Two loci control tuberculin skin test reactivity in an area hyperendemic for tuberculosis

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Approximately 20% of persons living in areas hyperendemic for tuberculosis (TB) display persistent lack of tuberculin skin test (TST) reactivity and appear to be naturally resistant to infection by *Mycobacterium tuberculosis*. Among those with a positive response, the intensity of TST reactivity varies greatly. The genetic basis of TST reactivity is not known. We report on a genome-wide linkage search for loci that have an impact on TST reactivity, which is defined either as zero versus nonzero (TST-BINa) or as extent of TST in millimeters (TST–quantitative trait locus [QTL]) in a panel of 128 families, including 350 siblings, from an area of South Africa hyperendemic for TB. We detected a major locus (*TST1*) on chromosomal region 11p14 ($P = 1.4 \times 10^{-5}$), which controls TST-BINa, with a lack of responsiveness indicating T cell–independent resistance to *M. tuberculosis*. We also detected a second major locus (*TST2*) on chromosomal region 5p15 ($P < 10^{-5}$), which controls TST-QTL or the intensity of T cell–mediated delayed type hypersensitivity (DTH) to tuberculin. Fine mapping of this region identified *SLC6A3*, encoding the dopamine transporter DAT1, as a promising gene for further studies. Our results pave the way for the understanding of the molecular mechanisms involved in resistance to *M. tuberculosis* infection in endemic areas (*TST1*) and for the identification of critical regulators of T cell–dependent DTH to tuberculin (*TST2*).

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Abbreviations used: BCG, Bacillus Calmette-Guérin; DTH, delayed type hypersensitivity; GWL, genome-wide linkage; IC, information content; LD, linkage disequilibrium; PPD, purified protein derivative; QTL, quantitative trait locus; SNP, single nucleotide polymorphism; TB, tuberculosis; TST, tuberculin skin test.

Only an estimated 10% of individuals infected with *Mycobacterium tuberculosis* develop clinical tuberculosis (TB), whether it is primary TB, which is typically an acute systemic disease of children, or reactivation TB, which is typically a chronic pulmonary disease of adults (Stewart et al., 2003). It has long been suspected that interindividual variability in progression from infection to clinical TB disease is under tight genetic control (Neel and Schull, 1954). Genetic epidemiological evidence in support of this view includes the large interpopulation variability in both incidence and severity of natural TB disease, an equally remarkable interindividual variability

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after the accidental inoculation of babies with *M. tuberculosis*, a higher incidence risk of the disease in first-degree relatives of TB cases as compared with first-degree relatives of unaffected individuals, and a higher concordance rate of TB among identical twins than among fraternal twins (Casanova and Abel, 2002). Studies of experimental infection in animal models, in the mouse in particular, have provided the first molecular evidence underlying genetic predisposition to TB (Schurr and Kramnik, 2008). Subsequent human molecular genetic studies have identified candidate genetic risk factors for pulmonary TB in adults (Schurr and Kramnik, 2008) and have documented Mendelian predisposition, IL-12Rβ1 deficiency in particular, to disseminated TB in some children (Alcaïs et al., 2005). Collectively, these studies have provided the long-awaited molecular proof of principle for the contribution of human genetic factors to TB susceptibility.

There is also interindividual variability at the earlier initial step of the infectious process, as ∼20% of long-exposed persons appear to be naturally resistant to infection by *M. tuberculosis* (Rieder, 1999). This estimate is based on the detection of *M. tuberculosis*–infected and –noninfected persons by means of the tuberculin skin test (TST) or Mantoux. The test measures induration of the skin after intradermal inoculation of *M. tuberculosis* purified protein derivative (PPD). The TST triggers a classical T cell–mediated delayed type hypersensitivity (DTH) reaction against mycobacterial antigens (Vukmanovic-Stejic et al., 2006). In hyperendemic areas for TB, a complete lack of TST reactivity is therefore suggestive of a T cell–independent resistance to infection by *M. tuberculosis* (Rose et al., 1995).

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

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

Interestingly, several genetic epidemiological studies in endemic areas have reported high levels of heritability for TST, which is considered either as a binary or quantitative trait, after *M. tuberculosis* exposure (Sepulveda et al., 1994; Jepson et al., 2001). For example, there was 92% heritability for quantitative TST in household children exposed to an adult TB case in Chile (Sepulveda et al., 1994). These observations suggested an important contribution of host genetic factors to resistance to *M. tuberculosis* infection and to the immune reactions underlying TST intensity.

However, surprisingly few studies have aimed to dissect the underlying genetic variants. A candidate gene study failed to identify significant association between quantitative TST and IL-1 receptor antagonist (Wilkinson et al., 1999). This gene does not appear to be critically involved in TST.

