Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia

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A better understanding of the role of CD4+CD25+ regulatory T cells in disease pathogenesis should follow from the discovery of reliable markers capable of discriminating regulatory from activated T cells. We report that the CD4+CD25+ population in synovial fluid of juvenile idiopathic arthritis (JIA) patients comprises both regulatory and effector T cells that can be distinguished by expression of CD27. CD4+CD25+CD27+ cells expressed high amounts of FoxP3 (43% of them being FoxP3+), did not produce interleukin (IL)-2, interferon-γ, or tumor necrosis factor, and suppressed T cell proliferation in vitro, being, on a per cell basis, fourfold more potent than the corresponding peripheral blood population. In contrast, CD4+CD25−CD27+ cells expressed low amounts of FoxP3, produced effector cytokines and did not suppress T cell proliferation. After in vitro activation and expansion, regulatory but not conventional T cells maintained high expression of CD27. IL-7 and IL-15 were found to be present in synovial fluid of JIA patients and, when added in vitro, abrogated the suppressive activity of regulatory T cells. Together, these results demonstrate that, when used in conjunction with CD25, CD27 is a useful marker to distinguish regulatory from effector T cells in inflamed tissues and suggest that at these sites IL-7 and IL-15 may interfere with regulatory T cell function.

There is now clear evidence that a distinct population of naturally occurring regulatory T cells, which can be identified by the constitutive expression of CD4 and CD25, plays an essential role in controlling autoimmunity (1). Regulatory T cells are generated in the thymus or in periphery (2, 3) and, once activated, suppress other T cells by an as yet uncharacterized contact-dependent, cytokine-independent mechanism (4). A functional result of suppression is impaired production of IL-2 (4), although evidence has been provided that an initial IL-2 production by responder cells is necessary for expansion of CD4+CD25+ T cells and induction of their suppressor function (5). The suppressor function of regulatory T cells can be relieved by exogenous IL-2 that acts on both regulatory and responder T cells and by IL-6 that blocks suppression at the level of responder cells (6, 7).

The development and function of regulatory T cells is critically dependent on the transcriptional repressor FoxP3 (8–10). Mice and humans that lack Foxp3 die from severe autoimmune diseases (11–14), whereas transduction of Foxp3 in naïve CD4+ T cells is sufficient to convert these cells into regulatory T cells (8, 9). Because Foxp3 is the master control gene, it is in principle the most specific marker for regulatory T cells. However, the facts that FoxP3 is expressed exclusively intracellularly and that reliable reagents for staining are not yet available prevent its use for the identification and isolation of regulatory T cells.

CD25 is the hallmark antigen of regulatory T cells in mice and humans (15–19). In normal conditions, CD25 appears to identify a relatively homogeneous population of anergic regulatory T cells, although some heterogeneity may exist. For instance, it has been reported that among CD4+CD25+ T cells those expressing CD103 or CD62L are more suppressive than their negative counterparts (20–22). Other useful markers of regulatory T cells under normal conditions include GITR, CTLA-4, and, in mice, neuropilin-1 (23–25).
There is growing interest in the identification of regulatory T cells in various pathological conditions and recent studies indicate that CD4⁺CD25⁺ cells with regulatory function can be indeed detected in inflamed tissues (26–28). However, the identification of regulatory T cells in an ongoing immune response or in inflamed tissues is complicated by the fact that all the aforementioned markers, including CD25, are also expressed on activated T cells (29). A possible heterogeneity of the CD4⁺CD25⁺ subset in inflamed tissues has not been addressed so far and the problem, therefore, remains how to discriminate in an ongoing immune response regulatory from activated effector T cells.

Here, we report that CD27 is stably expressed on regulatory T cells and can be used in conjunction with CD25 expression to discriminate in inflamed synovial regulatory T cells, expressing high amounts of FoxP3 and endowed with potent suppressive activity, from FoxP3⁺ effector T cells devoid of suppressor activity. We also show that IL-7 and IL-15 are present in synovial fluid and in vitro abrogate the suppressive function of regulatory T cells.

