

Distribution of Myeloid Dendritic Cells and Plasmacytoid Dendritic Cells in the Synovial Tissues of Rheumatoid Arthritis

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ABSTRACT. Objective. To examine the precise tissue distribution of dendritic cells (DC) and indoleamine 2,3-dioxygenase (IDO)-expressing cells in synovial tissue and synovial fluid (SF) from patients with rheumatoid arthritis (RA) and osteoarthritis (OA).

Methods. Synovial tissues from 30 patients with RA and 7 with OA were immunohistochemically stained for DC markers. The examined areas were classified into 5 categories based on pathobiological staging and histopathological grading systems. Myeloid DC (mDC) and plasmacytoid DC (pDC) were isolated using positive and negative magnetic sorting systems, respectively, from SF samples (7 patients with RA and 4 with OA) and synovial tissues (3 RA, 4 OA).

Results. mDC were mainly observed in lymphoid aggregations. pDC were scattered around perivascular infiltration areas, and small and large lymphoid aggregations in RA. The mDC/pDC ratio increased significantly, with higher grading in RA SF tissues compared to OA synovial tissues ($p < 0.05$). IDO-immunoreactivity was detected in pDC by serial sectioning and staining of RA synovial tissues.

Conclusion. Our results indicate that mature mDC play a central role in the RA inflammatory process. Although there were fewer pDC than mDC, the presence of IDO-positive pDC suggests a possible tolerance mechanism in RA synovial tissues. However, it is probably modest due to the marked inflammation in RA, in which mDC are dominant. (First Release Sept 1 2008; J Rheumatol 2008;35:1919–31)

Key Indexing Terms:

DENDRITIC CELLS
DISTRIBUTION

RHEUMATOID ARTHRITIS

SYNOVIAL TISSUE
INDOLEAMINE 2,3-DIOXYGENASE

Rheumatoid arthritis (RA) is a severe and chronic autoimmune inflammatory disease that principally affects multiple joints simultaneously. In the joints, an immune response predominantly occurs in the synovial tissue and fluid^{1,2}. The onset and progression of RA depend on multiple factors, and many types of cells are involved in the immune reaction process¹. Antigen-presenting cells can strongly activate autoreactive T cells via abnormal presentation of some self-

antigens in RA and other autoimmune diseases involving these cells³. It is generally accepted that antigen-presenting cells process the antigen, which is the first step in an immune response. Dendritic cells (DC) are well established as the most potent cell type among antigen-presenting cells. DC represent a specialized antigen-presenting cell system that is critical for the initiation of T cell responses in lymphoid and nonlymphoid organs⁴. After uptake of an antigen, DC migrate through afferent lymph vessels to T cell-dependent areas of secondary lymphoid organs and present their antigens to naive T cells. Through this process, DC become mature and act as potent antigen-presenting cells, with increased expressions of costimulatory molecules on their cell surface and the secretion of cytokines³.

DC have various abilities depending on their subsets and state of maturation. Myeloid DC (mDC) and plasmacytoid DC (pDC) have been identified as the 2 major subsets, and pDC are also known as interferon- $\alpha\beta$ -producing cells^{5,6}.

Recently, it has become evident that DC not only have immunostimulatory functions but also tolerogenic functions, since some immature DC were found to induce tolerance^{7,8}. In addition, a recent study demonstrated that regu-

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Accepted for publication May 20, 2008.

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latory pDC involved in tolerogenic functions express indoleamine 2,3-dioxygenase (IDO)⁹. These cells can deplete tryptophan and are involved in toxic tryptophan metabolism, both of which have strong inhibitory effects on T cell proliferation and survival^{9,10}.

We examined the precise distributions of DC and IDO-expressing cells in synovial tissue and synovial fluid (SF) from patients with RA and osteoarthritis (OA). The ratios of mDC/pDC and mature mDC/immature mDC *in loco* were also examined.

MATERIALS AND METHODS

Patients and specimens. Synovial tissues for immunohistochemical analysis were obtained from 30 patients with RA and 7 with OA treated by total knee arthroplasty or synovectomy of a hand, elbow, finger, or knee (Table 1). SF samples (7 patients with RA and 4 with OA) and synovial tissues (3 patients with RA and 4 with OA) for cellular immunophenotyping of isolated mDC and pDC were obtained from patients treated by arthrocentesis or total knee arthroplasty (Table 2).

All the patients with RA fulfilled the American College of Rheumatology criteria for RA¹¹. Regarding the patients with RA, 39 patients had been treated with disease modifying antirheumatic drugs (DMARD: methotrexate, salazosulfapyridine, or bucillamine), low-dose glucocorticoids, and nonsteroidal antiinflammatory drugs (NSAID), while 1 patient had been treated with NSAID alone. None of the patients had received any biological agents. Further, none had injected corticosteroid into their joints within at least 3 months of the study entry. All patients provided informed consent and our study was approved by the Yamagata University School of Medicine Ethics Committee.

Immunohistochemistry. All tissues were embedded in Tissue-Tek OCT compound, snap-frozen, and stored at -80°C until analysis. Serial sections (5-µm thick) were cut with a cryostat, mounted on silane-coated slides, dried for 1 h at room temperature, and fixed in acetone for 10 min at 4°C. After blocking endogenous peroxidase activity with 0.1% hydrogen peroxide (H₂O₂) in phosphate buffered saline (PBS) for 10 min, the sections were incubated with 4% skim milk for 1 h and then incubated with monoclonal antibodies (mAb) against CD3 (PS1; anti-mouse; Nichirei, Tokyo, Japan),

DC-LAMP (104.G4; mouse IgG1; Immunotech, Marseille, France), DC-SIGN (CD209, H-200; rabbit IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD123 (IL-3R, S-12; mouse IgG1; Santa Cruz Biotechnology), BDCA2 (AC144; mouse IgG1; Miltenyi Biotec, Gladbach, NRW, Germany), or IDO (10.1; mouse IgG3; Chemicon, Temecula, CA, USA) diluted 1:50–1:100 in PBS containing 0.5% fetal calf serum (FCS; Gibco, Grand Island, NY, USA) overnight at 4°C. The binding of biotinylated secondary antibodies was visualized by the avidin-biotin-horseradish peroxidase complex technique (ABC Vectastain Elite Kit; Vector Laboratories, Burlingame, CA, USA) using 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) as the substrate. The sections were counterstained with Mayer's hematoxylin and viewed under an Axiophot photomicroscope (Carl Zeiss, Postfach, KDZ, Germany). Negative controls included the use of IgG isotype controls and omission of the primary antibodies¹².

Saponin (0.1%; Junsei Chemical, Tokyo, Japan) was used to permeabilize the cell membrane to investigate DC-LAMP and IDO expressions, since these molecules are localized in the cytoplasm¹³.

Evaluation of the pathobiological staging of RA and inflammation in synovial tissues. The pathobiological stage was estimated in hematoxylin-eosin-stained sections using the Harris staging system¹⁴, while inflammation was estimated in CD3-stained sections using the Krenn histopathological grading system¹⁵, under a light microscope (data not shown).

Immunohistochemical evaluation of positive cells and immunoreactivity. Immunopositive cells for DC-LAMP, DC-SIGN, CD123, BDCA2, and IDO were independently counted by 2 observers in at least 5 fields under a light microscope (×200 magnification) in 5 different areas, as described below. Each count was then standardized using the dimensions of the whole area of a television screen. The dimensions were calculated using the National Institutes of Health Image software (version 1.61; NIH, Bethesda, MD, USA). The 5 areas were defined as follows: no or little T cell infiltration area; lining layer area; perivascular infiltration area; small lymphoid aggregation area with high endothelial venules; and large lymphoid aggregation area with high endothelial venules. Small lymphoid aggregates were defined as aggregates with dimensions of less than one-quarter relative to the whole area of the television screen. The mean number of positive cells per television screen in the microscopic high-magnification field of an Axiophot photomicroscope (Carl Zeiss) was calculated for each area. The counts were categorized by the Harris RA stage and Krenn inflammatory grade, respectively^{14,15}.