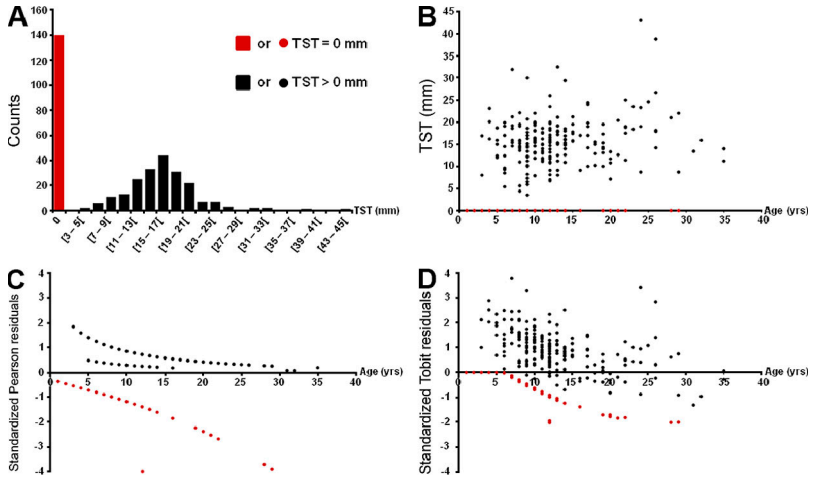

Figure 1. Distribution of TST according to age among the 350 children used for the linkage analysis before and after adjustment on relevant covariates. (A) Distribution of TST values among the 350 children used for the linkage analysis. A total of 140 subjects had no measurable reaction (red bar) and 210 subjects had TST induration > 0 mm (black bars). (B) Distribution of TST values among the 350 children according to age in years (same color coding as in A). Note that the red dots reflect variable numbers of subjects with TST = 0. Overall, 3, 2, 6, 2, 5, 22, 10, 20, 17, 11, 11, 13, 6, 2, 2, and 3 subjects had a TST = 0 at the ages of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 19, and 20 yr, respectively. (C) Distribution of the Pearson residuals obtained by logistic regression of TST-BINa on age, sex, and previous TB according to age in years. Color coding indicates those subjects with TST = 0 (red) or TST > 0 (black). As detailed in B, red dots, i.e., subjects with TST = 0, usually represent multiple persons. The two red dot outliers correspond to two subjects with previous TB and TST = 0. (D) Distribution of the residuals obtained by Tobit regression of TST-QTL on age, sex, and previous TB according to age in years. Color coding indicates those subjects with TST = 0 (red) or TST > 0 (black). As detailed in B, red dots, i.e., subjects with TST = 0, usually represent multiple persons. The two red dot outliers correspond to two subjects with previous TB and TST = 0.

In contrast, a recent study in Ghana showed that an *IL10* haplotype associated with low IL-10 production was significantly less frequent in PPD-negative controls than in the group of PPD-positive controls (Thye et al., 2009). A genome-wide linkage (GWL) study in an endemic area reported suggestive linkage of persistently low TST with chromosome regions 2q21-2q24 and 5p13-5q22 (Stein et al., 2008). The absence of significant linkage peaks in the latter study might be explained by the threshold-dependent categorization of the TST phenotype that was used to define infection status (as opposed to zero vs. nonzero) and by the heterogeneous Bacillus Calmette-Guérin (BCG) vaccination and/or HIV infection status of the subjects enrolled. Better controlled studies exploiting the full range of TST reactivity are thus needed because there is considerably more information for linkage analysis in quantitative variation than there is in any binary trait (Duggirala et al., 1997).

To address this question, over the last five years we have collected a unique population sample of 128 large nuclear families from a hyperendemic suburb of Cape Town, South Africa, with an estimated TST-based annual risk of TB infection as high as 4%, despite rates of HIV infection <2% in the pediatric population (Kritzinger et al., 2009). Out of 22 hyperendemic countries, South Africa has the highest TB annual incidence rate at 940/100,000, with children constituting up to 39% of this case load (World Health Organization, 2008). The Western Cape offers the advantage that detection of TB infection by TST in children is not significantly confounded by cross-reactivities to environmental mycobacteria and BCG vaccination, as the latter is done at birth, a situation where the impact of the BCG vaccination on the TST is known to vanish after 12–18 mo (Menzies, 2000). Indeed, in this setting there are virtually no TST results in the low reading range (1–5 mm; Kritzinger et al., 2009; Nicol et al., 2009; and unpublished data), making the TST a sensitive and specific test for *M. tuberculosis* infection. We took advantage of the unique Western Cape epidemiological setting and undertook the first GWL scan for both binary (zero vs. nonzero) and quantitative (in millimeters) TST reactivities to decipher the molecular basis of T cell–independent resistance to *M. tuberculosis* in endemic areas and the molecular basis of T cell–dependent TST intensity.

