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Genetic Variation in *TLR1* is associated with Pam_3CSK_4-induced effector T cell resistance to regulatory T cell suppression

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Abstract

Toll-like receptors (TLRs) play essential roles in the initiation and modulation of immune responses. TLR1/TLR2 heterodimers recognize tri-acylated bacterial lipopeptides, including the synthetic TLR1/2 lipopeptide, Pam_3CSK_4. 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 of varying 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 Pam_3CSK_4. 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.

Introduction

Toll-like receptors (TLRs) are a family of germline-encoded pattern recognition receptors essential to the detection of microbial components (1). Known for their role in innate immunity, TLRs detect a wide range of pathogen-associated molecular patterns (PAMPs). The genes encoding human TLRs 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 PAMPs (2–4). Pertinent to our current study, the TLR1/2 heterodimer recognizes tri-acylated lipopeptides isolated from bacterial pathogens or produced synthetically (Pam_3CSK_4).

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 has been highlighted by very

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strong associations with *Helicobacter pylori* seroprevalance ($p=1.42\times10^{-18}$) (5), self-reported allergy ($p=5.3\times10^{-21}$) (6), and allergic sensitization ($p=5.2\times10^{-11}$) (7). Multiple high frequency missense variants and varying LD structure across populations has made it difficult to pinpoint a single causative variant (8). However, two non-synonymous coding variants associated with clinical phenotypes rs4833095 (Asn248Ser) and rs5743618 (Ser602Ile) may play a role. In Caucasians, 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 messenger RNA or total cell protein levels (9–12). The rs5743618 (Ser602Ile) coding variant has also been shown to confer increased Pam3CSK4-induced NFκB reporter activity in transfected epithelial cell lines that do not express TLR1 (9, 13). We have also identified a SNP in the *TLR1* promoter in high linkage disequilibrium (LD) with these SNPs in which the minor allele rs5743551G is highly associated with death in sepsis (9). To date, the consequences of these polymorphisms with respect to T cell function have yet to be explored.

Multiple different TLRs are expressed in T cells and are capable of modulating the function of various T cell subsets. $CD4^+CD25^+FoxP3^+$ regulatory T cells are pivotal to the suppression of cellular immune responses. Important to these studies, TLR1/2 agonists have been shown in humans to impair Treg suppressive capacity (14, 15). Of interest, this effect was observed in only a subset of subjects implying inter-individual variation (14). In this study, we tested whether common genetic variation in *TLR1* affects TLR1/2 agonist-induced changes in Treg and 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 University of Washington human subjects committees.

Genotyping

We genotyped DNA for three SNPs in *TLR1*: rs5743618, rs4833095, and rs5743551 by Taqman PCR-based allelic discrimination. We identified Caucasian individuals who either 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).

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 percent of $FoxP3^+$ T Cells was 90% on average.

CFSE based suppression assay

$CD4^+CD25^-$ T effector cells (Teff) were isolated from thawed frozen autologous or heterologous donors' PBMC by negative selection with microbeads to CD4 and CD25 (Miltenyi Biotec) and CFSE labeled (16). nTregs (above) and Teff were co-cultured 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, Pam_3CSK_4 (1μg/ml; Invivogen), 0114:B4 LPS (1μg/ml; Invivogen), PGN (100ng/ml; Sigma-Fluka) or exogenous IL-6 (50ng/mL; BD Pharmigen). 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 less than 10% in media treated cultures. For STAT3 inhibition, $CD4^+$ T cells were incubated with phosphorylation inhibitor of STAT3 (Stattic V, Santa Cruz Biotechnology) at 1200ng/ml for one hour, then washed and cultured as above.

We calculated percent reduction in suppression in two stages (Supplemental Figure 1). First, percent suppression was calculated as [(%Teff alone proliferation - %Treg:Teff co-culture proliferation)/%Teff alone proliferation] × 100. We then determined the difference in percent suppression between co-cultures treated with PAMPs or media alone and expressed this as a percent 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 (25μL) were collected at 48 hours of autologous co-culture of Treg:Teff or Teff alone cultures described above. Cytokines were measured by electrochemiluminesence multiplex immunoassay (Meso Scale Discovery, Rockville, MD).

