Identification and Characterization of the Genetic Component of Differential Susceptibility to Mouse Tuberculosis

Loukia-Maria Mitsos

Department of Biochemistry, McGill University
Montreal

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ABSTRACT

Genetic control of susceptibility to tuberculosis in resistant C57BL/6 (B6) and susceptible DBA/2 (D2) mice is multigenic. Susceptibility in D2 is associated with a unique phenotype which includes unrestricted pulmonary replication, severe lung pathology, extensive tissue necrosis and early death. Quantitative trait linkage mapping in 2 genome wide scans was used to determine the number and location of genes controlling differential susceptibility to pulmonary tuberculosis in D2 and B6. In a first scan, 95 informative (B6XD2) F2 mice were infected *i.v* with 10^5 virulent *M. tuberculosis* H37Rv, and survival time was used as a phenotypic measure of susceptibility. These studies identified 3 significant linkages on chromosomes 1,3 and 7 designated *Trl-1/2/3* respectively. In a second genome scan, 104 (B6XD2) F2 mice were infected with 10^2 *M. tuberculosis* H37Rv by the aerosol route, and the extent of bacterial replication in the lungs (CFU) at 90 days was used as a phenotypic measure of susceptibility. Results from this analysis identified a major linkage on chromosome 19 *(Trl-4)* accounting for 25% of the phenotypic variance. In this scan *Trl-3* was confirmed as not only playing a role in overall survival to infection but also bacterial growth in the lungs. Our results suggest a strong genetic interaction between *Trl-3* and *Trl-4* in regulating pulmonary replication of *M. tuberculosis*. To further characterize the mechanistic basis of action of the *Trl-1/2/3/4* effect on *M. tuberculosis* infection, global expression analysis of approximately 15 250 genes was utilized to identify genes differentially expressed between the lungs of B6 and D2 mice prior to and during infection with *M. tuberculosis*. Our data suggests that the classical complement pathway and the apoptotic pathway plays a role in the differential susceptibility between B6 and D2 mice, with these pathways being upregulated in B6. Moreover the increase in neutrophil associated gene expression corroborates with our histological finding of increased neutrophil cell counts in D2 mice. Finally these studies have provided candidate genes that map in the *Trl-1* and *Trl-4* regions (*Cfh* and *Scd2* respectively).

RÉSUMÉ

Le contrôle génétique de l'infection à *Mycobacterium tuberculosis* entre les souris résistantes C57BL/6 (B6) et les souris susceptibles DBA/2 (D2) est polygénique. La susceptibilité chez la souris D2 est associée à une multiplication bactérienne incontrôlée, une pathologie pulmonaire sévère, une nécrose tissulaire extensive et une mort précoce. Des études de liaison génétique à l'aide de deux cartographies du génome entier ont été utilisées pour déterminer le nombre et la localisation des gènes contrôlant cette réponse différentielle entre les souris D2 et B6.Dans la première cartographie, 95 souris du type (B6XD2) F2 ont été infectées avec 10^5 *M. tuberculosis* H37Rv et la survie des souris a été suivie comme marqueur phénotypique de susceptibilité. Cette analyse a identifié 3 loci sur les chromosomes 1,3 et 7 désignés *Trl-1/2/3* respectivement. Dans la deuxième cartographie, 104 souris (B6XD2) F2 ont été infectées par voie aérienne avec10^2 *M. tuberculosis* H37Rv et la multiplication bactérienne dans les poumons, 90 jours après l'infection, a été utilisée comme marqueur phénotypique de susceptibilité. Cette étude a identifié un locus sur le chromosome 19(*Trl-4*) qui explique 25% de la variance phénotypique. Dans le deuxième cas, *Trl-3* a non seulement confirmé son rôle dans la survie à l'infection mais a également un rôle dans la croissance bactérienne dans les poumons. Nos résultats suggèrent une forte interaction génétique entre *Trl-3* et *Trl-4* qui régule la multiplication pulmonaire de *M. tuberculosis.* Pour caractériser l'effet de *Trl1/2/3* sur l'infection, une analyse de l'expression de 15 250 gènes a été faite pour identifier les gènes ayant une expression différentielle entre les poumons des souris B6 et D2 avant et après infection avec *M. tuberculosis*. Nos résultats suggèrent que la voie apoptotique et la voie classique du complément jouent un rôle dans la susceptibilité différentielle entre les souris B6 et D2. De plus, une augmentation dans l'expression des gènes associés aux neutrophiles dans la souris D2 supporte nos trouvailles histologiques antérieures où D2 a un décompte augmenté de neutrophiles. Finalement, nos études ont ciblé des gènes candidats pour les régions de *Trl-1* et *Trl-4* (*Cfh* et *Scd2* respectivement).

PREFACE

The work described in Chapter 2 and 3 have been published as follows:

Chapter 2 — Mitsos, L-M., Cardon, L.R., Fortin, A., Ryan, L., LaCourse, R., North, R.J. and P. Gros (2000) Genetic control of susceptibility to infection with *Mycobacterium tuberculosis* in mice. *Genes and Immunity*, **1:** 467- 477. © Nature Publishing Groups

Chapter 3 — Mitsos, L-M., Cardon, L.R., Ryan, L., LaCourse, R., North, R.J. and P. Gros (2003) Susceptibility to tuberculocis: a locus on mouse chromosome 19 (*Trl-4*) regulates *M. tuberculosis* replication in the lungs. *PNAS*, **100:** 6610-6615. ©National Academy of Science

Chapter 4 — has not been published:
Mitsos, L-M., Nantel, A., Ryan, L., LaCourse, R., North, R.J. and P. Gros. Multistage gene expression profiling in a differentially susceptible mouse model of tuberculosis infection.

CONTRIBUTION OF CO-AUTHORS

The work described in Chapter 2 has been performed in collaboration the co-authors as follows: Anny Fortin was responsible for my training in the genotyping techniques. The group of Robert J. North, Lynn Ryan and Ronald LaCourse were responsible for the infection of the (B6 X D2) F2 mice and the histopathology. I performed the genotyping of all the F2 mice. I was also responsible for maintaining the phenotypic and genotypic data sets. Lon Cardon performed the genetic analysis. I prepared all the figures and tables of the manuscipt and participated in its writing.

The research described in chapter 3. The group of Robert J. North, Lynn Ryan and Ronald LaCourse were responsible for the infection of the animals as well as providing the phenotypic data (CFU counts). I extracted the DNA from the tails of these F2 animals as well as performed the genotyping of all the F2 mice. I was also responsible for maintaining the phenotypic and genotypic data sets. Lon Cardon performed the genetic analysis. I prepared the figures and tables for the manuscript and wrote the first version.

Finally for the work in chapter 4, the group of Robert J. North, Lynn Ryan and Ronald LaCourse were responsible for the infection of the DBA/2J and C57BL/6 J mice and assisted me in the extraction of the RNA from the organs of the infected animals (this

work was done at the Trudeau institut in Saranac Lake, NY). I was responsible for the RNA extraction, veryfying the quality of the RNA, all the microarray experiments. André Nantel helped me with the analysis of the microarray experiments.

Philippe Gros provided expert supervision throughout all the studies.

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Chapter 1

Introduction and Literature Review

Tuberculosis is one of the oldest recorded human diseases, with the first recorded case documented more than 4 000 years ago. The disease may have been largely forgotten by many people living in developed countries but this ancient scourge is still one of the major killers amongst infectious diseases worldwide today, responsible for 4% of annual mortality amounting to 2.2 million deaths per year (one death every 10 seconds). *Mycobacterium tuberculosis* is one of the most effective human pathogens, with one-third of the world's population being infected and 8 million new tuberculosis cases per year (1 new case every 4 seconds) [1]. The largest number of cases occurs in the Southeast Asia and in sub-Saharan Africa, but no country is immune, as travel and immigration have brought the disease to all corners of the globe. Widespread resistance to antibiotics [2] as well as increased prevalence of tuberculosis infection in HIV patients have increased the severity of the problem and has prompted the search for alternative strategies for intervention [3]. The current vaccine against tuberculosis, Bacille Calmette-Guérin (BCG), was developed in the early 1900s, and while it helps protect against childhood forms of tuberculosis, it provides variable protection in adults. A better understanding of the natural mechanism of host defense against tuberculosis can provide novel targets for therapeutic intervention.

1.1 *Mycobacterium Tuberculosis*

In 1882, in a remarkable achievement of microbiology, Robert Koch isolated *M. tuberculosis* for the first time, and conclusively demonstrated in the guinea pig that this slow-growing mycobacterium was the agent of a human disease [4]. Mycobacteria belong to the family Mycobacteriaceae and the order Actinomycetales. *Mycobacterium tuberculosis*

is a slender, curved rod, gram- positive aerobic bacterium that does not form spores. The cell wall of the mycobacterium is composed of mycolic acids, glycolipids and complex waxes. The mycobacterial cell wall is acid-fast, that is, it retains carbolfuchsin dye when decolorized with acid. This is an important property since it permits differential staining of the mycobacterium in contaminated clinical specimens. This unique, robust cell wall structure endows mycobacteria with resistance to dehydration, acids, alkalis, contributes to the capacity of *M. tuberculosis* to survive in host phagocytes and provides the source of glycolipid antigens for T cells. Some of the more intriguing observations about cell wall components are those showing that CD1-restricted T-cells recognize mycolic acids [5], that one of the most potent protective antigens of *M. tuberculosis*, antigen 85 is a mycolyltransferase [6], and that capped lipoarabinomannan (LAM) plays a role in *M. tuberculosis* phagocytosis and facilitates the survival *of M. tuberculosis* within macrophage [7,8]. Several proteins characteristic of *M. tuberculosis* include those in purified protein derivative (PPD) tuberculin, a precipitate of non-species-specific molecules obtained from filtrates of heat-sterilized, concentrated broth cultures. Infection in an asymptomatic individual can be diagnosed with the intradermal introduction of PPD. *M. tuberculosis* is a slow-growing mycobacterium, with a replication time of 20 hours approximately. This slow growth may play a role in the chronic nature of infection and disease, complicates diagnosis and demands long-term drug treatment.

Many different strains of *M. tuberculosis* exist that are distinguished from one another on the basis of restriction fragment length polymorphism. This method is mostly based on the polymorphism and copy number of the IS6110 chromosomal insertion sequence that inserts in different numbers and sites along the chromosome. It is used to determine whether tuberculosis outbreaks are caused by the one or multiple *M.*

tuberculosis strains or recurrent disease is caused by endogenous or exogenous re-infection. However, other molecular methods are available to assist in strain identification[9].

The *M. tuberculosis* genome has been sequenced and is 4.41Mb in size. It contains 3995 protein-coding genes out of which it is possible to assign a function to 2058 proteins (52%) and only 376 putative proteins are seemingly unique to *M. tuberculosis.* A large proportion of the genes is devoted to the production of enzymes involved in cell wall metabolism and of glycine-rich proteins that are probably responsible for antigenic variations [10]. The importance of this information lies in the identification of genes that code for virulence factors and antigens against which host immunity is directed. The genome sequence is also important for identifying new targets for therapeutic intervention.

1.2. Tuberculosis Pathophysiology

A new infection is often detected by the conversion of the intradermal skin test with purified protein derivative (PPD) to positive or incidentally by detecting a lesion in a chest x-ray on an otherwise asymtomatic person. Clinical signs and symptoms are only seen in 5-10 % of infected healthy patients that usually manifest with pulmonary disease. Classical symptoms of tuberculosis infection are chronic, productive cough, low-grade fever, night sweats, fatigability, and cachexia. Tuberculosis manifests itself mainly in the lung (which is also the main port of entry) disease but can also manifest itself in any tissue. In less than 10% of all cases tuberculosis may also exhibit extrapulmonary manifestations such as lymphadenitis, meningitis, kidney/ bone or joint involvement or

disseminated disease. Extrapulmonary manifestations are increased among immunocompromised patients such as the elderly, poor, alcoholics, malnourished and the HIV-infected [11].

M. tuberculosis is most commonly transmitted from an infected patient with pulmonary tuberculosis to other people by airborne droplet nuclei, produced by coughing or speaking. Up to 3000 infectious nuclei are counted per cough. Small droplets (<5 to 10 μm in diameter) can remain suspended in the air for several hours and enter the terminal air passages when inhaled by other individuals. Transmission of the bacilli by other routes such as the skin or placenta are uncommon. Years ago, raw milk containing *M. bovis* from tuberculous cows was a frequent source of infection.

1.2.1 Early Events: A Mycobacterium's Intracellular Life

1.2.1.1 Entry into the Cell

M. tuberculosis infections occur by airborne transmission of droplet nuclei containing a few viable, virulent organisms produced by a sputum-positive individual. The bacilli are deposited in the alveolar spaces of the lungs, where they are engulfed by alveolar macrophages or possibly alveolar epithelial type II pneumocytes. *M. tuberculosis* replicates ex vivo in pneumocytes, and this cell type is more abundant in alveoli than macrophages [12,13]. Dendritic cells also play a role in the early stages of infection since they are better antigen presenting cells than macrophages [14]and play a key role in activating T cells with specific *M. tuberculosis* antigens[15,16]. Dendritic cells are also

migratory cells, more capable to migrate from peripheral tissues to secondary lymphoid tissues and therefore can play an important role in mycobacterial dissemination[17]. However we will focus on macrophage-mycobacterium interaction since these are more extensively studied and better understood.

Macrophages, as professional phagocytes, have several surface receptors that allow antigen uptake. Therefore, blocking individual receptors does not significantly alter *M. tuberculosis* intracellular fate [18]. The complement receptors are widely used receptors for mycobacteria, for both opsonized and non-opsonized entry [19,20]. However, mannose receptors [21], Fc receptors [22], scavenger receptors [18]and toll like receptors [23] have also been implicated in mycobacterial uptake. Cholesterol rich lipid domains have been shown to behave as a docking site for the pathogen, promoting receptor–ligand interactions [24]. A glycoprotein found on alveolar surfaces, surfactant protein A [25], can enhance binding and uptake of *M. tuberculosis* by upregulation of mannose receptors. On the other hand, surfactant protein D, arrests *M. tuberculosis* phagoctosis and interaction with the mannose receptor by blocking mannosyl oligosaccharide residues on the bacterial cell surface [26,27].

1.2.1.2 Antimycobacterial Mechanisms of Free Radicals

Upon entry in the host macrophage, *M. tuberculosis* resides in an endocitic vacuole: phagosome. If normal maturation of the phagosome occurs, the mycobacteria encounter a hostile environment: acid pH, reactive oxygen intermediates, lysosomal enzymes and toxic peptides. Reactive nitrogen intermediates produced by the activated murine macrophages are important determinants in antimicrobial activity[28]. Mice mutated for the inducible nitric oxide synthase gene are more susceptible to various pathogens

including *M. tuberculosis* [29]. Resistance to reactive nitrogen intermediates among various strains of *M. tuberculosis* correlates with virulence [30,31]. The role of reactive nitrogen intermediates in the control of human tuberculosis remains controversial, however, alveolar macrophages of the majority of tuberculosis-infected patients exhibit inducible nitric oxide synthase activity [32]. While the importance of nitric oxide in host defense against *M. tuberculosis* is well demonstrated, that of oxygen species remains controversial. Cytokine activated macrophages produce H_2O_2 and oxygen radicals that are mycobactericidal [33]. Mycobacteria are able to evade reactive oxygen intermediates by various mechanisms[34]: a) mycobacterial lipoarabinomannan and phenolicglycolipid I are oxygen radical scavengers [35,36] b) mycobacterial sulfatides hinder oxygen radical-dependent antimicrobial mechanism of macrophages. Also NADPH oxidase knockout mice are slightly more susceptible to *M. tuberculosis* infection than wild type mice. More studies need to be conducted before exclusion of reactive oxygen intermediates from antimycobacterial defense can be concluded[37,38].

1.2.1.3 The Phagolysosome

The mastery of *M. tuberculosis* in maintaining a vast reservoir of infected individuals lies in its ability to parasitize macrophages. In the 1970's D'Arcy-Hart *et al.* demonstrated that *M. tuberculosis*–containing phagosomes fail to fuse with lysosomes after internalization by macrophages [39] and that this absence of fusion was associated with the viability of the infecting mycobacteria. However the vacuoles do retain their capability to fuse with other intracellular vesicles[40,41]. Essentially, the mycobacteria arrest maturation of the phagosome and maintain access to the rapid recycling endosome system of their host cell

[40-43]. The restricted progression of *Mycobacterium*-containing vacuoles limits the hydrolytic capacity of the compartment, facilitates access to nutrients internalized by the host cell, and restricts the interface between the pathogen and the antigen processing and presentation machinery of the macrophage [44].

The major defining features of the unique *M. tuberculosis* phagosomal compartments are fewer or less-active H^+/ATPase molecules exhibiting limited acidification of the vacuole [45], a clear absence of the mature lysosomal hydrolases such as cathepsin D [40,42], the phagosome remains associated with the small GTPase Rab 5 that is known to modulate the fusion behavior of early endosomes and does not recruit Rab 7, fail to acquire EEA 1, the rab 5- and PI_3P-binding protein [46,47], and degradation of the vesicular soluble *N*-ethylmaleimide attachment protein receptor [48]. Mycobacterial phagosomes also show aberrant trafficking of plasma membrane markers [42], and taco [49], a factor identical to the previously characterized generic phagocytosis protein coronin [50] that seems to be important for the uptake of mycobacteria [51].

Ca^{2+} signaling is inhibited when live but not killed or antibody-opsonized *M. tuberculosis* are phagocytosed [52]. A decreased Ca^{2+} level helps *M. tuberculosis* avoid host defense mechanisms such as respiratory burst, NO and cytokine production. High Ca^{2+} levels are associated with phagolysosome maturation and trafficking to the late endosome.

Several studies also support a role for Nramp1 in antagonizing the ability of *mycobacteria* to block phagosome maturation. The effect of Nramp1 recruitment to the phagosomal membrane on mycobacterial phagosome maturation has been studied for *M. bovis* [53] and for *M. avium* [54,55]. Nramp1-positive phagosomes were significantly more acidic than those formed in Nramp1-negative cells due to increased concanamycin-

sensitive H^+ -pumping across the phagosomal membrane, and enhanced recruitment of V-ATPase-positive endosomes and/or lysosomes that was only seen with live mycobacteria and not with dead *M. bovis* or latex beads. These results suggest that Nramp1 action may antagonize an active microbial-encoded inhibitory process required for modulation of phagosome fusion and intracellular survival. Studies with *M. avium* showed increased bacteriostatic activity of Nramp1-positive macrophages, increased fusion to lysosomes, and increased acidification as compared to Nramp1-negative cells.

1.2.1.4 Innate Immunity through Tlr

Toll like receptor (*Tlr*) proteins enable the host to recognise a large number of pathogen-associated molecular patterns ensuring an appropriate immune response and proper defence against pathogens. Mycobacteria express a plethora of lipid, lipoprotein and glycolipid molecules that participate in these early recognition events, and there is increasing evidence that the resulting combination of signals are important in directing the course of infection. In vitro studies show that [23,56,57] *Tlr-2* and *Tlr-4* are essential in resistance of both mice and humans to *M. tuberculosis* infection.

Tlr-2 induced by lipoproteins triggers a proinflammatory response, which can promote mycobacterial killing [23] and promote apoptosis of infected cells [58]. It has also been suggested in studies showing a decrease in MHC class II proteins and MHC-II presentation of antigens in macrophages after *M. tuberculosis* infection, that a selective advantage to *M. tuberculosis* in staying in an early endosome would result in less host immunosurveillance by $CD4^+$ T cells [59]. This effect seems to be induced by the 19-kDa

lipoprotein of the mycobacterium through interaction with the *Tlr-2* receptor at the early stages of infection [23].

The importance of *Tlr-4* was shown in *in vivo* studies. *Tlr-4* -/- mice are significantly more susceptible to aerosol infection than wild type mice as evidenced by their increased lung bacterial growth, extensive pathology and shorter overall survival to infection [60]. However there are contradicting studies showing that after aerosol infection *Tlr-4-/-* are as capable as wild-type mice to control infection[61]. Along the same lines, a study of *Tlr4*-/-and *Tlr2*-/- mutant mice showed [62] that mice deficient in either of these *Tlrs* were no less capable than wild-type mice in defending against airborne infection, albeit *Tlr2* deficiency resulted in less resistance against a much higher dose of mycobacteria. Studies by Drennan *et al.* show that in aerosol infection with 100 or 500 live mycobacteria, *Tlr2*-/- mice display reduced bacterial clearance, a defective granulomatous response suggesting that *Tlr-2* may function as a regulator of inflammation, and in its absence an exaggerated immune inflammatory response develops [63]. Sugawara *et al.* suggest that *Tlr-2* does not play an essential role in tuberculosis pathogenesis but does play a role in defense against infection [64]. The subject is controversial and in need of further investigation. Besides *Tlr-2* and *Tlr-4*, other *Tlrs* may be involved in immune recognition of *M. tuberculosis*: heterodimerization of *Tlr-2* with *Tlr-6* or *Tlr-1* is necessary for signal transduction [65,66]. At the same time as *Tlr* signaling, mannose-capped lipoarabinomannan can deliver an anti-inflammatory signal through DC-SIGN, a C-type lectin receptor on dendritic cells, thereby reducing antimycobacterial activity and stimulating the release of IL-10 [67,68]

The balance in innate immune signaling regulates the strength of the initial inflammatory response and so has an important effect on the acute phase of infection.

Although the extent to which persistent bacteria continue to stimulate signaling through these innate immune receptors is unknown, the early response is clearly important in shaping the subsequent adaptive immune response.

1.2.2 Late Events

How does the mycobacterium survive and grow during the later stages of infection? Infected macrophages in the lung through chemokine production attract monocytes, neutrophils and lymphocytes to the site of infection, however these cells are unable to kill the mycobacteria [69]. In order to contain the spreading of the bacilli, a granuloma is formed: the accumulating mycobacteria stimulate an inflammatory focus, which matures into a granulomatous lesion characterized by a mononuclear cell infiltrate surrounding a core of degenerating epithelioid and multinucleated giant Langhans cells [70]. This tubercle may become enveloped by fibroblasts, and its center often progresses to caseous necrosis. Caseous tissue has low oxygen availability, acidic pH, and toxic fatty acids. However some bacteria can remain dormant under these conditions for years. *In vitro* experiments indicate that mycobacteria switch to lipid catabolism and nitrate respiration to ensure their survival [71,72]. Lipids are abundant in the caeseous detritus of granulomas, providing a rich source of nutrients during persistence. These bacteria can persist without producing disease and therefore create a state of latency. In the resistant host, the tubercle eventually becomes calcified. Nevertheless, the risk of disease outbreak at a later time remains. If an infected person can not control the initial infection or a person's immune system is compromised by HIV infection, age or malnutrition, liquefaction of the caseous material and erosion of the tubercle can serve as a rich

medium for bacterial replication. This can spread to other areas of the lung or spread via the lymphatic or hematogenous system to produce extrapulmonary or miliary disease respectively[70]. Once the alveoli are reached, the patient becomes infectious, and a person with active disease infects up to 15 people annually [73].

1.3 Vaccine Development

The first attempt for a vaccine against tuberculosis began by Robert Koch in 1890, several years after he identified the mycobacterium, however this attempt completely failed. Calmette and Guérin initiated the second attempt 10 years later. BCG (Bacille Calmette–Guérin) was derived from a virulent *M. bovis* isolate by 230 serial passages in a broth containing glycerol, potato-extract and bile salts [74]. During the course of these passages, the *M. bovis* strain progressively lost its virulence for animals and was first shown to be harmless and protective in a child in 1921. Since that time BCG has been used extensively and is still being used today as a live vaccine against tuberculosis with more than 3 billion administrations worldwide [75]. However, this vaccine has not met expectations it evoked. Reported BCG vaccination efficacy rates vary from 0% to 80% [76-78]. Possible explanations for the inconsistency in BCGs protective efficacy are: a) genetic host susceptibility, b) virulence of *M. tuberculosis* strains, c) gradual loss of BCG capacity to stimulate a durable immune response e) predominance of other mycobacterial infections in the population studied, f) discrepancy in protection against different forms of tuberculosis infection and g) degree of exposure to environmental mycobacteria [76,78,79].

Certain issues for new vaccine development require consideration such as the fact that even though BCG prevents disseminated tuberculosis in newborns, it fails to protect

against pulmonary tuberculosis in adults, which is the most common form of the disease. However, there is no proof that the current tuberculosis vaccine's protection lasts for more than 15 years in any population [77,80]. Other issues requiring consideration is the potential that a new vaccine has for clearing the infection since numerous cases of active tuberculosis arise from reactivation of an infection acquired years earlier and reflect the re-emergence of the actively growing organism from an apparently non-replicative state infection [81]. In new vaccine developments, two main strategies can be distinguished: the subunit strategy and the whole viable vaccine strategy. The subunit vaccine consists of a single protective antigen. The deciphering of the *M. tuberculosis* genome has opened the way for candidate identification [82]. Proteins absent in BCG however present in *M. tuberculosis* represent putative protective antigens. The two most studied antigens in DNA-vaccination in experimental animals are antigen 85 and heat-shock protein 60 [83,84]. However there is a need for good adjuvants, delivery systems to enhance their immunogenicity. The second concept is the viable vaccine concept. Neither BCG nor *M. tuberculosis* can generate an immune response that can control, let alone eradicate the mycobacterium in humans[73]. Strategies to improve BCG or attenuate *M. tuberculosis* need to be exploited. This could be done by over-expression of defined protective antigens and by the targeted deletion of antigens that contribute to virulence.

A new vaccine that will be more effective than BCG will require not only a clear understanding of genes and products of the mycobacterium that contribute to pathogenesis, but also a precise comprehension of the immune system and how it develops a protective response. Finally understanding the immune evasion of the mycobacterium is an important key to vaccine development.

1.4 Genetic Factors of Susceptibility to Tuberculosis in Humans

Most humans are resistant to tuberculosis infection presumably due to the generation of a successful immune response that prevents infection from being established after mycobacterial implantation in the lungs. Only 10- 30% of all people exposed to the mycobacterium actually become infected. Of those that do become infected over 90% do not develop clinical disease and are able to resolve the infection completely or to an extent where it is incapable of causing disease. In the latter case, the infection is latent and capable of reactivation disease in the future. From the 10% that are susceptible to disease 50% develop active disease within the year whereas the other 50% reactivates at a later time. However once diseases develops, if untreated more than 50% of individuals will succumb to the infection[11]. Humans therefore present a wide spectrum of susceptibility with the majority having effective innate resistance to tuberculosis. This susceptibility is genetically determined with multiple genes being involved.

1.4.1 Evidence That Genetic Factors Influence Resistance to Tuberculosis

A genetic component of susceptibility to infection in humans has been suggested in population and twin studies [85]. Racial differences in susceptibility to disease have been known for more than 100 years. This was seen very clearly in studies of first contact epidemics in isolated populations with no ancestral experience of this infection: Yanomami, Qu'Appelle Indians [86,87]. Initially, the death rate was 10% and decreased to 0.2% after 40 years. This rapid decrease in deaths caused by tuberculosis are probably due to selective pressure against mycobacteria-susceptibility genes. Moreover it has been

observed that Whites are more resistant to infection than Blacks[88]. These differences are not due to social factors. This is supported by studies of 25 000 nursing home residents in Arkansas and of male inmates of prisons in two states (in an outbreak situation) have found that Blacks were twice as prone as Whites to become infected with *M. tuberculosis* [89] and this difference was not credited to social or environmental factors.

Independent twin studies showing higher concordance rates for tuberculosis among monozygotic twins compared to dizygotic twins provide the most compelling evidence that host genes can affect outcome of infection with *M.tuberculosis* [90-92]. Therefore not only are host genetic factors important for tuberculosis susceptibility between ethnic groups but also within them. The genetic component responsible for susceptibility to tuberculosis is also supported by epidemiological data pointing to sex [93,94] as well as geographical distribution and familial aggregation [95].

1.4.2 Population-Based Studies and Polymorphisms

Molecular genetic methods such as association studies, linkage analysis and genetic analysis in animal models (will be looked at in section 1.5) have been applied to complex and quantitative traits in order to identify genes involved in susceptibility to tuberculosis.

1.4.2.1. Association Studies and Candidate Gene-Based Approach

One gene search method is association analysis. This approach involves the selection of a candidate gene by considering a variety of information such as the

presumed mechanism of disease, onset and pathology, and phenotypes of knockout and transgenic animals. This selection is followed by a search for variations/polymorphisms in the gene. The polymorphism is studied to see if there is a significant difference between unrelated patients (cases) and unrelated healthy individuals (controls), in terms of allele frequencies, allele carrier or genotype frequencies. Association studies can be designed between cases and unrelated controls, or within families. The most commonly used within-family association test uses an affected offspring and both parents to test if a particular allele is transmitted more frequently than would be expected under Mendelian inheritance. This is known as the transmission disequilibrium test (TDT).

This analysis has the highest detection power. However, it remains problematic because it is unlikely that all important susceptibility genes can be found *a priori*, and genes with major effects but unknown function can easily be unnoticed. Moreover, association studies results can often produce conflicting results. Some of the reasons are small numbers of individuals enrolled, variation of pathogens between geographically separated areas, an allele that shows association can be in linkage disequilibrium with another functional polymorphism, allelic heterogeneity and many other reasons. Therefore replication of these studies is necessary. We will now look at some of these genes.

HLA

Highly polymorphic Human leukocyte antigen (HLA) molecules present antigenic peptides to α/β T cells. Many association studies have been reported between HLA class I, class II and tuberculosis. No consistent findings were reported for HLA-I alleles.

Associations have been found consistently however between pulmonary tuberculosis and class II alleles, particularly the *HLA-DR2* subtype in patients from Indonesia and India [96-99]. However due to genetic heterogeneity, some populations such as the Chinese [100], Mexican [101] and Indian [102] populations do not demonstrate the *HLA-DR2* effect. Other case control studies have shown the importance of DQ1 alleles specifically of the *HLA-DQB1*0503* allele in tuberculosis progression in a Cambodian population and *DQB1*0501* in a Mexican population while no significant effect of *HLA-DR2* alleles was detected [103,104]. Another association study by Ravikumar et al. [105] on 126 patients with pulmonary tuberculosis and 87 endemic controls from India indicated that *HLADR2* subtypes *HLA-DRB1*1501* but not *HLA-DRB1*1502* is associated with pulmonary tuberculosis but *HLA-DQB1*0601* in strong linkage disequilibrium with *HLA-DRB1*1051* is also strongly associated. *HLA-DRB1*1051* has also been reported in leprosy and in North American AIDS patients with an accelerated onset of disseminated *Mycobacterium avium* complex diseases [106] suggesting that these three mycobacterial diseases have a common underlying mechanism. No connection has been found between an immunomodulatory gene within the HLA class III, *TNFα* promoter polymorphisms and tuberculosis risk in Cambodia and in Brazil [103,107].

NRAMP1

Natural resistance associated macrophage protein 1 (*Nramp1*), recently designated *Slc11a1* in the genome annotation, was isolated by positional cloning of *Bcg/Ity/Lsh* [108]. Naturally occurring [109] or experimentally induced loss of function mutations cause susceptibility to infection with antigenically unrelated intracellular pathogens such as

Mycobacterium, *Salmonella,* and *Leishmania* [110]. *Nramp1* codes for an integral membrane protein that depletes phagosomes of divalent metal cations essential for microbial survival [111]. The human ortholog *NRAMP1* of mouse *Nramp1* was therefore studied. In a large case-control study conducted in The Gambia, performed on 410 smear-test-positive tuberculosis patients and 417 ethnically matched healthy controls a highly significant association was found between tuberculosis and *NRAMP1* polymorphisms. The polymorphic variants analyzed are a dinucleotide CA repeat in the 5' region, a single nucleotide polymorphism in intron 4 (469 + 14G/C), a nonconservative single-base change at codon 543 (D543N), and a 4-base pair deletion in the 3' region. For all polymorphisms the rare alleles increased the risk for developing tuberculosis. Individuals with tuberculosis had more than four times the odds of having the disease associated *NRAMP1* genotypes compared to healthy controls. [112]. *NRAMP1* polymorphisms have also been confirmed in smaller populations following the Gambian study in patients from Japan[113], Cambodia [114], Guinea-Cornaky [115] , Denmark [116], Korea[117], Texas [118], South Africa [119] and the Gambia [120]. Studies in Taiwan [121] and Morroco [122] failed to detect an association of *NRAMP1* with tuberculosis.

A linkage study performed in a large Aboriginal Canadian pedigree found a major locus of susceptibility to tuberculosis in chromosomal region 2q35 where *NRAMP1* is located [123]. Carriers of the dominant susceptibility allele had a ten times higher than normal risk of contracting tuberculosis. However, linkage studies provide inconsistent evidence for a role of *NRAMP1* to tuberculosis susceptibility. Linkage between tuberculosis and *NRAMP1* was not seen in Brazilian [107], West African and South African populations [124]. *NRAMP1* is involved in mycobacterial susceptibility but its effect is very

weak to be the only gene involved. What remains to be elucidated is the causal relationship between *NRAMP1* and tuberculosis disease.

Recently, Malik *et al.* studied 184 families from the greater Houston area in order to identify the host genetic component of pediatric tuber'culosis susceptibility. They found that the most common alleles of *NRAMP1* polymorphisms were risk factors for pediatric tuberculosis disease. They hypothesize that the *NRAMP1*'s effect is most pronounced in the absence of prior exposure to mycobacteria (primary tuberculosis) and that *NRAMP1* influences the speed of progression from infection to tuberculosis disease[125].

MLB

Mannose binding lectin (MBL) also known as mannose binding protein (MBP) is a serum protein produced by the liver that can opsonize pathogens and activate the complement pathway. Mutations at codon 52, 54 or 57 of this gene produces little or no protein [126] and therefore has impaired opsonisation of some pathogens. A significantly increased genotype frequency of mutant homozygotes was noted in 202 pulmonary tuberculosis patients compared with109 healthy controls in India[127]. However codon 54 heterozygotes are protected from tuberculous meningitis in a South African population[128].

VDR

The active form of vitamin D 1,25 di-hydrocholecalciferol (1,25 $(OH)D_3$) is an immunomodulatory hormone. Its actions include suppression of lymphocyte proliferation,

immunoglobulin production, cytokine synthesis, alteration of dendritic cell maturation and impairment of growth of *M. tuberculosis* in macrophages[129]. Vitamin D exerts its effect through the vitamin D receptor present on macrophages, B and T lymphocytes. VDR receptor polymorphisms were typed in the same Gambian case-control study where NRAMP1 was found to be associated with tuberculosis. In that study, homozygotes for the rarer t allele at a Taq 1 site in codon 352, were reduced in frequency in the tuberculosis cases. The rare genotype is the one producing higher levels of $1,25(OH)D_3$, suggesting a protective effect of vitamin D against tuberculosis[130]. Although not statistically significant, another study of Gujurati Indian tuberculosis patients in West London also found the tt allele and deficiency of 25-hydroxycholecalciferol associated with tuberculosis[131].

$P2X_7$

Adenosine triphosphate ligation of P2X(7) receptors expressed on macrophages that are infected with both virulent and avirulent mycobacteria induces cell death and subsequent loss of intracellular bacterial viability. Among 300 Gambian patients with tuberculosis and 160 ethnically matched control subjects, a protective effect was associated with expression of the C allele at position -762 in the *$P2RX_7$* promoter region, which supports a putative role for this receptor in human immunity to tuberculosis[132].

IL-1b and IL-1Ra

The proinflammatory cytokine *IL-1b* has been shown to play an important role in defense against mycobacterial infections as seen in *IL-1R* knockout mice that are more susceptible to pulmonary tuberculosis, as reflected by an increased mortality and mycobacterial growth and defective granuloma formation as compared to wild type mice[133]. Moreover tuberculosis patients were found to have higher serum concentrations of *IL-1b* and *IL-1* receptor antagonist (*IL-1Ra*, specific inhibitor for *IL-1* activity) [134]. Two biallelic polymorphisms were found at position -511 and +3953 in the *IL-1b* gene whereas an 86-base pair variable number of tandem repeats polymorphism with five different alleles is found in the *IL-1Ra* gene. Investigation of a population of 89 Gujarati Indians living in England and 114 controls found that subjects carrying the *IL1Ra A2* allele produced 1.9 times more antagonists than the rest of the subjects. No difference was observed between the 2 groups with regards to *IL1b* and other *IL1Ra* polymorphisms. However, in patients carrying a certain *IL1b* +3953 allele and not the *IL1Ra A2* allele were overrepresented among patients with tuberculous pleurisy (92%) [135].

1.4.2.2 Genome Wide Scan

Most contributory genes identified to date by association studies account for a small proportion of the genetic variability in susceptibility to tuberculosis. The best known method to identify disease genes is 'linkage analysis'. Although there are several methods for linkage analysis, the nonparametric affected sib-pair method, which is

performed without any assumed mode of inheritance, is often used for investigating multifactorial diseases. This approach entails collection of as many samples from affected siblings as possible, analysis of 300 polymorphic microsatellite markers distributed throughout the human genome, and then data analysis with linkage analysis programs. The method involves a systematic search through the genome looking for the non-random segregation of microsatellite markers from parent to child. If the genetic marker and disease are linked with a disease phenotype, the affected children will have a greater likelihood of sharing markers than would be expected by chance. If this is observed in a sufficient number of families that are affected by disease, the observed segregation is non-random and deviates from what is expected under Mendel's law of independent assortment. A statistical analysis is then used to estimate the likelihood that the observed linkage has occurred by chance.

