CD30+ T Cells in Rheumatoid Synovitis: Mechanisms of Recruitment and Functional Role

Roberto Gerli, Costantino Pitzalis, Onelia Bistoni, Brunangelo Falini, Vincenzo Costantini, Anna Russano and Claudio Lunardi

J Immunol 2000; 164:4399-4407; doi: 10.4049/jimmunol.164.8.4399
http://www.jimmunol.org/content/164/8/4399
CD30$^+$ T Cells in Rheumatoid Synovitis: Mechanisms of Recruitment and Functional Role

Roberto Gerli, Costantino Pitzalis, Onelia Bistoni, Brunangelo Falini, Vincenzo Costantini, Anna Russano and Claudio Lunardi

High serum levels of soluble CD30 (sCD30) have been reported to better predict the response to second line therapy in rheumatoid arthritis (RA). It is believed that sCD30 is released by CD30$^+$ T cells present in the RA synovium. However, both the mechanism of recruitment to the joint and the functional role of this T cell subset in the pathogenesis of the disease remain unknown. This study confirmed higher levels of sCD30 in the serum and synovial fluid (SF) of RA patients compared with normal controls. However, analysis of mRNA and cell surface CD30 expression showed that CD30$^+$ T cells are detectable in the SF, but not in the synovial membrane. In contrast, T cells expressing the CD30 transcript, but not the surface molecule, were found in the peripheral blood of both RA and normal controls. CD30 surface expression was up-regulated by adhesion and migration through endothelium in vitro and in a delayed-type hypersensitivity model in vivo. Although the great majority of fresh or cloned CD30$^+$ T cells from SF produced both IFN-$\gamma$ and IL-4, CD30 expression strictly correlated with IL-4 synthesis in synovial T cell clones. In addition, CD30$^+$ T cell clones also produced high amounts of the anti-inflammatory cytokine IL-10. On this basis, we would like to propose that synovial CD30$^+$ cells may play a role in the control of the inflammatory response. Serum sCD30 may reflect such cell activity and, therefore, explain the previously demonstrated correlation between high sCD30 serum levels and positive response to therapy.

The Journal of Immunology, 2000, 164: 4399–4407.

C

d30 is a member of the tumor necrosis/nerve growth factor receptor superfamily, originally described as a marker for Hodgkin and Reed-Sternberg cells in Hodgkin’s disease, but subsequently found on various non-Hodgkin lymphomas and on activated T and B cells (1, 2). During mitogenesis induced by nonspecific stimuli, CD30 expression appears to be restricted to a minority of activated CD45R0$^+$ T cells (2, 3), whose functional role is still controversial. It has been reported that the CD30 ligand acts both as a costimulator for T cell proliferation and a mediator of cytotoxicity through apoptosis (4, 5). Moreover, CD30 was consistently expressed by human CD4$^+$ Th2 and Th0 and by CD8$^+$ type 2 cytotoxic T cell clones, whereas CD4$^+$ Th1 and CD8$^+$ type 1 cytotoxic clones usually exhibited poor or no CD30 expression (6, 7). Although the preferential expression of the CD30 molecule on Th2 cells has not always been confirmed in vitro (8), in vivo analysis of CD30 surface expression as well as detection of soluble CD30 (sCD30) provide additional evidence for a strict association between CD30$^+$ T cells and disorders in which a Th2 polyclonal activation prevails (9–11).

Some of us have recently reported high levels of sCD30 in both serum and synovial fluid (SF) of patients with rheumatoid arthritis (RA) (12). This increase seems to reflect a recruitment of CD30$^+$ T cells into the inflamed joints. However, the mechanisms by which CD30$^+$ T cells are enriched at the inflammation site are not clear, since circulating CD30$^+$ T cells are usually scarcely detectable in the peripheral blood (PB) of RA patients (12). In addition, the pattern of cytokine secretion and the possible functional role of these cells during inflammatory processes remain unclear.

In the present study we aimed to address some of these points. First, we wanted to confirm that sCD30 levels were elevated in both serum and SF in this study population in comparison to control levels. Second, we analyzed the distribution of CD30$^+$ T cells in these compartments as well as in the SM of some of these patients. Third, we investigated the mechanisms involved in regulating the expression of the CD30 molecule on the surface of T cells in relation to the processes of activation and localization to an inflammatory site. To this end, we analyzed the effects of cell adhesion and migration through endothelium using both an in vitro and an in vivo model. In addition, we examined the capacity of SF itself to induce CD30 surface expression. Moreover, CD30 surface expression and its specific mRNA transcript were analyzed in fresh and cloned T cells from PB and SF of patients with early compared with long-standing RA. Fourth, in an attempt to clarify the functional role of CD30$^+$ T cells in the pathogenesis of RA, we correlated the levels of sCD30 with the production of some Th1 and Th2 cytokines in the serum of these patients. Finally, this was complemented by analysis of the pattern of cytokine production (Th1/Th0/Th2) by the same above-mentioned T cell clones in patients with early and long-standing RA.

