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Interaction With Activated Monocytes Enhances Cytokine Expression and Suppressive Activity of Human CD4+CD45RO+CD25+CD127^{low} Regulatory T Cells

Gina J. Walter,[1] Hayley G. Evans,[1] Bina Menon,[2] Nicola J. Gullick,[3] Bruce W. Kirkham,[4] Andrew P. Cope,[2] Frédéric Geissmann,[1] and Leonie S. Taams[1]

Objective. **Despite the high frequency of CD4+ T cells with a regulatory phenotype (CD25+CD127^{low} FoxP3+) in the joints of patients with rheumatoid arthritis (RA), inflammation persists. One possible explanation is that human Treg cells are converted into proinflammatory interleukin-17 (IL-17)–producing cells by inflammatory mediators and thereby lose their suppressive function. The aim of this study was to investigate whether activated monocytes, which are potent producers of inflammatory cytokines and are abundantly present in the rheumatic joint, induce proinflammatory cytokine expression in human Treg cells and impair their regulatory function.**

Methods. **The presence and phenotype of CD4+CD45RO+CD25+CD127^{low} T cells (memory Treg cells) and CD14+ monocytes in the peripheral blood (PB) and synovial fluid (SF) of patients with RA were investigated by flow cytometry. Memory Treg cells obtained from healthy control subjects underwent fluorescence-activated cell sorting and then were cocultured with autologous activated monocytes and stimulated with anti-CD3 monoclonal antibodies. Intracellular cytokine expression, phenotype, and function of cells were determined by flow cytometry, enzyme-linked immunosorbent assay, and proliferation assays.**

Results. **In patients with RA, the frequencies of CD4+CD45RO+CD25+CD127^{low} Treg cells and activated CD14+ monocytes were higher in SF compared with PB. In vitro–activated monocytes induced an increase in the percentage of IL-17–positive, interferon-γ (IFNγ)–positive, and tumor necrosis factor α (TNFα)–positive Treg cells as well as IL-10–positive Treg cells. The observed increase in IL-17–positive and IFNγ-positive Treg cells was driven by monocyte-derived IL-1β, IL-6, and TNFα and was mediated by both CD14+CD16− and CD14+CD16+ monocyte subsets. Despite enhanced cytokine expression, cells maintained their CD25+FoxP3+CD39+ Treg cell phenotype and showed an enhanced capacity to suppress T cell proliferation and IL-17 production.**

Conclusion. **Treg cells exposed to a proinflammatory environment show increased cytokine expression as well as enhanced suppressive activity.**

Immune regulation is essential for the maintenance of peripheral tolerance, prevention of autoimmune diseases, and limitation of chronic inflammation. Human CD4+CD25+CD127^{low} Treg cells, which are characterized by expression of the lineage-specific transcription factor FoxP3, are important immune regulators through their ability to suppress activation, proliferation, and effector functions of a wide range of immune cells including CD4+ and CD8+ T cells, B cells, natural killer cells, and antigen-presenting cells (for review, see refs. 1 and 2). The notion that CD4+CD25+CD127^{low}FoxP3+ T cells are terminally differentiated suppressor cells has been challenged by

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[1]Gina J. Walter, MSc, Hayley G. Evans, PhD, Frédéric Geissmann, MD, PhD, Leonie S. Taams, PhD: King's College London, London, UK; [2]Bina Menon, MBBS, MSc, MRCP, Andrew P. Cope, MD, PhD: King's College London and Guy's and St Thomas' NHS Foundation Trust, London, UK; [3]Nicola J. Gullick, MRCP, PhD: King's College Hospital NHS Foundation Trust, London, UK; [4]Bruce W. Kirkham, MD, FRCP: Guy's and St Thomas' NHS Foundation Trust, London, UK.

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Address correspondence to Leonie S. Taams, PhD, Centre for Molecular and Cellular Biology of Inflammation, 1st Floor New Hunt's House, Room 1.26F, Guy's Campus, King's College London, London SE1 1UL, UK. E-mail: leonie.taams@kcl.ac.uk.

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studies showing that Treg cells can display significant plasticity during development and differentiation in the periphery in response to extrinsic cues (for review, see refs. 3 and 4). The concept of Treg cell plasticity has raised fundamental questions regarding the significance of the presence of CD4+CD25+CD127^{low}FoxP3+ Treg cells at sites of inflammation as well as the stability and safety of ex vivo–expanded human Treg cells for use in immunotherapy.

A landmark study by Miyara et al (5) revealed that Treg cells from human peripheral blood (PB) are heterogeneous, comprising at least 3 phenotypically and functionally distinct subpopulations. The so-called population III (CD45RA−FoxP3^{low}) was shown to be nonsuppressive and able to convert into interleukin-17 (IL-17)–producing cells. The in vivo existence of IL-17–positive Treg cells has been demonstrated in human PB (6,7) as well as in periodontitis lesions (8) and skin lesions of patients with severe psoriasis (9). Several groups of investigators have identified the proinflammatory cytokine IL-1β as a critical mediator in the conversion of human Treg cells into IL-17–producing cells in vitro (6,7,10–13). As yet, data are conflicting regarding whether the regulatory function of these proinflammatory cytokine–producing Treg cells is impaired. Furthermore, because most of these studies have been performed using anti-CD3/CD28 beads and recombinant cytokines, data on human Treg cell conversion in a physiologic context are scarce.

IL-17 has been associated with inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease, multiple sclerosis, asthma, systemic lupus erythematosus, psoriasis, and type 1 diabetes mellitus (for review, see ref. 14). A previous study by our group showed that CD14+ cells are present in large numbers in the synovial fluid (SF) of patients with RA, and that these cells preferentially promote Th17 responses in CD4+ T cells (15). CD14+ monocytes are important contributors to inflammation through the production of proinflammatory cytokines such as IL-1β. Based on these findings, we sought to determine whether activated monocytes drive the expression of IL-17 in highly purified CD4+CD45RO+CD25+ CD127^{low} Treg cells (memory Treg cells), and whether this affects Treg cell phenotype and function. We report here that human memory Treg cells, in the presence of activated monocytes, show increased expression of both proinflammatory and antiinflammatory cytokines. These cells maintain their Treg phenotype and exert enhanced suppressive effects on T cell proliferation and cytokine production.

PATIENTS AND METHODS

Patients and healthy volunteers. Peripheral blood (n = 29) and SF (n = 12) samples were obtained from patients with RA recruited from Guy's and St Thomas' Hospital NHS Trust. Peripheral blood was also collected from adult healthy control subjects. The mean ± SEM ages of the patients and healthy control subjects were 58 ± 2.8 years and 36 ± 2.2 years, respectively. The female-to-male ratios were 26:3 and 24:12, respectively. The Disease Activity Score in 28 joints (16), which was available for 18 patients, was 5.2 ± 0.3 (mean ± SEM). Among 29 patients, 5 were receiving tumor necrosis factor (TNF) inhibitor therapy, 18 were receiving disease-modifying antirheumatic drugs, and 3 were receiving steroids or nonsteroidal antiinflammatory drugs. All participants gave written informed consent. Ethics approval for this study was given by the Bromley Research Ethics Committee (reference 06/Q0705/20). Mononuclear cells were isolated from PB and SF using Ficoll-Hypaque (LSM 1077; PAA Laboratories) density-gradient centrifugation.