Statistical Analysis

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

Results and Discussion

Minor allele haplotype is associated with enhanced surface expression of TLR1

We and others have previously shown the minor alleles of non-synonymous coding polymorphisms in *TLR1* (rs5743618T and rs4833095C) are associated with altered cell surface expression of TLR1 on monocytes (9–11). We used flow cytometry to determine whether these *TLR1* alleles also alter cell surface expression of TLR1 on T lymphocytes. We obtained PBMCs from Caucasian 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±14yrs. 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 (Figure 1A) than those homozygous for the *TLR1* major allele haplotype (p=0.004). Cell surface expression of TLR5 did not differ by *TLR1* haplotype (Figure 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 Figure 2A, B). 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 Teff.

The minor allele haplotype of *TLR1* is associated with greater Pam_3CSK_4 – induced impairment of Treg suppression of Teff

We tested the effects of treatment with Pam_3CSK_4, a TLR1/2 agonist, on Treg function in subjects of differing *TLR1* haplotype. $CD4^{+}CD25^{HI}$ 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 $CD4^{+}CD25^{-}$ Teff co-cultured with anti-CD3/anti-CD28 coated beads in the presence or absence of various TLR agonists for 96 hours. We found that treatment of co-cultures with Pam_3CSK_4 decreased the average suppression of Teff by Treg (mean of 20%) compared to co-cultures treated with media alone (mean of 41%; p = 0.01; data not shown) which is consistent with previous reports (14, 15). We then compared the magnitude of this Pam_3CSK_4-induced effect in cultures of cells from subjects with either the minor or major allele *TLR1* haplotypes. We found that in the presence of Pam_3CSK_4, Treg suppression of Teff was impaired to a greater degree in cells from subjects harboring the *TLR1* minor allele haplotype as compared to those with the major allele haplotype (Figure 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 co-cultures were treated with LPS (p=0.48), a TLR4 agonist, or peptidoglycan, a TLR2 agonist that does not require TLR1 (p=0.51)(2). Our data show that Pam_3CSK_4 impairs Treg 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 Pam_3CSK_4 –induced effect on Teff suppression in absence of Tregs

The proliferation or activation state of effector T cells can change the sensitivity of Teff to Treg-mediated suppression. In order to assess whether Pam_3CSK_4 altered the proliferation of Teff in the absence of Tregs, we measured 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- or any of the TLR-treated cultures (Figure 2B). Thus, the genotypic differences in Pam_3CSK_4-induced modulation of Treg suppression of Teff are not due to alteration of autonomous Teff proliferation.

TLR1 minor allele haplotype is associated with Teff resistance

For subjects harboring the *TLR1* minor allele haplotype, greater Pam_3CSK_4-induced reduction of Treg suppression could be attributed to impaired Treg suppression or increased Teff resistance to Treg suppression. To address these possibilities we performed allogeneic co-culture experiments where either the genotype of the Treg or Teff population was held constant as the major allele haplotype. We have previously demonstrated that Treg suppression is not different in autologous versus heterologous assays (17). When we incubated Teff isolated from a major allele haplotype subject with Tregs from subjects carrying either the minor or major allele haplotype in the presence of Pam_3CSK_4, we observed no significant association between *TLR1* haplotype and Treg suppressive capacity (Figure 2C). This suggests that increased TLR1 surface expression on Tregs is not sufficient to observe the effect of *TLR1* variants on Pam_3CSK_4-induced alteration of Treg suppression. In contrast, when we incubated Tregs isolated from a subject carrying the major haplotype with Teff from subjects carrying either the minor or major allele *TLR1* haplotype, the haplotype-dependent Pam_3CSK_4-induced reduction in Treg suppression was observed ($p=0.04$, Figure 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

