This information is current as of January 1, 2021.

CD4+CD25bright Regulatory T Cells Actively Regulate Inflammation in the Joints of Patients with the Remitting Form of Juvenile Idiopathic Arthritis

Ismé M. de Kleer, Lucy R. Wedderburn, Leonie S. Taams, Alka Patel, Hemiata Varsani, Mark Klein, Wilco de Jager, Gisela Pugayung, Francesca Giannoni, Ger Rijkers, Salvatore Albani, Wietse Kuis and Berent Prakken

*J Immunol* 2004; 172:6435-6443; doi: 10.4049/jimmunol.172.10.6435

http://www.jimmunol.org/content/172/10/6435

References

This article cites 41 articles, 17 of which you can access for free at: http://www.jimmunol.org/content/172/10/6435.full#ref-list-1

Why *The JI*? Submit online.

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
CD4⁺CD25bright Regulatory T Cells Actively Regulate Inflammation in the Joints of Patients with the Remitting Form of Juvenile Idiopathic Arthritis

Ismé M. de Kleer,§ Lucy R. Wedderburn,† Leonie S. Taams,‡ Alka Patel,† Hemlata Varsani,† Mark Klein,* Wilco de Jager,* Gisela Pugayung,§ Francesca Giannoni,¶ Ger Rijkers,* Salvatore Albani,¶‖ Wietsje Kuis,* and Berent Prakken*†

This study investigates the role of CD4⁺CD25⁺ regulatory T cells during the clinical course of juvenile idiopathic arthritis (JIA). Persistent olioarticular JIA (pers-OA JIA) is a subtype of JIA with a relatively benign, self-remitting course while extended olioarticular JIA (ext-OA JIA) is a subtype with a much less favorable prognosis. Our data show that patients with pers-OA JIA display a significantly higher frequency of CD4⁺CD25bright T cells with concomitant higher levels of mRNA FoxP3 in the peripheral blood than ext-OA JIA patients. Furthermore, while numbers of synovial fluid (SF) CD4⁺CD25bright T cells were equal in both patient groups, pers-OA JIA patients displayed a higher frequency of CD4⁺CD25int T cells and therefore of CD4⁺CD25total in the SF than ext-OA JIA patients. Analysis of FoxP3 mRNA levels revealed a higher expression in SF CD4⁺CD25bright T cells of both patient groups and also significant expression of FoxP3 mRNA in the CD4⁺CD25int T cell population. The CD4⁺CD25bright cells of both patient groups and the CD4⁺CD25int cells of pers-OA JIA patients were able to suppress responses of CD25⁻ cells in vitro. A markedly higher expression of CTLA-4, glucocorticoid-induced TNFR, and HLA-DR on SF CD4⁺CD25bright regulatory T cells compared with their peripheral counterparts suggests that the CD4⁺CD25⁺ Treg cells may undergo maturation in the joint. In correlation with this mature phenotype, the SF CD4⁺CD25bright T cells showed an increased regulatory capacity in vitro compared with peripheral blood CD4⁺CD25bright T cells. These data suggest that CD4⁺CD25bright Treg cells play a role in determining the patient’s fate toward either a favorable or unfavorable clinical course of disease. The Journal of Immunology, 2004, 172: 6435–6443.

The adaptive immune system represents a highly effective and dynamic system that can protect the host from a wide array of pathogens. However, the establishment of a full repertoire of pathogen-specific lymphocytes is coincident with the formation of T cells able to recognize self-Ag. Some of these potentially autoreactive T cells escape negative selection in the thymus and are released into the periphery. To maintain peripheral tolerance, the immune system has evolved a number of regulatory mechanisms. Failure of this regulatory network can result in autoimmune disease.

One of the key players of immune regulation is the CD4⁺CD25⁺ regulatory T cell (Treg). These spontaneously occurring T cells can actively and dominantly prevent both the activation and the effector function of autoreactive T cells that escape other mechanisms of tolerance. CD4⁺CD25⁺ Treg cells were initially identified in mice and rats by their ability to inhibit the development of autoimmune gastritis following neonatal thymectomy (1) and since then to inhibit autoimmune diabetes (2, 3), prevent inflammatory bowel disease (4), impede antitumor immunity (5), prevent the expansion of other T cells in vivo (6), and inhibit T cell activation in vitro (7, 8).

It is now clear that CD4⁺CD25⁺ Treg cells are also part of the normal human immune repertoire, and it seems likely that the constitutive presence of this regulatory T cell subset also controls autoaggressive T and B cells in humans (9–13). There is a remarkable similarity between CD4⁺CD25⁺ Treg cells in mice and humans. Therefore, the current challenge is to investigate what role human CD4⁺CD25⁺ Treg cells play in the prevention of human autoimmune diseases and whether these cells can limit and perhaps reverse existing immune pathology.