**RESULTS**

**CD4⁺CD25⁺ T cells from synovial fluid of juvenile idiopathic arthritis (JIA) patients express high amounts of FoxP3 and show suppressor activity in vitro**

Mononuclear cells were isolated from both synovial fluid and peripheral blood of 15 JIA patients (7 with polyarticular and 8 with oligoarticular disease course) and analyzed for the expression of CD4 and CD25 (Fig. 1, A and B). The percentage of CD25⁺ cells within the CD4⁺ population ranged from 4 to 11.6 (median, 8.6) in peripheral blood, a value comparable to that found in healthy donors (not depicted), whereas in synovial fluid it was significantly higher ranging from 6.5 to 35.2 (median, 12.3; Fig. 1 B).

CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were isolated by cell sorting from blood and synovial fluid of the same individual and analyzed for expression of the transcription factor FoxP3 using quantitative real-time PCR. FoxP3 mRNA was higher in CD4⁺CD25⁺ as compared with CD4⁺CD25⁻ cells in both peripheral blood and synovial fluid (Fig. 1 C). However, the amount of FoxP3 mRNA was much higher in the two populations isolated from synovial fluid than in those isolated from peripheral blood (Fig. 1 C). These findings suggest that synovial CD4⁺CD25⁺ T cells may be activated in vivo and that some regulatory T cells may be present within the CD4⁺CD25⁻ subset. Indeed, upon in vitro stimulation peripheral blood CD4⁺CD25⁺ regulatory T cells, and to a lower extent CD4⁺CD25⁻ T cells (30), rapidly up-regulated FoxP3 mRNA (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050085/DC1).

To examine the suppressive activity in vitro, synovial and peripheral blood CD4⁺CD25⁺ T cells were added at a 1:1 ratio to cultures of CFSE-labeled autologous peripheral blood CD4⁺CD25⁻ responder T cells stimulated by DCs and anti-CD3. As control, responder T cells were cultured in the absence or presence of unlabeled CD4⁺CD25⁻ T cells. 4 d later, cultures were analyzed by FACS and the total number of responder T cells that had performed one or more cell divisions was determined by CFSE dilution analysis. Responder T cells proliferated to a similar extent in the absence of CD4⁺CD25⁻ control T cells (not depicted). The percentage inhibition was calculated from the number of dividing responder cells in presence of CD4⁺CD25⁺ T cells as compared with their number in presence of CD4⁺CD25⁻ control cells. One representative experiment out of five is shown.

![Figure 1](image-url)
CD27 discriminates between regulatory and activated T cells in synovial fluid

Because CD25 is an activation marker, the pool of CD4+CD25+ cells in synovial fluid may contain not only regulatory T cells but also activated T cells. To dissect a possible heterogeneity, we separated synovial CD4+CD25+ T cells according to the expression of several cell surface markers and used FoxP3 mRNA to monitor the presence of regulatory T cells. Subsets defined by presence or absence of CTLA-4, CD62L, CD69, and GITR contained comparable amounts of FoxP3 mRNA (Fig. 2 A). Similar data were obtained using CCR4, VLA-4, and CD103 (unpublished data). Thus, these markers, known to be expressed on circulating regulatory T cells (16–19, 31–33), did not segregate with FoxP3 expression in cells of inflamed synovia.

CD27 is a TNFR-family member that is expressed on naive and subsets of memory T cells and is lost on terminally differentiated effector T cells (34, 35). Because the latter are highly enriched in synovial fluid of adult rheumatoid arthritis and JIA patients (36, 37), we asked whether CD27 may discriminate regulatory from activated effector T cells in inflamed joints. Indeed, in some synovial samples, we noted that a high proportion (up to 50%) of CD4+CD25+ T cells were CD27− (Fig. 2 B and see Table II). CD4+CD25+CD27− cells were also found in peripheral blood of patients and healthy adults although at lower frequency (mean 15.6%, range 8.3–33 in 14 patients and 7.9%, range 2.6–11.3 in 5 controls).