Table 1. Characteristics of the RA and OA patients evaluated by immunohistochemistry.

	Age, yrs	Sex, female/male	Duration, yrs	CRP, mg/dl	RF, IU/ml	Steroids, +/-	DMARD, +/-
RAST, n=30	62.8 (40–78)	24/6	14.1 (3–30)	3.86 (0.2–18.2)	150.73 (8–805)	24/6 (0–10 mg)	30/0
OAST, n=7	72 (68–75)	5/2	13.5 (10–20)	0.08 (0–0.11)	—	—	—

With the exception of sex, steroids, and DMARD, the numbers represent the mean (range). ST: synovial tissue; CRP: C-reactive protein; RF: rheumatoid factor; steroids (+/-): corticosteroids (users/nonusers); DMARD (+/-): disease modifying antirheumatic drugs (methotrexate, salazosulfapyridine, or bucillamine) (users/nonusers).

Table 2. Characteristics of the RA and OA patients used for isolation of DC.

	Age, yrs	Sex, female/male	Duration, yrs	CRP, mg/dl	RF, IU/ml	Steroids, +/-	DMARD, +/-
RAST, n=7	57.5 (36–78)	7/0	12.8 (8–30)	4.25 (0.1–11.75)	347 (46–1450)	5/2 (0–7.5 mg)	6/1
OASF, n=4	74 (68–81)	4/1	16 (13–18)	0.1 (0.1)	—	—	—
RAST, n=3	64 (47–67)	3/0	22.5 (15–30)	5.08 (0.4–8.2)	439 (75–528)	3/0 (3–7.5 mg)	3/0
OAST, n=4	74 (68–81)	4/0	16 (13–18)	0.1 (0.1)	—	—	—

With the exception of sex, steroids, and DMARD, the numbers represent the mean (range). SF: synovial fluid; ST: synovial tissue; DC: dendritic cells; CRP: C-reactive protein; RF: rheumatoid factor; steroids (+/-): corticosteroids (users/nonusers); DMARD (+/-): disease modifying antirheumatic drugs (methotrexate, salazosulfapyridine, or bucillamine) (users/nonusers).

Isolation of DC. All cells were washed with RPMI-1640 (Gibco, Grand Island, NY, USA). The methods for isolating DC from SF were modified as described¹⁶. Briefly, RA and OA synovial fluid samples were enzymatically digested to reduce their viscosities using the following 3-step method: (1) hyaluronidase (15 g/ml; Sigma-Aldrich) for 15 min at 37°C; (2) collagenase type II (250 ng/ml; Worthington, Lakewood, NJ, USA) for 15 min at 37°C; and (3) hyaluronidase as in step 1. After each step, the cells were centrifuged at 300 ×g for 10 min. After the final resuspension, each digested fluid was passed through a nylon column with 50-µm pores, followed by density gradient centrifugation using Ficoll-Paque™ PLUS (Amersham Biosciences AB, Uppsala, Sweden) to isolate the mononuclear cell fraction. pDC were purified by positive sorting using anti-BDCA4-conjugated magnetic microbeads (Miltenyi Biotec). The residual cells were pruned by negative selection using Dynabeads M-450 CD2 (Pan T; 1 × 10⁷ beads/ml; Dynal, Oslo, Norway), Dynabeads M-450 CD19 (Pan B; 1 × 10⁷ beads/ml; Dynal), and Dynabeads M-450 CD14 (Monocyte; 1 × 10⁷ beads/ml; Dynal) to isolate mDC¹⁷.

The methods used for isolating DC from synovial tissues were modified as reported regarding T cell selection from RA synovial tissue¹⁸. Briefly, resected RA and OA synovial tissues were cut into pieces, and enzymatically digested with collagenase type II (4 mg/ml; Worthington) and DNase I (40 ng/ml; Roche, Mannheim, Germany) for 90 min at 37°C. The digested tissues were passed through a nylon column with 133-µm pores, followed by passage through a nylon column with 50-µm pores. The selected cells were centrifuged at 300 ×g for 10 min after each step. After the last resuspension, the cells were isolated by density gradient centrifugation using Ficoll-Paque™ PLUS. The isolated mononuclear cells were incubated overnight in RPMI-1640 containing 10% FCS to remove any fibroblasts by allowing their adhesion to the base of the dish¹⁹. pDC were collected from the supernatants of the dishes by positive sorting using anti-BDCA4-conjugated magnetic microbeads (Miltenyi Biotec). The residual cells were pruned by negative selection using the same Dynabeads described above for the SF samples in order to isolate mDC¹⁶.

Fluorescence activated cell sorting (FACS) analysis. The primary antibodies used for the flow cytometry analyses were as follows: anti-CD1a mAb (O10; mouse IgG1κ; Immunotech); anti-CD11c mAb (BU15; mouse IgG1; Serotec, Oxford, UK); anti-CD40 mAb (LOB-11; mouse IgG; Santa Cruz Biotechnology); anti-CD80 mAb (MAB104; mouse IgG1; Immunotech); anti-CD83 mAb (HB15a; mouse IgG2b; Santa Cruz Biotechnology); anti-CD86 mAb (BU63; mouse IgG1; YLEM, Rome, Italy); anti-CD123 mAb (IL-3R, S-12; mouse IgG1; Santa Cruz Biotechnology); anti-CD205 polyclonal antibody (LY75; goat IgG; Santa Cruz Biotechnology); anti-IDO mAb IDO, anti-DC-SIGN mAb, anti-DC-LAMP mAb, pure anti-BDCA2 mAb and anti-BDCA2-phycoerythrin (PE) mAb; anti-CD123-PE mAb (AC145; mouse IgG2a; Miltenyi Biotec). IgG1 and IgG2a control isotypes were purchased from Santa Cruz Biotechnology.

The cells were washed once with 0.01 M PBS (pH 7.4) containing 2% FCS, and incubated with pure antibodies or conjugated antibodies at 4°C for 30 min. After washing to remove unbound primary antibodies, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulins (Dako, Carpinteria, CA, USA) as the secondary antibody.

The cells were washed again and sorted using a FACSCalibur (BD Biosciences, Mountain View, CA, USA). Data were analyzed using the CellQuest software® (BD Biosciences). The physical measures of the DC, forward scatter (FSC) signals, and side scatter (SSC) signals were also examined.

Cytospin and morphological analyses. Following isolation by positive sorting using anti-BDCA4-conjugated magnetic microbeads, DC from RA and OA synovial fluid and synovial tissue samples were washed by centrifugation and cytocentrifuged onto microscope slides. After fixation in acetone (Nacalai Tesque, Kyoto, Japan) for 10 min at 4°C, the cytospin specimens were incubated with 1.14 mg/ml periodic acid dihydrate in PBS for 30 min to block endogenous peroxidase activity, followed by incubation with 4% skim milk for 1 h to block nonspecific antibody binding. Next, the speci-

mens were incubated with anti-CD123, anti-BDCA2, and anti-IDO mAb (diluted 1:50–1:100 in PBS containing 0.5% FCS) overnight at 4°C, followed by incubation in biotinylated secondary antibodies. The bound antibodies were visualized using horseradish peroxidase-conjugated F(ab')₂ fragments of a combination of goat anti-mouse IgG and IgM (Jackson Immuno Research, West Grove, PA, USA) with 3,3'-diaminobenzidine (Sigma-Aldrich) as the substrate. Sections were counterstained with Mayer's hematoxylin and viewed using an Axiophot photomicroscope. Negative controls included the use of IgG isotype controls and omission of the primary antibodies. In double-labeling experiments, the sections were incubated with anti-IDO mAb overnight at 4°C as described above, and then incubated with Alexa Fluor® 488-conjugated F(ab')₂ fragments of goat anti-mouse IgG (H+L) (Molecular Probes, Eugene, OR, USA) and anti-CD123-PE mAb.