We report here data on CD4⁺CD25⁺ Treg cells in juvenile idiopathic arthritis (JIA) patients and healthy children. JIA is the most frequent rheumatic disease of childhood and is defined as the development, in children under the age of 16 years, of arthritis in one or more joints lasting for >6 wk (14, 15). Two major reasons make JIA an ideal model for the study of immune regulatory

*University Medical Center Utrecht, Wilhelmina Children’s Hospital, Department of Pediatric Immunology and Immunology Advanced Center on Preclinical Immuno-genomics Institute for Translational Medicine, Utrecht, The Netherlands; † Institute of Child Health/Great Ormond Street Hospital, London, United Kingdom; ‡Infection and Immunity Research Group, Department of Life Sciences, King’s College, London, United Kingdom; §Department of Medicine and Pediatrics, University of California, San Diego, and Immunology Advanced Center on Preclinical Immuno-genomics Institute for Translational Medicine, La Jolla, CA 92039; ¶Torrey Pines Institute for Molecular Science, Viral Immunology Division, San Diego, CA 92121; and ‖Androclus Therapeutics, Milan, Italy.

Received for publication October 17, 2003. Accepted for publication February 17, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grants from the Dutch Arthritis Foundation, the University Medical Center Utrecht (The Netherlands, Sparks, U.K.), and the Cathal Hayes Foundation and National Institutes of Health Grants 5P50 AR44850-04, N01-AR-9-2241, 2R01 AI41721-05, and 1R01AR48084-01.
2 Address correspondence and reprint requests to Dr. Berent J. Prakken, Department of Pediatric Immunology, University Medical Center Utrecht, Wilhelmina Children’s Hospital, P.O. Box 85090; 3508 AB Utrecht, The Netherlands. E-mail address: b.prakken@w.kz.azu.nl
3 Abbreviations used in this paper: Treg, T regulatory; JIA, juvenile idiopathic arthritis; pers-OA, persistent oligoarticular; ext-OA, extended oligoarticular; SF, synovial fluid; SFMC, SF mononuclear cell; PB, peripheral blood; CD40L, CD40 ligand; GITR, glucocorticoid-induced TNFR, β2m, β2-microglobulin; Ct, mean threshold cycle; RA, rheumatoid arthritis; MTX, methotrexate.
Proliferation assays and coculture experiments

PBMC and SF mononuclear cells (SFMC) were isolated using Fiocoll-Hypaque density centrifugation (Fiocoll-Paque; Pharmacia, Uppsala, Sweden). For the analysis of the proliferative response of peripheral blood (PB) and SF, CD4^+CD25^- and CD4^+CD25^bright T cells were sorted by FACS (EPICS ALTRA; Beckman Coulter, Fullerton, CA) and subsequently incubated with 1 µM CFSE (Molecular Probes, Eugene, OR) for 5 min at 37°C. The cells were washed and cultured for 5 days in the presence or absence of 1 µg/ml PHA (Sigma-Aldrich, Gillingham, U.K.) and/or 10 ng/ml IL-2 (Eur setResulting from both The Netherlands (University Medical Center, Utrecht, The Netherlands) and Great Britain. The diagnosis of the patients was defined according to the revised criteria for JIA (16). Blood samples were obtained by venipuncture. SF was obtained at the time of a diagnostic or therapeutic joint aspiration. Informed consent was obtained either from parents/guardians or from the children directly when they were older than 12 years (The Netherlands) or 16 years (Great Britain). Table I shows the clinical characteristics of the included children at the time of blood draw and/or SF aspiration.

### Methods

#### Patients

Sixty patients with JIA with a pers-OA course, 34 patients with an ext-OA course, and 34 healthy children were evaluated for this study. Patients were included from both The Netherlands (University Medical Center, Utrecht, The Netherlands) and Great Britain (Great Ormond Street Hospital, London, U.K.). The diagnosis of the patients was defined according to the revised criteria for JIA (16). Blood samples were obtained by venipuncture. SF was obtained at the time of a diagnostic or therapeutic joint aspiration. Informed consent was obtained either from parents/guardians or from the children directly when they were older than 12 years (The Netherlands) or 16 years (Great Britain). Table I shows the clinical characteristics of the included children at the time of blood draw and/or SF aspiration.

#### Flow cytometry

PBMC and SFMC were washed twice in PBS containing 2% FCS (PBS-FCS) and adjusted to 0.5–1 × 10^6 cells/ml in staining buffer (PBS-FCS containing 0.1% sodium azide) and blocked with the appropriate normal serum (30 min at 4°C). Subsequently, the cells were incubated in 50 µl of FACS buffer containing three or four appropriately diluted PE-, FITC-, CyChrome, or allophycocyanin-labeled mAbs against human CD4 (clone RPA-T4), CD25 (clone 2A3), HLA-DR (clone L243), CD40 ligand (CD40L; clone ACT35), CD45RA (clone L48), CD45RO (clone 4CHL-1), CD69 (clone L78), CCR4 (clone 1G1), or glucocorticoid-induced TNFR (GITR; clone 110416). For cytoplasmic staining of CTLA-4 (clone BN13), CD25 (clone 2A3), HLA-DR (clone L243), CD40 ligand (CD40L; clone ACT35), CD45RA (clone L48), CD45RO (clone 4CHL-1), CD69 (clone L78), CCR4 (clone 1G1), or glucocorticoid-induced TNFR (GITR; clone 110416). For the analysis of the proliferative response, (mean ± SEM) of triplicate wells) were cultured in the presence of 5 × 10^5 PBMCs and SFMCs of CD4^+CD25^- or CD4^+CD25^bright T cells at the same ratio. The negative fraction of cells obtained after MACS sorting was used as APC after depletion of T cells by a second round of MACS sorting using anti-CD3 beads followed by irradiation (3500 rad). Three × 10^4 APCs were added to each well. The cells were incubated at 37°C for 6 days, the last 18 h in the presence of [3H]thymidine (1 µCi/well). The suppressive activity was determined by calculating the relative difference in proliferative response (mean ± SEM) of triplicate wells) between CD4^+CD25^- T cells cultured alone and CD4^+CD25^bright T cells cultured in the presence of CD4^+CD25^bright or CD4^+CD25^- T cells.