The four subsets of CD4+ cells identified according to the expression of CD25 and CD27 were isolated from the synovia and blood of JIA patients and from the blood of adult healthy controls and tested for FoxP3 mRNA expression. FoxP3 mRNA was at least 10-fold higher in CD4+CD25+CD27+ cells as compared with CD4+CD25+CD27− cells (Fig. 2 C and Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050085/DC1). Low amounts of FoxP3 mRNA were also found in CD4+CD45RO+CD27+ T cells, whereas FoxP3 was not detected in CD4+CD25+CD27− cells.

To estimate the frequency of FoxP3-expressing synovial T cells within the four subsets, we used a sensitive PCR method to detect FoxP3 in replicate samples containing limiting numbers of T cells (five cells per sample). The highest frequency of FoxP3+ cells (41.6%) was found in the CD4+CD25+CD27+ subset, whereas the CD4+CD25+CD27− and the CD4+CD25+CD27− subsets showed much lower frequencies (6.2% and 2.7%, respectively) and the CD4+CD25−CD27− subset was negative (Fig. 3). Considering that frequencies are underestimated, these results suggest that CD27 marks a rather homogeneous population of FoxP3 expressing cells in inflamed synovia.

Figure 2. CD27 identifies FoxP3-expressing cells within CD25+ synovial CD4+ T cells. (A) CD4+CD25+ SFMCs were stained with antibodies to CTLA-4, CD62L, CD69, and GITR. Positive and negative subsets were sorted and analyzed for FoxP3 mRNA. Mean ± SD of three separate experiments. (B) PBMCs and SFMCs from patient 12 were stained with antibodies to CD4, CD25, and CD27. The histograms show the expression of CD27 on gated CD4+CD25− or CD4+CD25+ T cells. (C) SFMCs were sorted according to the expression of CD4, CD25, and CD27 and analyzed for expression of FoxP3 mRNA. For comparison, the value in CD25+CD27+ was set to 100. Mean ± SD of three separate experiments.

Figure 3. FoxP3 mRNA in CD4+CD25+ T cell subsets in samples containing limiting cell numbers. CD4+ T cell subsets were sorted according to the expression of CD25 and CD27 and resorted, collecting 16 replicates of five cells each that were subsequently analyzed for expression of FoxP3 by PCR. Amplification of CD3 was used as control.
GITR and CTLA-4 were expressed at comparable levels on CD4⁺CD25⁺CD27⁺ and CD4⁺CD25⁺CD27⁻ cells, whereas CD62L and CCR4 were expressed on a higher proportion of CD27⁺ cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20050085/DC1). In addition, upon TCR triggering CD4⁺CD25⁺CD27⁺ T cells did not express IL-2, TNF, or IFN-γ mRNAs, whereas CD4⁺CD25⁺CD27⁻ cells expressed high amounts of cytokine mRNAs (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20050085/DC1). Together, these findings are consistent with the notion that CD4⁺CD25⁺CD27⁺ cells are regulatory cells, whereas CD4⁺CD25⁺CD27⁻ represent activated effector cells.

To directly test the suppressive function, the four subsets identified by the expression of CD25 and CD27 were isolated by cell sorting from the synovial fluid and added to cultures of CFSE-labeled peripheral blood CD4⁺CD25⁺ T cells. As shown in Fig. 4 A, suppressive activity was restricted to the CD4⁺CD25⁺CD27⁺ subset. The other three subsets either did not interfere or even enhanced T cell proliferation (Fig. 4 A). Synovial CD4⁺CD25⁺CD27⁺ T cells were, on a per cell basis, fourfold more potent as compared with CD4⁺CD25⁺CD27⁻ T cells isolated from peripheral blood of the same patient (Fig. 4 B). We conclude that based on four different criteria (expression of FoxP3 mRNA, expression of surface markers, lack of cytokine production, and suppression of T cell proliferation) CD27 expression in the context of CD25 expression allows discrimination of regulatory from activated/effector T cells in inflamed joints.

