberculosis disease[7]. The detailed study of linkage disequilibrium pattern in pediatric tuberculosis patients[7] and the recent discovery that silent nucleotide changes can have substantial biological effects[31] make the *NRAMP1*-274C/T polymorphism a strong causative candidate for a tuberculosis susceptibility variant. Overall, the present results, once validated in larger data sets, will provide the mechanistic framework for the identification of physiological pathways that need boosting in individuals at high risk of developing primary tuberculosis disease.

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Legends to figures

Figure 1: *NRAMP1* expression in U-937 and U-937+*NRAMP1* cells before and after PMA stimulation. Wildtype U-937 cells show no detectable *NRAMP1* expression either by gel electrophoresis following 30 rounds of amplification (left) or by real time PCR (right). In PMA stimulated U-937 cells, low expression of *NRAMP1* is detectable by both methods. By contrast, significantly higher expression is observed in the c-Myc-*NRAMP1*-transfected cells before and after PMA stimulation. In the real time PCR graph, RNA obtained from U-937+*NRAMP1* expressing cells treated with PMA (yellow lines), U-937+*NRAMP1* expressing cells (green lines), PMA treated U-937 cells (red lines), and U-937 cells (purple lines). Internal standard is *GAPDH* expression. Each experimental condition is reflected by three independent RNA samples. On average, we observed a difference of 10 c_t between U-937 and U-937+*NRAMP1* PMA stimulated cells across repeat experiments.

Figure 2: NRAMP1 and LAMP1 are recruited to the SCV membrane. Recruitment to SCVs is given as the percentage of *Salmonella* co-localizing with either (A) NRAMP1 or (B) LAMP1. Mean recruitment is indicated by a small horizontal line. PMA differentiated U-937 and U-937+*NRAMP1* cells were infected with GFP-expressing *Salmonella* (green) and immunolabeled for LAMP1 or c-Myc-tagged NRAMP1 (red). SCVs are enriched for LAMP1 in both cells lines and for c-Myc-tagged-*NRAMP1* in the U-937+*NRAMP1* cells (arrows).

Figure 3: Recruitment of M6PR to phagocytosed *Salmonella* in PMA differentiated U-937 and U-937+*NRAMP1* cells. (A) Recruitment of M6PR to SCVs is given as the

percentage of *Salmonella* co-localizing with M6PR. Although there is inter-experiment variation, each experiment consistently shows increased recruitment of M6PR to the SCV in U-937+*NRAMP1*. Mean recruitment for either cell line is indicated by a small horizontal line. The difference in recruitment between parental and *NRAMP1*-transfected U-937 cells was highly significant at $P = 0.0002$. (B) U-937 and U-937+*NRAMP1* cells were infected with GFP-expressing *Salmonella* (green). Non-phagocytosed bacteria were immunolabeled with an anti-LPS antibody (blue) to allow discrimination of phagocytosed from extracellular bacteria (not shown). Cells were immunolabeled for M6PR (red). Use of low infectious load facilitated the accurate counting of co-localizing *Salmonella* and M6PR in individual vacuoles. Arrows point to typical recruitment events between M6PR and the SCV. SCVs without arrows are non-recruiting and are shown for comparison.

Figure 4: Intracellular growth of *Salmonella* bacteria in U-937 and U-937+*NRAMP1* cells**.** In six independent experiments, each cell line was infected with *Salmonella* bacteria. CFUs were counted at 0 h and 24 h post-infection and are represented graphically as LOG CFU difference between 24 h and 0 h. In U-937 cells (closed circle), *Salmonella* consistently showed growth at 24 h post-infection. In comparison, U-937+*NRAMP1* cells (open circle) either maintain the bacterial load (similar number of CFUs at 0 h and 24 h post-infection) or show bacterial clearance. The difference in CFU growth is highly significant at $P = 0.0052$.

Figure 5: Recruitment of M6PR to SCVs in MDM from pediatric tuberculosis disease patients according to their *NRAMP1* genotypes. (A) Recruitment of M6PR to SCVs is given as the percentage of SCV positive for M6PR. Recruitment levels are indicated for

pediatric tuberculosis patients who are homo- or heterozygous for the *NRAMP1*-274C high risk allele. Mean recruitment for the groups of homozygous and heterozygous patients is indicated by a small horizontal line. The difference in recruitment between the two patient groups was significant at $P = 0.0129$. (B) Shown are MDM from two different pediatric tuberculosis disease patients that are heterozygous *NRAMP1*-274C/T. MDM were obtained by standard protocols from peripheral blood leucocytes and infected with a low dose of GFP-expressing *Salmonella* bacteria (green) in order that each cell was infected with 1-3 bacteria. Arrows point to typical recruitment events between M6PR and the SCV. SCVs without arrows are non-recruiting and are shown for comparison.

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Table 1: Intra-individual M6PR recruitment variability to SCV in MDM obtained from healthy control individuals

Healthy Control	*NRAMP1 274 Genotype*	*% M6PR Recruitment to SCV*
1	CC	29, 36
2	CC	28, 35
3	CT	43, 45
4	CT	41, 39, 43, 46
5	TT	45, 46, 43

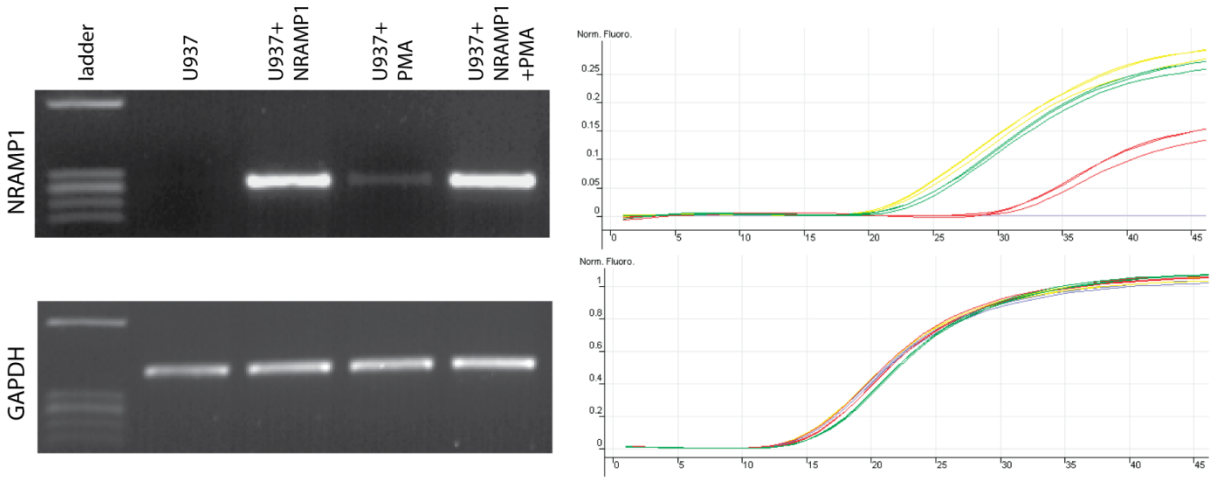
ladder
U937
U937+ NRAMP1
U937+ PMA
U937+ NRAMP1 +PMA
NRAMP1
GAPDH
Norm. Fluoro.
Norm. Fluoro.

Figure 1

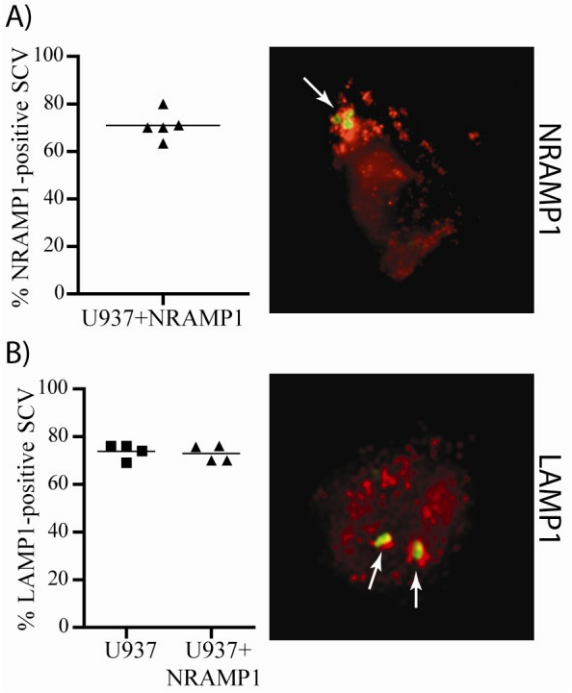
A)
% NRAMP1-positive SCV
100
80
60
40
20
0
U937+NRAMP1
NRAMP1
B)
% LAMP1-positive SCV
100
80
60
40
20
0
U937
U937+
NRAMP1
LAMP1