The advantage of this method is that it can search for locations for susceptibility genes over the entire genome without any knowledge of structure or function. This approach provides the possibility of identifying genes that modulate susceptibility to infectious diseases that had previously not been suspected of playing such a biological role. Since the detection power of this approach is low, even if hundreds of family samples are obtained, theoretically, it could detect only strongly contributing susceptibility variants. Association based candidate gene studies have much greater power and identify effects which would be missed in genome wide linkage studies. However if candidate gene approach is used alone, it can fail to detect genes exerting the largest effects on disease susceptibility.

In a study by Bellamy *et al.* 173 sib-pairs from South Africa and The Gambia were studied in a two-stage genome wide search for tuberculosis susceptibility genes. In

the first stage, 92 sib-pairs and their parents were typed with 299 chromosomal markers covering the entire genome[124]. Seven regions were retained showing limited evidence of cosegregation. Twenty-two markers from these seven regions were typed in a second round screen in 81 sib-pairs and suggestive linkage was found on chromosome 15q11-13 and Xq27. Further support of the susceptibility genes was provided by common ancestry mapping. Interestingly, there is a well documented excess of males with tuberculosis in Africa and an X-chromosomal major gene effect could contribute to this. Fine mapping for the 15q locus suggest that UBE3A or a closely flanking gene may be a tuberculosis-susceptibility locus [136]. Interestingly, the *NRAMP1* and *VDR* gene regions, for which associations were previously reported in the Gambian population [112,130], were not picked up in this study indicating that these genes had no major effect on tuberculosis in this population.

1.5 Mouse Model of Tuberculosis

Genetic control of susceptibility to tuberculosis in humans is known to be complex. Variable pathogenicity determinants of the bacterium, unequal exposure, incomplete penetrance, phenocopies, locus heterogeneity and polygenic effects make it extremely difficult to map single gene effects in human populations, even if they are major. Such complex genetic traits can however be studied in mouse models of disease, where environmental and genetic components can be best controlled, and where single gene effects may have become fixed in inbred, recombinant inbred/congenic and congenic strains of mice. These genes can then be localized by linkage studies and identified by positional cloning. The mouse is the animal model of choice for numerous

reasons: a) they are available as numerous wild type or mutant inbred strains that differ in terms of resistance and survival to infection b) they are available as targeted mutant strains that have functionally been deleted of one or more genes involved in the generation and expression of immunity, c) many more analytical reagents with which immunity can be investigated d) knock-in and knock-out mice can be created by gene targeting in order to analyze genotype/phenotype correlation e) the infectious agent, the dose, the route of infection can all be controlled reducing microbial-induced variability and f) they are cost effective.

1.5.1 Experimental Infection in Mice

Experimental tuberculosis infection in mice can be performed by multiple routes but the most commonly used ones are the intravenous and the aerogenic routes. With a direct injection of the pathogen into the blood stream, the majority of the i.v. inoculum implants in the liver (95%) and spleen (4%) with only 0.1% implanting in the lungs (the lung being the responsible organ for the disease). Therefore an i.v. dose of 10^5 is needed in order to get an implantation of 10^2 in the lungs. The aerogenic infection is carried out either by inhalation or by a nose-only apparatus[137] and this method most closely mimics the human infection reality and the mycobacteria get seeded in the lungs.

The virulence of *M. tuberculosis* depends on many factors and can vary depending on the pathogen strain, the route of infection (aerosol vs. i.v.), the manner of preparation of the inoculum, and the dose. The course and rate of mycobacterial infection are modulated by 1) the virulence of the organism, 2) the organism in which the bacilli are

lodged 3) the size of the inoculum and 4) the intensity of the immune response of the mouse strain.

Following infection, bacterial replication in the lungs, liver, spleen and kidney can be observed. Bacterial growth is sustained for 20 days post-infection in the lung of the mice after which in some mice infection is maintained at a stationary level and in others replication continues unabated.

1.5.2 Mouse pathology

There are multiple strains of *M. tuberculosis* currently used in laboratories. The common laboratory strains are H37Rv, Erdman, and NyH-27. However clinical isolates are also being used in mouse models of infection such as HN878[138] and CDC1551[139]. All the infections described in this work were performed with H37Rv. H37Rv strain of *M. tuberculosis* is similar to the lately isolated C strain that is responsible for a large number of tuberculosis cases throughout the New York metropolitan area [140]. It therefore seems reasonable to assume that H37Rv is still virulent even though it was isolated over 60 years ago.

The pathophysiology of *M. tuberculosis* H37Rv infection in the mouse, even though not identical to human tuberculosis, shares many similarities, which make it a useful model to study human disease. Firstly, in the mouse, as in more than 85% of susceptible humans, tuberculosis is exclusively a disease of the lungs. Secondly, lung pathology develops progressively in both species, in spite of the ability of anti- *M. tuberculosis* defenses to exert a growth inhibitory influence on the mycobacterium. Thirdly, the fact that mice are generally not as sensitive to the disease as other animal

models and can become chronically infected is more like the human situation. Also, in immunocompromised mice, a more progressive and quickly lethal disease is seen, as is seen in HIV immunocompromised patients. Finally, the progression of tuberculosis in mice is unlike that of humans in that the granulomas formed are not as distinct. In mice, the macrophages that accumulate at sites of infection are distributed among intact air sacs but humans form anatomically distinct structures composed of compact aggregates of *M. tuberculosis* -infected macrophages. The centers of granulomas can undergo necrosis and liquefaction, and this can lead to the formation of cavities, but not in mice.

1.5.3 Immune Response to M. Tuberculosis Infection

Knockout mice of many components of the immune and inflammatory response exist. Does knocking out a specific gene affect the outcome of MTB infection or not? (Table I).

1.5.3.1 Cytokines

Cytokines are an important part of the immune response to pathogens and regulate all the cells of the immune system. *M. tuberculosis* is a potent inducer of cytokine production and the subsequent inflammatory response is very important for the control

Table 1. Effect of *M. tuberculosis* infection on various knockout mice

Knockout gene	Effect of MTB infection	Reference
Toll like receptors		
Tlr-2	Conflicting	Sugawara et al. 2003 [64], Reiling et al. 2002[62], Drennan 2004[63]
Tlr-4	Conflicting	Reiling et al. 2002[62], Abel et al. 2002[60], Kamath et al. 2003[61]
Tlr-6	No	Sugawara et al. 2003[64]
MyD88	Yes	Scanga et al. 2004[141]
Cytokines		
IL-1αβ	Yes	Yamada et al. 2000[142]
IL-4	conflicting	Saunders et al. 2000[143], North et al. 1998[144]
IL-6	No	Saunders et al.2000[143]
IL-10	confliting	Roach et al. 2001[145], North et al. 1998[144], Murray et al. 1999[145]
IL-12	Yes	Kawakami et al. 2004[147]
IL-12α	No	Cooper et al. 2002[148]
IL-12β	Yes	Cooper et al. 2002[148]
IL-18	Yes	Sugawara et al. 1999[149]
IL-4/13	No	Jung et al. 2002[150]
IL-1R1	Yes	Sugawara et al. 2001[151]
IL-4 Rα	No	Jung et al. 2002[150]
Irf-1	Yes	Yamada et al. 2002[152]
IFN-γ	Yes	Flynn et al. 1993[153], Cooper et al. 1993[154]
Lymphotoxin α	Yes	Roach et al.2001[155]
Lymphotoxin β	Conflicting	Ehlers et al. 2003[156], Roach et al. 2001[155]
Lymphotoxin β R	Yes	Ehlers et al. 2003[156]
TNF-α	Yes	Bean et al. 1999[157]
TNF-α R	Yes	Flynn et al. 1995[158]
Immunology		
NK T cells	No	Sugawara et al. 2002[159]
αβ T cells	Yes	Mogues et al. 2001[160]
B cell	No	Turner et al. 2001[161]
γδ T cell	No	D'Souza et al. 1997[162]
CD1d	No	Sousa et al. 2000[163], Behar et al. 1999[164]
CD4	Yes	Caruso et al. 1999[165]
CD 8	Controversial	D'Souza et al. 2000[166], Sousa et al. 2000[163]
CD 8α	Yes	Sousa et al. 2000[163]
CD14	No	Reiling et al.2002[62]
CD40	Yes	Lazarevic et al. 2003[167]
CD40L	No	Lazarevic et al. 2003[167], Campos-Neto et al.1998[168]
CD44	No	Kipnis et al.2003[169]
CD95/CD95L	Yes	Turner et al. 2001[161]
MHC Class Iα	Yes	Urdahl et al. 2003[170]
MHC Class Iβ	No	Urdhal et al. 2003[170]
MHC Class II	Yes	Caruso et al. 1999[171]
MHC Class II transactivator	Yes	Repique et al. 2003[170]
β2-microglobulin	Yes	Flynn et al. 1992[172]
TAP-1	Yes	Sousa et al.2000[163]
Perforin	Controversial	Sousa et al. 2000[163], Cooper et al. 1997[173]
Chemokines		
CCR2	Yes	Peters et al. 2001[174]
CCL2	No	Kipnis et al. 2003[175]
Others		
Mrp-1	No	Verbon et al. 2002 [176]
C5 (Natural deficiency)	Yes	Actor et al 2001[177]
Dopamine β hydroxylase	Yes	Alaniz et al.1999[178]
Granzyme	No	Cooper et al.1997[173]
ICAM-1	No	Johnson et al. 1998
NF IL-6	Yes	Sugawara et al. 2001[180]
NF KB P50	Yes	Yamada et al. 2001[181]
NOS 2	Yes	Jung et al. 2002[182]
Phox	No	Cooper et al. 2000[37]
Stat 1	Yes	Sugawara et al. 2004[183]
Stat 4	Yes	Sugawara et al. 2003[184]
Stat 6	No	Sugawara et al. 2003[184]

and ultimate outcome of infection. The most important cytokines are reviewed in this section.

1.5.3.1.1 Pro-Inflammatory Cytokines

IL-12

IL-12 is a key player in host defense against *M. tuberculosis*. Macrophages and dendritic cells produce IL-12 in response to *M. tuberculosis* infection [185,186]. IL-12 drives the Th1 response by producing IFN-γ. Exogenous administration of IL-12 to *M. tuberculosis*-infected BALB/c mice resulted in significantly decreased mycobacterial counts and increased overall survival to infection [187], however only marginal effects where seen with C57BL/6 mice[188]. The most compelling evidence of the importance of IL-12 in resistance to tuberculosis was provided by IL-12p40 knockout mice[189]. These mice proved more susceptible to infection showing greatly increased bacterial replication and decreased survival to infection, as compared to control mice. This can probably be explained by the significantly reduced IFN-γ production in IL-12p40 knockout mice. IL-12 is a regulatory cytokine which connects the innate and adaptive host response to mycobacteria [190,191] and which exerts its protective effects mainly through the induction of IFN-γ [189].

IFN-γ

IFN-γ is produced by $CD4^+$, $CD8^+$, CD1-restricted T cells as well as NK cells after tuberculosis infection [192-194]. Lung macrophages were also found to produce IFN-γ in *M. tuberculosis*-infected mice [195]. IFN-γ's key role in immunity is seen in that IFN-γ knockout mice are most susceptible to *M. tuberculosis* infection more so than any other mouse strain [153,154]. Inadequate macrophage activation and low Nos2 expression in these mice [153,196] are possible factors that contribute to the exceptional susceptibility of these mice and the uncontrollable bacterial replication that is observed in their organs.

Mutations in five autosomal genes in the IL-12/IFN-γ pathway have been found to cause recurrent disseminated mycobacterial infections in humans. These defects are found in IFN-γ R1, IFN-γ R2, STAT1, IL-12p40 and IL-12Rβ1[95]. All the mutations result in impaired IFN-γ immunity with the IFN-γR1 mutation being the most severe.

TNF-α

TNF-α is a prototype proinflammatory cytokine for control *M. tuberculosis* infection with many complex immune and pathologic roles. *M. tuberculosis* induces TNF-α secretion by T cells, macrophages and dendritic cells [185,186,194,197]. *M. tuberculosis* infection in TNF-α or the 55-kDa TNF receptor knockout mice results in rapid death and increased bacterial loads when compared to control mice [157,158]. This increased susceptibility to infection may be due to the role of TNF-α as a mediator of macrophage activation. Moreover in TNF-α or the 55-kDa TNF receptor KO mice, the granulomatous

response is impaired [157,158] which is to be expected since TNF-α perturbs cell migration and localization within tissues and affects expression of adhesion molecules, chemokines and chemokine receptors.

IL-6

The roles of IL-6 in immune response include inflammation, hematopoiesis, and T cell differentiation. After aerosol infection of IL-6-/- mice with a low dose of mycobacteria, early increases in lung bacterial replication, in addition to decreased IFN-γ production were observed as compared to control mice. This suggests that IL-6 is important in the initial innate response to the pathogen. However once acquired immunity developed, the IL-6-/- mice controlled the infection and the mice survived. [143].

IL-18

In addition to IL-12, IL-18 is important in the IFN-γ axis. IL-18 was initially discovered as an IFN-γ-inducing factor, synergistic with IL-12 [191] but also stimulates the production of other cytokines, chemokines, and transcription factors [198]. The protective role of IL-18 during mycobacterial infections is seen in IL-18 deficient mice that are highly susceptible to BCG and *M. tuberculosis* [149]. IL-18's major effect in this model seems to be the induction of IFN-γ.

1.5.3.1.2 Anti-Inflammatory Cytokines

The proinflammatory response initiated by *M. tuberculosis* is antagonized by anti-inflammatory mechanisms. For example IL-1ß is counteracted by IL-1R antagonist (a naturally occurring antagonist of IL-1). Furthermore, proinflammatory cytokine production and their effects can be inhibited by anti-inflammatory cytokines such as IL-4 and IL-10 in tuberculosis.

IL-4

The destructive effects of IL-4 have been ascribed to this cytokine's suppression of IFN-γ production and macrophage activation [199,200]. A shift to a Th2 response was not observed in IFN-γ or IL-12p40-/- mice infected with *M. tuberculosis* suggesting that the absence of a Th1 response to *M. tuberculosis* does not necessarily promote a Th2 response. IL-4-deficiency in mice is controversial. IL-4 -/- mice displayed normal instead of increased susceptibility to mycobacteria in two studies, suggesting that IL-4 may be a consequence rather than the cause of tuberculosis infection[18,144]. In contrast, another study on IL-4 -/- mice showed increased granuloma size and mycobacterial growth after aerosol infection[201]. Thus, the role of IL-4 in tuberculosis susceptibility is not yet entirely resolved although these differences can be due to the different genetic backgrounds of the knockout mice (C57BL/6 vs Balb/c).

IL-10

This cytokine is produced by macrophages and T cells during *M. tuberculosis* infection [194]. IL-10 antagonizes the proinflammatory cytokine response by downregulation of TNF-α and of IL-12 production which in turn decreases IFN- γ production by T cells. Also, IL-10 directly inhibits $CD4^+$ T cell responses, as well as inhibiting APC function of cells infected with mycobacteria [202]. IL-10 therefore interferes with host defense against *M. tuberculosis*. In fact, IL-10 transgenic mice with mycobacterial infection develop a larger bacterial burden [203]. IL-10-/- mice were not more resistant to acute *M. tuberculosis*, compared to wild-type mice in one study [144] while another report showed a lower bacterial burden early after infection [146]. The role for IL-10 in tuberculosis therefore awaits further studies.

1.5.3.2 T Cells

Protective response to *M. tuberculosis* infection requires cell-mediated immunity since it is an intracellular pathogen, residing ordinarily within macrophages, and therefore T cell effector mechanisms rather than antibody are required to control infection. $CD4^+$ and $CD8^+$ T cells are fundamental for effective control against *M. tuberculosis* infection. Enumeration of the cells at the site of infection in mice show double the amount of $CD4^+$ than that of $CD8^+$ cells [204]. Moreover, the effect of a complete loss of $CD4^+$ is a more noticeable effect than that of $CD8^+$ T cell elimination on infection control. Mycobacteria-specific $CD4^+$ T cells are more important than Th2 cells for tuberculosis control as seen by the fact that infection progresses rapidly in IFN-γ deficient mice as compared to Th2

cytokine depleted mice, such as interleukin IL-4 [143,144]. The predominant $CD4^+$ and $CD8^+$ T cells, that express α/β antigen receptor, are however not the only cell types involved in tuberculosis infection in mice [205]. γ/δ T cells have also been found to have both a protective and anti-inflammatory role in *M. tuberculosis* infection [162,206].

$CD4^+$ T cells

M. tuberculosis rests primarily within a macrophage vacuole and consequently MHC class II presentation of mycobacterial antigens to $CD4^+$ T cells is an expected outcome of infection. Antibody depletion of $CD4^+$ T cells [207], adoptive transfer [208],or the use of knockouts [165] in mice have shown that the $CD4^+$ T cell subset is required for control of infection. The main function of $CD4^+$ T cells is to produce IFN-γ in order to activate macrophages to eliminate intracellular pathogens. In fact, IFN-γ levels are severely diminished very early in infection in MHC class II-/- or CD4-/- mice[208]. Aside from their crucial role of IFN-γ production, $CD4^+$ T cells might exert other roles [209] such as apoptosis conditioning of antigen presenting cells, help for B cells and CD8 cells and production of other cytokines.

$CD8^+$ T cells

It has been shown that mycobacterial antigens derived from infected cells can be presented by MHC class I to $CD8^+$ T cells in humans and mice. Studies in knockout mice looking at importance of $CD8^+$ T cells are conflicting [160,163,164,172,210,211]. $CD8^+$ T cells

recognize MHC Class I molecules and these are frequently derived from the cytoplasm of the cells. Despite the residence of *M. tuberculosis* within phagosomes, it is capable of stimulating MHC-class-I-restricted $CD8^+$ T cells, and compelling evidence shows that these T cells participate in protection [212]. Studies have suggested that *M. tuberculosis* remains inside the phagosome and perforates the phagosomal membrane to gain access to cytosolic nutrients[212]. This mechanism could also permit mycobacterial antigens to access the MHC class I pathway. Other alternative methods have also been suggested [213]. Mice that are deficient in classical MHC class I, transporter of antigen processing or CD8, are more resistant to *M. tuberculosis* infection than β_2-microglobulin (β_2m) knockout mice[163,164,172,211]. β_2m has multiple roles: 1) it stabilizes MHC class I surface expression, and hence its deficiency results in the lack of mature $CD8^+$ T cells 2) supports the surface expression of non-classical MHC class Ib (CD1) and classical polymorphic Class Ia (H2-K,D) T cells and 3) is involved in iron metabolism [214]. The increased susceptibility of β_2m-deficient mice to tuberculosis is therefore caused by a number of factors, and is only partially due to the absence of $CD8^+$ T cells.

$CD8^+$ T cells, like $CD4^+$ T cells, can produce IFN-γ and TNF-α, but their key role is target cell killing [212]. Lysis of targeted cells by $CD8^+$ T cells can occur via perforin and granzymes or the Fas/FasL pathway. Lysis can result in the apoptotic pathway or can release the bacteria from the infected cell or could facilitate the translocation of *M. tuberculosis* from incapacitated cells to more proficient effector cells.

When Class I and Class II knockout mice were compared for their ability to defend against tuberculosis infection, Class II dependent immunity was shown to be more important. In Class II deficient mice infection was progressive and quicker as compared

to controls whereas Class I knockouts had a stationary level of infection with only 1 log higher CFUs than control mice [160]. Thus studies over the past few years of anti-*M. tuberculosis* immunity in mice show that immunity is mediated predominantly by $CD4^+$ Th1 cells with the aid of $CD8^+$ T cells. The protective role of $CD4^+$ and $CD8^+$ T cells lies on their ability to synthesize and secrete IFN-γ, the key Th1 cytokine, and subsequently activate the mycobacteriostatic function of macrophages at the infection site. Anti-*M. tuberculosis* immunity is considered to be a Th1 mediated, as opposed to Th2 mediated, because of the fact [153,154] that knockout mice incapable of making IFN-γ are unable to inhibit mycobacterial growth in their organs. Moreover IL-12 knockout mice are incapable of expressing immunity against tuberculosis [189]. IL-12 secretion by antigen-presenting cells is essential for the induction and generation of Th1 immune responses.

1.5.4 Strain Variation in Susceptibility to Tuberculosis

Differences in susceptibility to tuberculosis have been noted over the past 50 years in a variety of inbred and outbred strains of mice using different mycobacterial isolates, different infection routes and doses and different evaluations of susceptibility [215-218]. Using survival time after i.v. injection with 1×10^5 *M. tuberculosis* H37Rv, inbred mouse strains are classified [219] into either highly susceptible (CBA, C3H, DBA/2, 129SvJ) with a mean survival time of 100 days or highly resistant (C57BL/6, BALB/c) with a mean survival time of 250 days. Similar results were obtained with mice infected with 10^2 MTB via the airborne route [219]. In an independent study using 10^6 *M. tuberculosis* Erdman strain also i.v. the results were consistent with those of North and colleagues with C3H, DBA/2, and CBA as susceptible and C57BL/6, C57BL/10 and BALB/c as resistant as

measured by survival times. A/SnYCit (A/Sn) and I/StSnEgYCit (I/St) were classified as polar extremes with regards to survival time mycobacterial lung load, histology and weight loss after intravenous infection with *M. tuberculosis* H37Rv with I/St as susceptible and A/Sn as resistant [220-222]. However A/J mice seem to have conflicting results: in one study A/J was shown as resistant whereas in another study it is shown as susceptible with infection of Erdman through the intravenous or aerosol route with regards to bacterial replication [223,224]. A study by Musa et al.[215] showed no difference in the number of tubercule bacilli in the lungs, spleens of BCG resistant and BCG susceptible mice after aerosol infection with 5 to 10 *M. tuberculosis* bacilli via the aerosol route. These results differ from those of Brown et al.[225] where the Bcg locus is shown to control mycobacterial replication using a different dose (10^3) and different route of infection (i.v.).

In mice, speaking of susceptibility and resistance is a relative term because eventually all strains succumb to infection-induced disease, with the difference that susceptible strains succumb earlier. Moreover, demonstrating a difference between resistant and susceptible mouse strains is dose dependent: for example an i.v. dose of 10^5 revealed a considerable difference between susceptible and resistant mice a dose of 10^7 reveals no difference at all. Overdosed resistant mice die of a lung disease that is different from that induced by a smaller dose i.e. they die before chronic lung pathology develops.

F1 hybrids between resistant and susceptible strains were highly resistant, as were hybrids between resistant strains showing that resistance is dominant and that complementation of parental susceptibility loci occurs in the hybrid. Male mice are more susceptible to bacterial load than female mice[226].

1.5.5 Differential Response of DBA/2 and C57Bl/6 Mice to H37Rv Infection

As discussed in section 1.5.4, C57BL/6 mice are more resistant to infection than DBA/2 mice after aerosol infection with 10^2 *M. tuberculosis* H37Rv with mean survival times of approximately 250 days as compared to 100 for DBA/2. Moreover, resistant C57BL/6 animals exhibit a 20-day period of log linear bacterial growth in the lungs that results in 6.5 $\log_{10}$ CFUs. This linear phase is followed by a stationary phase that persists from day 20 to the time of death (approximately 250 days post-infection) where there is inhibition of further mycobacterial growth. In the DBA/2 mice, lung infection progresses at the same rate as C57BL/6 for the first 20 days. However after day 20 of infection growth in the lungs of susceptible mice is not inhibited and progresses at a lower rate till death. At day 100 post-infection, susceptible mice have a 2 log higher level of bacterial load in the lungs than resistant mice do. All this holds true for infections initiated through the i.v. route as well. Lung pathology between resistant and susceptible mice is also different in day 100 post- infection. The resistant mice have smaller, fewer, more compact lesions in their lungs than susceptible mice do.

Infection through the aerosol route does not remain confined in the lungs but disseminates via the lymph nodes to infect the liver, spleen and kidney. Infection of these organs is evident at day 15 days-post infection and progresses till day 20 after which growth ceases and stationary infection begins in both mouse strains. Bacterial levels reached in these organs are much lower than in the lungs since infection is only apparent 15 days. Stationary infection in these organs does not cause progressive pathology. Sites of infection appear as small macrophage dominated granulomas that appear stable in number and size throughout the course of infection [140,227].Macroscopically and

microscopically the lungs are the only organs that show signs of progressive disease during the course of infection. Similar results are obtained in i.v. infection.

Comparison of cytokine profiles of C57BL/6 and DBA/2 mice [228] show that mice exhibited different induction kinetics for ICAM-1, MCP-3, MIP-2, RANTES, IFN-γ and iNOS. Higher RANTES, ICAM-1, and IFN-γ mRNA levels are seen in C57BL/6 mice. ICAM-1 which facilitates the ability of T lymphocytes to enter the lung, RANTES that is associated with a Th1-related immune response in mice and IFN-γ can explain the better control of bacillary concentrations and pulmonary infiltration observed in C57BL/6 mice. On the other hand, DBA/2 mice have higher levels, in their lungs, of MIP-2 and MCP-3 than C57BL/6 mice do. Since MIP-2 is a potent neutrophil-recruiting chemokine and MCP-3 exerts selective effects on circulating monocytes this can result in a stronger lung recruitment of macrophages and neutrophils in the lungs of DBA/2 mice as seen in previous studies[229].

The marked inter-strain differences in susceptibility to *M. tuberculosis* H37Rv infection in DBA/2 and C57BL/6 mice suggested that a genetic approach could identify key host defence mechanisms against this infection.

1.6 Genetic Dissection of Complex Traits Using Experimental Crosses

A complex trait is a form of genetic inheritance in which a direct and complete relationship does not exist between a gene and its phenotypic consequences. This is partly because complex trait phenotypes are more multifaceted than those of simple traits are. They represent the aggregate effects of many cellular, molecular and environmental

processes on diseases that are quantitative in nature such as disease susceptibility. QTL mapping is a genome-wide inference of the relationship between genotype at various genomic locations and phenotype for a set of quantitative traits in terms of the number, genomic positions, effects and interaction of QTLs. The primary purpose of QTL mapping is to localize chromosomal regions that significantly affect the variation of quantitative traits in a population. This localization is important for the ultimate identification of responsible genes and also for our understanding of genetic mechanisms of the variation.

1.6.1 Tools For Gene Mapping in Mice

In animal models, the availability of laboratory inbred mouse strains allows researchers to control the genetic contribution to a complex trait empirically. Although there is no allelic variation within an inbred strain, controlled matings amongst strains and their progeny create segregating populations in which trait loci can be mapped. Typically backcross (N_2 generation), intercross (F_2 generations) or recombinant inbred/recombinant congenic strains are used. In this thesis work, F2 mice were used where parental mouse strains [in this case C57BL/6 (B) and DBA/2 (D)] are first intercrossed to produce an F1 population. F2 mice are obtained by breeding the F1 mice to one another and there are three genotypes possible at each locus (BB, BD or DD). The F2 animals are the mapping tool of choice when the trait you are studying is possibly recessive or unknown, as compared to backcross animals that are used when the trait of interest is expected to be directed by a major dominant locus.

1.6.2 Gene Mapping by Whole Genome Scan Approach and Genetic Linkage Analyses

By the methods described in the previous section, a number of genetically and phenotypically diverse mice have been created. The phenotype of each individual must be determined as well as the genotypes. In order to determine the genotype, i.e. whether the progeny inherited a B or D allele, DNA is isolated from each individual mouse. Markers that are polymorphic between the two parental strains are chosen to cover the whole genome at 10cM spacing. Proper spacing of the markers is important to insure sufficient linkage detection power. The centi-Morgan, the linkage distance between two markers on the same chromosome, is the mean number (*M*) of nonsister exchanges in the interval between the markers per haploid meiotic product. A meiotic recombination frequency of 1% is equal to 1cM and equivalent to about 2 million base pairs in physical distance. A known genetic map can be used to align markers along the mouse chromosomes, or can be estimated in such a way to minimize the number of recombinations. Knowing the order as well as the distance between markers is necessary. The next step is linkage analysis, which permits us to identify genomic regions for which there is a correlation of the genotype with the phenotype. Once the data are collected on each individual, linkage analysis between the markers and quantitative trait are established through statistical approaches that range from simple techniques, such as analysis of variance (ANOVA), to models that include multiple markers and interactions.

1.6.2.1 Analysis of Variance

The simplest test to carry out QTL linkage analysis is a single-marker test called analysis of variance at marker loci. In this method, for each marker, the mice are segregated according to their genotypes and the phenotypes are subsequently compared (using means and variances). If a given marker is not linked to a phenotype, the genotype groups will have approximately the same distribution whereas if the marker is linked to the phenotype, the genotype groups will have significantly different distributions. One important issue that should be considered when using this method is sample size. The number of individuals studied provides information for the estimation of phenotypic means and variances. A large sample of individuals provides the opportunity to estimate parameters with greater accuracy and, therefore, a greater ability to detect QTL through a single-marker test. However, this method fails to provide an estimate of QTL location or recombination frequency between the marker and the QTL. This is because the QTL effect and the location are confounded, or are unable to be estimated separately. Confounding, in these situations, is addressed by (incrementally) fixing the location of the QTL and estimating the QTL effect between intervals of markers. These intervals of markers lead to a method that estimates both QTL effect and the location, known as interval mapping [230] and is the most widely used today.

1.6.2.2 Interval Mapping

Interval mapping uses an estimated genetic map as the framework for the location of QTL. The intervals that are defined by ordered pairs of markers are searched in

increments, and statistical methods are used to test whether a QTL is likely to be present at the location within the interval or not. Interval mapping statistically tests for a single QTL at each increment across the ordered markers in the genome. The algorithm first calculates the probability that an individual has a certain genotype at every chosen position of the interval. Possible genotypes at the putative gene position depends on the genotypes and the distances of the flanking markers. Assuming a putative QTL gene at several estimated positions and given the probable genotypes, the LOD score is calculated. The results of the tests are expressed as LOD (logarithm of the odds) scores, which compare the evaluation of the likelihood function under the null hypothesis (no QTL) with the alternative hypothesis (QTL at the testing position) for the purpose of locating probable QTL. The null distribution of maximum LOD score, influences on the threshold of significance of linkage and varies with the type of cross, the size of the genome, number, spacing of markers used and the pattern of missing genotypes and the phenotype distribution[231]. LOD score of 3 and over (1000:1) is generally taken to indicate linkage (compared to the 50:1 probability that any random pair of loci will be unlinked). The probability of obtaining a LOD score under the null hypothesis that would be as large or larger than the observed one for a given interval is called *P* value. Large LOD scores give small *P* values and provide support for the rejection of the null hypothesis.

The effect of a QTL is also measured as the proportion of the total phenotypic variance that is attributable to the QTL without factoring in environmental variation, measurement error, and other QTLs controlling the same trait.

DBA/2 suscpetibility is associated with unrestricted replication of *M. tuberculosis* in the lungs, severe lung pathology and early death. Our goal in chapter 1 is to identify the genes that cause this unique susceptibility in DBA/2 mice.

Chapter 2

Genetic control of susceptibility to infection with *Mycobacterium tuberculosis* in mice

ABSTRACT

Genetic factors play a key role in host response, disease severity, and ultimate outcome of infection with *Mycobacterium tuberculosis* in humans. In the mouse, the DBA/2J strain is very susceptible to *M. tuberculosis* H37Rv infection, while the C57Bl/6J strain is resistant. In DBA/2J, a heavier bacterial burden causes a unique phenotype, that includes very severe and rapidly fatal pulmonary disease with extensive exudation of neutrophils and tissue necrosis, as opposed to slower progressive pulmonary disease characterized by the accumulation of epithelioid macrophages with protective immune and inflammatory responses in C57Bl/6J. To identify the genes responsible for differences in host response to *M. tuberculosis* in these two strains, ninety five animals of an informative (C57Bl/6J X DBA/2J) F2 cross were infected intravenously with *M. tuberculosis* (1×10^5 CFU) and duration of survival was used as a quantitative phenotypic measure of susceptibility in a whole genome scan. Quantitative trait locus analysis (QTL) showed that the genetically controlled susceptibility was multigenic. QTL analysis identified two significant linkages on the distal portion of chromosome 1 (*Trl-1*, LOD, 4.80) and on the proximal portion of chromosome 7 (*Trl-3*, LOD, 4.66) that each account for approximately 21% of the phenotypic variance. A third suggestive linkage was identified on the proximal portion of chromosome 3 (*Trl-2*, LOD, 3.93; additional 18% of the variance). At each locus, homozygosity for the parental C57Bl/6J alleles was associated with increased resistance to infection. These novel mouse loci provide the basis for evaluating a possible association of the corresponding syntenic chromosomal regions in humans with susceptibility to tuberculosis.

INTRODUCTION

It is estimated that one third of the world population is infected with *Mycobacterium tuberculosis*. Infection with *M. tuberculosis* causes a wide spectrum of pathologies, from asymptomatic infection, to progressive pulmonary or extrapulmonary tuberculosis, including disease caused by reactivation. Tuberculosis is the leading cause of death due to infectious disease worldwide, with an estimated 8 million new cases and 3 million deaths each year [232,233]. Recently, the problem of tuberculosis has been exacerbated by the emergence of isoniazid, rifampin and ethambutol resistance in *M. tuberculosis* [234]. The host defense mechanisms against this infection and the mechanisms underlying long-term persistence and replication of mycobacteria in mononuclear phagocytes remain unclear and need to be better understood. Such host defense mechanisms can manifest themselves as genetic determinants of innate resistance or susceptibility in human populations from endemic areas of disease and during epidemics, as well as by strain variation in animal models of such infections. A genetic component of susceptibility to tuberculosis in humans has been suggested by population studies and studies in twins [85]. Indeed, studies of residents of nursing homes in Arkansas and of male inmates of prisons from two states (in an outbreak situation) show that blacks have a two fold increase in relative risk of becoming infected with *M. tuberculosis*, compared to whites, and as measured by conversion to a positive tuberculin test [89]. Furthermore, a recent study of first contact epidemics in Yanomami indians of Brazil, identified both a unique high prevalence of active disease and high prevalence of tuberculin skin test anergy when compared to controls of different ethnic origin from the same region [87]. Finally, results from independent studies in twins showing higher concordance rates of tuberculosis in monozygous twins vs dizygous twins [90,91] provide the most compelling evidence that host genes can affect outcome of infection with *M. tuberculosis*. The genetic analysis of a

complex trait such as susceptibility to tuberculosis is extremely difficult in humans due to variable pathogenicity determinants of the bacterium, unequal exposure, incomplete penetrance, phenocopies, locus heterogeneity, and polygenic effects [85]. Case control studies in endemic areas of tuberculosis using candidate genes have revealed association of susceptibility to disease with alleles of genes encoding the macrophage natural resistance protein *NRAMP1* [112], the vitamin D receptor [130] and HLA-DQ [103] in specific populations. However, the overall effect of these genes is acknowledged to be small, and other genes are certain to play a role in susceptibility to human tuberculosis.

Complex traits may be studied in genetically well defined inbred, recombinant or congenic strains of mice where single gene effects have either naturally segregated or have been experimentally isolated by breeding. Results from these studies can in turn provide candidate genes that can then be tested in humans. Differences in susceptibility to tuberculosis have been noted over the past fifty years in a variety of inbred and outbred mouse strains, using different *M. tuberculosis* isolates, different infection routes and doses, and different evaluations of susceptibility [215-218,235]. When studied, genetic control of susceptibility appeared multigenic [216], including a recent study that suggested 6 chromosomal regions contributing to the reported difference between the A/Sn and I/St strains in *M. tuberculosis* infection-induced body weight loss [236]. Although a role for H-2 genes in genetic susceptibility (H-2k protective over H-2b) has been identified [219,237], additional genes have yet to be identified.

Using survival time after infection with 1×10^5 *M. tuberculosis* i.v. as an expression of susceptibility / resistance, a recent study classified inbred mouse strains into either highly susceptible (CBA, C3H, DBA/2J, 129SvJ) or highly resistant (C57Bl/6J, BALB/c) to tuberculosis with median survival time varying between 67-114 days and 239-251 days for

susceptible and resistant strains, respectively [219]. Similar results were obtained by aerosol infection (1 X 10^2 CFU), with DBA/2J being very sensitive (MST, 110 days) and C57Bl/6J and BALB/c being very resistant (MST, 245 and 315 days, respectively) [219]. Susceptibility in DBA/2J mice is associated with severe lung pathology concomitant to rapid and unrestricted replication of *M. tuberculosis* in this organ, resulting in 100 fold more CFUs at 100 days after infection, when compared to resistant BALB/c. The enhanced bacillar replication in DBA/2J is specific to the lungs, as both DBA/2J and BALB/c can stabilize infection in liver and spleen to the same extent [238]. Histopathology analysis indicates a greater number of larger acid-fast bacilli-containing lesions and severe tissue necrosis in DBA/2J mice when compared to resistant animals [227,238]. In the present study, we have used a whole genome scan and a quantitative trait locus (QTL) analysis in an informative F2 population derived from susceptible DBA/2J and resistant C57Bl/6J to localize the genes that control the unique susceptibility of DBA/2J animals to *M. tuberculosis* replication in the lungs.