Materials and Methods

Patients

Serum samples were obtained from the PB of 59 patients (15 men and 44 women; age range, 21–72 years) who fulfilled the American College of
Rheumatology diagnostic criteria for RA (13). Disease duration ranged from 2–32 years in 38 of these subjects, who were classified as having long-standing RA. Twenty-one were studied at the diagnosis (early RA), before starting corticosteroids or disease-modifying anti-rheumatic drugs, and were then followed up for at least 1 year to confirm the diagnosis. Thirty-six age- and sex-matched healthy subjects acted as NC. SF were obtained from 16 patients with long-standing RA and from 8 subjects with early RA. Only two patients with effusions agreed to have artificial skin blisters formed (see below). SM samples were obtained from synoviectomy of the knee performed in 6 patients with long-standing RA. Written informed consent was obtained from each subject enrolled in the study.

General reagents and mAbs

The levels of sCD30 were tested in serum and SF samples with a commercially available ELISA kit (Ki-1 Ag ELISA, Dako, Glostrup, Denmark), according to the manufacturer’s instructions. ELISA kits (Genzyme, Cambridge, MA) were also used to detect IL-4, IL-10, and IFN-γ in both serum and T cell clone supernatants. A Cy-chrome-conjugated anti-human IFN-γ mAb (BioErgononics, St. Paul, MN) and a PE-conjugated anti-human IL-4 mAb (Serotec, Oxford, U.K.) were simultaneously employed to assess intracytoplasmic cytokines production. Anti-CD3, -CD4, and -CD8 mAbs were purified from supernatant of hybridoma cells obtained from American Type Culture Collection (Manassas, VA). Anti-CD45RO (UCHL-1) and FITC-conjugated anti-CD30 mAb were purchased from Dako (Glostrup, Denmark). Anti-CD30 mAb was purchased from Ortho (Raritan, NJ), and anti-CD25 (IL-2R) and anti-CD69 were obtained from Becton Dickinson (San Jose, CA). PHA was obtained from Life Technologies (Gaithersburg, MD). PMA, calcium ionophore A23187, monensin, and saponin were purchased from Sigma (St. Louis, MO). Human rIL-2 (sp. act., 9 × 10^6 U/mg) was obtained from Janssen (Beerse, Belgium), and rTNF-α (sp. act., 10^9 U/mg) was provided by Dr. E. Allevi (Knoll, Milan, Italy). Type I collagen solution extract from porcine skin (Cellmatrix I-A) were obtained from Nitta Gelatin (Osaka, Japan). Endothelial cell growth factor was purified from bovine hypothalamus extract, as previously described (14).

Cell isolation, phenotypic analysis, and total RNA extraction

PB from NC and paired PB and SF samples from RA patients were collected in preservative-free heparin (5 U/ml); SF samples were then incubated with 3000 U of hyaluronidase (Sigma) for 30 min at room temperature. SM was cut in small pieces and then incubated in RPMI medium containing 2.5 mg of collagenase and 0.10 mg of DNase (Sigma)/ml of medium for 30 min at 37°C. Samples were filtered through a cell strainer (Becton Dickinson), and the cells obtained were washed twice with RPMI 1640. PBMC were isolated by density gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Nycomed, Oslo, Norway) and depleted of adherent cells by incubation in tissue culture flasks for 90 min at 37°C in a 5% carbon dioxide atmosphere. T cells were enriched from the nonadherent cell fraction by E-rosetting with sheep erythrocytes and treatment with OKM1 mAb plus rabbit complement (Cedarlane, Hornby, Ontario, Canada) (15). T cell suspensions were then passed over a nylon wool column to eliminate residual contaminating B cells and monocytes. On the basis of their reactivity with anti-CD3 mAb, >98% of these mononuclear cells were T lymphocytes. Cell samples were then resuspended in RPMI supplemented with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium; Life Technologies).

The simultaneous presence of the CD30 and other Ags on the T cell surface was evaluated by flow cytometry (FACScan, Becton Dickinson) using a two-color immunofluorescence staining technique that employed an isotype-specific goat anti-mouse Ab (Southern Biotechnology Associates, Birmingham, AL) conjugated with either FITC or PE as developing agents for each mAb. This procedure has been reported in detail previously (16). Negative controls and tests to prove the specificity of the isotype-specific Abs were performed for each experiment as previously described (16). In some experiments, FITC-labeled CD30^+ T cells were isolated by cell sorting (FACSCalibur, Becton Dickinson) for intracytoplasmic cytokine evaluation. RNA was obtained from cell samples using the cesium chloride-guanidinium thiocyanate method (17).