Pro-inflammatory cytokine production, particularly IL-6, is associated with impaired regulatory T cell 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 has been 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 Pam_3CSK_4-induced cytokine levels in the T cell co-cultures. We measured cytokine levels in supernatants collected after 48 hours of incubation from co-cultures 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 co-cultures of Teff with Treg there was a trend towards increased IL-6 production in subjects with the minor allele haplotype but this difference did not achieve statistical significance (Figure 3A). We found that Pam_3CSK_4-induced IL-6 levels were significantly higher in Pam_3CSK_4-treated Teff isolated from subjects carrying the minor allele haplotype ($p=0.03$, Figure 3B). IL-2 and TNF-α levels did not differ by *TLR1* genotype for either cultures of Teff alone or co-cultures of Treg with Teff (Supplemental Figure 2C, D). These data support a model whereby Teff-driven differences in Pam_3CSK_4-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 co-culture assays, we observed that IL-6 levels in Teff:Treg co-cultures were strongly positively correlated with inhibition of Treg suppression (Spearman's $r=0.85$, $p=0.002$, Figure 4A).

To determine if 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 Pam_3-CSK_4-mediated impairment in Treg suppression (Figure 4B) in co-cultures with Teff for subjects of both *TLR1* haplotype (p=0.005). Addition of exogenous IL-6 to co-cultures increased major allele Teff resistance partially abrogating the difference between genotypes (Supplemental Figure 2E). In another published study, blockade of IL-6 independently reversed the effects of TLR1/2-mediated impaired Treg suppression (15). While other pro-inflammatory 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 have highlighted a potential role for genetic variation in *TLR1* in various immune-mediated and inflammatory diseases. Here, we have shown 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. detected a strong association between the *TLR10/1/6* genetic locus and seroprevalence for *Helicobacter pylori* (5). The most highly associated *TLR1* SNP in this study, rs10004195, is in high LD with the two non-synonymous coding SNPs included in the haplotype in our study (rs4833095 r^2=1 and rs5743618 r^2=0.95). Our data suggests that Teff from subjects bearing the minor alleles at these loci could be resistant to Treg suppression after chronic exposure to TLR1/2 ligands that 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 have shown that Caucasian 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 Pam_3CSK_4. This effect is due to Teff resistance and is largely mediated by IL-6 signaling, though other factors may also play a role. These studies highlight the potential importance of common genetic variation in *TLR1* in mediating inter-individual differences in Treg effects on Teff and provide a new mechanism through which SNPs in *TLR1* might alter susceptibility to disease states in which Treg:Teff interactions play a key role.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TLR	Toll-like Receptor
Pam_3CSK_4	N-palmitoyl-S-dipalmitoylglyceryl Cys-Ser-$(Lys)_4$
Treg	regulatory T cell
Teff	effector T cell
PGN	Peptidoglycan

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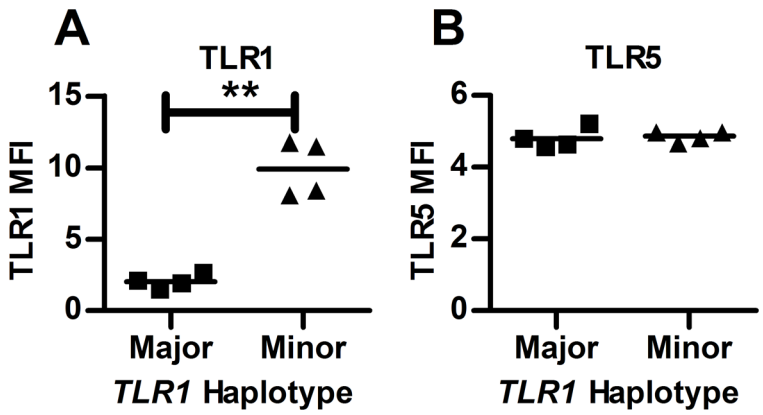

Figure 1. Variant *TLR1* polymorphisms are associated with enhanced TLR1 surface expression on CD4+ T cells

Frozen, thawed PBMC were stained with anti-CD4 and anti-TLR1 or anti-TLR5 for 4 subjects of the major and minor allele *TLR1* haplotype. Data are depicted as median fluorescence intensity (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 with **$p<0.01$ by paired t test.