MATERIALS AND METHODS

Animals

Inbred, pathogen free, 8-week-old C57Bl/6J and DBA/2J mice were purchased from Trudeau Animal Breeding Facility. Ninety-five (C57Bl/6J X DBA/2J)F2 progeny were bred by systematic brother sister mating of (C57Bl/6J X DBA/2J) F1. All mice were housed under standard laboratory conditions and were free of common viral pathogens according to the results of routine testing performed by the Research Animal Diagnostic and Investigative Laboratory, University of Missouri, (Columbia, MO). Mice between 8 and 10 weeks of age were used for infection.

Mycobacteria

M. tuberculosis H37Rv (TMC #102) was obtained from the Trudeau Institute Mycobacterial culture (TMC) collection. It was supplied as a frozen stock (- 70° C) log phase dispersed culture in Proskauer and Beck medium (Difco Laboratories inc., Detroit, MI) containing 0.01% Tween 80. For each experiment, a vial was thawed, subjected to 5 seconds of ultrasound to break up aggregates, and diluted appropriately in PBS containing 0.01% Tween 80. Mice were inoculated with ~10^5 CFU of bacilli in 0.2 ml of PBS via a lateral tail vein. Infected animals were then housed in a BL3 biohazard facility and monitored daily for the presence of severely moribund animals, which were sacrificed. Survival time were recorded and used for statistical analysis.

Genotyping

Prior to infection, tail biopsies were obtained from all animals. Genomic DNA was prepared from tail tips of individual F2 mice by incubation (12-16 h, 55 ^{o}C) in 700 µl of a buffer (100 mM Tris-HCl, pH8.0.5 mM EDTA, 200 mM NaCl, 0.2% SDS) containing 0.5 mg/ml Proteinase K, followed by RNase treatment (0.3 mg/ml; 2 h at 37^{o}C). DNA was purified by serial phenol-chloroform extractions and ethanol precipitation. DNA was dissolved in 10mM TRIS (pH8.0), 1 mM EDTA and stored frozen. Microsatellite markers (total of 163, with approximate 10cM spacing in the genome) were selected from the Mouse Genome Database (www-genome.wi.mit.edu), because they revealed polymorphisms in DBA/2J and C57Bl/6J strains. Oligonucleotide primer pairs defining these simple sequence length polymorphisms (SSLPs) were purchased from Research Genetics (Huntsville, AL). For PCR amplification of SSLPs from genomic DNA, a 50 ng aliquot of genomic DNA dissolved in PCR buffer (50mM KCl, 10mM Tris-HCl, pH 9.0, 1.5mM $MgCl_2$) was used in a 12 µl volume reaction, after addition of sequence-specific oligonucleotide primers (100 nmol), dNTPs (200 µM each), and 1 unit of *Thermophylus aquaticus* (Taq) DNA polymerase (BIOCAN, Montreal). The PCR reaction included trace amount (120 nCi) of [^{32}P]α-dATP (specific activity 3000 Ci/mmole, Amersham) to label the amplification products. The thermocycling program was one initial denaturation at 94°C for 2 min, 30 three-step cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by a final cycle at 72°C for 7 min. [^{32}P]-labeled PCR products were diluted two-fold in 100 % formamide, denatured 3 min at 90°C and electrophorezed in denaturing 8 % polyacrylamide gels containing 8 M urea and TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8). Gels were autoradiographed using Kodak BioMax films for a period of 12-16 h.

Statistical Analysis

The genetic markers were assigned to and mapped within the chromosomes by multipoint linkage analysis, using the Mapmaker/EXP [239]. The order of markers onto chromosomes was identical to those in published maps [240]. As can be seen in Fig. 1, the distribution of survival time was slightly skewed in the F2 data (skewness = 0.789). Simple $\log_{10}$ transformation of the data removed much of the skewness, resulting in a close approximation to a normal distribution (skewness = 0.160; Shapiro-Wilk deviation from normality W = 0.974, $p > .25$). Genome-wide interval mapping analysis between the transformed quantitative trait phenotype and genetic markers for the identification of QTLs was performed using Mapmaker/EXP version 3.0 and Mapmaker/QTL 1.1 [239]. LOD scores were calculated as $\chi^2/2\ln(10)$, with χ^2 values computed using the expectation/maximization algorithm in Mapmaker/QTL. Upon initial identification of linked regions, multi-locus models were fitted using Mapmaker/QTL to obtain estimates of the total phenotypic variance explained by the different chromosome regions. To evaluate possible epistasis, the most informative markers were chosen from each linked region and each pair of markers was examined using two-way factorial analysis of variance in SAS [241]. Combined epistatic effects were assessed for significance in these ANOVA models on the basis of the interaction F-ratios calculated by SAS.

Histology

Major organs were removed at the time of sacrifice and were fixed in 10% phosphate-buffered formalin, dehydrated in graded ethanol baths and embedded in paraffin by standard procedures, or in glycol methacrylate (JB-4 embedding kit; Polysciences Inc., Warrington, PA)

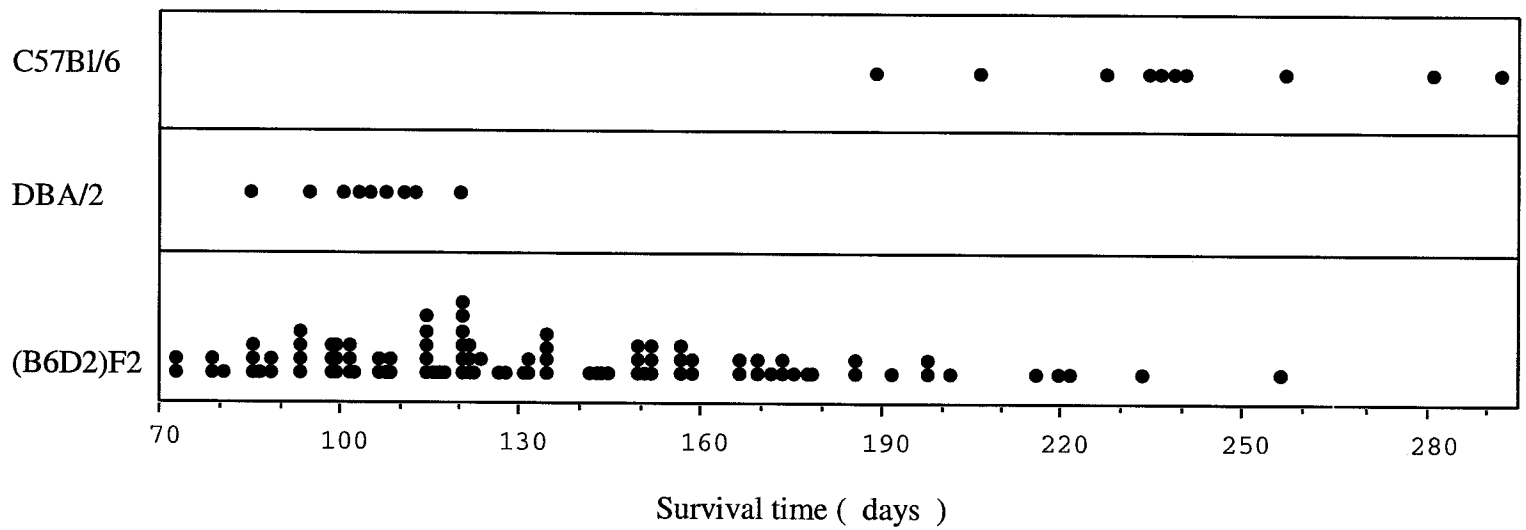
according to the supplier's instructions. Paraffin section 5 to 10μm were stained for acid-fast bacilli using Ziehl Nielsen stain and counterstained with methylene blue. Photomicrographs were taken using a Nikon Optiphot-2 photomicroscope.

RESULTS

The DBA/2J mouse strain is very susceptible to acute infection with *M. tuberculosis*, when introduced either by the intravenous or aerosol route, with very severe lung pathology associated with rapid bacterial replication in this organ [219]. This uncontrolled replication is specific to the lungs, is not seen in other test organs such as liver and spleen, and thus resembles the pathology of tuberculosis associated with immunosuppression in humans. Although the infection is also lethal for C57Bl/6J mice, this strain like BALB/c is more resistant to infection as measured by bacillar replication, type of lesions in the lungs and median survival time [219]. The aim of this study was to analyze the complexity of the genetic component and map individual gene(s) controlling the pronounced susceptibility of DBA/2J mice. For this, an informative (DBA/2J X C57Bl/6J) F2 cross was generated, and 95 male and female animals of this cross, as well as DBA/2J and C57Bl/6J controls, were infected by the intravenous route with 1×10^5 live *M. tuberculosis* H37Rv. Infected mice were observed daily and severely moribund animals were sacrificed. The survival time of individual animals from the (B6D2) F2 cross as well as parental controls is shown in Fig. 1. In this experiment, susceptible DBA/2J mice succumbed to infection between day 85 and 127 with a median survival time (MST) of 105 days. On the other hand, resistant C57Bl/6J mice did not start dying until day 188 with the last mouse dying at day 293, with a corresponding MST of 240 days (Fig. 1). Survival times in individuals from the (B6D2) F2 population showed a continuous distribution (day 71 to day 255) between that of resistant C57Bl/6J and DBA/2J susceptible progenitors, and all animals died. The MST value for the F2 cross was 130 days, a number closer to that seen in the susceptible than resistant progenitor. This suggests that susceptibility does not segregate as a recessive trait in this cross. In addition, the distribution of survival times in the (B6D2) F2 mice did not appear to be multimodal, suggesting that the differential resistance of B6 and D2 mice is not caused by a single gene. No effect of sex on survival was noted in the (B6D2) F2 population (t-test of mean differences = 0.650, $p > .50$).

Lungs from (B6D2) F2 mice succumbing early or late during infection in the range of susceptible and resistant parents were examined macroscopically and microscopically. At the

Figure 1: *Survival Time of C57Bl/6J, DBA/2J and (B6D2)F2 Mice to Infection with Mycobacterium tuberculosis.* Resistant C57Bl/6J and susceptible DBA/2J animals, as well as 95 animals from a (C57Bl/6J X DBA/2J) F2 cross were infected intravenously with 1 X 10^5 live *Mycobacterium tuberculosis* H37Rv, and survival times were recorded.


C57Bl/6
DBA/2
(B6D2)F2
70
100
130
160
190
220
250
280
Survival time (days)

time of death, lungs of (B6D2) F2 mice that died early (day 71) were strikingly similar in appearance to lungs of DBA/2J mice that died at the same period (day 85). They were edematous and swollen and showed numerous white lesions at their surface. Histological examination revealed large lesions throughout the lungs that appeared to collectively occupy about 50% of the lung volume. Each lesion was seen as an area of exudative, necrotic alveolitis in which contiguous air sacs were swollen and filled with degenerating neutrophils replete with acid-fast bacilli (Fig. 2a-2f). In contrast, histological appearance of lungs of B6 mice sacrificed on day 80 of infection (Fig. 2g-2i) was strikingly different, with small lesions that consisted of areas of alveolitis (Fig. 2g) populated by macrophages and lymphocytes and associated with large aggregates of lymphoid cells (Fig. 2h); some of the macrophages contained acid-fast bacilli, but the lesions contained relatively few bacilli (Fig. 2i). The lungs of long-lived (B6D2) F2 mice could not be analyzed at day 70-80, since these animals could not be identified at the time of testing moribund, susceptible (B6D2) F2 individuals. However lungs from long-surviving (B6D2) F2 mice were analyzed at the time of death (day 232) (Fig. 3), and were found to be extensively consolidated by interstitial expansion and occupation of air sacs with epithelial and mononuclear cells, some of which were positive for acid-fast bacilli. The histopathology profile of long-surviving (B6D2) F2 mice (day 230) is strikingly similar to that we have previously reported for innately resistant (B6D2) F1 mice sacrificed at day 250 [140]. Thus, histopathological analysis indicates that the distinct phentoypes of the DBA/2J and C57Bl/6J parental strains with respect to host response and lung pathology are recapitulated in (B6D2) F2 animals dying early and late after *M. tuberculosis* infection.

The continuous distribution seen in (B6D2) F2 mice with respect to survival suggests that survival to infection behaves as a quantitative trait amenable to study by quantitative trait locus (QTL) mapping. QTL mapping was carried out by a whole genome scan approach using survival time ($\log_{10}$) as a phenotypic trait. For this, genomic DNAs isolated from individual F2 mice prior to infection were genotyped with a total of 163 polymorphic dinucleotide repeat markers informative for DBA/2J and C57Bl/6J

Figure 2: *Histopathology Analysis of Lungs from Individual Mice Infected with Mycobacterium tuberculosis.* Sections of lungs from either a (B6D2) F2 mouse that succumbed early (day 80) after infection (a,b,c), a DBA/2J mouse that died at day 85 (d,e,f) and a C57Bl/6J mouse sacrificed at day 80 (g,h,i) were fixed, sectioned and stained for acid-fast bacilli followed by counter-straining with methylene blue. Magnifications from left to right are X 16.5, X 135, and X 400. Lung lesions of the (B6D2) F2 and DBA/2J mice are similar at all magnifications, consisting of an exudative, necrotic alveolitis. The extended, cell-filled air sacs (arrows) are seen at the lowest magnification (a,d) and can be seen to be filled predominantly with neutrophils replete with acid-fast bacilli at high magnification (c,f). In contrast, the lung pathology at sites of infection in the B6 mouse can be seen at low (g) and medium (h) magnification to consist of a macrophage-dominated alveolitis (M) with associated aggregates of lymphoid cells (L). At high power (I), some of the epithelioid macrophages can be seen to contain acid-fast bacilli (arrows).

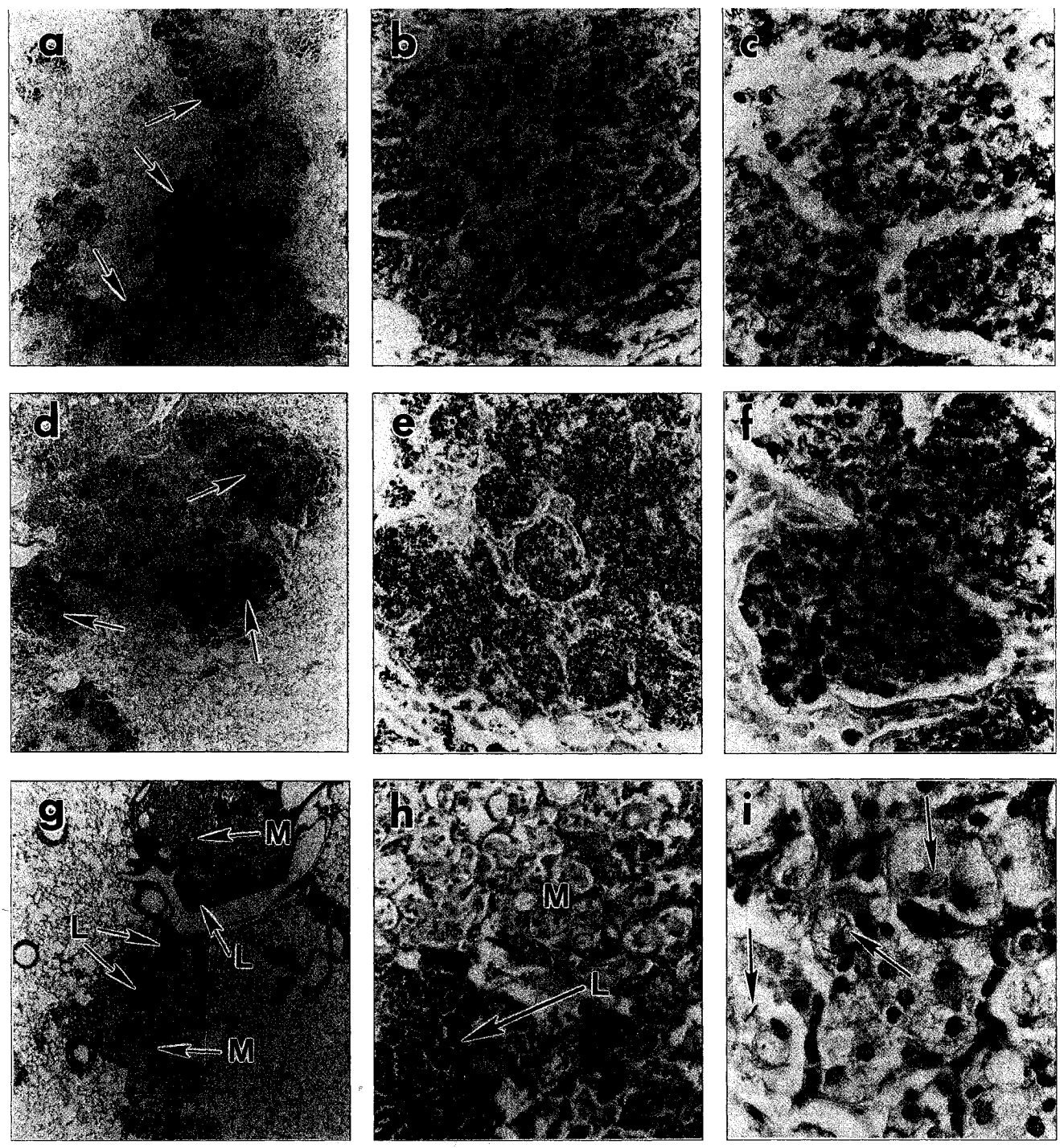
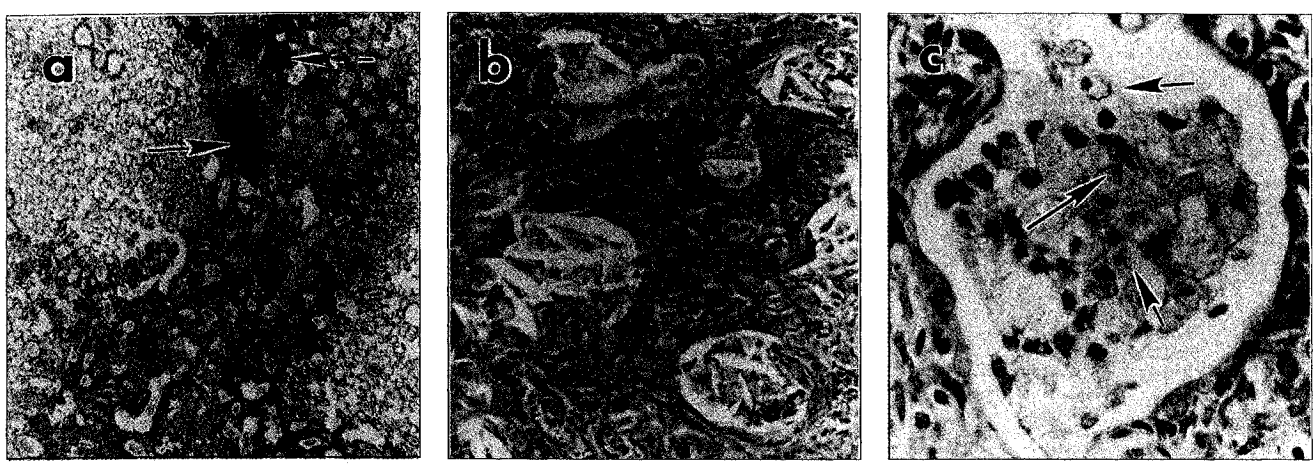

a
b
c
d
e
f
g
M
L
L
M
h
M
L
i

Figure 3: *Histopathology Analysis of Lungs from a (B6D2) F2 Mouse that Succumbed 232 days after Infection with Mycobacterium tuberculosis*. Tissue preparation, staining and magnification used are as described in the legend to Figure 2. Most of the lung is consolidated by a highly cellular and expanded interstitium. Conspicuous aggregates of lymphoid cells (arrows) are seen at low power (a). Consolidated lung tissue is frequently interrupted by the presence of extended air sacs containing epithelioid macrophages (b), some of which are positive for acid fast bacilli (c, arrow).


a
b
c

(http://carbon.wi.edu:8000/cgi-bin/mouse/index), using standard procedures. These markers provided an average coverage of 10 cM along each chromosome. In addition, a single non-polymorphic marker for Chr.Y (*SRY* [242]) was used to ascertain sex (markers are listed in Table 1). The largest distance separating any two markers or one marker from chromosome end was 18 cM for the proximal portion of Chr.X, and 18.6 cM for the distal portion of Chr.7. The genetic markers were assigned to, and mapped within the chromosomes by multipoint linkage analysis, using the Mapmaker/EXP version 3.0. The order of markers onto chromosomes was identical to those in published maps (www-genome.wi.mit.edu). Genome-wide multipoint linkage analysis was performed using Mapmaker/EXP version 3.0 and Mapmaker/QTL 1.1 [239]. The results from this analysis are shown as multiple point LOD score traces (Fig. 4), and numerical data for individual intervals shown in Table 2. Statistically significant linkages were identified on two chromosomes, with a third chromosome showing suggestive evidence by genome-wide criteria [243].

A significant linkage was identified on the distal portion of Chr.1, overlapping a region of 25-30cM with maximum linkage to markers *D1Mit265/396/425* ($\chi^2(2) = 21.2$-22.1; LOD = 4.80, $p<0.000015$; Table 2). This locus accounted for approximately 21% of the phenotypic variance. A second linkage was identified on the proximal portion of Chr.7, overlapping a region of 25cM with maximum linkage to markers *D7Mit117/270/228* ($\chi^2(2) = 21.4$; LOD = 4.66, $p<0.000015$; Table 2). This locus accounted for approximately 21% of the variance. A two-locus assessment for the Chr. 1 and Chr. 7 loci showed a LOD score of 9.01 ($\chi^2(4) = 41.5$; 41.5% of phenotypic variance). Finally, a weaker linkage was identified in the central portion of Chr.3, with maximum linkage to markers *D3Mit241/51* ($\chi^2(2) = 17.4$; LOD = 3.93, $p<0.0002$; Table 2), and explaining 17% of the variance. Fitting the data to a three-locus model yielded a maximum LOD score of 12.06 ($\chi^2(6) = 55.54$, $p < 4 \times 10^{-10}$), explaining 45.3% of the variance. Therefore, the three loci mapped in this study account for approximately half of the phenotypic variance between the two parents, with respect to survival time after infection and were given the provisional appellation *Trl-1*, *Trl-2*, and *Trl-*

TABLE 1. Polymorphic microsatllite markers used for whole genome scan

D1Mit66, 374, 213, 156, 19, 181, 415, 493, 218, 196, 265
396, 425, 109, 145, 206, 541, 150, 361, 221, 209
D2Mit1, 32, 367, 61, 91, 436, 164, 401, 225, 311, 200
D3Mit176, 306, 69, 241, 51, 72, 156, 106, 17, 128
D4Mit101, 39, 237, 214, 152, 303, 124, 148, 33
D5Mit348, 233, 201, 157, 406, 95, 168, 409
D6Mit86, 33, 9, 230, 25, 137
D7Mit178, 117, 270, 228, 158, 193, 30, 220, 330, 333
D8Mit3, 191, 25, 195, 266, 211, 200, 156
D9Mit90, 66, 154, 102, 196, 12, 116
D10Mit298, 51, 44, 15, 186, 42, 133, 179
D11Mit226, 19, 20, 349, 194, 39, 67, 224, 103
D12Mit12, 153, 34, 14, 118, 233, 20
D13Mit271, 60, 139, 250, 145, 293, 35
D14Mit207, 45, 18, 5, 39, 193, 161, 265, 266
D15Mit252, 229, 63, 118, 72, 171, 41
D16Mit55, 100, 101, 84,158
D17Mit100, 11, 68, 159, 127, 129
D18Mit94, 89, 33, 213
D19Mit109, 127, 106, 66, 38, 1, 137
DXMit166, 115, 117, 186
Chromosome Y : SRY*

*Not a polymorphic marker but was used to verify sex.

Figure 4: *Linkage Analysis by Whole Genome Scan of Susceptibility to Infection with Mycobacterium tuberculosis*. LOD (logarithm of odds ratio) score plots along chromosomes for which significant and suggestive quantitative trait loci (LOD scores superior to 3.0) controlling survival to *M. tuberculosis* H37Rv in (B6D2) F2 mice were detected. Traces are shown for chromosomes 1 (A), 3 (B) and 7 (C). The map positions of microsatellite markers used in the study are indicated, and chromosomal length are shown to scale.

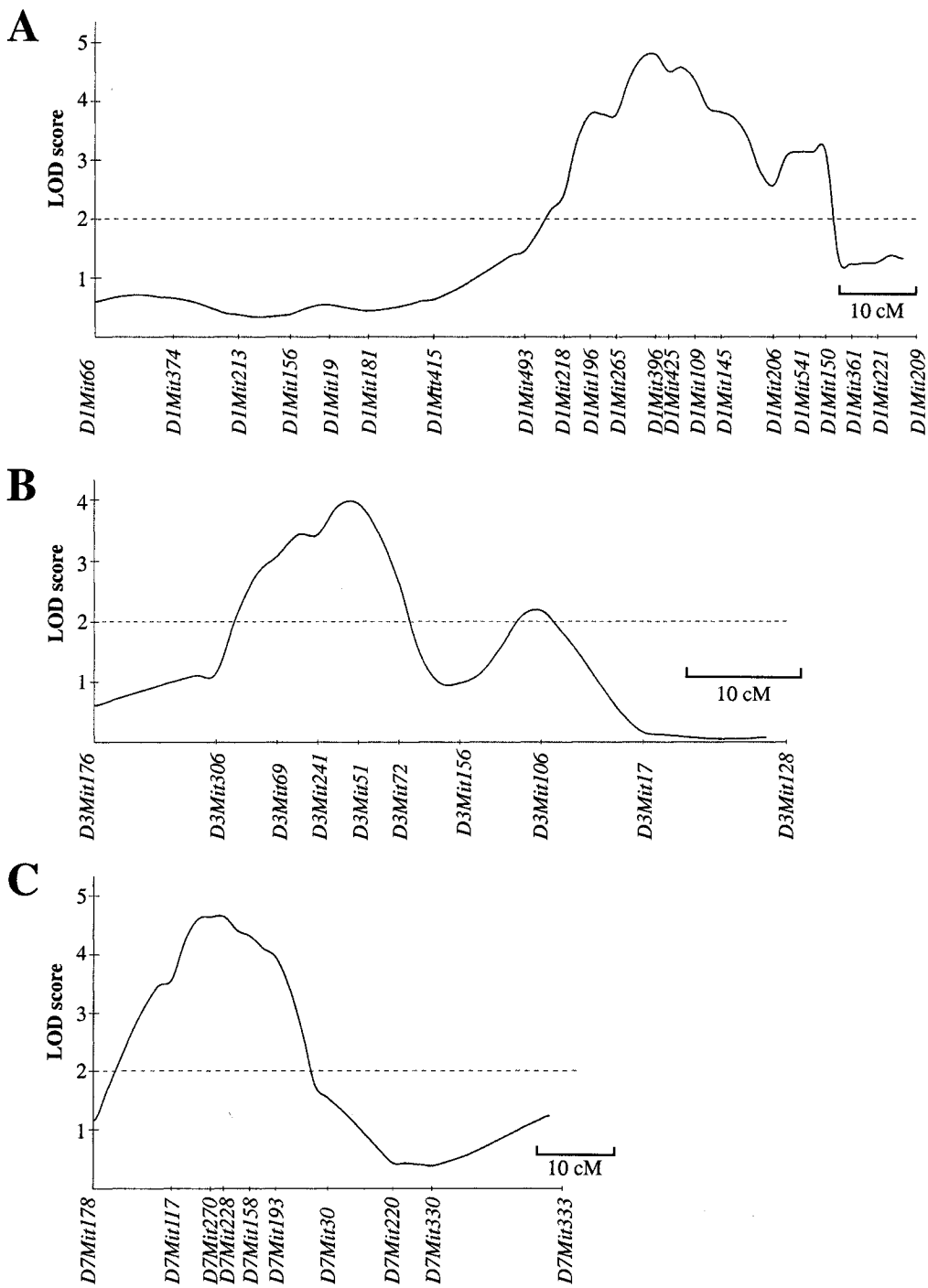

A
LOD score
5
4
3
2
1
10 cM
D1Mit66
D1Mit374
D1Mit213
D1Mit156
D1Mit19
D1Mit181
D1Mit415
D1Mit493
D1Mit218
D1Mit196
D1Mit265
D1Mit396
D1Mit425
D1Mit109
D1Mit145
D1Mit206
D1Mit541
D1Mit150
D1Mit361
D1Mit221
D1Mit209
B
LOD score
4
3
2
1
10 cM
D3Mit176
D3Mit306
D3Mit69
D3Mit241
D3Mit51
D3Mit72
D3Mit156
D3Mit106
D3Mit17
D3Mit128
C
LOD score
5
4
3
2
1
10 cM
D7Mit178
D7Mit117
D7Mit270
D7Mit228
D7Mit158
D7Mit193
D7Mit30
D7Mit220
D7Mit330
D7Mit333

TABLE 2. Statistical values for suggestive and significant linkages obtained from the whole genome scan

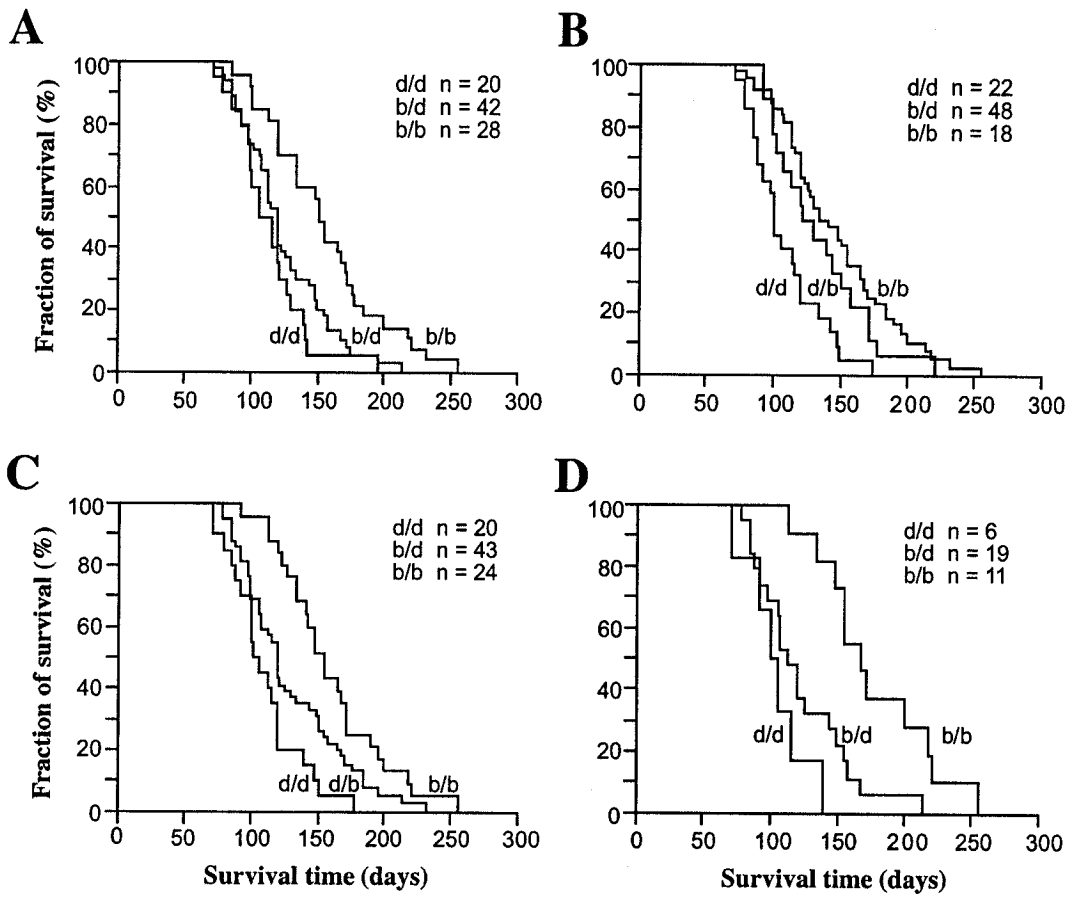
Markers	cM[a]	% variance explained[b]	$\chi 2$	LOD	P value
D1Mit415	51.4	6.9	6.69	1.45	0.035
D1Mit493	64.5	11.0	11.12	2.41	0.0039
D1Mit196	73.2	16.8	17.41	3.78	0.00017
D1Mit265	76.5	20.8	22.11	4.80	0.000016
D1Mit396	80.9	20.9	22.06	4.79	0.000016
D1Mit425	82.0	*21.0	21.26	4.62	0.000024
D1Mit109	85.2	19.7	20.06	4.35	0.000044
D1Mit145	87.4	17.4	17.46	3.81	0.00016
D1Mit150	99.5	14.3	14.40	3.13	0.00075
D1Mit361	101.6	5.9	5.75	1.25	0.057
D3Mit176	5.5	5.4	5.26	1.13	0.072
D3Mit69	21.9	15.4	15.73	3.41	0.00038
D3Mit241	24.0	17.4	18.12	3.93	0.00012
D3Mit51	26.2	*17.6	18.08	3.92	0.00012
D3Mit72	29.5	12.0	12.04	2.61	0.0024
D3Mit106	40.4	10.1	10.03	2.18	0.0066
D7Mit178	0.0	16.0	16.41	3.56	0.00027
D7Mit117	10.9	20.2	21.33	4.63	0.000023
D7Mit270	15.3	20.2	21.39	4.64	0.000023
D7Mit228	16.4	*20.8	21.48	4.66	0.000021
D7Mit158	18.6	18.9	19.85	4.31	0.000049
D7Mit193	21.9	17.5	18.14	3.94	0.00012
D7Mit30	28.4	7.2	7.04	1.53	0.03
Total Effect[c]		45.3		12.06	

[a] The chromosome position of each marker is expressed as distance from telomere (in cM), according to 1999 Chromosome Committee Reports. [b] The proportion of total phenotypic variance explained by each QTL is indicated as a percentage. [c] The total contribution of the 3 maximum QTL's (indentified by *) to overall phenotype has been calculated using a three locus model.

3 (Tuberculosis resistance locus) for linkages on chromosomes 1, 3 and 7, respectively. A parallel analysis was conducted using weight at the time of death as a phenotypic marker; this analysis detected a strong association due to sex on chromosome X only. Evaluation of the weight data after controlling for phenotypic sex differences by linear regression eliminated these apparent effects.

To visualize the effect of parental alleles combination (DBA/2J, d; C57Bl/6J, b) at each locus on survival to infection, the survival fraction of groups of individual (B6D2) F2 either homozygous (d/d, b/b) or heterozygous (b/d) for parental alleles is shown in Fig. 5. For *Trl-1*, resistance was associated with the C57Bl/6J allele and behaved as recessive, with homozygous b/b animals showing an MST of 149 days, while homozygous d/d animals showed and MST of 105 days (Fig. 5A). Similarly, resistance for *Trl-3* was associated with the C57Bl/6J allele (additive mode of inheritance), with homozygous b/b animals (MST, 154 days) surviving significantly longer than homozygous d/d mice (MST, 105 days; Fig. 5C). Finally, the weaker QTL, *Trl-2*, was inherited in a dominant fashion, with the C57Bl/6J allele also encoding resistance, and with MST values of 133 days for b/b animals vs. 99 days for d/d animals (Fig. 5B). Analysis of the combined effect of the two strongest QTLs (*Trl-1* and *Trl-3*) on survival indicated a completely additive and very strong effect of these two QTLs, with animals bearing b/b alleles at each locus (MST, 170 days) surviving significantly longer than animals bearing d/d alleles at both loci (MST, 99 days) (Fig. 5D). Pairwise analyses of other two-locus combinations failed to identify any major epitstatic effect, with each locus contributing in an additive fashion *Trl-1* and *Trl-2*, $F(4,86) = 0.65$, $p > 0.60$; *Trl-1* and *Trl-3*, $F(4,86) = 0.12$, $p > 0.95$; *Trl-2* and *Trl-3*, $F(4,86) = 0.59$, $p > .65$). Finally, not enough (B6D2) F2 animals were available for analysis to assess the combined effect of homozygosity at all three loci in the same animal on survival to infection.

Figure 5: *Effect of Haplotype Combination at Individual Identified QTLs on Survival to M. tuberculosis Infection.* The effect of parental alleles combinations at *Trl-1* (**A**, marker *D1Mit425*), *Trl-2* (**B**; marker *D3Mit69*), *Trl-3* (**C**; marker *D7Mit270*), and combined *Trl-1* and *Trl-3* (**D**) on survival to infection is shown. The data are expressed as the proportion of mice surviving the infection at each time point and are shown as a percentage of survival. Survival curves are shown for B6D2 progeny with genotype B6/B6 (b/b), D2/D2 (d/d) or B6/D2 (b/d) for the above mentioned markers.


