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Cutting Edge: Genetic Variation in *TLR1* Is Associated with Pam3CSK4-Induced Effector T Cell Resistance to Regulatory T Cell Suppression

Carmen Mikacenic*, Anya Schneider,† Frank Radella,* Jane H. Buckner,†,1 and Mark M. Wurfel*1

TLR play essential roles in the initiation and modulation of immune responses. TLR1/TLR2 heterodimers recognize triacylated bacterial lipopeptides, including the synthetic TLR1/2 lipopeptide Pam3CSK4. Genetic variation in *TLR1* is associated with outcomes in diseases in which regulatory T cells (Treg) play a role, including asthma and allergy. To determine whether genetic polymorphisms in *TLR1* are associated with alterations in Treg suppression of effector T cells (Teff), we performed in vitro suppression assays in healthy individuals with various haplotypes in *TLR1*. We show that functional genetic polymorphisms in *TLR1* modify surface expression of TLR1 on T lymphocytes and confer enhanced Teff resistance to Treg suppression in the presence of Pam3CSK4. These effects are mediated, in part, by IL-6 and inhibited by blocking IL-6 signaling through STAT3. These findings suggest that *TLR1* polymorphisms could influence immune-related disease through Teff resistance to Treg suppression. *The Journal of Immunology*, 2014, 193: 5786–5790.

 Toll-like receptors are a family of germline-encoded pattern recognition receptors essential to the detection of microbial components (1). Known for their role in innate immunity, TLR detect a wide range of pathogen-associated molecular patterns (PAMP). The genes encoding human TLR are dispersed throughout the genome, with the exception of *TLR1*, *TLR6*, and *TLR10*, which lie in a single locus on chromosome 4. TLR2, in combination with TLRs 1, 6, and possibly 10, recognize different PAMP (2–4). Pertinent to our current study, the TLR1/2 heterodimer recognizes triacylated lipopeptides isolated from bacterial pathogens or produced synthetically (Pam3CSK4).

Common genetic variation in the TLR10/1/6 locus is associated with multiple disease states and functional changes in immune responsiveness. Recently, in multiple genome-wide association studies, the importance of the TLR10/1/6 locus was highlighted by very strong associations with *Helicobacter pylori* seroprevalence (*p* = 1.42 × 10⁻¹⁸) (5), self-reported allergy (*p* = 5.3 × 10⁻²¹) (6), and allergic sensitization (*p* = 5.2 × 10⁻¹¹) (7). Multiple high-frequency missense variants and varying linkage disequilibrium (LD) structures across populations have made it difficult to pinpoint a single causative variant (8). However, two nonsynonymous coding variants associated with clinical phenotypes rs4833095 (Asn248Ser) and rs5743618 (Ser602Ile) may play a role. In whites, the minor alleles of these coding variants (rs5743618T and rs4833095C) have been associated with increased whole-blood cytokine responses and increased TLR1 surface expression on monocytes due to enhanced trafficking to the cell surface rather than changes in mRNA or total-cell protein levels (9–12). The rs5743618 (Ser602Ile) coding variant also was shown to confer increased Pam3CSK4-induced NF-κB reporter activity in transfected epithelial cell lines that do not express TLR1 (9, 13). We also identified a single nucleotide polymorphism (SNP) in the TLR1 promoter in high LD with these SNPs in which the minor allele rs5743551G is highly associated with death in sepsis (9). The consequences of these polymorphisms with respect to T cell function have yet to be explored.

Multiple TLR are expressed in T cells and are capable of modulating the function of various T cell subsets. CD4+CD25+FoxP3+ regulatory T cells (Treg) are pivotal to the suppression of cellular immune responses. Important to these studies, TLR1/2 agonists were shown to impair Treg suppressive capacity in humans (14, 15). Of interest, this effect was only observed in a subset of subjects, implying interindividual variation (14). In this study, we tested whether common genetic variation in *TLR1* affects TLR1/2 agonist-induced changes in Treg and effector T cell (Teff) function.
Materials and Methods

Study subjects
We obtained fresh peripheral blood, frozen PBMC, and DNA from healthy volunteers from whom written informed consent was obtained. This work was approved by the Benaroya Research Institute and the University of Washington human subjects committees.

