Interleukin-6 Receptor Blockade Enhances CD39+ Regulatory T Cell Development in Rheumatoid Arthritis and in Experimental Arthritis

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A. Thiolat,[1] L. Semerano,[2] Y. M. Pers,[3] J. Biton,[1] D. Lemeiter,[1] P. Portales,[4] J. Quentin,[5] C. Jorgensen,[3] P. Decker,[1] M.-C. Boissier,[2] P. Louis-Plence,[5] and N. Bessis[1]

Objective. **The rationale for blocking interleukin-6 (IL-6) in rheumatoid arthritis (RA) lies chiefly in the proinflammatory effect of this cytokine. Few studies have evaluated the consequences of anti–IL-6 receptor (IL-6R) antibody treatment on Treg cells. This study was undertaken to elucidate the mechanism of action of anti–IL-6R antibody treatment by studying the effects on Treg cells in an experimental arthritis model and in patients with RA.**

Methods. **Mice with collagen-induced arthritis (CIA) were treated with a mouse anti–IL-6R antibody (MR16-1), and changes in Treg, Th1, and Th17 cells were assessed at key time points during the course of the disease. Peripheral blood from 15 RA patients was collected on day 0 and after 3 months of tocilizumab treatment for flow cytometry analysis of Th17 and Treg cells.**

Results. **In MR16-1–treated mice, Th17 cell frequencies were unchanged, whereas Treg cell frequencies were increased. The Treg cell phenotype showed marked changes, with an increase in the frequency of CD39+ Treg cells in the lymph nodes and spleen. Interestingly, similar CD39+ Treg cell expansion was observed in RA patients who were tocilizumab responders at 3 months, with no change in Th17 cell frequency. Moreover, fluorescence-activated cell–sorted CD39+ Treg cells from responder RA patients were functionally able to suppress the proliferation of conventional T cells.**

Conclusion. **In both CIA and RA, the frequency of functionally suppressive CD39+ Treg cells is increased as a result of anti–IL-6R treatment, whereas Th17 cells are unaffected. The modification of Treg cell frequency and phenotype may be one of the mechanisms involved in the therapeutic effect of IL-6 blockade in RA.**

Interleukin-6 (IL-6) plays a central role in the pathogenesis of rheumatoid arthritis (RA). This cytokine targets multiple cells, including B cells, T cells, macrophages, neutrophils, synoviocytes, and osteoclasts. Signaling through the IL-6 receptor (IL-6R) leads to a combination of systemic and joint inflammation, resulting in pannus formation, osteoclast activation, and chronic synovitis. IL-6–deficient mice are resistant to collagen-induced arthritis (CIA) (1), and early administration of the anti-mouse IL-6R monoclonal antibody MR16-1 inhibits CIA (2). IL-6 is abundantly expressed in the synovium of RA patients (3). These data provide a strong rationale for targeting IL-6, and the humanized anti–IL-6R antibody tocilizumab is now used to treat moderate-to-severe RA (4).

IL-6 is also involved in the adaptive immune response. IL-6 influences T cell differentiation into effector T cells (Th1, Th2, and Th17 cells) or regulatory T cells (Treg cells) (5). Th17 cells and Treg cells have opposite roles in rheumatoid inflammation (6). Th17 cells are important mediators of inflammation in animal

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models of RA (7–9). In contrast, CD4+CD25+FoxP3+ Treg cells are essential for maintaining immune homeostasis, preventing autoimmunity, and limiting chronic inflammatory diseases. A Treg/Th17 cell imbalance has been documented in RA (10). In mice, Th17 cells and induced Treg (iTreg) cells arise from the same CD4+ naive T cell precursor in distinct cytokine milieus. IL-6, and probably other proinflammatory cytokines, may determine whether an immune response is dominated by either Treg cells or Th17 cells (11). Treg cells express IL-6R (12,13), and administration of a mouse monoclonal antibody to inhibit IL-6R in CD4+ T cell cultures potently inhibits Th17 cell differentiation in response to transforming growth factor β and conditioned medium, and partially restores the induction of Treg cells (14).

The role of Treg cells in RA has been investigated in both patients and animal models. In murine CIA, Treg cell depletion aggravates the disease (15), whereas adoptive Treg cell transfer has a protective effect. Similarly, the absence of Treg cells in K/BxN scurfy mice is associated with faster onset and greater severity of arthritis (16). The proportion of Treg cells in RA patients is increased in the synovium but unchanged in the peripheral blood, when compared to that in healthy individuals and patients with other joint diseases (17,18). Importantly, the Treg cell phenotype and suppressive activity of Treg cells are altered in RA. Rheumatoid Treg cells exhibit diminished suppressive capabilities, are unable to inhibit the production of tumor necrosis factor α (TNFα) and interferon-γ (IFNγ) by CD4+ conventional T (Tconv) cells or macrophages (19,20), and do not accumulate CTLA-4 on their surface (21). Similarly, another study showed resistance of CD4+ T cells to Treg cell–mediated suppression in the peripheral blood of RA patients (22). Moreover, TNFα compromises Treg cell function in RA (19,23).

Our group (24,25) and other investigators (23) have shown that TNFα antagonist therapy leads to a modification in the Treg cell phenotype, accompanied by an increase in suppressive activity. In this study, we hypothesized that IL-6R blockade could act via effects on Treg cells. Most of the studies on the effects of anti–IL-6R treatment on Treg cells have focused only on Treg cell frequency (10,14,26).

Our objective in the present study was to elucidate the consequences of anti–IL-6R treatment in terms of its effects on the Treg cell phenotype and the activation of Treg cells in both a mouse model of RA (CIA) and in human patients with RA (23), in order to determine whether Treg cells are involved in the mode of action of this targeted therapy.

PATIENTS AND METHODS

Patients. Patients meeting the American College of Rheumatology/European League Against Rheumatism (EULAR) revised criteria for RA (27) and qualifying for anticytokine therapy were invited to participate in a prospective, open-label, 12-week study of intravenous tocilizumab. Eligible patients were required to have a 28-joint Disease Activity Score (DAS28) of $\geq$3.2 and to have been treated unsuccessfully with at least 2 synthetic and/or biologic disease-modifying antirheumatic drugs (DMARDs). The study was approved by the local ethics committee (approval no. DC-2012-1579), and informed consent was obtained from all patients prior to study entry. Tocilizumab was given with approval of the French Drug Agency, in a dose of 8 mg/kg as a 60-minute intravenous infusion every 4 weeks.

We included 15 patients (13 women, 2 men) whose median age was 58 years and mean disease duration was 17.6 years (a detailed list of the patients' main characteristics at baseline is available from the corresponding author upon request). A history of an inadequate treatment response to methotrexate was noted in all 15 patients, an inadequate response to leflunomide was observed in 7 patients, and an inadequate response to TNFα antagonist therapy was observed in 8 patients (etanercept in 8, infliximab in 2, and adalimumab in 8). A list of the concomitant therapies (steroids or DMARDs) is available from the corresponding author upon request. After 12 weeks of tocilizumab therapy, 7 patients were good responders and 8 were nonresponders, according to the EULAR response criteria (28). For the functional suppression assay, 8 patients were recruited, including 6 of the 7 good responders in the CD39 expression study, and 2 other patients whose disease was in sustained remission or whose disease activity had been low for at least 6 months.