A
B
C
D
Fraction of survival (%)
Survival time (days)
d/d n = 20
b/d n = 42
b/b n = 28
d/d n = 22
b/d n = 48
b/b n = 18
d/d n = 20
b/d n = 43
b/b n = 24
d/d n = 6
b/d n = 19
b/b n = 11
d/d
b/d
b/b
0
20
40
60
80
100
0
50
100
150
200
250
300

DISCUSSION

Clear inter-strain differences in susceptibility to infection with *M. tuberculosis* have been described in mice and can thus be used for the possible mapping of individual loci affecting host defense mechanisms against this infection. In turn, these newly identified loci may provide valuable tools to study parallel effects of syntenic chromosomal regions in humans by association and linkage studies in individuals from endemic areas of disease. Ultimately, the molecular cloning of such genes may identify new potential targets for therapeutic intervention in tuberculosis. We used a genetic analysis to map individual loci contributing to the dramatic differential susceptibility of DBA/2J and C57Bl/6J to infection with *M. tuberculosis*, that is phenotypically expressed as differences in microbial replication in the lung, severity and type of lung pathology, and overall survival time, the phenotypic measure used in this study. Histopathological analysis indicates that the distinct phenotypes of the DBA/2J and C57Bl/6J parental strains with respect to host response and lung pathology are recapitulated in (B6D2) F2 animals dying early and late after *M. tuberculosis* inoculation. Lung lesions in susceptible animals (DBA/2J parent, short-lived F2 survivors) were seen as regions of exudative necrotic alveolitis as we previously described [227]. On the other hand, lesions in resistant C57Bl/6J mice sacrificed at the same time (day 80) were totally different and dominated by epithelioid macrophages and lymphocytes, similar to those we have previously described for resistant strains BALB/c and C57Bl/6J [227]. Because survival time was used as a phenotypic measure of susceptibility, the histopathology of long-surviving (B6D2) F2 mice could not be analyzed at day 80, but is presumed to resemble that detected in resistant C57Bl/6J (Fig. 2g-2i), BALB/c [219,238] and (B6D2) F1 resistant progenitors [140], namely a macrophage –dominated alveolitis with few acid-fast bacilli. Indeed, at the time of death of resistant (B6D2) F2 mice (day 230), the chronic histopathology in the lungs was similar to that we previously observed for resistant (B6D2) F1 progenitors sacrificed at day 250 [140].

Using a whole genome scan in (B6D2) F2 mice, we have localized three loci : *Trl-1*, *Trl-2* and *Trl-3* that together account for almost half of the phenotypic variance observed in an

informative (B6D2) F2 population of segregating animals. The remaining 50% of the phenotypic variance is likely to be accounted for by the action of additional genes indicating that, as is the case for humans, susceptibility to tuberculosis is a highly complex and polygenic trait in mice. Haplotype analysis at the three loci reveals that the resistance alleles are contributed by the resistant C57Bl/6J parent. In addition, analysis of the data using 2 and 3 loci models indicate that the contribution of the three loci is additive with little epistasis detected. Thus, these three loci together provide a major contribution and play a major role in regulating susceptibility to *M. tuberculosis* infection.

The three loci mapped in this study appear to be novel loci. Indeed, only two published studies document whole genome scans for QTLs that may affect host resistance to *M. tuberculosis* infection [236,244] and the loci mapped in our study appear distinct from those mapped by these two groups. The Chr.1 *Nramp1* locus plays a key role in host resistance against several intracellular infections in mice, including several mycobacterial species [245] and *NRAMP1* alleles have been associated with susceptibility to tuberculosis [112] and leprosy in humans [246]. However, *Trl-1* on Chr.1 identified in our study (map position 74cM; 1999 Chr.1 committee report; www.informatics.jax.org) maps approximately 35cM distal from the *Nramp1* locus (Map position 40cM) [247]. On the other hand, linkage of susceptibility to infection with high doses of *M. tuberculosis* (1 X 10^6, *i.v*) was recently reported with the Chr.1 marker *D1Mit49* (Map position 54cM) in a study using a (C3H X C57Bl/6J) F2 cross [244]. The relationship between the *D1Mit49* linkage and *Trl-1* mapped in the present study is difficult to establish with certainty, however they appear to be different as *D1Mit49* maps 25cM proximal to *Trl-1*. Interestingly, *D1Mit49* maps very close to another host resistance locus recently mapped in a (*M. molossinus* X C57Bl/6J) F2 intercross as a genetic modifier of *Nramp1*, and that controls susceptibility to infection with *Salmonella typhimuirum* [248]. Finally, a locus (*Tir3*) which affects susceptibility to trypanosomiasis as measured by survival time was recently mapped in a BALB/c X C57Bl/6J cross to the distal part of Chr.1 [249].

Lavebratt *et al.* recently studied genetic control of body weight loss, following infection with high doses of *M. tuberculois* (> 10^6 CFU) [236]. In this infection model, "susceptible" I/St mice and "resistant" A/Sn mice show MST values of 21.5/ 26.3 days (females/males) and 45.4/45.2, respectively. They identified significant linkages to chromosomes 3 and 9 in females only, and suggestive linkages on Chrs 8 and 17 (site of the H-2 locus) in females and on Chrs. 5 and 10 for males. The Chr.3 linkage identified by Lavebratt *et al.* maps to the very distal portion of the chromosome (Map position 55cM), as opposed to the proximal portion that carries *Trl-2*. We did not detect any linkage to chromosomes 5, 8, 9, 10 and 17 in our study. In addition, we did not observe any effect of sex on susceptibility to infection, and body weight at the time of death did not show any specific linkage in the (B6D2) F2 analyzed here, once data was regressed to account for gender of the animals. The lack of correlation in the QTLs mapped in the two studies indicate unrelated genetic controls that can be accounted for by differences in a) mouse strains used, b) infection protocol and infectious inoculum size, c) phenotypes analyzed, and d) pathologies observed in the two parental strains causing much more rapid death following infection. Finally, *Trl-3* identified here does not appear to map near any host resistance locus identified to date.

The genetic basis of differential susceptibility to infection in susceptible mouse strains such as DBA/2J and resistant strains such as BALB/c [20, 22] and C57Bl/6J (Fig. 2) is phenotypically expressed in the lungs as i) a more rapid growth and more extensive replication of the bacilli, and ii) a very different immune and inflammatory host responses to bacterial load leading to distinct pathologies and types of lesions. Although the linkages identified here cover substantial chromosomal segments (15-25cM), it is possible to consider potential candidate genes in these regions that may, by virtue of their known function in host immune or inflammatory responses, contribute to the observed phenotypes. *Trl-1* overlaps several interesting mapped genes that fall in this category. The chemokine receptor CXCR4 (map position 67cM) is expressed on T lymphocytes and binds to the SDF-1α/β ligand, and plays an important role in the activation of naïve $CD45RA^+$ T cells [250]. It has been proposed that antigen-presenting, SDF-1

secreting dendritic cells can activate naïve CXCR4-positive naïve T cells upon migration to lymph nodes [251]. This region also contains the gene for interleukin 10 (IL-10; map position 70cM), a cytokine produced by T cells (Th2) and macrophages, that blocks IFN-γ synthesis by T cells, and cytokine synthesis by macrophages. It is a potent suppressor of effector function of macrophages, T cells, B cells and NK cells [252]. Studies in IL-10-deficient mice show that it has potent anti-inflammatory activity, and plays an important role in balancing protective and pathologic immune response during infection with intracellular parasites [253,254]. Indeed, IL-$10^{-/-}$ mice show increased susceptibility to infection with *Plasmodium chabaudi* [255], *Toxoplasma gondii* [256] and *Trypanosoma cruzi* [257] associated with increased expression of inflammatory mediators such as IFN-γ, TNF-α, and IL-12. On the other hand, the same exacerbated response has beneficial effects in that it increases resistance of IL-10 mutant mice to infection with *Listeria monocytogenes* [258], *Chlamydia trachomatis* [259] and *Mycobacterium bovis* (BCG) [146]. *Trl-1* also overlaps the structural genes for the neutrophil cytosolic factor 2 (*Ncf2*, map position 76cM), the Fas ligand (FasL, map position 85cM), and a cluster of three selectins (*Sele, Sell, Selp,* map position 86 cM). *Ncf2* encodes the $p67^{phox}$ subunit of the multisubunit complex NADPH-dependent oxidase [260]. This key enzyme is abundant in neutrophils and is delivered to microbe-containing phagosomes, where it plays a central role in the generation of superoxide which in turn combines with water to produce the bactericidal species H_2O_2. In humans, mutations in $p67^{phox}$ cause chronic granulomatous disease, a complex pathology associated with recurrent infections with catalase-positive bacteria and fungi [260]. On the other hand, Fas-L is expressed by activated T cells, and together with its receptor Fas play an important role in down-regulation of immune responses, through apoptosis of antigen-stimulated T cells [261]. Naturally occurring mutation at Fas (*lpr*) or in Fas-L (*gld*) have been characterized and shown to cause lymphoadenopathy and inflammatory disease. Infection of either *lpr* or *gld* mice with *Leishmania major* results in progressive lesions that do not heal when compared to syngeneic controls [262]. Finally, selectins (L, P and E) are a group of cell surface adhesion molecules expressed on leukocytes and on endothelial cells which play an important role in leukocyte adherence to and

migration across endothelial cells during inflammatory responses. Mutant mice defective in both P and E-selectin show extreme leukocytosis, but become susceptible to cutaneous infections [263].

With respect to the weaker QTL, *Trl-2* (peak position 33cM), it overlaps three interleukin and interleukin receptor genes, including IL-2, IL-12 ("a" subunit), and IL-6 receptor α chain (Map positions, 19, 37 and 42cM, respectively), all of which participate in host immune response. IL-2 is produced by antigen- or mitogen-stimulated T cells, and has many biological activities including induction of growth and activation of T cells, B cells and NK cells. Deficiency in IL-2 and its receptor result in generalized fatal immunoproliferative disorder (XSCID) involving multiple organs [264] and loss of self tolerance, including severe fatal colitis in the mouse [265]. Of particular interest is IL-12, which is produced by mononuclear phagocytes in response to a variety of pathogens, and induces differentiation of naïve CD4+ T cells into antigen-specific, IFN-γ producing Th1 cells. This response is essential to host bactericidal activity against obligate intracellular pathogens [266]. In humans, mutations in the IL-12R cause severe immunodeficiency, associated with recurrent intracellular infections with *Mycobacterium* and *Salmonella* [267,268]. Introduction of a null allele at the IL-12 locus on a C57Bl/6J genetic background abrogates natural resistance to infection with *M. tuberculosis* [189] and *Leishmania major* [269]. Finally, IL-6 and its receptor (IL-6R, one subunit of which maps on Chr. 3) act synergistically with a number of other growth factors and cytokines to sustain normal proliferation and maturation of macrophages, T and B cells, and megakaryocytes [266]. IL-6 also behaves as an anti-inflammatory cytokine to control local and systemic inflammatory responses [270]. Additional experiments are required to formally test the candidacy of any of these genes as *Trl-1* and *Trl-2*. Finally, *Trl-3* (peak position 18cM) which accounts for 20% of the phenotypic variance does not show any obvious candidate genes and proteins possibly associated with host inflammatory or immune responses. The phenotypic contribution of each of these loci to the overall susceptibility of DBA/2J and resistance of C57Bl/6J can now be studied in reciprocal congenic mouse lines, where each locus has been fixed on the opposite genetic background by continuous backcrossing.

The results presented in this study now permit the evaluation of a possible association of the corresponding syntenic human chromosomal regions in susceptibility to tuberculosis. The proximal portion of the Chr.1 *Trl-1* is syntenic with human 2qter, while the more distal portion is syntenic with human 1q21-1q31. The Chr.3 *Trl-2* is syntenic with human 3q25 and 4q31, while the Chr.7 *Trl-3* is syntenic with human 19q13. A possible association of these chromosomal regions with susceptibility to tuberculosis in humans can now be tested in population studies from endemic areas of disease.

Our next question is how does *Trl-1/2/3* or perhaps additional loci regulate alone or in combination *M.tuberculosis* populations in the lungs.

Chapter 3

Susceptibility to tuberculocis: a locus on mouse chromosome 19 (*Trl-4*) regulates *M. tuberculosis* replication in the lungs.

ABSTRACT

The mouse DBA/2 (D2) strain is very susceptible to infection with virulent *Mycobacterium tuberculosis*, while C57BL/6 (B6) is much more resistant. Infection of D2 and B6 mice with *M. tuberculosis* H37Rv by the respiratory route is biphasic: during the first 3 weeks, there is rapid bacterial growth in the lung of both strains, while beyond this point replication stops in B6 but continues in D2 causing rapidly fatal pulmonary disease. To identify the genes regulating growth of *M. tuberculosis* in the lungs of these two strains, 98 informative (B6 X D2) F2 mice were infected by the respiratory route with *M. tuberculosis* H37Rv (2 X 10^2 CFU), and the extent of bacterial replication in the lungs at 90 days was used as a quantitative measure of susceptibility in a whole genome scan. Quantitative trait locus mapping identified a major locus on chromosome 19 (Tuberculosis resistance locus-4, *Trl-4*; LOD 5.6) which regulated pulmonary replication of *M. tuberculosis* and accounted for 25% of the phenotypic variance. B6 alleles at *Trl-4* were inherited in an incompletely dominant fashion and associated with reduced bacterial replication. An additional effect of a locus (*Trl-3*), previously shown to affect survival to *i.v.* infection with *M. tuberculosis*, was also noted. F2 mice homozygous for B6 alleles at both *Trl-3* and *Trl-4* were as resistant as B6 parents, while mice homozygous for D2 alleles were as susceptible as D2 parents. These results suggest a strong genetic interaction between *Trl-3* and *Trl-4* in regulating pulmonary replication of *M. tuberculosis*.

INTRODUCTION

The global prevalence of *Mycobacterium tuberculosis* infection was recently estimated at 32% (1.9 billion people), with an evaluated 8 million new cases of active tuberculosis (TB) and 2 million deaths that year [271]. Only a small proportion of individuals that come in contact with *M. tuberculosis* develop active TB and a wide clinical spectrum of disease severity is observed in such individuals. This is determined in part by microbial virulence determinants [234] and by environmental and host factors, such as social conditions and immune status, most critically by presence of concomitant HIV infection [272]. An important genetic component of vulnerability to TB in humans affecting susceptibility *per se*, disease progression and ultimate outcome has been well documented [95]. This includes epidemiological data pointing to sex [93,94] and racial differences in susceptibility [89], as well as geographical distribution and familial aggregation [95]. In addition, studies of first contact epidemics in isolated populations with no ancestral experience of this infection (Yanomami, Qu'Appelle indians) [86,87], survival data from accidental injection of virulent *M. tuberculosis* during a BCG vaccination trial (Lubeck disaster) [273], and studies in twins showing higher concordance rates of TB in monozygotic vs. dizygotic twins [90-92], provide compelling evidence that host genes affect the outcome of *M. tuberculosis* infection.

To date, inherited mutations in gp91/phox [274], IL-12 [275] and in the IFN-γ receptor genes [276] have been found in a few rare familial cases of infantile TB or of disseminated *M. bovis* (BCG) infection [95,277]. However, such Mendelian disorders are exceedingly rare. The genetic component of TB susceptibility has been investigated in population and family-based studies. Case control studies in areas of endemic disease have pointed to several gene variants contributing to TB risk, including the human leukocyte antigen (HLA) [95], the natural resistance associated macrophage protein NRAMP1 [112,113,115,117,278], the vitamin D receptor [130,131], and the mannose binding protein [127]. A strong association (LOD 3.8) of *NRAMP1* alleles on 2q35 with

susceptibility to TB was independently found by linkage analysis in a large Aboriginal Canadian pedigree in the outbreak situation [123]. Major gene effects were recently investigated by whole genome scan using 173 affected sib pairs from The Gambia and from South Africa; this analysis identified suggestive linkages (LOD ~ 2) on Chromosomes 15q and Xq [124,136]. These studies suggest that the genetic control of susceptibility to TB in humans is complex.

Such complex genetic traits can be studied in mouse models of disease, where environmental and genetic components can be best controlled, and where single gene effects may have become fixed in inbred, recombinant inbred and recombinant congenic strains of mice [279]. Genetic control of susceptibility to TB is complex in mouse and is influenced by the *M. tuberculosis* isolate, the route and dose of infection, the mouse strains used and the phenotypic measure of susceptibility [215-218,236,237]. Using survival time after *i.v.* injection of 1 X 10^5 *M. tuberculosis*, inbred mouse strains are classified [219] into either highly susceptible (CBA, C3H, DBA/2, 129svJ) or highly resistant (C57BL/6, BALB/c). Susceptibility of DBA/2 (D2) and resistance of C57BL/6 (B6) and BALB/c strains, as measured by mean survival time, is also observed after airborne infection with 10^2 bacilli [238]. Using informative backcross and F2 mice issued from C3HeB/Fe and B6 parents, and infected *i.v.* with 10^6 *M. tuberculosis* Erdman, Kramnik et al. have mapped a locus on distal Chr.1 (*sst1*; position 49-58cM) that controls the rate of bacterial replication and granuloma formation in the lung [280].

Susceptibility of D2 mice to *M. tuberculosis* infection is characterized by a) progressive bacterial replication in the lung, b) extended neutrophil-dominated lung pathology including large numbers of acid-fast bacilli and areas of necrosis, and c) early death [219,229,238]. We have previously mapped 3 quantitative trait loci (QTLs) on distal Chr. 1 (*Trl-1*; LOD 4.8), proximal Chr. 7 (*Trl-3*; LOD 4.7), and proximal Chr. 3 (*Trl-2*; LOD 3.9) that affect survival time of (B6 X D2) F2 mice following *i.v.* infection with 1 X 10^5 *M. tuberculosis* H37Rv [229]. In this report, the

genetic analysis of D2 susceptibility to pulmonary TB was expanded using an infection protocol more closely related to the human situation than the previously used *i.v.* model [229,236,280]. (B6 X D2) F2 mice were infected by the respiratory route with 2 X 10^2 *M. tuberculosis* H37Rv and pulmonary bacterial load at 90 days was used as a quantitative measure of susceptibility. A whole genome scan revealed a major locus on Chr. 19 (designated *Trl-4*) regulating replication of *M. tuberculosis* in the lung.

MATERIALS AND METHODS

Animals

Inbred, pathogen free, 8-week-old C57BL/6J (B6) and DBA/2J (D2) mice were purchased from the Trudeau Institute Animal Breeding Facility. Ninety-eight (B6 X D2) F2 progeny were bred by systematic brother sister mating of (B6 X D2) F1.

Mycobacteria

Mycobacterium tuberculosis strain H37Rv (TMC #102) was obtained from the Trudeau Mycobacterial culture collection as a frozen (-70° C) log phase dispersed culture in Proskauer and Beck medium (Difco Laboratories Inc., Detroit MI) containing 0.01%Tween 80. For each experiment, a vial was thawed, subjected to 5 sec ultrasound to break up aggregates, and diluted appropriately in PBS containing 0.01% Tween 80. Mice (8-10 weeks of age) were inoculated with 2 X 10^2 CFU by aerosol in a Middlebrook airborne infection apparatus (Tri instruments, Jamaica, NY). Bacilli were enumerated in the lungs of infected mice 90 days post-infection, by preparing lung homogenates in PBS containing 0.05% Tween 80 and by plating 10-fold serial dilutions of the homogenates on enriched agar (Middlebrook 7H11; Difco laboratories Inc.). Colony Forming Units (CFUs) were enumerated after 3-4 weeks of incubation at 37°C, and the data is presented as log of total CFU count per lung.

Genotyping

Prior to infection, tail biopsies were obtained, and genomic DNA was prepared [229]. A total of 151 microsatellite markers distributed over all chromosomes except Chr. Y (approximate 10cM coverage) were selected (www-genome.wi.mit.edu) and purchased from Research Genetics (Huntsville, AL). Genotyping was performed by standard PCR-based method using

trace amount of [^{32}P]α-dATP, followed by separation on denaturing polyacrylamide gels, exactly as described [229]. Some markers were genotyped using primer pairs fluorescently labeled, either commercially available or custom-synthesized by Applied Biosystems (Foster City, CA). In this case, one of the primers was synthesized and labeled with either FAM, HEX or NED phosphoramides. Products were analyzed by capillary separation using an ABI prism 3700 automated DNA sequencer.

Statistical Analysis

Genome-wide interval mapping analysis between lung CFUs (log), and genetic markers for the identification of QTLs was performed using Mapmaker/EXP version 3.0 and Mapmaker/QTL 1.1 [239]. LOD scores were calculated as $\chi^2/2\ln(10)$. Permutations of the phenotypes in this sample were conducted using linear regression in QTL Cartographer [281,282] to obtain empirical significance levels at each locus (10,000 iterates unless noted otherwise). Genome-wide significance levels were also obtained using QTL Cartographer, providing thresholds appropriate for this particular study. Initial linkage analyses were conducted using a "free" model involving co-dominance and dominance effects, yielding χ^2 statistics and LOD scores with 2 df. Tests of specific genetic sub-models were conducted by fitting each 1 df model (dominant, recessive, co-dominant) and comparing the likelihood ratios ($\chi^2(1) = 2$[log-likelihood(free) – log-likelihood(nested)]). For significant linkage regions, similar likelihood-ratio tests were conducted to test whether apparent linkage results were due to mean trait differences between sexes: heterogeneity test for sex – specific effects, $\chi^2 = 2$[LL(combined) – (LL(male) + LL(female))]. Significant results in this test indicate that the linkage results differ between males and females; non-significant results indicate that apparent differences in LOD scores are consistent with random variation.

The distribution of lung CFUs (log) in (B6 X D2) F2 mice closely followed a normal distribution (see Fig. 1). Significant differences were noted between mean CFU counts in female vs. male mice, (Table I; males mean = 7.11, females mean = 6.57; two tailed $t = 6.92$, $p < 0.001$) [241]. Linkage to chromosome X was initially detected (LOD 4.56), suggesting a possible sex effect. However, further analysis indicated no significant linkage to chromosome X in either males or females when analyzed independently (LOD of 0.25 and 0.81, respectively), suggesting that the original linkage simply reflects mean trait differences between genders instead of the influence of any chromosome X loci. To compensate for sex effects, the lung CFU counts (log) were then adjusted by subtracting the gender-specific mean from each individual to create a "sex-adjusted CFU" value. This measure allows the male and female CFU data to be analyzed jointly on the same scale, free of gender-specific influences.

Figure 1: *Replication of Mycobacterium tuberculosis in the lungs of B6, D2 and (B6 X D2) F2 mice.* Resistant B6 and susceptible D2 control mice were infected via the aerosol route with 2 X 10^2 *M. tuberculosis* H37Rv. The number of *M. tuberculosis* present in the lungs was determined (log10 CFU) at preset time points where each data point corresponds to the mean CFU counts for groups of 5 mice (A). Lung CFUs (log) of B6 and D2 controls, and of individual (B6 X D2) F2 mice 90 days following aerosol infection with 2 X 10^2 *M. tuberculosis* H37Rv. Horizontal bars represent means of CFUs for each group (B).

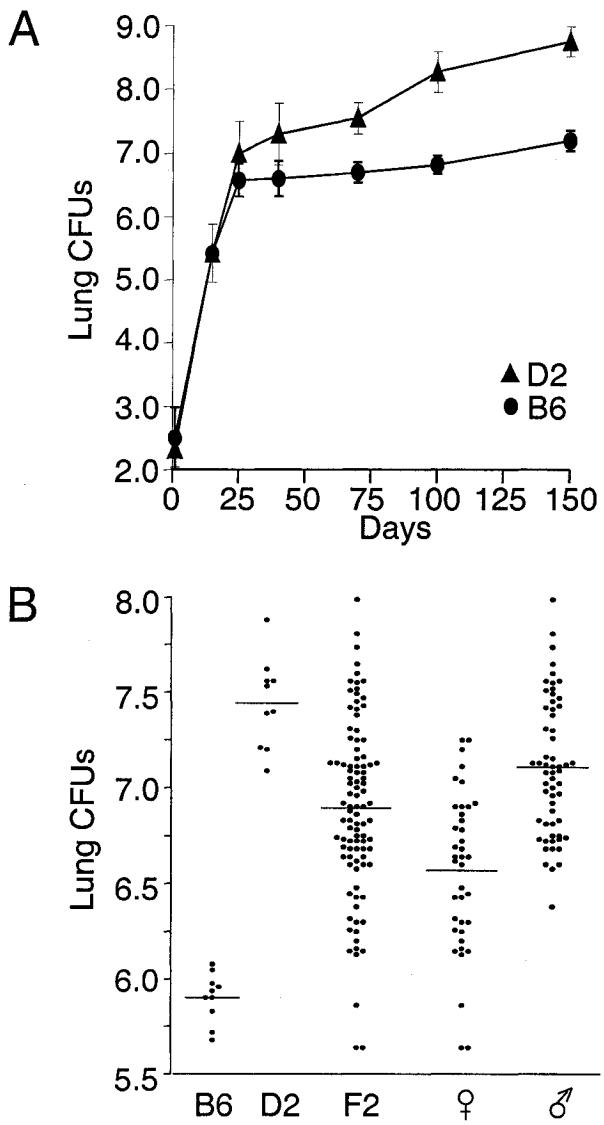

A
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
Lung CFUs
0
25
50
75
100
125
150
Days
D2
B6
B
8.0
7.5
7.0
6.5
6.0
5.5
Lung CFUs
B6
D2
F2
♀
♂

TABLE 1. Distribution of the CFU counts phenotype in the 98 (B6 X D2) F2 population

Trait	n	Mean	SD	Skewness	Kurtosis	Normality test (D)*	Nornmality test p*
CFU counts	98	6.89	0.46	-0.25	0.12	0.068	>0.15
Female CFU counts	39	6.57	0.40	-0.37	-0.35	0.095	>0.15
Male CFU counts	59	7.11	0.35	0.30	-0.62	0.100	>0.14
Sex adjusted CFU counts	98	0.00	0.37	-0.03	-0.43	0.068	>0.15

*Normality tests conducted using the Kolmogorov-Smirnov test as implemented in the SAS computer package (43)

RESULTS

Groups of DBA/2 (D2) and C57BL/6 (B6) mice were infected with 2 X 10^2 CFU of highly virulent *M. tuberculosis* H37Rv by the aerosol route and at predicted times (0 to 150 days) the number of CFUs recovered from infected lungs was determined (Fig. 1A). Pulmonary replication of *M. tuberculosis* in the 2 mouse strains was biphasic. In the early phase (up to 3-4 weeks), the pathogen grew to a similar extent in B6 and D2 lungs. In the second phase (4 weeks to 5 months), the infection was held stationary in B6 mice with only a 2 fold increase in the lung CFUs between day 25 (5 X 10^6) and day 150 (1 X 10^7). Histological analysis has shown that limited growth of *M. tuberculosis* in the lungs of B6 mice late in infection is associated with pathology dominated by macrophages in proximity of large aggregates of lymphoid cells [229]. B6 mice ultimately succumb to infection with a mean survival time of 239 days (range: day 147-292). In contrast, there is progressive replication of *M. tuberculosis* in the lungs of D2 mice between days 25 and 150, with an approximately 50-75 fold increase in lung bacterial load and some associated mortality. Histological examination shows large diffuse lesions, dominated by neutrophils and containing areas of tissue necrosis [227,229,238]. This severe lung pathology leads to early and uniform death in this group with a mean survival time of 102 days (range: day 88-126)(data not shown).

The genetic control of differential lung replication in the late phase of infection was investigated. For this, 98 informative male and female (B6 X D2) F2 animals, as well as D2 and B6 controls were infected by the respiratory route with 2 X 10^2 live *M. tuberculosis* H37Rv, and 90 days later the extent of bacterial replication in the lung was determined (Fig. 1B). The 90 day time point was chosen, because a) parental strains show clear differences in lung CFUs at this time, and b) D2 mice begin dying beyond 90 days. In this experiment, there was a highly significant 50 to 100 fold difference in log CFU counts recovered from susceptible D2, (X =

7.45; range 7.1 to 7.9) when compared to resistant B6 controls (X = 5.9; range 5.6 to 6.1). CFU counts in (B6 X D2) F2 showed a continuous distribution (log CFUs 5.55 to 8.0) between that of resistant B6 and susceptible D2 parents (Fig. 1B) with minor deviations from normality (Table I) [241]. The mean log CFUs in the F2 was at 6.89, a value closer to susceptible D2 than to resistant B6 controls, suggesting that susceptibility does not segregate as a recessive trait in this cross. Comparison of CFU counts in male and female (B6 X D2) F2 mice showed a clear gender effect, with females more resistant to *M. tuberculosis* replication (X = 6.57; range 5.55-7.25) than males (X = 7.11; range 6.35-8.0). No significant deviations from normality were observed for the full or gender-specific distributions of lung CFUs (Table I).

The raw CFU data for the total F2 cross behaves as a quantitative trait amenable to study by quantitative trait locus (QTL) analysis. QTL mapping was carried out by a whole genome scan approach, using a total of 152 polymorphic markers providing an average coverage of ~10cM along each chromosome (http://www-genome.wi.mit.edu/cgi-bin/mouse/index) (Table III). The largest gap was estimated at ~ 18.5 cM for chromosomes 7 and X. Genome-wide multipoint linkage analysis was performed using Mapmaker/EXP version 3.0 and Mapmaker/QTL 1.1 [239] and QTL Cartographer [282]. Results of this analysis are shown as multipoint LOD score traces in Fig. 2A and 2B, and numerical data for individual intervals are shown in Table II. Using raw CFU (log) data as a quantitative trait, one highly significant linkage was identified on the distal portion of Chr. 19, with maximal LOD score obtained for the interval defined by markers *D19Mit91* and *D19Mit54* (χ^2= 25.74; LOD 5.59, p = 0.000005). This level of significance was not observed one time in 50,000 permutations of the data. This QTL overlaps approximately 10cM on Chr. 19, explains 24.1% of the total phenotypic variance in the F2 cross, and was given the designation *Trl-4* (for *T*uberculosis *r*esistance *l*ocus *4*). Several weaker

Figure 2: *Linkage analysis by whole genome scan of susceptibility to infection with M. tuberculosis.* LOD (logarithm of odds ratio) score traces along chromosomes 19 (A) and 7 (B) for which highly significant (LOD > 5.0) and suggestive (LOD >3) QTL controlling bacterial replication of *M. tuberculosis* H37Rv following aerosol infection with 2 X 10^2 CFUs in (B6 X D2) F2 mice were detected. For comparison, LOD score plot for chromosome 7 from an independent genome scan for a significant QTL (*Trl-3*) that controls survival of (B6 X D2) F2 mice following *i.v.* infection with 1 X 10^5 CFUs of *M. tuberculosis* H37Rv is shown (C^{229}). The map positions of microsatellite markers used are indicated and chromosomal lengths are shown to scale.

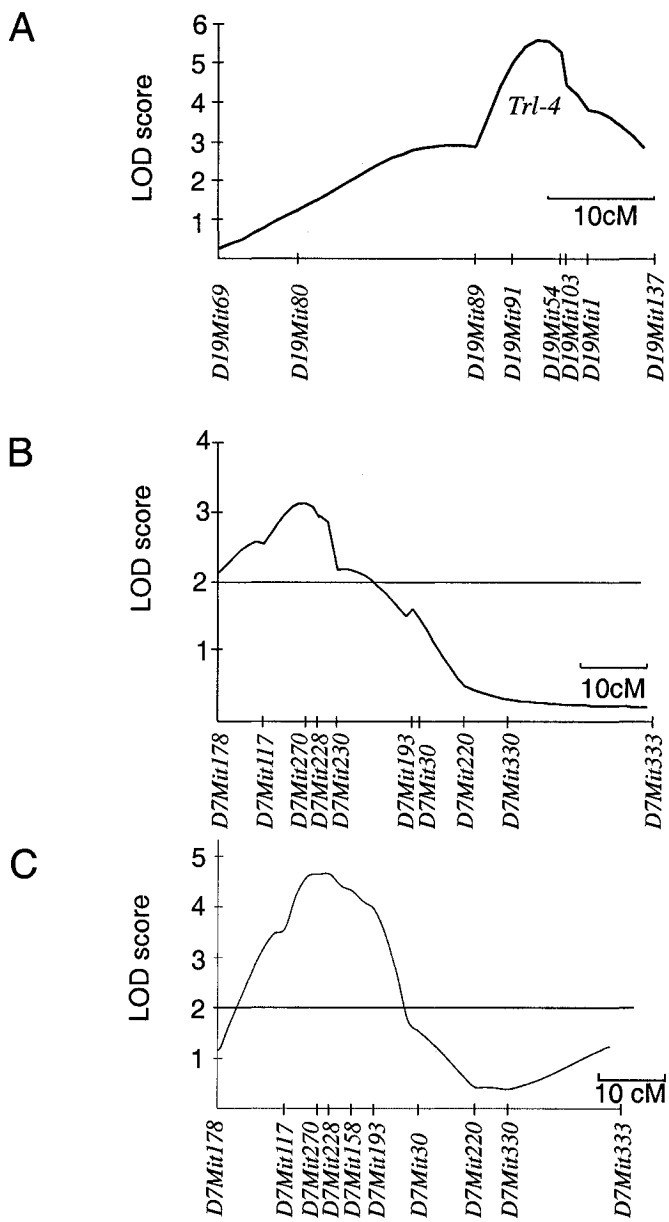

A
LOD score
6
5
4
3
2
1
Trl-4
10cM
D19Mit69
D19Mit80
D19Mit89
D19Mit91
D19Mit54
D19Mit103
D19Mit1
D19Mit137
B
LOD score
4
3
2
1
10cM
D7Mit178
D7Mit117
D7Mit270
D7Mit228
D7Mit230
D7Mit193
D7Mit30
D7Mit220
D7Mit330
D7Mit333
C
LOD score
5
4
3
2
1
10 cM
D7Mit178
D7Mit117
D7Mit270
D7Mit228
D7Mit158
D7Mit193
D7Mit30
D7Mit220
D7Mit330
D7Mit333

TABLE 2. Statistical values for linkages obtained from the (B6 X D2) F2 population

Marker	cM	LOD	χ^2	% Variance explained
CFU counts				
D5Mit297	17.5	3.37	15.50	15.7
D5Mit254	25.1	3.78	17.42	17.6
D5Mit201	28.4	3.24	14.90	14.2
D5Mit95	57.9	2.11	9.71	9.5
D5Mit216	62.3	3.42	15.77	14.8
D5Mit168	66.7	3.12	14.35	14.0
D7Mit117	10.9	2.61	12.01	12.8
D7Mit270	15.3	3.14	14.44	16.2
D7Mit228	16.4	2.85	13.11	12.5
D10Mit194	18.5	3.40	15.83	18.5
D10Mit186	36.1	2.58	11.87	13.1
D19Mit89	28.4	2.85	13.11	13.2
D19Mit91	35.0	5.06	23.28	21.6
D19Mit54	39.3	5.59	25.74	24.1
D19Mit103	40.4	5.44	25.02	22.6
D19Mit1	43.7	4.64	21.34	19.6
Sex Adjusted				
D5Mit297	19.7	2.66	12.24	11.8
D5Mit254	25.1	3.10	14.26	13.5
D5Mit201	28.4	2.89	13.29	12.7
D19Mit91	35.0	3.59	16.51	15.8
D19Mit54	39.3	4.00	18.40	18.0
D19Mit103	40.4	3.88	17.85	16.7
D19Mit1	43.7	3.29	15.13	14.3

Supplementary table

TABLE 3. Polymorphic microsatellite markers used for whole genome scan

D1Mit66, 122, 213, 156, 19, 181, 415, 387, 218, 425, 206, 150, 221	*D12Mit12, 153, 34, 14, 118, 233, 20*
D2Mit1, 81, 367, 91, 436, 164, 401, 225, 311, 200	*D13Mit60, 139, 250, 107, 262, 35*
D3Mit304, 306, 69, 241, 51, 72, 106, 17, 147	*D14Mit207, 45, 5, 39, 193, 265, 266*
D4Mit101, 39, 214, 152, 303, 279, 233, 33	*D15Mit252, 60, 67, 72, 171, 41*
D5Mit180, 297, 233, 254, 201, 309, 155, 157, 406, 95, 216, 168, 222, 409	*D16Mit100, 101, 84,158*
D6Mit86, 33, 9, 103, 135, 14	*D17Mit62, 68, 152, 94, 221*
D7Mit178, 117, 270, 228, 230, 193, 30, 220, 330, 333	*D18Mit94, 89, 33, 213*
D8Mit3, 191, 25, 266, 211, 200, 326	*D19Mit69, 80, 89, 91, 54, 103, 1, 137*
D9Mit188, 66, 154, 300, 196, 12, 19	*DXMit166, 84, 117, 186*
D10Mit 51, 194, 186, 42, 95, 70, 180	Chromosome Y : SRY❖
D11Mit226, 19, 20, 349, 194, 39, 67, 224, 103	

❖Not a polymorphic marker but was used to verify sex.

