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Cell-contact dependent activation of CD4+ T cells by adhesion molecules on synovial fibroblasts
（接着分子を介した滑膜線維芽様細胞との細胞接触による CD4 陽性 T 細胞の活性化）
Cell-contact dependent activation of CD4+ T cells by adhesion molecules on synovial fibroblasts

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Abstract

[Introduction] To determine how cell-cell contact with synovial fibroblasts (SF) influence on the proliferation and cytokine production of CD4+ T cells.

[Methods] Naïve CD4+ T cells were cultured with SF from rheumatoid arthritis patients, stimulated by anti-CD3/28 antibody, and CD4+ T cell proliferation and IFNγ/IL-17 production were analyzed. To study the role of adhesion molecules, cell contact was blocked by transwell plate or anti-intracellular adhesion molecule-1 (ICAM-1)/vascular cell adhesion molecule-1 (VCAM-1) antibody. To study the direct role of adhesion molecules for CD4+ T cells, CD161+ or CD161- naïve CD4+ T cells were stimulated on plastic plates coated by recombinant ICAM-1 or VCAM-1, and the source of IFN-γ/IL-17 were analyzed.

[Results] SF enhanced naïve CD4+ T cell proliferation and IFN-γ/IL-17 production in cell-contact and in part ICAM-1/VCAM-1-dependent manner. Plate-coated ICAM-1 and VCAM-1 enhanced naïve CD4+ T cell proliferation and IFN-γ production, while VCAM-1 efficiently promoting IL-17 production. CD161+ naïve T cells upregulating LFA-1 and VLA-4 were the major source of IFN-γ/IL-17 upon interaction with ICAM-1/VCAM.

[Conclusion] CD4+ T cells rapidly expand and secrete IFN-γ/IL-17 upon cell-contact with SF via adhesion molecules. Interfering with ICAM-1/VCAM-1 may be beneficial for inhibiting RA synovitis.

Key words
rheumatoid arthritis, synovial fibroblasts, CD4+ T cells, intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), CD161, IFN-γ, IL-17
Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that primarily affects the joints leading to their progressive destruction. The pathogenesis of RA is complex and includes many cell types, such as T cells, B cells, macrophages, and synovial fibroblasts (SF) (1).

SF are the major effector cells in RA synovitis (2). They secrete large amount of inflammatory cytokines and proteolytic enzymes that lead to the formation of pannus and joint destruction. One crucial characteristic of RA-SF is the expression and upregulation of adhesion molecules that mediate the attachment of these cells to the cartilage, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (1). These adhesion molecules not only mediate the adhesion to the cartilage, but also deliver a signal for other lymphocytes such as CD4+ T cells (3,4).

CD4+ T cells also play a critical role in the pathogenesis of RA. Classically, it has been hypothesized that IFN-γ producing Th1 cells play a major role in RA (5). Recently, a new helper T cell subset secreting IL-17, Th17 cells, have been identified as the main effector cells in various autoimmune diseases in animal models (6,7), although the role of Th17 cells in human RA is still controversial (8–10). It is reported that human naive CD4+ T cells contain a Th17 progenitor cells that is characterized by the expression of CD161 (11). CD161+ cells secrete not only IL-17 but also IFN-γ and are accumulated in the inflamed tissue of autoimmune diseases (11–13).

In rheumatoid synovium, CD4+ T cells reside in close proximity to hyperplastic SF(14,15). SF in RA synovium upregulate ICAM-1 and VCAM-1, while CD4+ T cells in RA synovium upregulates their ligands, such as lymphocyte function associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), suggesting the possible interaction via adhesion molecules (15). Previous works have revealed the bidirectional interaction between CD4+ T cells and SF, including cell surface expressed molecules and soluble factors (16). For example, effector CD4+ T cells stimulate SF to produce inflammatory cytokines and proteolytic enzymes via several mechanisms (14,15,17,18). Conversely, SF influence on CD4+ T cell cytokine production via several mechanisms(17,19–21). Interestingly, it is reported that SF have a unique character to induce the preferential production of IL-17 from CD4+ T cells, although the molecular mechanism have not been fully determined (17,19–21).

