Genetics of Host Innate Immune Factors in Tuberculosis Susceptibility

by

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January 2005

A thesis submitted to the Faculty of Graduate Studies and Reseach in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 0-494-12898-4
Our file Notre référence
ISBN: 0-494-12898-4

Abstract

Tuberculosis, caused by *Mycobacterium tuberculosis*, is globally a leading cause of morbidity and mortality. While a host genetic contribution to tuberculosis susceptibility is known to occur, the extent and nature of host genetic variability to tuberculosis pathogenesis are unknown. Thus, to better understand the role of host genetic factors in tuberculosis susceptibility, we have tested the contribution of candidate gene variants to tuberculosis susceptibility in three geographically, epidemically, and clinically distinct populations. We chose four candidate genes involved in the innate immune response, namely, *NRAMP1, MBL, SFTPA1,* and *SFTPA2.*

Since the global spread of tuberculosis is highly dependent on HIV/AIDS pandemic, we investigated the association of genetic *MBL* variants and HIV-1 infection in 278 Colombian HIV-infected and control individuals. *MBL* genotype frequencies were similar for both groups, and no association was detected between *MBL* alleles B, C, and D and susceptibility to HIV-1 infection ($P = 1.0$). These results do not support the hypothesis that MBL levels are a risk factor for HIV-1 infection in Colombia. Moreover, we were able to show that *MBL* variants do not contribute to tuberculosis susceptibility in this population.

In a pediatric population composed of 184 families we found allelic variants in the *NRAMP1* gene to be associated with tuberculosis disease ($P = 0.01$; Odds ratio [OR] = 1.75 [95% confidence interval: 1.10 – 2.77]). Common *NRAMP1* alleles were identified

as risk factors for pediatric tuberculosis while these same alleles were reported to be protective in adult cases, suggesting that the common alleles promote rapid progression from infection to tuberculosis disease. Furthermore, the association of *NRAMP1* with pediatric tuberculosis disease was significantly heterogeneous ($P = 0.01$) between simplex ($P < 0.0008$; OR = 3.13 [1.54– 6.25]) and multiplex families ($P = 1$) suggesting an interplay between mechanisms of genetic control and exposure intensities. Finally, we tested the correlation between the *NRAMP1* risk and NRAMP1 functional activity by measuring the recruitment efficiency of mannose-6-phosphate receptor (M6PR) to *Salmonella* containing vacuoles (SCV) in monocyte-derived-macrophages (MDM). We show that recruitment of M6PR to SCV is significantly lower ($P = 0.024$) in MDM from patients homozygous for the risk allele as compared to MDM from heterozygous patients. Thus, altered function of NRAMP1 appears to modulate the rate of progression from infection to disease.

Next, we investigated polymorphisms in the *SFTPA1* and *SFTPA2* genes for association with tuberculosis in 181 Ethiopian families. Three polymorphisms, *SFTPA1* 307A, *SFTPA1* 776T and *SFTPA2* 751A, were associated with tuberculosis ($P = 0.003$; $P = 0.006$ and $P = 0.012$, respectively). Moreover, subgroup analysis in male, female and more severely affected patients provided evidence for *SFTPA1/2*-covariate interaction. Finally, out of five intragenic haplotypes identified in the *SFTPA1* gene and nine identified in the *SFTPA2* gene only $1A^3$ was significantly associated with tuberculosis susceptibility ($P = 0.017$). These findings suggest that *SFTPA1* and *SFTPA2* modify the risk of tuberculosis susceptibility and that this risk is influenced by additional covariates.

Together, these results show the importance of the epidemiologic setting and the clinical phenotype chosen for the study of genetics of complex traits as well as the need to support findings from genetic investigations with functional studies.

Résumé

La tuberculose, causée par *Mycobacterium tuberculosis*, est une cause majeure de morbidité et de mortalité. Si la composante génétique de la susceptibilité de l'hôte à la tuberculose est reconnue, le degré et la nature de la variabilité génétique de l'hôte demeurent inconnus. Ainsi, afin de mieux comprendre le rôle des facteurs génétiques dans la susceptibilité à la tuberculose, nous avons testé la contribution de variants de gènes candidats à la susceptibilité dans trois populations géographiquement, épidémiquement et cliniquement distinctes. Nous avons choisi quatre gènes candidats impliqués dans l'immunité innée, soit *NRAMP1, MBL, SFTPA1* et *SFTPA2.*

Puisque la propagation de la tuberculose dépend largement de la pandémie VIH/SIDA, nous avons cherché une association entre des variants génétiques du gène *MBL* et l'infection au VIH-1 de 278 Colombiens infectés par le VIH et des individu non infectés comme groupe contrôle. La fréquence du génotype *MLB* était similaire pour chaque groupe et aucune association n'a été détectée entre les allèles B, C, et D de *MBL* et la susceptibilité à l'infection au VIH-1 en Colombie ($P = 1.0$). Ces résultats ne supportent pas l'hypothèse selon laquelle le niveau de *MBL* sont un facteur de risque pour l'infection au VIH-1 en Colombie. De plus, nous avons été capable de démontrer que les variants de *MBL* ne contribuent pas à la susceptibilité à la tuberculose dans cette population.

Dans une population infantile composée de 184 familles, nous avons trouvé que des variants alléliques du gène *NRAMP1* sont associés avec la tuberculose ($P = 0.01$;Odds

ratio [OR]=1.75 [intervalle de confiance de 95%; 1.10-2.77]. Les allèles communes du gène *NRAMP1* ont été identifiées comme facteur de risque de la tuberculose infantile, tandis qu'il a été décrit que les mêmes allèles ont un rôle protecteur dans des cas chez l'adulte, suggérant que les allèles communes favorisent une progression rapide de l'infection jusqu'au développement de la maladie. En outre, l'association de *NRAMP1* et la tuberculose chez l'enfant était significativement hétérogène ($P = 0.01$) entre les familles simplex ($P < 0.0008$;OR = 3.13 [1.54-6.25]) et multiplex ($P = 1$), suggérant un effet entre les mécanismes du contrôle génétique et l'intensité de l'exposition. Finalement, nous avons testé la corrélation entre le risque *NRAMP1* et l'activité fonctionnelle NRAMP1 en mesurant l'efficacité de recrutement du récepteur mannose-6-phosphate (M6PR) aux vacuoles contenant Salmonella (SCV) dans les macrophages dérivés de monocytes (MDM). Nous savons montré que le recrutement de M6PR à SCV est significativement inférieur ($P = 0.024$) dans les MDM de patients homozygotes pour l'allèle à risque comparé aux MDM de patients hétérozygotes. Ainsi, la fonction altérée de NRAMP1 semble moduler le taux de progression de l'infection vers la maladie.

Ensuite, nous avons examiné les polymorphismes des gènes *SFTPA1* et *SFTPA2* pour leur lien avec la tuberculose dans 181 familles éthiopiennes. Trois polymorphismes, *SFTPA1* 776T et *SFTPA2* 751A, ont été associés à la tuberculose ($P = 0.003$; $P = 0.006$; $P = 0.012$, respectivement). De plus, une analyse des sous-groupes homme, femme et patient plus sévèrement atteints a démontré l'évidence d'une interaction des covariants *SFTPA1/2*. Finalement, de cinq haplotypes intragéniques identifiés dans le gène *SFTPA1* et neuf identifiés dans le gène *SFTPA2*, seul $1A^3$ a été associé de façon significative avec

la susceptibilité à la tuberculose ($P = 0.017$). Ces résultats suggèrent que *SFTPA1* et *SFTPA2* modifient le risque de susceptibilité à la tuberculose et que ce risque est influencé par d'additionnel covariants. L'ensemble de ces résultats démontre l'importance de la base épidémiologique et du phénotype clinique choisi pour l'étude de la génétique de traits complexes aussi bien que la nécessité d'appuyer les résultats des recherches en génétique avec les études fonctionnelles.

Acknowledgements:

I am grateful to Dr. Erwin Schurr for all his support and supervision throughout my studies and for giving me the rare opportunity to conduct part of my research in Ethiopia. I appreciate very much his patience and willingness to help and guide me no matter how busy he was. I would also like to thank Dr. Ellen Buschman for her taking the time to advise and comment on my thesis.

Leah Simkin had the daunting task of keeping my data organized. She is one of the most genuine and caring people I know and I thank her so much for all that she has done for me over the years.

I would like to thank Dr. Celia Greenwood for her advice and explanations in statistical genetics.

I wish to thank Post-Doc Emeritus, Dr. Charlie Di Flumeri, for making these past several years dynamic and entertaining, to say the least. While I hold his friendship in the highest regard, he has grown to be more like family to me.

I wish to thank my very close friend, Dr. Tewodros Eguale, for our spirited talks especially while writing our theses. He made the stressful times easier to handle and was always willing to help anyway he could.

I wish to thank all the current and former members of the lab who all are very special to me: Audrey Poon, Tania Di Pietrantonio, Manon Girard, Annie Verville, Andrea Alter, Caroline Gallant, Dr. Naima Abbadi, Dr. Marcelo Mira, Dr. Jamila El Baghdadi, Dr. Sandrine Marquet, Dr. Zbynek Bozdech, Dr. Fabio Sanchez, Dr. Ulrike Delling, and Anne Miller. I would also like to thank Line Larivière for the French translation of my thesis abstract.

I dedicate my thesis to my parents, Ashok and Vibha, who were always there for me with support, encouragement, and love.

List of Abbreviations

AFB	Acid-fast bacillus
BCG	Bacillus Calmett-Guerin
CMI	Cell-mediated immunity
DTH	Delayed-type hypersensitivity
GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HWE	Hardy-Weinberg equilibrium
IL1B	*Interleukin-1 beta gene*
IL1RN	*Interleukin 1 receptor antagonist gene*
IL-1β	Interleukin-1 beta
IL-1Ra	Interleukin 1 receptor antagonist
IL-12	Interleukin-12
IL-12R	Interleukin-12 receptor
IL12B	*Interleukin-12p40 gene*
IL12RB1	*Interleukin 12 receptor, beta 1 gene*
IFNγ	Interferon gamma
IFNGR1	*Interferon gamma receptor 1 gene*
IFNGR2	*Interferon gamma receptor 2 gene*
LD	Linkage disequilibrium
M6PR	Mannose 6-phosphate receptor

MBL	*Mannose binding lectin gene*
MBL	Mannose binding lectin
MDM	Monocyte-derived macrophages
NRAMP1	*Natural resistance associated macrophage protein 1 gene*
NRAMP1	Natural resistance associated macrophage protein 1
PPD	Purified protein derivative
RC-TDT	Reconstruction-combined TDT
S-TDT	Sib-TDT
SCV	*Salmonella*-containing vacuole
SFTPA1	*Surfactant protein A1 gene*
SFTPA2	*Surfactant protein A2 gene*
SFTPD	*Surfactant protein D gene*
SNP	Single nucleotide polymorphism
SP-A	Surfactant protein A
TACO	Tryptophan aspartate-containing coat protein
TDT	Transmission disequilibrium test
TST	Tuberculin skin test
VDR	*Vitamin D receptor gene*
VDR	Vitamin D receptor

Guidelines Regarding Doctoral Thesis

Manuscripts and Authorship

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation." The thesis must include: a Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authos of the co-authored papers.

* * *

In accordance with the above guidelines, I hereby state that:

Chapter 1 of this thesis is partially comprised of a published manuscript entitled, "Genetic susceptibility to tuberculosis" by Suneil Malik and Erwin Schurr in *Clinical Chemistry and Laboratory Medicine* 40: 863-868 (2002). The manuscript was written by myself and edited by Erwin Schurr.

Chapter 2 of this thesis is text and results of a published manuscript entitled, "Absence of association between mannose-binding lectin gene polymorphisms and HIV-1 infection in a Colombian population" by Suneil Malik, Arias M, Celestino Di Flumeri, Luis F. Garcia, and Erwin Schurr which was published in *Immunogenetics* 55:49-52 (2003). M. Arias and LF Garcia provided the clinical data and DNA samples for the Colombian population. Celestino Di Flumeri participated in proofreading of the genotyping results. Genotyping of the markers and cloning and sequencing of compound heterozygotes were performed by myself. The manuscript was written by myself and edited by Erwin Schurr.

Chapter 3 of this thesis is text and results of a submitted manuscript entitled, "Functionally Deficient Alleles of the *NRAMP1* Gene are Major Risk Factors for Pediatric Tuberculosis Disease" by Suneil Malik, Caroline Gallant, Laurent Abel, Heather Tooker, Audrey Poon, Leah Simkin, Manon Girard, Nada Jabado, Gerald J. Adams, Jeffrey R. Starke, B. Brett Finlay, Kimberly C. Smith, Philippe Gros, Edward A. Graviss, James M. Musser, and Erwin Schurr. Caroline Gallant, with the help of Nada Jabado, B. Brett Finlay, and Philippe Gros, adapted and carried out the M6PR recruitment assay to assess NRAMP1 function in human cells. Celestino Di Flumeri genotyped the 5' polymorphisms of the healthy controls for the functional assays. Laurent Abel was the senior statistician on the paper who proof-read, corrected, and expanded my initial analysis. Jeffrey R. Starke provided unparalleled expertise in the diagnosis of pediatric tuberculosis. Kimberly C. Smith participated in the enrollment of study participants. James M. Musser and Edward Graviss coordinated sample collection. Gerald Adams and Heather Tooker were responsible for entering and processing patient information at the BCM site. Audrey Poon performed genotyping data for *NRAMP1-1729+55del4* and *NRAMP1-D543N.* Leah Simkin generated and maintained the BCM-McGill TB Gene Project website. Manon Girard participated in the genotyping of samples. Erwin Schurr coordinated the study and edited the manuscript. I selected all the *NRAMP1* SNP markers for the LD analysis and generated the LD map. Furthermore, I carried out the majority of genotyping and participated in the genetic analysis of the data and the writing of the manuscript.

Chapter 4 of this thesis is text and results of a submitted manuscript entitled, "Variants of the *SFTPA1* and *SFTPA2* Genes and Susceptibility to Tuberculosis in Ethiopia" by Suneil Malik, Celia. M. T. Greenwood, Tewodros Eguale, Alemayehu Kifle, Abebe Habte, Azeb Tadesse, Celestino Di Flumeri, Tania Di Pietrantonio, Sven Britton, and Erwin Schurr. Celia M.T. Greenwood performed conditional logistic regression analysis to provide odds-ratio estimates for tested markers as well as covariate analysis. Tewodros Eguale recruited families and provided clinical data on all participants of the study. Alemayehu. Kfile, Abebe Habte, and Azeb Tadesse extracted DNA and entered sample information in the AHRI-McGill TB Gene Project website. Sven Britton coordinated the study between AHRI and Hossana Hospital. Celestino Di Flumeri, Tewodros Eguale, and Tania Di Pietrantonio participated in proofreading of the genotypes. I performed all genotyping and all aspects of the non-conditional logistic regression analysis. Finally, the manuscript was written by myself and edited by Erwin Schurr.

Original Contribution to Knowledge

1. Excluded *MBL* as an HIV infection susceptibility gene in a Colombian population.

2. Identified *NRAMP1* as a major risk factor for pediatric tuberculosis disease.

3. Provided evidence that the rate of progression from *M. tuberculosis* infection to tuberculosis disease is influenced by the *NRAMP1* gene.

4. Provided evidence that the effect of *NRAMP1* on risk of pediatric tuberculosis disease is dependent upon intensity of exposure to *M. tuberculosis.*

5. Identified *SFTPA1* and *SFTPA2* gene variants as risk factors for tuberculosis susceptibility, among adult Ethiopian tuberculosis cases.

6. Demonstrated that *SFTPA1/SFTPA2* mediated risk of tuberculosis is sensitive to gender of patients.

7. Demonstrated that *SFTAP1/SFTPA2* alleles are genetic modulators of the severity of tuberculosis disease.

Publications

Suneil Malik and Erwin Schurr. (2002) Genetic susceptibility to tuberculosis. *Clinical Chemistry and Laboratory Medicine* 40: 863-868.

Suneil Malik, Arias M, Celestino Di Flumeri, Luis F. Garcia, and Erwin Schurr. (2003). Absence of association between mannose-binding lectin gene polymorphisms and HIV-1 infection in a Colombian population. *Immunogenetics* 55:49-52 (2003).

Suneil Malik, Caroline Gallant, Laurent Abel, Heather Tooker, Audrey Poon, Leah Simkin, Manon Girard, Nada Jabado, Gerald J. Adams, Jeffrey R. Starke, B. Brett Finlay, Kimberly C. Smith, Philippe Gros, Edward A. Graviss, James M. Musser, and Erwin Schurr. Functionally Deficient Alleles of the *NRAMP1* Gene are Major Risk Factors for Pediatric Tuberculosis Disease. Submitted.

Suneil Malik, Celia. M. T. Greenwood, Tewodros Eguale, Alemayehu Kifle, Abebe Habte, Azeb Tadesse, Sven Britton, and Erwin Schurr. Variants of the *SFTPA1* and *SFTPA2* Genes and Susceptibility to Tuberculosis in Ethiopia. Submitted

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Chapter 1 Introduction and Literature Review

1.1 History of tuberculosis

It is likely that tuberculosis has been prevalent among human populations for several thousand years. Pathological and molecular evidence has strongly suggested the presence of tuberculosis in ancient human remains. For example *Mycobacterium* DNA was identified in a 5400-year old Egyptian skeleton with spinal deformities consistent with tuberculosis of the spine (Pott's disease) (Crubezy et al. 1998). Moreover, in South America, acid-fast bacilli, presumed to be mycobacteria, were recovered from the lungs of a mummy dating back to 700 A.D (Allison, 1973). Finally, DNA specific for *Mycobacterium tuberculosis* was recovered from a lung lesion of a spontaneously mummified 1000-year old Peruvian female (Salo et al. 1994)

From as far back as 4000 years ago texts addressing the epidemiology and pathogenesis of tuberculosis are available (Herzog, 1998). The oldest recorded text describing a chronic lung disease, thought to be tuberculosis, was written by the Babylonian monarch, Hammurabi in 1948 and 1905 B.C. About 1500 years later in 460 B.C. Hippocrates referred to Phthisis (Greek:*wasting*), believed to be tuberculosis, as the most widespread disease leading to almost certain death. The 17th century brought about more definitive pathological descriptions of tuberculosis. In 1679 Sylvius de la Boe of Amsterdam first described tubercle lesions in the lungs and other organs of consumptive patients as well as later stage tuberculosis lung characteristics such as cavities and abscesses (Herzog,

1998). Ten years later Richard Morton described three stages of the disease: i) inflammation leading to the formation of tubercles, ii) ulcers or abcesses, and iii) phthisis. However, it was not until 1720 when the English physician Benjamin Marten offered novel epidemiological insight into the transmissibility of Phthisis. He suggested that prolonged contact with an infected person is "dangerous". Furthermore, Marten believed that physical closeness to a phthisis victim, including conversation, may allow expired air from the phthisis patient into the lungs of the contact which may lead to disease (Herzog, 1998).

The mid-1800's was an era stimulated by the germ theory of disease (Baxter, 2001), the establishment of infectious disease epidemiology and great advances in the microscopical techniques. In 1882 Robert Koch, who was awarded the Nobel Prize in Physiology and Medicine in 1905, isolated *M. tuberculosis* from guinea pigs and was able to propagate the bacilli in culture (Collins, 1998). Moreover, Koch was not only able to isolate the bacterium from infected animals, but also established disease in naïve animals using the bacterial isolates, thus definitively proving, by the so called 'Koch's Postulates', that *M. tuberculosis* was the causative agent of tuberculosis (Collins, 1998).

1.2 Epidemiology

1.2.1 Global epidemiology

Tuberculosis was the number one killer of all causes in the United States at the turn of the nineteenth century with a death rate of 188/100 000 (Bates and Stead, 1993). However, death rates were tempered in the early 1900s to 100/100 000 due to improved hygiene and living conditions, general better health among the population and the growing practice of isolating tuberculosis patients in sanatoriums (Bates and Stead, 1993). Between 1953 and 1985 a reduction of 74 percent of tuberculosis cases (4/100 000) was observed largely due to the advent of effective antibiotics (Committee on the Elimination of Tuberculosis in the United States, 2000). After 1985 the decline leveled off (Raviglione et al. 1993) and by 1988 tuberculosis rates in the United States were on the increase again. At the same time European nations were experiencing a stagnation in the decline of case notification rates. The interruption of this decline in developed countries was attributed to increased poverty in urban centers and the emergence of the human immunodeficiency virus (HIV) (Bloom and Murray, 1992) but, most notably, imported tuberculosis cases from developing countries (McKenna et al. 1995; Raviglione et al. 1993; Bloom and Murray, 1992). It became evident that controlling tuberculosis in developed countries would be difficult without addressing the global tuberculosis crisis.

Worldwide, it was estimated that there were 8.3 million new cases of tuberculosis and 1.8 million deaths in 2000. Eighty percent of the global burden was attributed to 22

countries, mostly in sub-Saharan Africa and Asia (WHO Report, 2004). Of these high burden countries (HBC), India and China harbour most new cases of tuberculosis, however, incidence rates are highest among African countries, ranging from 304/100 000 in Nigeria to 683/100 000 in Zimbabwe. Smaller countries in Africa such as Swaziland have estimated case rates of 1067/100 000. However, these countries are not considered HBC due to their small population. While the global incidence rate of tuberculosis is increasing by 1.1 percent (WHO Report, 2004), the number of tuberculosis cases in the former Soviet Union and sub-Saharan Africa is increasing more dramatically, with estimates of 6.0 percent per year (Corbett et al. 2003). The pandemic of HIV/AIDS has dramatically increased the tuberculosis notification rate in several countries, primarily in sub-Saharan Africa. In 2000, 11 percent of all new tuberculosis cases and 13 percent of deaths due to tuberculosis occurred in adults infected with HIV (Corbett et al. 2003).

The Centers for Disease Control (CDC) reported significant declines in tuberculosis cases in the United States from 1992 to 2002 with a decline of at least 40 percent in all races/ethnicities (Centers for Disease Control, 2003). Despite these trends, the rate of tuberculosis cases for 2002 was reported at 5.2/100 000, above the CDC's preset target of 3.5/100 000 by 2000. In 2002, tuberculosis rates among non-hispanic blacks and hispanics were reported at 12.3/100 000 and 11.3/100 000, respectively, the highest of any other race/ethnicity in the United States. The same report showed that Texas and California had the highest reported numbers of cases of tuberculosis in 2002, followed closely by New York (Centers for Disease Control, 2003).

Most alarming is the relative neglect and underreporting of pediatric tuberculosis by public health officials. The World Health Organization (WHO) estimates 1.2 million cases of pediatric tuberculosis worldwide with 450 000 new pediatric tuberculosis related deaths per year (Holmes et al. 1998), however, these figures may be grossly underestimated (Starke, 2003). A staggering tuberculosis disease incidence of 3588/100 000 was reported for children less than 5 years of age in a South African population (van Rie et al. 1999a). Likewise, annual rates of infection can reach 3.5 percent among schoolchildren, and many of these infected children will significantly contribute to the adult tuberculosis epidemic (Rieder, 1999).

Multidrug-resistant tuberculosis (MDR-tuberculosis) is defined as tuberculosis disease that is resistant to at least rifampicin and isoniazid. Currently, MDR-tuberculosis is not a concern from a global perspective as most MDR-tuberculosis cases are confined to local epidemics in a few regions including Estonia, Latvia, the Oblasts of Ivanovo and Tomsk in Russia as well as Henna and Zhejiang Provinces in China, however, available data are not exhaustive and half of the 22 HBC have yet to provide data regarding MDR-tuberculosis (Espinal, 2003). Mathematical modeling, however, suggests that 3 percent of new tuberculosis cases in 2000 were MDR-tuberculosis (Espinal, 2003).

In 1994 the WHO implemented a tuberculosis control strategy that comprised of five key elements (Elzinga et al. 2004): i) government cooperation and commitment to an ongoing tuberculosis control policy, ii) diagnosis by sputum smear microscopy, iii) standard short course chemotherapy, including directly observed treatment (DOT), iv)

availability of drugs, and v) accurate record keeping to assess treatment success. These five key elements constitute a tuberculosis control package referred to as "DOTS". Significant declines in tuberculosis rates have been observed in several countries who have adopted the DOTS program. The WHO had set a goal of achieving 70 percent case notification and 85 percent cure rate by 2000, however, case notification rates fell well short of this goal as did cure rates in sub-Saharan Africa and DOTS is difficult to implement in areas with increased HIV coinfection rates (Elzinga et al. 2004).

1.2.2 Risk factors for tuberculosis infection and disease

Development of tuberculosis disease may be considered a two-tier process whereby a susceptible individual must first acquire infection before progressing to disease (Comstock, 1975). Since infection and development of disease involve distinct physiological processes, it follows that some risk factors may also be distinct (Comstock, 1975).

Classification of an infected individual is based on a positive tuberculin skin test (TST) in the absence of any other disease manifestations. A number of characteristics/factors of the exposed contact contribute to the risk of infection including age, gender (Sutherland, 1976) and HIV infection (Centers for Disease Control, 2000). Specific genetic factors underlying tuberculosis infection have yet to be identified, however, a twin study demonstrating that the magnitude of the DTH response to mycobacterial antigens is heritable suggests that tuberculosis is, at least, partly under genetic control (Jepson et al.

2001). Furthermore, risk of infection is dependent on the intensity of exposure of a naïve individual to the source (Lienhardt et al. 2003). Exposure intensity is comprised of several factors: i) physical proximity of the source and contact (Comstock, 1975) (Lienhardt et al. 2003; Loudon et al. 1958) ii) frequency of cough from the source (Lienhardt et al. 2003; Loudon et al. 1958; Loudon et al. 1958), iii) density of bacilli in the sputum of the source (Shaw and Wynn-williams, 1954; Rathi et al. 2002), although this has not been consistently shown (Lienhardt et al. 2003), iv) number of persons in a household, v) presence of cavities in the lungs of source cases and vi) number of lung zones affected in the source (Lienhardt et al. 2003).

Once infected with *M. tuberculosis* the cumulative lifetime risk of an individual to develop disease is 10 percent (Horsburgh, 2004). A strong risk factor in the development of tuberculosis is age where at least two distinct peaks of high incidence occur (Comstock, 1975). The first peak occurs at infancy and early childhood followed by a second peak at adolescence (Comstock, 1975). The risk among the elderly however remains uncertain (Comstock, 2000). Furthermore, sex differences in case rates have been reported in several ethnically and geographically diverse populations, typically showing a 2:1 male-to-female ratio (Holmes et al. 1998). This is supported by mouse studies showing that females were more resistant to *mycobacterium spp.* than males (Yamamoto et al. 1990; Yamamoto et al. 1991). Nevertheless, whether the sex effect in humans is due to a true biological etiology or whether cultural or socioeconomic status biases case notification rates among sexes is unclear (Holmes et al. 1998).

Generally speaking, an inadequate host immune response may favour the survival and proliferation of the bacilli in an infected person and consequently lead to progression to tuberculosis disease. Factors that tend to influence progression to disease include diabetes mellitus, silicosis, chronic renal failure, jejunioleal bypass, renal and cardiac transplantation, head and neck carcinomas, and other neoplasms (Centers for Disease Control, 2000) and HIV infection (Davies, 2003).

1.3 Pathogenesis

1.3.1 The etiological agent

M. tuberculosis belongs to the genus *Mycobacterium*, the only genus in the family *Mycobacteriaceae*. Included in this genus are *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*, all of which represent the *M. tuberculosis* complex (Grosset et al. 2000). The tubercle bacterium is a long thin rod shaped bacillus with rounded extremities, typically 2-5 μm long and 0.2-0.3 μm thick. However, three physiological features of the bacterium may significantly impact on the pathogenesis of tuberculosis disease.

Firstly, *M. tuberculosis* is an obligate aerobe, possessing the Embden Meyerhof pathway and Kreb's cycle, generating energy from the oxidation of carbon sources (McKinney et al. 1998). The requirement of the bacterium for oxygen may, in part, explain its predominance in oxygen rich tissues like the lung, especially the apices of the lung which have the highest oxygen concentrations.