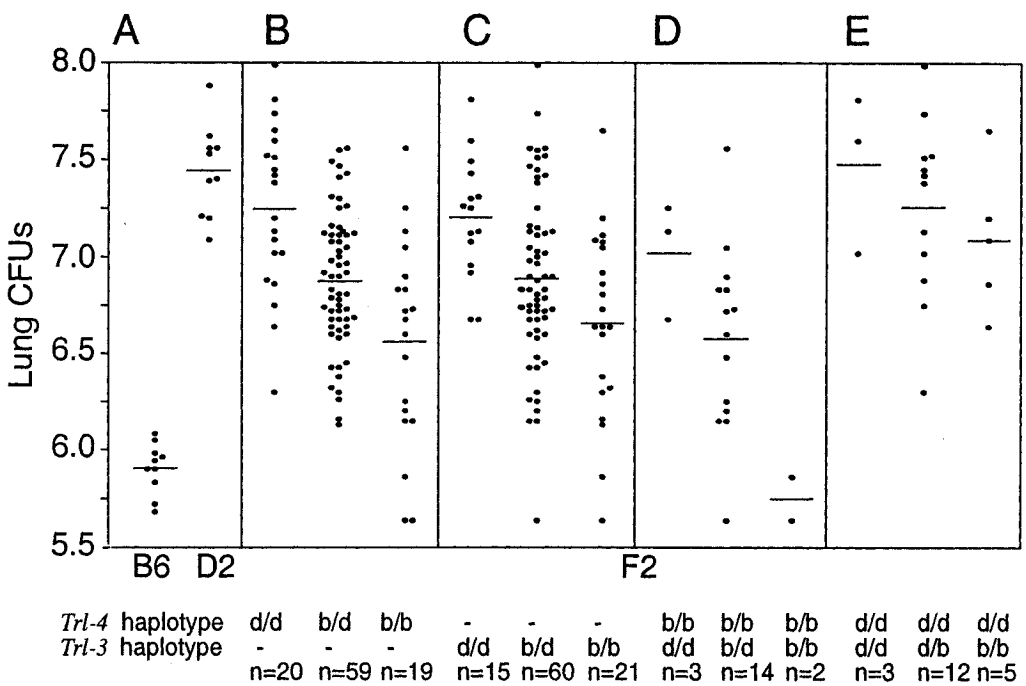
linkages were also detected on proximal (*D5Mit254;* χ^2= 17.42; LOD 3.78, empirical p = 0.0002) and distal (*D5Mit216*; χ^2= 15.77; LOD 3.42, empirical p= 0.04) portions of Chr. 5, Chr. 7 (*D7Mit270*; χ^2= 14.44; LOD 3.14, empirical p = 0.0005), and Chr. 10 (*D10Mit194;* χ^2= 15.83; LOD 3.4, empirical p = 0.02). However, none of these linkages reached genome-wide statistical significance at the 0.01 level.

Possible linkage was initially detected to all Chr. X markers tested (LOD 3.3 to 4.6). However, further analyses failed to reveal significant linkage to Chr. X in either males or females alone when analyzed independently (LOD of 0.25 and 0.81, respectively), suggesting that the original linkage reflects mean differences between genders instead of any Chr. X loci. In this gender-specific analysis, evidence for linkage to *Trl-4* was stronger in males (n = 59; LOD 3.8, empirical p = 0.0002), than in females (n = 39; LOD 1.59, empirical p = 0.02). A likelihood-ratio heterogeneity test (see Methods) indicated that these apparent differences were not statistically significant ($\chi^2(2) = 2(25.7-(17.54+7.37) = 1.66$, p = 0.44) suggesting that the differences in LOD scores between males and females may be due to differences in sample size and random variation. To further explore gender specificity in linkage to Chr. 19 QTL (*Trl-4*), residual CFU values for F2 mice were examined after controlling for sex effects (Table I; see Materials and Methods). This sex-adjusted CFU counts transformation provided a means to retain all data, while it eliminated the effects of baseline sex differences in bacterial replication. Analysis of the sex-adjusted CFUs retained solely the Chr.19 hit as significant (χ^2 =18.40; LOD 4.00; explaining 18% of the phenotypic variance). A result this large was not observed in 50,000 permutations of the data. Conditioning on the genotypes at each of the chromosome X markers genotyped revealed no evidence for improved linkage at any autosomal loci [283]. Thus, the linkage to *Trl-4* did not appear to be sex-specific.

To visualize the effect of parental alleles (D2, d; B6, b) at *Trl-4* on lung CFUs, F2 animals were separated according to their genotype at *D19Mit54* (Fig. 3B). B6 alleles were associated with reduced bacterial replication and were inherited in an incompletely dominant fashion, with mean CFU counts (log) of 7.25 ("d/d" homozygotes), 6.85 ("b/d" heterozygotes), and 6.55 ("b/b" homozygotes) (Fig. 3B). We have previously detected a QTL on Chr. 7 (*Trl-3*) that affects survival of (B6 X D2) F2 mice following *i.v.* injection of 1 X 10^5 *M. tuberculosis* (*D7Mit270;* χ^2= 21.4; LOD 4.66) [229]. In the present study, *D7Mit270* shows suggestive linkage to lung CFUs after aerosol infection (Table II; LOD 3.14; 16.2% of the variance), and alignment of LOD score plots from both studies (Fig. 2B vs. 2C) suggests that *Trl-3* may indeed affect both phenotypes. Thus, F2 animals were also separated according to their genotype for *D7Mit270* (*Trl-3*). Results in Fig. 3C also suggest that "b" alleles at the Chr. 7 linkage are associated with reduced *M. tuberculosis* replication in the lung and are inherited in a co-dominant fashion as previously noted for their effect on survival [229].

Analysis of combined effects of Chr. 19 and Chr. 7 loci on lung CFUs suggested an additive and very strong effect of the two QTLs. Two-loci linkage analysis yielded LOD of 10.09 for the combined QTLs, explaining 38% of the variation in raw CFUs. Regression tests of lung CFUs on both *D7Mit270* and *D19Mit54* did not indicate a significant interaction between the loci (t = -1.46, p = 0.15). However, although few animals were available for analysis, mice homozygous for "b/b" alleles at both loci were completely resistant and showed CFU counts (5.55, 5.80) in the range of B6 controls (range 5.6 to 6.1), while mice homozygous for "d/d" alleles were completely susceptible and showed CFU counts (7.0, 7.6, 7.8) similar to those seen in the susceptible D2 controls (range 7.1 to 7.9) (Fig. 3D, 3E). This effect was specific and was not seen when Chr. 19 haplotypes were analyzed in conjunction with any of the weaker linkages

Figure 3: *Effect of haplotype combination at individual QTLs on M. tuberculosis replication in the lungs.* The effect of parental allele combinations at *Trl-4* (B, *D19Mit54*), *Trl-3* (C, *D7Mit228*) as well as *Trl-3* and *Trl-4* in combination (D and E) on bacterial replication (log10 CFU at day 90 post-infection) is shown. The D2 and B6 parental alleles are identified as (d) and (b), respectively, and the number of animals (n) in each group is shown at the bottom. Each data point represents a single mouse, and horizontal bars indicate mean CFUs in each group. The log10 CFU in the parental B6 and D2 groups are shown in (A) for comparison.


A
B
C
D
E
8.0
7.5
7.0
6.5
6.0
5.5
Lung CFUs
B6
D2
F2
Trl-4 haplotype d/d b/d b/b - - - b/b b/b b/b d/d d/d d/d
Trl-3 haplotype - - - d/d b/d b/b d/d b/d b/b d/d d/b b/b
n=20 n=59 n=19 n=15 n=60 n=21 n=3 n=14 n=2 n=3 n=12 n=5

Chr. 5; Chr. 10) detected in this study (data not shown). Finally, presence of permissive homozygous "d/d" haplotypes at *Trl-4* largely dissipated the protective effect of "b" alleles at *Trl-3*.

DISCUSSION

Compared to other inbred strains, D2 is uniquely sensitive to infection with virulent isolates of human *M. tuberculosis* H37Rv, with a concomitant pathology and outcome that resemble *M. tuberculosis* infection in many AIDS patients. This includes unrestricted pulmonary microbial replication, massive inflammatory response *in situ*, and early death. Differential pulmonary growth of *M. tuberculosis* in B6 and D2 mice is associated with dramatic differences in histopathology starting 3 weeks post-infection. Whereas B6 lungs show well formed granulomas containing *M. tuberculosis* infected epithelioid macrophages in close proximity to aggregates of lymphocytes, D2 lungs show extensive diffuse lesions containing a large number of degenerating neutrophils replete with acid-fast bacilli [227,229,238]. Thus, the genetic control appears to regulate aspects of lung-specific host immune response that are triggered at 3-4 weeks [150,160]. This mechanism may be impaired in D2 (and replaced by a pronounced inflammatory response) possibly through impairment of mononuclear, polymorphonuclear and/or lymphocytic lineages.

To examine the genetic control of susceptibility of D2 mice, we have carried out independent genome scans in informative (B6 X D2) F2 mice. To sample different aspects of host response to infection that may be under unique or common genetic controls, we used different doses (2×10^2, 1×10^5) and routes of infection (aerosol, *i.v.*), and monitored different quantitative measures of susceptibility (bacterial replication in the lung, overall survival time). The major conclusions of these experiments are that 1) the genetic control of susceptibility in D2 is complex, 2) that individual QTLs affecting this trait can be mapped in this experimental setting, with 4 significant loci mapped to date (*Trl-1* to *Trl-4*), and 3) that different infection models reveal different gene effects with little overlap between the different experimental protocols. In a first genome scan [229], we infected (B6 X D2) F2 mice with 1×10^5 *M. tuberculosis* H37Rv by the *i.v.* route and used time of survival (log) as a quantitative measure of

susceptibility. The genetic control was found to be complex, with 2 significant linkages mapping on distal Chr. 1 (*Trl-1*; LOD 4.8) and proximal Chr. 7 (*Trl-3*; LOD 4.7), each accounting for 21% of the phenotypic variance. A third suggestive linkage was mapped to proximal Chr. 3 (*Trl-2*; LOD 3.9) [229]. In the present study, *M. tuberculosis* was introduced by the respiratory route (which closely resembles the mode of infection in humans) and the extent of pulmonary replication (log CFUs at day 90) was used as a quantitative measure of susceptibility. QTL mapping using either raw or sex adjusted CFU counts revealed a highly significant linkage on Chr. 19 (*Trl-4*; *D19Mit91* and *D19Mit54*; χ^2= 25.67; LOD 5.58, p = 0.000005), which explains 24.1% of the total phenotypic variance in the F2 cross. The *Trl-4* QTL is novel and distinct from other QTLs previously mapped in murine models of *M. tuberculosis* infection, including the *sst1* locus (*susceptibility to tuberculosis 1*) [280] originally mapped on Chr. 1 (*D1Mit49*) in a (C3H X C57BL/6) F2 cross, and which regulates pulmonary growth, inflammatory response, and overall survival following *i.v.* infection with high dose (1 X 10^6 *i.v.*) of *M. tuberculosis*. *Trl-4* is also distinct from the QTLs mapped by Lavebratt et al. [236], on Chrs. 3, 5, 9, and 10, that regulate in a gender-specific fashion differential body weight loss following *i.v.* infection with high doses of *M. tuberculosis* (> 10^6 CFU). Importantly, *Trl-4* is the first locus mapped to date that regulates replication of *M. tuberculosis* in the lung following aerosol infection with small numbers of the pathogen.

The interval for *Trl-4* on Chr. 19 is ~ 10cM, and contains an estimated 70 transcription units (data not shown). Several of these may be potential candidates by virtue of their established role in host immune and inflammatory responses. *Trl-4* maps to a QTL designated *Pgia12* [284], previously shown to control onset of arthritis induced by injection of human cartilage-derived proteoglycan (PGIA), a known mouse model of human rheumatoid arthritis. The relationship

between *Trl-4* and *Pgia12* is unknown, but it is interesting to note that both QTLs seem to affect host inflammatory responses. The *Trl-4* interval also contains the *NF-κB* (position 45.8cM) and *Ikkα* (*chuk*; position 45cM). *NF-κB* (p52) is a subunit of the *NF-κB* factors, a group of transcription factors implicated in the induction of numerous genes in response to inflammatory stimuli, as well as pathogen-derived or stress signals (LPS, IL-1 or TNF-α) [285]. Mouse mutants lacking functional *NF-κB* (p52) show absence of B cell follicles in secondary lymphoid organs and cannot produce antibodies to T-dependent antigens [286]. *NF-κB* mutant mice become susceptible to *Leishmania major* infection which is associated with uncontrolled parasite replication, non-healing lesions, and failure to develop an IFN-γ response [287]. *NF-κB* mutant mice are also susceptible to *Toxoplasma gondii* [288]. Ikkα kinases that phosphorylate IκBs, and thus acts as a regulatory subunit of NF-κB factors. *Ikkα* mutant mice (studied in chimeras) show a phenotype similar to *NF-κB* mutants, with respect to impaired B-cell function [289]. Finally, the *Trl-4* region also contains the α chain of the cell surface receptor for granulocyte-macrophage colony-stimulating factor (GM-CSF), known as GM-CSFR α (*CSF2rα*; position 51cM). GM-CSF, IL-3 and IL-5 are related cytokines that bind to cell surface receptors composed of a cytokine-specific α chain and a β chain common to the 3 receptors [290]. GM-CSF acts as a growth factor for macrophages and granulocytes, and GM-CSF mutant mice show increased susceptibility to pneumonia caused by *Pasteurella pneumotropica*, group B *Streptococcus*, *Penumocytis carinii*, and others [291-294]. They also display reduced pathogen killing by isolated alveolar macrophages [295], which is concomitant to exaggerated inflammatory response in the lungs [293]. Importantly, the only "constitutive" phenotype displayed by GM-CSF and GM-CSFRβ mutant mice is "pulmonary alveolar proteinosis" (PAP), a condition characterized by abnormal catabolism of lung surfactant by lung epithelial cells and by alveolar macrophages [290]. Together with the recent discovery of a GM-CSFRβ mutation in a human PAP patient [296], these findings

suggest that GM-CSF plays a major role in lung surfactant homeostasis by these cells. Interestingly, both alveolar type II epithelial cells and alveolar macrophages are invaded *in vivo* by *M. tuberculosis* [297], and are key hosts to this pathogen early in infection.

In 2 genome scans conducted to date in (B6 X D2) F2 mice (scan 1: *i.v.* infection, survival; scan 2: aerosol, lung replication), *Trl-4* constitutes the strongest linkage identified to date with a LOD ~ 5.6. Interestingly, the *Trl-4* linkage was only detected in scan 2, while the Chr. 1 (*Trl-1*) and Chr. 3 (*Trl-2*) QTLs were only detected in scan 1. This suggests that *Trl-4* may affect pulmonary replication *per se* (after aerosol infection), while *Trl-1/Trl-2* may influence time of death in the presence of high lung bacterial load. Alternatively, *Trl-1/2/4* may be statistical accidents that await validation in larger groups of mice of the same cross or of a different cross. This explanation is unlikely for *Trl-4* which reaches a very high degree of significance and which explains a large proportion of the phenotypic variance in the cross. Thus, we believe that *Trl-4* is a major determinant of *M. tuberculosis* replication in the lungs, following infection by the respiratory route. The *Trl-3* QTL was the only QTL detected in both scans 1 [229] and 2 (this study). The observation that in both scans the *Trl-3* alleles of B6 are protective and inherited in a co-dominant fashion suggests that the gene effect is real and thus this QTL affects both the extent of *M. tuberculosis* replication (scan 2) and the survival to infection (scan 1). Furthermore, a major additive effect of *Trl-3* and *Trl-4* on pulmonary replication of *M. tuberculosis* (LOD 10.09; ~40% of variance) was detected in scan 2 (this study). Remarkably, and although only a few animals were available for study, mice homozygous for B6 alleles at *Trl-3/Trl-4* were completely resistant to infection and phenotypically undistinguishable from B6 parents. Also, mice homozygous for D2 alleles at both loci were completely susceptible and similar to susceptible D2 controls (Fig. 3D, 3E). The independent and combined contribution of *Trl-3* and *Trl-4* to

regulation of *M. tuberculosis* replication in the lung is currently being investigated in congenic mice.

The Chr. 19 *Trl-4* region is syntenic with human 10q, while the Chr.7 *Trl-3* is syntenic with human 19q13. A possible association of these chromosomal regions with susceptibility to TB in humans can now be tested in population studies from areas where the disease is endemic.

The next step is to identify the genes underlying our QTL's. Expression profiling can identify potential candidates for the *Trl 1/2/3/4* regions, validate cell type involved as well as suggest possible cellular pathways differentially regulated in the lugs of B6 and D2 mice

Chapter 4

Multistage Gene Expression Profiling in A Differentially Susceptible Mouse Model of Tuberculosis Infection

ABSTRACT

The reasons underlying the differential response and the molecular mechanisms involved during infection with *Mycobacterium tuberculosis* remain unclear between tuberculosis susceptible DBA/2 (D2) and tuberculosis resistant C57BL/6 (B6) mice. In this study, microarray analysis was employed in order to 1) analyze genome-wide gene expression pre- and post- infection to better understand the molecular process of disease and 2) identify candidate genes located within the *Trl-1/2/3/4* regions formerly identified. Total RNA was isolated from lungs of B6 and D2 mice at day 0, 1, 20, and 70 post-aerosol infection with 10^2 *M. tuberculosis* H37Rv. RNA was reverse transcribed and cDNA samples labeled with Cy3 and Cy5 were hybridized to a glass chip containing 15250 mouse cDNA duplicate clones. Using the one class algorithm in Significance Analysis of Microarrays (SAM) with a false discovery rate below 5%, 56 transcripts were found to be differentially expressed in D2 vs. B6 prior to *M. tuberculosis* infection. A different set of 24 transcripts were found to be differentially expressed between B6 and D2 at 70 days post infection using a two-class, unpaired data algorithm in SAM. Overall our data suggests that the classical complement pathway and the apoptotic pathway seem to play a role in differential susceptibility between B6 and D2 mice, with these pathways being upregulated in B6. Moreover the increase in neutrophil associated gene expression (S100A9, Gro1) corroborates with our previous histological finding of increased neutrophil cell counts in D2 mice. Finally these studies have provided genes that map in the *Trl-1* and *Trl-4* regions (*Cfh1* and *Sdf4* respectively) that need to be further studied.

INTRODUCTION

The global prevalence of *Mycobacterium tuberculosis* infection was recently estimated at 32% (1.9 billion people), with an evaluated 8 million new cases of active tuberculosis (TB) and 2 million deaths in 1999 [271]. A significant host genetic component to variable susceptibility to tuberculosis has been demonstrated in epidemiological data pointing to sex [298,299], racial differences [300], geographical distribution and familial aggregation[95], first contact epidemics [86,87] and studies in twins [90,92,301]. Therefore, several independent studies strongly support the fact that tuberculosis has an inheritable component in humans but this is also seen in murine models of infection.

Among inbred mouse strains, C57BL/6 (B6) has been found to be resistant to *M. tuberculosis* infection while DBA/2 (D2) mice are susceptible. In D2, a heavier bacterial burden causes very severe and rapidly fatal pulmonary disease with extensive exudation of neutrophils and tissue necrosis, as opposed to slower progressive pulmonary disease characterized by the accumulation of epithelioid macrophages with protective immune and inflammatory responses in B6 [302]. These inbred murine strains have been used for quantitative trait loci (QTL) mapping and it has been determined that susceptibility to tuberculosis infection is polygenic. Four major tuberculosis resistance genes termed *Trl1/2/3/4* for Tuberculosis resistant loci have been localized on mouse Chr 1/3/7/19 respectively. Three QTLs are responsible for overall survival to *M. tuberculosis* infection (*Trl-1/2/3*) whereas two are responsible for bacterial replication in the lung (*Trl-3/4*). However, the gene identity of each QTL and the molecular mechanism whereby each QTL gene acts to regulate susceptibility has not yet been identified.

Identifying the genes underlying QTLs has been difficult, but occasionally successful, the first one having been identify is the *APP* gene in Alzheimer's disease in

1991 (reviewed in [303]). QTL mapping in mouse may be useful to find genes responsible for various aspects of human disease. However, one major difficulty of the QTL approach is the rather large genomic regions that define a QTL, as anywhere from tens to hundreds of genes may reside in a locus, or *a priori* candidate genes based on a biological model do not exist in the region. Trying to narrow down this QTL region and identify candidate genes has proven to be difficult. In addition, for a complex disease, many loci may contribute to the phenotype. With additional sources of information about the genes that reside in a genomic interval, such as gene expression, it may be possible to identify potential candidates.

Global expression analysis of approximately 15 250 genes was utilized to identify genes differentially expressed between the lungs of B6 and D2 mice prior to and during infection with *M. tuberculosis*. Our goal is to get insight into the physiological response pathway (inflammatory, immune) and cell populations (macrophages, lymphocytes, others) involved. Numerous genes involved in immune response, and complement activation were identified post-infection, and apoptotic pathway pre-infection providing potentially novel insights into mechanisms of tuberculosis susceptibility. Microarray analysis, combined with QTL analysis, can be employed to identify candidate genes for polygenic diseases.

MATERIALS AND METHODS

Animals

Inbred, pathogen free, 8-week-old C57BL/6J (B6) and DBA/2J (D2) mice were purchased from the Trudeau Institute Animal Breeding Facility.

Mycobacteria

M. tuberculosis strain H37Rv (TMC #102) was obtained from the Trudeau Mycobacterial culture collection as a frozen (-70° C) log phase dispersed culture in Proskauer and Beck medium (Difco Laboratories Inc., Detroit MI) containing 0.01%Tween 80. For each experiment, a vial was thawed, subjected to 5 sec ultrasound to brake up aggregates, and diluted appropriately in PBS containing 0.01% Tween 80. Mice (8-10 weeks of age) were inoculated with 2 X 10^2 CFU by aerosol in a Middlebrook airborne infection apparatus (Tri instruments, Jamaica, NY). Two mice were sacrificed at each time point: at 1, 20 and 70 days.

RNA extraction

Total cellular RNA was extracted from lung using a commercial TRIZOL reagent and following the manufacturer's recommended protocol (Invitrogen, Burlington, Ontario). Tissues were snap frozen in liquid nitrogen, and 100 mg of tissue was homogenized by mechanical disruption with a Polytron (Brinkmann Instruments, Mississauga, Ontario), in a final 1ml volume of TRIZOL reagent. The samples were incubated for 5 min at room temperature, followed by chloroform extraction. The aqueous

phase was removed and nucleic acids were precipitated with isopropanol. Pellets were washed with 75% ethanol, and dissolved in ribonuclease-free water treated with diethlypyrocarbonate (0.1%). RNA integrity and yield was assessed using Agilent BioAnalyser RNA LabChips and by electrophoresis on formaldehyde-containing agarose gel prior to use.

RNA labelling

15-25 μg of total RNA was converted into cDNA using reverse transcriptase (Super Script II, Invitrogen) and either Cy5- or Cy3-labeled dCTP (1 mM, Perkin Elmer-Cetus/NEN) in a reaction mixture containing 1.5 μl oligo(dT) (100 pmol/μl), 3 μl dNTP-dCTP (6.67 mM each), 1 μl dCTP (2 mM), 4 μl dithiothreitol (100 mM) and 8 μl 5 X RT Buffer (Invitrogen). The reactions were carried out at 42 °C for 3 h, and the RNA was then degraded by the addition of 0.5 μl RNase A (1 μg/μl) and 1.5 μl RNaseH (5 units/μl). We separated labeled cDNA from unincorporated nucleotides and further concentrated it by evaporation under vacuum.

Hybridization

We used labeled cDNA to hybridize Mouse 15k v.1 and 3 cDNA spotted arrays purchased from the UHN Microarray Facility (J. Woodgett, Toronto, Ontario) containing 15 250 expressed-sequence tags (NIA clone set) spotted in duplicate. Briefly, the arrays were prehybridized for 1–2 h with DIGEasy hybridization buffer (Roche) containing 10 g/ml denatured salmon sperm DNA and 10 g/ml yeast tRNA. We combined the Cy5- and

Cy3-labeled cDNAs, hybridized them in the same medium and incubated them with the arrays for 16–18 h at 37 °C. Finally, we washed the arrays three times for 10 min each in 0.1 saline sodium citrate (SSC; 20 SSC is 3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) and 0.1% SDS at 50 °C and four times for 3 min each in 0.1 SSC at room temperature and then dried them by centrifugation. We scanned the slides, acquired digitized images using a ScanArray 5000 instrument (Perkin Elmer) and quantified the intensity of individual spots from 16-bit TIFF images using the QuantArray software package (Perkin Elmer)

Microarray data analysis

We normalized the raw data generated by QuantArray (Perkin Elmer) using the GeneSpring software package (Silicon Genetics) by the Lowess scatter smoothing algorithm. We analyzed eight hybridizations consisting of dye-swap hybridizations of four biological replicates for D2 versus B6 at day 0. All other hybridizations consisted of technical replicates for day 1, 20 and 70; with 4 hybridizations (dye swaps of two technical replicates) D2 versus B6 at day 1, 6 hybridization (3 technical replicates) for day 20 and 4 hybridizations (2 technical replicates) for day 70. We used tissues from individual mice for each biological replicate, and a pool of two tissues for all technical replicates. We identified genes with reproducible changes in transcript abundance with the 'one class' algorithm in the SAM (Significance Analysis of Microarrays) [304] application. SAM assigns a score to each gene on the basis of the change in expression relative to the standard deviation of repeated measurements for that gene. We chose significant genes with a false discovery rate below 5% for day 0. Furthermore, significant

genes at day 1 to day 70 were obtained by comparing their ratios to the ratios at day 0. Genes were selected by using a two-class, unpaired data algorithm in SAM. These genes have at least 1.5-fold difference with day 0 and a false discovery rate below 5%. The results were visualized with GeneSpring.

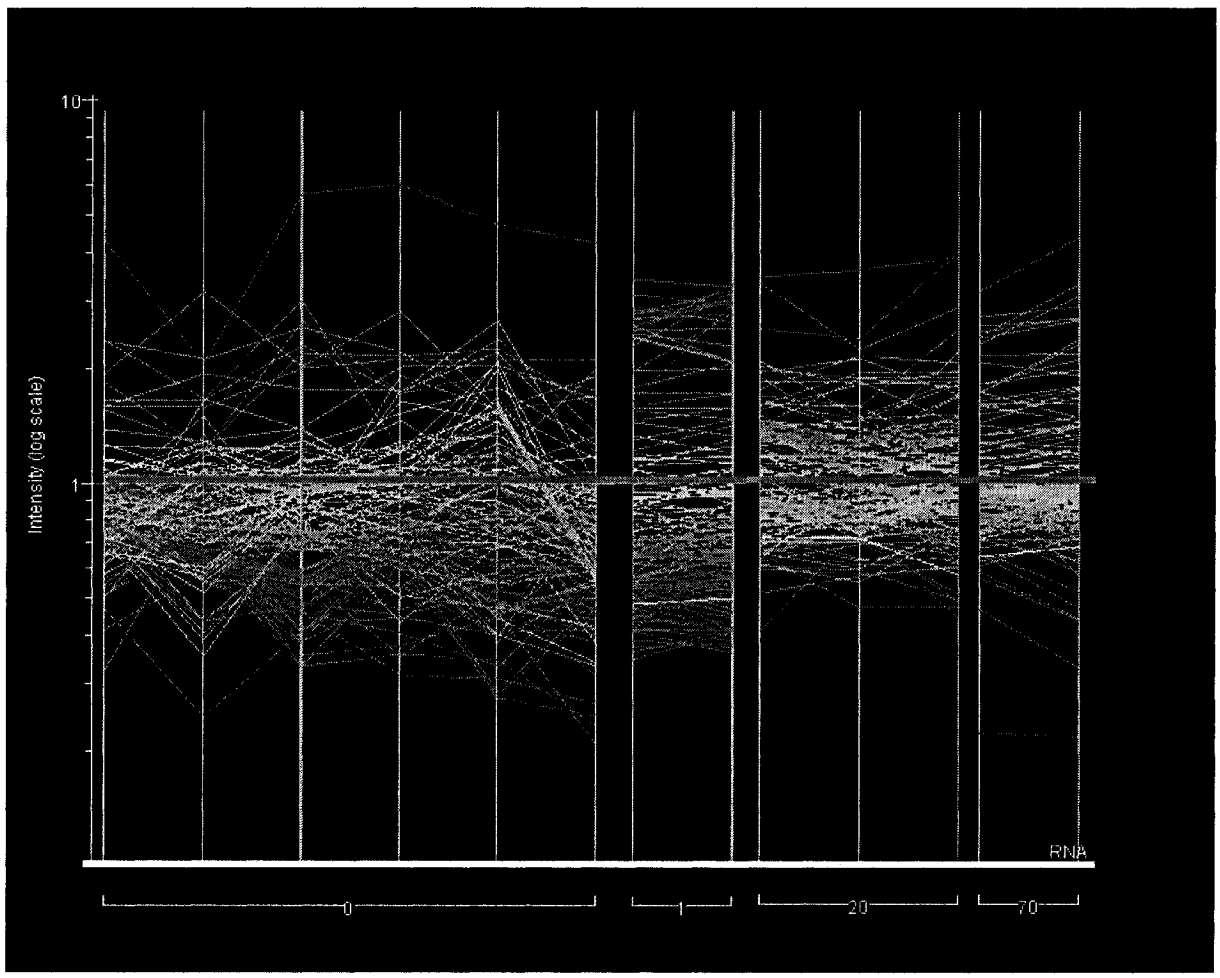
RESULTS

Groups of D2 and B6 mice were infected with 2 X 10^2 CFU of highly virulent *M. tuberculosis* H37Rv by the aerosol route and were sacrificed at different time points, in order to collect their lungs. Mice were sacrificed at day 0 (used as control), day 1 (used to see the immediate response), day 20 (immunity reaches a plateau and stationary infection begins) and day 70. The 70 day time point was chosen, because a) parental strains show clear interstrain differences in lung CFUs at this time, b) D2 mice begin dying beyond 70 days and c) highly significant 50 to 100 fold difference in log CFU counts are recovered from susceptible D2 when compared to resistant B6 lungs. RNA was then prepared from lungs of B6 and D2 mice.

These RNAs passed quality control (RNA integrity and yield was assessed using Agilent BioAnalyser RNA LabChips and by electrophoresis on formaldehyde-containing agarose gel prior to use) and were used to synthesize Cy3- or Cy5-labeled cDNAs that were then hybridized on cDNA microarrays. Reproducibility of mRNA expression profiles was assessed. The variability of expression profiles in individual lungs of day 0 mice was assessed by comparing expression profiles obtained from control subjects to each other or pools at day 1 through 70 (Figure 1). Identification of differentially regulated transcripts in D2 vs B6 mice was then looked at through two different angles.

Firstly, of the 15 250 spotted cDNAs contained on the mouse chip used in this study, we looked at transcripts that are differentially expressed between resistant B6 and susceptible D2 mice at day 0 (pre-infection). We were interested in genes that were differentially expressed at day 0 and whose change in transcript abundance was maintained throughout time. Genes were selected with the one class algorithm in SAM

Figure 1. Technical reproducibility of hybridization experiments. Fluorescence ratios were obtained for a random subset of significant genes independently at day 0,1, 20 and 70 post-infection. The ratios are compared for multiple hybridization RNAs hybridized to 8 (day 0), 4 (days 1, 70) or 6 (day 20) independent chips.


10
1
Intensity (log scale)
RNA
0
1
20
70

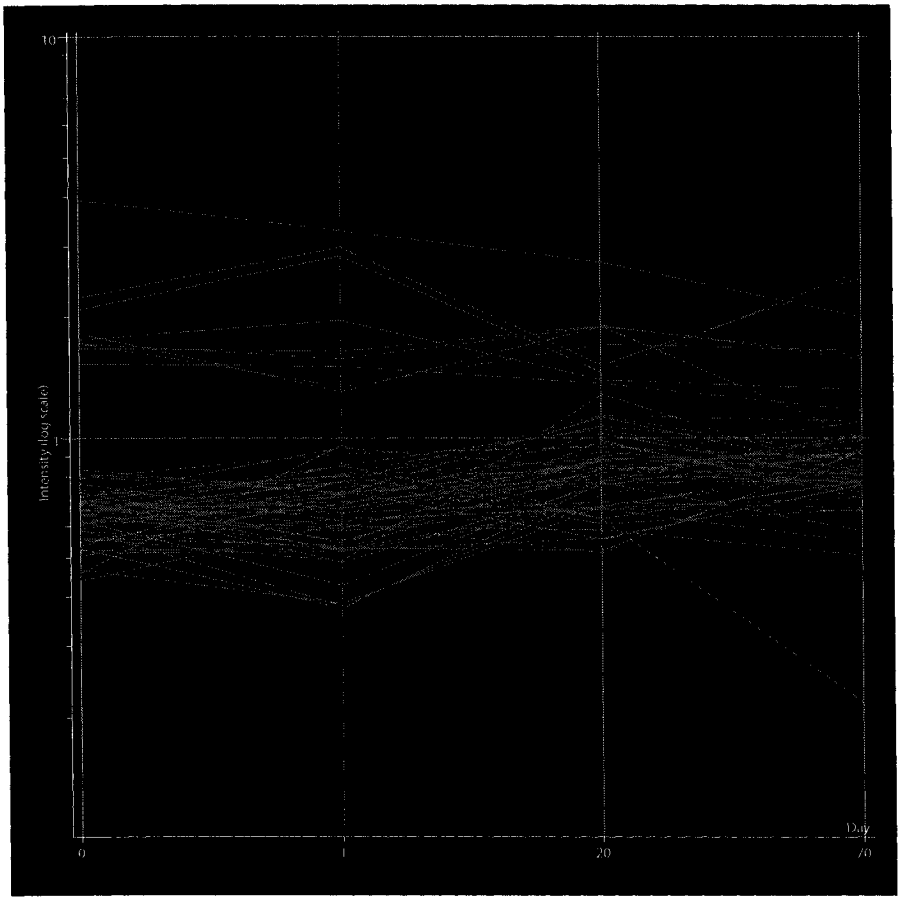
with a False Discovery Rate < 5%. Fifty-six transcripts had significantly different expression levels between B6 and D2 and all results are shown as a DBA/B6 ratio (Figure 2A and corresponding gene list Table 1). Of the 56 transcripts, 9 were up- regulated in D2 (colored in red) or down- (47 colored in green) regulated in D2 as compared to B6 (Figure2A). These transcripts were then grouped in 10 functional categories (Table 2). An additional 29 transcripts, including genes and ESTs, were differentially expressed but could not be functionally classified. Of the transcripts that were either up- or down-regulated in D2 as compared to B6, 5 were related to transcription, 5 to metabolism, and 4 to biosynthesis. Other genes were notably related to replication (2), cell adhesion (2) and signal transduction (2). These genes depict strain specific differences, innate differences, which are not affected from the infection process. These genes may not have a role in the differential susceptibility to tuberculosis but one cannot completely assume that these 56 transcripts do not play a role in the differential susceptibility of tuberculosis between B6 and D2 mice despite the lack of gene expression change following infection. In these innate differences we see an increase in genes involved in apoptosis in B6 mice.

Secondly we were interested in finding genes that were significantly different between B6 and D2 that are infection specific at day 70 i.e. genes that are differentially expressed between D2 and B6 by a minimum of 1.5 fold (FDR<5%) at day 70 and that are not strain specific differences (not detected at day 0). For this, 24 transcripts were selected by using a two-class, unpaired data algorithm in SAM (Table 3, Figure 2B). The list contains 24 transcripts, 20 that are up regulated in D2 (colored in red) and 4 up regulated in B6 (colored in green). Genes were once again classified in different functional classes that differ from the strain specific gene classes. Amongst those classes

we have the complement component class which contains 3 genes (C1qc, C1qb, C1S like), immune response class (containing S100a9, Gro1) and other genes involved in blood coagulation, transport and protein binding (Table 3). Overall our data shows that the complement pathway plays a significant role in differential susceptibility to infection. Moreover, our data suggests a neutrophil involvement in D2 mice.

Figure 2. Genes differentially modulated in B6 and D2 mice. **A.** Genes differentially modulated across time in B6 and D2 mice independently of infection. Expression profile of a subset of 56 genes that are differentially expressed in D2 vs. B6 prior to infection, and which ratio does not change during *M. tuberculosis* infection (red, overexpressed in D2; green, underexpressed in D2) Genes were selected with the one class algorithm in SAM with a false discovery rate below 5%. **B.** Genes differentially modulated in B6 and D2 mice after aerosol infection with 10^2 *M. tuberculosis* H37Rv. Genes overexpressed in D2 are colored in red and those overexpressed in B6 are colored in green. Significant genes at t= 70 were obtained by comparing their ratios to the ratios at t=0 and have at least 1.5-fold difference with t=0 and a false discovery rate below 5%, using a two-class unpaired data algorithm in SAM.