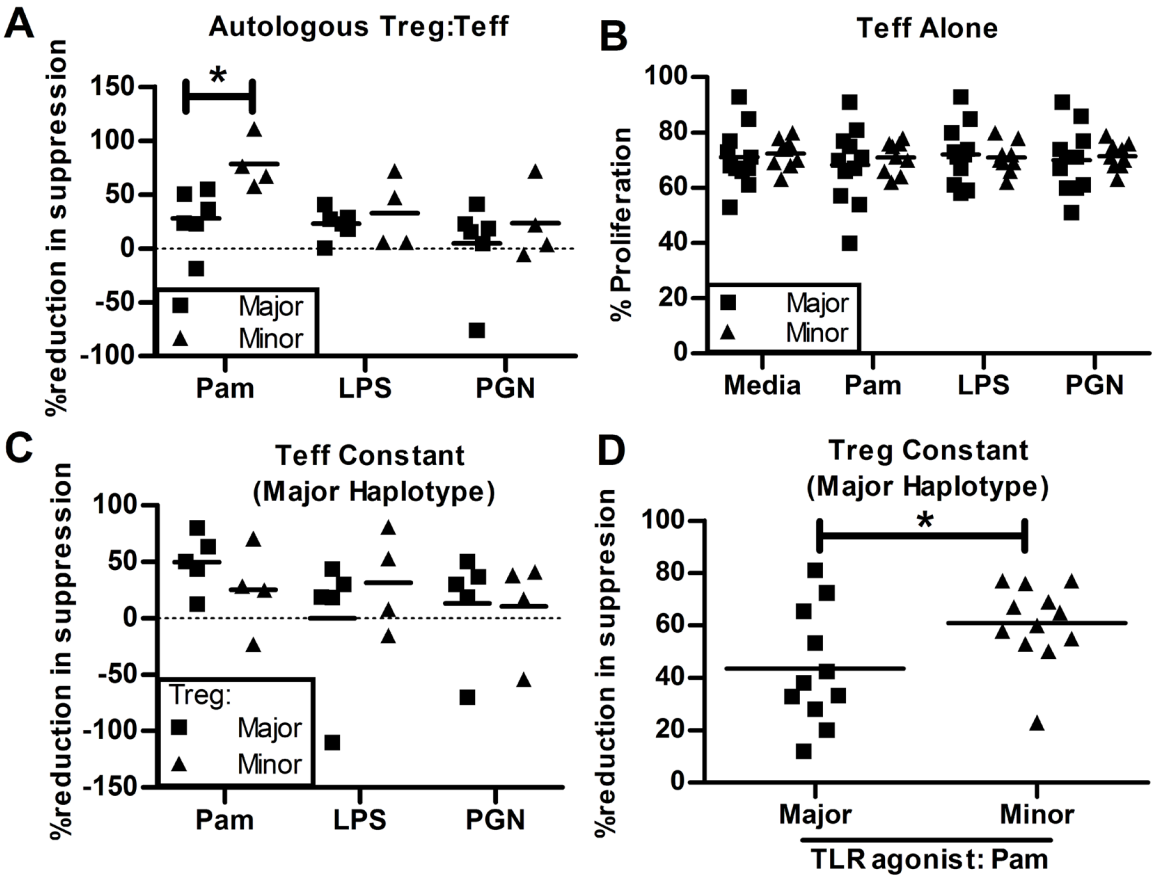

Figure 2. *TLR1* minor allele haplotype is associated with greater Pam_3CSK_4-induced Teff resistance to Treg suppression

(A) Freshly isolated nTreg were co-cultured at a ratio of 1:2 with CFSE-labeled $CD4^+CD25^-$ Teff from frozen autologous PBMC. (B) CFSE-labeled $CD4^+CD25^-$ Teff from frozen PBMC were cultured alone (no Tregs) and percent proliferation determined. (C) Freshly isolated nTreg from minor or major allele haplotype subjects were co-cultured at a ratio of 1:2 with CFSE-labeled CD4+CD25− Teff (major allele haplotype). (D) nTreg (major allele haplotype) were co-cultured at a ratio of 1:2 with CFSE-labeled $CD4^+CD25^-$ Teff isolated from subjects of either the major or minor allele haplotype in the presence of media or Pam_3CSK_4. Cultures were performed in the presence of anti-CD3 and anti-CD28 beads for 4 days ± TLR agonists. Percent reduction in suppression represents the difference in percent suppression between co-cultures treated with TLRs or media alone and expressed as a percent of media alone. Each graph represents data from at least 4 independent experiments. Statistical significance was determined using an unpaired Students t-test. *, $P<0.05$.

