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Chapter 6: Host Genetics and Susceptibility

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6.1. The difficulty in proving a genetic component for human susceptibility

6.1.1. Introduction

Tuberculosis (TB), “The White Plague” was a predominant public health problem in Europe and America in the 18th, 19th, and early 20th centuries, and considerable effort was spent trying to understand it. With the advent of effective antibiotic therapy in the '50s, the prevalence of the disease, and research on it, declined precipitously. Since the late '80s, however, there has been a resurgence of TB in urban settings in developed countries as well as in the developing world and Eastern Europe (Bloom 1992), and concomitantly, there has been a revival of research on TB and its causative agent, *M. tuberculosis*. Many of the questions investigated in the past are now being re-addressed at the molecular level.

One of the principal questions that occupied earlier researchers was the interplay of bacterial and host factors that determines who becomes infected and who develops TB. The discussion over the causes of TB goes back at least as far as the ancient Greeks and Romans, and basically consists of three different explanations: an inherited disorder; a contagious disease; and a disease caused by poor living conditions. Hippocrates thought it was inherited, while Aristotle and Galen believed it was contagious (Smith 2003). As the disease was most common in the urban poor, crowded into the rapidly growing cities of the recently industrialized Europe, social reformers of the time believed that TB was caused by the deplorable living conditions of the working class and rejected a contagious explanation. Although this chapter will present the published evidence supporting a heritable component to TB susceptibility, really all three explanations are correct and inter-related, which makes it difficult to separate, evaluate, and define the heritable genetic component.

While the discovery of the TB bacillus by Koch in 1882 disproved the notion that the disease had a purely hereditary etiology, or was caused solely by the unhealthy living conditions of the lower classes in the early industrial age (Hass 1996), several aspects of TB epidemiology are not explained by the germ theory and suggest that there are individual differences in susceptibility: not everyone exposed to *M. tuberculosis* becomes infected; even when infection can be demonstrated with a positive tuberculin skin test (TST), only about one in ten infected individuals becomes ill; the course of the disease varies in different individuals - before antibiot-

ics some tuberculars died rapidly of “galloping consumption” while others recovered or lived a relatively long life with chronic disease; and some infected individuals develop the disease only many years after the initial infection (Rich 1951). Without treatment, TB is fatal in about half of the patients who develop the disease.

As the disease was more common in particular families and racial or ethnic groups, a heritable component to susceptibility was a plausible assumption, but one that has defied solid experimental proof, perhaps due to the difficulty in eliminating the confounding biases of environment and exposure. In 1912, the statistician Karl Pearson, attempting to demonstrate racial differences in TB susceptibility, stated the basic question, “*We have to inquire whether persons living habitually in the same environment and with practically the same risk of infection have the same chance of developing phthisis whatever be their stock*” [cited in (Puffer 1946)].

Since the mid '80s, there have been many studies that have tried to identify genes that might be associated with TB susceptibility, as well as those testing the validity of published associations. While there are several recent reviews of the subject (Bellamy 2005, Bellamy 2006, Fernando 2006, Hill 2006, Ottenhoff 2005, Remus 2003), it is hard to come to definitive conclusions on most of the genes, because the accumulated literature is often contradictory. Studies showing that a polymorphism in a plausible gene is associated with TB susceptibility are often contradicted by subsequent work in other populations that finds no association. This has led to the recent publication of meta-analyses attempting to examine the body of published work on particular genes to determine whether a convincing consensus emerges (Kettaneh 2006, Lewis 2005, Li 2006). This chapter will attempt to summarize the current, inconclusive state of investigation on genetic determinants of TB susceptibility. In addition, it will review studies performed prior to the molecular era to illustrate the history of the field, which may help to clarify why finding genetic determinants has been elusive. It will focus on human susceptibility to *M. tuberculosis*, and will not consider susceptibility to leprosy (Geluk 2006, Schurr 2006) or other mycobacteria, except in the discussion of immune deficiencies (Casanova 2002).

The basic epidemiological designs employed in studies of genetic association, in approximate decreasing order of confidence that the results obtained are free of the complicating influences of environment and exposure are:

- twin studies comparing disease concordance in monozygotic vs. dizygotic pairs
- family linkage studies that associate the occurrence of TB in family members with the inheritance of a particular genetic marker

- case-control studies showing that, compared to controls, individuals with TB are different in some particular variable, such as exposure, race, HLA type, or the presence of polymorphisms in genes encoding elements of the immune system, such as cytokines or macrophage receptors, etc.
- anecdotal reports of family or ethnic clusters of TB cases, suggesting an increased susceptibility

As will be seen, the details and rigor of experimental design and the selection of control populations greatly affect the ability to discover associations, and the validity of the results obtained.

This tour of the literature on the genetic basis of human susceptibility to TB begins with a review of older family and twin studies that provide the basis for the belief that there is a significant component of genetic susceptibility to TB, and that show the difficulties in proving it. This is followed by an examination of racial differences in TB susceptibility, and then a summary of immunological defects, both general and specific, that confer extreme susceptibility to mycobacteria. After this comes a review of studies associating specific genes with susceptibility to common TB: first those looking at different human leukocyte antigen system (HLA) alleles; then studies on other genes thought to be important in human defense mechanisms against TB. Finally, after a review of human studies of genes equivalent to those altering TB susceptibility in mice, and work employing genomic scans, is an attempt to summarize the state of the field and put it into perspective. While this tour is not exhaustive, it attempts to critically present most of the relevant published work.

TB in famous families

French Royal Bourbon Family

Louis XIII—(1601-1643) died of galloping consumption

TB affected: His wife
 His son – Louis XIV (1638-1715)

Simon Bolivar (1783 – 1830) died of TB

TB deaths: Father – Juan Vincente (1786)
 Mother – Maria de la Concepción (1792)

Brontës

Chronic TB Father (died 1861)

TB deaths: His wife
 His four children:
 Charlotte (1816-1855) ("Jane Eyre")
 Emily (1816-1848) ("Wuthering Heights")
 Anne (1818-1848) ("Agnes Grey")
 Patrick (1817-1848)

Ralph Waldo Emerson (1803-1882) Chronic TB
(Romantic and Transcendentalist Poet)

TB deaths: Father
 Two brothers

Chronic TB: One brother

In 1949, a descendent wrote that TB had claimed lives and caused illness in 10 generations.

Henry David Thoreau (1817-1862) died of TB
(“Walden” "On Civil Disobedience")

TB deaths: Grandfather
 Father
 Sister

6.1.2. Early family and twin studies

Many early studies of TB in families compared the cumulative incidence of disease in the offspring of couples where one, both, or neither had TB, also noting other family history of TB, and whether cases were sputum positive (Puffer 1946, Stocks 1928, Frost 1933). While these studies clearly demonstrated that living in a house with a tubercular person increased the chances of developing TB, most investigators accepted that their results represented a combination of the effects of exposure and hereditary predisposition. Two examples illustrate the difficulty in separating these components.

Stocks and Karn (Stocks 1928) devised a correlation coefficient based on sibling disease concurrence expected by chance. They then used family records of 4,000 Belfast TB patients to demonstrate an excess of sibling cases occurring in families with a prior history of TB, as evidence of an inheritable factor in susceptibility. Although the attempt was interesting in its design, it could not assure comparability of environment and exposure, as a tuberculous relative could have had a confounding effect, either as a source of exposure or as a marker for lower socioeconomic status.

Puffer (Puffer 1946) attempted to separate exposure from heredity by comparing the incidence of TB in the spouses of tuberculous individuals with that in their children and siblings. Although an increased incidence of TB in the spouse of sputum positive tuberculars suggested the importance of exposure, TB was more common in consorts who additionally had a family history of TB, suggesting the greater importance of familial susceptibility. To address the obvious criticism that the spouses could have been exposed in childhood from the affected relative, Puffer stated that two thirds had no known household contact, although the contact may have been forgotten or missed. Overall, due to the near impossibility of controlling for household exposure, the family studies failed to convincingly demonstrate a genetic predisposition.

Twin studies (Table 6-1) have an experimental design that should control for the effects of environment and exposure more reliably, and several have studied inheritance of TB susceptibility. Monozygotic twins are genetically identical, while dizygotic twins are only as genetically similar as other siblings. If it can be assumed that both types of twins will share the same environments and exposures, a difference in concordance rates of TB – both twins with TB or both healthy – between the two types of twins can be attributed to the genetic components, even if multiple gene causality is suspected. The concordance in monozygotic twins can also serve as a measure of penetrance – the proportion of gene carriers who express the trait (Cantor 1992). In a large study (Kallmann 1943) performed in the United States (US) nearly three-fold greater concordance was found in monozygotic twins than in dizygotic twins, whether or not there was a history of exposure (69.2 % vs. 26.3 % with known exposure; 61.5 % vs. 12.7 % without known exposure). The concordance in dizygotic twins was the same as seen with other non-twin siblings. This study would appear to be solid evidence supporting hereditary influences, but it is weakened by several sources of potential bias specific to twin studies (Cantor 1992, Fine 1981) that are worth examining in detail because they again illustrate the difficulties in isolating genetic components from differences in exposure, and the importance of experimental design.

First, to assure validity, all affected twin pairs in the base population must be obtained. Kallman and Reisner relied upon reporting of twins from the active patients in various TB treatment facilities in New York City and New York State, a procedure that could lead to reporting bias favoring “novel” concordant monozygotic pairs, especially if there is no assurance that all twin pairs were identified. The study states that 657 twin pairs were identified, but the final analysis contained only 308 cases of “reinfection” TB, without a clear explanation of the exclusion criteria. The validity of twin studies depends upon the assumption of the equiva-

lence of environmental and exposure components, but in Kallman and Reisner's study there were more monozygotic pairs with TB in their direct ancestry (36.6 % vs. 13.4 %). They also failed to report on whether twins were living together, which tends to be more common in monozygotic pairs, and would be a source of increased concordance for uniformity of exposure, or if TB was spread from one twin to the other. In addition, even though they mention that TB is more common in females in the age group 20-35 years, the percentage of females in the two groups was not reported where the twin pairs were clustered.

Table 6-1: Twin studies

Reference	Monozygotic		Dizygotic		Monozygotic		Dizygotic	
	Total Pairs		Concordant pairs					
	N	%	N	%	N	%	N	%
Diehl 1936	80	39	125	61	52	65	31	25
Dehlinger 1938	12	26	34	74	7	58	2	6
Kallman 1943	78	25	230	75	52	66	53	23
Harvald 1956	37	26	106	74	14	38	20	19
Simonds 1963	55	27	150	73	18	32	21	14

The Proffit study set out to re-examine the conclusions of Kallman and Reisner's study by trying to correct all its shortcomings (Simonds 1963). It exhaustively searched for all twins among active patients in English TB clinics, determined if the twin pairs were living together at the time of onset of TB in the index cases, whether the index case was sputum positive, and reported on the sex of all subjects. Although more concordance was found in monozygotic than in dizygotic pairs (32 % vs. 14 %), the authors believed that this difference could be explained by other factors: more female monozygotic than dizygotic twins (68 % vs. 43 %), especially in the susceptible 20-30 years age group; more monozygotic twins living together (58 % vs. 50 %); more TB concordance among those living together (42.4 % vs. 18.4 % for monozygotic, 16.3 % vs. 10.3 % for dizygotic); more sputum positive index cases among concordant pairs (72 % vs. 49 % for monozygotic, no difference for dizygotic); and more TB in parents of monozygotic than dizygotic twins (57.2 % vs. 43.5 %) – even though most of these differences were not statistically significant. Comstock's re-analysis of the data (Comstock 1978), using multiple regression to control for the sex of co-twins, age at diagnosis, type of TB,

sputum positivity of index twin, TB contact of co-twin, twins living together, and years between diagnosis of the twin pairs, still found a two-fold difference in concordance between twin types (31.4 % in evidence for monozygotic vs. 14.9 % for dizygotic; $p \leq 0.05$).

Twin studies constitute the strongest evidence for a genetic component to TB susceptibility because they control for bias better than any other experimental study design, and because there is relative consistency of the findings in most studies (Table 6-1) (Dehlinger 1938, Diehl 1936, Harvald 1956). A conservative conclusion might be that some inheritable component exists, but it has a maximal penetrance of only 65 %, and the most careful study ever performed found only 31.4 % penetrance. In other words, in as few as only a third of cases, two individuals with exactly the same genes and similar exposures will either both develop or both not develop TB.