CD30 mRNA analysis

Approximately 4 μg of total RNA was used for RT-PCR, with 50 U of avian myeloblastoma virus reverse transcriptase (Life Technologies), and 300 ng of oligo(dT) (Pharmacia, Uppsala, Sweden). Five microliters was then added to a 50-μl reaction for PCR. The sequences of PCR primers used are the following: 5’ primer, 5’-GGAAGCGAAATGGGCGAAGA-3’; and 3’ primer, 5’-TACCGGTGTCGCCCTTCATG-3’, with an amplified fragment of 345 bp (18). After a 5-min denaturation step at 94°C, each PCR cycle consisted of a denaturation step at 72°C for 1 min for a total of 25, 30, and 35 cycles on a DNA thermal cycler 480 (Perkin-Elmer, Milan, Italy). After the chosen number of cycles, a final 10-min elongation step at 72°C was performed. Paired samples of SF and PB or SM and PB were amplified in the same experiments as well as NC. Negative controls (without cDNA added) were included for each set of experiments. Actin was used as an internal control. Ten of the 50 μl of the PCR amplified product was run and visualized only after 35 cycles as a single band on ethidium bromide-stained 1% agarose gel under UV light. The identity of each PCR product was confirmed by transfer onto a nitrocellulose membrane (Hybond-N, Aylesham, Cambridgeshire, U.K.) and hybridized with a specific radiolabeled probe. Filters were then exposed for autoradiography for 4 h (at −80°C (Hyperfilm, Amersham) with intensifying screen, and the presence of the hybridization signal was evaluated on the film.

To evaluate the CD30 transcript in T cell clones, total RNA was obtained from each clone (−1 × 10^6 cells) following the RNAzol B method according to the manufacturer’s instructions (Tel-Test, Friendswood, TX) (17). The RT-PCR was conducted using the RT-PCR kit (Superscript, Life Technologies). Briefly, ~200 ng of total RNA was used in a 50-pmol RT-PCR with 100 ng of CD30-specific oligonucleotides. After a 30-min incubation at 42°C, 35 cycles were conducted under the above-mentioned conditions. Negative and positive controls were added to each set of experiments. Ten microliters of the amplified product was transferred to a nylon membrane (Hybond-N) using a slot-blot apparatus (Hybri-Blot Manifold, BRL-Life Technologies). PCR products were hybridized with a [32P]CTP-radiolabeled CD30-specific probe, and the signal was visualized on an x-ray film after a 3- to 4-h exposure. RT-PCR for actin was performed to control that negative amplifications were not due to RNA degradation.

Immunohistology

SM specimens were embedded in optimal temperature cutting compound (OCT, Miles Laboratories, Elkhart, IN) and snap-frozen in liquid nitrogen. Samples were stored at ~70°C until sectioned for immunohistologic staining. Five-micron-thick sections were cut with a cryostat (Leitz, Wetzlar, Germany) at ~22°C. Sequential sections were mounted on poly-l-lysine-coated slides and dried overnight at room temperature. Sections were fixed in acetone for 10 min, wrapped in aluminum foil, and stored at ~70°C until further use.

CD30^+ T cells were identified by the immunohistological technique, as previously described (19). In brief, frozen sections were incubated with the primary mAb, followed by rabbit anti-mouse Ig (Dako) and immunohistological complex. To maximize the sensitivity of the method, steps 2 and 3 were repeated once each. All Ab steps were performed for 30 min with washing 5 min in between. All slides were buffered saline, pH 7.6. Endogenous alkaline phosphatase was blocked with 1 mM/l levamisole (20). Slides were then counterstained for 5 min in Gill’s hematoxylin and mounted in Kaiser’s glycerol gelatin (Merck, Darmstadt, Germany). Staining of negative control samples was performed on all specimens studied. A procedure identical with that previously described was followed: except that the primary mAb was substituted with an irrelevant Ab of the same isotype. Normal human tonsils and lymph node biopsies from cases of Hodgkin’s disease were used as positive controls for the CD30 immunolabeling (1, 2).

T cell activation by anti-CD3 mAb or autologous SF

Purified T cells were incubated in complete medium containing 25 or 50% autologous inflammatory SF for 12, 24, and 72 h at 37°C. In parallel experiments, T cells were stimulated with plastic immobilized anti-CD3 mAb plus 20 U/ml of rIL-2 as previously described (15). At the end of the culture period, cells were extensively washed and phenotypically analyzed as described above.