RESULTS AND DISCUSSION

The distribution of TST is bimodal

We studied 128 informative families (including 186 parents and 350 children) comprising two to six children with available TST induration size readings (Table I). TST reactivity of the 350 children is shown in Fig. 1 A. Reactivity extended from 0 to 43 mm with a median size of 11.15 mm. Mean age at the time of TST was 11.5 yr (SD, 4.9 yr) and the sex-ratio was 1. As expected as a result of the cumulative exposure to *M. tuberculosis* in this high incidence area, age had an important impact on TST positivity (Fig. 1 B). Closer inspection of the extent of TST reactivity showed a clear bimodal distribution with 140 individuals (40%) having a value of zero and 210 (60%) having values that approximately followed a normal distribution centered around 16 mm. Only two children had a TST between 1 and 5 mm. Given the age distribution of study subjects (i.e., mean age of 11.5 yr), such a high proportion of TST^+ individuals is consistent with an annual risk of TB infection that is >4% in the study area (Kritzinger et al., 2009). This TST distribution is strongly suggestive of a gene or group of genes having an impact on TST positivity by

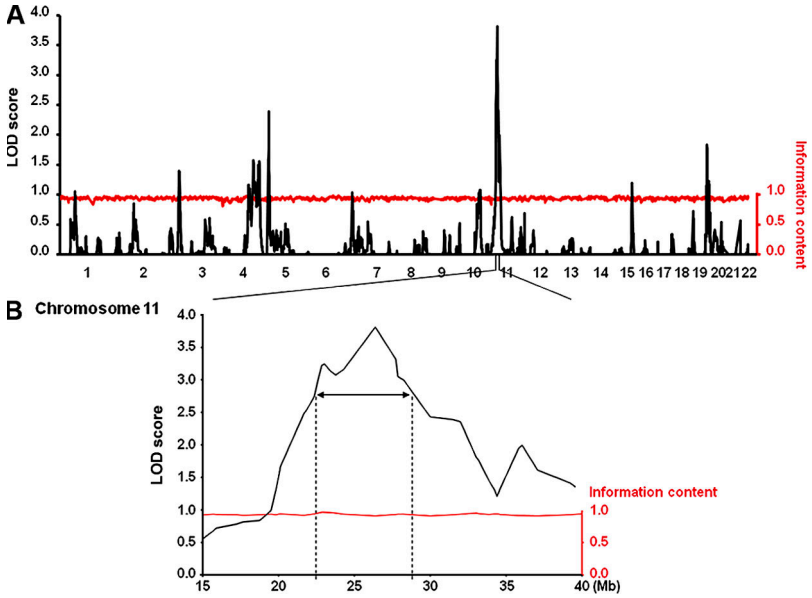

Figure 2. Genome-wide model-free linkage analysis of TST-BINa in a panel of 128 families including 350 siblings. (A) Multipoint LOD score (black line; left y-axis) and IC (red line; right y-axis) are plotted along the 22 autosomes. (B) Expanded view of the region with the highest LOD score on chromosome 11. The multipoint LOD score (black line), IC at marker positions (red line), and 90% confidence intervals for the location of the QTL (arrow and dashed lines) are given. Left and right y-axes indicate LOD score and IC, respectively. Chromosomal positions are given in megabases (Mb).

itself and a different gene or set of genes controlling the extent of TST reactivity. To test this hypothesis, we genotyped ~6,000 single nucleotide polymorphism (SNPs) in all 536 individuals of the 128 nuclear families and we performed two complementary linkage analyses. The first analysis focused on the phenotype of positivity by itself, i.e., TST = 0 versus TST > 0 (TST-BINa). The second analysis focused on the quantitative phenotype of extent of TST reactivity (TST quantitative trait locus [QTL]), with a particular emphasis on individuals with a TST > 0. Before linkage analysis, the two phenotypes were adjusted on age, gender, and previous clinical TB to remove as many environmental or nonspecific genetic sources of TST variation as possible. Subsequent linkage analyses were therefore performed on the resulting adjusted residuals shown in Fig. 1 C (TST-BINa) and Fig. 1 D (TST-QTL) that are, by definition, quantitative traits.

A major locus for TST positivity by itself maps to chromosome region 11p14

We first searched for genetic factors controlling TST-positive response by itself. Results of the TST-BINa linkage analysis are shown in Fig. 2. Information content (IC) was very high across all autosomes with mean genome-wide information of 94.4% (from 80.6 to 98.7%). This IC is excellent and corresponds to the level only accomplished at the fine-mapping stage in microsatellite-based genome scans. Linkage analysis of TST-BINa identified a significant linkage signal on chromosomal region 11p14 (LOD score = 3.81) at chromosomal position 26.37 Mb (IC = 91.15%, P = 1.4×10^{-5}; Fig. 2, bottom). This level of statistical support exceeds the stringent threshold of significance (LOD score = 3.6) for GWL scans (Lander and Kruglyak, 1995). The 1-LOD confidence interval for the location of the major locus (corresponding to the 90% confidence interval for the location of the QTL underlying the linkage peak) spanned from 22.35 to 28.82 Mb (Fig. 2, bottom). A suggestive linkage signal was also observed on chromosomal region 5p15 (LOD score = 2.39, P = 0.0005, IC = 90.5%) at the same position as the TST-QTL locus (see following paragraph). In addition, five weaker linkage peaks with P < 0.01 (i.e., LOD score > 1.17) were observed on chromosomal regions 3p24 (LOD score = 1.40, P = 0.0056, IC = 96.8%), 4q28 (LOD score = 1.57, P = 0.0036, IC = 94.7%), 15q26 (LOD score = 1.20, P = 0.0094, IC = 93.4%), 19q13 (LOD score = 1.83, P = 0.0018, IC = 92.4%), and 20p13 (LOD score = 1.23, P = 0.0087, IC = 93.9%; Fig. 2, top). As a substantial proportion of TST = 0 persons are most likely resistant to infection with *M. tuberculosis* (see Concluding remarks), these data therefore point toward the identification of one major locus (*TST1*) controlling human resistance to *M. tuberculosis*. A list of the known genes located in the 1-LOD confidence interval is given in Table S1.