Statistical analysis. Spearman's rank correlation coefficient analysis, Mann-Whitney U-tests, repeated-measures analysis of variance (ANOVA), and Tukey-Kramer tests were performed using the StatView-J software (Abacus Concepts, Berkeley, CA, USA). Differences of $p < 0.05$ were considered statistically significant.

RESULTS

Pathologic stages and inflammatory grades of RA and OA synovial tissues. The pathobiological stages and inflammatory grades of the RA and OA synovial tissues were estimated using hematoxylin-eosin-stained sections and CD3-stained sections, respectively^{14,15}. The staging system and inflammatory grading system were correlated with each other (Spearman's rank correlation: $p < 0.0001$). RA synovial tissues were classified into stage 2: 12 cases, stage 3: 14 cases, stage 4: 4 cases; and grade 1: 15 cases, grade 2: 14 cases, grade 3: 1 case. Mean stage and grade of RA synovial tissues were 2.7 (2–4) and 1.43 (1–3), respectively. OA synovial tissues were classified into grade 0: 4 cases, grade 1: 3 cases, and the mean grade was 0.42 (0–1) (Figure 1A, 1B). The synovial tissue obtained from patients with advanced disease treated by total knee replacement mainly showed fibrosis and little or no inflammation under light microscopy. Their inflammation was estimated as relatively low stage and grade with serologically lower level in many cases and moderate in some cases [$n = 11$, mean stage 2.3 (2–3), mean grade 1.1 (1–2), and mean value of C-reactive protein (CRP) 2.2 mg/dl (0.2–5.4)]. Mean value of CRP was 4.9 mg/dl (1.5–7.8) in stage 4, 4.3 mg/dl (0.4–18.3) in stage 3, 2.2 mg/dl (0.2–3.1) in stage 2, 5.1 mg/dl in grade 3, 4.8 mg/dl (0.4–18.3) in grade 2, 2.2 mg/dl (0.2–5.4) in grade 1. However, staging system and inflammatory grading system were not correlated with the level of CRP (3.86 ± 14.3 mg/dl) (Spearman's rank correlation: 0.17, 0.11, respectively).

Localizations of mDC and pDC in synovial tissues. Mature mDC, identified by DC-LAMP-positivity²⁰, were abundantly detected in the perivascular infiltration, small lymphoid aggregation, and large lymphoid aggregation areas (Figure 2A). mDC were more frequently found in RA synovial tissues than in OA synovial tissues, but the difference was not significant (Mann-Whitney U-test; Figure 3A). The number of mature mDC increased according to progression of the stage and grade (repeated-measures ANOVA and Tukey-

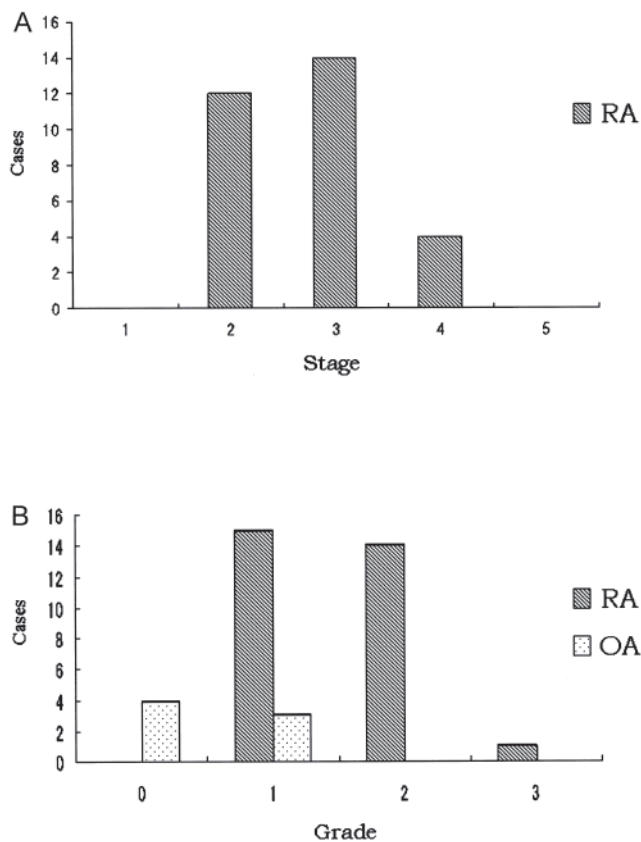


Figure 1. Pathobiologic stages of rheumatoid arthritis (RA) (A). Histopathological grades of the inflammations in RA and osteoarthritis (OA) (B). The staging system and inflammatory grading system were correlated (Spearman's rank correlation: $p < 0.0001$).

Kramer test: $p < 0.01$; Figure 3B, 3C). The numbers of cells positive for DC-SIGN, a marker of immature mDC²⁰, increased in the lining layer (Figure 2C) and perivenular areas, although statistical significance was not found ($p = 0.3$; Figure 3D). DC-SIGN-positive cells were scarce in large lymphoid aggregation areas. The mature/immature DC ratios were increased in the large aggregation areas compared with the no infiltration and lining layer areas, but the difference was not significant ($p = 0.56$; Figure 3F). DC-SIGN was previously reported to be expressed on pDC²¹, in addition to CD1a on immature DC²²⁻²⁴, but these findings were not observed in our study (data not shown).

Small numbers of cells positive for CD123 and BDCA2, markers of pDC⁸, were found around the perivenular infiltration, small aggregation, and large aggregation areas (Figure 2D, 2E, 3D). IDO-positive cells were found together with CD123- and BDCA2-positive cells (Figure 2D, 2E, 2F), but not DC-LAMP- and DC-SIGN-positive cells (Figure 2A, 2B, 3D), on serial sections. pDC were more frequently found in RA synovial tissues than in OA synovial tissues, but the difference was not significant (Figure 3A).

mDC were observed mainly in the small and large lymphoid aggregation areas (Figure 3D). pDC were scattered

around the perivenular infiltration, small lymphoid aggregation, and large lymphoid aggregation areas of RA tissues (Figure 3D). The numbers of mature mDC, but not immature mDC or pDC, increased with progression of staging and grading in RA tissues (Figure 3E). The mDC/pDC ratios increased significantly, with higher grading scores in RA tissues compared to OA tissues ($p < 0.05$; OA data not shown).

Isolation of mDC and pDC from RA and OA SF and synovial tissue samples. pDC isolated from RA SF samples were strongly positive for CD123, BDCA2, IDO, and costimulatory molecules (CD40, CD80, and CD86; Figure 4A). mDC isolated from RA SF samples were strongly positive for CD11c, CD83, and CD205, moderately positive for costimulatory molecules (CD40, CD80, and CD86), and weakly positive for CD1a (Figure 4B). Counts of mononuclear cells revealed higher counts per unit from synovial fluid of the patient receiving only NSAID ($n = 1$, $31.5 \times 10^6/\text{mm}^3$) than that of the patients receiving DMARD [$n = 6$, $9.1 \times 10^6/\text{mm}^3$ (3.2–13.5)]. Since mDC were selected by negative selection using CD2 (Pan T), CD19 (Pan B), and CD14 (Monocyte), the mDC fractions, especially those from synovial tissues, included contaminants such as small granulocytes, basophils, residual T cells, B cells, monocytes/macrophages, and fibroblasts. Therefore, the mDC-rich fraction area was estimated by gating using CellQuest software (Figure 4C, 4D). No pDC or mDC were detected in the fractions isolated by positive and negative selection from OA SF samples (Figure 4E, 4F).

