CD4+ PD-1+ T Cells Accumulate as Unique Anergic Cells in Rheumatoid Arthritis Synovial Fluid

SAORI HATACHI, YOSHIKO IWAI, SEIJI KAWANO, SAHOKO MORINOBU, MASAYA KOBAYASHI, MASAHIRO KOSHIBA, RYUICHI SAURA, MASAHIRO KUROSAKA, TASUKU HONJO, and SHUNICHI KUMAGAI

ABSTRACT. ***Objective.*** The PD-1 receptor, whose deficiency in mice causes autoimmune diseases such as arthritis, is considered to be a negative regulator of activated T cells and to play a crucial role in peripheral tolerance. To clarify the involvement of the PD-1 system in rheumatoid arthritis (RA), we investigated PD-1 expression on synovial fluid (SF) T cells from patients with RA.

Methods. FACS analysis for PD-1 was performed on SF T cells from 44 patients with RAand 6 with osteoarthritis (OA), and also on peripheral blood (PB) T cells from 12 RA patients and 7 healthy controls. Two-color analysis of cell surface PD-1 expression and the intracellular concentration of cytokine production was used to investigate CD4+ T cells from SF of patients with RAand PB from controls.

Results. Scarcely any PD-1 expression was detected on control PB T or OASF T cells. In contrast, PD-1+ cells made up 20.9 ± 8.6% (mean ± SD) of RA SF T cells. In RA SF, PD-1 was expressed more predominantly on CD4+ T cells than on CD8+ T cells. As well as expressing CD45RO and CXCR3, CD4+ PD-1+ T cells were mostly CTLA-4 positive and CD26 negative, and were enriched in CD45RB^{low} cells. Intracellular cytokine staining revealed that CD4+ PD-1+, but not CD4+ PD-1–, T cells produced interleukin 10 (IL-10), and that CD4+ PD-1+ T cells produced less IL-2 than CD4+ PD-1– T cells.

Conclusion. PD-1+ T cells in RA SF are enriched, and phenotypic analysis suggests that these cells constitute a unique anergic T cell subset in RASF. (J Rheumatol 2003;30:1410–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS SYNOVIALFLUID T CELLS FLOW CYTOMETRY PD-1

Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis characterized by marked hyperplasia of synovial lining cells, neovascularization, and intense infiltration of mononuclear cells, predominantly CD4+ T cells, into the synovium[1,2]. Recently, it has been proposed that RA consists of 3 separate, albeit interrelated, processes: disease initiation, perpetuation, and terminal destruction[3]. T cells are thought to participate mainly in the perpetuation process of RA[4]. Synovial tissue and synovial fluid (SF) cells from patients with RA have been found to be enriched with memory (CD45RA–, CD45RO+, CD11a^{high}, CD44^{high}) and activated (CD69+) T cells. Moreover, these cells are enriched in differentiated CD4+ CD45RB^{low} T cells, a subset of mature memory T cells that develop after prolonged antigenic stimulation and that have enhanced capacity to provide help for B cells[5].

PD-1 was originally identified by Ishida, *et al* as a molecule linked to *in vitro* induction of apoptotic cell death in murine lymphoid cell lines[6]. Recently, PD-1 has been regarded as a novel member of the CD28 family because, in the extracellular domain, the PD-1 molecule has 24% amino acid homology to CTLA-4. PD-1 delivers a negative signal by recruitment of SHP-2 to the phosphorylated tyrosine residue in the cytoplasmic region[7]. PD-1$^{-/-}$ mice with a C57BL/6 background develop lupus-like glomerulonephritis and arthritis as they age[8], while those with a BALB/c background develop fatal autoimmune dilated cardiomyopathy[9]. In 2C × PD-1$^{-/-}$ mice, autoreactive T cells expressing activated memory markers (CD45RB–, CD62L–) were found to have infiltrated into epidermis without undergoing negative selection in thymus[8]. These reports suggest that the PD-1 molecule negatively regulates immune responses, and may play a critical role in the establishment and/or maintenance of peripheral tolerance and autoimmune diseases[6,8,10,11]. We describe infiltration of PD-1+ T cells in the synovia of RA patients and analyze the properties of PD-1+ T cells.

MATERIALS AND METHODS

Subjects. Fresh SF cells were isolated from 44 patients with RA(mean age

From the Department of Clinical Pathology and Immunology, Kobe University Graduate School of Medicine, Kobe, Japan.

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S. Hatachi, MD; S. Kawano, MD, PhD; S. Morinobu, MD; M. Kobayashi, MS; M. Koshiba, MD, PhD; S. Kumagai, MD, PhD, Department of Clinical Pathology and Immunology, Kobe University Graduate School of Medicine; Y. Iwai, MD; T. Honjo, MD, PhD, Department of Medical Chemistry, Graduate School of Medicine, Kyoto University, Kyoto; R. Saura, MD, PhD; M. Kurosaka, MD, PhD, Department of Orthopedics, Kobe University Graduate School of Medicine.

Address reprint requests to Dr. S. Kumagai, Department of Clinical Pathology and Immunology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. E-mail: kumagais@kobe-u.ac.jp

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57.8 ± 12.0 yrs, range 27–87) and 6 with OA (mean age 64.5 ± 17.4 yrs, range 40–81) undergoing therapeutic joint fluid aspiration, while fresh peripheral blood (PB) cells were isolated from 12 patients with RA by venipuncture. Diagnosis of RAwas based on the revised criteria established by the American College of Rheumatology in 1987[12]. At the time of this study, all patients were orally taking nonsteroidal antiinflammatory drugs, 43 disease modifying antirheumatic drugs, and 27 prednisolone. Blood was also donated by 7 healthy controls (4 men and 3 women, mean age 30.1 ± 4.1 yrs, range 27–40) and 12 patients with RA(all women, mean age 54.6 ± 12.8 yrs, range 27–74). Fresh synovial tissue cells were isolated from 5 RApatients who were undergoing therapeutic surgery of the knee joints (4 women, one man, mean age 64.4 ± 13.3 yrs, range 41–74).