Figure 2

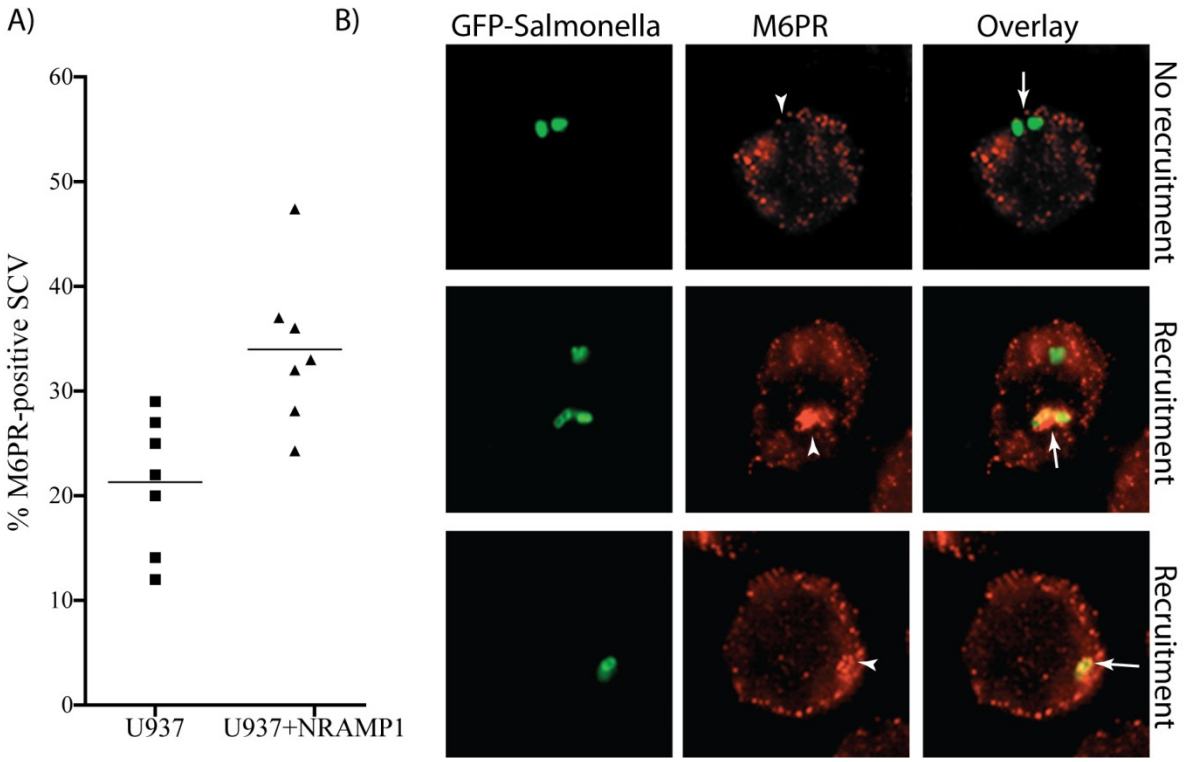
A)
B)
% M6PR-positive SCV
60
50
40
30
20
10
0
U937
U937+NRAMP1
GFP-Salmonella
M6PR
Overlay
No recruitment
Recruitment
Recruitment

Figure 3

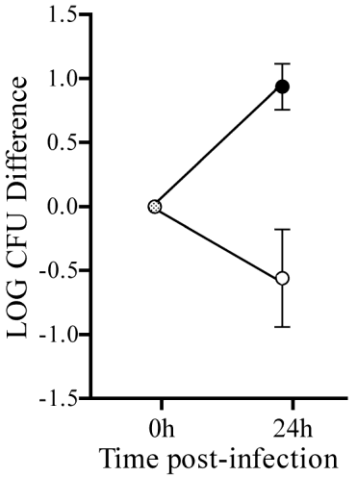
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
LOG CFU Difference
0h
24h
Time post-infection

Figure 4

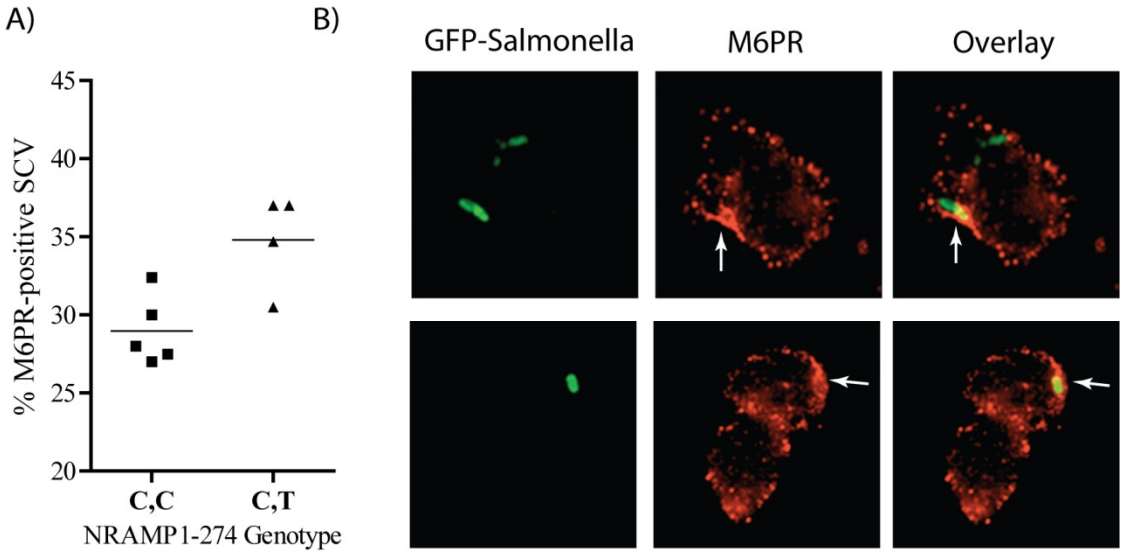
A)
B)
% M6PR-positive SCV
45
40
35
30
25
20
C,C
C,T
NRAMP1-274 Genotype
GFP-Salmonella
M6PR
Overlay

Figure 5

Chapter 7
Discussion and Conclusion

We performed a large, population-based comparative study of baseline anti-mycobacterial immunity in a TB endemic setting. This was the first simultaneous measurement of TST reactivity, effector molecules (IFNγ and TNFα) and effector cells ($CD4^+$ and $CD8^+$) in a population-based investigation for the comparative analysis of different aspects of the anti-mycobacterial immune response. We showed that *in vitro* IGRAs and the *in vivo* TST, two assays to detect *M. tuberculosis* infection, are non-redundant measures of anti-mycobacterial immunity. In addition, we observed a significant impact of age, but not sex, on both the extent of immune reactivity and assay positivity. The studies provide greater insight into the biology of the measured immune responses, particularly in children and young adults, an age group with a substantial gap in our knowledge of anti-TB immunity.

We calculated the heritability of the uni- and multivariate anti-mycobacterial immune response and obtained high estimates**.** Therefore, in the study population, the measured immunes phenotypes are highly heritable and amenable to gene cloning experiments. Moreover, the high heritability of the multivariate traits supports a model of pleiotropic gene regulation. We performed the first genome-wide linkage scan of the quantitative trait TST reactivity and demonstrated that reactivity is under sequential genetic control. We identified a major locus on 11p14 impacting on TST positivity *per se* (i.e. T-cell-independent resistance to *M. tuberculosis*), and another major locus on 5p15 impacting on the extent of the TST response (i.e. T-cell mediated DTH to tuberculin). The results from the genome scan suggest that host genetics may confound the use of the

TST in TB control programs, may confound the suggested relationship between TST size and risk of disease, and critically, modulates a protective response to *M. tuberculosis*. In addition, we demonstrated that mechanistic insight into disease pathogenesis is possible by dissecting the genetic control of key intermediate phenotypes. Finally, we developed an assay to test the functional activity of NRAMP1, a well established TB susceptibility protein, and provided evidence for the impact of *NRAMP1* TB disease risk alleles on protein function.

7.1. TUBERCULIN SKIN TEST AND INTERFERON-GAMMA RELEASE ASSAYS

Testing for TB infection is an integral part of TB control programs[53,140]. For nearly a century, the TST has been the only test available to detect TB infection yet it is an imperfect diagnostic tool. BCG vaccination and infection with NTM affect the specificity of the assay, TB patients display anergy and the TST cannot distinguish whether an infection is a result of recent exposure where symptoms have not yet developed, or from a long term infection where the host successfully contained the pathogen[112,113,115,116,123,136]. In response to the TST's disadvantages, a new class of tests have been developed – the IGRAs. The new tests are based on *in vitro* measures of anti-mycobacterial immunity analysed in our study and are proposed to replace or complement the TST[139]. Yet, before our population study of quantitative anti-mycobacterial immunity, we knew very little about the biology of the immune responses underlying IGRAs and importantly, how these responses related to the well established TST.

A number of studies have compared the performance of TST reactivity and IGRA[62,140]. Consistently, these studies identified groups of discordant individuals that are responders in one test but non-responders in the other (IGRA+/TST- and IGRA-/TST+)[93,140,144]. Deciphering the underlying cause(s) of the discordance is not straightforward as there is no gold standard to detect TB infection. However, a common explanation is that the IGRAs are more specific and sensitive to detect *M. tuberculosis* infection than the TST, especially in BCG vaccinated persons[62,140]. In our comparative analysis, we showed that discordant responders reflect an overall poor correlation of both assays. We demonstrated that the discordance is not simply a matter of antigens employed or assay specificity as we observed significant discordance using the same antigen (i.e. PPD) across assays. Finally, we also showed that intrinsic host characteristics, including age group and genetics, impact on assay outcome. Specifically, TST and ESAT-6-specific IFNγ release displayed distinct patterns of reactivity across age groups with TST showing an increase in reactivity in the older than 20 years age group whereas for ESAT-6, an increase was only observed in the 10-15 years group. As for host genetics, we observed high heritability estimates for the anti-mycobacterial immune measures and identified a major locus impacting on the extent of TST reactivity. Therefore, discordant groups of responders between the TST and IGRAs are at least in part caused by different aspects of the anti-mycobacterial immune response measured by the *in vivo* and *in vitro* assays, and the impact of host factors on these immune responses, rather than only reflecting higher specificity or sensitivity of IGRAs.