In this study, we tried to determine how cell-contact with SF influence on the proliferation and cytokine production of CD4+ T cells. To see the direct effect of SF for CD4+ T cells, we cultured naïve CD4+ T cells from healthy volunteers with SF from RA.
patients and assessed the proliferation and cytokine production after polyclonal anti-CD3 and/or anti-CD28 stimulation. We focused on how adhesion molecules which are highly upregulated on RA-SF act on the proliferation and cytokine production from CD4+ T cells.
Methods

Ethics
The study was designed in accordance with the declaration of Helsinki and was approved by the ethics committee of Kyoto University Graduate School and Faculty of Medicine. Written informed consent was obtained from all the participants.

Isolation of CD4+ T cells
Blood samples were obtained from healthy volunteers and RA patients. Peripheral blood mononuclear cell (PBMC) was isolated by Ficol (GE Healthcare Life Bioscience, Uppsala, Sweden) gradient centrifugation. Purification of CD4+ T cells and CD62L+ naïve CD4+ T cells and separation of CD161+ CD62L+ and CD161- CD62L+ CD4+ T cells were done by MACS (Miltenyi Biotech, Bergsh Gladbach, Germany) as manufacturer’s instructions. The purity of naïve CD4+ T cells was 95~97 %.

Preparation of fibroblasts
SF was obtained from surgical specimens in the total knee replacement surgery for RA or osteoarthritis (OA) patients at Kyoto University Hospital. The RA patients fulfilled the American college of Rheumatology criteria for RA (22). The OA patients were diagnosed according to the typical clinical signs and symptoms of American college of Rheumatology criteria for OA (23). Briefly, tissue was minced and treated with 100 μg/ml Liberase (Roche, Basel, Switzerland) and RPMI (Gibco, Carlsbad, USA) and rocked at 37 °C for 1h. The cell suspension was poured on 6 well treated plate (Greiner, Frickenhausen, Germany). SF was maintained in DMEM (Gibco) supplemented with 20% fetal bovine serum (Sigma Aldrich). SF on 4 to 10 passages were used as SF as previously described(2). Dermal fibroblast (DF) obtained from healthy volunteer was kindly provided by Dr. Akihiko Kito (Department of Dermatology, Graduate School of Medicine, Kyoto University). Similar to SF, skin sample was minced and treated by Liberase and RPMI and rocked at 37 °C for 1h. The cell suspension was poured on 6 well treated plate. DF was maintained in DMEM supplemented with 20% fetal bovine serum (24).

Cell culture
Naïve CD4+ T cells were stimulated by plate-coated anti-CD3 (Biolegend, San Diego, CA, USA) (10 μg/ml) and/or soluble anti-CD28 (BD biosciences, Franklin Lakes, New Jersey, USA) (1 μg/ml) antibodies, cultured in 96 well flat bottom plates with a final volume of 200 μL of RPMI supplemented with 10% fetal bovine serum (Sigma Aldrich, St. Louis,
MO, USA). 2.5 × 10⁴ of CD4⁺ T cell and 2.5 × 10³ of SF or DF were applied per well and proliferation and cytokine production was analyzed on day 3. For transwell experiment, CD4⁺ T cells and SF were co-cultured in 96 well transwell plates separated by the membrane in lower and upper chambers, respectively (Costar, New York, USA). For blockade experiments, CD4⁺ T cells and SF were cultured with 25 μg/ml anti-ICAM-1 (CD54) and/or anti-VCAM-1 (CD106) antibodies (R&D Systems, bingdon, UK). In experiments to see the direct effect of ICAM-1 and VCAM-1 for CD4⁺ T cell proliferation and cytokine production, 2.5 × 10⁴ naïve CD4⁺ T cells were stimulated by anti-CD3/28 Abs on 96 well flat bottom plates pre-coated by recombinant ICAM-1 or VCAM-1 (Peprotech, Rocky Hill, NJ, USA) in PBS at 37 °C for 1 hour, or soluble recombinant ICAM-1 and VCAM-1 were added at the time of co-culture at indicated doses.

**CFSE proliferation assay**
CD4⁺ T cells were labelled with 1 μM carboxyfluorescein succinimidyl ester (CFSE) (Dojindo, Kumamoto, Japan) and cell division was analyzed by flow cytometry. Samples were analyzed on FACSCalibur equipment (BD biosciences).

**Intracellular cytokine staining**
CD4⁺ T cells were stimulated with 20 ng/ml phorbol 12-myristate 13 acetate (PMA) (Sigma and Aldrich) and 1 μM ionomycin (Sigma and Aldrich) in the presence of Golgi stop (BD Biosciences) for 4h. Cells were stained for anti-CD4 Ab, and fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences), followed by anti-IFN-γ, anti-IL-17 and anti-GM-CSF staining. Samples were analyzed on FACSCalibur equipment (BD biosciences).