Sample preparation. SF cells were collected in the presence of heparin and EDTA-2Na, washed twice with PBS buffer, and passed through a nylon mesh. PB mononuclear cells (PBMC) and SFMC were separated from the heparinized PB or SF cells by Ficoll-Paque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). CD4+ T cells, CD8+ T cells, and CD19+ B cells were purified from PBMC and SFMC by positive selection using magnetic beads. Briefly, for separation of CD4+ T, CD8+ T, and CD19+ B cells, PBMC and SFMC were enriched for lymphocytes by removal of cells adhering to plastic tissue culture dishes (for 30 min at 37°C). The nonadherent cells were then magnetically labeled with MACS CD4, CD8, or CD19 MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and separated on a column placed in the magnetic fields of a MACS separator (Miltenyi Biotec). Fibroblast-like synoviocytes (FLS) were isolated from RAsynovial tissue as described with modifications[13].

Monoclonal antibodies (Mab). FITC or phycoerythrin (PE) conjugated anti-human mouse Mab against CD3 (UCHT), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD16 (3G8), CD19 (HIB19), CD26 (M-A264), CD28 (CD28.2), CD45RO (UCHL1), and CTLA-4 (CD152, BNI3) were obtained from PharMingen (San Diego, CA, USA). FITC or PE conjugated antibodies against CD25 (3G10), CD45RB (MEM55), CD69 (CH/4), interferon-γ (B27), interleukin 2 (IL-2) (MQ1-17H12), IL-4 (MP4-25D2), and IL-10 (JES3-9D7) were obtained from Caltag Laboratories (Burlingame, CA, USA), and FITC conjugated anti-CXCR3 (49801. 111) Mab and carboxyfluorescein succinimidylester (CFS) conjugated anti-Fas (DX2) Mab from Dako (Glostrup, Denmark). Purified Mab against B7.1 (CD80, MAB104) were supplied by Immunotech SA(Marseille, France) and B7.2 (CD86, 2331, FUN-1) by PharMingen. Anti-human mouse Mab against PD-1 (J116) and PD-L1 (#184) were established and biotinylated with Sulfo-NHS-Biotin (Pierce Chemical, Rockford, IL, USA) as described[14]. Isotype matched control mouse Mab were also obtained from PharMingen and Caltag Laboratories.

FACS analysis. Separated SF or PB cells were washed and resuspended in phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) (PBS/2% FBS), and immediately used for immunofluorescent staining. Half a million cells from SF or PB were incubated with PBS containing human AB serum (20%) for 5 min on ice, and then with either FITC or PE conjugated Mab together with biotin conjugated anti-PD-1 Mab for 30 min on ice. Biotinylated anti-PD-1 Mab or mouse IgG1 (isotype control) was developed with streptavidin Cy-chrome (Dako) for 30 min on ice. Control samples consisted of cells incubated with FITC, PE, or biotin conjugated mouse IgG.

For immunofluorescent staining, incubated FLS were resuspended with PBS containing human AB serum (20%) or human IgG1 (Chemicon, Temecula, CA, USA) for 5 min on ice, and then with purified Mab against CD80 and CD86, or biotin conjugated anti-PD-L1 Mab for 30 min on ice. Purified Mab against CD80 and CD86 were developed with goat anti-mouse IgG FITC (Southern Biotechnology, Birmingham, AL, USA). Biotinylated anti-PD-L1 Mab or human IgG4 (isotype control) was developed with streptavidin Cy-chrome for 30 min on ice.

Stained cells were analyzed with a FACSCalibur® (Becton Dickinson, Palo Alto, CA, USA) flow cytometer and its software program using electronic gating and compensation. Lymphocytes, monocytes, and granulocytes were identified by their characteristic forward angle and side scatter profiles, and their profile was confirmed by FACS analysis using the respective Mab against CD3 (Tcells), CD19 (B cells), CD14 (monocytes), and CD16 (granulocytes) as shown in Figure 1A. FACS analysis of lymphocytes was performed by setting appropriate gates corresponding to R1 (Figure 1A).

Cell culture. CD4+ T cells from RAPB, control PB, and RASF were resuspended in RPMI-1640 medium supplemented with L-glutamine (Nissui Pharmaceutical, Tokyo, Japan), 10% heat inactivated FBS, and 25 mM HEPES. In order to detect CD45RB expression on cell surfaces or the intracellular cytokine production, CD4+ T cells (1×10^6/ml) were activated with 50 ng/ml phorbol myristate acetate (PMA; Sigma, St. Louis, MO, USA) and 0.5 µg/ml ionomycin (Io; Sigma) in plastic tissue culture dishes at 37°C for 24 h. For CD45RB detection, cells were incubated 24 h, and for cytokine detection, monensin (GolgiStop, PharMingen) was added for the last 4 h of incubation.

Induction of cytokines and intracellular cytokine staining. After activation, CD4+ T cells were washed twice in PBS and incubated with PBS containing human AB serum (20%) for 5 min on ice, and then with biotin conjugated anti-PD-1 Mab or mouse IgG1 for 30 min on ice. Biotinylated anti-PD-1 Mab or mouse IgG1 (isotype control) was developed with streptavidin Cy-chrome (Dako) for 30 min on ice in the dark. These cells were then fixed with 1% paraformaldehyde-PBS overnight. The next day, the cells were washed twice with PBS and then permeabilized with 0.5% saponin (Sigma) in PBS supplemented with 2% FCS (PBS/2% FCS/0.5% saponin). The cells were treated with each of the anticytokine Mab for 30 min on ice in the dark, washed with PBS/2% FCS/0.5% saponin twice and then washed with PBS/2% FCS, and finally resuspended in PBS/2% FCS.

Statistical analysis. Statistical analysis used StatView software (Abacus Concepts, CA, USA). Data are given as the mean ± SD of the indicated number of samples studied. Statistical comparative analysis of 2 independent groups was performed with the Mann-Whitney U-test and that of multiple, independent diagnostic groups with the Kruskal-Wallis test. Correlation coefficients were determined with Spearman's rank correlation method, and results were considered statistically significant at $p < 0.05$.

RESULTS

Phenotypic characterization of each group was carried out by staining SF cells with Mab against CD3 (T cells), CD19 (B cells), CD14 (monocytes), and CD16 (granulocytes) (Figure 1A). For the next series of experiments, appropriate gates corresponding to R1 were set for FACS analysis of lymphocytes.

