(See figure on previous page.)
Figure 2 Effect of lysophosphatidic acid receptor 1 on pseudoemperipolesis and migration of rheumatoid arthritis fibroblast-like synoviocytes. After preincubation of cocultured rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs) and $CD4^+$ T cells **(A)** and **(B)** or $CD8^+$ T cells **(C)** and **(D)** or $CD19^+$ B cells **(E)** and **(F)** with a lysophosphatidic acid (LPA) receptor 1 inhibitor (LA-01; 0, 1 or 10 nM) for 30 minutes, the cells were stimulated with 10 μM LPA for 12 hours. Control: no stimulation with LPA. After the cells were washed, the number of lymphocytes beneath FLSs was counted. Representative photomicrographs of three independent experiments are shown **(A, C and E)**. Arrows indicate the lymphocytes beneath FLSs. Original magnification, ×200. Data on the number of lymphocytes beneath FLSs are presented as one of three independent experiments analyzed in triplicate **(B, D, and F)**. Data are presented as the mean ± SEM. *$P < 0.05$ vs control or LA-01 0 nM **(B, D, F)**.

the expression level was substantially higher than that of OA FLSs (Figure 1B).

Lysophosphatidic acid receptor 1 inhibitor suppressed lysophosphatidic acid–induced proliferation and cytokine production in RA fibroblast-like synoviocytes

We analyzed the effects of LPA on the proliferation and production of inflammatory mediators by RA FLSs. Stimulation with LPA dose-dependently induced the proliferation of FLSs (Figure 1C). LPA stimulation also induced the production of IL-6 and CCL2 from FLSs in a dose-dependent manner (Figures 1E and 1G), which supports a previous report that LPA upregulated IL-6 mRNA expression by RA FLSs [18]. Stimulation with LPA also induced the production of VEGF and MMP-3 by RA FLSs *in vitro* (Figures 1I and 1J).

Next, we analyzed the effect of an LPA_1 inhibitor on LPA stimulation for RA FLSs. Enhanced cell proliferation by 10 μM LPA was significantly suppressed by LA-01, the LPA_1-selective antagonist (Figure 1D). The treatment with LA-01 significantly reduced the production of IL-6, CCL2, VEGF and MMP-3 by LPA-stimulated RA FLSs (Figures 1F and 1H through 1J). In contrast, the production of CXCL12 by RA FLSs was not altered by stimulation with LPA (Figure 1K). We used Ki16425, another LPA_1 antagonist, to confirm the effects of LPA_1 inhibition on IL-6 production from LPA-stimulated RA FLSs. Incubation with Ki16425 suppressed IL-6 production from LPA-stimulated RA FLSs as well as LA-01 (IL-6 concentrations: vehicle = 299.413 ± 28.084 pg/ml; Ki16425 = 116.785 ± 11.162 pg/ml ($P < 0.05$ vs vehicle); LA-01 = 145.715 ± 15.921 pg/ml ($P < 0.05$ vs vehicle)). These results suggest that LPA–LPA_1 signaling plays important roles in proliferation and cytokine production of RA FLSs *in vitro*.

LPA–LPA_1 signaling promoted pseudoemperipolesis

RA FLSs have been shown to promote the spontaneous migration of leukocytes beneath them, a process termed *pseudoemperipolesis* [21]. We examined the effect of LPA on pseudoemperipolesis. Stimulation with 10 μM LPA significantly increased the number of $CD4^+$ and $CD8^+$ T cells, as well as $CD19^+$ B cells, beneath RA FLSs (Figures 2A to 2F). Moreover, incubation with LA-01 suppressed the LPA-enhanced pseudoemperipolesis of $CD4^+$ and $CD8^+$ T and $CD19^+$ B cells (Figures 2A through 2F), suggesting that interaction of LPA and LPA_1 promotes pseudoemperipolesis of leukocytes.

LPA–LPA_1 signaling promoted cell motility of RA fibroblast-like synoviocytes

We also analyzed the effect of LPA_1 on RA FLS migration by scrape motility assay. Incubation with 10 μM LPA significantly decreased the cell-free area, indicating that LPA induced cell migration *in vitro* (Figures 3A and 3B), as reported previously [22]. In addition, LA-01 significantly increased the cell-free area of RA FLSs (Figures 3A and 3B), suggesting that LPA–LPA_1 signaling also contributes to the promotion of RA FLS motility.

LPA–LPA_1 signaling induced adhesion molecule expression on RA fibroblast-like synoviocytes

It has been reported that signaling from VCAM and ICAM in RA FLSs supports pseudoemperipolesis [21]. Therefore, we next analyzed the expression of VCAM and ICAM on RA FLSs by flow cytometry. We found that stimulation with 10 μM LPA induced the expression of VCAM and ICAM on RA FLSs (Figure 4). Moreover, LA-01 decreased the expression of VCAM and ICAM induced by LPA on RA FLSs (Figure 4). However, the expression of E-selectin on RA FLSs was not altered by LPA simulation (data not shown).

Discussion

In this study, we found that LPA_1 was highly expressed in RA FLSs. LPA stimulated RA FLSs to enhance proliferation, production of inflammatory mediators, pseudoemperipolesis, migration and the expression of adhesion molecules, which are attributable to signaling through LPA_1.

RA FLSs express inflammatory cytokines, chemokines and matrix-degrading enzymes, which contribute to the pathogenesis of RA. LPA has been reported to induce IL-6 mRNA expression on RA FLSs, as well as cell motility [13]. However, the corresponding LPA receptor on RA FLSs has not been identified. We show that LPA augmented IL-6, CCL2, VEGF and MMP-3 production by RA FLSs. Moreover, the LPA-induced production of the inflammatory mediators was inhibited by a LPA_1-selective inhibitor. Therefore, the LPA–LPA_1 cascade plays an important role in cytokine, chemokine and matrix-degrading enzyme production by RA FLSs. Although IC_{50} of LA-01 was 86 nM, which was determined by using LPA_1-transfected CHO cells, 10 nM LA-01 significantly inhibited stimulation of LPA in RA FLSs. The IC_{50} may be dependent on cell type or on the expression level of LPA_1.

Pseudoemperipolesis contributes to the chronic inflammation induced by lymphocyte recruitment in the inflamed joints and protects lymphocytes from apoptosis [21,23,24]. We show that LPA enhanced the pseudoemperipolesis of T and B cells, which is also attributable to LPA_1. It has been reported that stimulation with CXCL12 and signaling from VCAM and ICAM in RA

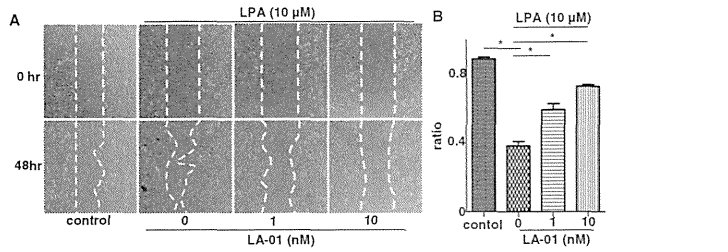


Figure 3 The effect of lysophosphatidic acid receptor 1 on the migration of rheumatoid arthritis fibroblast-like synoviocytes. A scraped cell-free area was created on cultured RA FLSs. After preincubation with a lysophosphatidic acid (LPA) receptor 1 inhibitor (LA-01; 0, 1 or 10 nM) for 30 minutes, cells were stimulated with 10 μM LPA for 48 hours. Control: no stimulation with LPA. **(A)** Representative photomicrographs of three independent experiments are shown. Original magnification, ×40. **(B)** The cell-free area was assessed, and a ratio (cell-free area in 48 hours to cell-free area in 0 hours) was defined. Data are presented as the means (±SEM) of one of three independent experiments analyzed in triplicate. *$P < 0.05$ vs control or LA-01 0 nM. Upper dashed line indicates the cells are stimulated with LPA 10 uM, and lower dashed line indicates LA-01 is added with indicated concentration.