Phenotypic analysis. The following monoclonal antibodies were used: Pacific Blue–conjugated CD2 (clone TS18), allophycocyanin (APC)–Cy7–conjugated CD3 (clone HIT3a), PerCP–Cy5.5–conjugated CD4 (clone SK3), APC–Cy7–conjugated CD14 (clone HCD14), Alexa Fluor 488–conjugated CD16 (clone 3G8), phycoerythrin (PE)–Cy7–conjugated CD39 (clone A1), Pacific Blue–conjugated CD45RO (clone UCHL1), Alexa Fluor 647–conjugated CD54 (clone HCD54), Pacific Blue–conjugated CD86 (clone IT2.2), Alexa Fluor 488–conjugated CD127 (clone HCD127), and Alexa Fluor 647–conjugated CD161 (clone HP-3G10) (all from BioLegend), PE-conjugated CD25 (clone 4E3; Miltenyi Biotec), PE-conjugated CD40 (clone LOB7/6) and fluorescein isothiocyanate (FITC)–conjugated CD69 (clone FN50; both from AbD Serotec), and PerCP–Cy5.5–conjugated HLA–DR (clone G46-6; BD Biosciences). For intracellular cytokine staining, cells were stained for CD2 and CD14, followed by fixation with 2% paraformaldehyde. Cells were then stained intracellularly with Alexa Fluor 488–conjugated IL-10 (clone JES3-9D7), PE-conjugated IL-17A (clone BL168), APC-conjugated TNFα (clone MAb11), and PerCP–Cy5.5–conjugated interferon-γ (IFNγ; clone 4S.B3) (all from BioLegend) using 0.5% saponin. For intranuclear staining, cells were extracellularly stained and fixed as described above followed by permeabilization with 1× FoxP3 Perm Buffer (BioLegend). Cells were then stained with Alexa Fluor 647–conjugated FoxP3 (clone 259D) and Alexa Fluor 488–conjugated Ki-67 (clone Ki-67; both from BioLegend) in combination with PE-conjugated IL-17. Cells were acquired on a BD FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.1 software (Tree Star).

Cell isolation. Peripheral blood mononuclear cells were incubated with CD14 MicroBeads (Miltenyi Biotec) for positive selection of CD14+ monocytes using magnetic-activated cell sorting. Purity was confirmed by flow cytometry and was consistently >97%. The CD14− fraction was used for memory CD4+ T cell isolation by negative selection (Miltenyi Biotec) according to the manufacturer's instructions, and the purity of cells was always >96%. Memory CD4+ T cells were stained with PE-conjugated CD25 and FITC-conjugated CD127 (clone A019D5; BioLegend) and sorted into

CD25+CD127^{low} Treg cells and CD25$^{low/negative}$CD127+ T cells (Teff cells), using a BD FACSAria II cell sorter (purities >97%). When indicated, isolated CD14+ monocytes were stained with Alexa Fluor 488–conjugated CD16 after incubation with FcR blocking reagent (Miltenyi Biotec) and sorted into CD16+ and CD16− cells.

Coculture experiments. Cells were cultured in RPMI 1640 (Gibco), 20 m*M* L-glutamine (Gibco), 1% penicillin/streptomycin (Gibco), and 10% fetal calf serum (Sigma) at 37°C in an atmosphere of 5% CO_2. Monocytes (10×10^6/ml) were preincubated with medium, 100 ng/ml lipopolysaccharide (LPS; Sigma), or a cytokine cocktail consisting of rhIL-1β, IL-6, IL-10, IL-17, TNFα, osteopontin (OPN) (all 10 ng/ml), and 10 units/ml IFNγ (all from R&D Systems) for 30 minutes. Following incubation, cells were washed twice with 5–10 ml of medium and recounted.

Monocytes (1×10^5) were cocultured (1:1 ratio) with sorted CD4+CD45RO+CD25+CD127^{low} Treg cells or CD4+CD45RO+CD25$^{low/negative}$CD127+ Teff cells and 100 ng/ml soluble anti-CD3 monoclonal antibodies (OKT3; Janssen-Cilag) in a total volume of 250 μl. On day 3, cells were stimulated with 50 ng/ml phorbol myristate acetate (PMA; Sigma) and 750 ng/ml ionomycin (Sigma) for 6 hours, with GolgiStop (BD Biosciences) present for the last 3 hours. When supernatants of activated monocytes were transferred, they were collected from autologous or allogeneic LPS-preactivated monocytes after 40 hours and added 1:1 (volume/volume) to cocultures. For blocking experiments, neutralizing antibodies against IL-1β (1 μg/ml; clone 8516, mIgG1), IL-6 (10 μg/ml; clone 1936, mIgG2b), and TNFα (1 μg/ml; clone 1825, mIgG1) (all from R&D Systems) were added at the start of culture.

Suppression assay. Freshly sorted Teff cells ($2–5 \times 10^6$/ml) were labeled with 2 μM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) according to the manufacturer's instructions. Cells were washed, recounted, and plated in a 24-well plate (1×10^6/ml). At the same time, unlabeled, sorted Treg cells at various concentrations ($1–5 \times 10^4$) were cultured with anti-CD3 monoclonal antibody and 5×10^4 monocytes pretreated with either LPS or medium in a total volume of 100 μl. The next day, CFSE-labeled Teff cells were taken off the plate, washed, and recounted, and 5×10^4 cells were added to the Treg cell–monocyte cocultures. To determine proliferation, fluorescence was assessed 2 days later by flow cytometry, and the percentage of suppression was calculated using the following formula: 100 − ([% dividing cells (condition with Treg cells)]/[% dividing cells (condition without Treg cells)]) × 100.

Cytokine analysis. Supernatants of cocultures were collected on day 3 after restimulation with PMA and ionomycin, centrifuged to remove cell debris, and stored at −80°C until analyzed. The levels of IL-1β (R&D Systems), IL-6, IL-17, IFNγ, and TNFα (all from BioLegend) were determined using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Supernatants from monocytes stimulated with either medium or LPS for 40 hours were analyzed using a Cytokine Human 25-Plex Panel for the Luminex platform (Invitrogen Life Technologies). For detection of cytokines in the SF of patients with RA, a FlowCytomix Th1/Th2 assay (eBioscience), and IL-17 and OPN ELISAs (R&D Systems) were used.

Statistical analysis. Data were tested for normality using a D'Agostino-Pearson omnibus normality test, followed by the appropriate parametric or nonparametric test, as indicated in the figure legends. Statistical analysis was performed using Prism 5 software (GraphPad).