PD-1 was almost exclusively expressed on T cells from RA SF, but not from OASF. We used 2-color analysis to examine the cell surface expression of PD-1 on CD3+ T cells from 10 RAPB, 32 RA SF, and 6 OASF samples. Table 1 shows the clinical characteristics of 32 RA patients who underwent CD3 analysis. The representative data of PD-1 expression on CD3+ T cell samples are shown in Figure 1B. PD-1+ cells made up 20.9 ± 8.6% of RASF T cells, but only 1.2 ± 0.7% of RA PB and 3.6 ± 4.0% of OA SF T cells (Figure 1C). Statistical analysis showed that the percentage of PD-1+ cells on T cells from RASF was significantly higher than on T cells from RA PB ($p < 0.0001$) and OA SF ($p = 0.0002$).

PD-1+ cells are enriched in CD4+ Tcells from RASF. Two-color FACS analysis of RASF cells, using Mab against PD-1, CD4, or CD8, showed that PD-1+ cells made up 34.5 ±

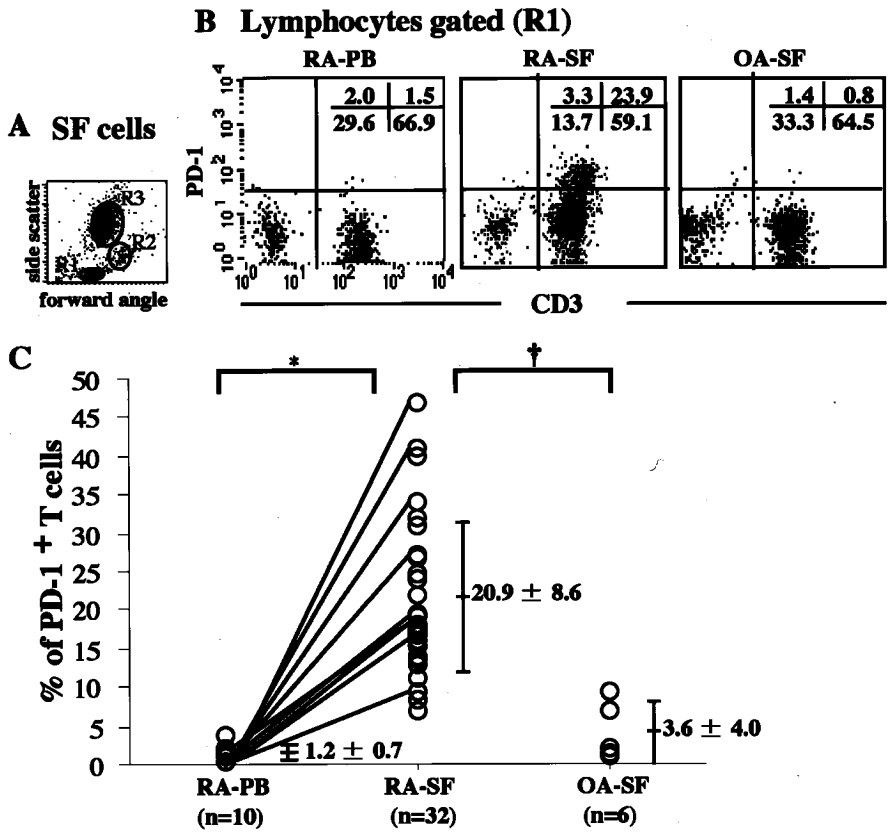

Figure 1. PD-1 expression on synovial fluid (SF) lymphocytes from patients with RA. (A) Differences in leukocytes analyzed by flow cytometry. Three groups of SF cells (R1–R3) were identified with forward angle and side scatter profiles. CD3+ T cells accounted for 79.5 ± 23.9% (mean ± SD), but CD19+ B cells accounted for only 1.7 ± 1.4% of region 1 (R1), CD14+ monocytes for 82.8 ± 11.8% of region 2 (R2), and CD16+ granulocytes for 98.4 ± 3.5% of region 3 (R3). (B) Representative data for PD-1 expression on peripheral blood (PB) and SF T cells from an RApatient, as well as SF T cells from a patient with OA (disease control). Percentages of PD-1+ cells among CD3+ T cells are shown on the vertical axis. (C) Distribution of PD-1 expression on T cells from RA PB, RASF, and OASF. Percentages of PD-1+ cells among CD3+ T cells are plotted on the vertical axis. Eight paired samples of RA PB and RA SF are connected by lines. Values show means ± SD. *$p < 0.0001$, †$p = 0.0002$.

12.9% of CD4+ T cells, but only 9.3 ± 5.1% of CD8+ T cells (Figure 2A). Statistical analysis showed that the percentage of PD-1+ cells on CD4+ T cells was significantly higher than that of CD8+ T cells from RA SF ($p < 0.0001$). Next, we purified CD4+ T, CD8+ T, and CD19+ B cells by using magnetic beads, and performed single-color staining with an isotype control (thin line) or anti-PD-1 Mab (thick line) (Figure 2B). PD-1 was found to be expressed strongly on CD4+ T cells and moderately on CD8+ T cells from RASF, only weakly on T cells from RAPB, and scarcely at all on T cells from control PB samples.

PD-1 is mainly expressed on the CD45RB^{low} population

Table 1. Clinical characteristics of patients with RA(CD3$^+$ SF T cell samples). Results are shown as the mean ± SD of each 32 experiments according to Larsen grade of knee joints where joint fluid aspiration was performed.

Larsen Grade	Age, yrs	Disease duration, yrs	Pred, n	MTX, n	ESR, mm/h	CRP, mg/ml	WBC, x 10^9/l	RF, x 10^2 IU/ml
0 (n = 0)	—	—	—	—	—	—	—	—
I (n = 5)	57 ± 6	7 ± 4	2	2	33 ± 21	1.7 ± 1.4	7.6 ± 1.0	3.0 ± 3.8
II (n = 7)	64 ± 12	14 ± 8	4	6	62 ± 29	3.3 ± 2.4	8.8 ± 7.8	2.0 ± 1.4
III (n = 13)	57 ± 8	17 ± 14	8	6	59 ± 23	4.1 ± 2.6	9.8 ± 1.7	3.0 ± 4.0
IV(n = 7)	57 ± 12	16 ± 14	6	2	49 ± 17	2.4 ± 2.2	8.3 ± 2.3	2.6 ± 3.3
V (n = 0)	—	—	—	—	—	—	—	—

Pred: prednisolone, MTX: methotrexate, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, RF: rheumatoid factor.