We recommend caution in directly replacing the TST with IGRAs before we have a greater understanding of the underlying biology of the new class of assays. Although we did not employ the commercial IGRAs in our study, we measured the quantitative immune responses underlying the new class of assays which allowed us to compare the immune responses to the TST. Additional characterization of the quantitative immune response would help explain the considerable variation in IGRA specificity and sensitivity observed across conditions (e.g. in contacts with recent TB exposure)[142] and populations (e.g. HIV+, young children, TB endemic)[119,145,148]. In addition, several studies have investigated the predictive value of IGRA positivity for the development of TB disease[156,409], yet the characteristics of the IGRA immune response related to disease progression are unknown[93]. Finally, we provided evidence that the two assays measure different aspects of anti-mycobacterial immunity and therefore TST test characteristics (e.g. predictive value for progression to disease[64,130,131]) are not immediately applicable to IGRAs.

7.2. TUBERCULOSIS IMMUNITY

It is well established that exposure to *M. tuberculosis* is necessary but not sufficient to cause infection and disease[52]. The different stages of disease pathogenesis – susceptibility to infection and progression to disease – are thought to be controlled by two different arms of the immune system. Innate immunity is proposed to independently modulate protection in the early stages of TB infection whereas an adaptive cell-mediated immune response is largely thought to be responsible for protection against development of active TB disease. While considerable work has been devoted to understanding

susceptibility to progression to disease following infection[9,64,68,77,80,83,87,88], the importance of the initial encounter between the host and pathogen on the outcome of TB exposure is only now emerging.

We know that close contacts of TB patients and persons living in communities with high TB prevalence are at high risk of becoming infected with *M. tuberculosis*[40,41,57]. However, across TB endemic populations there is evidence of individuals who remain negative in diagnostic tests of TB infection despite repeated exposure to the bacteria and who most likely represent individuals naturally resistant to TB infection[61,62,73]. Recently, a number of studies have highlighted the importance of innate immunity (i.e. T-cell independent immunity) in modulating protection in the early stages of TB infection[54,56,199,200,405,410]. The majority of these studies have been performed using *in vitro* tests or *in vivo* animal models. In contrast, our study was performed in a TB endemic human population. The identification of a major locus impacting on T-cell independent immunity in this study population provides evidence *"in natura"* - in natural, non-experimental conditions with humans[249] – of the importance of innate immunity modulating the initial encounter with the pathogen. The identification of the genetic variants and the pathways impacting on TB-specific innate immunity will contribute directly to efforts to develop novel therapeutics and vaccines to control TB infection.

For logistical reasons, the number of immune phenotypes analysed in this thesis was limited and there is a need for additional population-based investigations. For example, we did not measure the frequency of ESAT-6-specific IFNγ^+ T cells. Such data

would allow to determine if ESAT-6 IFNγ production is dependent on the frequency of antigen-specific T cells, which in turn would contribute to our understanding of the relationship between ELISA- and ELISPOT-based IGRAs that employ the ESAT-6 antigen. In addition, we did not determine if individual T cells were polyfunctional, i.e. expressed more than one cytokine (IFNγ, TNFα and IL-2), or if they were primarily effector or central memory cells. Recent studies have shown that polyfunctional and central memory T cells are associated with protection against disease[161,411]. Finally, we do not know whether distinct anti-mycobacterial immune profiles measured in our studies are correlated with progression to disease. Large population-based prospective studies where anti-mycobacterial immunity, including antigen-specific polyfunctional T-cells, is measured in healthy individuals and the subjects are monitored for progression to disease are needed.

7.3. GENOME-SCAN OF THE IMMUNE PHENOTYPES AS UNIVARIATE AND MULTIVARIATE TRAITS

The combination of the high heritability estimates and the identification of two major loci impacting on TST supports the use of intermediate phenotypes to dissect the mechanistic aspects of diseases susceptibility. Genetic analysis of the other key anti-mycobacterial immune responses studied in this thesis is therefore warranted and the logical next step will be to perform linkage analysis of antigen-specific IFNγ and TNFα production and the frequency of antigen-specific $IFN\gamma^+$ lymphocytes both independently and together as multivariate traits.

For the univariate traits, we expect to detect a number of independent loci impacting on anti-mycobacterial immunoreactivity. A previous genome-wide linkage study of lymphocytes identified loci impacting on variation in T cell subpopulations[408] which provides support for the presence of genetic control elements modulating the frequency of antigen-specific T cells. In addition, a genome-wide linkage analysis conducted in an Ugandan population reported suggestive linkage with a locus impacting on TB-specific TNFα production[405]. It is not clear if we will detect the same significant loci identified in the TST scan. If the locus linked to TST positivity *per se* truly reflects genetically controlled T-cell independent resistance, then we would expect, at least theoretically, to also detect linkage with positivity of antigen-specific IFNγ release or antigen-specific $IFN\gamma^+$ T cells. These immune-phenotypes, like the TST, detect TB infection based on T cell sensitization, and persistent lack of sensitization in this endemic setting most likely represents resistance. However, in our data, ESAT-6 displayed low immunogeneticity and may therefore lack sensitivity to detect TB infection when used alone (i.e. without other *M. tuberculosis*-specific stimulating antigens). In addition, BCG and PPD antigens are not specific to *M. tuberculosis* and therefore positivity in these assays do not necessarily reflect TB infection[123]. Finally, it is not clear if the locus impacting on the extent of TST reactivity will also be involved in the control of antigen-specific IFNγ production or the frequency of $IFN\gamma^+$ T cells as our results suggest that the locus may be specific to DTH response to mycobacteria.

Antigen-specific IFNγ production and the frequency of antigen-specific IFNγ$^+$ lymphocytes are both measures of anti-mycobacterial immunity and are therefore correlated to some degree. As multivariate traits we observed high heritability estimates, however, we do not know the genetic components that underlie the significant estimates. A possible and attractive explanation of the correlation between phenotypes is the existence of immune regulator genes with pleiotropic effects on different immune pathways[412]. This would be the first report of pleiotropic QTLs impacting on TB immunity. Along with the results of the TST linkage study, genetic analysis of the remaining immune phenotypes will help determine the effect of host genetics on inter-individual variability of key anti-mycobacterial immune responses, and whether genetically-controlled variability impacts on disease pathogenesis and the use of immune-based diagnostics to detect TB infection.

7.4. REPLICATION, VALIDATION AND FUNCTIONAL CHARACTERIZATION

A key aspect of genotype-phenotype association studies in complex traits is the replication, validation and functional characterization of the genetic findings. The terms validation and replication are often used interchangeably but are suggested to represent different methods[413]. With replication, the aim is to reproduce the association of a genetic marker with a phenotype – same SNP allele with the same phenotype in the same direction under the genetic model – in an independent sample but from the same population background as the initial study was performed. With validation, the

confirmation sample is drawn from an independent population that is different from the original study[413]. Both replication and validation greatly increases confidence in the importance of observed associations (i.e. association is unlikely a false positive) and provide the impetus for functional studies that are required for mechanistic understanding of the studied trait.

Replication and validation studies will be used to confirm any variants identified by fine mapping to be significantly associated with TST reactivity. First, association will be confirmed in an independent sample of individuals recruited from the same ethnic group and area of Cape Town. Second, validation will be sought in different populations where TST reactivity is measured. For the locus on chromosome region 11p14 shown to modulate T-cell independent immunity, alternative study designs can be pursued for the validation studies. Many of the subjects recruited in the current study were young (< 15 years) and thus it is possible that a small number of individuals with a negative TST were never exposed to *M. tuberculosis* and do not represent innately resistance individuals. Therefore, it will be necessary to more directly control for TB exposure. For example, recruitment could focus on contacts of TB cases with prolonged and sustained exposure that either remain TST and IGRA negative or become positive in both tests of infection. Alternatively, recruitment could focus specifically on older subjects (> 35 years) living in a TB endemic setting with a very high annual risk of infection - the incidence of infection in a given population. At least theoretically, all individuals living in the area with an annual risk of infection of 3-4% would have been exposed by the age of 35 years.

Following validation and replication, the most challenging next step will likely be functional studies to determine if any associated variant is truly causal and to determine its role in modulating innate resistance to *M. tuberculosis* infection or T-cell mediated DTH to tuberculin. At present, we do not know what genes or SNP alleles will be found to be associated with TST reactivity. The associated SNP(s) could be non-synonymous[414] and therefore possibly directly impact on protein function. More likely, the associated SNP(s) could be synonymous or found in intergenic regions. If this is the case, identifying the causal variant and determining its impact will be more difficult as they present no obvious effect on a gene or protein. The associated SNP could be regulatory[245,415] and therefore an appropriate assay to test gene expression would need to be designed. The associated SNP may be in linkage disequilibrium with the causal mutation. This was seen for a SNP within the *IRGM* gene associated with Crohn's disease – it was discovered that the associated SNP was strongly correlated with a mutation upstream of the gene affecting tissue-specific expression[269]. It is also possible that the causal SNP is perfectly correlated with other SNPs in close proximity[308] making it impossible to distinguish them without designing allele-specific assays[416,417]. Following testing of associated variants for causation, experiments will be designed to test the role of the implicated genes on the trait of interest (i.e. innate resistance or TST reactivity).