**ELISA and cytokine beads array**
IL-17 and GM-CSF levels in supernatant were assessed by ELISA kits as manufacturer’s instructions. (R&D Systems). The minimum detectable level of IL-17 and GM-CSF were 15 and 3 pg/mL, respectively. IFN-γ, IL-2, and TNF-α levels were assessed by cytokine beads array as manufacturer’s instructions (BD biosciences). Samples were analyzed on FACSCalibur equipment (BD biosciences), and data were analyzed with FCAP array. The minimum detectable level of IFN-γ, IL-2, and TNF-α were 3.7, 2.6 and 3.8 pg/mL, respectively.

**Flow cytometry**
The following antibodies were purchased from BD biosciences: IL-17-Alexa647 (SCPL1362), IFN-γ-PE (4S-B3), CD80-PE (2D10), CD86-APC (2331), CD54-PE (LB-2), CD106-APC (51-10C9), CD62L (DREG-56), CD11a-PE (G43-25B). The following
antibodies were purchased from Biolegend: CD4-PerCP (SK3), CD49d-PE (9F10). The following antibodies were purchased from Milteny Biotech: CD161-APC (191B8).

Confocal microscopy
Anti-CD3/28 stimulated CD4+ T cells labelled with CFSE and SF were cultured on Glass Bottom Culture Dish (MatTek, Ashland, MA, USA). Images of co-culture samples were inspected by confocal microscopy (LSM700: Zeiss, Jena, Germany) and the images were analyzed using Zen images analysis software 2009 (Zeiss).

Statistical analysis.
Data were presented as the mean ± SEM and statistical difference was analyzed by student t-test. P values less than 0.05 were considered significant.
Results

Cell-contact with synovial fibroblasts enhances CD4+ T cell proliferation and cytokine production.

To determine how SF influence on the proliferation and cytokine production of CD4+ T cells, we first cultured CD4+ T cells from RA patients with or without SF obtained from the same donor and stimulated them by anti-CD3 and CD28 antibodies (Figure 1a). As assessed by CFSE labeling, co-culture with SF markedly increased the proliferation of anti-CD3/28 stimulated CD4+ T cells (Figure 1a). Next, to study the mechanism how SF influence on the proliferation and cytokine production of CD4+ T cells, we cultured naïve CD4+ T cells from healthy individuals with or without SF (Figure 1b). Similar to the experiment with RA CD4+ T cells, co-culture with SF markedly increased the naïve CD4+ T cell proliferation (Figure 1b). On day 3 when almost no proliferation was observed for naïve CD4+ T cells cultured without SF, more than 40 % of naïve CD4+ T cells cultured with SF proliferated, and reached the plateau on day 7. Because the proliferation of CD4+ T cells on day 7 highly varied depending upon the expansion of SF derived from different RA donors, we performed the subsequent experiments on day 3. Interestingly, anti-CD3 antibody stimulated naïve CD4+ T cells cultured with SF proliferated even in the absence of anti-CD28 antibody, suggesting that SF may have co-stimulatory effect for CD4+ T cells (Supplementary Figure 1).

Next, we assessed the cytokine production of naïve CD4+ T cells stimulated by anti-CD3/28 in the presence or absence of SF (Figure 1c). Co-culture with SF markedly enhanced IFN-γ/IL-17 production from anti-CD3/28 stimulated CD4+ T cells, as assessed by intracellular staining (Figure 1c) and cytokine concentration in the culture supernatant (Figure 1d).

After the co-culture, CFSE-labelled CD4+ T cells aggregated on SF as inspected by confocal microscope (Figure 1e). Interestingly, only CD4+ T cells attached to SF showed blast like morphology and lost CFSE staining (Figure 1e), suggesting the proliferation of CD4+ T cells in contact with SF. To determine whether the SF mediated enhancement of CD4+ T cell proliferation and cytokine production was mediated by cell-cell contact or soluble factors, we performed transwell experiments (Figure 1f, g). When cell-cell contact was blocked by the transwell, SF-mediated enhancement of CD4+ T cell proliferation on day 3 was almost completely inhibited (Figure 1f). IFN-γ/IL-17 production in the culture supernatant was also inhibited (Figure 1g), suggesting that SF-mediated activation of CD4+ T cells critically depends upon cell-cell contact but not soluble factors.
ICAM-1 and VCAM-1 are involved with cell-contact dependent activation of CD4+ T cells with synovial fibroblasts