It was difficult to isolate DC from RA synovial tissues for accurate assessment by FACS analysis due to contamination by variable numbers of fibroblasts and other cell types compared to synovial fluid and peripheral blood. pDC (CD123- and BDCA2-positive cells) only represented about 30% of the fraction purified by positive sorting, and IDO staining was not strongly positive (Figure 4C). mDC (CD11c- and CD83-positive cells) represented 20%–40% of the fraction purified by negative selection (Figure 4D). We could not estimate many markers, such as costimulatory molecules, in the RA synovial tissues because we were unable to obtain sufficient nucleated cells for FACS analysis. Regarding the OA synovial tissues, sufficient cells for FACS analysis were not obtained, since the tissues did not contain abundant nucleated primitive cells.

Morphology of pDC purified from RA SF samples. Almost pure populations of pDC purified from RA SF samples, as determined by FACS analysis, were cytocentrifuged onto microscope slides. pDC were detected by staining for CD123 and BDCA2 (Figure 5A, 5B; brown staining with diaminobenzidine), and exhibited dendritic features by light microscopy. Similar dendritic-shaped cells were also strongly positive for IDO by light microscopy (Figure 5C). Double-immunofluorescence staining for CD123 and IDO was performed to reveal their colocalization (Figure 6A-6C; red staining for CD123, green staining for IDO, yellow staining for overlay of CD123, and IDO staining).

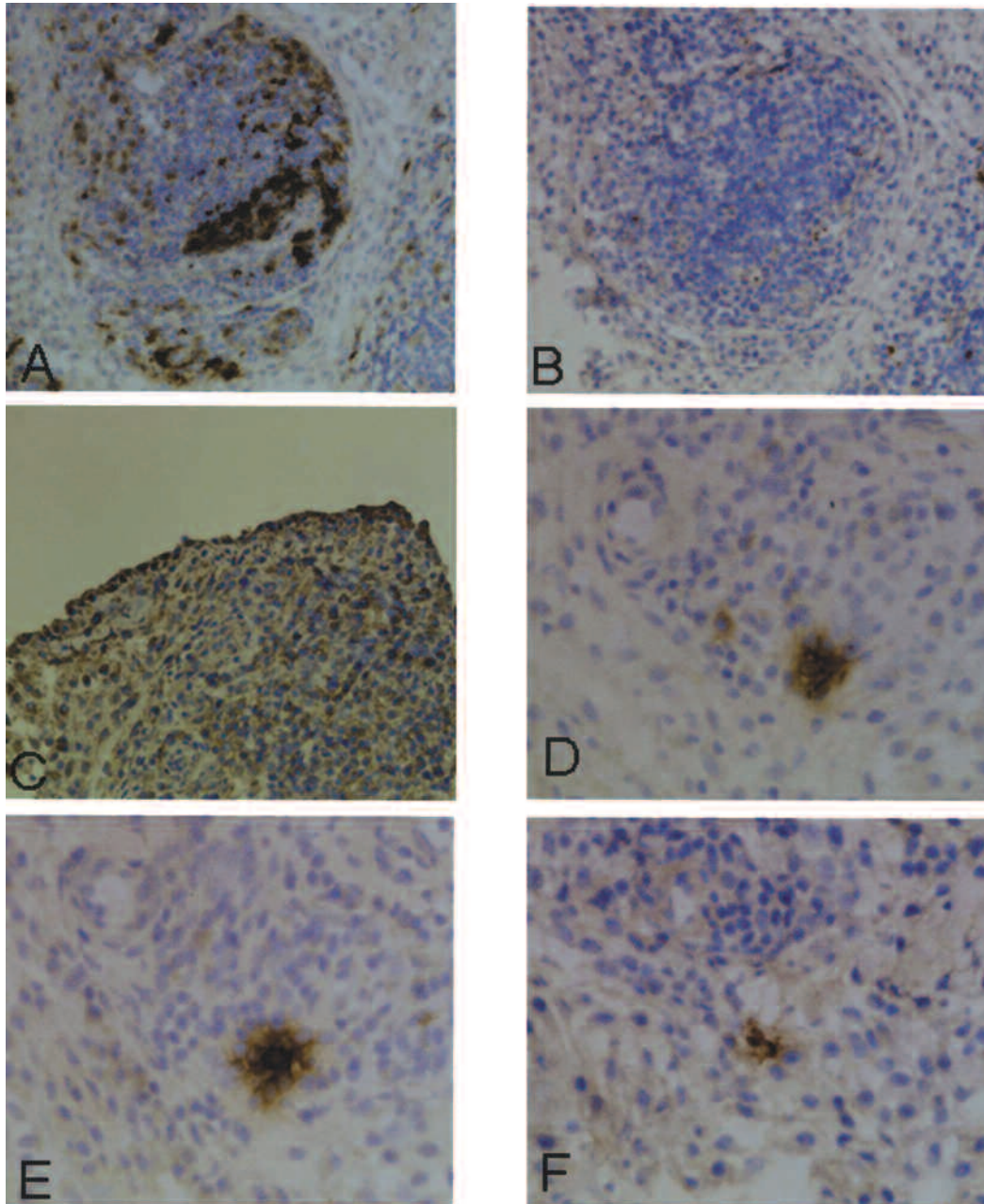


Figure 2. Representative images of 5 different areas of RA synovial tissues examined by light microscopy. Cells positive for DC-LAMP, mature myeloid dendritic cell (DC) marker, are expressed around the large lymphoid aggregation area (A). Indoleamine 2,3-dioxygenase (IDO) is expressed in the large aggregation area (B). DC-SIGN is expressed in the lining layer area (C). CD123, BDCA2, and IDO are expressed in the small lymphoid aggregation area (D, E, and F, respectively). The cells expressing DC-LAMP and IDO are different (A, B). Cells positive for CD123 and BDCA2, plasmacytoid DC markers, exhibit dendritic shapes and are related to IDO-positive cells. Original magnification: $\times 200$ (A, B, C); $\times 400$ (D, E, F).

DISCUSSION

We demonstrated that mature mDC possibly play the main role in rheumatoid inflammation, since mDC increased significantly according to progression of grading, especially lymphoid cell infiltration. The proportion of IDO-positive pDC infiltration was not correlated with staging or grading,

suggesting that pDC could not fully suppress the cellular activity and progression of rheumatoid inflammation *in situ*.

Chronic inflammatory arthritis is associated with migration of mDC and leukocytes in SF and synovial tissue²⁰⁻²⁷. DC, which can affect both T and B cell functions, control immunity and tolerance^{28,29}. Recently, it has become evi-