6.1.3. Racial differences

Much of the controversy about genetic susceptibility to TB in the early part of the 20th century was concerned with allegations of racial differences, or more specifically, that Asians and especially Africans and African Americans had less innate resistance than Whites. While the near fixation on this topic by authors such as Rich (Rich 1951) might be ascribed to the prevailing racism of the period, the assumption of greater susceptibility of Africans and African Americans continues to be cited in current literature, with investigators now using molecular findings to try to explain it (Liu 2006). While Rich gave equal credit to “*the marked influence of environment... in different economic strata of individual communities within a given country*” for Whites, he attributed the higher rates in Africans and African-Americans predominantly to the effects of genetic composition. Although he cited examples of higher TB rates in Africans, he really concentrated on the more severe nature of the pathology of the disease. He proposed that because of Africa’s short history of exposure to TB, Africans have not developed genetic resistance to the bacillus, and therefore many Africans, even as adults, develop a systemic, overwhelming form of the disease usually seen only in White children. While Rich states that he “*has no intention of minimizing the importance of adverse economic and environmental conditions as factors that influence the TB mortality rate of the Negro,*” one cannot help but recall the work of Dr. James McCune Smith in debunking the notion that African Americans were genetically predisposed to rickets by showing that whites of the same low socioeconomic status were similarly predisposed (Krieger 1992).

Stead and Bates (Bates 1993, Stead 1992, Stead 1997) cite several examples to support their argument that Africans and Native Americans have less resistance to TB: the greater TB mortality of the Sudanese conscripted into the Egyptian army compared to the Egyptian soldiers; the similar fate of Senegalese soldiers sent to France in the first world war; and the decimation by TB of the American Indians forced to live on US military bases. It's interesting that these three commonly cited examples all involve foreign conscripts or internees on a colonizer's military base, and rely on the dubious assumption that their physical and emotional environments were the same as those of the host soldiers.

Stead and Bates expound on the often-cited theory for the existence of racial differences in susceptibility - the duration of the exposure to endemic TB in Africa and Asia has not been long enough to select for a resistant gene pool. They postulate that a TB epidemic has a 300-year cycle, in which the more resistant survivors reproduce and increase the proportion of naturally resistant individuals in the population, so that after 50-100 years, the mortality, and subsequently morbidity, reach a peak and then progressively decline. The White populations in Western Europe and the US, where the epidemic peaked in the late 1700's and early 1800's, are now composed of individuals with a relatively resistant genetic make-up. The Africans, Eskimos and other Native Americans, however, were only exposed to TB much later, so their gene pool has yet to complete the selection for resistant individuals (Stead 1992). This theory, though still cited in current literature (Fernando 2006), is completely unproven and will likely remain so. Indeed, how could it be proven that the progressive lowering of rates for TB and other infectious diseases in Western Europe and the US, prior to the introduction of antibiotics, was the result of a changing gene pool and not of improvements in nutrition, housing, and working conditions, whose influences could outweigh any putative inheritable component (McKeown 1978)? Nonetheless, the abundance of literature describing increased susceptibility and a more progressive disease course in Africans and Native Americans suggests that some racial difference may, in fact, exist. Putting aside the theory for the origin of racial differences, are there any studies that have sufficiently controlled for environment and exposure, in order to credibly document a difference?

Kushigemachi et al. critically reviewed the epidemiological studies that relate to this question (Kushigemachi 1984). They reasoned that the only studies that could provide usable information are those that follow TST-positive groups of people for the development of disease. They cite several relevant studies from the literature (see Table 6-2), mostly isoniazid (INH) prophylaxis or bacille Calmette-Guérin (BCG) vaccine trials. In the two studies done on Eskimos, the average annual case

rates of 936 and 725 per 100,000 were much higher than rates seen in any other study, but there is no data on other risk factors. In studies predominantly involving Whites, the annual case rates varied from 29-79/100,000. The few BCG trials that included more than one race tended to show higher rates in Blacks than Whites, but both the absolute rates and the racial differences varied. In the Alabama study, the overall racial difference was predominantly due to very high rates in young Black women. The best single study was among Navy recruits, because the environment and follow-up were usually equivalent, at least once they were in the Navy. In that study, African Americans had an annual rate only 17 % higher than whites (91/78), but the Asians (195) had a rate more than double that of African Americans. It was also noted that upon entry into the Navy, highly positive purified protein derivative (PPD) reactions (> 20 mm) were more common in African Americans, which suggested that some may have entered with active disease.

Because of the variability of the rates in Whites, the small difference found in the Navy study, and the lack of data on other risk factors, the authors concluded, "*assertions that certain racial groups possess a "natural resistance" to TB are clearly unwarranted on the basis of available evidence.*" The high rates in Asians and Eskimos compared to both Whites and Blacks seem less convincingly dismissed than the differences between Blacks and Whites, but risk factors, such as nutritional state, lack of a TB control program, or crowded and closed living conditions may explain the differences. In fact, after the implementation of intensive TB control measures in the Eskimo (Inuit) population in Canada, their TB rates, which had been the highest recorded in the world, showed the fastest rate of decline on record (Enarson 1986).

Stead attempted to eliminate exposure and environmental bias by studying 1,786 documented TST conversions among 13,122 residents of integrated nursing homes in Arkansas, with a similar analysis of approximately 2,000 inmates from integrated prisons in Minnesota and Arkansas (Stead 1990). The results were analyzed using multivariate analysis with a proportional-hazards model, adjusting for covariates of age, sex, and percentage of nursing home residents who were TST positive at entry. They found that Blacks had a higher rate of TST conversions (7.2 % in Whites vs. 13.8 % in Blacks overall, $P < 0.001$) regardless of the percentage of Black residents of the facility, and regardless of the race of the potential source patient. In fact, Blacks had higher rates of TST conversions even when the presumed source case was White (8.4 % vs. 15.3 %; $P < 0.001$).

Table 6-2: Race differences and TB rates

Location	Criteria for a positive reactor	Observation period (years)	Age on entry (years)	Racial group	Average annual case rates per 100,000 reactors
Muscogee, George and Russell Counties, Alabama (20,21)	> 5 mm induration to 5 T.U. PPD (Mantoux)	20	20-29	WM	78
				BM	74
				WF	32
				BF	96
			30-39	WM	49
				BM	75
				WF	10
				BF	93
			40-49	WM	104
				BM	97
				WF	32
				BF	83
			50-59	WM	141
				BM	131
				WF	107
				BF	105
Puerto Rico (22)	≥ 6 mm induration to 1 T.U. or 10 T.U. PPD- (Mantoux)	18,87	1-19	White	91
				Black	87
U.S. Navy Recruits (23)	≥ 10 mm induration to 5 T.U. (Mantoux)	4	17-22	White	78
				Black	91
				Asian	195

WM: white males T.U. = tuberculin units
 BM: black males
 WF: white females
 BF: black females

Similar results were found in the prison populations. In contrast, however, there was no racial difference in the incidence of TB that developed in the nursing home

residents with positive skin tests. The authors interpreted this as evidence for the distinction of two aspects of TB, the initial infection and the development of disease, and concluded that Blacks have decreased resistance to the initial infection, but that once infected, they develop TB at the same rates as TST-positive Whites. This is consistent with the conclusions of the review by Kushigemachi *et al.* Although the nursing home setting convincingly controls for sources of bias, including age and sex, there is no data on the residents' weights, general health, or patterns of association and rooming. One other problem is that when no source patient was identified, the difference in TST conversion rates was greatest (4.4 % vs. 13.2 % $P < 0.001$), suggesting that the Blacks may have had some other source of infection, perhaps from visitors, which could explain all the differences. Even if African-Americans have a slightly increased rate of infection, the fact that there was no difference in the rate of progression to disease deflates the credibility of arguments that their immune system is less capable of controlling the infection. A separate study looked at TST conversion in school children exposed to a physical education teacher with TB. No racial differences were found, leading the authors to question the validity of the conclusions from the nursing home study (Hoge 1994).

The notion that the decline in TB in Europe was due to genetic selection runs counter to most thinking in the public health field. In the '70s, the historian Thomas McKeown (McKeown 1978) showed that the death rates in England and Wales from TB and other respiratory diseases declined precipitously from about 1830 to 1950, well before the advent of the BCG vaccine and anti-tuberculosis drugs (Figure 6-1). A similar decline also occurred in the United States.

McKeown concluded that improved nutrition was responsible for the decline in mortality and the increase in population, while others later argued that more important factors were the general improvements in living standards and such public health measures as improved housing, isolation of infectious individuals, clean drinking water, and improved sanitation (Szreter 2002). Nonetheless, it is generally accepted that this dramatic decrease was mainly the result of societal factors. This explanation appears plausible because: the decline was temporally linked to the improvements in living conditions and public health; the decline was too rapid and steep to be explained exclusively by genetic selection (Lipsitch 2002); the rate of decline remained steep even as the putative "selective pressure" decreased; and the dramatic decline in mortality was not limited to TB, but was also seen for many other infectious diseases (McKeown 1978). Although an element of genetic selection may also have played a role, the primacy of societal factors was demonstrated by the rise in TB rates in the US in the 1980s and '90s that accompanied the increase in homelessness and decrease in TB control measures, and set the stage for

the rampant spread of TB in the HIV-infected population (Frieden 1996). In New York City in the '90s, it was found that infection with a clustered TB strain, considered to be a marker of recent transmission, was associated with both homelessness and with being African-American. Can it then be argued that this demonstrates a genetic susceptibility to TB in the homeless? Taking into account the questionable hypothesis of extensive genetic selection for a TB-resistant population, and the lack of well-controlled, reproducible studies demonstrating that any racial group has either an increased susceptibility to infection or an increased propensity to develop disease, the notion of racial differences in susceptibility seems unproven. Nonetheless, it is certainly possible that distinct ethnic groups and populations may have different frequencies of polymorphisms that confer susceptibility or resistance to TB, and the frequency of alleles that conferred severe susceptibility in an endemic setting may be reduced over time. However, the danger in this line of thinking is that higher rates of TB in these populations may be accepted as the irremediable result of genetic make-up, rather than the consequence of lower socio-economic and health status along with poor TB control programs, which have the potential for improvement if, as demonstrated in New York City, sufficient financial and political resources are committed to the task.

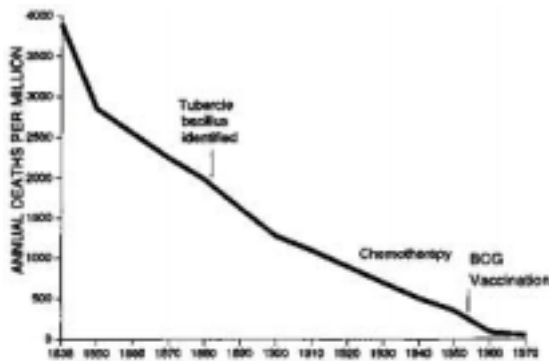


Figure 6-1: Fall in deaths from TB in England and Wales from 1838 to 1970. Most of the drop occurred before specific treatment or immunization was available. (Scrimshaw 1976). Available at: <http://www.unu.edu/unupress/food/V181e/p07a.gif>

6.2 Search for mutations and polymorphisms that increase susceptibility

6.2.1 Tuberculosis susceptibility in generalized immune deficiencies

Considerable insight has been obtained by studying humans with immunological deficiencies, and determining which genetic defects lead to increased risk of mycobacterial infections (Picard 2006). Undoubtedly, the largest group of highly susceptible persons are individuals infected with the HIV virus, who are prone to develop TB early in the course of the disease. After the onset of AIDS, they are also susceptible to atypical or environmental mycobacteria as well as many other pathogenic and opportunistic agents. TB takes the lives of a large percentage of AIDS patients in Africa (Cantwell 1996), and the early susceptibility underlines the overwhelming importance of CD4+ T cells in immunity to TB.

There are over 100 different primary genetic immunodeficiencies that predispose to infections with a variety of viruses, bacteria, fungi and protozoa, but only a few have been associated with severe mycobacterial infections (Casanova 2002). As might be imagined, children with severe combined immunodeficiency (SCID) who completely lack T cells are highly vulnerable to disseminated BCG infections after being vaccinated. Only a few cases of infections with atypical mycobacteria and *M. tuberculosis* have been described in these patients, but this may be due to lack of exposure, because without a bone marrow transplant, most of these children die within a year of birth.