Secondly, the cell envelope of the bacterium is composed of densely interconnected core proteins including peptidoglycans, arabinogalactans, and mycolic acids, that are adorned by more sparsely distributed lipoarabinomannon molecules (McNeil et al. 1996). The outermost layer of the cell envelope is made up of glycolipids that are complexed with and anchored by mycolic acids (McNeil et al. 1996). This complex lipid rich composition enhances resistance of the bacterium to chemical and physical stress and to several antimicrobial agents (McKinney et al. 1998). Moreover, the cell envelope enables the bacterium to persist and replicate in the hostile macrophage environment and to promote a strong inflammatory response contributing to tuberculosis pathogenesis (McKinney et al. 1998).

Thirdly, the tubercle bacterium has the slowest growth rate of any free-living bacterium with a doubling time of 20-24 hours which may allow prolonged host survival and a less vigorous antimicrobial response in the macrophage (McKinney et al. 1998).

1.3.2 Transmission

M. tuberculosis is transmitted through aerosolization from a diseased individual to persons in close proximity. A vigorous expulsion of air typically in the form of a cough expels droplet nuclei that are made up of two or three bacilli surrounded by a layer of moisture. Once airborne, the moisture layer evaporates leaving a smaller concentrated nucleus of bacteria that can remain suspended in air for longer periods of time. Moreover, the reduced size of the nucleus can penetrate the inner most reaches of the

recipient's airway. Successful transmission of infection depends on the viability, the number, and the virulence of the bacilli (Nardellj and Piessens, 2000). While the infective dose is uncertain it is estimated to be as few as than 10 bacilli (Riley, 1957).

Horizontal transmission of tuberculosis in children is uncommon as children rarely have cavitary tuberculosis or a sufficiently productive cough to aerosolize infectious nuclei droplets (Lawrence, 1996). Contact investigation studies suggest that tuberculosis in children is most often attributed to recent transmission from an infectious adult source (Nolan, 1986; Starke, 1989).

Rarely, tuberculosis in humans is attributed to *Mycobacterium bovis*. Transmission of *M. bovis* occurs primarily through the ingestion of unpasteurized dairy products, but may also be transmitted person to person through aerosolization (Dankner et al. 1993).

The recent emergence of molecular fingerprinting has offered greater insight into disease transmission. For example, shared DNA fingerprints among *M. tuberculosis* strains, classified as clusters, are attributed to recently transmitted or primary tuberculosis disease whereas unique isolates are consistent with reactivated tuberculosis (Murray and Alland, 2002). Since clinical presentation does not clearly delineate primary and reactivated tuberculosis, DNA fingerprinting is of obvious value. Furthermore, molecular fingerprinting showed that persons that are negative for microscopic detection of *M. tuberculosis* in stained sputum samples (so-called AFB negative) but culture positive for *M. tuberculosis* account for at least 17 percent of all transmissions (Behr et al. 1999).

This finding toppled the long held belief that disease transmission occurs almost exclusively from smear positive sources and put forth an important precedence to re-evaluate the existing public health infrastructure with regard to tuberculosis control.

1.3.3 Clinical forms

As mentioned, once infected with *M. tuberculosis* the lifetime risk of developing tuberculosis disease is 10 percent, equally distributed among primary and reactivated disease types (Horsburgh, 2004). Primary tuberculosis disease occurs within 2-5 years of initial infection and most often develops in children, however, it may also occur in those with conditions affecting their immune system such as HIV/AIDS patients, diabetic patients, those on corticosteroids, cancer patients undergoing chemotherapy, alcoholics, and those with poor nutritional habits (Milburn, 2001). Reactivated tuberculosis disease occurs from an endogenous infection at any point 2 years post-initial infection and most often is represented by adult tuberculosis cases.

Both primary and reactivated disease include a broad spectrum of clinical forms of tuberculosis defined by the location and pathogenesis of tuberculosis disease. The most common clinical form is pulmonary tuberculosis where disease is restricted to the lungs. Primary pulmonary tuberculosis is typically characterized by pleural effusion, hilar and mediastinal lymphadenopathy, and involvement of the lower and middle lobes or the anterior segments of the upper lobes (Woodring et al. 1986). On the other hand, reactivation disease most often produces fibroproductive parenchymal densities (nodules)

typically in the apical and posterior segments of the upper lobes of the lung (Woodring et al. 1986). Extrapulmonary forms of tuberculosis are less common than pulmonary tuberculosis, accounting for 20 percent of adult cases of tuberculosis (Fanning, 1999) and nearly 30 percent of pediatric cases (Graham et al. 2004). Moreover, extrapulmonary tuberculosis is most often a manifestation of primary disease type. Although extrapulmonary tuberculosis can appear anywhere in the body other than the lung, the most common sites of disease include bone and joints, lymphatics, miliary, genitourinary, peritoneal, and meningeal (Yang et al. 2004). Children who develop disease often suffer from the most serious forms of tuberculosis such as meningitis and acute miliary tuberculosis (Chaulet, 1992).

1.3.4 Diagnosis

There are typically four steps in diagnosing tuberculosis: 1) medical history, 2) tuberculin skin test, 3) chest-x-ray, and 4) microscopic detection of stained bacilli in sputum samples and/or bacterial culture from sputum or blood (Dunlap et al. 2000; U.S. Department of Health and Human Services, 1994)

A medical history is used to determine whether patients have any risk factors for tuberculosis such as exposure to a tuberculosis case or whether they have ever had tuberculosis infection or tuberculosis disease. Typical symptoms of pulmonary tuberculosis include persistent cough, chest pain, and hemoptysis accompanied by

systemic symptoms such as fever, chills, night sweats, fatigue, loss of appetite and weight loss (U.S. Department of Health and Human Services, 1994).

The standard method for identifying *M. tuberculosis* infection in persons who have not developed disease is the tuberculin skin test (TST) (Horsburgh, 2004). Given that *M. tuberculosis* infection is marked by a delayed type hypersensitivity (DTH) reaction, specific antigenic components, coined purified protein derivative (PPD) of the bacterium are used to test for the presence of infection (Dunlap et al. 2000). Typically, five tuberculin units of PPD are administered intradermally. Forty eight to 72 hours post injection a DTH response in the form of a palpable swelling or area of induration may be observed. A positive tuberculin test is based on the diameter of the induration area in millimeters. Three cut-off sizes have been established based on the risk of acquiring infection:1) $\geq$ 5 mm for high risk persons, i.e. HIV infection, recent contact with cases, immunosuppression, 2) $\geq$ 10 mm medium risk persons i.e., IV drug users, recent immigrants, employees of high risk congregate settings such as jails, AIDS hospices, people with preexisting conditions such as silicosis, diabetes, and cancers, and 3) $\geq$ 15 mm for low risk persons (Dunlap et al. 2000). However, the TST should be interpreted with caution since false-positive reactions may occur in those with previous exposure to nontuberculous mycobacteria or BCG vaccination (Dunlap et al. 2000). Conversely, false-negative reactions may occur in the very young (less than 6 months), those with recent tuberculosis infection (within 10 weeks of infection), reading and administration of the test, coinfections, and those who are anergic (Dunlap et al. 2000).

Although chest X-rays may not be used to confirm diagnosis of tuberculosis, they can rule out pulmonary tuberculosis in persons testing positive for TST (U.S. Department of Health and Human Services, 1994). Moreover, chest X-rays may show abnormalities in the lung which may also determine whether primary or secondary tuberculosis exists (McGuinness and Naidich, 1996).

Definitive diagnosis of tuberculosis is contingent upon a positive culture of *M. tuberculosis* or detection of bacilli. In the case of pulmonary tuberculosis the sample is obtained from sputum while in the case of extrapulmonary tuberculosis, it may be taken from any region of the body (U.S. Department of Health and Human Services, 1994).

Diagnosing tuberculosis in children presents challenges (Khan and Starke, 1995; Shingadia and Novelli, 2003). Often symptoms are either absent or nonspecific. Moreover, less than 20 percent of proven childhood pulmonary tuberculosis cases are AFB smear positive, with even less success for extrapulmonary tuberculosis cases (Lipsky et al. 1984; Strumpf et al. 1979). While *M. tuberculosis* detection from gastric aspirates are considerably higher, ranging from 23 to nearly 50 percent (Lobato et al. 1998; Starke and Taylor-Watts, 1989), and even up to 75 percent in infants of less than 1 year (Vallejo et al. 1994), such detection rates are still modest. A positive TST is most commonly used to indicate infection, however, in children less than 5 years old, a positive TST may take up to 3 months to develop (Khan and Starke, 1995). Unfortunately, severe forms of tuberculosis such as menigitis and miliary tuberculosis may develop within 3 months of infection. Still, the current set of criteria for diagnosing

pulmonary tuberculosis in children requires that either a positive AFB smear is obtained or at least two out of the following criteria must be met: i) history of a contact with an adult case, ii) coughing lasting longer than 2 weeks, iii) a positive TST, iv) radiological findings compatible with tuberculosis, or v) clinical improvement upon treatment (Khan and Starke, 1995).

1.3.5 Treatment

Tuberculosis patients are typically treated with four drugs which act on three forms of bacilli: i) bactericidal killing on actively multiplying bacteria, ii) sterilization to kill semidormant bacteria, and iii) drug resistant bacteria (Hershfield, 1999). Isoniazid is a potent antituberculosis drug highly effective on actively dividing bacteria and acts by targeting cell wall biosynthesis (Rozwarski et al. 1998). Rifampicin and pyrazinamide kill the more slowly dividing bacterium which 'sterilizes' the infection (Hershfield, 1999). Rifampicin works by inhibiting DNA dependent RNA polymerase, effectively interfering with RNA transcription (Hershfield, 1999) while pyrazinamide disrupts membrane transport in *M. tuberculosis* (Zhang et al. 2003). Moreover, the four drug combination reduces the likelihood that drug resistant strains will develop. Although antituberculosis regimens vary, a six month course of treatment is the minimum to achieve acceptable cure rates of culture confirmed tuberculosis patients (Hershfield, 1999). Typically isoniazid and rifampin administered for nine months is curative, however, with the addition of pyrazinamide in the first two months of treatment the regimen can be shortened to six months. Multidrug-resistance cases are typically treated with a

combination of second-line drugs such as ethionamide, capreomycin, kanamycin, and amikacin for 12 months (Hershfield, 1999).

1.3.6 Molecular mechanisms of tuberculosis pathogenesis

Once inhaled, *M. tuberculosis* bacilli reach the alveolar sacs and are engulfed by resident alveolar macrophages. Since the natural reservoir of *M. tuberculosis* is the macrophage, the key to controlling infection lies primarily in this cell. While quiescent macrophages are permissive for bacterial replication, activated macrophages impart bacteriostatic or bacteriocidal activity (Dannenberg, 1994; Flesch and Kaufmann, 1990). Typically, resident alveolar macrophages are constituitively activated and can therefore confine and neutralize the bacilli immediately (Dannenberg, 1993). However, a minority of bacilli may be taken up by unactivated macrophages which subsequently migrate into the interstitium of the lung, establishing infection (Dannenberg, 1993). Given that unactivated macrophage are unable to mount effective bacteriocidal activity, bacilli grow unconstrained eventually leading to necrosis of the macrophage and release of the bacilli (Dannenberg, 1993). Released bacilli serve as chemoattractants for naïve circulating monocytes and macrophages that accumulate and engulf the bacilli, forming the early stages of the tubercle or granuloma (Dannenberg, 1993). Three weeks following initial infection the host develops delayed-type hypersensitivity (DTH), a "nuisance" version of cell-mediated immunity (CMI) (Kobayashi et al. 2001). DTH is initiated by $CD4^+$ T-cells and mediates direct cell cytotoxicity by $CD8^+$ T-cells, typically resulting in host tissue damage (Kobayashi et al. 2001). Antigen-specific T-cells produce, most notably,

IFNγ which is critical to control *M. tuberculosis* infection, yet not sufficient by itself (Algood et al. 2003; Ma et al. 2003; Bonecini-Almeida et al. 1998). In turn, activated macrophages produce chemokines such as interleukin (IL)-8, (macrophage inflammatory protein (MIP) - 1α, monocyte chomoattractant protein (MCP)-1 that attract T-cells, natural killer (NK) cells, and monocyte/macrophages to the focus of infection, further developing the granuloma (Algood et al. 2003; Sadek et al. 1998). Moreover, activated macrophages produce IL-1, IL-6, and tumour necrosis factor (TNF)-α that augment chemokine production from lymphocytes. Perhaps central to DTH is the production of IL-12, of which mycobacterium infected activated macrophages are potent inducers (Kobayashi et al. 2001; Cooper et al. 1997; Flynn and Chan, 2001; Gately et al. 1998). Interleukin-12 stimulates the differentiation of naïve $CD4^+$ lymphocytes into IFNγ producing T-helper (Th)-1 cells and potentiates cytolytic effects of $CD8^+$ T-cells. Macrophages not able to successfully contain infection are subject to $CD8^+$ T-cells dependent cytolytic activity. $CD8^+$ T-cells produce perforin which permeabilizes the membrane of the infected macrophage, allowing granulysin and granzyme into the macrophage to directly kill the bacilli (Browne et al. 1999; Stenger et al. 1998). Such destruction creates a caseous centre in the tubercle generating an anoxic and acid environment laden with toxic fatty acids causing bacilli to either die or remain dormant. However, over time bacilli are released from this caseous centre only to be engulfed by newly activated macrophages (Dannenberg, 1993). On the other hand, hosts unable to control intracellular replication must rely on continuous $CD8^+$ T-cell dependent cytolytic killing, effectively enlarging the caseous centre of the granuloma leading to more extensive lung damage (Dannenberg, 1993).

Although chemokines, proinflammatory cytokines, and toxic and lytic compounds are effective means at controlling the bacilli, these factors also cause host tissue damage at the site of infection. For this reason anti-inflammatory cytokines including IL-10, TGFβ, and IL-4 temper the host immune response in order to limit tissue injury to the host, effectively maintaining a delicate balance of host-pathogen destruction (Sharma and Bose, 2001; Wigginton and Kirschner, 2001).

1.3.6.1 Interplay between *M. tuberculosis* and its macrophage host

Phagocytosis is initiated by the invagination of the plasma membrane, forming a discrete and mildly bacteriocidal vacuole, referred to as a phagosome. Typically, the phagosome undergoes fusion events with early and late endosomes and lysosomes transforming the phagosome into a bacteriocidal organelle termed the phagolysosome (Beron et al. 1995; Deretic and Fratti, 1999; Tjelle et al. 2000). The mechanism of sequential fusion between phagosomes and other vacuoles is not fully understood, however, a subset of small GTPases from the Ras superfamily has been shown to mediate these processes. For example, phagosome interaction with endosomes and lysosomes are mediated, at least in part, by Rab5 and Rab 7 GTPases, respectively. Furthermore, *in vitro* studies show a role for Nramp1 in phagolysosomal fusion events (Frehel et al. 2002). Nramp1 knockout macrophages are unable to process mycobacteria to the phagolysosomal compartment, allowing only phagosome-endosome fusion events while normal maturation of the phagosome was observed in Nramp1 expressing cells (Frehel et al. 2002).

Upon fusion with early endosome, late endosome, and lysosome, the maturing phagosome becomes richer in hydrolytic enzymes and progressively more acidic (Botelho et al. 2000; Clemens and Horwitz, 1995; Jahraus et al. 1998; Via et al. 1997). The specific effect of such a hostile environment on the pathogen is not fully characterized, however, several proposals have been made (Hackam et al. 1998). Since mycobacteria grow optimally in a pH range of 6.5-7.9, acidification may directly compromise bacterial growth. Furthermore, lowered pH can activate microbicidal enzymes, delivered by the lysosomal compartment. Next, the protonation of nitrite is favoured by an acidic milieu, resulting in nitrous acid. The latter undergoes dismutation which converts to bactericidal reactive nitrogen species. Finally, lowered pH promotes dismutation of superoxide resulting in hydrogen peroxide, which in turn, in the presence of myeloperoxidase, produces strongly antiseptic hypochlorite ions (Hackam et al. 1998; Bokoch, 2002).

While acidification of the bacteria containing vacuole is critical in controlling infection, limiting access of divalent cations to the pathogen is of equal importance in this regard. In this respect, the Nramp1 protein is an important cation transporter. Although some studies have suggested that Nramp1 functions as an antiporter of divalent cations (i.e. transport into the phagosome) (Goswami et al. 2001; Kuhn et al. 1999; Kuhn et al. 2001; Zwilling et al. 1999), compelling *in situ* evidence suggests the contrary (i.e. transport out of the phagosome) (Forbes and Gros, 2003; Jabado et al. 2000). While the bacteriocidal effects of divalent cation depletion on the bacterium is not fully understood, three hypotheses have been put forth: 1) the bacterium requires divalent metals for

general metabolic activity, 2) divalent metals are required cofactors for bacterial superoxide dismutase activity, and 3) divalent cation depletion could affect bacterial virulence factors that may mediate phagosome maturation (Frehel et al. 2002).

Successful establishment of infection by the bacteria requires that they survive and persist within the host macrophage. Consequently, mycobacteria have evolved survival strategies primarily to circumvent contact with late endosomes/lysosomes. For example, *in vitro* studies found that *M. avium* prevented phagolysosomal fusion in macrophages not expressing Nramp1 while phagosomes matured and fused extensively with lysosomes in Nramp1 expressing macrophages, suggesting an interplay between mycobacteria and Nramp1 (Frehel et al. 2002). Furthermore, a tryptophane aspartate-containing coat protein (TACO) present on the plasma membrane of macrophages is recruited and actively maintained on phagosomes containing live mycobacteria but not dead mycobacteria (Ferrari et al. 1999). The presence of TACO on the phagosome prevents lysosomal fusion thus allowing the mycobacterium to survive. Finally, a less popular evasion proposal suggests that *M. tuberculosis* escape from the phagolysosomal vacuole (McDonough et al. 1993).

The interplay between macrophage and *M. tuberculosis* reveals a constant battle for control. An understanding of this dynamic interchange may allow for novel chemotherapeutic targets which may ultimately tip the balance of control in favour of the host.

1.4 Genetics of complex traits

A complex trait, of which tuberculosis is a prime example, refers to a phenotype that does not follow a classic Mendelian inheritance pattern. It is becoming apparent that most, if not all, genetic diseases fall into the class of complex traits. For example, additional genetic factors, called modifier genes, have been identified for sickle cell disease (SCD), traditionally thought of as the "prototypic" monogenic disease (Nagel, 2001). While SCD is characterized by a single point mutation in the β-globin chain in adult hemoglobin causing red blood cell sickling (Ingram, 1956), it is not sufficient to explain the wide spectrum of clinical presentations or severity of SCD (Nagel, 2001). It has been shown that fetal hemoglobin, which lacks the β-globin chain (Platt et al. 1994) and therefore the single point mutation, resists red blood cell sickling (Goldberg et al. 1977) and improves life expectancy in SCD patients (Platt et al. 1994). Interestingly, the relative proportion of fetal hemoglobin containing cells is largely genetically determined (Garner et al. 2000). More recently, polymorphisms in the human leukocyte antigen *(HLA)* and vascular cell adhesion molecule-1 *(VCAM1)* genes have been shown to influence the clinical outcome of SCD (Hoppe et al. 2003; Taylor et al. 2002).

1.4.1 Basic definitions

Genetic epidemiology studies are based on polymorphisms present in a particular population. A polymorphism is the existence of more than one allele or gene variant

present in a given population at frequencies above 1 percent. A particularly common type of polymorphism is a single nucleotide polymorphism (SNP) which differs by only one base at a given nucleotide position. Another type of polymorphism is a microsatellite in which the number of tandem repeats of simple sequences distinguishes one allele at a particular locus from another. The combination of two alleles at a particular locus comprise an individual's genotype whereby the individual is homozygous when both alleles are the same and heterozygous when the alleles differ. Nonrandom association of alleles at linked loci is referred to as linkage disequilibrium. Finally, combinations of alleles found on linked loci on a single chromosome are termed haplotypes.

1.4.2 Strategies employed to dissect complex traits

The two approaches most often used to search for specific genetic factors of complex diseases are candidate gene studies and genome wide linkage analyses. Both methods are complimentary in the sense that the candidate gene approach is best suited to detect moderate genetic effects while the genome–wide approach is used to detect major susceptibility genes in an unbiased fashion. Candidate genes are selected based on their known or putatively known relevance in the pathogenesis of disease. Polymorphisms within genes are tested, most commonly, by association studies employing either population-based or family-based case-control designs. Genome wide linkage analysis studies are most often performed on nuclear families with pairs of affected offsprings (Nyholt, 2000). Under the assumption of no linkage and across a large panel of affected sibpair families, two affected sibs share 0, 1, or 2 parental alleles identical by descent

(IBD) at a given locus with frequencies of 0.25, 0.5, and 0.25, respectively (Nyholt, 2000). Significant deviations from this distribution indicate evidence of linkage between marker and disease loci. In tuberculosis genetics, susceptibility loci have so far only been identified by employing candidate gene strategies (Malik and Schurr, 2002).

Population Case-Control design

Association studies determine whether a given allele is overrepresented or underrepresented in cases as compared to controls. A significant association between marker and disease can occur for three reasons: i) the given marker allele is the actual cause of the phenotype, ii) the marker allele is in linkage disequilibrium with the causative allele, or most concerning, iii) the significance of association arose spuriously due to undetected population substructure (Abel and Dessein, 1998). To circumvent the problem of population substructure, a family based approach is often taken.

Family-Based Association design

The transmission disequilibrium test (TDT), which was first formally introduced in 1993 (Spielman et al. 1993), illustrates a very popular family based approach for the detection of associations. The test determines whether allelic transmissions from heterozygous parents to affected offspring deviate from the 50/50 ratio, expected under the null hypothesis of no association between marker and disease.

Although the TDT is typically designed for nuclear families with two unaffected parents and one affected child (trios or simplex families) the reality of association studies is that both parents may not be available for the study especially when studying late onset types of diseases. The traditional TDT cannot be performed without the genotypic information of the parents consequently an alternative to the traditional TDT uses genotypic information from affected and non-affected siblings (Spielman and Ewens, 1998). This alternative to the TDT is suitably named "sib-TDT" or S-TDT and does not attempt to reconstruct parental genotypes . The S-TDT compares marker allele frequencies among affected and unaffected sibs and determines whether a statistical difference between the two groups exist. It would be tempting to simply compare the total number of alleles between affected and nonaffected and to test for deviation from the null hypothesis using a χ^2 table, similar to that of the TDT. However, because of non-independence (sibs of the same family) a χ^2 is not valid under these circumstances. According to Spielman et al, (1998), two valid methods of determining significance are the permutation and z-score methods.

The more common permutation method employs a within-family Monte Carlo permutation procedure. Consider a family with five siblings of which 2 are "affected" and 3 are "unaffected". The sibs are then "shuffled" and their affection status ignored. For a single replicate, 2 sibs will be randomly chosen and are assigned to the affected category while the remaining 3 sibs will be assigned to the unaffected category. The genotypes of the "affected" and "unaffected" are then scored. Genotypes are scored as opposed to alleles since permuting alleles could lead to the creation of genotypes not

originally observed in the families. Upon completion of the first replicate the sibs are then "shuffled" again and 2 new randomly chosen sibs are categorized as "affected" and the remaining 3 as "unaffected". A large number of repeat shufflings can be generated using the Monte Carlo permutation procedure. The total number of alleles are then determined over all families. Then if, for example, allele M is the putative susceptible allele, the proportion of replicates in which the number of M alleles in the "affected" category is equal or more to the observed value generates an appropriate and very precise p-value. Alternatively, reconstructive combined (RC)-TDT may be used when parental genotypes can be recontructed, with certainty, from sib information (Knapp, 1999)

1.4.3 Evidence for a role of genetic factors in tuberculosis susceptibility

There is considerable evidence from epidemiologic and twin studies in favour of host genetic factors modulating tuberculosis susceptibility. As far back as the 17th century evidence of familial clustering of tuberculosis has suggested a human heredity factor in disease susceptibility (Dubos, 1987). Recently, a study that considered a large population of racially mixed nursing home residents, showed that blacks were about twice as likely to become infected with *M. tuberculosis* than whites (Stead et al. 1990). Similarly, in this same report, tuberculosis outbreaks in prison populations showed that blacks have a two fold increased risk of infection over whites (Stead et al. 1990). However, in these studies unidentified exposures specific to blacks were likely. Furthermore, socioeconomic differences, prevalence of underlying immunosuppressive diseases, and institutional living conditions were not measured. Importantly, other investigators have

disputed such increased susceptibility to *M. tuberculosis* infection according to racial group (Hoge et al. 1994). The reason for this discrepency is not clear, however, both authors attribute their conflicting results to environmental and socioeconomic differences. A formal evaluation of the impact of socioeconomic status on tuberculosis risk among various U.S. born ethnicity concluded that socioeconomic status accounted for approximately half of the increased risk of tuberculosis previously associated with race/ethnicity (Cantwell et al. 1998). Nevertheless, twin studies have consistently shown that concordance rates for tuberculosis are significantly higher in monozygotic twins than in dizygotic twins (Kallmann and Reisner, 1943; Comstock, 1978). These latter studies provide strong evidence that tuberculosis susceptibility has a genetic component.

1.4.4 Candidate genes implicated in tuberculosis susceptibility

1.4.4.1 *MBL*

The mannose binding lectin (MBL) is an important complement activating protein as well as an opsoninzing agent (Turner and Hamvas, 2000). Three SNPs in codons 52, 54, and 57, of the *MBL* gene, result in lowered serum MBL (Madsen et al. 1994). In a pediatric South African population a protective effect for pulmonary tuberculosis (79 controls vs 91 pulmonary tuberculosis patients) was observed in individuals who were homozygote for the *MBL-G54D* allele (P=0.017) (Hoal-Van Helden et al. 1999). In a much larger adult case-control study involving a Gambian population of 844 controls and 794 tuberculosis patients, the *MBL-G57D* variant allele was found to be weakly associated

with tuberculosis resistance (P = 0.037) (Bellamy et al. 1998d). Conversely, results based on an adult case-control study involving an East Indian population of 109 controls and 202 pulmonary tuberculosis patients reported that subjects who were homozygous for the variant alleles (predominantly the *MBL-G54D* allele) were more likely to develop tuberculosis than those with any other genotype ($P = 0.008$) (Selvaraj et al. 1999). Furthermore, low levels of MBL, but not undetectable levels, are associated with protection against tuberculosis. A Danish study showed that a low expressing promoter allele in front of a normal MBL gene in combination with a dysfunctional allele on the other chromosome was more frequently observed in controls than patients (Soborg et al. 2003). The inverse directions of associations in different populations, and the observation in the Gambian study that serum MBL levels are not associated with tuberculosis susceptibility (Bellamy et al. 1998c), point to the need for additional studies of MBL in tuberculosis susceptibility.

1.4.4.2 *VDR*

Numerous epidemiologic studies have consistently shown that vitamin D deficiency leads to tuberculosis susceptibility (Chan, 2000). Also, the active form of vitamin D, $1,25(OH)_2D_3$, plays a part in immunoregulation and aids in suppressing intracellular growth of *M. tuberculosis* in monocytes (Rook et al. 1986). An SNP in codon 352 of the vitamin D receptor with alleles designated "T" for the common allele and "t" for the rare allele has been examined with respect to pulmonary tuberculosis in a Gambian population comprising 408 pulmonary tuberculosis patients and 414 controls

(Bellamy et al. 1999). Results from this study showed that the "tt" genotype was over-represented in healthy individuals (P = 0.01, OR=0.53, 95% CI=0.31-0.88), suggesting a protective effect of the t allele. By contrast, an analysis involving 200 pulmonary tuberculosis patients and 108 controls of an Indian population showed a lack of association between the *VDR-TT/tt* genotype and tuberculosis (Selvaraj et al. 2000). However, upon stratifying this population by gender, the "tt" genotype was found to be over-represented in female tuberculosis patients (P = 0.02). Correcting for multiple tests would eliminate the formal significance of this finding. A study involving a Gujarati Indian population showed that undetectable levels or deficiency of serum 25-hydoxycholecalciferol were strongly associated (OR=9.9 and OR=2.9, respectively) with tuberculosis (Wilkinson et al. 2000). This same study considered two polymorphisms, one described previously (T/t) and the other at nucleotide position 117, designated F for the wild type. Analysis of the two polymorphisms showed no association between tuberculosis and SNP genotypes, however, a combination of genotypes TT/Tt and vitamin D deficiency, and ff genotype and undetectable serum levels of vitamin D were associated with tuberculosis (OR = 2.9 and OR = 5.1, respectively). Taken together, these somewhat divergent results suggest that further studies are required to better assess a physiological and gender role of VDR in tuberculosis susceptibility.