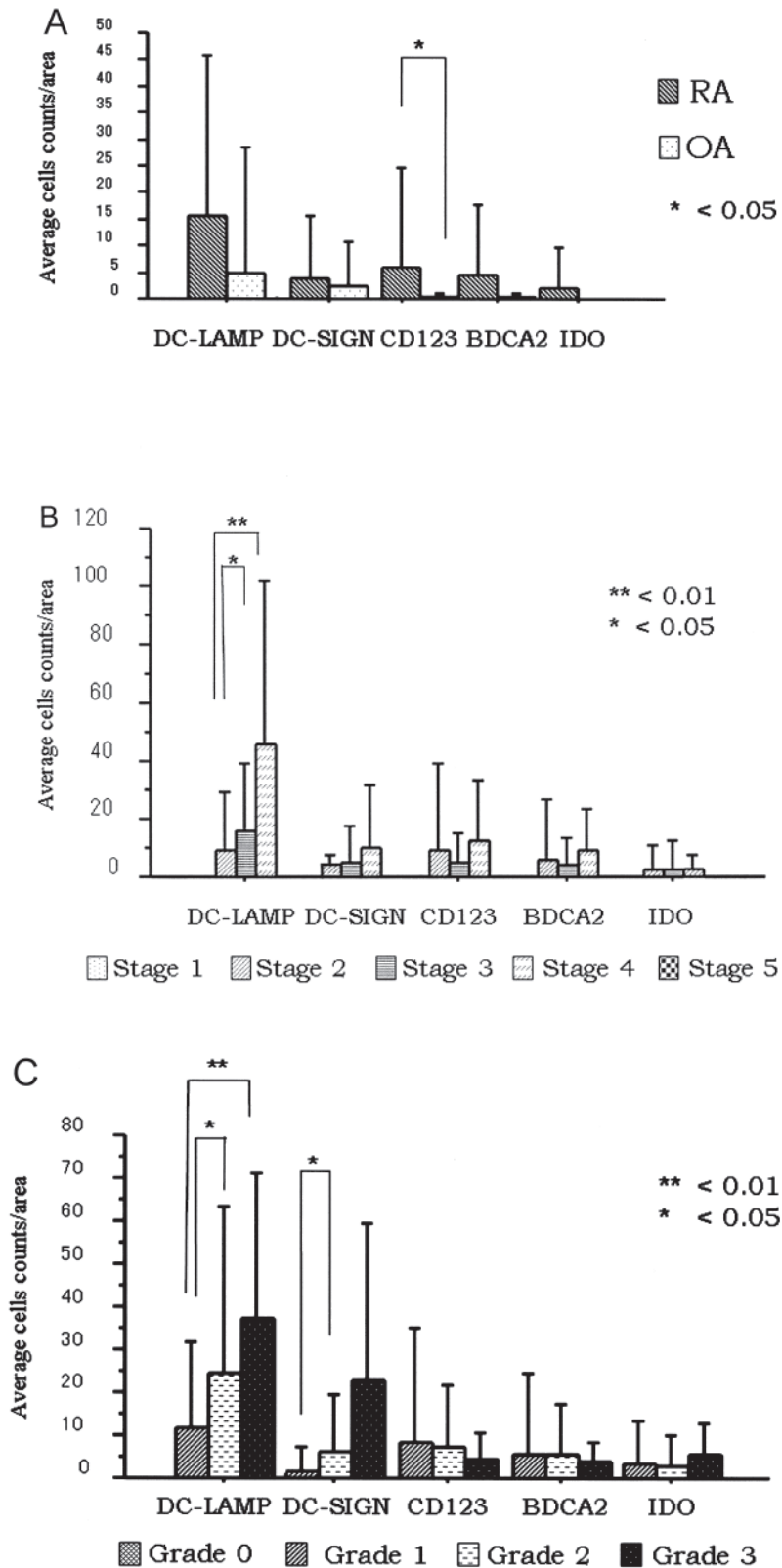
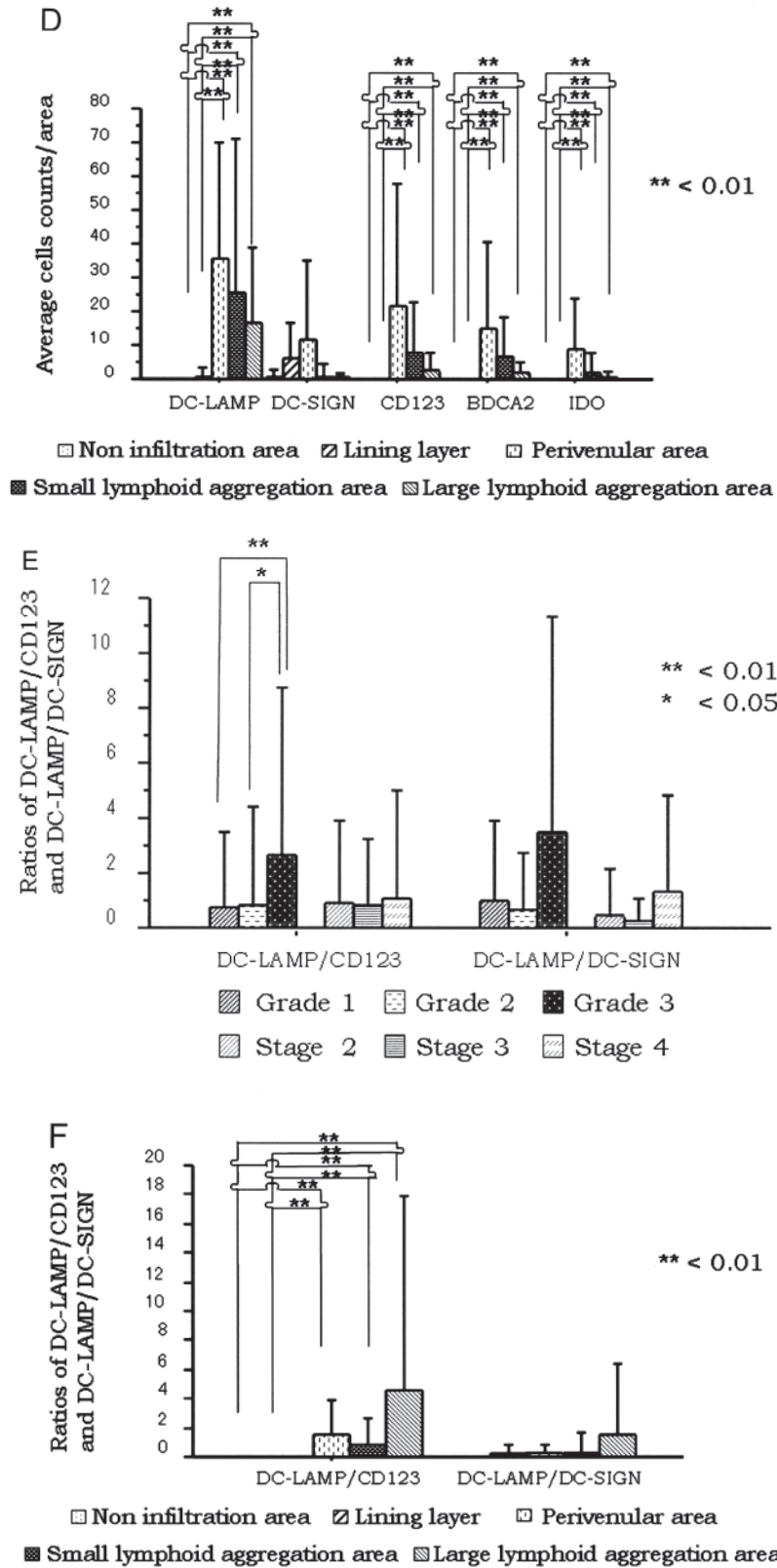


Figure 3. Cellular ratios of dendritic cell marker-positive cells in rheumatoid arthritis (RA) and osteoarthritis (OA) (A). Evaluation of the staging (B), inflammatory grading (C), localization of dendritic cells (D), and ratio of DC-LAMP/CD123 and DC-LAMP/DC-SIGN (E, F) in RA and OA synovial tissues. Synovial tissues from 30 patients with RA and 7 with OA were examined by immunohistochemistry and estimations were made in 5 different areas in each section. DC-LAMP is a marker for mature myeloid DC, DC-SIGN for immature myeloid DC, and CD123 for plasmacytoid DC. The following significant



differences were detected: RA vs OA for CD123 ($*p < 0.05$) (A); stage 2 vs stages 3 and 4 for DC-LAMP ($**p < 0.01$ and $*p < 0.05$, respectively) (B); grade 1 vs grades 2 and 3 for DC-LAMP and ratio of DC-LAMP/CD123 ($**p < 0.05$) and grade 1 vs grade 2 for DC-SIGN ($**p < 0.05$) (C, E); and no infiltration and lining layer areas vs perivenular infiltration, small aggregation, and large aggregation areas for DC-LAMP, CD123, BDCA2, indoleamine 2,3-dioxygenase (IDO) and ratio of DC-LAMP/CD123 ($**p < 0.01$) (D, F).