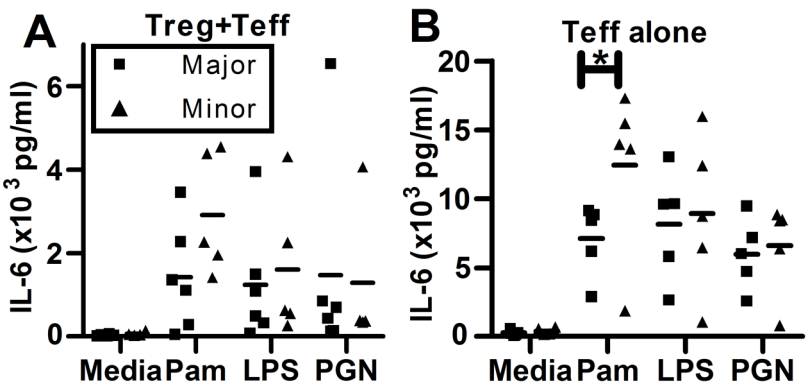

Figure 3. Higher IL-6 production is seen in co-cultures of cells from subjects harboring the *TLR1* minor allele haplotype

IL-6 was measured by multiplex immunoassay in 48 hour supernatants from autologous co-cultures of Treg:Teff cultured at a ratio of 1:2 (A) and Teff alone (B) in the presence of anti-CD3/anti-CD28 beads ± TLR agonists. Data are from 10 subjects, 5 of each genotype. Statistical analysis was performed using a paired t test. *, $P<0.05$.

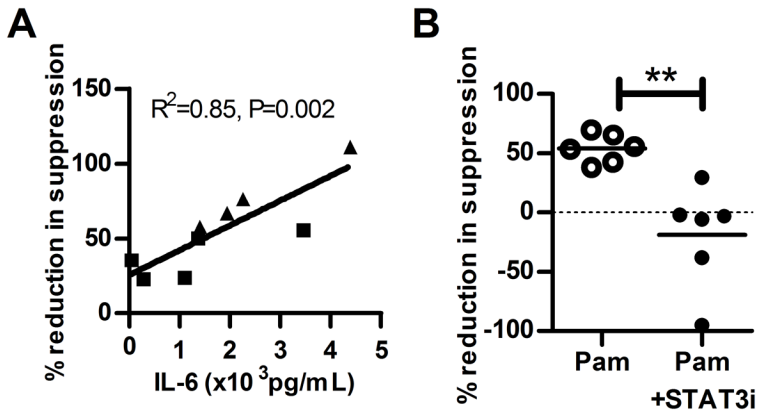

Figure 4. Co-culture IL-6 production correlates with greater reduction in Treg suppression in the presence of Pam_3CSK_4

(A) The correlation (Spearman's) between IL-6 production at 48 hours in autologous co-culture supernatants and percent reduction of suppression from the corresponding assays was performed. (B) Treg and Teff were incubated ± STAT3i (Stattic V), an inhibitor of STAT3 phosphorylation, at 1200ng/ml for one hour, then washed and co-cultured in a Treg suppression assay at a ratio of 1:2 in the presence of anti-CD3/anti-CD28 beads and media or Pam_3CSK_4. '%Reduction in suppression' corresponds to the difference in % suppression between co-cultures treated with Pam_3CSK_4 or media alone expressed as a percent of media alone. The Pam_3CSK_4 + STAT3i culture is normalized to a media + STAT3i treated culture. Data shown are from 6 subjects, 3 of each genotype. The Pam_3CSK_4-induced reduction of suppression is blocked in the presence of inhibition of STAT3 phosphorylation (**$p<0.01$).