TLR2 Stimulation Regulates the Balance between Regulatory T Cell and Th17 Function: A Novel Mechanism of Reduced Regulatory T Cell Function in Multiple Sclerosis

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CD4+CD25hi FOXP3+ regulatory T cells (Tregs) maintain tolerance to self-Ags. Their defective function is involved in the pathogenesis of multiple sclerosis (MS), an inflammatory demyelinating disease of the CNS. However, the mechanisms of such defective function are poorly understood. Recently, we reported that stimulation of TLR2, which is preferentially expressed by human Tregs, reduces their suppressive function and skews them into a Th17-like phenotype. In this study, we tested the hypothesis that TLR2 activation is involved in reduced Treg function in MS. We found that Tregs from MS patients expressed higher levels of TLR2 compared with healthy controls, and stimulation with the synthetic lipopeptide Pam3Cys, an agonist of TLR1/2, reduced Treg function and induced Th17 skewing in MS patient samples more than in healthy controls. These data provide a novel mechanism underlying diminished Treg function in MS. Infections that activate TLR2 in vivo (specifically through TLR1/2 heterodimers) could shift the Treg/Th17 balance toward a proinflammatory state in MS, thereby promoting disease activity and progression.

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Reduced regulatory T cell (Treg) function has been associated with a number of autoimmune diseases, including multiple sclerosis (MS), an inflammatory demyelinating disease of the CNS, which is thought to be initiated by myelin-reactive T cells (1, 2). Thymus-derived natural CD4+CD25hi FOXP3+ Tregs (nTregs) play an important role in maintaining tolerance to self-Ags and preventing autoimmune responses. Depletion of Tregs contributes to the induction of severe autoimmune diseases in animal models, and several studies reported a defect of Tregs in various human autoimmune diseases, including MS (1, 3–5). Tregs are characterized by their expression of the transcription factor FOXP3, which is a master regulator in their development and function (6). The levels of FOXP3 in the CD4+CD25hi population were reported to be decreased in MS (5, 7). In addition, a reduced regulatory function of peripheral blood CD4+CD25hi Tregs was shown in patients with MS compared with healthy subjects (1–3, 5).

Recent studies succeeded in dividing human Tregs into more homogeneous subsets on the basis of cell surface marker expression. The most common approach to defining human Treg subsets is based on combining CD25 and CD127 expression with expression of the classic markers for naïve (CD45RA) and memory (CD45RO) conventional T cells (8, 9). In addition, based on the expression of CD25 and CD45RA, we (10) and other investigators (8) classified human CD4+ T cells into six subpopulations, fractions (Fr.) I–VI. Fr. I, II, and III are FOXP3+, and the degree of FOXP3 protein expression is proportional to CD25 expression. Fr. I and II are highly suppressive when cocultured with responder T cells (Tresps) (Fr. VI), whereas Fr. III cells are nonsuppressive (8, 10, 11).

Several factors may be responsible for the loss of suppression by Tregs, including the presence of proinflammatory stimuli as a result of clinical or subclinical infections. One such proinflammatory stimulus is the cytokine IL-6 (12), which can reduce or abolish the suppressive function of mouse (13) and human (10, 14) Tregs. Stimulation of IL-6R leads to activation of several transcription factors, most notably STAT3 (15). IL-6 and STAT3 are also required for the commitment of naïve T cells toward the differentiation of Th17 cells (16, 17). Th17 cells produce several proinflammatory cytokines, including IL-17, IL-6, IL-21, IL-22, and TNF-α. Although Th17 cells play a key role in the protection against bacterial infections, they also may mediate pathogenicity in organ-specific autoimmune diseases (18). Indeed, it was shown that human Th17 lymphocytes can promote blood–brain barrier disruption and kill human neurons in vitro, suggesting a pathogenic role in MS (19). IFN-β1a, a commonly used disease-modifying immunotherapy for patients with relapsing-remitting MS (RRMS)
(the phase of disease characterized by clinical relapses and remissions), is thought to exert at least part of its ameliorating effect through Th17 inhibition (20). In addition, complete abrogation of relapsing disease (both clinically and radiologically) after bone marrow transplantation was associated with selective reduction of Th17, but not Th1, responses (21).

TLRs are pattern recognition receptors that play a central role in the initiation of innate immunity against invading pathogens (22). Individually, or in combination with other TLRs, they recognize a spectrum of pathogen-associated molecular patterns, including lipids, lipoproteins, nucleic acids, and proteins (22). TLR2 forms heterodimers with either TLR1 or TLR6. The identification of TLRs on T cells, and particularly on Tregs, was an important development in the field of innate immune-regulation of adaptive T cell responses (10, 13, 23, 24). We (10) and others (14, 25) demonstrated that TLRs modulate the functions of Tregs. We showed that stimulation of TLR2 with Pam3Cys, a synthetic triacylated lipopeptide agonist (26), reduces Treg suppressive function and induces them to release IL-17A and IL-17F (10). These TLR2-mediated effects on Tregs are dependent, at least in part, on IL-6, supporting an important role for this cytokine in balancing Treg and Th17 functions (27). Pam3Cys is a model for the effect of bacterial lipopolysaccharides, and, more generally, it is a strong and selective TLR2 agonist of target cell functions (28).

In contrast to Pam3Cys, which stimulates heterodimers composed of TLR2 and TLR1, diacylated lipopeptides, such as Pam2Cys-Ser-Lys(4) and FSL-1, stimulate heterodimers composed of TLR2 and TR6 (29). Of note, the latter are not effective modulators of Treg function (10, 14). The effect of TLR2 stimulation on the suppressive functions of Tregs from MS patients has not been studied. Because infections are thought to influence both susceptibility to MS (30) and the occurrence of clinical exacerbations (31, 32), studies that would unravel the relationship among infections, TLR2 activation, and MS pathogenesis are warranted. We hypothesized that Tregs in patients with MS are more susceptible to TLR2-induced modulation of their function and to Th17 differentiation than in healthy controls (HC). We compared the effect of TLR2 stimulation on the suppressive functions of CD4+CD25+CD127low Tregs and other subsets (including CD4+CD25++CD45RA- naive Tregs and CD4+CD25++CD45RA- effector Tregs) in patients with RRMS and HC. A T cell–suppression assay was used to measure the suppressive function of Tregs (10). Stimulation of TLR2 in RRMS patients reduced Treg suppressive function more potently than in HC. In addition, Treg populations isolated from RRMS patients produced more IL-17 and IL-22 than did those from HC upon TLR2 stimulation. CD4+ T cells from RRMS patients also expressed higher IL-17 and IL-22 than did those from HC upon TLR2 stimulation (10).

