

Original Article

Pathophysiological Functions of CD30⁺ CD4⁺ T Cells in Rheumatoid Arthritis

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High levels of soluble CD30 (sCD30) were detected in the serum and synovial fluid of patients with rheumatoid arthritis (RA), indicating the involvement of CD30⁺ T cells in the pathogenesis. We investigated the induction of CD30 and its functions in CD4⁺ T cells from patients with established RA (disease duration ≥ 2 years). CD4⁺ T cells from both the peripheral blood (PB) and synovial tissue (ST) of RA patients expressed surface CD30 when stimulated with anti-CD3 antibody (Ab) and anti-CD28 Ab, but their CD30 induction was slower and weaker compared with PB CD4⁺ T cells of healthy controls (HC). Immunohistochemical analysis showed that only a small proportion of lymphocytes expressed CD30 in the ST (-1%). RA PB CD4⁺ T cells, after recovery from 6-day stimulation with anti-CD3 Ab and anti-CD28 Ab, showed in intracellular cytokine staining that CD30⁺ T cells could produce more interleukin-4 (IL-4) but less interferon- γ . In the culture of RA PB CD4⁺ T cells with anti-CD3 Ab and anti-CD28 Ab, blocking anti-CD30 Ab similarly inhibited the cell proliferation and activation of nuclear factor- κ B on day 4 in RA and HC, but inhibited the apoptotic cell death on day 6 only in RA. These results indicate that despite high-level expression of sCD30, the anti-inflammatory activity of IL-4-producing CD30⁺ CD4⁺ T cells may be limited in the ST due to a poor induction of surface CD30 and a susceptibility to CD30-mediated cell death.

Key words: apoptosis, CD4⁺ T cells, CD30, interleukin-4 (IL-4), rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic inflammatory disease that primarily affects multiple synovial joints. The synovial membrane is characterized by massive infiltration with CD4⁺ T cells and macrophages, together with proliferation of synovial fibroblast-like cells. CD4⁺ T cell infiltrates are composed primarily of the Th1-phenotype that preferentially produces IFN- γ and IL-17 [1, 2]. These cytokines, in association with

surface molecules expressed on activated T cells, have been indicated to activate synovial macrophages, fibroblast-like cells, and osteoclasts, thereby contributing to both chronic inflammation and joint destruction [3].

CD30 is a member of the tumor necrosis factor (TNF)/nerve growth factor receptor superfamily [4]. The CD30 antigen (Ki-1) was originally identified as a surface marker for Hodgkin and Reed-Sternberg cells in Hodgkin's disease [5]. However, this molecule is normally expressed on a small subpopulation of activated CD45RO⁺ memory T cells [6], and regulates their proliferation, differentiation, and apoptotic cell death [7].

Interestingly, CD30 expression has been associated with Th2-type cytokine responses. Human Th2- and Th0-type T cell clones consistently express high levels of CD30 [8–10]. CD30 costimulation promotes the proliferation and cytokine secretion of Th2 and Th0 clones and the development of Th2 cells [11]. Surface expression of CD30 on T cells is upregulated by interleukin-4 (IL-4), but downregulated by interferon- γ (IFN- γ) [12].

The soluble form of CD30 (sCD30) is released after activation from the cell surface by proteolytical cleavage [13]. Elevated serum or plasma levels of sCD30 have been found not only in Th2-dominated disease conditions such as Omenn's syndrome, a congenital Th2-mediated immunodeficiency disease [14], allergic disorders [15–18], and systemic sclerosis [19, 20], but also in Th1-dominated inflammatory diseases such as tuberculosis [21] and Wegener's granulomatosis [22].

High levels of sCD30 have also been detected in the peripheral blood (PB) and synovial fluid (SF) of patients with active rheumatoid arthritis (RA) [23]. In early RA, sCD30 elevation indicates a good response to disease-modifying antirheumatic drug (DMARD) therapy [24, 25]. CD30⁺ T cells capable of producing IL-4 and IL-10 in combination with IFN- γ , thus classified as Th0 cells, are found at significant levels in the SF compartment, and the ratio of their IFN- γ to IL-4 synthesis is lower in early than in late disease [25]. These findings suggest that

IL-4-producing CD30⁺ T cells may play a counter-regulatory role during disease progression in early RA.

This regulatory activity of CD30⁺ T cells may be restricted in late disease because IL-4 expression is absent or extremely low in chronically inflamed joints [26, 27]. To analyze the induction of CD30⁺ CD4⁺ T cells and their functions in patients with late RA, we investigated the induction of surface CD30 molecule on CD4⁺ T cells from the PB and ST of RA patients with a disease duration of ≥ 2 years when stimulated with anti-CD3 antibody (Ab) and anti-CD28 Ab; the infiltration of CD30⁺ lymphocytes in the synovial tissue (ST); IL-4 production by PB CD30⁺ CD4⁺ T cells; and the effects of CD30-mediated signaling on proliferation, apoptotic cell death, and the activation of nuclear factor κ B (NF- κ B) in PB CD4⁺ T cells.