Disseminated BCG infection, pneumonia with *M. intracellulare*, and a *M. tuberculosis* brain abscess (Metin 2004) have been described in individuals with hyper-IgE syndrome, a rare autosomal dominant disorder characterized by high serum IgE levels, eczema, and susceptibility to bacterial and fungal infections (Casanova 2002). Reports have described low levels of interleukin 12 (IL-12) and interferon gamma (IFN- γ) in several of these patients (Netea 2005), but this defect must be mild or variable, as many hyper IgE patients have been vaccinated with BCG and survived into adulthood without mycobacterial infections. A patient was recently described, who had been clinically diagnosed with hyper IgE syndrome and was unusually susceptible to various microorganisms including mycobacteria, as well as virus and fungi (Minegishi 2006). A mutation was found in the gene for tyrosine kinase 2 (Tyk2), a non-receptor tyrosine kinase of the Janus kinase family. The patient's cells showed defects in multiple cytokine signaling pathways, including IFN- γ , which were restored by transducing an intact Tyk2 gene.

The neutrophils of patients with chronic granulomatous disease lack the oxidative burst associated with ingestion of microorganisms, due to mutations in the NADPH oxidase complex. This defect in neutrophil killing makes them susceptible to severe recurrent bacterial and fungal infections. Both disseminated and local infections with BCG are fairly common in these patients (Jacob 1996), but disseminated infections with atypical mycobacteria (Moskaluk 1994, Ohga 1997), and TB (Baresi 2004) have also been described, demonstrating that the phagocytic respiratory burst plays a role in the control of mycobacterial infections.

Mutations that impair signaling and activation of gene transcription promoted by Nuclear Factor kappa B (NF- κ -B) cause a rare disorder called anhydrotic ectodermal dysplasia with immunodeficiency. Affected patients are predisposed to disseminated infections with atypical mycobacteria, septicemia from pyogenic bacteria, and viral infections. This syndrome has been associated with X-linked hypomorphic (reduced function) mutations in NF- κ -B essential modulator (NEMO), and autosomal dominant hypermorphic mutations in the inhibitor of NF- κ -B (von Bernuth 2005).

Overall, mycobacterial infections occur in perhaps a third of patients with severe combined immunodeficiency and anhydrotic ectodermal dysplasia with immunodeficiency. However, it is only seen in a small percentage of patients with chronic granulomatous disease or hyper IgE, suggesting that the susceptibility is only partial. None of the other primary immunodeficiencies, including defects of HLA classes I and II, complement, B-cells, T-cells, or Toll-like receptor (TLR) signaling seem to predispose to mycobacterial infections (Ottenhoff 2005). Thus it appears that the TLR and NF- κ -B pathways are important for immunity to many types of pathogens, while, as described in the following section, the IL-12/23-IFN- γ pathway is critical specifically for immunity to mycobacteria and Salmonella.

6.2.2. Mendelian susceptibility to mycobacterial disease

Perhaps the most convincing evidence for genes involved in human susceptibility to mycobacteria has come from studying those rare patients with genetic mutations that selectively increase their susceptibility to mycobacteria, salmonella and occasionally virus (Casanova 2002, Fernando 2006, Ottenhoff 2005). Most of the mycobacterial infections in these unfortunate children and adolescents are not caused by *M. tuberculosis*, but rather by BCG after being vaccinated, or by the atypical mycobacteria that are ubiquitous in the environment (Petriani 2006). Presumably,

they are also very susceptible to *M. tuberculosis* but simply not exposed, because disease with *M. tuberculosis* has been reported in several of these patients.

The mutations responsible for this susceptibility have been identified in many afflicted individuals, and found to be transmitted by classic Mendelian inheritance. Although the defects are heterogeneous, they often occur in children of consanguineous parents, with several cases in the same family. The syndrome has been termed Mendelian susceptibility to mycobacterial diseases (MSMD). The mutations encountered are defining the human immunological response essential for controlling mycobacterial infections, which appears to be based upon the production of IFN- γ , the receptors for IFN- γ , and the subsequent downstream signal transduction that promotes the expression of the largely unidentified genes that confer immunologic protection.

Mutations causing MSMD have been found in five different genes: IFN- γ R1 and IFN- γ R2, the two chains of the IFN- γ receptor; IL-12B, encoding the p40 subunit of IL-12; IL-12RB1, the β 1 subunit of the IL-12 receptor; and signal transducer and activator of transcription 1 (STAT1). The inheritance is most commonly autosomal recessive, but autosomal dominance has been reported in some families, and there is at least one example of X-linked recessive inheritance. In addition, the defects can be partial or complete, leading to at least 10 different disorders. Complete defects cause more severe disease than partial defects, and children with complete IFN- γ R deficiencies are the most severely affected. The severity and prognosis correlate with the immune response to the infections: children who form lesions typical of lepromatous leprosy - poorly defined, with many mycobacteria but no epithelioid or giant cell - generally succumb to overwhelming infections that are often resistant to cure even with intensive antibiotic therapy. In contrast, patients who form granulomas similar to those of tuberculoid leprosy - paucibacillary, well defined, with giant and epithelioid cells - generally respond to therapy and survive (Ottenhoff 2005).

The first genetic defect identified in these patients was a complete deficiency of the IFN- γ receptor ligand-binding chain (IFN- γ R1). Subsequently, kindreds were found with mutations in the IFN- γ receptor signaling chain (IFN- γ R2). The recessive forms of these defects are null mutations - no IFN- γ receptor is found on the surface, and there is no response to IFN- γ in vitro. In other kindreds with complete defects, the children have IFN- γ receptors on the cell surface, but amino acid substitutions in the IFN- γ R1 prevent binding of IFN- γ .

Table 6-3: MSMD, immune defects and TB susceptibility

Condition	Defect	Infections
Generalized Immune Deficiencies		
SCID	No T-cells	BCG / NTM / <i>M. tuberculosis</i> / virus / bacteria / fungi / protozoa
Hyper IgE EDA-ID	Low IFN- γ /IL-12	BCG / NTM / bacteria / fungi
NEMO I κ Ba	Low NF- κ -B function	BCG / NTM / <i>M. tuberculosis</i> Other bacteria
CGD	No oxidative burst	BCG / NTM / <i>M. tuberculosis</i> / other bacteria / fungi
MSMD		
No response to IFN- γ		
IFN- γ R1	Complete	BCG / NTM / <i>M. tuberculosis</i> / Salmonella
IFN- γ R2	Complete	BCG / NTM / <i>M. tuberculosis</i> / Salmonella
Impaired response to IFN- γ		
IFN- γ R1	Partial	BCG / NTM / <i>M. tuberculosis</i> / virus
IFN- γ R2	Partial	BCG/NTM
STAT1	Complete	BCG/Virus
STAT1	Partial	BCG/NTM
Reduced production of IFN- γ		
IL-12p40	Complete	BCG / NTM / <i>M. tuberculosis</i> / Salmonella / other infections
IL-12RB1	Complete	BCG / NTM / <i>M. tuberculosis</i> / Salmonella
IL-12RB1	Partial	BCG
NEMO	Partial	NTM

Table modified from Casanova 2002, Ottenhoff 2005, and Fernando 2006

NTM: Non-tuberculous mycobacteria

Although patients with complete IFN- γ R deficiencies have very high blood levels of IFN- γ (Casanova 2002), they all had disseminated atypical mycobacterial infections before reaching the age of 3 years; and all those who were BCG vaccinated developed disseminated BCG disease. The mycobacteria involved were both slow- and fast-growing species, and even included the generally innocuous *M. smegmatis* (Casanova 2002). These infections are life threatening and often incurable. Patients with complete INF- γ R deficiencies can also be subject to severe viral infections (Dorman 1999).

Partial IFN- γ R deficiencies have been attributed to mutations in both IFN- γ R1 and IFN- γ R2. In one form, a mutation in the extracellular segment of IFN- γ R1 reduces the affinity of IFN- γ binding, while in other kindreds a mutation in the cytoplasmic domain perturbs the receptor's recycling/internalization and signaling. A single amino acid mutation in the extracellular domain of IFN- γ R2 has been described that impairs but doesn't abolish the response to IFN- γ . A partial dominant IFN- γ R1 deficiency, caused by a small frameshift deletion in IFN- γ R1, has been described in several unrelated kindreds. The truncated proteins accumulate at the cell surface and bind IFN- γ , but lack an intracellular recycling site, so exert a dominant negative effect. Most of the IFN- γ R1 dimers in heterozygotes will have at least one defective subunit and be nonfunctional, but the few normal dimers that are present are functional. The prognosis for patients with partial IFN- γ R is relatively good, and many have survived into young adulthood, often without treatment.

Patients with IL-12B mutations have a complete IL-12p40 deficiency, with neither monocytes nor dendritic cells secreting IL-12 upon stimulation. Their lymphocytes secrete less IFN- γ than normal, but can be complemented by treatment with exogenous recombinant IL-12. BCG infections have occurred in all patients with complete IL-12 deficiency, and a minority also had infections with atypical mycobacteria or Salmonella.

Mutations in IL-12RB1 generally cause a complete lack of IL-12 receptor subunit IL-12R β 1, resulting in low IFN- γ production that doesn't respond to exogenous IL-12. These patients have curable BCG and atypical mycobacterial infections, and about half have Salmonella infections. An X-linked recessive partial defect has also been described. Only one death has been reported, and there is wide variation in the clinical presentation between family kindreds and even among family members affected by the same mutation. Some individuals with documented mutations have been completely asymptomatic while their siblings have had disseminated BCG infections. This variation suggests that there may be other cytokine inducers of IFN- γ that can compensate in the absence of IL-12 signaling (Casanova 2002). The

p40 subunit of IL-12 is shared by IL-23, and the IL-23 receptor shares the IL-12 β 1 subunit, so defects in these genes also lead to deficiencies in IL-23 signaling (Ottenhoff 2005).

Complete STAT-1 deficiency has been described in two unrelated infants. Although they survived disseminated BCG infections, both died of severe viral infections, presumably the result of a defect in the STAT-1 mediated signaling of interferon alpha (IFN- α) through ISRE (IFN- α sequence response element), which is the key to anti-viral immunity. Partial STAT-1 deficiencies have been found to be caused by different mutations that impair either STAT-1 phosphorylation, DNA-binding (Chapgier 2006), or dimerization and translocation to the nucleus (Dupuis 2001). Some of these mutations confer susceptibility to mycobacterial infections in the heterozygous state (dominant trait), but susceptibility to viral infections only when homozygous (recessive trait).

Very recently, three kindreds were described, each with one male patient having sporadic mycobacterial infections and an X-linked recessive defect in the leucine zipper domain of NEMO (NF- κ -B essential modulator). Surprisingly, they did not display the classical features of anhydrotic ectodermal dysplasia with immunodeficiency mentioned above. In vitro studies showed a defect in IL-12 production after stimulation of monocytes and dendritic cells by CD40L-expressing T cells and fibroblasts (Filipe-Santos 2006), due to a defect in NEMO and NF- κ B/c-Rel-mediated CD40 signaling.

There are other kindreds with marked susceptibility to mycobacterial infections whose genetic defect has yet to be identified, and it has been argued that genetic defects may be responsible for a much larger percentage of childhood TB than previously thought. TB in seemingly normal children in endemic areas can present as a disseminated form that is rare in adults, who tend to have only pulmonary disease. By estimating the frequency of disseminated disease in children (2×10^{-4}) the frequency of Mendelian type TB susceptibility mutations in the population (10^{-4} to 10^{-5}), and the cumulative incidence of disseminated TB among these individuals (0.5 to 0.9 cumulative penetrance), it has been postulated that mutations conferring Mendelian predisposition could be responsible for between 3 and 45 % of disseminated TB in children (Alcais 2005). While this remains unproven, the hypothesis could be experimentally tested. One recent, relevant study of children with non-tuberculous mycobacterial cervical lymphadenitis found no evidence of abnormalities in the IL-12/IFN- γ pathway (Serour 2006).