To determine the molecules involved with the rapid, cell-contact dependent activation of CD4+ T cells by SF, we analyzed the cell surface molecules on SF. It is known that SF do not express classical co-stimulatory molecules such as CD80/86, while they highly express adhesion molecules such as ICAM-1 or VCAM-1(1). Consistent with previous reports, SF did not express CD80 and CD86 even after co-culture with anti-CD3/28 stimulated CD4+ T cells (Figure 2a). On the other hand, SF from both of RA and OA patients highly expressed ICAM-1 and VCAM-1, which were further up-regulated after co-culture with anti-CD3/28 stimulated CD4+ T cells (Figure 2a) or after the addition of TNF-α (Supplementary Figure 2). Interestingly, although dermal fibroblasts (DF) also expressed ICAM-1, they scarcely express VCAM-1 at the steady state and even after co-culture with anti-CD3/28 stimulated CD4+ T cells (Figure 2a) or after the addition of TNF-α (Supplementary Figure 2).

To determine whether ICAM-1 and VCAM-1 are involved with the SF-mediated enhancement of CD4+ T cell proliferation and cytokine production, ICAM-1 and VCAM-1 interaction was blocked by the addition of anti-ICAM-1 and/or VCAM-1 antibody. As assessed by CFSE dilution, SF-mediated enhancement of CD4+ T cell proliferation was significantly suppressed by the addition of anti-ICAM-1, anti-VCAM-1, and their combination (Figure 2b). In accord with the proliferation, anti-ICAM-1 and anti-VCAM-1 significantly suppressed the IFN-γ/IL-17 production in the culture supernatants (Figure 2c). Interestingly, IFN-γ production was more suppressed by anti-ICAM-1 antibody than by anti-VCAM-1 antibody, while IL-17 production was suppressed more by anti-VCAM-1 antibody than by anti-ICAM-1 antibody (Figure 2c).

ICAM-1 and VCAM-1 directly promote CD4+ T cell proliferation and cytokine production

Because IFN-γ and IL-17 production from CD4+ T cells were differently modulated by anti-ICAM-1 and anti-VCAM-1 antibody, it is possible that ICAM-1 and VCAM-1 interaction may have differential effect for CD4+ T cell cytokine production. Because cell-cell adhesion by ICAM-1 or VCAM-1 may upregulate other cell-surface molecules or soluble factors that could indirectly affect CD4+ T cell cytokine production, we asked whether ICAM-1 and VCAM-1 interaction has a direct effect for naïve CD4+ T cell proliferation and cytokine production. To see it, we stimulated naïve CD4+ T cells alone with anti-CD3/28 antibody on plastic plates pre-coated by recombinant ICAM-1 or VCAM-1, or added soluble recombinant ICAM-1 or VCAM-1 to the culture (Figure 3).

On plates pre-coated by ICAM-1 or VCAM-1 but not on plates added with soluble ICAM-1 or VCAM-1, proliferation of anti-CD3 or anti-CD3/CD28 stimulated naïve CD4+
T cells was markedly enhanced, suggesting that cell-surface expressed ICAM-1 and VCAM-1 have a direct co-stimulatory effects for CD4+ T cells (Figure 3 a, b). In accord with proliferation, IFN-γ/IL-17 production of anti-CD3/28 stimulated CD4+ T cells were enhanced by plate-coated ICAM-1 or VCAM-1 (Figure 3c). Similar to IFN-γ, production of IL-2, GM-CSF, or TNFα was also enhanced by plate-coated ICAM-1 or VCAM-1 (Supplementary Figure 3). Interestingly, IL-17 production was markedly enhanced by plate-coated VCAM-1, but was scarcely induced by ICAM-1(Figure 3c), suggesting that VCAM-1 interaction is efficient for promoting IL-17 production from naïve CD4+ T cells.

CD161+ CD4+ T cells are the major source of IFN-γ and IL-17 upon interaction with ICAM-1 and VCAM-1.

