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Association of CD90 Expression by CD14$^+$ Dendritic-Shaped Cells in Rheumatoid Arthritis Synovial Tissue With Chronic Inflammation

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Objective. CD14$^+$ dendritic-shaped cells show a dendritic morphology under the electron microscopy and engage in a pseudoemperipolesis phenomenon with lymphocytes. CD90 has been used as a marker of a major subset of fibroblast-like synoviocytes in rheumatoid arthritis (RA). In this study, we investigated the significance of CD90 expression in CD14$^+$ dendritic-shaped cells and its correlation with RA chronic inflammation.

Methods. Double immunofluorescence staining for CD14 and CD90 was performed in the collected tissues, including 12 active RA synovial tissues. The localization of CD14$^+$CD90$^+$ cells, the percentages of CD14$^+$CD90$^+$ cells and vascular areas, the degree of synovitis, and clinical data were investigated. Furthermore, CD14$^+$CD90$^+$ cells analyzed by flow cytometry (CD14^{high}CD90$^{intermediate\ (int)}$ cells) were sorted from RA synovial cells, and we examined their potential to differentiate into dendritic cells.

Results. Double immunofluorescence staining showed that CD14$^+$CD90$^+$ cells were abundant in RA synovial tissues. The percentages of CD14$^+$CD90$^+$ cells and vascular areas correlated with some of the Krenn synovitis scores, but neither showed a strong correlation with RA disease activity parameters. Flow cytometry analysis indicated that CD14^{high}CD90^{int} cells were more abundant in both peripheral blood samples and synovial tissues in patients with active RA. CD14^{high}CD90^{int} cells were more likely to differentiate into dendritic cells in vitro.

Conclusion. CD14$^+$ dendritic-shaped cells expressed CD90 in the perivascular areas of RA synovial tissues. These findings suggest that CD14$^+$CD90$^+$ dendritic-shaped cells migrate from the peripheral blood to the synovial tissue, the site of inflammation, and may contribute to the chronic inflammation of RA as dendritic progenitor cells.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease, and prolonged arthritis leads to joint destruction, followed by functional limitations that interfere with daily life. Inflammation in human RA is thought to originate from the synovial tissue in joints (1). Normal synovial membrane that lies along the cavity is smooth and consists of one to two layers of synovial cells. In contrast, RA synovial tissue shows a villous proliferation of synovial lining cells, hyperplasia of the synovial lining layer, and increased vascularity histopathologically. In addition, various immune cell types are observed, including infiltrating inflammatory cells, such as lymphocytes, neutrophils, macrophages, and spindle-shaped fibroblasts. Notably, many spindle-shaped fibroblasts are prominent in the sublining layer, and they are known as fibroblast-like synoviocytes (FLSs) (2,3). FLSs have many functions, including the production of inflammatory cytokines and proteolytic enzymes (4), promotion of differentiation into osteoclasts (5), and regulation of various processes in RA. Thus, FLSs are one of the cells that characterize RA.

We have previously reported electron microscopic observations of RA synovial tissues (6,7). Many dendritic-shaped cells are found in RA synovial tissues, and we confirm that they are positive for CD14 via enzyme electron microscopy. Also, our

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studies using electron microscopy have revealed that $CD14^+$ dendritic-shaped cells display a pseudoemperipolesis phenomenon. They extend their long protrusions to patronize plasma cells or lymphocytes three-dimensionally and form cell-to-cell contact with adjacent cells. Furthermore, we have investigated that most of the $CD14^+$ dendritic-shaped cells express human leukocyte antigen-DR isotype (HLA-DR), and some of them also express CD68. These results suggest that there are some $CD14^+$ dendritic-shaped cells that may have two cell types simultaneously, namely, the $HLA\text{-}DR^+$ immune response type and the $CD68^+$ phagocytic macrophage type. Additionally, based on the morphology of these cells, we were interested in investigating whether they had mesenchymal functions. In our preliminary experiments, immunohistological staining for vimentin showed that almost all cells in RA synovial tissues were positive, including $CD14^+$ dendritic-shaped cells. In contrast, immunohistological staining for CD90 showed that $CD14^+$ dendritic-shaped cells were strongly positive in the perivascular areas of RA synovial tissues. Although the number of $CD14^+CD90^+$ cells was not high in the whole RA synovial tissues, they were abundant in the perivascular areas. Therefore, we consider that they might be involved in their spreading and T-cell activation or have multipotent differentiation ability like monocyte-derived multipotential cells (MOMCs) (8), and then we focus on $CD14^+CD90^+$ cells.

CD90 (also known as Thy-1) is a 25- to 37-kd, heavily N-glycosylated, glycophosphatidylinositol-anchored, conserved cell surface protein with a single V-like immunoglobulin domain, originally discovered as a thymocyte antigen (9). Recently, CD90 has been touted as a stem cell marker in various tissues, such as a hematopoietic stem cell marker in combination with CD34 and as a mesenchymal stem cell (MSC) marker in combination with CD105 (10). In addition, recent reports suggest that CD90 is a marker for a subset of FLSs that proliferate in the perivascular areas of RA synovial tissues and is considered to be involved in tissue destruction by highly expressed genes related to osteoclast differentiation or activation, such as receptor activator of nuclear factor-κB ligand (RANKL) (11). Furthermore, another article reports that $CD90^+HLA\text{-}DR^{high}$ fibroblasts express interleukin-6 (IL-6) and are involved in the pathogenesis of RA (12). In this study, we investigated the expression of CD90 in $CD14^+$ dendritic-shaped cells immunohistochemically and correlation between $CD14^+CD90^+$ cells and RA pathogenesis.

MATERIALS AND METHODS

Synovial tissues. We obtained synovial tissues from 17 patients with active RA with serum C-reactive protein (CRP) levels at greater than 2 mg/dl at the time of joint surgeries, such as synovial biopsies, synovectomy, and replacement. As controls, six osteoarthritis (OA) synovial tissues from joint replacement surgeries and four granulation tissues from fracture surgeries or revision surgeries were also collected. Among the collected tissues, five RA synovial tissues, four OA synovial tissues, and one granulation tissue were prepared for electron microscopy. Among the collected tissues, 12 RA synovial tissues, two OA synovial tissues, and three granulation tissues were prepared in paraffin blocks for histological staining and also for cell culture in vitro. The diagnosis of RA was based on the American College of Rheumatology (ACR) criteria in 1987 (13) or the ACR/European League Against Rheumatism (EULAR) classification criteria in 2010 (14). This study was approved by the Ethics of Human Experiments Committee at Hirosaki University Graduate School of Medicine, Hirosaki, Japan; Hirosaki Memorial Hospital, Hirosaki, Japan; and Iwate Medical University School of Medicine, Morioka, Japan. Informed consent was obtained from all patients.

