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Genes and Immunity (2012) **13**, 275 – 281

www.nature.com/gene

ORIGINAL ARTICLE

Epiregulin (EREG) variation is associated with susceptibility to tuberculosis

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Although host genetics influences susceptibility to *Mycobacterium tuberculosis*, the human genes regulating pathogenesis remain largely unknown. We used *M. tuberculosis*-stimulated macrophage gene expression profiling in conjunction with a case–control genetic association study to discover epiregulin (EREG), as a novel candidate tuberculosis (TB) susceptibility gene. Using a genome-wide association study dataset, we found that among the 21 genes with greater than 50-fold induction, EREG had the most polymorphisms associated with TB. We genotyped haplotype-tagging polymorphisms in discovery ($N = 337$ cases, $N = 380$ controls) and validation ($N = 332$ cases) datasets and an EREG polymorphism (rs7675690) was associated with susceptibility to TB (genotypic comparison; corrected $P = 0.00007$). rs7675690 was also associated more strongly with infections caused by the Beijing lineage of *M. tuberculosis* when compared with non-Beijing strains (controls vs Beijing, OR 7.81, $P = 8.7 \times 10^{-5}$; non-Beijing, OR 3.13, $P = 0.074$). Furthermore, EREG expression was induced in monocytes and peripheral blood mononuclear cells stimulated with *M. tuberculosis* as well as TLR4 and TLR2/1/6 ligands. In murine macrophages, EREG expression induced by *M. tuberculosis* was MYD88- and TLR2-dependent. Together, these data provide the first evidence for an important role for EREG as a susceptibility gene for human TB.

Genes and Immunity (2012) **13,** 275–281; doi:10.1038/gene.2011.83; published online 15 December 2011

Keywords: tuberculosis; TB; macrophage; *Mycobacterium tuberculosis*; SNP; genetic association

INTRODUCTION

Tuberculosis (TB), a common and deadly infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*), infects one third of the world's population. Pulmonary TB (PTB) accounts for ~80% of all forms of active TB[1] and the remaining 20% constitutes disseminated extra-pulmonary TB. TB meningitis (TBM) is the most severe form of TB with a mortality rate of 30% (65% in HIV co-infected) and significant morbidity in those who survive.[2,3] The incidence of TB in Vietnam in 2008 was 200/100 000 and TB mortality was 34/100 000.[4] It is unknown why some individuals are more susceptible to disseminated TB, whereas others can localize the infection and prevent spread beyond the lungs.

Studies suggest that host genetics influences susceptibility to TB, including data from twins, family-based genome-wide linkage scans and candidate gene approaches.[5–8] Furthermore, immunological and genetic studies suggest that innate immunity is important in the control of *Mtb*.[6,9] One hypothesis is that extra-pulmonary TB develops because of the failure of the innate immune response genes to control *Mtb* replication and dissemination.[10] Although some innate immune response genes are known to be involved in TB pathogenesis, genes involved in the pathogenesis of TBM are poorly described. We previously identified polymorphisms in TIRAP/Mal and TLR2 which are preferentially associated with TBM in comparison to PTB.[11,12] To identify additional genes involved in TB pathogenesis, we recently examined macrophage gene expression profiles of individuals with latent, pulmonary and meningeal TB.[13] From this study we identified epiregulin (EREG) as a candidate gene to investigate further and determine its functional role in TB pathogenesis.

EREG is a member of the epidermal growth factor (EGF) family and a ligand for the EGF receptor. EREG and EGF receptor regulate cell differentiation, growth and homeostasis and have been intensively examined for their role in cancer pathogenesis.[14,15] EREG is expressed in epithelial cells, including keratinocytes, where it regulates skin homeostasis as suggested by the development of chronic dermatitis in *Ereg−/−* mice. EREG is also expressed in macrophages where it modulates the immune response to TLR ligands such as peptidoglycan and lipopolysaccharide.[16,17] Recent data suggest that *Mtb* stimulates expression of EREG in the lungs during an *in vivo* mouse infection model of TB.[18] To our knowledge, the role of EREG in the human immune response to TB has not been examined and the role of genetic variation of EREG and disease susceptibility has not been reported. We hypothesized that (1) EREG polymorphisms are associated with susceptibility to different clinical phenotypes of TB and (2) EREG modulates the innate immune responses to TB.