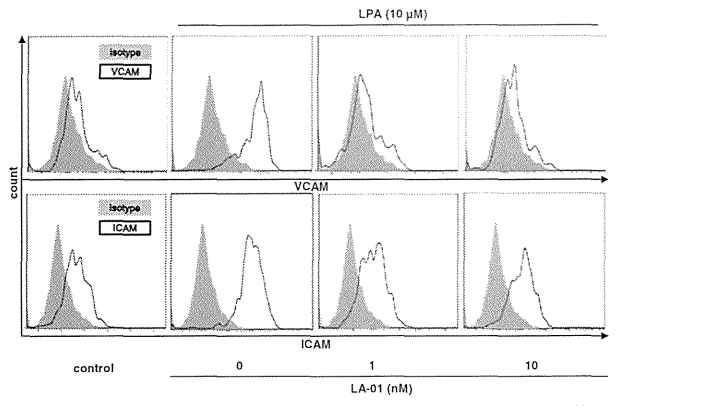


Figure 4 The effect of lysophosphatidic acid receptor 1 on the expression of adhesion molecules on fibroblast-like synoviocytes. Rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs) were pretreated with a lysophosphatidic acid (LPA) receptor 1 inhibitor (LA-01; 0, 1 or 10 nM) for 30 minutes, then the cells were stimulated with 10 μM LPA for 12 hours. Cells were stained with allophycocyanin-conjugated monoclonal antibody (mAb) against vascular cell adhesion molecule (anti-VCAM) or phycoerythrin-conjugated mAb against intercellular adhesion molecule (anti-ICAM). Allophycocyanin- or phycoerythrin-conjugated mouse immunoglobulin G1 (IgG1) was used as a control. The expression of VCAM and ICAM on FLSs was analyzed by flow cytometry. Filled histogram (gray): isotype control; open histogram (black line): VCAM or ICAM.

FLSs support pseudoemperipolesis [21]. Our results indicate that LPA upregulated the expression of VCAM and ICAM on RA FLSs, which was blocked by the LPA_1 antagonist. Thus, LPA may enhance pseudoemperipolesis via the upregulation of VCAM and ICAM expression on RA FLSs through LPA_1. Interestingly, CXCL12 production by RA FLSs was not altered by LPA simulation. Stimulation of lymphocytes by LPA via LPA_1 may also contribute to the enhanced pseudoemperipolesis. In this regard, it has been reported that LPA induced chemokinesis in T cells [25] and lymphocyte transmigration through high endothelial venules [26,27]. Further studies are needed to clarify the effects of LPA–LPA_1 signaling for the lymphocytes on pseudoemperipolesis.

The hyperplastic rheumatoid pannus is characterized by an overabundance of FLSs [2]. This cellular excess stems largely from an imbalance between the proliferation and apoptosis of FLSs [2]. The migration of RA FLSs may also contribute to pannus formation [2]. Our results show that LPA induced the proliferation and migration of FLSs, which was inhibited by the LPA_1 antagonist. Moreover, in a recent study, researchers reported that LPA suppressed tumor necrosis factor–induced apoptosis on RA FLSs via LPA_1 [28]. Therefore, it is suggested that the LPA–LPA_1 signaling also contributed to the cellular excess and migration of FLSs in the RA synovium.

In this study, we show that there are important roles of LPA–LPA_1 signaling on RA FLS stimulation. However, the effects of LPA signals via LPA_{2-6} remain unclear, although RA FLSs also expressed LPA_{2-6}. Further studies are warranted to elucidate the roles of LPA_{2-6} in LPA stimulation of FLSs by using each of the LPA receptor–specific antagonists or FLSs from each LPA receptor–deficient mouse.

It was shown that conditional genetic ablation of ATX, which generates LPA via hydrolysis of lysophosphatidylcholine, in mesenchymal cells resulted in disease attenuation in animal models of arthritis [12]. We have also found that LPA_1 is essential for the development of arthritis in collagen-induced arthritis [15]. The ATX–LPA–LPA_1 axis may play an important role in the development of arthritis.

Conclusion

Our study suggests that LPA–LPA_1 signaling in FLSs may contribute to the pathogenesis of RA by inducing proliferation, production of inflammatory mediators, pseudoemperipolesis and migration on RA FLSs. Thus, LPA_1 could be a promising therapeutic target for RA.

Abbreviations

ATX: Autotaxin; CCL2: Chemokine (C-C motif) ligand 2; CXCL12: Chemokine (C-X-C motif) ligand 12; DMEM: Dulbecco's modified Eagle's medium; Edg: Endothelial cell differentiation gene; ELISA: Enzyme-linked immunosorbent assay; FCS: Fetal calf serum; FLS: Fibroblast-like synoviocyte; ICAM: Intercellular adhesion molecule; IL: Interleukin; LPA: Lysophosphatidic acid; mAb: Monoclonal antibody; MMP-3: Metalloproteinase 3; OA: Osteoarthritis; RA: Rheumatoid arthritis; SEM: Standard error of the mean; VCAM: Vascular cell adhesion molecule; VEGF: Vascular endothelial growth factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YM participated in the design of the study, carried out the experiments and statistical analysis and drafted the manuscript. CM and CS assisted in carrying out the experiments and with manuscript preparation. YI assisted in data interpretation and manuscript preparation. WY and KS collected the clinical materials and assisted in data interpretation and manuscript preparation. MH, MM, NM and TN conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Prof. Timothy J Wright (Otsuma Women's University, Tokyo, Japan) for his helpful advice and for improving the readability of this manuscript. This work was supported in part by the Japanese Ministry of Education, Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases, and Takeda Science Foundation.

Author details

[1]Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. [2]Department of Dermatology, Tokyo Medical University, 6-7-1, Nishi-Shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan. [3]Department of Molecular Biology, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu, Fukuoka 807-8555, Japan. [4]Department of Clinical Research Medicine, Teikyo University, 2-11-1, Kaga, Itabashi-ku, Tokyo 173-8605, Japan. [5]Sonoda Joint Replacement and Sports Medical Center, 1-21-10, Hogima, Adachi-ku, Tokyo 121-0064, Japan. [6]Department of Pharmacovigilance, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. [7]Interdisciplinary Program for Biomedical Sciences, Osaka University, 2-2, Yamada-oka, Suita, Osaka 565-0871, Japan.