1.4.4.3 *IL1B* and *IL1RN*

Interleukin-1 is a potent proinflammatory cytokine implicated in a number of inflammatory diseases (Dinarello, 1996). Assessment of cytokine profiles determined

from several studies have consistently shown that interleukin-1β (IL-1β) is significantly elevated in the bronchoalveolar lavage fluid (BALF) in patients with active tuberculosis (Casarini et al. 1999; Condos et al. 1998; Tsao et al. 1999) suggesting that lung inflammation in these patients is at least partly due to IL-1β. To downregulate the proinflammatory response to IL-1β, an antagonist, IL-1Ra, competes for the IL-1 receptor. It is for this reason that tuberculosis susceptibility may be influenced by the IL-1β/ IL-1Ra ratio. Genetic analysis of the 86-bp variable number tandem repeat (VNTR) in intron 2 of the *IL1RN* gene (coding for IL-1Ra) showed that, in response to *M. tuberculosis* stimulation of PBMC from healthy subjects, allele 2 (two 86bp repeats) was associated with 1.9 fold greater IL-1Ra production as compare to non allele 2 genotypes (Wilkinson et al. 1999). Furthermore, two biallelic markers at positions -511 and +3943 were analyzed in the *IL1B* (coding for IL-1β). While *M. tuberculosis* stimulated production of IL-1β did not differ with respect to allelic variants of either marker, allele +3953 A2 was associated with depressed levels of *IL1B* mRNA (P=0.04). In this same investigation a small population case-control study of 114 controls and 89 patients with varying tuberculosis forms showed no association either allelic or genotypic, with *IL1B* or *IL-1RN* polymorphisms and increased risk of tuberculosis. However the *IL1RN* $A2^-$ */IL1B* (+3953) $A1^+$ haplotype was somewhat over represented in patients with pleural disease (P=0.028). A larger case-control study in The Gambia considered a population made up of 400 tuberculosis patients and 400 healthy controls (Bellamy et al. 1998a). In agreement with the study by Wilkinson and colleagues (Wilkinson et al. 1999) allele 2 of *IL1RN* was found to be less common among tuberculosis patients (P=0.03) suggesting a

protective affect. Together these studies suggest that polymorphisms governing IL-1 activity may have a modest effect on the clinical presentation of tuberculosis.

1.4.4.4 *HLA*

Many genetic studies of tuberculosis focused primarily on the human leukocyte antigen (HLA) complex, however, findings have been varied and conflicting. Serological based HLA typing was performed on a modest number of multicase families to follow HLA-DR2 segregation in tuberculosis patients of an Indian population (Singh et al. 1983). Although the specific mode of inheritance was not established, it was clear that DR2 was linked with tuberculosis susceptibility. Likewise, there was evidence for distortion of transmissions ratios with DR2 being transmitted from heterozygous parents to affected children at an average of 83 percent while only 51 percent of the time to healthy children (Singh et al. 1983). Similar results were found in a case-control study of an Indian population with 153 pulmonary tuberculosis patients and 289 healthy controls where HLA-DR2 was over represented among the tuberculosis patients (P_{corr}=0.029, RR=1.8) (Rajalingam et al. 1996). However, upon molecular subtyping of DR2 performed on DR2-positive healthy controls (n=81) and tuberculosis patients (n=61), the most frequent alleles, *DRB1*1501* and*DRB1*1502*, were not associated with pulmonary tuberculosis (PTB) (66.7 percent in controls vs 77 percent in PTB, and 37 percent in controls vs 28 percent in PTB, respectively). In contrast, among patients, significant enrichment of the *HLA-DRB1*1501* DR2 allele was observed in a study of Mexican tuberculosis patients and healthy controls (Teran-Escandon et al. 1999). A South Indian case-control study

examining 126 sputum positive pulmonary tuberculosis patients and 87 endemic controls revealed two polymorphic variants associated with patients, *HLA-DRB1*1501* (OR=2.68, 95% CI=1.30-5.89, P=0.013), and DQB1*0601(OR=2.32, 95% CI=1.29-4.27, P=0.008), while a third allele,*DPB1*04,* was enriched in healthy controls suggesting a protective effect (OR=0.45, 95% CI=0.21-0.95, *P*=0.036) (Ravikumar et al. 1999). However, in this and other studies significance values would be diminished if corrections for multiple testing were taken into consideration. The problem of multiple testing and the concomitant loss of power has been elegantly addressed in a case-control study involving 126 Cambodian pulmonary tuberculosis patients and 88 healthy controls. Using a two-stage study design, the authors found a significant association between the *HLA-DQB1*0503* allele and pulmonary tuberculosis (P=0.005) (Goldfeld et al. 1998).

1.4.4.5 *SFTPA1, SFTPA2,* and *SFTPD*

Pulmonary surfactant proteins (SP)-A and SP-D are essential for the normal functioning of lungs and play a role in innate immune defense as well as the regulation of inflammatory response (rev in Khubchandani and Snyder, 2001). It has been shown that SP-A can mediate attachment of *M. tuberculosis* to alveolar macrophages (Pasula et al. 1997) while SP-D mediates agglutination of the bacteria, thereby reducing phagocytosis of the bacteria (Ferguson et al. 1999). This suggests that although SP-A and SP-D have differing roles in the innate immune response to *M. tuberculosis*, both may play an important role in the outcome of tuberculosis infection. On the genetic level the *SP-A* locus consists of two genes, *SFTPA1* and *SFTPA2*, in opposite transcriptional orientation

(Hoover and Floros, 1998) and separated by 54 kb, while *SFTPD* is located 324 kb 5' of *SFTPA1*. Specifically, diallelic variants in the *SPTPA1* gene occur at amino acids 19, 50, 62, 133, and 219 while variants in the *SFTPA2* gene occur at amino acids 9, 91, 140, and 223 (DiAngelo et al. 1999). Patterns of various combinations of SNPs at given amino acids determine a specific allele. For example allele $6A^4$ refers to the *SP-A1* gene *(6A)* with the superscript "4" representing the fourth allele, which differs from all other common alleles at amino acid 219 (Trp for $6A^4$ and an Arg for all others). Similarly, allele $1A^3$ differs from other common *SP-A2* alleles at amino acid 223 (with the exception of allele $1A^1$) while the *SP-D* marker, *DA11*, is a biallelic polymorphism corresponding to a C to T base change (*DA11_C*:threonine to *DA11_T*: methionine) at amino acid 11. A case-control study of a Mexican population assessed polymorphic variants in the *SFTPA1*, *SFTPA2*, and *SFTPD* genes with respect to risk of pulmonary tuberculosis (Floros et al. 2000). Logistic regression analyses showed that allele $1A^3$ of the *SFTPA2* gene and the $6A^4$ allele of the *SP-A1* gene were significantly overrepresented in the Mexican tuberculosis patients relative to healthy controls (OR=9.28, 90% CI=1.61-53.39, P=0.018; OR=2.71, 90 percentCI=1.25-5.87, P=0.033, respectively), while allele *DA11_C* of the *SFTPD* gene was associated with tuberculosis susceptibility when compared to tuberculosis skin-positive controls (OR=2.66, 90% CI=1.57-4.49, P=0.002) (Floros et al. 2000).

1.4.4.6 *NRAMP1*

Inbred strains of mice revealed that natural resistance to intracellular pathogens such as

M. bovis (Bcg) (Gros et al. 1981), *M. avium* complex (Goto et al. 1989), *M. lepraemurium* (Skamene et al. 1984), *Leishmania donovani* (Bradley, 1977) and *Salmonella typhimurium* (Plant and Glynn, 1976) is influenced by a single dominant acting gene, designated *Bcg*. Positional cloning of *Bcg* isolated the natural resistance associated macrophage protein 1*(Nramp1)* gene and showed that natural resistance to infection was influenced by a single G169D amino acid substitution in the encoded protein (Vidal et al. 1993; Vidal et al. 1995). Subsequently, with numerous polymorphisms identified in the human homologue *NRAMP1* (Buu et al. 1995; Liu et al. 1995), a number of studies have implicated *NRAMP1* with tuberculosis susceptibility in humans. A large case-control study examining 410 smear positive pulmonary tuberculosis patients ethnically matched with 417 healthy controls showed that *NRAMP1* variants were associated with susceptibility to tuberculosis in a West African population (Bellamy et al. 1998b). Specifically, four variants of the *NRAMP1* gene were examined: a dinucleotide CA-repeat at the 5' promoter region, $(GT)_n$, a single nucleotide change in intron 4 *(469+14G/C)*, a single nonsynonymous nucleotide substitution in codon 543 *(D543N)*, and a TGTG deletion in the 3' untranslated region *(UTR; 1729+55del4)*. Results showed that subjects heterozygous for both *469+14G/C* and *1729+55del4* polymorphisms were particularly overrepresented among tuberculosis patients (OR=4.07; 95% CI, 1.86-9.12; chi-square=14.58, P<0.001) (Bellamy et al. 1998b). A smaller family based study, set in Guinea-Conakry, West Africa, was carried out using 26 one parent and 17 two parent nuclear families to assess three *NRAMP1* polymorphisms: GT_n, *1729+55del4*, and *469+14G/C* (Cervino et al. 2000). This study reported that 469+14G/C was significantly associated with tuberculosis (P=0.036), further supporting

the role of *NRAMP1* in tuberculosis. The *1729+55del4* TGTG deletion in the 3'UTR of *NRAMP1* was evaluated with respect to tuberculosis susceptibility in a Korean population involving 192 tuberculosis patients and 193 healthy individuals (Ryu et al. 2000). Results showed that the *3'UTR* polymorphism was indeed weakly associated with tuberculosis susceptibility (*P*=0.02) with an excess of heterozygotes in the tuberculosis patient population. Likewise, analysis of two Japanese populations from Tokyo and Osaka demonstrated a strong association of the *5'(GT)$_n$ NRAMP1* promoter polymorphism with tuberculosis (P=0.0003, OR=1.86, 95% CI=1.32-2.61) (Gao et al. 2000). Conversely, a linkage study assessing 37 multicase families of a Brazilian population did not show evidence of a tuberculosis susceptibility locus linked to *NRAMP1,* however, two markers (*IL8RB* and *D2S1471*) tightly linked to *NRAMP1* were shown to be weakly linked to disease susceptibility (P=0.038; P=0.025, respectively) (Shaw et al. 1997). Together, these studies provide strong evidence that *NRAMP1* influences tuberculosis susceptibility, however, genetic heterogeneity cannot be ruled out in some populations as indicated by a Moroccan study (El Baghdadi et al. 2003). Here, a family based approach examining 116 nuclear families with respect to *NRAMP1* polymorphisms spanning the entire gene failed to implicate *NRAMP1* as a susceptibility gene for tuberculosis.

A more detailed view of the gene-environment interactions that impact on tuberculosis susceptibility was provided by a parametric linkage study employing liability classes according to recorded clinico-epidemiological data. Linkage of *NRAMP1* and adjacent markers with tuberculosis susceptibility was analysed in a large aboriginal Canadian

pedigree that experienced a tuberculosis outbreak (Greenwood et al. 2000). Linkage analysis was conducted assuming a major tuberculosis susceptibility locus with a relative risk of 10 for the high risk over the low risk allele. This analysis resulted in maximum LOD (logarithm of the odds) scores of 3.55, 3.21, and 3.36 for linkage between tuberculosis and intragenic polymorphisms *NRAMP1-(GT)n* , *D543N* and an *NRAMP1* haplotype of 10 variants, respectively. Furthermore, a maximum multipoint LOD score of 4.2 was obtained with the susceptibility gene locating on top of *NRAMP1* in the multilocus genetic map (Greenwood et al. 2000). If the sample was analysed without specification of liability classes there was no evidence for linkage. These results strongly suggest that even major genetic effects can be missed if gene environment interactions are neglected in the genetic analysis.

1.4.4.7 Familial atypical mycobacteriosis and tuberculosis susceptibility

Rare Mendelian disorders predisposing individuals to hypersusceptibility to relatively avirulent mycobacterial species have offered insight into genes which may play a role to tuberculosis on the population level. *In vivo* and animal studies have shown a critical role of IFN-γ for mycobacterial host defense, presumably due to its ability to activate macrophages (rev in Schluger and Rom, 1998). Likewise, IL-12 is crucial in the mediation of optimal production of IFNγ, and IL-12 deficiency in mice demonstrated its importance in *M. tuberculosis* infections (rev in Gately et al. 1998). Therefore, genes included in the signaling cascade of IFNγ and IL-12, namely, *IFNGRI, IFNGR2, IL12B,* and *IL-12RB1* were examined and found to be the cause of idiopathic-disseminated

mycobacterial infections.

Molecular analysis of a Tunisian female infant with fatal BCG infection showed a substantial decrease in *IFNGR1* mRNA due to a homozygous missense mutation in exon 2 (Jouanguy et al. 1996). Moreover, disseminated atypical mycobacteriosis in four Maltese children was attributable to a point mutation at nucleotide 395 of *IFNGR1* which truncates the protein and effectively abrogates its expression on the cell surface (Newport et al. 1996). Furthermore, partial *IFNGR1* deficiency due to a homozygous missense mutation at nucleotide position 260 has been described in siblings, one with disseminated BCG infection with tuberculoid granulomas and the other sibling with clinical tuberculosis who had not previously received BCG innoculation (Jouanguy et al. 1997). Whereas the two previous studies considered consanguinous kindreds, a case study of an Italian infant, born to two nonrelated parents, afflicted with disseminated *M. smegmatis* was examined (Altare et al. 1998b). Results from this study found that the child carried two novel mutations, a 4-bp insertion in exon 2 and an SNP at the splice-site of intron 3, revealing the first compound heterozygote patient.

A case study describing a child with disseminated *M. fortuitum* and *M.avium* complex infections showed that the immunodeficiency was due to a mutation in the *IFNGR2* gene (Dorman and Holland, 1998). Specifically, sequence analysis revealed a homozygous dinucleotide deletion at nucleotides 278 and 279, introducing a premature stop codon resulting in a truncated protein.

A female infant born to consanguineous Pakistani parents with *BCG* and *Salmonella enteritidis* infection was found to carry a large homozygous deletion encompassing two coding exons within the *IL12P40* gene (Altare et al. 1998c). Consequently, IFNγ production after PBMC stimulation was markedly reduced, most likely due to IL-12 deficiency (Altare et al. 1998c). Also, IL-12Rβ1 deficiency in three unrelated kindreds caused disseminated mycobacterial and non-typhi salmonella infections in otherwise healthy individuals (Altare et al. 1998a). Three distinct mutations were identified in each of these patients: a homozygous nonsense mutation at position 913 resulting in a premature stop codon, a framshift mutation causing the skipping of the exon spanning nucleotides 701 to 783, and a missense mutation at nucleotide position 641 (Altare et al. 1998a). All three mutations led to complete IL-12Rβ1 deficiency although this deficiency led to less severe infections than those patients with IFNγR1 deficiency, and, unlike complete deficiency of *IFNGR1*, did not compromise the formation of mature granulomas (Altare et al. 1998c). Similarly, in three unrelated individuals with severe mycobacterial and *Salmonella* infections it was shown that mutations in the *IL12RB1* subunit encoding gene resulted in a lack of functional IL-12R complexes (de Jong et al. 1998). Three unique mutations were responsible for IL-12R deficiency: nonsense mutations at nucleotide positions 94 and 1126, and a deletion spanning from nucleotide position 409 to 549. All patients were homozygous for the mutations and all mutations led to premature stop codons effectively abrogating IL-12R cell surface expression.

Together, the above summarized studies indicated that *IFNGR1, IFNGR2, IL12B*, and *IL-12RB1* are strong candidate genes for tuberculosis susceptibility. This conclusion was

further supported by a recent report describing a patient with disseminated BCG disease displaying tuberculoid granulomas who carried a heterozygous mutation in *STAT1* (Dupuis et al. 2001). STAT1 is a critical mediator of IFN-mediated cellular processes and the report highlights the pivotal role that is played by genetic variations of genes positioned along *IFNG* response pathway. Interestingly, the common genetic variant in *IFNG* has recently been identified and implicated with tuberculosis susceptibility. A two-tier study design that was carried out in a South African coloured population examined an SNP located in the first intron of *IFNG* (+874 A/T) in a case-control and a family based setting (Rossouw et al. 2003). The first tier, a case-control study design comprising 313 tuberculosis patients and 235 controls, showed that allele T was overrepresented in the control group (P=0.0055) suggesting a protective role for this allele. This finding was confirmed a family-based study of 131 tuberculosis families (P=0.005), arguing against the possibility that the case-control association was spurious due to population substructure. A further replication of this finding was carried out in a case-control Spanish population composed of 113 culture proven tuberculosis cases, 207 healthy close contacts and 100 healthy tuberculin-negative control subjects, showing a 3.75 fold increase risk in individuals homozygous for the A allele (Lopez-Maderuelo et al. 2003). The latter study provided evidence that the 874T/A polymorphism influences IFNγ production in PPD-induced individuals, with carriers homozygous for allele A showing depressed IFNγ levels. Finally, a Sicilian study made up of 45 tuberculosis cases and 97 controls, matched for age and sex, also implicated 874T/A with tuberculosis susceptibility showing an overrepresentation of TT homozygotes present in tuberculosis patients (P=0.02) (Lio et al. 2002). Together these studies provide compelling evidence

that the +874T/A polymorphism is a genetic susceptibility factor for tuberculosis that directly influences the production of IFNγ, a critical cytokine in tuberculosis infection and disease.

Recently, four common polymorphisms were identified in *IL12RB1* and were examined in a case-control design composed of 98 tuberculosis patients and 197 controls (Akahoshi et al. 2003). Three missense variants (Q214R, M365T, and G378R) and one synonymous substitution were found to be in very strong linkage disequilibium (D'=0.98). Homozygosity for allele 2 (R214-T365-R378) was associated with tuberculosis (OR=2.45; 95% CI: 1.20-4.99; P=0.013). Furthermore, genotype 2/2 showed depressed levels of IL-12 induced signaling as measured by IFNγ production from CD2 cells (T-cells and NK cells).

1.4.4.8 Genome wide studies

A two step genome-wide scan using 299 highly informative markers was performed on 92 sibpairs with tuberculosis from the Gambia and South Africa (Bellamy et al. 2000). Seven chromosomal regions that showed putative linkage were further genotyped in 81 additional sibpairs using 22 markers for each region. Weak evidence for linkage was indicated on chromosome regions 15p and Xq (multipoint MLS_{PT} = 2.00 and 1.77, respectively). These results argue against a major gene control of tuberculosis susceptibility in the families studied. Nevertheless, a follow-up study on the chromosome 15 linkage interval identified the *UBE3A* as a possible tuberculosis

susceptibility gene (Cervino et al. 2002)

1.4.4.9 Conclusion

It is apparent that host genetic profiles play a pivotal role in susceptibility to tuberculosis and, consequently, identification of candidate genes responsible for susceptibility is of importance for a better understanding of tuberculosis pathogenesis. Armed with new knowledge from the Human Genome Project, an increasing understanding of disease mechanism, and high-throughput genotyping technology such as high-density variation-detection DNA chips (Wang et al. 1998), novel candidate genes and SNPs will allow us to build a more comprehensive genetic profile of tuberculosis susceptibility.

1.5 Connecting text

HIV/AIDS represents a formidable challenge in the global control of tuberculosis, as discussed in chapter 1. With HIV infection accounting for up to 31% of new tuberculosis cases and close to a quarter of a million tuberculosis deaths (Corbett, 2003), it is clear that identifying risk factors for HIV/AIDS is crucial to address the major public health concern of tuberculosis.

Consequently, we tested a previously identified HIV susceptibility gene, the mannose binding lectin *(MBL)* in a Colombian HIV population. MBL is a serum pattern recognition protein, known to be part of the innate immune system. Specifically, three polymorphisms in exon 1, known to impact on MBL serum levels, were tested under a population case-control design. The results of this study are described in chapter 2.

Abstract

Mannose-binding lectin (MBL) is a calcium-dependent lectin shown to play an important role in innate immunity to infection by activating the classical complement pathway and phagocytosis. In vitro studies have shown that MBL is able to bind to the gp120 HIV-1 surface antigen, and variants of the gene are associated with increased risk of HIV infection among Scandinavians. We investigated the association of genetic *MBL* variants and HIV-1 infection in 278 Colombian HIV-infected and control individuals. *MBL* genotype frequencies were similar for both groups, and no association was detected between *MBL* alleles B, C, and D and susceptibility to HIV-1 infection (P=1.0). Since there is a well-documented link between the tested *MBL* alleles and very low MBL serum concentration, these results do not support the hypothesis that MBL levels are a risk factor for HIV-1 infection in Colombia.

Keywords: Innate immunity · HIV infection · Mannose-binding lectin gene · Candidate gene · Association study

Introduction

Acquired immunodeficiency syndrome (AIDS) is caused by infection with human immunodeficiency virus type 1 (HIV-1). There is clear evidence for the involvement of host genetic factors in modulating the pathogenesis of HIV-1 infection (Hogan and Hammer, 2001; Roger, 1998). Specifically, genetic variants of chemokines and their receptors have been implicated in modulating transmission of HIV-1. Homozygosity for a 32-bp deletion in CC chemokine receptor 5 (CCR5) is highly protective for HIV-1 infection (Dean et al. 1996). Homozygosity at the *CCR5-59356T* promoter polymorphism is a strong risk factor for perinatal transmission of HIV-1 (Kostrikis et al. 1999) and a compound heterozygote carrying a rare point mutation at position 303 of *CCR5* and the 32-bp *CCR5* deletion has been strongly implicated in resistance to HIV-1 infection (Quillent et al. 1998). Interestingly, a promoter variant in the *CCR5* using chemokine RANTES was also associated with increased risk of HIV-1 infection in the United States Multicenter AIDS Cohort Study (McDermott et al. 2000). Polymorphisms in the promoter region that drive reduced expression of the NRAMP1 gene (Searle and Blackwell, 1999) are associated with reduced risk of HIV infection (Marquet et al. 1999). Finally, several studies in Scandinavian populations detected a significant effect of serum levels of mannose-binding ligand (MBL) on susceptibility to HIV-1 infection. A Danish study found that undetectable levels of serum MBL were more frequently observed in HIV-infected persons (Nielsen et al. 1995). Moreover, two independent genetic studies in Danish and Finnish populations observed an excess of *MBL* gene variants that result in low serum MBL levels among HIV-infected persons (Garred et al.

1997; Pastinen et al. 1998). The *MBL* gene, also known as mannose-binding protein *(MBP)* gene, is located in chromosome region 10q11.21. MBL is a multichain serum lectin made up of a variable number of 96-kDa subunits, each composed of three identical 32-kDa polypeptide chains covalently linked by disulfide bonds (Taylor et al. 1989). Mannose glycans present in the cell walls of certain gram-negative bacteria, parasites, fungi, as well as in viral envelopes of HIV-1 serve as initial binding sites for MBL. By binding to these residues, MBL acts as an opsonin that promotes pathogen phagocytosis (Kuhlman et al. 1989). A second independent function of MBL is the activation of the complement cascade via binding of MBL-associated serine proteases (Matsushita et al. 2000). Changes of the primary amino acid sequence of MBL due to common nucleotide substitutions in codon 52 (52C, allele D), codon 54 (54D, allele B), and codon 57 (57E, allele C) impede the trimerization of MBL polypeptides (Lipscombe et al. 1995). Such mutant MBL trimers are less stable and display reduced polymerization, leading to enzymatic degradation of MBL polypeptide and functional deficiency of MBL due to very low MBL serum levels (Lipscombe et al. 1995). Heterozygous carriers for any of the codon variants have approximately 20% of wildtype MBL serum levels, while homozygous or compound heterozygous carriers display very low or even undetectable MBL serum levels in Eskimos, Northern Europeans, and West Africans (Lipscombe et al. 1992; Madsen et al. 1995). In addition, this strong correlation between codon variants and reduced MBL serum levels has been confirmed for populations residing at geographically diverse locations, including Africa (Bellamy et al. 1998), the United Kingdom (Crosdale et al. 2000), China (Ip et al. 2000), Japan (Hakozaki et al. 2002), Australia (Minchinton et al. 2002), and Brazil (Santos et al.

2001). This demonstrates that the codon variants can be used as genetic indicators of low MBL levels.

Materials and methods

In the present study, 278 adult individuals were enrolled from the urban region of Medellin, Antioquia, Colombia. Of these, 138 persons were HIV positive, while 140 control DNA samples were obtained from the general population. HIV infection was determined by standard immune assays. Genotyping for *MBL* variants was performed by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) (Madsen et al. 1995). Primers 5-AGTCGACCCAGATTGTAGGACAGAG-3 (forward) and 5-AGGATCCAGGCAGTTTCCTCTGGAAGG-3 (reverse) were used to amplify a 349-bp fragment of exon 1. Allele B was detected using BanI restriction digestion of the 349-bp fragment. BanI cleaves the normal allele into two fragments of 260 bp and 89 bp while leaving the B allele uncut. MboII cleaves the C allele into two fragments of 279 bp and 70 bp. Detection of allele D was performed by RFLP on fragments (125 bp) amplified by site-directed mutagenesis PCR (SDM-PCR) using a unique set of primers (Madsen et al. 1995). Fragments specific for the D allele were cleaved with MluI into two fragments of 100 bp and 25 bp. Ambiguous RFLPs were confirmed by direct sequencing. Compound heterozygotes were determined by cloning exon 1 into pBluescript SK, followed by direct sequencing employing the reverse primer used to amplify exon 1.

Results

In the control population, the allele frequencies for the wildtype allele (52R, 54G, 57G, allele A) were 71.8%, for the 52C variant (allele D) 3.6%, for the 54D variant (allele B) 21.4%, and for the 57E variant (allele C) 3.2%. Hence, the composite frequency of structural *MBL* variant alleles was 28.2%. Genotype frequencies did not deviate from Hardy-Weinberg expectations in the HIV-positive (P=0.78) and control groups (P=0.26). Results of *MBL* genotyping are shown in Table 1. The frequency of the homozygous or compound heterozygous variants in the HIV-1-infected and control group was 5.8% and 5.7%, respectively, which was not significant (P=1.0, two-sided Fisher's exact test). Moreover, the frequencies of homozygote normals and heterozygotes did not differ significantly between the groups (Table 1). Further stratification of the groups by sex, age, gender, or the known presence of bacterial infections did not provide evidence of an association between *MBL* alleles or genotypes and HIV infection (data not shown).

Table 1.

Distribution of *MBL* genotypes among HIV-positive individuals and controls from the general population.