Mice. *Clinical and histologic assessments of CIA.* DBA/1 mice (6 weeks old) were purchased from Janvier. All procedures were approved by the Animal Care and Use Committee of the Université Paris 13 (Bobigny, France).

Arthritis was induced with native bovine type II collagen (CII) (MD Biosciences) in male DBA/1 mice. Clinical and histologic scores of arthritis were evaluated in each mouse, as described elsewhere (29,30). Briefly, the animals were killed and their legs were dissected free and processed for histologic studies. At least 4 serial sections were cut from each paw to ensure extensive evaluation of the arthritic joints. The lesions were evaluated in each joint as previously described, using a 4-point scale (scores of 0–3, where 0 indicates normal and 3 indicates severe). This global histologic score reflects both the extent of synovitis (synovial proliferation, inflammatory cell infiltration) and the extent of joint destruction (bone and cartilage thickness and irregularity and presence of erosions). We also separately evaluated the presence of articular destruction, by taking into account the degradation of bone and cartilage regardless of inflammation. For this assessment, we again used a 4-point scale (scores of 0–3, where 0 indicates no destruction and 3 indicates the presence of subchondral bone erosions).

Treatment of mice. IL-6 blockade was achieved using an intraperitoneal injection of 8 mg of MR16-1, a rat IgG1-specific monoclonal antibody against murine IL-6R. Control animals received an intraperitoneal injection of IgG purified

from the serum of unimmunized rats or an intraperitoneal injection of phosphate buffered saline (PBS). The injection was given on the day of CII immunization (day 0) to enable assessment of therapeutic effects in early disease. MR16-1 was a gift from Chugai Pharma France, and control IgG was purchased from Jackson ImmunoResearch.

Cell and tissue preparation. *Mice.* Spleen leukocytes were prepared using a homogenizer, and red blood cells were lysed in hemolysis buffer (NH_4CL, $KHCO_3$, and EDTA). Afferent popliteal and axillary lymph nodes were dissected out of the limbs, and node leukocytes were prepared using a homogenizer. Thymus leukocytes were prepared using a homogenizer, and then frozen at −80°C in heat-inactivated fetal calf serum (FCS) containing 10% dimethyl sulfoxide until staining. Blood was collected by heart puncture.

RA patients. For monitoring of the immune response, blood samples from RA patients were collected just before the first and fourth tocilizumab infusions (8 mg/kg each). For the functional suppression assay, 25–40 ml of blood from responder patients was collected and peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll gradient centrifugation.

Flow cytometry. *Mice.* The cell surfaces were stained with one of the following: fluorescein isothiocyanate (FITC)–labeled anti-CD25 (clone 3C7), FITC-labeled anti-CD4 (clone RM4-5), R-phycoerythrin (PE)–labeled anti–CTLA-4 (clone UC10-4F10-11), PE-labeled anti-CD62L (clone MEL-14) (all from BD Biosciences), PE-labeled anti–glucocorticoid-induced TNF receptor (anti-GITR; clone DTA-1), PE-labeled anti–inducible T cell costimulator (anti-ICOS; clone 7E.17G9) (all from eBioscience), PE-labeled anti-CD39 (clone 24 DMS1; eBioscience) or PE-labeled anti-Helios (clone 22F6; BioLegend), and PerCP–Cy5.5–labeled anti-CD4 (clone RM4-5) or PerCP–Cy5.5–labeled anti-CD8 (clone 53-6.7) (both from BD Biosciences). The cells were stained at 4°C in PBS containing 4% heat-inactivated FCS and 0.01*M* sodium azide, and then incubated for 30 minutes with appropriate dilutions of the various monoclonal antibodies coupled to FITC, PE, or PerCP–Cy5.5. The allophycocyanin (APC)–labeled anti-FoxP3 (clone FJK-16s) staining set (eBioscience) was used for intracellular staining, in accordance with the manufacturer's recommendations. Staining for intracellular cytokines (IL-17 and IFNγ) was performed as described previously (24).

Flow cytometry was performed on a 4-color FACSCalibur (Becton Dickinson). Dead cells were excluded on the basis of forward-scatter and side-scatter characteristics. Reported statistical data are based on at least 1,000 events gated on the cell population of interest. Results were analyzed using CellQuest Pro software (BD Biosciences). The WEASEL program (version 2.3; Walter + Eliza Hall, Institute of Medical Research, Parkville, Australia) was used for graphic representations.

Patients with RA. For assessment of cells from RA patients, we used antibodies to surface markers, from either BD PharMingen (PE-labeled anti-CD127 [clone HIL-7R-M21], PE-labeled anti-CCR6 [clone 11A9], PerCP–Cy5.5–labeled anti-CD25 [clone M-A251], V450-conjugated anti-CD4 [clone RP-T4], and V500-conjugated anti-CD45 [clone HI30]) or eBioscience (PE–Cy7–labeled anti-CD39 [clone A1], Alexa Fluor 647–conjugated anti-FoxP3 [clone 259D/C7], and anti–IL-17 [clone SCPL1362]). The antibodies were detected after fixation and permeabilization with reagents from eBioscience. For Th17 cell staining, PBMCs were first stimulated with anti-CD3/anti-CD28–coated beads (Invitrogen) for 24 hours.

Flow cytometry was performed on a FACSCanto II (BD Biosciences). Data were analyzed using DIVA version 6, and further processed using Prism version 5.0 (GraphPad Software).

Assessment of the suppressive effects of Treg cells on CD4+CD25− Tconv cells. Spleen CD4+CD25− and CD4+CD25+ T cells were purified using a Regulatory T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol (24). Flow cytometry demonstrated 90–95% purity of the CD4+CD25− and CD4+CD25+ T cell–enriched fractions. The proliferation of carboxyfluorescein succinimidyl ester (CFSE)–stained CD4+CD25− T cells (Tconv cells) with or without CD4+CD25+ cells was then assessed, as described previously (24).

Assessment of the suppressive effects of CD39+ and CD39− Treg cells on CD4+CD25− Tconv cells. Isolation of human T cell subsets was carried out by labeling 25–80 million PBMCs with APC–H7–conjugated anti-CD4, PerCP–Cy5.5–conjugated anti-CD25, PE-conjugated anti-CD127, and PE–Cy7–conjugated anti-CD39. Viable cells were cell-sorted on a FACSAria (MRI platform) in 3 various populations: CD4+CD127+CD25− T cells (Tconv cells), CD4+CD127^{low}CD25+CD39+ T cells (CD39+ Treg cells), and CD4+CD25+CD127^{low}CD39− T cells (CD39− Treg cells). Cell-sorted populations were routinely >98% pure.