In vitro T cell adhesion to and migration through HUVEC monolayers

HUVEC were harvested from fresh human umbilical cords treated with 0.1% collagenase (type I; Roche, Mannheim, Germany) according to a previously described method (21) and cultured as previously reported (22). HUVEC monolayers (passages 2–3) were cultured for at least 3 days on collagen gels in plastic 24-well plates as described previously (23), then stimulated with rTNF-α (10 ng/ml) at 37°C for 4 h and finally extensively washed. HUVEC monolayers were then incubated with T cell suspensions (2.0 × 10^6/well) at 37°C under static conditions. After 4 h unbound T cells were removed from the surface of the HUVEC monolayers by gently washing with warmed medium 199 and 0.1% BSA. Then,
HVEUC were incubated for 20 min at 37°C with 0.4% EDTA in PBS to remove adherent T cells. Almost all adherent T cells could be detached from the surface of HVEUC with this treatment. Monolayers were then treated for another 30 min with 0.4% EDTA to remove HVEUC from the surface of collagen gels; this process was monitored with phase microscopy to confirm removal of HVEUC. After monolayers were washed out with PBS, the collagen gels containing migrating T cells were treated with 0.05% collagenase/HBSS at 37°C for 3 min two to three times, with continuous pipetting to release the T cells. After each pipetting with collagenase, PBS (10% final concentration) was added to the solution containing cells to diminish the enzyme activity. The numbers of adherent and migrating T cells were counted by hemocytometer and analyzed by flow cytometry. No changes in the surface expression of CD3 or CD30 molecules were found on T cells following collagenase treatment.

In vivo T cell migration into artificial skin blisters

Intraepidermal blisters were formed by negative suction on the forearm of two RAs, sites of DTH skin reactions to Mycobacterium tuberculos is purified protein derivative (PPD), as previously reported (24). Briefly, 1000 U of PPD in 100 μl of sterile saline was injected into five sites on the volar aspects of the forearm at time zero. The following day, blisters were formed by 1–2 h of constant suction using a vacuum pump. Twenty-four hours later blister fluids were gently collected with a sterile 0.5-ml syringe and diluted 1/5 into heparinized PBS (20 U/ml heparin). Cells were then pelleted in Eppendorf tubes by centrifugation at 3000 rpm for 10 min at a benchfuge, resuspended in PBS supplemented with 0.2% BSA and 1 mM CaCl2, and characterized by double immunofluorescence and FACS analysis as described above. Paired PB and SF were collected at the same time.

Evaluation of intracytoplasmic cytokines in fresh SF T cells

Purified total T cells or sorted CD30+ T cells were incubated in complete medium, supplemented with a combination of 25 ng/ml of PMA and 1 μg/ml of calcium ionophore A23187 for 6 h in the presence of 2.5 μM monensin. Cells were then washed, permeabilized by preincubation with Ca2+- and Mg2+-free PBS containing 0.1% saponin and 0.01 M HEPES (saponin buffer) for 10 min, and incubated with Cy-chrome-conjugated anti-human IFN-γ mAb and PE-labeled anti-human IL-4 mAb. Cy-chrome- or PE-conjugated isotype-matched mAbs were used as negative controls. After 30-min incubation at room temperature, cells were washed in saponin buffer, resuspended in PBS, and analyzed by flow cytometry.

T cell cloning and cytokine production pattern analysis (IL-4, IFN-γ, and IL-10)

Mononuclear cells from both SF and PB of two patients, one with early RA, and the other with long-standing RA, were cultured with 25 IU/ml IL-2 in complete medium for 14 days before cloning to select for activated T cells. T cells were then cloned by limiting dilution as previously described (25). In brief, viable expanding cells were isolated by Ficoll-Hypaque density gradient, resuspended in complete medium, and finally seeded at 0.3 cells well in 96-well round-bottomed plates (Nunclon, Nunc, Kamstrup, Denmark) in the presence of 1 × 105 irradiated (5000 rad) allogeneic PBMC as feeder cells, PHA (1/200), and rIL-2 (25 IU/ml). Growing microcultures were further expanded at weekly intervals with irradiated feeder cells and rIL-2 for 14 days. Viable T cell clones were finally transferred into fresh complete medium and phenotypically analyzed by flow cytometry using anti-CD4 and anti-CD8 mAb. To measure cytokine levels in the supernatants, T cell clones (1 × 106/ml) were cultured for 24 h in complete medium in the presence of PMA (10 ng/ml) and anti-CD3 (50 ng/ml). Cultures were then centrifuged, and supernatants were collected, filtered through a 0.22-μm filter and stored at −20°C, until use. The levels of IL-4, IFN-γ, and IL-10 in T-cell clone supernatants were detected by the above-described ELISA.

Statistical analysis

Wilcoxon’s two-tailed test-normal approximation for paired data, Spearman’s rank correlation coefficient, and simple linear regression were adopted for the statistical analysis of the results. Values of p < 0.05 were chosen for rejection of the null hypothesis.

Results

Levels of sCD30

Analysis of PB sCD30 confirmed that patients with long-standing RA (n = 38) had higher serum levels than NC (n = 36; 99.5 ± 20 U/ml vs 12.2 ± 3; p < 0.001). Notably, even higher values were detected in the serum of patients with early RA (n = 21; 123.4 ± 37 U/ml; p < 0.001 vs NC). Furthermore, and of most importance, these values were significantly higher than those in patients with long-standing RA (p < 0.05). In addition, the sCD30 values were always higher in the SF than in the serum of both early RA (156.7 ± 22 vs 123.4 ± 37; p < 0.05) and long-standing RA (139.4 ± 22 vs 99.5 ± 20; p < 0.05).

