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A Common Haplotype of the TNF Receptor 2 Gene Modulates Endotoxin Tolerance

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Endotoxin tolerance is characterized by the suppression of further TNF release upon recurrent exposure to LPS. This phenomenon is proposed to act as a homeostatic mechanism preventing uncontrolled cytokine release such as that observed in bacterial sepsis. The regulatory mechanisms and interindividual variation of endotoxin tolerance induction in man remain poorly characterized. In this paper, we describe a genetic association study of variation in endotoxin tolerance among healthy individuals. We identify a common promoter haplotype in *TNFRSF1B* (encoding TNFR2) to be strongly associated with reduced tolerance to LPS ($p = 5.82 \times 10^{-6}$). This identified haplotype is associated with increased expression of *TNFR2* ($p = 4.9 \times 10^{-5}$), and we find basal expression of *TNFR2*, irrespective of genotype and unlike *TNFR1*, is associated with secondary TNF release ($p < 0.0001$). Functional studies demonstrate a positive-feedback loop via TNFR2 of LPS-induced TNF release, confirming this previously unrecognized role for TNFR2 in the modulation of LPS response. *The Journal of Immunology*, **2011, 186: 3058–3065.**

The inflammatory response to LPS, an invariant component of the outer membrane of Gram-negative bacteria, is mediated via a TLR-dependent transduction cascade (1, 2). This process is tightly regulated and characterized by the coordinate expression and release of multiple cytokines including TNF, IL-6, and IL-1β. Much of the early morbidity associated with Gram-negative bacterial infections is attributable to LPS-induced TNF release (3). Therefore, mechanisms controlling this response in man have vital implications in health and disease. Repeated or chronic exposure to LPS, or indeed other TLR ligands, leads to rapid tolerance to their proinflammatory actions both at a cellular level and at the level of the organism. This tolerance to LPS is referred to as endotoxin tolerance and was described over a century ago (4, 5). However, only recently have the underlying molecular mechanisms begun to be elucidated (6). Endotoxin tolerance is now recognized as a multifactorial process characterized by the induction of negative regulators of TLR signaling that can be proteins such as SHIP (7) and IL-1R–associated kinase-M (8) or microRNAs including micro (mi)R146, miR155, and miR9 (9, 10). Concomitant to this, chronic exposure to LPS leads to chromatin remodeling and the selective silencing of proinflammatory genes, resulting in a more profound state of tolerance (11).

The multiple conserved biological mechanisms of tolerance across species underline the importance of homeostatic regulation of innate immunity. In animal models, induction of endotoxin tolerance can render lethal doses of LPS sublethal, primarily by restricting further LPS-induced TNF release. Failure to silence innate immune activity is implicated in many acute and chronic inflammatory states, ranging from sepsis and autoimmune conditions to cancer. Identification of mechanisms regulating innate immune tolerance in man provides targets for therapeutic intervention to silence dysregulated innate immune responses.

Genetic polymorphisms play a significant role in determining interindividual variation in cytokine responsiveness to LPS (12, 13), but the phenotype of endotoxin tolerance has not been characterized in this respect. Genome-wide association analyses have demonstrated the impact of common genetic variants on multifactorial traits, highlighting the importance of noncoding sequence variants that modulate gene expression (14). For a number of common diseases such as type 1 diabetes and Crohn's disease, genome-wide association studies have isolated genes for which encoded products had not been previously considered in disease pathophysiology (15). In this study, we sought to define the extent of variation in endotoxin tolerance to repeated stimulation with LPS based on TNF release among healthy volunteers. We aimed to identify functional genetic variants associated with tolerance induction and, in doing so, gain novel insights into the regulation of tolerance induction in man. We show that a common haplotype spanning *TNFRSF1B*, the gene encoding TNFR2 (denoted by its alternative gene name *TNFR2* in the following text), is strongly associated with reduced tolerance to LPS. We proceed to demonstrate that this haplotype is associated with differential nuclear factor binding and is specifically associated with increased basal expression of *TNFR2*. We subsequently show that LPS-induced TNF activity can positively feedback through TNFR2, impacting upon the duration and magnitude of TNF release.

Materials and Methods

Ethics statement

This study was approved by the Oxfordshire Research Ethics Committee (REC reference 06/Q1605/55). All volunteers gave written informed consent.

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Abbreviations used in this article: CI, confidence interval; CRE, cAMP response element; LD, linkage disequilibrium; mi, micro; SNP, single nucleotide polymorphism; TRAF, TNFR-associated factor; VNTR, variable number tandem repeat.

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Study volunteers, PBMC purification, and LPS tolerance assays