Flow cytometric analysis of T cells

CD4+ enriched T cells, CD4+CD25+CD127low Tregs, and subpopulations of CD4+ T cells (CD4+CD25++CD45RA- cells, Fr. I; CD4+CD25++CD45RA- cells, Fr. II; CD4+CD25++CD45RA- cells, Fr. III; CD4+CD25+CD45RA- cells, Fr. IV; CD4+CD25+CD45RA- cells, Fr. V; and CD4+CD25+CD45RA- naive Tresp, Fr. VI) from MS patients and HC were cultured alone or cocultured in triplicate wells at 1:16, 1:8, and 1:4 Treg/Tresp ratios. Cultures were carried out in the absence or presence of Pam3Cys or stimulation with the TLR2 agonist, fibroblast-stimulating ligand-1 (FSL-1) (both at 5 × 10−9 m). Where indicated, neutralizing Abs to TLR2 (10 μg/ml; R&D Systems), IL-6 (5 ng/ml), or neutralizing anti-IL-6 (1 μg/ml; R&D Systems) were added to cultures on day 0. Cells were surface stained with anti-CD3 (BD Biosciences) and anti-CD4 (BD Biosciences), and anti–RAR-related orphan receptor C (RORC; R&D Systems). Purity of sorted cells was always >95% (Supplemental Fig. 1C, 1D).

Materials and Methods

Study participants

MS patients attending outpatient clinics at Nottingham University Hospitals were recruited for this study. The study included 35 adult MS patients with clinically definite MS, according to the McDonald Criteria (33), aged 23–51 y (mean: 36.5 ± 6.4 SEM). Patients had not been treated with any immunomodulatory drugs or corticosteroids within 3 mo of study entry. In addition, 47 HC aged 23–52 y (mean: 34.0 ± 8.3 SEM) were recruited. HC had no history of autoimmune disease or recent symptomatic infections. There was no significant age difference between MS patients and HC (p = 0.153). All MS patients and HC gave written informed consent prior to blood sampling. The study was approved by the Nottingham Research Ethics Committee and by Nottingham University Hospitals National Health Service Trust Research and Innovation Services.

Purification of CD4+CD25+CD127low Tregs and subpopulations of CD4+ T cells by FACS sorting

PBMCs were isolated from 50 ml peripheral blood from each study participant. CD4+ T cells were then isolated from PBMCs by negative selection using MACS MicroBeads (Miltenyi Biotec). CD4+ T cells were stained with CD4-allophycocyanin-Cy7 (BD Biosciences), CD25-PE (Miltenyi Biotec), and CD127-FITC (eBioscience) and then sorted into CD4+CD25+CD127low Tregs and CD4+CD25 DC127+ Tresp (Supplemental Fig. 1A). In separate experiments, CD4+ T cells were stained with CD44-allophycocyanin-Cy7, CD25-PE, and CD45RA-FITC (eBioscience) and classified into six subpopulations of CD4+ T cells (8, 10, 34). The following populations of CD4+ T cells were sorted: CD4+CD25++CD45RA- (naive or resting Tregs; Fr. I), CD4+CD25++CD45RA- (effector or activated Tregs; Fr. II), CD4+CD25+CD45RA- (memory-like non-Tregs; Fr. III), and CD4+CD25+CD45RA- (naive Tcells; Fr. VI; in our coculture experiments these cells are designated as Tresps). The other fractions consist of memory-like CD4+CD45RA- FOXP3- non-Tregs (Fr. IV and V together), as previously described by our group (10) and other investigators (8) (Supplemental Fig. 1B). The percentage frequency of each subset in RRMS patients (n = 7) and HC (n = 7) is as follows: CD4+CD25++CD45RA- (naive or resting Tregs; Fr. I); HC = 2.1 ± 0.5, RRMS = 2.2 ± 0.8, p = 0.544; CD4+CD25+CD45RA- (effector or activated Tregs; Fr. II); HC = 2.1 ± 0.5, RRMS = 0.361; CD4+CD25CD45RA- (memory-like non-Tregs; Fr. III); HC = 4.1 ± 2.5, RRMS = 5.6 ± 3.1, p = 0.019; CD4+CD25+CD45RA- (memory-like non-Tregs; Fr. IV); HC = 13 ± 1.5, RRMS = 13 ± 2.4, p = 0.507; CD4+CD25+CD45RA- (memory-like non-Tregs; Fr. V); HC = 29.1 ± 1.5, RRMS = 28.5 ± 2.6, p = 0.296; and CD4+CD25+CD45RA- (naive Tresps, Fr. VI); HC = 48.9 ± 2.2, RRMS = 48.7 ± 2.6, p = 0.508. We used a MACS XP cell sorter (Becton Coulter) in all cell-sorting assays. Purity of sorted cells was always >95% (Supplemental Fig. 1C, 1D).

Suppression assays

The suppressive functions of Tregs and the effect of TLR2 stimulation were studied in coculture suppression assays. CD4+CD25+CD127low Tregs (2.5 × 106) and CD4+CD25+CD127+ Tresp (2.5 × 106) were cultured alone or cocultured in triplicate wells at 1:16, 1:8, and 1:4 Treg/Tresp ratios. Where indicated, neutralizing Abs to TLR2 (10 μg/ml; R&D Systems), anti-CD28 in the absence or presence of Pam3Cys or stimulation with the TLR2 agonist, fibroblast-stimulating ligand-1 (FSL-1) (both at 5 × 10−9 m). Where indicated, neutralizing Abs to TLR2 (10 μg/ml; R&D Systems), IL-6 (5 ng/ml), or neutralizing anti-IL-6 (1 μg/ml; R&D Systems) were added to cultures on day 0. Cells were surface stained with anti-CD3 (BD Biosciences) and anti-CD4 (BD Biosciences), and anti–RAR-related orphan receptor C (RORC; R&D Systems). Purity of sorted cells was always >95% (Supplemental Fig. 1C, 1D).
used in all FACS analyses. Cells were acquired using an LSR II flow cytometer (BD Biosciences), collecting a minimum of $1 \times 10^5$ events in each sample, and analyzed using FlowJo software (version X.O.7; TreeStar).