In mice, it is known that naïve CD4+ T cells do not produce effector cytokines unless they have differentiated into helper T cells in the presence of a combination of various inflammatory cytokines, for example, Th17 cells by IL-6 and TGF-β (6). In contrast with the mouse CD4+ T cells, human naïve CD4+ T cells contain CD161+ cells that act as the progenitor Th17 cells and have the potential to secrete not only IL-17 but also IFN-γ (11,12). To determine the source of IFN-γ and IL-17 production from naïve CD4+ T cells interacting with plate-coated ICAM-1 or VCAM-1, we first analyzed LFA-1 and VLA-4 expression of CD161+ and CD161- naïve CD62L+ CD4+ T cells (Figure 4a). VLA-4 expression and, to a lesser extent, LFA-1 expression was upregulated on CD161+ cells compared with CD161- cells (Figure 4a). Then, we separated naïve CD4+ T cells into CD161+ and CD161- cells, and analyzed the proliferation and cytokine production of each population. After the culture on ICAM-1 or VCAM-1 coated plates, both CD161+ and CD161- cells proliferated by anti-CD3/28 stimulation, but the proliferation was more enhanced on CD161+ cells. (Figure 4b). When the cytokine production from each population was analyzed, IFN-γ and IL-17 production was observed mostly from CD161+ cells but not from CD161- cells (Figure 4c). Again, IL-17 production was predominantly observed on VCAM-1-coated plates compared with ICAM-1-coated plates (Figure 4c). These results suggested that CD161+ CD4+ T cells were the major source of IFN-γ/IL-17 upon interaction with ICAM-1/VCAM-1 (Figure 4 b, c).
Discussion

In this study, we have shown that cell-contact with SF rapidly promote the proliferation and IFN-γ/IL-17 production of CD4+ T cells in cell-contact dependent and in part ICAM-1- and VCAM-1-dependent manner. ICAM-1 and VCAM-1 had a direct co-stimulatory effect for CD4+ T cells and differentially regulated IFN-γ and IL-17 production. CD161+ cells upregulating LFA-1 and VLA-4 were the major source of IFN-γ and IL-17 upon ICAM-1/VCAM-1 ligation. This in vitro study shows that adhesion molecules on SF has a strong co-stimulatory activity for CD4 T cells and help understanding the interaction between SF and CD4+ T cells (14–21).

Our transwell experiments suggested the critical importance of cell-cell contact for the rapid CD4+ T cell activation upon interaction with SF (Figure 1). Because the interaction between SF and T cells is complex, involving many receptor-ligand pairs, our results do not exclude other possibilities involved with the activation of CD4+ T cells by SF (16,17,19–21). In our study, IFN-γ and IL-17 production in CD4+ T cells and SF co-culture was not completely inhibited by anti-ICAM-1/VCAM-1 antibodies (Figure 2). The result suggests that other redundant mechanisms may also play a role in SF-mediated activation of CD4+ T cells in vivo (17,19–21).

Our study suggested that adhesion molecules on SF not only mediate the adhesion to extracellular matrix but also actively involved with the activation of CD4+ T cells in RA synovitis. Because cell-cell adhesion between CD4+ T cells and SF may upregulate other receptors or ligand pairs that may further augment the activation of both cells, strategy to suppress or block ICAM-1 or VCAM-1 interaction may be beneficial for inhibiting RA synovitis. In this regard, several current ant-rheumatic may act on RA at least in part, by downregulating ICAM-1 and VCAM-1. For example, TNF inhibitors may down regulate ICAM-1 or VCAM-1 on SF because TNF-α upregulates the expression of these molecules (Supplementary Figure 2). Methotrexate, the most commonly used conventional synthetic DMARDs, or COX2 inhibitor might also have potential to down-regulate ICAM-1 and VCAM-1 expression on SF as shown for other cell types (25,26). Anti-ICAM-1 monoclonal antibody has been used for RA and were effective for suppressing RA and IFN-γ production (27). Anti-VLA-4 antibody has been used for multiple sclerosis patients in which the role of Th17 cells are implicated for its pathogenesis (28). The combined blockade of ICAM-1 and VCAM-1 might be more beneficial for inhibiting RA synovitis.