The recruitment and demographics of the healthy volunteer cohort, sample collection, PBMC purification, and cell culture conditions have been previously described (13). In summary, healthy volunteers without a history of recent viral or bacterial infection were venesected in the morning and PBMCs purified using a Ficoll-paque PLUS (GE Healthcare, Chalfont St Giles, U.K.) gradient within 2 h. Cells were counted with a hemocytometer and resuspended at 2.5×10^6 cells/ml in RPMI 1640 growth medium (Sigma-Aldrich) supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 U/ml penicillin, 0.1 mg/ml streptomycin), and 10% v/v heat-inactivated gold-standard FCS, with three biological replicate assays per condition set up for each volunteer. After overnight incubation, cells were washed in fresh media then resuspended in media alone or with LPS at 2 ng/ml (L-4391, lot 114K4133; Sigma-Aldrich), for 6 h prior to separation of cells from supernatant by centrifugation. To assess tolerance, additional naive or LPS-treated cells (2 ng/ml for 6 h) were exposed to LPS (20 ng/ml) for a further 6 h. Three biological replicate assays per condition were set up for each volunteer and the mean used in subsequent statistical analysis. Supernatants for ELISA were stored at −80°C prior to quantification; cell pellets for RNA extraction were resuspended in RLT buffer (Qiagen, Valencia, CA) supplemented with 2-beta-mercaptoethanol. Where indicated, a TNFR2 allosteric modulator Ab (anti-human TNF-R II clone 80M2, catalog number HM2022; Hycult Biotech, Uden, The Netherlands) or mouse IgG1 isotype control (functional-grade) mAb (catalog number M075-3M2; Medical and Biological Laboratories, Nagoya, Japan) was added to cultures at a concentration of 2.5 μg/ml for the times indicated. For the experiments involving purified monocytes, CD^{14+} monocytes were positively selected with Miltenyi CD^{14+}-labeled microbeads (catalog number 130-050; Miltenyi Biotec, Bergisch Gladbach, Germany) according to instructions, with all steps performed at 4°C or on ice. Experiments with monocytes were carried out at the lower concentration of 5×10^5 cells/ml.

ELISAs

ELISAs were performed on all samples in duplicate as described (13) using TNF, IL-6, and soluble TNFRII Duoset kits (DY 206, DY210, DY726; R&D Systems, Minneapolis, MN).

RNA extraction, cDNA synthesis, and quantitative PCR

Total RNA was prepared using the RNeasy Mini kit (Qiagen) as described with cDNA prepared with SuperScript III (Invitrogen, Paisley, U.K.) using random hexamers (13). Quantitative PCR was carried out using SYBR Green Supermix (Bio-Rad, Hemel Hempstead U.K.) on the CFX96 C1000 Thermal Cycler (Bio-Rad). PCR efficiency was determined and melt curve analysis performed for gene-specific primer sets (Supplemental Table I). Relative gene transcript levels were determined by the ΔΔCt method.

Genotyping, haplotype, and statistical analysis

The *TNFR2* variable number tandem repeat (VNTR) was genotyped by amplification of a 150-bp fragment by PCR (primer sequences shown in Supplemental Table I), and the number of repeats was directly identified from electrophoresis of a 3% agarose gel. Genotyping of the volunteer cohort using the human CVD bead array chip (Illumina, San Diego, CA), computation of linkage disequilibrium (LD) and haplotypes using the HaploView 3.3.2 program (16), and expression quantitative trait mapping using PLINK (17) was performed as previously described (13) with verification using SPSS (SPSS), R package, and SNPTest. Standard quality control measures were used with exclusion criteria of maximum per single nucleotide polymorphism (SNP) missing (geno >0.1 and maf <0.03). The genotyping rate was >98.8%. For each SNP analyzed, the phenotypic mean for the three genotypic states was compared using the Wald test statistic to generate a p value that does not require that the data fit a normal distribution. Covariates age, ethnicity, and sex were included in PLINK analysis to further interrogate observed associations. Nonparametric statistics and log transformation were applied where data were not normally distributed, and otherwise unpaired t tests were used for analysis of expression data for specific SNPs, data passing tests of normality. Permutation analysis was performed using a label-swapping procedure and an adaptive algorithm. Analysis was performed with one million permutations using a within-cluster algorithm as described (13). The empirical p value is robust with respect to normality of phenotype and multiple testing issues. All correlations were carried out using linear regression least-square fit models with statistical relevance and correlation coefficients described for each association. Where indicated, two-tailed t tests were used.

Nuclear extracts and EMSAs

Nuclear extracts were prepared, oligonucleotide probes were radiolabeled with [^{32}P]deoxycytidine 5′-triphosphate (PerkinElmer, Beaconsfield, U.K.) by fill-in of 5′ overhanging ends using Klenow and unlabeled 2'-deoxyadenosine 5′-triphosphate, deoxyguanosine 5′-triphosphate, and deoxythymidine 5′-triphosphate, and EMSA performed as previously described (18, 19). Probes were generated by annealing forward and reverse oligonucleotides. Sequences are shown in the Supplemental Table I.

Results

Variation in endotoxin tolerance at the level of TNF release in response to LPS

Similar to previous studies (20), we noted that a 6 h pretreatment of cells with LPS established marked tolerance to further LPS-induced TNF release. We also observed a degree of interindividual variation at this time point, permitting investigation of underlying genetic contributions to this. We therefore based our assay on TNF release in response to LPS stimulation for 6 h comparing naive versus pretreated cells. We were interested in the individual silencing of TNF production after LPS treatment, and so to normalize for variation in initial response, due to either interindividual variation in PBMC composition or other intrinsic sensitivities to LPS, values were expressed relative to the naive response. This approach allowed us to focus upon silencing of TNF responses in a sample most representative to that in circulation. We proceeded to recruit a cohort of 96 healthy volunteers and to define variation in tolerance to LPS based on three replicate stimulation assays for a given volunteer on a particular day, with the mean value used in subsequent analyses.