**Flow cytometric analysis of p-STAT3 (p-Y705) and p-STAT1 (p-Y701) expression**

CD4$^+$ T cells and FACS-sorted subpopulations of CD4$^+$ T cells (CD4$^+$ CD25$^{++}$CD45RA$^-$naive Tregs), CD4$^+$CD25$^{++}$CD45RA$^-$ [effector Tregs]), CD4$^+$CD25$^{+}$CD45RA$^-$ [memory non-Tregs], and CD4$^+$CD25$^{-}$CD45RA$^+$ [naive Tregs]) were cultured for 1 h in the presence or absence of Pam3Cys (5 μg/ml) and FSL-1 (5 μg/ml), with or without neutralizing anti–TLR2 Ab (10 μg/ml; R&D Systems). Cells were fixed with 1.5% formaldehyde (final concentration) for 10 min at room temperature and then permeabilized in 100% ice-cold methanol for 10 min at 4°C. Cells were then washed twice with FACS buffer (PBS with 1% FCS) and stained with PE-conjugated p-STAT3 (Y705) and allophycocyanin-conjugated p-STAT1 (p-Y701) Abs (all from BD Biosciences) for 1 h at room temperature. Flow cytometric analysis was performed using an LSR II flow cytometer (BD Biosciences) and FlowJo software (version X.O.7; TreeStar).

**Analysis of cytokine production in culture supernatants**

Human cytokine multiplex kits (eBioscience) were used to determine IL-1β, IL-17A, IL-21, IL-22, and IL-6 in the supernatants from cultures of CD4$^+$ T cells, CD4$^+$CD25$^{++}$CD45RA$^+$ [naive Tregs], CD4$^+$CD25$^{++}$CD45RA$^-$ [effector Tregs], CD4$^+$CD25$^{+}$CD45RA$^-$ [memory non-Tregs], and CD4$^+$CD25$^{-}$CD45RA$^+$ [naive Tregs]). We recently reported that the human CD4$^+$CD25$^{+}$ Treg population expresses higher levels of TLR2 compared with non-Treg fractions (10). In this study, we compared the expression of TLR2 by CD4$^+$CD25$^{+}$CD127$^{hi}$ Tregs from HC and patients with RRMS. We FACS sorted CD4$^+$CD25$^{+}$CD127$^{hi}$ Tregs as previously described (10). First, the comparison showed that the expression of TLR2 density in unstimulated Tregs was higher in patients than in HC patients.

**Quantitative immunoblotting**

CD4$^+$ enriched T cells were cultured or not with anti-CD3 and anti-CD28 (1 μg/ml; R&D Systems) were added to cultures on day 0.

**Statistical analysis**

The mean (± SEM) cpm measured by thymidine uptake of triplicate cultures was calculated for each coculture condition. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Comparisons between groups were made using the Mann–Whitney U test. The p values < 0.05 were considered significant.

**Results**

**Higher density of TLR2 expression by Treg populations from patients with RRMS compared with HC**

We recently reported that the human CD4$^+$CD25$^{+}$ Treg population expresses higher levels of TLR2 compared with non-Treg fractions (10). In this study, we compared the expression of TLR2 by CD4$^+$CD25$^{+}$CD127$^{hi}$ Tregs from HC and patients with RRMS. We FACS sorted CD4$^+$CD25$^{+}$CD127$^{hi}$ Tregs as previously described (10). First, the comparison showed that the expression of TLR2 density in unstimulated Tregs was higher in patients than in HC patients.
in HC (Fig. 1A, 1C; \( p = 0.032 \)). Next, these cells were stimulated for 48 h by plate-bound anti-CD3 and anti-CD28 (1 \( \mu \text{g/ml} \)) in the absence or presence of 5 \( \mu \text{g/ml} \) Pam3Cys (an agonist of TLR1/2 heterodimers) or FSL-1 (which stimulates TLR2/6 heterodimers). Cells from patients expressed higher levels of TLR2 than did those from HC (Fig. 1B, 1D; \( p = 0.024 \)). In addition, the expression of TLR2 was enhanced in both patients and controls after stimulation with Pam3Cys (Fig. 1B, 1D; HC: \( p = 0.013; \) RRMS: \( p = 0.001 \)), with higher levels observed in the MS group (Fig. 1D; \( p = 0.001 \)). In contrast, stimulation with FSL-1 did not enhance TLR2 expression in either group (Fig. 1D).

We then compared the expression of TLR2, TLR1, and TLR6 in subpopulations of CD4\(^+\) T cells, as defined on the basis of CD25 and CD45RA expression (8, 10), between HC and patients. In both groups, there was a clear pattern of higher expression of TLR2 in naive (Fr. I) and effector (Fr. II) Tregs and in memory non-Tregs (Fr. III) compared with other T cell populations. In addition, Fr. I–III from patients expressed higher levels of TLR2 compared with HC (Fig. 1E). The expression of TLR1 and TLR6 was also higher in Treg than in non-Treg T cell subsets in both patients and HC. We also found that TLR1, but not TLR6, was expressed more in Treg fractions from MS patients than in HC (Fig. 1F, 1G). Together, these data demonstrate that Treg populations are the preferred target of TLR2 agonists and that higher expression of TLR2 and TLR1 on Tregs from MS patients may render them more responsive to TLR2 agonists than those from HC.

**TLR2 stimulation preferentially reduces the suppressive functions of CD4\(^+\)CD25\(^{hi}\)CD127\(^{neg/low}\) Tregs from RRMS patients**

We compared the effect of TLR2 stimulation on the suppressive functions of CD4\(^+\)CD25\(^{hi}\)CD127\(^{neg/low}\) Tregs between MS patients and HC. We were particularly interested in this subset of Tregs because a previous report showed that, when CD4\(^+\)CD25\(^{hi}\) T cells expressing the IL-7R \( \alpha \)-chain (CD127) were included in the Treg population, the suppressive function of such Tregs was weaker in MS patients than in controls, whereas the function of CD4\(^+\)CD25\(^{hi}\)CD127\(^{neg/low}\) Tregs did not differ between the two groups (37).