6.3. Candidate genes in common tuberculosis

The identification of the genes where mutations lead to extreme susceptibility has helped to identify the essential components of the human immune defense to mycobacteria. These genes, and several others thought to play a role in the human defense against TB, have also been studied to see if there might exist different alleles or polymorphisms that cause subtle changes in function that could account for individual variation in susceptibility to common TB. The polymorphic human leukocyte antigens (HLA) were the first proteins to be examined for associations with TB susceptibility, and reports continue to appear, making this the largest group of studies.

6.3.1 Human leukocyte antigens (HLA)

For a summary of HLA studies see Table 6-4 at <http://www.tuberculosis textbook.com/pdf/Table 6-4.pdf>.

HLA alleles have been associated with susceptibility to several infectious diseases, including severe malaria, HIV progression, and hepatitis B and C persistence (Hill 2006, Yee 2004). HLA studies have also shown an association of HLA-DR2 with either leprosy per se or the type of leprosy - tuberculoid or lepromatous - in both case-control and family linkage studies, and in Asian, African, and American populations (Geluk 2006). Many studies have looked for associations of TB susceptibility with particular HLA alleles of the major histocompatibility complex (MHC). The MHC loci are divided into class I and class II alleles. The class I, HLA-A, B, and C, are thought to be principally involved in the presentation of peptides generated in the cytosol by virus-infected cells to CD8+ T cells, while the class II molecules, DR, DQ and DP, present antigens of phagocytosed pathogens, such as mycobacteria, to CD4+ T cells. In the human immune response to TB, CD4+ cells seem to be of primary importance (Flynn 2001), although CD8+ T cells probably also play a role.

While earlier studies found associations of TB susceptibility with class I alleles, there were several problems: the alleles that were found to be associated varied from study to study; the studies performed before the early '90s determined the HLA phenotype using the lymphocytotoxic method, which had a 25 % misclassification rate compared with PCR-based techniques (Rajalingam 1996); and the studies often tested for associations to many different alleles without employing a correction for multiple testing (Bland 1995). A correction is necessary because when the statistical significance is defined at the 95 % level, as many as one in 20 alleles

tested can appear, by pure chance, to be associated. One means of correction is to multiply the probability of the association by the number of alleles tested. If the resulting probability is still below 0.05, there is more confidence that the association is real. This is termed a Bonferroni correction.

A recent meta-analysis (Kettaneh 2006) examined many of the earlier studies reporting HLA associations with TB susceptibility, but included only work involving mostly or exclusively adults, pulmonary TB, and serological determination of MHC alleles. The meta-analysis concluded that there was no significant association of pulmonary TB with class I antigens of either the A or C loci, but there was a protective effect for HLA B13 (OR 0.64, 95 % CI 0.50-0.81; $P = 0.0001$). OR is the odds ratio, which means that individuals carrying the HLA B13 allele have only a 64 % chance of developing TB compared to those without this allele. The confidence interval (CI) shows the boundaries in which the true value will be found 95 % of the time. The P value is the level of statistical confidence that the result obtained did not occur by chance. For the class II DR locus, lower risks of pulmonary TB were found for carriers of DR3 (OR 0.72, 95 % CI 0.59-0.89; $P = 0.002$) and DR7 (OR 0.65, 95 % CI 0.53-0.80; $P < 0.0001$), and a higher risk for carriers of DR8 (OR 1.72, 95 % CI 1.21-2.46; $P = 0.003$). In other words, individuals with DR8 have a 72 % greater probability of developing TB. The results for DR2 were heterogeneous, and evidence for it conferring a greater risk of pulmonary TB fell just short of statistical significance (fixed more stringently at 0.005 after a Bonferroni correction) (OR 1.67, 95 % CI 1.16-2.41; $P = 0.006$) (Kettaneh 2006). While this meta-analysis is very useful for analyzing the confusing early HLA literature, and questioning the validity of the varied associations, it is rather divorced from the recent literature because it excludes studies that use more accurate DNA-based methods for determining MHC alleles. The reason given for this is that the nomenclature varied in reports using the different methods, making comparisons very difficult. The HLA-B13, DR3, DR7, and DR8 associations have received scant attention in recent years, but the borderline DR2 association is frequently cited and is worth examining to illustrate the difficulties in unequivocally establishing associations.

The association with HLA-DR2 was reported in several studies of Asian subjects, mostly from a single group of investigators in New Delhi. In 1983, two studies from New Delhi reported an association of DR2 with TB (Singh 1983, Singh 1983). A case-control study then compared North Indian sputum-positive pulmonary TB patients with controls matched for age, sex and socioeconomic status. The difference in DR2 distribution between TB patients and controls was not significant after correction for the number of antigens tested, and the OR was only 1.6

(Bothamley 1989). A family study looked at HLA class I and II haplotype segregation in 25 multi-case families and found a significantly skewed transmission of DR2 from parents with pulmonary TB to offspring with pulmonary TB, using the method of Weitkamp (Weitkamp 1981). A re-analysis of the same family data with the LOD score method, published the following year (Singh 1984), found no evidence of HLA linkage to pulmonary TB. Because family studies generally have fewer subjects, they have less statistical power than case-control studies to find significant associations, and 100-200 families might be regarded as a minimum required to obtain reliable data.

Subsequently, a case-control study in an Indonesian population reported an association of TB with HLA-DR2 and DQw1 (Bothamley 1989), but it was not clear whether the controls came from the same community as the patient population. In addition, if only 10 % of the DR2 determinations were misclassified by the error-prone lymphocytotoxic method, the association disappears (Rajalingam 1996). These caveats are also relevant to other studies reporting an association with DR2 (Brahmajothi 1991).

A Russian study then looked at the presence of various HLA antigens in pulmonary TB patients and controls from six ethnic groups (Khomeenko 1990). Different HLA antigens were found associated with pulmonary TB in the different ethnic groups, but in five of the six groups, a positive association was found for DR2, and a protective effect for the presence of DR3. After correcting the probabilities for the number of antigens tested (Bonferroni), the association with DR2 will probably maintain significance in only two of the groups.

A DR2 association was also reported in Tuvian Mongol children (Pospelov 1996). Both HLA-DR2 and DRw53 were increased in children with TB when compared with healthy children, but not when compared to children with other chronic lung diseases. After correcting for the number of antigens tested, only the DRw53 association remained significant.

Using the more accurate oligonucleotide hybridization technique to identify the 11 subtypes of the DR2 antigen (Mehra 1995), a significant increase of DRB1*1501 was noted in pulmonary TB patients compared to controls ($p < 0.05$), and a subsequent report from the same group (Rajalingam 1996) found a slightly higher frequency of HLA-DR2 in pulmonary TB patients than in controls ($P_c = 0.029$, $RR = 1.8$ [P_c indicates a P value after a Bonferroni correction]) and a stronger association in drug-failure patients with extensive disease ($P_c = 0.0001$), but no association with any particular DR subtype. More recent studies have found DRB1*1501 associated with TB in India (Sriram 2001) and Mexico ($OR \sim 8$) (Teran-Escandon 1999),

and with rapid onset of disease with *M. avium* in US patients with AIDS (LeBlanc 2000). The association of HLA-DR2 with pulmonary TB was not found in case-control studies of South Indians (Sanjeevi 1992), African Americans (Hwang 1985), Hong Kong Chinese (Hawkins 1988), Egyptians (Hafez 1985), Mexican-Americans (Cox 1988), Cambodians (Goldfeld 1998), or in a family study from Northern Brazil (Blackwell 1998). It is hard to reconcile the reports of associations found with DR2 and alleles such as DRB1*1501 with the many reports finding no association. Could the differences be attributable to ethnic differences, multiple testing, or study design?

In contrast to the questionable DR2 associations, a study in rural Cambodia (Goldfeld 1998) found a significant association of HLA-DQB1*0503 with pulmonary TB ($P = 0.005$), but no significant association with either DR2 or Tumor Necrosis Factor alpha (TNF- α) alleles. The study design strengthened the case for this association because it was done in two stages with two separate groups of patients. In the first stage, a large number of HLA alleles were tested but only HLA-DQB1*0503 appeared to be associated. Because the second stage only tested for this subtype, no statistical correction was necessary. In addition, the controls were patients seen for minor illnesses at the same hospitals, so were likely to be representative of the TB patient population. As this is a highly TB-exposed population, the authors interpreted the DQ allele as an association with development of clinical TB rather than susceptibility to infection (Goldfeld 1998). This association also appears more solid than others because there is a functional explanation for the increased TB susceptibility. The β subunit of DQB1*0503 has aspartic acid instead of alanine at amino acid 57 (Delgado 2006), which changes the charge in the cleft of the peptide binding pocket (Kwok 1996). This alters its binding affinity for antigenic peptides, reducing the affinity by five-fold for a peptide from the central region of the important TB antigen 6-kDa early secretory antigenic target (ESAT-6) (Brodin 2004). In a follow-up study, progression to TB was also found to be associated with homozygosity of other alleles with aspartic acid in position 57 of the HLA-DQB peptide (6-4): DQB1*0301, 0303; DQB1*04 (-0401, 0402); DQB1*0503; DQB1*0601, -0602, -0603. Compared with HLA β 57-Ala alleles, presentation of the ESAT-6 peptide by HLA-DQB β 57-Asp resulted in less IFN- γ production by CD4+ T cells from TB patients (Delgado 2006). Remarkably, 41 HLA-DQB1*0503* alleles were found among TB patients, but the allele was not detected in any of the 107 TST-positive controls, suggesting that this allele in particular could have a near Mendelian effect. This allele has not been reported to be associated with TB in any other population, so could be specific for Cambodians.

However, a study of TB patients in the Venda population of South Africa (Lombard 2006) found an association of TB with some of the other β 57-Asp haplotypes identified in the Cambodian study, DRB1*1302-DQB1*0602, DRB1*1302-DQB1*0603, DRB1*1101-1121-DQB1*0301-0304, and DRB1*1101-1121-DQB1*05. Seven other studies have also found some of these same alleles to be associated with TB susceptibility (Dubaniewicz 2000, Dubaniewicz 2005, Goldfeld 1998, Kim 2005, Pospelova 2005, Teran-Escandon 1999, Wang 2001), while three studies reported protective effects (Dubaniewicz 2005, Vejbaesya 2002, Wang 2001).

It has been suggested (Lombard 2006) that the haplotypes conferring increased susceptibility to TB may be maintained in the population because some of the same alleles also protect from severe malaria (DRB1*1302-DQB1*0501) (Hill 1991), from persistent hepatitis B (HLA-DRB1*1302) (Thursz 1995) (Hill 2001, Wang 2003), and from chronic hepatitis C (DRB1*1101 and DQB1*0301) (Hong 2005). Perhaps heterozygosity for these HLA alleles could protect Africans from both malaria and TB, as well as chronic hepatitis. Conversely, although the HLA-DQ β -57-Asp is associated with susceptibility to TB, it is also associated with resistance to autoimmune type-1 diabetes. Therefore, by the putative genetic selection in the European population for HLA-DQ β 57-Ala alleles (HLA-DQ2 and -DQ8), Europeans, while increasing their resistance to TB, could have become more likely to develop autoimmune type-1 diabetes (Delgado 2006).

6.3.2. Cytokines and cytokine receptors

Many studies have looked for an association of TB susceptibility with polymorphisms in genes encoding other elements of the immune system thought to be important in controlling mycobacterial infections. These different polymorphisms, or alleles, which coexist in the population, are generally changes in a single nucleotide (Single Nucleotide Polymorphism, or SNP). They have mainly been evaluated in case control studies, but some have also been tested in family studies correlating the inheritance of particular parental alleles with the development of TB in both the parents and offspring.

For a summary of studies on candidate genes see Table 6-5 at <http://www.tuberculosis textbook.com/pdf/Table 6-5.pdf>.