Over a 6-h period, LPS treatment (2 ng/ml) resulted in a pronounced release of TNF into cell culture supernatants, with significant interindividual variability in induced TNF release (geometric mean 664 pg/ml; 95% confidence interval [CI] 593–743 pg/ml; range 118–3511 pg/ml) (Fig. 1). Cells were subsequently washed extensively and restimulated with a 10-fold higher LPS concentration (20 ng/ml) for a further 6 h. In tandem with this, naive cells that had been treated identically (washed in same manner), but had been exposed to media alone, were stimulated with LPS at the higher concentration. Cells pre-exposed to LPS showed a pronounced reduction in secondary release of TNF upon restimulation and also showed significant variability across the cohort (geometric mean 83 pg/ml; 95% CI 69–100 pg/ml; range 11–550 pg/ml) (Fig. 1).

Although most individuals showed a high level of silencing on secondary stimulation (mean 15.8%; SD 10.8; range 1.4–59), several individuals showed reduced induction of tolerance with secondary responses 30–50% that of their primary response (Fig. 1). Interestingly, we did not note an association between the magnitude of the primary and secondary TNF response (data not shown). When we analyzed the effect of secondary stimulation on IL-6 production at 6 h, we did not see tolerance, with most individuals releasing a similar amount or more IL-6 (Fig. 1), confirming continued cell viability and reflecting mechanistically different silencing mechanisms between TNF and other acute-phase cytokines at this relatively early time point in endotoxin tolerance.

Common genetic markers associated with reduced endotoxin tolerance

To determine genetic modulators of endotoxin tolerance, we proceeded to map tolerance as a quantitative trait among the cohort of 96 healthy volunteers. Individuals were genotyped at 45,237 SNPs using the Illumina HumanCVDv1 beadchip (Illumina), which has high density coverage of ~2000 genes implicated in immune and inflammatory responses of specific relevance to vascular pathology, metabolic disorders, and inflammatory disease states (21).

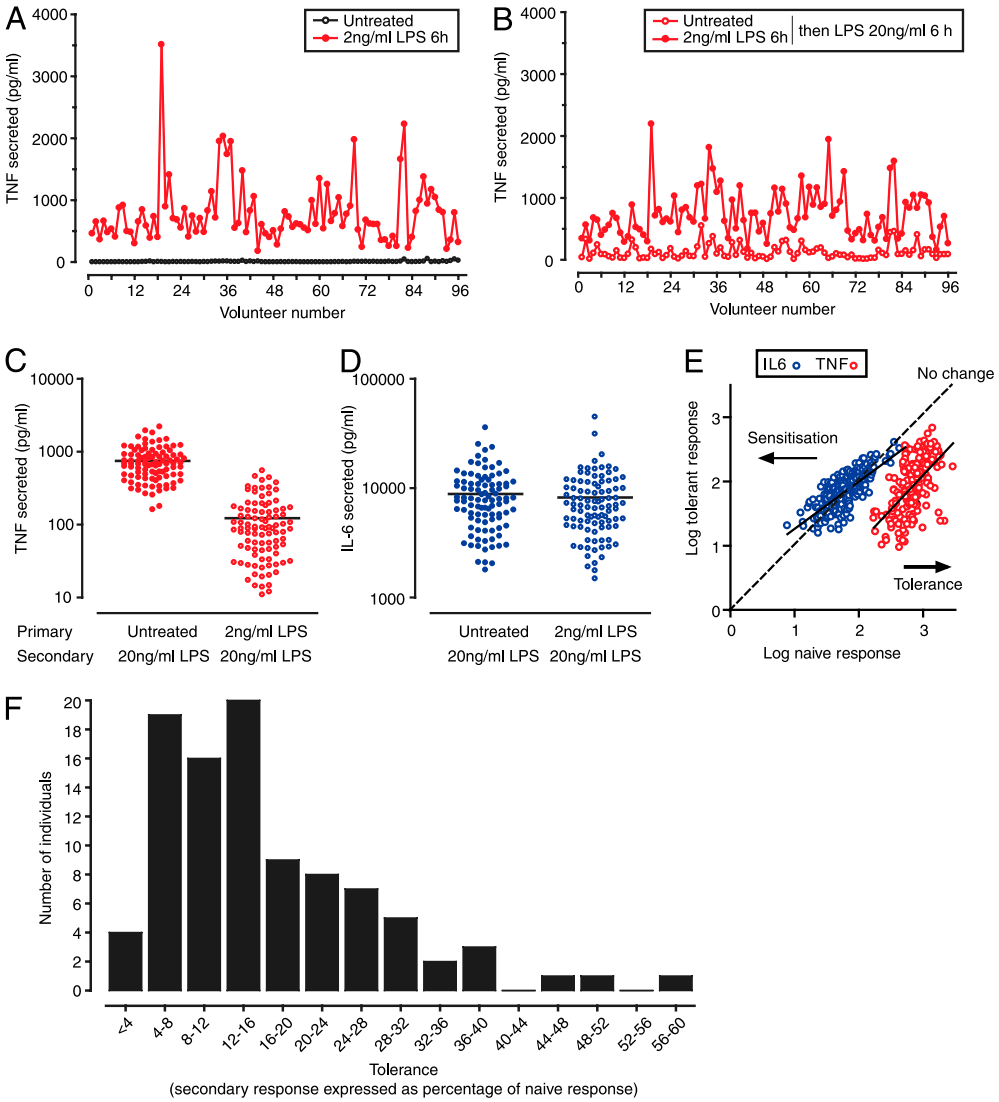

FIGURE 1. LPS-induced TNF release among 96 healthy volunteers. TNF levels in culture supernatant for untreated and treated PBMCs (2ng/ml LPS for 6 h) (*A*) or comparing response to 20 ng/ml LPS for 6 h between naive cells and cells pretreated with LPS 2 ng/ml for 6 h (*B*). Scatter plot for all volunteers showing TNF secretion (*C*) and IL-6 secretion (*D*). *E*, Comparison of TNF and IL-6 naive versus tolerant response. *F*, Frequency distribution of tolerance across the cohort.