Figure 4. CD27 identifies potent suppressor cells within CD4⁺CD25⁺ synovial T cells. (A) Proliferation of CFSE-labeled CD4⁺CD25⁺ peripheral blood T cells stimulated by anti-CD3 and DCs in the absence (dashed lines) or presence (solid line) of equal numbers of the indicated autologous CD4⁺ T cells isolated from synovial fluid. The percentage inhibition was calculated as in Fig. 1. Comparable results were obtained using synovial T cells isolated from patient no. 14 (depicted) and nos. 2, 5, and 7. (B) Proliferation of CFSE-labeled CD4⁺CD25⁻ peripheral blood T cells in the presence of serial twofold dilutions of autologous CD4⁺CD25⁺CD27⁺ T cells from peripheral blood (open circle) or synovial fluid (closed square). Shown is the total number of responder T cells that had undergone more than one cell division. Mean ± SD of triplicate cultures. Comparable results were obtained with samples from patient no. 15 (depicted) and nos. 5 and 7.

Figure 5. CD27 is stably expressed in proliferating regulatory T cells, whereas it is down-regulated on activated conventional T cells. (A) CD4⁺CD25⁺CD27⁺ regulatory T cells and CD4⁺CD25⁻CD27⁻ T cells (comprising naive and memory cells) were sorted from peripheral blood to >99% purity. (B and C) Cells were labeled with CFSE and stimulated with plastic-bound CD3 antibodies in the absence or presence of IL-2 or CD28 antibodies. CD27 expression was measured as a function of cell division on days 5 (B) and 10 (C). One representative experiment out of three performed is shown.
CD27 is retained on regulatory T cells after activation and expansion

It is known that CD27 is lost upon terminal differentiation of conventional CD4⁺ T cells (34). To investigate whether regulatory T cells may lose or retain CD27 expression upon activation, we isolated CD4⁺CD25⁺CD27⁺ regulatory T cells and CD4⁺CD25⁺ naïve and memory T cells from peripheral blood (Fig. 5 A). Cells were labeled with CFSE and stimulated with anti-CD3 in the absence or presence of exogenous IL-2 or anti-CD28. As shown in Fig. 5 (B and C), CD4⁺CD25⁺ T cells proliferated extensively and down-regulated CD27 expression as a function of cell division. In contrast, CD4⁺CD25⁺ regulatory T cells proliferated poorly in the absence of IL-2, whereas

Figure 6. Differential distribution of CD25⁺CD27⁺ regulatory and CD25⁺CD27⁻ activated effector cells within synovial CD4⁺ T cells of JIA patients with oligoarticular or polyarticular disease course. (A) Percentages of total CD4⁺CD25⁺ T cells and CD27⁺ and CD27⁻ subsets in 13 JIA patients with oligoarticular disease course (white bars) and 12 JIA patients with polyarticular disease course (gray bars). (B) Ratio between regulatory and activated effector CD4⁺ cells as defined by CD25 and CD27 expression in oligoarticular (white bars) or polyarticular (gray bars) JIA patients. Boxes contain values falling between the 25th and 75th percentiles. Lines that extend from the boxes represent the highest and the lowest values from each subgroup. The lines within the boxes represent median values. p-values were determined by Mann-Whitney U test. Also, see Table II for data from individual patients.