CFSE-labeled Tconv cells (10^5) were cultured with titrated numbers of CD39+ Treg cells, CD39− Treg cells, or unlabeled Tconv cells in the presence of allogeneic APC (5×10^5) and 10 μg/ml anti-CD3ε monoclonal antibody 2C11. After 4 days of culture, proliferation of Tconv cells was assessed by fluorescence-activated cell sorting (FACS) analysis. Data were analyzed using FlowJo software to determine the percentage of original cells that divided, and further processed using Prism version 5.0 (GraphPad Software).

ATP hydrolysis assay. The ATPase activity of Treg cells from MR16-1–treated or control IgG-treated mice was evaluated 9 days after the induction of CIA. CD4+CD25+ cells were purified as described above, and ATPase activity was determined as described previously (31).

Cytokine assay. For detection of TNFα in the sera, a FlowCytomix kit (eBioscience) was used in accordance with the manufacturer's instructions. Samples were analyzed by flow cytometry. FlowCytomix Pro software (eBioscience) was used for cytokine quantification.

Enzyme-linked immunosorbent assay (ELISA) for IFNγ. Levels of IFNγ in the culture supernatants were measured using commercially available ELISA kits (R&D Systems), in accordance with the manufacturer's instructions.

Statistical analysis. Depending on the distribution of the data, parametric or nonparametric tests with appropriate comparisons were used to compare groups. Clinical scores and log-transformed histologic scores were compared using one-way analysis of variance (ANOVA) with comparison using post hoc Student-Newman-Keuls test.

The impact of MR16-1 treatment during the course of CIA on thymus, spleen, and lymph node Treg cells or Th17 cell

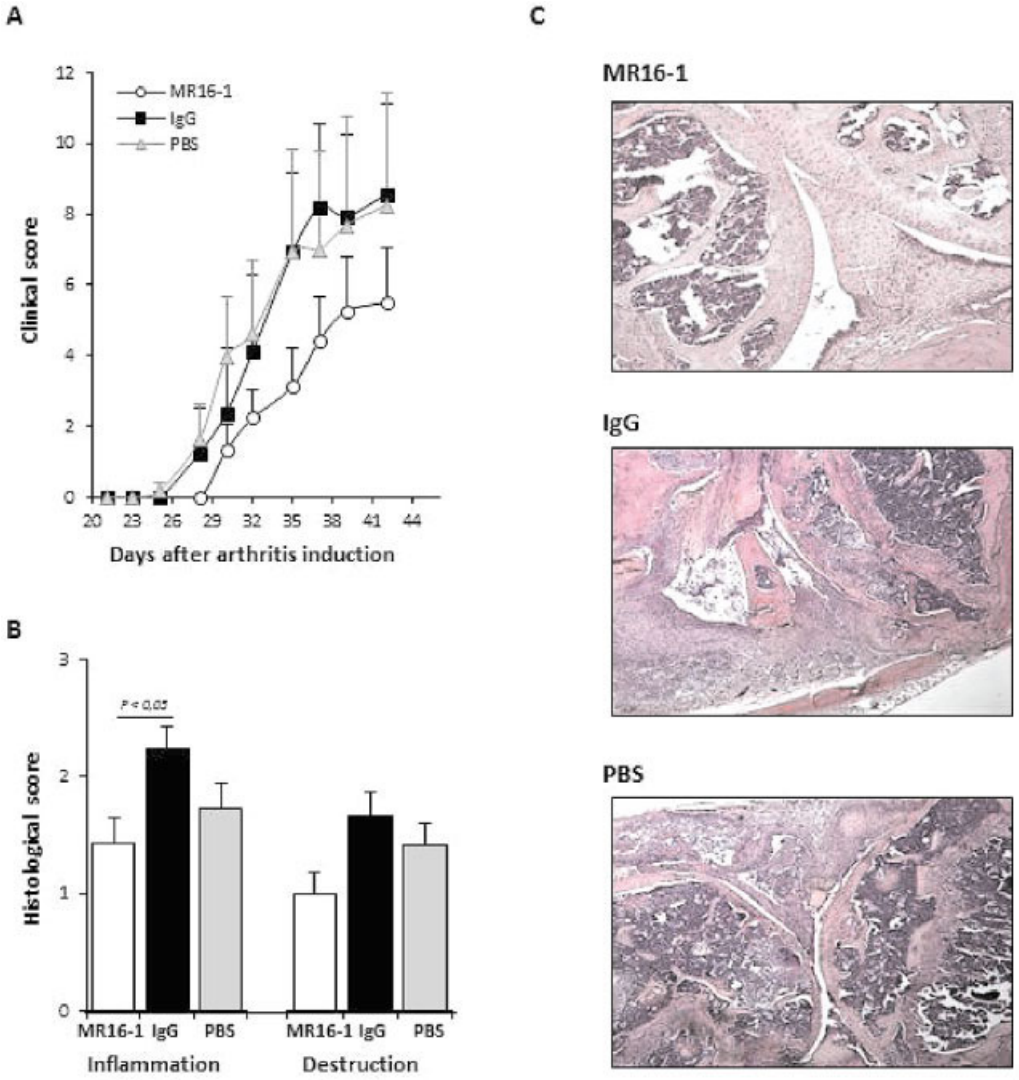

Figure 1. Interleukin-6 receptor (IL-6R) blockade with anti–IL-6R monoclonal antibody inhibits murine collagen-induced arthritis (CIA). On day 0, type II collagen was injected into mice to induce CIA, followed by intraperitoneal injection with anti–IL-6R monoclonal antibody MR16-1 (8 mg; n = 8) or with IgG (8 mg; n = 8) or phosphate buffered saline (PBS; n = 8) as controls. **A** and **B**, Clinical arthritis scores (**A**) and histologic joint inflammation and destruction scores (**B**) were compared between groups. The incidence of CIA was 6 (75%), 6 (75%), and 5 (62%) in the MR16-1, IgG, and PBS groups, respectively. Results are the mean ± SEM. *P* values were determined by one-way analysis of variance. **C**, Representative histologic slides of knee sections (stained with hematoxylin and eosin) show the extent of inflammatory synovitis and joint destruction in mice treated with MR16-1, compared with mice in the IgG or PBS control groups. Original magnification × 50. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38246/abstract.

populations, in terms of the number, frequency, ratio, and expression of various molecules (assessed as the mean fluorescence intensity [MFI]), was evaluated using two-way ANOVA (treatment group and time). Serum cytokine (TNFα) levels were compared between MR16-1– and IgG-treated mice using the Mann-Whitney test with Bonferroni correction. All statistical analyses were performed with MedCalc software version 10.4, unless otherwise specified.

