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A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis

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Toll-like receptors (TLRs) and members of their signaling pathway are important in the initiation of the innate immune response to a wide variety of pathogens[1–3]. The adaptor protein Mal (also known as TIRAP), encoded by *TIRAP* (MIM 606252), mediates downstream signaling of TLR2 and TLR4 (refs. 4–6). We report a case-control study of 6,106 individuals from the UK, Vietnam and several African countries with invasive pneumococcal disease, bacteremia, malaria and tuberculosis. We genotyped 33 SNPs, including rs8177374, which encodes a leucine substitution at Ser180 of Mal. We found that heterozygous carriage of this variant associated independently with all four infectious diseases in the different study populations. Combining the study groups, we found substantial support for a protective effect of S180L heterozygosity against these infectious diseases ($N = 6{,}106$; overall $P = 9.6 \times 10^{-8}$). We found that the Mal S180L variant attenuated TLR2 signal transduction.

TLRs recognize a diverse array of pathogens and initiate intracellular signaling via their Toll/interleukin-1 receptor (TIR) domains, leading to differential effects on gene expression and the generation of an inflammatory host response. TLR2 has been shown to recognize, among other agonists, lipoteichoic acid, lipoarabinomannan and mycobacterial lipopeptides[1], whereas TLR4 recognizes lipopolysaccharide (LPS)[7–9]. Both TLR2 and TLR4 sense *P. falciparum* glycosylphosphatidylinositol (GPI) (C. Aucan *et al.*, unpublished data)[10]. The adaptor protein Mal (TIR domain–containing adaptor protein), encoded by the gene *TIRAP*, is essential for MYD88-dependent signaling downstream of TLR2 (with TLR1 and TLR6 as coreceptors) and TLR4 (refs. 1,5). After stimulation of TLR2 or TLR4, Mal triggers a signaling cascade, which culminates in the activation of the transcription factor NF-κB and the subsequent activation of proinflammatory genes. Based on the central position of Mal in the TLR2 and TLR4 pathways and our knowledge of the microbial components that associate with these TLRs, we hypothesized that genetic variation at *TIRAP* might underlie susceptibility to common infectious diseases.

TIRAP spans 14,500 bp on chromosome 11q24.2 and encodes a protein of 221 amino acids. We analyzed 33 SNPs in *TIRAP* and the surrounding region in multiple populations and found that *TIRAP* S180L consistently associated with disease (**Supplementary Note**, **Supplementary Fig. 1**, **Supplementary Table 1** and **Supplementary Table 2** online). We genotyped *TIRAP* S180L in a total of 6,106 individuals with four different diseases: two UK populations of European ancestry with invasive pneumococcal disease (IPD); a Kenyan population with bacteremia; three populations with malaria (from the Gambia, Kenya and Vietnam) and two populations with tuberculosis (from Algeria and West African populations of the Gambia, Guinea-Bissau and the Republic of Guinea).

In the UK population, heterozygosity at *TIRAP* S180L was associated with protection from invasive pneumococcal disease

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Table 1 ***TIRAP*** **S180L genotype frequencies in individuals with infectious disease and controls**