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

Results of the TST-QTL linkage analysis are shown in Fig. 3. A highly significant linkage signal was observed on chromosomal region 5p15 with a multipoint LOD score of 4.00 (P = 9×10^{-6}) at position 2.70 Mb (*TST2*). Again, this is substantially above the commonly accepted threshold for significance in GWL studies (Lander and Kruglyak, 1995). The 1-LOD confidence interval for location of the QTL was small, spanning ~2 Mb from 1.07 Mb to 3.23 Mb (Fig. 3, bottom).

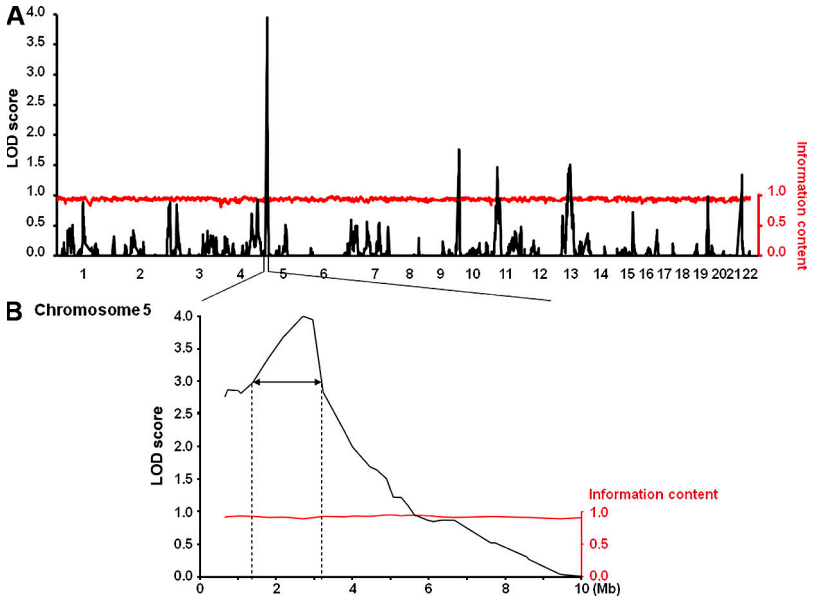

Figure 3. Genome-wide model-free linkage analysis of TST-QTL in a panel of 128 families including 350 siblings. (A) Multipoint LOD score (black line; left y-axis) and IC (red line; right y-axis) are plotted along the 22 autosomes. (B) Expanded view of the region with the highest LOD score on chromosome 5. The multipoint LOD score (black line), IC at marker positions (red line), and 90% confidence intervals for the location of the QTL (arrow and dashed lines) are given. Left and right y-axes indicate LOD score and IC, respectively. Chromosomal positions are given in megabases (Mb).

In addition to this major locus, four weaker linkage peaks with P < 0.01 were observed in chromosomal regions 11p14 (LOD score = 1.47, P = 0.0046, IC = 93.1%), at the same position as the TST-BINa locus 10p15 (LOD score = 1.76, P = 0.002, IC = 93.3%), 13q21 (LOD score = 1.51, P = 0.0042, IC = 96%), and 22q11 (LOD score = 1.34, P = 0.0065, IC = 97%). Therefore, these results support the hypothesis of a second major locus (*TST2*) on chromosomal region 5p15 controlling the intensity of TST reactivity as a quantitative trait, i.e., the intensity of T cell–mediated DTH to tuberculin. An interesting observation is the mirror effect between the two previous analyses with a suggestive linkage signal on chromosomal region 5p15 in the analysis of TST-BINa and on chromosomal region 11p14 in the analysis of TST-QTL. This is suggestive of some level of redundancy between the two phenotypes with the most straightforward explanation being individuals coded as 0 in both analytical approaches. To further explore this observation, we performed a linkage analysis of TST-QTL among individuals with TST > 0 mm only. Despite a dramatic reduction in family numbers (68 families comprising 164 children), we still found significant evidence of linkage at chromosomal region 5p15 (LOD score = 2.17, $P < 8 \times 10^{-4}$). In contrast, the linkage signal on 11p14 totally disappeared (LOD score = 0.14, P = 0.21). These results may reflect a sequential genetic control of TST reactivity, with *TST1* controlling TST positivity by itself and, presumably, T cell–independent innate resistance to *M. tuberculosis*, and *TST2* controlling the intensity of TST reactivity as a quantitative trait and, presumably, the intensity of T cell–mediated DTH to tuberculin.