Genotype	HIV-infected individual (n=138)	Controls (n=140)
Homozygous normal (52 C/C, 54 G/G, 57 G/G)	74 (54%)	69 (49%)
Heterozygous	56 (40.6%)	63 (45%)
52 C/T	8	8
54 G/A	38	48
57 G/A	10	7
Homozygous and compound heterozygote variants	8 (5.8%)	8 (5.7%)
52 T/T	0	0
54 A/A	5	5
57 A/A	0	1
52T/54A	2	2
54A/57A	1	0

Discussion

Several independent studies have provided evidence that MBL plays a protective role in HIV-1 infection in Scandinavian populations, although the precise nature of this relationship is unclear (Garred et al. 1997; Nielsen et al. 1995; Pastinen et al. 1998). In vitro studies have shown that MBL can bind efficiently to the carbohydrate moieties present on gp120/gp41 (Saifuddin et al. 2000). It seems plausible, therefore, that low serum MBL may result in opsonization deficiency, and thus increased susceptibility to primary HIV-1 infection. Our results, however, do not support this hypothesis, showing equal prevalence of homozygote variants in control and HIV-infected individuals. This is in agreement with other groups who failed to detect an association between *MBL* polymorphisms or serum levels and HIV-1 infection in European HIV patients (Amoroso et al. 1999; Senaldi et al. 1995). Discrepancies between our results and the Scandinavian studies may be due to differences in ethnicity, environmental and social conditions of the populations. A noteworthy difference is the decreased allele frequency of variant alleles in the control group in the Scandinavian studies (16%-20%) relative to a significantly higher frequency of 28.3% in the Colombian controls. The selective pressure that maintains a high proportion of non-functional *MBL* alleles is unknown. However, it is conceivable that constitutive or acquired immune mechanisms exist that can complement or take up the innate defense function provided by MBL. Such redundant defense systems are likely favored under conditions of high pathogen exposure and high frequency of non-functional MBL alleles. Compared with Scandinavian populations, both conditions seem present in the Colombian population, possibly

explaining the divergent results of a contribution of MBL to susceptibility to HIV infection.

ADDENDUM 1.

Distribution of *MBL* genotypes among tuberculosis patients and controls from the study population described in Chapter 2.

Genotype	Tuberculosis patients (n=44)	Controls (n=210)
Homozygous normal (52 C/C, 54 G/G, 57 G/G)	22 (50%)	113 (53.8%)
Heterozygous	20 (45.4%)	87 (41.4%)
52 C/T	3	14
54 G/A	13	61
57 G/A	4	12
Homozygous and compound heterozygote variants	2 (4.5%)	10 (4.8%)
52 T/T	0	0
54 A/A	2	6
57 A/A	0	0
52T/54A	0	4

Connecting text

It is widely accepted that identifying risk factors for the HIV/AIDS pandemic may dramatically impact the global spread of tuberculosis. In chapter 2, I show that functional polymorphisms in exon 1 of *MBL* are not associated with HIV infection in a Colombian population. Furthermore, I show that *MBL* polymorphisms are not associated with tuberculosis susceptibility in this population. Thus, MBL serum levels may not be critical in the overall control of tuberculosis in this population.

However, to more directly elucidate risk factors for tuberculosis we tested a well-known tuberculosis susceptibility gene, *NRAMP1*, in a family-based pediatric tuberculosis population. This population represents a less variable phenotype with minimal gene-environment interactions as compared to adult patients. Alleles showing evidence of association were further analyzed with respect to covariates (family structure, gender, ethnicity, site of disease-pulmonary/extrapulmonary, and age of onset) under a conditional logistic regression framework. Finally, in order to correlate *NRAMP1* polymorphisms with function, we adapted the M6PR recruitment assay, previously designed to measure murine Nramp1 activity (Cuellar-Mata, 2002), to human cells. These results are presented in Chapter 3.

Abstract

Tuberculosis, caused by *Mycobacterium tuberculosis*, is a major cause of morbidity and mortality worldwide. To study the host genetic component of tuberculosis susceptibility, we enrolled 184 ethnically diverse families from the Greater Houston area with at least one child affected by pediatric tuberculosis disease. Employing a family-based control design, we found allelic variants of the *NRAMP1* gene (alias *SLC11A1*) significantly associated with tuberculosis disease in this pediatric patient population ($P = 0.01$; Odds ratio [OR] = 1.75 [95% confidence interval: 1.10 – 2.77]). The association of *NRAMP1* with pediatric tuberculosis disease was significantly heterogeneous ($P = 0.01$) between simplex ($P < 0.0008$; OR = 3.13 [1.54– 6.25]) and multiplex families ($P = 1$) suggesting an interplay between mechanisms of genetic control and exposure intensities. To test for correlation of the *NRAMP1* tuberculosis susceptibility allele with altered NRAMP1 function, we measured recruitment of mannose-6-phosphate receptor (M6PR) to *Salmonella*-containing vacuoles in monocyte-derived-macrophages (MDM) from pediatric tuberculosis patients. M6PR recruitment was significantly lower ($P = 0.024$) in MDM from patients homozygous for the *NRAMP1* risk allele as compared to MDM from patients heterozygous for the risk allele. Hence, impairment of NRAMP1 function appears to result in fast progression from infection to tuberculosis disease.

Words: 200

Introduction

The human pathogenic bacterium *Mycobacterium tuberculosis*, the causative agent of tuberculosis, infects an estimated one third of the world's population resulting in over 8 million tuberculosis cases and 2 million deaths each year (WHO, 2001). The rate of progression from infection to disease is highly variable, and approximately 90% of infected individuals never develop clinical disease. Of the 10% of *M. tuberculosis* infected persons who do develop clinically-overt disease, approximately half will be diagnosed within less than 2 years of infection and can be considered fast progressors. This so-called primary tuberculosis disease is particularly common among children, and the majority of pediatric cases present with primary tuberculosis disease. Tuberculosis patients who progress more slowly from infection to tuberculosis disease and develop clinical disease more than two years after infection are somewhat arbitrarily referred to as "reactivation" cases. Little is known about the mechanisms that influence the rate of progression from infection to disease. For example, it is unknown if different mechanisms of pathogenesis operate in individuals who progress at different rates from infection with *M. tuberculosis* to clinical tuberculosis disease.

Many lines of evidence support an important role of host genetic variation in tuberculosis susceptibility, including animal models of the disease (Lavebratt et al., 1999; Kramnik et al., 2000; Mitsos et al., 2000; Sanchez et al., 2003), ethnic clustering of tuberculosis cases (Stead et al., 1990), increased concordance rates of tuberculosis among monozygotic versus dizygotic twins (Kallmann and Reisner, 1943; Comstock, 1978),

evidence that certain gene variants are associated or linked with increased risk of tuberculosis (Marquet and Schurr, 2001; Casanova and Abel, 2002), and the demonstration that patients with Mendelian disorders of the interleukin 12-interferon-γ axis are hyper-susceptible to *M. tuberculosis* (Casanova and Abel, 2002). However, most genetic studies have investigated adult pulmonary tuberculosis cases whereas few studies have focused on pediatric or primary tuberculosis disease (Hoal-Van Helden et al., 1999; Greenwood et al., 2000). From a genetic point of view, pediatric tuberculosis disease patients represent a better defined age-of-onset group as compared to adult patients who can vary considerably in terms of age-at-infection and time to onset-of-disease, suggesting that genetic factors (as in many other diseases) should have a stronger effect on childhood disease. Hence, to obtain a less variable phenotype of tuberculosis susceptibility and to minimize gene-environment interactions for increased susceptibility gene penetrance, we studied pediatric cases.

To elucidate the host genetic factors that impact on disease susceptibility, linkage or association studies can be pursued. Non-parametric linkage studies are generally employed for genome scanning but have the disadvantage that only strong genetic effects can be detected. Genome scans have been conducted for both leprosy (Siddiqui et al., 2001; Mira et al., 2003) and tuberculosis (Bellamy et al., 2000) but significant linkage hits were detected only in the two leprosy scans. Hence, a strategy like the one successfully employed to identify variants in the *PARK2/PACRG* genes as universal risk factors of

leprosy (Mira et al., 2004) presently can not be pursued in tuberculosis. Candidate gene analysis can be performed by testing for association or linkage of specific variants with disease susceptibility. Yet, the majority of all studies in infectious diseases employ association tests. Association studies can be based on classical case-control designs, however, cryptic population substructures may result in inflated type I errors (Cardon and Palmer, 2003). Alternatively, family-based designs can be used, especially in regions where the likelihood of population admixture and stratification is high. The general principle of the family-based association study is to search for a distortion of the transmission of candidate gene alleles from parents to affected children, a strategy that has been termed "Transmission Disequilibrium Test" (TDT) (Spielman et al, 1993). Families with missing parental data can be analyzed either by reconstructing parental genotypes from children (RC-TDT) (Knapp, 1999)or by using unaffected sibs as controls (Sib-TDT) (Spielman and Ewans, 1998).

The Natural Resistance-associated Macrophage Protein gene 1 (*NRAMP1,* alias *SLC11A1*) is a well known tuberculosis susceptibility candidate gene (Gros and Schurr, 2004). The interest in *NRAMP1* leading to the molecular cloning of the gene (Cellier et al., 1994) was stimulated by the importance of murine *Nramp1* for innate resistance/susceptibility to infection with *Mycobacterium bovis* (BCG) and other atypical mycobacteria such as *M. smegmatis/intracellulare* (Goto et al, 1989) and *M. avium* (Gomes and Appelberg, 1998). Human *NRAMP1* consists of 15 exons that span 13 kb (Marquet et al., 2000) and encode a 550 amino acid polytopic membrane protein with 12

predicted transmembrane domains (Cellier et al., 1995). Owing to the high degree of homology between mouse and human Nramp1 proteins (Cellier et al., 1994) and supported by preliminary experimental data (Goswami et al., 2001) it is generally assumed that Nramp1/NRAMP1 fulfill the same cellular function. In mouse macrophages Nramp1 is expressed in the phagosomal membrane where in analogy to Nramp2 the protein acts as transporter of divalent cations (Gruenheid et al., 1997). How Nramp1-mediated changes in cationic fluxes translate into the phenotype of innate resistance/susceptibility to multiple macrophage pathogens is currently under intense investigation. Likewise, it is presently not known if or how *NRAMP1* alleles associated with risk of various human diseases impact on NRAMP1 function (Poon and Schurr, 2004).

Here, we report strong association between *NRAMP1* alleles and pediatric tuberculosis disease specifically among ethnically-diverse individuals that are likely to lack previous exposure to *M. tuberculosis*. Moreover, the *NRAMP1* risk alleles displayed reduced protein function as measured by the capacity of NRAMP1 to antagonize *Salmonella*-dependent blockade of phagosome maturation. Indeed, *Salmonella*-containing vacuoles (SCVs) formed in monocyte-derived macrophages (MDM) from individuals bearing risk alleles showed reduced recruitment of mannose-6-phosphate receptor (M6PR) (Cuellar-Mata et al., 2002). These results shed new light on the role of *NRAMP1* in risk of tuberculosis disease and provide a plausible explanation for *NRAMP1* genetic heterogeneity in tuberculosis susceptibility.

Materials and Methods

Families: The diagnostic criteria for pediatric cases were culture confirmation of tuberculosis (78 patients) or clear clinical criteria of disease (Starke and Taylor-Watts, 1989; Starke, 2000). All parental cases were culture positive. Information regarding BCG vaccination and previous tuberculosis disease were obtained by interview or by visual inspection of skin scars. Ethnicity was self-reported. Mantoux status of family members was determined as part of routine patient care and contact tracing. Blood (2 ml to 10 ml) was obtained by venipuncture and used for extraction of genomic DNA with the Nucleon extraction kit (Pharmacia-Amersham). Written informed consent was obtained from all study participants. The study was approved by the Institutional Review Board at Baylor College of Medicine, Houston, TX, USA; and the Ethics Committee at the Research Institute of the McGill University Health Centre, Montreal, Que, Canada.

Genotyping: The intragenic *NRAMP1* polymorphisms *274C/T, 469+14G/C, D543N,* and *1729+55del4* were determined as previously described (Liu et al, 1995). The *3'UTR* (N10) insertion/deletion polymorphism was amplified with the ^{32}P-labelled forward primer reported by Buu et al (1995) employing 5'-TCAAGCTCCAGTTTGGAGCCT-3'as reverse primer and resolved as length variants on 6% polyacrylamide gels. The same conditions were used to genotype the promoter $(GT)_n$ [N01] polymorphism except primers 5' GACATGAAGACTCGCATTAG 3' and 5'TACCCCATGACCACACCC 3' were used as described by Marquet et al. (1999).

Markers *D543N* and *1729+55del4* (N09) were also genotyped with Taqman assays. The primers and probes used in the assays were designed by using the software PRIMER3. The following primers were synthesized: (c.D543N): 5'CCACCACCACTTCCTGTATG 3' and 5'CACGTCATACATGCCACTCC 3', (c.1729+del4): 5'GGGAGTGGCATGTATGACG 3' and 5' TCTATCCTGCTGCCTGCAC 3'. The following probes were synthesized and labelled with fluorescent dyes: (D543N): 5'-FAM-CCCTTTCTGGTCCTCTTCAAGGA-TAMRA, 5'-TET-CCCTTTCTGGTTCTCTTCAAGGAGC-TAMRA; (c.1729+del4): 5'- FAM-TGGCCTGCTGGATGTGGAG-TAMRA, 5'-TET-TGACTGGCCTGCTGGAGAGG-TAMRA. PCR reactions were performed in a volume of 45 μl containing 5μl of 10 X PCR buffer (Invitrogen), 5 μl of $MgCl_2$ (50 mM) (Invitrogen), 1 μl of dNTPs (10mM) (Invitrogen), 0.75 μl of forward primer (20 mM), 0.75 μl of reverse primer (20 mM), 0.60 μl of FAM probe (2 M) (Research Genetics), 0.60 μl of TET probe (2 M) (Research Genetics), 0.1 μl of Platinum *Taq* polymerase (Invitrogen) and 5 μl of DNA (10 ng/μl). Three non-template controls were included on each plate. All PCRs were carried out in transparent 96-well plates with caps (Applied Biosystem). DNAs were amplified in MJ PT-100 machines (MJ Research) under the following conditions: 1) 96 °C for 10 min; 2) 96 °C for 25 sec; 3) 60 °C for 1 min; 4) repeat step 2 to 3 for 39 times; 5) 72 °C for 5 min; 6) 10 °C ambient time. PCR products

were analyzed with an Applied Biosystem 7700 Sequence Detector (Applied Biosystem) spectrophotometer equipped with Sequence Detector v1.7 software. Fluorescence readings were exported to a spreadsheet and graphed as a scatter plot.

Markers rs2292555, rs1017698, and rs9076 were genotyped by PCR-RFLP under identical conditions. The PCR reaction mixture included 100 ng of genomic DNA, 1X Buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.09 mM dNTPs, 0.2 μM of each primer (rs2292555: 5'-AGCCAGGGTAGGCAGGATAC-3'; GGCATTCACGATTGCTTTTC-3'; rs1017698: 5'-CCACCATAGCCAAACCATTC-3', 5'-GGGATGTGATACCCTTCCAG; rs9076: 5'-GTTTTATCCGCAGCCCTTTT-3', 5'- CCAGTCGGAAGAAACAGCAT-3'), and 1 unit of *Taq* polymerase. Cycling conditions included an initial denaturation at 95 °C for 3 min, followed by 25 cycles of 95 °C for 50 sec, 50 °C for 50 sec, then 72 °C for 50 sec, and a final extension at 72 °C for 10 min. For marker rs2292555 a total of 5 μl of PCR product was added to 5 μl of digestion mix which contained 1 X Buffer 4 (NEB Biolabs), and 0.3 units of *Dde*I, followed by incubation at 37 °C for 4 hrs. Conditions for DNA restriction were identical for markers rs1017698 and rs9076 except that 0.4 units of *Bts*I were used for marker rs1017698 and 0.1 units of *Bsg*I and 1X were used for marker rs9076. All banding patterns were resolved on a 2% agarose gel stained with ethidium bromide. Markers rs2104615, rs4324314 and rs4674297 were genotyped on the UHT

Orchid genotyping platform (Bell et al., 2002) as previously described in detail (Mira et al., 2004).

M6PR and GFP-*Salmonella typhimurium* co-localization assay: Human U-937 and transfected U-937 cells overexpressing a *NRAMP1*-c-Myc-tagged construct (Roig et al., 2002) were seeded (4 x10^5 cells/well) in a 12-well plate on glass coverslips. The cells were differentiated using 20 nM phorbol 12-myristate 13-acetate (PMA; Sigma) for 48 hrs followed by a 24 hrs incubation without stimulation. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (inactivated, endotoxin tested), 2 mM L-glutamine and 1 mM sodium pyruvate (Gibco). The cells were infected with *Salmonella enterica* serovar *typhimurium* (*Salmonella)* containing a plasmid expressing a green fluorescent protein (GFP). The bacteria were grown in Luria-Bertani broth supplemented with tetracycline (12 μg/ml) overnight with shaking at 37 °C. The bacteria were subcultured at a 1:33 dilution for 3 hours at 37 °C 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; Gibco) to give an OD_{600} of approximately 0.2 before being added to the differentiated cells for 20 min at 37 °C, 5% CO_2 at a MOI 100:1. After invasion, the cells were washed gently 3 times with PBS to remove non-internalized bacteria.

For experiments with MDM, a total of 10 ml of blood were taken from control individuals and patients for each experiment. The blood was diluted 1:2 using RPMI 1640 with no supplements and layered over Ficoll-Paque (Amersham Bioscience). The diluted blood and Ficoll-Paque were centrifuged for 30 min at 400 x g. The layer containing the mononuclear cells was removed. The mononuclear cells were washed with RPMI and centrifuged for 10 min at 400 x g twice and resuspended in RPMI with supplements. The macrophages 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, and infected as described for U937 such that a majority of infected cells phagocytose 1-2 GFP expressing *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. To inhibit further maturation, all subsequent steps were done on ice unless otherwise noted. 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 dilution in PBS plus 1% normal goat serum; Coxtex Biochem) for 15 min followed by a 20 min incubation with Alexa Fluor 350 labeled goat anti-rabbit secondary antibodies (1:500; Jackson ImmunoResearch). Cells were washed between each subsequent labeling procedure with ice-cold PBS for 2 x 5 min. Cells were fixed with 4% paraformaldehyde for 1 hr at room temperature and blocked overnight with 5% normal goat serum plus 0.2% Triton X-100

in PBS. All subsequent antibodies were diluted in PBS plus 1% normal goat serum and 0.2% Triton X-100. To label late-endosomes, cells were incubated the following day with a mouse anti-M6PR antiboby (1:50; Affinity BioReagents) or mouse anti-LAMP1 antibody (1:300; Developmental Studies Hybridoma Bank) for 1 hr followed by a Cy-3 conjugated goat anti-mouse 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 co-localization between M6PR or LAMP1 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 Inc.). Only cells containing 1-2 bacteria were considered. All counting was done with the investigator being blinded for the genotypes of the studied cells. One hundred bacteria per slide were counted in five independent counts.

Statistical analysis: The association study was mainly performed by the family-based method implemented in the FBAT program (Horvath et al., 2001). The FBAT statistic combined the three different methods described in the text (TDT, RC-TDT, and Sib-TDT). Furthermore, it allows the use of an empirical variance-covariance estimator for

the statistic which is consistent when sibling marker genotypes are correlated (e.g. when the analysis include multiplex families) (Lake et al., 2000). Exact *P*-values also were computed using the RC-TDT software (Knapp, 1999). Finally, alleles with evidence for association also were analyzed by conditional logistic regression as described (Schaid and Rowland, 1998) assuming a multiplicative effect of alleles on the disease relative risk. This analysis allowed us to provide odds-ratio estimates, and to test for differences in the regression coefficients associated with selected polymorphisms according to five binary criteria described in the text.

To test for heterogeneity of the sample according to a binary criterion (e.g. simplex/multiplex), the analysis was performed on the whole sample (184 families), and separately on the two subsamples (143 simplex and 41 multiplex families). Under the hypothesis of homogeneity, twice the difference between the likelihood of the whole sample and the summed likelihoods of the two subsamples is distributed as a chi square with one degree of freedom.

Hardy-Weinberg Equilibrium (HWE) was tested at each SNP for the subset of all parents across ethnicities, and for the groups of Black and Hispanic parents independently. No significant deviations from HWE were observed. The strength of LD between pairs of SNPs was measured as D prime (D') (Lewontin, 1988), using

Haploview (http://www.broad.mit.edu/personl/jcbarret/haplo/). LD blocks were inferred from the definition proposed by Gabriel et al. (2002) as implemented in Haploview with D' confidence bounds of 0.7 – 0.92.

Results

Description of patients and their families. All families enrolled in the study were from greater Houston, TX. The Houston metropolitan area historically has had a high rate of pediatric tuberculosis cases and is ethnically very diverse (Starke and Taylor-Watts, 1989). To avoid possible confounding of gene-phenotype associations due to inappropriately chosen controls or population substructures we conducted a family-based association study. This design is particularly robust in an ethnically and racially mixed community like that of the greater Houston area. We enrolled 184 nuclear families with at least one child with pediatric tuberculosis (Table 1). The majority of families (n=143) were composed of only a single tuberculosis case (simplex families) whereas more than one case was diagnosed in 41 families (multiplex families). In 73 of the 184 families in our sample one parent was not available for analysis. With regard to ethnicity, we enrolled 136 Hispanic, 69 Black, 13 Asian, 7 White, and 9 tuberculosis patients of mixed ethnic origin. Of these 236 tuberculosis cases, 30 were adult cases and 206 were children. The disease manifestation was classified as pulmonary in 57.3%, extrapulmonary in 37.5%, and mixed pulmonary and extrapulmonary in 11.2% of all pediatric cases. There were no statistically significant differences in the proportion of simplex versus multiplex families and in pulmonary versus extrapulmonary involvement across ethnic groups (data not shown).

***NRAMP1* is associated with pediatric tuberculosis disease.** Over all families, the common "C" allele of the *NRAMP1* N02 polymorphism was significantly associated with increased risk of pediatric tuberculosis disease ($P = 0.01$; Figure 1). Under a multiplicative genetic model, the odds ratio (OR) of tuberculosis for C/C homozygotes vs. C/T heterozygotes or C/T heterozygotes vs. T/T heterozygotes was 1.75 [95% confidence interval: 1.10 – 2.77]. In addition, there was a trend ($0.028 < P > 0.075$) in favor of a positive association between the *NRAMP1* N01 promoter polymorphism and tuberculosis (Figure 1). There was no significant association between the *NRAMP1* polymorphisms located in the 3' region of the gene and pediatric tuberculosis disease.

Linkage disequilibrium among markers in the *NRAMP1* genome region. To better define the observed association of *NRAMP1* alleles with pediatric tuberculosis disease we computed linkage disequilibrium (LD), measured as D', among the five tested markers in the group of Hispanic parents, the largest ethnic group among the enrolled families. We found that the three 5' markers (N01 – N03) were in strong LD among them but observed only weak to moderate LD with markers N09 and N10 in the 3' *NRAMP1* region. To better delimit the LD pattern of the tuberculosis associated markers N01-N03, we genotyped 7 additional markers flanking the 5' *NRAMP1* region. Five of these SNPs were used to tag the *MGC581* open reading frame (rs4674297 and rs4324314), and the *MR-1* gene (rs9076, rs2014615, rs1017698), the two closest neighbours located 15 kb and 65 kb upstream of *NRAMP1*, respectively. The two remaining SNPs were located in intron 6 (rs2290708) and exon 15 (NRAMP1 D543N) of the *NRAMP1* gene. Over the entire

interval of approximately 80 kb, we were able to identify four haplotype blocks (Figure 2). Although clearly not part of the same haplotype blocks there was substantial LD between pairs of SNPs among 5' *NRAMP1* markers, the two *MGC581* tag SNPs, and, to a lesser degree, the *MR-1* located SNPs (Figure 2). None of the additional markers showed significant evidence for association with pediatric tuberculosis disease and all pediatric tuberculosis associated SNPs localized to the 5 kb haplotype block 3 (Figure 2). The variable strength of association with tuberculosis among those markers is likely explained by the fact that LD is not complete between markers of block 3, and the differences in allele frequencies (especially between N02 and N03). A similar pattern of pairwise D' values was also observed for the parents of the Black families. However, due to the reduced number of informative chromosomes, confidence intervals were too large to allow for the definition of haplotype block structures (data not shown).

Family characteristics and strength of association between *NRAMP1* alleles and pediatric tuberculosis disease. To test whether the association observed between the *NRAMP1* N02 polymorphism and tuberculosis was influenced by family or case characteristics, we performed heterogeneity tests in the conditional logistic regression analysis framework (Schaid and Rowland, 1998). Specifically, we tested for differences in the regression coefficient associated with each of the two polymorphisms according to five binary criteria: family structure (simplex/multiplex), ethnicity of family (Hispanics/others), sex of affected child (male/female), anatomic site of tuberculosis (pulmonary/extra-pulmonary), and age-of-onset (≤ 5years/>5 years). Due to small

numbers, Asian and White families could not be tested independently for *NRAMP1* N02 association heterogeneity, and it is formally possible that *NRAMP1* N02 is not a significant risk factor for pediatric patients from these racial backgrounds. Only the sex of pediatric patient and family structure were found to have significant effects. The association of the *NRAMP1* N02 polymorphism and tuberculosis was stronger in males (OR for C/C vs. C/T = 2.82 [1.44-5.61]), and the difference in transmission between male and female patients was borderline significant ($P<0.04$).

Next, the association of *NRAMP1* with tuberculosis was analyzed separately in simplex and multiplex and families. Independent of the mode of analysis, there was a highly significant distortion ($P < 0.0008$) of the *NRAMP1* N02 polymorphism transmission in simplex families (OR for C/C vs C/T or C/T vs T/T = 3.13 [1.54– 6.25] that was not detected in multiplex families (Table 2). Formal testing of variable strength of association between N02 and pediatric tuberculosis in simplex vs multiplex families clearly revealed a significant heterogeneity ($P < 0.01$). This result argues that differences in *NRAMP1* N02 transmission to tuberculosis-affected children in simplex and multiplex families represent a true effect and are not simply a reflection of different numbers of informative simplex and multiplex families. When focusing only on the 17 informative simplex families with male pediatric patients, the effect of N02 on tuberculosis risk was very highly significant ($P < 0.00004$) with an estimated OR for C/C vs. C/T of 20.0 (2.69-148). Those 17 families include 20 heterozygous C/T parents who transmitted the C allele to their affected child 19 times.

Family exposure to *M. tuberculosis* and strength of association of *NRAMP1* with pediatric tuberculosis disease. To follow-up on the restriction of *NRAMP1* tuberculosis association, we decided to investigate the heterogeneity of family structure in *NRAMP1* mediated risk on pediatric tuberculosis disease (Table 2). The majority (26/41) of multiplex families in our family collection included adult infectious cases. Children living in close proximity to adult cases are expected to have increased exposure to *M. tuberculosis*. Hence, we used the PPD skin test conversion among all unaffected co-sibs as a measure of exposure intensity in individual families, and found a substantial higher proportion of co-sibs that tested PPD+ in multiplex families with at least one affected parent (62.5%) as compared to simplex families (36.5%). Since pediatric cases generally have a low infectious potential, there was no significant difference in the proportion of PPD+ co-sibs among multiplex families without adult cases (31%) and simplex families.

To further test the resulting hypothesis that *NRAMP1* effects on tuberculosis disease risk are most readily detectable under conditions of low *M. tuberculosis* transmission, we selected all simplex families comprising at least one child in addition to the affected sib. Of the available 71 families, 32 families included at least one additional PPD+ co-sib ("high exposure families"), whereas among 39 families no PPD+ co-sib was identified ("low exposure families"). Family size was not a confounding factor for classification into high and low exposure families ($P > 0.9$; Table 3). Among the entire subsample of 71 simplex families with at least one additional co-sib, there was strong

evidence for an association of *NRAMP1* N02 alleles with pediatric tuberculosis disease ($P = 0.006$). When separated into high- and low-exposure families there was less evidence of significant distortion of *NRAMP1* N02 allele transmission among high exposure families ($P = 0.17$) as compared to low exposure families ($P = 0.01$). Since less than 10% of cases had been vaccinated with BCG, these findings strongly suggest that *NRAMP1* alleles have their highest impact on risk of tuberculosis disease under conditions of low transmission/exposure of *M. tuberculosis*.

Quantitation of NRAMP1 functional activity. Significant associations between marker alleles and phenotype do not implicate the marker in pathogenesis. To correlate NRAMP1 function with *NRAMP1* polymorphisms, we adapted the M6PR recruitment assay, previously developed to determine murine Nramp1 functional activity (Cuellar-Mata, et al., 2002), to human cells. Following infection of cells with GFP *Salmonella* bacteria, the assay estimates Nramp1-modulated intracellular vesicle flow by quantitating the number of phagocytosed GFP-expressing *Salmonella* that co-localize with the late endosomal marker M6PR. Increased Nramp1 activity results in significantly increased M6PR recruitment to SCVs (Cuellar-Mata, et al., 2002).

