Altered Memory T Cell Differentiation in Patients with Early Rheumatoid Arthritis

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Altered Memory T Cell Differentiation in Patients with Early Rheumatoid Arthritis

Alla Skapenko,* Jörg Wendler, † Peter E. Lipsky, ‡ Joachim R. Kalden,* and Hendrik Schulze-Koops²*

The chronic immune response in rheumatoid arthritis (RA) might be driven by activated Th1 cells without sufficient Th2 cell differentiation to down-modulate inflammation. To test whether disordered memory T cell differentiation contributes to the typical Th1-dominated chronic inflammation in RA we investigated differentiation of resting CD4⁺ memory T cells in patients with early (6 wk to 12 mo) untreated RA and in age- and sex-matched healthy controls in vitro. No difference in cytokine secretion profiles of freshly isolated memory T cells was detected between patients and controls. A cell culture system was then employed that permitted the differentiation of Th effectors from resting memory T cells by short term priming. Marked differences were found in response to priming. Th2 cells could be induced in all healthy controls by priming with anti-CD28 in the absence of TCR ligation. By contrast, priming under those conditions resulted in Th2 differentiation in only 9 of 24 RA patients. Exogenous IL-4 could overcome the apparent Th2 differentiation defect in seven patients but was without effect in the remaining eight patients. In all patients a marked decrease in IL-2-producing cells and a significant increase in well-differentiated Th1 cells that produced IFN-γ but not IL-2 were evident after priming with anti-CD3 and anti-CD28. The data suggest that CD4⁺ memory T cells from patients with early untreated RA manifest an intrinsic abnormality in their ability to differentiate into specific cytokine-producing effector cells that might contribute to the characteristic Th1-dominated chronic (auto)immune inflammation in RA. The Journal of Immunology, 1999, 163: 491–499.

Although the mechanisms regulating the chronic autoimmune immune response in rheumatoid arthritis (RA) are not completely understood, considerable evidence supports the conclusion that activated CD4⁺ T cells play a central role in initiating and perpetuating the chronic inflammation characteristic for the disease (1–4). Based on their distinctive cytokine secretion pattern and effector functions, human CD4⁺ T cells can be divided into at least three different subsets. Th1 cells produce the pro-inflammatory cytokine IFN-γ, IL-2, and TNF-β, promote macrophage activation, induce delayed-type hypersensitivity, and are involved in cell-mediated immunity. Th2 cells have been associated with down-modulation of macrophage effector functions, they produce the anti-inflammatory cytokine IL-4, IL-5, and IL-13, and mediate allergic immune responses (5–8). A third subset, designated T regulatory cells, produces mainly IL-10 and might play a role in maintaining peripheral tolerance (9).

Imbalances in the ratio of activated Th1 vs Th2 cells have been associated with the development of a variety of pathologic inflammatory responses (10–12). For example, Th1-mediated immunity is associated with the pathogenesis of several organ-specific autoimmune diseases in animals (13–15) and has recently been implicated in mediating human autoimmune diseases such as RA (16, 17). In this regard, elevated levels of IL-2 and IFN-γ mRNA have been detected in the peripheral circulation of patients with RA (17). Moreover, T cells from human rheumatoid synovial tissue produce mainly IFN-γ and IL-2 (11, 16, 18–21). By contrast, T cells producing cytokines characteristic of activated Th2 cells, in particular IL-4, were rarely found in the peripheral circulation or in inflamed synovial tissue from patients with RA (17, 21, 22).

Given the significance that differentiated effector cells play in the outcome of an immune response, it is important to understand the mechanisms that regulate the development of T effector cells. Cytokines appear to be the major factor in determining Th cell polarization from naive T cells. In particular, Th2 cells arise after priming in the presence of IL-4 (23–27), whereas priming in the absence of IL-4 initiates Th1 cell development (25, 26, 28) that is greatly enhanced by IL-12 (29, 30). Other factors with a regulatory capacity for Th cell differentiation from naive T cells have been identified, such as the intensity of TCR ligation during priming (31, 32) or the nature and intensity of costimulatory signals (33–36). Recently, it has been shown that in humans, resting memory T cells can also be primed to differentiate into IL-4-producing Th2 cells. Generation of Th2 memory effector cells from early CD27⁺ memory T cells was dependent on stimulation by CD28, was enhanced by exogenous IL-4, and was inhibited by TCR ligation (37).

As chronic autoimmune responses are perpetuated by repeatedly activated memory T cells, disordered regulation of memory T cell differentiation might promote the pathogenesis of autoimmune diseases. In this regard, large numbers of mature memory T cells have been detected in the peripheral circulation and the synovial tissue of RA patients (38). Although phenotypically mature (CD4⁺CD27⁺CD45RBlow) memory T cells from patients with...
RA fail to produce IL-4, in contrast to those from healthy individuals (39). Thus, mature memory T cells from patients with RA appear to differ functionally from mature memory T cells in healthy individuals. However, the mechanisms responsible for these functional differences have yet to be elucidated.