Surface and mRNA CD30 expression

Similarly to the results of a previous study (12), in the present series of RA subjects and NC we found only negligible numbers of PB CD30+ T cells (<2%), but confirmed the increased numbers of CD30+ T cells in the SF (median, 7%; range, 1–16%) from both early and long-standing RA patients (Fig. 1). In contrast, to our surprise, immunostaining for CD30 of the SM of RA patients did not demonstrate any positive cells (data not shown). Nevertheless, this histological observation was supported by the very sensitive CD30-specific mRNA analysis. As shown in Fig. 2, this confirmed the presence of CD30 mRNA transcripts in SF T lymphocytes but not in SM cells, indicating that in this latter compartment there were no cells in which this molecule was actively transcribed. The sensitivity of our molecular methodology was further confirmed by the fact that this could detect CD30 transcripts in considerable amounts in the PB of both RA and NC, where, as mentioned, surface expression was largely negative. It is of note that CD30 mRNA was detected after hybridization with a radiolabeled CD30-specific probe even after 25 cycles of amplification, when the product was not detectable on an ethidium bromide agarose gel in the majority of the PB samples analyzed.

Coexpression of activation molecules on CD30+ T cells in RA SF

To establish which activation molecules were coexpressed on the surface of CD30+ T cells from RA SF, purified T lymphocytes from six subjects with long-standing RA and from four patients with early RA were analyzed by a two-color immunofluorescence technique. The results showed that the majority of CD30+ T cells were positive for HLA-DR (median, 77.5%; range, 59–84%), CD45R0 (median, 94.3%; range, 89–99%), and CD69 (median, 90.1%; range, 83–96%). Notably, most of CD30+ T cells also coexpressed the IL-2R (median, 89.8%; range, 75–95%), although...
and migration, lymphocytes can be separated into nonadherent, actuated in a similar fashion. In this static in vitro model of adhesion
37°C to investigate the possibility that CD30 could be also mod-
activated HUVEC monolayer cultured on collagen gel for 4 h at
T lymphocytes from normal donors were incubated on a cytokine-

A SF did not lead to significant induction of surface CD30 (Fig. 3A).

The difference in CD30 expression between PB and SF T cells in
RA patients with different concentrations of autologous inflamma-
ry SF. Although about 20% of T cells expressed the CD30 after
activation stimuli inducing the molecule act either during the migratory process or directly at the
site of inflammation. Thus, in an attempt to verify the mechanisms by which CD30 is induced on the T cell surface, we firstly eval-
uated its expression after incubation of purified PB T cells from RA patients with different concentrations of autologous inflamma-
tory SF. Although about 20% of T cells expressed the CD30 after
24 h of culture upon T cell activation triggered by anti-CD3 mAb, incubation of T cells for a period ranging from 12–72 h in complete
medium containing 25 or 50% of autologous inflammatory SF did not lead to significant induction of surface CD30 (Fig. 3A).

Since it is known that the simple contact with activated endo-
thelial cells can activate resting T cells (22, 26, 27), unstimulated T lymphocytes from normal donors were incubated on a cytokine-
activated HUVEC monolayer cultured on collagen gel for 4 h at
37°C to investigate the possibility that CD30 could be also mod-
ulated in a similar fashion. In this static in vitro model of adhesion and migration, lymphocytes can be separated into nonadherent,

adherent, and migrated cell fractions. As shown in a representative example of four separate experiments in Fig. 3B, we demonstrated
that the migration process causes a considerable enrichment of T

cells expressing high levels of surface CD30 (median, 31%; range, 19–44%) compared with the nonadherent fraction (<2%). In addi-
tion, the process of adhesion in itself appears to be sufficient to
induce the expression of CD30 in some T cells (median, 7%; range, 3–11%).

To investigate whether similar mechanisms could be operating
in vivo, we examined the expression of CD30 in lymphocytes mi-

gated into artificial skin blisters raised over DTH skin reactions in
two patients with RA. The results were compared with paired PB
and SF. As demonstrated in Fig. 3C, a marked accumulation of
CD30+ T cells occurred at DTH sites at 24 h, with 43.0 and 50.4%
of blister T lymphocytes being positive for this marker.