Association between *NRAMP1* and TB disease susceptibility has been independently replicated across numerous different populations making it an established TB susceptibility gene[90,368-371]. Despite the numerous associations, no study before ours had shown a relationship between *NRAMP1* gene variants and protein function. In a

study of paediatric TB patients from Houston, Texas, a common *NRAMP1* allele was identified as a major TB disease risk factor[90]. The polymorphism corresponds to a silent nucleotide substitution with no obvious effect on protein structure or expression, however a previous study had shown that silent nucleotide changes can have substantial biological effects[418]. We therefore decided to develop a gene-specific assay to test the impact of the polymorphism on protein function. We exploited NRAMP1's role modulating the recruitment of the M6PR protein to the phagosomal membrane as a means to test its function[419]. A key aspect of functional assays is that they need to be sensitive and precise enough in order to detect significant differences in function if the measured allelic impact is small and possibly additive. In our assay, we were able to detect a significant difference in NRAMP1 function between individuals homozygous or heterozygous for the risk allele. The next and crucial step that has yet to be performed will be to establish the link between variation in NRAMP1 function and impact on TB disease risk.

7.5. NEXT STEP: HIGH RESOLUTION LINKAGE DISEQUILIBRIUM MAPPING

The immediate next step in our study of anti-mycobacterial immunity will be high resolution linkage disequilibrium mapping, or fine mapping, to identify the genetic variants underlying the TST linkage peaks on chromosome regions 5p15 and 11p14. Briefly, the strategy we intend to pursue includes defining the 90% confidence interval of the linkage peak (corresponds to the 1-LOD down interval) and identifying all the genes and open reading frames within the interval. SNPs tagging bins - groups of correlated SNPs - across the gene intervals are chosen in order to capture > 90% of the overall SNP

genetic variation reported in the Yoruba HapMap population (representative African population). If no significant association with TST reactivity or positivity *per se* is detected with the chosen SNP gene variants, intergenic regions are targeted next. To "ultra-fine" map regions showing significant association, comparative sequencing of approximately 20-25 unrelated subjects is done and newly discovered and uncorrelated polymorphic SNPs are included in the next round of association testing. It is possible that polymorphic structural variants, including small insertions-deletions or inversions, will also be detected during comparative sequencing experiments[261]. These variants will also be investigated for association to disease and functional impact. The fine mapping method described here has successfully led to the positional cloning of underlying susceptibility variants for leprosy[279,308] and asthma[420].

7.6. CONCLUSION

Despite the long co-evolution of humans and *M. tuberculosis*, we still cannot answer some of the critical questions concerning detection and protection from TB infection and susceptibility to TB disease. Determining who is infected, and among the infected who will progress to disease is instrumental to control a pathogen that is estimated to have infected one-third of the human population. Beyond understanding the dynamics of TB infection, the ultimate goal of TB control is to prevent infection altogether. Preventative therapy based on innate resistance may offer an attractive avenue of TB control for persons at high risk of progressing to severe disease following infection, most notably children and HIV/AIDS patients. However, to date little research has been dedicated to understanding the mechanisms modulating the initial encounter between the

host and pathogen. The research in this thesis contributes significantly to our understanding of the anti-mycobacterial immune responses underlying detection of infection and has laid the ground-work for dissecting the pathways related to innate resistance to TB infection.

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Original Contributions to Knowledge

1. Performed the first, population-based comparative study of key anti-mycobacterial immune responses in young individuals living in a region hyper-endemic for tuberculosis;

2. Revealed low redundancy between *in vitro* and *in vivo* measures of anti-mycobacterial immunity;

3. Identified an effect of age on anti-mycobacterial immunity measured by IFNγ T cell assays. This finding has important implications for the use of tuberculosis immune-based diagnostics and vaccine design;

4. Calculated the heritability of multivariate anti-mycobacterial immune responses and observed high estimates. The results suggest an effect of pleiotropic regulatory genes and therefore open up a potential new of avenue of research in host genetic control of tuberculosis immunity;

5. Performed the first genome-wide linkage scan of TST reactivity and identified a sequential model of genetic control;

6. Identified for the first time a major locus impacting on resistance to tuberculosis infection and a second major locus controlling the extent of T-cell mediated delayed type hypersensitivity to tuberculin;

7. Designed and successfully applied a functional assay to test *NRAMP1* tuberculosis disease risk alleles.

Appendix I
Research Ethics Board Approval Letters

Centre universitaire de santé McGill
McGill University Health Centre
Les meilleurs soins pour la vie
The Best Care for Life

October 24, 2008

Dr. Erwin Schurr
Research Institute
Montreal General Hospital

RE: GEN#06-030 entitled "Cloning of Genes Impacting on Human Immune Responses to Mycobacteria."

Dear Dr. Schurr:

We have received an Application for Continuing Review of the Montreal General Hospital Research Ethics Committee for the research study referenced above. The report was found to be acceptable for continued conduct at the McGill University Health Centre.

At the MUHC, sponsored research activities that require US federal assurance are conducted under Federal Wide Assurance (FWA) 00000840.

We are pleased to inform you that re-approval for the above-mentioned study was provided via review by the Co-Chair on October 24, 2008, valid until October 23, 2009. It is cited in your report that the study is active and subjects have been and continue to be enrolled in the study.

Please take note that all research involving human subjects requires review at a regular interval and it is the responsibility of the principal investigator to submit an application for Continuing Review before the expiration of the study approval. However, should the research be concluded for any reason prior to the next review, a Termination Report is required for submission to the Committee once the study analysis is complete, to give an account of the study findings and publication status.

Should any revision to the research or other unanticipated development occur prior to the next required review, please advise the REB promptly and prior to initiating a proposed revision.

We trust this will prove satisfactory to you.

Sincerely,

Denis Cournoyer, M. D.
Co-Chairman
GEN-Research Ethics Board
MUHC-Montreal General Hospital

UNIVERSITEIT VAN STELLENBOSCH
UNIVERSITY OF STELLENBOSCH

5 October 2001

Prof N Beyers
Department of Paediatrics and Child Health

Dear Prof Beyers

RESEARCH PROJECT: "AN EVALUATION OF SOCIAL AND GENETIC FACTORS INFLUENCING THE SUSCEPTIBILITY OF INDIVIDUALS TO TUBERCULOSIS"
PROJECT NUMBER : 95/072

Your letter dated 28 June 2001 refers.

I hereby confirm that the Manager: Research Development and Support (Tygerberg), approved the following documents on 5 October 2001 in accordance with the authority granted to him by Subcommittee C of the Research Committee as it is in compliance with the guidelines of the Committee:

1. The proposed amendment as set out in your letter.

2. The amended patient information and consent forms.

Yours faithfully

CJ VAN TONDER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)

CJVT/ev

cc Dr EG Hoal-Van Helden

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2 November 2001

Prof N Beyers
Department of Paediatrics and Child Health

Dear Prof Beyers

RESEARCH PROJECT: **"AN EVALUATION OF SOCIAL AND GENETIC FACTORS INFLUENCING THE SUSCEPTIBILITY OF INDIVIDUALS TO TUBERCULOSIS"**
PROJECT NUMBER : **95/072**

My letter dated 5 October 2001 refers.

At a meeting that was held on 29 October 2001 Subcommittee C of the Research Committee ratified the approval of the amendment and the amended patient information and consent documents by the Manager: Research Development and Support (Tygerberg).

Yours faithfully

CJ VAN TONDER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)

CJVT/ev

cc Dr EG van Helden

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27 June 2002

Dr EG van Helden
Dept of Medical Physiology and Biochemistry

Dear Dr Van Helden

RESEARCH PROJECT: **"AN EVALUATION OF SOCIAL AND GENETIC FACTORS INFLUENCING THE SUSCEPTIBILITY OF INDIVIDUALS TO TUBERCULOSIS"**
PROJECT NUMBER : **95/072**

Your letter dated 26 June 2002 refers.

The Manager: Research Development and Support (Tygerberg), approved the proposed amendment as well as the amended Afrikaans and English patient information and consent forms in accordance with the authority given to him by Subcommittee C of the Research Committee.

This approval will be submitted to the Committee for ratification.

Yours faithfully

CJ VAN TONDER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)

CJVT/ev

cc Prof N Beyers

Fakulteit Gesondheidswetenskappe • Faculty of Health Sciences

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Appendix II
Paper Reprint and Licence

Genes and Immunity (2007) 8, 691–698

www.nature.com/gene

ORIGINAL ARTICLE

Reduced in vitro functional activity of human NRAMP1 (SLC11A1) allele that predisposes to increased risk of pediatric tuberculosis disease

CJ Gallant[1,2], S Malik[1,2], N Jabado[3], M Cellier[4], L Simkin[1], BB Finlay[5], EA Graviss[6,7], P Gros[3], JM Musser[8] and E Schurr[1,2]

[1]McGill Centre for the Study of Host Resistance, Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada; [2]Departments of Medicine and Human Genetics, McGill University, Montreal, Quebec, Canada; [3]Department of Biochemistry, McGill University, Montreal, Quebec, Canada; [4]Institut National de la Recherche Scientifique, INRS-Institut Armand-Frappier, Laval, Quebec, Canada; [5]Biotechnology Laboratory, Department of Biochemistry, University of British Columbia, Vancouver, BC, Canada; [6]Center for Human Bacterial Pathogenesis Research, Houston, TX, USA; [7]Department of Pathology, Baylor College of Medicine, Houston, TX, USA and [8]Center for Molecular and Translational Human Infectious Disease Research, Department of Pathology, The Methodist Hospital Research Institute, Houston, TX, USA

Polymorphic variants within the human natural resistance-associated macrophage protein-1 (NRAMP1, also known as SLC11A1) gene have been shown to impact on susceptibility to tuberculosis in different human populations. In the mouse, Nramp1 is expressed at the macrophage phagosomal membrane and its activity can be assayed by the relative acquisition of mannose 6-phosphate receptor (M6PR) in Salmonella-containing vacuoles. Based on this M6PR recruitment assay, we have now developed an assay in primary human macrophages to test the function of human NRAMP1 gene variants. First, we established that M6PR acquisition was significantly higher ($P = 0.002$) in human U-937 monocytic cell lines transfected with NRAMP1 as compared to untransfected U-937 cells. Second, the M6PR assay was shown to be highly reproducible for NRAMP1 activity in monocyte-derived macrophages (MDM) from healthy volunteers. Finally, the assay was investigated in MDM from pediatric tuberculosis patients and significantly lower NRAMP1 activity was detected in MDM from individuals homozygous for the NRAMP1-274 high-risk allele (CC genotype) in comparison to heterozygous individuals (CT genotype; $P = 0.013$). The present study describes both an assay for human NRAMP1 functional activity and concomitant evidence for reduced NRAMP1 function in the common genetic variant shown to be associated with tuberculosis susceptibility in pediatric patients.