To test the hypothesis that altered memory effector cell differentiation contributes to the typical Th1-dominated immune response in RA, we investigated memory T cell differentiation in RA patients in vitro. A culture system was employed to assess Th1 and Th2 cell differentiation in vitro using highly purified populations of CD4+ memory T cells that has previously been used to identify the signals controlling this process effectively (37). As it has been demonstrated that chronic activation might cause fixation of the Th phenotype (40), RA patients were chosen with early disease (6 wk to 12 mo). To eliminate the influence of drugs on the generation of polarized effector cells, patients were excluded who had previously been treated with disease-modifying anti-rheumatic drugs (DMARDs), including methotrexate, or corticosteroids. Whereas Th2 cell differentiation could be induced in all healthy individuals, RA patients were identified with an impaired ability to generate Th2 effectors. In some of the patients exogenous IL-4 could overcome the apparent T cell differentiation defect. However, in eight of 24 patients, no Th2 effectors could be induced. On the contrary, all RA patients manifested increased in vitro differentiation into specialized IFN-γ-positive Th1 cells. The data imply an abnormal memory Th effector cell differentiation in RA that might contribute to the characteristic Th1-dominated rheumatoid inflammation and, thus, to the pathogenesis of RA.

Materials and Methods

Abs and reagents

The following mAbs were used for purification of cells, for cell culture and staining: anti-CD3 (OKT3), anti-CD8 (OKT8), anti-HLA-DR (L243), and P1.17 (control IgG2a mAb, American Type Culture Collection, Manassas, VA); anti-CD11b, anti-CD19, FITC-conjugated anti-CD25, FITC-labeled anti-CD30, and FITC-labeled anti-HLA-DR (Dako Diagnostika, Hamburg, Germany); anti-CD45RA (11-1C5, a gift from Dr. Ramon Vilella, Barcelona, Spain); FITC-conjugated anti-CD3, PE-labeled anti-CD4, and PE-labeled anti-CD45RO (UCHL-1, Sigma-Aldrich, Deisenhofen, Germany); and anti-CD28 (28.2), FITC-labeled anti-CD69, PE-labeled anti-IL-4 (MP4-25D2), PE-labeled anti-IL-2 (MQ1–17H12), and FITC-labeled anti-IFN-γ (4S.B3, Pharmingen, San Diego, CA). Abs used for immunoprecipitation and immunoblotting were anti-STAT5 (8-20 and M-20) and anti-phosphotyrosine mAb (PY20; all from Santa Cruz Biotechnology, Santa Cruz, CA).

Study population

The study population consisted of 24 patients with an established diagnosis of RA as defined by the 1987 revised criteria of the American College of Rheumatology for the classification of RA (41). All patients were required to have symptoms of the disease for <12 mo and had not previously been treated with corticosteroids, DMARDs, or methotrexate. The mean age of the study population was 52.9 yr (range, 30–71 yr) with a mean disease duration of 5.9 ± 3.8 mo (range, 6 wk to 12 mo). Sixteen of the twenty-four patients had elevated erythrocyte sedimentation rates (ESR; ≥20 mm/h; Westergren), 14 patients had increased levels of C-reactive protein (CRP; ≥5 mg/l), and 17 of the patients were seropositive for IgM rheumatoid factor. The clinical and demographic data of the patients are summarized in Table I. For the healthy age- and sex-matched volunteers used as controls. The study protocol was approved by the review board of the University of Erlangen-Nuremberg, and written informed consent was obtained from all individuals before entering the study.

Memory T cell preparation

PBMC were obtained from heparinized venous blood by centrifugation (30 min, 400 × g) over a Ficoll-Hypaque gradient (Sigma-Aldrich). Cells were washed with saline and subsequently incubated with neuraminidase-treated sheep RBC (42). The rosette-positive cells were further purified by negative selection panning as previously described (37). In brief, cells were incubated with saturating amounts of mAb against CD8, CD19, CD16, HLA-DR, and CD45RA for 15 min on ice in RPMI 1640 medium (Life Technologies, Eggenstein, Germany) containing 2% pooled normal human serum (NHS), washed and allowed to bind to plastic petri dishes (Greiner, Frickenhausen, Germany) coated with goat anti-mouse IgG (Cappel, West Chester, PA) for 30 min at room temperature. Recovered cells were washed with PBS and resuspended in RPMI containing 10% NHS. The homogeneity and purity of the recovered cells were assessed by flow cytometry. Typically, ≥95% of the cells were positive for CD3 and CD4, ≥90% stained brightly with an mAb to CD45RO, and >98% of the cells were viable after the purification procedure. The cells were negative for the activation markers CD25, CD30, CD69, and HLA-DR.