6.3.2.1. IFN- γ

From studies in mice (Flynn 1993) and investigations of humans with MSMD, it is clear that IFN- γ is critical for the defense against mycobacteria, and therefore, the

gene encoding it was an obvious candidate for polymorphisms that might slightly affect its function and alter susceptibility to common TB. At nucleotide +874, a SNP was identified that can present either a thymidine (T) or an adenine (A). In studies in Sicily (Lio 2002), South Africa (Rossouw 2003), Hong Kong (Tso 2005), and Spain (Lopez-Maderuelo 2003), the A allele, thought to produce less IFN- γ , was more common in patients with TB, whereas the T allele was more common in controls. The increase in the chances of having TB in an individual with an adenine at +874 compared to the chances in a patient with two thymidines at +874 (TT) varied from ~ 1.5 to 4.6 fold. In Croatia, an association was found only with microscopy or culture positive vs. negative TB cases (Etokebe 2006), and no association emerged from work performed in Turkey (Oral 2006), Malawi (Fitness 2004), Houston, Texas (Moran 2007) or West Africa (Cooke 2006). The study in West Africa found a minimal increase in susceptibility with two other IFN- γ alleles (OR ~ 1.45) (Cooke 2006). Thus, despite its importance in TB immunity, published studies can only suggest that polymorphisms in the IFN- γ gene might influence susceptibility to TB in some populations, but the data is inconclusive. There is even less evidence that different alleles of the IFN- γ receptor affect susceptibility. Only two (Cooke 2006, Fraser 2003) of six studies (Awomoyi 2004, Mirsaeidi 2006, Park 2004, Rosenzweig 2004) found an association of TB susceptibility with polymorphisms in the gene encoding the IFN- γ receptor 1 protein.

6.3.2.2. IL-12, IL-1, IL-10, TNF- α , IL-8

Other attractive candidate genes with contradictory studies are those encoding IL-12 and the IL-12 receptor. A study in Hong Kong Chinese (Tso 2004) found an association with two different polymorphisms in IL-12B, the gene encoding the p40 subunit of IL-12, while a study in Texas found no association with SNPs in the 3' untranslated region (3'UTR) (Ma 2003). In studies on the IL-12RB1 gene, encoding the beta subunit of the IL-12 receptor, associations with TB were found in both Morocco (Remus 2004) and Japan (Akahoshi 2003), but the associated IL-12RB1 SNPs were different in the two countries. A subsequent study confirmed the associations in Japanese patients (Kusuhara 2007), while a study in Korea tested several IL-12RB1 SNPs and found no association (Lee 2005).

Of several studies on polymorphisms in the IL-1B gene, encoding the beta chain of IL-1, two (Awomoyi 2005, Gomez 2006) found different SNPs to be associated with TB, while three found no associations (Bellamy 1998, Delgado 2002, Wilkinson 1999). A few studies have looked at polymorphisms in the IL-1 receptor, but just one found an association, and that was only with pleural TB (Wilkinson 1999).

Studies of polymorphisms in the gene for IL-10 found that in the -1082 SNP, the G allele was more common in TB patients in Cambodia (Delgado 2002), Sicily (Scola 2003), and Turkey (Oral 2006), with odds ratios of around 2. In Colombian patients, pleural TB was associated with SNPs at both -1082 and +874 (Henao 2006). No association for the -1082 SNP was found in studies in Gambia (Bellamy 1998), Korea (Shin 2005) or Spain (Lopez-Maderuelo 2003). In Korea, the C allele at IL-10 -592 and the ht2 haplotype (Shin 2005) showed slight protection (OR = 0.69). Overall, there is a suggestion of an association of TB with IL-10, especially the -1082 SNP, but the differences in susceptibility were quite modest.

The TNF- α -308 G-A polymorphism was found to protect against TB in Sicily (Scola 2003); and the -308A-238G haplotype was protective in Colombia (Correa 2005), but no association was found in studies from Turkey (Oral 2006), India (Selvaraj 2001) or Cambodia (Delgado 2002). An IL-8 polymorphism at -251 was found to confer 3.5 fold susceptibility to TB in Texas (Ma 2003), but no association with this SNP was found in the Gambia (Cooke 2004). Studies looking at other cytokines have failed to demonstrate convincing associations. Although it is always possible to suggest that differences in ethnic genetic make-up, or in study design or selection of controls can account for contradictory findings in distinct populations, the high degree of heterogeneity in the results, and the modest effects in most studies finding associations, make the putative influence of different cytokine SNPs on TB susceptibility less credible. One exception is MCP-1, described with genomic screens, below.

6.3.2.3. Vitamin D receptor

In the pre-chemotherapy era, TB was treated with vitamin D supplements, vitamin D-rich diets, and the sunlight that was the basis of the sanatorium movement (Evans 1994). As synthesis of vitamin D₃ is dependent on cutaneous exposure to ultraviolet light, which is blocked by melanin, it was postulated that the putative increased susceptibility to TB (Liu 2006) in more pigmented races could be related to a relative vitamin D deficiency, especially when living in less sunny climates (Wilkinson 2000). In vitro studies have shown that the addition of vitamin D to infected macrophages augments their ability to eliminate *M. tuberculosis*, although it is not clear whether this is due to the induction of increased expression of reactive nitrogen intermediates (Rockett 1998), reactive oxygen intermediates (Sly 2001), or the anti-microbial peptide cathelicidin (Liu 2006).

Several polymorphisms were found in the gene for the Vitamin D Receptor (VDR) (Uitterlinden 2004). They were initially thought to influence bone density and osteoporosis (Sainz 1997), but subsequent studies found no convincing evidence

that they are associated with an increase in fractures (Uitterlinden 2006). However, studies have associated the different polymorphisms with susceptibility to osteoarthritis, diabetes, cancer, cardiovascular disease (Uitterlinden 2004), and TB (Bellamy 1999). Work on TB associations has focused on four polymorphisms that determine the presence or absence of four restriction enzyme sites, FokI, TaqI, BsmI, and ApaI, respectively, so that each of the four polymorphisms can have two possible alleles, designated F/f, T/t, B/b, and A/a (Bornman 2004).

An early study found that the homozygous tt genotype was underrepresented in Gambian patients with pulmonary TB, suggesting that it might be protective (OR = 0.53) (Bellamy 1999). This created sufficient interest to motivate at least eight other studies, which have reported diverse results. In Gujarati Indians in London, serum vitamin D deficiency was associated with susceptibility to TB, which appeared to be synergistically increased in individuals with the Tt or TT VDR genotypes, suggesting, again, that tt is protective (Wilkinson 2000). In contrast, the TT genotype was associated with decreased susceptibility in South Indian females (Selvaraj 2000), while the FF genotype was associated with resistance to TB in Han Chinese soldiers (Liu 2004). Six studies found no association of any individual vitamin D receptor with TB susceptibility (Bornman 2004, Delgado 2002, Lombard 2006, Roth 2004, Soborg 2007, Wilkinson 2000), although a study in Peru (Roth 2004) found that the Tt and FF genotypes were associated with faster sputum culture conversion after initiation of therapy.

Work in West Africa found that the SNPs had no independent associations with TB, but were in strong linkage disequilibrium, and the FA haplotype was transmitted more frequently than expected from TB parents to TB affected offspring (Bornman 2004). This means that the FA pair of SNPs was inherited together, perhaps with another linked gene that affects TB immunity. Finally, a study in the Venda population of South Africa found that the haplotype FbAT appeared to be protective (Lombard 2006). This would be consistent with the association of the f allele with TB in the Han Chinese (Liu 2004) and Gujarati Asians (Wilkinson 2000), and the protective effect of the TT genotype in South Indian women (Selvaraj 2000), but conflicts with the protective association of tt in the Gambia (Bellamy 1999) and Gujarati Asians (Wilkinson 2000), and the transmission of the FA haplotype to affected offspring in West Africa (Bornman 2004).

A meta-analysis of studies on the FokI and TaqI polymorphisms found the results to be inconclusive, and that the studies had too few participants (low statistical power) to prove the weak increases or decreases in susceptibility identified in those studies that found associations (Lewis 2005). In summary, while there is evidence that vitamin D promotes macrophage killing of *M tuberculosis* (Liu 2006, Rockett

1998, Sly 2001) the effector mechanism is not clear, and the association of VDR polymorphisms with susceptibility to TB remains unproven. Should there be an association of particular haplotypes with susceptibility to, or protection from TB, it seems likely to be the result of varying linkage disequilibrium with a nearby polymorphism that has functional significance in the human defense against TB. However, the relevant gene does not appear to be the vitamin D receptor, or else its effect is so minimal that it is easily obscured by other genetic or environmental factors. It is also possible that the affects of VDR polymorphisms on TB susceptibility are manifest only when the serum vitamin D levels are very low (Wilkinson 2000).

6.3.3. Pattern recognition receptors

One of the first lines of defense of the immune system is the recognition and uptake of microorganisms by professional phagocytes: macrophages and dendritic cells. On the surface of phagocytic cells are several different pattern recognition receptors, which, in the absence of adaptive immunity, bind to different patterns on microbes to promote phagocytosis and activate signaling that leads to cytokine production, antigen presentation, and the development of adaptive immunity. These pattern recognition receptors include Toll-like receptors (TLR), scavenger receptors, the complement receptors, the macrophage mannose-binding lectin (MBL), the dendritic-cell-specific intercellular adhesion molecule-3, called DC-SIGN, and others. Several of these have been shown to mediate the phagocytosis of *M. tuberculosis* (Ernst 1998), and have been studied to see whether different polymorphisms might affect TB susceptibility (Neyrolles 2006).

6.3.3.1. Toll-like receptors

Human TLRs are a family of proteins that recognize different pathogen-associated molecular patterns and stimulate signaling pathways that activate the innate immune response, cytokine production, and the process of adaptive immunity (Cook 2004). Mycobacteria are recognized by TLR1, 2, 4, and 6, which interact with the adaptor proteins MyD88 and Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP), to activate macrophages and dendritic cells (Heldwein 2002). Signaling occurs when the TIR domain of the TLR interacts with the TIR domain of TIRAP (Yamamoto 2002). A few studies have looked at the possibility that polymorphisms in elements of the TLR system might influence susceptibility to TB. Three studies, in Turkey (Ogus 2004) Korea (Yim 2006), and Tunisia (Ben-Ali 2004) looked at different polymorphisms in TLR-2, and all found alleles that occurred more frequently in TB patients than in controls. A TLR4 polymorphism

was found to have no association with TB in a study in the Gambia (Newport 2004). A study in Vietnam found that the C558T polymorphism in TIRAP was associated with TB. The association was stronger in homozygote TT individuals, and was more pronounced when only patients with TB meningitis were considered (Hawn 2006). Interestingly, this TIRAP polymorphism appeared to impair signaling only with TLR2 ligands, but not those binding to TLR4 (Hawn 2006). These preliminary studies need to be repeated in other settings, but there is a clear suggestion that polymorphisms in the TLR2 pathway of innate immunity may influence how TB infections evolve.

6.3.3.2. Mannose-binding lectin (MBL)

MBL is a collagenous serum protein produced by the liver that participates in the innate immune system (Neth 2000). On binding to its ligands, it activates the complement cascade. Low levels of MBL in humans are caused by any of three structural mutations in codons 52, 54, or 57. The latter two polymorphisms are present at a fairly high frequency in sub-Saharan African and Eurasian populations, and have been associated with an increased risk of infection (Neth 2000). As mannose is abundant in the *M. tuberculosis* cell wall component manlam, and MBL has been shown to be a receptor for *M. tuberculosis*, studies have looked at the effect of the levels of MBL on susceptibility. While a study in India (Selvaraj 1999) found that more TB patients than controls were homozygous for low-producing polymorphisms, studies in Gambia (Bellamy 1998), China (Liu 2006), Poland (Druszczynska 2006), Turkey (Ozbas-Gerceker 2003) and Malawi (Fitness 2004) found no association. In contrast, although MBL was thought to aid in the immunity to infections, especially meningococcus (Abel 2002) (Neth 2000), studies in Denmark (Soborg 2003), Tanzania (Garred 1997) and South Africa (Hoal-Van Helden 1999) found that the lower MBL levels occurring with the alternative polymorphisms appear to be protective against TB. In the South African study, the protection was more pronounced against TB meningitis. Protection afforded by low levels of MBL is consistent with a recent finding that the binding of MBL by manlam during phagocytosis is key in limiting phago-lysosomal fusion (Kang 2005), which is thought to be important for intracellular survival of the bacteria. However, other work suggests that there has been no population selection either for or against the low producing alleles, and that MBL is probably redundant and not important in human defenses (Abel 2002, Verdu 2006).