Genes and Immunity (2007) **8,** 691–698; doi:10.1038/sj.gene.6364435; published online 4 October 2007

Keywords: *tuberculosis; NRAMP1; functional assay; complex traits*

Introduction

According to recent figures, approximately 2 billion people worldwide are infected with *Mycobacterium tuberculosis*, the bacterium causing tuberculosis. The ongoing prevalence of tuberculosis results in nearly 9 million new tuberculosis disease cases and 2 million deaths each year.[1] In addition, there is an alarming rise in multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis*,[2] which points to the urgent need to develop treatment strategies outside of the antibacterial spectrum. One avenue toward new therapeutic targets is based on identifying host genetic factors underlying susceptibility to infection.

Correspondence: Dr E Schurr, Departments of Medicine and Human Genetics, Montreal General Hospital Research Institute, McGill University, 1650 Cedar Avenue, Room L11-521, Montreal, Quebec, Canada H3G 1A4.
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Received 21 June 2007; revised and accepted 4 September 2007; published online 4 October 2007

Numerous genetic studies of tuberculosis disease have now been conducted, primarily in adults with pulmonary tuberculosis where the time lapse from infection with *M. tuberculosis* to clinically evident disease is unknown. Recently, we have focused on studies of primary tuberculosis disease (development of disease within 2 years of infection) on the assumption that early- and rapid-onset of tuberculosis disease may be less confounded by unmeasured environmental influences. In our studies, we have analyzed the human natural resistance-associated macrophage protein-1 gene (*NRAMP1*, also known as *SLC11A1*) as a candidate gene, as several groups have identified an impact of *NRAMP1* on susceptibility to tuberculosis, as well as leprosy and inflammatory diseases.[3–5] We have identified common alleles of *NRAMP1* polymorphisms as major risk factors for primary tuberculosis in two exposure settings: among previously unexposed adults in a tuberculosis disease outbreak in Northern Alberta, Canada,[6] as well as among children in a low *M. tuberculosis* exposure setting in Houston (TX, USA).[7] Specifically, the *NRAMP1*-274C

allele was identified as a major pediatric tuberculosis disease risk factor in families with low exposure intensities to *M. tuberculosis*.[7]

The *NRAMP1*-274C/T polymorphism corresponds to a silent nucleotide substitution in codon 66 (Phe) in exon 3 of the gene. In order to study the possible impact of the *NRAMP1*-274C allele on protein function, we have turned to the murine model of Nramp1 *in vitro* activity. Murine Nramp1 is primarily expressed at the phagosomal membrane of macrophages and functions as a pH-dependent divalent cation pump.[8,9] A single G169D mutation in murine Nramp1 leads to loss of a mature protein and causes increased susceptibility to intracellular pathogens, such as *Mycobacterium bovis* bacillus Calmette-Guerin, *Salmonella typhimurium* and *Leishmania donovani*.[10] These pathogens avoid host killing by sequestration in phagosomes, albeit through different evasion mechanisms. Once *Salmonella* species invade their host cells, the bacteria are able to modify the composition and location of their phagosomal vacuoles in the cell to form an intracellular replication niche called the *Salmonella*-containing vacuole (SCV).[11–13] The late-endosomal/lysosomal marker mannose 6-phosphate receptor (M6PR) is largely excluded from vacuoles containing live *Salmonella* bacteria.[14,15] However, there is greater recruitment of the M6PR to the SCV in *Nramp1*-expressing murine macrophages compared with nonexpressing cells. Therefore, the influence of Nramp1 on recruitment provides a quantitative measure of Nramp1 activity.[14]

In the present study, we have adapted the recruitment of M6PR to the SCV in human macrophages as a measure of NRAMP1 activity. Specifically, we have developed the SCV-M6PR assay in macrophages derived from blood as a method to study the impact on NRAMP1 function of known genetic variants. The results suggest that the *NRAMP1*-274C allele may be linked with low innate macrophage function.

Results

Expression of NRAMP1 *in U-937 cells*

The first series of experiments were undertaken in the human monocytic cell line U-937, both untransfected and stably expressing an *NRAMP1*-c-Myc-tagged construct (U-937 + *NRAMP1*), to establish assay parameters. The level of *NRAMP1* mRNA expression in both cell types was determined before and after PMA treatment of U-937 cells, which is required for differentiation and adhesion of U-937 cells.[16–18] In wild-type U-937 cells there was no *NRAMP1* expression in PMA nonstimulated cells and very low expression after PMA stimulation. In contrast, there was strong *NRAMP1* expression in U-937 transfectants both before and after PMA stimulation as shown by PCR (Figure 1). The results of the mRNA expression studies were confirmed by immunolabeling with an *NRAMP1*-specific antibody. Employing immunofluorescence microscopy, wild-type U-937 expressed no or very low levels of NRAMP1, while U-937 + *NRAMP1* PMA-stimulated cells expressed clearly detectable levels of c-Myc-NRAMP1 protein (Figure 2).

Recruitment of Salmonella *with the late-endosomal marker LAMP1 and NRAMP1 in U-937 cells*

In these experiments, we tested the intracellular localization of NRAMP1-c-Myc protein in the U-937 transfectants. U-937 cells were infected with *S. typhimurium* and SCVs were identified by green fluorescent protein (GFP)-expressing *Salmonella*. NRAMP1-SCV recruitment was compared with the SCV marker lysosomal-associated membrane protein-1 (LAMP1), a late-endosomal/lyso-

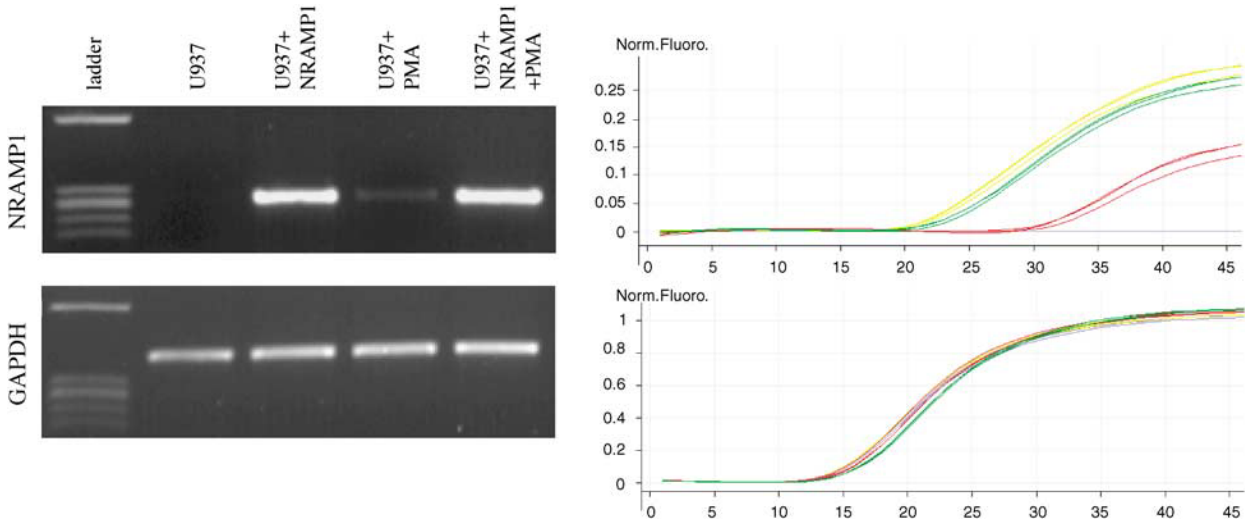

Figure 1 *NRAMP1* expression in U-937 and U-937 + *NRAMP1* cells before and after PMA stimulation. Wild-type U-937 cells show no detectable *NRAMP1* expression either by gel electrophoresis following 30 rounds of amplification (left) or by real-time PCR (right). In PMA-stimulated U-937 cells, low expression of *NRAMP1* is detectable by both methods. By contrast, significantly higher expression is observed in the c-Myc-*NRAMP1*-transfected cells before and after PMA stimulation. In the real-time PCR graph, RNA obtained from U-937 + *NRAMP1*-expressing cells treated with PMA (yellow lines), U-937 + *NRAMP1*-expressing cells (green lines), PMA-treated U-937 cells (red lines), and U-937 cells (purple lines). Internal standard is *GAPDH* expression. Each experimental condition is reflected by three independent RNA samples. On average, we observed a difference of 10 threshold cycles (C_t) between U-937 and U-937 + *NRAMP1* PMA-stimulated cells across repeat experiments.