We FACs sorted highly pure CD4\(^+\)CD25\(^{hi}\)CD127\(^{neg/low}\) Treg and CD4\(^+\)CD25\(^{−}\) Tresp subsets. Tregs from HC and patients with RRMS did not proliferate in response to plate-bound anti-CD3/anti-CD28, with or without Pam3Cys (data not shown). By contrast, Tresps from both groups proliferated after stimulation with plate-bound anti-CD3/anti-CD28, and the presence of Pam3Cys did not significantly increase such proliferation in either group (data not shown).

In the absence of Pam3Cys, there was no significant difference in the suppressive capacity of Tregs between MS and HC groups (Fig. 2), whereas stimulation with Pam3Cys reduced the suppressive activity of Tregs in both groups (Fig. 2). However, this effect was more potent in patients than in controls at the tested Treg/Tresp ratios (1:16, \( p = 0.004; \) 1:8, \( p = 0.021; \) and 1:4, \( p = 0.041 \)) (Fig. 2). These observations are consistent with the higher expression of TLR2 by Treg populations in MS patients (Fig. 1). Together, these data show that TLR2-induced loss of suppressive function is more prominent in CD4\(^+\)CD25\(^{hi}\)CD127\(^{neg/low}\) Tregs from MS patients compared with HC.

**Naive and effector Tregs from patients with RRMS are more susceptible to TLR2-mediated reduction of suppressive function**

Our next aim was to examine the effect of TLR2 stimulation on distinct subsets of human Tregs (8, 10). To this aim, we FACs sorted highly pure subsets of CD45RA\(^+\)CD25\(^−\) naive Tresp, CD45RA\(^+\)CD25\(^{+}\) naive Tregs, and CD45RA\(^−\)CD25\(^{++}\) effector Tregs from HC and patients with RRMS. Naive Tresp from both groups proliferated after stimulation with plate-bound anti-CD3/anti-CD28. The addition of Pam3Cys did not induce significant proliferation in either group (data not shown). By contrast, naive and effector Tregs from both groups did not proliferate after stimulation with plate-bound anti-CD3/anti-CD28 in the absence or presence of Pam3Cys (data not shown).

To measure the effect of TLR2 stimulation on Treg suppression, naive Tregs or effector Tregs were cocultured with naive Tresp at 1:16, 1:8, and 1:4 ratios on plate-bound anti-CD3/anti-CD28 in the absence or presence of Pam3Cys. Naive Tregs from HC and RRMS patients suppressed the proliferation of naive Tresp at 1:16, 1:8, and 1:4 ratios (Fig. 3A–C, Table I). Of note, naive Tregs from RRMS patients were less potent suppressors of Tresp proliferation (\( p = 0.024, p = 0.042, \) and \( p = 0.031 \) at 1:16, 1:8, and 1:4 Tregs/naive T cell ratio, respectively; Fig. 3A–C, Table I).
Although stimulation with Pam3Cys led to a reduction in the suppressive function of naive Tregs from both HC and RRMS patients, as indicated by the increased proliferation of naive Tresps (HC: $p = 0.013$, $p = 0.022$, and $p = 0.011$; RRMS: $p = 0.001$, $p = 0.001$, and $p = 0.002$ at 1:16, 1:8, and 1:4 naive Treg/Tresp ratio, respectively; Fig. 3A–C, Table I), the magnitude of Pam3Cys-induced loss of Treg suppressive function was significantly greater in patients than in HC (Fig. 3A–C, Table I). Similarly, effector Tregs from both HC and patients with RRMS were more susceptible to TLR2-induced loss of suppression than those from HC ($n = 13$) (Tables I, II). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns, not significant.

**FIGURE 3.** TLR2 stimulation reduces the suppressive function of CD4$^+$CD25$^{++}$CD45RA$^+$ naive and CD4$^+$CD25$^{+++}$CD45RA$^-$ effector Tregs from both HC and MS patients. FACSt-sorted CD4$^+$CD25$^-$CD45RA$^+$ cells (naive Tresps, Fr. VI) were cocultured with CD4$^+$CD25$^{++}$CD45RA$^+$ cells (naive Tregs, Fr. I) or CD4$^+$CD25$^{+++}$CD45RA$^-$ cells (effector Tregs, Fr. II) from HC and RRMS patients. Cells were cultured in triplicate wells at a naive Treg/Tresp ratio of 1:16, 1:8, and 1:4, respectively (A–C) and at an effector Treg/Tresp ratio of 1:16, 1:8, and 1:4, respectively (D–F), in the absence or presence of Pam3Cys (5 μg/ml) on plate-bound anti-CD3 and anti-CD28 (1 μg/ml). Cells were pulsed with $[^{3}H]$thymidine for the last 16 h of the 6-d culture. Data represent lymphocyte proliferation expressed as cpm. Naive and effector Tregs from MS patients ($n = 11$) were more susceptible to TLR2-induced loss of suppression than those from HC ($n = 13$) (Tables I, II). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns, not significant.

**TLR2 stimulation preferentially enhances Th17 differentiation of Tregs from RRMS patients**

Th17 cells play a pathogenic role in several autoimmune diseases, including MS (18, 38). We recently showed that TLR2 stimulation enhances IL-17 production and promotes a Th17 shift in human Tregs (10). We analyzed the effect of TLR2 stimulation on the differentiation of CD4$^+$CD25$^+$CD127$^{low}$ Tregs (10, 37) after TLR2 activation in both study groups. Stimulation with Pam3Cys significantly enhanced the expression of IL-17A, RORC, and CCR6 in Tregs isolated from patients ($p = 0.031$, $p = 0.004$, $p = 0.002$, respectively; Fig. 4A–C) and controls ($p = 0.043$, $p = 0.012$, $p = 0.032$, respectively; Fig. 4A–C). However, the magnitude of stimulation was significantly greater in MS patients (IL-17A, $p = 0.033$; RORC, $p = 0.041$; CCR6, $p = 0.031$; Fig. 4A–C).
Next, we investigated the secretion of Th17 cytokines by Tregs cultured in the same conditions. Treatment with Pam3Cys enhanced the production of IL-17A, IL-17F, IL-22, and IL-6 by Tregs in both patients and controls (Fig. 4D–G). However, the effect in patients was stronger than in controls (IL-17A, p = 0.032; IL-17F, p = 0.043; IL-22, p = 0.021; IL-6, p = 0.031; Fig. 4D–G). TLR2 stimulation did not affect the Th1 signature cytokine, IFN-γ, or the Th1 transcription factor, T-bet, in either HC (10) or RRMS patients (data not shown). Together, these data indicate that TLR2 stimulation leads to enhanced expression and secretion of Th17 markers in Treg populations, with a more potent effect in MS patients.