Fine mapping of *TST2* identifies *SLC6A3* as a promising candidate gene

Encouraged by the narrow linkage peak, we decided to fine map the *TST2* locus. Based on the 1-LOD confidence interval for the location of the underlying QTL for *TST2*, we selected a chromosomal region extending from 1.1 to 3.2 Mb on the physical map of chromosome 5 (Fig. 4) for association studies of TST-QTL in a familial sample including the 128 nuclear families used in the linkage study and two additional trios (one child and her/his two parents). This interval contains 13 genes (*NKD2→C5orf38*; National Center for Biotechnology Information build 36 [http://www.ncbi.nlm.nih.gov]). We genotyped 133 SNPs in the targeted interval, 113 of which were considered suitable for association analysis (Fig. 4 and Table S2). To optimize gene coverage, we used a genocentric strategy for SNPs selection. Tag SNPs were identified for each known gene including 2 kb of their 5′ and 3′ regulatory regions. To capture most genetic variation, a very stringent tag-SNP selection scheme was used (r^2 = 0.8 with all

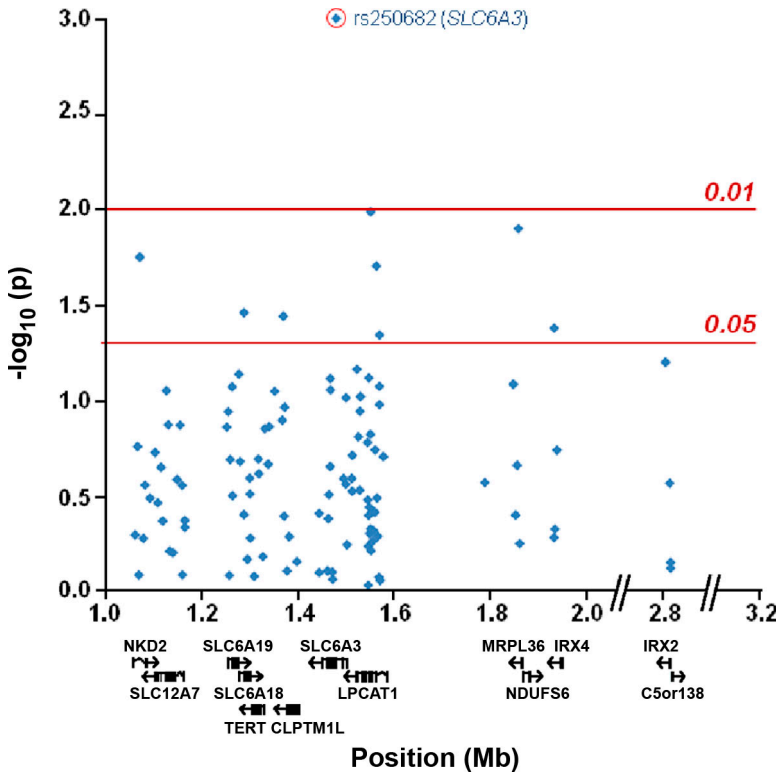

Figure 4. Fine mapping of the 90% confidence interval for the location of *TST2* locus in a panel of 128 families including 350 siblings. Evidence for association with TST-QTL of 113 SNPs located in the 90% confidence linkage interval is given as $-\log_{10}P$ and plotted against SNP position (blue diamonds). The locations of the 13 genes of this region are provided. Two intervals with no annotated genes (2–2.7 Mb and 2.9–3.2 Mb) are not shown (//). The red lines indicate the P = 0.05 and P = 0.01 significance thresholds.

tagged SNPs and minor allele frequency > 5%). Because no genes have been annotated on chromosomal intervals 2–2.7 Mb and 2.9–3.2 Mb, few SNPs were genotyped in these chromosomal segments and none were significant. In univariate analysis, nine SNPs (rs4975579, rs6554677, rs1801075, rs250682, rs10475030, rs11747565, rs2922061, rs1018120, and rs2232376) were significantly associated with TST-QTL at the 0.05 level (Fig. 4). Analysis of the linkage disequilibrium (LD) patterns between these nine SNPs showed no LD, supporting the hypothesis of independent effects even though it is likely that some of these signals are false positives (unpublished data). Out of these nine SNPs, rs250682 located in *SLC6A3* (solute carrier family 6 member 3) displayed the strongest association with TST-QTL (P = 0.001). Allele G of rs250682 (q = 0.36) has a dominant effect and is associated with lower values of the TST-QTL. To ensure that SNP rs250682 was responsible for the observed association, we screened the Yoruba sample of the HapMap database (www.hapmap.org) in a region spanning 5 Mb around rs250682. Only four SNPs (rs250681, rs40358, rs403636, and rs464049) were correlated with an $r^2 > 0.5$ with rs250682. All of these four SNPs are located in the *SLC6A3* gene. These results identified *SLCA63* as a promising target for further study on the intensity of T cell–mediated DTH to tuberculin.