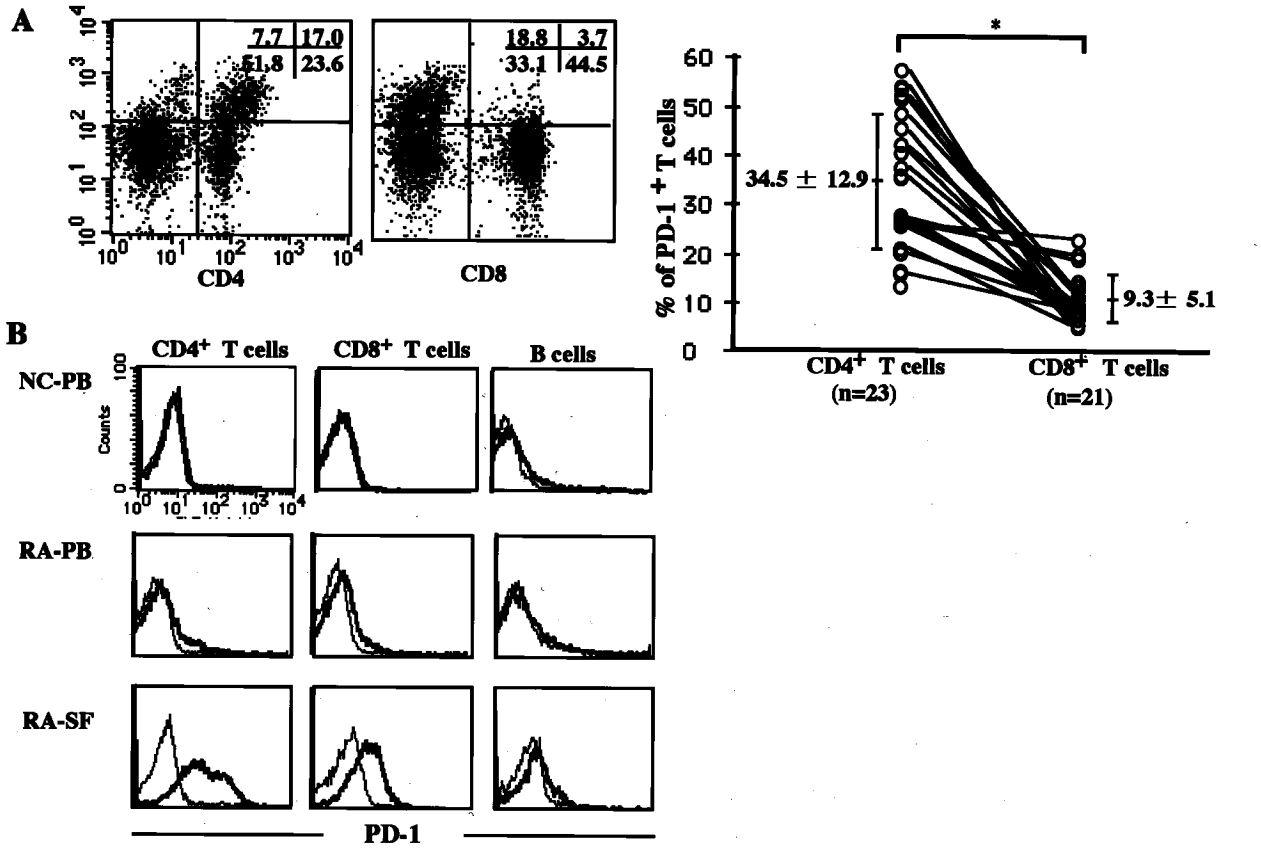

Figure 2. PD-1 expression on each of the subpopulations of SF or PB T cells from RApatients. (A) Two-color staining of PD-1 and CD4 or CD8. Quadrants were located according to the staining for isotype control samples. The right panel shows percentages of PD-1+ cells on CD4+ or CD8+ T cells from RASF. Twenty-one paired samples are connected by lines. Values show means ± SD. *p < 0.0001. (B) Comparison of PD-1 expression on CD4+ T cells, CD8+ T cells, and CD19+ B cells from control (NC) PB, RAPB, and RASF. Cells were analyzed for single-color staining with an isotype control (thin line) or anti-PD-1 Mab (thick line). Representative data are shown.

of CD4+ T cells from RA SF. To clarify the differentiation status of PD-1+ cells in RA joints, 2-color FACS analysis was performed using Mab against PD-1 and CD45RB, a cell-surface marker for T cell differentiation (Figure 3). When PD-1/CD45RB expression on CD4+ T cells from PB was analyzed before and after activation, the PD-1+ CD45RB^{low} population was found to have increased on CD4+ T cells after activation in both RAPB and control PB. In contrast, most of the PD-1-expressing cells in RA SF were CD4+ CD45RB^{low} T cells.

Phenotypic characterization of CD4+ PD-1+ T cells from RA SF. To clarify the composition of PD-1+ cells in CD4+