RESULTS

Abundant presence of CD4+CD45RO+ T cells with a regulatory phenotype and activated CD14+ monocytes at the site of inflammation in patients with RA. Various studies, including studies by our group, have demonstrated increased frequencies of CD4+CD25+ Treg cells in the SF compared with the PB of patients with RA (17–24). However, these studies did not account for the fact that virtually all T cells in SF have a memory CD45RO+ phenotype, while their blood counterparts comprise both CD45RO+ and CD45RO− T cells. We therefore determined the percentage of memory CD4+ T cells with a regulatory phenotype (CD25+CD127^{low}) in PB and SF from patients with RA and in PB from healthy controls (additional information regarding the gating strategy is available from the corresponding author).

Our results showed no significant difference in the percentage of CD25+CD127^{low} cells within the CD3+CD4+CD45RO+ T cell population in the PB of patients with RA (n = 29) and healthy controls (n = 36) (mean ± SEM 11 ± 0.7% versus 10 ± 0.5%) (Figure 1A), even after correcting for age differences between the groups (data not shown). However, the percentage of CD25+CD127^{low} cells among CD3+CD4+CD45RO+ T cells was significantly increased in SF (n = 12) compared with PB (n = 29) from patients with RA (23 ± 2.0%; $P < 0.0001$) (Figure 1A). A similar increase was observed when analyzing paired PB and SF samples only (n = 11; $P = 0.0005$). We also determined the presence of CD14+ monocytes in healthy control subjects and patients with RA and observed a significant increase in the percentage of CD14+ monocytes in the PB of patients compared with the PB of control subjects (19 ± 2.3% versus 13 ± 1.0%; $P < 0.05$) and an even higher percentage at the site of inflammation (26 ± 3.5%) (Figure 1B).

We performed flow cytometry to assess the phenotype of PB monocytes from patients with RA and healthy control subjects. The expression of CD14, CD16, CD40, CD54, and HLA–DR in PB monocytes was significantly higher in patients with RA compared with control subjects (Figure 1C), indicating an activated phenotype. CD14+ monocytes were shown to be further

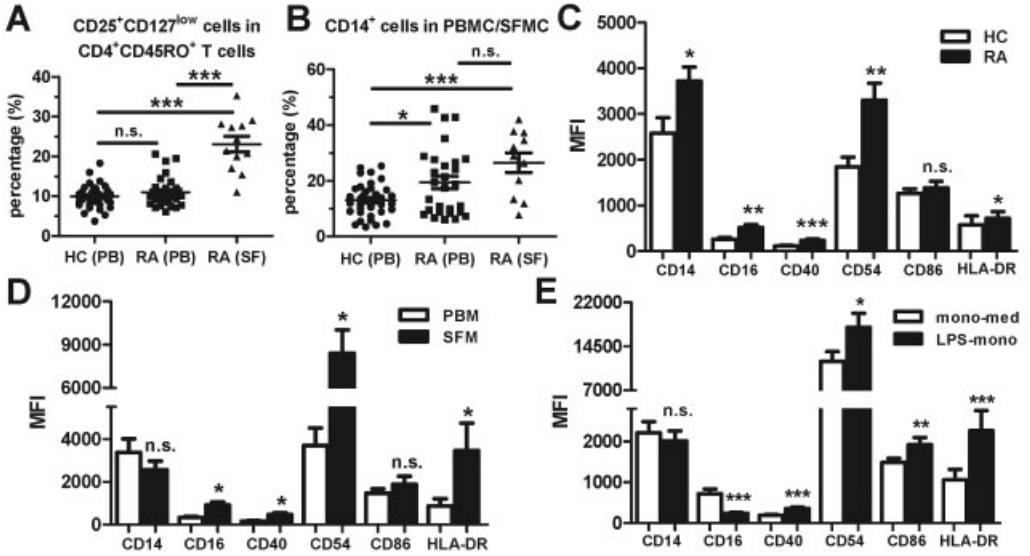

Figure 1. Increased frequencies of memory CD4+ T cells with a Treg phenotype and activated CD14+ monocytes at the site of inflammation in patients with rheumatoid arthritis (RA). **A** and **B,** Mononuclear cells (MCs) from the peripheral blood (PB) of healthy controls (HCs; n = 36) and from the PB (n = 28–29) and synovial fluid (SF) (n = 11–12) of patients with RA were analyzed for the percentage of CD25+CD127^{low} cells within CD4+CD45RO+ T cells (**A**) and for the percentage of CD14+ cells in PBMCs/SFMCs (**B**). Each data point represents an individual subject; horizontal bars and error bars show the mean ± SEM. * = $P < 0.05$; *** = $P < 0.001$, by analysis of variance. **C–E,** Surface expression of the indicated markers was determined in PB CD14+ monocytes (PBMs) from patients with RA (n = 17) and healthy control subjects (n = 16) (**C**), in paired PBMs and SF-derived CD14+ monocytes (SFMs) from patients with RA (n = 7) (**D**), and in PBMs from healthy control subjects (n = 18) that were treated with medium (mono-med) or lipopolysaccharide (LPS-mono) for 30 minutes and cultured for 16 hours (**E**). Bars show the mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ versus HCs (**C**), PBMs (**D**), and medium-treated monocytes (**E**), by Wilcoxon's matched pairs signed rank test. NS = not significant; MFI = mean fluorescence intensity.

activated in SF from patients with RA, as shown by significantly increased expression of CD16, CD40, CD54, and HLA–DR by SF-derived monocytes relative to paired PB monocytes (n = 7) (Figure 1D). In order to investigate the effect of monocytes with an activated phenotype on human Treg cells, we established an in vitro system using 100 ng/ml of LPS to activate monocytes from healthy control subjects. These LPS-treated monocytes (n = 18) showed a significant up-regulation of the activation markers CD40, CD54, CD86, and HLA–DR but a down-regulation of CD16 compared with medium-treated monocytes (Figure 1E), indicating an in vitro–activated phenotype that is similar but not identical to that of in vivo–activated SF monocytes.

In vitro–activated monocyte-induced cytokine expression in CD4+CD45RO+CD25+CD127^{low} Treg cells. We next assessed the effects of activated monocytes on Treg cell phenotype and function. CD4+CD45RO+CD25+CD127^{low} T cells (memory Treg cells) from healthy control subjects were sorted to high purity and cocultured with autologous CD14+ monocytes that had been pretreated with either 100 ng/ml LPS or medium for 30 minutes, followed by extensive washing. Soluble anti-CD3 monoclonal antibodies were added to the cocultures to activate Treg cells. In the presence of LPS-treated monocytes, a significant increase in the percentage of memory Treg cells expressing IL-17, IFNγ, or TNFα was observed relative to medium-treated monocytes (Figures 2A–D). Notably, the presence of LPS-treated monocytes also significantly increased the percentage of IL-10–positive Treg cells (Figures 2A and E).