We found the most significant associations were with a cluster of SNPs at or near to *TNFR2* at chromosome 1p36.22 (Fig. 2). Moreover, analysis of quantile–quantile plots highlighted that SNPs in TNFR-related genes or TNFR-associated factor (TRAF) family genes accounted for the majority of the overdispersion of *p* values over the expected in terms of statistical association (Fig. 2). Three SNPs in complete LD upstream of the *TNFR2* gene (rs522807, rs5745938, and rs625847) showed the strongest association with endotoxin tolerance; the mean tolerance was 31.3% (95% CI 17.2–45.5) among individuals possessing a copy of the haplotype defined by rs522807 compared with 14.4% (95% CI 12.5–16.3) among those without (Supplemental Fig. 1). By contrast, we found no association with initial TNF release (Fig. 2). The reduced tolerance-associated SNP rs522807 shows marked differences in allele frequency among different populations worldwide. The tolerance-associated A allele (denoted as the ancestral allele as this is the allele observed in chimp, orangutan, and macaque genomes) was present at a frequency of 8% in northern European-derived populations, absent in Asian populations but present at a frequency of near 50% in equatorial Africa (Supplemental Fig. 2).

rs522807 is associated with increased expression of TNFR2 in the basal state

Given the association of SNPs spanning *TNFR2* with endotoxin tolerance, we sought to define if the same SNPs were modulating expression of this gene locally in a *cis*-acting manner. Using data from real-time quantitative RT-PCR, we proceeded to carry out an expression quantitative trait analysis for *TNFR2*. Interestingly, we found that rs522807 marked an eQTL for *TNFR2* ($p = 4.9 \times 10^{-5}$). Individuals possessing a copy of the endotoxin-associated SNP rs522807 had significantly increased basal expression of *TNFR2* (Fig. 2, Supplemental Fig. 1). This indicated that the promoter polymorphisms associated with decreased tolerance enhanced basal expression of the receptor, suggesting the hypothesis that irrespective of genotype, basal expression of *TNFR2* positively influences secondary responses by regulating tolerance.

TNFR2 expression correlates with secondary, but not primary, TNF release

We proceeded to test this hypothesis by investigating whether there was any correlation between the expression of *TNFR2* and LPS-induced TNF response. We postulated that feedback of LPS-

A

SNP	Gene	Chromosome	Location	Unadjusted P value	Empirical P value from permutation analysis
rs522807	*TNFRSF1B*	1p36.22	Promoter	5.8×10^{-6}	7.9×10^{-5}
rs12652669	*SGCD*	5q33.3	Intron	8.5×10^{-5}	7.0×10^{-4}
rs9332553	*F5*	1q24.2	Intron	2.2×10^{-4}	2.1×10^{-4}
rs2806424	*DDR2*	1q23.3	Intron	2.2×10^{-4}	2.8×10^{-4}
rs6041750	*FKBP1A*	20p13	3' UTR	3.2×10^{-4}	2.5×10^{-4}
rs9465838	*CDKAL1*	6p22.3	Intron	3.3×10^{-4}	4.9×10^{-4}