### Table I. Clinical characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>pers-OA JIA (n = 60)</th>
<th>ext-OA JIA (n = 34)</th>
<th>Healthy controls (n = 34)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td>20 (33%)</td>
<td>9 (26%)</td>
<td>24 (62%)</td>
</tr>
<tr>
<td><strong>Age (mean, range)</strong></td>
<td>11 y 3 m (2 y 7 m–25 y 4m)</td>
<td>10 y 3 m (2 y 7 m–19 y 9 m)</td>
<td>7 y 3 m (2 y 9m–17 y 3m)</td>
</tr>
<tr>
<td><strong>Duration of disease</strong></td>
<td>5 y 6 m (2m–17 y 8m)</td>
<td>6 y 6 m (1m–14y)</td>
<td>2.7 (0–10)</td>
</tr>
<tr>
<td><strong>Joint count</strong> (mean, range)</td>
<td>1.1 (1–4)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Medication</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisone, low dose, orally</td>
<td>0 (0%)</td>
<td>1 (4%)</td>
<td>NA</td>
</tr>
<tr>
<td>MTX, low dose</td>
<td>8 (13%)</td>
<td>9 (26%)</td>
<td>2 (6%)</td>
</tr>
<tr>
<td>MTX, high dose</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>NA</td>
</tr>
<tr>
<td>Anti-TNF-α and/or other</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>NA</td>
</tr>
<tr>
<td>immunosuppressives</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salazopyrine</td>
<td>1 (2%)</td>
<td>1 (4%)</td>
<td>21 (62%)</td>
</tr>
</tbody>
</table>

Nonsteroidal anti-inflammatory drug

[^a]: NA, Not applicable.
[^b]: Two and one-half to 12.5 mg/wk.
[^c]: More than 12.5 mg/wk.
mRNA analysis by quantitative PCR

From three ext-OA and nine pers-OA JIA patients, CD4+CD25bright and CD4+CD25total T cells were isolated from PB and SF by FACS sorting. Total RNA was isolated using Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 500 ng/ml. First-strand cDNA was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison WI) with 1 μg/μl oligo(dT) and 10 mM dNTP (both Amersham Pharmacia Biotech, Uppsala, Sweden). The reaction mixture was incubated at 40°C for 90 min followed by incubation at 70°C for 15 min.

To ensure the fidelity of mRNA extraction and reverse transcription to first-strand cDNA, all samples were subjected to real-time PCR amplification with primers specific for the constitutively expressed gene GAPDH or β-actin (20), mRNA expression levels of the cytokines IL-10, IFN-γ, and TGF-β and of the transcription factors GATA3 and T-bet were determined by real-time quantitative PCR on a TaqMan instrument (ABI PRISM 7700 thermal cycler; PerkinElmer, Wellesley, MA). GAPDH was analyzed as a housekeeping gene. The following combination of primers and probes were used: IFN-γ forward, 5'-CCA ACG CAA ACG AAT ACA TGA TGA-3'; IFN-γ reverse, 5'-TTT TCG CTT CCC GTC TTG AGC T-3'; IFN-γ probe JOE, 5'-TCA CAG TGA TCG AAC TAT GTC C-3'; IL-10 forward, 5'-TGA GAA CAG CTC CAC CAA CTT-3'; IL-10 reverse, 5'-GCT GAA GGC ACT TCG GAG AT-3'; IL-10 probe FAM, 5'-CAG GCA ACC TGC CTA ACA TGC TTC GA-3'; T-bet forward, 5'-GGT GGT GGA GGT GGT GCT GGT CTT-3'; T-bet reverse, 5'-CTT TCA ACA CTG CAC CCA CTT-3'; T-bet probe JOE, 5'-GCT GAA ACC ACT GCC GGT ACC AGA G-3'; GAPDH forward, 5'-CCA CCT ACC GAT GAA TCC-3'; GAPDH reverse, 5'-TGG GAT TTC CAT TGA TGA CAA G-3'; and GAPDH probe TET, 5'-TGG CAC CTG CAA GGC GAA GGA C-3'. To quantify mRNA amounts of IL-10, IFN-γ, T-bet and GATA3 induction indices were calculated using the comparative cycle threshold method (20). Differences in the mean threshold cycle (Ct) for the target gene and Cc for the housekeeping gene (GAPDH), indicated as ΔCt, were calculated to normalize differences in the mRNA extractions and the efficiency of reverse transcription. The relative mRNA amount for each target gene in CD4+CD25bright and CD4+CD25total Treg cells is calculated as ΔΔCt and expressed as n-fold difference relative to CD4+CD25 T cells in the same compartment (induction index).