We first tested M6PR recruitment in *Salmonella* infected U937 control cells and *NRAMP1* overexpressing U937 transfectants. Both wildtype cells and transfectants were differentiated by exposure to PMA. Following differentiation, low levels of *NRAMP1* were detected in control transfected wildtype cells while a strong overexpression of

NRAMP1 was observed in the *NRAMP1*-transfectants regardless of their PMA differentiation status (data not shown) (Roig et al., 2002). Differentiated cells were infected with GFP-*Salmonella* and the proportion of SCV that showed co-localization with M6PR was determined (Figure 3A). In 8 independent experiments we recorded a significantly higher M6PR recruitment by *NRAMP1* overexpressing cells (mean M6PR recruitment: 35.1%) versus *NRAMP1* non-overexpressing controls transfectants (mean: 22.1%; $P < 0.0001$; Figure 3B). Next, we obtained monocyte-derived macrophages (MDM) from 5 healthy donors, infected the cells with GFP-expressing *Salmonella* bacteria and determined the proportion of SCVs that had recruited M6PR. To obtain an estimate of assay variability, the experiment was 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 assay could be used for NRAMP1 functional activity comparisons among individuals carrying distinct *NRAMP1* genotypes (Table 4). Moreover, we noticed that M6PR recruitment was lower among homozygous carriers of the high risk N02-C (*NRAMP1*-274C) allele. Colocalization of LAMP1 and GFP expressing *Salmonella* was 75% to 80% (data not shown).

Recruitment of M6PR to SCVs formed in MDM from pediatric tuberculosis patients. In an effort to correlate the results of the genetic association study with NRAMP1 function, we traced families that had participated in the genetics study and invited them to participate in the functional replication study. Of those that could be contacted, 12 families agreed to participate and a blood sample was obtained from the

pediatric cases. We were able to obtain MDM from 11 pediatric tuberculosis patients and infected the cells with GFP-expressing *Salmonella* bacteria. Care was taken to infect the MDM under conditions to obtain a large proportion of MDM infected with 1-2 bacteria (Figure 4A). In such cells M6PR recruitment was determined by counting a minimum of 100 SCVs. Examples of SCVs recruiting M6PR are shown in Figure 4. Over all pediatric tuberculosis cases, recruitment efficiency varied from 26.7% to 42%. Of the randomly selected patients, 3 children belonged to Black families, 6 to Hispanic families, 1 child belonged to a White and 1 child to an Asian-Pacific family. Since the number of White and Asian children in the genetics study had been too small to allow tests for ethnicity specific effects of *NRAMP1* and since both the White and Asian child were homozygous for the high risk *NRAMP1-274C* allele, i.e. we had no ethnically matched heterozygous samples for M6PR recruitment, these two children were excluded from genotype-function comparisons. Among the remaining 9 children, 5 individuals were homozygous for the high risk *NRAMP1-274C* allele whereas four were *NRAMP1-274C/T* heterozygotes. The mean M6PR recruitment efficiency of *NRAMP1-274C/C* homozygotes was 29.0% whereas 274C/T heterozygotes recruited M6PR to 34.8% of SCVs (Figure 4B; $P = 0.024$). There was no discernible difference of genotype or M6PR recruitment between Black and Hispanic children; i.e. of the 4 *NRAMP1-274C/C* homozygotes, 1 was Black and 3 were Hispanic and M6PR recruitment overlapped between cells obtained from children of both ethnic backgrounds. Overall, these findings are in good agreement with the results of the genetic analysis. The data suggest that lower

NRAMP1 functional activity is underlying the observed association of the *NRAMP1-274C* allele with risk of pediatric tuberculosis disease.

Table 1: Characteristics of 184 nuclear tuberculosis families comprising 737 individuals enrolled for the present study.

	Total	TB Affected Patients				
		All Patients	Parents	Mean Age (Years) at Diagnosis (± SD)	Children	Mean Age (Years) at Diagnosis (± SD)
Females	353	118	18	25.4 ± 6.8	100	5.3 ± 5.7
Males	384	116	10	29.2 ± 6.3	106	6.2 ± 5.4
Combined	737	234	28	28.8 ± 6.6	206	5.7 ± 5.6

Table 2: Association between susceptibility to pediatric tuberculosis disease and *NRAMP1* alleles stratified by family structure.

		Simplex Families				Multiplex Families			
			P-Value				*P*-Value		
GENE	Polymorphism	Informative Families	FBAT	RCTDT	Conditional Logistic Regression	Informative Families	FBAT	RCTDT	Conditional Logistic Regression
NRAMP1	N01	39	0.020	0.048	nd	22	0.594	0.697	nd
	N02	36	0.00045	0.00059	0.00080	19	1.000	1.000	nd
	N03	37	0.014	0.021	nd	17	0.7546	0.876	nd

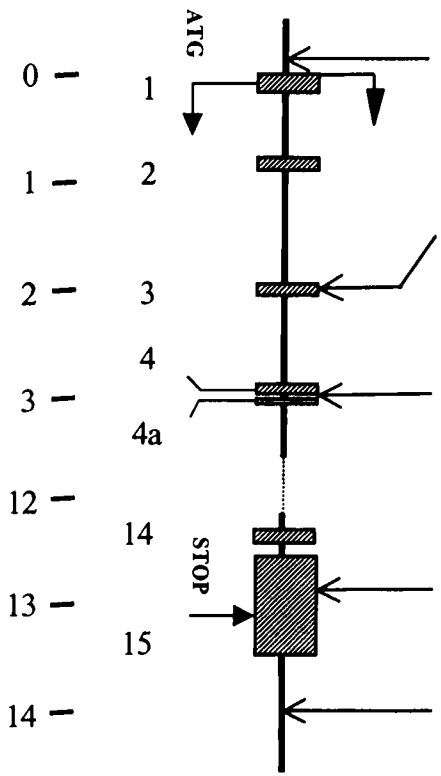
Table 3 : Family sibship size and proportion of families with at least one unaffected co-sib (UCS).

Number of families	Number of UCS	Number of families with UCS-PPD^{+} $\geq$ 1	Number of families with only UCS-PPD^{-}
38	1	14	24
24	2	12	12
6	3	4	2
3	4	2	1

Table 4: M6PR recruitment variability in MDM obtained from adult healthy control individuals

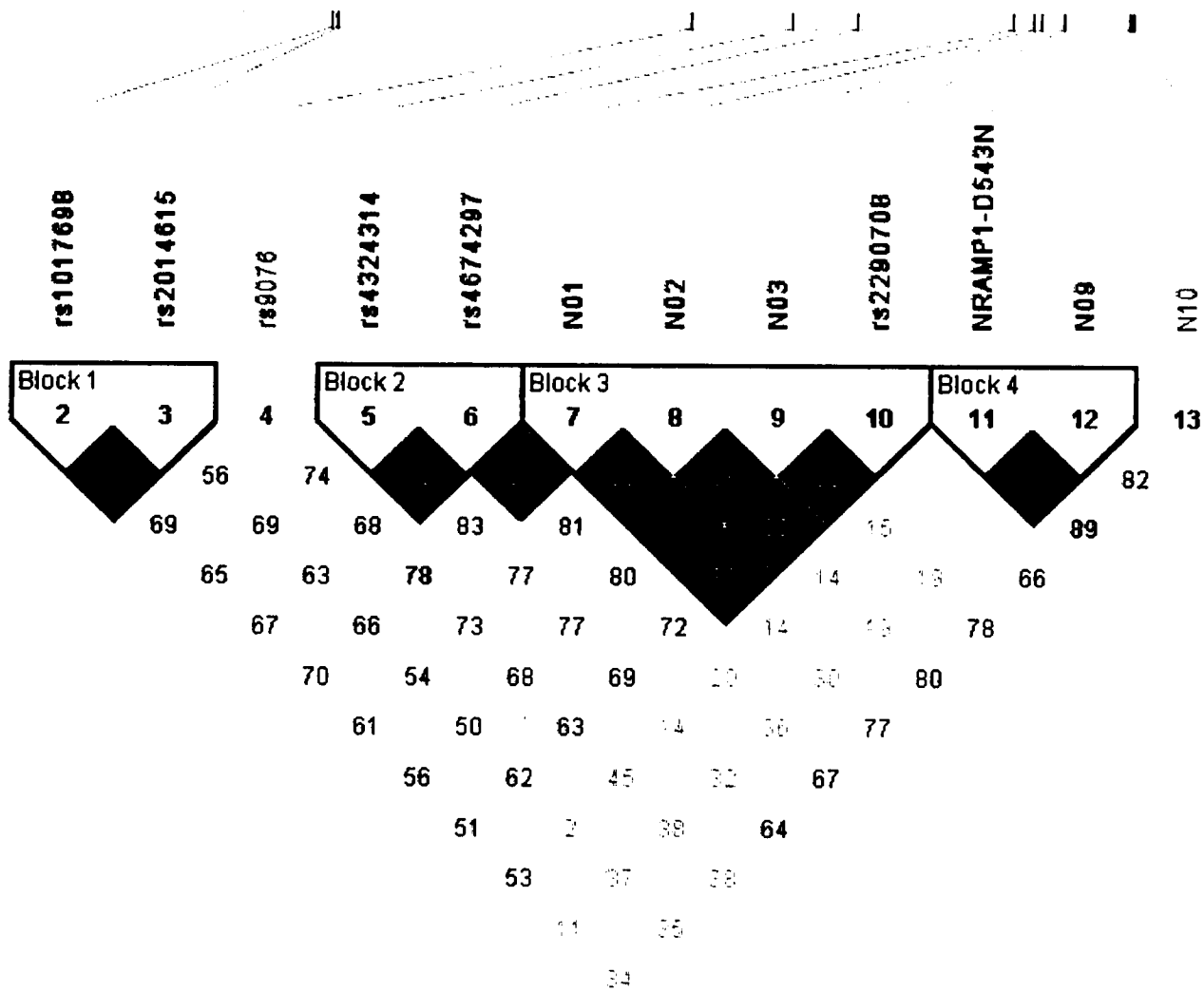
Control	N02 Genotype	% M6PR Recruitment to SCV
1	CC	29, 36
2	CC	28, 35
3	CT	43, 45
4	CT	41, 39, 43, 46
5	TT	45, 46, 43

Figure 1: Schematic presentation of the *NRAMP1* candidate gene and intragenic location of gene polymorphisms. The genomic distance spanned by *NRAMP1* in kilobase pairs (kb), the exon numbers, the translational initiation (ATG) and termination sequences (Stop), and the location of distinct gene polymorphisms with respect to the exon-intron organization are given on the left side of the diagram. Designation of gene polymorphisms either adopted names already established in the literature or followed standard nomenclature rules (Antonarakis, 1998). The type of polymorphism - microsatellite repeat, single nucleotide polymorphism (SNP), insertion/deletion polymorphism (INS/DEL) - together with a simple polymorphism alias as well as the identity and frequency of the common allele are also indicated. Finally, the number of families comprising at least one parent heterozygous for the polymorphisms, i.e. a parent for which preferential allele transmision can be monitored, is given. *P*-values indicating evidence for distortion of allele transmissions are given for the FBAT (Horvath, et al., 2001) and RCTDT (Knapp, 1999) analytical procedures.



Distance (kb)	Exon	Genomic Organization	Polymorphism	Type	Alias	Common Allele (Frequency)	Informative Families	P-values	
								FBAT	RCTDT
0	1		5' (GT)n	Micro-satellite	N01	133bp (0.765)	62	0.043	0.075
1	2								
			274C/T	SNP	N02	C (0.768)	55	0.010	0.0134
2	3								
	4								
3			469+14G/C	SNP	N03	G (0.793)	54	0.129	0.159
	4a								
12									
	14								
13			1729+59del4	INS/DEL	N09	+TGTG (0.859)	42	0.373	0.449
	15								
14			276del4	INS/DEL	N10	+CAAA (0.640)	77	0.265	0.307

Figure 2: LD pattern in the hispanic population between pairs of SNPs spanning the *NRAMP1* gene and its upstream genomic region. The *NRAMP1* 3' region is located on the right end of the schematic chromosome line indicated on top of the graph. Consequently, the *NRAMP1* gene orientation is in the 3'to 5' orientation from right to left. The telomere of chromosome 2q is located towards the right. Names of polymorphisms used for the LD matrix of pairs of markers are given and their chromosomal locations are indicated by solid lines. Haplotype blocks according to Gabriel et al (2002) are indicated, and names of markers that are part of haplotype blocks are indicated in bold. Each square represents the magnitude of pairwise LD. Each pairwise D' measure is shown as D'x 10^{-2} within the corresponding square. Squares without D' written on them represent D'of 1.0 Black squares indicate pairwise LD that is strong (lower confidence interval (CI), 0.7, upper CI > 0.92), light grey squares represent intermediate strength LD, white squares represent weak LD.


rs1017698
rs2014615
rs9076
rs4324314
rs4674297
N01
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N03
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NRAMP1-D543N
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Block 1
Block 2
Block 3
Block 4
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Figure 3: Colocalization of M6PR with SCVs in U937 cells and U937-*NRAMP1* cells overexpressing the *NRAMP1* gene. Control cells and *NRAMP1* overexpressing tranfectants were infected with a low dose of GFP-expressing *Salmonella* bacteria. Non-phagocytosed bacteria were labelled with Alexa Fluor 350 (blue) coupled anti-LPS antibody to allow discrimination of phagocytosed from external bacteria. **A:** For U937 and U937-*NRAMP1* cells, ingested GFP expressing Salmonella bacteria are shown on the left, distribution of M6PR is depicted in the central panels and the overlay between ingested Salmonella bacteria and M6PR is show in the right panels. **B:** The proportion of SCVs that colocalized with M6PR was determined in six independent experiments for U937 and U937-*NRAMP1* cells. Results for individual experiment are indicated by dots, and mean of SCVs colocalizing with M6PR is indicated by a short horizontal line for each cell type.

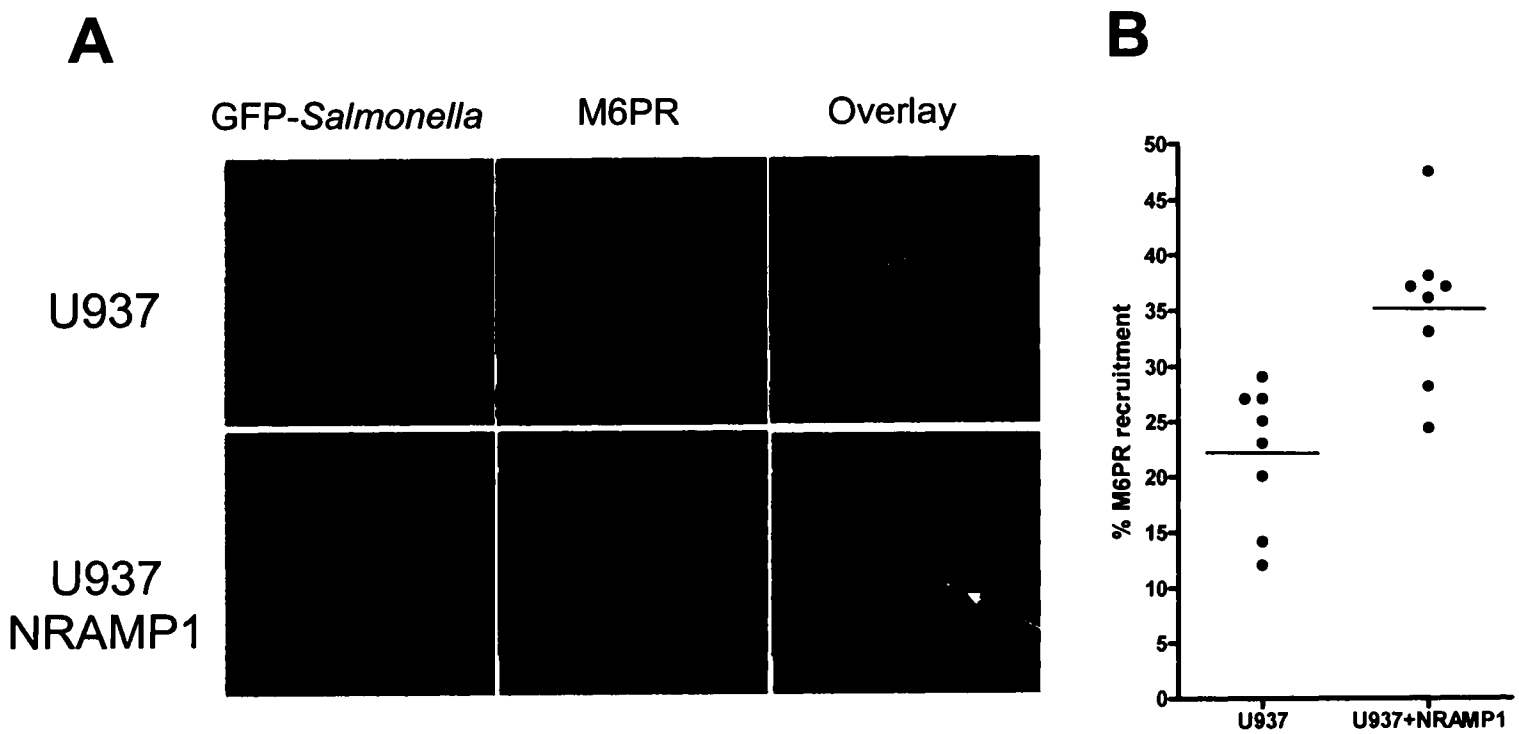
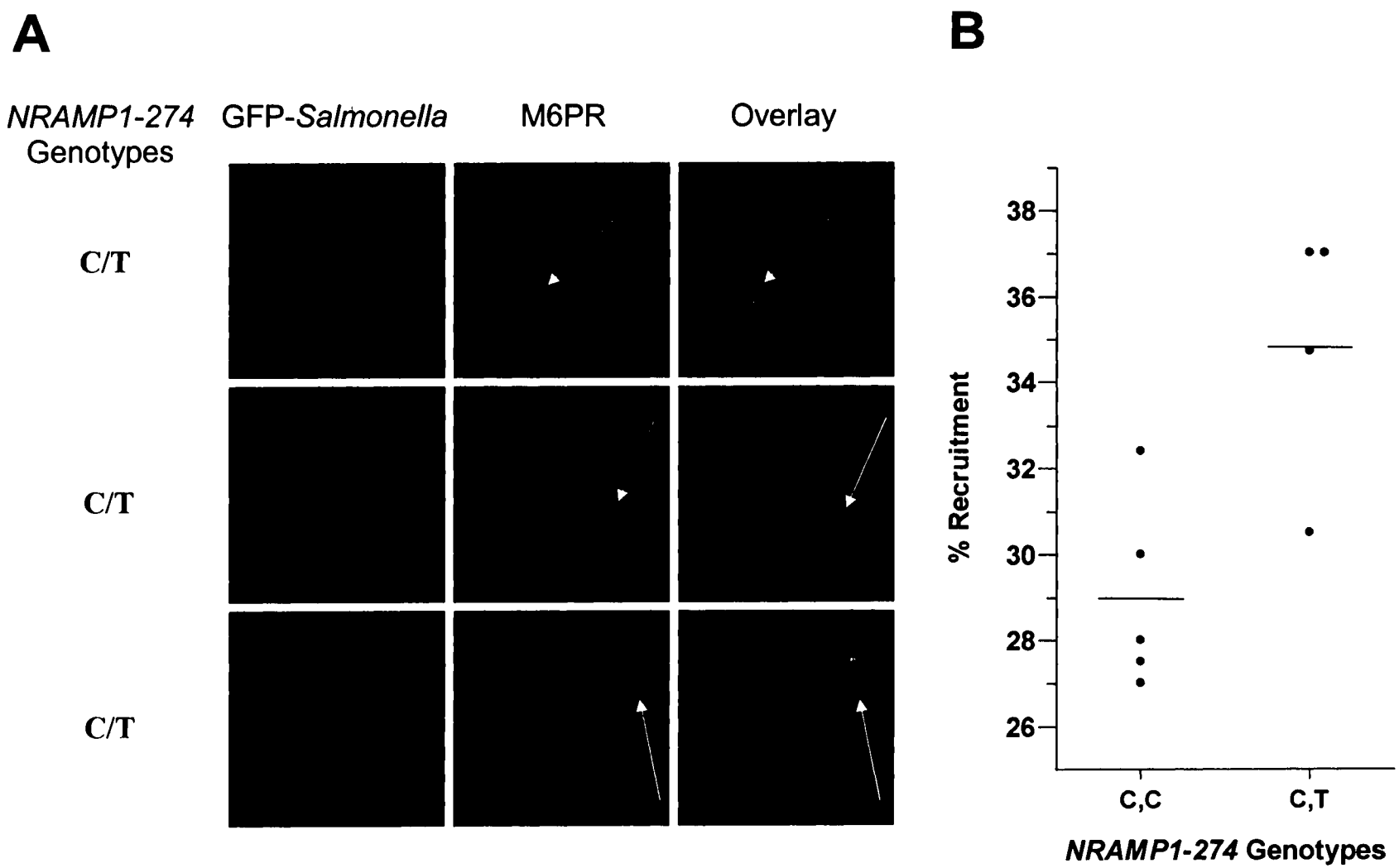

A
GFP-Salmonella
M6PR
Overlay
U937
U937
NRAMP1
B
% M6PR recruitment
50
45
40
35
30
25
20
15
10
5
0
U937
U937+NRAMP1

Figure 4: Colocalization of M6PR with SCVs in MDM obtained from pediatric tuberculosis patients with C/T *NRAMP1* N02 genotypes. MDM were obtained by standard protocols from peripheral blood leucocytes. **A:** Examples of M6PR recruitment to SCVs. The location of internalized GFP *Salmonella* bacteria is indicated on the left, M6PR staining is shown in the centre panel and the overlay between GFP-*Salmonella* and M6PR stain is given on the right. Use of low infectious load facilitated the accurate counting of co-localizing GFP-expressing *Salmonella* and M6PR in individual vesicles. The *NRAMP1* 274 C/T genotype for each donor is given on the right of the panel. **B:** Recruitment efficiency of M6PR to SCVs in MDM from pediatric tuberculosis disease patients according to their *NRAMP1* N02 genotypes. Recruitment of M6PR to SCVs is given as the percentage of GFP-expressing *Salmonella* co-localizing with M6PR. Recruitment levels are indicated for Black and Hispanic 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 was significant at $P = 0.024$ (two-tailed t test).


A
NRAMP1-274 Genotypes
GFP-Salmonella
M6PR
Overlay
C/T
C/T
C/T
B
% Recruitment
38
36
34
32
30
28
26
C,C
C,T
NRAMP1-274 Genotypes

Discussion

The human *NRAMP1* gene has been implicated in increased risk of tuberculosis disease in several studies. For example, polymorphisms in the 5' and 3' regions of *NRAMP1* have been found to be linked or associated with tuberculosis disease susceptibility in Guinea Conakry (Cervino et al., 2000), Japan (Gao et al., 2000), The Gambia (Bellamy et al., 1998; Awomoyi et al., 2002), Canada (Greenwood et al., 2000), Texas (Ma et al., 2002) and Denmark (Soborg et al., 2002) but not in Taiwan (Liaw et al., 2002) or Morocco (El Baghdadi et al., 2003). The focus of most studies was on susceptibility to smear positive tuberculosis among adult populations. The results of a Danish study (49) suggested a predominant impact of *NRAMP1* on bacterial replication but not cavity formation whereas a Japanese study (Abe et al., 2003) provided weak evidence for an impact of *NRAMP1* on cavity formation. Interestingly, *NRAMP1* also has been linked or associated with leprosy (Mira et al., 2003; Abel et al., 1998; Meisner et al., 2001) and visceral leishmaniasis (Bucheton et al., 2003; Mohamed et al., 2004) but not typhoid fever (Dunstan et al., 2001). Taken together, these studies suggest that *NRAMP1* mediated effects on susceptibility to intracellular diseases show heterogeneity across populations, epidemiological settings, and infectious agents.

Our findings demonstrate that the *NRAMP1* gene, previously implicated in the genetic control of adult tuberculosis, also influences the risk of pediatric tuberculosis disease. Surprisingly, our results demonstrate the direction of *NRAMP1* allele association with pediatric tuberculosis disease was inverted compared to previous studies in adult

pulmonary tuberculosis. While among adult patients the common 5' *NRAMP1* alleles had been found associated with protection, i.e. depleted among cases, (Cervino et al., 2000; Gao et al., 2000; Bellamy et al., 1998; Ma et al., 2002), in the pediatric cases the common alleles are risk factors, i.e. enriched among cases. The enrichment of the common N02 C-allele, and to a lesser degree the N01 and N03 common alleles, in early onset cases and the corresponding depletion of the same allele in late onset patients suggests that the N02 C-allele promotes rapid progression from infection to disease. In agreement with the suggestion that common *NRAMP1* alleles are risk factors for early-onset tuberculosis was a previous genetic analysis of tuberculosis in a large Canadian Aboriginal family that experienced a tuberculosis outbreak (Greenwood et al., 2000). In this outbreak, individuals had limited prior exposure to mycobacteria and all tuberculosis cases were diagnosed within a maximum time of 2 years from the index case with the majority of cases occurring within 6 – 9 months after diagnosis of the index case (Mah and Fanning, 1991). Consequently, all patients clinically diagnosed with tuberculosis could be classified as fast progressors or primary tuberculosis disease cases. The analysis revealed that common *NRAMP1* alleles were preferentially transmitted to tuberculosis patients. Considering that only approximately 10% of individuals infected with *M. tuberculosis* advance to clinical forms of tuberculosis, the involvement of genes controlling the rate of progression rather than *bona fide* susceptibility to tuberculosis may offer more effective genetic control of disease risk.

Among pediatric tuberculosis disease cases, *NRAMP1* alleles have different strength of association among different patient subgroups. The observation that

NRAMP1 association with tuberculosis disease is more readily detected among male than female pediatric cases is interesting in light of the known gender-specific differences in frequencies among adult tuberculosis cases. However, since evidence for such a gender-specific effect of *NRAMP1* alleles on pediatric disease was weak in our sample, additional studies are required to confirm the significance of this observation. By contrast, our data suggest that gene-environment interactions are critical for the appropriate selection of efficient host responses and, hence, genetic control mechanisms. This is illustrated by our observation that genetic control of tuberculosis disease by *NRAMP1* was most easily detected in families that experienced low exposure intensities to *M. tuberculosis.* Under conditions of increased exposure, the *NRAMP1* effect became less strong, suggesting that mechanisms independent of the *NRAMP1* gene become more prominent in this instance. This finding may reflect different epidemiologic settings and *M. tuberculosis* transmission rates experienced by pediatric cases in simplex and multiplex families. That exposure history is a critical parameter for *NRAMP1*-linked risk of tuberculosis also has been suggested by the previous genetic analysis of a tuberculosis outbreak in a Native Canadian community (Greenwood et al., 2000). Employing parametric linkage analysis, highly significant evidence for linkage of *NRAMP1* to tuberculosis susceptibility was found when a model of liability classes according to the mycobacterial exposure status of tuberculosis patients was employed. By disregarding exposure history, significant linkage of *NRAMP1* with tuberculosis susceptibility was lost (Greenwood et al., 2000). Hence, the genetic analysis of *NRAMP1* in primary tuberculosis disease during an outbreak in Northern Canada and in the present study, employing entirely different study designs on different ethnic backgrounds, reached the

same conclusions, i.e. *NRAMP1* genetic control is a function of previous exposure to mycobacteria and common *NRAMP1* alleles are risk factors of disease.