Figure 7. Distribution of CD4⁺CD25⁺CD27⁺ regulatory T cells in lymph nodes and synovial tissues. (A–C) Serial sections of a reactive lymph node stained with antibodies to CD4 (A), CD25 (B), and CD27 (C). (D–H) Serial sections of synovial biopsy from a JIA patient showing a T and B lymphoid aggregate stained with antibodies to CD3 (D), CD20 (E), CD27 (F), CD25 (G), and CD4 (H). (I) A consecutive section stained with CD25 (red) and CD27 (green) by two-color fluorescence.
they performed several cell divisions when exogenous IL-2 or costimulation was provided. Remarkably, in both conditions, proliferating regulatory T cells retained high expression of CD27. We conclude that CD27 is a stable marker of regulatory T cells that is retained after activation and expansion.

Different proportions of CD4+CD25+ subsets in JIA patients with polyarticular or oligoarticular disease

Some of the patients in this study had the disease limited to a few joints (persistent oligoarticular), whereas others showed a more aggressive polyarticular involvement (extended oligoarticular, polyarticular, and systemic forms; Tables I and II). Although the percentage of total CD4+CD25+ cells did not differ significantly between the two groups, the relative proportion of CD27+ and CD27− cells within the CD4+CD25+ subset in affected joints was significantly different (Fig. 6 A). Patients with polyarticular disease showed higher proportion of CD27− activated/effecter cells and slightly lower proportion of CD27+ regulatory T cells as compared with patients with oligoarticular disease that showed a significantly higher ratio of regulatory to activated cells (Fig. 6 B).

IL-7 and IL-15 are present in synovial fluid and limit the suppressor activity of regulatory T cells

Biopsies of synovial tissues were obtained from three JIA patients. When analyzed by immunohistochemistry, regulatory T cells coexpressing CD4, CD25, and CD27 were found in lymphoid aggregates (Fig. 7) consistent with the possibility that they may participate in the ongoing immune response in the inflamed tissue. To investigate the possibility that cytokines that are present in the inflamed tissue may interfere with regulatory T cell function, we tested a large panel of recombinant cytokines for their capacity to block the suppressive function of regulatory T cells in vitro. The activity of regulatory T cells was strongly reduced in the presence of IL-2, IL-7, and IL-15 and virtually abolished when IL-7 and IL-15 were added together (Fig. 8 A). In contrast, the proinflammatory cytokines IL-6, IL-12, TNF, and IFN-γ, as well as another γ–common–dependent cytokine, IL-4, were ineffective, whereas TGF-β and IL-10 showed direct suppressive activity even in the absence of regulatory T cells (Fig. 8 A). Remarkably, IL-7 and IL-15 were detected in the synovial fluid of JIA patients, IL-7 being significantly higher in patients with polyarticular as compared with patients with

Table I. Clinical characteristics of JIA patients included in the study

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*aDefined as presence of swelling and/or limitation of motion with tenderness.

CS, corticosteroids; CyA, cyclosporin A; ESR, erythrocyte sedimentation rate; MTX, methotrexate; NSAID, nonsteroidal antiinflammatory drugs; RF, rheumatoid factor.
ARTICLE

oligoarticular disease (Fig. 8 B). Together, these results suggest that in inflamed tissues IL-7 and IL-15 may substantially limit the suppressor function of regulatory T cells.

DISCUSSION

In this study, we addressed the problem of identifying naturally occurring regulatory T cells in inflamed tissues. We found that in synovial fluid of JIA patients, CD27 expression can be used to discriminate, within the CD4+CD25+ subset, regulatory T cells from activated effector T cells. CD27 is expressed on regulatory T cells in both peripheral blood and synovial tissues and is retained by these cells after activation and clonal expansion in vitro, whereas CD27 is absent on effector T cells and is rapidly lost on CD27 naive and memory T cells after activation (38, 39). Thus, although CD27 is not a specific marker for regulatory T cells, the differential regulation of expression in regulatory and conventional T cells makes it a suitable marker for the identification of regulatory T cells in inflamed tissues.