Next, from PB and SF of four pers-OA and four ext-OA JIA patients and from PB of four healthy controls, CD4+CD25bright and CD4+CD25total T cells were isolated by FACS sorting and mRNA was isolated as described above. For Fox-P3 and β2m transcripts, real-time quantitative PCR was performed with a LightCycler (Roche Diagnostics) based on specific primers and general fluorescence detection with SYBR Green I. For the following primer combinations were used: Fox-P3 forward, 5'-TCA AGC ACT GCC AGG CG-3'; Fox-P3 reverse, 5'-CAG GCA CCC TTT TCG GAT G-3' and β2m forward, 5'-CCA GAG AAT GGA AAG TCC-3'; and β2m reverse, 5'-GAT GCT GCT TAC ATG TCT CG-3'. All PCR were performed using LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics). A pool of cDNA from tetanus-stimulated human PBMC was used as a standard and normalization to β2m was performed for each sample. Semi-quantitative levels of Fox-P3 are expressed as percentage of the Fox-P3 expression of the control pool.

Statistical analysis

Basic descriptive statistics were used to describe the patient population. A Mann-Whitney U test and a paired t test were used to compare numbers of CD4+CD25+ T cells in the PB and SF of the different patient groups. A paired t test was used to compare the expression of molecules on PB and SF CD4+CD25+ T cells.

Results

Numbers of CD4+CD25bright T cells in PB and SF of JIA patients and in PB of healthy controls

Sixty PB and 35 SF samples from 60 pers-OA JIA patients, 34 PB, and 26 SF samples from 34 ext-OA JIA patients and 34 PB samples from healthy children were evaluated on frequencies of CD4+CD25+ T cells. Since the regulatory CD4+ T cells preferentially reside within the CD4+CD25bright population (21), we analyzed PB and SF samples for the relative numbers of both CD4+CD25bright T cells and CD4+CD25total T cells (Fig. 1, A and B). The range of the PBL values of the patients whose SF values are shown fall in the overall distribution of PBL values. The number of CD4+CD25bright and CD4+CD25total T cells is expressed as a percentage of all CD4+ T cells.

Our results reveal a significantly lower number of CD4+CD25bright T cells in the PB of pers-OA JIA patients (mean ± SEM, 1.2 ± 0.2%) as well as in the PB of ext-OA JIA patients (mean ± SEM, 0.5 ± 0.2%) when compared with healthy controls (mean ± SEM, 1.6% ± 0.1%) (p < 0.001 and p < 0.0001, respectively, Fig. 1A). The difference in PB CD4+CD25bright T cell frequency between pers-OA and ext-OA JIA patients was also significant (p < 0.05).

In the SF of both pers-OA and ext-OA JIA patients, the number of CD4+CD25bright T cells was highly increased (mean ± SEM, 6.2 ± 0.7%; mean ± SEM, 5.2 and 0.9%, respectively, Fig. 1B) when compared with PB (p < 0.0001 and p < 0.0001, respectively). Also, the CD25 density on the SF cells was brighter than on cells from PB as reflected by a higher mean fluorescence intensity (Fig. 1D). There was no significant difference in the number of SF CD4+CD25bright T cells between pers-OA and ext-OA JIA patients. However, when CD4+CD25total T cells were analyzed as a whole, patients with pers-OA JIA had significantly higher numbers of CD25-positive cells within the joints (mean ± SEM, 30.5 ± 2.3%) than patients with ext-OA disease (mean ± SD, 20.3 ± 1.7%; p < 0.01) and this was explained by a higher frequency of CD4+CD25* T cells.

The difference in relative numbers of PB CD4+CD25bright Treg cells between pers-OA and ext-OA JIA patients seems not to be the result of a difference in the number of aggressor cells, since the absolute CD4+ T cell counts were equal in both patient groups (Fig. 1C). Data on absolute blood counts in the healthy controls and absolute cell counts in the SF were not available.

Furthermore, more ext-OA JIA patients are treated with the immunosuppressive drug methotrexate (MTX). We therefore analyzed whether this difference in treatment can explain the differences in CD4+CD25+ T cell frequencies. However, no significant differences in absolute and relative numbers of PB or SF CD4+CD25bright and CD4+CD25total T cells could be found between patients treated with or without MTX.

High expression of the regulatory cell marker FoxP3 in PB and SF CD4+CD25bright T cells but also significant expression in CD4+CD25total T cells

Recently Fox-P3 has been identified as a specific marker of CD4+CD25+ Treg cells, distinguishing them from recently activated, nonregulatory CD4+CD25+ T cells (22, 23). We therefore sorted CD4+CD25-, CD4+CD25int, and CD4+CD25bright T cells from the PB and SF of four pers-OA and four ext-OA JIA patients and from the PB of four healthy controls by FACS and evaluated the sorted populations for the expression of Fox-P3 mRNA by quantitative PCR. To obtain clear populations we defined CD4+CD25bright T cells as the 1.4% CD25 brightest of the CD4+ T cells.