Population	Status	Number of individuals in each genotype (%)				
		Ser/Ser	Ser/Leu	Leu/Leu	*P* value[a] (adjusted *P* value[b])	OR for heterozygote advantage model (95% c.i.)
UK	Control (1)	527 (71.1%)	199 (26.9%)	15 (2.0%)		
	IPD	146 (76.4%)	36 (18.8%)	9 (4.7%)	**0.013**[h] (**0.031**)	0.65 (0.44–0.97)[c,d]
	Control (2)	246 (68.1%)	107 (29.6%)	8 (2.2%)		
	Pneumococcal empyema	25 (69.4%)	8 (22.2%)	3 (8.3%)	0.080[i] (0.080)	0.74 (0.32–1.68)[e]
Kenyan	Control	398 (94.1%)	25 (5.9%)	0 (0%)		
	Overall bacteremia[m]	719 (97.6%)	18 (2.4%)	0 (0%)	**0.003**[j] (**0.003**)	0.34 (0.17–0.69)
	Pneumococcal bacteremia[g]	161 (98.2%)	3 (1.8%)	0 (0%)	**0.024**[l] (0.056)	0.30 (0.06–0.99)
Gambian	Control	172 (96.6%)	6 (3.4%)	0 (0%)		
	General malaria	652 (99.1%)	6 (0.9%)	0 (0%)	**0.025**[f] (**0.004**)	0.15 (0.043–0.55)
	Severe malaria	413 (99.3%)	3 (0.7%)	0 (0%)	**0.024**[f] (**0.003**)	0.04 (0.004–0.34)
Vietnamese	Control	81 (95.3%)	4 (4.7%)	0 (0%)		
	Severe malaria	357 (98.9%)	4 (1.1%)	0 (0%)	**0.046**[l] (**0.041**)	0.23 (0.056–0.94)
Kenyan	Control	398 (94.1%)	25 (5.9%)	0 (0%)		
	Severe malaria	599 (96.5%)	22 (3.5%)	0 (0%)	0.035[k,l] (0.035)	0.58 (0.31–1.09)
West African (the Gambia,	Control	593 (98.0%)	12 (2.0%)	0 (0%)		
Guinea-Bissau, Republic of Guinea)	Tuberculosis	671 (99.4%)	4 (0.6%)	0 (0%)	**0.041**[f] (**0.013**)	0.23 (0.07–0.73)

[a]2 × 2 or 3 × 2 χ^2 comparisons of genotypes, depending on the presence or absence of mutant homozygotes in the different populations. [b]Logistic regression for confounding factors in the different diseases (sickle cell trait, age, ethnicity and homestead for malaria; HIV status, malnutrition and age for bacteremia; HIV status, ethnicity, age and sex for tuberculosis; comorbid conditions and age for IPD). The Hosmer-Lemeshow goodness of fit tests was not significant ($P > 0.05$) for any of the logistic regression models, indicating that the phenotypes predicted by the logistic regression models fitted with the observed phenotypes. [c]Logistic regression using the wild-type Ser/Ser homozygotes as reference. [d]Odds ratio of the Leu/Leu homozygotes = 2.39 (95% c.i.: 0.95–5.92). [e]Odds ratio of the Leu/Leu homozygotes = 4.01 (95% c.i.: 0.65–17.66). [f]Two-tailed Fisher's exact test. [g]The Kenyan pneumococcal bacteremia cases are a subset of the Kenyan bacteremia case population, and the result here is analyzed against the same Kenyan controls. The data relating to pneumococcal cases were not included separately in the combined analysis; only the overall Kenyan bacteremia case-control data were included. [h]3 × 2 $\chi^2 = 8.72$. [i]3 × 2 $\chi^2 = 5.05$. [j]2 × 2 $\chi^2 = 9.05$. [k]2 × 2 $\chi^2 = 3.28$. [l]One-tailed *P* values were used for replication sets. [m]Protection afforded by the heterozygote variant was seen for individuals suffering from both Gram-positive bacteremia (2 × 2 $\chi^2 = 5.18$, $P = 0.023$, OR = 0.40, 95% c.i.: 0.16–0.95) and Gram-negative bacteremia (2 × 2 $\chi^2 = 6.82$, $P = 0.009$, OR = 0.38, 95% c.i.: 0.17–0.85). Correction for the effects of HIV status malnutrition, and age using binary logistic regression did not affect the observed associations.

(3 × 2 $\chi^2 = 8.72$, $P = 0.013$, **Table 1**). We also observed an excess of mutant homozygotes among individuals with IPD (**Table 1**) in this UK population. We then examined *TIRAP* S180L in a separate group of individuals from the UK with thoracic empyema and a second control group. Although we did not observe any association between genotype and susceptibility to thoracic empyema overall ($n = 584$, 3 × 2 $\chi^2 = 0.63$, $P = 0.73$), analysis of the small subgroup of individuals with pneumococcal empyema uncovered a nonsignificant trend toward association (3 × 2 $\chi^2 = 5.05$, $P = 0.080$; **Table 1**). Again, we observed an excess of mutant homozygotes among this second group of IPD cases (**Table 1**).