6.3.3.3. DC-SIGN

DC-SIGN (dendritic-cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin) is a lectin present on macrophages and monocyte derived

dendritic cells that recognizes many pathogens, and may modify the pathogenesis of HIV and dengue (Neyrolles 2006). It was shown to be a major receptor for *M. tuberculosis* on dendritic cells, presumably by binding to the mannose in the *M. tuberculosis* cell wall manlam (Tailleux 2003). Its expression is scant on alveolar macrophages from healthy individuals, but is variably induced after TB infection (Tailleux 2005). Two variants (-871G and -336A) have been identified in the promoter region of CD209, the gene for DC-SIGN, and the -336A allele has been shown to increase expression. In a study in South African Coloreds, these two variants were associated with a lower risk of developing TB, and the alternate nucleotides with an increased risk (-871A OR = 1.85; -336G OR = 1.48) (Barreiro 2006). The protective allele, -871G, was present in 21 % and 38 % of Asians and Europeans respectively, but was absent in Africans, which, it was postulated, could contribute to the putative increased TB susceptibility in this ethnic group (Barreiro 2006). A subsequent study from Colombia found no significant association of TB with the -336 allele, although the frequency of this allele was very low in the population studied (Gomez, Anaya et al. 2006).

DC-SIGN is an attractive candidate for influencing TB susceptibility, but more work is needed to prove an association, and there are inconsistencies in understanding how DC-SIGN might affect susceptibility. TB protection was associated with the high expression allele, -336A, suggesting that phagocytosis by DC-SIGN may somehow give the phagocytosing cell an advantage over the pathogen. In contrast, other studies suggest that phagocytosis mediated by DC-SIGN allows the pathogen to circumvent antigen processing. It was reported that DC-SIGN binding of ManLam prevents DC maturation through TLR-mediated signaling, thereby diminishing T-cell responses and fostering pathogen survival (van Kooyk and Geijtenbeek 2003). Interestingly, mice lacking SIGNR1, the murine homolog of DC-SIGN, show increased T cell activity early after infection, but no apparent alteration in susceptibility to TB (Wieland 2007).

6.3.3.4 The purinergic P2X7 receptor

Purinergic P2X7 receptors are cationic channels present on the cells in the blood and immune systems, and highly expressed on macrophages (Gu 2001). The P2X7 receptor is activated by extracellular ATP, which causes an opening of their cation-selective channel, leading to an influx of calcium and an induction of the caspase cascade, resulting in apoptosis. A calcium-dependent phospholipase D pathway is also activated, promoting phago-lysosomal fusion and mycobacterial killing.

A study of normal subjects found a polymorphism with a 1513 A-C change that causes the glutamic acid at residue 496 to be replaced by alanine, which results in a

marked decrease in the ability of the P2X7 pore to open after ATP activation (Gu 2001). Although this polymorphism was not associated with pulmonary TB in a case-control study in Gambia (Li 2002), this study identified five SNPs in the putative promoter region of the gene for P2X7, and in one, at -762, the presence of a C showed significant protection against TB, with an OR of 0.7 (C.I: 0.54 - 0.89; P = 0.003) for the heterozygote and 0.545 (CI: 0.318-0.934 P = 0.027) for CC homozygotes. It was suggested that the C at -762 could affect the level of P2X7 expression by altering the binding of a transcription factor. Other loss of function polymorphisms have been identified in the P2X7 coding region, but their frequency is too low to be analyzed in association studies (Fernando 2005). However, the importance of P2X7 polymorphisms is not clear, as the differences in TB susceptibility appeared slight, at most two fold, and mice lacking P2X7 are as capable as wild-type mice in controlling pulmonary infections with *M. tuberculosis* (Myers 2005).

A study of two cohorts of Southeast Asian refugees in Australia found no association of the 1513 SNP with pulmonary TB, but, surprisingly, found a strong association of the C polymorphism with extrapulmonary TB (Fernando 2006). The odds ratio for a C at 1513 was 3.8 (CI 1.6 – 9.0; p < 0.01) in one cohort, and 3.7 (1.7 – 8.1; p = 0.001) in the second. Furthermore, in vitro studies showed that the ATP-mediated killing of mycobacteria was absent in macrophages from patients homozygous for the 1513 C allele, and impaired in macrophages from heterozygous subjects. There was a strong correlation between the capacity for mycobacterial killing and ATP-induced apoptosis. The authors postulated that decreased macrophage apoptosis leads to decreased killing of mycobacteria, permitting the bacillus to spread to other organs, both in recent infection as well as in reactivation. In one cohort, 35 % of reactive disease was extrapulmonary and showed a strong association with the 1513 C allele. While this association with extrapulmonary TB is interesting, it must be confirmed in other studies, along with the associations of MBL (Hoal-Van Helden 1999) and TIRAP (Hawn 2006) with TB meningitis, and SCL11A1 (Kim 2003), and IL-1Ra with pleural TB (Wilkinson 1999).

6.3.3.5. NOD2, surfactant proteins, complement receptor 1

Another pattern recognition receptor is the nucleotide oligomerization-binding domain 2 (NOD2). The caspase recruitment domain-containing protein 15 (CARD15) gene which encodes the NOD2 protein, is implicated in susceptibility to Crohn's disease, a granulomatous, chronic inflammatory gastrointestinal disorder for which *M. avium* subsp. *paratuberculosis* has been proposed as a causative agent (Behr 2006). The recognition of mycobacterial components by the NOD2 receptor was shown to be important for the induction of pro-inflammatory cytokines by mononuclear cells stimulated with *M. tuberculosis* (Ferwerda 2005). However,

large case-control studies in both Gambia (Stockton 2004) and South Africa (Moller 2006, Stockton 2004) failed to find an association between NOD2 and TB.

The surfactant proteins A (SP-A) and D (SP-D) are other pattern recognition elements of innate immunity that contribute to protection against virus, bacteria, and fungi (Kishore 2005). These lung surfactant-associated proteins are collagen containing calcium-dependent lectins, called collectins, and are structurally similar to MBL. They recognize many pathogens via their lectin domains and activate immune cells through their collagen regions. Surfactant protein A is a multi-chain protein encoded by the SFTP-A1 and SFTP-A2 genes, and several polymorphisms have been found in each. Polymorphisms in the SFTP-A2 gene were found to be associated with susceptibility to TB in Ethiopia (Malik 2006), Mexico (Floros 2000), and India (Madan 2002). It is likely that other studies will try to confirm these interesting associations.

Yet another of the many receptors on the surface of macrophages that have been shown to mediate the phagocytosis of *M. tuberculosis* (Ernst 1998) is the complement receptor 1 (CR1) (Schlesinger 1990). A recent large-scale study in Malawi found that homozygotes in one of five CR1 polymorphisms (Q1022H) had an increased TB risk (OR = 3.12). The SNP causes an amino acid change that may alter ligand binding, perhaps reducing the phagocytosis of *M. tuberculosis* (Fitness 2004).

6.4 Genes from mouse genetic susceptibility studies

6.4.1 *bcg*/NRAMP1/SLC11A1

Over half a century ago Lurie bred strains of rabbits that showed differing levels of resistance to infections with *M. tuberculosis* (Dorman 2004, Lurie 1952). While it is unfortunate that these rabbit strains were lost, it would have been difficult to identify the relevant genetic determinants. However, there are also susceptible and resistant strains of mice, and mouse genetics have developed sufficiently to have allowed some of the putative genes responsible for the differences to be identified.

The first gene identified was the NRAMP1 (natural resistance-associated macrophage protein 1), originally termed the *bcg* gene (Skamene 1994). It was found to be responsible for the abnormal sensitivity of a strain of mice to infections with BCG, *Salmonella*, and *Leishmania*. The encoded protein is a divalent cation transporter that appears to play a role in macrophage activation (Nevo 2006). It may also alter the phagosome environment to affect anti-microbial capacity, and regulate the levels of cations, especially iron. The gene was found only after a long

process that mirrored the development of mouse genetics (Liu 1995), but subsequently, the equivalent human gene, SLC11A1 (Solute carrier family 11, member 1), was identified rapidly (Cellier 1994). Since then, a number of studies have looked at genetic markers to see whether in humans, as in mice, there are variants that confer different levels of resistance to TB. Initial studies suggested a minor effect, with about a two-fold increase in susceptibility to TB for each of two polymorphisms, and a four-fold difference when both were present (Bellamy 1998). Results of subsequent studies have varied, and several have shown no effect (Li 2006). Doubts about the role of NRAMP1 in TB susceptibility increased after it was shown that mice that deleted for the NRAMP1 gene were as resistant to *M. tuberculosis* as wild-type mice (North 1999). Interest in the gene was renewed when a study found a SLC11A1 polymorphism to be strongly associated with susceptibility to TB in an extended indigenous Canadian family (Relative Risk = ~ 10). Stratifying the family members for TB exposure was critical in revealing the association (Greenwood 2000).

A recently published meta-analysis (Li 2006) analyzed 17 case-control studies on the association of TB susceptibility with four SLC11A1 polymorphisms (Figure 6-2):

- 5' (GT) n (a micro-satellite with a variable number (n) of GT repeats immediately 5' of the SLC11A1 gene)
- INT4 (a single nucleotide change in intron 4: 469+14G/C)
- D543N (an aspartic acid to asparagine substitution at codon 543 in exon 15)
- 3' UTR (a TGTG deletion located 55 bp downstream of the last codon in exon 15:1729+55del4)

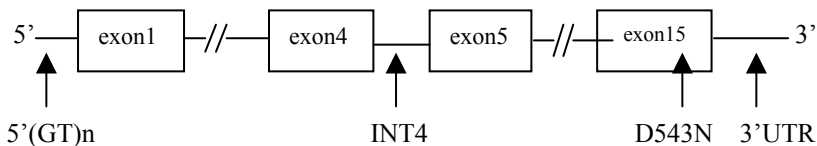


Figure 6-2: Sites of four frequently studied SLC11A1 polymorphisms

The results of the different studies were compared, the data were combined and analyzed as if they were a single group, as well as being analyzed separately for

associations in Africans, Asians, and Europeans. When the subjects in all the studies were considered as a whole, the less common alleles at the 3'UTR, D543N and 5' (GT)_n showed significant associations with pulmonary TB, and individuals with these SNPs had a 27 %, 61 % and 25 % higher risk of developing TB than those with the common alleles. When only Asian populations were considered, these three polymorphisms were significantly associated with an increased risk of TB (39 %, 59 %, and 65 % respectively). Of the four polymorphisms, only the 3'UTR failed to show a significant association in African populations, but none of the four was associated with TB in Europeans. However, this lack of association may be due to the low proportion of Europeans (9.3 %) included in the studies analyzed (Li 2006). Although the association of SLC11A1 with TB is not found in all studies, there seems to be enough evidence to suggest that some of the variants have a mild effect on susceptibility. However, perhaps the true magnitude of the effect is apparent only if, as in the study of the large indigenous Canadian family, the subjects can be classified by exposure to TB, and other variables, including genetic background and general environment, can be controlled.

6.4.2 *Ipr/sst1/SP110*

Another potential gene candidate for genetic resistance was identified in a search for the genetic determinants responsible for the extreme sensitivity to *M. tuberculosis* shown by mouse strain C3HeB/FeJ (Kramnik 2000). This strain succumbs to infection within 4-5 weeks after infection, compared to 6-8 months for normal mice (Pan 2005). The TB lesions show pronounced necrosis, and infected macrophages display a pattern of necrosis rather than the apoptosis seen in macrophages from resistant strains (Pan 2005). This marked sensitivity, thought to involve innate immunity, was seen after infection with virulent *M. tuberculosis*, but there was little difference for infections with avirulent strains. The locus identified, termed *sst1* (super susceptibility to TB), was located on mouse chromosome 1, 10 cM distal to the NRAMP1 gene.

The responsible gene was subsequently identified and termed *Ipr1*, for intracellular pathogen resistance 1. Its expression is stimulated by IFN- γ and upregulated after infection in the *sst1* resistant mice, but it is not expressed in *sst1* sensitive strains of mice. Expression of *sst1* limits intra-macrophage replication of *Listeria monocytogenes* (Boyartchuk 2004) by a mechanism thought to involve innate immunity, which is dependent upon IFN- γ and reactive oxygen intermediates, but independent of nitric oxide.