RESULTS

Genetic variation and TB susceptibility of genes highly induced *ex vivo* by *Mtb* in human macrophages

We hypothesized that variation in genes that are highly induced by *Mtb* is associated with susceptibility to TB. We previously used

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Received 14 September 2011; revised 8 November 2011; accepted 14 November 2011; published online 15 December 2011

microarrays to examine gene expression in human macrophages stimulated with soluble extracts of *Mtb* and found 21 genes with greater than 50-fold induction[13] (Table 1), of which EREG was the 11th highest expressed gene with 96-fold induction. Using RTqPCR we confirmed that EREG is highly induced following *Mtb* stimulation of macrophages, especially in MDMs from TBM patients (Supplementary Figure 1).

We analyzed data from a genome-wide genetic association study (GWAS) to examine whether polymorphisms in these 21 genes were associated with susceptibility to TB.[19] Among 273 cases with TB and 188 population controls, eight genes had polymorphisms associated with TB using an allelic model for analysis (IL-1A, PTX3, EREG, CXCL1, F3, TNFAIP6, INHBA and SERPINB2) (Table 1). Among the eight genes, *EREG* had the greatest number (6) and percentage (50%) of polymorphisms associated with TB. The GWAS dataset included 12 single-nucleotide polymorphisms (SNPs) across a 60-kb region around the *EREG* gene on chromosome 4 of which six were associated with TB disease (combining TBM and PTB sub-phenotype groups) in both allelic and genotypic comparisons (Supplementary Table 1). In contrast, 8 genes had only 1 or 2 polymorphisms per gene associated with susceptibility to TB (Table 1). For 11 of the genes, no polymorphisms were available in the GWAS dataset to assess their association.

Discovery and validation of EREG SNPs associated with susceptibility to TB

To investigate the association in more detail, we genotyped seven haplotype-tagging SNPs in the *EREG* gene region [1 SNP, rs12641042 had also been genotyped by Affymetrix (Santa Clara, CA, USA) array in the GWAS] in our discovery sample set of 337 TB cases and 380 controls. A total of three SNPs were intronic and four were upstream within the promoter region (Table 2). In the discovery sample set 3/7 SNPs (rs12641042, rs1563826 and rs7675690) were associated with susceptibility to TB when comparing the frequencies of these SNPs in controls with the frequencies in TB cases. To validate these associations and avoid false discoveries from multiple comparisons, we genotyped these seven SNPs in a second independent validation sample of TB cases ($N = 332$). The allelic and genotypic frequencies were compared between the validation cases and the same controls ($N = 380$). The results confirmed in an independent case set that rs1563826 in intron 1 and rs7675690 in intron 4 were associated with susceptibility to TB (Table 2). Combined data from both discovery and validation was used to obtain a larger sample size and a more accurate estimation of allele and genotype frequencies. The associations of rs1563826 and rs7675690 with TB were significant in the combined sample set with $P = 0.005$ and $P = 0.00001$, respectively (Table 2), however, only rs7675690 remained significant after Bonferroni correction for multiple tests (0.00001×7 SNPs $= 0.00007$). SNP rs7675690 showed strong evidence for an increased risk of TB disease under the recessive model of inheritance ($P = 0.0009$, OR 4.95, 95% CI (1.74–14.07) (data not shown)).

Relationship between rs7675690 and the *Mtb* Beijing lineage

We have previously reported associations between lineages of *Mtb* and different TB clinical phenotypes.[20] We next examined whether the EREG SNP, rs7675690 was associated with infection by any particular bacterial lineage, defined by large sequence

Table 1. Polymorphisms associated with TB in genes induced in Mtb-stimulated macrophages