Generation of memory effector T cells

All cell cultures were conducted in RPMI 1640 medium supplemented with penicillin G (100 IU/ml), streptomycin (100 µg/ml), l-glutamine (2 mM), and 10% NHS at 37°C in a humidified atmosphere containing 5% CO2. For the generation of memory effector T cells, a previously described multistep ex vivo cell culture system was used (37) that permitted the differentiation of effector T cells by short term mitogenic stimulation. In brief, mature effector cells were generated by priming purified CD4+ resting memory T cells in flat-bottom cell culture plates (Costar, Cambridge, MA) at a concentration of 0.5 × 10^6 cells/ml with 1 µg/ml anti-CD28 mAb and 10 U/ml recombinant human IL-2 in the presence or the absence of recombinant human IL-4 (31.25 ng/ml). When anti-CD3 stimulation was used during priming, the plates were coated with OKT3 (1 µg/ml in 50 ml Tris-HCl, pH 9.5) for 24 h at room temperature. Unbound mAb was removed by washing the plates with PBS immediately before the cells were washed. After 5 days of priming, the cells were harvested, counted, washed, and rested for 60 h at 37°C in RPMI containing 5 × 10^5 cells/ml in medium supplemented with 10 U/ml IL-2. The phenotype of the effector populations as defined by the ability of the cells to produce cytokines was determined by intracellular flow cytometry after maximal mitogenic stimulation and compared with that of the starting population. By assessing Th cell differentiation in serial controls, the assay was found to be highly reproducible in individual healthy donors and stable over time.

Flow cytometry

Cells (1 × 10^6/sample) were stained with saturating amounts of directly labeled mAb and analyzed by FACS (EPICS, Beckman Coulter, Fullerton, CA). To assess the inherent capacity of the cells for cytokine production and to bypass proximal signaling events after TCR/CD3 activation that have been suggested to be impaired in synovial T cells from RA patients (43), cells were stimulated with ionomycin (1 µg/ml; Calbiochem, San Diego, CA) and PMA (20 ng/ml; Sigma-Aldrich) for 5 hi nt h e presence of 10 µM monensin (Sigma-Aldrich) to prevent secretion of cytokines. Cells were harvested, washed, fixed with 4% paraformaldehyde (Sigma-Aldrich) for 5 h in the presence of 2 µM monensin (Sigma-Aldrich) to prevent secretion of cytokines. Cells were harvested, washed, fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 min at 37°C, and stored at −70°C in 10% DMSO/PBS until analysis. Cells were permeabilized with 0.1% (w/v) saponin (Sigma-Aldrich) in 2% FCS/PBS. Non-specific binding sites were blocked with 4% mouse and rat serum. Cytoplasmic cytokines were detected by staining with directly labeled mAb against human cytokines for 25 min on ice. Cells were washed with 0.1% saponin/2% FCS/PBS, resuspended in 2% FCS/ PBS, and analyzed by flow cytometry. Unstimulated cells that were identically stained and stimulated cells that were permeabilized or not but stained with irrelevant mAb were used as controls for determining background fluorescence.

Immunoprecipitation and Western blotting

Purified CD4+ memory T cells (2–5 × 10^6; 100 × 10^6/ml) were depleted of serum for 4 h at 37°C and were stimulated with PMA (100 ng/ml) for 10 min. The cells were lysed in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS, 10 mM NaF, 5 mM EDTA, 1 mM Na3VO4, 10

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Table I. Study population

<table>
<thead>
<tr>
<th>n</th>
<th>Sex (M/F)</th>
<th>Age (yr)</th>
<th>Disease duration (mo)</th>
<th>Erythrocyte sedimentation rate (mm/h)</th>
<th>C-reactive protein (mg/l)</th>
<th>Rheumatoid factor (IU/ml)</th>
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<tr>
<td>24</td>
<td>8/16</td>
<td>52.9 ± 13.4</td>
<td>5.9 ± 3.8</td>
<td>22 [1; 60]</td>
<td>8 [3; 82]</td>
<td>22 [10; 339]</td>
</tr>
</tbody>
</table>

* Data are presented as absolute values, mean ± SD or median [range].
Table II. Frequency of cytokine producing peripheral blood CD4+ memory T cells*

<table>
<thead>
<tr>
<th></th>
<th>RA Patients</th>
<th>Healthy Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>78.5 ± 5.4</td>
<td>79.2 ± 8.2</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>30.2 ± 7.0</td>
<td>30.0 ± 8.7</td>
</tr>
<tr>
<td>IFN-γ+IL-4 (Th1)</td>
<td>28.6 ± 6.6</td>
<td>28.3 ± 8.1</td>
</tr>
<tr>
<td>IL-4</td>
<td>4.6 ± 1.5</td>
<td>4.7 ± 1.9</td>
</tr>
<tr>
<td>IL-4+IL-2, IFN-γ (Th2)</td>
<td>3.0 ± 0.9</td>
<td>3.2 ± 1.6</td>
</tr>
</tbody>
</table>

*Highly purified resting peripheral blood CD4+ memory T cells were stimulated for 5 h with PMA and ionomycin, double stained with mAbs to IFN-γ and IL-2 or IL-4, and intracellular cytokines were detected by flow cytometry. The differences between RA patients (n = 24) and healthy controls (n = 10) were not statistically significant (two-tailed Student’s t test).