Materials and Methods

Patients and samples. Serum samples were obtained from 61 patients with RA (52 women and 9 men), 13 patients with osteoarthritis (OA), and 15 healthy controls (HC). Serum and SF samples were stocked at -30°C until the sCD30 measurement. Study patients were diagnosed according to the American College of Rheumatology (formally, the American Rheumatism Association) criteria for the classification of RA

Table 1 Profile of patients with rheumatoid arthritis (RA) and osteoarthritis (OA) and healthy subjects

Subjects	sCD30 determination			Functional studies of CD30 ⁺ CD4 ⁺ T cells	
	RA patients	OA patients	HC	RA patients	HC
Number (female/male)	61 (52/9)	13 (13/0)	15 (5/10)	50 (36/14)	19 (7/12)
Age (range)	60.0 \pm 11.2 years (20–82 years)	68.2 \pm 13.0 years (51–84 years)	40.1 \pm 1.4 years (38–42 years)	61.8 \pm 10.6 years (36–80 years)	48.4 \pm 3.3 years (44–57 years)
Disease duration (range)	8.2 \pm 5.8 years (2–31 years)	n.a.	n.a.	10.5 \pm 6.8 years (2–33 years)	n.a.
CRP	41.9 \pm 40.0 mg/l	n.a.	n.a.	23.3 \pm 21.2 mg/l	n.a.
RF	142 \pm 158 U/ml	n.a.	n.a.	258 \pm 766 U/ml	n.a.
Number of Prednisolone users (female/male)	40 (32/8)	1 (1/0)	0	37 (31/6)	0
Number of DMARDs users (female/male)	57 (50/7)	0	0	47 (35/12)	0

CRP, C-reactive protein; DMARDs, disease-modifying antirheumatic drug; na, not applicable; RF, IgM class rheumatoid factor. Values are expressed as the mean \pm SD.

[28] and OA [29]. Their clinical profiles are provided in Table 1. SF samples were also aspirated from the knee joint during therapeutic arthrocentesis in 40 RA patients and 13 OA patients. Most RA patients were receiving DMARD and ≤ 5 mg/day of prednisolone.

Both PB and ST samples from 6 RA patients and PB samples from 7 additional RA patients and 7 HC were examined for the induction of CD30 expression on CD4⁺ T cells; ST samples from 6 patients for immunohistochemical staining and mRNA detection for CD30; PB samples from 9 patients for IL-4 production by CD30⁺ CD4⁺ T cells; and PB samples from 22 RA patients and 13 HC for the effects of CD30 costimulation on CD4⁺ T cell functions. ST samples were obtained at the time of surgical treatment. Their profiles are provided in Table 1. All patients and HC gave informed consent.

Immunoassay for sCD30. The concentrations of sCD30 were measured in duplicate with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (DAKO, Glostrup, Denmark). The detection limit was 5 U/ml.

Isolation and culture of CD4⁺ T cells. PB mononuclear cells were prepared from heparinized blood samples by Ficoll-Hypaque density gradient centrifugation. ST mononuclear cells were prepared, as previously described [30]. Fresh ST samples were fragmented and digested with collagenase and DNase for 1 h at 37 °C. After removing tissue debris, cells were washed well with RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA). PB and ST cells were resuspended in complete medium: RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 25 mM HEPES (Life Technologies), 100 IU/ml penicillin, and 100 μ g/ml streptomycin. CD4⁺ T cells were purified by positive selection using anti-CD4 monoclonal Ab (mAb)-coated magnetic beads (Dynabeads M-450 CD4; Dynal, Oslo, Norway) and detaching solution (DETACHaBEAD CD4/CD8; Dynal) according to the manufacturer's instructions. The resultant CD4⁺ T cell populations were dispensed into the wells of 24-well microtiter plates (Coster, Cambridge, MA, USA), which were coated with 10 μ g/ml anti-CD3 mAb (Immunotech, Marseille, France) [1], at a density of 4×10^5 cells/ml in 0.5 ml of complete medium with 1 μ g/ml anti-CD28 mAb (Immunotech). The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were harvested 2, 4, and 6 days later and measured for CD30 expression by flow cytometry.

Flow cytometry. The surface expression of CD30 on CD4⁺ T cells was analyzed by direct staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD30 mAb (BerH8; Becton Dickinson, Franklin Lakes, NJ, USA) and phycoerythrin (PE)-conjugated anti-CD4 mAb (SK3; Becton Dickinson). Cells were incubated with saturating concentrations of FITC-anti-CD30 mAb, PE-anti-CD4 mAb, and FITC- and PE-isotype-matched control mAb (PharMingen), washed well with phosphate-buffered saline (PBS), and resuspended in PBS/1% FCS. Analysis of cell surface expression of CD4 and CD30 was performed using a FACScan cytometer (Becton Dickinson).