Gene ID	*Ratios Mtb/PBS stimulation*	*No. screened SNPs*	*No. associated SNPs*	*SNP ID*	*Allelic comparisons*[a]		
					P *(CvTB)*	P *(CvP)*	P *(CvM)*
IL1A	424	9	2	rs17598291	**0.034**	**0.035**	**0.189**
				rs11680809	**0.041**	**0.045**	**0.189**
CCL20	355	0					
LOC341720	337	0					
PTGS2	311	0					
IL1B	272	0					
ELOVL7	203	0					
TNF	128	0					
SGPP2	117	0					
PTX3	103	14	1	rs16827657	**0.028**	**0.035**	0.088
EREG	96	12	6	rs2125398	**0.009**	**0.007**	0.081
				rs1812149	**0.018**	**0.011**	0.155
				rs6846401	**0.026**	**0.014**	0.16
				rs12646889	**0.026**	**0.014**	0.16
				rs12646908	**0.008**	**0.005**	0.11
				rs12641042	**0.018**	**0.012**	0.143
CXCL1	93	18	2	rs6858086	0.06	**0.048**	0.24
				rs2886927	0.086	**0.042**	0.42
F3	89	14	2	rs3917605	$\mathbf{5.8 \times 10^{-5}}$	$\mathbf{9.4 \times 10^{-5}}$	$\mathbf{6.5 \times 10^{-5}}$
				rs698944	**0.049**	**0.015**	0.4
ANKRD15	83	0					
TNFAIP6	72	9	1	rs12619870	0.415	**0.008**	0.218
CCL4	68	0					
IL8	66	13	0				
INHBA	58	15	2	rs10951655	**0.021**	**0.025**	0.088
				rs6463037	0.075	**0.038**	0.375
SERPINB2	57	9	1	rs2758313	0.118	0.411	**0.049**
SOD2	55	0					
HEY1	51	0					

Abbreviations: CvM, controls vs TB meningitis; CvP, controls vs pulmonary; CvTB, control vs tuberculosis; *Mtb, Mycobacterium tuberculosis*; SNP, single-nucleotide polymorphism; TB, tuberculosis. [a]*P*-value for allelic associations between controls and clinical TB phenotypes; controls vs combined pulmonary TB and TB meningitis (CvTB), controls vs pulmonary TB (CvP) and controls vs TB meningitis (CvM). Values in bold represent *P* values < 0.05.

Table 2. EREG polymorphisms are associated with susceptibility to TB

SNP	Position	Discovery			Validation[a]			Combined		
		Allelic		Genotypic	Allelic		Genotypic	Allelic		Genotypic
		OR[b] (95%CI)	P-value	P-value	OR (95%CI)	P-value	P-value	OR (95%CI)	P-value	P-value
rs12641042	4:75432202; 5′ UTR	0.80 (0.64–0.99)	**0.047**[c]	0.198	1.07 (0.87–1.33)	0.491	0.745	1.16 (0.97–1.39)	0.107	0.3
rs6446991	4:75447118; 5′ UTR	0.84 (0.65–1.08)	0.165	0.215	0.99 (0.77–1.26)	0.946	0.441	0.91 (0.73–1.12)	0.367	0.281
rs6446992	4:75447236; 5′ UTR	0.90 (0.70–1.15)	0.391	0.547	0.97 (0.75–1.24)	0.822	0.693	0.93 (0.75–1.14)	0.471	0.61
rs3806794	4:75449166; 5′ UTR	1.26 (0.93–1.70)	0.139	0.237	0.95 (0.69–1.30)	0.761	0.919	1.14 (0.88–1.49)	0.326	0.561
rs1460006	4:75450108; intron1	0.91 (0.71–1.17)	0.467	0.362	1.09 (0.86–1.39)	0.46	0.389	1.00 (0.81–1.23)	0.996	0.406
rs1563826	4:75454388; intron1	0.82 (0.66–1.03)	0.087	**0.022**	0.96 (0.77–1.20)	0.759	**0.028**	0.89 (0.74–1.08)	0.247	**0.005**
rs7675690	4:75467515; intron4	1.02 (0.70–1.48)	0.101	**0.0001**	1.08 (0.70–1.54)	0.725	**0.0002**	1.03 (0.75–1.41)	0.857	**0.00001**

Abbreviations: CI, confidence interval; EREG, epiregulin; OR, odds ratio, SNP, single-nucleotide polymorphism; TB, tuberculosis. [a]Validation analysis includes comparison of new case group with original control group. [b]For odds ratio calculation each group was compared with the control group. [c]Numbers in bold represent *P* values <0.05.