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μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg/ml PMSE in PBS). The lysates were precleared with normal mouse serum and incubated with primary Ab for 1 h at 4°C. Immune complexes were precipitated with recombinant protein A agarose (Pharmacia, Uppsala, Sweden) for 1 h at 4°C, washed three times in lysis buffer, and eluted with SDS-PAGE loading buffer. Proteins were separated in a 7% polyacrylamide gel and transferred onto nitrocellulose. After blocking of nonspecific binding sites (5% milk powder and 0.05% Tween-20 in PBS), the blots were probed with specific Abs for STAT6 or phosphotyrosine (PY20) and developed with enhanced chemiluminescence (Santa Cruz Biotechnology).

Statistical analysis

Differences in data distribution were analyzed by two-tailed Student’s t tests for comparison of frequencies of cells producing particular cytokines between healthy individuals and RA patients, by paired two-tailed t tests for comparison of Th subsets in effector populations with the starting populations within the same group of patients or healthy controls, and by Fisher’s exact test for comparison of the frequencies of HLA-DRB1*01- or *04-positive individuals within different patient groups. p < 0.05 was considered significant.

Results

Th1/Th2 cytokines in resting CD4+ peripheral blood memory T cells after in vitro stimulation

To assess the inherent capacity of resting peripheral blood CD4+ memory T cells from RA patients and healthy controls to produce cytokines, freshly purified cells were stimulated in vitro and analyzed by flow cytometry. As it has been suggested that TCR-mediated signaling in RA synovial T cells might be impaired with respect to phosphorylation of the TCR ζ-chain (43), the cytokine secretion pattern of cells was analyzed throughout the study after stimulation with PMA and ionomycin, which bypasses the most proximal signaling events after TCR/CD3 engagement. No difference in the cytokine secretion pattern was found between cells from RA patients and cells isolated from healthy controls (Table II). Moreover, the frequencies of Th1 cells (IFN-γ-positive, IL-4-negative) or Th2 cells (IL-4-positive, IFN-γ-negative) in resting memory T cells were similar in patients and controls (Table II). Consequently, resting CD4+ memory T cells from patients and healthy individuals expressed similar Th1/Th2 cell ratios after in vitro stimulation (10.5 ± 5.4 vs 9.1 ± 3.6 in RA patients and controls, respectively). Furthermore, no differences were detected between RA patients and controls with respect to cells producing IL-4 and IFN-γ, IL-2 and IFN-γ, IFN-γ but no IL-2, or IL-2 but no IFN-γ (data not shown).

Th2 memory effector cell generation in healthy controls

To analyze Th2 memory cell differentiation in RA patients we first established the optimal conditions for Th2 generation in a group of healthy individuals who were age- and sex-matched with RA patients. Purified CD4+ memory T cells from the healthy individuals were primed under various conditions that have previously been demonstrated to regulate Th2 effector generation from resting memory T cells, namely stimulation with CD28 and IL-4 in the absence of the presence of TCR ligation (37). The cytokine secretion pattern of freshly isolated and ex vivo differentiated memory effector cells from one healthy individual is shown in Fig. 1. Th2 cell frequencies could not be increased by priming with anti-CD3 and anti-CD28 (3.1 ± 1.2% in primed cells compared with 3.2 ± 1.6% in freshly isolated cells in the entire group of healthy individuals; Table III) and were only slightly increased when exogenous IL-4 was added during priming (4.2 ± 1.8%; data not shown). By contrast, priming with anti-CD28 in the absence of TCR ligation induced Th2 cell differentiation and significantly increased the Th2 cell population to 5.7 ± 1.3% (p < 0.04, compared with fresh cells). Priming with anti-CD28 in the presence of exogenous IL-4 further increased the Th2 cell frequencies to 9.7 ± 2.7% (p < 0.006 vs fresh cells; Table III). It should be stressed that in all healthy individuals, increased numbers of Th2 effector cells could be generated from resting peripheral blood CD4+ memory T cells by priming with anti-CD28 in the absence of TCR engagement, with an increase in Th2 cells of at least 50% routinely noted. Furthermore, exogenous IL-4 enhanced the efficiency of
anti-CD28-induced Th2 cell differentiation in all healthy individuals and resulted in an additional increase in the number of Th2 cells of ≥50%.

Impaired Th2 memory effector cell differentiation in RA patients

Analysis of Th2 effector cell differentiation in RA patients revealed striking differences compared with that in healthy individuals. Statistical examination of the Th2 cell differentiation data from RA patients in response to priming under Th2-inducing conditions suggested that the data were not normally distributed and therefore were unlikely to be derived from a single entity but, rather, represented different cohorts. Patients could be grouped according to the ability of their memory T cells to differentiate into Th2 effectors under optimal Th2-inducing conditions. In nine RA patients Th2 effectors were generated by priming with anti-CD28 in the absence of TCR stimulation in a way comparable to that in healthy controls, e.g., an increase of Th2 cell frequencies of at least 50% was initiated by stimulation with anti-CD28 in these patients (Fig. 2A). When IL-4 was added during priming, a further increase in the Th2 cell frequencies within the primed population of ≥50% was achieved (p < 0.02; Table III). In contrast to healthy individuals, however, in this group of RA patients priming with anti-