Received: 11 April 2014 Accepted: 22 September 2014
Published online: 02 October 2014

References

1. Kinne RW, Bräuer R, Stuhlmüller B, Palombo-Kinne E, Burmester GR: **Macrophages in rheumatoid arthritis.** *Arthritis Res* 2000, **2**:189–202.
2. Bottini N, Firestein GS: **Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors.** *Nat Rev Rheumatol* 2013, **9**:24–33.
3. Aoki J: ***Mechanisms of lysophosphatidic acid production.*** *Semin Cell Dev Biol* 2004, **15**:477–489.
4. Kawagoe H, Stracke ML, Nakamura H, Sano K: **Expression and transcriptional regulation of the PD-Iα/autotaxin gene in neuroblastoma.** *Cancer Res* 1997, **57**:2516–2521.
5. Yang SY, Lee J, Park CG, Kim S, Hong S, Chung HC, Min SK, Han JW, Lee HW, Lee HY: **Expression of autotaxin (NPP-2) is closely linked to invasiveness of breast cancer cells.** *Clin Exp Metastasis* 2002, **19**:603–608.
6. Stassar MJ, Devitt G, Brosius M, Rinnab L, Prang J, Schradin T, Simon J, Petersen S, Kopp-Schneider A, Zöller M: **Identification of human renal cell carcinoma associated genes by suppression subtractive hybridization.** *Br J Cancer* 2001, **85**:1372–1382.
7. Umezu-Goto M, Kishi Y, Taira A, Hama K, Dohmae N, Takio K, Yamori T, Mills GB, Inoue K, Aoki J, Arai H: **Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production.** *J Cell Biol* 2002, **158**:227–233.
8. Liu S, Umezu-Goto M, Murph M, Lu Y, Liu W, Zhang F, Yu S, Stephens LC, Cui X, Murrow G, Coombes K, Muller W, Hung MC, Perou CM, Lee AV, Fang X, Mills GB: **Expression of autotaxin and lysophosphatidic acid receptors increases mammary tumorigenesis, invasion, and metastases.** *Cancer Cell* 2009, **15**:539–550. A published erratum appears in *Cancer Cell* 2009, **16**:172.
9. Masuda A, Nakamura K, Izutsu K, Igarashi K, Ohkawa R, Jona M, Higashi K, Yokota H, Okudaira S, Kishimoto T, Watanabe T, Koike Y, Ikeda H, Kozai Y, Kurokawa M, Aoki J, Yatomi Y: **Serum autotaxin measurement in haematological malignancies: a promising marker for follicular lymphoma.** *Br J Haematol* 2008, **143**:60–70.
10. Schleicher SM, Thotala DK, Linkous AG, Hu R, Leahy KM, Yazlovitskaya EM, Hallahan DE: **Autotaxin and LPA receptors represent potential molecular targets for the radiosensitization of murine glioma through effects on tumor vasculature.** *PLoS One* 2011, **6**:e22182.
11. Houben AJ, Moolenaar WH: **Autotaxin and LPA receptor signaling in cancer.** *Cancer Metastasis Rev* 2011, **30**:557–565.
12. Nikitopoulou I, Oikonomou N, Karouzakis E, Sevastou I, Nikolaidou-Katsaridou N, Zhao Z, Mersinias V, Armaka M, Xu Y, Masu M, Milles GB, Gay S, Kollias G, Aidinis V: **Autotaxin expression from synovial fibroblasts is essential for the pathogenesis of modeled arthritis.** *J Exp Med* 2012, **209**:925–933.
13. Kehlen A, Lauterbach R, Santos AN, Thiele K, Kabisch U, Weber E, Riemann D, Langner J: **IL-1β- and IL-4-induced down-regulation of autotaxin mRNA and PC-1 in fibroblast-like synoviocytes of patients with rheumatoid arthritis (RA).** *Clin Exp Immunol* 2001, **123**:147–154.
14. Bourgoin SG, Zhao C: **Autotaxin and lysophospholipids in rheumatoid arthritis.** *Curr Opin Investig Drugs* 2010, **11**:515–526.
15. Miyabe Y, Miyabe C, Iwai Y, Takayasu A, Fukuda S, Yokoyama W, Nagai J, Jona M, Tokuhara Y, Ohkawa R, Albers HM, Ovaa H, Aoki J, Chun J, Yatomi Y, Ueda H, Miyasaka M, Miyasaka N, Nanki T: ***Necessity of lysophosphatidic* acid receptor 1 for development of arthritis.** *Arthritis Rheum* 2013, **65**:2037–2047.
16. Cader MZ, Filer A, Hazlehurst J, de Pablo P, Buckley CD, Raza K: **Performance of the 2010 ACR/EULAR criteria for rheumatoid arthritis: comparison with 1987 ACR criteria in a very early synovitis cohort.** *Ann Rheum Dis* 2011, **70**:949–955.
17. Kaneko K, Miyabe Y, Takayasu A, Fukuda S, Miyabe C, Ebisawa M, Yokoyama W, Watanabe K, Imai T, Muramoto K, Terashima Y, Sugihara T, Matsushima K, Miyasaka N, Nanki T: **Chemerin activates fibroblast-like synoviocytes in patients with rheumatoid arthritis.** *Arthritis Res Ther* 2011, **13**:R158.
18. Watanabe K, Penfold ME, Matsuda A, Ohyanagi N, Kaneko K, Miyabe Y, Matsumoto K, Schall TJ, Miyasaka N, Nanki T: **Pathogenic role of CXCR7 in rheumatoid arthritis.** *Arthritis Rheum* 2010, **62**:3211–3220.
19. Tanaka M, Nakaide S, Takaoka Y: **Compounds having lysophosphatidic acid receptor antagonism and uses thereof.** Patent Version Number: WO/2005/058790 (2005-06-30) [http://patentscope.wipo.int/search/en/WO2005058790] (accessed 9 October 2014).
20. Ishii S, Noguchi K, Yanagida K: **Non-Edg family lysophosphatidic acid (LPA) receptors.** *Prostaglandins Other Lipid Mediat* 2009, **89**:57–65.
21. Bradfield PF, Amft N, Vernon-Wilson E, Exley AE, Parsonage G, Rainger GE, Nash GB, Thomas AM, Simmons DL, Salmon M, Buckley CD: **Rheumatoid fibroblast-like synoviocytes overexpress the chemokine stromal cell-derived factor 1 (CXCL12), which supports distinct patterns and rates of CD4+ and CD8+ T cell migration within synovial tissue.** *Arthritis Rheum* 2003, **48**:2472–2482.
22. Zhao C, Fernandes MJ, Prestwich GD, Turgeon M, Di Battista J, Clair T, Poubelle PE, Bourgoin SG: **Regulation of lysophosphatidic acid receptor expression and function in human synoviocytes: implications for rheumatoid arthritis?** *Mol Pharmacol* 2008, **73**:587–600.
23. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ: **Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1.** *Blood* 2000, **96**:2655–2663.
24. Burger JA, Zvaifler NJ, Tsukada N, Firestein GS, Kipps TJ: **Fibroblast-like *synoviocytes support* B-cell *pseudoemperipolesis via a stromal* cell-derived factor-1– and CD106 (VCAM-1) –dependent mechanism.** *J Clin Invest* 2001, **107**:305–315.
25. Kanda H, Newton R, Klein R, Morita Y, Gunn MD, Rosen SD: **Autotaxin, an ectoenzyme that produces lysophosphatidic acid, promotes the entry of lymphocytes into secondary lymphoid organs.** *Nat Immunol* 2008, **9**:415–423.
26. Zhang Y, Chen YC, Krummel MF, Rosen SD: **Autotaxin through lysophosphatidic acid stimulates polarization, motility, and transendothelial migration of naive T cells.** *J Immunol* 2012, **189**:3914–3924.
27. Bai Z, Cai L, Umemoto E, Takeda A, Tohya K, Komai Y, Veeraveedu PT, Hata E, Sugiura Y, Kubo A, Suematsu M, Hayasaka H, Okudaira S, Aoki J, Tanaka T, Albers HM, Ovaa H, Miyasaka M: **Constitutive lymphocyte transmigration across the basal lamina of high endothelial venules is regulated by the autotaxin/lysophosphatidic acid axis.** *J Immunol* 2013, **190**:2036–2048.
28. Orosa B, González A, Mera A, Gómez Reino JJ, Conde C: **Lysophosphatidic acid receptor 1 suppression sensitizes rheumatoid fibroblast-like synoviocytes to tumor necrosis factor-induced apoptosis.** *Arthritis Rheum* 2012, **64**:2460–2470.

doi:10.1186/s13075-014-0461-9
Cite this article as: Miyabe *et al.*: Activation of fibroblast-like synoviocytes derived from rheumatoid arthritis via lysophosphatidic acid–lysophosphatidic acid receptor 1 cascade. *Arthritis Research & Therapy* 2014 **16**:461.