We then studied *TIRAP* S180L in a second population with invasive bacterial disease: Kenyan children with well-defined bacteremia. Although the mutant allele was less common in the Kenyan population than in UK individuals, we observed the same pattern of association. The *TIRAP* S180L heterozygotes were significantly more common among community controls (5.9%) compared with individuals with bacteremia (2.4%) (2 × 2 $\chi^2 = 9.05$, $P = 0.003$; **Table 1**). The protective effect of heterozygosity at the S180L locus was also significant within the subgroup of 164 Kenyan children with pneumococcal bacteremia ($F_{exact} = 0.024$, **Table 1**), thus replicating the findings in the UK studies.

In the Gambian malaria case-control study, *TIRAP* S180L heterozygosity showed a significant protective effect against both general malaria (Wald = 8.35, $P = 0.004$, **Table 1**) and severe malaria (Wald = 8.706, $P = 0.003$, **Table 1**). This result was replicated in a second malaria case-control study of Vietnamese individuals with severe malaria. Again, we found that *TIRAP* S180L heterozygotes were more prevalent among the controls (4.7%) than among the severe malaria cases (1.1%) ($F_{exact} = 0.046$, **Table 1**). An attempt to replicate this in a third malaria case-control study (from Kenya) demonstrated similar results (2 × 2 $\chi^2 = 3.28$, $P = 0.035$; **Table 1**).

Finally, we examined the possible effect of the *TIRAP* S180L polymorphism on susceptibility to tuberculosis in 1,280 individuals from the Gambia, Guinea-Bissau and the Republic of Guinea (collectively known as the EUTB West African study), again with local controls matched for similar ancestry. The *TIRAP* S180L heterozygous genotype was found to be protective against clinical tuberculosis (Wald = 6.23, $P = 0.013$). We then replicated this finding in two family-based studies on tuberculosis (the EUTB West African study and a study in Algeria). In both studies, we found that the variant *TIRAP* S180L was undertransmitted in individuals with clinical tuberculosis compared with individuals free from clinical tuberculosis (using the analysis program TRANSMIT (see Methods); $P = 0.075$ and $P = 0.038$, respectively; **Table 2**). When we combined all the study groups and stratified them for the different populations, there was a clear association between heterozygosity at the *TIRAP* S180L locus and protection against multiple infectious diseases ($N = 6{,}106$; $P \leq 9.6 \times 10^{-8}$). Consequently, we undertook studies on the functional effect of the S180L variant.

We first assayed the effect of the S180L polymorphism on TLR2 signaling using transfected mouse embryonic fibroblasts (MEFs) from *Tirap* knockout mice (**Fig. 1a**). Treatment of wild-type MEFs with the TLR2 ligand macrophage-activating lipopeptide 2 (Malp2) induced IκBα degradation at 30 and 50 min. This effect was absent in *Tirap*-deficient MEFs. However, transfection of *Tirap* knockout cells with the wild-type Ser180 fully reconstituted TLR2 signaling, with degradation of IκBα occurring at 30 and 50 min after treatment. Transfection with

Table 2 Transmission disequilibrium test (TDT) results of *TIRAP* S180L in West Africa and Algeria

	Algeria				West Africa			
	All sibs		One sib		All sibs		One sib	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
180-SS	159	150.92	74	70.346	259	258.23	259	258.23
180-SL	15	23.079	10	13.654	1	1.779	1	1.779
P^a	0.020		0.038		0.075		0.075	

Combined Algerian and West African family study: $P = 0.016$ (one-tailed P values). Combined data from all studies: $P = 9.6 \times 10^{-8}$. 180-SS: Ser180/Ser180 homozygotes; 180-SL: Ser180/Leu180 heterozygotes.
[a]One-tailed P values.

the mutant Leu180 did not result in IκBα degradation after TLR2 stimulation. Both wild-type Ser180 and the variant Leu180 were expressed at similar levels in the cells (data not shown).