Table III. Frequency of Th2 cells in resting and effector memory populations

<table>
<thead>
<tr>
<th></th>
<th>Resting Cells</th>
<th>Anti-CD28 anti-CD3</th>
<th>Anti-CD28</th>
<th>Anti-CD28 rIL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>10</td>
<td>3.2 ± 1.6</td>
<td>3.1 ± 1.2</td>
<td>5.7 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>RA patients&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>9</td>
<td>3.1 ± 1.0</td>
<td>7.9 ± 5.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.6 ± 8.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group II</td>
<td>7</td>
<td>3.2 ± 0.5</td>
<td>2.8 ± 1.0</td>
<td>3.4 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III</td>
<td>8</td>
<td>3.0 ± 1.2</td>
<td>3.1 ± 1.5</td>
<td>3.2 ± 1.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Intracellular cytokines were detected in freshly isolated and primed effector memory T cells by flow cytometry.

<sup>b</sup> Statistically significant difference compared to the starting population within the same group (paired two-tailed Student’s t-test).

<sup>c</sup> Group I, patients with anti-CD28 inducible Th2 differentiation; group II, patients with IL-4 dependent Th2 differentiation; group III, patients with Th2 differentiation deficiency.

FIGURE 2. Generation of Th2 effectors in RA patients. CD4<sup>+</sup> memory T cells were isolated from the peripheral blood from RA patients. Effector cells were generated by priming for 5 days with anti-CD28 without ligation of the TCR in the absence or the presence of rIL-4. Cytoplasmic cytokines were detected by flow cytometry after maximum stimulation as described in Materials and Methods. The numbers indicate the percentage of cytokine-producing cells from the total population of gated viable lymphocytes. Demonstrated is a representative cytokine secretion pattern of freshly isolated and primed memory effector T cells from one of the nine RA patients in whom Th2 effectors could be generated in a manner similar to that in healthy controls (A), one of the seven RA patients in whom Th2 effector generation was dependent on the presence of exogenous IL-4 (B), and one of the eight RA patients with defective induction of Th2 effector cells (C).
CD28 in the presence of immobilized anti-CD3 also significantly increased the IL-4-producing cells ($p < 0.05$; Table III).

In the remaining 15 RA patients, Th2 memory effectors could not be generated by priming of CD4$^+$ memory T cells with anti-CD28 alone (Table III). There was a significant difference in the frequencies of Th2 cells after priming with anti-CD28 between this group of patients and healthy individuals ($3.2 \pm 1.2$ vs $5.7 \pm 1.3$; $p < 0.001$). Priming in the presence of exogenous IL-4 induced Th2 cell differentiation in 7 of these 15 patients (Fig. 2B). In these seven patients a significant increase in Th2 cells was achieved after culture with anti-CD28 and rIL-4 compared with that in the starting population ($p < 0.005$; Table III). In contrast, however, in eight RA patients, exogenous IL-4 was without effect with respect to the generation of Th2 effectors (Fig. 2C). In this group of patients, the Th2 cell frequencies were $3.0 \pm 1.1$% in freshly isolated cells, $3.2 \pm 1.5$% in CD28-primed effectors, and $3.6 \pm 1.4$% in effectors that were primed with anti-CD28 and IL-4 (Fig. 2C). In contrast to the nine patients described above but similar to healthy individuals, Th2 cell differentiation was not observed after engagement of the TCR in these 15 patients (Table III).

**FIGURE 3.** Altered Th1 differentiation in RA. CD4$^+$ memory T cells were isolated from the peripheral blood of RA patients. Th1 cells were generated in vitro by priming for 5 days with immobilized anti-CD3 and a mAb to CD28. After rest, cells were stimulated with PMA and ionomycin and double stained with mAb to IL-2 and IFN-γ, and cytoplasmic cytokines were detected by flow cytometry. The mean ± SD of the frequencies of cells producing IL-2 and/or IFN-γ from healthy controls ($n = 10$; stippled bars) or RA patients ($n = 24$; solid bars) are shown. Comparison of the data was performed by two-tailed $t$ test. ns, Not significant.

**FIGURE 4.** Th1 cytokines in ex vivo generated memory effector cells. CD4$^+$ memory T cells were isolated from the peripheral blood, and effector cells were generated by priming for 5 days with immobilized anti-CD3 and a mAb to CD28. Cytosplasmic cytokines were detected by flow cytometry after maximum stimulation as described in Materials and Methods. The numbers indicate the percentage of cytokine-producing cells from the total population of gated viable cells. Demonstrated is a representative cytokine secretion pattern of one of the 10 healthy controls (A), one of the nine RA patients in whom Th2 effectors could be generated in a manner comparable to that in the healthy controls (B), and one of the 15 RA patients with impaired anti-CD28-induced Th2 effector generation (C).