ICAM-1 and VCAM-1 had a differential effect for the rapid IFN-γ/IL-17 production from naïve CD4+ T cells. Although ICAM-1 is known to have the capacity to promote IL-17 production from CD4+ T cells (29), our work suggested that VCAM-1/VLA-4 interaction may be more efficient for promoting the rapid production of IL-17 from
human CD4+ T cells. Specific expression of VCAM-1 on SF but not dermal fibroblast might in part explain why SF have a unique character to promote preferential IL-17 production from CD4+ T cells as previously reported (17,19–21). CD161+ naïve CD4+ T cells which expressed high level of VLA-4 and, to a lesser extent, LFA-1 were the main source of ICAM-1- and VCAM-1-mediated IFN-γ/IL-17 production. We speculate that IL-17 production from CD161+ CD62+ naïve CD4+ T cells is not the contamination of effector/memory CD4+ T cells, because CD161+ T cells are present even in truly naïve T cells in the cord blood (11). However, in vitro induction of human Th17 cells from naïve CD4+ T cells is known to be challenging (30). Therefore, it should be determined in the future whether truly naïve cord blood CD161+ cells have the capacity to respond ICAM-1/VCAM-1 and secrete IFN-γ/IL-17. The rapid expansion of CD161+ cells upon interaction with ICAM-1 and VCAM-1 may be one reason why CD161+ cells are accumulated in the inflamed tissues of RA and other autoimmune diseases (11–13).

Because this is an in vitro experiment culturing naïve CD4+ T cells with SF and stimulated with polyclonal anti-CD3/28 antibody, the in vivo relevance of this study remains to be determined. This is the major limitation of our study. For example, we used naïve CD4+ T cells from healthy volunteers to analyze the mechanism how SF influence on CD4+ T cell proliferation and cytokine production. Although the majority of CD4+ T cells infiltrating in the synovium are effector/memory CD4+ T cells (31), naïve CD4+ T cells can also be recruited to the inflamed joint by chemokine gradients because naïve CD4+ T cells express CCR7 while CCR7 ligands such as CCL19 or CCL21 are abundantly present in RA synovium (32). In addition, we analyzed the CD4+ T cell proliferation and cytokine production on day 3, because the addition of SF dramatically enhanced CD4+ T cell proliferation and the proliferation on day 7 highly varied depending upon the SF derived from different RA donors. However, the day 3 time point might be short to evaluate the full differentiation of naïve CD4+ T cells into helper T cells (33). Considering these limitations of the cell preparation and experimental time bias, it remains to be determined in the future how effector memory CD4+ T cells from RA patients interact with SF and adhesion molecules at longer time period.

In conclusion, we have shown that CD4+ T cells in contact with SF rapidly expand and secrete IFN-γ/IL-17 in cell-contact dependent, and in part ICAM-1- and VCAM-1-dependent manner. Our findings may help designing the strategy to control RA synovitis by inhibiting adhesion molecules.

**Abbreviation**

RA, Rheumatoid arthritis; SF, synovial fibroblasts; RA-SF, synovial fibroblasts of Rheumatoid arthritis; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; LFA-1, lymphocyte function-associated antigen-1; VLA-4, very
late antigen-4, PBMC, peripheral blood mononuclear cell; OA, osteoarthritis; DF, Dermal fibroblast; CFSE, carboxyfluorescein succinimidyl ester; PMA, phorbol 12-myristate 13 acetate; OA-SF, synovial fibroblast of osteoarthritis; DMARDs, disease modified anti-rheumatic drugs; APC, antigen presenting cells.

**Competing Interests**

MH, T. Fujii, MF, HI, and T. Mimori are affiliated with a department that is supported financially by five pharmaceutical companies (Mitsubishi Tanabe Pharma Co., Bristol-Myers K.K., Chugai Pharmaceutical Co., Ltd., AbbVie GK., Eisai Co., Ltd.). MH and MF has received grant and research support from Astellas Pharma Inc. and Pfizer Japan Inc. JH is a person in Astellas Pharma Inc. HI has received grant and research support from Astellas Pharma Inc., Pfizer Japan Inc., NTT communications, and Takeda Pharmaceutical Co., Ltd. T. Fujii has received grant and research support from Takeda Pharmaceutical Co., Santen Pharmaceutical Co., Ltd., Astellas Pharma Inc., Asahi Kasei Pharma Corporation, and Daiichi Sankyo Co., Ltd. T. Mimori has received grant and research support from Asahi Kasei Pharma Corporation, Astellas Pharma Inc., Bristol-Myers Squibb K.K., Chugai Pharmaceutical Co., Ltd., Eisai Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Pfizer Japan Inc., Santen Pharmaceutical Co., Ltd., and Takeda Pharmaceutical Co., Ltd., and speakers bureau from AbbVie GK., Bristol-Myers Squibb K.K., Chugai Pharmaceutical Co., Ltd., Eisai Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Pfizer Japan Inc., and Takeda Pharmaceutical Co., Ltd.; The sponsors were not involved in the study design; in the collection, analysis, interpretation of data; in the writing of this manuscript; or in the decision to submit the article for publication. The authors, their immediate families, and any research foundations with which they are affiliated have not received any financial payments or other benefits from any commercial entity related to the subject of this article.