The function of the mouse Nramp1 protein has been subject of considerable discussion and controversy. While it is generally agreed that Nramp1, by analogy to Nramp2, is involved in metal transport across the phagosomal membrane, varying conclusions with respect to direction of transport, role of the proton gradient and topology of the protein have been reached by different investigators. One set of data suggested that that under physiological conditions Nramp1/NRAMP1 function as cation/proton antiporter at the late phagosomal membrane (Goswami et al., 2001; Zwilling et al., 1999; Kuhn et al., 2001; Kuhn et al., 1999). The resulting excess Fe^{2+} in the phagosome was suggested to contribute to the formation of microbicidal reactive oxygen intermediates via the Fenton/Haber-Weiss reactions (Zwilling et al., 1999). In contrast, a second set of data suggested that Nramp1/NRAMP1, like Nramp2/NRAMP2, function as metal/proton symporters (Gomes et al., 1998; Atkinson and Barton, 1999; Gomes and Appelberg, 2002). Specifically, real-time measurement of Mn^{2+} accumulation in distinct phagosomes within primary macrophages obtained from Nramp1-expressing and -deficient mice detected significant depletion of Mn^{2+} in the Nramp1 expressing phagosomes dependent on an intact proton-gradient across the vesicular membrane (Jabado et al., 2000). Finally, by redirecting an Nramp1 chimeric protein to the plasma membrane of transfected CHO cells it was possible to directly demonstrate that Nramp1, as Nramp2, mediates cation uptake along proton gradients (Forbes and Gros, 2003).

The survival of pathogens in their host cells may depend on altered vesicle maturation. In this regard, it has been shown that Nramp1 influences vesicle maturation in both *Salmonella* and *Mycobacteria* infected cells (Forbes and Gros, 2001). For example, survival of *Salmonella* in host cells depends on exclusion of the SCV from the lysosomal environment which in turn is a function of the exclusion of M6PR from the SCV (Garcia-del Portillo and Finlay, 1995). It has been shown that Nramp1 can mediate recruitment of M6PR to the SCV providing an assay for Nramp1 biological activity (Cuellar-Mata et al., 2002). Likewise, the application of membrane permeable iron chelators in Nramp1-deficient cells can mediate M6PR recruitment to the SCV suggesting that Nramp1 impacts on vesicle maturation via changes in intra-phagosomal iron concentrations (Jabado et al., 2003). Hence, Nramp1 appears to mediate its anti-microbial effects by two complementary strategies: reduction of essential ionic nutrients from the phagosomal environment of ingested pathogens and restoration of pathogen-diverted vesicle maturation.

Little is known about the biological function of NRAMP1 or how *NRAMP1* alleles linked or associated with infectious disease modulate protein function. Employing an *in vitro* reporter construct expression system, a common *NRAMP1* promoter length polymorphism was driving variable reporter gene expression in transiently transfected cells (Searle and Blackwell, 1999). In addition, it has been suggested that among adult tuberculosis patients an important facet of *NRAMP1* activity on tuberculosis susceptibility is mediated by changing IL10 expression levels (Awomoyi et al., 2002).

However, studies that correlate cellular phenotypes known to be under control of NRAMP1 with specific *NRAMP1* alleles are so far missing. Here, we provide direct evidence that the function of Nramp1 in murine cells is reflected by its human NRAMP1 orthologue. In perfect analogy to the function of mouse Nramp1 (Cuellar-Mata et al., 2002; Jabado et al., 2003), NRAMP1 also impacts on intracellular vesicle trafficking as shown by differential recruitment of M6PR to SCVs in PMA differentiated U937 cells with different levels of NRAMP1 expression. Moreover, 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 results directly link an *NRAMP1* genetic risk factor with a known function of the NRAMP1 protein. Not surprisingly, M6PR recruitment in U937 tranfectants and MDMs is slightly different from the one reported for mouse cells. However, a range of recruitment from 20% to 45% for human cells is in reasonably good agreement with recruitment of ~15% to ~60% and ~15% to ~40% in RAW cells vs Nramp1 RAW transfectants, and Nramp1$^{0/0}$ vs Nramp1$^{r/r}$ macrophages, respectively Nramp1 (Cuellar-Mata et al., 2002; Jabado et al., 2003).

The present study is one of an increasing number of positive-association reports between risk of tuberculosis disease and NRAMP1 alleles in populations of vastly different ethnic background The strong impact that race can have on LD pattern makes it unlikely that these associations are caused by a disease variant outside of *NRAMP1*. The replication of association findings in a range of populations employing different study designs together with the correlation of *NRAMP1* risk alleles and impaired *NRAMP1*

function demonstrated in the present report make a strong case for *NRAMP1* as a tuberculosis disease risk factor. More specifically, our results strongly suggest that *NRAMP1* is a modulator of the speed of progression from infection with *M. tuberculosis* to tuberculosis disease. The detailed molecular events that trigger the more rapid progression to clinically evident disease are presently unknown. However, a protein that limits multiplication of ingested *M. tuberculosis* bacilli due to restricted access to essential nutrients seems a reasonable candidate for containing the spread but not the initial infection by the bacterium.

Acknowledgments

We sincerely thank all families that agreed to participate in the present study. We also like to thank our colleagues J.L. Casanova, K. Morgan, and E. Skamene for critical comments on earlier versions of the manuscript. This work was supported by National Institutes of Health grant AI41168-01 to J.M.M. Work in the lab of P.G. was supported by NIAID grant AI35237. L.A. was supported in part by program PRFMMIP from the Ministère Français de l'Education Nationale de la Recherche et de la Technologie. B.B.F and P.G. are Distinguished Scientists and E.S. is an Investigator of the Canadian Institutes of Health Research.

ADDENDUM 2

NRAMP1 polymorphisms were tested in an adult Ethiopian population comprised of 140 families. Under an additive model no alleles were associated with tuberculosis susceptibility

Allele frequencies and association analysis of polymorphisms in *NRAMP1* with tuberculosis in an adult Ethiopian population as described in Chapter 3.

Polymorphism	Alias	Common Allele (Frequency)	Number of Families	*P*-value (FBAT)
5' (GT)n	N01	0.70	79	0.72
274 C/T	N02	0.73	61	0.40
469+14 G/C	N03	0.77	62	0.62
3'UTR	N10	0.53	84	0.70

Connecting text

Chapter 3 describes that altered phagosome maturation in the macrophage is correlated with a tuberculosis risk allele *(NRAMP1-274_C)*. Furthermore, we provide evidence that *NRAMP1* influences the rate of progression from initial infection to tuberculosis disease. Finally, we show that *NRAMP1* effects on disease risk are best detected in low *M. tuberculosis* exposure intensity environments.

While *NRAMP1* influences phagosome maturation in the macrophage, factors mediating pathogen entry into the macrophage is equally critical to the overall survival and proliferation of the pathogen. Thus, we selected two candidate genes, *SFTPA1* and *SFTPA2* that code for the multimeric protein, SP-A. Chapter 4 discusses evidence implicating SP-A as a pro/anti-inflammatory switch as well as an opsonin for *M. tuberculosis* internalization into macrophages. A total of nine intragenic polymorphisms were tested in the context of a family based study in an adult Ethiopian tuberculosis population. Covariates (severity of disease, age, and gender) were analyzed by conditional logistic regression to further dissect the role of the genes in tuberculosis susceptibility. Results of this study are reported in chapter 4.

Abstract

Lungs are the central organ affected and targeted by *Mycobacterium tuberculosis* and immune processes in the lung are of critical importance in the pathogenesis of tuberculosis. A major lung defense against invading pathogens is provided by surfactant protein A, a multi-chain protein encoded by the *SFTPA1* and *SFTPA2* genes. Here, we investigated polymorphisms in the *SFTPA1* and *SFTPA2* genes for association with tuberculosis in 181 Ethiopian families comprising 226 tuberculosis cases. Three polymorphisms, *SFTPA1* 307A, *SFTPA1* 776T and *SFTPA2* 751A, were associated with tuberculosis ($P = 0.003$; $P = 0.006$ and $P = 0.012$, respectively). Additional subgroup analysis in male, female and more severely affected patients provided evidence for *SFTPA1/2*-covariate interaction. Finally, out of five intragenic haplotypes identified in the *SFTPA1* gene and nine identified in the *SFTPA2* gene only $1A^3$ was significantly associated with tuberculosis susceptibility ($P = 0.017$). These findings suggest that *SFTPA1* and *SFTPA2* modify the risk of tuberculosis susceptibility and that this risk is influenced by additional covariates.

Introduction

Almost one-third of the world population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis resulting in approximately 8 million tuberculosis cases and 1.87 million tuberculosis-related deaths per year (Corbett et al. 2003). Human immunodeficiency virus (HIV) co-infection greatly increases the rate of progression from infection to clinical tuberculosis disease and an increasing number of tuberculosis cases are recorded among AIDS patients (Corbett et al. 2003). The global number of cases of tuberculosis is increasing by 2% per year and tuberculosis cases caused by multidrug resistant strains of the tubercle bacillus are expected to rise even faster (Dye et al. 2002). Nevertheless, in the absence of AIDS, approximately of 90% of persons infected with *M. tuberculosis* never advance to clinical forms of the disease suggesting that effective anti-*M. tuberculosis* immunity exits in the majority of individuals. This natural immunity to tuberculosis is thought to be strongly influenced by host genetic factors, however, the molecular identity and function of such genetic factors remain largely unknown (Casanova and Abel, 2002; Malik and Schurr, 2002).

The main route of transmission of *M. tuberculosis* is via respiratory droplets that are inhaled into alveoli of exposed individuals. Inhalation of *M. tuberculosis* triggers a chain of poorly understood events that may result in invasion of lung alveolar macrophages by tubercle bacilli (El-Etr and Cirillo, 2001). Consequently, local innate immune responses in the lungs may be crucial to prevent infection of alveolar macrophages and also delay progression from infection to disease. Surfactant protein A (SP-A) is part of the innate

immune system in the lung and has been implicated in inflammation and host defense against a wide range of unrelated pathogens (rev in Haagsman, 2002). SP-A is most commonly produced by alveolar type-II epithelial cells and nonciliated (Clara) cells (rev in Madsen et al. 2003) and is secreted into the fluid lining on the surface of these cells. SP-A has been shown to enhance attachment of *M. tuberculosis* to alveolar macrophages (Downing et al. 1995). It is controversial if SP-A acts as a proinflammatory (Kremlev et al. 1997; Song and Phelps, 2000; Wang et al. 2002; Wang et al. 2000; Kremlev and Phelps, 1994; Blau et al. 1997) or anti-inflammatory mediator (Pasula et al. 1999; Borron et al. 2000; Rosseau et al. 1999; Hussain et al. 2003; Sano et al. 1999). However, a recent report suggested a dichotomous role of SP-A as a pro- and anti-inflammatory agent. SP-A exerts anti-inflammatory effects on macrophages when unbound to pathogen but exerts pro-inflammatory effects when the globular head of SP-A becomes pathogen bound (Gardai et al. 2003).

The primary structure of SP-A can be divided into four domains, an N-terminal domain involved in intermolecular disulfide bonding, a flexible collagen region, a hydrophobic neck region containing an amphipathic helix directing protein trimerization, and a C-terminal globular domain involved in lipid binding and Ca^{2+} dependent carbohydrate binding (Garcia-Verdugo et al. 2002; Palaniyar et al. 2001). It is thought that native SP-A is an octadecamer of six trimers, each trimer consisting of SP-A1 and SP-A2 molecules in a 2:1 ratio (Voss et al. 1991; Voss et al. 1988). In humans, the *SP-A* locus has been mapped to chromosome region 10q22-q23 and consists of two functional genes, *SFTPA1* and *SFTPA1* encoding SP-A1 and SP-A2, respectively (Hoover and Floros, 1998).

Several synonymous and non-synonymous polymorphisms have been identified in the *SFTPA1* and *SFTPA2* genes (DiAngelo et al. 1999) and *SFTPA1/2* alleles have been shown to be associated with infectious and noninfectious pulmonary events including respiratory distress syndrome (RDS) (Floros et al. 2001a; Ramet et al. 2000; Floros et al. 2001b; Haataja et al. 2001; Haataja et al. 2000; Kala et al. 1998; Marttila et al. 2003), respiratory syncytial virus infection in infants (Lofgren et al. 2002), allergic bronchopulmonary aspergillosis (ABPB) (Saxena et al. 2003) and pulmonary tuberculosis (Floros et al. 2000; Madan et al. 2002). To further test the hypothesis that *SFTPA1/2* polymorphisms may represent global tuberculosis risk factors we investigated the association of pulmonary tuberculosis with *SFTPA1/2* in patients from South-Eastern Ethiopia. Here, we report the systematic analysis of *SFTPA1* and *SFTPA2* polymorphisms that detected significant associations between tuberculosis and polymorphism within *SFTPA1* and *SFTPA2*.

Patients and Methods:

Study population: A total of 181 nuclear families were enrolled at Hossana Hospital in Ethiopia from 1997 through 2001. Hosanna is a rural administrative and agricultural center located approx. 250 km south-west of Addis Ababa. Annually, approximately 3000 new TB cases are registered by the Hosanna hospital and its affiliated primary health care clinics. At the time of enrolment, the prevalence of HIV+ individuals among TB patients was estimated at approximately 3 % - 5 %. The low incidence of HIV positive individuals was the main reason for selecting Hosanna as study area.

The majority (>90%) of the population in the study area of Hosanna are members of the Hadiya ethnic group. Minority ethnic groups are Gurage, Gurage-Silti, Kembata, and Amhara. In addition, a low proportion of families may represent inter-group marriages. A major advantage of family-based designs for association studies is that ethnicity can be excluded as a confounding factor.

The phenotype analyzed in the present study was adult pulmonary tuberculosis. Consequently, inclusion criteria for patients were detection of acid fast bacilli in sputum and/or successful cultivation of *M. tuberculosis* from sputum samples. All subjects were interviewed about duration of common symptoms of tuberculosis. Severity of tuberculosis disease was assessed by recording weight loss over the last two months. All subjects were enrolled in the study after written informed consent was obtained. The study was approved by the National Ethics Commission of Ethiopia and the Ethics

Committee at the Research Institute of the McGill University Health Centre.

Genotyping analysis: Genomic DNA was extracted from whole blood employing the Nucleon DNA extraction kit (Amersham Biosciences). *SFTPA1* and *SFTPA2* genotyping was based on methods described by DiAngelo et al. (1999). Briefly, a 3.3kb fragment was amplified from the *SFTPA1* or *SFTPA2* gene. The 3.3kb fragment served as a template for subsequent genotyping (PCR-RFLP) of five single nucleotide polymorphisms (SNPs) in the *SFTPA1* gene (codons 19, 60, 62, 133, 219) and four SNPs in the *SFTPA2* gene (codons 9, 91, 140, 223). Digested products were run on 12 % PAGE to resolve banding patterns.

Statistical analysis: Allele-phenotype associations were investigated employing a family-based association design. Family-based studies avoid confounding of gene-phenotype associations due to inappropriately chosen controls or population substructures. The general principle of this study is to search for a distortion of the transmission of alleles from parents to affected offspring, a strategy that has been termed "Transmission Disequilibrium Test" (TDT). Data were analyzed by the family-based method implemented in the FBAT program which allows tests of association in instances of incomplete parental data (Rabinowitz and Laird, 2000). Furthermore, FBAT allows the use of an empirical variance-covariance estimator for the statistic which is consistent when sibling marker genotypes are correlated, i.e. when families with multiple affected children are being analyzed, and can be used to study haplotype phenotype associations. Finally, alleles showing some evidence for association were also analyzed by means of

conditional logistic regression employing the GASSOC program (Schaid, 1996) and SAS (Statistical Analysis System, Cary NC). This analysis allowed us to provide odds-ratio estimates and confidence intervals for tested markers, and to investigate whether the associations varied with age, sex or weight (Schaid, 1996). For these conditional models, only families with two genotyped parents were included. Furthermore, one affected child per family was randomly selected when there were multiple affected siblings.

Results

We enrolled a total of 181 families with at least one offspring affected by pulmonary tuberculosis (Table 1). The majority of families belonged to the Hadiya ethnic group, 14 families were of mixed ethnic background. and 41 families belonged to 5 ethnic groups other than Hadiya. Of the 226 tuberculosis patients, 119 (53%) were male 107 (47%) were female, 69% of patients belonged to 2 parent families whereas 31% belonged to 1 parent families (Table 1). All one parent families included a minimum of two genotyped children. Of the 38 multiplex families, 15 comprised one tuberculosis case in the parental generation. Tuberculosis patients with weight loss of more than 10 kg during the last two months were considered as severely affected, and 17.7% (40/226) of all patients confirmed to this definition (Table 1). There was no significant heterogeneity of patient gender, severe weight loss or age across ethnic groups or family structure (data not shown).

The *SFTPA1* and *SFTPA2* genes are separated by 50.5 kb of genomic sequence with opposite transcriptional orientation (Figure 1). There are no additional functional genes located between the two *SFTPA* genes. A series of single nucleotide polymorphisms (SNP) are found in the coding regions of both genes. Here, we genotyped five SNPs located in the coding region of *SFTPA1* and four SNPs located in the coding region of *SFTPA2*. Non-synonymous polymorphisms occur at amino acid positions A19V (*177C/T*), V50L (*269G/C*), and R219W (*776G/A*) in *SFTPA1* and at amino acid positions N9T (*110A/C*), P91A (*355C/G*), and K223Q (*751A/C*) in *SFTPA2*. Synonymous

polymorphisms were also examined and were identified at amino acid positions 62P (*307G/A*) and 133T (*520G/A*) in *SFTPA1* and amino acid position 140S (*504T/C*) in *SFTPA2* (Figure 1). In contrast to most other genes, it is common practice for *SFTPA1* and *SFTPA2* to refer to intragenic haplotypes, rather than individual SNP variants, as "alleles." However, in this study, we will adhere to the standard nomenclature and refer to distinct SNP variants as alleles. The intragenic haplotypes ("alleles") found in the Hosanna study families with minor frequencies > 1% are indicated in Figure 1.

The subjects enrolled in the study were genotyped for all SNPs. All genotyped markers were in HWE and segregation was consistent with Mendelian rules. Among the unrelated parents of all families, SNPs located within each gene were in strong linkage disequilibrium (LD). However, there was only weak LD (D' = 0.1 -0.3) between alleles in the two genes (data not shown). To test for their contribution to risk of tuberculosis, we tested the *SFTPA1* and *SFTPA2* SNPs individually for association with tuberculosis. Across all markers, *SFTPA1* alleles 307A ($P = 0.003$) and 776T ($P = 0.006$) as well as *SFTPA2* allele 751A ($P = 0.012$) provided the strongest evidence for association (Table 2). If applying the Bonferoni correction for multiple comparisons, only alleles *SFTPA1* 307A ($P_{Bonf} = 0.02$) and 776T ($P_{Bonf} = 0.05$) remained significantly associated with tuberculosis although this correction is certainly overly conservative due to the strong linkage disequilibrium among the tested SNPs within each gene. To obtain estimates of the strength of the genetic effects we also employed conditional logistic regression on the subset of two parent families. Since the analysis could be done only on a family subset

there was a drop in *P*-values. Nevertheless, the trend of association between *SFTPA* markers and tuberculosis was the same with *SFTPA1* alleles 307A and 776T as well as *SFTPA2* allele 751A being the strongest risk factors (Table 2).

We decided to further explore the observed association of both *SFTPA* genes with tuberculosis separately in male and female patients, in patients with severe forms versus less severe forms of tuberculosis, and in patients older or younger than 20 years of age. The hypothesis being that differences in incidence rates (male vs female) or disease manifestations are the result of different pathways of pathogenesis, and, hence, genetic control. The measure of severity used was excessive weight loss over the last two months. There was evidence for a difference in transmission ratios for *SFTPA2* allele 751A in patients with excessive weight loss ($P = 0.001$; $P_{Bonf} = 0.036$) as well as evidence for gender specific effects of *SFTPA2* alleles 355C and 751A ($P = 0.0048$; $P_{Bonf} = 0.17$ and$P = 0.0018$; $P_{Bonf} = 0.065$, respectively). There was also strong evidence for preferential transmission ratio distortion among patients older than 20 years for *SFTPA1* allele 776T ($P = 0.00053$; $P_{Bonf} = 0.019$; Table 3). T obtain estimates of the subgroup-specific strength of the identified *SFTPA1/2* polymorphisms, we conducted subgroup analysis in the conditional logistic regression model. A summary of the significant results is presented in Table 4. Surprisingly, the strong effect of the *SFTPA1*_776T allele in patients older than 20 years was not found by conditional logistic regression. By contrast, group-specific effects of *SFTPA1*_307G and *SFTPA2* alleles 355G, 504C and 751 were reproduced in the conditional logistics framework (Table 4). The strongest risk factors are *SFTPA2*_504C in severe cases (OR = 6.35 for genotypes CC vs CT and TT in

the subgroup of patients with weight loss > 10 kg) and SFTPA2_751A in the age group older than 20 years (OR = 4.66 for genotypes AA vs CA and CC). Finally, we conducted tests to assess the impact of age, gender and body weight on *SFTPA1/2* allele transmission and found significant effects for all three co-variates for a *SFTPA1/2* alleles (data not shown). However, age, gender and weight are correlated: males tended to have higher weights than females, and older cases tended to weigh more than younger children (data not shown). Therefore, these data do not permit a conclusive interpretation of the effects of gender, body weight and age in affecting *SFTPA* genes transmission ratios to tuberculosis patients.

Derived from SNP combinations, five intragenic haplotypes ("alleles") in *SP-A1* ($6A^2$, $6A^3$, $6A^{18}$, $6A^{13}$, and $6A^{19}$) and ten intragenic haplotypes ("alleles") in *SP-A2* ($1A^1$, $1A^2$, $1A^0$, $1A$, $1A^{10}$, $1A^6$, $1A^5$, $1A^3$, $1A^8$, and $1A^9$) were observed in the present study at frequencies > 1%. Only the $1A^3$ haplotype was significantly overrepresented in patients ($P = 0.017$) while none of the *SP-A1* intragenic haplotypes were significantly associated with tuberculosis susceptibility (Table 5). If corrected for multiple testing none of the intragenic haplotypes was significantly associated with tuberculosis. Extended haplotypes spanning the *SFTPA1* and *SFTPA2* genes were distributed equally among patients (data not shown). The pronounced drop in strength of association from individual SNPs to intra- and intergenic haplotypes implicates individual tested SNPs in the pathogenesis of tuberculosis.

Table 1: Characteristics of 181 families comprising 566 individuals enrolled in the present study.

Family Structure (number of families)	Gender	Number of Tuberculosis Patients (number of families)			
		2 Parent Families (125)	Weight Loss > 10kg	1 Parent Families (56)	Weight Loss > 10kg
Simplex (143)	male	51	9	24	3
	female	47	9	21	5
Multiplex (38)	male	31	6	13	2
	female	27	6	12	0
Total Number of Tuberculosis Patients		156	30	70	10

Table 2: Allele frequencies and univariate association analysis of polymorphisms in *SFTPA1* and *SFTPA2* with tuberculosis.

Gene	Amino Acid Polymorphism	Marker	Allele	Allele Frequency	Number of Families	FBAT *P*-values	GASSOC *P*-values	GASSOC* Odds Ratio (95% CI)
SFTPA1								
	A19V	177C/T	C	0.07	28	NS	NS	ND
	V50L	269G/C	G	0.69	38	NS	NS	ND
	P62P	307G/A	A(A=1)	0.77	17	0.003	0.015	1.78 (1.11-2.85)
	T133T	520G/A	G	0.05	23	NS	NS	ND
	R219W	776C/T	T(C=2)	0.10	42	0.006	0.0043	2.64 (1.25-5.59)
SFTPA2								
	N9T	110A/C	A	0.17	51	NS	NS	ND
	P91A	355C/G	C	0.22	48	0.10	NS	ND
	S140S	504T/C	T(C=1)	0.51	50	0.07	0.073	1.94 (0.99-3.79)
	K223Q	751A/C	A(C=1)	0.49	49	0.012	0.016	2.28 (1.16-4.48)

NS: non-significant at $P > 0.1$

* For the GASSOC results, the following models were used: The p-values and odds ratios for P62P were estimated under an additive model, i.e. risk increasing with each copy of allele '1' (A?); for R219W, S140S, and K223Q, recessive models for alleles '2'(C), '1'(C) and '1' (C) , respectively, were used. P-values are based on score tests; confidence intervals are based on Wald tests.

Table 3: FBAT assessment of the sensitivity of the associations due to covariates.

Gene	Allele	P-values (number of informative families)					
		Gender		Weight Loss		Age	
SFTPA1		Male	Female	Weight loss>10kg	Weight loss<10kg	Age > 20	Age < 20
	177C	0.63 (18)	0.083 (12)	0.26 (7)	0.65 (19)	0.41 (13)	NS
	269G	0.27 (24)	0.38 (14)	0.56 (14)	0.15 (20)	0.07 (30)*	NS
	307A	0.033(12)	ND (7)	0.16 (6)	0.011 (10)	0.020 (12)	NS
	520G	0.48 (13)	0.043 (11)	0.81 (5)	0.32 (15)	NS (10)	NS
	776T	0.053 (26)	0.050 (19)	0.16 (8)	0.042 (26)	0.00088 (18)	NS
SFTPA2							
	110A	0.67 (23)	0.50 (28)	0.88 (12)	0.19 (31)	NS (19)	NS
	355C	0.23 (18)	0.0048 (32)	0.89 (13)	0.20 (30)	ND (7)	NS
	504T	0.044 (28)	0.50 (22)	0.023 (8)	0.24 (33)	NS (19)	NS
	751A	0.0018 (29)	0.81 (20)	0.0010 (8)	0.47 (31)	0.012 (18)	NS

NS: Non-significant at P>0.1; ND: Less than 10 families; not considered.

Table 4: Subgroup estimates of odds ratios.

Allele	Number of families	Model (Allele)	Subgroup	Odds Ratio (95%CI)
SFTPA1-307G/A	70	Additive (A=1)	WL < 10kg	2.13 (1.16-3.94)
	56	Recessive(A)	Male	1.88 (0.95-3.73)
SFTPA1-269G/C	45	Dominant(C=1)	Age≥20	3.10 (1.25-7.68)
SFTPA1-776C/T	41	Recessive(C=2)	Age≥20	4.95 (1.38-17.74)
SFTPA2-355C/G	43	Recessive(C=2)	Male	N/A (p=.008)
	51	Recessive(G=1)	Female	2.43 (1.04-5.72)
SFTPA2-504T/C	17	Dominant (T)	WL > 10kg	6.35 (1.15-35.06)
SFTPA2-751A/C*	88	Recessive (C=1)	All	2.28 (1.16-4.48)
	47	Recessive (C=1)	Male	2.95 (1.19-7.34)
	19	Recessive (C=1)	WL > 10kg	3.25 (1.06-9.97)
	39	Recessive (C=1)	Age ≥20 years	4.69 (1.23-17.81)

WL: Weight loss

N/A: Wald tests gave estimates of infinity for the odds ratio since too few individuals were homozygous for allele "C".

* Conditional logistic models with covariates age, weight at interview, or weight loss showed marginal significance for heterogeneity at 751A/C (age: p=.051; weight at interview: p=.033; weight loss>10kg: p=.064). Three other markers also demonstrated some heterogeneity with weight at interview (110A/C :p=.036, 307G/A: p=.032; 177C/T: p=.067).

Table 5: Allele frequencies and association analysis of intragenic haplotypes in the *SFTPA1* and *SFTPA2* genes with tuberculosis.