**Altered Th1 cell differentiation in RA patients**

TCR stimulation of T cells has been shown to be critical for the induction of Th1 cell differentiation (31, 37). Thus, Th1 cell differentiation of RA memory T cells was analyzed after priming with anti-CD3 and anti-CD28 and was compared with that in healthy controls. A significant increase in the numbers of IFN-γ-producing cells occurred after priming with anti-CD3 and anti-CD28 in controls and patients, with no apparent difference between the groups (data not shown). To provide a more detailed analysis of the cytokine secretion profile of individual Th1 cells, freshly isolated CD4$^+$ memory T cells and effector cells were double stained with mAb to IL-2 and IFN-γ and analyzed for the distribution of IL-2 and IFN-γ double-producing Th1 cells and more specialized IFN-γ single producers. In freshly isolated memory T cells, no differences were detected between healthy controls and RA patients or the patient groups in which Th2 cells could or could not be induced (data not shown). However, after priming, a markedly reduced number of cells that produced IL-2 only and a significant shift in the balance of IFN-γ/IL-2 double producers to IFN-γ single-producing Th1 cells were noted in RA patients compared with controls (Fig. 3). Whereas in controls, 18.6 ± 4.6% of the primed effector cells were IL-2 single producers, only 14.2 ± 7.2% of the cells from RA patients exclusively contained IL-2. Moreover, significantly fewer effector cells were IFN-γ/IL-2 double producers in RA patients (23.6 ± 8.2%) compared with those in healthy individuals (33.1 ± 7.0%; $p < 0.003$). Concomitantly, specialized IFN-γ single producers were found more frequently among cells from RA patients than in healthy individuals (29.0 ± 11.5% vs 22.3 ± 7.5%; $p < 0.05$). When patients with impaired Th2 cell differentiation were analyzed, an even more pronounced shift toward specialized Th1 cells became obvious (Fig. 4): 18.1 ± 4.7% of the effector cells from this group of patients were positive for both IFN-γ and IL-2, and 32.1 ± 12.4% were specialized IFN-γ single-producing Th1 cells.

**Expression and phosphorylation of STAT6 in RA CD4$^+$ T cells**

Exogenous IL-4 had no effect with respect to Th2 cell differentiation in eight RA patients. To examine whether an altered expression or activation of STAT6, which is the critical signaling molecule downstream of the IL-4R (44–46), might underlie the impaired cellular response to IL-4 in these patients, CD4$^+$ T cells from them and from controls were purified and stimulated with IL-4. STAT6 was immunoprecipitated and probed with an Ab to...
STAT6 or a mAb to phosphotyrosine (PY20). No differences in the expression of STAT6 and its phosphorylation after activation were found between RA patients and healthy controls (Fig. 5).

Correlation of clinical parameters and T cell differentiation

The ex vivo T cell differentiation data were compared with the clinical parameters, such as disease duration, ESR, CRP, and rheumatoid factor, and the demographic data of the patients, such as age, sex, and genetic background. No significant correlation to any of these parameters was found. It should be noted, however, that the frequency of patients with an HLA-DR allele that is associated with aggressive forms of RA (HLA-DRB1*01 and HLA-DRB1*04) was 0.67 (10 of 15) in the patients with impaired Th2 differentiation compared with 0.22 (two of nine) in the group of patients in whom Th2 effectors could be induced (p = 0.09, by Fisher’s exact test).

Discussion

Many aspects of the chronic inflammation in RA have been attributed to a dominance of activated Th1 effector cells (17, 20, 47, 48). As disordered differentiation of memory effector T cells might contribute to the development of the characteristic Th1 bias in RA, we investigated Th effector cell differentiation from resting memory T cells in patients with early, untreated active RA in vitro. In the majority of patients, Th2 effector cells could not be generated under priming conditions that were optimal for Th2 cell differentiation in healthy individuals, e.g., priming with anti-CD28 in the absence of TCR ligation.

Th2 cell differentiation could be induced by anti-CD28 in the absence of TCR ligation in all of 10 healthy individuals from the current study in a rather homogeneous fashion. A similar pattern was observed in a group of healthy volunteers that were not age matched with the study population (data not shown), indicating that the induction of Th2 cell differentiation is a common result of priming of memory Th cells from healthy individuals with anti-CD28. The intracellular signals induced by the interactions between CD28 and its natural ligands CD80 and CD86 have been shown to not only complement TCR-mediated signals to allow full activation of T cells but also to play a central role in Th2 cell differentiation. Whereas immune responses driven by Th1 cells could be induced in CD28-deficient mice (49), strong (co)stimulatory signals through CD28 were required to induce Th2 differentiation (34, 35, 50). The proximal and distal events of CD28 signaling are just beginning to be understood. Multiple signaling cascades that may be independent of or dependent on protein tyrosine kinase activation have been demonstrated to be activated by CD28, including activation of phosphoinositide 3-kinase, extracellular signal-regulating kinases 1 and 2, stress-activated protein kinase/c-jun amino terminal protein kinase, and mitogen activation protein kinase 38 (p38/HOG1) (reviewed in Refs. 51 and 52), and it has been shown that CD28-activated signaling cascades regulate several transcription factors involved in cytokine transcription (51, 52). Recently, some evidence has been provided demonstrating that signaling through CD28 in the absence of TCR ligation is sufficient to activate resting T cells, as indicated by cell proliferation, increase of intracellular calcium, and induction of cytokine gene transcription (37, 53). The precise mechanisms, however, that contribute to anti-CD28-induced Th2 cell differentiation remain to be elucidated.