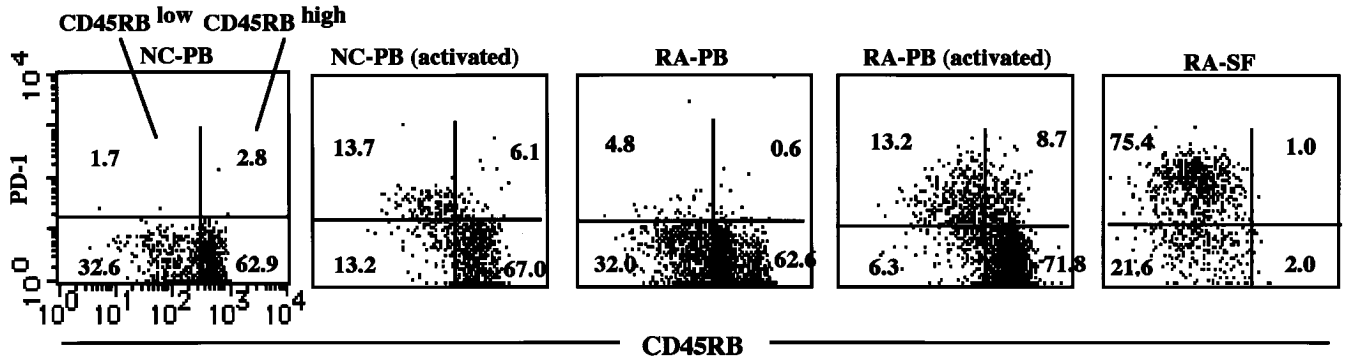

Figure 3. PD-1 is mainly expressed on the CD45RB^{low} fraction of CD4+ T cells. PD-1 expression on CD4+ T cells from control (NC) PB and RAPB samples was analyzed before and after stimulation with PMAand ionomycin. Two-color staining of PD-1 (vertical axis) and CD45RB (horizontal axis) on CD4+ T cells is illustrated. Quadrants are located according to the staining with control Mab.

SF T cells, 2-color FACS analysis was performed using Mab against PD-1 and other cell-surface antigens (CD45RO, CD28, CXCR3, Fas, CD69, CD25, CTLA-4, or CD26). Representative data are shown in Figure 4. Almost all CD4+ T cells from RA SF were positive for CD45RO, CD28, CXCR3, CD69, and Fas. PD-1+ T cells made up about 30–40% of the population positive for each antigen. Most CD4+ CTLA-4+ SF T cells were positive for PD-1, but CD4+ CD26+ SF T cells rarely coexpressed PD-1, while PD-1+ cells were not always CD25 positive (Table 2).

Intracellular cytokine analysis of CD4+ PD-1+ RA SF T cells. CD4+ T cells from control PB and RA SF were cultured with PMA/Io for 4 or 24 h to examine their capacity for cytokine production in relation to PD-1 expression (Figure 5). We determined that normal CD4+ PB T cells could produce cytokines including interferon-γ (IFN-γ), IL-2, IL-4, and IL-10 after stimulation for 4 h with PMA/Io (Figure 5A). Twenty to thirty percent of CD4+ PB T cells became PD-1 positive after 24 h stimulation and produced IFN-γ and IL-2, but not IL-4 or IL-10.

Analysis of cytokine production from CD4+ PD-1+ T cells from RASF showed that those cells produced IL-10 in addition to IFN-γ and IL-2, but not IL-4 (Figure 5B). Comparison of cytokine production by PD-1+ and PD-1– T cells revealed that CD4+ PD-1+ T cells tended to produce less IFN-γ and IL-2 than did CD4+ PD-1– T cells (Figure 5C). On the other hand, PD-1+ cells but not PD-1– cells produced IL-10. Statistical analysis showed that IL-2 production from CD4+ PD-1+ T cells detected by mean fluorescence intensity (MFI) was significantly lower than that from CD4+ PD-1– T cells (MFI 39.9 ± 21.2 vs 172.3 ± 86.8; p = 0.002) (Table 3), and IL-10-producing cells were found in CD4+ PD-1+ T cells much more than in CD4+ PD-1– T cells (8.3 ± 3.8% vs 0.9 ± 0.7%, p = 0.002) (Table 3).

PD-L1, but not CD80 or CD86, is induced on RA synoviocytes by IFN-γ. We examined the expression of the B7 family (CD80, CD86, and PD-L1) on FLS from RA synovial tissues. While there was no expression of CD80, CD86, or PD-L1 detected on FLS cultured with medium, PD-L1 but not CD80 or CD86 was significantly induced on FLS after stimulation with IFN-γ (Figure 6). PD-L1 was not induced on FLS after either tumor necrosis factor-α (TNF-α) or IL-1ß stimulation (data not shown).

Correlation between PD-1 expression on Tcells and clinical features. Patients with RA were classified into 6 groups according to the Larsen grade[15] of the knee joints where joint fluid aspiration was performed (Table 1). The

Table 2. CTLA-4, CD26, and CD25 expression on CD4+ PD-1± T cells in synovial fluid from RA patients. Results are shown as the mean ± SD percentages of cells in each population obtained from 5 experiments represented in Figure 4.

	CD4+ T Cells	
	PD-1+	PD-1–
CTLA4+	13.7 ± 7.0**	2.5 ± 1.7**
CTLA4–	37.4 ± 9.0	38.8 ± 10.5
CD26+	3.8 ± 2.5†	25.4 ± 9.4†
CD26–	37.4 ± 12.3	33.4 ± 20.3
CD25+	15.9 ± 10.8	12.5 ± 4.8
CD25–	33.9 ± 8.8	37.8 ± 13.5