RESULTS

Improvement in CIA following early IL-6R blockade. A single intraperitoneal injection of 8 mg of MR16-1 on day 0 attenuated the clinical manifestations of arthritis ($P < 0.05$) (Figure 1A) and the histologic arthritis scores (Figures 1B and C), as compared to IgG-

Table 1. Effects of anti–interleukin-6 receptor monoclonal antibody MR16-1 on thymic Treg cells in mice with collagen-induced arthritis*

	Days after arthritis induction			
	8	18	28	42
Frequency of CD4+CD8−FoxP3+ cells				
MR16-1	2.22 ± 0.15	1.28 ± 0.10	2.08 ± 0.18	1.55 ± 0.13
IgG control	1.07 ± 0.10	1.24 ± 0.12	1.91 ± 0.13	0.97 ± 0.10
Number of CD4+CD8−FoxP3+ cells (10^{-4})				
MR16-1	16.7 ± 1.3	4.6 ± 0.5	11.3 ± 2.4	2.9 ± 0.4
IgG control	8.1 ± 1.4	3.1 ± 0.4	7.7 ± 1.0	1.6 ± 0.2
Frequency of Helios+ cells among CD4+CD8−FoxP3+ cells				
MR16-1	98.25 ± 0.19	98.23 ± 0.16	98.38 ± 0.18	96.01 ± 0.46
IgG control	97.44 ± 0.42	97.58 ± 0.25	97.90 ± 0.32	93.78 ± 0.90
MFI of Helios expression among CD4+CD8−FoxP3+ cells				
MR16-1	572.96 ± 42.42	650.04 ± 13.34	450.10 ± 21.97	434.39 ± 18.78
IgG control	477.33 ± 37.05	563.32 ± 14.54	403.89 ± 12.43	318.04 ± 28.25

* The MR16-1 treatment group comprised 8 mice, and the IgG control group comprised 10 mice on all days except day 42 (n = 8). Thymic Treg cells, defined as CD4+CD8−FoxP3+ cells, were monitored using flow cytometry in all mice on days 8, 18, 28, and 42 after arthritis induction. Helios expression was assessed on gated CD4+CD8−FoxP3+ cells. Results are the mean ± SEM. Differences between groups were significant ($P < 0.05$ by two-way analysis of variance) on all days throughout the development of collagen-induced arthritis. MFI = mean fluorescence intensity.

and PBS-treated control mice. TNFα was detectable in the serum of mice with late-stage CIA (after day 28), and TNFα levels were higher in the IgG group than in the MR16-1 group (mean ± SEM 25.4 ± 2.4 pg/ml versus 13.9 ± 11.1 pg/ml; $P < 0.05$) on day 42.

Increased frequency of thymic Treg cells in MR16-1–treated mice. Treg cells can be generated either in the thymus from CD4+CD8+ double-positive thymocytes (natural Treg [nTreg] cells) or peripherally (induced Treg [iTreg] cells) from conventional CD4+ FoxP3− T cells (Tconv cells) (32). In MR16-1–treated mice compared to IgG-treated control mice, we found increases in both the count and frequency of nTreg cells (CD4+CD8−FoxP3+ cells) among whole thymocytes during the course of arthritis (Table 1), mainly attributable to differences observed at the begining (day 8) and at the end (day 42) of the analysis. Similarly, the frequency of thymic CD4+CD8−FoxP3+ cells expressing Helios and the level of Helios expression by thymic Treg cells were significantly increased in MR16-1–treated mice compared to controls (Table 1).

Increased Treg:Th17 and Treg:Tconv cell ratios in MR16-1–treated mice. In the spleen, the percentage of Tconv cells among leukocytes from day 18 to day 42 was lower in MR16-1–treated mice compared to IgG-treated controls, whereas the percentage of Treg cells was similar in both groups. Thus, the Treg:Tconv cell ratio was unmodified (results available from the corresponding author upon request). In the lymph nodes, MR16-1 treatment decreased the Tconv cell percentage among leukocytes from day 18 to day 42 as compared to that in the control groups, whereas the Treg cell per-

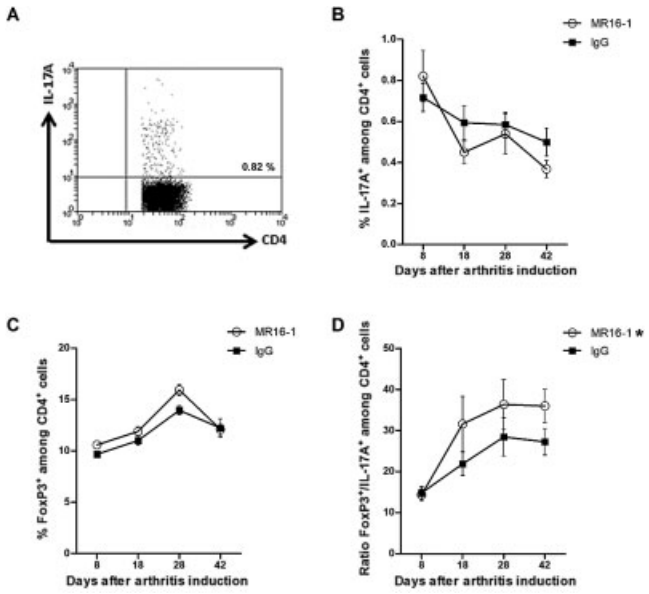

Figure 2. Interleukin-6 receptor (IL-6R) neutralization increases the Treg:Th17 cell ratio in murine collagen-induced arthritis (CIA). Lymph node cells (from the same mice as described in Figure 1) were labeled with fluorochrome-conjugated anti-CD4, anti–IL-17, and anti-FoxP3. Th17 and Treg cells, defined as CD4+IL-17+ cells and CD4+FoxP3+ cells, respectively, were monitored in the lymph nodes. **A**, Representative dot plot showing the percentage of IL-17+ cells on gated CD4+ cells in an MR16-1–treated mouse on day 8. **B** and **C**, Percentages of IL-17+ (**B**) and FoxP3+ (**C**) cells among CD4+ cells in the lymph nodes of MR16-1–treated mice and IgG control mice during the course of CIA. **D**, Ratio of FoxP3+ cells to Th17+ cells among CD4+ cells in the lymph nodes from MR16-1–treated mice compared with IgG controls during the course of CIA. Results are the mean ± SEM. * = $P < 0.05$ versus IgG, by two-way analysis of variance.