**Table I. Intracytoplasmic detection of IFN-γ and IL-4 in the entire T cell population or in the purified CD30+ T cell subset from the SF of three different RA patients**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>SF CD30+ T Cells (%)</th>
<th>IFN-γ+ IL-4*</th>
<th>IFN-γ+ IL-4+</th>
<th>IFN-γ+ IL-4*</th>
<th>IFN-γ+ IL-4+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>56</td>
<td>&lt;1</td>
<td>6.5</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>49</td>
<td>&lt;1</td>
<td>9.9</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>64</td>
<td>&lt;1</td>
<td>3.1</td>
<td>17</td>
</tr>
<tr>
<td>Mean±SE</td>
<td>7.7 ± 2</td>
<td>56.3 ± 4</td>
<td>&lt;1</td>
<td>6.5 ± 2</td>
<td>20.6 ± 12</td>
</tr>
</tbody>
</table>

*, p < 0.001 in comparison to the correspondent value of the total T cell population.
Soluble CD30 levels and cytokine production pattern in RA serum

To shed some light on the functional role of CD30+ cells in the pathogenesis of RA, we verified whether the CD30 molecule expression on T cells was associated with a preferential production of Th1- or Th2-type cytokines. The levels of sCD30 were initially compared with those of IFN-γ or IL-10 detected in the serum of all 21 patients with early RA and with the levels of IL-4 detected in 6 of the 21 patients. Patients with early RA were selected to avoid as much as possible drug interference on the experimental observations. The results, however, did not show any correlation between sCD30 and cytokine serum levels (data not shown).

Intracytoplasmic cytokine production pattern in fresh SF T cells

The lack of correlation between the levels of sCD30 and Th1/Th2-type cytokines in RA sera prompted us to initially examine intracytoplasmic IFN-γ and IL-4 at the single-cell level in fresh T lymphocytes from the SF of long-standing RA patients. No cytokines were detectable in unstimulated SF T cells (data not shown). As shown in Table I, SF T cells stimulated with PMA and A23187 mainly produced IFN-γ (62.8 ± 2%). Some of these (~10%) were also producing IL-4, whereas there were no cells which produced IL-4 alone. Almost all purified SF CD30+ T cells were producing IFN-γ (93.0 ± 3%). However, in contrast to the whole T cell population, the great majority of these (~90%) were Th0, since they produced both IFN-γ and IL-4.

CD30 expression and cytokine production pattern in T cell clones

CD30 is expressed at a relatively low intensity of expression on a small number of RA SF T cells. This may be due to rapid shedding of the molecule after cell activation. Thus, to better analyze the correlation between CD30 expression and cytokine production, T cell clones were generated from either PB or SF of two RA patients, and CD30 expression was evaluated both on the cell membrane and at the mRNA level. As it is believed that the immune process at the basis of the disease can change during its course, we selected one patient with early and one with long-standing RA. In addition, because our results showed that almost all CD30+ T cells also expressed CD25, our clonal strategy was that T cells were initially cultured with rIL-2 at low concentration (25 IU/ml), so that the in vivo activated CD30+ T cells would be preferentially expanded. T cell blasts were then recovered and cloned by limiting dilution. A total of 18 (CD4+/CD8−, 16/2) and 21 (19/2) randomly selected clones from SF and 16 (13/3) and 14 (10/4) from PB of the long-standing and early RA patients, respectively, were then evaluated for both CD30 surface expression and IFN-γ, IL-4, or IL-10 secretion.

There was a wide range of CD30 expression on T cell clones from SF or PB, although the mean intensity of expression in each clone was relatively low. Interestingly, parallel CD30 mRNA analysis revealed a good correlation between expression of the CD30

![Figure 4](image.png)

**Figure 4.** CD30 surface expression in CD30 mRNA-positive or -negative T cell clones generated from RA PB or SF.

![Figure 5](image.png)

**Figure 5.** IL-4 and IFN-γ production by T cell clones from SF and PB of long-standing and early RA patients. ●, CD30-positive clones; ○, CD30-negative clones.
transcript and mean fluorescence intensity values in both SF and PB clones (Fig. 4).

Regarding the analysis of cytokine production, as shown in Fig. 5, no Th2 cell clones, i.e., producing IL-4 but not IFN-\(\gamma\), were generated from either the SF or the PB of the two patients. T cell clones expressing the CD30 molecule were more frequent in the SF (14/39, 36%) than in the PB (7/30, 23%) and were all producing both IL-4 and IFN-\(\gamma\) (Th0), except two clones from the long-standing RA PB, which were producing only IFN-\(\gamma\) (Th1). The long-standing SF RA clones produced very high amounts of IFN-\(\gamma\). Consequently, the IFN-\(\gamma\)/IL-4 ratio in these clones (median, 4.2; range, 1.6 –32.5) was much higher than that in early SF RA clones (1.2, 0.4 –11.3; \(p\), 0.001).

The low number of CD30\(^+\) cell clones from the PB did not allow to draw any conclusion on possible relationship between the expression of the molecule and cytokine production in this compartment. In the SF T cell clones, the CD30 mean fluorescence intensity expression was strictly correlated with the IL-4 synthesis in both long-standing (\(r = 0.67; p < 0.006\)) and early (\(r = 0.71; p < 0.001\); Fig. 6). In addition, the highly IL-4-producing CD30\(^+\) clones also produced high levels of IL-10 (Fig. 7). Finally, it is important to note that the production of IL-10 by CD30\(^+\) clones was much higher in early than in late RA (mean \(\pm\) SE, 877 \(\pm\) 197 vs 438 \(\pm\) 103 pg/ml; \(p < 0.001\)).