Concluding remarks

We report in this paper that absence of TST reactivity (zero induration) has a major human genetic component and that the corresponding locus, *TST1*, maps to chromosome region 11p14. As the risk of developing TB for persons with *M. tuberculosis* exposure but TST = 0 was previously shown to be extremely small, such TST = 0 persons are most likely not infected with *M. tuberculosis* (Rose et al., 1995; ATS-CTC-IDSA, 2000). Persons who experience continued high exposure to *M. tuberculosis* and display persistent lack of T cell sensitization are much more likely to be naturally resistant to infection with *M. tuberculosis* than intrinsically deficient in mounting a DTH response. An estimate of infection-resistant persons can be obtained from countries where TB is highly endemic. In such conditions, where exposure to the tubercle bacillus is intense and sustained, ∼20% of individuals remain TST negative (Rieder, 1999). In our families, it is not possible to distinguish between individuals who have not been exposed to *M. tuberculosis* and those who have been exposed but are naturally resistant to infection. However, because the lack of exposure is difficult to reconcile with a genetic component, the most parsimonious explanation of our data is that the *TST1* locus reflects T cell–independent resistance to *M. tuberculosis* infection. Consequently, a substantial fraction of TST = 0 persons in the study families must have been exposed to *M. tuberculosis* but, as a result of very effective T cell–independent responses, those persons were resistant to *M. tuberculosis* before T cell sensitization could occur. Our findings make it likely that being spared from infection with *M. tuberculosis* is not simply a matter of lack of exposure but reflects genetically controlled profound differences of T cell–independent resistance among exposed persons. Although it is possible that this innate resistance can be overcome by extreme exposure pressure, the targeted strengthening of T cell–independent resistance loci in infection susceptible persons offers an attractive avenue of protection from TB disease. The identification of the *TST1* locus that belongs to a much larger region than *TST2* will follow the strategy successfully applied in leprosy (Mira et al., 2004; Alcaïs et al., 2007).

Our study also provides strong evidence that a major QTL (*TST2*) mapping to chromosome region 5p15 is involved in the control of TST extent in families living in a hyperendemic region for TB. Consistent with our results, the same region showed some evidence of linkage (P < 0.05) with persistently low TST reactivity in a familial sample from Uganda (Stein et al., 2008). *TST2* is the first non-MHC locus that has an impact on intensity of T cell–mediated DTH to tuberculin. This identifies *TST2* as important immune modulator and possible confounder in the numerous studies that have aimed at correlating extent of TST with risk for future clinical TB disease. A more direct link of *TST2* with risk of disease is perhaps provided by the repeated observation of the 5p15 region as location of a sarcoidosis susceptibility locus (Iannuzzi et al., 2007). Sarcoidosis is characterized by the immune paradox of extensive local inflammation (granuloma and cytokine secretion) associated with suppression of the immune response to tuberculin (Miyara et al., 2006). Multiple studies have investigated the association between mycobacterial antigens and sarcoidosis but no clear consensus has emerged (Iannuzzi et al., 2007). Our results suggest that human genetics could serendipitously connect the mechanisms governing sarcoidosis and T cell–dependent DTH to mycobacteria. The only other example of DTH for which response regulators have been reported in humans is the Montenegro skin test to leishmania antigen (Jeronimo et al., 2007a,b). However, none of these regulators mapped to the 5p15 (or the 11p14) chromosomal region, suggesting that *TST2* immune regulation involves an element of specific antigen recognition. Our first genocentric association scan of the *TST2* interval identified *SLC6A3* as a promising candidate for *TST2*. Experimental deletion of this gene in mice resulted in their significantly reduced ability to mount a DTH reaction against ovalbumin (Kavelaars et al., 2005). *SLC6A3* is related to *NRAMP1* (alias *SLC11A1*), which has previously been shown to have a major impact on the extent of the granulomatous response to *Mycobacterium leprae* antigen (Alcaïs et al., 2000). Nevertheless, additional studies will be required to fully understand the molecular basis of the *TST2* locus.

The gene regions previously reported to play a role in clinical TB (Schurr and Kramnik, 2008), including the only major TB susceptibility locus on chromosome 8p13 (El Baghdadi et al., 2006), do not overlap with the two main chromosomal regions identified in this study. For the *TST1* locus on chromosome 11p14, this is not surprising because genetic studies are generally done on TST-positive (i.e., infected) subjects, which precludes identification of infection resistance genes. For the *TST2* region on chromosome 5p15,