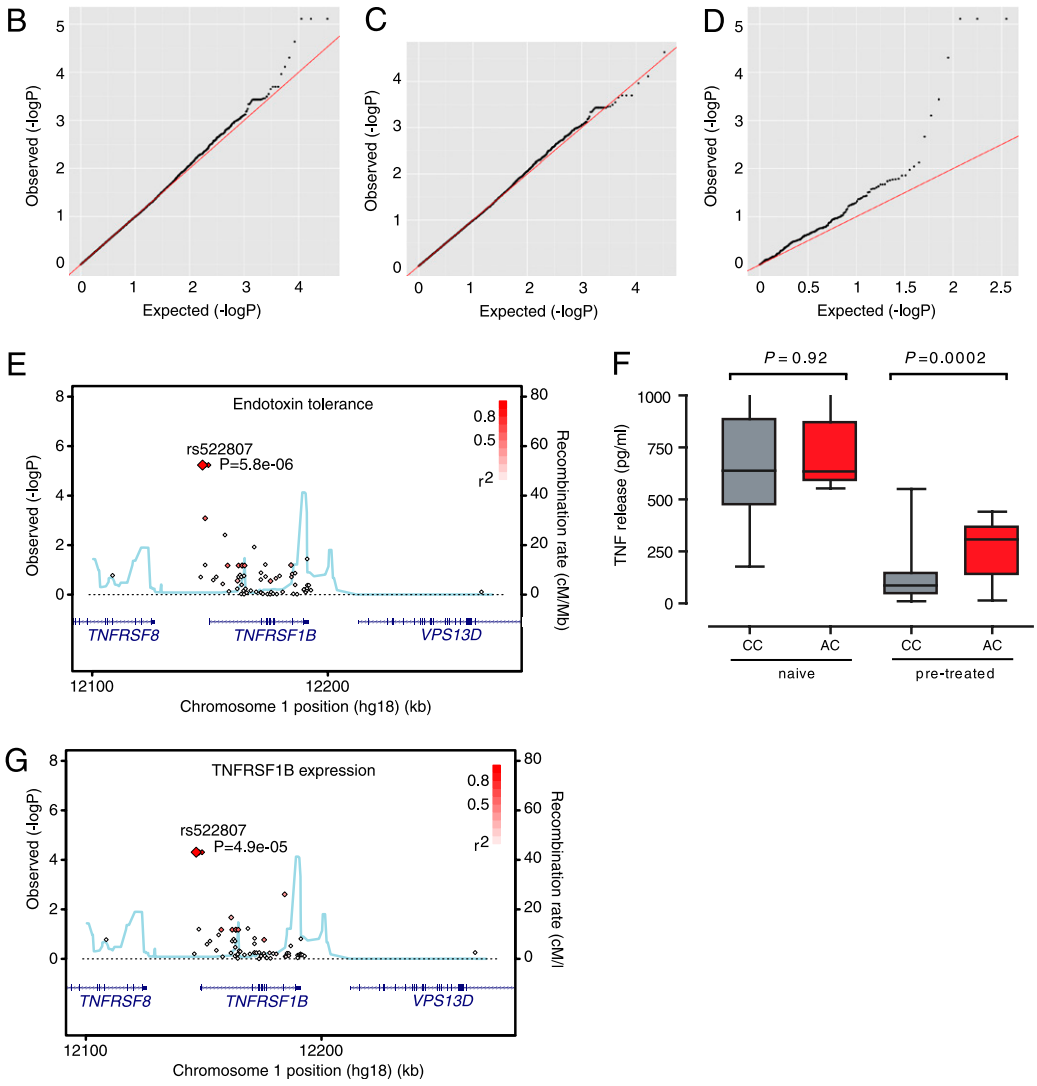

FIGURE 2. Expression quantitative trait mapping of endotoxin tolerance. *A*, Top SNP associations with endotoxin tolerance. Quantile–quantile plots considering all genotyped SNPs (*B*), SNPs excluding those involving all TNF/TRAF genes (*C*), or including only those SNPs relating to TNF/TRAF genes (*D*). *E*, Regional association plot for endotoxin tolerance. *F*, Allelic association for rs522807 with TNF release following LPS 20 ng/ml for 6 h, with and without LPS 2 ng/ml pretreatment for 6 h. *G*, Regional association plot for *TNFR2* expression.

induced TNF through basally expressed *TNFR2* may influence the secondary response, although we were uncertain whether this would contribute to desensitization and tolerance or have the opposite effect. Using real-time quantitative RT-PCR, we found there was no association between basal expression of either *TNFR1* or *TNFR2* and primary release of TNF (data not shown). However, we found a highly significant correlation between the basal expression of *TNFR2* mRNA and secondary TNF protein release ($p < 0.0001$) (Fig. 3). LPS stimulation resulted in a robust induction of *TNFR2* expression (4.8-fold induction; 95% CI 4.2–5.4), but whereas an association between induced *TNFR2* and tolerance remained, this was far more modest ($r^2 = 0.09$; $p = 0.002$). This implied that the level of basal expression is of greater biological relevance in the determination of later responses. Notably, the association with tolerance was not seen for *TNFR1* (Fig 3), suggesting this is a *TNFR2*-specific phenomenon. Analysis of expression levels of *TNF* mRNA rather than protein gave similar results (Supplemental Fig. 3).

Fine mapping association at TNFRSF1B and analysis of transcription factor binding

To fine map the causative functional genetic variant, we resequenced a 3.5-kb region of the *TNFR2* promoter that included