Healthy controls and pers-OA JIA patients displayed higher amounts of mRNA Fox-P3 in PB CD4+CD25total and CD4+CD25bright T cells than ext-OA JIA patients (Fig. 2B). In the SF of all pers-OA as well as ext-OA JIA patients, each cell population analyzed displayed higher levels of mRNA Fox-P3 than the same PB population. Especially the SF CD4+CD25+ T cells showed a high expression of the Fox-P3 gene, but also the SF CD4+CD25* T cells expressed much higher levels of mRNA Fox-P3 than their PB counterparts. Comparing the two patient groups, pers-OA JIA patients displayed higher amounts of mRNA Fox-P3 in both SF CD4+CD25int and SF CD4+CD25bright T cells. These differences in mRNA Fox-P3 expression between the two patient groups were not statistically significant due to the low number of patients analyzed. However, when analyzing more closely at clinical outcome of the affected joint, the correlation of mRNA Fox-P3 levels with clinical outcome seemed to be
further confirmed. Each SF aspiration was followed by an intra-articular injection of corticosteroids. Interestingly, the two pers-OA JIA patients with the lowest numbers of CD4+CD25int and CD4+CD25bright T cells in the SF relapsed very soon (within 3 mo) after the local immune-suppressive treatment. Likewise, the ext-OA JIA patient with the highest number of CD4+CD25int and CD4+CD25bright T cells in the joint showed a long-term remission (>6 mo; data not shown).

Altogether these findings indicate that the accumulation of CD4+CD25bright T cells in the SF of JIA patients is attributable to the accumulation of professional CD4+CD25+ Treg cells. The Tregs do not only reside within the CD4+CD25bright T cell population but can also be found in the CD4+CD25int T cell population. A good correlation was found between the measured amounts of mRNA FoxP3 and the relative numbers of CD4+CD25int and CD4+CD25bright T cells in the PB and the SF (Fig. 1). Also, the measured amounts of mRNA FoxP3 seem to correlate well with clinical subtype and clinical course after local treatment.

**SF CD4+CD25bright Treg cells show a state of functional maturation when compared with PB**

We investigated the expression of a number of molecules on CD4+CD25− and CD4+CD25total T cells in the PB and on CD4+CD25−, CD4+CD25int, and CD4+CD25bright T cells in the SF (Fig. 3). In all children evaluated, the phenotype of PB CD4+CD25+ T cells differed from that of CD4+CD25− T cells in a manner consistent with published literature, i.e., by the constitutive expression of CTLA4 and GITR (4, 24–26). Comparing PB CD4+CD25bright Treg cells with their synovial counterparts, the SF populations showed a significantly higher expression of the activation marker HLA-DR (p < 0.01) and a significantly higher expression of CTLA-4 (p < 0.0001) and GITR (p < 0.0001). CTLA-4 density on SF CD4+CD25bright T cells was 10–15 times higher when compared with their peripheral counterparts and 2 times higher when compared with SF CD4+CD25−CD69+ T cells.

We observed a gradual increase in the expression of HLA-DR, CTLA-4, and GITR with the increase in the expression of CD25 (Fig. 3), which was consistent for all samples measured. PB as well as SF CD4+CD25bright T cells were highly positive for the chemokine receptor CCR4. No statistically significant difference was found in the expression of CCR4 between PB and SF CD4+CD25bright T cells.

Beside the markers shown in Fig. 3, we evaluated the expression of CD69, CD45RB, CD40L, and CD134. PB CD4+CD25− T cells showed no expression of CD69 while the SF CD4+CD25+ T cells showed a high expression of CD69 (mean ± SEM, 53 ± 5.2%). The
expression of CD40L and CD134 did not differ significantly between the different subsets. In both PB and SF, all CD4+CD25high T cells were CD45RBlow. There were no differences in phenotype of the CD4+CD25high and CD4+CD25int T cells between pers-OA and ext-OA JIA patients.

**Hyporesponsiveness of SFMC is due to active suppression by CD4+CD25bright T cells and can be overcome by IL-2**

In rheumatoid arthritis (RA) as well as in JIA, SF-derived T cells show a state of hyporesponsiveness (27, 28) and few cells in active cell division (29). Thus, although these cells have a high activation status they appear relatively inactive in situ and respond only weakly to diverse stimuli ex vivo. We hypothesized that this relative hyporesponsiveness could be due to the presence of CD4+CD25+ Treg cells in the SF. We therefore compared the proliferative response of PB and SF T cells from children with pers-OA and ext-OA JIA to the T cell mitogen PHA. A representative hyporesponsive state could be due to suppression by the CD4+CD25+ Treg cells, which are abundantly present in the SF. Since high doses of IL-2 can abrogate the suppressive activity and the anergic phenotype of CD4+CD25+ Treg cells (8, 10, 30), we evaluated whether the addition of IL-2 to the cultures could overcome the hyporesponsiveness of SF T cells. The addition of 10 ng/ml IL-2 was found to overcome the hyporesponsive state of the SF T cells (Fig. 4A, right panel). Depletion of the CD4+CD25bright T cells from SF by FACS sorting resulted in an increased response of the remaining SF CD4+ T cells (Fig. 4B, right panel). The CD4+CD25bright T cells alone were profoundly anergic (Fig. 4B, middle panel) compared with unsorted SFMC (Fig. 4B, left panel).