** p = 0.016, † p = 0.009.

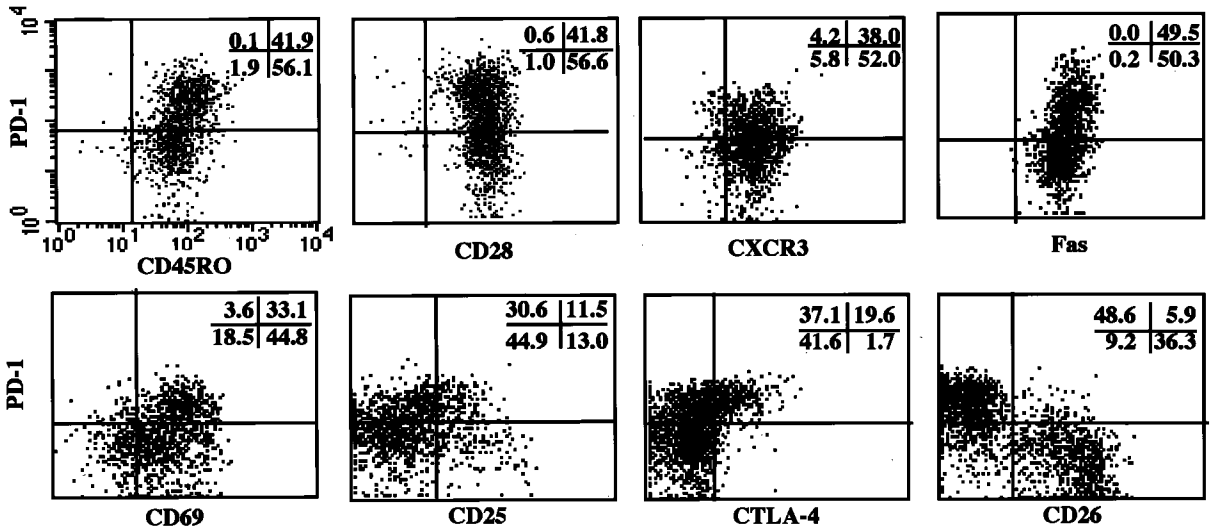

Figure 4. Two-color staining of PD-1 and activation/memory markers. PD-1 expression (vertical axis) and expression of activation/memory markers (horizontal axis: CD45RO, CD28, CXCR3, Fas, CD69, CD25, CTLA-4, or CD26) on the CD4+ subset of SF lymphocytes are plotted. Representative data are shown. Quadrants were located according to staining with control Mab.

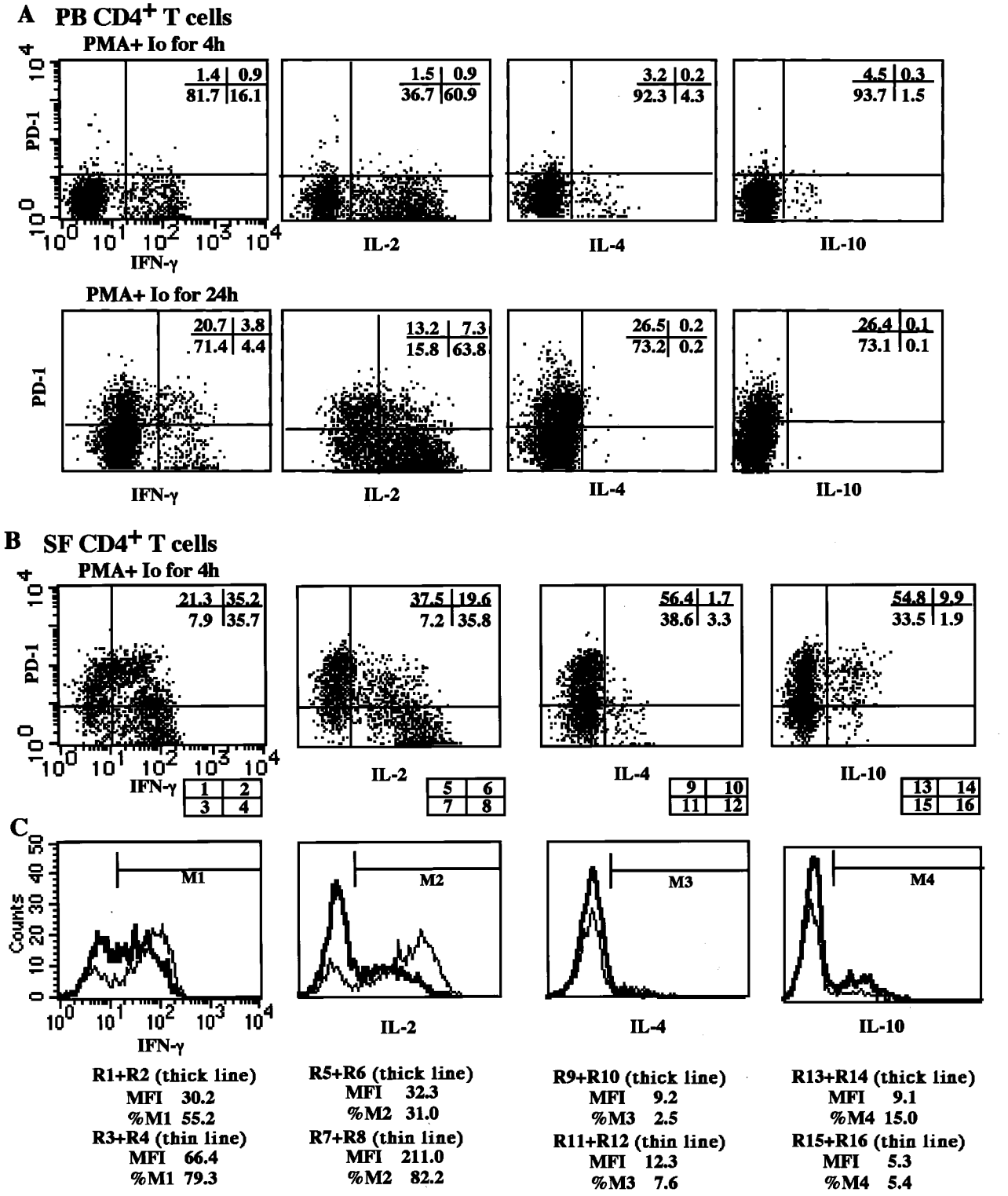

Figure 5. Intracellular staining for cytokine production on *in vitro* activated CD4+ T cells in control PB and RASF. (A) CD4+ T cells from control PB were stimulated for 4 or 24 h with PMA/ionomycin (Io) in the presence of monensin. PD-1 expression on cell surface (vertical axis) and intracellular expression of IFN-γ, IL-2, IL-4, or IL-10 (horizontal axis) are shown. (B) CD4+ T cells from RASF were cultured for 4 h with PMA/Io and monensin. PD-1 expression on cell surface (vertical axis) and intracellular expression of IFN-γ, IL-2, IL-4, or IL-10 (horizontal axis) are shown. Quadrants inside the scattergram were located according to the staining with control Mab. Quadrants in the lower panel define the gates for Figure 5C. (C) Comparison between PD-1+ and PD-1– cells for IFN-γ, IL-2, IL-4, and IL-10 production in CD4+ T cells from RASF. Gates are located as in B (lower quadrants), and results are shown as histograms for each of the cytokines. MFI and positive percentages for each cytokine (%M1–4) are shown.

Table 3. Cytokine production from CD4+ T cells after PMA plus ionomycin stimulation in synovial fluid from RApatients. Results are shown as the mean ± SD percentages of positive cells obtained from 7 individuals. Representative data are illustrated in Figure 5B. Relative percentages for PD-1+ and PD-1– cells are shown in parentheses.