When a transgenic strain of mice was constructed with the resistant form of *lpr1* replacing the sensitive form in the sensitive C3HeB/FeJ mouse, the level of resistance to *M. tuberculosis* was improved, but was not restored to that of the resistant mouse strains. This suggested that there are other genetic determinants responsible for the extreme sensitivity of the C3HeB/FeJ mouse. Transgenic studies have subsequently identified four other putative loci on chromosomes 7, 12, 15, and 17, confirming the multigenic determination of TB susceptibility in mice (Yan 2006).

In humans, the closest homologue of the predicted *lpr1* protein is SP110, which is only 41 % identical but found in a region of human chromosome 2 that is syntenic to the *sst* region on mouse chromosome 1. SP110 has some of the same protein motifs as *lpr1* (SP100 and SAND domains), and is a component of the nuclear body, a multi-protein complex believed to be involved in regulation of gene transcription (Thye 2006). It is predominantly expressed in leukocytes and spleen cells, with lower levels of expression in other tissues, and expression is regulated by interferon. SP110 interacts with viral proteins, and polymorphisms in SP110 have been associated with susceptibility to hepatitis C virus (Tosh 2006).

A study to determine whether variants in the SP110 gene are associated with susceptibility to TB was carried out in three West African countries (Tosh 2006). Families of TB patients were analyzed with transmission disequilibrium testing to see if certain variants were transmitted more frequently to affected offspring. Three SNPs were significantly associated with TB in Gambia, but only one of these, rs2114592, also showed a significant association in families from both Guinea-Bissau and the Republic of Guinea, while another, rs3948464, showed associations that were significant in the Republic of Guinea, but not in Guinea-Bissau. Several of the associations would not be significant after a Bonferroni correction for the 20 SNPs tested. The variants were found to be in strong linkage disequilibrium in a region of low haplotype diversity, so it is possible that it is actually another polymorphism in this region that has a functional role in altering TB susceptibility.

However, the role of SP110 in human susceptibility to TB remains unproven. A recent study described cohorts of children with mutations in SP110 who suffered from an autosomal recessive disorder of hepatic vascular occlusion, severe hypogammaglobulinemia, combined T and B cell immunodeficiency, absent lymph node germinal centers, and absent tissue plasma cells (Roscioli 2006). Their immunodeficiencies made them prone to infections with *Pneumocystis jirovecii*, enterovirus, and mucocutaneous candidiasis, but not mycobacterial infections. While this would seem contradictory with the role of *lpr1* in mice, it should be recalled that its effect in mice was only seen with virulent *M. tuberculosis*, and not avirulent BCG (Pan 2005). Thus, the unfortunate children with the SP110 mutations may not be

abnormally susceptible to BCG or atypical mycobacteria, and may not have been exposed to *M. tuberculosis*. Further doubts about SP110 come from a large case-control study in Ghana that examined 21 SNP variants and found no association (Thye 2006). Additional surveys are needed to determine whether SP110 is truly associated with TB susceptibility.

6.4.3. Genomic screens - family studies revisited

Blackwell *et al.* “scanned” the mouse genome for loci involved in susceptibility to leishmaniasis by comparing sensitive and resistant strains of inbred mice for hundreds of genetic markers across the entire genome (Bellamy 2006, Blackwell 1996). Five major chromosomal regions were identified, including the NRAMP1 locus. Possible associations with the homologous regions of the human chromosome were then examined in a large study in Belem, Brazil (Blackwell 1997), using multicase families for TB (98 families; 704 individuals), leprosy (72 families; 389 individuals), and leishmaniasis (89 families, 638 individuals), all from the same socio-economic strata (Shaw 1997). All relevant family members were studied using a combination of gene polymorphisms and microsatellite markers to trace the inheritance of the human chromosomal regions equivalent to those identified in mouse studies.

Leprosy was associated with both TNF- α and DR2 (Blackwell 1998), but there was no association of TB with TNF- α or any HLA locus, and only a weak association with NRAMP1, or a gene close to it. An association with TB susceptibility was found for the locus 17q11.2-q12 (LOD-score 1.3 $P=0.01$), a region that is similar (syntenic) to a region on mouse chromosome 11 that is associated with susceptibility to leishmaniasis. Genes in this region encode several proteins that could be plausibly linked to TB immunology: NOS2A (Rockets 1998), encoding the inducible form of nitric oxide synthetase (Blackwell 1998); chemokines CCL2/MCP-1 (monocyte chemoattractant protein-1) (Lu 1998), CCL3/MIP-1 α (macrophage inflammatory protein-1 α), CCL4/MIP-1 β , CCL5/RANTES (regulated on activation, normal T cell expressed and secreted), CCR7, the receptor for CCL19/21, and several genes encoding signal transducers and activators of transcription: *STAT3*, *STAT5A*, and *STAT5B*. A subsequent study of 92 multicase families looked for associations with particular genes in this region. Using 16 microsatellites and 69 SNPs, associations were found for four genes that are separated by fairly large intervals (NOS2A-8.4 Mb-CCL18-32.2 kb-CCL4-6.04 Mb-STAT5B). Conditional logistic regression using a case/pseudo-control data set showed that each gene contributed separately, suggesting that this is a cluster of susceptibility genes.

However, after correcting for the number of markers tested, the only significant association was with a SNP in cytokine CCL18 (Jamieson 2004). The authors calculated that in order to detect a gene that causes at least a two-fold or greater effect on TB susceptibility or resistance among variant alleles with < 0.2 frequency, a study would need 400 affected families composed of at least one patient and their parents. If the effect was less than two-fold, and the variant allele frequency < 0.1 , the association would not be found even with 800 parent/child trios (Jamieson 2004).

A separate two-stage study of 16 Brazilian multi-case TB families first used 405 markers to scan the whole genome for regions associated with TB, and then further examined the 58 markers that produced a positive result, using a second set of 22 families and additional markers (Miller 2004). Associations were confirmed for three chromosomal regions, 10p26.13, 11q12.3, and 20p12.1, but no association was found for the 17q11.2-q12, perhaps because of the limited study size.

A large case-control study then looked for associations with different genes at the 17q11.2 locus in Mexican and Korean TB patients (Flores-Villanueva 2005). No association was found for NOS2A, RANTES or MIP-1 α alleles, but a significant association was found for the adenine (A) to guanine (G) change in the -2518 promoter polymorphism of monocyte chemoattractant protein-1 (MCP-1), also known as, SCYA2 and CCL2. Compared with AA homozygotes, both Mexican and Korean AG heterozygotes had an increased risk for developing TB of 2.3 and 2.8 fold respectively, while GG homozygotes were 5.4 fold and 6.9 fold more likely to develop TB. The G allele increases production of MCP-1, and the higher blood levels of MCP-1 in the GG homozygotes were correlated with lower levels of IL-12 subunit IL-12p40, which has been shown in Mendelian disease to be critical for control of the infection. Furthermore, the hypersusceptible GG allele was present in 53 % of Mexican TB patients, compared with 27 % of controls, and 36 % of Korean cases, compared with 14 % of controls. This allele could thus be responsible for as much as 64 % of TB cases in the Mexican population, and the TB rates might be 64 % lower in a population without the G allele (Alcais 2005).

In mice, CCL2, the equivalent of MCP-1, and its receptor, CCR2, are important for protection from high dose *M. tuberculosis* infections (Peters 2001), but CCL2 $^{-/-}$ or CCR2 $^{-/-}$ mice infected with lower doses of *M. tuberculosis* have outcomes similar to wild-type mice, despite impaired macrophage recruitment to the lungs (Scott 2002, Kipnis 2003). Mice expressing high levels of MCP-1 show increased susceptibility to TB (Gu 1997).

Why was there no association with MCP-1 seen in the Brazilian study? Perhaps the ability to demonstrate this association in the Mexican/Korean study was aided by its strict criteria for study participants: sputum smear-positive new adult TB patients with culture-confirmed disease, excluding those with chronic illnesses, including malnutrition, or previous episodes of TB. The patients also had “*clinical and epidemiological features suggestive of active TB of recent evolution after recent exposure*” (Flores-Villanueva 2005). Controls were healthy TST-positive and TST-negative persons not vaccinated with BCG, who had had recent contact with a TB case. The three groups were similar in demographics and body mass index (before developing TB), household income, and consumption of cigarettes and alcohol. Using these strict inclusion criteria, the study could distinguish the predisposition for progression to clinical disease from susceptibility to infection. The MCP-1 G allele was as common in TST-positive as in TST-negative persons, showing that it had no effect on susceptibility to infection (Flores-Villanueva 2005).

A larger, two-stage genome wide study (Bellamy 2000) was performed by analyzing families from Gambia and South Africa that had at least two siblings with TB - 83 families in the first stage and 53 in the second. Associations were only found for two chromosomal regions, 15q and Xq, with LOD scores of 2.00 and 1.77 respectively. LOD scores of at least 3.0 are generally considered to indicate a strong association, so this, as well as the Belem work, suggested that there are no dominant genes responsible for TB susceptibility. Instead, perhaps many genes are involved, each exerting a small effect. This study found no association with NRAMP1 or the vitamin D receptor, both of which were found to increase susceptibility to TB about two fold in case control studies in the same Gambian population (Bellamy 1998, Bellamy 1998). However, as mentioned above, for a family association study to identify genes with such weak effects, an unreasonable number of families would be required (Bellamy 2000). A subsequent study fine-mapped the 15q region and found that the strongest association was with a region containing the gene UBE3A, which encodes a ubiquitin ligase involved in the ubiquitination and degradation of specific proteins, including the T lymphocyte src kinase Lck (Cervino 2002). There have been no subsequent reports associating this gene with susceptibility to TB.

A different conclusion came from a whole genome scan of 96 multi-case Moroccan families, each having at least two siblings with pulmonary TB (Baghdadi 2006). No associations were seen for the 15q and Xq loci, the 17q11-q21 locus (Flores-Villanueva 2005, Jamieson 2004) or the 10p26.13, 11q12.3, and 20p12.1 loci (Miller 2004) found in the Brazilian studies, but a strong association (LOD = 3.49)

was found for a single region of chromosome 8q12-q13. Although this locus was not associated with TB in all families, the association was especially strong (LOD 3.94) when the families had at least one parent with TB, suggesting that predisposition to TB is inherited as an autosomal dominant trait. In contrast to the notion that TB susceptibility is determined by the sum of many genes, each exerting only small effects, this study suggests that there are genes with large, dominant effects. This model bridges the gap between Mendelian susceptibility mutations and determination of susceptibility by a quorum effect involving multigenic determinants (Casanova 2007).

6.5. The good, the bad and the maybe, in perspective

While the work on the Mendelian inheritance of genes responsible for extreme susceptibility to mycobacterial infections is clear, convincing and informative with respect to the human immune response to TB, the genetic components of common TB infections remain unclear. Considering all of the genes that have been tested for association with susceptibility to TB, in many diverse populations, using a variety of study designs and exclusion criteria, is there any way to make sense of the varied and often contradictory results? While the frequent lack of clear and reproducible associations may result from the difficulty in isolating the effects of a particular gene from the background of many genes involved in determining susceptibility to TB, there is also a suggestion that some genes may have large, dominant effects, at least in particular populations (Casanova 2007).

One approach to try to understand the literature might be to classify or stratify the genes into different categories. The first group would contain genes that have never or have only rarely shown an association, generally of small effect. This group would include most HLA alleles from the early studies, TNF, NOD, TLR-4, and probably MBL, although its association with TB meningitis deserves further study. There are also other genes, not reviewed here that have failed to show evidence of an association (Gomez 2006, Rajalingam 1997).

A second group, also fairly easy to identify, are those few genes with alleles that appear to confer important increases in susceptibility: HLA-DQB1*0503 and HLA-DQB1 alleles with an aspartic acid at position 57; MCP-1 (Flores-Villanueva 2005); and an as yet unidentified gene in locus 8q12-q13 (Baghdadi 2006). In two carefully performed studies of adult pulmonary TB in Cambodia, the HLA-DQB1*0503 allele was found to have near Mendelian effects, being present in less than 10 % of patients with TB but in none of the controls (Delgado 2006, Goldfeld

1998). When this allele was excluded, individuals homozygous with other HLA-DQ β 57Asp alleles had a 2.35-fold increased risk of developing TB, an effect also seen in South Africa (Lombard 2006) and several other populations (see Table 6-4). However, Asp/Asp homozygosity was present in only 26 % of the Cambodian TB patients, and was also found in 10 % of PPD+ healthy controls. While the Asp at aa 57 of the HLA-DQ beta chain reduces antigen presentation in a manner consistent with an increased risk of developing TB (Delgado 2006), the increase in susceptibility is only about two-fold, so other factors must be involved. The much greater effect on susceptibility conferred by the HLA-DQB1*0503 allele remains unexplained, and has only been reported in Cambodians.