Peripheral blood samples and synovial cells derived from synovial tissues for flow cytometry analysis. We obtained peripheral blood samples from seven patients with active RA who were not treated with glucocorticoids or antirheumatic drugs. In addition, peripheral blood samples from seven patients with RA in clinical remission with methotrexate (MTX) and biological originator and targeted synthetic disease-modifying anti-rheumatic drugs (bo/tsDMARDs) therapy and peripheral blood samples from five patients with OA were collected. All peripheral blood samples were used for flow cytometry analysis. Synovial cells were prepared by immediately shredding synovial tissue of five patients with RA collected at the time of surgery and used for flow cytometry analysis. Informed consent was obtained from all patients.

Electron microscopy. Collected tissues were immediately processed into 1 × 1 mm pieces. Prefixation was performed in 2% paraformaldehyde (PFA) and 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 hours at 4°C. After osmication in 1% osmium tetroxide, the specimens were dehydrated and embedded. To obtain a wide field of view images using multiscale electron microscopy (MS-EM), slices of resin-embedded tissues were cut using an ultramicrotome (Leica EM-UC6), placed on conductive, hydrophilic, coated glass slides, and stained with 1% toluidine blue. The images were digitalized using NanoZoomer software (NanoZoomer Digital Pathology, NDP view 2.2.1; Hamamatsu Photonics) and saved as digital slides. The same slice was stained with 1% uranyl acetate and lead and viewed on a scanning electron microscope (Hitachi SU8010). Tiling images were constructed with ImageJ software (National Institutes of Health). Image data were saved as virtual slides for MS-EM. NanoZoomer software (Hamamatsu Photonics) was used for a wide field of view images.

Double immunofluorescence staining. For immunofluorescence staining of paraffin sections, the collected tissues were fixed in 4% PFA for 2 hours at room temperature, dehydrated

through a series of graded ethanol, and then embedded in paraffin blocks. The paraffin sections were cut at a 4-μm thickness and routinely deparaffinized. After heat-induced antigen retrieval, fluorescent immunostaining was conducted using an anti-CD14 rabbit monoclonal antibody (1:200; Abcam plc) and an anti-CD90 mouse monoclonal antibody (1:1000; ProteinTech) and then visualized using goat anti-rabbit immunoglobulin G (IgG) (1:200, Alexa Fluor 594; Abcam plc) and goat anti-mouse IgG (1:200, Alexa Fluor 488; Abcam plc) as second antibodies. These sections were observed using a fluorescence microscopy (All-in-One Fluorescence Microscope, BZ-X710; Keyence). In addition, 4′, 6-diamidino-2-phenylindole dihydrochloride was used as a counterstain.

Histological evaluation. We used the Krenn synovitis score with hematoxylin and eosin (HE) stained sections (15). The Krenn synovitis score consists of three items, each graded from 0 to 3: enlargement of the synovial lining cell layer (0, one layer; 1, two to three layers; 2, four to five layers; and 3, more than five layers), the density of the resident stromal cells (0, normal; 1, slightly; 2, moderately; and 3, greatly), and inflammatory infiltrates (0, no; 1, few; 2, numerous; and 3, numerous). Moreover, we originally graded the total score as a Krenn inflammation score from 0 to 3 (0, sum 0-1; 1, sum 2-4; 2, sum 5-7; and 3, sum 8-9).

The percentages of CD14^{+}CD90^{+} cells and vascular areas. Ten fields of view were randomly extracted from each section of double-immunofluorescence stained tissues to investigate the percentages of CD14^{+}CD90^{+} cells and vascular areas in a fluorescence microscopy (Keyence). Each fluorescence color was carefully extracted, and their areas were calculated using the cell count function attached to the fluorescence microscope system. The setup of color density was kept constant so as to avoid differences among tissues and the percentages of CD14^{+}CD90^{+} cells and vascular areas were calculated in RA synovial tissues (n = 12) and controls (n = 5).

Cell culture and flow cytometry analysis. The collected synovial tissues were digested with 3 mg/ml collagenase type V (Wako Pure Chemical Industries) for 3 hours at 37°C, washed with phosphate buffered saline, centrifuged at 400*g* for 5 minutes, and then resuspended in α Modified Eagle Minimum Essential Medium (Sigma-Aldrich) containing 10% fetal bovine serum (Thermo Fisher Scientific) and antibiotics (100 units/ml penicillin G and 100 μg/ml streptomycin) (Thermo Fisher Scientific). Synovial cells were then seeded in 100-mm culture dishes and cultured at 37°C in a 5% carbon dioxide incubator. The medium was replaced twice a week, and cultured cells were passaged at subconfluency. Cultured synovial cells of passage zero (P0) and passage one (P1) from patients with RA were analyzed by flow cytometry (FACScan, Becton Dickinson) to investigate the number of CD14^{+}CD90^{+} cells. Fluorescein isothiocyanate–coupled anti-CD14 antibody (Abcam) and phycoerythrin-coupled anti-CD90 antibody (Abcam) were used, and the fluorescence intensity was compared with each isotype control (Abcam).

Differentiation potential of CD14^{high}CD90$^{intermediate\ (int)}$ cells into dendritic cells. To investigate the potential of CD14^{high}CD90^{int} cells to differentiate into dendritic cells, CD14^{high}CD90^{int} cells were sorted from cultured synovial cells derived from RA synovial tissues of five patients with RA and cultured in vitro. Both sorted CD14^{high}CD90^{int} and non-CD14^{high}CD90^{int} cells were cultured using a dendritic differentiation medium (Promocell) in a 24-well dish for 7 days. To prove the differentiation of each cell to dendritic cells, the expression of CD83, which is an original marker of dendritic cells, was analyzed using PerCP with a cyanine dye (PerCP–Cy5.5)–coupled anti-CD83 antibody (Biolegend) by flow cytometry.