Isolation of mRNA and reverse transcriptase-polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from fresh ST cells using an RNA isolation kit (RNeasy Midi kit; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The mRNA expression of CD30 and CD30 ligand (CD30L; CD153) was detected by RT-PCR, as previously described [31]. Complementary DNA (cDNA) was synthesized from total RNA with Molony murine leukemia virus reverse transcriptase (US Biochemical, Cleveland, OH, USA) and oligo-(dT)15 primers (Promega, Madison, WI, USA). Samples of cDNA were PCR-amplified for 25 cycles for β -actin and 30 cycles for CD30 and CD30L with *rTaq* DNA polymerase (Promega) and specific primers in a thermal cycler (iCycler™, Bio-Rad Laboratories, Hercules, CA, USA). Each cycle consisted of 1 min of denaturation at 95 °C, 1 min of annealing at 60 °C for CD30, or at 65 °C for β -actin and CD30L, and 1 min of extension at 72 °C. PCR products were electrophoresed on 1.0% agarose gel and visualized by ethidium bromide staining. The sequences of oligonucleotide primers were as follows: for β -actin 5'primer GTGGGGCGCCCCAGGCACCA and 3'primer CTCCTTAATGTCACGCACGATTTTC; for CD30 5'primer CCTACCCAATCTGTGCAGCAG, 3'primer GGAATCCACAAGCTCTAGC; and for CD30L 5'primer CTCCTGGAGACACAGCC, 3' primer GGTGCTTGTATCTATGTACT.

Immunohistochemistry. The expression of CD30 protein in ST samples was detected by immunohistochemical staining with anti-CD30 mAb (BerH2; NeoMarkers, Union City, CA, USA). Next, 4- μ m sections were prepared from paraffin-embedded ST blocks. Deparaffinized tissue sections were boiled in 1 mM EDTA, pH 8.0, for 10 min, and then blocked with

10% normal goat serum (Nichirei, Tokyo, Japan) for 30 min. Sections were incubated with 2.5 $\mu\text{g}/\text{ml}$ anti-CD30 mAb or control mAb for 30 min, followed by 30-min incubation with biotinylated goat anti-mouse IgG (Vector, Burlingame, CA, USA). After the addition of avidin-biotin-alkaline phosphatase complex (Vector), slides were developed with 3 amino-ethylcarbazol substrate solution (Nichirei). Finally, tissue sections were counterstained with hematoxylin.

Intracellular cytokine staining. PB CD4⁺ T cells were harvested after stimulation with anti-CD3 mAb and anti-CD28 mAb for 6 days, and washed well with RPMI-1640 medium. The production of IL-4 and IFN- γ by CD4⁺ T cells was detected by intracellular cytokine staining, as previously described [1]. The cells were incubated with 50 ng/ml phorbol myristate acetate (PMA; Sigma, St. Louis, MO, USA) and 1 $\mu\text{g}/\text{ml}$ calcium ionophore A23187 (Wako Pure Chemical) in the presence of 10 $\mu\text{g}/\text{ml}$ brefeldin A (Sigma) for 4 h. Cells were stained with FITC-anti-CD30 mAb, peridium chlorophil protein (PerCP)-anti-CD4 mAb (Becton Dickinson), and FITC- and PerCP-control mAb (PharMingen), followed by fixation with 1% paraformaldehyde. Cells were then stained with PE-anti-IFN- γ mAb (4S.B3; PharMingen), PE-anti-IL-4 mAb (8D4-8; PharMingen), and PE-control mAb (PharMingen) for 30 min. After being washed well with PBS/ 1% FCS /0.5% saponin and then with PBS/1% FCS, cells were resuspended in PBS/1% FCS for flow cytometry.

Electrophoretic mobility shift assay (EMSA) for NF- κ B. For studying the effect of anti-CD30 mAb (BerH8; PharMingen) on NF- κ B activation in CD4⁺ T cells, PB CD4⁺ T cells were incubated in 24-well microtiter plates at a density of 4×10^5 cells/ml in complete medium with immobilized anti-CD3 mAb and anti-CD28 mAb with 20 $\mu\text{g}/\text{ml}$ anti-CD30 mAb or control mAb (107.3; Becton Dickinson), and cells were harvested 4 and 6 days later.

Nuclear extracts were prepared by the mini-extraction procedure and EMSA was performed, as previously described [32]. The nuclear protein supernatant was harvested by centrifugation. Double-stranded oligonucleotides for NF- κ B were labeled with [α -³²P] dATP (Amersham, Little Chalfont, UK) with Klenow fragment (Takara, Tokyo, Japan); the sequences were as follows: 5' GATCCGAGGGGACTTTCCGCTGGGGACTTTC CAGG, 3' GATCCCTGGAAAGTCCCCAGCGGAA AGTCCCCTAG. The binding reaction was performed,

and samples were electrophoresed on 5% polyacrylamide gel, followed by autoradiography. To verify the specificity of NF- κ B protein binding, unlabeled competitor oligonucleotides and 2.0 μg of rabbit polyclonal anti-NF- κ B p65 Ab (H-286; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the binding reaction and incubated on ice for 30 min prior to addition of the probe.

Proliferation assay. PB CD4⁺ T cells (4×10^4 cells/100 μl) were incubated in triplicate in 96-well microtiter plates with anti-CD3 mAb and anti-CD28 mAb with anti-CD30 mAb (BerH8) or control mAb. After 4 and 6 days of culture with a final 16-h incubation with 1 μCi ³H-thymidine (Amersham), cells were harvested onto glass-fiber filters. The incorporation of radioactivity was measured in a liquid scintillation counter.