The intensity of CD25 expression is considered a reliable marker for regulatory T cells in peripheral blood (16). However, this may not be sufficient to identify regulatory T cells in inflamed tissues. Indeed, a recent study demonstrated that CD25dim cells from synovial fluid express FoxP3 and have suppressive activity (40). In this regard, we found that CD27 is expressed not only on all CD25bright cells, but also on a sizeable proportion of CD25 dim cells and that CD25 dimCD27+ and CD25 brightCD27+ T cells express comparable amounts of FoxP3 mRNA (unpublished data). We conclude that the combination of CD25 and CD27 allows identification of most of regulatory T cells, while effectively excluding effector T cells.

Growing evidence over the past few years indicates that CD27, as well as other members of the TNFR family, such as OX40 (CD134) and 4-1BB (CD137), plays an important role for the effective generation of many types of T cell responses (41). It remains to be established what role CD27 may play in regulatory T cell function and whether sustained expression of CD27 on regulatory T cells contributes to their maintenance in vivo.

Using limiting dilution analysis, we could estimate that 41% of CD4+CD25+CD27+ T cells isolated from inflamed

Table II. Distribution of CD27 in synovial CD4+CD25+ and CD4+CD25− T cells subsets in JIA patients with oligoarticular and polyarticular disease course

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aPercentages within CD4+ synovial cells.
bPercentages within CD4+CD25+ synovial cells.
cPercentages within CD4+CD25− synovial cells.
presence (shaded histograms) of equal numbers of the CD4+
stimulated by TSST-pulsed DCs in the absence (unshaded histograms) or
blood regulatory T cells (42), these findings suggest that the
both FoxP3 expression and suppressor function of peripheral
tory T cells. As activation by anti-CD3 and IL-2 increases
suppressing T cell proliferation than peripheral blood regula-
FoxP3 and were on a per cell basis fourfold more potent in
direct comparison of regulatory T cells from synovial fluid
compared with regulatory T cells from peripheral blood. A
tissues was instrumental to establish their relative potency as
mogeneous population of regulatory T cells from inflamed
joints express FoxP3. The possibility of isolating a rather ho-
omegous population of regulatory T cells from inflamed
tissues was instrumental to establish their relative potency as
com pared with regulatory T cells from peripheral blood. A
direct comparison of regulatory T cells from synovial fluid and
peripheral blood of the same patient revealed that syn-
ovial regulatory T cells expressed much higher levels of
and varies with disease state, it will be important to reassess
the presence of regulatory T cells in other pathological tis-
ues (26, 28) by using a combination of markers as those de-
scribed in the present study.

Although regulatory T cells can be found in tissues un-
dergoing chronic inflammation it remains to be established
whether they exert their function in vivo. Besides possible
intrinsic defects in regulatory T cells (43–45), there may be
several mechanisms that limit the efficacy of regulatory T cells
in peripheral inflamed tissues. For instance, in vitro preacti-
vated T cells become resistant to suppression and this resis-
tance is dependent on the strength and duration of the stimu-
lus (28, 42). We also found that naive T cells become
completely refractory to suppression 24 h after TCR stimula-
tion (unpublished data). In addition, exogenous signals such as
those provided by GITR-L and IL-6 render responsive T
cells resistant to suppression mediated by CD4+CD25+ regu-
lar T cells (7, 46). We screened a large panel of cytokines
for their capacity to relieve suppression in vitro and found
that IL-7 and IL-15, and more effectively a combination of
the two, can counteract the suppressor function of regulatory
T cells. Based on these results and on the finding that both
IL-7 and IL-15 can be detected in the joint fluid of JIA pa-
tients, we suggest that in target tissues the function of regula-
tory T cells may be substantially limited by these cytokines
and that therapies that aim at neutralizing such cytokines
may not only decrease bystander T cell activation but also reconsti-
ute the suppressor function of regulatory T cells.