Data collection. The following demographic data were recorded at the time of tissue collection: gender, age, disease duration, serum CRP levels, erythrocyte sedimentation rate (ESR), serum rheumatoid factor positivity (normal limit <15 IU/ml), serum anti-cyclic citrullinated peptide antibody positivity (normal limit <4.5 U/ml), serum matrix metalloproteinase-3 levels, disease activity scores in 28 joints with four variables, including CRP, disease activity scores in 28 joints with four variables, including ESR, simplified disease activity index (SDAI), clinical disease activity index, and medication (MTX, bo/tsDMARDs, and glucocorticoids).

Statistical analysis. Mann–Whitney *U* test and Spearman's rank correlation coefficient were used to assess significant differences. *P* values less than 0.05 were considered statistically significant.

RESULTS

CD14^{+} dendritic-shaped cells in RA synovial tissue. Figure 1A, B, and C show HE staining of RA synovial, OA synovial, and granulation tissues, respectively. These are pathologically referred to as nonspecific inflammation, but the findings under electron microscopy are markedly different among them. RA synovial tissues are rich in various cells, including lymphocytes, neutrophils, plasma cells, and spindle-shaped fibroblasts (Figure 1D, G, and H). In OA synovial tissues, the surface of the synovial membrane is flattened, and the number of cells is low compared with RA synovial tissues (Figure 1E). In granulation tissues, the number of cells is high, but the types of cells are scarce (Figure 1F). In particular, many CD14^{+} dendritic-shaped cells are detected adjacent to the plasma cells in the sublining layer of RA synovial tissues (pseudoemperipolesis phenomenon) (Figure 1G). In addition, CD14^{+} dendritic-shaped cells are also detected in the perivascular areas of RA synovial tissues (Figure 1H). Figure 1I

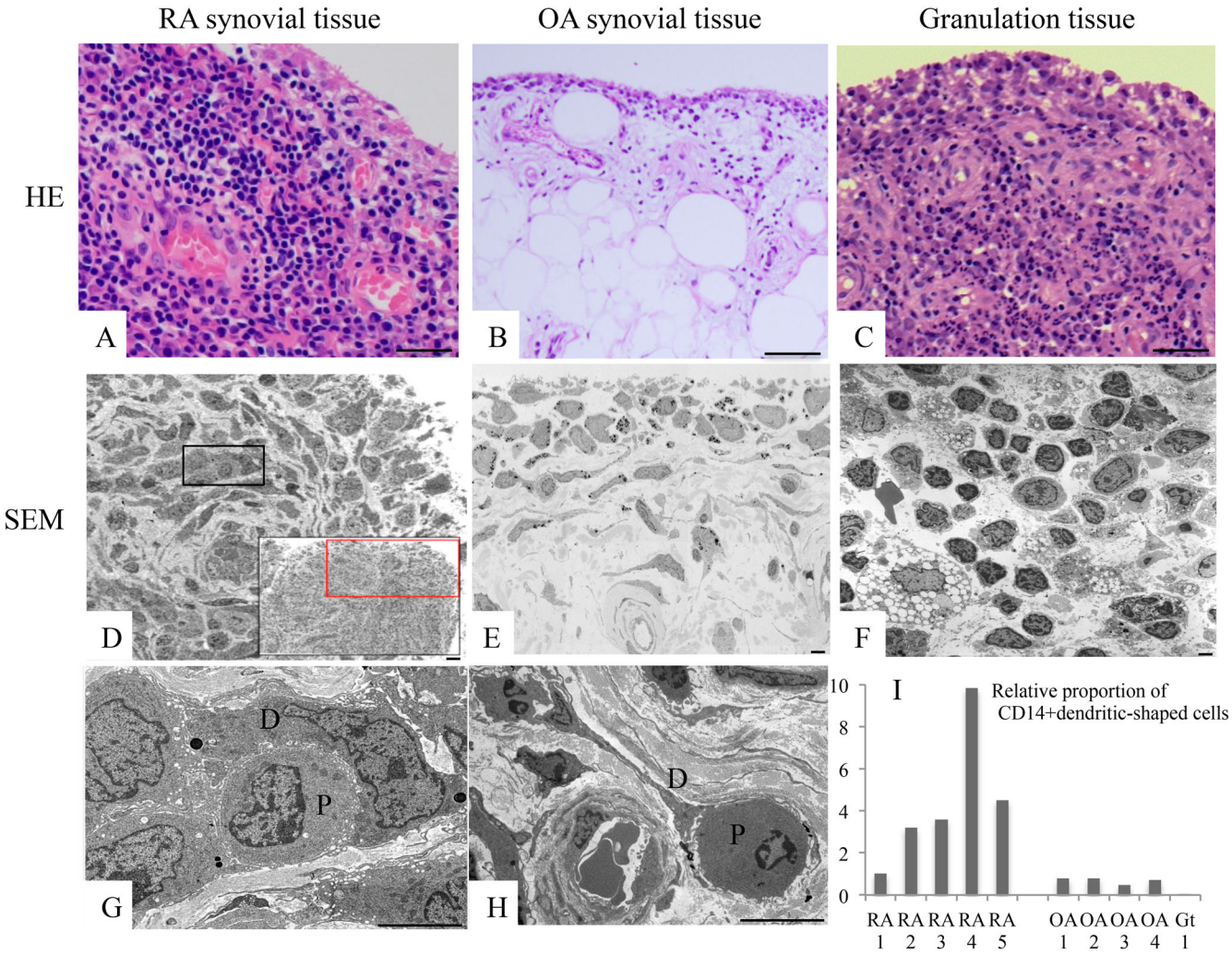

Figure 1. Histological and electron microscopic findings. Hematoxylin and eosin (HE) findings of human rheumatoid arthritis (RA) synovial (**A**), human osteoarthritis (OA) synovial (**B**), and human granulation tissues (**C**) are pathologically referred to as nonspecific inflammation. However, findings by scanning electron microscopy (SEM) of human RA synovial (**D**), human OA synovial (**E**), and human granulation tissues (**F**) were markedly different. **G,** The magnification of the square area in **D**. **H,** The magnification of the perivascular area in RA synovial tissues. **A-C**, Scale bars = 100 μm. **D-H**, Scale bars = 5 μm. "D" represents $CD14^+$ dendritic-shaped cells. "P" represents plasma cells. **I**, In SEM findings of five RA synovial tissues (RA 1-5), four OA synovial tissues (OA 1-4), and one granulation tissue (Gt 1), we randomly extracted 10 fields of view from each section, counted the number of $CD14^+$ dendritic-shaped cells showing a pseudoemperipolesis phenomenon, and compared the proportion of them with case RA-1 as 1.

shows the relative proportion of $CD14^+$ dendritic-shaped cells showing a pseudoemperipolesis phenomenon under electron microscopy. The number of $CD14^+$ dendritic-shaped cells was markedly higher in RA synovial tissues and almost absent in the OA synovial tissues and granulation tissue.