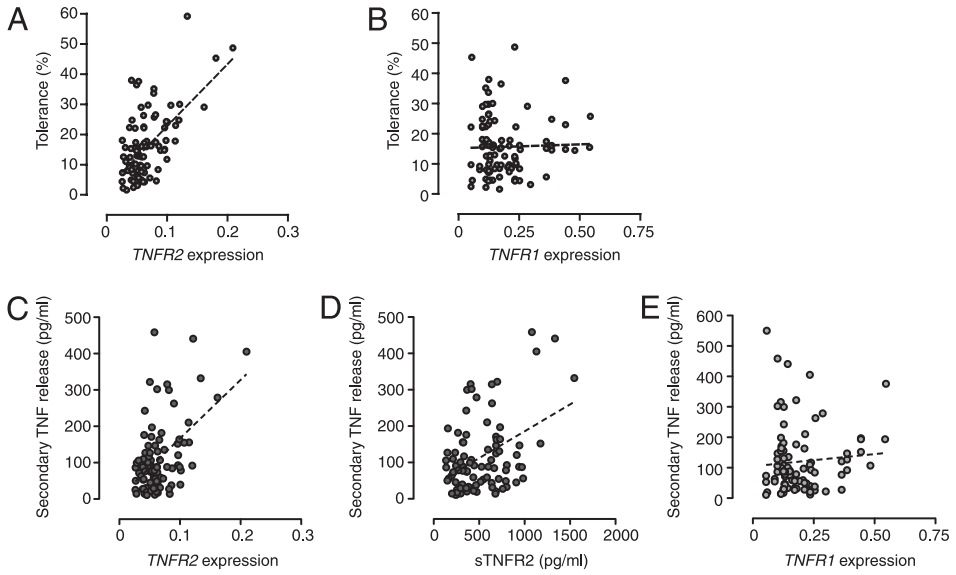

FIGURE 3. Baseline expression of *TNFR2* predicts tolerance to LPS irrespective of genotype. Scatter plots of tolerance assayed at the level of TNF protein secretion showing correlation with *TNFR2* mRNA expression (r^2 = 0.36; $p < 0.0001$) (*A*) and with *TNFR1* mRNA expression ($r^2 < 0.01$; p = NS) (*B*). Correlation of secondary TNF protein with *TNFR2* mRNA expression (r^2 = 0.88; $p < 0.0001$) (*C*), sTNFR2 protein (r^2 = 0.18; $p < 0.0001$) (*D*), and *TNFR1* mRNA expression (r^2 = 0.02; p = NS) (*E*). All transcript abundance expressed relative to *ACTB*.

rs522807 (chr1: 12,146,659–12,149,575). In addition to the three single nucleotide substitutions identified from the array (rs522807, rs5745938, and rs625847), a further single nucleotide substitution (rs520916) and insertion (rs35752907) were found to be in complete LD with the haplotype defined by rs522807. Additionally, we noted a previously described VNTR comprising a 15-bp insertion within the promoter that exists in two allelic forms (either one or two repeats) (22) that has been associated with both infective and autoimmune disease processes (23, 24), although as yet it is unclear whether it influences expression of *TNFR2*. To answer this question, we genotyped this VNTR and found a weak association between possession of a single copy of the VNTR and *TNFR2* expression, but this effect was lost when the analysis was conditioned on rs522807.

To determine if any of the SNPs within the endotoxin tolerance-associated haplotype modulated allele-specific protein–DNA interactions, EMSAs were performed. Radiolabeled oligonucleotides corresponding to each SNP allele were incubated with nuclear extracts from the human leukemic monocyte lymphoma cell line U937. We observed that rs522807 showed markedly different binding profiles between alleles using nuclear extracts from U937 cells that were specific on competition EMSA (Fig. 4). In silico analysis shows that the associated A allele of rs522807 introduces a cAMP response element (CRE) binding site, and competition experiments demonstrated that the upper allele-specific complex I is specifically competed by an oligonucleotide containing a CRE binding site, but the identity of the allele-specific CRE binding protein recruited at rs522807 remains to be defined.

As this study neared completion, data from the 1000 Genomes Project (http://www.1000genomes.org) became available. This shows that the haplotype tagged by rs522807 extends for 15.9 kb upstream of *TNFR2* with 17 SNPs in complete LD (Supplemental Table II), which will require further characterization to define the causative regulatory variant(s).

Allosteric modulation demonstrates a role for TNFR2 in augmenting LPS responsiveness

Our data suggest that the expression of TNFR2 plays a direct role in modulating tolerance to LPS-induced TNF release. However, the overriding effect of increased basal expression of TNFR2 was unclear. TNFR2 is cleaved at the cell surface by ADAM 17 (ADAM metallopeptidase domain 17), a disintegrin and metalloproteinase also referred to as TACE (TNF-α converting enzyme) to form soluble TNFR2 that can antagonize the activity of TNF at its receptors with important functional consequences (25, 26). It was therefore possible that the predominant effect of overexpression of TNFR2 would be to antagonize the action of TNF. To dissect out the potential effect of TNFR2 activity in the development of endotoxin tolerance, we took advantage of an allosteric modulating mAb raised against this receptor (anti-human TNF-R II clone 80M2). Although 80M2 has no independent agonist activity, it markedly increases the affinity for TNF to TNFR2 and thus confers enhanced TNFR2 signaling in the presence of TNF only (27).

Prior to LPS application, cells were treated with 80M2 or isotype control IgG1 for 1 h before extensive washing to remove any unbound Ab. Cells were then treated with LPS for up to 10 h, with supernatants and RNA harvested in the absence of LPS and at 2, 6, and 10 h. Pretreatment with Abs did not elicit TNF release in the absence of LPS. When LPS was applied, 80M2 had no effect on early induced TNF release at 2 h, but at later time points, cells pretreated with 80M2 showed significantly enhanced LPS-induced TNF release (Fig. 5). Furthermore, expression of TNF, which was maximal at 2 h, was not significantly different from IgG_1. However, at 6 h, there was delayed normalization in TNF expression (Fig. 5).

In addition to modulating the primary response to LPS, we were keen to investigate whether 80M2 could modify secondary responses to LPS and hence tolerance. Use of 80M2 in crude PBMC populations had variable effects, with cells from some individuals showing pronounced alteration in tolerance, whereas others were not affected (data not shown). We reasoned that this may be secondary to varying cellular composition of PBMC fractions. 80M2 has been shown to have activity at specific T cell populations (28), and it was reasoned that activity of the Ab at nonmonocyte populations may confound effects on tolerance. To avoid this possibility, we purified CD^{14+} monocytes from the PBMC fraction of unrelated healthy volunteers, thus focusing on the cells mainly responsible for LPS-induced cytokine release. Similar to the PBMC fraction, we found pretreatment with 80M2 had no independent agonist activity (data not shown), but greatly enhanced LPS-induced TNF release (2363 ng versus 1010 ng; n = 4, $p < 0.05$) (Fig. 5). After 6 h of 2 ng/ml LPS, monocytes were washed and restimulated for a further 3 h with 20 ng/ml LPS before measuring secondary responses. We found that this pretreatment significantly enhanced secondary TNF release in response to LPS, demonstrating activity through TNFR2 can modulate tolerance