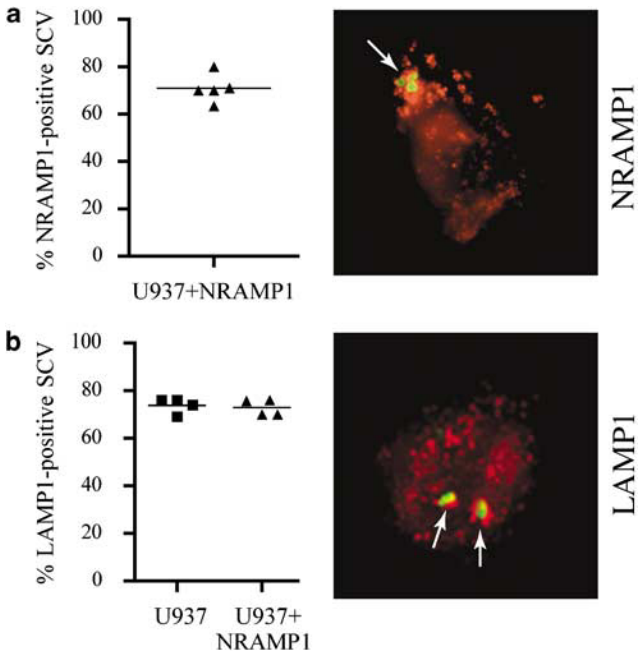

Figure 2 NRAMP1 and LAMP1 are recruited to the SCV membrane. Recruitment to SCVs is given as the percentage of *Salmonella* co-localizing with either (**a**) NRAMP1 or (**b**) LAMP1. Mean recruitment is indicated by a small horizontal line. PMA-differentiated U-937 and U-937 + *NRAMP1* cells were infected with GFP-expressing *Salmonella* (green) and immunolabeled for LAMP1 or c-Myc-tagged NRAMP1 (red). SCVs are enriched for LAMP1 in both cells lines and for c-Myc-tagged *NRAMP1* in the U-937 + *NRAMP1* cells (arrows).

somal protein.[14,19] LAMP1 was immunolabeled with anti-LAMP1 and NRAMP1 was detected with an anti-c-Myc antibody since the use of NRAMP1 antibodies did not produce a signal of sufficient strength for recruitment experiments. *Salmonella* bacteria were verified to be internalized by immunolabeling extracellular bacteria with anti-LPS antibodies in the absence of host cell permeabilization. After immunolabeling the cells, we measured recruitment of LAMP1 and NRAMP1 to the GFP-expressing SCV in PMA-differentiated U-937 and U-937 + *NRAMP1* cells (Figure 2). We found in four independent experiments that LAMP1 co-localized with 73.8 ± 1.7% of SCV in U-937 cells and 72.9 ± 1.7% in U-937 + *NRAMP1* cells. To obtain an estimate of NRAMP1 recruitment to the SCV in U-937 + *NRAMP1* cells, recruitment was quantified between the SCV and c-Myc-tagged *NRAMP1* in five independent experiments. The majority (70.9 ± 2.7%) of SCVs in PMA-stimulated U-937 + *NRAMP1* cells show NRAMP1 recruitment (Figure 2). Nearly identical recruitment of LAMP1 to the SCV in U-937 and U-937 + *NRAMP1* cells, and the efficient recruitment of NRAMP1 to the SCV suggest that over-expression of *NRAMP1* does not cause a generalized deregulation of endocytic vesicle trafficking.

Recruitment of Salmonella *with the late-endosomal marker M6PR in U-937 cells*

We assayed the number of phagocytosed GFP-expressing *Salmonella* that co-localize with M6PR in human PMA-differentiated wild-type U-937 and *NRAMP1* transfectants. Care was taken to analyze host cells that had phagocytosed only 1–3 *Salmonella* bacteria. Quantification of M6PR recruitment to SCV was done by counting at least 100 internalized bacteria per experiment in seven independent experiments. As in the LAMP1/NRAMP1 recruitment experiments, *Salmonella* bacteria were confirmed to be internalized by immunolabeling of extracellular bacteria with anti-LPS antibodies. Subsequently, U-937 wild-type and *NRAMP1* transfectant cells were permeabilized and the proportion of SCV that had recruited M6PR was determined. A significantly higher percentage of recruitment between *Salmonella* and M6PR was reproducibly obtained in PMA-differentiated U-937 + *NRAMP1* cells (34.0 ± 2.9%) as compared to U-937 wild-type cells (21.3 ± 2.42%, $P = 0.0002$) (Figure 3).

Salmonella *growth assay in U-937 cells*

We next asked if there were differences in intracellular *Salmonella* growth in PMA-differentiated U-937 + *NRAMP1* tranfectants and U-937 wild-type cells. In six independent experiments, PMA-differentiated U-937 and U-937 + *NRAMP1* cells were infected with *Salmonella* grown to late-log phase and colony forming units (CFU) were determined at 0 and 24 h postinfection. The differences in *Salmonella* growth were measured as log CFU 24–0 h for U-937 and U-937 + *NRAMP1* cells. In U-937 cells, *Salmonella* consistently showed growth at 24 h postinfection. In comparison, U-937 + *NRAMP1* cells either maintained their bacterial load (similar number of CFU at 0 and 24 h post infection) or showed bacterial clearance ($P = 0.0052$; Figure 4). Taken together with the M6PR recruitment studies, these results demonstrated that both M6PR recruitment and *Salmonella* survival are modulated by the NRAMP1 protein.

Recruitment of Salmonella *bacteria with the late-endosomal marker M6PR in monocyte-derived macrophages (MDM) from healthy volunteers*

In the next series of experiments, we tested if recruitment of M6PR to the SCV could also be detected in primary macrophage explants. We obtained MDM from blood of healthy volunteers and normalized the number of cells per well before they were infected with GFP-expressing *Salmonella* bacteria at an approximate MOI 100:1. As in the U-937 + *NRAMP1* transfectant system, a relatively weak immunofluorescence signal obtained with NRAMP1 antibodies precluded the determination of NRAMP1 recruitment. Recruitment of M6PR with the SCV was determined as for the U-937 cell experiments. We observed recruitment of M6PR to the SCV in MDM obtained from all five donors with experiments repeated at least twice for each donor. Overall, there was good inter-experimental reproducibility of M6PR recruitment by cells from the same donor suggesting that the observed differences in M6PR recruitment reflected intrinsic properties of the MDM (Table 1). Interestingly, recruitment efficiencies of M6PR to SCV in MDM from donors with the *NRAMP1*-274CC genotype did not overlap with those obtained in MDM from *NRAMP1*-274CT and *NRAMP1*-274TT donors.

Recruitment of Salmonella *with the late-endosomal marker M6PR in MDM from pediatric tuberculosis patients*

In a previous study, we had shown that among pediatric tuberculosis disease patients, *NRAMP1* was a strong risk factor for tuberculosis disease. Specifically, we showed that the *NRAMP1*-274C allele predisposed to tubercu-

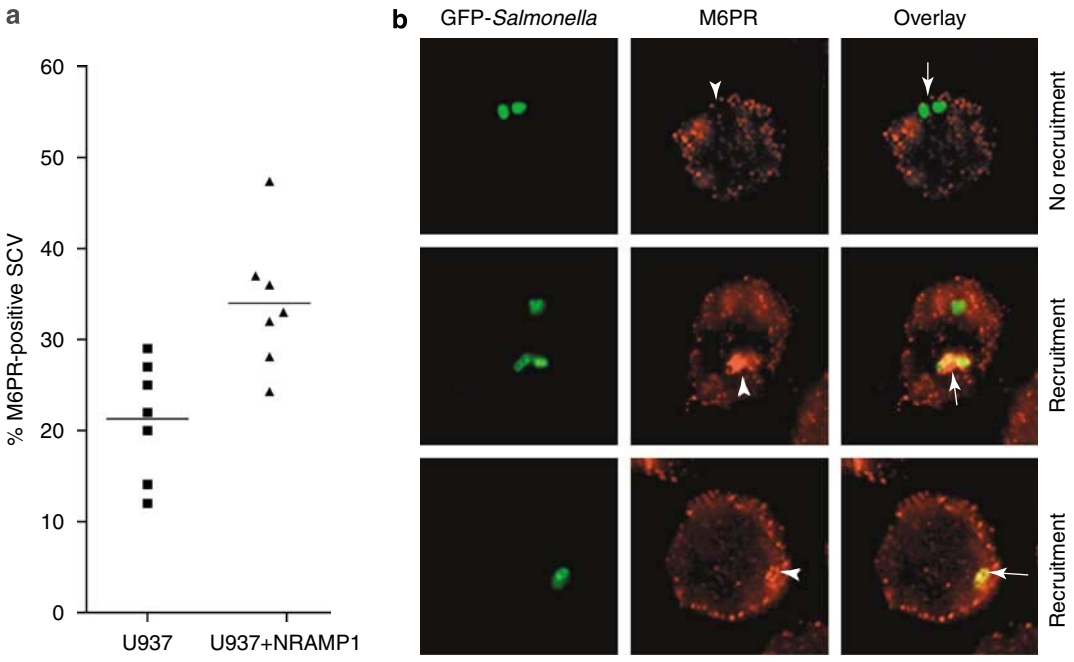

Figure 3 Recruitment of M6PR to phagocytosed *Salmonella* in PMA-differentiated U-937 and U-937 + *NRAMP1* cells. (**a**) Recruitment of M6PR to SCVs is given as the percentage of *Salmonella* co-localizing with M6PR. Although there is inter-experiment variation, each experiment consistently shows increased recruitment of M6PR to the SCV in U-937 + *NRAMP1*. Mean recruitment for either cell line is indicated by a small horizontal line. The difference in recruitment between parental and *NRAMP1*-transfected U-937 cells was highly significant at $P = 0.0002$. (**b**) U-937 and U-937 + *NRAMP1* cells were infected with GFP-expressing *Salmonella* (green). Nonphagocytosed bacteria were immunolabeled with an anti-LPS antibody (blue) to allow discrimination of phagocytosed from extracellular bacteria (not shown). Cells were immunolabeled for M6PR (red). Use of low infectious load facilitated the accurate counting of co-localizing *Salmonella* and M6PR in individual vacuoles. Arrows point to typical non-recruitment (upper panel) and recruitment events (lower panels) between M6PR and the SCV.