To demonstrate active suppression by CD4+CD25bright T cells, we mixed CD4+CD25− and CD4+CD25bright T cells (ratio, 2:1) and evaluated the proliferative response (Fig. 4C). The mixed cells proliferated poorly (Fig. 4C, right panel) compared with CD4+CD25− cells alone (Fig. 4C, middle panel), indicating an active suppressive role for the SF CD4+CD25bright T cells.

**Suppressive function of SF CD4+CD25bright T cells of both persistent oligoarticular and extended oligoarticular patients in vitro**

We next investigated the suppressive capacity in vitro of CD4+CD25bright T cells by a direct functional comparison of CD4+CD25bright T cells from PB and SF of JIA patients and from PB of healthy children. We sorted CD4+CD25+ and CD4+CD25bright T cells from PB and SF. To obtain enough cells, the gate for each sort of SF as well as PB CD4+CD25+ T cells was set to include the brightest 1.4% of CD4+CD25+ T cells. The regulatory capacity of the CD4+CD25bright T cells was measured in cocultures. The suppressive activity was determined by calculating the relative difference in proliferative response to plate-bound anti-CD3 between CD4+CD25+ T cells cultured alone and CD4+CD25− T cells cultured in the presence of CD4+CD25bright T cells (ratio, 1:1). We tested the PB of 10 healthy children, 13 pers-OA, and 6 ext-OA JIA patients and the SF of 6 pers-OA and 3 ext-OA JIA patients.

In two healthy children (20%), three pers-OA (23%), and three ext-OA JIA patients (50%), the PB CD4+CD25bright T cells were not suppressive when tested in the above-described assays. The PB CD4+CD25bright T cells of the remaining 8 healthy controls, 10 pers-OA JIA, and 3 ext-OA JIA patients showed a suppressive activity of 44 ± 7%, 68 ± 7%, and 62 ± 14% (mean ± SEM), respectively. The SF CD4+CD25bright T cells of the six pers-OA and the three ext-OA JIA patients all showed good suppressive effect (mean ± SEM, 82 ± 0.1% and 80 ± 0.01%, respectively). There was no significant difference in the percentage of inhibition between the different study groups.

**SF CD4+CD25int T cells of pers-OA JIA patients are able to suppress**

Pers-OA JIA patients harbor significantly higher amounts of mRNA FoxP3 expressing CD4+CD25int T cells in the SF than ext-OA JIA patients. To test whether the SF CD4+CD25int T cell compartment of pers-OA JIA patients contain enough regulatory T cells to suppress, we performed a coculture experiment with SF
CD4^+CD25^- and CD4^+CD25^int T cells of three pers-OA JIA patients. In all three patients, the SF CD4^+CD25^- T cells were able to suppress CD4^+CD25^- responder T cells, although to a lesser extent than SF CD4^+CD25^bright T cells (Fig. 5).

**SF CD4^+CD25^bright T cells express higher IL-10, lower IFN-γ, and similar TGF-β mRNA levels when compared with SF CD4^+CD25^- T cells**

The role of immunosuppressive cytokines such as TGF-β and IL-10 in relationship to CD4^+CD25^- Treg cells is still unclear, but has been implicated in suppressive function in some reports. We therefore evaluated the expression of mRNA TGF-β, IL-10, and IFN-γ in FACS-sorted SF CD4^+CD25^bright T cells and CD4^+CD25^- T cells by quantitative PCR. The expression of mRNA in CD4^+CD25^- T cells was compared with the expression of mRNA in CD4^+CD25^bright T cells, and expressed as the mean induction index relative to CD4^+CD25^- T cells (Fig. 5).

**FIGURE 3.** Phenotype of PB and SF CD4^+CD25^- and CD4^+CD25^int T cells. PB CD4^+CD25^- and CD4^+CD25^int T cells and SF CD4^+CD25^- and CD4^+CD25^int T cells were evaluated on the expression of molecules associated with regulatory T cells. The dot plots indicate the sorting gates used for the different subpopulations. There were no differences in the phenotype of the CD4^+CD25^bright and CD4^+CD25^int T cells of the two patient groups. The depicted FACS plots were obtained from a pers-OA JIA patient. Mean fluorescence intensity (MFI) values of the whole population are shown in each plot.

CD4^+CD25^- and CD4^+CD25^int T cells of three pers-OA JIA patients. In all three patients, the SF CD4^+CD25^- T cells were able to suppress CD4^+CD25^- responder T cells, although to a lesser extent than SF CD4^+CD25^bright T cells (Fig. 5).

**SF CD4^+CD25^bright T cells express higher IL-10, lower IFN-γ, and similar TGF-β mRNA levels when compared with SF CD4^+CD25^- T cells**

The role of immunosuppressive cytokines such as TGF-β and IL-10 in relationship to CD4^+CD25^- Treg cells is still unclear, but has been implicated in suppressive function in some reports. We therefore evaluated the expression of mRNA TGF-β, IL-10, and IFN-γ in FACS-sorted SF CD4^+CD25^bright T cells and CD4^+CD25^- T cells by quantitative PCR. The expression of mRNA in CD4^+CD25^- T cells was compared with the expression of mRNA in CD4^+CD25^bright T cells, and expressed as the mean induction index relative to CD4^+CD25^- T cells (Fig. 5).