A


10
1
Intensity (log scale)
0
1
20
70
Day

B

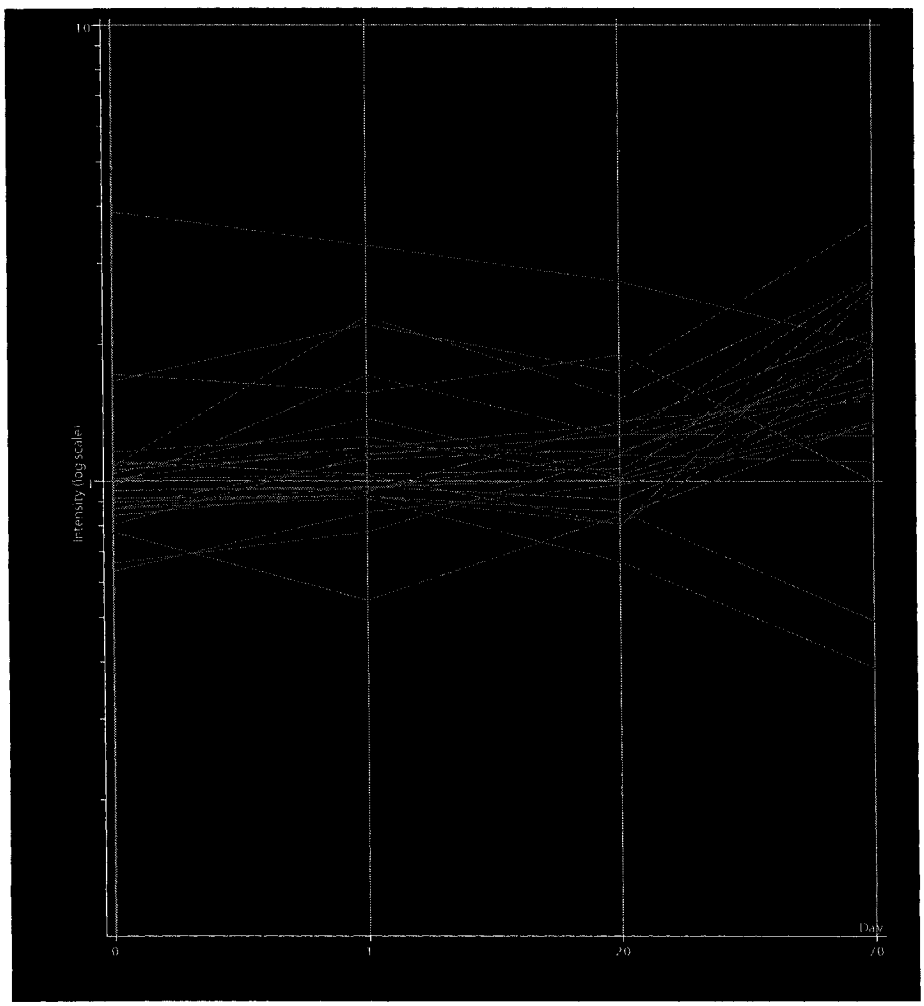

10
1
Intensity (log scale)
0
1
20
70
Day

Table 1. Gene expression differences between DBA/2 and C57BL/J mice (raw data)

0 Normalized	1 Normalized	20 Normalized	70 Normalized	Common	Genbank	Map	Description
1.53	1.50	1.37	1.17		AW556467		Mus musculus 10, 11 days embryo whole body ,t-complex-associated testis expressed 3
1.67	1.63	1.90	1.57	1110008E19Rik	AW539494	8	RIKEN cDNA 1110008E19 gene
1.82	1.30	1.89	1.60	Alad	AW539820	4 30.6 cM	aminolevulinate, delta-, dehydratase
3.90	3.29	2.74	2.00		AW550806		Mus musculus, clone IMAGE:4191452, mRNA,
1.76	1.97	1.40	1.32	Cd209c-pending	AW539111	8	CD209c antigen
1.72	1.56	1.89	1.00		AW538548		Mus musculus, Similar to phosphatidylserine decarboxylase
2.24	2.98	1.51	2.51		AU040774	12	AU040774 Mouse four-cell-embryo cDNA Mus musculus cDNA
2.08	2.85	1.45	2.61	4932443L08Rik	AU040460	3	RIKEN cDNA 4932443L08 gene
1.72	1.34	1.71	1.64				No Hits Found
0.58	0.52	0.67	0.81	Pttg1	AU015203	11	pituitary tumor-transforming 1
0.65	0.78	0.85	0.95	5730557K01Rik	AW551740	8	RIKEN cDNA 5730557K01 gene
0.66	0.77	1.14	1.10	Rex3	AU015646	X 57.5 cM	reduced expression 3
0.47	0.38	0.67	0.86		AU024765		AU024765 Mouse unfertilized egg cDNA Mus musculus cDNA
0.62	0.66	0.76	0.78	AA415817	AW554943	16	expressed sequence AA415817
0.76	0.62	0.67	0.83	Mad5	AW556185	2	Max dimerization protein 5
0.44	0.61	0.51	0.93				Mus musculus DNA, 3' flanking region of interleukin 12 receptor beta
0.64	0.53	0.52	0.92	Trim30	AU022388	7 50.4 cM	tripartite motif protein 30
0.52	0.52	0.66	0.55	AW550831	AW550831	1	expressed sequence AW550831
0.55	0.68	0.81	0.78	Tjp2	AU044024	10	tight junction protein 2
0.80	0.73	0.79	1.03		AW547635		EST
0.70	0.81	1.07	0.93	AI115388	AW553758	6	EST AI115388
0.66	0.69	0.77	1.01	Hdc	AU042518	2 71.0 cM	histidine decarboxylase cluster
0.64	0.85	1.06	0.77	1300002F13Rik	AW543971	10	RIKEN cDNA 1300002F13 gene
0.64	0.71	0.87	0.99	Cfh	AW556041	1 74.1 cM	complement component factor h
0.79	0.91	0.95	0.89	Sdf4	C78930	10	stromal cell derived factor 4
0.81	0.75	0.86	0.77	Eif3	C78481	19	eukaryotic translation initiation factor 3
0.61	0.80	0.89	0.91	Bach-pending	AW550836	4	brain acyl-CoA hydrolase
0.71	0.94	0.83	0.92	2310051E17Rik	AW536738	8	RIKEN cDNA 2310051E17 gene
0.74	0.66	0.88	0.85		AW547791	11	Mus musculus, Similar to mutL (E. coli) homolog 3
0.71	0.80	0.97	0.73	Klf4	AU018863	4 19.7 cM	Kruppel-like factor 4 (gut)
0.62	0.58	0.67	0.71	Arl6ip	AW537237	10	ADP-ribosylation-like factor 6 interacting protein
0.53	0.37	0.77	0.58				Mus musculus solute carrier family 34 (sodium phosphate), member 2
0.54	0.78	0.61	0.66		AW544134		Mus musculus, clone IMAGE:1265114, mRNA, partial cds
0.55	0.63	0.66	0.65				
0.83	0.72	0.84	0.74	1810045K07Rik	AW538602	3	RIKEN cDNA 1810045K07 gene
0.50	0.72	0.92	0.81	AW539211	AW539211	3	expressed sequence AW539211
0.46	0.96	0.62	0.22	AI326478	AU015491	12	expressed sequence AI326478
0.64	0.59	0.83	0.88		AW558849		Mus musculus, Similar to hypothetical protein FLJ13213, clone MGC:28555 IMAGE:4206928, mRNA, complete cds
0.66	0.64	0.87	0.90	Ctsc	C86712	7 D3-E1.1	cathepsin C
0.69	0.65	0.69	0.66	Arl6ip	AU044379	10	ADP-ribosylation-like factor 6 interacting protein
0.68	0.68	1.03	0.78		AW558717		ESTs, Moderately similar to Z298_HUMAN ZINC FINGER PROTEIN 298 (PR-DOMAIN ZINC FINGER PROTEIN 15) [H.sapiens]
0.59	0.72	1.13	0.89	Ppid	AA407023	10	peptidylprolyl isomerase D (cyclophilin D)
0.70	0.60	0.59	0.51	Sh3bgrl3	AW538026	4	SH3 domain binding glutamic acid-rich protein-like 3
0.66	0.73	1.12	0.82	Scd2	AW536336	19 43.0 cM	stearoyl-Coenzyme A desaturase 2
0.60	0.73	0.55	0.76				No Hits Found
0.68	0.55	0.82	0.99	P4ha1	AW548258	4	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide
0.59	0.38	0.72	0.93	Snta1	AW536987	2 84.0 cM	syntrophin, acidic 1
0.62	0.42	0.78	0.83	4921517N04Rik	AA410046	10	RIKEN cDNA 4921517N04 gene
0.69	0.85	0.63	0.77				No Hits Found
0.56	0.54	0.98	0.70	D5Ertd227e	C79033	5 31.0 cM	DNA segment, Chr 5, ERATO Doi 227, expressed
0.69	0.52	0.56	0.76	1200015M12Rik	AU022808	3	RIKEN cDNA 1200015M12 gene
0.56	0.49	1.28	0.80	Gpx3	AW550656	11 B3-B5	glutathione peroxidase 3
0.52	0.52	0.84	0.81	Sftpd	AU042350	14 14.0 cM	surfactant associated protein D
0.73	0.54	0.90	1.10	Fbxl5	AW538739	5	f-box and leucine-rich repeat protein 5
0.68	0.80	0.63	0.78	1200015M12Rik	C87085	3	RIKEN cDNA 1200015M12 gene
0.69	0.49	0.76	0.92				Mus musculus mitochondrion, complete genome

Table 2. Gene expression differences between DBA/2 and C57BL/J mice (classified)

Day 0	Day 1	Day 20	Day 70	Common	Genbank	Map	Description
Metabolism							
1.72	1.56	1.89	1.00		AW538548		Mus musculus, Similar to phosphatidylserine decarboxylase
0.68	0.55	0.82	0.99	P4ha1	AW548258	4	procollagen-proline, 2-oxoglutarate 4-dioxygenase, alpha 1 polypeptide
0.66	0.69	0.77	1.01	Hdc	AU042518	2 71.0 cM	histidine decarboxylase cluster
0.61	0.80	0.89	0.91	Bach	AW550836	4	brain acyl-CoA hydrolase
0.59	0.72	1.13	0.89	Ppid	AA407023	10	peptidylprolyl isomerase D (cyclophilin D)
Transcription							
0.76	0.62	0.67	0.83	Mad5	AW556185	2	Max dimerization protein 5
0.71	0.80	0.97	0.73	Klf4	AU018863	4 19.7 cM	Kruppel-like factor 4 (gut)
0.71	0.94	0.83	0.92	2310051E17Rik	AW536738	8	RIKEN cDNA 2310051E17 gene, Kruppel-like factor 9 (Klf9)
0.65	0.78	0.85	0.95	5730557K01Rik	AW551740	8	RIKEN cDNA 5730557K01 gene (PRDM16)
0.64	0.53	0.52	0.92	Trim30	AU022388	7 50.4 cM	tripartite motif protein 30
Biosynthesis							
1.82	1.30	1.89	1.60	Alad	AW539820	4 30.6 cM	aminolevulinate, delta-, dehydratase
0.81	0.75	0.86	0.77	Eif3	C78481	19	eukaryotic translation initiation factor 3
0.66	0.73	1.12	0.82	Scd2	AW536336	19 43.0 cM	stearoyl-Coenzyme A desaturase 2
Replication							
0.74	0.66	0.88	0.85		AW547791	11	Mus musculus, Similar to mutL (E. coli) homolog 3
0.58	0.52	0.67	0.81	Pttg1	AU015203	11	pituitary tumor-transforming 1
Signal Transduction							
0.70	0.60	0.59	0.51	Sh3bgrl3	AW538026	4	SH3 domain binding glutamic acid-rich protein-like 3
0.44	0.61	0.51	0.93				Mus musculus DNA, 3' flanking region of interleukin 12 receptor beta
Cell Adhesion							
1.76	1.97	1.40	1.32	Cd209c	AW539111	8	CD209c antigen
0.52	0.52	0.84	0.81	Sftpd	AU042350	14 14.0 cM	surfactant associated protein D
Complement component							
0.64	0.71	0.87	0.99	Cfh	AW556041	1 74.1 cM	complement component factor h
Oxidative stress							
0.56	0.49	1.28	0.80	Gpx3	AW550656	11 B3-B5	glutathione peroxidase 3
Extracellular							
0.79	0.91	0.95	0.89	Sdf4	C78930	10	stromal cell derived factor 4
0.59	0.38	0.72	0.93	Snta1	AW536987	2 84.0 cM	syntrophin, acidic 1
Integral Membrane protein							
0.69	0.65	0.69	0.66	Arl6ip	AU044379	10	ADP-ribosylation-like factor 6 interacting protein
0.55	0.68	0.81	0.78	Tjp2	AU044024	10	tight junction protein 2
0.53	0.37	0.77	0.58				Mus musculus solute carrier family 34 (sodium phosphate), member 2
Other Genes							
0.73	0.54	0.90	1.10	Fbxl5	AW538739	5	f-box and leucine-rich repeat protein 5
0.66	0.77	1.14	1.10	Rex3	AU015646	X 57.5 cM	reduced expression 3
0.66	0.64	0.87	0.90	Ctsc	C86712	7 D3-E1.1	cathepsin C
Ests							
3.90	3.29	2.74	2.00		AW550806		Mus musculus, clone IMAGE:4191452, mRNA,
2.24	2.98	1.51	2.51		AU040774	12	AU040774 Mouse four-cell-embryo cDNA Mus musculus cDNA
2.08	2.85	1.45	2.61	4932443L08Rik	AU040460	3	RIKEN cDNA 4932443L08 gene
1.72	1.34	1.71	1.64				No Hits Found
1.67	1.63	1.90	1.57	1110008E19Rik	AW539494	8	RIKEN cDNA 1110008E19 gene
1.53	1.50	1.37	1.17		AW556467		Mus musculus 10, 11 day embryo whole,t-complex-associated testis expressed 3
0.83	0.72	0.84	0.74	1810045K07Rik	AW538602	3	RIKEN cDNA 1810045K07 gene
0.80	0.73	0.79	1.03		AW547635		EST
0.70	0.81	1.07	0.93	AI115388	AW553758	6	EST AI115388
0.69	0.85	0.63	0.77				No Hits Found
0.69	0.52	0.56	0.76	1200015M12Rik	AU022808	3	RIKEN cDNA 1200015M12 gene
0.69	0.49	0.76	0.92				Mus musculus mitochondrion, complete genome
0.68	0.80	0.63	0.78	1200015M12Rik	C87085	3	RIKEN cDNA 1200015M12 gene
0.68	0.68	1.03	0.78		AW558717		ESTs, Moderately similar to Z298_HUMAN ZINC FINGER PROTEIN 298 (PR-DOMAIN ZINC FINGER PROTEIN 15) [H.sapiens]
0.64	0.85	1.06	0.77	1300002F13Rik	AW543971	10	RIKEN cDNA 1300002F13 gene
0.64	0.59	0.83	0.88		AW558849		Mus musculus, Similar to hypothetical protein FLJ13213, clone MGC:28555 IMAGE:4206928, mRNA, complete cds
0.62	0.66	0.76	0.78	AA415817	AW554943	16	expressed sequence AA415817
0.62	0.42	0.78	0.83	4921517N04Rik	AA410046	10	RIKEN cDNA 4921517N04 gene
0.56	0.54	0.98	0.70	D5Ertd227e	C79033	5 31.0 cM	DNA segment, Chr 5, ERATO Doi 227, expressed
0.55	0.63	0.66	0.65				
0.54	0.78	0.61	0.66		AW544134		Mus musculus, clone IMAGE:1265114, mRNA, partial cds
0.52	0.52	0.66	0.55	AW550831	AW550831	1	expressed sequence AW550831
0.50	0.72	0.92	0.81	AW539211	AW539211	3	expressed sequence AW539211
0.47	0.38	0.67	0.86		AU024765		AU024765 Mouse unfertilized egg cDNA Mus musculus cDNA
0.46	0.96	0.62	0.22	AI326478	AU015491	12	expressed sequence AI326478

Table 3. Day 70 modulated genes

Normalized	Common	Genbank	Map	Description	GO
3.69	1300007C21Rik	AU040207	10	RIKEN cDNA 1300007C21 gene	
2.79		C87015		C87015 Mouse fertilized one-cell-embryo cDNA Mus musculus cDNA	
2.76		AU015042		AU015042 Mouse two-cell stage embryo cDNA Mus musculus cDNA	
2.64	Fabp3	AU045254	4 61.0 cM	fatty acid binding protein 3, muscle and heart	Transport
2.57	S100a9	AW546964	3 43.6 cM	S100 calcium binding protein A9 (calgranulin B)	Immune Response
2.14	Gro1	C85477	5 51.0 cM	GRO1 oncogene	Immune Response
2.00		AW550806		Mus musculus, clone IMAGE:4191452, mRNA, partial cds	
1.98	F3	AW542396	3 50.0 cM	coagulation factor III	Blood coagulation
1.97	AW107722	AU042389	16	expressed sequence AW107722	
1.93	1190003K14Rik	AU019271	3	RIKEN cDNA 1190003K14 gene	
1.87	AW536807	AW536807	15	MARCKS-like protein	Calmodulin binding
1.69		AU020603	1	AU020603 Mouse eight-cell stage embryo cDNA Mus musculus cDNA	
1.63	Mlp	AW543640	4 59.0 cM	MARCKS-like protein	Calmodulin binding
1.58	AU018381	AU018381	4	AU018381 Mouse eight-cell stage embryo cDNA Mus musculus cDNA	
1.57	Fhl2	C76204	1	four and a half LIM domains 2	Transcription
1.51	AI255193	AU018982	6	Mus musculus, Similar to complement component 1, s subcomponent	Complement component
1.36		AU024446		ESTs	
1.35	Slc16a1	C81388	3	solute carrier family 16 (monocarboxylic acid transporters), member 1	Transport
1.32	Lpl	AW537964	8 33.0 cM	lipoprotein lipase	Metabolism
1.26	H1f0	AU042799	15 46.75 cM	H1 histone family, member 0	Transcription
1.10	Rex3	AU015646	X 57.5 cM	reduced expression 3	Protein Binding
1.00		AW538548		Mus musculus, Similar to phosphatidylserine decarboxylase	Metabolism
0.49	C1qc	AW547306	4 66.1 cM	complement component 1, q subcomponent, c polypeptide	Complement component
0.39	C1qb	AW555781	4 66.1 cM	complement component 1, q subcomponent, beta polypeptide	Complement component

DISCUSSION

Our goal in this study, through the microarray technique, is to 1) determine strain-specific and infection-specific differences in gene expression between tuberculosis-susceptible D2 and tuberculosis-resistant B6 mice, in order to provide insight into novel pathways or genes involved in infection and 2) to identify candidate genes located within the QTL regions that are expressed differentially between B6 and D2. The unselected cDNAs present on the microarray chip were not designed to focus on lung-specific gene expression. Consequently, we did not bias the data derived from these studies to demonstrate a disproportionate number of anatomic, physiologic, or biochemical class of genes. Moreover, this study contains a partial set of genes and not a complete one therefore there can be important information missing.

After stringent statistical analysis for determining significance, a small fraction of transcripts showed differences in expression levels between B6 and D2 mice at day 70. Since this study shows significant modifications with a small number of mice, the 24 transcripts likely include those with the most persistent and possibly the greatest alterations. Interestingly, the list of modified genes included more genes with higher expression in D2 as compared to B6 mice. Moreover, our study has identified overexpression of rather few genes coding for inflammatory mediators and immune response.

Gro1 is upregulated in D2 mice as compared to B6 by 2 fold at day 70 post-infection. Gro1 (also known as CXCL1) plays a role in neutrophil influx to sites of injury[305]. Gro1 also plays a role in exocytosis and respitratory burst. It activates polymorphonculear granulocytes and NK cells. A study by Riedel et al. showed that *M.*

tuberculosis- and LAM-activated human polymorphonuclear granulocytes secrete the leukocyte attractant interleukin-8 and the polymorphonuclear granulocyte-specific chemokine GRO-1 in a dose-dependent manner[306]. Another gene that has been upregulated in D2 by 2.5 fold as compared to B6 is S100A9. Myeloid-related proteins (MRPs) [307]are involved in neutrophil migration. The MRPs S100A8 and S100A9 (known as MRP 8 and 14) are calcium-binding proteins that belong to the S100 protein family are expressed almost exclusively by cells of myeloid lineage. The MRPs are constitutively expressed in the cytosol of neutrophils. High serum concentrations of MRPs have been found in advanced HIV infections, adult and juvenile rheumatoid arthritis, chronic bronchitis, cystic fibrosis, systemic lupus erythematosus, and granulomatous conditions, such as tuberculosis and sarcoidosis [308-314]. Newton and Hogg [315] recently demonstrated that human S100A9 stimulates neutrophil adhesion to fibrinogen by activating the β_2 integrin Mac-1 (CD11b/CD18). S100A8/9 might also serve as a reservoir for arachadonic acid that is used to activate O_2^- generating NADPH oxidase which is responsible for microbicidal function of neutrophils and macrophages [316]. Histological response at sites of *M. tuberculosis* infection in the lungs of susceptible D2 [229,238] is similar to that in the lungs of resistant mice up to days 40-50 of infection, and mononuclear cells dominate. As time progresses, the dominant cell type, macrophages are replaced progressively by neutrophils. Eventually the lung becomes occupied by a fulminating neutrophil-dominated necrotizing pathology that causes early death. However B6 mice after day 50 have macrophage-dominated alveolitis with associated aggregates of lymphoid cells. Therefore we are able to see that 70 days post-infection there is an upregulation in D2 mice of neutrophil associated genes as we had seen in the histopathology of D2 mice.

The first component of the classical pathway of complement activation is C1, which is a complex of 3 proteins: C1q, C1r and C1s. C1q, the first subcomponent of C1, has a complicated 18-chain structure: 6 a, 6 b, and 6 c chains. In the microarray experiment at day 70, 3 components of the C1 complex seem to be differentially expressed between B6 and D2 mice: C1qb, C1qc and C1s. The C1qb and c chains are up regulated in B6. Antibody dependent- [317] and independent C1q binding to microorganisms has been described and has the capacity to activate the classical complement pathway [318,319]. The complement system plays a role in the pathogenesis of tuberculosis during innate immune response. A recent study has shown that when *M. tuberculosis* is first inhaled in the lungs, entry into alveolar macrophages the bacterium is opsonised through the classical pathway of activation [320]. Moreover, the structure of mannose binding protein resembles that of C1q component of complement although the two proteins do not share sequence homology. Mannose binding protein (MBP) does not appear in the genes that are differentially modulated during the infection, however previous studies have shown that MBP is known to play a role in tuberculosis infection [127,128]. Finally *Cfh1*, which is a gene upregulated in B6 pre-infection, is involved in the complement pathway maps to the *Trl-1* region. *Cfh1*'s candiacy needs to further be studied.

Moreover, from the strain-specific (pre-infection) genes whose expression does not change throughout time there is a group of genes that are involved in apoptosis (*Pttg1, Clathrin D, Ppid and Scd2*) All these genes are upregulated in B6 as opposed to D2. Moreover *Scd2* maps in the *Trl-4* region. During tuberculosis infection, there is dysregulation of the mechanisms involved in the cell death of mononuclear phagocytes and which may partly explain the tissue damage and bacterial dissemination that occurs.

Necrosis of infected macrophages may result in the release of mycobacterial and proinflammatory molecules leading to bacterial dissemination, tissue damage and disease. In apoptosis of infected macrophages a non-permissive environment is created for bacterial replication and no enzymes are released in the extracellular milieu. A recent study suggests that apoptosis of monocytes exposed to mycobacteria may partly explain the protective immune response found in PPD-positive control subjects, whereas necrosis may be determinant of the bacterial dissemination and tissue damage that occur in patients with active tuberculosis [321]. It has been suggested that the suppression of the death of host cells by the pathogen might represent a pathogen strategy for survival. There are several mechanisms whereby macrophage apoptosis might act to limit *M. tuberculosis* replication in the lung, in fact it is suggested that bacilli in apoptotic cells are killed when they are ingested by fresh macrophages [322]. Recent findings also show that infection with virulent *M. tuberculosis* alters the expression of genes involved in apoptosis and necrosis of host cells, and the bacterial genes responsible for this are currently being identified [323].

Immune response to *M. tuberculosis* in both humans and mouse is associated with the production of IFN-γ by $CD4^+$ T cells with IL-12 being a key cytokine for the differentiation of IFN- γ producing Th1 cells [324]. Mutations in five autosomal genes in the IL-12/IFN-γ pathway have been found to cause recurrent disseminated mycobacterial infections. These defects are found in IFN-γ R1, IFN-γ R2, STAT1, IL-12p40 and IL-12Rβ1[95]. All the mutations result in impaired IFN-γ immunity with the IFN-γR1 mutation being the most severe. Mutations in the gene encoding for IL12Rβ1 were found in patients causing complete IL12Rβ1 deficiency [267,325]. A role for IL-12 in resistance to *M.*

tuberculosis was suggested by the improved clinical outcome observed when the cytokine was combined with drug therapy [326]. The IL-12Rβ (Table 1) is one of the strain specific genes that is known to play a role in tuberculosis and appeared in our experiment. This further strengthens the association of gene expression and tuberculosis susceptibility.

Another way to elucidate the molecular mechanism whereby our most important QTLs act to produce their phenotype is through the construction of congenic mice. In order to isolate each individual QTL for study, we are producing several mouse congenic strains that carry whole chromosomes of QTL identified in an F2 population between B6 and D2 strains of mice. The congenic strains were created by transferring a region containing a QTL on a single chromosome from the donor strain, B6, to the D2 (recipient) background. The QTL on chromosome 19 (*Trl-4*) contributes approximately 25% to the total variance between B6 and D2 mice, and it has a LOD score of 5.6, which is the statistically strongest QTL we have identified from B6 and D2 mice. Also, a congenic line for the chromosome 7 QTL has been created (*Trl-3*, LOD 4.6, accounting for 21% of the variance) since this QTL has been detected in two studies showing an effect on both bacterial replication in the lungs and overall survival to infection. Moreover a double congenic line has been created for *Trl-3* and *Trl-4*. A strong additive effect was detected between *Trl-3* and *Trl-4*, with two-loci linkage analysis yielding a LOD of 10.09 explaining 48% of the variation in raw CFUs in lungs. Since the difference between the genome of B6 and congenic mice is only in the QTL region, the differential expression of genes outside of the QTL region would be, presumably, downstream or upstream of the QTL gene. Therefore, the expression profile of genes outside the QTL

region could provide important mechanistic information on the molecular basis of the QTL gene.

The results obtained using microarrays need to be validated with other techniques (for example northern blots or immunohistochemistry, RT-PCR should allow a better definition of specific cell types involved in differential gene expression). Nonetheless, this study displays distinct expression profiles between resistant and susceptible mouse strains to *M. tuberculosis* infection. These differences provide a global view of the lung pathophysiology and can provide invaluable insight to the infection process and different host responses associated with resistance or susceptibility to infection. Gene-expression profiling, combined with genetic mapping data, has been proven to identify candidate genes; already microarrays have been used to identify a gene at a QTL that influences susceptibility in a model of asthma [327], lipoxygenase (*Alox15*) has been proposed as a candidate gene for bone mass [328], an interferon-inducible gene for susceptibility to an autoimmune condition (systemic lupus) [329] and glutathione *S*-transferase M2 has been identified as a gene that might be involved in hypertension [330]. Although array technology is valuable, as regards gene function, it only provides one more piece (that is, transcriptional profile) of the puzzle. The flow of information that is stored in a gene, transcribed into RNA and finally translated into protein are key step in gene action and it will be essential to subject protein synthesis, as well as protein interaction, to the same genome-wide analysis, to understand how genotype can influence a complex phenotype.

Now that we have identified 4 loci involved in susceptibility to infection, and have a few candidate for our regions, what is going to be done next.

Chapter 5

General Discussion and Conclusion

5.1 General Discussion

5.1.1 Mouse Model Limitations

Mice, although not naturally subject to tuberculosis, have proven an invaluable tool for genetic analysis of susceptibility to infection. However, the mouse model does have its difficulties and limitations. Manner of preparation of the inoculum and its size, route of infection and the test organ can affect the growth of the organism *in vivo*. The dissemination of the infectious inoculum is not the same when introduced via the i.v. or the aerosol route: depending on the route of infection, mice may receive inocula varying by as much as 1 million fold [331]. When introduced through the i.v. route, the inoculum seeds mostly in the liver with only a very small fraction reaching in the lung after all the routes passed. Quite the opposite happens with an aerosol infection, which most closely mimics human disease and bacteria are seeded directly in the lungs.

Also, overall survival to infection is a complex trait it can be influenced by multiple factors such as bacterial replication, host inflammatory and immune response as well as general fitness traits. Survival time can demonstrate the presence of an immune response yet tell very little since all of the challenged animals eventually succumbed to infection-induced disease, the difference being that susceptible strains succumb much earlier. However bacterial counts in the lung assesses the immune response in terms of growth or inactivation of the inoculum within the organs rather than merely recording time of death.

Moreover a sex effect has been seen in mouse models of infection, with females generally more resistant than males. This can lead to a false linkage to chromosome X

unless one uses sex adjusted CFUs (as seen in Chapter 4). This problem could be overcome by not pooling female and male mice together or using male or female mice only. Also because inbred mouse strains only represent a fraction of the genetic variation presented in the wild, a QTL segregating in one cross may not be detected in other inbred strains.

5.1.2 Genetic Linkage Studies in Mouse Models: Trls and Other QTL

QTL mapping in 2 genome wide scans was used to determine the number and location of genes controlling differential susceptibility to pulmonary tuberculosis in DBA/2 and C57BL/6 mice. In these 2 scans, we have used different doses (1 X 10^5, 2 X 10^2 CFU), different routes of infection (intravenous, aerosol) and independent phenotypic measurements of susceptibility (survival, pulmonary replication) to map genes that influence common or unique aspects of tuberculosis pathophysiology. In a first scan, 95 informative (C57BL6/J X DBA/2) F2 mice were infected *i.v* with 10^5 virulent *M. tuberculosis* H37Rv, and survival time ($\log_{10}$) was used as a phenotypic measure of susceptibility. These studies identified 2 significant linkages on the distal portion of Chr.1 [***Trl-1;*** LOD = 4.80, $p<1.5x10^{-5}$], and on the proximal portion of Chr.7 [***Trl-3;*** LOD = 4.66, $p<1.5x10^{-5}$] and one weaker linkage in the central portion of Chr.3 [***Trl-2;*** LOD = 3.93, $p<2x10^{-4}$]. These QTLs control 21%, 21% and 17% of the variance respectively, with B6 resistance alleles at the 3 loci being inherited in a semi-dominant fashion (Chapter 2). At each locus, homozygosity for parental C57BL/6 J alleles was associated with resistance. The effect of the three loci was additive (no epistasis detected), and

explained 45% of the total variance. In a second genome scan, 104 (C57BL6/J X DBA/2) F2 mice were infected with 10^2 *M. tuberculosis* H37Rv by the aerosol route, and the extent of bacterial replication in the lungs (CFU) at 90 days was used as a phenotypic measure of susceptibility (Chapter 3). Results from this analysis identified a major linkage on the central portion of Chr. 19 [***Trl-4***; LOD = 5.6, $p<1x10^{-6}$; 24% of the variance], along with weaker linkage with the *Trl-3* region (LOD = 3.1), but also with Chr. 5 (LOD = 3.7) and Chr. 10 (LOD = 3.4). In addition, strong additive effect was detected between *Trl-3* and *Trl-4*, with two-loci linkage analysis yielding a LOD= 10.09 explaining 48% of the variation in raw CFUs. Remarkably, mice homozygous for B6 alleles at *Trl-3* and *Trl-4* were completely resistant and showed CFU counts (5.55, 5.80) in the range of B6 controls (range 5.6 to 6.1), while mice homozygous for D2 alleles at both loci were completely susceptible and showed CFU counts (7.0, 7.6, 7.8) similar to those seen in the susceptible D2 controls (range 7.1 to 7.9). These results strongly suggest that combined action of *Trl-3* and *Trl-4* is a major regulator of MTB replication in the lungs. Also a third and final whole genome scan has also been completed where 104 mice of the same strain combination were infected by the aerosol route with10^2 *M. tuberculosis* H37Rv and survival time was used as a phenotypic measure of susceptibility, in order to help validate *Trl-1/2/3/4* (data not shown). Mice were genotyped for a total of 175 markers. QTL analysis failed to identify any significant linkage. These studies demonstrate the importance of *Trls* for regulation of bacterial replication and survival to infection. *Trl-3* is of particular interest since it has been detected by different doses and by different routes of infection. Validation of *Trl-3* suggests that its genetic effect is robust, possibly affecting important host defense mechanisms.

It is important to note that the *Trl* loci mapped by our group are different from those mapped by other groups (Table 1). Using F2 mice derived from resistant C57BL/6 J and susceptible C3HeB/FeJ progenitor strains, and infected with 10^6 *M. tuberculosis* Erdman i.v., Kramnik et al.[280] mapped a locus on distal chromosome (Chr.) 1 (*sst1*; position 49-58 cM) that controls the rate of bacterial replication and granuloma formation in the lung, and survival time. *Sst1* is located approximately 15 cM distal of the *Nramp1* gene, and both loci appear different as the allele which confers short survival times (susceptibility) is derived from the C3H strain which carries an *Nramp1*-resistant allele. Recently a candiadate gene, *Ipr 1* for intracellular pathogen resistance 1, has been identified in the *sst1* locus in a congenic mouse strain in which the *sst1* locus from the C3HeB/FeJ mice has been replaced with the same region from C57BL/6J mice. Macrophages from mice having the resistant *sst1* locus have an increased capacity to resist intracellular replication of *M. tuberculosis.* Moreover resistant mice have induced the apoptotic pathway in macrophages after infection as compared to susceptible mice that undergo necrosis[332].

Lavebratt et al.[236] studied genetic control of body weight loss, following i.v. infection with 10^6 CFU of *M. tuberculois* in progeny of a (A/SnxI/St) F1xI/St backcross. Suceptible I/St mice and resistant A/Sn mice show MST values of 21.5/26.3 days (females/males) and 45.4/45.2 respectively. They identified significant linkages to chromosomes Chr. 3 and 9 in females only, and suggestive linkages on Chr. 8 and 17 in females and on Chr. 5 and 10 for males.

It would be interesting to study whether the corresponding syntenic chromosomal regions in humans are associated with disease susceptibility, in population studies from endemic areas of disease.

TABLE 1. Linkage mapping for tuberculosis susceptibilitiy in mice

	Chromosomal location	Locus	Susceptible Mouse strain	Resistant Mouse strain	Type of cross	*M. tuberculosis* strain and dose	Infection route	Effect of resistant parent allele	Reference
*	1	*Sst1*	C3HeB/FeJ	C57BL/6	Intercross	Erdman 10^6	Intravenous	Resistance	Kramnik et al. 2000
*	3	*Tbs1*	I/St	A/Sn	Backcross	H37Rv 10^6	Intravenous	Body weight loss prevention	Lavebratt et al. 1999
		Tbs2	"	"	Backcross	"	"	Body wight loss prevention and resistance	Sanchez et al. 2003
*	1	*Trl-1*	DBA/2	C57BL/6	Intercross	H37Rv 10^5	Intravenous	Resistance	Mitsos et al. 2000
	3	*Trl-2*	"	"	"	"	"	"	"
	7	*Trl-3*	"	"	"	"	"	"	"
	19	*Trl-4*	"	"	"	H37Rv 10^2	Aerosol	"	Mitsos et al. 2003

5.1.3 Microarray analysis

Microarray analysis has allowed us to determine strain-specific and infection-specific differences in gene expression between tuberculosis-susceptible D2 and tuberculosis-resistant B6 mice, and provided insight into novel pathways involved in infection. Our data suggests that the classical complement pathway and the apoptotic pathway seem to play a role in differential susceptibility between B6 and D2 mice, with these pathways being upregulated in B6. Moreover the increase in neutrophil associated gene expression (S100A9, Gro1) corroborates with our previous histological finding of increased neutrophil cell counts in D2 mice. Finally these studies have provided candidate genes that map in the *Trl-1* and *Trl-4* regions (*Cfh1* and *Sdf4* respectively). The candidacy of these genes needs to be further studied.