	CD4+ T cells	
	PD-1+	PD-1–
IFN-γ		
MFI	20.3 ± 8.5	35.5 ± 20.9
%	28.9 ± 12.0	15.2 ± 12.0
IL-2		
MFI	39.9 ± 21.2**	172.3 ± 86.8**
%	15.4 ± 7.0	17.4 ± 10.6
IL-4		
MFI	8.2 ± 1.9	11.0 ± 3.6
%	2.7 ± 2.0	2.0 ± 1.8
IL-10		
MFI	8.1 ± 2.2†	5.7 ± 1.6†
%	8.3 ± 3.8††	0.9 ± 0.7††

** p = 0.006, † p = 0.04, †† p = 0.002. PMA: phorbol myristate acetate, MFI: mean fluorescence intensity.

percentage of PD-1 expression on CD3+ SF T cells tended to increase as the knee erosion progressed from grade I to grade III, and was significantly higher for grade III than grade IV (p = 0.043) (Figure 7). On the other hand, in joints of Larsen grade IV, activation markers of RA tended to decrease, reflecting the diminished inflammation. We found no significant correlation between PD-1+ percentages and other clinical features such as age, sex, medication (methotrexate or steroids), disease duration, or activation markers [erythrocyte sedimentation rate, C-reactive protein (CRP), white blood cell (WBC) count, hemoglobin level, platelet count, and rheumatoid factor (RF) of peripheral blood] in patients with RA(data not shown).

DISCUSSION

We determined that PD-1+ T cells accumulate in the synovial fluid of patients with RA. The percentages of PD-1 expression on CD3+ SF T cells were significantly higher for Larsen grade III, the most active state, than for grade IV, the less inflammatory state, while there was no correlation between PD-1 expression and systemic markers for the activation status of RA such as CRP, WBC counts, and RF. These findings suggest that PD-1 expression on synovial fluid T cells may reflect local rather than systemic inflammation in RA.

PD-1 was mainly expressed on CD4+ CD45RO+ CD45RB^{low} CXCR3+ T cells. In addition, the increase in PD-1 expression was associated with the shift of CD4+ T cells from CD45RB^{high} to CD45RB^{low} phenotype upon activation in both RA and control peripheral blood cells. These results indicate that PD-1+ cells have the characteristics of memory/activated T cells. PD-1 has been shown to be involved in the negative regulation of immune responses and is supposed to play a role in peripheral tolerance[6,8,10,11]. If activated T cells in RA joints expressed PD-1 in the course of chronic inflammation and were inactivated by a negative signal through PD-1, then it is puzzling that so many CD4+ PD-1+ T cells remained accumulated. One

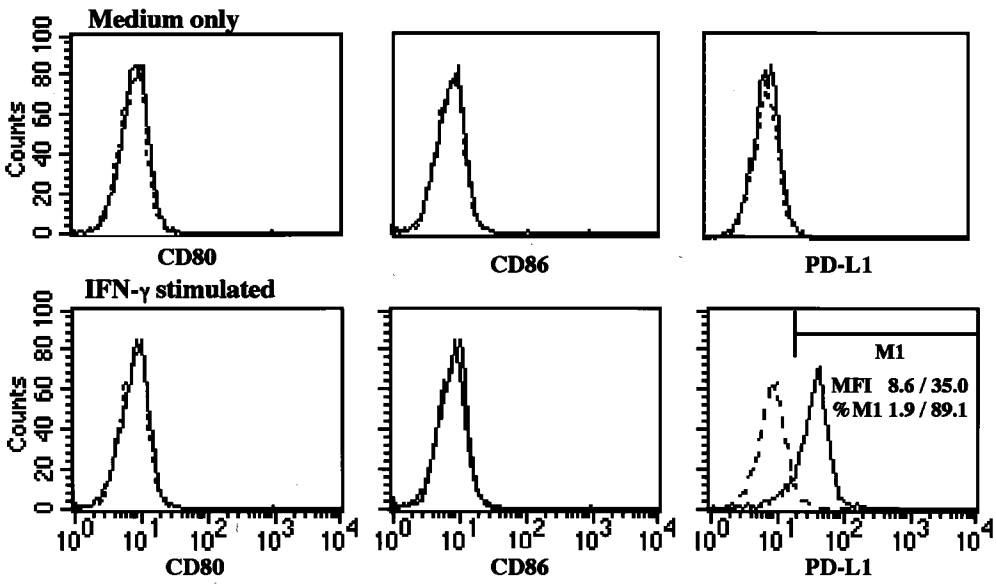

Figure 6. Expression of CD80, CD86, and PD-L1 on synoviocytes from RApatients. Fibroblast-like synoviocytes (FLS) from a patient with RAwere incubated with or without IFN-γ (100 ng/ml) for 24 h. Cells were stained with an isotype control (broken line) or anti-human Mab against CD80, CD86, and PD-L1 (solid line). MFI (broken line/solid line) and positive percentages for each of the molecules (%M1; broken line/solid line) are shown.

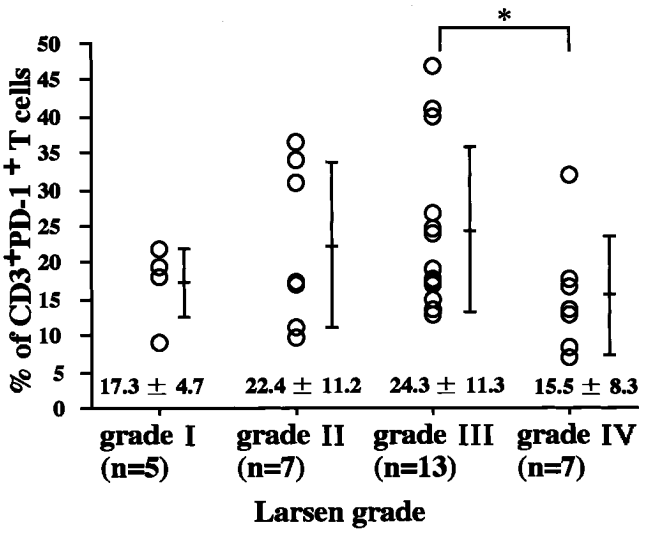

Figure 7. Correlation between PD-1 expression on CD3+ SF T cells and Larsen grade for RApatients. Percentages of PD-1+ cells among CD3+ T cells from RA SF are plotted for each Larsen grade group. Values show means ± SD. *p = 0.043.

explanation is that PD-1+ cells may survive because the PD-1-mediated system causes cell cycle arrest but does not mediate a death signal[16].