Analysis of freshly isolated resting memory T cells with flow cytometry after staining with mAbs to CD28 revealed no differences in the density of CD28 on the surface of cells from healthy individuals and patients or between individual patient groups (data not shown), confirming previous reports in which no abnormalities in surface CD28 were detected in RA patients (54). Moreover, CD28 signaling in CD4+ memory T cells in RA has been suggested to be functionally intact, as determined by proliferative responses to anti-CD3/anti-CD28-induced activation (55, 56). Consistent with these data, no differences between RA patients and healthy controls in the proliferation of memory cells and the viability of recovered effector cells were observed in the current study after stimulation with anti-CD28 alone or with anti-CD3 and anti-CD28 in combination (data not shown). However, despite similar proliferative responses and comparable densities of surface CD28, memory T cells from the majority of RA patients failed to differentiate into IL-4-producing Th2 effector cells after CD28 stimulation. Thus, CD28-mediated signals in those patients were apparently intact with respect to proliferation of memory T cells, but were impaired with regard to the induction of Th2 differentiation.

An indication of the nature of the defect in CD28 signaling, however, might derive from the observation that in seven of the 15 patients with impaired Th2 cell differentiation, priming in the presence of exogenous IL-4 could overcome the failure to induce Th2 cell differentiation. It has been demonstrated in healthy individuals that Th2 effector generation from resting memory T cells is IL-4 dependent (37). Thus, it can be surmised that in those patients, IL-4R-mediated signals triggered by exogenous IL-4 are required to supplement CD28 signals for the initiation of Th2 cell differentiation. Strikingly, however, in the remaining eight of the 15 patients, even large concentrations of exogenous IL-4 did not yield Th2 effectors. It has been shown that in RA patients with long standing disease, T cells derived from the synovial tissue predominantly produced IL-2, IFN-γ, and IL-10 (11, 16, 18–22). Moreover, it was found that those cells were also resistant to modulation of their phenotype after stimulation under Th2-inducing conditions (57). However, those cells might have been primed and activated in vivo and therefore might have lost the flexibility of functional modulation, as has been demonstrated for repeatedly activated T cells lines (40). In the current study resting memory T cells from the peripheral blood of patients with early RA were analyzed. Some of the patients had symptoms of the disease for as little as 6 wk, which makes it unlikely that the impairment of Th2 differentiation is secondary to long standing inflammation. Thus, the finding that the cells from these patients were unable to generate Th2 effector cells even in the presence of exogenous IL-4 strongly suggests that a disordered potential for memory cell differentiation contributes not only to the perpetuation but, more importantly, to the initiation of the unresolved Th1-mediated inflammation in some patients with RA.
The data imply that in some RA patients memory T cells not only failed to initiate Th2 effector cell differentiation after stimulation with anti-CD28, but also expressed a reduced cellular response to IL-4. Several molecules have been identified that are involved in transmission of signals from the IL-4R to the nucleus. In particular, binding of IL-4 to its receptor initiates phosphorylation of JAK3 and JAK1 that, in turn, phosphorylate STAT6, which exists in a latent form in the cytoplasm (46, 58, 59). Whereas JAK1 and JAK3 are activated by several cytokines, STAT6 confers specificity to IL-4R-mediated signals and is the only presently known STAT protein that is activated in response to IL-4 (58, 59). It has been conclusively shown that STAT6 is required for IL-4 responses and for the development of Th2 cells (44–46). Moreover, developing Th cells express a reduced phosphorylation of JAK3 and subsequently of STAT6, and it has been speculated that this might be a mechanism of phenotype determination (60). Therefore, an alteration in the expression and/or activation of STAT6 in resting memory T cells from RA patients with impaired Th2 differentiation might contribute to reduced IL-4 responsiveness. However, no differences were detected in STAT6 expression and phosphorylation after IL-4-induced activation between patients with impaired Th2 differentiation and controls. Thus, the precise molecular mechanisms of reduced functional cellular responsiveness to IL-4 in these RA patients still must be identified.

