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

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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.

TLR are 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 (5). Pertinent to our current study, the TLR10/1/6 locus was shown to be 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.
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 major alleles (rs5743618T, rs5743551G, rs4833095C) and age- and gender-matched controls carrying two copies of the haplotype for the three major alleles (rs5743618G, rs5743551A, rs4833095T).

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+ T cells were isolated from thawed frozen autologous or heterologous donors’ PBMC by negative selection with MicroBeads to CD4 and CD25 (Miltenyi Biotech) and labeled with CFSE (16). nTreg and T eff 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 phosphorylation 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 [(%Treg alone proliferation − %Treg:Teff coculture proliferation)/%Treg 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 T eff-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 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). Thus, TLR1 variants associated with increased expression of TLR1 on peripheral blood monocytes are also associated with enhanced TLR1 surface expression on both Treg and T eff.

The minor allele haplotype of TLR1 is associated with greater Pam3CSK4-induced impairment of Treg suppression of Teff

We tested the effects of treatment with Pam3CSK4, a TLR1/2 agonist, on Treg function in subjects with different TLR1 haplotypes. CD4+CD25+ Treg (mean FOXP3+ 90%) were isolated from each subject. Using an in vitro CFSE-based suppression assay, we compared the ability of Treg to suppress the proliferation of autologous CD4+CD25− T eff co-cultured with anti-CD3/anti-CD28-coated beads in the presence or absence of various TLR agonists for 96 h. We found that treatment of cocultures with Pam3CSK4 decreased the average suppression of Teff by Treg (mean, 20%) compared with cocultures treated with media alone (mean, 41%; p = 0.01, data not shown), which is consistent with previous reports (14, 15). We then compared the magnitude of this Pam3CSK4-induced effect in cultures of cells from subjects with either the minor or major allele TLR1 haplotypes. We found that, in the presence of Pam3CSK4, Treg suppression of Teff was impaired to a greater degree in cells from subjects harboring the TLR1 minor allele haplotype compared with those with the major allele haplotype (Fig. 2A, p = 0.02). Evidence that the effect of the TLR1 haplotypes is specific to TLR1/2-mediated responses was provided by the finding that there were no haplotype-specific effects observed when cocultures were treated with LPS (p = 0.48), a TLR4 agonist, or peptidoglycan, a TLR2 agonist that does not require TLR1 engagement for TLR1/2-mediated responses. Statistically, these results are significant (p < 0.01) and indicate that there is an association between TLR1 minor allele haplotype and reduced Treg suppression of Teff. **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 any of the TLR-treated cultures (Fig. 2B). Thus, the genotypic differences in Pam3CSK4-induced modulation of Treg suppression of Teff are not due to alterations in autonomous Teff proliferation.

**TLR1 minor allele haplotype is associated with Teff resistance**

For subjects harboring the *TLR1* minor allele haplotype, a greater Pam3CSK4-induced reduction in Treg suppression could be attributed to impaired Treg suppression or increased Teff resistance to Treg suppression. To address these possibilities, we performed allogeneic coculture experiments in which either the genotype of the Treg or Teff population was held constant as the major allele haplotype. We demonstrated previously that Treg suppression is not different in autologous versus heterologous assays (17). When we incubated Teff isolated from a major allele haplotype subject with Treg from subjects carrying either the minor or major allele haplotype in the presence of Pam3CSK4, we did not observe any significant association between *TLR1* haplotype and Treg-suppressive capacity (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 (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 T 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...
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 (rs4833095 $r^2 = 0.95$; 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 T effector 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.

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Disclosures

The authors have no financial conflicts of interest.

References