In order to determine the relative magnitude of the percentage of cytokine-expressing Treg cells, we performed a similar analysis within memory Teff cells by setting up parallel cocultures of CD4+CD45RO+ CD25$^{low/negative}$CD127+ T cells (Teff cells) with medium-treated or LPS-activated monocytes. This analysis showed that although the percentage of proinflammatory cytokine–expressing Treg cells was increased in the presence of activated monocytes, the percentage was still relatively low compared with the percentage of proinflammatory cytokine–expressing Teff cells (mean ± SEM 3.8 ± 0.4% versus 17 ± 1.6% IL-17–positive cells) (Figures 2B–D). The percentage of proinflammatory cytokine–expressing Treg cells was also

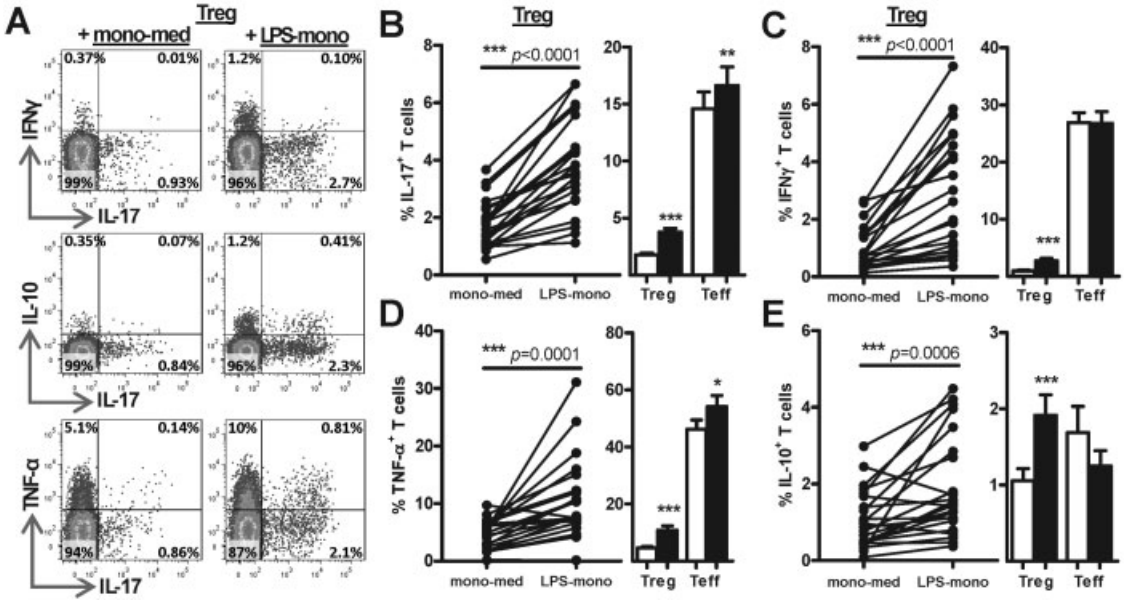

Figure 2. Induction of cytokine expression in CD4+CD45RO+CD25+CD127^{low} Treg cells by in vitro–activated monocytes. **B–E,** Sorted CD4+CD45RO+CD25+CD127^{low} T cells (Treg cells) or CD4+CD45RO+CD25$^{low/negative}$CD127+ T cells (Teff cells) were cocultured with autologous CD14+ monocytes, which were pretreated with either medium (open bars) or LPS (solid bars), in the presence of anti-CD3 monoclonal antibodies. After 3 days, cells were restimulated with phorbol myristate acetate and ionomycin for 6 hours, with GolgiStop present for the last 3 hours, and stained intracellularly for interleukin-17 (IL-17) (**B**), interferon-γ (IFNγ) (**C**), tumor necrosis factor α (TNFα) (**D**), and IL-10 (**E**). Results from 18–23 experiments with either Treg cell or Teff cell cocultures with monocytes are shown. Each pair of symbols represents one experiment. Bars show the mean ± SEM. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$ versus medium-treated monocytes, by Wilcoxon's matched pairs signed rank test. **A,** Results of a representative experiment are shown as flow cytometric dot plots. See Figure 1 for other definitions.

low compared with the percentage of unsorted memory CD4+ T cells (data not shown). In contrast, the percentage of IL-10–positive cells was highest in Treg cells following incubation with activated monocytes (Figure 2E). Of note, although proinflammatory cytokine–expressing regulatory T cells are not "Treg cells" per definition, for the purpose of clarity, we will continue to refer to these cytokine-expressing CD4+CD45RO+CD25+CD127^{low} T cells as Treg cells in this report.

Effect of cytokine-activated monocytes and CD14+CD16− and CD14+CD16+ subsets on proinflammatory cytokine expression in memory Treg cells. The composition of RA SF differs between patients, because the SF contains varying levels of proinflammatory and antiinflammatory cytokines (Figure 3A) (25). To mimic the environment to which monocytes may be exposed in the inflamed joint, we incubated monocytes with either medium or a cocktail of cytokines commonly associated with RA SF (IL-1β, IL-6, IL-17, IFNγ, IL-10, TNFα, and OPN) for 30 minutes, followed by extensive washing. Monocytes were cultured overnight, and their phenotype was assessed. A consistent increase in expression of the monocyte activation markers CD40, CD54, CD86, and HLA–DR, but a concomitant decrease in the expression of CD16, was observed in cytokine-activated monocytes compared with medium-treated monocytes (n = 5) (Figure 3B), indicating that these cytokine-activated monocytes are similar but not identical to in vivo–activated SF monocytes (Figure 1D). Coculture experiments revealed that cytokine-activated monocytes also increased the percentage of memory Treg cells expressing IL-17, IFNγ, TNFα, and, to a lesser extent, IL-10 (Figure 3C).

It was shown recently that the frequency of CD14+CD16+ monocytes is increased in the PB of patients with RA, and that these cells are "potent inducers" of IL-17 production by CD4+ T cells (26). In agreement with that study, we observed a significant increase in the percentage of CD14+CD16+ monocytes in the PB of patients with RA (n = 22) compared with that in the PB of healthy controls (n = 26) (mean ± SEM 10 ± 1.2% versus 7 ± 0.6% of CD14+ monocytes; $P = 0.016$) (data not shown). Because CD14+ monocytes isolated by magnetic-activated cell sorting contain both "proinflammatory" CD14+CD16+ and "classic" CD14+CD16− populations, we sorted CD14+ mono-