We then assessed the expression of Th17 markers in CD4+CD25++CD45RA+ naive Tregs, CD4+CD25++CD45RA− effector Tregs, CD4+CD25++CD45RA− memory non-Tregs (Fr. III), and CD4+CD25−CD45RA− naive Tregs in both groups. The expression of IL-17A and IL-22 was assessed in the described subpopulations of CD4+ T cells after culture for 72 h in the presence or absence of Pam3Cys on plate-bound anti-CD3 and anti-CD28. Effector Tregs and memory non-Tregs in the MS group had higher baseline expression of IL-17A (p = 0.013 and p = 0.021, respectively; Fig. 5B, 5C) and IL-22 (p = 0.0023 and p = 0.011, respectively; Fig. 5F, 5G) than HC. Treatment with Pam3Cys enhanced the expression of IL-17A and IL-22 by naive Tregs, effector Tregs, and memory non-Tregs (for IL-17A HC: p = 0.006, p = 0.043, p = 0.011, respectively; RRMS patients: p = 0.002, p = 0.003, p = 0.005, respectively; Fig. 5E–G, Supplemental Fig. 4). Of note, the magnitude of TLR2-induced IL-17 expression by naive Tregs, effector Tregs, and memory non-Tregs in RRMS patients was higher than in HC (p = 0.022, p = 0.027, p = 0.013, respectively; Fig. 5A–C, Supplemental Fig. 4). The same pattern was found for IL-22 in naive and effector Tregs (p = 0.002 and p = 0.002, respectively; Fig. 5E, 5F, Supplemental Fig. 4). TLR2 stimulation did not induce IL-17 and IL-22 expression in naive Tregs (Fig. 5D, 5H). These data are consistent with observations by Miyara et al. (8) showing low levels of RORC and AHR transcripts in Fr. VI. More recent studies also demonstrated that memory non-Tregs (Fr. III) produce IL-17 (11).

We also studied the effect of TLR2 stimulation on Th17 differentiation by CD4+ T cells and subpopulations of T cells by analyzing mRNA expression of Th17 transcription factors, AHR, RORγt, and STAT3, and the Th1 transcription factor, T-bet, by RT-PCR (data not shown). CD4+ T cells or the indicated subsets were cultured for 72 h on plate-bound anti-CD3 and anti-CD28 Abs in the presence or absence of Pam3Cys. Following RNA extraction and cDNA synthesis, we performed RT-PCR and found that TLR2 stimulation augmented the expression of STAT3, AHR, and RORγt mRNAs, but not T-bet, in CD4+ T cells. TLR2 stimulation upregulated the expression of STAT3 mRNA in all T cell subsets and the expression of AHR and RORγt mRNAs in naive and effector Tregs and memory non-Tregs. Expression of T-bet mRNA was not affected in the T cell subsets (data not shown).

**TLR2 stimulation preferentially enhances IL-6 expression by CD4+ T cells and Treg subsets from RRMS patients**

Although it is known that TLR2 stimulation enhances the production of IL-6 by Tregs in HC (10), we extended our previous investigation to T cells from RRMS patients. CD4+ T cells were cultured for 24 h on plate-bound anti-CD3 and anti-CD28 in the presence or absence of Pam3Cys. CD4+ T cells from MS patients expressed higher baseline levels of IL-6 and IL-6Rα, and stimulation with Pam3Cys increased IL-6, IL-6Rα, and gp130 expression in naive Tregs and CD4+ T cells from RRMS patients to

### Table I. The effect of Pam3Cys on the suppressive functions of naive Tregs (Fr. I) from MS patients and HC

<table>
<thead>
<tr>
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<th>HC</th>
<th>MS Patients</th>
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<tr>
<td>% Suppression (medium)</td>
<td>42.20%</td>
<td>68.32%</td>
</tr>
<tr>
<td>% Suppression (Pam3Cys)</td>
<td>23.46%</td>
<td>55.27%</td>
</tr>
<tr>
<td>% Loss of suppression</td>
<td>18.74%</td>
<td>13.05%</td>
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Data are expressed as average percentage of suppression in the presence or absence of Pam3Cys. Percentage of suppression was calculated using the following formula: [1 − (average cpm incorporated in the coculture)/cpm of responder population alone] × 100%. Percent loss of suppression was calculated as follows: (percent suppression with medium − percent suppression in the presence of Pam3Cys). Significance of the difference in the percent loss of Treg suppression between HC (n = 13) and RRMS patients (n = 11): 1:16, p = 0.002; 1:8, p = 0.001; 1:4, p = 0.04, paired t test.

### Table II. The effect of Pam3Cys on the suppressive functions of effector Tregs (Fr. II) from MS patients and HC

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<th></th>
<th>HC</th>
<th>MS Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Suppression (medium)</td>
<td>51.34%</td>
<td>65.17%</td>
</tr>
<tr>
<td>% Suppression (Pam3Cys)</td>
<td>29.67%</td>
<td>46.82%</td>
</tr>
<tr>
<td>% Loss of suppression</td>
<td>22.67%</td>
<td>18.35%</td>
</tr>
</tbody>
</table>

Data are expressed as average percentage of suppression in the presence or absence of Pam3Cys. Percentage of suppression was calculated using the following formula: [1 − (average cpm incorporated in the coculture)/cpm of Tresp population alone] × 100%. Percent loss of suppression was calculated as follows: (percent suppression with medium − percent suppression in the presence of Pam3Cys). Significance of the difference in the percent loss of suppression between HC (n = 13) and RRMS patients (n = 11): 1:16, p = 0.002; 1:8, p = 0.001; 1:4, p = 0.04, paired t test.