Expression of CD90 in $CD14^+$ dendritic-shaped cells. Double immunofluorescence staining for CD14 and CD90 in each paraffin section was performed (Figure 2). Figure 2A-E show the images of double immunofluorescence staining of different synovial tissues of five patients with RA. In all cases, many $CD14^+CD90^+$ cells were detected in the perivascular areas of RA synovial tissues (yellow). Red, signifying CD14 expression, appeared to predominate away from the blood vessels. In contrast, in control OA synovial tissues (Figure 2F and G) and granulation tissues (Figure 2H and I), $CD14^+CD90^+$ cells were barely detected in the whole tissues, including perivascular areas.

Correlation between the percentages of $CD14^{+-}$ $CD90^+$ cells and vascular areas with synovitis and RA disease activity. The clinical data of 12 patients with RA and five control patients at the time of tissue collection were gathered to investigate the correlation between the percentages of $CD14^{+-}$ $CD90^+$ cells and vascular areas with synovitis and RA disease activity (Table 1). Figure 3A shows the percentages of $CD14^+$ $CD90^+$ cells in all tissues. The results showed that $CD14^+CD90^+$

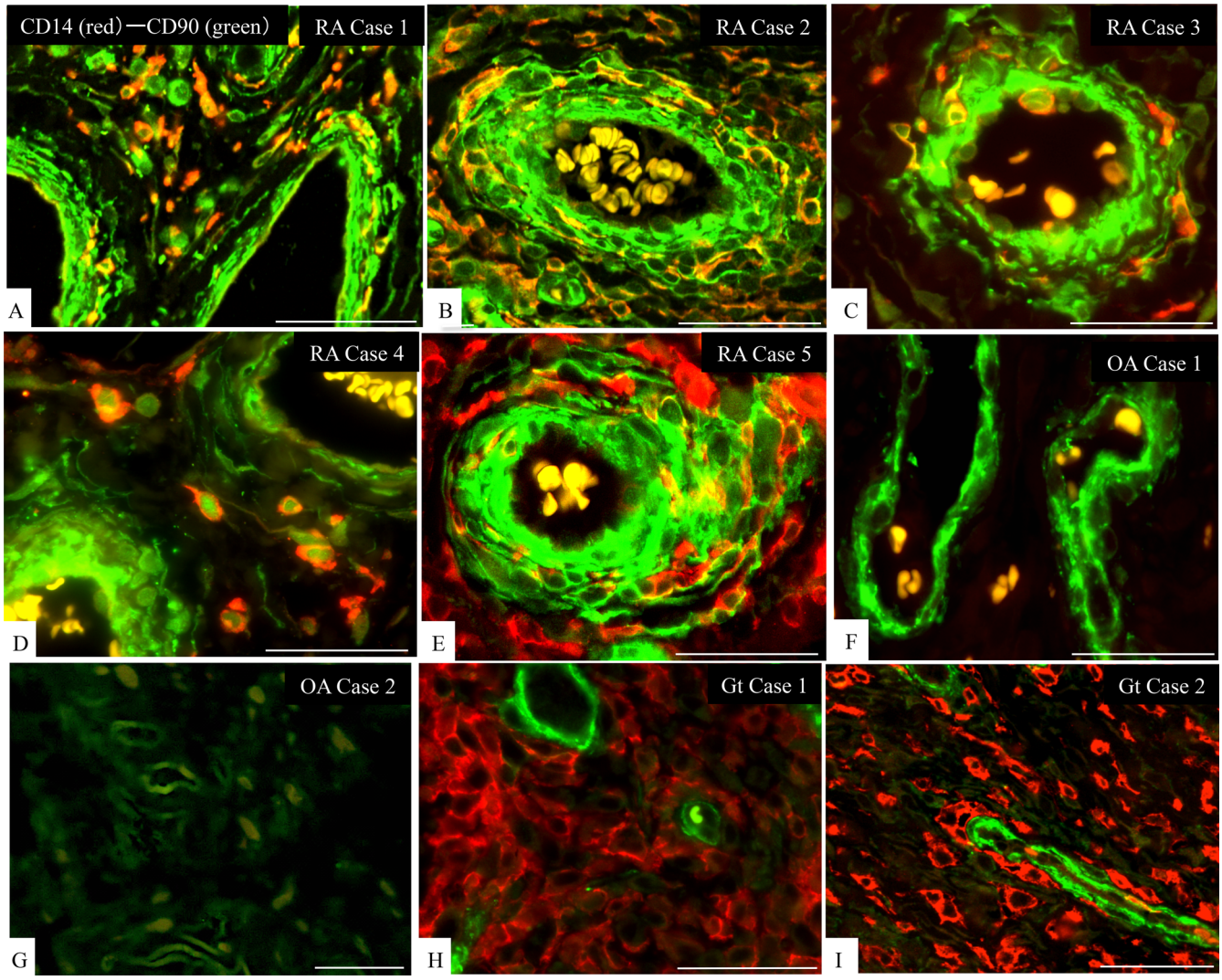

Figure 2. Double immunofluorescence staining for CD14 (red) and CD90 (green). **A-E**, Representative five different rheumatoid arthritis (RA) synovial tissues (RA case 1-5). In RA synovial tissues, many CD14$^+$ dendritic-shaped cells expressed CD90 (yellow), especially, in the perivascular areas. In contrast, in control osteoarthritis (OA) synovial tissues (**F** and **G)** (OA cases 1 and 2) and granulation tissues (**H** and **I)** (Gt cases 1 and 2), CD14$^+$CD90$^+$ cells were barely detected in the whole tissues, including the perivascular areas. 4′, 6-diamidino-2-phenylindole dihydrochloride (blue) was used as a counterstain. Scale bar = 100 μm.

cells were significantly more abundant in RA synovial tissues than in controls. Figure 3B-E show the correlation between the percentages of CD14$^+$CD90$^+$ cells and the degree of synovitis using the Krenn synovitis score in 12 RA synovial tissues. The percentages of CD14$^+$CD90$^+$ cells were weakly correlated with the synovial lining cell layer score, inflammatory infiltrate score, and Krenn inflammation score of the Krenn synovitis score (Figure 3B, D, and E). Figure 3F shows the percentages of vascular areas in all tissues. The results showed that there was no significant difference between RA tissues and controls. Figure 3G-J show the correlation between the percentages of vascular areas and the degree of synovitis using the Krenn synovitis score in 12 RA synovial tissues. The percentages of vascular areas were weakly correlated with the stromal cell density and inflammatory infiltrate of the Krenn synovitis score (Figure 3H and I). Table 2 and Figure 4 show the correlation between the percentages of CD14$^+$CD90$^+$ cells and the percentages of vascular areas with clinical parameters. The percentages of CD14$^+$CD90$^+$ cells had a weak correlation with the SDAI score but did not correlate with other parameters of RA disease activity. Also, the percentages of vascular areas did not correlate with any of these parameters.