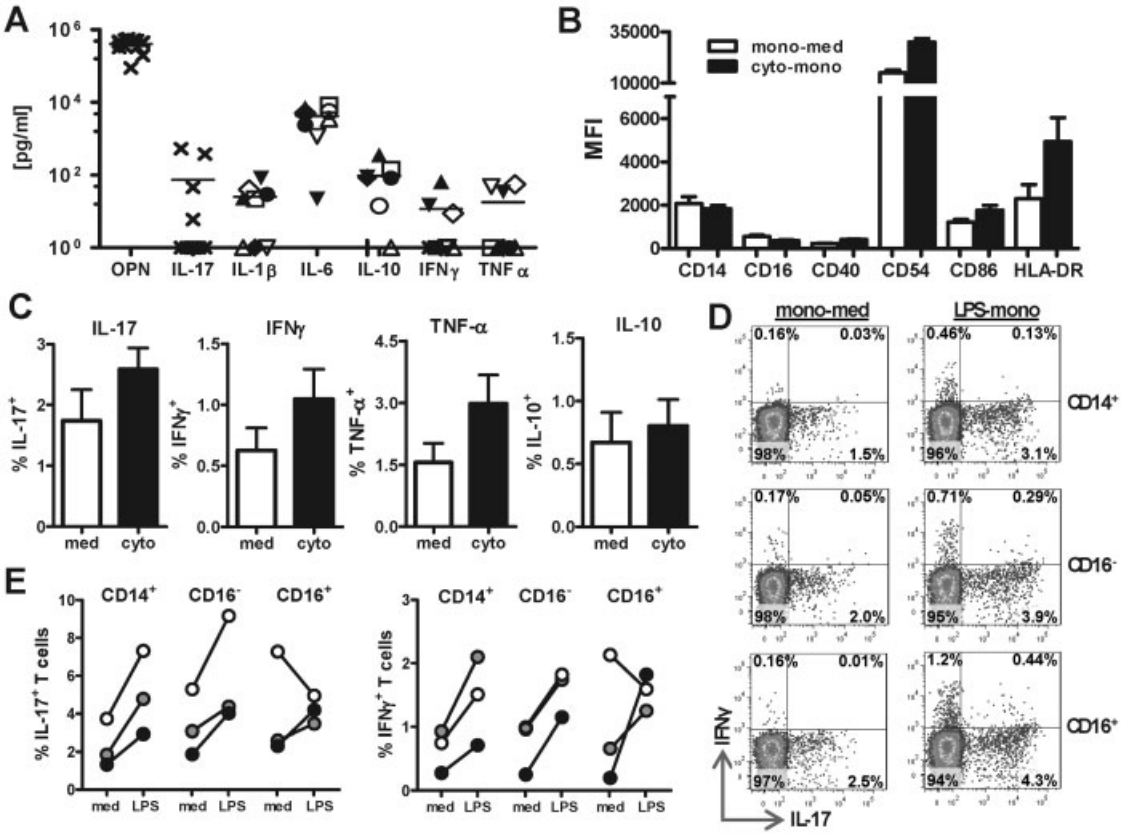

Figure 3. Induction of proinflammatory cytokine expression in memory Treg cells by cytokine-activated monocytes as well as CD14+CD16− and CD14+CD16+ subsets. **A,** The presence of cytokines in rheumatoid arthritis synovial fluid (n = 8–13) was determined by enzyme-linked immunosorbent assay or FlowCytomix Th1/Th2 assay for the indicated cytokines. For IL-1β, IL-6, IL-10, IFNγ, and TNFα, identical symbols reflect the same sample; horizontal lines show the mean. **B,** Expression of the indicated markers was determined on cytokine-activated monocytes (cyto-mono) and compared with medium-treated monocytes (mono-med) from healthy control subjects (n = 5). **C,** Memory Treg cells were cocultured with medium-treated (med) or cytokine-activated (cyto) monocytes from healthy control subjects in the presence of anti-CD3 monoclonal antibodies for 3 days, and the percentage of cytokine-expressing T cells was assessed by intracellular cytokine staining (n = 5). Bars in **B** and **C** show the mean ± SEM. **D** and **E,** Memory Treg cells were cocultured with total CD14+ monocytes isolated by magnetic-activated cell sorting or with sorted CD14+CD16− or CD14+CD16+ monocytes, in the absence (med) or presence of lipopolysaccharide (LPS), with anti-CD3 monoclonal antibodies added. The percentage of IL-17–positive or IFNγ–positive T cells was analyzed on day 3 by intracellular cytokine staining after restimulation with phorbol myristate acetate and ionomycin. Results of 1 representative experiment are shown in **D**; individual data from 3 experiments are shown in **E**. OPN = osteopontin; MFI = mean fluorescence intensity (see Figure 2 for other definitions).

cytes into CD16− and CD16+ cells and cocultured them with memory Treg cells with or without LPS. Both CD16− and CD16+ monocytes showed a capacity similar to that of CD14+ monocytes to induce proinflammatory IL-17 and IFNγ expression in memory Treg cells (Figures 3D and E). Taken together, these findings indicate that both Toll-like receptor 4–stimulated monocytes and cytokine-activated monocytes can induce proinflammatory cytokine expression in CD4+CD45RO+ CD25+CD127^{low} Treg cells, and that this is not a unique feature of a particular CD14+ monocyte population.

Increased expression of IL-17 and IFNγ in memory Treg cells driven by monocyte-derived IL-1β, IL-6, and TNFα. To determine the mechanism by which activated monocytes drive the observed increase in the frequency of cytokine-expressing Treg cells, we first investigated whether soluble factors were involved. Monocytes were preactivated with LPS, extensively washed, and cultured for 40 hours. Supernatants were collected and transferred to monocyte–Treg cell cocultures, and intracellular cytokine expression was determined on day 3. The addition of supernatants from