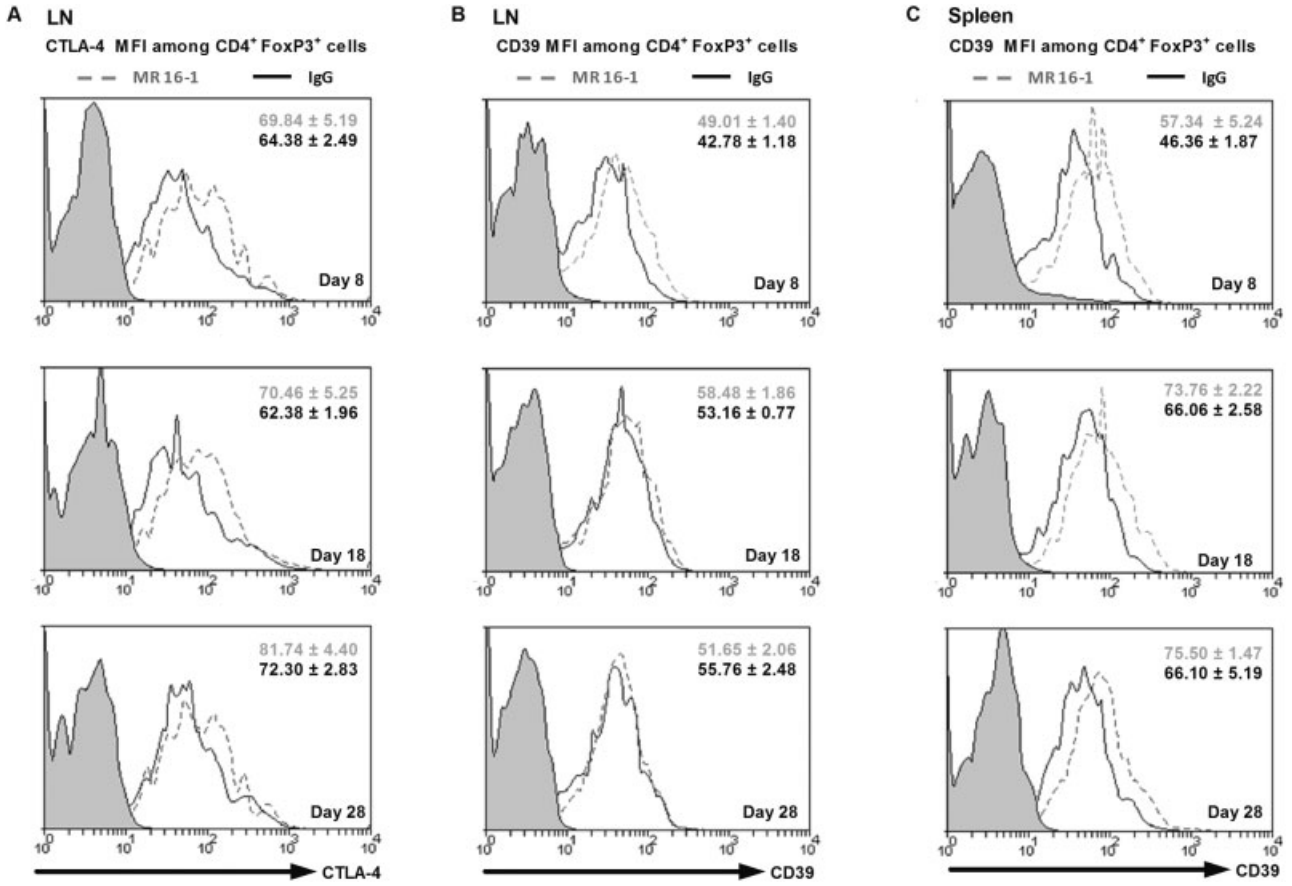

Figure 3. Interleukin-6 receptor (IL-6R) blockade in mice with collagen-induced arthritis (CIA) increases the intensity of CD39 Treg cell expression in the spleen. Leukocytes in the lymph nodes (LN) (**A** and **B**) or spleen (**C**) from mice with CIA (same mice as described in Figure 1) were labeled with fluorochrome-conjugated anti-CD4, anti-FoxP3, and anti–CTLA-4 (**A**) or anti-CD39 (**B** and **C**), and expression of CTLA-4 and CD39 on gated CD4+FoxP3+ cells was analyzed using flow cytometry. For each day, histograms are shown for 1 representative mouse in the MR16-1–treated group and control IgG group, as well as the isotype control (shaded histograms). Mean ± SEM values are the mean fluorescence intensity (MFI) of CTLA-4 (**A**) or CD39 (**B** and **C**) expression. Differences between groups were significant ($P < 0.05$ by two-way analysis of variance) throughout the course of CIA.

centage was not different between groups. Thus, the Treg:Tconv cell ratio increased in the lymph nodes of MR16-1–treated mice compared to control mice globally over time (results available from the corresponding author upon request).

IL-6 acts as a decision point that determines whether the immune response is dominated by either Treg cells or Th17 cells. We found no significant change in the frequency of either Th17 cells (Figures 2A and B) or Treg cells (Figure 2C) among CD4+ cells in the lymph nodes after MR16-1 treatment. Nevertheless, the ratio of Treg cell frequencies to Th17 cell frequencies among CD4+ cells was significantly increased in favor of Treg cells in the lymph nodes of MR16-1–treated mice (Figure 2D).

Modification of the Treg cell phenotype by IL-6R neutralization in mice with CIA. We characterized the Treg cell phenotype throughout the development of CIA, from day 8 to day 28, by evaluating the expression levels of CTLA-4, CD62L, GITR, ICOS, Helios, and CD39. Levels of ICOS, GITR, and Helios were not modified on Treg cells from the lymph nodes or spleen of mice at any time after MR16-1 treatment (results not shown). In contrast, MR16-1 treatment increased the percentage of CTLA-4+ Treg cells (results available from the corresponding author upon request) and in-

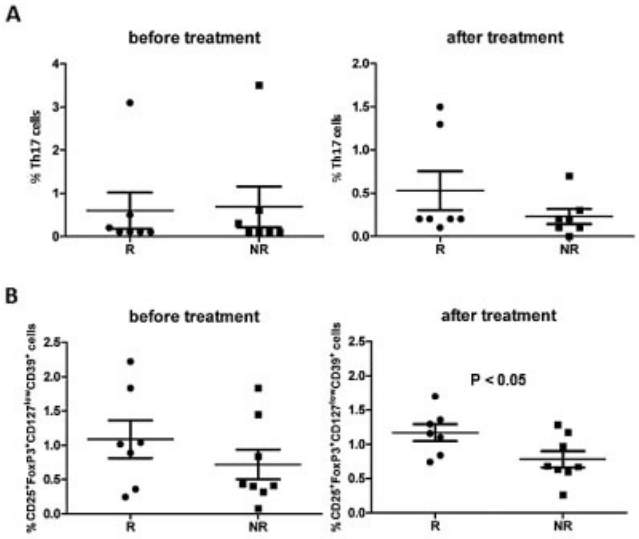

Figure 4. Tocilizumab treatment does not affect the frequency of Th17 cells, but increases the frequency of CD39+ Treg cells, in patients with rheumatoid arthritis (RA). Flow cytometry was used to analyze the percentage of Th17 cells (CD4+IL-17+CCR6+ cells) (**A**) and Treg cells (CD4+CD25+FoxP3+CD127^{low}CD39+ cells) among CD4+ cells (**B**) from 15 patients with RA at baseline and 3 months after treatment with tocilizumab. Each symbol represents an individual patient. Bars show the mean ± SEM. After treatment, the frequency of CD39+ Treg cells differed significantly (as determined by Mann-Whitney test) between responders (R) and nonresponders (NR).

creased the intensity (MFI) of CTLA-4 expression in the lymph nodes (Figure 3A), but not in the spleen (results not shown), when compared to IgG treatment during the course of CIA.