We observed a similar lack of reconstitution when we measured IL-6 (**Fig. 1b**). Both LPS and Malp2 induced IL-6 in wild-type MEFs, causing a fourfold increase over untreated cells. However, the effect of both stimuli was abolished in *Tirap*-deficient cells (second bar in each set). Transfection of the *Tirap*-deficient cells with a plasmid encoding wild-type Mal Ser180 reconstituted the effect of both LPS and Malp2, leading to a threefold stimulation over untreated cells. However, the variant Mal Leu180 was unable to reconstitute the effect of either stimulus. Both wild-type Ser180 and variant Leu180 were expressed at similar levels in the cells. We also tested the functional consequences of coexpression of wild-type Ser180 and variant Leu180 in Mal-deficient cells. Expression of wild-type Ser180 Mal activated an NF-κB-linked reporter gene (**Fig. 1c**). However, the variant Leu180 form was inactive in cells lacking endogenous Mal, attesting further to the functional deficiency of this form of Mal. Notably, the variant form inhibited the ability of the wild-type form of Mal to activate NF-κB. This suggests that Mal signaling will be directly impaired in heterozygotes, as the variant might interfere with signaling by the wild-type form.

Wild-type Mal interacts with TLR2 (ref. 11). We generated models of the wild-type and mutant protein in order to assess the effect of S180L on Mal structure. According to our model (**Fig. 2**), Ser180 is located on the edge of a surface-exposed loop opposite the BB loop, the region that contains a critical proline residue that has been shown for other TIR-containing proteins to form a point of contact with downstream signaling molecules. The model predicts that the electrostatic surface potential does not differ significantly between the wild-type and mutant protein. However, the leucine residue appears to be more exposed (**Fig. 2**). Previous modeling and interaction studies