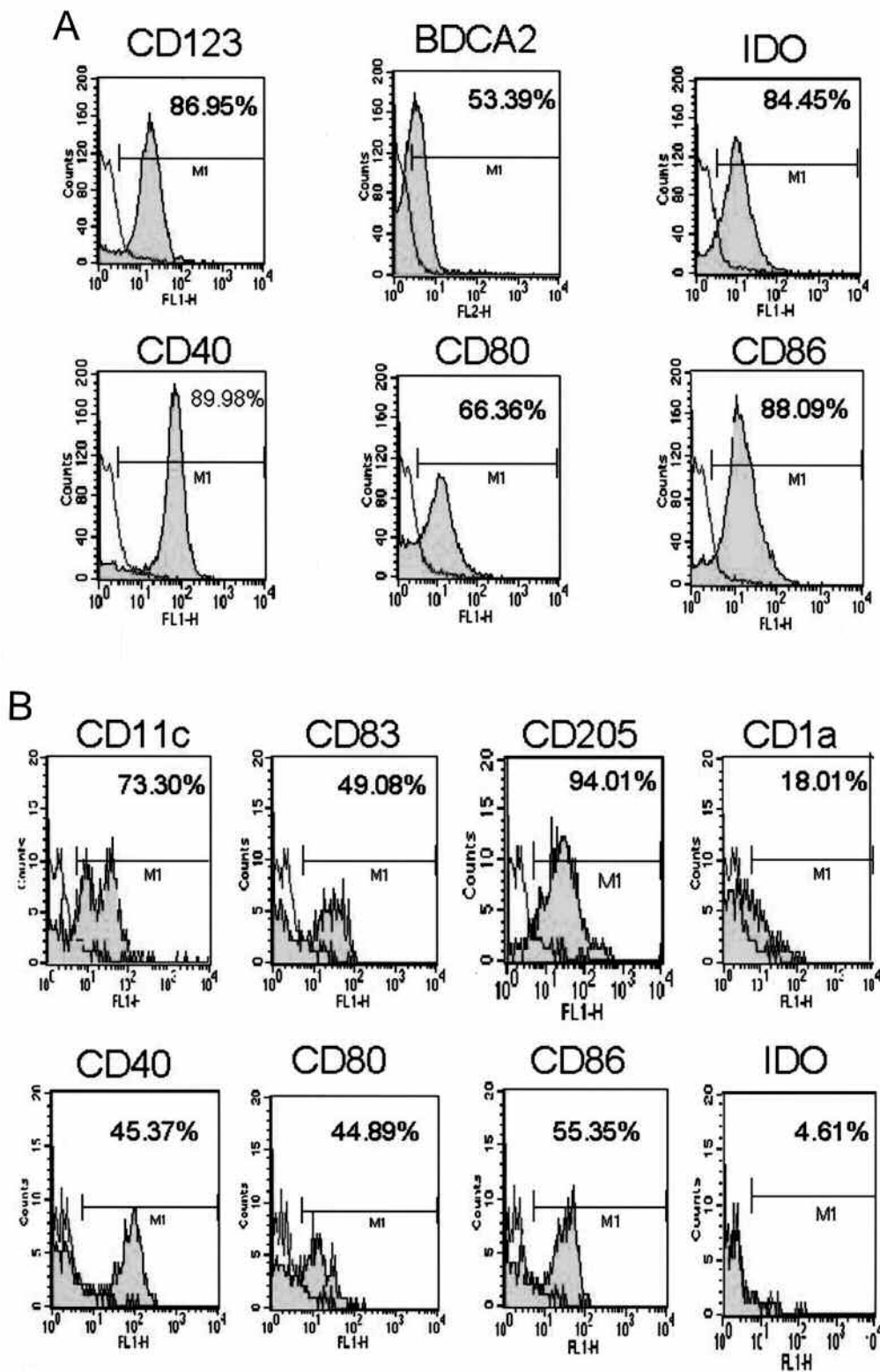


Figure 4. Cellular profiles of positively and negatively sorted cells from RA synovial fluid samples (A, B), RA synovial tissues (C, D), and OA synovial fluid samples (E, F). Positively sorted cells were analyzed for their expressions of CD123, BDCA2, IDO, CD40, CD80, and CD86, while negatively sorted cells were analyzed for expressions of CD11c, CD83, CD205, CD1a, CD40, CD80, CD86, and IDO, by flow cytometry. Results are shown as percentages of the positive cells. Representative cases are presented.