MATERIALS AND METHODS

Patients. 25 consecutive JIA patients diagnosed according to ILAR
Durban’s criteria (47) were included in the study. All patients had active
disease and underwent synovial fluid aspiration for steroid injection. To
be included in the study, patients with persistent oligoarticular course
should have presented a disease duration >1 yr. In all cases, a steroid in-
jection in the same joint in the previous 6 mo was considered as an exclud-
cation criterion. The patients tested in the functional studies were under
NSAID and/or methotrexate treatment. The main clinical and laboratory
features and the ongoing treatment at the moment of the study are re-
ported in Table I. Patients’ samples were taken after parents’ permission
according to the informed consent approved by the ethical committee of
the G. Gaslini Institute.

Media and reagents. The medium used throughout the experiments was
RPMI 1640 supplemented with 10% fetal calf serum, 1% Glutamax, 1%
onessential amino acid, 1% pyruvate, 50 U/ml penicillin, 50 μg/ml strep-
tomycin (all obtained from Invitrogen), and 5 × 10−5 M 2-mercaptoethanol
(Merck). Recombinant human IL-6, IL-7, IL-10, IL-12, IL-15, IFN-γ,
TNF, and TGF-β were purchased from BD Biosciences. IL-2 and IL-4 were produced in our laboratory using the myeloma-based expression system.

**FACS analysis.** The following monoclonal antibodies were used: mouse CD4-FITC, CD4-APC, CD27-FITC, CD27-PE, CD62L-PE, CD69-PE, CTLA4-PE, CCR4-PE (all obtained from Becton Dickinson), CD25-FITC (DakoCytomation), CD25-PE (Miltenyi Biotec), and GITR-PE (R&D Systems).

**Cell isolation.** PBMCs and synovial fluid mononuclear cells (SFMCs) were isolated by Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation. After staining for various surface markers, subpopulations of CD4+ T cells were sorted by FACS Viaantage (Becton Dickinson). Purity of cell preparations was typically >95%. Monocytes were isolated from PBMCs by positive selection using CD14 microbeads (Miltenyi Biotec). The purified monocytes were cultured for 3–5 d in RPMI 1640 10% FCS containing 50 ng/ml GM-CSF (Leukomax; Novartis) and 1,000 U/ml IL-4.

**Proliferation assay.** Peripheral blood CD4+CD25− T cells were labeled with 0.5 μM CFSE (Molecular Probes) for 8 min at room temperature. After quenching of the labeling reaction by addition of RPMI 1640 10% FCS, cells were washed extensively. 1.5 × 10^5 cells were cultured alone or together with different numbers of unlabelled regulatory or control cells in the presence of 10^5 immature DCs and either 2 ng/ml TST (Toxin Technology) or 0.25 ng/ml CD3 antibodies (supernatant from clone OKT3). Proliferation was measured on day 4 on a FACS Calibur (Becton Dickinson) using propidium iodide (Sigma-Aldrich) to exclude dead cells. In some experiments, T cells were stimulated with plate-bound CD3 antibodies (2 μg/ml, from clone Tr66) in the absence or presence of 100 U/ml of recombinant IL-2 or 2 μg/ml plate-bound CD28 antibodies (BD Biosciences).

**Real-time PCR.** For quantitative assessment of relative mRNA levels, total RNA was prepared from sorted subpopulations using Trizol LS reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed using M-MLV RT reverse transcriptase kit with random hexamer primers (Invitrogen). The relative level of FoxP3 mRNA in each subset was determined by real-time PCR on an ABI PRISM 7700 sequence detector (Applied Biosystems) using the Assay-On-Demand product (Hs00174128_m1), and IFN-γ (Hs00203958_m1) and universal PCR master mix for FoxP3 detection (Hs00203958_m1) and universal PCR master mix (4327052F).