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a greater extent compared with the control group (Fig. 6B, Supplemental Fig. 2). Similarly, higher expression of IL-6 was observed in naive Tregs, effector Tregs, and memory non-Tregs (Fig. 6B–D). Neutralizing anti-TLR2, but not anti-TLR1, blocked the effects of TLR2 activation by Pam3Cys (Fig. 6A–D). Both anti-TLR2– and anti-TLR1–neutralizing Abs did not significantly block Pam3Cys-induced IL-6 expression in the memory non-Treg subset (Fr. III) from RRMS patients (Fig. 6D). Stimulation with Pam3Cys did not enhance IL-6 expression by naive Tresps (Fig. 6E). Together, these data demonstrate greater background levels of IL-6, as well as TLR2-induced IL-6 production, in CD4⁺ T cell subsets in RRMS patients.

**TLR2 stimulation induces p-STAT3 expression in CD4⁺ T cell subsets**

Because IL-6 activates STAT3 (15), we next assessed the effect of TLR2 stimulation on the phosphorylation of STAT3 protein. STAT3 is an important transcription factor known to bind both the IL-17A and IL-17F promoters (39). CD4⁺ T cells and FACS-sorted subpopulations of CD4⁺ T cells (CD4⁺CD25hiCD127neg/low Tregs, CD4⁺CD25hiCD127neg/low Tregs from HC and RRMS patients were cultured in the absence or presence of Pam3Cys on plate-bound anti-CD3 and anti-CD28. Cultures were incubated for 96 h, and the cells were harvested and stained for the expression of IL-17A (A), CCR6 (B), and RORC (C). The percentage of cells expressing IL-17A, RORC, and CCR6 was compared between HC and RRMS patients. Culture supernatants were analyzed by ELISA for the production of IL-17A (D), IL-17F (E), IL-22 (F), and IL-6 (G). Cytokine production was compared between the two groups. Data represent average concentrations ± SE of the indicated cytokines and markers in n = 13 HC and n = 15 RRMS patients. *p < 0.05, **p < 0.01. ns, not significant.
anti-TLR1 Abs failed to block Pam3Cys-induced p-STAT3 expression by memory non-Tregs in both groups (Fig. 7D). Neutralizing anti–IL-6 Abs blocked Pam3Cys-induced phosphorylation of STAT3 in CD4+ enriched T cells, as well as in naive and effector Treg, memory non-Treg, and naive Tresp subsets (Supplemental Fig. 3).

Next, we confirmed our observation that Pam3Cys-induced phosphorylation of STAT3 is IL-6 dependent using Western blotting experiments. Enriched CD4+ T cells from HC were activated with anti-CD3 and anti-CD28 in the presence or absence of Pam3Cys, IL-6, and a neutralizing anti–IL-6 Ab for 2 or 6 h. Neutralization of IL-6 blocked Pam3Cys-induced phosphorylation of STAT3 at both time points. At 6 h, activation of the TCR with costimulation through CD28 was sufficient to induce IL-6–dependent phosphorylation of STAT3 (Fig. 8), suggesting that Pam3Cys-induced IL-6 accelerates phosphorylation of STAT3 but is not strictly required (Fig. 8). Of note, we observed a degree of interindividual variability in STAT3 phosphorylation in response to Pam3Cys-induced IL-6 in T cell subsets from HC and RRMS patients. Data obtained from n = 7 HC and n = 7 RRMS patients are shown. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant.
Neutralization of TLR2 blocks Pam3Cys-induced, but not cytokine-induced, production of Th17 cytokines

Next, we investigated the mechanism of TLR2-induced differentiation of Tregs into Th17 cells in our system. For this purpose, CD4+ enriched T cells were cultured on plate-bound anti-CD3 and anti-CD28 for 72 h in the absence or presence of Pam3Cys or Th17-differentiation mixture of cytokines and Abs (10 ng/ml IL-1β, 30 ng/ml IL-6, 10 ng/ml IL-23, 0.5 ng/ml TGF-β, and 10 µg/ml neutralizing anti–IL-4 and anti–IFN-γ), with or without 10 µg/ml neutralizing anti-TLR2 Ab. The expression of IL-17 and IL-22 was assessed using flow cytometry, and culture supernatants were assessed for the production of Th17-associated cytokines (IL-17A, IL-17F, IL-21, and IL-22). Consistent with the FACS data (Fig. 9A–C), supernatants of cells from RRMS patients contained higher levels of the above cytokines than did cells isolated from controls (Fig. 9D–G). We next tested whether a neutralizing anti-TLR2 Ab would block Th17 differentiation induced by the standard Th17-differentiation mixture of cytokines and Abs in our cohorts. The TLR2-neutralizing Ab blocked TLR2-induced Th17 differentiation (Fig. 9); however, it did not significantly neutralize Th17 differentiation induced by the Th17-differentiation mixture of cytokines and Abs described above (Fig. 9). Pam3Cys induced greater levels of IL-17A, IL-17F, IL-21, and IL-22 in patients than in controls. However, the cytokine/Ab mixture induced similar levels of Th17 cytokine production in both groups (Fig. 9D–G). Together, these data indicate that different pathways are activated in the two differentiation protocols induced by Pam3Cys and by conventional Th17-inducing cytokines and Abs, and that a number of Th17 cytokines are more potently induced by Pam3Cys in patients than in controls. These data also suggest that cytokine- and TLR2-induced pathways of Th17 differentiation are distinct.

Discussion

In this study, we investigated the mechanisms of action that underpin TLR2-induced loss of Treg activity and shift toward Th17-like phenotype and function in MS patients. TLR expression by Tregs suggests that these cells may respond directly to microbial molecular patterns, thus linking innate signals with adaptive regulatory responses. Such regulation of adaptive immune responses by innate stimuli may explain, in part, how systemic infections influence phases of immune activation (including clinical relapses).
in immune-mediated diseases, such as MS. Our key findings are:
(1) TLR2 is expressed at higher levels by Tregs of RRMS patients than Tregs of HC. Consistent with this observation, CD4+CD25hi 
CD127neg/low Tregs, as well as naive and effector Tregs, from RRMS patients were more susceptible to Pam3Cys-induced re-
duction of suppressive functions; similar to our previous report 
in HC, it is the TLR1/2 heterodimer that mediates the effects 
of TLR2 stimulation in our experimental paradigm, rather than 
TLR2/6 (10). (2) Induction of IL-6, a key mediator of TLR2 
effects on Tregs (10, 40), was more pronounced in the MS group.
(3) Activation of the STAT3 signaling pathways by Pam3Cys 
requires the secretion of IL-6 by target cells, and (4) people with 
MS may be more susceptible than HC to the effects of TLR2-
induced Th17 differentiation. These findings have implications in 
the mechanisms of infection-induced inflammatory activity in 
RRMS.