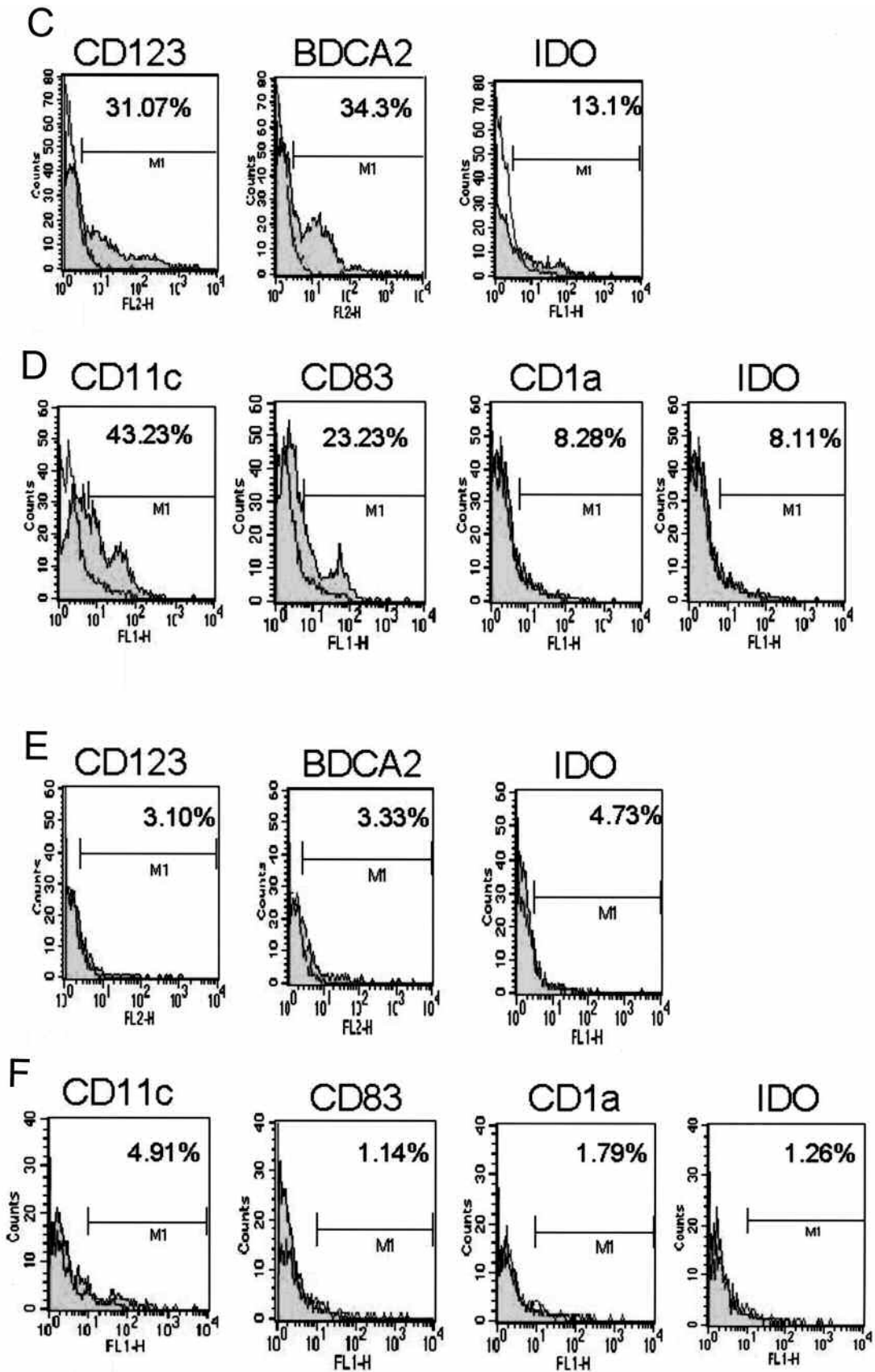


Figure 4. Continued

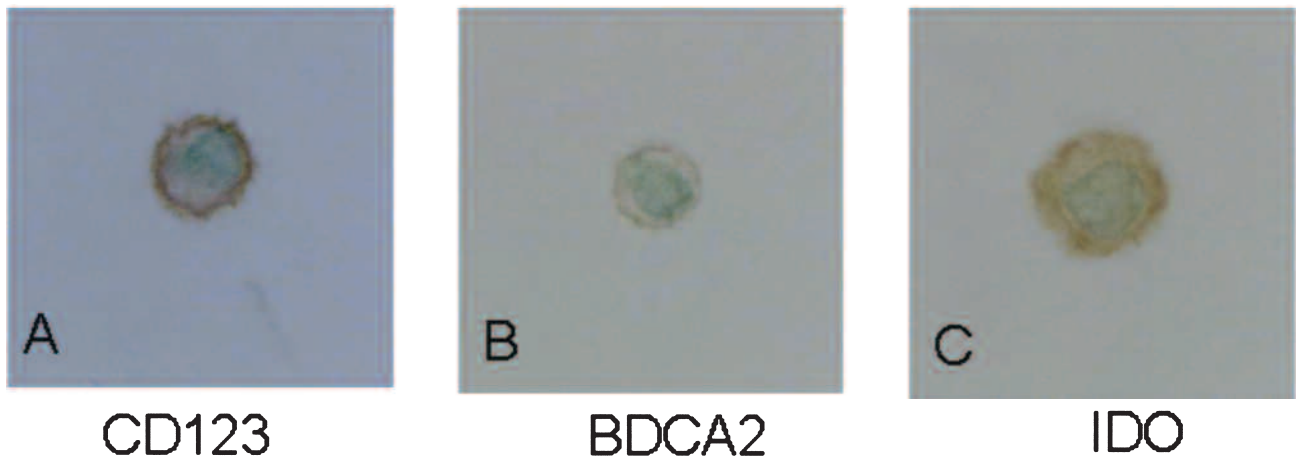


Figure 5. Immunohistochemical images show expression of plasmacytoid dendritic cell markers CD123 (A) and BDCA2 (B) and IDO (C) in positively sorted cells from RA synovial fluid.

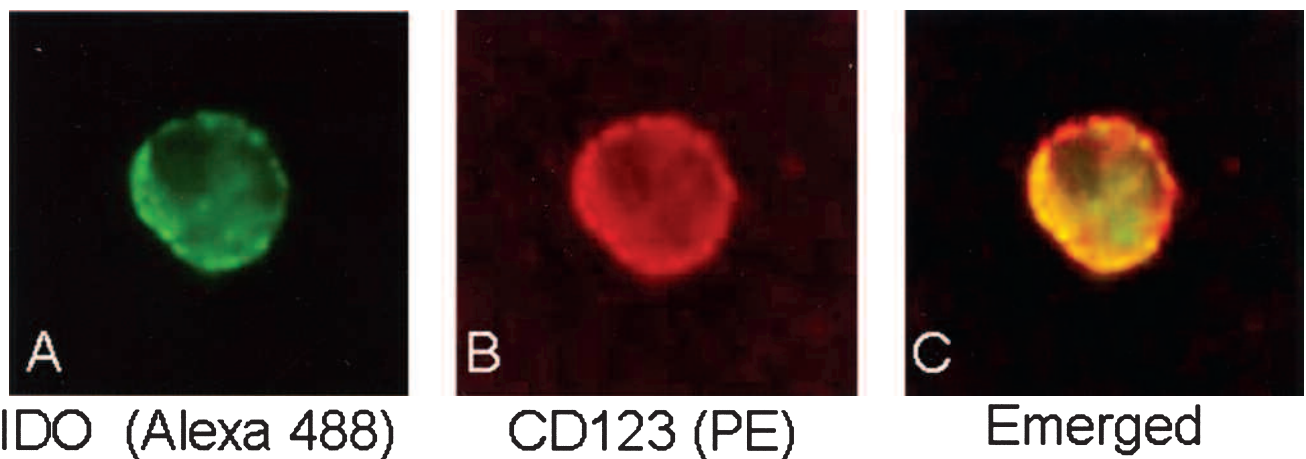


Figure 6. Double-immunofluorescence staining of positively sorted cells from RA synovial fluid for IDO (green; A) and CD123 (red; B). The overlaid image reveals that these 2 molecules show similar immunostaining patterns (yellow; C). Original magnification: $\times 800$ (A–C).

dent that IDO-expressing regulatory DC play a role in tolerance, in addition to their roles in the manner in which the complex balance between immunity and tolerance is maintained in health and broken in autoimmune diseases such as RA, systemic lupus erythematosus, psoriasis, and thyroiditis³⁰.

In our study, cells positive for DC-LAMP, a marker for mature mDC²⁰, were mainly observed in small and large lymphoid aggregation areas of RA synovial tissues, while cells positive for CD123, a marker of pDC⁸, were scattered around perivascular infiltration, small lymphoid aggregation, and large lymphoid aggregation areas. The mDC/pDC ratios were significantly increased along with progression of grading in RA tissues. Staging system and inflammatory grading system seemed to be linked with levels of CRP, but not significantly. The discrepancy between the serologic and histopathological status may be due to the cases of advanced disease and difference of the treatment received.

Thus, it seems evident that mDC, especially mature mDC, are dominant in the immune balance of RA, as reported³⁰. An mDC/pDC ratio of approximately 2.3 has been reported for RA synovial tissue^{26,27}. However, these previous reports did not provide any details regarding the pathological staging or inflammatory grading. Cells positive for DC-SIGN, a marker of immature mDC²⁰, tended to increase in the lining layer and perivascular areas with progression of grading and staging in our study. In contradiction to our immunohistochemical results on serial sections, Soilleux, *et al*²¹ have reported that a small subset of BDCA-2-positive pDC express DC-SIGN. pDC may not express detectable levels of DC-SIGN²⁰, because DC-SIGN-positive pDC were a minimal part of total pDC²¹. It is possible that immature DC may be on standby in their area for stimulation by certain kinds of antigens. The mature/immature mDC ratio tended to increase with advancing stage and grade. Previous