Genotyping
We genotyped DNA for three SNP in TLR1: rs5743618, rs4833095, and rs5743551 by TaqMan PCR-based allelic discrimination. We identified white individuals who carried two copies of the haplotype for the three minor alleles (rs5743618T, rs5743551G, rs4833095C) and age- and gender-matched controls carrying two copies of the haplotype for the three major alleles (rs5743618G, rs5743551A, rs4833095T). The study subjects were predominantly male (63%) and had a mean age of 36 ± 14 y. PBMC were stained for surface expression of CD4 and TLR1. Subjects homozygous for the TLR1 minor allele haplotype had a significantly higher TLR1 median fluorescence intensity (MFI) on CD4+ T cells (Fig. 1A) than did those homozygous for the TLR1 major allele haplotype (p = 0.004). Cell surface expression of TLR5 did not differ by TLR1 haplotype (Fig. 1B). When we further differentiated T cells by FOXP3 staining, we found that both CD4+FOXP3+ and CD4+FOXP3− T cells from subjects homozygous for the TLR1 minor allele haplotype had a significantly higher percentage of cells expressing TLR1 relative to the major allele haplotype (Supplemental Fig. 2A, 2B).

Treg isolation
Natural Treg (nTreg) from freshly isolated PBMC were sorted by flow cytometry for the CD4+ cells expressing the highest 3–5% of CD25 (16). The mean percentage of FOXP3+ T cells was 90%.

CFSE-based suppression assay
CD4+CD25+ Teff were isolated from thawed frozen autologous or heterologous donors’ PBMC by negative selection with Microbeads to CD4 and CD25 (Miltenyi Biotec) and labeled with CFSE (16). nTreg and Teff were cocultured, at a ratio of 1:2, with anti-CD3/anti-CD28-coated Dynabeads (Invitrogen) at a ratio of 1:10 (beads:Teff) in the presence of media, Pam3CSK4 (1 μg/ml; InvivoGen), 0114:B4 LPS (1 μg/ml; InvivoGen), peptidoglycan (100 ng/ml; Sigma-Fluka), or exogenous IL-6 (50 ng/ml; BD Pharmingen). On day 4, cells were stained for anti-CD4 and anti-CD25 (BioLegend) and analyzed by flow cytometry. Data were excluded from analysis when suppression was <10% in media-treated cultures. For STAT3 inhibition, CD4+ T cells were incubated with phosphorlyation inhibitor of STAT3 (Stattic V; Santa Cruz Biotechnology) at 1200 ng/ml for 1 h, washed, and cultured as above.

We calculated the percentage reduction in suppression in two stages (Supplemental Fig. 1). First, the percentage suppression was calculated as [(%Teff alone proliferation − %Treg:Teff coculture proliferation)/%Teff alone proliferation] × 100. We then determined the difference in the percentage suppression between cocultures treated with PAMP or media alone and expressed this as a percentage of media alone: [(% suppression media treated − % suppression PAMP treated)/% suppression media treated] × 100.

TLR1 staining
PBMC were stained with human anti-CD4 (RPA-T4; BioLegend) and anti-TLR1 (GD2.F4; BioLegend), anti-TLR5 (624915; R&D Systems), or isotype control. For a subset, this was followed by intracellular staining using anti-FOXp3 (206D; BioLegend) and a FOXp3 Fix/Perm Buffer Set (BioLegend).

Cytokine measurement
Cell culture supernatants (25 μl) were collected after 48 h of autologous coculture of Treg:Teff or Teff-alone cultures described above. Cytokines were measured by electrochemiluminescence multiplex immunoassay (Meso Scale Discovery, Rockville, MD).

Statistical analysis
We used two-tailed unpaired t tests, two-tailed paired t tests, or a two-tailed nonparametric (Spearman) correlation, as indicated.

Results and Discussion

Minor allele haplotype is associated with enhanced surface expression of TLR1
We (9) and other investigators (10, 11) showed that the minor alleles of nonsynonymous coding polymorphisms in TLR1 (rs5743618T and rs4833095C) are associated with altered cell surface expression of TLR1 on monocytes. We used flow cytometry to determine whether these TLR1 alleles also alter cell surface expression of TLR1 on T lymphocytes. We obtained PBMCs from white subjects carrying two copies of the TLR1 “minor” allele haplotype (rs5743618T, rs5743551G, rs4833095C) and control subjects carrying two copies of the “major” allele haplotype (rs5743618G, rs5743551A, rs4833095T). The minor allele haplotype (rs5743618T, rs5743551G, rs4833095C) and control subjects carrying two copies of the “major” allele haplotype (rs5743618G, rs5743551A, rs4833095T).

FIGURE 1. Variant TLR1 polymorphisms are associated with enhanced TLR1 surface expression on CD4+ T cells. Thawed frozen PBMC were stained with anti-CD4 and anti-TLR1 or anti-TLR5 for four subjects carrying the major and minor allele TLR1 haplotype. Data are shown as MFI after subtraction of MFI of isotype control. Subjects carrying two copies of the minor allele haplotype showed significantly increased surface expression of TLR1 (A), but not TLR5 (B), in the CD4+ T cell population. **p < 0.01, paired t test.
suppression of Teff proliferation to a greater extent in subjects who harbor the TLR1 minor allele haplotype that confers higher TLR1 surface expression.