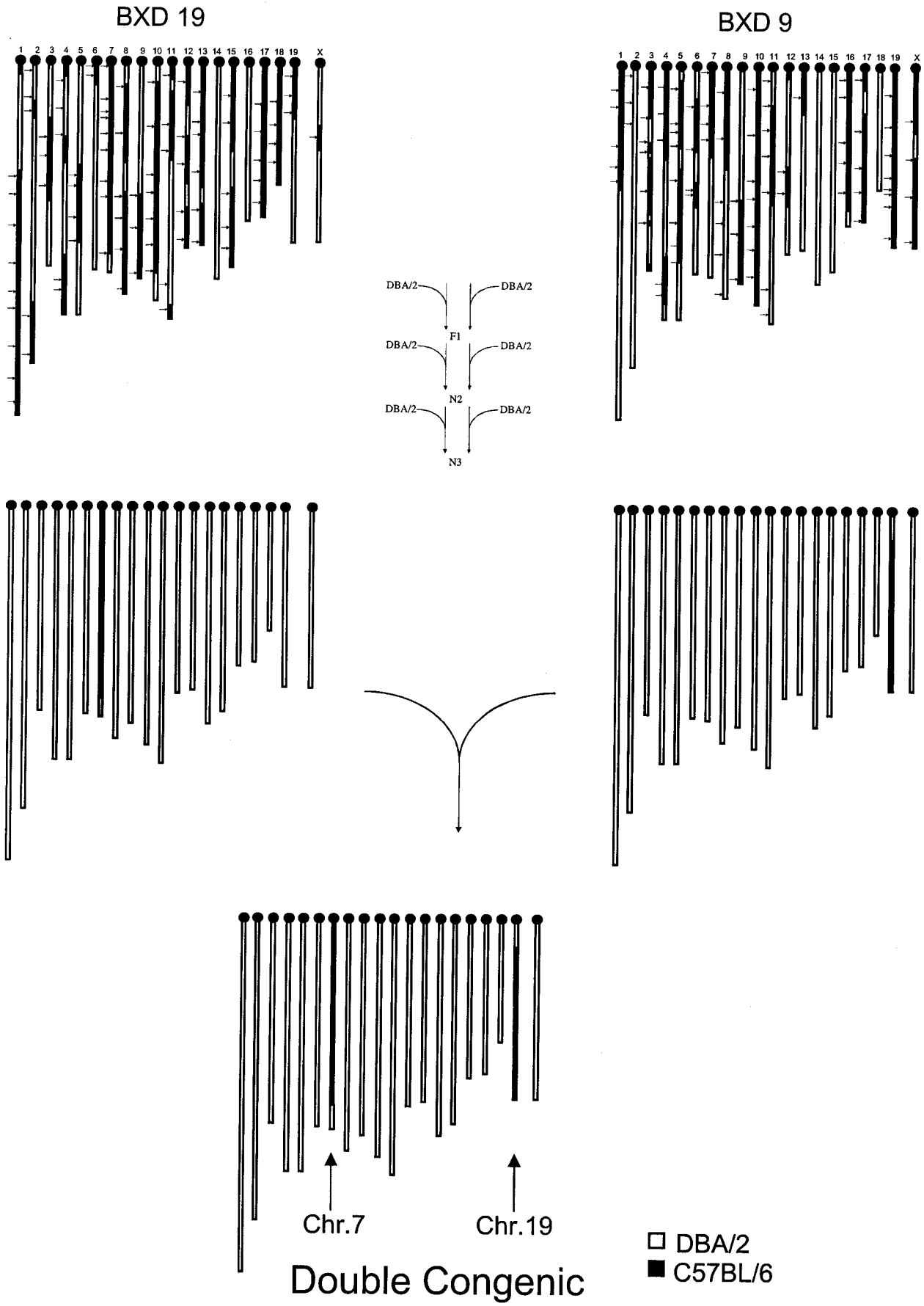
Validation of these gene lists will be provided in microarray studies on congenic mice (see below). Since the congenic strains have inherited approximately 95% of their genome the background strain, its gene expression profiles in different tissues and cell types of will be very similar to those seen in the parental strains except for the genes involved in the congenic segment.

5.2 Future Perspectives

5.2.1 Validation of the Individual and Combined Effect of Trl-3/Trl-4 on MTB replication in the Lungs

The next step would be to get insight into mechanistic basis of action of the major loci mapped. The focus is put on 2 QTLs *Trl-3* and *Trl-4*: a) *Trl-3* appears to affect both

the rate of pulmonary bacterial replication and survival to infection, b) *Trl-4* is the strongest QTL detected to date (LOD = 5.6) and c) the combined effect of both loci (LOD = 11) explains ~50% of the phenotypic variance in the (C57BL6/J X DBA/2) F2 cross used. A necessary pre-requisite to the phenotypic analysis of single locus effects is the isolation of the susceptibility (D2) or the resistance (B6) alleles at these loci on opposite genetic backgrounds in reciprocal congenic mouse lines. We have already initiated the breeding of individual mouse lines congenic for the *Trl-3* (Chr. 7) and *Trl-4* (Chr. 19) loci. In these mice, the chromosome(s) carrying the QTL(s) of interest (chromosomes 7 and 19) from the resistant strain (C57BL/6) is transferred by breeding to the genetic background of the susceptible strain (DBA/2). Such mice allow one to directly establish the independent contribution of each of the mapped QTLs to the overall phenotype of resistance/susceptibility to *M. tuberculosis* infection. We used the "speed congenic" [333] approach to construct congenic lines containing over 95% of one of the parental genome and the QTL of interest from the other parent. We have independently introgressed (Figure 1) the entire B6-derived Chr. 7 of the recombinant inbred strain BXD19, and Chr. 19 of BXD9 onto DBA/2 background (consomic lines) using a speed congenic protocol [333]. In this protocol, successive F1 backcross males are genotyped with 40 informative markers in order to identify males with the lowest B6 content that are then selected for further backcrossing. This process is repeated at each generation intercalating two sets of 40 informative markers and allows one to generate congenic lines in a reduced period of time. This task is facilitated by the fact that the entire genomic haplotypes of BXD9 and BXD19 for B6 and D2 segments is known. Once at the N4 generation, heterozygotes are intercrossed to generate the homozygote congenic lines at each locus (*Trl-3*, *Trl-4*), and also to produce the doubly congenic line. We only constructed congenic lines where the


BXD 19
BXD 9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 X
DBA/2
DBA/2
F1
DBA/2
DBA/2
N2
DBA/2
DBA/2
N3
Chr.7
Chr.19
Double Congenic
DBA/2
C57BL/6

B6 segment was introgressed in an otherwise susceptible background because the reciprocal mouse lines are available [334] .

Then the aspect of host response that is under the control of either *Trl-3 or Trl-4* or both will be functionally characterized in these congenic lines. Insight into the physiological response pathway (inflammatory, immune) and cell populations (macrophages, lymphocytes, others) involved will be seeked. Mice will be infected (2 X 10^2 CFU, aerosol), and at pre-determined time intervals (0 to 90 days), lungs (also spleen, liver and bone marrow) will be harvested, fixed and analyzed for gross differences in histology, with respect to number, type, morphology, and organization of lesions (granulomas), including presence and location of acid-fast bacilli. The host cell types involved will be monitored by direct morphological examination of tissue sections stained by standard procedures, but also after immunostaining with markers specific for T cells (CD4), B cells (surface Ig), macrophages (Mac1), dendritic cells (CD11c), and PMN (GR1). The type and magnitude of the host inflammatory and immune response will be estimated in congenic lines at different times following *M. tuberculosis* infection: a) *in situ* by immunostaining fixed sections with antibodies directed against major cytokines such as IL12-p40, IL-15 and IFN-γ, b) parallel measures for presence and amount of Th1 cytokines, IL-12, TNF-α, and IFN- γ, in serum by ELISA, or using spleen or lung RNA using commercially available panels of cRNA probes (RNAse protection), c) distribution of T, B, Mac, PMN, erythroid precursors by FACS analysis. These studies should not only clarify the aspect of pathophysiology affected by *Trl-3/Trl-4* but should also help prioritize positional candidates in the search for the genes involved.

5.2.2 Positional cloning

First, the genetic interval delineating each QTL should be reduced. Because the trait involved is complex, classical segregation analyses in large numbers of (C57BL6/J X DBA/2) F2 mice (>1000) are unlikely to refine appreciably the confidence interval. Rather, the ability of chimeric chromosomes carrying different B6-derived segments of chromosomes 7 (*Trl-3*) and 19 (*Trl-4*) to correct the recessive D2 susceptibility phenotype in a series of D2 congenic mouse lines should be tested. In a preferred scenario, recombinant chromosomes would consist of B6 DNA of increasing size and anchored at the telomeric (*Trl-4*) or centromeric end (*Trl-3*) of the chromosome. The position of the locus can be regionally assigned either directly upon phenotyping the resulting strains for susceptibility to *M. tuberculosis*, or indirectly by phenotyping progenies issued from mice carrying the recombinant chromosome (progeny testing) in order to increase statistical power of the analysis, and as described in Ref. [335]. Studying the minimal genetic interval at each locus for the presence of "haplotype blocks" conserved between B6 and D2 [336], and that are unlikely to harbor a major distinguishing trait between the 2 strains [337], thus reducing the confidence limits of the genetic interval(s), can be very beneficial. As the mouse haplotype map is improving, this approach is gaining popularity and has been used successfully to recently clone *Stk6/STK15* as a QTL affecting susceptibility to skin tumor (papilloma) formation [338].

5.2.3 Validation of Candidate Genes

Positional candidates will be further analyzed in a series of consecutive validation steps. This will include: a) verifying the pattern of tissue, temporal and quantitative mRNA expression either prior to or during *M. tuberculosis* infection by Northern analysis and by quantitative RT-PCR, using RNA from various normal or infected organs obtained from congenic and control animals; b) systematic sequencing of positional candidates and looking for presence of susceptibility-associated mutations; c) strong candidates identified by sequence analysis will be validated through the creation of loss-of-function or gain of function mutations. Genes such as such as *Tlr-2* have already been looked at (data not shown).

5.3 Final Conclusion

With a potential global impact on up to two billion people, there is a strong case for the development of intervention in tuberculosis. The development of tuberculosis is the result of a complex interaction between the host and pathogen influenced by environmental factors. Numerous host genes are likely to be involved in this process. The rapid development of a diversity of genetic and molecular biological tools lately, and the determination of the nucleotide sequence of the *M. tuberculosis* genome, have enabled investigators to make significant progress in understanding some aspects of *M. tuberculosis* persistent infection. Analysis in humans have started to unveil the complexity of interaction between the mycobacterium and its host. Genetic studies in the mouse model have also begun to catalogue a number of loci controlling susceptibility to

tuberculosis infection. Identification of the genes underlying these loci will hopefully identify novel defense mechanisms that could be targeted for therapeutic intervention in a fundamental problem of human health that has preoccupied humanity for hundreds of years.

ORIGINAL CONTRIBUTION TO KNOWLEDGE

0) Identification of 3 loci *Trl-1/ 2/3* controlling overall survival to infection after infection with *M. tuberculosis.*

0) Identification of 2 loci Trl-3 /4 controlling bacterial replication in the lungs of mice infected with M. tuberculosis.

0) Initiation of breeding of a congenic mouse line for Trl-3/4 and double congenic.

0) Identification, through microarray experiments, of physiological response pathways (classical complement pathway as well as the apoptotic pathway upregulated in tuberculosis resistant mice) and cell populations (increased neutrophil associated genes in tuberculosis susceptible mice) involved in differential susceptibility to *M. tuberculosis* infection between susceptible and resitant mouse strain prior to and during infection.

LIST OF ABBREVIATIONS

B6: C57Bl/6

BCG: Bacillus Calmette-Guérin

D2: DBA/2

LOD: Logarithem of odds ratio

MTB: *Mycobacterium tuberculosis*

TB: tuberculosis

Trl: Tuberculosis resistance locus

Tlr:Toll like receptors

QTL: quantitative trait locus

BIOHAZARDS INVENTORY
INVENTAIRE DES RISQUES BIOLOGIQUES

Investigator/Chercheur Dr. P. Gros **Date** August 12, 2003

Location/Lieu McIntyre 907-910, 925 / Biochemistry

Level 1 (non-pathogenic) Material
Substances (non-pathogènes) du niveau 1

Yeast, non-pathogenic bacteria (rooms 907-910 and 925)

Spill Response – Disinfection Protocol
Protocole de désinfection en cas de déversement

Wipe with paper towels, then disinfect with bleach. Bag and autoclave contaminated items.

Level 2 Material
Substances du niveau 2

Mammalian cell lines (room 907-910)

Spill Response – Disinfection Protocol
Protocole de désinfection en cas de déversement

Contain spill. Wipe with paper towels, then disinfect with 70% ethanol. Bag and autoclave contaminated items.

Emergency Contacts / À contacter en cas d'urgence

Name/nom	Dr. P. Gros	Name/nom
Tel/Tél	Local 7291 450-466-5593	Tel/Tél

CAMPUS EMERGENCY NUMBERS: DOWNTOWN 3000, MACDONALD 7777
NUMÉROS À COMPOSER EN CAS D'URGENCE: CENTRE-VILLE 3000, MACDONALD 7777

REFERENCES

1. Organisation, W.H. The World Health Report 2000. Health Systems: Improving Performance.
2. Maartens, G. Advances in adult pulmonary tuberculosis. *Curr Opin Pulm Med* **8**, 173-7 (2002).
3. Waugh, A.C. & Long, P.F. Prospects for generating new antibiotics. *Sci Prog* **85**, 73-88 (2002).
4. Koch, R. Die Aetiologie der Tuberculose. *Berl Klin Wochenschr* **19**, 221-230 (1882).
5. Beckman, E.M. et al. Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells. *Nature* **372**, 691-4 (1994).
6. Belisle, J.T. et al. Role of the major antigen of Mycobacterium tuberculosis in cell wall biogenesis. *Science* **276**, 1420-2 (1997).
7. Taylor, M.E. Recognition of complex carbohydrates by the macrophage mannose receptor. *Biochem Soc Trans* **21**, 468-73 (1993).
8. Schluger, N.W. & Rom, W.N. The host immune response to tuberculosis. *Am J Respir Crit Care Med* **157**, 679-91 (1998).
9. Mostrom, P., Gordon, M., Sola, C., Ridell, M. & Rastogi, N. Methods used in the molecular epidemiology of tuberculosis. *Clin Microbiol Infect* **8**, 694-704 (2002).
10. Camus, J.C., Pryor, M.J., Medigue, C. & Cole, S.T. Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv. *Microbiology* **148**, 2967-73 (2002).

11. Harrison. *Harrison's PRINCIPLES OF INTERNAL MEDICINE*, (McGraw Hill, 2005).

12. Mehta, P.K., King, C.H., White, E.H., Murtagh, J.J., Jr. & Quinn, F.D. Comparison of in vitro models for the study of Mycobacterium tuberculosis invasion and intracellular replication. *Infect Immun* **64**, 2673-9 (1996).

13. Bermudez, L.E. & Goodman, J. Mycobacterium tuberculosis invades and replicates within type II alveolar cells. *Infect Immun* **64**, 1400-6 (1996).

14. Tascon, R.E. et al. Mycobacterium tuberculosis-activated dendritic cells induce protective immunity in mice. *Immunology* **99**, 473-80 (2000).

15. Bodnar, K.A., Serbina, N.V. & Flynn, J.L. Fate of Mycobacterium tuberculosis within murine dendritic cells. *Infect Immun* **69**, 800-9 (2001).

16. Gonzalez-Juarrero, M. & Orme, I.M. Characterization of murine lung dendritic cells infected with Mycobacterium tuberculosis. *Infect Immun* **69**, 1127-33 (2001).

17. Lipscomb, M.F. & Masten, B.J. Dendritic cells: immune regulators in health and disease. *Physiol Rev* **82**, 97-130 (2002).

18. Ernst, J.D. Macrophage receptors for Mycobacterium tuberculosis. *Infect Immun* **66**, 1277-81 (1998).

19. Schlesinger, L.S. Entry of Mycobacterium tuberculosis into mononuclear phagocytes. *Curr Top Microbiol Immunol* **215**, 71-96 (1996).

20. Schorey, J.S., Carroll, M.C. & Brown, E.J. A macrophage invasion mechanism of pathogenic mycobacteria. *Science* **277**, 1091-3 (1997).

21. Schlesinger, L.S. Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors. *J Immunol* **150**, 2920-30 (1993).

22. Armstrong, J.A. & Hart, P.D. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival. *J Exp Med* **142**, 1-16 (1975).

23. Thoma-Uszynski, S. et al. Induction of direct antimicrobial activity through mammalian toll-like receptors. *Science* **291**, 1544-7 (2001).

24. Gatfield, J. & Pieters, J. Essential role for cholesterol in entry of mycobacteria into macrophages. *Science* **288**, 1647-50 (2000).

25. Gaynor, C.D., McCormack, F.X., Voelker, D.R., McGowan, S.E. & Schlesinger, L.S. Pulmonary surfactant protein A mediates enhanced phagocytosis of Mycobacterium tuberculosis by a direct interaction with human macrophages. *J Immunol* **155**, 5343-51 (1995).

26. Ferguson, J.S., Voelker, D.R., Ufnar, J.A., Dawson, A.J. & Schlesinger, L.S. Surfactant protein D inhibition of human macrophage uptake of Mycobacterium tuberculosis is independent of bacterial agglutination. *J Immunol* **168**, 1309-14 (2002).

27. Ferguson, J.S., Voelker, D.R., McCormack, F.X. & Schlesinger, L.S. Surfactant protein D binds to Mycobacterium tuberculosis bacilli and lipoarabinomannan via carbohydrate-lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages. *J Immunol* **163**, 312-21 (1999).

28. Nathan, C.F. & Hibbs, J.B., Jr. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr Opin Immunol* **3**, 65-70 (1991).

29. MacMicking, J.D. et al. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci U S A* **94**, 5243-8 (1997).

30. Chan, J., Tanaka, K., Carroll, D., Flynn, J. & Bloom, B.R. Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis. *Infect Immun* **63**, 736-40 (1995).

31. O'Brien, L., Carmichael, J., Lowrie, D.B. & Andrew, P.W. Strains of Mycobacterium tuberculosis differ in susceptibility to reactive nitrogen intermediates in vitro. *Infect Immun* **62**, 5187-90 (1994).

32. Nicholson, S. et al. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med* **183**, 2293-302 (1996).

33. Walker, L. & Lowrie, D.B. Killing of Mycobacterium microti by immunologically activated macrophages. *Nature* **293**, 69-71 (1981).

34. Chan, J. & Kaufmann, S. Immune mechanisms of protection. in *Tuberculosis: Pathogenesis, Protection and Control* (ed. Bloom, B.) 389-415 (Am. Soc. Microbiol., Washington, DC, 1994).

35. Chan, J., Fan, X.D., Hunter, S.W., Brennan, P.J. & Bloom, B.R. Lipoarabinomannan, a possible virulence factor involved in persistence of Mycobacterium tuberculosis within macrophages. *Infect Immun* **59**, 1755-61 (1991).

36. Chan, J. et al. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. *Proc Natl Acad Sci U S A* **86**, 2453-7 (1989).

37. Cooper, A.M., Segal, B.H., Frank, A.A., Holland, S.M. & Orme, I.M. Transient loss of resistance to pulmonary tuberculosis in p47(phox-/-) mice. *Infect Immun* **68**, 1231-4 (2000).

38. Adams, L.B., Dinauer, M.C., Morgenstern, D.E. & Krahenbuhl, J.L. Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to

Mycobacterium tuberculosis using transgenic mice. *Tuber Lung Dis* **78**, 237-46 (1997).

39. Hart, P.D., Armstrong, J.A., Brown, C.A. & Draper, P. Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. *Infect Immun* **5**, 803-7 (1972).

40. Sturgill-Koszycki, S., Schaible, U.E. & Russell, D.G. Mycobacterium-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. *Embo J* **15**, 6960-8 (1996).

41. Russell, D.G., Dant, J. & Sturgill-Koszycki, S. Mycobacterium avium- and Mycobacterium tuberculosis-containing vacuoles are dynamic, fusion-competent vesicles that are accessible to glycosphingolipids from the host cell plasmalemma. *J Immunol* **156**, 4764-73 (1996).

42. Clemens, D.L. & Horwitz, M.A. Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med* **181**, 257-70 (1995).

43. Clemens, D.L. & Horwitz, M.A. The Mycobacterium tuberculosis phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. *J Exp Med* **184**, 1349-55 (1996).

44. Russell, D.G. Mycobacterium tuberculosis: here today, and here tomorrow. *Nat Rev Mol Cell Biol* **2**, 569-77 (2001).

45. Sturgill-Koszycki, S. et al. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* **263**, 678-81 (1994).

46. Fratti, R.A., Backer, J.M., Gruenberg, J., Corvera, S. & Deretic, V. Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *J Cell Biol* **154**, 631-44 (2001).

47. Via, L.E. et al. Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. *J Biol Chem* **272**, 13326-31 (1997).

48. Fratti, R.A., Chua, J. & Deretic, V. Cellubrevin alterations and Mycobacterium tuberculosis phagosome maturation arrest. *J Biol Chem* **277**, 17320-6 (2002).

49. Ferrari, G., Langen, H., Naito, M. & Pieters, J. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell* **97**, 435-47 (1999).

50. Maniak, M., Rauchenberger, R., Albrecht, R., Murphy, J. & Gerisch, G. Coronin involved in phagocytosis: dynamics of particle-induced relocalization visualized by a green fluorescent protein Tag. *Cell* **83**, 915-24 (1995).

51. Schuller, S., Neefjes, J., Ottenhoff, T., Thole, J. & Young, D. Coronin is involved in uptake of Mycobacterium bovis BCG in human macrophages but not in phagosome maintenance. *Cell Microbiol* **3**, 785-93 (2001).

52. Malik, Z.A., Denning, G.M. & Kusner, D.J. Inhibition of Ca(2+) signaling by Mycobacterium tuberculosis is associated with reduced phagosome-lysosome fusion and increased survival within human macrophages. *J Exp Med* **191**, 287-302 (2000).

53. Hackam, D.J. et al. Host resistance to intracellular infection: mutation of natural resistance-associated macrophage protein 1 (Nramp1) impairs phagosomal acidification. *J Exp Med* **188**, 351-64 (1998).

54. Frehel, C., Canonne-Hergaux, F., Gros, P. & De Chastellier, C. Effect of Nramp1 on bacterial replication and on maturation of Mycobacterium avium-containing phagosomes in bone marrow-derived mouse macrophages. *Cell Microbiol* **4**, 541-56 (2002).

55. de Chastellier, C., Frehel, C., Offredo, C. & Skamene, E. Implication of phagosome-lysosome fusion in restriction of Mycobacterium avium growth in bone marrow macrophages from genetically resistant mice. *Infect Immun* **61**, 3775-84 (1993).

56. Means, T.K. et al. Differential effects of a Toll-like receptor antagonist on Mycobacterium tuberculosis-induced macrophage responses. *J Immunol* **166**, 4074-82 (2001).

57. Means, T.K. et al. Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. *J Immunol* **163**, 3920-7 (1999).

58. Lopez, M. et al. The 19-kDa Mycobacterium tuberculosis protein induces macrophage apoptosis through Toll-like receptor-2. *J Immunol* **170**, 2409-16 (2003).

59. Noss, E.H. et al. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of Mycobacterium tuberculosis. *J Immunol* **167**, 910-8 (2001).

60. Abel, B. et al. Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. *J Immunol* **169**, 3155-62 (2002).

61. Kamath, A.B., Alt, J., Debbabi, H. & Behar, S.M. Toll-like receptor 4-defective C3H/HeJ mice are not more susceptible than other C3H substrains to infection with Mycobacterium tuberculosis. *Infect Immun* **71**, 4112-8 (2003).

62. Reiling, N. et al. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. *J Immunol* **169**, 3480-4 (2002).

63. Drennan, M.B. et al. Toll-like receptor 2-deficient mice succumb to Mycobacterium tuberculosis infection. *Am J Pathol* **164**, 49-57 (2004).

64. Sugawara, I. et al. Mycobacterial infection in TLR2 and TLR6 knockout mice. *Microbiol Immunol* **47**, 327-36 (2003).

65. Ozinsky, A. et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A* **97**, 13766-71 (2000).

66. Bulut, Y., Faure, E., Thomas, L., Equils, O. & Arditi, M. Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. *J Immunol* **167**, 987-94 (2001).

67. Maeda, N. et al. The cell surface receptor DC-SIGN discriminates between Mycobacterium species through selective recognition of the mannose caps on lipoarabinomannan. *J Biol Chem* **278**, 5513-6 (2003).

68. Geijtenbeek, T.B. et al. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med* **197**, 7-17 (2003).

69. van Crevel, R., Ottenhoff, T.H. & van der Meer, J.W. Innate immunity to Mycobacterium tuberculosis. *Clin Microbiol Rev* **15**, 294-309 (2002).

70. Dannenberg, A.M. & Rook, J.A. *Pathogenis of pulmonary tuberculosis: an interplay of tissue damaging and macrophage activating immune responses. Dual*

mechanism that control bacillary multiplication., 459-483 (American Society for Microbiology, Washigton, DC, 1994).

71. Weber, I., Fritz, C., Ruttkowski, S., Kreft, A. & Bange, F.C. Anaerobic nitrate reductase (narGHJI) activity of Mycobacterium bovis BCG in vitro and its contribution to virulence in immunodeficient mice. *Mol Microbiol* **35**, 1017-25 (2000).

72. McKinney, J.D. et al. Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**, 735-8 (2000).

73. Kaufmann, S.H. Is the development of a new tuberculosis vaccine possible? *Nat Med* **6**, 955-60 (2000).

74. Calmette, A. La Vaccination Préventive contra la Tuberculosis. (ed. Cie, M.e.) 259 (Paris, 1927).

75. Fine, P.E. Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* **346**, 1339-45 (1995).

76. Buu, N., Sanchez, F. & Schurr, E. The Bcg host-resistance gene. *Clin Infect Dis* **31 Suppl 3**, S81-5 (2000).

77. Colditz, G.A. et al. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. *Jama* **271**, 698-702 (1994).

78. Brandt, L. et al. Failure of the Mycobacterium bovis BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infect Immun* **70**, 672-8 (2002).

79. Buddle, B.M., Wards, B.J., Aldwell, F.E., Collins, D.M. & de Lisle, G.W. Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine* **20**, 1126-33 (2002).

80. Li, H. et al. Evidence for absence of the MPB64 gene in some substrains of Mycobacterium bovis BCG. *Infect Immun* **61**, 1730-4 (1993).

81. van Rie, A. et al. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. *N Engl J Med* **341**, 1174-9 (1999).

82. Cole, S.T. et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* **393**, 537-44 (1998).

83. Tascon, R.E. et al. Vaccination against tuberculosis by DNA injection. *Nat Med* **2**, 888-92 (1996).

84. Barry, C.E., 3rd. Interpreting cell wall 'virulence factors' of Mycobacterium tuberculosis. *Trends Microbiol* **9**, 237-41 (2001).

85. Bellamy, R.J. & Hill, A.V. Host genetic susceptibility to human tuberculosis. *Novartis Found Symp* **217**, 3-13; discussion 13-23 (1998).

86. Motulsky, A.G. Metabolic polymorphisms and the role of infectious diseases in human evolution. *Hum Biol.* **32**, 28-62 (1960).

87. Sousa, A.O., Salem, J.I., Lee, F.K., Vercosa, M.C., Cruaud, P., Bloom, B.R., Lagrange, P.H. & David, H.L. An epidemic of tuberculosis with a high rate of tuberculin anergy among a population previously unexposed to tuberculosis, the Yanomami Indians of the Brazilian Amazon. *Proc. Natl. Acad. Sci* **94**, 13227-13232 (1997).

88. Budd, W. The nature and the mode of propagation of phthisis. *Lancet* **ii**, 451-452. (1867).

89. Stead, W.W., Senner, J.W., Reddick, W.T. & Lofgren, J.P. Racial differences in susceptibility to infection by Mycobacterium tuberculosis. *N Engl J Med* **322**, 422-7 (1990).

90. Comstock, G.W. Tuberculosis in twins: a re-analysis of the Prophit survey. *Am. Rev. Resp. Dis* **117**, 621-624 (1978).

91. Kallmann FJ & D, R. Twin studies on the significance of genetic factors in tuberculosis. *Am Rev Tuberc* **47**, 549-574 (1942).

92. Simmonds, B. *Tuberculosis in twins*, (London, UK, 1963).

93. Rieder, H.L., Kelly, G.D., Bloch, A.B., Cauthen, G.M. & Snider, D.E., Jr. Tuberculosis diagnosed at death in the United States. *Chest* **100**, 678-81 (1991).

94. Hinman, A.R., Judd, J.M., Kolnik, J.P. & Daitch, P.B. Changing risks in tuberculosis. *Am J Epidemiol* **103**, 486-97 (1976).

95. Casanova, J.L. & Abel, L. Genetic dissection of immunity to mycobacteria: the human model. *Annu Rev Immunol* **20**, 581-620 (2002).

96. Singh, S.P., Mehra, N.K., Dingley, H.B., Pande, J.N. & Vaidya, M.C. Human leukocyte antigen (HLA)-linked control of susceptibility to pulmonary tuberculosis and association with HLA-DR types. *J Infect Dis* **148**, 676-81 (1983).

97. Bothamley, G.H. et al. Association of tuberculosis and M. tuberculosis-specific antibody levels with HLA. *J Infect Dis* **159**, 549-55 (1989).

98. Brahmajothi, V. et al. Association of pulmonary tuberculosis and HLA in south India. *Tubercle* **72**, 123-32 (1991).

99. Rajalingam, R., Mehra, N.K., Jain, R.C., Myneedu, V.P. & Pande, J.N. Polymerase chain reaction--based sequence-specific oligonucleotide hybridization

analysis of HLA class II antigens in pulmonary tuberculosis: relevance to chemotherapy and disease severity. *J Infect Dis* **173**, 669-76 (1996).

100. Hawkins, B.R. et al. HLA typing in the Hong Kong Chest Service/British Medical Research Council study of factors associated with the breakdown to active tuberculosis of inactive pulmonary lesions. *Am Rev Respir Dis* **138**, 1616-21 (1988).

101. Cox, R.A. et al. Immunogenetic analysis of human tuberculosis. *J Infect Dis* **158**, 1302-8 (1988).

102. Sanjeevi, C.B. et al. No association or linkage with HLA-DR or -DQ genes in south Indians with pulmonary tuberculosis. *Tuber Lung Dis* **73**, 280-4 (1992).

103. Goldfeld, A.E. et al. Association of an HLA-DQ allele with clinical tuberculosis. *Jama* **279**, 226-8 (1998).

104. Teran-Escandon, D. et al. Human leukocyte antigen-associated susceptibility to pulmonary tuberculosis: molecular analysis of class II alleles by DNA amplification and oligonucleotide hybridization in Mexican patients. *Chest* **115**, 428-33 (1999).

105. Ravikumar, M. et al. Associations of HLA-DRB1, DQB1 and DPB1 alleles with pulmonary tuberculosis in south India. *Tuber Lung Dis* **79**, 309-17 (1999).

106. LeBlanc, S.B., Naik, E.G., Jacobson, L. & Kaslow, R.A. Association of DRB1*1501 with disseminated Mycobacterium avium complex infection in North American AIDS patients. *Tissue Antigens* **55**, 17-23 (2000).

107. Shaw, M.A. et al. Evidence that genetic susceptibility to Mycobacterium tuberculosis in a Brazilian population is under oligogenic control: linkage study of the candidate genes NRAMP1 and TNFA. *Tuber Lung Dis* **78**, 35-45 (1997).

108. Vidal, S.M., Malo, D., Vogan, K., Skamene, E. & Gros, P. Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. *Cell* **73**, 469-85 (1993).

109. Malo, D. et al. Haplotype mapping and sequence analysis of the mouse Nramp gene predict susceptibility to infection with intracellular parasites. *Genomics* **23**, 51-61 (1994).

110. Vidal, S. et al. The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. *J Exp Med* **182**, 655-66 (1995).

111. Jabado, N. et al. Natural resistance to intracellular infections: natural resistance-associated macrophage protein 1 (Nramp1) functions as a pH-dependent manganese transporter at the phagosomal membrane. *J Exp Med* **192**, 1237-48 (2000).

112. Bellamy, R. et al. Variations in the NRAMP1 gene and susceptibility to tuberculosis in West Africans. *N Engl J Med* **338**, 640-4 (1998).

113. Gao, P.S. et al. Genetic variants of NRAMP1 and active tuberculosis in Japanese populations. International Tuberculosis Genetics Team. *Clin Genet* **58**, 74-6 (2000).

114. Delgado, J.C., Baena, A., Thim, S. & Goldfeld, A.E. Ethnic-specific genetic associations with pulmonary tuberculosis. *J Infect Dis* **186**, 1463-8 (2002).

115. Cervino, A.C., Lakiss, S., Sow, O. & Hill, A.V. Allelic association between the NRAMP1 gene and susceptibility to tuberculosis in Guinea-Conakry. *Ann Hum Genet* **64**, 507-12 (2000).

116. Soborg, C. et al. Natural resistance-associated macrophage protein 1 polymorphisms are associated with microscopy-positive tuberculosis. *J Infect Dis* **186**, 517-21 (2002).

117. Ryu, S. et al. 3'UTR polymorphisms in the NRAMP1 gene are associated with susceptibility to tuberculosis in Koreans. *Int J Tuberc Lung Dis* **4**, 577-80 (2000).

118. Ma, X. et al. 5' dinucleotide repeat polymorphism of NRAMP1 and susceptibility to tuberculosis among Caucasian patients in Houston, Texas. *Int J Tuberc Lung Dis* **6**, 818-23 (2002).

119. Hoal, E.G. et al. SLC11A1 (NRAMP1) but not SLC11A2 (NRAMP2) polymorphisms are associated with susceptibility to tuberculosis in a high-incidence community in South Africa. *Int J Tuberc Lung Dis* **8**, 1464-71 (2004).

120. Awomoyi, A.A. et al. Interleukin-10, polymorphism in SLC11A1 (formerly NRAMP1), and susceptibility to tuberculosis. *J Infect Dis* **186**, 1808-14 (2002).

121. Liaw, Y.S. et al. Variations in the NRAMP1 gene and susceptibility of tuberculosis in Taiwanese. *Int J Tuberc Lung Dis* **6**, 454-60 (2002).

122. El Baghdadi, J. et al. Variants of the human NRAMP1 gene and susceptibility to tuberculosis in Morocco. *Int J Tuberc Lung Dis* **7**, 599-602 (2003).

123. Greenwood, C.M. et al. Linkage of tuberculosis to chromosome 2q35 loci, including NRAMP1, in a large aboriginal Canadian family. *Am J Hum Genet* **67**, 405-16 (2000).

124. Bellamy, R. et al. Genetic susceptibility to tuberculosis in Africans: a genome-wide scan. *Proc Natl Acad Sci U S A* **97**, 8005-9 (2000).

125. Malik, S. et al. Alleles of the NRAMP1 gene are risk factors for pediatric tuberculosis disease. *Proc Natl Acad Sci U S A* **102**, 12183-8 (2005).

126. Turner, M.W. Mannose-binding lectin (MBL) in health and disease. *Immunobiology* **199**, 327-39 (1998).

127. Selvaraj, P., Narayanan, P.R. & Reetha, A.M. Association of functional mutant homozygotes of the mannose binding protein gene with susceptibility to pulmonary tuberculosis in India. *Tuber Lung Dis* **79**, 221-7 (1999).

128. Hoal-Van Helden, E.G. et al. Mannose-binding protein B allele confers protection against tuberculous meningitis. *Pediatr Res* **45**, 459-64 (1999).

129. Rook, G.A. et al. Vitamin D3, gamma interferon, and control of proliferation of Mycobacterium tuberculosis by human monocytes. *Immunology* **57**, 159-63 (1986).

130. Bellamy, R. et al. Tuberculosis and chronic hepatitis B virus infection in Africans and variation in the vitamin D receptor gene. *J Infect Dis* **179**, 721-4 (1999).

131. Wilkinson, R.J. et al. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. *Lancet* **355**, 618-21 (2000).

132. Li, C.M. et al. Association of a polymorphism in the P2X7 gene with tuberculosis in a Gambian population. *J Infect Dis* **186**, 1458-62 (2002).

133. Juffermans, N.P. et al. Interleukin-1 signaling is essential for host defense during murine pulmonary tuberculosis. *J Infect Dis* **182**, 902-8 (2000).

134. Juffermans, N.P. et al. Tumor necrosis factor and interleukin-1 inhibitors as markers of disease activity of tuberculosis. *Am J Respir Crit Care Med* **157**, 1328-31 (1998).

135. Wilkinson, R.J. et al. Influence of polymorphism in the genes for the interleukin (IL)-1 receptor antagonist and IL-1beta on tuberculosis. *J Exp Med* **189**, 1863-74 (1999).

136. Cervino, A.C. et al. Fine mapping of a putative tuberculosis-susceptibility locus on chromosome 15q11-13 in African families. *Hum Mol Genet* **11**, 1599-603 (2002).

137. Schwebach, J.R. et al. Infection of mice with aerosolized Mycobacterium tuberculosis: use of a nose-only apparatus for delivery of low doses of inocula and design of an ultrasafe facility. *Appl Environ Microbiol* **68**, 4646-9 (2002).

138. Manca, C. et al. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha /beta. *Proc Natl Acad Sci U S A* **98**, 5752-7 (2001).

139. Manca, C. et al. Mycobacterium tuberculosis CDC1551 induces a more vigorous host response in vivo and in vitro, but is not more virulent than other clinical isolates. *J Immunol* **162**, 6740-6 (1999).

140. Dunn, P.L. & North, R.J. Virulence ranking of some Mycobacterium tuberculosis and Mycobacterium bovis strains according to their ability to multiply in the lungs, induce lung pathology, and cause mortality in mice. *Infect Immun* **63**, 3428-37 (1995).

141. Scanga, C.A. et al. MyD88-deficient mice display a profound loss in resistance to Mycobacterium tuberculosis associated with partially impaired Th1 cytokine and nitric oxide synthase 2 expression. *Infect Immun* **72**, 2400-4 (2004).