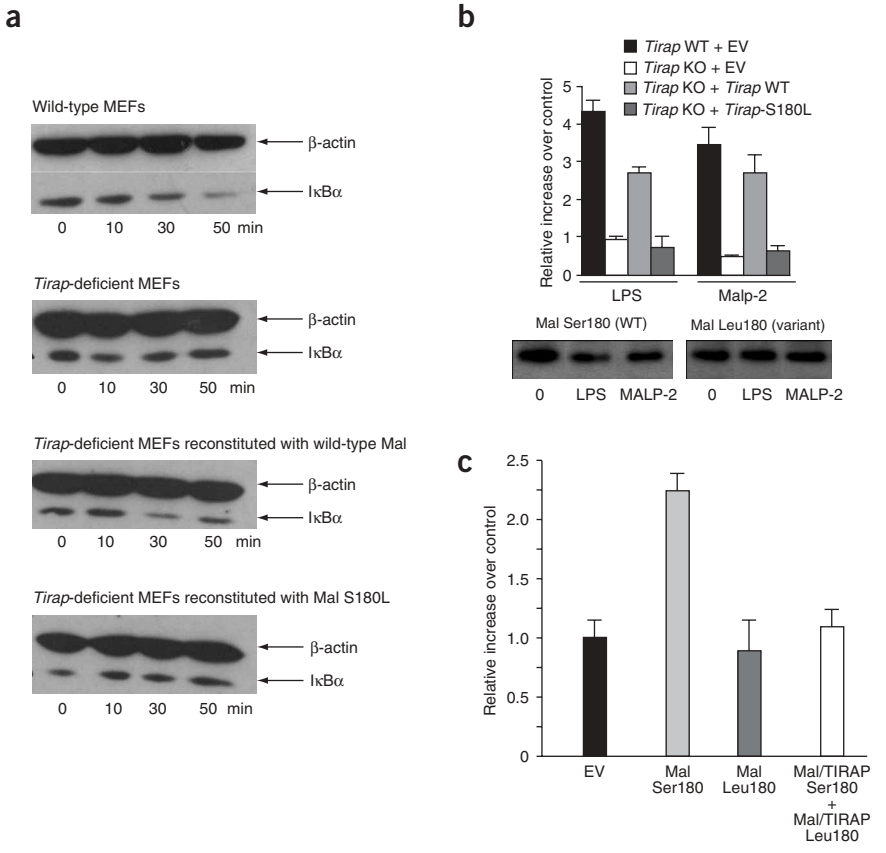

Figure 1 Functional analysis of Mal Ser180 and Mal Leu180. (**a**) We assayed TLR2 signaling by measuring degradation of IκBα induced by Malp2. Top: wild-type MEFs stimulated with 5 nM Malp2. Second row: Malp2-stimulated *Tirap* knockout cells. Third row: Malp2-stimulated *Tirap* knockout cells transfected with *Tirap* Ser180. Fourth row: Malp2-stimulated *Tirap* knockout cells transfected with *Tirap* Leu180. Samples were immunoblotted with an antibody to IκBα. We used β-actin as a loading control. Results shown are representative of three separate experiments. (**b**) Wild-type MEFs or *Tirap* knockout MEFs (transfected with empty vector or plasmids encoding Mal Ser180 or Mal Leu180) were treated with LPS (1 μg/ml) or Malp2 (5 nM). After 24 h, supernatants were removed and assayed for IL-6 by ELISA (mean ± s.e.m. of triplicate experiments; we obtained similar results in two additional experiments). Expression levels of Mal Ser180 and Mal Leu180 (untreated or treated with LPS or Malp2 for 24 h) are shown in the lower panel. (**c**) *Tirap* knockout MEFs were transfected with 100 ng empty vector or with plasmids encoding Mal Ser180 or Mal Leu180 (50 ng of each, supplemented to 100 ng with 50 ng of empty vector) or a combination of plasmids encoding Mal Ser180 and Mal Leu180 (50 ng of each) in combination with a plasmid containing an NF-κB-linked luciferase reporter gene (80 ng per well) and a *Renilla* luciferase reporter gene (40 ng) as an internal control. Cell lysates were prepared as previously described[4]. Results shown are mean + s.d. from triplicate experiments.

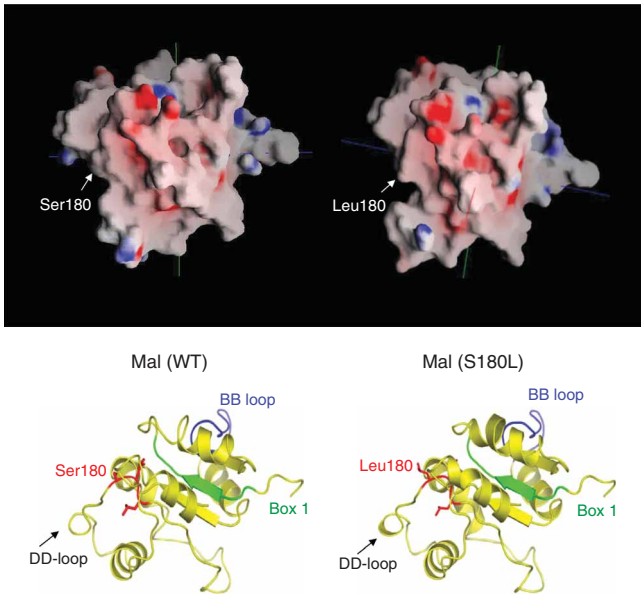

Figure 2 Molecular models of wild-type and mutant Mal. The computational utility Molecular Operating Environment (MOE2006.02) (see URLs section of Methods) was used to generate the models. Electrostatic surface potentials are highlighted in blue (positive) and red (negative) (top panel). Structural features (including box 1 of the TIR domain, the Mal BB loop and the position of Ser180) are highlighted in the lower panel.