Analysis of apoptotic cell death. PB CD4⁺ T cells (2×10^5 cells/0.5 ml) were incubated in 24-well microtiter plates with anti-CD3 mAb and anti-CD28 mAb with blocking anti-CD30 mAb or control mAb, and harvested 6 days later. Apoptosis was measured by flow cytometry with PE-conjugated Annexin V (PharMingen) and 7-amino-actinomycin D (7-AAD; PharMingen) staining, as described previously [33, 34]. Cells were washed with PBS, and incubated for 15 min at a density of 1×10^6 cells/ml in 100 μl of Annexin V labeling solution (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) with 7-AAD. The test volume was made up to 500 μl with incubation buffer, and analyzed by flow cytometry. Annexin V + 7-AAD-cells were considered apoptotic. The apoptotic effects of CD30 were expressed as the CD30-inducing rate (%) = [apoptotic cells with control mAb (%) - apoptotic cells with anti-CD30 mAb (%)] / [apoptotic cells with control mAb (%)] $\times 100$.

Statistical analysis. Data were expressed as the mean value \pm SD of the number of samples indicated. The statistical significance of differences between the 2 groups was determined by the Mann-Whitney *U* test or the Wilcoxon signed rank test. The correlation coefficient was analyzed by Spearman's rank correlation. *P* values less than 0.05 were defined as significant.

Results

High levels of sCD30 in active RA. The sCD30 levels in serum ($n = 61$) and SF ($n = 40$) of patients with late RA (disease duration ≥ 2 years) were

compared by ELISA with the serum and SF levels of patients with OA (n = 13) and the serum levels of HC (n = 15). The serum levels of sCD30 were significantly higher in RA patients (mean \pm SD; 68 ± 93 U/ml) than in OA patients (5 ± 7 U/ml) and HC (6 ± 10 U/ml) (Fig. 1). SF levels of sCD30 were markedly elevated in RA patients (142 ± 207 U/ml) than in OA patients (11 ± 29 U/ml). In paired RA samples, sCD30 levels were always increased in the SF compared with the serum (142 ± 207 U/ml versus 68 ± 93 U/ml), with a positive correlation between their levels (n = 40, r = 0.460, $P < 0.005$). In addition, serum sCD30 levels had a positive correlation with both C-reactive protein (CRP) values (r = 0.458, $P < 0.05$) and IgM class rheumatoid factor (RF) titers (r = 0.465, $P < 0.001$).

CD30 expression on activated CD4⁺ T cells in RA. Purified CD4⁺ T cells from the PB (n = 13) and ST (n = 6) of RA patients and the PB of HC (n = 7) were stimulated with immobilized anti-CD3 Ab and anti-CD28 Ab, and the mean fluorescence intensity (MFI) of CD30 expression on days 2, 4, and 6 of culture was measured by flow cytometry. The expression of surface CD30 was negligible on freshly isolated CD4⁺ cells, but was significantly induced after stimulation with anti-CD3 Ab and anti-CD28 Ab. There was a marked difference in the kinetics of CD30 induction between RA and HC, although the CD30 expression levels varied among individuals. In HC PB CD4⁺ T cells, surface CD30 induction was evident on day 2 (mean MFI ratio; 1.9 ± 0.6), rapidly peaked on day 4 (6.6 ± 6.8), and declined by day 6 (2.1 ± 1.0) (Fig. 2). In contrast, CD30 expression was slowly induced through day 6 in both RA PB and ST CD4⁺ T cells (day 2, 1.3 ± 0.4 and 1.2 ± 0.3 ; day 4, 2.8 ± 2.5 and 1.8 ± 0.8 ; day 6, 3.4 ± 3.7 and 2.1 ± 1.2 , respectively). Accordingly, the peak mean level of CD30 expression was much lower in RA than in HC CD4⁺ T cells.

CD30⁺ lymphocytes in RA ST. The levels of mRNA expression for both CD30 and CD30L were all detectable by RT-PCR in ST sections from 3 RA patients (Fig. 3a). By immunohistochemical staining, CD30-positive cells with cytoplasmic staining were distributed in the sublining layer, mostly at the periphery of lymphoid aggregates (Fig. 3b). Histological analysis of ST sections from 3 different RA patients showed that ST lymphocytes contained ~1.0% of CD30⁺ cells. In OA ST, lymphocyte infiltrates were rarely found with no CD30 staining (data not shown).

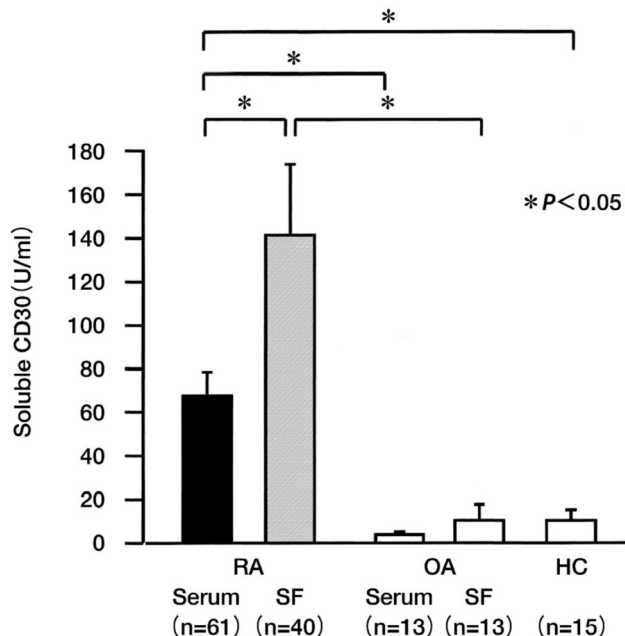


Fig. 1 Levels of soluble CD30 (sCD30) in rheumatoid arthritis (RA), osteoarthritis (OA), and healthy controls (HC), as determined by enzyme-linked immunosorbent assay. Serum and synovial fluid (SF) samples were obtained from patients with RA (disease duration \geq 2 years) and OA, and serum samples from healthy controls (HC). Values are the mean \pm SEM. n, number of subjects.