Discussion

The present study confirmed that patients with RA have significantly higher levels of sCD30 in serum and SF compared with NC. Notably, this difference was more pronounced in the group of patients with very early RA studied at the moment of the diagnosis before starting corticosteroids and/or disease-modifying anti-rheumatic drugs. In agreement with the findings of a previous study (12), the CD30 molecule was scarcely detectable on the surface of PBL of both RA patients and NC, but was expressed by a subset of RA SF T cells. Surprisingly, we did not find CD30\(^+\) cells in the SM by immunostaining analysis. Because the CD30 molecule could be difficult to detect when expressed at relatively low density on the T cell surface (28), specific mRNA analysis was conducted. The results showed that CD30 mRNA can be found in PB circulating cells, even in the absence of surface expression of the molecule, in both RA and NC. On the other hand, the negative CD30 surface expression by SM T cells was confirmed by the absence of specific mRNA transcripts, while both surface expression and transcripts of the molecule were detected in SF T cells. These results appear to rule out the possibility that the lack of CD30\(^+\) cells in the SM is due to technical failure of immunohis- tologic analysis or shedding of the molecule from the tissue-infiltrating cells. An alternative explanation may include the presence of transcription inhibitory factors within the SM or the lack/down-modulation of adhesion molecules able to anchor CD30\(^+\) cells to the inflamed tissue (29). Our mRNA data are different from those of other researchers who failed to detect CD30 transcript in PBL (30). We believe that this discrepancy is due to the far more sensitive method used in our study. Indeed, when the amplified product was evaluated on an ethidium bromide-stained agarose gel, it could be seen in most of the cases analyzed only after an amplification of 35 cycles. In addition, the subsequent hybridization with a CD30-radiolabeled probe always confirmed the presence of the specific transcript.

In the SF, CD30 was expressed by a subset of activated T cells, as demonstrated by the coexpression of CD45R0, CD25, HLA-DR, and CD69. This is in agreement with in vitro observations, which showed that high expression of CD30 is induced by activation on a subset of T cells that derives exclusively from CD45R0\(^+\) T cells and coexpresses the p55 IL-2R (CD25) (3).
The extent to which activation of synovial T cells occurs before entry, during the transendothelial migratory process, or following entry into the inflamed RA synovial tissue is not yet completely understood (31). Only a minority of RA SF T cells, which express the high affinity IL-2R, are oligoclonally activated via the TCR/CD3 complex (32–34). The majority of the T cells entering inflammatory foci, in fact, appears to be preactivated, and their activation is enhanced by contact with endothelium and by a cytokine-enriched synovial environment (22, 26, 27, 31). The present study has shown that CD30 can be induced on a T cell subset with strong migratory ability by contact with cytokine-activated endothelial cells. However, the coexpression of both CD25 and CD69 by synovial CD30+ T cells is against a simple nonspecific activation induced by endothelial contact, because the latter has been reported to be associated with the persistent expression of CD69, but not CD25 (27). In contrast, T cells activation through the TCR rapidly induces the expression of CD69, quickly followed by CD30 and maintaining CD69 expression. Moreover, our observations appear to support the concept that SF T cells positive for CD30 have been recently activated. Indeed, it has been previously reported that in skin blisters induced in vivo, PPD-induced DTH reactions, CD69/CD25 coexpressing T cells are about 50% of the entire infiltrating T cell population at 24 h, but only 10% at 96 h (26). Here, we have shown, using the same model, that >50% of blister T cells highly expressed the CD30 molecule at 24 h despite the presence of very few CD30+ T cells in the PB. Whether such high expression in vivo is driven by the migration process itself, the TCR-mediated activation in response to the recall Ag PPD, or the combination of the two still remains to be established. In addition, inflammatory cytokines may also contribute to CD30 induction, as reported for IL-15 (27). Indeed, although in our hands cytokine-rich RA SF did not induce in vitro CD30 expression, we think that a role for cytokines in vivo in contributing to the modulation of this molecule cannot be completely ruled out.