the lack of overlap with TB susceptibility genes is more surprising. However, because *TST2* has a strong impact on TST induration size, which is correlated with risk of subsequent TB disease, a further study of *TST2* on susceptibility to clinical TB may provide new insights into the mechanism of advancement from infection to TB disease. HLA class II genes are validated TB susceptibility loci, and MHC alleles are known from the mouse model to have a strong impact on DTH (Schurr and Kramnik, 2008). These observations made the chromosome 6 HLA region a prime candidate for loci impacting on TST induration size. Yet we failed to observe any evidence for a role of HLA genes on TST. That the strongest genetic effect on tuberculin reactivity is caused by a non-HLA locus directly leads to the question of whether this or other non-HLA loci will be modulators of vaccine-induced anti-mycobacterial protective immunity. Although the impact of the *TST1* and *TST2* loci on progression from infection to TB disease is presently unknown, the two loci which control T cell–independent resistance to infection with *M. tuberculosis* (*TST1*) and T cell–dependent intensity of tuberculin reactivity (*TST2*) represent an important expansion in our understanding of TB immunity.

MATERIALS AND METHODS

Subjects and families. Nuclear families (i.e., parents and offspring) with at least two children were enrolled from Ravensmead and Uitsig. Ravensmead/Uitsig (R/U) is a suburban area of Cape Town, South Africa, with a population of 38,656 living in a 3.5-km^2 area. HIV prevalence at the time of family enrolment was 5.2% in the overall population and <2% in the pediatric population. The population is stable and there are few homeless people or migratory workers. All individuals of the sample belong to the Cape Colored ethnic group. There was no requirement for subjects to be household contacts of TB cases. However, this area has a notification rate of TB (all cases) of 761/100,000 and of new bacteriologically confirmed cases of 313/100,000 (Verver et al., 2004), which is suggestive of a high level of exposure to *M. tuberculosis*. The enrolment strategy was to target large households to allow later reconstruction of nuclear families. In addition, if TST reactivity was known at time of enrollment, households that contained both strongly TST-positive and TST-negative subjects were prioritized for enrollment, as this sampling strategy has been shown to be the most powerful for linkage analysis.

Subjects who had had clinical TB disease in the 2 yr preceding the study were excluded. It is of note that the distribution of TST values among siblings of individuals who were excluded because they developed TB in the last two years did not differ from the overall distribution (i.e., 33 vs. 40% of null values and mean TST of 15.8 vs. 16 mm). Individuals who were HIV positive, pregnant, or using immunomodulatory chemotherapy were also excluded at the time of enrolment. BCG vaccination at birth is routine in the study area and was therefore not a confounding factor in our study because several studies have shown that the impact of BCG at birth on the TST vanishes rapidly (Menzies, 2000). Similarly, all individuals belong to the Cape Colored ethnic group, therefore limiting the risk of genetic heterogeneity. Informed consent was obtained from all study participants. Protocols involving human subjects were approved by the Stellenbosch University Health Research Ethics Committee (Tygerberg, South Africa), the University of Cape Town (Cape Town, South Africa), and the Research Ethics Board at the Research Institute of the McGill University Health Centre (Montreal, Canada).

Phenotype and covariates. TSTs were performed by specially trained health care providers using the Mantoux method with *M. tuberculosis* PPD RT23 (2 tuberculin units; Statens Serum Institut, Copenhagen, Denmark). TST reactivity was read between 48 and 72 h after the skin test was performed and the diameter of induration was measured in millimeters using a set of calipers calibrated to the nearest 0.5 mm. Two phenotypic definitions were used (Fig. 1). First, we dichotomized the TST distribution using 0 mm as the threshold to study the TST positivity (TST-BINa). Second, we analyzed the extent of the TST reactivity as a quantitative trait (TST-QTL) by Tobit regression.

Before linkage analysis, TST-BINa and TST-QTL phenotypes were all adjusted on previous clinical TB (at least 2 yr preceding the study), sex (male or female), and age (in years). Pearson and Tobit residuals were used for linkage analysis of TST-BINa and TST-QTL, respectively. TST-BINa was adjusted by means of logistic regression (Fig. 1 C), as implemented in the PROC LOGISTIC of the SAS software v9.1 (SAS institute, Cary, NC). TST-QTL was adjusted by means of the Tobit censored regression as implemented in the PROC QLIM of the SAS software, with the censoring threshold fixed at 0 (Fig. 1 D). Note that because the linkage analysis was performed on the residuals generated by the adjustment procedure, both phenotypes under study (TST-BINa and TST-QTL) were analyzed as quantitative traits. For each phenotype, the best fitting model for the age effect was determined among a set of multivariate fractional polynomials (FP) models as proposed in (Royston et al., 1999). First (FP1) and second (FP2) degree FP models were fitted with power p (i.e., age to the power p) for FP1 and p and q for FP2 chosen from −2, −1, −0.5, 0, 0.5, 1, 2, and 3, with 0 denoting log transformation. Among the 44 possible combinations, the model providing the smaller Akaïke Information Criteria was selected. A simple FP1 model was retained with power p = 0.5 for TST-BINa, whereas a FP2 model was retained for TST-QTL (power p = 1 and q = 2).