Absence of Pam3CSK4-induced effect on Teff suppression in absence of Treg

The proliferation or activation state of Teff can change their sensitivity to Treg-mediated suppression. To assess whether Pam3CSK4 altered the proliferation of Teff in the absence of Treg, we measured the proliferation of Teff with anti-CD3/anti-CD28–coated beads in the presence of TLR agonists or media alone. There was no significant difference in proliferation of Teff between TLR1 haplotypes for media-treated or Pam3CSK4-treated Teff (Fig. 2C). This suggests that increased TLR1 surface expression on Treg is not sufficient to observe the effect of TLR1 variants on Pam3CSK4-induced alteration of Treg suppression. In contrast, when we incubated Treg isolated from a subject carrying the major haplotype with Teff from subjects carrying either the minor or major allele TLR1 haplotype, the haplotype-dependent Pam3CSK4-induced reduction in Treg suppression was observed (*p = 0.04, Fig. 2D). These data suggest that increased stimulation of Teff conferred by the minor allele haplotype of TLR1 causes Teff resistance to Treg suppression.

Impaired Treg suppression correlates with higher IL-6 production and is reversed by STAT3 inhibition

Proinflammatory cytokine production, particularly IL-6, is associated with impaired Treg suppression (18). Teff resistance to Treg suppression has been implicated in the pathogenesis of several disease states, including psoriasis, diabetes, and relapsing remitting multiple sclerosis, and the IL-6 pathway was shown to mediate this resistance (17, 19, 20). We reasoned that enhanced cell surface expression of TLR1 on Teff cells from subjects carrying the minor allele haplotype would result in increased Pam3CSK4-induced cytokine levels in the T cell cocultures. We measured cytokine levels in supernatants collected after 48 h of incubation from cocultures of autologous Treg and Teff and the cultures of Teff alone stimulated with TLR agonists in the presence of anti-CD3/anti-CD28–coated beads. In supernatants from cocultures of Teff with Treg, there was a trend toward increased IL-6 production in subjects with the minor allele haplotype; however, this difference did not achieve statistical significance (Fig. 3A). We found that Pam3CSK4-induced IL-6 levels were significantly higher in Pam3CSK4-treated Teff isolated from subjects carrying the minor allele haplotype (*p = 0.03, Fig. 3B). IL-2 and TNF-α levels did not differ by TLR1 genotype for either cultures of subjects harboring the TLR1 minor allele haplotype. IL-6 was measured by multiplex immunoassay in 48-h supernatants from autologous cocultures of Treg:Teff cultured at a ratio of 1:2 (A) and Teff alone (B) in the presence of anti-CD3/anti-CD28 beads with or without TLR agonists. Data are from 10 subjects (5 of each genotype). *p < 0.05, paired t test.
Treg alone or cocultures of Treg with Teff (Supplemental Fig. 2C, 2D). These data support a model whereby Teff-driven differences in Pam3CSK4-induced IL-6 production participate in Teff resistance to Treg suppression. Further support for this model was demonstrated through the observed relationship between IL-6 production and the impairment in autologous Treg suppression of Teff. Although IL-6 levels were not significantly different by genotype from the coculture assays, we observed that IL-6 levels in Teff:Treg cocultures had a strong positive correlation with inhibition of Treg suppression (Spearman’s $r = +0.85$, $p = 0.002$, Fig. 4A).

To determine whether IL-6 is directly involved in the impairment of Treg suppression in our system, we tested whether inhibition of STAT3 phosphorylation, a key event in IL-6 intracellular signaling, abrogated Teff resistance. We found that STAT3 inhibition decreased the average Pam3CSK4-mediated impairment of Treg suppression (Fig. 4B) in cocultures with Teff for subjects of both TLR1 haplotypes ($p = 0.005$). Addition of exogenous IL-6 to cocultures increased major allele Teff resistance, partially abrogating the difference between genotypes (Supplemental Fig. 2E).

In another published study, blockade of IL-6 independently reversed the effects of TLR1/2-mediated impaired Treg suppression (15). Although other proinflammatory cytokines may be involved in modulating Teff resistance in this system, these data demonstrate that IL-6 signaling participates in the induction of Teff resistance to Treg suppression in response to TLR1/2 stimulation.