polymorphism typing and whether the relationship influenced disease phenotype. rs7675690 displayed a strong association with TB disease in general in a genotypic comparison (All TB; controls vs all isolates, $P = 3 \times 10^{-5}$) and remained associated with TB regardless of whether the patients were infected with non-Beijing isolates (All TB; controls vs non-Beijing isolates $P = 0.0003$) or Beijing isolates (All TB; controls vs Beijing isolates $P = 0.0002$) (Table 3). rs7675690 was associated more strongly to TBM (TBM; controls vs all isolates $P = 1.4 \times 10^{-5}$) than to PTB (PTB; controls vs all isolates $P = 0.015$). The association was the most strong in TBM patients that had been infected with *Mtb* of the Beijing lineage (TBM; controls vs Beijing $P = 4.0 \times 10^{-5}$) compared with the non-Beijing lineage (TBM; controls vs non-Beijing $P = 0.001$) (Table 3). When using a recessive model, rs7675690 GG homozygotes had a higher risk of TBM when caused by Beijing compared with non-Beijing *Mtb* strains (TBM; controls vs Beijing, OR 10.83 (95% CI, 2.95–29.71), $P = 1.1 \times 10^{-5}$). These results indicate that rs7675690 GG homozygotes infected with Beijing *Mtb* strains, have a higher risk of developing meningitis than PTB.

EREG cellular function

To investigate the role of EREG in *Mtb* pathogenesis, we examined regulation of its expression. We found that EREG mRNA basal expression was elevated in HeLa (cervical) and A549 (lung) human epithelial cell lines (Figure 1a). In contrast, basal expression was low in THP1 and U937 monocyte cell lines as well as peripheral blood mononuclear cells (Figure 1b). However, EREG was induced when these cells were stimulated with LPS (TLR4 ligand), whole cell lysate of *Mtb*, the di-acylated lipopeptide PAM2 (TLR2/6 ligand) and the tri-acylated lipopeptide PAM3 (TLR2/1 ligand) (Figure 1b). The level of induction by LPS was lower than other stimuli. We examined EREG expression in murine peritoneal macrophages (PM) and bone marrow macrophages from WT C57B1/6J (Figure 1c). EREG was strongly induced when macrophages were stimulated with *Mtb* and TLR ligands. Relative comparisons of EREG expression show that the EREG expression level in PM was >5 fold higher than that in bone marrow macrophages (Figure 1c). Therefore, in experiments using macrophages from knockout mice below, we only examined EREG expression in PM.

We next investigated whether the TLR pathway regulates expression of EREG.[21,22] EREG expression was impaired in PMs isolated from *Tlr2−/−*, *Tlr4−/−*, or *Myd88−/−* mice, when cells were stimulated with their respective ligands [LPS for TLR4, PAM2 and PAM3 for TLR2, all ligands for Myd88 (Figure 1d)]. EREG expression was impaired in LPS stimulated PMs from *Tlr4−/−* mice ($P < 0.0001$ for comparison of WT and *Tlr4−/−*), in PAM2/PAM3 stimulated PMs from *Tlr2−/−* mice ($P < 0.00001$ for comparison of WT and *Tlr2−/−*), and in PMs from *Myd88−/−* mice stimulated with all three ligands ($P < 0.001$ for comparison of WT and *Myd88−/−* when cells stimulated with LPS, and $P < 0.00001$ when cells stimulated with PAM2/PAM3). *Mtb* induction of EREG expression was Myd88 and TLR2-dependent ($P < 0.001$ for comparison of WT and *Myd88−/− or Tlr2−/−* (Figure 1d)). Together, these results suggest that EREG expression is stimulated by *Mtb* and regulated by the TLR pathway.

DISCUSSION

Using a genome-wide expression study with *Mtb*-stimulated macrophages, we identified EREG as a highly induced TLR-dependent gene with a human polymorphism associated with susceptibility to TB. In addition, the risk of TB was further increased in individuals who had EREG polymorphism rs7675690 and were infected with the Beijing strain of *Mtb*.

As a member of the epidermal growth factor (EGF) family, EREG is better known for its role in cell growth and homeostasis.

Table 3. Association of EREG Polymorphism rs7675690 with *Mtb* Bacterial Strain Type