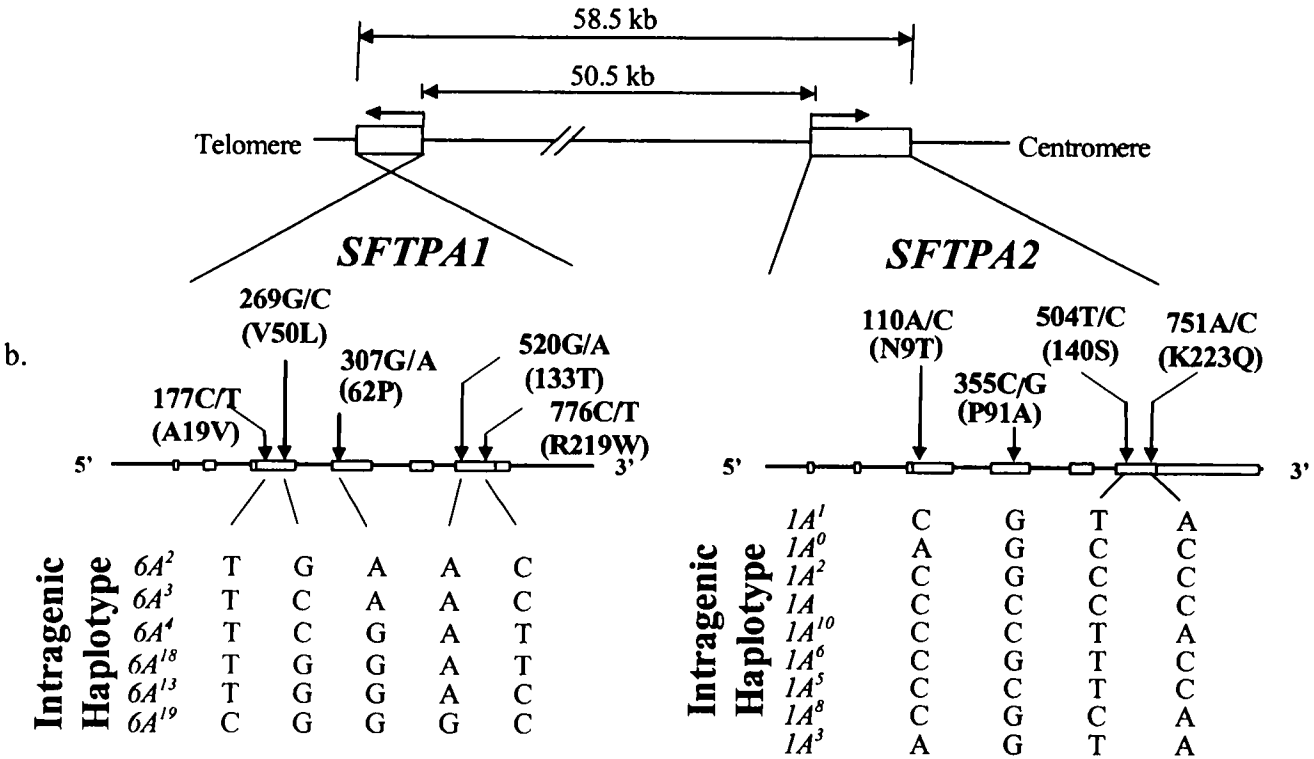
Gene	Intragenic Haplotypes	Haplotype Frequency	Number of Families	*P*-value
SFTPA1				
	$6A^{2}$	0.47	57	0.18
	$6A^{3}$	0.29	67	0.77
	$6A^{18}$	0.11	35	0.32
	$6A^{13}$	0.06	29	0.82
	$6A^{19}$	0.05	20	0.97
SFTPA2				
	$1A^{1}$	0.37	50	0.27
	$1A^{0}$	0.17	39	0.30
	$1A^{2}$	0.17	45	0.44
	1A	0.11	29	0.55
	$1A^{10}$	0.09	20	0.51
	$1A^{6}$	0.02	15	0.58
	$1A^{5}$	0.02	8	ND
	$1A^{8}$	0.02	10	0.42
	$1A^{3}$	0.01	13	0.01

Figure 1:

a) Schematic representation of relative distances between *SFTPA1* and *SFTPA2*. Red arrows represent transcriptional directions of each gene based on telomere and centromere orientation.

b) Coding exons are represented by solid boxes, and non-coding exons as hatched boxes. Blue arrows represent relative location of polymorphisms and respective amino acids at each gene. Combinations of SNPs are represented by intragenic haplotypes indicated by *6A*^{n} for *SFTPA1* and *1A*^{n} for *SFTPA2* based on previously adopted nomenclature (DiAngelo, et al. 1999). All intragenic haplotypes were observed at frequencies > 1% except *6A*^{4} which was added for discussion purposes.

a.


58.5 kb
50.5 kb
Telomere
Centromere
SFTPA1
SFTPA2
b.
269G/C
(V50L)
177C/T
(A19V)
307G/A
(62P)
520G/A
(133T)
776C/T
(R219W)
5'
3'
Intragenic Haplotype
6A2 T G A A C
6A3 T C A A C
6A4 T C G A T
6A18 T G G A T
6A13 T G G A C
6A19 C G G G C
110A/C
(N9T)
355C/G
(P91A)
504T/C
(140S)
751A/C
(K223Q)
5'
3'
Intragenic Haplotype
1A1 C G T A
1A0 A G C C
1A2 C G C C
1A C C C C
1A10 C C T A
1A6 C G T C
1A5 C C T C
1A8 C G C A
1A3 A G T A

Discussion

We examined 9 exonic SNPs in *SFTPA1* and *SFTPA2* and found strong association between *SFTPA1* alleles 307A ($P = 0.003$) and 776T ($P = 0.006$), and *SFTPA2* allele 751A ($P = 0.012$) with tuberculosis. Since there is limited LD between polymorphisms in the *SFTPA1/2* genes these results indicate independent association of both *SFTPA* genes with tuberculosis. These data replicate previous findings in a Mexican tuberculosis cohort (Floros et al. 2000), and, hence, implicate *SFTPA1/2* alleles as risk factors of tuberculosis in two ethnically and geographically distinct populations. Specifically, in the Mexican population the $6A^4$ and $1A^3$ intragenic haplotypes had been found to be risk factors of tuberculosis. In our study, the only *SFTPA1/2* intragenic haplotype that reached borderline significance was also $1A^3$. The association of $1A^3$ with tuberculosis in the Ethiopian family panel is likely a result of the strong and consistent association across subgroups of *SFTPA2* allele 751A which forms part of the 1A3 haplotype. Yet, in the Mexican population *SFTPA2* 751A was not associated with tuberculosis. This divergence may be a consequence of changed LD pattern across the *SFTPA2* gene between Mexicans and Ethiopians implying that the true "causative" polymorphism is in very tight LD with allele 751A in Ethiopians but not in Mexicans. Alternatively, it is conceivable that allelic heterogeneity for *SFTPA1/2* exists across ethnic groups. Extensive allelic heterogeneity has recently been described for *TLR4* and meningococcal disease (Smirnova et al. 2003). Finally, it is noteworthy that the *SFTPA1* 776T polymorphism associated with tuberculosis in Ethiopians is part of the $6A^4$ haplotype and that the same arguments given for $1A^3$ and *SFTPA2* 751A also apply to $6A^4$ and *SFTPA1*

776T.

Assuming that *SFTPA1/2* allelic heterogeneity for tuberculosis susceptibility exits between Ethiopian and Mexican patients and considering the stronger tuberculosis association of individual polymorphisms as compared to intragenic haplotypes observed in our study, this implicates the tested *SFTPA1/2* variants in tuberculosis susceptibility among Ethiopian patients. Unfortunately, little is known about specific *SFTPA1/2* genotypes and their impact on SP-A1/2 function. For example, genotypic variants in the *SFTPA 1/2* genes have been shown to mediate differential *SFTPA 1/2* mRNA expression, *SFTPA1/SFTPA2* mRNA ratios, and alternative splicing (Wang et al. 2003; Karinch et al. 1997). Furthermore, alleles in the *SFTPA1* and *SFTPA2* genes influence the ability of SP-A to self-aggregate and to induce LPS aggregation (Garcia-Verdugo et al. 2002), and, consistent with an inflammatory role of SP-A, *SFTPA1* and *SFTPA2* polymorphisms have been shown to influence the production of TNF-α and IL-8 in THP-1 cells (Wang et al. 2002; Wang et al. 2000). Among the *SFTPA1/2* polymorphisms associated with tuberculosis in the present study, the *SFTPA2* 751A>C polymorphism results in a lysine to glutamine change. Glutamine is a neutral amino acid with a polar amide group, whereas lysine is a positively charged amino acid with a basic side chain. Given the differences in physiochemical properties of the two allelic amino acids and their location in the carbohydrate recognition domain (CRD) of SP-A, (Garcia-Verdugo et al. 2002) the *SFTPA2*-751A/C polymorphism may influence the attachment of *M. tuberculosis* to alveolar macrophages. It is not known how the synonymous P62P polymorphism, *SFTPA1* 307G/A, might affect SP-A function . However, this

polymorphism is in close proximity to the exon-intron splice junction and may impact on mRNA maturation. It is well documented that silent mutations that affect splicing regulation can result in serious disorders such as spinal muscular atrophy, frontotemporal dementia, and parkinsonism (Cartegni et al. 2002). The *SFTPA2* 355C>G polymorphism, identified in a previous small study (Madan et al. 2002) and by subgroup analysis in the Ethiopian patients, results in a proline to alanine change. It is known that proline normally stabilizes collagen triple helices due to conformational restrictions of the pyrrolidine ring and the presence of tertiary amides while alanine substitutions tend to destabilize the triple helix (Kersteen and Raines, 2001). Likewise, it seems possible that a non-conserved arginine to tryptophane substitution at amino acid position 219 located in the CRD of SP-A1 (*SFTPA1* 776C>T) might impact on protein function but direct experimental evidence is lacking.

The data obtained by subgroup analysis suggest that the impact of genetic *SFTPA1/2* risk factors is modulated by additional covariates. For example, when stratifying the present study by gender, 355C (proline) was significantly overrepresented in female patients (P = 0.005). The gender specificity of risk factors for tuberculosis was confirmed by logistic regression analysis for additional *SFTPA1/2* markers. Since it has been reported that the synthesis of surfactant is stimulated by estrogen (Chu and Rooney, 1985), it is conceivable that the interplay between estrogen and *SFTPA1/2* polymorphisms may result in gender specific SP-A activity in tuberculosis pathogenesis. Our data suggest that two *SFTPA2* polymorphisms, 504 and 751A, have their strongest effect among patients with more severe forms of tuberculosis. A recent study demonstrated that SP-A is potent

modulator of inflammation in the lung, and that presence of SP-A is critical for protection of lung segments not involved in tuberculosis pathogenesis from inflammatory damage (Gold et al. 2004). Hence, any polymorphism resulting in reduced lung SP-A concentrations or activity can significantly add to tuberculosis pathogenicity.

Interestingly, the intragenic haplotype $1A^3$, as well as the *SFTPA2* polymorphisms 355C and 751A have been shown to be associated with pulmonary infections other than tuberculosis. For example, *355C* was shown to be a risk factor for allergic bronchopulmonary aspergillosis, caused by the pathogenic fungi *Aspergillus fumigatus* (Saxena et al. 2003), and $1A^3$, *355C*, and *751A* are risk factors for severe respiratory syncytial viral (RSV) infection in infants (Lofgren et al. 2002). This implies that SP-A may operate in a similar fashion for all pathogens since the same genetics variants predispose individuals to bacterial, viral, and fungal infections (Floros et al. 2000; Lofgren et al. 2002; Saxena et al. 2003). On the other hand, while*SFTPA2* polymorphisms such as $1A^3$, *355C*, *751A* are associated with susceptibility to pulmonary infections, these polymorphisms are not implicated in diseases that are not triggered by pathogens such as respiratory disease syndrome and idiopathic pulmonary fibrosis (Haataja, 2001; Floros, J., 2001; Kala, 1998; Sleman, 2003). For example idiopathic pulmonary fibrosis is associated with *SFTPA1* polymorphisms that are part of the $6A^4$ intragenic haplotype (Selman et al 2003). Polymorphisms consistently associated with RDS are represernted by $1A^0$ and $6A^2$ intragenic haplotypes (Floros et al. 2001a; Floros et al. 2001b; Haataja et al. 2001; Haataja et al. 2000; Kala et al. 1998; Marttila et al. 2003; Ramet et al. 2000) . While there is some overlap in risk alleles between infectious

and non-infectious diseases, e.g. the *SFTPA1* 776T allele is a risk factor for tuberculosis and idiopathic pulmonary fibrosis, the dichotomy in risk factors for infectious and non-infectious lung diseases is pronounced suggesting different mechanisms of SP-A mediated pathogenesis.

Here, we show that genetic polymorphisms in the *SFTPA1* and *SFTPA2* genes are risk factors for pulmonary tuberculosis in an Ethiopian population. This finding confirms a previous investigation in a Mexican case control population (Floros et al. 2000), and therefore identifies *SFTPA* polymorphisms as global risk factors for tuberculosis. We also provide evidence that the genetic risk conferred by distinct *SFTPA* variants is sensitive to severity of disease, gender and age. This finding points to the possibility of complex gene-environment interactions. A focus for future studies will be to understand the functional consequences of the identified *SFTPA1/2* polymorphisms for pathogenesis of tuberculosis.

Chapter 5

General Discussion and Conclusion

Several lines of evidence suggest that intervention of the innate immune system is perhaps the best chance at controlling infection and preventing tuberculosis disease. For example, classic experiments by Lurie demonstrated that only 7 days after aerosol infection with tubercle bacilli of susceptible and resistant rabbits, the former had 20-3-fold more viable bacteria in the lunges compared to the latter (Lurie, et al. 1952). Such a difference is unlikely due to T-cell function since acquired immunity generally develops 2 to 3 weeks after infection (Dannenberg, 1993). More recently, BCG vaccination in mice and humans was shown to be more effective at protecting against disseminated tuberculosis than initial pulmonary disease (Colditz et al. 1994; North et al. 1999) indicating that sensitized T-cells were lacking at the initial stage of infection. Interestingly, naturally acquired immunity was unable to prevent exogenous reinfection with *M. tuberculosis* (van Rie et al. 1999b) further suggesting that innate immunity plays a substantial role in controlling infection. Finally, polymorphisms in genes involved in innate immunity have been found to influence susceptibility to tuberculosis disease (Malik and Schurr, 2002) directly implicating innate immunity with tuberculosis disease. Thus, we set out to examine a set of genes that had previously been found to be associated with tuberculosis and that are part of the innate immune system, namely *NRAMP1, MBL, SFTPA1,* and *SFTPA2.* Furthermore, since the spreak of tuberculosis is potentiated by the HIV/AIDS pandemic, especially in developing countries, we have chosen to test *MBL* as a risk factor for HIV infection.

NRAMP1

In mice natural resistance to Mycobacteria and other intracellular pathogens was shown to be modulated by *Nramp1* (Skamene et al. 1998). While mouse models have been used extensively to delineate genes involved in latent tuberculosis infection and reactivated tuberculosis disease in humans, parallels between the mouse model and humans in this respect are imperfect (Ramakrishnan, 2004). Typically, mice inoculated with *M. tuberculosis* are characterized by high bacterial burden, the absence of cavitation, and rapidly progressive disease and death (Manabe and Bishai, 2000; Mustafa et al. 1999). While guinea pigs show more of the pathological features of human tuberculosis, they are extremely sensitive to progressive pulmonary infection (Ramakrishnan, 2004). Thus, the emerging theme with common laboratory animal models is that their phenotype most closely resembles that of human "primary" tuberculosis disease rather than latent infection or reactivation tuberculosis. Therefore, it is a natural progression to test the human *Nramp1* orthologue, *NRAMP1*, with respect to primary tuberculosis, here represented by a pediatric tuberculosis population.

NRAMP1 findings

Our findings indicate that the common alleles of the 5' region of *NRAMP1* are associated with pediatric tuberculosis, which is in contrast to previous findings where the rare alleles were associated with adult tuberculosis disease (Soborg et al. 2002; Bellamy et al. 1998b; Cervino et al. 2000; Gao et al. 2000; Soborg et al. 2002) Together these

findings indicate that *NRAMP1* influences the rate of progression from *M. tuberculosis* infection to tuberculosis disease, rather than simple susceptibility to disease. While it is possible that a major susceptibility gene exists, it is equally possible that other genes, acting either in concert or independently with *NRAMP1*, slow the rate of progression from infection to disease to such an extent that tuberculosis is not manifested within one's lifetime. These conclusions open the possibility that genes impacting on susceptibility to infection function as classical susceptibility factors that define the sub-population in which progressor genes can act. Interestingly, NRAMP1 may operate in a similar fashion in other infectious diseases. Like the chronic nature of tuberculosis, chronic hepatitis C infection offers an interesting parallel to tuberculosis in that the immune response promotes tissue damage and progressive fibrosis in chronic infection. In a Spanish population the rare allele of the $(GT)_n$ repeat of *NRAMP1* was not associated with susceptibility to chronic hepatitis infection but was associated with a lack of advanced fibrosis (Romero-Gomez et al. 2004). More recently, the rare $(GT)_n$ repeat was shown not to influence HIV susceptibility but in a small subset of the population, the rare allele was overrepresented in those progressing slowly from HIV infection to AIDS (Donninger et al. 2004). In contrast, the common *NRAMP1* alleles in the 5' region have been reported to be risk factors for HIV infection (Marquet et al. 1999). However, without following the patients, this study could not demonstate a role for *NRAMP1* as a progressor gene. Together these studies points to the ubiquitous nature of *NRAMP1* as a progressor gene for macrophage tropic infectious diseases.

In our experiments, covariate analysis showed that family structure influenced the association between the *NRAMP-274_C* polymorphism and tuberculosis. Analyzing simplex and multiplex families separately yielded a highly significant allelic distortion of the risk allele in simplex families only ($P < 0.0008$). Given that the majority of adult cases was observed in multiplex families and that children in close and frequent contact with adult cases are expected to experience increased exposure intensity, it follows that multiplex families represent higher exposure intensity environments than simplex families. Since PPD status is correlated with exposure intensity, PPD status of unaffected sibs were used to categorize simplex families into high and low intensity families. Analysis performed separately on both types of families revealed clear *NRAMP-274_C* allelic transmission only in low exposure families ($P=0.01$). These findings show that NRAMP1 is most readily detected in low exposure environments, likely a reflection of limited exposure episodes or "casual transmission". Clearly, cases pose a serious risk of tuberculosis transmission to naïve members within intimate settings such as households (Murray and Alland, 2002; Loudon et al. 1958b), however, casual transmission may lead to dissemination of tuberculosis within and among populations (Aparicio et al. 2000; Raffalli et al. 1996), contributing significantly to the tuberculosis epidemic.

Interestingly, the observation that NRAMP1 operates only in low exposure environments may explain the failure of some studies to implicate NRAMP1 in tuberculosis susceptibility. Several linkage analysis studies have been carried out in order to identify major genetic determinants in tuberculosis susceptibility, however, all but one failed to identify *NRAMP1* as a major susceptibility gene in tuberculosis (Shaw et al. 1997;

Greenwood et al. 2000; Bellamy et al. 2000; Blackwell, 1998; Miller et al. 2004). Only when considering exposure intensity in the form of liability classes is the *NRAMP1* locus linked to tuberculosis (Greenwood et al. 2000). Thus, *NRAMP1* cannot be discounted as having a major gene effect in tuberculosis. While most association based studies have found an association between *NRAMP1* and tuberculosis susceptibility, two published reports failed to confirm this (El Baghdadi et al. 2003; Liaw et al. 2002). Furthermore, we examined 140 families in an adult Ethiopian tuberculosis population with respect to *NRAMP1*, however, none of the polymorphisms was found to be associated with tuberculosis susceptibility (Addendum 2). Although the authors of the former two studies attribute these inconsistencies to sample size and genetic heterogeneity, we cannot rule out the role of exposure intensity of *M. tuberculosis* for failing to find an association with *NRAMP1* and tuberculosis disease susceptibility. Moreover, since the Ethiopian study examined a large number of families, our failure to find an association between tuberculosis disease and *NRAMP1* is less likely a function of sample size and more likely due to a high exposure intensity or genetic heterogeneity. Together, these observations highlight the importance of carrying out genetic studies in the proper epidemiologic context.

Although sex bias is not typically observed in pediatric tuberculosis populations (Sutherland, 1976) our study revealed that the effect *of NRAMP1-275_C* on tuberculosis risk is highly significant when considering only male patients in simplex families ($P < 0.00004$). The implication of this finding is that sex bias does exist among pediatric tuberculosis cases, and that case notification rates showing a lack of such a bias may by a

function of social circumstance whereby female infants are more often brought for medical attention. However, given that only 17 informative simplex families with male pediatric cases were assessed, there is a clear need for replication of the observation with larger sample sizes. Moreover, the sex-specificity of the *NRAMP1*-mediated risk must be taken with caution since formal heterogeneity testing was only borderline significant. Nevertheless, if correct, the finding might imply that different mechanisms of pathogenesis are present in boys and girls.

Genetic epidemiology studies cannot implicate alleles with altered biological functions. In order to study the potentially divergent roles of NRAMP1 alleles in human cells, we adapted a murine mannose-6-phosphate receptor (M6PR) recruitment assay to measure NRAMP1 activity. The premise of this assay is based on the observation that *Salmonella typhimurium* selectively blocks fusion of salmonella containing vacuoles (SCV) with vesicles containing the late-endosomal marker, M6PR, in Nramp1$^{-/-}$ murine macrophages. This blockade is overcome in NRAMP1 expressing cells from Nramp1$^{+/+}$ cells (Cuellar-Mata et al. 2002). Here we report the mean recruitment efficiency for monocyte-derived macrophages (MDM) from homozygous *NRAMP1-274C/C* carriers to be 30 percent while the recruitment of M6PR cells from in heterozygote carriers was significantly higher at 34.8 percent (P=0.024), suggesting that phagosome interaction with the endocytic pathway is, at least, partly under the control of NRAMP1. Thus, the overall individual bacterial burden may be reduced due to the greater interaction of phagosome and the late endocytic pathway in those with the protective allele by restricting bacterial replication in the macrophage. It is not known what the functional consequence of

274C/T is in this context but preliminary *in vitro* studies in our laboratory show that the risk allele is correlated with reduced expression of NRAMP1. Given this, it is likely that reduced numbers of NRAMP1 molecules reduce the fusogenic efficiency of phagosomes to the late endocytic pathway. Moreover, fewer NRAMP1 molecules may result in reduced expulsion efficiency of divalent cations from the phagolysosomal lumen. In the presence of divalent cations, pathogens may be able to initiate divalent cation dependent superoxide dismutase and catalase activity, both of which defend the pathogen against reactive oxygen intermediates generated by the macrophage (Agranoff and Krishna, 1998).

Surfactant protein A (SP-A) is the multimeric gene product of the *SFTPA1* and *SFTPA2* genes (Hoover and Floros, 1998) (Voss et al. 1991; Voss et al. 1988). It is well established that SP-A maintains surface tension of the air-liquid interface in the alveolus and deficiency of SP-A is the main cause of respiratory disease syndrome (RDS), most commonly afflicting premature infants (Avery and Mead, 1959). Recent studies have shown that SP-A has the ability to bind to a variety of unrelated pathogens (Haagsman, 2002) and enhance attachment of *M. tuberculosis* to alveolar macrophages (Downing et al. 1995). Moreover, the complex nature in which SP-A acts on immune function is underscored by two recent reports showing that SP-A acts as a pro-inflammatory and anti-inflammatory agent (Gardai et al. 2003b; Gold et al. 2004). These studies suggest that, in the presence of *M. tuberculosis*, the globular head of SP-A binds the bacterium and the collagenous tail interacts with a macrophage membrane complex, calreticulin/CD91, effectively activating the macrophage. Conversely, in the case of

casual stimuli of inhaled particles, the globular head binds to the signal inhibitory regulatory protein α (SIRPα) causing a depression of proinflammatory mediators (Gardai et al. 2003a), presumably to minimize tissue injury and maintain normal lung homeostasis.

We examined 9 polymorphic markers in the *SFTPA1* and *SFTPA2* genes with respect to tuberculosis disease susceptibility in an adult Ethiopian population. Overall, alleles *307_A, 776_T,* and*751_A* provided the strongest evidence of association (P=0.003, P=0.006, and P=0.012, respectively). Moreover, the intragenic haplotype, $1A^3$, was suggestively associated with tuberculosis susceptibility (P=0.017). Interestingly, these polymorphisms have all been previously implicated with tuberculosis disease and other infectious diseases.

Covariate analysis suggests that alleles *504_T* and *751_A* are associated with weight loss, characteristic of severe tuberculosis disease (Tsao et al. 2002), however, the underlying mechanism is unknown. Previous studies have indicated that IFNγ is, at least in part, responsible for weight loss in diverse illnesses, including tuberculosis (Tsao et al. 1999; Matthys et al. 1995; Madihally et al. 2002; Tsao et al. 2002). Consequently, we hypothesize that alleles *504_T* and *751_A* may disrupt normal lung homeostasis by preventing the globular head of the protein from efficiently binding to SIRPα, thereby allowing unregulated production of IFNγ during infection.

Furthermore, we report a very clear association between *776_A* and tuberculosis susceptibility in patients aged >20 years old (p=0.00053) and, moreover, we show that allele *751_A* is a strong risk factor for tuberculosis in this same age group (OR=4.66; CI: 1.23-17.72). It is paradoxical as to why surfactant protein plays a more prominent role in older patients than in younger patients since younger patients may have a less developed acquired immune system due to limited exposure events to pathogens and may need to rely more on innate immunity. It is conceivable that because younger patients rely less on acquired immunity they may have a larger arsenal of innate immune proteins in operation, thereby creating a redundancy and diluting the effect of any one protein.

Finally, we report that *751_A* shows an increased risk for tuberculosis in male patients, consistent with previous epidemiological observations (rev in Holmes et al. 1998). It is unclear how surfactant polymorphisms relate to the sexual dimorphism, however, previous reports show that estrogen and androgen differentially regulate surfactant protein production (Gross et al. 1979; Hart et al. 1998; Khosla et al. 1983; Khosla et al. 1980).

Interestingly, genetic risk factors for tuberculosis and other infectious diseases, including respiratory syncytial virus (RSV), and allergic bronchopulmonary aspergillosis (ABPA) are distinct from genetic risk factors for RDS. Given that RDS is generally a function of surfactant deficiency (Lacaze-Masmonteil, 2003), it apprears possible that the polymorphisms associated with RDS operate by modulating protein levels, whereas

polymorphisms influencing susceptibility to tuberculosis and other infectious diseases mediate binding efficiency to the pathogen.

From an epidemiological perspective, the spread of HIV, predominantly in Sub-Saharan Africa, has fueled the tuberculosis epidemic. Identifying genetic factors predisposing persons to HIV may have a profound impact on the incidence of tuberculosis especially in developing countries. Interestingly, MBL variants have been implicated in HIV susceptibility and progression as well as in protection against tuberculosis (Mombo et al. 2003), however many of these studies have been weak or contradictory and have yet to been replicated (Casanova and Abel, 2004).

Thus, we assessed a Colombian HIV population, examining three SNPs in exon 1, previously found to influence MBL serum levels. We show that none of the 3 polymorphisms are associated with HIV susceptibility in the Colombian population. These findings are contrary to a Danish (Garred et al. 1997), Finnish (Pastinen et al. 1998) and a Gambonese study (Mombo et al. 2003). However, our results are in agreement with an Italian report (Amoroso et al. 1999). It is noteworthy that the allele frequency of the variant alleles in the Colombian population (28 %) was significantly higher than in the Danish, Finnish and Gambonese studies (14-20 %), although the Italian study did not report allele frequencies of all structural variants. Altogether, it appears that the high frequency of the variant alleles in the Colombian population, and consequently, low serum levels, preclude MBL from playing a significant role in HIV infection in this population and that other components of the innate immune system may

compensate in this respect, whereas in the Scandinavian and African populations, MBL was sufficiently present to influence HIV susceptibility.

The precise relationship between HIV and tuberculosis, with respect to MBL, is unclear, however, it is tempting to speculate that in African populations, with a long history of tuberculosis, variant alleles of MBL have been selected for and are protective against tuberculosis. The last 20 years of the AIDS epidemic may have taken advantage of the high frequency of such variant alleles, predisposing the population to the relatively recent HIV. Despite the relatively high frequency of variant alleles in these populations, tuberculosis is still growing at a similar rate to that of HIV (Corbett et al. 2003). This is most likely due to the severity of immune suppression in HIV/AIDS patients and thus the relative ease of tuberculosis infection. AIDS patients serve as reservoirs and transmission sources of *M. tuberculosis* within the population further fueling the spread of tuberculosis.