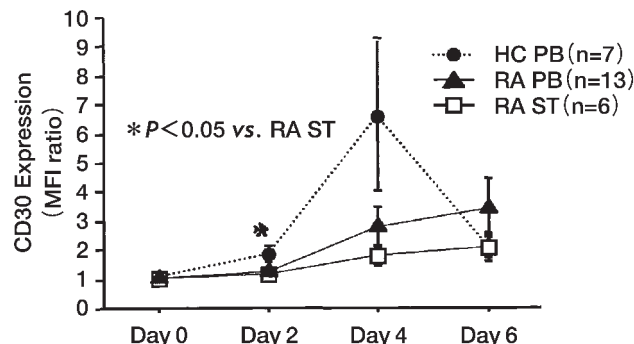


Fig. 2 Kinetics of surface CD30 induction on RA peripheral blood (PB) and synovial tissue (ST) CD4⁺ T cells and HC PB CD4⁺ T cells. Purified CD4⁺ T cells were incubated for 2, 4, and 6 days with immobilized anti-CD3 Ab and anti-CD28 Ab, and CD30 expression was measured by flow cytometry. Data are expressed as the mean \pm SEM of the mean fluorescence intensity (MFI) ratio of CD30 antigen divided by the MFI of isotype-matched control samples. n, number of studied subjects.

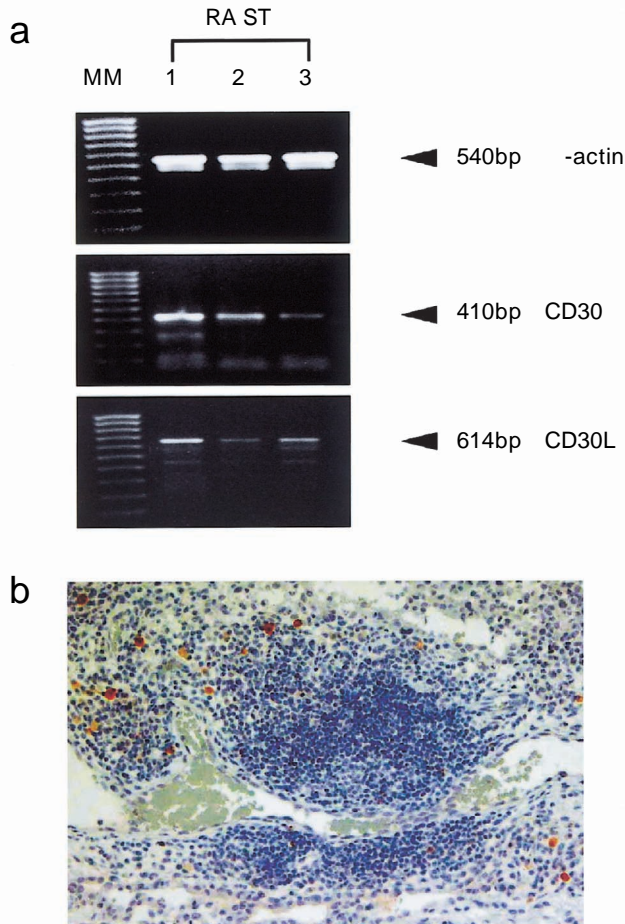


Fig. 3 (a) mRNA expression of CD30 and CD30 ligand (CD30L) in RA ST, determined by reverse-transcriptase-polymerase chain reaction. MM, molecular weight markers. (b) Immunohistological localization of CD30-expressing cells in RA ST. No isotype-matched control mAb staining was observed. CD30-positive cells were distributed in at the periphery of lymphoid aggregates.

IL-4 production by activated CD30⁺ CD4⁺ T cells in RA. PB CD4⁺ T cells from RA patients (n = 9) and HC (n = 4) were recovered after 6-day stimulation with anti-CD3 Ab and anti-CD28 Ab for analysis of intracellular cytokine staining. The frequency of CD30⁺ cells was decreased to a certain extent after activation with PMA and A23187, presumably due to the shedding of surface CD30. As shown in a representative experiment (Fig. 4a), RA CD30⁻ cells predominantly produced high levels of IFN- γ , but CD30⁺ cells produced low levels of both IL-4 and IFN- γ . The mean MFI ratio of surface CD30 expression was significantly greater in IL-4-producing cells (3.4 ± 2.7) than in IFN- γ -producing cells