Group	*AA/AG/GG*	*Allelic OR[a] (95%CI)*	P-*value*	*Genotypic* P-*value*	*Recessive Model (AA+AG vs GG)*	
					OR (95%CI)	P-*value*
Controls	306/55/4					
All TB						
All isolates	231/16/14	0.97 (0.65 - 1.46)	0.9	**3.0×10^{-5}**	5.11 (1.66 - 15.72)	**0.002**
Non-Beijing	138/5/5	0.56 (0.32 - 1.01)	**0.051**	**0.0003**	3.13 (0.84 - 11.92)	0.074
Beijing	93/11/9	1.56 (0.98 - 2.49)	0.061	**0.0002**	7.81 (2.36 - 25.86)	**8.7×10^{-5}**
PTB						
All isolates	126/9/4	0.69 (0.40 - 1.20)	0.187	**0.015**	2.67 (0.66 - 10.84)	0.153
Non-Beijing	78/3/1	0.33 (0.13 - 0.84)	**0.015**	**0.021**	1.11 (0.12 - 10.10)	0.923
Beijing	48/6/3	1.25 (0.65 - 2.39)	0.508	**0.053**	5.01 (1.09 - 23.02)	**0.022**
TBM						
All isolates	105/7/10	1.32 (0.82 - 2.12)	0.255	**1.4×10^{-5}**	8.06 (2.48 - 26.19)	**4.8×10^{-5}**
Non-beijing	60/2/4	0.87 (0.43 - 1.74)	0.689	**0.001**	5.82 (1.42 - 23.89)	**0.006**
Beijing	45/5/6	1.89 (1.06 - 3.37)	**0.028**	**4.0×10^{-5}**	10.83 (2.95 - 39.71)	**1.1×10^{-5}**

Abbreviations: CI, confidence interval; EREG, epiregulin; OR, odds ratio, *Mtb, Mycobacterium tuberculosis.* [a]For OR calculation the genotype distribution in each group was compared with the control group.

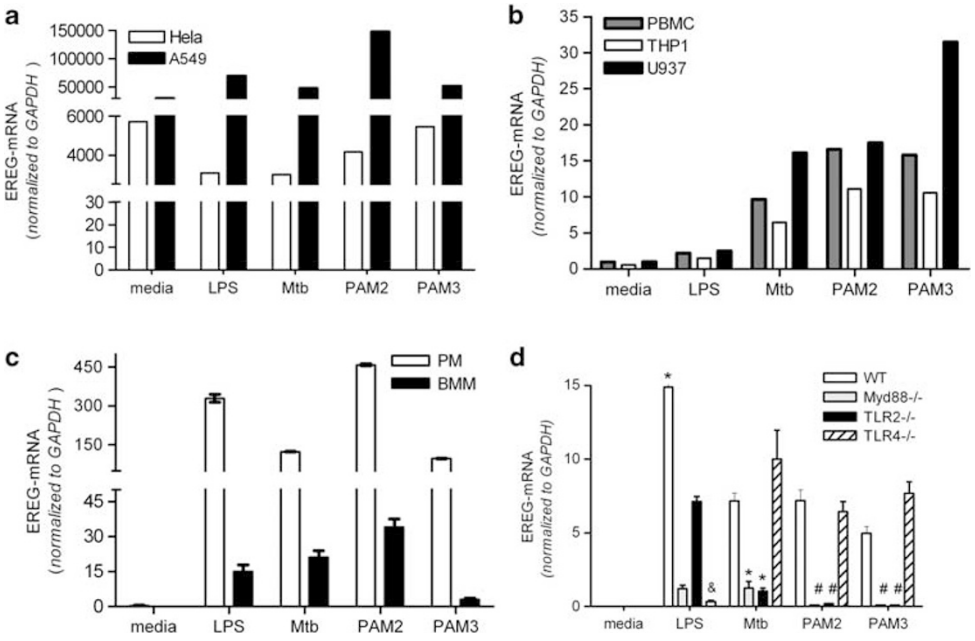

Figure 1. Regulation of EREG in mice and human cells. EREG mRNA expression in human cells stimulated with *Mtb* and TLR ligands; (**a**) in human epithelial cell lines Hela (open columns) and A549 (solid columns); (**b**) in human peripheral blood mononuclear cell (PBMC) (gray columns), THP1 (open columns) and U937 (solid columns). (**c**) EREG mRNA expression in PM (open columns) and bone marrow macrophages (BMM; solid columns) from wild-type C57Bl/six mice, which were stimulated with *Mtb* and TLR ligands. Cells were stimulated with LPS (100 ng ml^{-1}), whole cell lysates of H37Rv *Mtb* (50 μg ml^{-1}) and lipopeptides PAM2 or PAM3 (250 ng ml^{-1}). After stimulation for 4 h, mRNA was extracted and measured by real-time PCR. (**d**) EREG expression in PM from wild-type, *Myd88−/−, TLR2−/− and TLR4−/−* knockout mice. Peritoneal macrophages were stimulated with LPS (100 ng ml^{-1}), *Mtb* (100 μg ml^{-1}), PAM2 (250 ng ml^{-1}) or PAM3 (250 ng ml^{-1}) for 4 h. EREG expression (performed in triplicate, mean ± s.d.) was determined by real-time PCR. Student's *t*-test for comparisons of EREG expression between WT and KO mice stimulated with either media (non-stimulated), LPS, *Mtb*, PAM2 or PAM3 have $P < 0.001$ (*), < 0.0001 ($^{\&}$) and < 0.00001 ($^{\#}$).