CD14^{high}CD90^{int} cells in peripheral blood samples and synovial cells. Figure 5A-E are representative of flow cytometry analyses of the peripheral blood samples from OA controls (Figure 5A), patients with active RA (Figure 5B and C), and patients with RA in clinical remission with MTX or bo/tsDMARDs therapy (Figure 5D and E). CD14^{high} cells were mostly monocytes

Table 1. Clinical data of the patients

	RA (n = 12)	OA/Gt (n = 5)
Number of patients (male/female)	3/9	1/4
Age at surgery (years)	62 (36-73)	71 (65-76)
Disease duration (years)	5.7 (0-26)	
CRP at surgery (mg/dl)	4.4 (2.1-6.7)	
ESR at surgery (mm/hour)	72 (48-99)	
RF positive at surgery (%, median) (IU/ml)	54.5%, 217.9	
anti-CCP antibody (%, median) (U/ml)	27.3%, 160.6	
MMP-3 at surgery (ng/ml)	421 (90-742)	
DAS28CRP4 at surgery	3.88 ± 0.66 (2.26-5.25)	
DAS28ESR4 at surgery	4.54 ± 0.65 (3.47-5.57)	
SDAI at surgery	17.7 ± 4.6	
CDAI at surgery	13.3 ± 5.2	

Note: Medication at surgery: MTX (%, median), 33.3%, 7.5 mg/week; bo/tsDMARDs (%, n), 16.7%, 1 TCZ, 1 TOF; glucocorticoids (%, median) 8.3%, 9.0 mg/day.
Abbreviations: bo/tsDMARDs, biological originator and targeted synthetic disease modifying antirheumatic drugs; CCP, cyclic citrullinated peptide; CDAI, Clinical Disease Activity Index; CRP, C-reactive protein; DAS28CRP4, Disease Activity Scores in 28 joints with 4 variables, including CRP; DAS28ESR4, Disease Activity Scores in 28 joints with 4 variables, including ESR; ESR, erythrocyte sedimentation rate; MMP-3, matrix metalloproteinase-3; MTX, methotrexate; OA/Gt, osteoarthritis/granulation tissue; RA, rheumatoid arthritis; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index; TCZ, tocilizumab; TOF, tofacitinib.