Furthermore, IL-6R neutralization increased the frequency of CD39+ Treg cells in both the lymph nodes and spleen, as compared to that in the IgG control group (results available from the corresponding author upon request). The intensity (MFI) of CD39 expression on Treg cells from the spleen, but not the lymph nodes, was increased throughout the course of CIA in the MR16-1 group compared to the IgG control group (Figures 3B and C). MR16-1 treatment had no effect on the ability of Treg cells to suppress the proliferation of Tconv cells or production of IFNγ, nor did it have any effect on the ATPase activity of Treg cells (results not shown).

Lack of effect on Th17 cells, but increase in CD39+ Treg cells, following tocilizumab treatment in RA patients. Of the 15 RA patients treated with tocilizumab, 7 met the EULAR criteria for a very good response (defined as a change in the DAS28 of >1.2) (results available from the corresponding author upon request). The frequency of peripheral blood Th17 cells (defined as CD4+IL-17+CCR6+ cells) was not significantly modified by tocilizumab therapy. Moreover, the frequency of Th17 cells did not differ between responders and nonresponders (mean ± SEM 0.53 ± 0.22% versus 0.23 ± 0.09%) (Figure 4A).

With regard to Treg cells, CD39 is constitutively expressed on ~50% of human CD25+FoxP3+ nTreg cells in healthy controls (33). The percentage of CD39+ cells among CD4+CD25+FoxP3+ T cells was signifi-

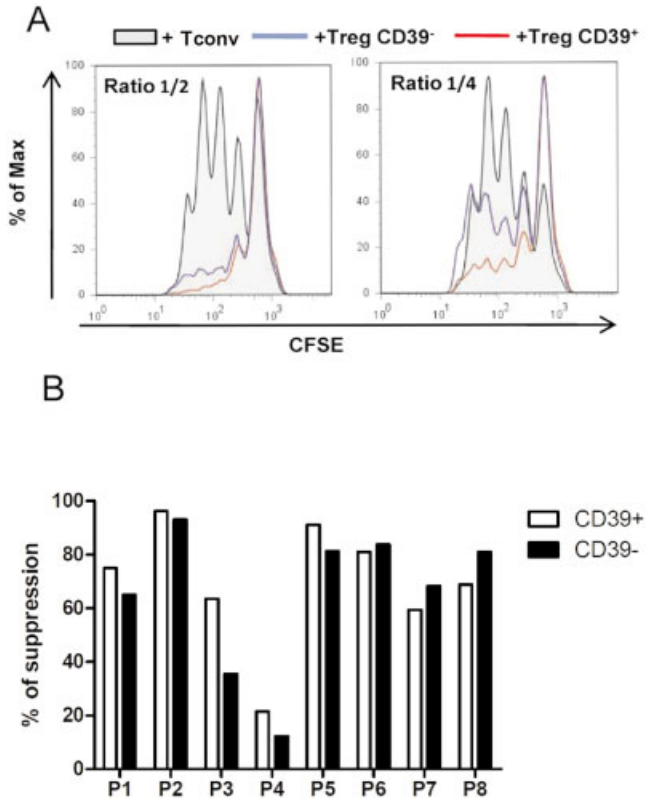

Figure 5. CD39+ Treg cells suppress the proliferation of conventional T (Tconv) cells in patients with rheumatoid arthritis (RA) who are tocilizumab responders. **A**, Unlabeled titrated numbers of CD4+ Tconv cells or CD39+ or CD39− Treg cells were cocultured with carboxyfluorescein succinimidyl ester (CFSE)–labeled Tconv cells (in ratios of 1:2 or 1:4) in the presence of allogeneic allophycocyanin and anti-CD3ε antibody. Gray histograms represent proliferation of activated CD4+ T cells cocultured with Tconv cells. Blue and red histograms represent proliferation of activated CD4+ T cells cocultured with fluorescence-activated cell–sorted CD39− Treg cells or CD39+ Treg cells, respectively. Representative results from 1 of 8 experiments are shown. **B**, CFSE dilution data obtained (at a ratio of 1 Treg cell to 2 Tconv cells) from 8 responder patients (P1–P8) were analyzed by FlowJo to determine the percentage of original cells that divided. Thereafter, the percentage of inhibition of proliferation was assessed following the addition of CD39+ Treg cells or CD39− Treg cells in 8 independent experiments.

cantly higher in the responders than in the nonresponders after 3 months of tocilizumab therapy (mean ± SEM 72 ± 4% versus 44 ± 8%; $P < 0.05$). Importantly, as a consequence, the frequency of CD39+ Treg cells among CD4+ cells was significantly higher in responders compared to nonresponders (mean ± SEM 1.17 ± 0.12% versus 0.78 ± 0.12%; $P < 0.05$) (Figure 4B). Interestingly, this increase in the frequency of CD39+ Treg cells was observed as early as 1 month (results not shown) following treatment with tocilizumab, but the increase only reached a statistically significant difference at 3 months of treatment.

Suppressive function of CD39+ and CD39− Treg cells in responder patients. To assess the functionality of Treg cells in a suppression assay, CD39+ and CD39− Treg cells from 8 responder patients (mean ± SEM DAS28 2.2 ± 0.8; mean ± SEM duration of tocilizumab treatment 22.6 ± 7.2 months) were FACS-sorted based on the expression of CD25, CD127, and CD39, to separate both Treg cell populations from Tconv cells. First, we found that in all tested patients, both Treg cell populations suppressed, in a very efficient manner, the proliferation of CD4+ Tconv cells, even at a Treg:Tconv cell ratio of 1:2 (Figure 5A).

Based on the percentage of original cells that had divided, we calculated the relative suppression activity of both Treg cell populations. FACS-sorted CD39+ Treg cells from the 8 responder RA patients were functionally able to suppress the proliferation of Tconv cells. It may be noted that in 5 of the 8 responders, the suppressive function of CD39+ Treg cells was higher than that of CD39− Treg cells (Figure 5B).

DISCUSSION

The results of the present study demonstrate, for the first time, the involvement of the CD39+FoxP3+ Treg cell population following administration of an anticytokine treatment in arthritis. We show that blockade of the IL-6 pathway modified the Th17/Treg cell balance and led to induction of a functional CD39+FoxP3+ Treg cell population that was associated with an attenuation of disease severity in both humans with RA and mice with CIA.

Since IL-6 is a key factor in Th17 cell differentiation (34), the protective effect of anti–IL-6R antibody treatment against arthritis was hypothesized to involve the suppression of Th17 cells (14,35), as has been seen in other models of autoimmune disease. Studies in various autoimmunity models, such as experimental autoimmune encephalomyelitis (36), uveoretinitis (37), CIA (2,38), and glucose-6-phosphate isomerase–induced arthritis (39), showed that anti–IL-6R antibody treatment suppressed antigen-specific Th17 cell differentiation. In our study, Th17 cell frequencies were not significantly different between tocilizumab responders and nonresponders, and no significant changes were observed throughout the course of CIA in MR16-1–treated mice. An important difference in comparison with other studies is that we evaluated non–antigen-specific Th17 cells. Therefore, our results in CIA may more accurately reflect the events in human RA, whose pathogenesis likely involves several different autoantigens.