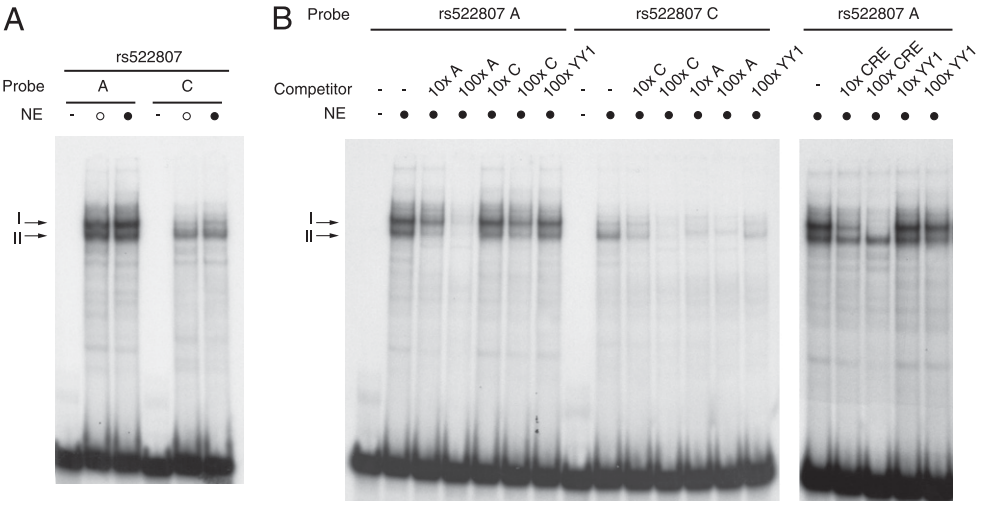

FIGURE 4. Allele-specific protein–DNA interactions. *A*, EMSA using radiolabeled probes corresponding to the two alleles of rs522807 show allele-specific differences in binding when incubated with nuclear extracts for U937 cells, either unstimulated (○) or after induction with LPS for 6 h (●). *B*, The specificity of these interactions was confirmed on competition with 10- or 100-fold molar excess of unlabeled probe corresponding to each of the two alleles, a CRE binding site, or an unrelated sequence (corresponding to a YY1 binding site).

(Fig. 5). These results supported the hypothesis generated from the genetic screen whereby LPS-induced TNF acts to further amplify late release of TNF in a TNFR2-specific manner. These data show that manipulation of basally expressed TNFR2 can dictate the both the magnitude and durability of LPS-induced TNF release.

Discussion

Although the phenomenon of endotoxin tolerance has been described for over a century, the biological mechanisms underlying this critical immunological process are only now beginning to be elucidated. These include the concomitant upregulation of negative regulators of TLR signaling, induction of miRNAs, which specifically target products of the NF-κB pathway, and the selective silencing of multiple proinflammatory genes. Most data emanate from mouse models, and there is a degree of discrepancy between genes implicated in the induction of endotoxin tolerance in animal studies and in man such that only IL-1R–associated kinase-M has consistently been demonstrated to play a role in both mice and men (8, 20). The significance of a more comprehensive understanding of the basis of endotoxin tolerance is underlined by the clear role for this phenomenon in the pathogenesis of diseases