In contrast, Th2 cell differentiation could be induced by priming with anti-CD28 in other patients from the current study and could be further enhanced by the addition of exogenous IL-4. We have previously shown that the increase in Th2 cell frequencies in CD28-primed populations is not simply caused by preferential survival of a subset of T cells but clearly results from expansion of early uncommitted CD27+ T cells (37). In these patients, therefore, CD28-mediated signaling appeared to be intact in resting, uncommitted memory T cells with regard to proliferation and the initiation of differentiation. Furthermore, cellular responsiveness to IL-4 in these patients was comparable with that in normal controls. It is interesting to note that in these patients even higher numbers of Th2 cells could be induced by priming with anti-CD28 or anti-CD28 and rIL-4 compared with healthy controls, and that, in contrast to controls, Th2 cells could also be generated when the TCR was stimulated during priming (Table III). These data imply that the cells from these patients were more sensitive to Th2-inducing priming conditions. It will be interesting to determine the consequences of increased Th2 generation. It was previously reasoned that RA is a heterogeneous entity with distinct histologically defined phenotypes and that several pathologic mechanisms may cause an RA-like syndrome (61, 62). In those studies variants of RA have been postulated based upon different cytokine secretion patterns in tissue sections of synovial biopsies that could be correlated with the clinical progress of the disease in the patients. Thus, it might be speculated that the RA patients with increased Th2 differentiation might suffer from a more self-limited disease(s), related clinically to RA but not pathogenetically. Follow-up evaluation of these patients should provide information to test this hypothesis. In this respect it is interesting to note that only two of those nine patients were positive for an HLA-DR allele that is associated with aggressive forms of the disease (HLA-DRB1*01 or HLA-DRB1*04) compared with 10 of the 15 patients with impaired Th2 differentiation.

Although the ability of memory T cells from RA patients to generate Th2 effectors allowed a clear separation of the patients, no correlations with clinical and demographic data were detected. However, it should be emphasized that the patients from the current study were required to have early disease with symptoms for 6 wk to 12 mo and no previous treatment with corticosteroids and DMARDs. Thus, the patients were analyzed upon their first visit to a rheumatologist when disease might not have proceeded far enough to manifest clear differences between individual patients. Nevertheless, it is clear from the data that patients with similar clinical symptoms might have different cellular pathogenetic mechanisms.

The cytokine secretion profiles of resting memory T cells from RA patients and healthy individuals stimulated immediately after purification were comparable with respect to the frequencies of cells producing IL-2, IL-4, or IFN-γ. As Th2 differentiation has been shown to be dependent on IL-4 and could not be induced in the absence of cells that were able to produce IL-4 (37), the lack of memory cells capable of producing IL-4 would have provided a simple explanation for the observed differences in Th2 cell generation. However, as the frequencies of cells producing IL-4 after stimulation with PMA and ionomycin were indistinguishable between RA patients and healthy individuals and among the different patient groups, impaired Th2 cell differentiation could not simply be attributed to a lack of cells capable of secreting IL-4 but, rather, is more likely to be related to an impairment of the cells’ capacity to respond appropriately to CD28 and/or IL-4 signaling. The finding that resting memory T cells from controls and patients were similar with respect to their cytokine secretion patterns also indicates that in RA patients Th cell differentiation was apparently normal to some extent before the disease had become manifest. Presumably, the resting memory T cells in the patients were generated in response to some antecedent antigenic exposure. Clinically, the immune responses of RA patients appear to be normal before the onset of the disease. It might be speculated, therefore, that Th cell differentiation is not significantly altered in RA patients before the initiation of the disease, which could indicate that the resting memory T cell population from patients with early RA was generated in response to peptides that were presented by non-RA-associated MHC class II alleles. Alternatively, the data might indicate that the T cell differentiation defect in RA is acquired rather than genetic, which might have substantial implications for the pathogenesis of the disease.

Supportive of recent suggestions of a Th1-dominated immune response in the pathogenesis of RA (17, 20, 47, 48), an alteration in the differentiation of resting memory T cells into Th1 effectors was obvious in all patients. Whereas the frequencies of cells producing IFN-γ were similar in patients and controls after priming with anti-CD3 and anti-CD28, a marked decrease in IL-2-producing cells and a significant increase in specialized Th1 cells that produced IFN-γ but not IL-2 were evident in RA patients. An even more pronounced differentiation into specialized Th1 cells producing only IFN-γ was noted in patients in whom Th2 cells could not be induced. These data indicate that an alteration of Th1 cell differentiation is a common finding in RA, but is enhanced in patients in whom a concomitant Th2 differentiation defect could be detected. In combination, these functional abnormalities might result in a marked shift of the Th1/Th2 balance in activated effector cells of these individuals.

In summary, we have provided evidence that memory T cells from patients with early RA express an altered ability to differentiate into Th effectors. RA patients could be grouped according to the ability of their memory T cells to differentiate into Th2 effectors. Whereas in some patients Th2 differentiation could be induced, the majority of patients were identified to have a decreased ability to generate Th2 effectors in response to CD28-mediated signals, and some of them also failed to respond to IL-4. These data suggest a reduced functional responsiveness to Th2-inducing signals, in particular CD28 engagement and IL-4, in patients with RA. Moreover, increased generation of Th1 cells specialized in
secretory IFN-γ, but not IL-2, was observed in all patients. Altered Th1 cell differentiation and impaired generation of Th2 cells might contribute to the characteristic Th1-dominated inflammation in RA. These results imply that abnormalities in the potential of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. Arthritis Rheum. 41:1609.