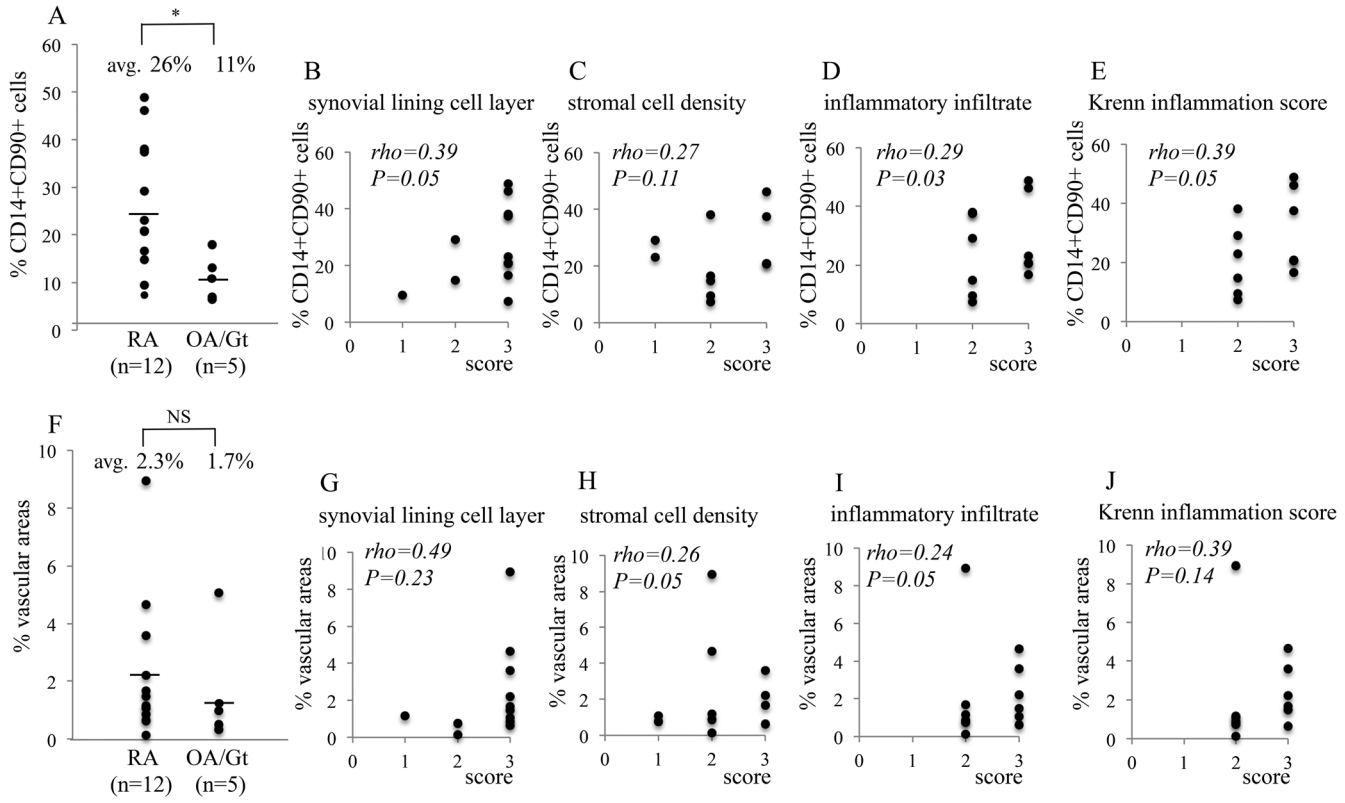

Figure 3. Correlation between the percentages of $CD14^+CD90^+$ cells and vascular areas with the grade of synovitis. **A**, The percentages of $CD14^+CD90^+$ cells in active rheumatoid arthritis (RA) synovial tissues and controls (osteoarthritis and granulation tissue [OA/Gt]) using cell analysis software attached to the fluorescence microscope system. $CD14^+CD90^+$ cells were significantly more abundant in RA synovial tissues than in controls. Bars show the average value (avg.). Mann–Whitney *U* test, $^*P < 0.05$. The percentages of $CD14^+CD90^+$ cells were weakly correlated with the synovial lining cell layer score (**B**), inflammatory infiltrate score (**D**), and Krenn inflammation score of the Krenn synovitis score (**E**). Spearman's rank correlation coefficient was used to assess significant differences. $P < 0.05$. **F**, The percentages of vascular areas in these tissues using cell analysis software attached to the fluorescence microscope system. There was no significant difference in the percentages of vascular areas between RA synovial tissues and controls. Bars show the average value. Mann–Whitney *U* test, $^*P < 0.05$. NS, not significant. The percentages of vascular areas were weakly correlated with the stromal cell density (**H**) and inflammatory infiltrate (**I**) of the Krenn synovitis score. No correlation was found for (**C**), (**G**), and (**J**). Spearman's rank correlation coefficient was used to assess significant differences. $P < 0.05$.

Table 2. Correlation between % CD14$^+$CD90$^+$ cells and % vascular areas with clinical parameter (n = 12)

	% CD14$^+$CD90$^+$ cells		% Vascular areas	
	r	*P*	*r*	*P*
Age	0.62	0.02	0.22	0.04
Disease duration	−0.12	0.01	−0.08	0.01
CRP	0.68	0.46	0.01	0.01
ESR	0.12	0.01	0.28	0.08
RF	0.14	0.02	0.10	0.01
anti-CCP antibody	0.17	0.03	0.12	0.01
MMP-3	0.13	0.01	0.11	0.01
DAS28CRP4	0.04	0.001	0.15	0.02
DAS28ESR4	0.16	0.02	0.06	0.003
SDAI	0.20	0.04	−0.34	0.11
CDAI	0.03	0.001	0.46	0.21

Note: Spearman's rank correlation coefficient was used. $P < 0.05$. Abbreviations: CCP, cyclic citrullinated peptide; CDAI, Clinical Disease Activity Index; CRP, C-reactive protein; DAS28CRP4, Disease Activity Scores in 28 joints with 4 variables, including CRP; DAS28ESR4, Disease Activity Scores in 28 joints with 4 variables, including ESR; ESR, erythrocyte sedimentation rate; MMP-3, matrix metalloproteinase-3; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index.

(gate P2 in Figure 5A). The frames in Figure 5A-E show CD14^{high}CD90^{int} cells. The regions of these frames were determined by comparison with OA controls. Figure 5F shows the percentages of CD14^{high}CD90^{int} cells in peripheral blood samples of

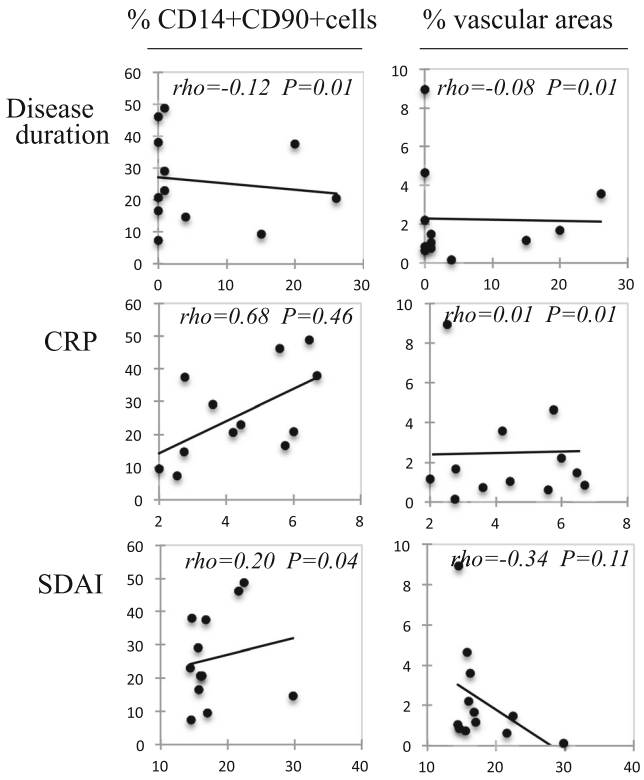

Figure 4. Representative correlation figures in table 2. Spearman's rank correlation coefficient was used. $P < 0.05$. CRP, C-reactive protein; SDAI, Simplified Disease Activity Index.

OA controls, patients with active RA, and patients with RA in clinical remission. CD14^{high}CD90^{int} cells were frequently detectable in active RA peripheral blood. On the other hand, they were little detected in peripheral blood samples of OA controls and patients with RA in clinical remission with MTX or bo/tsDMARDs therapy. Figure 5G and H shows representative flow cytometry analyses of RA synovial cells treated immediately after collecting synovial tissues. Figure 5I and J shows representative flow cytometry analyses of P0 and P1 cultured RA synovial cells, respectively. CD14^{high}CD90^{int} cells were clearly detectable in a higher percentage in synovial cells immediately after collection (Figure 5G and H) compared with in cultured cells (Figure 5I and J).

Differentiation potential of CD14^{high}CD90^{int} cells into dendritic cells. Cultured RA synovial cells (P0) were sorted by FACScan (Becton Dickinson), divided into CD14^{high}CD90^{int} cells and non-CD14^{high}CD90^{int} cells, and then compared for each differentiation potential into dendritic cells. Figure 5K shows representative data, and approximately 45% of CD14^{high}CD90^{int} cells were differentiated into CD83$^+$ cells in vitro. Figure 5L shows the data of five experiments for dendritic differentiation culture. The results showed that CD14^{high}CD90^{int} cells might have a higher potential of dendritic differentiation than non-CD14^{high}CD90^{int} cells.