**SF CD4^+CD25^bright T cells have an increased regulatory capacity when compared with PB CD4^+CD25^bright T cells**

The phenotype and in vitro suppressive activity of the CD4^+CD25^bright T cells in the SF suggests an increased regulatory capacity compared with PB CD4^+CD25^bright T cells. To test this hypothesis, we isolated CD4^+CD25^- and CD4^+CD25^bright T cells from two JIA patients. We cocultured a fixed number of SF CD4^+CD25^- T cells (5000/well) with increasing numbers of SF or PB CD4^+CD25^bright T cells as described above. Proliferative responses of SF CD4^+CD25^- T cells in the presence of SF CD4^+CD25^bright T cells were compared with proliferative responses of SF CD4^+CD25^- T cells in the presence of PB CD4^+CD25^bright T cells (Fig. 6). In each patient a clear enhanced suppression was observed when SF CD4^+CD25^- cells were cocultured with SF CD4^+CD25^bright T cells when compared with coculture with PB CD4^+CD25^bright T cells.
Treg cells were functional in most patients, we can only speculate whether or not this reduced number contributed to the development of the autoimmune disease. Despite this low frequency of CD4\(^+\)CD25\text{bright}\ Treg cells in the PB of JIA patients, the number of CD4\(^+\)CD25\text{bright}\ Treg cells in the SF was highly enriched. This enrichment at the site of inflammation is consistent with a recent study that showed an increased number of CD4\(^+\)CD25\text{bright}\ T cells in the SF of RA patients (32).

Interestingly, a comparison of the different patient groups of JIA showed that patients with pers-OA JIA display significantly higher frequencies of PB CD4\(^+\)CD25\text{bright}\ T cells with concomitant higher levels of mRNA FoxP3 than patients with ext-OA JIA. Furthermore, although the number of SF CD4\(^+\)CD25\text{bright}\ T cells was equal in both patient groups, pers-OA JIA patients displayed a higher frequency of CD4\(^+\)CD25\text{int}\ T cells, and thus a higher number of CD4\(^+\)CD25\text{+}\ cells in total in the SF than ext-OA JIA patients. Analysis of mRNA FoxP3 levels in SF CD4\(^+\)CD25\text{bright}\ and CD4\(^+\)CD25\text{int}\ T cells separately revealed a high expression of the gene in SF CD4\(^+\)CD25\text{bright}\ T cells of both patient groups, also significant levels of expression in the SF CD4\(^+\)CD25\text{+}\ T cell population. Again, pers-OA JIA patients displayed higher levels of mRNA FoxP3 than ext-OA JIA patients. Although it is expected that the SF CD4\(^+\)CD25\text{int}\ T cell population consists of a mixture of activated and regulatory T cells, suppression assays with SF CD4\(^+\)CD25\text{+}\ T cells of pers-OA JIA patients showed that even this mixture is able to suppress CD4\(^+\)CD25\text{+}\ responder T cells in vitro. This finding is especially interesting, since it shows that although, consistent with reports by Baecher-Allan et al. (21), most CD4\(^+\)CD25\text{+}\ Tregs reside in the CD4\(^+\)CD25\text{int}\ T cell population during inflammation, CD4\(^+\)CD25\text{+}\ Tregs can also be found among CD4\(^+\)CD25\text{+}\ T cells. Even more, low levels of mRNA FoxP3 were found in the SF CD4\(^+\)CD25\text{+}\ T cell population. Taking our results together, we propose that the differences in mRNA FoxP3 levels, number of PB CD4\(^+\)CD25\text{bright}\ T cells, and number of SF CD4\(^+\)CD25\text{+}\ T cells between pers-OA and ext-OA JIA patients contribute to the differences in clinical course. It needs to be noted however that, despite highly statistically significant differences in CD4\(^+\)CD25\text{+}\ T cell frequencies between the two patient groups, it will not be possible to use the analysis of PB and/or SF CD4\(^+\)CD25\text{+}\ T cell frequencies as a test to predict whether a patient with oligoarticular JIA will progress to ext-OA stages or persist as oligoarticular, as CD4\(^+\)CD25\text{+}\ T cell frequencies in the two patient groups are too much overlapping.

As in RA, the isolated SF CD4\(^+\)CD25\text{bright}\ T cells as well as the SF CD4\(^+\)CD25\text{+}\ T cells of pers-OA JIA patients exhibited an extremely potent suppressive capacity in vitro. Different groups previously reported a profound state of hyporesponsiveness of SFMC in RA (27, 28) as well as in JIA and that synovial T cells in JIA show little evidence of proliferation (29). Our results now show that this cellular hyporesponsiveness is the result of active suppression by highly activated CD4\(^+\)CD25\text{+}\ Treg cells present in the SF. Interestingly, beside depletion of the CD4\(^+\)CD25\text{bright}\ T cells from the SF also the addition of IL-2 could overcome this relative hyporesponsiveness. It has been shown that IL-2 can overcome the suppressive capacity of CD4\(^+\)CD25\text{+}\ Treg cells (8, 10, 30). It is therefore conceivable that the addition of IL-2 to our cultures caused a breakdown in the hyporesponsive state by abrogating the suppressive capacity of the SF CD4\(^+\)CD25\text{+}\ Treg cells.