ARTHRITIS & RHEUMATOLOGY
Vol. 66, No. 10, October 2014, pp 2751–2761
DOI 10.1002/art.38743

Macrophage-Derived Delta-like Protein 1 Enhances Interleukin-6 and Matrix Metalloproteinase 3 Production by Fibroblast-like Synoviocytes in Mice With Collagen-Induced Arthritis

Chiyoko Sekine,[1] Toshihiro Nanki,[2] and Hideo Yagita[3]

Objective. **We previously reported that blockade of the Notch ligand delta-like protein 1 (DLL-1) suppressed osteoclastogenesis and ameliorated arthritis in a mouse model of rheumatoid arthritis (RA). However, the mechanisms by which joint inflammation were suppressed have not yet been revealed. This study was undertaken to determine whether DLL-1 regulates the production of RA-related proinflammatory cytokines.**

Methods. **Joint cells from mice with collagen-induced arthritis (CIA) and mouse fibroblast-like synoviocytes (FLS) were cultured with or without stimuli in the presence of neutralizing antibodies against Notch ligands, and the production of proinflammatory cytokines was determined by enzyme-linked immunosorbent assay. The expression of Notch receptors and ligands on mouse joint cells was determined by flow cytometry.**

Results. **The production of interleukin-6 (IL-6) and granulocyte–macrophage colony-stimulating factor (GM-CSF) by mouse joint cells with or without stimulation was suppressed by DLL-1 blockade. DLL-1 blockade also suppressed the levels of IL-6 and matrix metalloproteinase 3 (MMP-3) in the joint fluid in a mouse model of RA. However, the production of tumor necrosis factor α and IL-1β was not suppressed by DLL-1 blockade. The production of IL-6 and MMP-3 by mouse FLS was enhanced by DLL-1 stimulation as well as Notch-2 activation. Among joint cells, DLL-1 was not expressed on mouse FLS but was expressed on macrophages.**

Conclusion. **These results suggest that the interaction of DLL-1 on mouse joint macrophages with Notch-2 on mouse FLS enhances the production of IL-6 and MMP-3. Therefore, suppression of IL-6, GM-CSF, and MMP-3 production by DLL-1 blockade might be responsible for the amelioration of arthritis in a mouse model of RA.**

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint inflammation and synovial hyperplasia leading to joint destruction. At the site of inflammation, inflammatory cells, such as T cells, B cells, macrophages, and neutrophils, are recruited and further activated. These cells also activate joint cells, including fibroblast-like synoviocytes (FLS), chondrocytes, and osteoclasts, which increase inflammation and induce cartilage degradation and bone erosion (1).

The intimal lining of the synovium is composed of macrophage-like synoviocytes (type A synoviocytes) and FLS (type B synoviocytes) in relatively equal proportions. Macrophage-like synoviocytes express markers similar to other tissue resident macrophages, such as CD11b, CD68, CD14, and class II major histocompatibility antigens. They have little capacity to proliferate. FLS are mesenchymal cells that display many characteristics of fibroblasts and secrete proteins critical for joint lubrication, including hyaluronan and lubricin.

The synovium plays a key role in the pathogenesis of RA. Highly activated macrophage-like synoviocytes and FLS interact with each other directly and through the secretion of mediators that contribute to the perpetuation of synovitis and destruction of the extracellular matrix (1,2). The macrophage-like synoviocytes produce proinflammatory cytokines, chemokines, and growth factors, while the activated hyperplastic FLS augment destructive inflammation by producing cytokines, prostaglandins, proteases, and cartilage-degrading enzymes. Actually, these mediators are dominated in RA synovium, and FLS are the primary source of interleukin-6 (IL-6) and matrix metalloproteinases (MMPs) in RA. Moreover, FLS in RA exhibit unique aggressive features and exacerbate joint damage by invading the bone matrix of the rheumatoid joint.

It is well established that tumor necrosis factor α (TNFα) and IL-6 play dominant roles in the pathobiology of RA (1). IL-1 also has a significant impact on the disease process. Therapeutic use of agents that inhibit TNFα, IL-6, or IL-1 ameliorates inflammation and joint destruction in RA. Collagenases (MMP-1 and MMP-13) and stromelysin (MMP-3) are also important for the degradation of cartilage in RA (2). Their synthesis and activation are induced by various factors, including proinflammatory cytokines and Toll-like receptor ligands.

The Notch pathway is known to regulate cell fate decision and differentiation during embryonic and postnatal development (3). Notch is conserved across species, and 4 mammalian Notch receptors (Notch-1,- 2, -3, and -4) have been identified. Notch ligands of the delta-like protein (DLL) and Jagged families (DLL-1, DLL-4, Jagged-1, and Jagged-2) are transmembrane proteins, and the extracellular domain contains the Delta/Serrate/Lag-2 domain that is required for their interaction with Notch receptors. Jagged-1 and Jagged-2 contain an additional cysteine-rich domain. Receptor–ligand engagement triggers the cleavage of Notch receptor extracellular domain, which facilitates a cleavage within the Notch transmembrane domain by a γ-secretase. This leads to release of the Notch intracellular domain (ICD) from the membrane, and the released Notch ICD translocates to the nucleus to drive the expression of Notch target genes (3,4).

The importance of Notch receptors in osteoclast differentiation has been reported, and we previously found that DLL-1 promoted osteoclastogenesis via Notch-2, and that Jagged-1 suppressed osteoclastogenesis via Notch-1 in both mice and humans (5). We also demonstrated that DLL-1 blockade reduced the number of osteoclasts in the affected joints in a mouse model of RA and suppressed ovariectomy-induced bone loss in mice (5). In addition, the arthritis score and histologic examination of joint sections indicated the amelioration of arthritis by DLL-1 blockade in a mouse model of RA, suggesting that DLL-1 contributes to inflammation in the joint.

Involvement of Notch-1 in the TNFα-induced proliferation of RA synoviocytes has been demonstrated (6). Notch has also been implicated in vascular endothelial growth factor/angiopoietin 2–induced angiogenesis and the production of IL-6, IL-8, MMP-2, and MMP-9 in RA synovium (7,8). It has also been shown that Notch signal inhibitors suppressed IL-6 production by TNFα-stimulated FLS and by lipopolysaccharide (LPS)/interferon-γ (IFNγ)–stimulated macrophages (9,10). Notch signaling has also been implicated in cytokine production by dendritic cells and T cells (11,12). Therefore, DLL-1 could modulate the production of inflammatory cytokines in the mouse model of RA, underlying the suppression of joint inflammation by DLL-1 blockade demonstrated in our previous study (5). In the current study, we investigated whether DLL-1 regulates RA-related cytokine production in the joints in a mouse model of RA.

MATERIALS AND METHODS

Mice. Seven-week-old male DBA/1 mice were purchased from Charles River. Collagen-induced arthritis (CIA) was induced by immunization with 150 μg of bovine type II collagen (Collagen Research Center) emulsified with Freund's complete adjuvant (Difco) on day 0 and day 21. Eight weeks after the second immunization, peripheral blood (PB), spleen, bone marrow (BM), and joint cells were prepared and used for experiments. Mice with CIA were injected intraperitoneally with 0.25 mg of HMD1-5 or control hamster IgG twice a week for 2 weeks. Treatment was begun on day 21. Arthritis was assessed clinically by visual scoring using a scale of 0–4 as previously described (5). All animal experiments were approved by the Juntendo University Animal Experimental Ethics Committee.