Higher expression of TLR2 by T cells in MS patients suggests 
that people with MS may be more susceptible than HC to the 
effects of microbial stimuli on Treg populations. This is consistent 
with our functional data showing that loss of suppressive function 
induced by TLR2 agonists is more prominent in the MS group.
Moreover, the ability of Pam3Cys to upregulate the expression of 
TLR2 in MS patients raises the possibility that specific infectious 
agents could exert such effect in vivo. Of note, higher expression of 
TLR2 and greater functional responsiveness to TLR2 agonists in 
MS patients compared with HC could account for the reported 
reduced Treg function in MS patients (1, 5, 41). TLR2 is a rela-
tively flexible innate immune receptor, with broad recognition 
potential that is due, at least in part, to the formation of hetero-
dimers with TLR1 and TLR6. The clear contrast between the 
biological action of Pam3Cys, a triacylated agonist of TLR1/2, 
and the weak or absent activity of FSL-1, a diacylated agonist of 
TLR2/6, in modulating human T cells could be useful in identi-
fying specific types of microbial agonists that may be exerting 
modulation of Treg function in vivo and, potentially, in defining 
therapeutic targets (29).

We examined the role of IL-6 in mediating the effects of TLR2 
stimulation. The importance of this pleiotropic cytokine in in-
flammatory demyelination is underscored by its high expression in 
the CNS (42) and, specifically, in MS lesions at sites of active 
demyelination (43). IL-6 is also one of few cytokines uncondi-
tionally required for the development of experimental autoimmune 
encephalomyelitis, an animal model of MS (44). With TGF-
β, it promotes the generation of Th17 cells while inhibiting TGF-β–
induced Treg differentiation (45), thereby regulating the balance 
between Th17 cells and Tregs (27). IL-6 plays a critical role in 
mediating the effect of TLR2 on Treg function (10, 40). In 
particular, IL-6 is required for TLR2-induced reduction of 
Treg suppressive function, as demonstrated by our neutralization 
experiments. Neutralization of IL-6 also reversed TLR2-induced 
expression of IL-17 and IL-22 in human Tregs (10). In addition to 
its direct effects on Tregs, IL-6 can enhance the resistance of ef-
fector T cells to Treg-mediated inhibition in patients with MS (46, 
47). Our observation of greater production of IL-6 by Tregs upon
stimulation with Pam3Cys in subjects with MS compared with controls suggests that IL-6 is essential in mediating TLR2-induced anti-regulatory and proinflammatory signals in MS. Such signals may be delivered in vivo by microbial stimuli, and neutralization of IL-6 may have therapeutic potential in MS as was demonstrated in other immune-mediated diseases, including juvenile arthritis and the MS-related neuroinflammatory disease, neuromyelitis optica (48, 49).

We observed increased production and secretion of Th17-associated cytokines and increased STAT3 protein phosphorylation in CD4+ T cells and subpopulations of T cells from RRMS patients in response to TLR2. Th17 cells are involved in the pathogenesis of autoimmune inflammatory demyelination and other organ-specific autoimmune diseases, particularly when they differentiate in the presence of TGF-β1 and IL-6 (50). The expression levels of RORC and the production of IL-17 and IL-22 in response to TLR2 activation by human T cells (Fig. 4) (10) suggest that they could have pathogenic potential in vivo (50). In human tissue, Th17 cells were identified in MS lesions (51), where IL-17 production has been associated with active inflammation (52). Our finding that, similar to observations in the mouse (53), TLR2 activation can promote human Th17 differentiation suggests that peripheral activation of T cells in the presence of microbial TLR1/2 stimuli could drive them to a Th17 phenotype and function. IL-22 has been associated with the myelin autoantigen MBP (54). Th22 cells expressed lower levels of IFNAR1 and were insensitive to IFN-β inhibition, suggesting that expansion of Th22 cells in MS could influence resistance to IFN-β therapy (54). Because naturally occurring Tregs express higher levels of TLR2 and TLR1 (10, 14, 24, 55), our data indicate that they may preferentially respond to microbial stimuli in vivo by switching to a Th17/Th22 phenotype and function. Plasticity of human Tregs also was reported by Hafler and colleagues, who observed that FOXP3+ Tregs cultured in the presence of IL-12 acquired a Th1-like phenotype associated with reduced suppressive activity (41). Of relevance to MS, human Th17 cells expressing IL-17 and IL-22 (Figs. 5, 9) have the capacity to cross the blood-cerebrospinal fluid barrier (19) and, therefore, could infiltrate the CNS and initiate tissue-specific inflammatory damage.

To further elucidate the mechanisms by which TLR2-induced IL-6 modulates T cell function, we studied the phosphorylation of the transcription factor STAT3 in response to TLR2 activation. STAT3 is a key mediator of IL-6–induced signaling and of Th17-differentiation programs (50, 56). Phosphorylation of STAT3 was induced by TLR2 activation and found to be IL-6 dependent in neutralization experiments (Figs. 7, 8, Supplemental Fig. 3). In naive and effector Tregs, phosphorylation was significantly greater in MS patients compared with HC (Fig. 7A–C), suggesting that these cells may be particularly susceptible to TLR2 stimuli in MS. Increased STAT3 signaling was observed in experimental CNS inflammatory diseases (16, 57), with a critical role played by its expression in CD4+ T cells (16). In addition, STAT3 phosphorylation in PBMCs was correlated with disease activity (58) and in mediating resistance of effector T cells to Treg-mediated inhibition in MS (46). These data suggest that inhibition of IL-6/STAT3 signaling can be a rational therapeutic strategy in MS. However, similar to previous reports, we observed a degree of variability in responsiveness to TLR2 stimuli in different individuals, possibly due to donor-dependent differences in the TLR expression pattern (10, 14) or polymorphisms in TLR2 or TLR1 (59, 60). This suggests that TLR-targeted treatments would need preliminary testing of TLR expression and responsiveness in individual patients.