The expression of FoxP3 suggests that the SF CD4\(^+\)CD25\text{bright}\ T cells are so-called professional Treg cells. Recently it has been shown, in both mice as well as in humans, that activation of CD4\(^+\)CD25\text{+}\ T cells can lead to expression of FoxP3 and the acquisition of T regulatory activity (33). Thus, clear evidence exists now that there are two pathways for the generation of CD4\(^+\)CD25\text{+}\ Tregs: one as a result of...
thymic selection and a distinct pathway as a consequence of immune responses in the periphery (33, 34).

Our data do not provide information on the origin of the CD4+CD25+ Treg cells in the SF. It has been suggested that thymus-derived Treg cells, which are autoreactive and have self-renewing capabilities, allow for regulation of autoactivity (35). Part of the SF CD4+CD25+ Tregs, presumably thymus-derived, may therefore have entered the joint in the initial phase of the autoimmune response. Since all SF CD4+CD25bright T cells express the chemokine receptor CCR4, CCR4 may have a role in the homing of CD4+CD25+ Treg cells from the periphery to the joint. The expression of CCR4 is an intrinsic feature of CD4+CD25+ Treg cells (36), and it has been suggested before that the presence of CCR4-expressing T cells in the SF of JIA patients may function in an anti-inflammatory capacity (17). In addition, it is possible that part of the CD4+CD25+ Tregs in the SF were generated during the excessive (bystander) activation of CD4+CD25− T cells at the site of inflammation. The presence of functional Tregs in the CD4+CD25int T cell population may then mirror an intermediate stage of development.

Our data on the phenotype of the SF CD4+CD25bright T cells suggest that, independent of the origin, the CD4+CD25+ Treg cells undergo maturation at the site of inflammation. The SF CD4+CD25bright Treg cells showed a marked increase in the expression of CD25, CTLA-4, and GITR compared with their peripheral counterparts. Furthermore, we observed a gradual increase in the expression of CTLA-4 and GITR on SF CD4+CD25+ T cells with the increase in the expression of CD25. It has been shown in human CD4+CD25+ T cell clones that the expression level of CTLA-4 and GITR clearly correlates with their suppressive capacity (37). Herewith consistent is our finding that the striking expression of CTLA-4 and GITR on the SF CD4+CD25bright T cells in JIA correlates with an increased regulatory capacity in vitro. Thus, both the local enlargement of the CD4+CD25+ Treg cell population, either due to homing or due to induction at the site of inflammation, and the change in phenotype leading to enhanced suppressive capacity seem to be mechanisms by which CD4+CD25bright Treg cells try to regulate local inflammation.

As well as the marked increase in the expression of CTLA-4 and GITR, we found that the SF CD4+CD25bright T cells of oligoarticular JIA patients contain an increased amount of mRNA IL-10 when compared with PB CD4+CD25bright T cells or to SF CD4+CD25− responder T cells. This finding suggests an active role for IL-10 during the suppression of inflammation by CD4+CD25− Treg cells in the joints of these patients. Previous studies on IL-10 and arthritis have suggested that insufficient IL-10 in the presence of inflammation could be one of the mechanisms allowing joint inflammation to continue (38–40). Although data on the secretion of the immunosuppressive cytokines IL-10 and TGF-β and their involvement in suppression by CD4+CD25+ T cells have been very controversial in literature (8, 13, 41), our findings are in line with data from Fontenot et al. (23) showing that expression of Foxp3 correlates with increased amounts of IL-10 mRNA in CD4+CD25+ Treg cells. The controversy surrounding the data concerning the function of IL-10 in suppression by CD4+CD25+ Treg cells in general may be partly due to differences in isolation techniques or the use of different in vitro assays. In the study of Fontenot et al. (23) and in our study, mRNA IL-10 was measured in CD4+CD25+ Treg cells that were taken directly ex vivo.
Altogether our data lead to the hypothesis that the outcome of disease may in part be a matter of balance. Although the CD4+/CD25bright Treg cells could not prevent the development of the disease in pers-OA JIA patients, the remitting clinical course in these patients suggests that they contribute to reversing ongoing inflammation. In the ext-OA JIA patients, the disease is neither prevented nor reversed, presumably because the number of CD4+/CD25bright T cells in the SF is not sufficient to suppress the inflammation.

In conclusion, pers-OA JIA is an example of a human disease in which CD4+/CD25+ Treg cells, in addition to other regulatory mechanisms, seem to play an active role in the limiting and even reversal of established autoimmune pathology. The progression to ext-OA JIA in some patients seems not the result of an intrinsic defect of the CD4+/CD25+ Treg cells but rather a failure to either home in or expand at the site of inflammation. Therefore, further studies need to be focused on finding ways to enhance local proliferation of CD4+/CD25+ Treg cells without abrogating their regulatory capacity.

Acknowledgments
We thank Ger Arkesten for his excellent help and advice on FACS sorting, Wilco de Jager and Brenda Hendriks for their help on the molecular work, and Erica Roks for excellent editorial assistance.

References