Genotyping. High-density genotyping for the linkage study was performed at the Centre National de Génotypage (Paris, France) with the linkage IVb panel (Illumina), containing >6,000 SNPs. 11 nonpolymorphic SNPs and 79 SNPs with a call rate <80% were excluded. None of the remaining SNPs showed departure from Hardy-Weinberg equilibrium among the founders at the 0.001 level. Pairwise LD analysis between adjacent SNPs was performed using Haploview software (Barrett et al., 2005) in the 186 parents of our sample. In our sample, pairwise LD between adjacent SNPs was very weak, with most of SNPs-pairs having an $r^2 < 0.1$ (mean pairwise $r^2 = 0.07$).

Genotyping for fine mapping of *TST2* on chromosome region 5p15 was performed at the McGill University and Genome Quebec Innovation Centre. 133 SNPs spanning the 2.2-Mb targeted interval on chromosome region 5p15 (i.e., the 1-LOD confidence interval for the location of the QTLs underlying the linkage peak) were selected on the basis of their location within known genes in the interval and on information regarding bin structure and allelic frequencies publicly available from the International HapMap project (http://www.hapmap.org/). These 133 SNPs were genotyped on the high-throughput MassARRAY platform (SEQUENOM, Inc.), which uses the iPLEX assay to incorporate mass-modified terminal nucleotides in the SBE step, which are then detected by MALDI-TOF MS (Griffin and Smith, 2000). 10 SNPs were excluded because they were nonpolymorphic and 1 SNP was excluded because of a call rate <80%. SNPs with a minor allele frequency <5% were also excluded. Finally, two SNPs that showed departure from Hardy-Weinberg equilibrium among the founders at the 0.01 level were excluded (Table S1).

Internal population structure analysis. As suggested by Thompson et al. (2006), we checked for population substructure to minimize genetic heterogeneity of the sample before linkage analysis. We performed a principal component analysis of the 5,567 autosomal SNPs in the 186 genotyped founders of our sample as implemented in the SMARTPCA software (Patterson et al., 2006). The principle of this method is to determine the major axes of genetic variation in the sample and to output each individual's coordinates along axes of variations, without formally clustering individuals into discrete population. No population substructure was found in our data but we identified five outliers distributed in four families that were excluded from the analysis.

Family-based analysis. We performed quantitative model-free multipoint linkage analysis on the Pearson residuals of the TST-BINa logistic

regression and on the residuals of the TST-QTL censored Tobit regression. As residuals were not normally distributed and pedigrees were extended, we used the maximum-likelihood binomial (MLB) method extended to quantitative trait linkage analysis (MLB-QTL v.3.0 available for download at www.hgid.net; Alcaïs and Abel, 1999) that has been implemented in an extension of the GENEHUNTER program.

The MLB approach considers the sibship as a whole and does not make any assumption about the distribution of the phenotype. The idea of the MLB method is to introduce an individual latent binary variable, which captures the linkage information between the observed quantitative trait and the marker. The probability that the latent variable for an individual takes the value 0 or 1 depends on the quantitative trait value of the individual and on a link function, which can be parametric (like the standard cumulative normal distribution) or empirical (derived from the observed phenotypic distribution).

The test of linkage is a maximum-likelihood ratio test that compares the likelihoods under the null hypothesis of no linkage (H_0) and the hypothesis of linkage. The test statistic is asymptotically distributed as a 50:50 mixture of χ^2 distribution with 0 and 1 degree of freedom and can be expressed as a classical LOD score. In this study, we used an empirical link function based on the deciles of the residuals distribution as suggested in Alcaïs and Abel (1999). Confidence intervals for the location of QTL underlying the linkage peaks were calculated by use of the support interval method in which a 1-LOD interval corresponds to a 90% confidence interval (Dupuis and Siegmund, 1999). LOD scores 3.6 ($P \leq 2 \times 10^{-5}$) and 2.2 ($P \leq 7 \times 10^{-4}$) were considered as significant and suggestive of linkage, respectively, as proposed in Lander and Kruglyak (1995).

It has been shown that linkage analysis using a dense SNP panel in familial samples with missing parents could inflate the type I error rate in regions where SNPs display high levels of LD (Huang et al., 2004). Therefore, although the LD was very weak at the genome-wide level and between markers underlying the two different linkage peaks (mean pairwise $r^2 = 0.07$), we removed the SNP with the lowest minor allele frequency for each pair of SNPs with an $r^2 > 0.1$. This had no impact on the LOD scores or the IC, ruling out the possibility of *TST1* and *TST2* being false positive linkage signals caused by LD patterns.

We performed family-based association testing on the 113 SNPs located in the 2.2-Mb targeted interval on chromosome region 5p15. The analysis was performed using the extension of the transmission disequilibrium test to quantitative phenotypes as implemented in FBAT software v2.0.3 (Laird et al., 2000).

Online supplemental material. Table S1 presents a list of the known genes that are located in the 1-LOD interval of *TST1*. Table S2 shows the SNPs that were genotyped for the fine mapping of *TST2*. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090892/DC1.

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