Cell preparation. Mouse joint cells were prepared from the hind legs of mice with CIA 8 weeks after the second immunization, as previously described (13). Briefly, the skin was removed from the hind legs, soft tissue and tendons were cleaned as possible, and bones were then separated at the hip joint without damaging the bones, to avoid contamination with BM cells. The knee and ankle tendons were dissected in phosphate buffered saline containing 0.5 mg/ml of collagenase (Wako) to open up the joint cavity, then incubated at 37°C for 1 hour. Joint cells were pipetted from the knee and ankle joint spaces and subjected to red blood cell lysis.

To establish mouse FLS as previously described (14), joint cells prepared as described above were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako) containing 10% fetal bovine serum (FBS; JRH Biosciences). Joint cells other than mouse FLS, including synovial macrophages, have a limited lifespan in vitro and rarely survive more than a few weeks in culture. Cells beyond the third passage were CD45-negative (99%). Cells from passages 6–11 were used.

Supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

[1]Chiyoko Sekine, PhD: Juntendo University School of Medicine, Tokyo, Japan, and Teikyo University School of Medicine, Tokyo, Japan; [2]Toshihiro Nanki, MD, PhD: Teikyo University School of Medicine, Tokyo, Japan; [3]Hideo Yagita, PhD: Juntendo University School of Medicine, Tokyo, Japan.

Address correspondence to Chiyoko Sekine, PhD, Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail: csekine@juntendo.ac.jp.

Submitted for publication September 16, 2013; accepted in revised form June 5, 2014.

Femoral BM cells from mice with CIA were cultured with 50 ng/ml of recombinant mouse macrophage colony-stimulating factor (M-CSF; Wako) in α-minimum essential medium (Wako) containing 10% FBS for 8 days and used as BM-derived macrophages (BMMs).

Antibodies. Generation and characterization of hamster IgG monoclonal antibodies (mAb) specific for mouse Notch-1 (HMN1-12), Notch-2 (HMN2-29), Notch-3 (HMN3-133), Notch-4 (*HMN4-14*), *DLL-1* (*HMD1-5*), *DLL-4* (HMD4-2), Jagged-1 (HMJ1-29), and Jagged-2 (HMJ2-1) have been described previously (15,16). The stimulating activity of the antireceptor mAb and the blocking activity of the antiligand mAb have been verified in vitro and in vivo (5,15,16). Fluorescein isothiocyanate (FITC)– or phycoerythrin (PE)–labeled mAb against mouse CD45 (30-F11), PerCP–Cy5.5–labeled mAb against mouse CD11b (M1/70), allophycocyanin-labeled mAb against mouse F4/80 (BM8), and PE-conjugated streptavidin were obtained from eBioscience. FITC-labeled mAb against mouse Ly6G (1A8) was from BD Bioscience. Antibodies against cleaved Notch-1 (Val1744) and α-tubulin (11H10) were purchased from Cell Signaling Technology. Antibody specific for Notch-2 ICD (411801) was from R&D Systems.

Reagents. The γ-secretase inhibitor DAPT was purchased from Calbiochem and used at 10 μ*M*. Recombinant mouse TNFα and IL-1β were purchased from Wako and used at 10 ng/ml. Recombinant mouse IFNγ (Wako) was used at 50 ng/ml, and LPS (Sigma) was used at 10 ng/ml.

Flow cytometry. Multicolor staining was conducted using combinations of the indicated mAb as previously described (16). The cells were analyzed on a FACSCalibur system and analyzed with CellQuest software (both from BD Biosciences).

Cell culture. Mouse joint cells (3×10^5/ml) or mouse FLS (5×10^4/ml) were cultured in DMEM containing 10% FBS in the presence of the indicated reagents. Cytokine concentrations in the culture supernatant were measured using a specific enzyme-linked immunosorbent assay (ELISA) kit according to the protocols recommended by the manufacturers (OptEIA for TNFα, IL-1β, and IL-6 [BD Biosciences] and Quantikine for MMP-3 and GM-CSF [R&D Systems]). Cell viability was accessed by water-soluble tetrazolium 8 assay using Cell Counting Kit 8 (Dojin).

Chinese hamster ovary (CHO) cells and mouse DLL-1–, DLL-4–, Jagged-1–, or Jagged-2–transfected cells (15) were fixed with 1% paraformaldehyde, then used as stimulators. Mouse FLS were cultured with 10 μ*M* DAPT or control DMSO for 2 hours, then paraformaldehyde-fixed CHO transfectants were added in the presence or absence of 10 ng/ml LPS. Cytokine concentrations in the culture supernatant after 24 hours were measured by ELISA as described above.

To purify joint macrophages, CD11b+ cells were sorted from the joint cells of mice with CIA using CD11b MicroBeads (Miltenyi Biotec). These CD11b+ joint macrophages (3×10^5/ml) were cultured with mouse FLS (5×10^4/ml) for 24 hours, and cytokine concentrations were analyzed as described above. BMMs, developed as described above, were stimulated with IFNγ for 24 hours and then cultured with mouse FLS for 24 hours. Cytokine concentrations were analyzed as described above.

Joint fluid collection. Serum from arthritic K/BxN mice (100 μl) was injected into the peritoneal cavity of C57BL/6 mice on day 0 and day 2 to induce arthritis. Anti-mouse DLL-1 mAb (HMD1-5) or control hamster IgG (eBioscience) was administrated intraperitoneally twice a week, beginning on day 3. Arthritis scores and hematoxylin and eosin staining of the arthritic joints have been described previously (5). Thirteen days after serum transfer, the knee joints were opened from the patellar tendon and washed in 500 μl of complete medium, and incubated for 1 hour at room temperature to allow the elution of cytokines, as previously described (17). Supernatants were then removed and stored at −20°C until assayed. Joint fluid from mice with CIA was collected 36 days after immunization.

Immunohistochemistry. Joint cell suspensions were centrifuged onto glass cytospin slides, fixed with acetone, and then incubated with 2.5 μg/ml of rat anti-mouse IL-6 mAb (MP5-20F3; BD Bioscience) for 2 hours. They were then incubated with biotinylated antibody against rat immunoglobulins (DakoCytomation), and Alexa Fluor 594–labeled streptavidin (Molecular Probes) was used for detection. They were further incubated with FITC-labeled mAb to CD11b, then Alexa Fluor 488–labeled anti-FITC (Molecular Probes) was used as secondary antibody. DAPI was used for nuclear counterstain. Fluorescent images were acquired on a microscope (Eclipse TE300; Nikon) equipped with a digital camera (C10600-10B; Hamamatsu Photonics) and processed using AquaCosmos (Hamamatsu Photonics).

Paraffin-fixed joint tissue sections from mice with K/BxN serum–induced arthritis (described above) were deparaffinized, pretreated in Liberate Antibody Binding Solution (Polysciences) for 5 minutes, and incubated with 2.5 μg/ml of biotin-labeled HMD1-5. A Catalyzed Signal Amplification System and peroxidase (DakoCytomation) were used for detection. Color was developed with diaminobenzidine, whereas the sections were counterstained with hematoxylin. Images were acquired on a microscope (Eclipse E800M; Nikon) equipped with a digital camera (DS-Fi1c; Nikon) and processed using DS-L2 (Nikon). For fluorescence immunohistochemistry, HMD1-5 was detected using a Tyramide Signal Amplification Kit (PerkinElmer). To detect FITC-labeled mAb to CD11b, Alexa Fluor 488–labeled anti-FITC (Molecular Probes) was used as a secondary antibody. For staining CD248 (Abnova), biotinylated anti-rabbit IgG (Dako) was used as a secondary antibody and detected by Alexa Fluor 594–labeled streptavidin (Molecular Probes). Fluorescent images were acquired as described above.

Reverse transcriptase–polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIzol reagent (Life Technologies) and was reverse-transcribed to complementary DNA with oligo(dT) and SuperScript RT (Invitrogen). PCR consisted of 35 cycles of 45 seconds at 94°C, 1 minute at 58°C, and 1 minute at 72°C. The primers have been described previously (5).