However, our study supports a broader biological role for EREG with evidence that it is involved in the macrophage response to *Mtb* infection. Unlike other EGF family members, EREG expression is not ubiquitous in normal human tissue at the mRNA level.[23] Interestingly, we found that EREG induction was selective for myeloid cells after TLR or *Mtb* stimulation. Previous studies have also found that EREG is induced in murine tissue and PM by the TLR agonists LPS and peptidoglycan. The molecular mechanism of how EREG modulates macrophage function is only partially understood. Shirawasa *et al.*[16] found that IL6 and TNF-α were reduced in PM from EREG knockout mice in response to LPS and PGN, compared with cytokine levels in wild-type mice. Along with our data, this suggests that EREG expression is dependent upon TLR activation and may also modulate TLR-mediated signaling in

macrophages. EREG has both a membrane-bound and a soluble form, which can act as an autocrine growth factor for keratinocytes *in vitro*.[24] The membrane-anchored form, but not the soluble form, regulates cytokine production in macrophages.[16,25] on the basis of the literature and our data,[16,25,26] we propose a multi-step model of EREG-dependent function in *Mtb*-infected macrophages. First, *Mtb* stimulates EREG expression and other inflammatory molecules in a TLR2-dependent manner. EREG subsequently acts in a membrane or soluble form to stimulate cells through EGF receptor and further amplify cytokine production in an autocrine or paracrine manner. Interestingly, EREG modulation of cytokine production may be dependent on the specific TLR which is stimulated (for example, regulation of EREG when TLR2 is stimulated with peptidoglycan, but not when TLR9 is stimulated with CpG DNA).[16,17] In addition, previous studies suggest that soluble EREG may modulate inflammatory pathways in a selective manner in some cell types by downregulating IL-18 expression.[16] How EREG modulates inflammatory pathways in *Mtb*-stimulated macrophages is incompletely understood and will require further study.

The mechanistic role of EREG in TB pathogenesis is not yet known. EREG expression was not only induced in *Mtb* stimulated macrophages, but displayed higher expression in macrophages from patients with TBM compared with PTB patients and LTB subjects. A recent study demonstrated that EREG expression is induced in murine interstitial lung macrophages quite some time after low dose aerosol infection of *Mtb* [(after 360 days, but not after 56 or 180 days)[18]]. Other studies have shown that a group of genes including *EREG*, *MMP1*, *MMP2* and *COX2* are expressed in human breast cancer cells. These genes are required to breach the lung vasculature and enable extravasation of cancer cells during their dissemination from mammary tumors to the lungs.[27,28] In *Mtb*-infected people, after bacilli invade the pulmonary alveolar macrophage within the lung, they replicate and disseminate to the regional lymph nodes. In the case of extra-pulmonary disease, bacteria spread haematogenously to other organs in the body. TBM subjects with elevated EREG expression may have a higher risk of dissemination due to an effect on one of these steps in pathogenesis.

The polymorphism associated with TB is located within an intronic region of the *EREG* gene and is likely a genetic marker in linkage disequilibrium with the functional and disease causative allele. The association of this polymorphism with TB was identified in our discovery cohort and was 'validated' in a second cohort with independent cases. This technically does not constitute a 'complete validation' cohort as the same controls were used for both cohorts. It is possible that random variation in this one control group could be responsible for the observed associations. However this is unlikely, and the combination of phenotype-genotype association and expression-genotype association provides a useful validation, especially as it suggests actual biological consequences with regard to altering gene expression. Although we cannot exclude that the causative variant is within a neighboring gene (EPGN encoding Epigen and AREG encoding Amphiregulin, both EGF family ligands for ERBB family receptors), it is unlikely because of low linkage disequilibrium between the associated EREG SNPs and the haplotype block defined by SNPs in EPGN and AREG ($r^2 < 0.50$). To our knowledge this is the first association between EREG SNPs and any disease.