**Cytokine detection assays.** IL-7 and IL-15 were measured using commercial ELISA (R&D Systems) in the sera and synovial fluid of 30 JIA patients (15 with oligoarticular course and 15 with polyarticular course) and in sera of 12 age-matched controls that were obtained for routinely preoperative examinations before minor surgery. The assay detection limit was 0.1 pg/ml for IL-7 and 2 pg/ml for IL-15.

**Five-cell PCR.** For determination of FoxP3 mRNA at a five-cell level, the four CD4+ T cell subsets were first sorted by flow cytometry. From each of the purified subpopulations, five-cell aliquots were resorted directly into wells of a 96-well conical plate. The subsequent procedures for cDNA preparation and nonspecific cDNA amplification were performed as described by Bigouret et al. (48). 1 μl of the nonspecifically amplified cDNA was used to amplify FoxP3 cDNA with 0.5 μM of the specific primers FoxP3-F (5′-CACCTACGCC-CACGCTCATC-3′) and FoxP3-R (5′-ACTAGGTTTGCCGGGATG-3′) (both obtained from Microsynth) in 1.5 mM MgCl₂. As a control, the expression of CD3δ was assessed using the primers CD3 S1 (5′-CGT-TCAGTTCCCTCTTTTCTT-3′) and CD3 AS1 (5′-GATTAAGGGGT-TGGAGGGAGTG-3′) (Microsynth). The program used for amplification was 3 min at 94°C, 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, 40 cycles.

**Immunohistochemistry.** Tissue specimens were prepared for immunohistochemistry according to standard technique. In brief, specimens were fixed in 10% formalin for 4 h, dehydrated, and embedded in paraffin. Paraffin sections were stained for 30 min at room temperature with mouse antibodies to CD4 (AB12), CD25 (25C04), CD27 (137B4; obtained from Neomarkers), CD20 (L26), and CD3 (polyclonal antisera; obtained from DakoCytomation) followed by anti-mouse Ig antibody conjugated to peroxidase-labeled dextran polymer (EnVision; DakoCytomation) and chromogenic diaminobenzidine substrate (DakoCytomation). Slides were counterstained with Mayer's hematoxylin. For double immunofluorescence, secondary labeling was performed for 30 min at room temperature with Alexa Fluor 594 goat anti–mouse IgG2b (Molecular Probes) and, subsequently, with Alexa Fluor 488 goat anti–mouse IgG1 (Molecular Probes) to label CD25 and CD27, respectively.

**Statistical analysis.** Differences in the percentages of matched peripheral blood and synovial fluid CD4+CD25+ T cells were analyzed by the Wilcoxon matched pairs signed rank test. Differences in the percentages of CD4+CD25+CD28+ and CD4+CD25−CD28− and in the amounts of IL-7 and IL-15 in oligoarticular and polyarticular JIA patients were analyzed by Mann-Whitney U test.

**Online supplemental material.** Fig. S1 shows the kinetics of FoxP3 mRNA accumulation in peripheral blood CD4+CD25+ regulatory T cells and CD4+CD25− naïve and memory T cells upon TCR stimulation. Fig. S2 depicts the amount of FoxP3 mRNA in peripheral blood CD4+ T cells sorted from JIA patients and adult healthy donors according to the expression of CD25 and CD27. Fig. S3 shows the expression of markers associated with regulatory T cells on the synovial CD4+CD25+CD27+ and CD4+CD25−CD27− subset. Fig. S4 depicts the amount of cytokine mRNA produced by synovial CD4+ T cell subsets isolated according to the expression of CD25 and CD27 upon TCR stimulation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050085/DC1.

We thank D. Jarrossay for cell sorting, M. Ugucioni for help with immunohistochemistry, and J. Geginat for critical reading of the text.

This work was supported by grants from the National Institute of Health (U19 AI057266-01) and the Swiss National Science Foundation (grant no. 3100-101962). The authors have no conflicting interests.

Submitted: 10 January 2005
Accepted: 20 April 2005

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