Immunoblotting. Immunoblotting was performed as previously described (5). Briefly, mouse FLS were lysed in whole cell lysis buffer (20 m*M* HEPES, pH 7.8, 420 m*M* NaCl, 0.5% Nonidet P40, 25% glycerol, 0.2 m*M* EDTA, 1.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 1 m*M* phenylmethylsulfonyl fluoride, and complete protease inhibitors) (Roche). The lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to PVDF membranes. These membranes were incubated with primary antibody, and then with horseradish peroxidase–conjugated secondary antibodies. The immunoreactive proteins were visualized using ECL Prime (GE Healthcare).

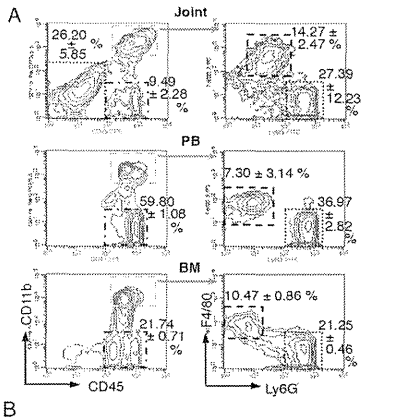
RNA interference. Small interfering RNAs (siRNAs) targeting mouse Notch-1 or Notch-2 and scrambled negative control siRNA were purchased from OriGene. Each siRNA was transfected into mouse FLS using Metafectene Pro (Biontex). Cell surface expression was analyzed by flow cytometry to validate the silencing of Notch-1 or Notch-2.

Statistical analysis. All comparisons between 2 groups were analyzed using Student's unpaired *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Mouse joint cell populations. Joint cells were prepared from the arthritic hind legs of mice with CIA as described in Materials and Methods. As shown in Figure 1A, CD45−CD11b− cells, which included mouse FLS, were detected in joint cells but few were detected in PB or BM cells. In contrast, the CD45+CD11b− cell population, mainly CD3+B220− T and CD3−B220+ B lymphocytes, was small in joint cells but abundant in PB cells. In the CD45+CD11b^{high} cell population, Ly6G^{high}F4/80− neutrophils were detected in similar percentages in joint, PB, and BM cells. While joint cells had Ly6G^{intermediate}F4/80^{high} tissue-resident macrophages, PB and BM cells had Ly6G−F4/80^{intermediate} monocyte-lineage cells in the CD45+CD11b^{high} compartment. As expected, the number of joint cells obtained from normal mice was 14 times lower than that obtained from mice with CIA, and few leukocytes were detected in normal joint cells (Figure 1B). The increase in neutrophils was most prominent in joint cells from mice with CIA, but the macrophage population and the fibroblast population were also increased considerably.

DLL-1 blockade suppresses IL-6 production by mouse joint cells. To address how the blockade of DLL-1 suppressed joint inflammation in K/BxN serum–transfer arthritis (5), a mouse model of RA (18,19), we determined the effect of DLL-1 blockade on the production of RA-related proinflammatory cytokines in a culture of mouse joint cells. Production of TNFα and IL-1β was not affected by the blockade of DLL-1, DLL-4, Jagged-1, or Jagged-2 (Figure 2A). Production of IL-6, GM-CSF, and MMP-3 was significantly suppressed by the blockade of DLL-1 but not DLL-4, Jagged-1, or Jagged-2 (Figure 2A). The production of IL-6, GM-CSF, and MMP-3 by mouse joint cells was enhanced by stimulation with TNFα and IL-1β, and this increase was also suppressed by DLL-1 blockade, while cell viability was not reduced (Figure 2B). IL-6 and GM-CSF production by LPS-stimulated mouse spleen cells was also suppressed by DLL-1 blockade, while TNFα and IL-1β levels were not affected (Figure 2C). MMP-3 production from LPS-stimulated spleen cells was not detected. These results indicate that the contri-



B

Joint cell populations	CIA	Normal
Total	4560 ± 611	320 ± 11
CD45$^-$CD11b$^-$Ly6G$^-$F4/80$^-$ (Fibroblasts)	1140 ± 115	138 ± 86
CD45$^+$CD11b^{hi}Ly6G^{int}F4/80^{hi}(Macrophages)	640 ± 128	26 ± 8
CD45$^+$CD11b^{hi}Ly6G^{hi}F4/80$^-$ (Neutrophils)	1080 ± 383	23 ± 17
CD45$^+$CD11b$^-$CD3$^+$B220$^-$ (T lymphocytes)	120 ± 38	7 ± 2
CD45$^+$CD11b$^-$CD3$^-$B220$^+$ (B lymphocytes)	190 ± 75	14 ± 1

Figure 1. Cell populations in the joints of mice with collagen-induced arthritis (CIA). **A,** Flow cytometric analysis of populations of joint cells, peripheral blood (PB), and bone marrow (BM) cells from mice with CIA. Right panels show staining of the CD45+CD11b^{high}-gated cells in the left panels. Values are the mean ± SD percent of total cells (n = 3 mice). **B,** Results of flow cytometric analysis of joint cell populations from mice with CIA and normal mice. Values are the mean ± SD cell number (× 10^2) per leg (n = 3 mice per group).

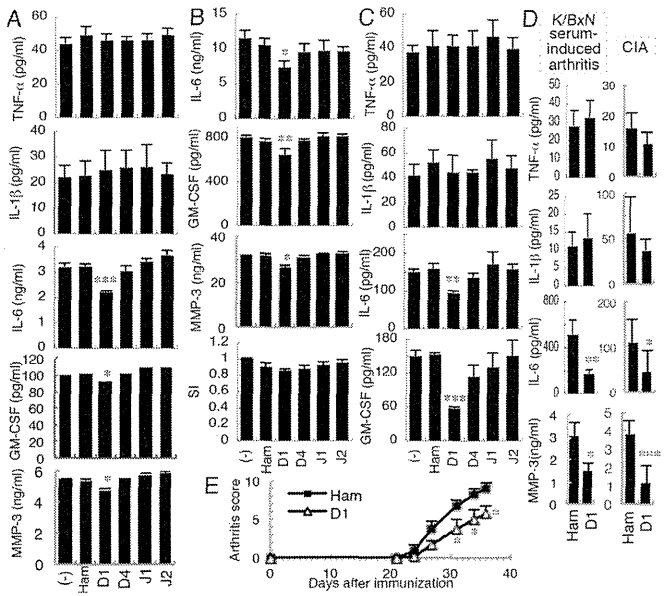


Figure 2. Effects of Notch ligand blockade on inflammatory cytokine production by joint cells from mice with collagen-induced arthritis (CIA). **A,** Levels of cytokines in the supernatants of joint cells from mice with CIA after 24 hours of culture with 20 μg/ml of the indicated monoclonal antibody (mAb) (HMD1-5 [D1], HMD4-2 [D4], HMJ1-29 [J1], or HMJ2-1 [J2]) or control hamster IgG (Ham), as measured by enzyme-linked immunosorbent assay (ELISA). **B,** Levels of cytokines in the supernatants of joint cells from mice with CIA after 24 hours of stimulation with tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) (10 ng/ml each) in the presence of the indicated mAb or control, as measured by ELISA. Cell viability was analyzed by water-soluble tetrazolium 8 assay and is indicated as the stimulation index (SI). **C,** Levels of cytokines in the supernatants of spleen cells from mice with CIA after 24 hours of stimulation with 10 ng/ml lipopolysaccharide in the presence of the indicated mAb or control, as measured by ELISA. In **A–C,** values are the mean ± SD from triplicate wells. **D,** Cytokines in the joint fluid of mice with K/BxN serum–induced arthritis and mice with CIA treated with control hamster IgG or HMD1-5, as measured by ELISA. Values are the mean ± SD (n = 7 mice per group). **E,** Severity of arthritis, assessed by arthritis score, in mice with CIA treated with control hamster IgG or HMD1-5. Values are the mean ± SEM (n = 7 mice per group). In **A–E,** results are representative of 3 independent experiments. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ versus control hamster IgG. GM-CSF = granulocyte–macrophage colony-stimulating factor; MMP-3 = matrix metalloproteinase 3.

bution of DLL-1 to IL-6 and GM-CSF production is not specific to the mouse joint cells.