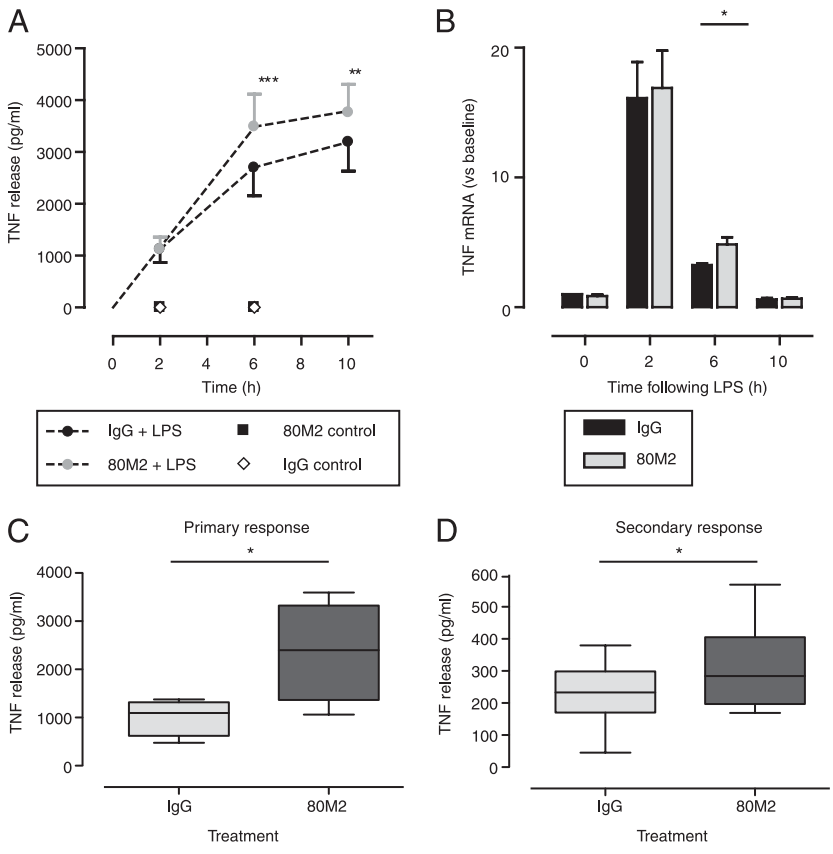

FIGURE 5. Allosteric modulator of TNFR2 enhances late LPS-induced TNF release. PBMCs were pretreated for 1 h with allosteric modulating Ab or isotype control (2 mg/ml) prior to extensive washing and LPS treatment (2 ng/ml). *A*, TNF protein ($n = 7$). *B*, TNF transcript (relative to baseline with IgG control) ($n = 5$). $CD14^+$ monocytes were pretreated for 1 h with allosteric modulating Ab or isotype control (2 mg/ml) prior to extensive washing and LPS treatment (2 ng/ml). TNF protein released was assayed. Following stimulation with LPS for 6 h (2 ng/ml) (*C*) or following pretreatment with LPS (2 ng/ml) for 6 h (*D*) then restimulation with LPS (20 ng/ml) for 3 h. $*p < 0.05$, $**p = 0.01$, $***p < 0.01$.

such as sepsis. The tolerant state is associated with increased susceptibility to subsequent infections and indeed may reflect an underlying proinflammatory but paradoxically immunosuppressed state (20). Nonetheless, the highly conserved nature of tolerance and the multiplicity of mechanisms whereby it can be achieved indicate it plays a key role in the regulation of the innate immune response. Indeed, it is postulated that endotoxin tolerance has evolved to act as a protective mechanism to prevent the cytokine storm associated with onset of sepsis and strongly associated with shock (6).

In this study, we were interested in addressing the degree to which individuals tolerized to LPS over a short period, thus reflecting silencing of TNF release. We specifically chose a 6-h pretreatment duration because in a clinical setting, the onset of sepsis can often be abrupt, with patients deteriorating rapidly over such a time period. Although studies of tolerance over 24 h show universal silencing of most inflammatory cytokines (11), we find there is a large degree of interindividual variability in the silencing of TNF over 6 h, and therefore, this time point is more applicable to an association study. It is of note that in our study we found little silencing of the IL-6 response, illustrating both the differential regulation of these genes and also the continued viability of cells.

Our interest in identifying genetic markers of endotoxin tolerance has been driven by the observations that responses to innate immune stimuli show a large degree of heritability (12, 29). We were curious to investigate whether there may be genetic variants associated with differential silencing of TNF release. Our initial finding that the most significantly associated SNP from a panel of over 48,000 SNPs was located in the promoter region for *TNFR2*, a receptor for the measured parameter (TNF release), was of high biological plausibility. We proceeded to demonstrate that this SNP, rs522807, marks a haplotype associated with increased basal expression of TNFR2 in our dataset and directly modulated allele-specific recruitment of a CRE binding protein. The observation that the basal expression of TNFR2 relates to secondary TNF release was replicated across the cohort, independently of genotype, with a highly significant correlation between expression and secondary response. By contrast, there was no association with the expression of TNFR1, suggesting a specific role for TNFR2 in the regulation of LPS-induced TNF release. This may reflect the differential signaling pathways of TNFR1 versus TNFR2, with TNFR2 specifically activated by membrane-bound TNF and showing relative insensitivity to free TNF (27). It is plausible that the early expression of membrane-bound TNF in response to LPS and subsequent signaling via TNFR2 has later effects on tolerance. This would also explain why the basal expression levels on TNFR2 seem to be of greatest importance in influencing subsequent later responses, with individuals expressing higher levels having consequentially reduced tolerance. We substantiated this finding by using mAbs specific for TNFR2 that enhance the affinity of the receptor for TNF (27). Pretreatment of cells with Ab has no stimulatory effect in its own right, demonstrating that TNFR2 activity only acts to amplify other responses in this experimental model. Moreover, at 2 h, cells pretreated with Ab show similar LPS-induced TNF release to those pretreated with isotype control. However by 6 h, a time when endotoxin tolerance has developed (20), there is a highly significant difference between isotype control-treated cells and those treated with TNFR2-specific Abs, indicating positive feedback of LPS-induced TNF release, acting through the receptor to enhance further TNF production. Using a purified monocyte subset, we were able to separately demonstrate that activity involving TNFR2 could modulate tolerance, with monocytes pretreated showing a significant increase in secondary TNF release compared with control cells.

Our data have highlighted the extent of individual variation in the endotoxin tolerance phenotype among healthy individuals and has implications for the tailored use of therapeutic interventions to modulate the inflammatory response. We have shown how expression quantitative trait mapping in primary human tissue can be used to identify functionally relevant polymorphisms for complex immunological responses. This unbiased approach allows the elucidation of pathway genes for which the role may have been previously unsuspected—in this case, the *TNFR2* gene in endotoxin tolerance. This result has significant physiological implications for our understanding of the biological mechanisms regulating endotoxin tolerance and highlights the value of large-scale genotyping in the identification of functional variation in innate immune responses in man.

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Disclosures

The authors have no financial conflicts of interest.

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