**Author Contributions**

MM carried out most of the experiments and wrote the manuscript. MH designed the experiments and wrote the manuscript. T. Matsuo helped immunoassays experiments. T. Fujii and T. Mimori helped to design the study and supervised the manuscript. MF and HI obtained clinical samples and helped preparing materials. HY and JH helped co-culture experiments. SA helped confocal microscopy experiments. Y. Ito, RN, Y. Imura, NY, HY, and KO helped the interpretation of data and critically revising the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Cell contact with synovial fibroblasts enhance CD4+ T cell proliferation and cytokine production. (a, b) CD4+ T cells of RA patients and naïve CD4+ T cells of healthy volunteers were labelled with CFSE and stimulated by anti-CD3 (10 µg/ml) and anti-CD28 (1 µg/ml) in the presence or absence of RA-SF. CFSE dilution was analyzed on day 3 and 7. (c) Intracellular cytokine staining of anti-CD3/28 stimulated CD4+ T cells after co-culture with or without RA-SF. (d) IFN-γ and IL-17 levels in the culture supernatant after co-culture of anti-CD3/28 stimulated CD4+ T cells with or without RA-SF. (e) Confocal microscopy of CFSE labelled CD4+ T cells stimulated with anti-CD3/28 after co-culture with RA-SF. (f) CFSE proliferation assay of anti-CD3/28 stimulated naïve CD4+ T cells after co-culture with RA-SF in the presence or absence of transwell membrane. (g) IFN-γ and IL-17 levels in the culture supernatants after co-culture of anti-CD3/28 stimulated naïve CD4+ T cells with RA-SF in the presence or absence of transwell membrane. Bars in (d) and (g) represent the mean value and SEM of 3 experiments. *p < 0.05.

Figure 2. ICAM-1 and VCAM-1 are involved with cell-contact dependent activation of CD4+ T cells by synovial fibroblasts. (a) Expression of CD80, CD86, ICAM-1, and VCAM-1 on SF from RA or osteoarthritis (OA) patients and dermal fibroblasts (DF) before and after co-culture with anti-CD3/28 stimulated naïve CD4+ T cells. Gray histogram represents the isotype control, dotted line represent SF before co-culture, and continuous line represent SF after co-culture. (b) CFSE proliferation assay of anti-CD3/28 stimulated naïve CD4+ T cells cultured with RA-SF in the presence or absence of anti-ICAM-1 and/or anti-VCAM-1 antibodies. (c) IFN-γ and IL-17 production in culture supernatant of the co-culture with naïve CD4+ T cells and RA-SF in the presence or absence of anti-ICAM-1 and/or anti-VCAM-1 antibodies. Bars in (c) represent the mean value and SEM of 3 experiments. *p < 0.05.

Figure 3. ICAM-1 and VCAM-1 differentially promote CD4+ T cell cytokine production. (a) CFSE proliferation assay of anti-CD3 or anti-CD3/28 stimulated naïve CD4+ T cells cultured with plate-coated ICAM-1 or soluble ICAM-1 at indicated dose. (b) CFSE dilution of anti-CD3 or anti-CD3/28 stimulated naïve CD4+ T cells cultured with plate-coated VCAM-1 or soluble VCAM-1 at indicated dose. (c) IFN-γ and IL-17 production from anti-CD3 (filled square) or anti-CD3/28 (filled triangle) stimulated CD4+ T cells on plate-coated ICAM-1/VCAM-1 at indicated doses. Bars in (c) represent the mean value and SEM of 3 experiments.
**Figure 4.** CD161+ naïve CD4+ T cells secrete IFN-γ and IL-17 production upon interaction with ICAM-1 and VCAM-1. (a) Expression of LFA-1 and VLA-4 on CD161+ or CD161- CD62+ CD4+ T cells. Gray histogram represents the isotype control, dotted line represents CD161- cells, and continuous line represents CD161+ cells. (b) CFSE proliferation assay of anti-CD3 or anti-CD3/28 stimulated CD161+ CD 4+ T cells or CD161+ CD4+ T cells cultured on plates coated by ICAM-1 or VCAM-1 at indicated doses. (c) IFN-γ and IL-17 production from anti-CD3/28 stimulated CD161+ (filled square) or CD161- (filled triangle) CD62L+ CD4+ T cells cultured on ICAM-1/VCAM-1 coated plate at indicated doses. Bars in (c) represent the mean value and SEM of 3 experiments.