Next, we measured the levels of TNFα, IL-1β, IL-6, GM-CSF, and MMP-3 in the sera and swollen joints of mice with K/BxN serum–induced arthritis that were treated with anti–DLL-1 mAb or control IgG as previously described (5). Although these cytokines were undetectable in the serum on day 13, the levels of IL-6 and MMP-3, but not TNFα or IL-1β, in the joints of anti–DLL-1 mAb–treated mice were significantly lower than those in the control IgG-treated mice (Figure 2D). The levels of IL-6 and MMP-3 in the joint fluid of mice with CIA were also suppressed by anti–DLL-1 mAb treatment (Figure 2D). GM-CSF was undetectable in the joints of these mice. In addition, in mice with CIA, arthritis was suppressed by DLL-1 blockade (Figure 2E). These results suggest that the suppression of IL-6, GM-CSF, and MMP-3 production in the mouse joint might be responsible for the suppression of arthritis by DLL-1 blockade.

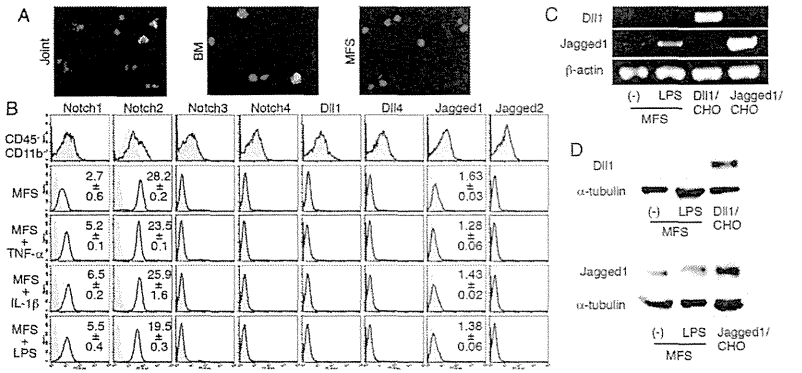


Figure 3. Mouse fibroblast-like synoviocyte (FLS) expression of Notch receptors and ligands. **A,** Immunohistologic staining of joint cells, bone marrow (BM) cells, and FLS (MFS) from mice with CIA. Green indicates CD11b, red indicates IL-6, and blue indicates DAPI. Original magnification × 200. **B,** Expression of Notch receptors and ligands on CD45−CD11b− mouse joint cells, analyzed by flow cytometry. Mouse FLS were cultured with or without the indicated stimulus for 6 hours, and then expression was analyzed. Shaded histograms indicate staining with control hamster IgG. Open histograms indicate staining with mAb specific for the indicated Notch receptor or ligand. Values are the mean ± SD mean fluorescence intensity of positive staining divided by control (n = 3 independent experiments). **C,** Levels of mRNA for delta-like protein 1 (DLL-1) and Jagged-1, measured by reverse transcriptase–polymerase chain reaction. Mouse FLS were cultured with or without lipopolysaccharide (LPS) for 6 hours. DLL-1–transfected Chinese hamster ovary (CHO) cells and Jagged-1–transfected CHO cells were used as positive controls. **D,** Western blotting of DLL-1 and Jagged-1 using HMD1-5 (for DLL-1) or HMJ1-29 (for Jagged-1). Mouse FLS were cultured with or without LPS for 6 hours. DLL-1–transfected CHO cells and Jagged-1–transfected CHO cells were used as positive controls, and α-tubulin was used as a loading control. See Figure 2 for other definitions.

Expression of Notch receptors and ligands by mouse FLS. The mouse joint cells produced a high level of IL-6, while levels of TNFα and IL-1β were low (Figure 2A). We found that IL-6 was produced by a subset of CD11b− nonmyeloid cells in the joints and cultured mouse FLS, while CD11b+ macrophages in the joint and BM did not secrete IL-6 (Figure 3A). IL-6 production by cultured BM cells was not detectable by ELISA (data not shown). Therefore, we next examined whether DLL-1 was involved in the production of IL-6 by mouse FLS.

As shown in Figure 3B, the CD45−CD11b− FLS population in the joint cells expressed Notch-2, and low levels of DLL-1 and Jagged-1 were also detected. Mouse FLS, which were established as described in Materials and Methods, also expressed Notch-2 and Jagged-1 but not DLL-1. Notch-1 expression was detected on mouse FLS and was up-regulated significantly by inflammatory stimulation with TNFα, IL-1β, or LPS ($P = 0.00031$, $P = 0.000029$, and $P = 0.000018$, respectively). In contrast, Notch-2 and Jagged-1 expression on mouse FLS were slightly down-regulated by such stimulation. Statistical analysis showed that these decreases in expression were also significant ($P = 0.0005$ for TNFα, $P = 0.027$ for IL-1β, and $P = 0.00025$ for LPS). DLL-1 expression on mouse FLS was not detectable even after stimulation. RT-PCR analysis showed that DLL-1 messenger RNA (mRNA) was not detectable in mouse FLS with or without LPS stimulation, while Jagged-1 mRNA was expressed in mouse FLS (Figure 3C). Immunoblot analysis supported these results, indicating that DLL-1 was not detectable, while Jagged-1 was expressed in mouse FLS (Figure 3D).

Enhanced production of IL-6 and MMP-3 by mouse FLS cultured with DLL-1. Since DLL-1 was not detected on mouse FLS, DLL-1 on other cells in the joints could stimulate Notch-1 and Notch-2 on mouse FLS. As shown in Figure 4A, IL-6 production by mouse FLS was enhanced by DLL-1 but not by any other Notch ligands. Levels of MMP-3, which is produced primarily

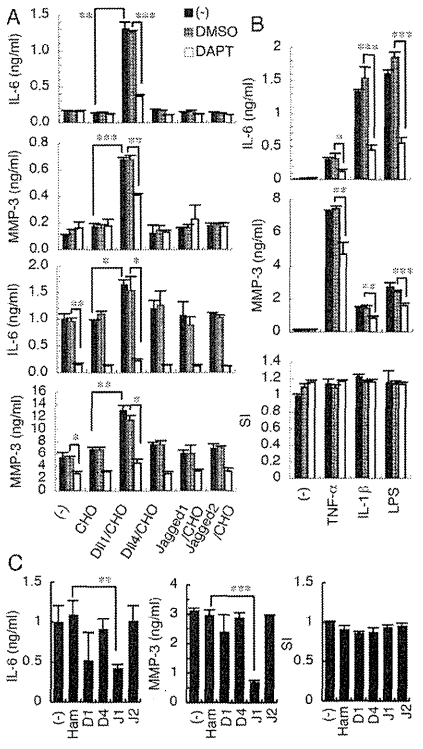


Figure 4. Increased production of IL-6 and MMP-3 by fibroblast-like synoviocytes (FLS) from mice with CIA cultured with delta-like protein 1 (DLL-1). **A,** Levels of IL-6 and MMP-3 in mouse FLS cultured with DLL-1–, DLL-4–, Jagged-1–, or Jagged-2–transfected Chinese hamster ovary (*CHO*) cells or control *CHO* cells in the presence of DAPT or DMSO control for 24 hours, as measured by ELISA. The 2 bottom panels show the results of experiments that also included stimulation with lipopolysaccharide (LPS). **B,** Levels of IL-6 and MMP-3 in mouse FLS cultured with the indicated stimulus for 24 hours in the presence of DAPT or DMSO control, as measured by ELISA. Cell viability was analyzed by water-soluble tetrazolium 8 assay and is indicated as the SI. **C,** Levels of IL-6 and MMP-3 in mouse FLS stimulated with LPS in the presence of the indicated mAb or control hamster IgG as described in Figure 1, as measured by ELISA. Results are representative of 3 independent experiments. Values are the mean ± SD from triplicate wells. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. See Figure 2 for other definitions.