have suggested that Mal may bind to TLR2 via a region termed the DD loop[11]. In our current model, Ser180 is located in close proximity to this DD loop (**Fig. 2**). Therefore, we performed an *in vitro* binding assay in order to assess the effect of the mutation on the Mal–TLR2 interaction. A glutathione *S*-transferase (GST)-tagged version of the Mal Leu180–containing allotype, unlike wild-type Mal, failed to bind to TLR2 (**Fig. 3**), suggesting that the defect in the reconstitution of signaling by Mal S180L occurs as a result of TLR2 not recruiting the variant. In addition to TLR2, Mal has also been shown to bind to both itself and MyD88 (ref. 4). Thus, we tested the effect of S180L on these interactions and found that it had no effect on the interaction of Mal with either itself or MyD88 (**Fig. 3**).

Studies in Mal-deficient mice have demonstrated impaired cytokine responses and NF-κB activation after stimulation with ligands at TLR4 and TLR2 as well as the TLR2 coreceptors TLR1 and TLR6 (refs. 5,6). Unlike bacterial components[1,12,13], the interaction of malaria parasites with TLRs is less well described, although it has been demonstrated that *P. falciparum*-derived GPI is a ligand for both TLR2 and TLR4 (ref. 10 and C. Aucan *et al.*, unpublished data). The association between *TIRAP* variants and malaria in Gambian and Vietnamese populations presented here provides further evidence for a role of the TLR pathway in malaria pathogenesis. In addition, a role for the TLR2 system in host defense against malaria is also suggested from mutations in CD36, which associate with susceptibility to severe malaria[14]. Recently, CD36 has been shown to be a coreceptor for TLR2 (ref. 15). Taken with the *TIRAP* variant presented here, the TLR2 system is clearly important for the host response in malaria.

Judging from our cotransfection experiments (**Fig. 1c**), the protective heterozygote state is likely to be associated with attenuated signaling and reduced NF-κB activation. This finding is consistent with increasing evidence that an excessive host inflammatory response may render individuals more vulnerable to developing severe forms of malaria and bacterial disease[16–18]. On the other hand, too little signaling would lead to an inadequate antipathogen response. This provides the opportunity for intermediate genotypes regulating signaling to be of optimal protective value. The mutant homozygotes are too rare to assess in African and Vietnamese populations, although in each of the UK studies there is a significant association with increased susceptibility to IPD among mutant homozygotes. This may have occurred by chance through analysis of the relatively small number of mutant homozygote individuals, or alternatively it may reflect a true heterozygote protective effect. Heterozygote protection against infectious disease is well recognized, with examples such as sickle cell trait and malaria, prion protein gene variation and spongiform encephalopathy and human leukocyte antigen (HLA) and the progression of HIV/AIDS[19–21]. If the *TIRAP* Leu180 homozygous state does indeed confer increased susceptibility to IPD, the mechanism by which this occurs is unclear, although we speculate that it may result in severely impaired or abolished signaling and increased susceptibility to severe infection, as has also been demonstrated in individuals with rare functional mutations in *IRAK4* and *NEMO* in the same pathway[22,23]. We suggest that heterozygosity at S180L may therefore confer a protective phenotype characterized by intermediate levels of pathway activation and an 'optimal', balanced inflammatory response. Additional host and microbial factors such as pathogen virulence and dose and prior immunity might be expected to interact with this model, as described in mouse models of pneumococcal susceptibility[24], and the optimal level of inflammatory response to a given exposure will probably vary depending on such factors.