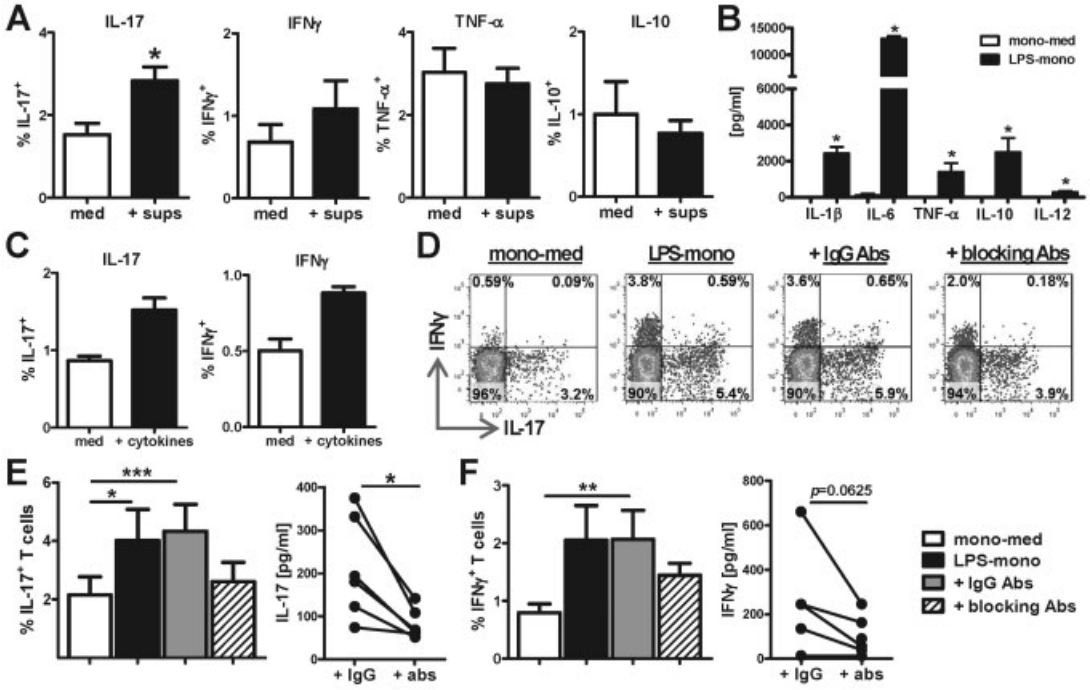

Figure 4. Effect of monocyte-derived IL-1β, IL-6, and TNFα on IL-17 and IFNγ expression in memory Treg cells. **A,** CD4+CD45RO+CD25+CD127^{low} Treg cells were cocultured with medium-treated monocytes (mono-med) alone or with the addition of supernatants (sups) from lipopolysaccharide (LPS)–pretreated monocytes (LPS-mono) in the presence of anti-CD3 monoclonal antibodies. The percentage of cytokine-expressing T cells was determined as described in Figure 2 (n = 6 experiments). $* = P < 0.05$ versus medium-treated monocytes. **B,** Monocytes from healthy control subjects were pretreated with medium or LPS (100 ng/ml) and cultured for 40 hours. Supernatants were quantified by 25-plex cytokine array (n = 6). $* = P < 0.05$ versus medium-treated monocytes. **C,** Sorted memory Treg cells were cocultured with medium-treated monocytes for 3 days with or without rhIL-1β, IL-6, and TNFα, and the expression of IL-17 and IFNγ was assessed (n = 3). **D–F,** Memory Treg cells were cocultured with medium-treated monocytes, LPS-treated monocytes, or LPS-treated monocytes and neutralizing antibodies against IL-1β, IL-6, and TNFα (blocking antibodies), or the appropriate IgG isotype control antibodies. On day 3, cells were analyzed for IL-17 (**E**) and IFNγ (**F**) expression by intracellular cytokine staining and for secretion by enzyme-linked immunosorbent assay. Results of 1 representative experiment are shown in **D**; cumulative data (n = 6 experiments) are shown in **E** and **F**. Bars show the mean ± SEM. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$, by Wilcoxon's matched pairs signed rank test or Friedman's test for repeated measures with Dunn's multiple comparison test. rhIL-1β = recombinant human interleukin-1β (see Figure 2 for other definitions).

activated monocytes led to a significant increase in the percentage of IL-17–positive Treg cells and a trend toward an increased percentage of IFNγ-positive Treg cells ($P = 0.09$; n = 6), while the expression of TNFα and IL-10 was not affected (Figure 4A).

To elucidate which soluble factors secreted by LPS-treated monocytes could drive the increased expression of proinflammatory IL-17 and IFNγ in memory Treg cells, supernatants from medium-treated monocytes and LPS-treated monocytes were analyzed using a 25-plex cytokine array. LPS-treated monocytes produced significantly increased amounts of proinflammatory (IL-1β, IL-6, TNFα, IL-12p40/p70) and antiinflammatory (IL-10) cytokines (Figure 4B), which are also present in the SF of patients with RA (Figure 3A, and results not shown). IL-1β, IL-6, and TNFα are known inflammatory mediators in the pathogenesis of RA and can drive the induction of IL-17–producing T cells (27). To examine the role of these cytokines, we performed reconstitution and blocking experiments. The addition of rhIL-1β, IL-6, and TNFα to monocyte–memory Treg cell cocultures led to an increase in the percentage of IL-17–positive and IFNγ-positive Treg cells compared with medium control (Figure 4C). Conversely, neutralization of IL-1β, IL-6, and TNFα during coculture of memory Treg cells with LPS-treated monocytes consistently prevented an increase in IL-17 expression and, to some extent, IFNγ expression (Figures 4D–F) but did not affect the expression of IL-10 or TNFα (results not shown). The addition of neutralizing antibodies also reduced IL-17 and, to a lesser extent, IFNγ secretion (Figures 4E and F).

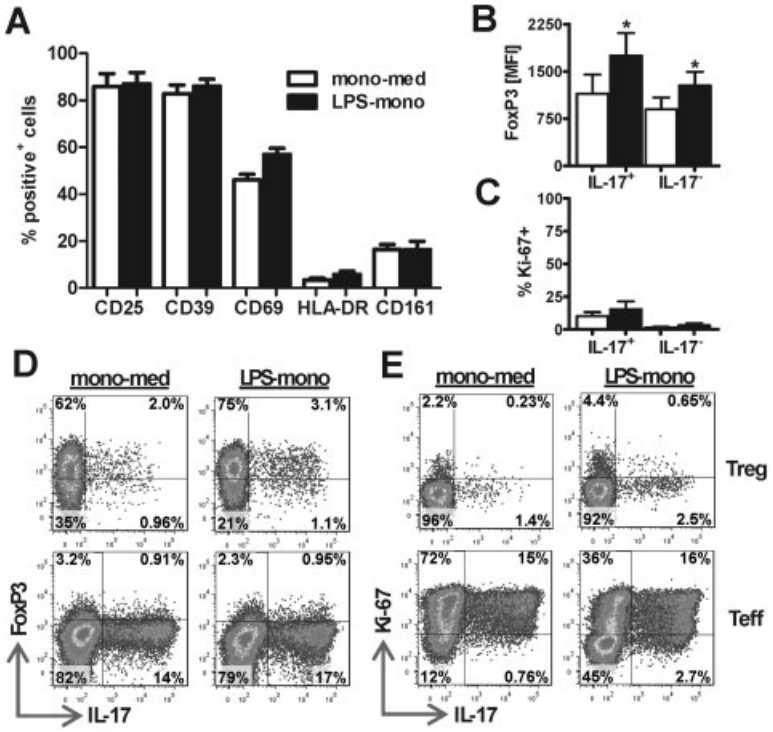

Figure 5. Phenotype of CD4+CD45RO+CD25+CD127^{low} T cells following incubation with activated monocytes. **A,** CD4+CD45RO+CD25+CD127^{low} Treg cells were cocultured with medium-treated monocytes (mono-med) or lipopolysaccharide (LPS)–treated monocytes (LPS-mono) in the presence of anti-CD3 monoclonal antibodies. On day 3, cells were stained for CD25, CD39, CD69, HLA–DR, and CD161. Results are shown as the percentage of CD2+CD14− cells expressing the indicated marker (n = 3). **B–E,** Memory Treg cells or Teff cells from a 3-day culture with monocytes underwent intranuclear staining for FoxP3, Ki-67, and interleukin-17 (IL-17) after restimulation with phorbol myristate acetate/ionomycin. Results in **B** and **C** represent the average FoxP3 expression (**B**) and the percentage of Ki-67–positive cells within IL-17–positive and IL-17–negative CD2+CD14− cells (**C**) (n = 6). Results of 1 representative experiment are shown in **D** and **E**. Bars show the mean ± SEM. $* = P < 0.05$ versus medium-treated monocytes, by Wilcoxon's matched pairs signed rank test. MFI = mean fluorescence intensity.