DISCUSSION

CD14$^+$ cells with various morphologies, such as round and spindle-shaped cells, are detected in RA synovial tissue histologically. They have been considered very important cells in the pathogenesis of RA (16). In our study using electron microscopy, we have reported that RA synovial tissue contains many CD14$^+$ dendritic-shaped cells, which are not simple spindle-shaped cells but three-dimensionally dendritic-shaped, HLA-DR$^+$, and/or CD68$^+$ and engage in a pseudoemperipolesis phenomenon (6,7). This pseudoemperipolesis phenomenon has been originally observed in the thymus (17–19), and in vitro experiments of leukemia have shown that cells differentiated from monocytes in the blood may protect leukocytes from apoptosis (20). This suggests that the pseudoemperipolesis phenomenon in RA may protect inflammatory cells such as plasma cells or lymphocytes from apoptosis, but it has not yet been elucidated.

CD90 is a glycosylphosphatidylinositol-linked cell surface glycoprotein expressed on multiple cell types, including neurons, thymocytes, fibroblasts, endothelial cells, mesangial cells, and some hematopoietic and stromal stem cells (21). It has been identified as a regulator of cell adhesion, migration, apoptosis, axonal growth, cell–cell and cell–matrix interactions, and fibrosis (22). In previous experiments using mouse models, it has been reported that CD90$^+$ cells could induce cell proliferation in the spleen and lymph nodes and was involved in the activation of T cells (23,24). In this study, the results of double immunofluorescence staining

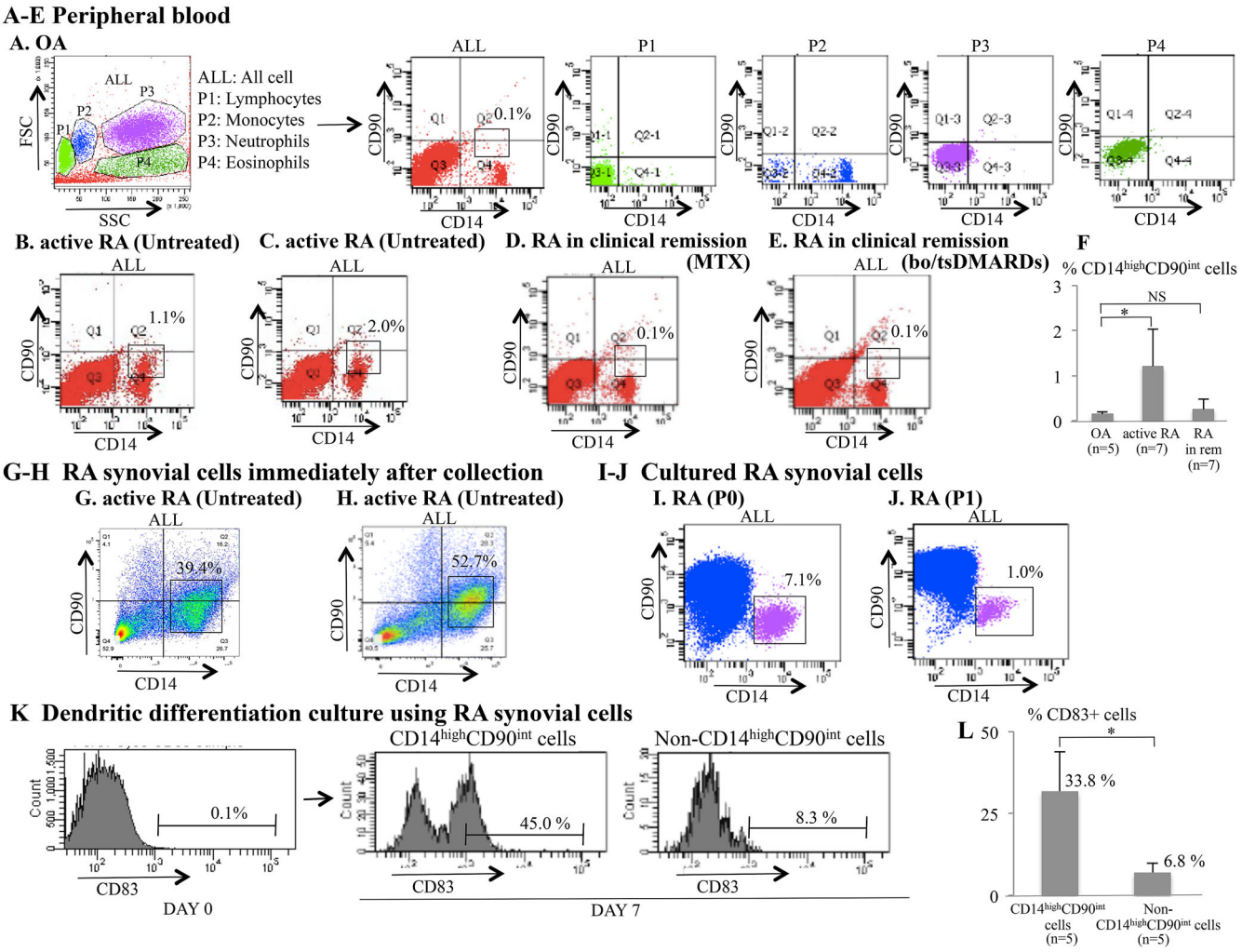

Figure 5. *In vitro* analyses. **A-E**, Representative flow cytometry analyses of the peripheral blood samples from osteoarthritis (OA) controls (**A**) (n = 5), patients with active rheumatoid arthritis (RA) who were not treated with glucocorticoids or anti-rheumatic drugs (**B and C**) (n = 7), and patients with RA in clinical remission with methotrexate (MTX) and biological originator and targeted synthetic disease-modifying anti-rheumatic drugs (bo/tsDMARDs) therapy (**D and E**) (n = 7). **F,** Percentages of $CD14^{high}CD90^{int}$ cells in peripheral blood. There were significantly more $CD14^{high}CD90^{int}$ cells in peripheral blood samples of patients with active RA compared with those of OA controls. Bars show the average value and standard deviation. Mann–Whitney *U* test, $^*P < 0.05$. **G and H**, Representative flow cytometry analyses of RA synovial cells immediately after collection (n = 5). **I and J**, Representative flow cytometry analyses of the cultured RA synovial cells (n = 5). **I and J** show passage 0 (P0) and passage 1 (P1) cultured cells, respectively. The frames in **A-J** show $CD14^{high}CD90^{int}$ cells. $CD14^{high}CD90^{int}$ cells were frequently detectable in active RA peripheral blood samples (**B and C**) and in a higher percentage in RA synovial cells immediately after collection (**G and H**) than in cultured RA synovial cells (**I and J**). **K**, Representative data of dendritic differentiation culture analyses of $CD14^{high}CD90^{int}$ cells derived from RA synovial cells. Approximately 45% of $CD14^{high}CD90^{int}$ cells were differentiated into $CD83^+$ cells in vitro. **L**, Average data of five experiments for dendritic differentiation culture. The results showed that $CD14^{high}CD90^{int}$ cells had a higher potential of dendritic differentiation than non-$CD14^{high}CD90^{int}$ cells. Bars show the average value and standard deviation. Mann–Whitney *U* test, $^*P < 0.05$.