MCP-1 (Flores-Villanueva 2005) is presumably the gene responsible for the association found at the 17q11.1-q12 locus (Jamieson 2004). Studies on the -2518 promoter SNP found that GG homozygotes and AG heterozygotes showed increased TB risk in both Mexican (OR = 2.3, 5.4) and Korean (OR = 2.8, 6.9) populations. The GG genotype was found in a striking 53 % of the Mexican TB cases (Alcais 2005) suggesting that the allele could have a major effect on the TB burden in the population. However, 27 % percent of PPD+ healthy controls were also GG homozygotes, and 48 % were AG heterozygotes. Although the GG genotype may confer a large increase in susceptibility, many TB infected GG homozygotes and AG heterozygotes don't develop TB, implying that other factors must be involved in determining who gets TB, both in the GG and the non-GG TB infected population.

The proposed autosomal dominant locus on chromosome 8q12-q13 gave a LOD score of 3.4 overall, and 3.9 in the Moroccan families with an affected parent. This association is stronger than reported for genes in any other affiliation study, although the locus was not associated with TB in all families examined (Baghdadi 2006). However, the reports describing the associations of this locus, and of MCP-1, are very recent and require confirmation, and the associated gene in the 8q12-q13 region remains to be identified.

The third group contains genes reported to be associated with susceptibility to TB, but whose associations either await confirmation or were not confirmed in all subsequent reports. These promising gene candidates requiring further study include: TLR-2, TIRAP, P2X7, DC-SIGN, Sst/SLC110, IL-12RB1, CR1 and the surfactant protein A subunits SFTPA1 and A2. More difficult to classify are the genes encoding IL-10, IL-8, IL-1, IL-12, VDR, and IFN- γ R1, which were associated with minor changes in TB susceptibility in some reports, but no association in several others studies. Similarly, associations with HLA alleles, such as B13, D2, D3 (Kettaneh 2006), or DRB1*1501*, have been found in some studies but not in others (see Table 6-4), and an association with the MHC region has not been found

in any genome scan. Because MSMD has shown the importance of IFN- γ , it was tempting to think that its polymorphisms might affect susceptibility to common TB, but the heterogeneity of results with the IFN- γ polymorphisms, especially the +874 SNP, make it hard to come to any conclusion. In addition, the supposedly susceptible +874 AA genotype is present in more than 40 % of controls.

Finally, there is NRAMP1/SLC110, which was identified by comparing innately susceptible and resistant mouse strains, and found to have minor effects in some of the many case-control studies that looked for an association, and a minor association in one genome screen (Blackwell 1997). However, it appeared to have a major effect (RR = 10) in one carefully studied extended indigenous Canadian family (Greenwood 2000). A meta-analysis concluded that three polymorphisms showed significant associations with pulmonary TB, but only increased susceptibility by 27 %, 61 % and 25 %.

While many of the first gene candidates tested, such as VDR, IFN- γ , and IL-10 have given very heterogeneous results, perhaps more recent candidates, such as TLR2, DC-SIGN or SFTPA1 and 2 will prove more robust. However, most of their effects are not large, being generally less than three-fold. Is there any way to explain the difficulty in conclusively identifying the genes that determine why not all those exposed to *M. tuberculosis* become infected, and why only 10 % of those infected develop the disease? Is it possible to explain why genes associated with susceptibility in some studies often fail to demonstrate an association in others? Some possible explanations include:

- TB susceptibility is cumulatively determined by the sum of many different genes, each having small effects, and the genes may be different in different populations. This could certainly be possible, and is consistent with data in mice (Yan 2006), but proof would likely require the technical capacity to sequence hundreds of genes in hundreds or thousands of individuals (Hill 2006).
- Different ethnic groups have major different determinants of susceptibility. This seems appropriate for the marked effect of HLA-DQB1*0503 in Cambodians, and perhaps other loci, but can't explain the varied results in similar ethnic groups, such as in different West African countries (Tosh 2006).
- Much of TB susceptibility is determined by the large effects of predominant genes, but most of these have not yet been identified (Casanova 2007).

While all of these explanations may be true to some extent, there are other important variables that could help account for the heterogeneity of results: exposure, strain virulence and general environment. These differences were recognized as nearly insurmountable confounding difficulties by the investigators of the early and mid 20th century, who knew that valid associations would only be detected if all epidemiologic variables were carefully controlled. Many of the molecular studies that showed the clearest associations had rigorous criteria for defining both cases and controls to ensure, for example, that the study was looking only at the development of pulmonary TB in recently infected adults of the same ethnic group, age, nutritional and socioeconomic status, with no complicating risk factors (Flores-Villanueva 2005, Moran 2007).

Even the most rigorous exclusion criteria can't control for all important variables. While in mouse experiments animals are infected with a uniform dose and delivery of a single strain of *M. tuberculosis*, human subjects in a study are generally infected with a variety of strains with varying levels of virulence (Lopez 2003). However, even if a study looking for associations were to perform molecular epidemiology on all the strains involved, and could assign a measure of relative virulence to each strain, how could it evaluate the differing intensities of exposure - the number of bacilli that each subject inhaled? Could it be possible that a particular genetic make-up would be able to avoid either infection or disease after a low-dose exposure to a low-virulence strain, but succumb to the same level of exposure to a more virulent strain, or a much higher dose of the less virulent strain? Perhaps there are alleles that make a person resistant to 80 % of the *M. tuberculosis* strains in a community but susceptible to the most virulent 20 %. While family studies should control for strain differences, the small effects of multiple genes would only be found if very large numbers of families were studied, and the most important genes may vary from family to family. To further complicate the analysis, the concordance rate in twin studies was, at most, about 50 % - so identical genes may not yield identical results at least half the time. Given the differences in the strain virulence and exposure within a population, and the genetic heterogeneity and apparent incomplete penetrance of the responsible genes, it should not be surprising that it is difficult to obtain clear, reproducible associations with specific alleles, even those that may have moderate effects.

While documenting or quantifying exposure to the bacillus, or strain virulence, may be difficult, their roles in pathogenesis are obvious. In contrast, environmental influences are not only difficult to document and quantify (Lienhardt 2001), but their effects have not been well studied and are poorly understood. Trudeau performed a classic study that demonstrated the importance of environment to the

development of TB. He compared two groups of infected rabbits: five animals were free to roam outdoors with ample food, while another five were kept in dark cages with minimal food. Within 3 months, four of the five caged rabbits died of TB, and the fifth developed serious illness. In contrast, only one of the free rabbits succumbed to TB, and the others remained healthy after six months (Smith 2003). The reasons for the difference - poor nutrition (Chan 1996, Dubos 1952), crowded living conditions, or emotional stress (Stansfeld 2002) - and the mechanism of their effects on the immune system, are unclear. Nonetheless, environmental influences and differences in exposure appear to affect susceptibility to TB, and their potential as confounders or effect modifiers can confuse, obscure, or invalidate attempts to identify genetic determinants.

The importance of environmental factors in human TB was illustrated by the change in TB mortality rates in Belgium and the Netherlands during the First World War. Before the war, in 1913, the rates were 118 and 142/100,000 for Belgium and the Netherlands, respectively, but by 1918, the rates had increased to 245 and 204/100,000 (Rich 1951). Another example was in Warsaw, Poland, where, in the early 20th century it was thought that there was a genetic explanation for the lower rates of TB mortality in the city's Jews, compared to non-Jewish Poles. However, the relative rates were inverted during the prolonged Nazi assault on the Warsaw Jewish Ghetto during World War II (Dubos 1952).

While the spread of TB, and the rates in a population - the likelihood that an individual will contract TB - are strongly influenced by general socioeconomic status, and possibly also stress levels (Farinpour 2003), perhaps the most important determinant is the quality of the local TB control program. It may be difficult to separate these factors however, because deteriorating and traumatic social conditions are often accompanied by a collapse of the healthcare system. A recent example is the dramatic rise in TB rates in Russia after the fall of the Soviet Union (Figure 6-3).

By the late '90s the death rate from TB for men aged 20-24 years was twice what it was in 1965, and while deaths from many causes began to fall in Russia after 1994, those from TB continued to rise. Part of this may be explained by falling living standards, prison TB, and mass migrations, but the most important component was likely to have been the crumbling healthcare system (Shilova 2001). While the Soviet system had kept TB under control, after its fall, as described by Professor Margarita Shilova, Head of the Tuberculosis Epidemiology Department at Moscow's Phthisiopulmonology Research Institute, "*suddenly, the money stopped. There were no drugs, communication with local hospitals broke down ... the system broke down*". The result was a 7.5 % annual increase in new cases from 1991-99 (Shukshin 2006).

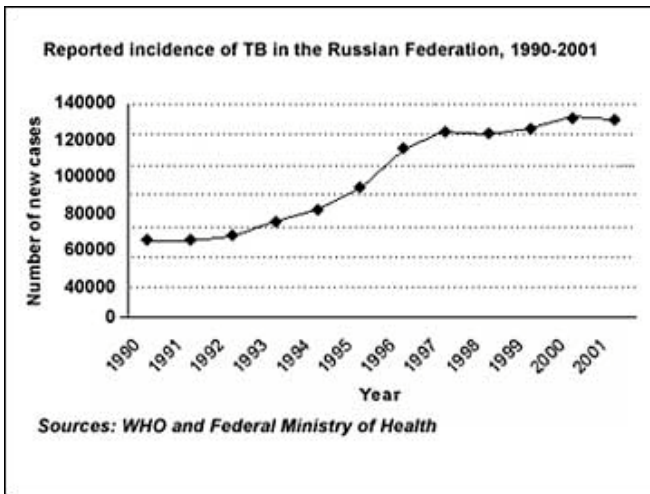


Figure 6-3 Increase in the reported incidence of TB in the Russian Federation, 1990-2001. Available at http://missinglink.ucsf.edu/lm/russia_guide/Russianhealth2.htm

Each of these examples illustrates how the TB rates in a population changed drastically due to changes in living conditions and TB control programs, while the genetic composition of the population remained constant. The point is that socioeconomic conditions and TB control programs can modify and perhaps override the effects of genetic composition in determining susceptibility to TB, except in cases of Mendelian inheritance of extreme susceptibility. Will identifying genetic determinants of susceptibility contribute to the control of TB? Given that susceptibility seems to be determined by a complex interplay of strain virulence, intensity of exposure and environmental factors, as well as human genetic composition, would it be feasible or advisable to target vaccines, prophylaxis, treatment, or control efforts based on the genetic composition of individuals, families or ethnic groups, instead of simply improving control programs (and socioeconomic status, although more difficult) for the entire population? In the resource-poor countries where TB is endemic, would it be feasible or ethical to screen the population to decide who to vaccinate, or who to treat with prophylaxis? Might it be more efficient and less costly simply to concentrate on diagnosing and effectively treating cases, and using extra funds for contact tracing? In populations with alleles that are highly associated with increased susceptibility, such as HLA-DQB1*0503 in Cambodians, the results of a cost-benefit analysis could depend upon the frequency of the relevant

alleles, the cost of detecting them, their importance in reactivation TB, and the target population to be tested - contacts, PPD positives, children, or the general population.

The identification of genes responsible for Mendelian inheritance of extreme susceptibility has helped identify the essential elements of human immune defense against mycobacteria, and the discovery of genetic determinants of susceptibility to common TB could further this knowledge and perhaps lead to the development of better vaccines, more precise evaluations of candidate vaccines, new diagnostic tests for active and latent disease, and therapeutic strategies for immunological intervention. In light of the continuing presence of multi-drug resistant strains (Raviglione 2006), and the difficulties in finding and bringing new drugs and vaccines into clinical use, further investigation in the field may be justified, despite the relatively disappointing results obtained so far.

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