**Supplementary Figure 1.** Naïve CD4+ T cells of a healthy volunteer were labelled with CFSE and stimulated by anti-CD3 (10 µg/ml) and/or anti-CD28 (1 µg/ml) in the presence or absence of RA-SF. CFSE dilution was analyzed on day 3. Flow cytometry analysis were representative data from the same donor CD4+ T cells.

**Supplementary Figure 2.** ICAM-1 and VCAM-1 expression on SF and DF before and after the addition of recombinant TNF-α (10 µg/ml) for 8 hours. Gray histogram represents isotype control, dotted line represents before the addition, continuous line represents after the addition of TNF-α.

**Supplementary Figure 3.** IL-2, GM-CSF, and TNF-α production from anti-CD3 (filled square) or anti-CD3/28 (filled triangle) stimulated CD4+ T cells on plate-coated ICAM-1/VCAM-1 at indicated doses. Naïve CD4+ T cells were obtained from a healthy volunteer. Bars represent the mean value and SEM of 3 experiments.
Fig. 1

(a) CD4+ T cells of RA
   day 3  day 7
   30%  85.8%

   (+)
   98.7%  99.9%

   (+RA-SF)

(b) naive CD4+ T cells of healthy volunteer
   day 3  day 7
   3.5%  43.8%

   (+)
   82.8%  99%

   (+RA-SF)

(c) Anti-CD3/28
   CD4+ T cells
   CD4+ T cells + RA-SF
   0.05%  0.0%
   0.14%  0.02%
   1.4%  13.5%

   IL-17

   (+IFN-γ)

(d) IFN-γ (pg/ml)
   0  5000
   10000
   15000
   N.D.  N.D.
   +  +
   +  +
   N.D.  N.D.

   IL-17 (pg/ml)
   0  50
   100
   150
   N.D.  N.D.
   +  +
   +  +
   N.D.  N.D.

(e) Image

(f) CD4+ T cells
   2.4%

   CD4+ T cells + RA-SF
   63%

   CD4+ T cells + RA-SF Transwell
   7.2%

(g) IFN-γ (pg/ml)
   0  2500
   5000
   * * *
   N.D.  N.D.
   -  -
   +  +
   N.D.  N.D.

   IL-17 (pg/ml)
   0  50
   100
   150
   * * *
   N.D.  N.D.
   -  -
   +  +
   N.D.  N.D.
Fig. 2

(a) RA-SF  RA-SF  OA-SF  DF

CD80  ICAM-1

CD86  VCAM-1

(b) CD4+ T cells  CD4+ T cells  CD4+ T cells  CD4+ T cells  CD4+ T cells
+RA-SF  +RA-SF  +RA-SF  +anti-ICAM-1  +anti-VCAM-1

(c) IFN-γ (pg/ml)

CD4+ T cells  RA-SF  anti-ICAM-1  anti-VCAM-1

N.D.  N.D.  +  -  +  +  +  +  +  +  +

IL-17 (pg/ml)

CD4+ T cells  RA-SF  anti-ICAM-1  anti-VCAM-1

N.D.  N.D.  +  -  +  +  +  +  +  +  +
Fig. 4

(a) 

CD161

CD62L

5%

LFA-1

VLA-4

Isotype

CD4⁺CD161⁻

CD4⁺CD161⁺

(b) 

CD161⁻

1.9%

64%

9.3%

46.6%

37.9%

plate-coated ICAM-1 (8µg/ml)

plate-coated VCAM-1 (8µg/ml)

(c) 

IFN-γ (pg/ml)

IL-17 (pg/ml)

ICAM-1 (µg/ml)

IL-17 (pg/ml)

IFN-γ (pg/ml)

VCAM-1 (µg/ml)
Sup. 1

CD4+ T cells

- (-) 0.2%
- Anti-CD3 0.5%
- Anti-CD28 0.4%
- Anti-CD3/28 1%

CD4+ T cells + RA-SF

- 0.3%
- 60%
- 0.5%
- 65%