A number of studies have examined the expression of costimulatory molecules, the CD28 family, and their ligands, the B7 family, in RA[17]. CTLA-4 is expressed on about 15% of SF T cells, but scarcely at all on PB T cells[18]. While we observed simultaneous expression of PD-1 and CTLA-4, the tissue distribution of their ligands is quite different. CD80 and CD86, common ligands for CD28 and CTLA-4, are expressed mainly on professional antigen-presenting cells in lymphoid organs[19,20]. On the other hand, mRNAof 2 ligands for PD-1 (PD-L), PD-L1/B7-H1[21,22] and PD-L2/B7-DC[16,23], are expressed in nonlymphoid organs such as placenta, heart, lung, kidney, and liver as well as on lymphoid organs. In human monocytes, IFN-γ but not TNF-α treatment results in expression of both ligands[22]. IFN-γ treatment can also induce PD-L1 expression in non-lymphoid cells such as keratinocytes and endothelial cells[22,24]. As for synoviocytes, FLS were negative for CD80 and CD86 in RA synovium even after IFN-γ treatment *in vitro* as reported[25]. On the other hand, PD-L1 expression was induced on FLS treated with IFN-γ but not with TNF-α or IL-1ß. These data imply that the PD-1/PD-L system rather than CTLA-4/B7 system may play a critical role in the maintenance of peripheral tolerance and/or establishment of autoimmune disease in local joints.

Intracellular cytokine staining showed that CD4+ PD-1+ T cells have unique characteristics. A sizable amount of IFN-γ production and no IL-4 production suggest that they are Th1-type cells. We observed low production of IL-2 from PD-1+ T cells in this study, which seems to imply that PD-1+ cells include anergic T cells. Howell, *et al*[26] postulated that T cells from RA SF are anergic because they lack IL-2 protein production despite the presence of IL-2 mRNA. They suggested that T cell anergy and lack of IL-2 production might be induced when Th1 clones are stimulated via their T cell receptors in the absence of costimulatory signals[26]. Since then, the large majority of reports of anergy have described the inhibition of IL-2 secretion by T cells that are normally capable of secreting IL-2 upon activation. Thus CD4+ PD-1+ T cells are believed to be involved in the establishment of anergy in RA joints.

CD4+ PD-1+ T cells produced a higher concentration of IL-10 than did CD4+ PD-1– cells in RAjoints. It should be noted that IL-10 production was detected only in RA SF T cells, but not in PB T cells *in vitro*. It is of interest that IL-10 has been identified as a critical cytokine in the suppression of multiple activities in the immune response[27,28]. Recent reports have highlighted the capacity of IL-10 to inhibit inflammation and joint destruction *in vitro* in patients with RA and *in vivo* in experimental arthritis models[29]. Thus, IL-10 may play an important role in the maintenance of peripheral tolerance, although IL-10 production from CD4+ PD-1+ T cells may be insufficient to downregulate chronic inflammation in RA joints. Another possibility is that they may secrete IL-10 in an autocrine or paracrine manner to inactivate themselves because they are autoreactive T cells. As antiinflammatory cytokines (IL-10, IL-13, transforming growth factor-ß) and antagonists of inflammatory mediators (IL-1Rα and soluble TNF receptor) are present in significant amounts in RAjoints[30], it should be of considerable interest to clarify the role of PD-1+ cells in producing such factors or their effect on PD-1+ cells.

CD4+ PD-1+ T cells from RA SF expressed CD45RB^{low} and CTLA-4 with IL-10 secretion, and their characteristics appear similar to that of CD4+ CD25+ regulatory T cells[31-33]. We do not think, however, that the former are identical to the latter, for 2 reasons. First, CD4+ CD25+ regulatory T cells are by definition nonprimed cells, while PD-1 is expressed only after activation on normal PB T cells[34]. Second, our data showed that CD4+ PD-1+ SF T cells did not always display CD25 expression.

Another CD4+ T cell subset similar to CD4+ PD-1+ T cells is Tr1. This subset is characterized by low proliferation capacity and by high IL-10 production with low production of IL-2 and IL-4[35]. Although involvement of Tr1 cells with autoimmune diseases is not determined, involvement in infectious diseases has been reported[36,37]. Thus, Tr1 cells may arise and contribute to the persisting immune response of the host. Tr1 cells reportedly consist of no more than 5% of CD4+ T cells of peripheral blood and synovial tissue from patients with RA[38]. This is in contrast to the abundance of PD-1+ cells from RA SF observed in this study. It would be highly relevant to investigate the mechanism of CD4+ PD-1+ T cells, and to clarify the correlation or distinction

between CD4+ PD-1+ T cells and CD4+ CD25+ regulatory T cells or Tr1 cells in RA joints.

While CD4+ T cells from RA-SF coexpressed PD-1 and memory/activation markers, PD-1+ cells also coexpressed CTLA-4, a receptor for negative regulation of activated T cells[39]. Thus, PD-1+ cells are likely to be susceptible to negative signals from B7 molecules or PD-L. On the other hand, CD26, another costimulatory molecule that mediates activation signals and is considered to be one of the markers for Th1-type cells[40,41], rarely coexpressed PD-1 on CD4+ T cells from RA SF. The different expressions of CD26 and PD-1 are thus likely to divide CD4+ T cells into 2 groups — activated Th1 cells and supposedly inactivated or inactivation-prone CD4+ T cells. Further characterization of these 2 possible CD4+ T cell subtypes may provide information about the role of CD4+ T cells in RA joints.

To summarize, CD4+ PD-1+ T cells from RA synovial fluid consist of a well differentiated and activated/memory population. Further, these cells are characterized by CTLA-4 but not CD26 coexpression, and by IL-10 production and to a lesser degree IL-2 production. Further examination of these unique cells may clarify the mechanism of T cell anergy and its breakdown in RA joints.

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