High levels of sCD30 have been also described in the serum of a number of other rheumatic conditions, and the involvement of CD30+ T cells in the pathogenetic mechanisms of these diseases has been suggested (35–39). However, the functional role exerted by CD30+ T cells in the course of these disorders is still unclear. There is in vitro evidence indicating that CD30 is preferentially expressed by Th2 cells, and some in vivo reports that support an association between CD30+ T cells and Th2-driven diseases (6, 7, 9, 11, 40–42). Thus, if CD30+ cells were involved in the rheumatoid process, this would be in apparent contrast with the widespread idea that the RA synovitis is a Th1-dominated disorder (31, 43–46). However, in RA synovitis a large number of cytokines is produced, and the degree of inflammation is probably the result of a balance between cytokines with pro- and anti-inflammatory properties (46). The fact that in RA serum sCD30 levels did not correlate with those of IFN-γ and IL-4, the hallmarks of, respectively, Th1 and Th2 responses (10), or with those of IL-10, a cytokine with strong anti-inflammatory activity mainly produced by Th2 cells (10, 46), prompted us to analyze the intracytoplasmic production of both IL-4 and IFN-γ in purified CD30+ fresh T cells from RA SF. Despite the fact that the large majority of synovial CD30+ T cells produced IFN-γ, similarly to the entire T cell population, these cells also produced IL-4 (Th0 type), in contrast to the predominance of Th1-type cells within the total T cell subset present in the RA SF. CD30 expression and the pattern of cytokine production were then evaluated in clones generated by IL-2-expanded T cells from the joint. The experiments were performed with low concentrations of rIL-2 to avoid alterations in cytokine synthesis (47–49). Furthermore, because cytokine production may vary according to the different stage and/or activity of the disease and may be influenced by therapy (31, 46, 50–52), our cloning experiments were performed in a patient with long-standing and in another subject with early, untreated, RA.

No pure Th2 clones, i.e., producing IL-4 only, were generated. In contrast, the great majority of them, particularly those from the SF of long-standing RA, produced high levels of IFN-γ. Although this would support the idea that RA synovitis is a Th1-driven condition (43–45), a consistent number of these clones also produced significant amounts of IL-4, putting them in the category of the Th0 phenotype. Similar results were obtained when the pattern of cytokine production of those clones expressing CD30 was analyzed. The majority of these clones, generated mainly from the SF (as expected from the rarity of CD30+ cells found in the PB), produced both IFN-γ and IL-4 (Th0 phenotype), similarly to fresh SF CD30+ T cells. Although the production of IFN-γ, a Th1 cytokine, by CD30+ T cells is not in keeping with a pure Th2 phenotype, this is not surprising, as similar observations have been reported after activation in vitro and in tuberculosis lung lesions in vivo (53, 54). Moreover, similarly to our findings in RA, the cytokine production of CD30+ T cells generated in response to Mycobacterium Ag, which recalls the CD30+ cells found in PPD-induced skin blisters, falls within the Th0 phenotype (54).

Considering the problem the other way around, i.e., the effects of different cytokines in regulating CD30 expression, there is in vitro evidence to suggest that the expression of the molecule is controlled by the balance between IL-4 and IFN-γ, namely, CD30 is up-regulated by the former and down-regulated by the latter (55). Our data from RA SF clones appear to support this concept, since IL-4 synthesis correlated well with CD30 expression in both early and long-standing RA. However, it is important to note that the IFN-γ/IL-4 ratio in T cell clones was lower in early than in late RA, perhaps indicating that as the disease progresses there is more of a shift toward a Th1 type of disease. Finally, the simultaneous synthesis of IFN-γ by IL-4-producing CD30+ clones may account for the relatively low density of CD30 surface expression, as suggested by published data (55). Thus, our findings argue against an exclusive correlation of CD30 expression with Th2 response, but confirm its dependence on IL-4, as previously demonstrated in both mice and humans (55, 56).

The role of IL-4 in the pathogenesis of the RA synovitis is controversial, mainly because of the very low/absent levels of both IL-4 and IL-4-producing cells within the inflamed synovium (31, 46). This finding is in line with our data showing the absence of CD30+ T cells in rheumatoid SM. Therefore, the hypothesis that IL-4 would exert a counter-regulatory activity in the attempt to balance the production of pro-inflammatory cytokines in the rheumatoid synovium is still under discussion (46). On the other hand, IL-4 has been shown to be protective in various experimental models of arthritis (46, 57–59). Such an effect is potentiated by IL-10, which is produced not only by macrophages, but also by T cells (47, 60–64), as confirmed at the clonal level in the present study. In this context it is interesting to emphasize the results of our experiments, which demonstrated that IL-4-producing CD30+ clones also produce high amounts of IL-10, particularly in early RA. We propose, therefore, that the high serum levels of sCD30 may represent a detectable marker of IL-4-producing activated T cells in the inflamed joint that are attempting to down-modulate inflammation, although this attempt may be hampered by the fact that the CD30+ T cells migrate into the SF, but are not retained in
In summary, we have provided data to support the hypothesis that a preactivated CD30-committed T cell subset, with high adhesion and migration ability, is recruited from the PB into the inflamed joint. Endothelial contact as well as other activation signals via the TCR/CD3 complex induce the expression of the CD30 molecule, which is then rapidly shed from the cell surface, causing inflammation in the joint. Endothelial contact as well as other activation signals may thus induce the expression of CD30 in the synovium, which is then rapidly shed from the cell surface, causing inflammation in the joint.

Acknowledgments

We thank Marusca Capanni (Department of Clinical and Experimental Medicine, Section of Hematology and Clinical Immunology, University of Perugia, Perugia, Italy) for technical assistance with cell sorting.

References