Rather than conducting separate measurements of the frequencies of Th17 cells and Treg cells, an assessment of the balance between these 2 T helper cell subsets is needed, since IL-6 is the key cytokine directing the differentiation process toward one or the other cell type (11). An appropriate Th17/Treg cell balance is crucial for maintaining immune homeostasis, whereas an excess of Th17 cells relative to Treg cells causes the onset of various autoimmune and chronic inflammatory diseases (40).

Our study shows that anti–IL-6R antibody treatment modifies the Th17/Treg cell balance by modifying the Treg:Tconv cell ratio, but not by acting on the Th17 cell subset (neither in mice nor in humans). In a study by Fujimoto et al (14), the Th17:Treg cell ratio was modified in mice with CIA treated with MR16-1, although contrary to our study findings, this modification could be attributed to a decreased Th17 cell frequency rather than a modified Treg cell percentage. In addition, in a study by Samson et al, treatment of RA patients with tocilizumab induced a decreased frequency of Th17 cells as compared to that in healthy donors (10). This apparent discrepancy with our data can be explained by the different types of controls used in the 2 studies. In our study, all patients received tocilizumab, and we compared responder RA patients to nonresponder RA patients. In contrast, in the study by Samson et al (10), tocilizumab-treated RA patients were compared to untreated patients with active RA. Nevertheless, similar to our findings, the study by Samson et al showed that tocilizumab induced an increased percentage of Treg cells, and in both studies, inhibition of the IL-6 pathway restored the Th17/Treg cell balance.

IL-6 pathway inhibition also modified the Treg cell phenotype. In particular, we found that the Helios+ Treg cell population was increased within the thymus of MR16-1–treated mice. Helios was initially described as a specific marker for discriminating thymus-derived nTreg cells (Helios+) from peripherally differentiated iTreg

cells (Helios−) (41). However, results of recent studies have suggested that Helios may be unable to distinguish nTreg cells from iTreg cells, as Helios expression may be induced during the activation of T cells regardless of their origin (42,43). The relevance of Helios as a marker for Treg cell activation needs to be further investigated. Nevertheless, assuming that Helios is a Treg cell activation marker, our results suggest that IL-6 pathway inhibition in CIA may lead to either increased re-entry of activated Treg cells into the thymus or, more probably, to increased intrathymic activation of Treg cells (42).

Given that MR16-1 inhibited the clinical, histologic, and biologic signs of CIA, we studied whether these effects were related to an enhanced Treg cell suppression of Tconv cell proliferation. In vitro, the degree of suppression in the spleen was similar in the MR16-1 and control mice (results not shown). To study another Treg cell suppressive phenotype, we investigated whether anti–IL-6R antibody treatment modified CD39 expression on Treg cells. In mice with CIA, MR16-1 treatment expanded the CD39+ Treg cell population. Interestingly, tocilizumab treatment induced a CD39+ Treg cell population in responder patients with RA after 1 month, and this increase reached a statistically significant difference at 3 months after treatment. This CD39+ Treg cell population was functionally able to suppress Tconv cell proliferation in responder patients.

Membrane-expressed CD39 catalyzes extracellular ATP hydrolysis and, together with CD73, results in production of the antiinflammatory mediator adenosine (31). CD39 expression on Treg cells has thus been associated with increased suppressive potency, one effect of which is suppression of Th17 cell activity (33,44). Moreover, alterations in the CD39/CD73 machinery lead to loss of Treg cell function and to autoimmunity (45). FoxP3+CD39+ Treg cells are impaired in patients with multiple sclerosis (33). Conversely, the CD39+ Treg cell population expands during remission of multiple sclerosis and after antiinflammatory treatment in CIA (46,47). Moreover, a very recent study has shown that an efficient therapy based on stem cell adoptive transfer mostly depended on CD39/CD73 signals and partially on the induction of CD4+CD39+FoxP3+ Treg cells in CIA (48). These findings are consistent with the increased CD39 expression on Treg cells in CIA as well as in responder patients after anti–IL-6R blockade. We can therefore hypothesize that this anti–IL-6R antibody allows the generation of a potent suppressive Treg cell population capable of inducing metabolic changes that protect against joint inflammation.

Overall, our results support the hypothesis that one mechanism underlying the control of arthritis via IL-6 pathway blockade may be both the restoration of a nonpathogenic Th17/Treg cell balance and the generation of a suppressive CD39+ Treg cell population. Further studies are warranted to determine whether IL-6R inhibition acts directly on Treg cells or involves other cell populations acting on Treg cells.

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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. Boissier 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.

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REFERENCES

1. Alonzi T, Fattori E, Lazzaro D, Costa P, Probert L, Kollias G, et al. Interleukin 6 is required for the development of collagen-induced arthritis. J Exp Med 1998;187:461–8.
2. Takagi N, Mihara M, Moriya Y, Nishimoto N, Yoshizaki K, Kishimoto T, et al. Blockage of interleukin-6 receptor ameliorates joint disease in murine collagen-induced arthritis. Arthritis Rheum 1998;41:2117–21.
3. Madhok R, Crilly A, Watson J, Capell HA. Serum interleukin 6 levels in rheumatoid arthritis: correlations with clinical and laboratory indices of disease activity. Ann Rheum Dis 1993;52:232–4.
4. Tak PP, Kalden JR. Advances in rheumatology: new targeted therapeutics. Arthritis Res Ther 2011;13 Suppl 1:S5.
5. Dienz O, Eaton SM, Bond JP, Neveu W, Moquin D, Noubade R, et al. The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells. J Exp Med 2009;206:69–78.