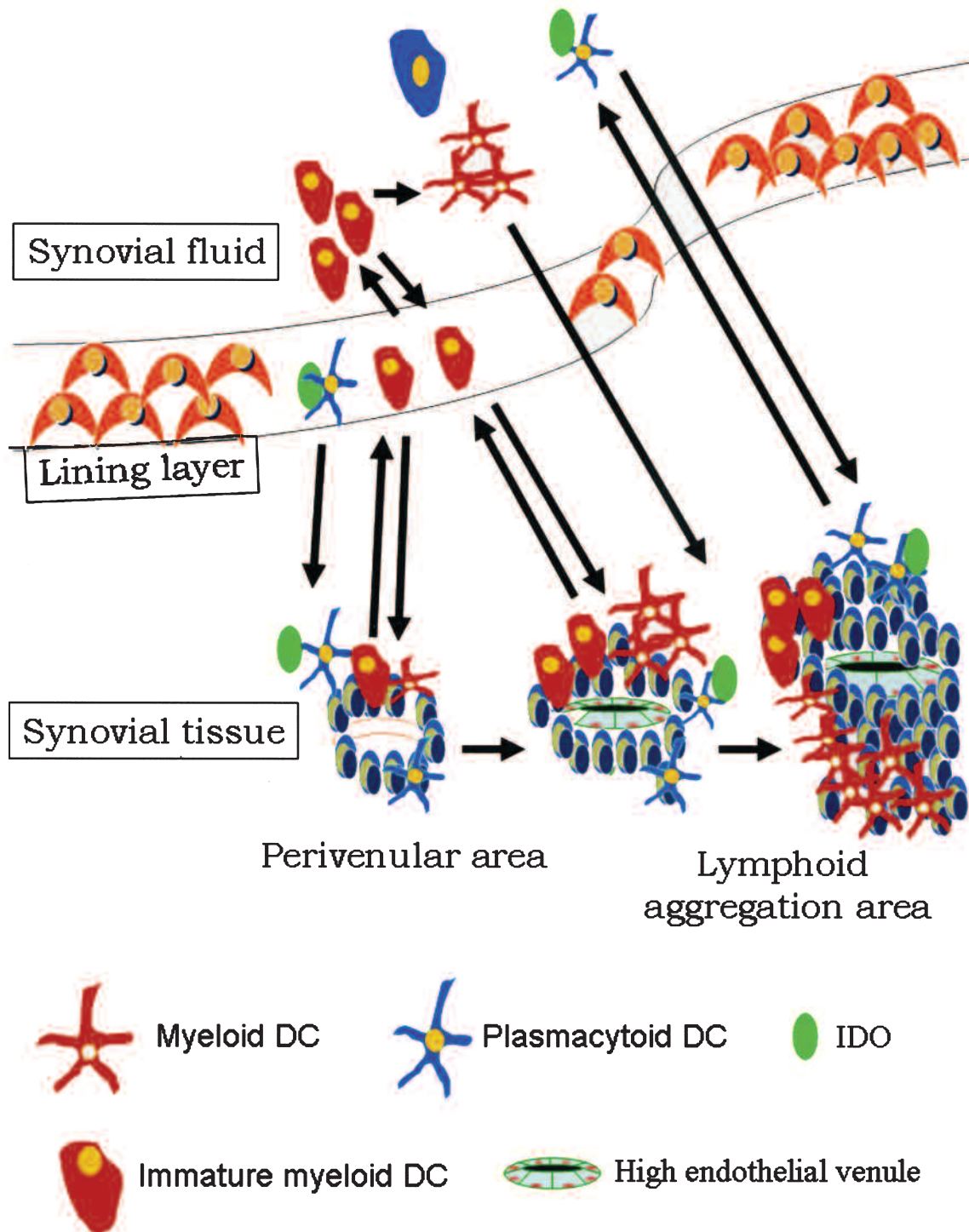


Figure 7. Putative schematic of the localization and migration of dendritic cells (DC) in RA. The count of mature myeloid DC increases with advanced staging and higher grading, whereas the counts of plasmacytoid DC and indoleamine 2,3-dioxygenase (IDO)-positive cells do not. IDO-positive plasmacytoid DC are scattered around the perivenular infiltration, small aggregation, and large aggregation areas.

studies have reported mature mDC (DC-LAMP-positive)/immature mDC (CD1a-positive) ratios of 0.8–1.1 in RA synovial tissues, but also did not provide any details of the pathological stage or inflammatory grade^{22,23}. There has

been one report in which fresh mDC were isolated from RA synovial tissue and analyzed by flow cytometry²⁵. However, the techniques used to isolate these cells from the RA synovial tissue were not described in detail²⁵. In addition, it is

difficult to estimate the maturity of pDC *in loco* by currently available immunohistologic methods, such as determining the amounts of interferon- α (IFN- α) and IFN- β production¹⁶.

In RA SF samples, the mDC/pDC ratios were markedly increased compared to those in OA SF samples. Studies have reported that the mDC/pDC ratio was about 10 in RA SF, and that the mDC and pDC were all immature or semi-mature, as evaluated by FACS analysis^{26,27,31,32}. In our study, the status of mDC was found to be immature or semi-mature due to their moderate positivities for costimulatory molecules⁷. The status of pDC was also found to be mature or semi-mature due to their strong positivities for costimulatory molecules by FACS analysis. Therefore, the patients in our study seemed to have active RA, but received no aggressive treatment. Indeed, we obtained abundant mononuclear cells from SF of the patient receiving only NSAID versus that of the patients receiving DMARD. In contrast, we tried to estimate the degree of inflammation in some samples having an aggressive treatment, such as in the patients receiving biologic agents and aggressive combination therapy of nonbiologic DMARD, but could not obtain sufficient nucleated cells for analysis (data not shown). As pharmacological approaches to the patients were not homogenous in our study, we have to study more patients and their correlation with each other.

IDO-expressing cells appeared to correspond well to CD123- and BDCA2-positive cells in RA SF and RA synovial tissue in our study. These data indicate that the positive cells were pDC, although IDO-expressing cells have been identified among some macrophages, skin fibroblasts, and syncytiotrophoblasts^{28,33,34}. IDO is known to be a potent immunoregulatory enzyme, which can deplete tryptophan and increase proapoptotic kynurenines, thereby regulating T cell proliferation and survival *in vitro* and *in vivo*^{35,36}. IDO is induced by IFN- γ in inflammatory states, such as microbial infections, and during immunosuppression in pregnancy^{33,36}. Certain human DC can constitutively express immunoreactive IDO protein, although IDO cannot normally be activated without a signal, such as CTLA4-CD80/CD86³⁷. IDO can also activate signaling systems, such as the GCN2 kinase pathway and mammalian target of rapamycin kinase pathway of T cells, and thus suppress T cell function²⁸.

We show a putative schematic of the localization and migration of DC and IDO-positive cells in RA in Figure 7. We detected IDO-expressing pDC in RA SF and examined their presence in the perivascular and small lymphoid aggregation areas, which are thought to represent states before the development of larger lymphoid aggregation areas, in RA synovial tissues by light microscopy. IDO-positive pDC may play roles in inducing and regulating the growth of lymphoid aggregation in some areas by initiating the tolerance of T cells, and also induce regulatory T cells, defined

as CD4+CD25+ T cells³⁸. However, pDC were so scarce in RA synovial tissues that they could not contribute to sufficient induction of the tolerance mechanism with the dominant presence of mDC. The low pDC and IDO-positive cell counts in all grades and stages also seem to support an insufficient regulatory mechanism of tolerance by IDO-positive pDC.

Increased serum kynurenine/tryptophan ratios have been reported in patients with RA compared to healthy controls³⁸. IDO plays a critical role in the regulation of T cell-mediated immune responses, since it catalyzes the oxidative catabolism of tryptophan to kynurenine²⁸. Thus, IDO may be more activated in the tryptophan metabolism pathway in RA than in healthy controls. The percentage of CD4+CD25+ T cells among the total CD4+ T cells was found to be increased in peripheral blood from patients with RA, and even further increased in RA SF, compared with the corresponding levels in peripheral blood from healthy controls³⁹. However, we found that the immune balance in RA showed a trend toward high inflammatory activity. We estimated that the induction of tolerance was not sufficient against the inflammatory activity, due to weakness of the capability, limited cell counts, or abnormal function of IDO-producing cells and regulatory T cells^{38,39}. CTLA4-Ig, which competes with CD28 for CD80 and CD86 on the surface of antigen-presenting cells and can therefore be used to selectively modulate T cell activation, was reported to show clinical benefits for patients with RA. Adjustment of the immune balancing system toward tolerance is important for therapy of RA³⁷.

Our results indicate that mature mDC play a central role in the inflammatory process in RA. The presence of IDO-positive pDC suggests a tolerance mechanism in RA, but this mechanism is not enough to suppress the marked inflammation of RA. IDO-positive pDC may be insufficient for functional induction of tolerance in active RA, due to their small population or malfunction. A better understanding of these cells could lead to the development of new therapeutic strategies to increase the orientation toward tolerance.

ACKNOWLEDGMENT

We thank Dr. Makoto Ohata, Yamagata Saisei Hospital, for obtaining the tissues, and Hitoshi Suzuki, Kumiko Abe, and Eiko Saito, Yamagata University School of Medicine, for staining and making tissue sections.

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