and flow cytometry analyses showed that $CD14^+$ dendritic-shaped cells in the perivascular areas of RA synovial tissues expressed CD90. There have been few previous reports suggesting that $CD14^+$ cells express CD90 simultaneously. On the other hand, progenitor cells, such as MOMCs (12,25) and $CD105^+$ blood-acquired mesenchymal progenitor cells (BMPCs) (26), have been shown to bear a mixed hematopoietic and mesenchymal phenotype. MOMCs are pluripotent cells derived from $CD14^+$ monocytes in peripheral blood that are positive for MSC markers, such as CD105, and have been reported to differentiate into mesenchymal cells. BMPCs have been reported to express CD14. Additionally, another article has reported that human umbilical cord–derived MSCs express CD90 and CD14 (27,28). Therefore, we consider that it is impossible to exclude the possibility that cells other than monocytes and macrophages express CD14. $CD14^+$ dendritic-shaped cells in our study might possess mesenchymal phenotype as well as immune response and phagocytosis. Also, they might be important cells associated with RA chronic inflammation, such as migration, adhesion, and activation of T cells.

Furthermore, the expression of CD90 suggests a different origin from the previously considered origins of CD14$^+$ dendritic-shaped cells. There have been several reports on the origin of CD14$^+$ dendritic-shaped cells, namely, cells differentiated from MSCs that migrated to the site of inflammation (29), cells that migrated to RA synovial tissue through the pannus from the bone marrow (30–32), cells differentiated into mesenchymal cells from circulating CD14$^+$ monocytes (33), and synovial cells activated by granulocyte–macrophage colony-stimulating factor in synovial fluid (34,35). In our study, CD14^{high}CD90^{int} cells were abundant in the samples of peripheral blood of patients with active RA and in the perivascular areas of RA synovial tissues. Furthermore, CD90 expression was weaker away from blood vessels. In recent reports (10,36), single-cell RNA sequencing of RA synovial fibroblasts revealed that Notch ligands (JAG1, JAG2, and DLL4) expressed on vascular endothelial cells stimulate NOTCH3 on fibroblasts, leading to their transformation into CD90$^+$ fibroblasts. Therefore, we assume that CD14^{high}CD90^{int} cells in peripheral blood migrate to synovial tissue, which is the site of inflammation, increase the expression of CD90 simultaneously, proliferate CD14^{high}CD90^{int} cells, are associated with lymphocytes by cell-to-cell contact, and are involved in the pathogenesis of RA. Activation of Notch signal might be involved in the spreading of CD14^{high}CD90^{int} cells.

In our previous studies using electron microscopy, tomograms with double-axis electron beam tomography showed that cell-to-cell contact occurred between CD14$^+$ dendritic-shaped cells and adjacent plasma cells or lymphocytes (6). This phenomenon of cell-to-cell contact is known as trogocytosis, and it has been reported that proteins are transferred between cells (37,38). In culture experiments using cancer cells, it is reported that the Ras protein migrates between cells (39). In addition, recent studies have focused on a dynamic immunological phenomenon independent of gene transcriptional regulation, namely, T or dendritic cells withdraw proteins, such as major histocompatibility complex class I, from the contacted cells and alter their native functions (40). Therefore, we consider that in RA, the inflammatory loop might be formed through the transfer of some kinds of proteins, such as those that inhibit the apoptosis of lymphocyte, promote antibody production, and secrete cytokines or chemokines after CD14^{high}CD90^{int} cells spread within the tissues.

In RA synovial tissue, there are few positive cells for CD83, the original mature dendritic cell marker. However, an average of 33.8% of the cultured CD14^{high}CD90^{int} cells were positive for CD83, suggesting that they were directed toward dendritic cells by differentiation culture (Figure 5L). CD83 is also reported to be expressed on T cell–associated dendritic cells and Langerhans cells (41). Therefore, CD14^{high}CD90^{int} cells may be related to these cells, and their immunopathology needs further investigation. Recently, it has been reported that CD90$^+$ and T cells in the RA synovial tissue express RANKL, which is involved in the differentiation into osteoclasts, leading to bone destruction (10,42). In addition, it has been reported that CD90$^+$HLA-DR^{high} fibroblasts express IL-6 (11). We consider that CD14^{high}CD90^{int} cells may be also related to bone destruction.

This study has limitations regarding the number of samples and patients. The association between the percentages of CD14$^+$CD90$^+$ cells and clinical parameters was mostly negative. A previous study has reported similar results, namely, that there was no association between CD90$^+$ cells and clinical parameters (10). However, we should analyze the statistics with a larger number of patients. In recent years, it has been difficult to obtain highly active RA samples, and we intend to continue the study to increase the number of samples. In the experiments of differentiation into dendritic cells, we did not compare with OA synovial tissues as controls. Our preliminary experiments in OA synovial tissues showed that CD14^{high}CD90^{int} cells differentiate into CD83$^+$ cells. However, experiments in OA synovial tissues are difficult because CD14^{high}CD90^{int} cells are not as abundant as in RA synovial tissues and CD14 expression decreases with culture passages.

Our findings suggest that CD14$^+$ dendritic-shaped cells are monocyte-derived immune response cells with phagocytosis ability, have mesenchymal phenotype, and have a potential to differentiate into dendritic cells. CD14$^+$ dendritic-shaped cells may play a crucial role for RA, and further studies of them may lead to novel understanding in the pathogenesis of RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. R. Kurose had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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