There is a evidence to support the hypothesis that some strains of *Mtb* are more virulent than others,[29] however, it remains unclear whether bacterial lineage influences the development of human TB disease. We previously found that Euro-American lineage strains of *Mtb* were significantly less likely to cause disseminated TB with meningitis than the East/Asian Beijing and Indo-Oceanic lineage strains.[20] In addition the combination of a particular host genotype (the C allele of TLR-2 T597C) with TB caused by the East-Asian/Beijing lineage increased the risk of TBM. Here, the association of the EREG SNP rs7675690 was the strongest in TBM patients that had been infected with *Mtb* of the Beijing lineage. This suggests that the causative variant of EREG may be associated with an impaired immune response to *Mtb* leading to more aggressive disease, prolonged bacteraemia and an increased chance of seeding to the meninges. This demonstrates a further significant interaction between host and bacterial genotypes and the development of TB.

Together, these data provide the first evidence to suggest an important role for EREG in the human immune response to TB. By combining macrophage transcriptional profiling with human genetic studies, we identified a potential human susceptibility gene that was not previously thought to be involved with TB pathogenesis.

MATERIALS AND METHODS

Human subjects

For the microarray study, 12 subjects were enrolled; TBM ($N = 4$), PTB ($N = 4$) and LTB ($N = 4$). An extended sample set of TBM ($N = 10$), PTB ($N = 12$) and LTB ($N = 12$) was used in validation experiments. The experiment is described elsewhere.[13]

For the case-control genetic association studies 294 TBM and 375 PTB patients and 380 population controls were used. Briefly, TBM subjects were recruited as part of clinical studies at the Hospital for Tropical Diseases, in Ho Chi Minh City (HCMC), Vietnam.[3] All subjects were >14 years of age and HIV-negative. 'Definite' TBM patients ($N = 162$) had clinical evidence of meningitis (nuchal rigidity and abnormal cerebrospinal fluid parameters), a positive Ziehl-Neelsen stain for acid-fast bacilli and/or *Mtb* cultured from the cerebrospinal fluid. 'Probable' TBM patients ($N = 132$) had one or more of the following; suspected active PTB on chest radiography, acid-fast bacilli found in any specimen other than the cerebrospinal fluid, and clinical evidence of other extra-pulmonary TB. PTB patients ($N = 375$) had no previous history of treatment for PTB, no evidence of miliary or extra-pulmonary TB, chest x-ray consistent with active disease (but not miliary disease), and *Mtb* cultured from the sputum. The control individuals were enrolled at Hung Vuong Hospital in HCMC where blood was collected from the umbilical cord of babies after birth ($N = 380$ population controls).

All samples came from unrelated individuals who were ethnic Vietnamese Kinh. Written informed consent was obtained from each volunteer. Protocols were approved by human subjects review committees at the Hospital for Tropical Diseases and Pham Ngoc Thach Hospital for TB and Lung Disease, HCMC, Vietnam. Ethical approval was also granted by the Oxford Tropical Research Ethics Committee (UK), The University of Washington Human Subjects Committee (USA) and the Western Institutional Review Board (USA).

Case-control genetic association study and statistics

Genomic DNA was extracted using Qiagen blood midi kit (Qiagen, Lewes, UK) and measured by picogreen (Molecular Probes Invitrogen, Paisley, UK). Firstly, genotyping was performed as part of a GWAS of TB using the Affymetrix 250 K NspI Chip.[19] To analyze *Mtb*-stimulated genes identified by gene expression aray, polymorphism frequencies of SNPs within and near (30 kb flanking the gene) the 21 genes in Table 1 were analyzed from the GWAS dataset. If a simple Bonferroni correction for multiple comparisons was applied to the SNP associations in Table 1 (17 SNPs multiplied by *P* value), then the majority of the associations would not be statistically significant. Rather than reject these initial findings with an overly conservative Bonferroni correction which does not account for linkage disequilbirum among polymorphisms, we proceeded to genotype individual EREG SNPs in 'discovery' and 'validation' cohorts using the previously described MassARRAY technique (Sequenom, San Diego, CA, USA).[11] SNPs were genotyped in population controls ($N = 380$) and two cohorts of TB patients (TB 'discovery' cohort ($N = 337$, including 162 definite TBM and 175 PTB); TB 'validation' cohort ($N = 332$, including 132 probable TBM and 200 PTB)). Based on the Han Chinese Beijing data from