Work from our laboratory and others highlighted a potential role for genetic variation in TLR1 in various immune-mediated inflammatory diseases. In this study, we showed that a haplotype in TLR1 composed of the minor alleles identified in these clinical-association studies is associated with increased Teff resistance to Treg suppression after stimulation with a TLR1/2 agonist. These findings provide a novel potential mechanism through which these TLR1 variants might affect clinical outcomes. For example, Mayerle et al. (5) detected a strong association between the TLR10/11/6 genetic locus and seroprevalence for *H. pylori*. The most highly associated TLR1 SNP in this study, rs10004195, is in high LD with the two nonsynonymous coding SNPs included in the haplotype in our study ($r^2 = +1$; rs5743618 $r^2 = +0.95$). Our data suggest that Teff from subjects bearing the minor alleles at these loci could be resistant to Treg suppression after chronic exposure to TLR1/2 ligands, which are abundant at these mucosal sites. Similar mechanisms could be responsible for associations with asthma through altered immune responses in the respiratory mucosa.

In summary, we showed that white subjects harboring the minor allele TLR1 haplotype rs5743618T, rs5743551G, rs4833095C have greater cell surface expression of TLR1 on both CD4+FOXP3+ and CD4+FOXP3+ T cells and greater impairment of Treg-mediated suppression of Teff proliferation after treatment with the TLR1/2 agonist Pam3CSK4. This effect is due to Teff resistance and is largely mediated by IL-6 signaling, although other factors may also play a role. These studies highlight the potential importance of common genetic variation in TLR1 in mediating interindividual differences in Treg effects on Teff and provide a new mechanism through which SNP in TLR1 might alter susceptibility to disease states in which Treg:Teff interactions play a key role.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1A. Equations used to calculate percent suppression and percent reduction in suppression. 

**S1A.**

\[
\text{% suppression} = \frac{\% \text{ proliferation Teff alone} - \% \text{ proliferation Treg:Teff}}{\% \text{ proliferation Teff alone}} \times 100
\]

\[
\text{% reduction in suppression} = \frac{\% \text{ suppression Media-treated} - \% \text{ suppression TLR-treated}}{\% \text{ suppression Media-treated}} \times 100
\]

**S1B.**

<table>
<thead>
<tr>
<th>Subject 1</th>
<th>Media-treated:</th>
<th>Pam3CSK4-treated:</th>
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<tbody>
<tr>
<td>T effectors Alone</td>
<td>Treg:Teff 1:2</td>
<td>Treg:Teff 1:2</td>
</tr>
<tr>
<td>(61%)</td>
<td>(22%)</td>
<td>(36%)</td>
</tr>
<tr>
<td>(% \text{ suppression Media-tx} = \frac{61% - 22%}{61%} \times 100 = 64%)</td>
<td>(% \text{ suppression Pam-tx} = \frac{61% - 36%}{61%} \times 100 = 41%)</td>
<td>(% \text{ reduction in suppression} = \frac{64% - 41%}{64%} \times 100 = 36%)</td>
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| Subject 2 | \(68\%\) | \(46\%\) | \(51\%\) |
| \(\% \text{ suppression Media-tx} = \frac{68\% - 46\%}{68\%} \times 100 = 32\%\) | \(\% \text{ suppression Pam-tx} = \frac{68\% - 51\%}{68\%} \times 100 = 25\%\) | \(\% \text{ reduction in suppression} = \frac{32\% - 25\%}{32\%} \times 100 = 22\%\) |

Examples of real CFSE data used to calculate the % suppression and % reduction in suppression for two subjects. Data shown are previously gated on lymphocytes and CD4+ cells.
**Supplemental Figure 2A.** Gated CD4⁺ lymphocytes were identified as FoxP3 positive or negative and analyzed for TLR1 staining. The proportion of TLR1-positive Tregs and Teff (mean percentage) represents the percentage of cells with fluorescence ≥95% of that seen with the isotype-control antibody. Pairwise flow cytometry data is shown for 10 subjects tested, 5 subjects per genotype. 

**S2B.** Summary of data in S2A showing subjects carrying two copies of the minor allele haplotype showed significantly increased surface expression of TLR1 in CD4⁺FoxP3⁺ and CD4⁺FoxP3⁻ populations with *p<0.05 by paired t test.

**S2C, S2D.** Secretion of IL-2 and TNF-α do not differ by genotype. IL-2 and TNF-α were measured by multiplex immunoassay in 48 hour supernatants from Teff alone (C) and autologous co-cultures of Treg:Teff cultured at a ratio of 1:2 (D). Cultures were performed in the presence of anti-CD3/anti-CD28 beads ± TLR agonists. Data are from 10 subjects, 5 of each genotype. No differences were significant by paired t test.

**S2E.** Addition of exogenous recombinant human IL-6 increases effector resistance in subjects of the major allele haplotype. Treg from a single major allele donor were co-cultured with Teff from either the major or minor allele haplotype in the presence of anti-CD3/anti-CD28 beads + Pam3CSK₄ ± exogenous IL-6 (50ng/ml). Addition of exogenous IL-6 increased effector resistance partially abrogating the difference between genotypes.