Sustained Treg cell phenotype after coculture with in vitro–activated monocytes. We determined whether CD4+CD45RO+CD25+CD127^{low} Treg cells still displayed a regulatory phenotype following incubation with activated monocytes. After coculture with LPS-treated monocytes, Treg cells remained positive for CD25 and CD39 (Figure 5A). The percentage of cells positive for CD69 and HLA–DR was slightly increased, indicating an activated status of the cells. Despite the increased frequency of IL-17–expressing cells, we did not observe an increased frequency of the Th17 marker CD161 (28) (Figure 5A). IL-17–positive Treg cells showed sustained FoxP3 expression following coculture with activated monocytes (Figures 5B and D). In fact, FoxP3 expression in LPS-treated monocyte–activated Treg cells was significantly higher in both IL-17–positive and IL-17–negative cells compared with their respective medium-treated monocyte–cultured counterparts (Figures 5B and D).

In contrast, virtually all IL-17–positive Teff cells, which were induced under the same conditions, were FoxP3 negative (Figure 5D). IL-17–positive Treg cells from cocultures with both medium-treated monocytes and LPS-treated monocytes contained a low percentage of Ki-67–positive cells (mean ± SEM 10 ± 3.1% and 15 ± 6.1%, respectively) (Figures 5C and E), while IL-17–positive Teff cells were predominantly Ki-67 positive (91 ± 2.7% and 75 ± 5.7%, respectively) (Figure 5E, and results not shown). Taken together, these data demonstrated that although CD4+CD45RO+CD25+CD127^{low} Treg cells showed increased proinflammatory cytokine expression following coculture with activated monocytes, their Treg phenotype was maintained.

Enhanced capacity of activated memory Treg cells to suppress cytokine secretion and T cell proliferation. Finally, we determined whether enhanced proinflammatory cytokine expression in CD4+CD45RO+CD25+CD127^{low} Treg cells impaired their ability to

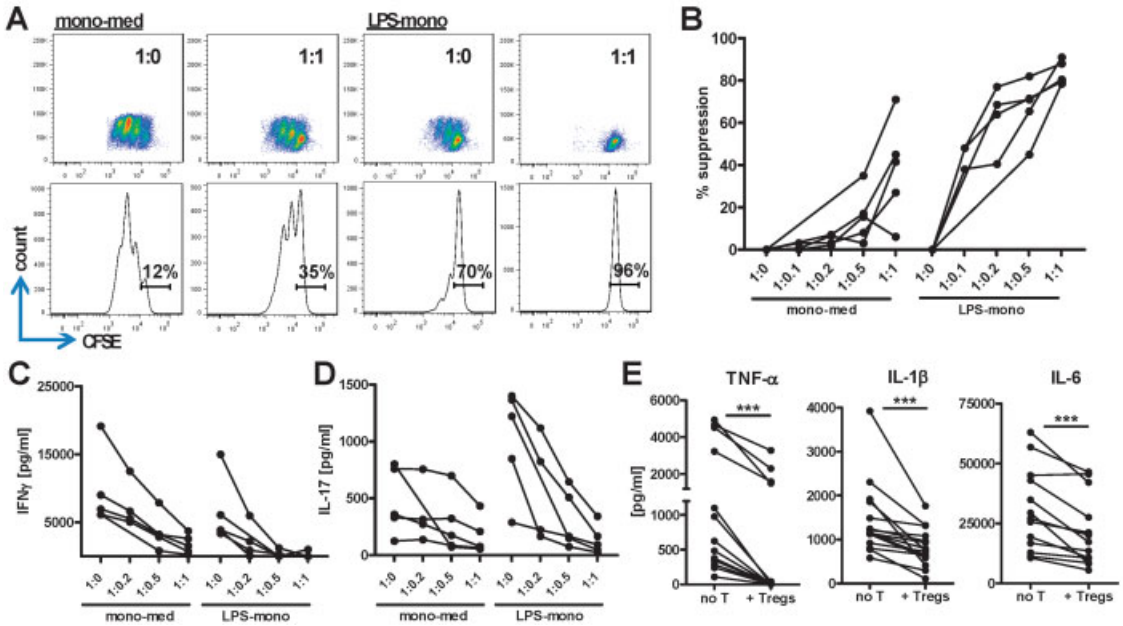

Figure 6. Enhanced capacity of activated memory Treg cells to suppress T cell proliferation and interleukin-17 (IL-17) production. **A–D,** Sorted CD4+CD45RO+CD25+CD127^{low} Treg cells (1–5 × 10^4) were cocultured overnight with 5 × 10^4 medium-treated monocytes or LPS-treated monocytes in the presence of anti-CD3 monoclonal antibodies at the indicated cell ratios. The next day, carboxyfluorescein succinimidyl ester (CFSE)–labeled memory Teff cells (5 × 10^4) were added to the cocultures, and proliferation was assessed on day 3. Supernatants from the cocultures in **A** and **B** were analyzed for interferon-γ (IFNγ) (**C**) and IL-17 (**D**) secretion by enzyme-linked immunosorbent assay. Results of 1 representative experiment are shown in **A**. **E,** Supernatants from cell cultures of monocytes with or without Treg cells (no T) were collected on day 3 and analyzed for tumor necrosis factor α (TNFα; n = 14), IL-1β (n = 15), and IL-6 (n = 13) secretion. *** = $P < 0.001$ versus without Treg cells, by Wilcoxon's matched pairs signed rank test. See Figure 1 for other definitions.

suppress T cell proliferation as well as monocyte- and T cell–derived cytokine production. We cocultured memory Treg cells with either medium-treated monocytes or LPS-treated monocytes in the presence of anti-CD3 monoclonal antibodies overnight, at various cell ratios to allow interaction between cells. The next day, CFSE-labeled autologous Teff cells were added to the cultures, and proliferation was assessed by flow cytometry 2 days later. Teff cell proliferation was strong in the presence of medium-treated monocytes and was suppressed in the presence of Treg cells (Figures 6A and B). Although Teff cells proliferated less profoundly in the presence of LPS-activated monocytes, their proliferation was still strongly suppressed by the presence of Treg cells. In fact, when we calculated the percent suppression of proliferation, we observed an increased suppressive capacity when Treg cells were precultured with LPS-treated monocytes, at all Teff cell:Treg cell ratios (Figure 6B).

We also assessed the suppressive effects of Treg cells on the secretion of IFNγ and IL-17 by Teff cells and observed that following interaction with LPS-treated monocytes, memory Treg cells were more efficient in suppressing cytokine secretion, which was particularly evident for IL-17 (Figures 6C and D). LPS-treated monocyte–activated Treg cells suppressed IL-17 secretion at a Teff cell:Treg cell ratio of 1:0.2, while medium-treated monocyte–cultured Treg cells, in most cases, suppressed IL-17 secretion only at a ratio of 1:1 (Figure 6D). Finally, the addition of memory Treg cells to cultures of LPS-activated monocytes significantly suppressed the secretion of TNFα, IL-1β, and IL-6 by monocytes (Figure 6E) but did not affect IL-10 secretion (results not shown). Overall, these data indicated that monocyte-activated memory Treg cells, despite an increased expression of proinflammatory cytokines, maintained their Treg phenotype and function and in fact showed an enhanced capacity to suppress T cell proliferation and IL-17 production.