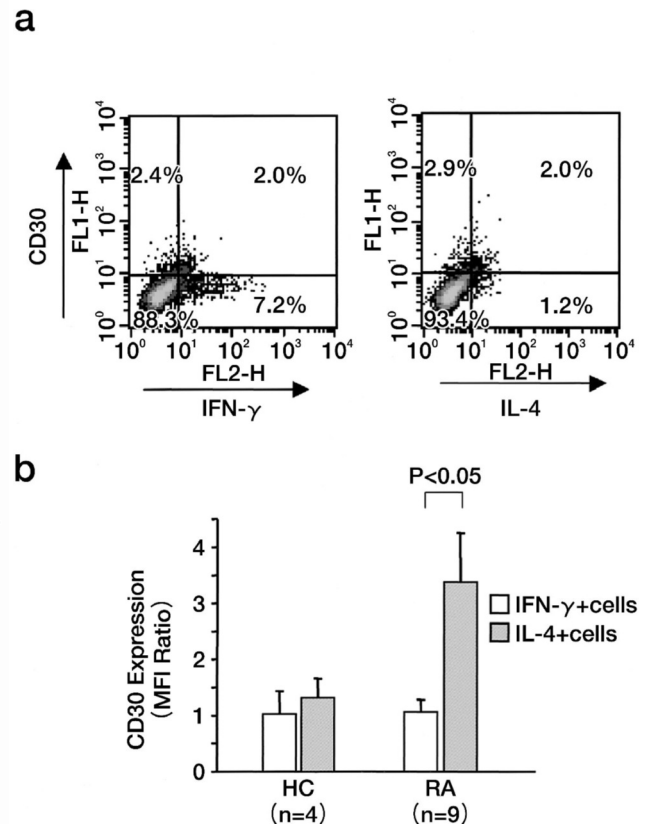


Fig. 4 IL-4 production by RA CD30⁺ CD4⁺ T cells. PB CD4⁺ T cells after stimulation with anti-CD3 Ab and anti-CD28 Ab for 6 days were determined for IFN- γ , IL-4, and CD30 production by intracellular cytokines and flow cytometry. (a) A representative result is shown. (b) The intensity of CD30 expression by RA PB CD4⁺ T cells producing IFN- γ or IL-4. Data are expressed as the mean \pm SEM of the MFI ratio of CD30 antigen divided by the MFI of control samples. n, number of studied subjects.

(1.0 ± 0.7) in RA. However, in HC, there was no difference in the MFI of CD30 expression between IL-4- and IFN- γ -producing cells (1.3 ± 0.7 vs. 1.0 ± 0.8) (Fig. 4b).

NF- κ B activation by CD30 costimulation in RA CD4⁺ T cells. The levels of NF- κ B activation in both RA and HC PB CD4⁺ T cells after stimulation with anti-CD3 Ab and anti-CD28 Ab were significantly detectable on day 4 but not on day 6. These activities were similarly inhibited in the presence of anti-CD30 Ab in RA and HC (Fig. 5).

CD30-induced proliferation and apoptosis in RA CD4⁺ T cells. PB CD4⁺ T cells from RA patients and HC were examined for the effects of blocking

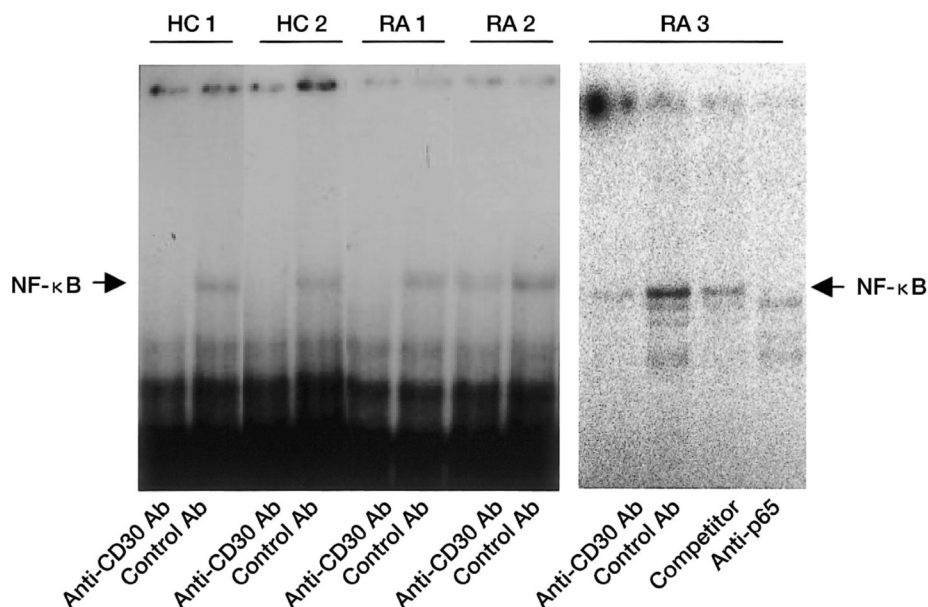


Fig. 5 Inhibition of NF- κ B activation by anti-CD30 Ab in RA PB CD4⁺ T cells. PB CD4⁺ T cells after stimulation with anti-CD3 Ab and anti-CD28 Ab with blocking anti-CD30 Ab or control Ab for 4 days were determined for NF- κ B activity by electrophoretic mobility shift assay. The specificity of NF- κ B protein binding was verified by anti-NF- κ B p65 Ab inhibition experiments.