The rarity of the mutant allele in African populations is also noteworthy. The burden of infectious disease mortality is very high in African populations, and this may strongly select against mutant homozygote individuals and act to reduce the Leu180 allele frequency. The observed allele frequency may reflect a balance between protection afforded to heterozygotes and increased susceptibility to infectious disease in mutant homozygotes. It is also possible that the leucine allele may be subject to ongoing selective pressure and may have not

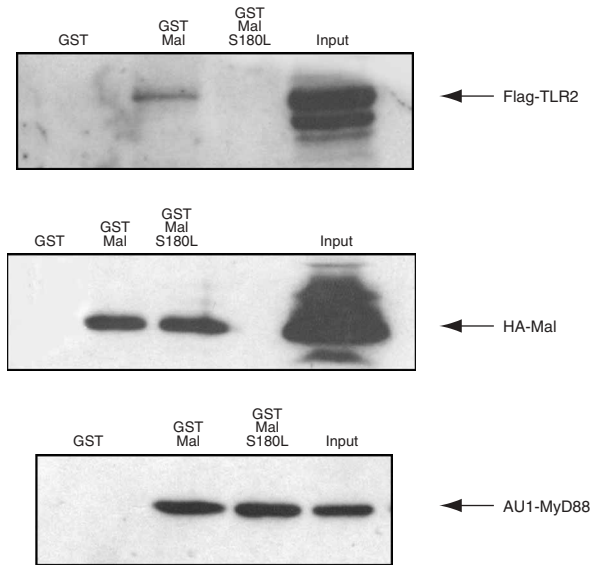

Figure 3 HEK293 cells (1×10^6) were transfected with 3 μg of Flag-tagged TLR2, hemagglutinin (HA)-Mal or AU1-tagged MyD88. Lysates were incubated with purified glutathione-coupled GST, GST-Mal and GST-Mal S180L for 2 h. After washing, the complex was analyzed by SDS-PAGE and protein blotting. Results shown are representative of three separate experiments.

yet achieved fixation in the African population. Furthermore, it is conceivable that *TIRAP* S180L may influence susceptibility to other common infectious and inflammatory causes of death and thus may be subject to multiple selective pressures which may differ between human populations and ecological settings. Investigation of the role of this functional variant of Mal in susceptibility to other diseases may shed light on this area. Here we give the first report of a single-nucleotide change that seems to have an impact on numerous major infectious diseases, roughly halving the risk in heterozygous individuals. This should represent a major evolutionary pressure, as these diseases together account for over 5 million deaths each year in the developing world.

METHODS

Sample collections. The UK IPD, thoracic empyema, Gambian malaria, West African tuberculosis and Kenyan bacteremia study groups have previously been described in detail (see **Supplementary Methods**). Informed consent and written approval from the relevant ethical review boards has been granted for all sample collections. Overviews of the sample collections are presented in **Supplementary Methods** online.

Sample genotyping. Direct sequencing of the *TIRAP* coding exons, a 1,500-bp region immediately 5′ to the transcription start site, and the 3′ UTR was performed in 48 Gambian individuals. The databases searched include dbSNP and Ensembl. The *TIRAP* S180L polymorphism was genotyped using the Sequenom MassArray MALDI-TOF primer extension assay for the Gambian malaria, Kenyan bacteremia and UK IPD study groups[25]. Confirmation via direct sequencing with BigDye v3.1 terminator mix (ABI) was then performed on 12 randomly selected wild-type and 12 heterozygous mutant samples from each of these populations. For the Vietnamese sample set, genotyping was performed using a site-directed mutagenesis–assisted restriction fragment length polymorphism (RFLP) assay, and for the Gambian tuberculosis study group, genotyping was performed using the RFLP assay and confirmed by both the Sequenom MassArray MALDI-TOF primer extension assay and direct sequencing. The rarity of the S180L polymorphism in the African populations studied increases the risk of bias due to genotyping error. To address this issue, all genotypes containing the mutant allele in these populations were confirmed by direct sequencing. The –80708, –68168, –52070, –29144, –23852, –18304, –12534, –5289, –4570, –1580, –386, –355, +177, +3977, +6032, +7685, R13W, S55N, Q101Q, S180L, A186A, V197I, +10122, +10610, +11347, +13466, +13955, +14472, +21162, +35464, +44402, +60339 and +71446 polymorphisms were genotyped via the Sequenom Mass-Array assay. Details of the genotyping methodology for all markers genotyped above are available in the **Supplementary Note**, **Supplementary Methods** and **Supplementary Tables 1** and **2** online. The individual assay details can be found in **Supplementary Table 3** online. All control genotype frequencies were in Hardy-Weinberg equilibrium.