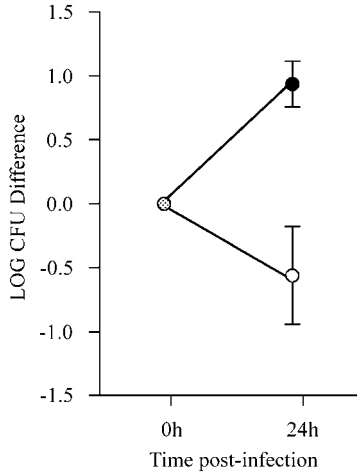

Figure 4 Intracellular growth of *Salmonella* bacteria in U-937 and U-937 + *NRAMP1* cells. In six independent experiments, each cell line was infected with *Salmonella* bacteria. CFUs were counted at 0 and 24 h postinfection and are represented graphically as LOG CFU difference between 24 and 0 h. In U-937 cells (closed circle), *Salmonella* consistently showed growth at 24 h postinfection. In comparison, U-937 + *NRAMP1* cells (open circle) either maintain the bacterial load (similar number of CFUs at 0 and 24 h postinfection) or show bacterial clearance. The difference in CFU growth is highly significant at $P = 0.0052$.

losis disease among simplex families with an odds ratio of 3.13 (95% CI: 1.54–6.25). We therefore applied the M6PR assay to MDM from pediatric tuberculosis patients to test if risk alleles correlated with reduced functional NRAMP1 activity. We were able to obtain a second blood sample from 10 pediatric tuberculosis disease patients enrolled in the initial genetic study. In total, we successfully derived and infected MDM of nine patients with GFP-*Salmonella* and determined the level of M6PR recruitment to the SCV for each individual.

Table 1 Intra-individual M6PR recruitment variability to SCV in MDM obtained from healthy control individuals

Healthy control	*NRAMP1-274 genotype*	*M6PR recruitment to SCV (%)*
1	CC	29, 36
2	CC	28, 35
3	CT	43, 45
4	CT	41, 39, 43, 46
5	TT	45, 46, 43

The *NRAMP1* genotype for each patient was obtained only after M6PR/SCV recruitment had been determined for all samples. None of the patients carried a homozygous protective *NRAMP1*-274TT genotype. Hence, patients were assigned to two groups: those that were homozygous for the high-risk C allele (CC) and those that were heterozygous (TC). There was a significant difference in M6PR recruitment to the SCV between both groups ($P = 0.0129$; Figure 5). Individuals homozygous for the high-risk *NRAMP1*-274C allele recruited $29.0 \pm 1.0\%$ while heterozygote 274CT individuals on average showed significantly higher recruitment of $34.8 \pm 1.5\%$. Representative experiments of SCVs recruit-

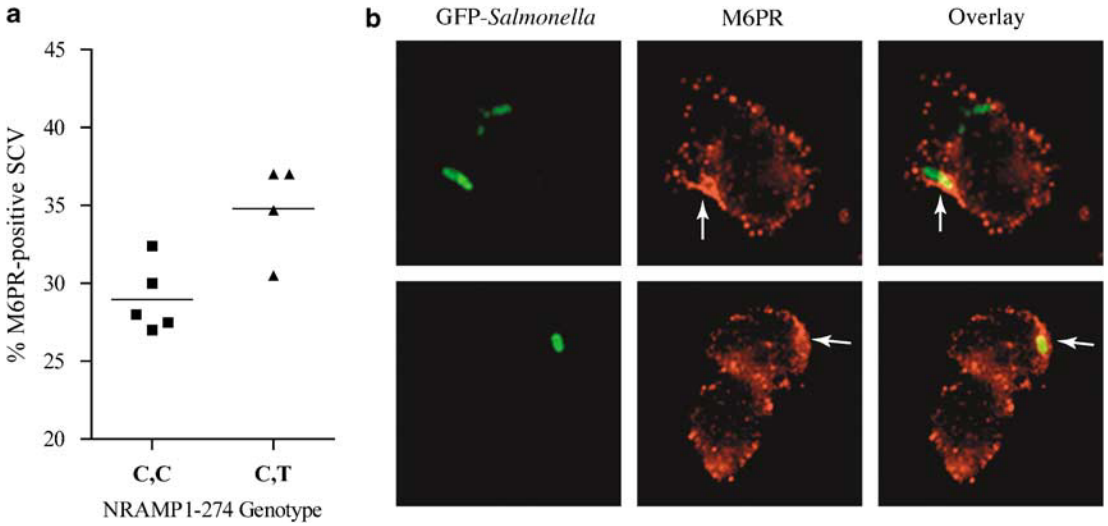

Figure 5 Recruitment of M6PR to SCVs in MDM from pediatric tuberculosis disease patients according to their *NRAMP1* genotypes. (**a**) Recruitment of M6PR to SCVs is given as the percentage of SCV positive for M6PR. Recruitment levels are indicated for pediatric tuberculosis patients who are homo- or heterozygous for the *NRAMP1*-274C high-risk allele. Mean recruitment for the groups of homozygous and heterozygous patients is indicated by a small horizontal line. The difference in recruitment between the two patient groups was significant at $P = 0.0129$. (**b**) Shown are MDM from two different pediatric tuberculosis disease patients that are heterozygous *NRAMP1*-274C/T. MDM were obtained by standard protocols from peripheral blood leukocytes and infected with a low dose of GFP-expressing *Salmonella* bacteria (green) in order that each cell was infected with 1–3 bacteria. Arrows point to typical recruitment events between M6PR and the SCV. SCV without an arrow is nonrecruiting and is shown for comparison.

ing M6PR in MDM from the pediatric tuberculosis patients are shown in Figure 5. There was no discernible difference of genotype or M6PR recruitment between Black and Hispanic children; that is, of the five *NRAMP1*-274CC homozygotes, two were Black and three were Hispanic and M6PR recruitment overlapped between cells obtained from children of both ethnic backgrounds.

Discussion

The human *NRAMP1* gene has been implicated with greater risk of tuberculosis disease by a number of studies and polymorphisms within *NRAMP1* have now been linked or associated with susceptibility to tuberculosis in at least 10 independent populations.[4,6,7,20–26] The focus of most studies was on susceptibility to smear-positive tuberculosis disease among adult populations. In order to dissect the genetic contribution to the onset of tuberculosis disease, we had previously analyzed the effect *NRAMP1* alleles had on risk of primary tuberculosis disease in a sample of pediatric patients from Houston (TX, USA). We identified the common *NRAMP1*-274C allele as major risk factor for early-onset pediatric tuberculosis.[7] Importantly, this finding replicated the results of the genetic analysis in an outbreak of primary tuberculosis among previously unexposed Canadian aboriginals.[6] Considering that approximately half of all tuberculosis disease cases qualify as primary tuberculosis, a better understanding of NRAMP1 function is of critical importance.

The mechanism by which human *NRAMP1* disease associated variants impact on NRAMP1 activity, and how this altered activity is translated into increased disease risk is presently unknown. Murine Nramp1, found in the membranes of the late endosome/lysosome, influences phagosome maturation in both *Salmonella*- and *Mycobacteria*-infected cells.[14,27] However, the impact of Nramp1 on phagosome maturation is likely a secondary effect of its function as a metal transporter across the phagosomal membrane.[9,28] Here, we show that in analogy to murine Nramp1,[14,28] NRAMP1 also impacts on intracellular vacuolar trafficking as revealed by differential recruitment of M6PR to SCVs at 90 min postinfection in PMA-differentiated *NRAMP1*-expressing or *NRAMP1*-deficient U-937 cells. We used one time point to quantify recruitment as established in the murine model. In this model, a difference of recruitment efficiency of approximately 20% was detected between cell lines expressing (33%) or devoid of *Nramp1* (13%).[14] It is possible that the smaller difference in recruitment between U-937 and U-937 + *NRAMP1* cells was due to background *NRAMP1* expression in PMA-stimulated U-937 cells or other background differences between human and murine cells. Nevertheless, the results obtained in the human system were remarkably consistent with the mouse model and strongly support that human and murine NRAMP1 proteins impact on the same biological pathways in phagocytic cells. Importantly, these results also support that, like in murine macrophages, the extent of recruitment of M6PR to SCVs is a valid assay for quantitation of human *NRAMP1* functional activity.