HapMap, 10 high frequency haplotype-tagging SNPs ($>10\%$, $r^2>0.9$) were chosen for genotyping across the gene region, which included 30 kb of the genomic region flanking the 3′ and 5′ boundaries of EREG. A total of 7/10 SNPs passed genotyping quality, were in Hardy–Weinberg Equilibrium ($P>0.01$) in the controls and were also genotyped in the validation TB case cohort ($N=361$) to confirm the association between the polymorphisms and TB (the 7 SNPs are shown in Table 2). The Sequenom genotyping quality for rs1563826 and rs7675690, the two EREG associated SNPs, was validated by a second technique, Taqman RT-PCR (Life Technologies, Carlsbad, CA, USA). Univariate analysis was performed for categorical variables using the χ^2-test.

Cellular studies

Human MDMs for expression microarray analysis. MDMs were prepared and stimulated with PBS or 5 μg ml^{-1} of an irradiated, soluble, whole-cell lysate of *Mtb* H37Rv (Colorado State University, CO, USA) for 4 h. RNA was extracted and hybridized to the Human Genome U133 Plus 2.0 Array (Affymetrix) as described elsewhere.[13]

Stimulation of human cells by Mtb. Human THP-1 and U137 cell lines and *ex-vivo* peripheral blood mononuclear cellss were plated in RPMI 1640, *HeLa* cells were cultured in DMEM and A549 cells were cultured in F12K, all containing 10% FCS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2 mM L-glutamine. 5×10^5 cells were plated in 24-well plates overnight and stimulated with either *Mtb*, LPS, PAM2 or PAM3 (*Mtb* is whole-cell lysate of *Mtb* H37Rv (Colorado State University); LPS from *Salmonella Minnesota* (List Biological Laboratories, Campbell, CA, USA); PAM2 and PAM3 bacterial lipopeptides (EMC Microcollections, Tuebingen, Germany). After stimulation for 4 h, RNA was extracted by Trizol and mRNA expression was determined by real-time PCR.

Mice peritoneal and bone marrow macrophages. Peritoneal and bone marrow macrophages were isolated from *TLR2−/−*, *Myd88−/−*, *TLR4−/−* and wild-type (WT) C57Bl/6J mice. BMDMs were used after 4–10 days of culture. To prepare for stimulation, the cells were counted, the concentration adjusted to 4×10^5 cells ml^{-1}, and 0.5 ml was added to each well in a 24-well plate and incubated over night at 37 °C with 5% CO_2. The cells were ready for stimulation the following day.

Microarray data processing and analyses. The microarray data is publicly available at http://www.tbdb.org. and the analysis is summarized in Thuong *et al*.[13]

Real-time quantitative PCR (RTqPCR). Taqman real time PCR was used to validate the expression level of genes selected from the expression microarray results as described in Thuong *et al*.[13] *EREG* gene expression was further examined by Taqman probes and primers (Applied Biosystems) designed for human (assay ID: Hs00914312-m1) and mice (assay ID: Mm00514794-m1) sequences. Samples were normalized to GAPDH and analyzed by using either Applied Biosystems SDS 2.1 Relative Quantification software or an Excel spreadsheet to perform relative quantification analysis.

Mtb genotyping. *Mtb* lineages were defined by large sequence polymorphism typing, as previously described.[19] Isolates were classified into one of the three principal lineages present in Vietnam: East-Asian (Beijing), Indo-Oceanic or Euro-American.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We would like to acknowledge the work of the clinical staff from the Hospital of Tropical Diseases and Pham Ngoc Thach Hospital who initially diagnosed and studied the patients with TBM and PTB. We would like to thank Dr Nguyen Thi Hieu from Hung Vuong Obstetric Hospital and all Vietnamese individuals who were recruited into this study. We also thank Drs Alan Aderem and Marta Janer from the Institute for Systems Biology for advice and Sarah Li for technical assistance. *Mtb* materials were received as part of NIH, NIAID Contract No. HHSN266200400091C, entitled 'uberculosis Vaccine Testing and Research Materials,'which was awarded to Colorado State University. This work was supported by The Dana Foundation (to TRH, SJD, and JJF), the Burroughs Wellcome Fund [to TRH], the Agency for Science Technology and Research, Singapore, (to MH and MS) and the Wellcome Trust of Great Britain (to MC and JJF).

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Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)