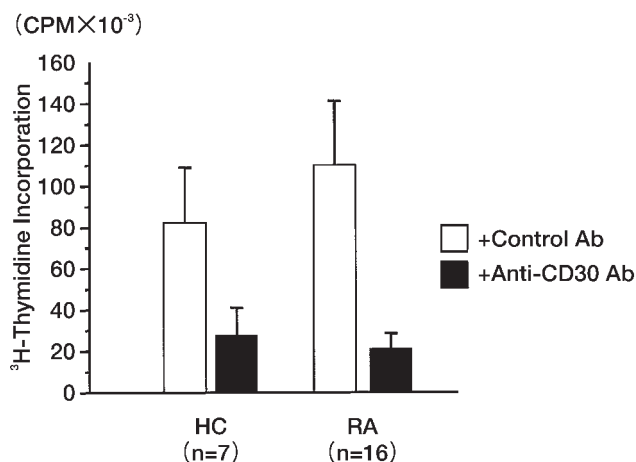


Fig. 6 Inhibition of proliferation of RA PB CD4⁺ T cells by anti-CD30 Ab. PB CD4⁺ T cells after stimulation with anti-CD3 Ab and anti-CD28 Ab with blocking anti-CD30 Ab or control Ab for 4 days were determined for ³H-thymidine incorporation. Values are the mean \pm SEM.

anti-CD30 Ab on their proliferation and apoptosis. The proliferative response of CD4⁺ T cells was found significantly on day 4, but only weakly on day 6. The proliferation was similarly inhibited by anti-CD30 Ab in RA and HC (Fig. 6). In contrast, the apoptotic response was observed not on day 4, but on day 6. Apoptotic cell death of CD4⁺ T cells was markedly decreased by the addition of anti-CD30 Ab in RA (22.6 \pm 18.9%), but not in HC (0.4 \pm 0.6%) (Fig. 7).

Discussion

CD30⁺ T cells have been proposed to play a counter-regulatory role during disease progression in early RA through their elaboration of anti-inflammatory cytokines such as IL-4 and IL-10 [35]. However, as previously reported [23], the levels of sCD30 were also increased in both serum and SF of patients with late RA (disease duration \geq 2 years), with the SF levels being higher than the serum levels. There was a positive correlation of serum sCD30 levels with CRP values and RF titers. These findings suggest that CD30⁺ T cells may be persistently induced in the chronically inflamed joints of RA, despite the Th1 predominance.

In the present study, the induction of CD30⁺ CD4⁺

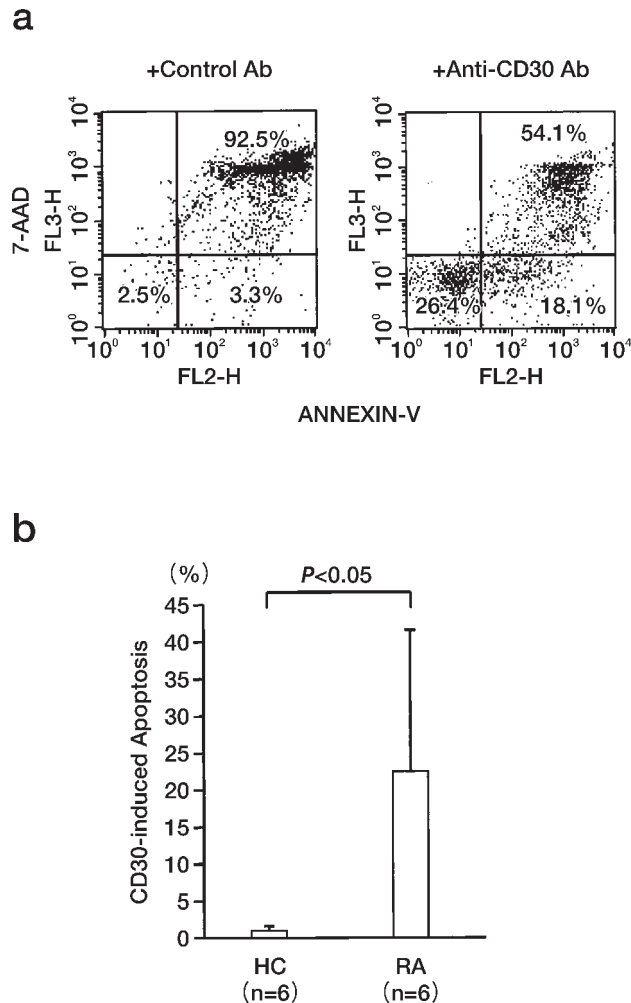


Fig. 7 Inhibition of apoptotic cell death of RA PB CD4⁺ T cells by anti-CD30 Ab. PB CD4⁺ T cells after stimulation with anti-CD3 Ab and anti-CD28 Ab with blocking anti-CD30 Ab or control Ab for 6 days were measured for cell death by Annexin V and 7-amino-actinomycin D staining (a) Representative data of flow cytometric analysis are shown. (b) The apoptotic effect of CD30 stimulation is expressed as the CD30-inducing rate (%). Values are the mean \pm SD.