Pam3Cys stimulation did not significantly affect the expression of the Th1 transcription factor T-bet and the production of IFN-γ (data not shown). This is in contrast to the clear effects on Treg and Th17 phenotype and function. These data indicate that TLR2
signaling preferentially enhances Th17 differentiation without significantly altering IFN-γ production (10, 53). This suggests that, in MS, IFN-γ is not directly involved in TLR2-induced reduction of Treg function, an effect it can play in response to IL-12.
The Journal of Immunology 5773

(41). The observation that TLR2 neutralization blocked Pam3Cys-induced, but not cytokine-induced, Th17 differentiation (Fig. 9) suggests that distinct pathways are involved in Th17 differentiation. We hypothesize that cytokine-induced Th17 development recruits Th17 transcription factors in a combinatorial program that is different from that induced by TLR2 activation and could be further elucidated by transcriptional regulation studies (56).

Our present findings provide a possible link between urinary and respiratory infections and T cell dysregulation that may lead to MS clinical exacerbations (relapses) (31, 32, 61, 62), because the cell walls or outer membranes of Gram-negative bacteria, such as Escherichia coli, as well as Staphylococcus saprophyticus and other Gram-positive bacteria, which are typically involved (61, 62), contain TLR2 agonists (29, 63). In particular, most in vivo relevant lipopeptides are triacylated and, therefore, are adequately modeled by Pam3Cys used in our study (28, 64). Respiratory viruses that can activate TLR2 (29, 63) also have been associated with MS exacerbations (31, 32, 61). In addition, EBV, which is strongly associated with MS susceptibility (30), is able to activate the transcription factor NF-κB and the production of the chemokine CCL2 in a TLR2-dependent manner (65, 66).

In conclusion, our data suggest that the occurrence of relapses in RRMS patients following specific bacterial or viral infections may be facilitated by stimulation of TLR2 on Tregs, leading to reduction of their suppressive functions and differentiation into a pathogenic Th17 lineage that can mediate tissue damage. The observation that background levels of TLR2 and its heterodimeric partner TLR1 are higher in the MS group and that stimulation with TLR2 agonists leads to upregulation of the receptor indicate that microbial stimuli may drive the higher levels of TLR2 in MS patients in vivo. We propose that, during infections, TLR2 could promote autoimmune responses by inducing IL-6 and tilting the balance between Tregs and Th17 cells toward the latter (10, 27). Thus, TLR2 effects on IL-6 and STAT3 could underpin such effects of infections in vivo. Therefore, acute infections could facilitate disease exacerbations, whereas repeated infections may contribute to the gradual disease progression that is commonly observed in MS patients (67).

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure 1. Subsets of Tregs and Tresp used in the present study. (A) MACS isolated CD4-enriched T cells were labelled with anti-CD4, CD25, CD45RA and CD127, and then FACS-sorted as CD4+CD25hiCD127neg/low Tregs and CD4+CD25-CD127+ Tresp. The percent frequencies of different subsets are summarized in the materials and methods. (B) In separate experiments, CD4+ T cells were FACS-sorted as CD4+CD25++CD45RA+ (Naïve Tregs, Fr. I), CD4+CD25+++CD45RA- (effector Tregs, Fr. II), and CD4+CD25-CD45RA+ (Naïve Tresp, Fr. VI) cells based on CD45RA and CD25 as indicated. The other fractions comprise CD4+CD25++CD45RA- (memory non-Tregs, Fr. III) and “memory-like” non-Tregs CD4+CD25-CD45RA- (Fr. IV and V together). (C, D) Purity of the sorted cells was confirmed by FACS.
Supplemental Figure 2. Pam3Cys enhances IL-6Rα and gp130 expression by CD4+ T cells. CD4+ enriched T cells were cultured for 48 h on plate-bound anti-CD3 and anti-CD28 (1 µg/ml) in the absence or presence of Pam3Cys (5 µg/ml). Cells were analysed by flow cytometry, and the percent expression of (A) gp130 and (B) IL-6Rα was compared between HC and RRMS. Data obtained from 7 HC and 7 RRMS patients are shown. P values < 0.05 were considered significant.
Supplemental Figure 3. Neutralization of IL-6 reduces TLR2-mediated expression of pSTAT3 by CD4+ enriched T cells and subpopulations of CD4+ T cells. CD4+ T cells from HC were cultured in the absence or presence of Pam3Cys, IL-6 and with or without anti-TLR2 antibody or a neutralizing anti-IL-6 antibody on plate-bound anti-CD3 and anti-CD28. Cultures were incubated for 1 h then harvested and stained for the expression of pSTAT3 by flow cytometry. The expression of pSTAT3 was assessed in (A) total CD4+ cells. pSTAT3 expression was also assessed on individual gated subsets of CD4+ T cells: (B) CD4+CD25++CD45RA+ (naïve Tregs, Fr. I), (C) CD4+CD25+++CD45RA- (effector Tregs, Fr. II), (D) CD4+CD25++CD45RA- (memory non-Tregs, Fr. III) and (E) CD4+CD25-CD45RA+ cells (naïve Tresp, Fr. VI). Data obtained from 4 HC are shown (*P < 0.05, ns = not significant).
**Supplemental Figure 4.** TLR2 stimulation enhances IL-17A and IL-22 expression by subpopulations of CD4⁺ T cells from RRMS patients and HC. FACS-sorted CD4⁺CD25⁺⁺CD45RA⁺ (naïve Tregs, Fr. I), CD4⁺CD25⁺⁺⁺CD45RA⁻ (effector Tregs, Fr. II), CD4⁺CD25⁺⁺CD45RA⁻ (memory non-Tregs, Fr. III) and CD4⁺CD25⁻CD45RA⁺ cells (naïve Tresp, Fr. VI) from HC and RRMS were cultured for 96 h in the absence or presence of Pam3Cys (5 µg/ml) on plate-bound anti-CD3 and anti-CD28. Flow cytometry dot plots from representative HC (n=6) and RRMS (n=7) depicting IL-17 and IL-22 expression by CD4⁺CD25⁺⁺CD45RA⁺ (naïve Tregs, Fr. I), CD4⁺CD25⁺⁺⁺CD45RA⁻ (effector Tregs, Fr. II), CD4⁺CD25⁺⁺CD45RA⁻ (memory non-Tregs, Fr. III) and CD4⁺CD25⁻CD45RA⁺ cells (naïve Tresp, Fr. VI). A minimum of 1 x 10⁵ events were acquired in each sample.