The biological consequence of increased NRAMP1-dependent M6PR recruitment is not clear. It is likely that increased M6PR recruitment mirrors changes in phagosomal maturation that correct the ability of *Salmonella* bacteria to subvert the normal degradative pathway of the phagosome to avoid killing by host cells. In addition, M6PR is an important mediator for the delivery of vacuoles containing lysosomal enzymes to the phagosome.[29] The observed difference in *Salmonella* bacterial growth between U-937 wild type and *NRAMP1* trans-

fectants supports the claim that altered vesicular trafficking, demonstrated by differential M6PR recruitment in this study, impacts on bacterial replication and survival. Further studies are required to fully elucidate the role of NRAMP1 in modulating bacterial replication by comparing intracellular growth of *Mycobacteria* and other pathogens.[27]

Here, we provided direct evidence that primary explanted macrophages from carriers of high-risk *NRAMP1* genotypes displayed a phenotype of reduced NRAMP1 function as compared to carriers of an intermediate-risk genotype. Specifically, we detected significant differences of M6PR recruitment to SCVs in MDMs from pediatric tuberculosis patients homozygous for the high-risk *NRAMP1*-274C allele as compared to heterozygous donors. These findings are in good agreement with the results of the genetic analysis done in this population and suggest that lower NRAMP1 activity is underlying the observed association of the *NRAMP1*-274C allele with risk of pediatric tuberculosis disease.[7] The detailed study of linkage disequilibrium pattern in pediatric tuberculosis patients[7] and the recent discovery that silent nucleotide changes can have substantial biological effects[30] make the *NRAMP1*-274C/T polymorphism a strong causative candidate for a tuberculosis susceptibility variant. Overall, the present results, once validated in larger data sets, will provide the mechanistic framework for the identification of physiological pathways that need boosting in individuals at high risk of developing primary tuberculosis disease.

Materials and methods

Subjects

Pediatric tuberculosis patients were enrolled by the Baylor College of Medicine. Clinical criteria for diagnosis have been previously described.[7] Informed consent was obtained from all study participants. Upon agreement to participate, 8–10 ml blood was obtained from 10 patients and 5 healthy controls. Of the 10 patient samples, one was lost during setup of recruitment experiments. The study received ethical clearance from the Institutional Review Board at Baylor College of Medicine, Houston, TX, USA, and the Research Ethics Committee at the Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada.

NRAMP1 expression

Reverse transcription (RT)–PCR and real-time PCR was performed to detect *NRAMP1* expression in U-937 and U-937 + *NRAMP1* cells before and after stimulation with phorbol 12-myristate 13-acetate (PMA). RNA was isolated from the cells using a Qiagen (Mississauga, Ontario, Canada) RNeasy Mini Kit and then 2 μg RNA was reversed transcribed into cDNA. RT–PCR was performed using specific NRAMP1 primers spanning intron 2 (forward: 5′-GAAGATCCCCATCCCAGAC-3′; reverse: 5′-CTTCCCTAGCCCAGCTCCT-3′) as well as GAPDH primers as a control. Real-time PCR was performed on the same cDNA using a specific *NRAMP1* primer and TaqMan probe spanning intron 11 (Hs00184453_m1, TaqMan Gene Expression Assay, Applied Biosystems, Foster City, CA, USA). Expression of GAPDH, TBP and 18 s genes were each tested to determine the best endogenous control (Hs99999905_m1, Hs99999910_m1, Hs99999901_s1, respectively, TaqMan Gene Expression Assay, Applied Biosystems). All three performed equally well but only GAPDH was subsequently used as an endogenous control for the expression experiments.

M6PR and GFP-S. typhimurium *recruitment assay*

Human U-937 and U-937 cells over-expressing an *NRAMP1*-c-Myc-tagged construct were seeded (5×10^5 cells per well) in a 12-well plate on glass cover slips. The *NRAMP1* construct was made with a pCB6 plasmid and a CMV-based promoter. Transfectant clones were verified for membrane protein expression via western blot (not shown). The clone with the highest expression was chosen to be used in our study. U-937 cells carry the *NRAMP1*-274CC genotype. The transfected construct also carries a 'C' at the nucleotide position 274. The cells were differentiated using 20 nM PMA (Sigma-Aldrich, St Louis, MO, USA) for 48 h followed by a 24 h incubation without stimulation. The cells were grown in antibiotic-free RPMI 1640 supplemented with 10% fetal calf serum (inactivated, endotoxin tested), 2 mM L-glutamine and 1 mM sodium pyruvate (Invitrogen, Burlington, Ontario, Canada). The cells were infected with *Salmonella enterica* serovar *Typhimurium* (*Salmonella)* containing a plasmid expressing the GFP. The bacteria were grown in Luria–Bertani broth supplemented with tetracycline (12 μg ml^{-1}) overnight with shaking at 37 °C. The bacteria were subcultured at a 1:33 dilution for 3 h at 37 °C to late-log phase. After harvesting by centrifugation, the bacteria were washed with phosphate-buffered saline (PBS) and resuspended in Earle's buffered saline solution (pH 7.7; Invitrogen) to give an OD_{600} of approximately 0.2 before being added to the differentiated cells for 10 min at 37 °C 5% CO_2 at a multiplicity of infection of approximately 100:1. Subsequently, the cells were washed gently three times with PBS to remove noninternalized bacteria.

For experiments with MDM, a total of 8–10 ml of blood was used for each experiment. The blood was diluted 1:2 using RPMI 1640 with no supplements and layered over Ficoll-Paque (GE Healthcare, Piscataway, NJ, USA) and centrifuged for 30 min at 400 *g*. The layer containing the mononuclear cells was removed. The cells were washed twice with RPMI for 10 min at 100 *g* before being resuspended in RPMI with supplements. Mononuclear cells were seeded in 3–4 wells of a 12-well plate over glass cover slips. Macrophages were allowed to adhere overnight, washed once with both RPMI and PBS before being infected as described for U-937 cells such that a majority of infected cells phagocytosed 1–3 *Salmonella* bacteria.

Immediately following invasion, the cells were incubated in RPMI 1640 with supplements for 90 min at 37 °C 5% CO_2 to allow for SCV maturation. Ice-cold blocking buffer (5% normal goat serum in PBS) was added for 10 min. Extracellular *Salmonella* were detected using a rabbit anti-*Salmonella* antibody (1:300; Cortex Biochem, San Leandro, CA, USA) for 15 min followed by 20 min incubation with Alexa Fluor 350-labeled goat anti-rabbit secondary antibodies (1:500; Jackson ImmunoResearch, West Grove, PA, USA). Cells were fixed with 4% paraformaldehyde for 45 min at room temperature, and blocked and permeabilized overnight with 5% normal

goat serum plus 0.2% Triton X-100 in PBS. To label late endosomes, cells were incubated the following day with a mouse anti-M6PR antibody (1:50; Affinity BioReagents, Golden, CO, USA), mouse anti-LAMP1 antibody (1:300; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), mouse anti-c-Myc (1:400; BD Biosciences, Mississauga, Ontario, Canada) or rabbit anti-NRAMP1 (1:200)[31] for 1 h followed by a Cy-3-conjugated goat anti-mouse or anti-rabbit secondary antibody for an additional 1 h (1:500; Jackson ImmunoResearch).

Mounted glass cover slips were analyzed using conventional epifluorescent microscopy with a $\times 63$ oil objective. To quantify the level of recruitment between M6PR, LAMP1 or c-Myc-tagged NRAMP1 and GFP-expressing *Salmonella* bacteria, photos of the *Salmonella* (green), the M6PR or LAMP1 (red) and the extracellular *Salmonella* (blue) were taken in the same plane and merged using the Northern Eclipse image software (Empix Imaging Inc., Mississauga, Ontario, Canada). Extracellular bacteria were labeled to allow discrimination from phagocytosed bacteria. Only cells containing 1–3 bacteria were considered. At least 100 randomly chosen bacteria per slide were counted for each experiment.

Salmonella *growth assay*

U-937 and U-937 + *NRAMP1* cells were seeded (2–3×10^5 cells per well) in a 12-well plate. The cells were differentiated and infected as described for the M6PR and GFP-*S. typhimurium* recruitment assay. Following infection, cells incubated for 24 h were maintained in RPMI with 100 μg ml^{-1} gentamicin for 2 h and then in 10 μg ml^{-1} gentamicin for the remaining time. At either 0 or 24 h postinfection, cells were washed once with PBS and then lysed (1% Triton X-100/0.1% sodium dodecyl sulfate in PBS) for 5 min at RT. The lysate was serially diluted and plated on Luria-Bertani agar plates. The CFUs were counted after incubating the plates for 24 h at 37 °C. Each time point was performed in triplicate and each individual experiment was repeated six times. To accommodate experimental variation of baseline uptake of *Salmonella* by U-937 cells, *Salmonella* CFU at $t = 0$ h was subtracted in all experiments.

Statistical analysis

The difference in mean percent recruitment of *Salmonella* and the endocytic marker M6PR in U-937 cells was analyzed using a paired *t*-test. Recruitment values are shown as the mean ± standard error. The differences in *Salmonella* growth measured as LOG CFU 24 h − CFU 0 h for U-937 and U-937 + *NRAMP1* cells, and M6PR recruitment among MDM obtained from carriers of different *NRAMP1*-274C/T genotypes were analyzed using a two-tailed *t*-test. *P*-values < 0.05 were considered significant.

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