T cells and their functions in patients with late RA were determined. We have demonstrated that surface CD30 is induced on both RA PB and ST CD4⁺ T cells after stimulation with anti-CD3 Ab and anti-CD28 Ab, but that the CD30 expression of these cells was slower and weaker when compared with PB CD4⁺ T cells of HC. By immunohistochemistry, it was found that only a small proportion of lymphocytes expressed CD30 in the ST. PB CD30⁺ CD4⁺ T cells of RA were able to predominantly produce IL-4, but were sensitive to CD30-

mediated apoptosis. It is therefore likely that the anti-inflammatory activity of CD30⁺ CD4⁺ T cells is restricted in the ST lesion by their poor CD30 expression and their susceptibility to CD30-induced cell death.

Like PB CD4⁺ T cells, freshly isolated ST CD4⁺ T cells expressed negligible levels of surface CD30. CD30 expression on T cells is dependent on cell activation, but CD3-mediated signaling alone is not sufficient. For the optimal expression of CD30, T cells require either CD28 costimulation or cytokines such as IL-2 and IL-4 [6, 12, 36]. Because CD28 ligands such as CD80 and CD86 are substantially expressed in RA ST [37–39], while both IL-2 and IL-4 are absent or at very low levels [26, 40, 41], we examined the kinetics of CD30 induction on CD4⁺ T cells after stimulation with anti-CD3 Ab and anti-CD28 Ab. The kinetics profiles of RA CD4⁺ T cells revealed their diminished CD30 expression. Interestingly, PB CD4⁺ T cells from early RA (disease duration \leq 6 months) showed the same kinetics as HC CD4⁺ T cells (data not shown). These findings suggest that CD30 induction on CD4⁺ T cells may be impaired during disease progression, according to the establishment of Th1 predominance.

High levels of sCD30 were found to be present in RA joints, whereas surface CD30 expression on activated ST CD4⁺ T cells was poor and the frequency of CD30-expressing lymphocytes was very limited in the ST (\sim 1%). Correspondingly, previous studies have shown that CD30⁺ T cells are detectable in the SF, but not in the ST [25], and that IL-2-expanded T cell lines from the ST express less CD30 than T cell lines from rheumatoid nodules [42]. Therefore, increased SF levels of sCD30 may represent CD30⁺ T cell activation in the SF compartment. However, given an abundance of CD4⁺ T cells in the ST, the persistency of a low level of CD30 induction in the inflamed ST lesion could be associated with sCD30 elevation in late, active RA because surface-expressed CD30 is quickly lost after activation due to the shedding [10].

CD30⁺ CD4⁺ T cells from late RA could produce both IL-4 and IFN- γ , in support of their anti-inflammatory role in the disease. Because IL-4 can potentially inhibit inflammatory cytokine production and synovial fibroblast proliferation [43], the presence of CD30⁺ lymphocytes in the ST may represent a homeostatic response to chronic inflammation and Th1 responses. However, their regulatory activity may be extremely limited, as judged from the paucity of both IL-4 and

CD30⁺ T cells at inflammatory sites [25, 26]. In this regard, monomeric sCD30 has recently been shown to bind CD30L with high affinity and to block transmembrane signaling by CD30, suggesting that high concentrations of sCD30 also may be involved in the inhibition of CD30⁺ T cell activation within RA joints [44].

CD30-mediated signaling induces the activation of NF- κ B through adaptor proteins such as TNF receptor-associated factor-2 (TRAF2) [45], and this NF- κ B activation is involved in various T cell functions such as their proliferation, differentiation, and apoptosis [7]. The activation of NF- κ B in CD4⁺ T cells after stimulation with anti-CD3 Ab and anti-CD28 Ab is thought to be induced by CD30 and CD30L interactions because CD30L is significantly induced on activated CD4⁺ T cells [46], and because this NF- κ B activation is reduced by anti-CD30 Ab.

A similar inhibition of both NF- κ B activation and proliferation of CD4⁺ T cells by anti-CD30 Ab was found in RA and HC. However, inhibition of apoptotic cell death by anti-CD30 Ab was observed in RA but not in HC CD4⁺ T cells. CD30 can induce cell death under some circumstances, although it does not possess the death domain [47–49]. It has been demonstrated that rapid depletion of TRAF1 and TRAF2 proteins during CD30 signaling not only limits its own ability to transduce cell survival signals, but also increases the sensitivity of lymphocytes to undergo apoptosis through activation of death-inducing receptors such as the type 1 TNF receptor (TNFR1) [50]. There is thus a possibility that other apoptotic signals such as the TNF-TNFR1 interaction may be induced by activated CD4⁺ T cells from RA but not HC. In any event, CD30-mediated apoptosis could facilitate removal of CD30⁺ T cells from the inflammatory site.

Our findings indicate that CD30⁺ CD4⁺ T cells can produce IL-4 in late RA, but that their anti-inflammatory activity may be limited in the ST due to a poor induction of surface CD30 and a susceptibility to CD30-mediated cell death, as well as high concentrations of sCD30, an *in vivo* inhibitor of the CD30-CD30L interaction. Such down-regulation of CD30⁺ T cell activity might be associated with the Th1 predominance in late, active RA.

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