6. Boissier MC. Cell and cytokine imbalances in rheumatoid synovitis. Joint Bone Spine 2011;78:230–4.
7. Hirota K, Hashimoto M, Yoshitomi H, Tanaka S, Nomura T, Yamaguchi T, et al. T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17+ Th cells that cause autoimmune arthritis. J Exp Med 2007;204:41–7.
8. Geboes L, Dumoutier L, Kelchtermans H, Schurgers E, Mitera T, Renauld JC, et al. Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice. Arthritis Rheum 2009;60:390–5.
9. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol 2003;171:6173–7.
10. Samson M, Audia S, Janikashvili N, Ciudad M, Trad M, Fraszczak J, et al. Inhibition of interleukin-6 function corrects Th17/Treg cell imbalance in patients with rheumatoid arthritis. Arthritis Rheum 2012;64:2499–503.
11. Bettelli E, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol 2007;8:345–50.
12. Doganci A. The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. J Clin Invest 2005;115:313–25.
13. Oberg HH, Wesch D, Grussel S, Rose-John S, Kabelitz D. Differential expression of CD126 and CD130 mediates different STAT-3 phosphorylation in CD4+CD25− and CD25high regulatory T cells. Int Immunol 2006;18:555–63.
14. Fujimoto M, Serada S, Mihara M, Uchiyama Y, Yoshida H, Koike N, et al. Interleukin-6 blockade suppresses autoimmune arthritis in mice by the inhibition of inflammatory Th17 responses. Arthritis Rheum 2008;58:3710–9.
15. Morgan ME, Sutmuller RP, Witteveen HJ, van Duivenvoorde LM, Zanelli E, Melief CJ, et al. CD25+ cell depletion hastens the onset of severe disease in collagen-induced arthritis. Arthritis Rheum 2003;48:1452–60.
16. Nguyen LT, Jacobs J, Mathis D, Benoist C. Where FoxP3-dependent regulatory T cells impinge on the development of inflammatory arthritis. Arthritis Rheum 2007;56:509–20.
17. Jiao Z, Wang W, Jia R, Li J, You H, Chen L, et al. Accumulation of FoxP3-expressing CD4+CD25+ T cells with distinct chemokine receptors in synovial fluid of patients with active rheumatoid arthritis. Scand J Rheumatol 2007;36:428–33.
18. Lin SC, Chen KH, Lin CH, Kuo CC, Ling QD, Chan CH. The quantitative analysis of peripheral blood FOXP3-expressing T cells in systemic lupus erythematosus and rheumatoid arthritis patients. Eur J Clin Invest 2007;37:987–96.
19. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J Exp Med 2004;200:277–85.
20. Cao D, van Vollenhoven R, Klareskog L, Trollmo C, Malmstrom V. CD25brightCD4+ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease. Arthritis Res Ther 2004;6:R335–46.
21. Flores-Borja F, Jury EC, Mauri C, Ehrenstein MR. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. Proc Natl Acad Sci U S A 2008;105: 19396–401.
22. Van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum 2004;50: 2775–85.
23. Nadkarni S, Mauri C, Ehrenstein MR. Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. J Exp Med 2007;204:33–9.
24. Biton J, Semerano L, Delavallee L, Lemeiter D, Laborie M, Grouard-Vogel G, et al. Interplay between TNF and regulatory T cells in a TNF-driven murine model of arthritis. J Immunol 2011;186:3899–910.
25. Biton J, Boissier MC, Bessis N. TNFalpha: activator or inhibitor of regulatory T cells? Joint Bone Spine 2011;79:119–23.
26. Aricha R, Mizrachi K, Fuchs S, Souroujon MC. Blocking of IL-6 suppresses experimental autoimmune myasthenia gravis. J Autoimmun 2011;36:135–41.
27. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO 3rd, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 2010;62: 2569–81.
28. Van Gestel AM, Prevoo ML, van 't Hof MA, van Rijswijk MH, van de Putte LB de, van Riel PL. Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis: comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism criteria. Arthritis Rheum 1996;39:34–40.
29. Clavel G, Valvason C, Yamaoka K, Lemeiter D, Laroche L, Boissier MC, et al. Relationship between angiogenesis and inflammation in experimental arthritis. Eur Cytokine Netw 2006;17: 202–10.
30. Denys A, Thiolat A, Descamps D, Lemeiter D, Benihoud K, Bessis N, et al. Intra-articular electrotransfer of mouse soluble tumour necrosis factor receptor in a murine model of rheumatoid arthritis. J Gene Med 2010;12:659–68.
31. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood 2007;110:1225–32.
32. Bilate A, Lafaille J. Induced CD4(+)Foxp3(+) regulatory T cells in immune tolerance. Ann Rev Immunol 2012;30:733–91.
33. Fletcher JM, Lonergan R, Costelloe L, Kinsella K, Moran B, O'Farrelly C, et al. CD39+Foxp3+ regulatory T cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. J Immunol 2009;183:7602–10.
34. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441: 235–8.
35. Yoshida H, Hashizume M, Mihara M. IL-6 blockade preferentially inhibits Th17 differentiation in collagen-induced arthritis. Rheumatol Int 2011;31:127–31.
36. Serada S, Fujimoto M, Mihara M, Koike N, Ohsugi Y, Nomura S, et al. IL-6 blockade inhibits the induction of myelin antigen-specific Th17 cells and Th1 cells in experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A 2008;105:9041–6.
37. Hohki S, Ohguro N, Haruta H, Nakai K, Terabe F, Serada S, et al. Blockade of interleukin-6 signaling suppresses experimental autoimmune uveoretinitis by the inhibition of inflammatory Th17 responses. Exp Eye Res 2010;91:162–70.
38. Lissilaa R, Buatois V, Magistrelli G, Williams A, Jones G, Herren S, et al. Although IL-6 trans-signaling is sufficient to drive local immune responses, classical IL-6 signaling is obligate for the induction of T cell-mediated autoimmunity. J Immunol 2010;185: 5512–33.
39. Iwanami K, Matsumoto I, Tanaka-Watanabe Y, Inoue A, Mihara M, Ohsugi Y, et al. Crucial role of the interleukin-6/interleukin-17 cytokine axis in the induction of arthritis by glucose-6-phosphate isomerase. Arthritis Rheum 2008;58:754–63.
40. Tanaka T, Kishimoto T. Immunotherapeutic implication of IL-6 blockade. Immunotherapy 2012;4:87–192.
41. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, et al. Expression of Helios, an Ikaros transcription

factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol 2010;184: 3433–41.
42. Akimova T, Beier U, Wang L, Levine MH, Hancock WW. Helios expression is a marker of T cell activation and proliferation. PloS One 2011;6:e24226.
43. Gottschalk RA, Corse E, Allison JP. Response to comment on "expression of Helios in peripherally induced Foxp3+ regulatory T cells." J Immunol 2012;189:500–1.
44. Ye ZJ, Zhou Q, Zhang JC, Li X, Wu C, Qin SM, et al. CD39+ regulatory T cells suppress generation and differentiation of Th17 cells in human malignant pleural effusion via a LAP-dependent mechanism. Respir Res 2011;12:77.
45. Sauer AV, Brigida I, Carriglio N, Hernandez RJ, Scaramuzza S, Clavenna D, et al. Alterations in the adenosine metabolism and CD39/CD73 adenosinergic machinery cause loss of Treg cell function and autoimmunity in ADA-deficient SCID. Blood 2012; 119:1428–39.
46. Kochetkova I, Golden S, Holderness K, Callis G, Pascual DW. IL-35 stimulation of CD39+ regulatory T cells confers protection against collagen II-induced arthritis via the production of IL-10. J Immunol 2010;184:7144–53.
47. Kochetkova I, Thornburg T, Callis G, Pascual DW. Segregated regulatory CD39+CD4+ T cell function: TGF-beta-producing Foxp3- and IL-10-producing Foxp3+ cells are interdependent for protection against collagen-induced arthritis. J Immunol 2011;187: 4654–66.
48. Chen M, Su W, Lin X, Guo Z, Wang J, Zhang Q, et al. Adoptive transfer of human gingiva-derived mesenchymal stem cells ameliorates collagen-induced arthritis via suppressing Th1 and Th17 cells and enhancement of regulatory T cell differentiation. Arthritis Rheum 2013;65:1181–93.