142. Yamada, H., Mizumo, S., Horai, R., Iwakura, Y. & Sugawara, I. Protective role of interleukin-1 in mycobacterial infection in IL-1 alpha/beta double-knockout mice. *Lab Invest* **80**, 759-67 (2000).

143. Saunders, B.M., Frank, A.A., Orme, I.M. & Cooper, A.M. Interleukin-6 induces early gamma interferon production in the infected lung but is not required for generation of specific immunity to Mycobacterium tuberculosis infection. *Infect Immun* **68**, 3322-6 (2000).

144. North, R.J. Mice incapable of making IL-4 or IL-10 display normal resistance to infection with Mycobacterium tuberculosis. *Clin Exp Immunol* **113**, 55-8 (1998).

145. Roach, D.R. et al. Endogenous inhibition of antimycobacterial immunity by IL-10 varies between mycobacterial species. *Scand J Immunol* **54**, 163-70 (2001).

146. Murray, P.J. & Young, R.A. Increased antimycobacterial immunity in interleukin-10-deficient mice. *Infect Immun* **67**, 3087-95 (1999).

147. Kawakami, K. et al. Interferon-gamma production and host protective response against Mycobacterium tuberculosis in mice lacking both IL-12p40 and IL-18. *Microbes Infect* **6**, 339-49 (2004).

148. Cooper, A.M. et al. Mice lacking bioactive IL-12 can generate protective, antigen-specific cellular responses to mycobacterial infection only if the IL-12 p40 subunit is present. *J Immunol* **168**, 1322-7 (2002).

149. Sugawara, I. et al. Role of interleukin-18 (IL-18) in mycobacterial infection in IL-18-gene-disrupted mice. *Infect Immun* **67**, 2585-9 (1999).

150. Jung, Y.J., LaCourse, R., Ryan, L. & North, R.J. Evidence inconsistent with a negative influence of T helper 2 cells on protection afforded by a dominant T

helper 1 response against Mycobacterium tuberculosis lung infection in mice. *Infect Immun* **70**, 6436-43 (2002).

151. Sugawara, I., Yamada, H., Hua, S. & Mizuno, S. Role of interleukin (IL)-1 type 1 receptor in mycobacterial infection. *Microbiol Immunol* **45**, 743-50 (2001).

152. Yamada, H., Mizuno, S. & Sugawara, I. Interferon regulatory factor 1 in mycobacterial infection. *Microbiol Immunol* **46**, 751-60 (2002).

153. Flynn, J.L. et al. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med* **178**, 2249-54 (1993).

154. Cooper, A.M. et al. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med* **178**, 2243-7 (1993).

155. Roach, D.R. et al. Secreted lymphotoxin-alpha is essential for the control of an intracellular bacterial infection. *J Exp Med* **193**, 239-46 (2001).

156. Ehlers, S. et al. The lymphotoxin beta receptor is critically involved in controlling infections with the intracellular pathogens Mycobacterium tuberculosis and Listeria monocytogenes. *J Immunol* **170**, 5210-8 (2003).

157. Bean, A.G. et al. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. *J Immunol* **162**, 3504-11 (1999).

158. Flynn, J.L. et al. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. *Immunity* **2**, 561-72 (1995).

159. Sugawara, I. et al. Mycobacterial infection in natural killer T cell knockout mice. *Tuberculosis (Edinb)* **82**, 97-104 (2002).

160. Mogues, T., Goodrich, M.E., Ryan, L., LaCourse, R. & North, R.J. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. *J Exp Med* **193**, 271-80 (2001).

161. Turner, J. et al. CD8- and CD95/95L-dependent mechanisms of resistance in mice with chronic pulmonary tuberculosis. *Am J Respir Cell Mol Biol* **24**, 203-9 (2001).

162. D'Souza, C.D. et al. An anti-inflammatory role for gamma delta T lymphocytes in acquired immunity to Mycobacterium tuberculosis. *J Immunol* **158**, 1217-21 (1997).

163. Sousa, A.O. et al. Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. *Proc Natl Acad Sci U S A* **97**, 4204-8 (2000).

164. Behar, S.M., Dascher, C.C., Grusby, M.J., Wang, C.R. & Brenner, M.B. Susceptibility of mice deficient in CD1D or TAP1 to infection with Mycobacterium tuberculosis. *J Exp Med* **189**, 1973-80 (1999).

165. Caruso, A.M. et al. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J Immunol* **162**, 5407-16 (1999).

166. D'Souza, C.D. et al. A novel nonclassic beta2-microglobulin-restricted mechanism influencing early lymphocyte accumulation and subsequent resistance to tuberculosis in the lung. *Am J Respir Cell Mol Biol* **23**, 188-93 (2000).

167. Lazarevic, V., Myers, A.J., Scanga, C.A. & Flynn, J.L. CD40, but not CD40L, is required for the optimal priming of T cells and control of aerosol M. tuberculosis infection. *Immunity* **19**, 823-35 (2003).

168. Campos-Neto, A. et al. CD40 ligand is not essential for the development of cell-mediated immunity and resistance to Mycobacterium tuberculosis. *J Immunol* **160**, 2037-41 (1998).

169. Kipnis, A., Basaraba, R.J., Turner, J. & Orme, I.M. Increased neutrophil influx but no impairment of protective immunity to tuberculosis in mice lacking the CD44 molecule. *J Leukoc Biol* **74**, 992-7 (2003).

170. Urdahl, K.B., Liggitt, D. & Bevan, M.J. CD8+ T cells accumulate in the lungs of Mycobacterium tuberculosis-infected Kb-/-Db-/- mice, but provide minimal protection. *J Immunol* **170**, 1987-94 (2003).

171. Repique, C.J. et al. Susceptibility of mice deficient in the MHC class II transactivator to infection with Mycobacterium tuberculosis. *Scand J Immunol* **58**, 15-22 (2003).

172. Flynn, J.L., Goldstein, M.M., Triebold, K.J., Koller, B. & Bloom, B.R. Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. *Proc Natl Acad Sci U S A* **89**, 12013-7 (1992).

173. Cooper, A.M., D'Souza, C., Frank, A.A. & Orme, I.M. The course of Mycobacterium tuberculosis infection in the lungs of mice lacking expression of either perforin- or granzyme-mediated cytolytic mechanisms. *Infect Immun* **65**, 1317-20 (1997).

174. Peters, W. et al. Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A* **98**, 7958-63 (2001).

175. Kipnis, A., Basaraba, R.J., Orme, I.M. & Cooper, A.M. Role of chemokine ligand 2 in the protective response to early murine pulmonary tuberculosis. *Immunology* **109**, 547-51 (2003).

176. Verbon, A., Leemans, J.C., Weijer, S., Florquin, S. & Van Der Poll, T. Mice lacking the multidrug resistance protein 1 have a transiently impaired immune response during tuberculosis. *Clin Exp Immunol* **130**, 32-6 (2002).

177. Actor, J.K. et al. A role for complement C5 in organism containment and granulomatous response during murine tuberculosis. *Scand J Immunol* **53**, 464-74 (2001).

178. Alaniz, R.C. et al. Dopamine beta-hydroxylase deficiency impairs cellular immunity. *Proc Natl Acad Sci U S A* **96**, 2274-8 (1999).

179. Johnson, C.M., Cooper, A.M., Frank, A.A. & Orme, I.M. Adequate expression of protective immunity in the absence of granuloma formation in Mycobacterium tuberculosis-infected mice with a disruption in the intracellular adhesion molecule 1 gene. *Infect Immun* **66**, 1666-70 (1998).

180. Sugawara, I., Mizuno, S., Yamada, H., Matsumoto, M. & Akira, S. Disruption of nuclear factor-interleukin-6, a transcription factor, results in severe mycobacterial infection. *Am J Pathol* **158**, 361-6 (2001).

181. Yamada, H., Mizuno, S., Reza-Gholizadeh, M. & Sugawara, I. Relative importance of NF-kappaB p50 in mycobacterial infection. *Infect Immun* **69**, 7100-5 (2001).

182. Jung, Y.J., LaCourse, R., Ryan, L. & North, R.J. Virulent but not avirulent Mycobacterium tuberculosis can evade the growth inhibitory action of a T helper

1-dependent, nitric oxide Synthase 2-independent defense in mice. *J Exp Med* **196**, 991-8 (2002).

183. Sugawara, I., Yamada, H. & Mizuno, S. STAT1 knockout mice are highly susceptible to pulmonary mycobacterial infection. *Tohoku J Exp Med* **202**, 41-50 (2004).

184. Sugawara, I., Yamada, H. & Mizuno, S. Relative importance of STAT4 in murine tuberculosis. *J Med Microbiol* **52**, 29-34 (2003).

185. Ladel, C.H., Szalay, G., Riedel, D. & Kaufmann, S.H. Interleukin-12 secretion by Mycobacterium tuberculosis-infected macrophages. *Infect Immun* **65**, 1936-8 (1997).

186. Henderson, R.A., Watkins, S.C. & Flynn, J.L. Activation of human dendritic cells following infection with Mycobacterium tuberculosis. *J Immunol* **159**, 635-43 (1997).

187. Flynn, J.L. et al. IL-12 increases resistance of BALB/c mice to Mycobacterium tuberculosis infection. *J Immunol* **155**, 2515-24 (1995).

188. Rhoades, E.R., Cooper, A.M. & Orme, I.M. Chemokine response in mice infected with Mycobacterium tuberculosis. *Infect Immun* **63**, 3871-7 (1995).

189. Cooper, A.M., Magram, J., Ferrante, J. & Orme, I.M. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. *J Exp Med* **186**, 39-45 (1997).

190. Trinchieri, G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* **13**, 251-76 (1995).

191. O'Neill, L.A. & Greene, C. Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. *J Leukoc Biol* **63**, 650-7 (1998).

192. Orme, I.M., Roberts, A.D., Griffin, J.P. & Abrams, J.S. Cytokine secretion by CD4 T lymphocytes acquired in response to Mycobacterium tuberculosis infection. *J Immunol* **151**, 518-25 (1993).

193. Orme, I.M. et al. T lymphocytes mediating protection and cellular cytolysis during the course of Mycobacterium tuberculosis infection. Evidence for different kinetics and recognition of a wide spectrum of protein antigens. *J Immunol* **148**, 189-96 (1992).

194. Barnes, P.F. et al. Patterns of cytokine production by mycobacterium-reactive human T-cell clones. *Infect Immun* **61**, 197-203 (1993).

195. Wang, J., Wakeham, J., Harkness, R. & Xing, Z. Macrophages are a significant source of type 1 cytokines during mycobacterial infection. *J Clin Invest* **103**, 1023-9 (1999).

196. Dalton, D.K. et al. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* **259**, 1739-42 (1993).

197. Serbina, N.V. & Flynn, J.L. Early emergence of CD8(+) T cells primed for production of type 1 cytokines in the lungs of Mycobacterium tuberculosis-infected mice. *Infect Immun* **67**, 3980-8 (1999).

198. Netea, M.G., Kullberg, B.J., Verschueren, I. & Van Der Meer, J.W. Interleukin-18 induces production of proinflammatory cytokines in mice: no intermediate role for the cytokines of the tumor necrosis factor family and interleukin-1beta. *Eur J Immunol* **30**, 3057-60 (2000).

199. Powrie, F. & Coffman, R.L. Inhibition of cell-mediated immunity by IL4 and IL10. *Res Immunol* **144**, 639-43 (1993).

200. Appelberg, R., Orme, I.M., Pinto de Sousa, M.I. & Silva, M.T. In vitro effects of interleukin-4 on interferon-gamma-induced macrophage activation. *Immunology* **76**, 553-9 (1992).

201. Sugawara, I., Yamada, H., Mizuno, S. & Iwakura, Y. IL-4 is required for defense against mycobacterial infection. *Microbiol Immunol* **44**, 971-9 (2000).

202. Rojas, M., Olivier, M., Gros, P., Barrera, L.F. & Garcia, L.F. TNF-alpha and IL-10 modulate the induction of apoptosis by virulent Mycobacterium tuberculosis in murine macrophages. *J Immunol* **162**, 6122-31 (1999).

203. Murray, P.J., Wang, L., Onufryk, C., Tepper, R.I. & Young, R.A. T cell-derived IL-10 antagonizes macrophage function in mycobacterial infection. *J Immunol* **158**, 315-21 (1997).

204. Gonzalez-Juarrero, M. et al. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with Mycobacterium tuberculosis. *Infect Immun* **69**, 1722-8 (2001).

205. Shen, Y. et al. Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections. *Science* **295**, 2255-8 (2002).

206. Ladel, C.H., Blum, C., Dreher, A., Reifenberg, K. & Kaufmann, S.H. Protective role of gamma/delta T cells and alpha/beta T cells in tuberculosis. *Eur J Immunol* **25**, 2877-81 (1995).

207. Muller, I., Cobbold, S.P., Waldmann, H. & Kaufmann, S.H. Impaired resistance to Mycobacterium tuberculosis infection after selective in vivo depletion of L3T4+ and Lyt-2+ T cells. *Infect Immun* **55**, 2037-41 (1987).

208. Tascon, R.E., Stavropoulos, E., Lukacs, K.V. & Colston, M.J. Protection against Mycobacterium tuberculosis infection by CD8+ T cells requires the production of gamma interferon. *Infect Immun* **66**, 830-4 (1998).

209. Balcewicz-Sablinska, M.K., Keane, J., Kornfeld, H. & Remold, H.G. Pathogenic Mycobacterium tuberculosis evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF-alpha. *J Immunol* **161**, 2636-41 (1998).

210. van Pinxteren, L.A., Cassidy, J.P., Smedegaard, B.H., Agger, E.M. & Andersen, P. Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells. *Eur J Immunol* **30**, 3689-98 (2000).

211. Rolph, M.S. et al. MHC class Ia-restricted T cells partially account for beta2-microglobulin-dependent resistance to Mycobacterium tuberculosis. *Eur J Immunol* **31**, 1944-9 (2001).

212. Flynn, J.L. & Chan, J. Immunology of tuberculosis. *Annu Rev Immunol* **19**, 93-129 (2001).

213. Canaday, D.H. et al. Activation of human CD8+ alpha beta TCR+ cells by Mycobacterium tuberculosis via an alternate class I MHC antigen-processing pathway. *J Immunol* **162**, 372-9 (1999).

214. Schaible, U.E., Collins, H.L., Priem, F. & Kaufmann, S.H. Correction of the iron overload defect in beta-2-microglobulin knockout mice by lactoferrin abolishes their increased susceptibility to tuberculosis. *J Exp Med* **196**, 1507-13 (2002).

215. Musa, S.A. et al. Response of inbred mice to aerosol challenge with Mycobacterium tuberculosis. *Infect Immun* **55**, 1862-6 (1987).

216. Lynch, C.J., Pierce-Chase, C.H. & Dubos, R. A Genetic Study of Susceptibility to Experimental Tuberculosis in Mice Infected with Mammalian Tubercle Bacilli. *J Exp Med* **121**, 1051-70 (1965).

217. Donovick, R., McKee, C.M., Jambor, W.P. & Rake, G. The use of the mouse on a standardized test for anti-tuberculous activity of compounds of natural or synthetic origin.II.Choice of mouse strains. *Am Rev Tub* **60**, 109-120 (1949).

218. Pierce, C., Dubos, R.J. & Middlebrook, G. Infection of mice with mammelian tubercule bacilli grown in Tween-albuminliquid medium. *J Exp Med* **86**, 159-174 (1947).

219. Medina, E. & North, R.J. Resistance ranking of some common inbred mouse strains to Mycobacterium tuberculosis and relationship to major histocompatibility complex haplotype and Nramp1 genotype. *Immunology* **93**, 270-4 (1998).

220. Nikonenko, B.V. et al. Influence of the mouse Bcg, Tbc-1 and xid genes on resistance and immune responses to tuberculosis infection and efficacy of bacille Calmette-Guerin (BCG) vaccination. *Clin Exp Immunol* **104**, 37-43 (1996).

221. Nikonenko, B.V., Averbakh, M.M., Jr., Lavebratt, C., Schurr, E. & Apt, A.S. Comparative analysis of mycobacterial infections in susceptible I/St and resistant A/Sn inbred mice. *Tuber Lung Dis* **80**, 15-25 (2000).

222. Apt, A.S. et al. Distinct H-2 complex control of mortality, and immune responses to tuberculosis infection in virgin and BCG-vaccinated mice. *Clin Exp Immunol* **94**, 322-9 (1993).

223. Actor, J.K., Olsen, M., Jagannath, C. & Hunter, R.L. Relationship of survival, organism containment, and granuloma formation in acute murine tuberculosis. *J Interferon Cytokine Res* **19**, 1183-93 (1999).

224. Nesbitt, M.N. & Skamene, E. Recombinant inbred mouse strains derived from A/J and C57BL/6J: a tool for the study of genetic mechanisms in host resistance to infection and malignancy. *J Leukoc Biol* **36**, 357-64 (1984).

225. Brown, D.H., Miles, B.A. & Zwilling, B.S. Growth of Mycobacterium tuberculosis in BCG-resistant and -susceptible mice: establishment of latency and reactivation. *Infect Immun* **63**, 2243-7 (1995).

226. Mitsos, L.M. et al. Susceptibility to tuberculosis: a locus on mouse chromosome 19 (Trl-4) regulates Mycobacterium tuberculosis replication in the lungs. *Proc Natl Acad Sci U S A* **100**, 6610-5 (2003).

227. Medina, E. & North, R.J. Genetically susceptible mice remain proportionally more susceptible to tuberculosis after vaccination. *Immunology* **96**, 16-21 (1999).

228. Cardona, P.J. et al. Widespread bronchogenic dissemination makes DBA/2 mice more susceptible than C57BL/6 mice to experimental aerosol infection with Mycobacterium tuberculosis. *Infect Immun* **71**, 5845-54 (2003).

229. Mitsos, L.M. et al. Genetic control of susceptibility to infection with Mycobacterium tuberculosis in mice. *Genes Immun* **1**, 467-77 (2000).

230. Lander, E.S. & Botstein, D. Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics* **121**, 185-99 (1989).

231. Broman, K.W. Review of statistical methods for QTL mapping in experimental crosses. *Lab Anim (NY)* **30**, 44-52 (2001).

232. Porter, J.D. & McAdam, K.P. The re-emergence of tuberculosis. *Annu Rev Public Health* **15**, 303-23 (1994).

233. Huebner, R.E. & Castro, K.G. The changing face of tuberculosis. *Annu Rev Med* **46**, 47-55 (1995).

234. Young, D.B. & Duncan, K. Prospects for new interventions in the treatment and prevention of mycobacterial disease. *Annu Rev Microbiol* **49**, 641-73 (1995).

235. Nickonenko BV, A.A., Moroz AM, Averbakh MM. Genetic analysis of susceptibility of mice to H37Rv tuberculosis infection: sensitivity versus relative resistance. In: Skamene. E (ed). Genetic Control of Host Resistance to Infection and Malignancy. A. R. Liss: Toronto, 1985, pp 291–298.

236. Lavebratt, C., Apt, A.S., Nikonenko, B.V., Schalling, M. & Schurr, E. Severity of tuberculosis in mice is linked to distal chromosome 3 and proximal chromosome 9. *J Infect Dis* **180**, 150-5 (1999).

237. Brett, S., Orrell, J.M., Swanson Beck, J. & Ivanyi, J. Influence of H-2 genes on growth of Mycobacterium tuberculosis in the lungs of chronically infected mice. *Immunology* **76**, 129-32 (1992).

238. Medina, E. & North, R.J. Evidence inconsistent with a role for the Bcg gene (Nramp1) in resistance of mice to infection with virulent Mycobacterium tuberculosis. *J Exp Med* **183**, 1045-51 (1996).

239. Lander, E.S. et al. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* **1**, 174-81 (1987).

240. Dietrich, W.F. et al. A comprehensive genetic map of the mouse genome. *Nature* **380**, 149-52 (1996).

241. SAS Institute. (1990) in SAS/STAT User's Guide, V., Fourth Edition. SAS Institute. (Cary, North Carolina).

242. Gubbay, J. et al. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* **346**, 245-50 (1990).

243. Lander, E. & Kruglyak, L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* **11**, 241-7 (1995).

244. Kramnik, I., Demant, P. & Bloom, B.B. Susceptibility to tuberculosis as a complex genetic trait: analysis using recombinant congenic strains of mice. *Novartis Found Symp* **217**, 120-31; discussion 132-7 (1998).

245. Skamene, E., Schurr, E. & Gros, P. Infection genomics: Nramp1 as a major determinant of natural resistance to intracellular infections. *Annu Rev Med* **49**, 275-87 (1998).

246. Abel, L. et al. Susceptibility to leprosy is linked to the human NRAMP1 gene. *J Infect Dis* **177**, 133-45 (1998).

247. Malo, D., Vidal, S.M., Hu, J., Skamene, E. & Gros, P. High-resolution linkage map in the vicinity of the host resistance locus Bcg. *Genomics* **16**, 655-63 (1993).

248. Sebastiani, G. et al. Mapping of genetic modulators of natural resistance to infection with Salmonella typhimurium in wild-derived mice. *Genomics* **47**, 180-6 (1998).

249. Kemp, S.J., Iraqi, F., Darvasi, A., Soller, M. & Teale, A.J. Localization of genes controlling resistance to trypanosomiasis in mice. *Nat Genet* **16**, 194-6 (1997).

250. Bleul, C.C., Fuhlbrigge, R.C., Casasnovas, J.M., Aiuti, A. & Springer, T.A. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med* **184**, 1101-9 (1996).

251. Ward, S.G. & Westwick, J. Chemokines: understanding their role in T-lymphocyte biology. *Biochem J* **333 (Pt 3)**, 457-70 (1998).

252. Moore, K.W., O'Garra, A., de Waal Malefyt, R., Vieira, P. & Mosmann, T.R. Interleukin-10. *Annu Rev Immunol* **11**, 165-90 (1993).

253. Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. & Muller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263-74 (1993).

254. Rennick, D.M., Fort, M.M. & Davidson, N.J. Studies with IL-10-/- mice: an overview. *J Leukoc Biol* **61**, 389-96 (1997).

255. Li, C., Corraliza, I. & Langhorne, J. A defect in interleukin-10 leads to enhanced malarial disease in Plasmodium chabaudi chabaudi infection in mice. *Infect Immun* **67**, 4435-42 (1999).

256. Gazzinelli, R.T. et al. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *J Immunol* **157**, 798-805 (1996).

257. Hunter, C.A. et al. IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi. *J Immunol* **158**, 3311-6 (1997).

258. Dai, W.J., Kohler, G. & Brombacher, F. Both innate and acquired immunity to Listeria monocytogenes infection are increased in IL-10-deficient mice. *J Immunol* **158**, 2259-67 (1997).

259. Yang, X., Gartner, J., Zhu, L., Wang, S. & Brunham, R.C. IL-10 gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following Chlamydia trachomatis lung infection. *J Immunol* **162**, 1010-7 (1999).

260. Malech, H.L. & Nauseef, W.M. Primary inherited defects in neutrophil function: etiology and treatment. *Semin Hematol* **34**, 279-90 (1997).

261. Nagata, S. & Golstein, P. The Fas death factor. *Science* **267**, 1449-56 (1995).

262. Conceicao-Silva, F., Hahne, M., Schroter, M., Louis, J. & Tschopp, J. The resolution of lesions induced by Leishmania major in mice requires a functional Fas (APO-1, CD95) pathway of cytotoxicity. *Eur J Immunol* **28**, 237-45 (1998).

263. Frenette, P.S., Mayadas, T.N., Rayburn, H., Hynes, R.O. & Wagner, D.D. Susceptibility to infection and altered hematopoiesis in mice deficient in both P- and E-selectins. *Cell* **84**, 563-74 (1996).

264. Noguchi, M. et al. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell* **73**, 147-57 (1993).

265. Sadlack, B. et al. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* **75**, 253-61 (1993).

266. Bagby GC, H.C.G.f., cytokines, and the control of Hematopoiesis. In: Hoffman R et al (eds). Hematology: Basic Principles and Practice. Churchill Livingstone: New York, 2000, pp 154–202.

267. Altare, F. et al. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science* **280**, 1432-5 (1998).

268. de Jong, R. et al. Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. *Science* **280**, 1435-8 (1998).

269. Mattner, F. et al. Genetically resistant mice lacking interleukin-12 are susceptible to infection with Leishmania major and mount a polarized Th2 cell response. *Eur J Immunol* **26**, 1553-9 (1996).

270. Xing, Z. et al. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* **101**, 311-20 (1998).

271. Dye, C., Scheele, S., Dolin, P., Pathania, V. & Raviglione, M.C. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA* **282**, 677- 868 (1999).

272. Lienhardt, C. From exposure to disease: the role of environmental factors in susceptibility to and development of tuberculosis. *Epidemiol Rev* **23**, 288-301 (2001).

273. Dubos, R. & Dubos, J. The White Plague: Tuberculosis, Man and Society. *Little, Brown and Co.: Boston* (1952).

274. Reichenbach, J. et al. Mycobacterial diseases in primary immunodeficiencies. *Curr Opin Allergy Clin Immunol* **1**, 503-11 (2001).

275. Picard, C. et al. Inherited interleukin-12 deficiency: IL12B genotype and clinical phenotype of 13 patients from six kindreds. *Am J Hum Genet* **70**, 336-48 (2002).

276. Jouanguy, E. et al. A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection. *Nat Genet* **21**, 370-8 (1999).

277. Doffinger, R. et al. Inherited disorders of IL-12- and IFNgamma-mediated immunity: a molecular genetics update. *Mol Immunol* **38**, 903-9 (2002).

278. Meisner, S.J. et al. Association of NRAMP1 polymorphism with leprosy type but not susceptibility to leprosy per se in west Africans. *Am J Trop Med Hyg* **65**, 733-5 (2001).

279. Fortin, A., Stevenson, M.M. & Gros, P. Complex genetic control of susceptibility to malaria in mice. *Genes Immun* **3**, 177-86 (2002).

280. Kramnik, I., Dietrich, W.F., Demant, P. & Bloom, B.R. Genetic control of resistance to experimental infection with virulent Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A* **97**, 8560-5 (2000).

281. Basten, C.J., Weir, B.S. & Zeng, Z-B. (1994) in Zmap - QTL cartographer. In Proceedings of the 5th World Congress on Genetics Applied to Livestock Production: Computing Strategies and Software, eds. C. Smith, C., Gavora, J.S., Benkel, B., Chesnais, J., Fairfull, W., Gibson, J.P., Kennedy, B.W. & Burnside, E.B. pp 65-66.

282. Basten, C.J., Weir, B.S. & Zeng, Z-B. (2002) in QTL Cartographer, Version 1.16. Department of Statistics. (Raleigh, NC.).

283. Sanchez, F. et al. Multigenic control of disease severity after virulent Mycobacterium tuberculosis infection in mice. *Infect Immun* **71**, 126-31 (2003).

284. Otto, J.M. et al. Identification of multiple loci linked to inflammation and autoantibody production by a genome scan of a murine model of rheumatoid arthritis. *Arthritis Rheum* **42**, 2524-31 (1999).

285. Baeuerle, P.A. & Henkel, T. Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol* **12**, 141-79 (1994).

286. Franzoso, G. et al. Mice deficient in nuclear factor (NF)-kappa B/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *J Exp Med* **187**, 147-59 (1998).

287. Speirs, K., Caamano, J., Goldschmidt, M.H., Hunter, C.A. & Scott, P. NF-kappa B2 is required for optimal CD40-induced IL-12 production but dispensable for Th1 cell Differentiation. *J Immunol* **168**, 4406-13 (2002).

288. Caamano, J. et al. Identification of a role for NF-kappa B2 in the regulation of apoptosis and in maintenance of T cell-mediated immunity to Toxoplasma gondii. *J Immunol* **165**, 5720-8 (2000).

289. Kaisho, T. et al. IkappaB kinase alpha is essential for mature B cell development and function. *J Exp Med* **193**, 417-26 (2001).

290. Trapnell, B.C. & Whitsett, J.A. Gm-CSF regulates pulmonary surfactant homeostasis and alveolar macrophage-mediated innate host defense. *Annu Rev Physiol* **64**, 775-802 (2002).

291. Stanley, E. et al. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A* **91**, 5592-6 (1994).

292. LeVine, A.M., Reed, J.A., Kurak, K.E., Cianciolo, E. & Whitsett, J.A. GM-CSF-deficient mice are susceptible to pulmonary group B streptococcal infection. *J Clin Invest* **103**, 563-9 (1999).

293. Paine, R., 3rd et al. Granulocyte-macrophage colony-stimulating factor in the innate immune response to Pneumocystis carinii pneumonia in mice. *J Immunol* **164**, 2602-9 (2000).

294. Zhan, Y., Lieschke, G.J., Grail, D., Dunn, A.R. & Cheers, C. Essential roles for granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF in the sustained hematopoietic response of Listeria monocytogenes-infected mice. *Blood* **91**, 863-9 (1998).

295. Shibata, Y. et al. GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. *Immunity* **15**, 557-67 (2001).

296. Dirksen, U. et al. Human pulmonary alveolar proteinosis associated with a defect in GM-CSF/IL-3/IL-5 receptor common beta chain expression. *J Clin Invest* **100**, 2211-7 (1997).

297. Sato, K. et al. Type II alveolar cells play roles in macrophage-mediated host innate resistance to pulmonary mycobacterial infections by producing proinflammatory cytokines. *J Infect Dis* **185**, 1139-47 (2002).

298. Hinman, A.R., Judd, J.M., Kolnick, J.P. & Daitch, P.B. Changing risks in tuberculosis. *Am. J. Epidemiol* **103**, 486-497 (1976).

299. Rieder, H.L., Kelly, G.D., Bloch, A.B., Cauthen, G.M.& Snider, D.E. Tuberculosis diagnosed at death in the United States. *Chest* **100**, 678-681 (1991).

300. Stead, W.W., Sener, J.W., Reddick, W.T. & Lofgren, J.P. Racial differences in susceptibility to infection by Mycobacterium tuberculosis. *New. Engl. J. Med* **322**, 1670-1673 (1990).

301. Kallmann, F.J.R., D. *Am. Rev. Tuberc* **47**, 549-574 (1942).

302. Mitsos LM, C.L., Fortin A, Ryan L, LaCourse R, North RJ, Gros P. Genetic control of susceptibility to infection with Mycobacterium tuberculosis in mice. *Genes Immun.* **1**, 467-477 (2000).

303. Korstanje, R. & Paigen, B. From QTL to gene: the harvest begins. *Nat Genet* **31**, 235-6 (2002).

304. Tusher VG, T.R., Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A* **98**, 5116-5121 (2001).

305. Li, Q., Park, P.W., Wilson, C.L. & Parks, W.C. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell* **111**, 635-46 (2002).

306. Riedel, D.D. & Kaufmann, S.H. Chemokine secretion by human polymorphonuclear granulocytes after stimulation with Mycobacterium tuberculosis and lipoarabinomannan. *Infect Immun* **65**, 4620-3 (1997).

307. Guignard, F., Mauel, J. & Markert, M. Identification and characterization of a novel human neutrophil protein related to the S100 family. *Biochem J* **309 (Pt 2)**, 395-401 (1995).

308. Haga, H.J. et al. Calprotectin in patients with systemic lupus erythematosus: relation to clinical and laboratory parameters of disease activity. *Lupus* **2**, 47-50 (1993).

309. Roth, J. et al. Complex pattern of the myelo-monocytic differentiation antigens MRP8 and MRP14 during chronic airway inflammation. *Immunobiology* **186**, 304-14 (1992).

310. Berntzen, H.B., Fagerhol, M.K., Ostensen, M., Mowinckel, P. & Hoyeraal, H.M. The L1 protein as a new indicator of inflammatory activity in patients with juvenile rheumatoid arthritis. *J Rheumatol* **18**, 133-8 (1991).

311. Barthe, C., Figarella, C., Carrere, J. & Guy-Crotte, O. Identification of 'cystic fibrosis protein' as a complex of two calcium-binding proteins present in human cells of myeloid origin. *Biochim Biophys Acta* **1096**, 175-7 (1991).

312. Strasser, F., Gowland, P.L. & Ruef, C. Elevated serum macrophage inhibitory factor-related protein (MRP) 8/14 levels in advanced HIV infection and during disease exacerbation. *J Acquir Immune Defic Syndr Hum Retrovirol* **16**, 230-8 (1997).

313. Frosch, M. et al. Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers

for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. *Arthritis Rheum* **43**, 628-37 (2000).

314. Pechkovsky, D.V., Zalutskaya, O.M., Ivanov, G.I. & Misuno, N.I. Calprotectin (MRP8/14 protein complex) release during mycobacterial infection in vitro and in vivo. *FEMS Immunol Med Microbiol* **29**, 27-33 (2000).

315. Newton, R.A. & Hogg, N. The human S100 protein MRP-14 is a novel activator of the beta 2 integrin Mac-1 on neutrophils. *J Immunol* **160**, 1427-35 (1998).

316. Bromberg, Y. & Pick, E. Unsaturated fatty acids stimulate NADPH-dependent superoxide production by cell-free system derived from macrophages. *Cell Immunol* **88**, 213-21 (1984).

317. Schlesinger, L.S. & Horwitz, M.A. A role for natural antibody in the pathogenesis of leprosy: antibody in nonimmune serum mediates C3 fixation to the Mycobacterium leprae surface and hence phagocytosis by human mononuclear phagocytes. *Infect Immun* **62**, 280-9 (1994).

318. Mintz, C.S., Arnold, P.I., Johnson, W. & Schultz, D.R. Antibody-independent binding of complement component C1q by Legionella pneumophila. *Infect Immun* **63**, 4939-43 (1995).

319. Leist-Welsh, P. & Bjornson, A.B. Immunoglobulin-independent utilization of the classical complement pathway in opsonophagocytosis of Escherichia coli by human peripheral leukocytes. *J Immunol* **128**, 2643-51 (1982).

320. Ferguson, J.S., Weis, J.J., Martin, J.L. & Schlesinger, L.S. Complement protein C3 binding to Mycobacterium tuberculosis is initiated by the classical pathway in human bronchoalveolar lavage fluid. *Infect Immun* **72**, 2564-73 (2004).

321. Gil, D.P. et al. Differential induction of apoptosis and necrosis in monocytes from patients with tuberculosis and healthy control subjects. *J Infect Dis* **189**, 2120-8 (2004).

322. Fratazzi, C., Arbeit, R.D., Carini, C. & Remold, H.G. Programmed cell death of Mycobacterium avium serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. *J Immunol* **158**, 4320-7 (1997).

323. Danelishvili, L., McGarvey, J., Li, Y.J. & Bermudez, L.E. Mycobacterium tuberculosis infection causes different levels of apoptosis and necrosis in human macrophages and alveolar epithelial cells. *Cell Microbiol* **5**, 649-60 (2003).

324. Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* **3**, 133-46 (2003).

325. Altare, F. et al. Interleukin-12 receptor beta1 deficiency in a patient with abdominal tuberculosis. *J Infect Dis* **184**, 231-6 (2001).

326. Greinert, U., Ernst, M., Schlaak, M. & Entzian, P. Interleukin-12 as successful adjuvant in tuberculosis treatment. *Eur Respir J* **17**, 1049-51 (2001).

327. Karp, C.L. et al. Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. *Nat Immunol* **1**, 221-6 (2000).

328. Klein, R.F. et al. Regulation of bone mass in mice by the lipoxygenase gene Alox15. *Science* **303**, 229-32 (2004).

329. Rozzo, S.J. et al. Evidence for an interferon-inducible gene, Ifi202, in the susceptibility to systemic lupus. *Immunity* **15**, 435-43 (2001).

330. McBride, M.W. et al. Microarray analysis of rat chromosome 2 congenic strains. *Hypertension* **41**, 847-53 (2003).

331. McMurray, D.N., Collins, F.M., Dannenberg, A.M., Jr. & Smith, D.W. Pathogenesis of experimental tuberculosis in animal models. *Curr Top Microbiol Immunol* **215**, 157-79 (1996).

332. Pan, H. et al. Ipr1 gene mediates innate immunity to tuberculosis. *Nature* **434**, 767-72 (2005).

333. Markel, P. et al. Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains. *Nat Genet* **17**, 280-4 (1997).

334. Iakoubova, O.A. et al. Genome-tagged mice (GTM): two sets of genome-wide congenic strains. *Genomics* **74**, 89-104 (2001).

335. Demant, P. Cancer susceptibility in the mouse: genetics, biology and implications for human cancer. *Nat Rev Genet* **4**, 721-34 (2003).

336. Wiltshire, T. et al. Genome-wide single-nucleotide polymorphism analysis defines haplotype patterns in mouse. *Proc Natl Acad Sci U S A* **100**, 3380-5 (2003).

337. Park, Y.G., Clifford, R., Buetow, K.H. & Hunter, K.W. Multiple cross and inbred strain haplotype mapping of complex-trait candidate genes. *Genome Res* **13**, 118-21 (2003).

338. Ewart-Toland, A. et al. Identification of Stk6/STK15 as a candidate low-penetrance tumor-susceptibility gene in mouse and human. *Nat Genet* **34**, 403-12 (2003).