Statistical methods. Statistical analysis of genotype frequencies was performed with χ^2 tests, two-tailed Fisher's exact tests if the χ^2 test was inappropriate, and forward, stepwise logistic regression, using SPSS. $P < 0.05$ was considered significant. One-tailed P values were computed if statistical testing was performed on a sample set replicating the same disease state. The overall two-tailed P value for the combined analysis was computed using a method similar to that previously described[26]. Analysis of linkage disequilibrium was performed using HaploView (v3.2)[27]. Analysis of transmission disequilibrium was performed using TRANSMIT[28].

Reporter gene assays. Mal-deficient MEFs (2×10^4 cells/well) were seeded into 96-well plates, transfected as described[4] on the following day with pcDNA3-Mal-hemagglutinin (HA) or pcDNA3-MalS180L-HA (generated using the Stratagene QuikChange kit) in combination with a luciferase reporter gene (80 ng/well NF κB luciferase reporter construct) and with a *Renilla* luciferase reporter gene (40 ng) as an internal control. Cell lysates were prepared as previously described[4].

Reconstitution assays. MEFs were transfected with plasmids encoding Mal, Mal-S180L or empty vector using metafectene essentially as described by the manufacturers (Biontex). After 48 h, cells were treated with LPS (1 μg/ml) or Malp2 (5 nM) for the indicated times. Immunoblot analysis was performed using a monoclonal antibody to IκBα, an antibody to phosphorylated p38 or an antibody to β-actin as loading control (all from Santa Cruz). IL-6 levels were measured by ELISA according to the manufacturer's recommendation (R & D Systems).

GST pulldown assays. The pGEX-Mal construct has been described previously[11]. The S180L point mutation was generated using the QuikChange site-directed mutagenesis kit (Stratagene) according the manufacturer's protocol. HEK293 cells were seeded (10^5 ml^{-1}) onto 100-mm dishes 24 h before transfection with 3 μg of Flag-tagged TLR2, HA-tagged Mal or AU1-tagged MyD88 using Genejuice (Novagen) according to the manufacturer's recommendations. We washed cells 24 h after transfection with 3 ml ice-cold PBS. Cells were lysed at 4 °C (1 h) in buffer containing 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP-40, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM Na_3VO_4 and 1 μg/ml leupeptin. GST pulldown assays were performed using the recombinant wild-type and mutant GST-Mal proteins coupled to glutathione (GSH)-Sepharose. Lysis extracts were incubated for 2 h with the fusion proteins described in the figure legends. The complexes were washed three times in lysis buffer and subjected to SDS-PAGE and protein blotting. Monoclonal antibodies against the Flag (12CA5) and AU-1 epitopes were obtained from Sigma and Berkeley Antibody Company (BAbCo), respectively. The polyclonal antibody against the HA epitope tag (Y-11) was obtained from Santa Cruz Biotechnology.

URLs. Ensembl: http://www.ensembl.org; Molecular Operating Environment (MOE2006.02): http://www.chemcomp.com.

Accession number. *TIRAP*: GeneID, 114609.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

C.C.K., S.J.C. and F.O.V. performed genotyping and wrote the article. A.D. and C.M. carried out functional experiments on Mal. O.K. and A.K. performed the modeling analysis on Mal. E.Y.L., A.J.F., A.J.W., C.A., S.S., C.E.M., K.K., S.J.C. and R.T.G. contributed to the experimental design of the genetic studies. C.L., A.S., P.A., O.Y.S., J.S., G.S., N.P., T.N.W., K.M., R.J.O.D., D.P.K., N.P.D., D.Y., D.W.C., K.M. and J.A.B. contributed to the design and collection of the case-control studies. All authors critically reviewed the manuscript. L.A.J.O'N. & A.V.S.H. led the functional and genetic efforts, respectively, and contributed equally to this work.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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