Next, we directly addressed the genetic role of *MBL* in tuberculosis susceptibility in a pediatric tuberculosis population, largely comprised of non-white Hispanics, as well as in a tuberculosis subset of the Colombian HIV population, however, we failed to show an association between the structural variants and tuberculosis susceptibility (Addendum 1). It was unexpected that no association was found between *MBL* and tuberculosis in the pediatric population since this age group generally relies on the their innate immune system rather than their relatively underdeveloped acquired immunity. Our findings are in contrast to previous studies implicating the structural variants with protection against

tuberculosis (Bellamy et al. 1999; El Sahly et al. 2004). It is not readily obvious why such a dichotomy exists, however, genetic heterogeneity may explain the lack of association in our families. On the other hand, the reported *MBL* associations were weak and only borderline significant. It is possible that the findings reflect primarily publication bias (it is difficult to publish negative studies) rather than a true genetic effect.

Whereas the precise biological mechanism is not understood, MBL is known to opsonize and facilitate phagocytosis of microorganisms (Turner, 1996) and, moreover, binds specifically to *M. tuberculosis* (Garred et al. 1994) and HIV (Saifuddin et al. 2000). Hence, MBL may promote internalization of *M. tuberculosis* and HIV into the macrophage. Whereas *M. tuberculosis* internalization by alveolar macrophages is required for establishing tuberculosis infection (Dannenberg, 1993b; Zahrt, 2003), it is less certain whether macrophages play an equally crucial role in establishing HIV infection. While studies indicate that macrophages serve as long term reservoirs for HIV (Aquaro et al. 2002; Blankson et al. 2002), HIV infected lymphocytes turnover quickly (Perelson et al. 1996) perhaps allowing a greater dissemination of the virus and, consequently, a better opportunity at establishing a stable infection. Thus, MBL sequestration of HIV to macrophages may decrease the availability of the virus to infect lymphocytes and, consequently, reduce the likelihood of a stable HIV infection.

Summary

The studies undertaken as part of this thesis provide an entirely new concept of NRAMP1 function in tuberculosis pathogenesis. Our work, in context of other genetic epidemiology studies, supports a role of *NRAMP1* in mediating progression from infection with *M. tuberculosis* to tuberculosis disease under low *M. tuberculosis* exposure intensity environments. Furthermore, a functional role of the common *NRAMP1-274_C* risk allele has been described, showing that *NRAMP1-274C/T* correlates with reduced phagolysosomal fusion in the macrophage in the presence of an intracellular parasite.

We also show that both *SFTPA1* and *SFTPA2* are associated with tuberculosis susceptibility in an adult tuberculosis population, supporting a role for collectins in the immune regulation of tuberculosis. Furthermore, for the first time, we have identified genetic variants in surfactant genes that may influence severity of tuberculosis disease. Also, for the first time, we report sex and age specific variants. These findings further emphasize the importance of gene-environment interactions in complex infectious diseases.

Clearly, from an epidemiological perspective, controlling HIV infection in developing countries would impact greatly on the spread of tuberculosis, therefore, identifying genetic factors impacting on HIV infection is of utmost importance. Here, we have ruled out a role for MBL in HIV susceptibility in an adult case-control study. While MBL is implicated in other HIV populations as a risk factor, our findings suggests that the

underlying molecular mechanisms in the studied Colombian population are distinct and that genetic heterogeneity exists for susceptibility to HIV infection.

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Appendix 1

Reprints of Articles

serum MBL levels among HIV-infected persons
arred et al. 1997; Pastinen et al. 1998).

The *MBL* gene, also known as mannose-binding
otein (*MBP*) gene, is located in chromosome region
q11.21. MBL is a multichain serum lectin made up of a
riable number of 96-kDa subunits, each composed of
ee identical 32-kDa polypeptide chains covalently
ked by disulfide bonds (Taylor et al. 1989). Mannose
cans present in the cell walls of certain gram-negative
cteria, parasites, fungi, as well as in viral envelopes of
V-1 serve as initial binding sites for MBL. By binding
these residues, MBL acts as an opsonin that promotes
hogen phagocytosis (Kuhlman et al. 1989). A second
lependent function of MBL is the activation of the
mplement cascade via binding of MBL-associated
ine proteases (Matsushita et al. 2000). Changes of the
mary amino acid sequence of MBL due to common
cleotide substitutions in codon 52 (52C, allele D),
don 54 (54D, allele B), and codon 57 (57E, allele C)
pede the trimerization of MBL polypeptides (Lip-
mbe et al. 1995). Such mutant MBL trimers are less
ble and display reduced polymerization, leading to
zymatic degradation of MBL polypeptide and func-
nal deficiency of MBL due to very low MBL serum
els (Lipscombe et al. 1995). Heterozygous carriers for
y of the codon variants have approximately 20% of
ldtype MBL serum levels, while homozygous or
mpound heterozygous carriers display very low or
en undetectable MBL serum levels in Eskimos, North-
... Europeans, and West Africans (Lipscombe et al.
1992; Madsen et al. 1995). In addition, this strong correlation between codon variants and reduced MBL serum levels has been confirmed for populations residing at geographically diverse locations, including Africa (Bellamy et al. 1998), the United Kingdom (Crosdale et al. 2000), China (Ip et al. 2000), Japan (Hakozaki et al. 2002), Australia (Minchinton et al. 2002), and Brazil (Santos et al. 2001). This demonstrates that the codon variants can be used as genetic indicators of low MBL levels.

Materials and methods

In the present study, 278 adult individuals were enrolled from the urban region of Medellin, Antioquia, Colombia. Of these, 138 persons were HIV positive, while 140 control DNA samples were obtained from the general population. HIV infection was determined by standard immune assays. Genotyping for MBL variants was performed by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) (Madsen et al. 1995). Primers 5'-AGTCGACCCAGATTGTAGGACAGAG-3' (forward) and 5'-AGGATCCAGGCAGTTTCCTCTGGAAGG-3' (reverse) were used to amplify a 349-bp fragment of exon 1. Allele B was detected using *Ban*I restriction digestion of the 349-bp fragment. *Ban*I cleaves the normal allele into two fragments of 260 bp and 89 bp while leaving the B allele uncut. *Mbo*II cleaves the C allele into two fragments of 279 bp and 70 bp. Detection of allele D was performed by RFLP on fragments (125 bp) amplified by site-directed mutagenesis PCR (SDM-PCR) using a unique set of primers (Madsen et al. 1995). Fragments specific for the D allele were cleaved with *Mlu*I into two fragments of 100 bp and 25 bp. Ambiguous RFLPs were confirmed by direct sequencing. Compound heterozygotes were determined by cloning exon 1 into pBluescript SK, followed by direct sequencing employing the reverse primer used to amplify exon 1.

Results

In the control population, the allele frequencies for the wildtype allele (52R, 54G, 57G, allele A) were 71.8%, for the 52C variant (allele D) 3.6%, for the 54D variant (allele B) 21.4%, and for the 57E variant (allele C) 3.2%. Hence, the composite frequency of structural MBL variant alleles was 28.2%. Genotype frequencies did not deviate from Hardy-Weinberg expectations in the HIV-positive (P=0.78) and control groups (P=0.26). Results of *MBL* genotyping are shown in Table 1. The frequency of the homozygous or compound heterozygous variants in the HIV-1-infected and control group was 5.8% and 5.7%, respectively, which was not significant (P=1.0, two-sided Fisher's exact test). Moreover, the frequencies of homozygote normals and heterozygotes did not differ significantly between the groups (Table 1). Further stratification of the groups by sex, age, gender, or the known presence of bacterial infections did not provide evidence of an association between *MBL* alleles or genotypes and HIV infection (data not shown).

Table 1 Distribution of *MBL* genotypes among HIV-positive individuals and controls from the general population

Genotype	HIV-infected individuals (n=138)	Controls (n=140)
Homozygous normal (52 C/C, 54 G/G, 57 G/G)	74 (54%)	69 (49%)
Heterozygous	56 (40.6%)	63 (45%)
52 C/T	8	8
54 G/A	38	48
57 G/A	10	7
Homozygous and compound heterozygote variants	8 (5.8%)	8 (5.7%)
52 T/T	0	0
54 A/A	5	5
57 A/A	0	1
52T/54A	2	2
54A/57A	1	0

scussion

veral independent studies have provided evidence that BL plays a protective role in HIV-1 infection in andinavian populations, although the precise nature of s relationship is unclear (Garred et al. 1997; Nielsen et 1995; Pastinen et al. 1998). In vitro studies have shown it MBL can bind efficiently to the carbohydrate ieties present on gp120/gp41 (Saifuddin et al. 2000). seems plausible, therefore, that low serum MBL may sult in opsonization deficiency, and thus increased sceptibility to primary HIV-1 infection. Our results, wever, do not support this hypothesis, showing equal evalence of homozygote variants in control and HIV-fected individuals. This is in agreement with other oups who failed to detect an association between MBL lymorphisms or serum levels and HIV-1 infection in ropean HIV patients (Amoroso et al. 1999; Senaldi et . 1995).

Discrepancies between our results and the Scandinavian studies may be due to differences in ethnicity, vironmental and social conditions of the populations. A teworthy difference is the decreased allele frequency of riant alleles in the control group in the Scandinavian dies (16%–20%) relative to a significantly higher equency of 28.3% in the Colombian controls. The lective pressure that maintains a high proportion of non-nctional *MBL* alleles is unknown. However, it is nceivable that constitutive or acquired immune mech-isms exist that can complement or take up the innate defense function provided by MBL. Such redundant defense systems are likely favored under conditions of high pathogen exposure and high frequency of non-functional *MBL* alleles. Compared with Scandinavian populations, both conditions seem present in the Colombian population, possibly explaining the divergent results of a contribution of MBL to susceptibility to HIV infection.

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MBL (8). In an adult South African population, a protective effect for pulmonary tuberculosis (PTB) (28% controls *vs.* 13% PTB patients) was observed in individuals who were homozygous for the *MBL-G54D* allele (p=0.017), whereas a stronger protective effect was observed for pediatric tuberculous meningitis (p=0.002) (9). Further implicating a role of MBL in tuberculosis, TB-negative controls had significantly lower serum MBL concentrations than fully recovered TB patients (p=0.004). In a much larger adult case-control study involving a Gambian population of 844 controls and 794 TB patients, the *MBL-G57D* variant allele was found to be associated with TB resistance (p=0.037) (10). Finally, results based on an adult case-control study involving a South Indian population of 109 controls and 202 PTB patients indicated that homozygotes for the variant alleles (predominantly the *MBL-G54D* allele) were more likely to develop tuberculosis (p=0.008) than those with any other genotype (11). The inverse directions of associations in different populations point to the need for additional studies of MBL in TB susceptibility.

Numerous epidemiological studies have consistently shown that vitamin D deficiency leads to TB susceptibility (12). Also, the active form of vitamin D, $1,25(OH)_2D_3$, plays a part in immunoregulation and aids in suppressing intracellular growth of *M. tuberculosis* in monocytes (13). A SNP in codon 352 of the vitamin D receptor (VDR) with alleles designated T for the common allele and t for the rare allele has been examined with respect to PTB in a Gambian population comprising 408 PTB patients and 414 controls (14). Results from this study showed that the tt genotype was over-represented in healthy individuals (p=0.01, OR=0.53, 95% CI=0.31–0.88), suggesting a protective effect of the t allele (14). By contrast, an analysis involving 200 PTB patients and 108 controls of an Indian population showed a lack of association between the *VDR-TaqI* genotype and tuberculosis (15). However, upon stratifying this population by gender, the tt genotype was found to be over-represented in female TB patients (p=0.02), yet correcting for multiple tests would eliminate the formal significance of this finding. A study involving a Gujarati Indian population showed that undetectable levels or deficiency of serum 25-hydroxycholecalciferol were strongly associated (OR=9.9 and OR=2.9, respectively) with tuberculosis (16). The same study considered two polymorphisms, one described previously (T/t) and the other at nucleotide position 117, designated F for the wild-type. Analysis of the two polymorphisms showed no association between tuberculosis and SNP genotypes, however, a combination of TT/Tt genotypes and vitamin D deficiency, and ff genotype and undetectable serum levels of vitamin D were associated with tuberculosis (OR=2.9 and OR=5.1, respectively) (16). Although the studies involving Gujarati and Indian populations share a common ethnicity, the inconsistent findings may point to the possibility that the Gujaratis may be deficient in vitamin D due to a lack of sunlight in the UK environment as opposed to being in the Indian environment. Taken together, these somewhat divergent results suggest that further studies are required to better assess a physiological, gender and environmental role of VDR and vitamin D in TB susceptibility.

Interleukin-1 is a potent pro-inflammatory cytokine implicated in a number of inflammatory-type diseases (17). Assessment of cytokine profiles determined from several studies have consistently shown that interleukin-1β (IL-1β) is significantly elevated in the bronchoalveolar lavage fluid (BALF) in patients with active tuberculosis (18–20), suggesting that lung inflammation in these patients is at least partly due to IL-1β. To downregulate the pro-inflammatory response to IL-1β, an antagonist, IL-1Ra, competes for the IL-1 receptor. It is for this reason that TB susceptibility may be influenced by the IL-1β/IL-1Ra ratio. Genetic analysis of the 86 bp VNTR in intron 2 of the *IL-1Ra* gene showed that, in response to *M. tuberculosis* stimulation of peripheral blood mononuclear cells (PBMC) from healthy subjects, allele 2 (two 86 bp repeats) was associated with 1.9-fold greater IL-1Ra production than that of non-allele 2 carriers (21). Furthermore, two biallelic markers at positions −511 and +3943 were analyzed in the *IL-1β* gene. While *M. tuberculosis*-stimulated production of IL-1β did not differ with respect to allelic variants of either marker, only allele +3953 A2 was associated with depressed levels of Il-1β mRNA (p=0.04). In the same investigation, a small population case-control study of 114 controls and 89 patients of varying TB forms showed no association, either allelic or genotypic, with *IL-1β* or *IL-1Ra* polymorphisms and increased risk of tuberculosis. However, the IL-1Ra A2⁻/IL-1β (+3953) A1⁺ haplotype was somewhat over-represented in patients with pleural disease (p=0.028). A larger case-control study in the Gambia considered a population made up of 400 TB patients and 400 healthy controls (22). In agreement with the study by Wilkinson and colleagues (21), allele 2 of IL-1Ra was found to be less common among TB patients (p=0.03) suggesting a protective affect. Together, these studies suggest that polymorphisms governing IL-1 activity may have a modest effect on the clinical presentation of tuberculosis.

Initial genetic studies of tuberculosis focused primarily on the human leukocyte antigen (HLA) complex, however, findings have been varied and conflicting. Serological-based HLA typing was performed on a modest number of multicase families to follow HLA-DR2 segregation in TB patients of an Indian population (23). Although the specific mode of inheritance was not established, it was clear that DR2 was linked with TB susceptibility. Likewise, there was evidence for distortion of transmission ratios with DR2 being transmitted from heterozygote parents to affected children at an average rate of 83%, while only 51% of the time to healthy children (23). Similar results were found in a case-control study of an Indian population concerning 153 PTB patients and 289 healthy controls where HLA-DR2 was over-represented among the TB patients (p_{corr}=0.029, relative risk (RR)=1.8) (24). However, upon molecular subtyping of DR2 performed on DR2-positive healthy controls (n=81) and TB patients (n=61), the

most frequent alleles, *DRB1*1501* and *DRB1*1502*, were not associated with PTB (66.7% in controls *vs.* 77% in PTB, and 37% in controls *vs.* 28% in PTB, respectively), yet small sample size may severely compromise the power of this study to reach statistical significance. Furthermore, it should be noted that the trend of the findings is consistent with other studies discussed below. Enrichment of the *HLA-DRB1*1501* DR2 allele was observed in a study of a Mexican population, however, the sample size was quite small (95 controls *vs.* 50 PTB patients) and reaching a conclusion, as the authors have stated, is difficult (25). A South Indian case-control study revealed two polymorphic variants associated with sputum positive PTB patients, *HLA-DRB1*1501* (OR=2.68, 95% CI=1.30–5.89, p=0.013), and *DQB1*0601* (OR=2.32, 95% CI=1.29–4.27, p=0.008), while a third allele, *DPB1*04*, was enriched in healthy controls suggesting a protective effect (OR=0.45, 95% CI=0.21–0.95, p=0.036). However, in this and other studies significance values would be diminished if corrections for multiple testing were taken into consideration. The problem of multiple testing and the concomitant loss of power have been elegantly addressed in a case-control study involving 126 Cambodian PTB patients and 88 healthy controls. Using a two-stage study design, the authors found a significant association between the *HLA-DQB1*0503* allele and PTB (p=0.005) (26).

Pulmonary surfactant proteins SP-A and SP-D are essential for the normal functioning of lungs and play a role in innate immune defense as well as in the regulation of inflammatory response (27). It has been shown that SP-A can mediate attachment of *M. tuberculosis* to alveolar macrophages (28), while SP-D results in agglutination of the bacteria, thereby reducing phagocytosis of the bacteria (29). This suggests that although SP-A and SP-D have differing, perhaps opposing roles in the innate immune response to *M. tuberculosis*, both may play an important role in the outcome of TB infection. On the genetic level the *SP-A* locus consists of two genes, *SP-A1* and *SP-A2*, in opposite transcriptional orientation (30). A case-control study of a Mexican population assessed polymorphic variants in the *SP-A1*, *SP-A2*, and *SP-D* genes with respect to PTB (31). Specifically, diallelic variants in the *SP-A1* gene occur at amino acids 19, 50, 62, 133, and 219, while variants in the *SP-A2* gene occur at amino acids 9, 91, 140, and 223 (32). Patterns of various combinations of SNPs at given amino acids determine a specific allele. For example, allele $6A^4$ refers to the *SP-A1* gene (6A) with the superscript "4" representing the fourth allele, which differs from all other common alleles at amino acid 219 (Trp for $6A^4$ and an Arg for all others). Logistic regression analyses showed that allele $1A^3$ of the *SP-A2* gene and the $6A^4$ allele of the *SP-A1* gene were significantly over-represented in the Mexican TB patients relative to healthy controls (OR=9.28, 90% CI=1.61–53.39, p=0.018; OR=2.71, 90% CI=1.25–5.87, p=0.033, respectively), while allele *DA11_C* of the *SP-D* gene was associated with TB susceptibility when compared to TB skin-positive controls (OR=2.66, 90% CI=1.57–4.49, p=0.002) (31). Here, allele $1A^3$ differs from other common *SP-A2* alleles at amino acid 223 (with the exception of allele $1A^1$), while the *SP-D* marker, *DA11*, is a biallelic polymorphism corresponding to a C to T base change (*DA11_C*: threonine to *DA11_T*: methionine) at amino acid 11. To allow a better assessment of the role of surfactant protein genes in TB pathogenesis, a replication of these interesting findings is required in other ethnic groups and epidemiological settings.

Inbred strains of mice revealed that natural resistance to intracellular pathogens such as *Mycobacterium bovis* (BCG) (33), *M. avium* complex (34), *M. lepraemurium* (35), *Leishmania donovani* (36) and *Salmonella typhimurium* (37) is influenced by a single dominant gene, designated *BCG*. Positional cloning isolated the natural resistance associated macrophage protein 1 (*Nramp1*) gene and showed that natural resistance to infection was influenced by a single G169D amino acid substitution in this gene (38, 39). Subsequently, with numerous polymorphisms identified in the human homologue *NRAMP1* (40, 41) a number of studies have implicated *NRAMP1* with TB susceptibility in humans. A large case-control study examining 410 smear-positive PTB patients ethnically matched with 417 healthy controls showed that *NRAMP1* variants were associated with susceptibility to tuberculosis in a West African population (42). Specifically, four variants of the *NRAMP1* gene were examined: a dinucleotide CA repeat at the 5′ promoter region $(GT)_n$, a single nucleotide change in intron 4 (469+14G/C), a single nonsynonymous nucleotide substitution in codon 543 (D543N), and a TGTG deletion in the 3′ untranslated region (UTR; 1729+55del4). Results showed that subjects heterozygous for both 469+14G/C and 1729+55del4 polymorphisms were particularly over-represented among TB patients (OR=4.07; 95% confidence interval, 1.86–9.12; chi-square=14.58, p<0.001) (42). A smaller family-based study, set in Guinea-Conakry, West Africa, was carried out using 26 one-parent and 17 two-parent nuclear families to assess three *NRAMP1* polymorphisms: GTn, 1729+55del4, and 469+14G/C (43). This study reported that 469+14G/C was significantly associated with tuberculosis (p=0.036), further supporting the role of *NRAMP1* in tuberculosis. The 1729+55del4 TGTG deletion in the 3′UTR of *NRAMP1* was evaluated with respect to TB susceptibility in a Korean population involving 192 TB patients and 193 healthy individuals (44). Results showed that the 3′UTR polymorphism was indeed weakly associated with TB susceptibility (p=0.02) with an excess of heterozygotes in the TB patient population. Likewise, analysis of two Japanese populations from Tokyo and Osaka demonstrated a strong association of the 5′$(GT)_n$ *NRAMP1* promoter polymorphism with tuberculosis (p=0.0003, OR=1.86, 95% CI=1.32–2.61) (45). Conversely, a linkage study assessing 37 multi-case families of a Brazilian population did not show evidence of a TB susceptibility locus linked to *NRAMP1*, however, two markers (*IL8RB* and *D2S1471*) tightly linked to *NRAMP1* were shown to be weakly linked to disease susceptibility (p=0.038; p=0.025, respectively) (46).

...ions that impact on TB susceptibility was provided
y a parametric linkage study employing liability
asses according to recorded clinico-epidemiological
ata. Linkage of *NRAMP1* and adjacent markers with
B susceptibility was analyzed in a large aboriginal
anadian pedigree that experienced a TB outbreak
7). Linkage analysis was conducted assuming a ma-
ir TB susceptibility locus with a relative risk of 10 of
ie high risk compared to the low risk allele. This analy-
s resulted in maximum LOD scores of 3.55, 3.21, and
.36 for linkage between TB and intragenic polymor-
hisms *NRAMP1-(GT)n* , *D543N* and an *NRAMP1* hap-
otype of 10 intergenic variants, respectively. Further-
iore, a maximum multipoint LOD score of 4.2 was
btained with the susceptibility gene locating on top of
IRAMP1. If the sample was analyzed without specifi-
ation of liability classes there was no evidence for link-
ge. These results strongly suggest that even major
enetic effects can be missed if gene-environment in-
eractions are neglected in the genetic analysis.

lare Mendelian Disorders

lare Mendelian disorders predisposing individuals to
ypersusceptibility to relatively avirulent mycobacter-
al species may offer insight into genes which may play
role in tuberculosis on the population level. *In vivo*
nd animal studies have shown a critical role for inter-
eron (IFN)-γ for mycobacterial host defense, presum-
bly due to its ability to activate macrophages (48),
whereas IL-12 is crucial in the mediation of optimal pro-
duction of IFN-γ, and further, IL-12 deficiency in mice at-
tests to its importance in *M. tuberculosis* infections
(49). Therefore, genes included in the signaling cas-
cade of IFN-γ and IL-12, namely, *IFNgRI, IFNgR2, IL-12p40*, and *IL-12Rβ1* genes, were examined and found to be associated with idiopathic disseminated my-
cobacterial infections.

Molecular analysis of a Tunisian female infant with fatal BCG infection showed a substantial decrease in *IFNgR1* mRNA due to a homozygote missense muta-
tion in exon 2 (50). Moreover, disseminated atypical mycobacterial in four Maltese children was attributable to a point mutation at nucleotide 395 of the *IFNgR1* gene which truncates the protein and effectively abro-
gates its expression on the cell surface (51). Further-
more, partial IFN-γR1 deficiency due to a homozygous missense mutation at position 260 has been described in siblings, one with disseminated BCG infection with tuberculoid granulomas, and the other sibling with clinical tuberculosis who had not previously received BCG inoculation (52). Whereas the two previous stud-
ies considered consanguineous kindreds, in a case-
study an Italian infant, born to two nonrelated parents, afflicted with disseminated *Mycobacterium smegmatis* was examined (53). Results from this study found that the child carried two novel mutations, a 4-bp insertion in exon 2 and a SNP at the splice-site of intron 3, re-
vealing the first compound heterozygote patient.

Mycobacterium fortuitum and *M. avium* complex in-
fections showed that the immunodeficiency was due to a mutation in the *IFNgR2* gene (54). Specifically, se-
quence analysis revealed a homozygous dinucleotide deletion at nucleotides 278 and 279, introducing a pre-
mature stop codon resulting in a truncated protein.

A female infant with BCG and *Salmonella enteritidis* infections, born to consanguineous Pakistani parents, was found to carry a large homozygous deletion encom-
passing two coding exons within the Il-12p40 subunit (55). Consequently, INF-γ production after PBMC stimu-
lation was significantly reduced. Also, IL-12Rβ1 defi-
ciency in three unrelated kindreds caused disseminated mycobacterial and non-typhi salmonella infections in otherwise healthy individuals (56). Three distinct muta-
tions were identified in each of these patients: a ho-
mozygous nonsense mutation at position 913 resulting in a premature stop codon, a frameshift mutation caus-
ing the skipping of the exon spanning nucleotides 701 to 783, and a missense mutation at nucleotide position 641 (56). All three mutations led to complete IL-12Rβ1 defi-
ciency, although this deficiency led to less severe infec-
tions than those in patients with IFN-γR1 deficiency, and, unlike complete deficiency of IFN-γR1, did not compro-
mise the formation of mature granulomas (55). Simi-
larly, in three unrelated individuals with severe my-
cobacterial and *Salmonella* infections it was shown that mutations in the IL-12Rβ1 subunit result in a lack of func-
tional IL-12R complexes (57). Three unique mutations were responsible for IL-12R deficiency: nonsense muta-
tions at nucleotide positions 94 and 1126, and a deletion spanning from nucleotide position 409 to 549. All pa-
tients were homozygous for the mutations and all muta-
tions led to premature stop codons effectively abrogat-
ing IL-12R cell surface expression.

Together, these studies indicate that *IFNgR1, IFNgR2, IL-12p40* and *IL-12Rβ1* are strong candidate genes for TB susceptibility and that population- and family-
based studies should be carried out using common ge-
netic polymorphisms in these genes. This conclusion is further supported by a recent report describing a pa-
tient with disseminated BCG disease displaying tuber-
culoid granulomas who carried a heterozygous muta-
tion in *STAT1* (58). STAT1 is a critical mediator of IFN-mediated cellular processes and the report high-
lights the pivotal role that is played by genetic varia-
tions of genes positioned along the IFN-γ response pathway.

Genome-wide Scan

A two-step genome-wide scan using 299 highly infor-
mative markers was performed on 92 sibpairs with tu-
berculosis from the Gambia and South Africa (59). Seven chromosomal regions that showed putative linkage were further genotyped in 81 additional sib-
pairs using 22 markers for each region. Weak evidence for linkage was indicated on chromosome regions 15q and Xq (multipoint MLS_{PT} =1.82 and =2.18, respec-

tively). These results argue against a major gene control of TB susceptibility in the families studied.

Conclusion

TB infections have risen at a dramatic rate within the past decade due to the alarming increase of HIV/AIDS, particularly in the developing countries, and the relative ineffectiveness of BCG vaccination. Studying why humans are naturally susceptible to tuberculosis can offer new clues for the development of more effective drugs and vaccines against tuberculosis. Since it is becoming increasingly clear that host genetics plays a pivotal role in susceptibility to tuberculosis, exploiting natural pathways of resistance/susceptibility may provide important clues for control of this disease. Armed with new knowledge from the Human Genome Project, an increasing understanding of disease mechanisms, and high-throughput genotyping technologies, such as high-density variation-detection DNA chips (60), novel candidate genes and SNPs will allow us to build a more comprehensive genetic profile of TB susceptibility. Consequently, drugs/vaccines tailored to host genetic profiles may offer a more specific and effective means of controlling tuberculosis by modern medicine.

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Appendix 2

Research Ethics Board Approval Letters