DISCUSSION

Here, we show that human CD4+CD45RO+CD25+CD127^{low} Treg cells can be induced to express

proinflammatory (IL-17, IFNγ, TNFα) as well as anti-inflammatory (IL-10) cytokines upon interaction with activated monocytes. Importantly, despite the observed increase in proinflammatory cytokine expression, Treg cells maintain their regulatory phenotype and appear to have an enhanced rather than an impaired ability to suppress T cell proliferation and cytokine production, particularly IL-17. Our data suggest that cytokine-expressing Treg cells at sites of inflammation may still exert potent immune suppression.

We observed that the increased frequency of IL-17–positive (and to a lesser extent IFNγ-positive) Treg cells was driven by monocyte-derived IL-6, TNFα, and IL-1β. TNFα was the most abundantly induced cytokine in Treg cells following interaction with activated monocytes, and TNFα-positive Treg cells have also been observed in other in vitro studies (6,7,11). However, the mechanism for induction of TNFα expression in Treg cells appeared different, because the addition of supernatants from activated monocytes did not increase TNFα expression. The induction of IL-10 expression in T cells was relatively low overall, but the percentage of IL-10–positive cells was highest in LPS-treated monocyte–activated Treg cells (mean $\pm$ SEM 1.9 $\pm$ 0.3%). It should be noted that the cytokine expression we report represents the cytokine profile following restimulation with PMA and ionomycin. Although this is a very commonly used system for the detection of cytokine-expressing cells, the levels reported may not reflect the actual levels of cytokine-secreting cells.

Various other studies indicate that human CD4+CD25+FoxP3+ Treg cells comprise a heterogeneous cell population that can display plasticity during development and differentiation and when exposed to a proinflammatory environment (for review, see refs. 3 and 4). It has been shown that human Treg cells can be induced in vitro to express the proinflammatory cytokine IL-17 following stimulation with anti-CD3/CD28 monoclonal antibody–coated beads in the presence of rhIL-1β and IL-2 (7,10–13). IL-17–positive Treg cells have also been observed in vivo at sites of inflammation, including periodontitis lesions (8), psoriatic skin (9), human tonsils (7), and the lamina propria of patients with Crohn's disease (29). Furthermore, Treg cells from patients with psoriasis showed an enhanced propensity to differentiate into IL-17–producing cells, which was accompanied by decreased FoxP3 expression and increased expression of retinoic acid receptor–related orphan receptor C2 (9). These findings overall suggest that Treg cells from sites of inflammation may convert into IL-17–producing cells.

The key question is whether human proinflammatory cytokine–expressing Treg cells maintain their suppressive capacity. Two groups of investigators reported that the induction of IL-17 expression in naive Treg cells under inflammatory conditions was accompanied by impaired suppressive function (11,12). Single-cell cloning experiments further suggested that Treg cells can transiently lose their suppressive function when actively secreting IL-17, but FoxP3 expression was not affected (13). In contrast, IL-17–positive Treg cell clones (6,7,13), as well as IL-17–positive Treg cells from the inflamed intestinal mucosa of patients with Crohn's disease, were shown to be suppressive (29). In patients with type 1 diabetes mellitus, the frequency of IFNγ-positive Treg cells was reported to be increased, but these cells expressed high levels of FoxP3 and possessed suppressive activity (30). Furthermore, IFNγ production by Treg cells was recently suggested to be essential for the prevention of graft-versus-host disease (31).

Although care needs to be taken when extrapolating in vitro data into the in vivo situation, as exemplified by a preclinical model for xenogeneic graft-versus-host-disease (32), our data suggest that Treg cells exposed to an inflammatory environment may have enhanced suppressive effects particularly on T cell proliferation and IL-17 production, despite the fact that the percentage of IL-17–positive Treg cells increases. Th17 cells are thought to be more resistant to Treg cell–mediated suppression (33–36), and our findings that an increase in the number of IL-17–positive Treg cells corresponds to enhanced suppression of Th17 cells are consistent with elegant studies in mice demonstrating that Treg cells adapt to their cytokine milieu through the up-regulation of specific transcription factors, thus ensuring appropriate Th cell–specific control of inflammation (37–39). Taken together, these data suggest that the induction of proinflammatory cytokine expression in Treg cells is not indicative, per se, of conversion toward a less suppressive and/or more pathogenic function. Instead, this should be seen in the context of the other cytokines produced by Treg cells (e.g., IL-10), their level of cytokine expression relative to that of Teff cells, and their regulatory phenotype and function.

Our findings may have physiologic relevance, because we show that memory Treg cells and activated monocytes are present in abundance in the inflamed rheumatic joint. The well-documented presence of T cells with a regulatory phenotype and function in SF from patients with RA (17–24) thus leaves the question as to why inflammation persists despite the presence of these potentially suppressive cells. It has been shown

that the addition of proinflammatory mediators such as IL-7 or TNFα in vitro can break down Treg cell function (40). TNFα was also shown to down-regulate FoxP3 expression and function in human Treg cells (41). Furthermore, dendritic cell–derived IL-6 was shown to abrogate Treg cell–mediated suppression in mice (42), to increase IL-17 production (43), and to induce a loss in FoxP3 expression, which was exacerbated in the presence of IL-1β (44).

Inhibition of IL-6 using tocilizumab, a humanized anti–IL-6 receptor antibody, was shown to correct the Th17/Treg cell imbalance in RA (45) and enhanced the suppressive capacity of SF-derived Treg cells (46). A recent study showed that the ability to control monocyte-derived IL-6 production was critical for the ability of Treg cells to suppress Th17 cell responses in patients with RA (47). It should be noted, however, that the aforementioned studies did not always allow a distinction between impaired Treg cell function and increased resistance of Teff cells to suppression. Our data also show a distinct effect of IL-1β, IL-6, and TNFα on Treg cells, because we observed that increased expression of IL-17 and IFNγ in Treg cells was driven by these cytokines. Notwithstanding the above-mentioned effects of TNFα, IL-6, and IL-1β on Treg cell phenotype and function in vitro, it is evident that SF-derived Treg cells are fully suppressive ex vivo (18–24,48) and, in fact, may even be more suppressive than their PB counterparts (18,48). Recent data also revealed a fully demethylated FoxP3 promoter region in SF-derived Treg cells, indicating that these cells may indeed be "true" Treg cells, despite low-level IL-17 and IFNγ expression upon stimulation (46). Thus, Treg cells at sites of inflammation may not be intrinsically defective. Instead, possible defects in immunoregulation may reside within the activated Teff cell population that becomes resistant to Treg cell–mediated suppression (18,49–51).

In conclusion, our data, together with the existing literature, provide evidence that Treg cells that have been exposed to an inflammatory environment express proinflammatory cytokines but may maintain their suppressive capacity, and that their function may, in fact, be strengthened rather than weakened.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Taams had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Walter, Evans, Cope, Geissmann, Taams.

Acquisition of data. Walter, Evans, Menon, Gullick, Kirkham.

Analysis and interpretation of data. Walter, Evans, Kirkham, Cope, Geissmann, Taams.

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