by FLS and is important for cartilage degradation in RA, were also increased by DLL-1. This enhancement by DLL-1 was abrogated by the Notch inhibitor DAPT, implying that DLL-1 activates Notch signaling. Stimulation of mouse FLS with LPS up-regulated the production of IL-6 and MMP-3, and DLL-1 further enhanced it (Figure 4A). The LPS-induced up-regulation, as well as the DLL-1–induced enhancement, was also abrogated by DAPT (Figure 4A), indicating that the LPS-induced up-regulation was also Notch dependent. In addition, TNFα-induced and IL-1β–induced up-regulation of IL-6 and MMP-3 was inhibited by DAPT, while cell viability was not affected (Figure 4B). These results imply that Notch signaling via mouse FLS–mouse FLS interactions is involved in the up-regulation of IL-6 and MMP-3 by these proinflammatory agents. Consistent with the expression of Notch ligands on mouse FLS (Figures 3B–D), blockade of Jagged-1 only inhibited the IL-6 and MMP-3 production by LPS-stimulated mouse FLS without affecting cell viability (Figure 4C). Production of GM-CSF by mouse FLS was undetectable even with stimulation in this culture condition (data not shown).

Expression of DLL-1 on mouse joint cells. Since DLL-1 was involved in the production of IL-6 by mouse FLS, we next determined the DLL-1–expressing cells in the joint. As shown in Figure 5A, DLL-1 was expressed on $CD11b^{high}Ly6G^{intermediate}F4/80^{high}$ macrophages but not on $CD11b^{high}Ly6G^{high}F4/80-$ neutrophils or CD45+CD11b− lymphocytes. Notch-2 was expressed on all of these cells. DLL-1 was also expressed on the $CD11b^{high}Ly6G-F4/80^{intermediate}$ monocyte/macrophage population in the PB and BM of arthritic mice (Figure 5B).

To examine the effect of inflammation on DLL-1 expression on macrophages, BMMs were stimulated with inflammatory cytokines. BMMs expressed DLL-1 at a low level (mean ± SD MFI 1.33 ± 0.049), and the expression was strongly up-regulated by IFNγ stimulation (mean ± SD MFI 7.21 ± 1.16; $P = 0.00091$), while TNFα or IL-1β stimulation had no effect (Figure 5C). Stimulation of BMMs with a combination of TNFα and IL-1β also did not affect the expression of Notch ligands (data not shown). Expression of Jagged-1 and Jagged-2 was also up-regulated by IFNγ (for Jagged-1, mean ± SD MFI 1.13 ± 0.025 versus 1.91 ± 0.14; $P = 0.00063$ and for Jagged-2, mean ± SD MFI 3.97 ± 0.39 versus 7.52 ± 0.70; $P = 0.0016$) (Figure 5C).

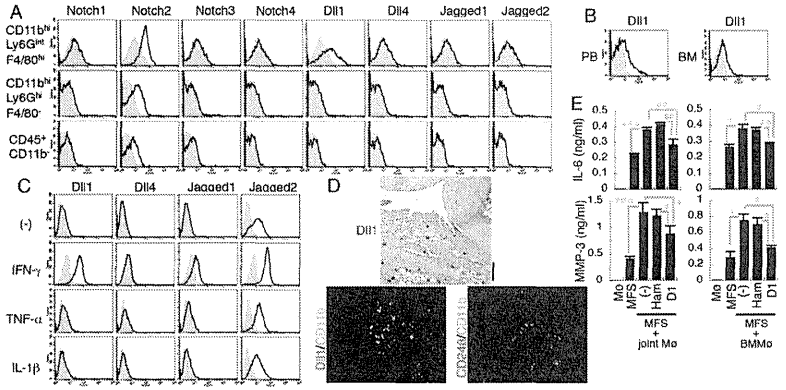


Figure 5. Expression of Notch receptors and ligands on macrophages (Mϕ). **A,** Expression of Notch receptors and ligands on the indicated joint cell populations from mice with CIA, analyzed by flow cytometry. **B,** Delta-like protein 1 (DLL-1) expression by the monocyte/macrophage population ($CD11b^{high}Ly6G-F4/80^{intermediate}$) in bone marrow (BM) and peripheral blood (PB) from mice with CIA. **C,** Expression of Notch ligands on BM-derived macrophages (BMMs). BMMs were stimulated with the indicated cytokine for 24 hours, and the expression of Notch ligands was analyzed by flow cytometry. In **A–C,** shaded histograms indicate staining with control hamster IgG, and open histograms indicate staining with mAb specific for the indicated Notch receptor or ligand. **D,** Immunohistochemical staining for DLL-1 expression in arthritic joints from mice with K/BxN serum–induced arthritis. Representative staining is shown. Bar = 100 μm in top panel. Original magnification × 200 in bottom panels. **E,** Levels of IL-6 and MMP-3 in the supernatants of fibroblast-like synoviocytes (MFS) from mice with CIA cultured with CD11b+ cells (macrophages) sorted from the joints of mice with CIA (left) or cultured with interferon-γ (IFNγ)–stimulated BMMs (right), as measured by ELISA. Cells were cultured in the presence of HMD1-5 or control hamster IgG. Results are representative of 3 independent experiments. Values are the mean ± SD from triplicate wells. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. See Figure 2 for other definitions.

DLL-1 expression in the joints of arthritic mice was determined by immunohistochemistry (Figure 5D). Double-labeling of arthritic joints revealed that some of the CD11b+ cells expressed DLL-1 and were in direct contact with CD248+ mouse FLS (13) (Figure 5D). To verify that the interaction of macrophages and mouse FLS induced IL-6 and MMP-3 production, CD11b+ cells in the joints were isolated and cultured with established mouse FLS. As shown in Figure 5E, stimulation of mouse FLS with CD11b+ joint cells enhanced the production of IL-6 and MMP-3, and these increases were suppressed by DLL-1 blockade. Consistent with the results shown in Figure 3A, IL-6 was not secreted from CD11b+ joint cells alone (Figure 5E). Stimulation of mouse FLS with IFNγ-stimulated BMMs, which expressed DLL-1 as shown in Figure 5C, also enhanced the production of IL-6 and MMP-3, and these increases were inhibited by DLL-1 blockade (Figure 5E).

Notch-2 signaling enhances the production of IL-6 and MMP-3 by mouse FLS. Although Notch-1 and Notch-2 were expressed on mouse FLS (Figure 3B), production of IL-6 and MMP-3 was enhanced only by anti–Notch-2 agonistic mAb with or without LPS stimulation (Figures 6A and B). GM-CSF production was undetectable with such stimulation (data not shown). Stimulation of Notch-1 and Notch-2 with each agonistic mAb was demonstrated by induction of Notch-1 ICD and Notch-2 ICD, respectively (Figure 6C). Notably, LPS stimulation of mouse FLS preferentially induced Notch-2 ICD, and DAPT suppressed this generation of Notch-2 ICD as well as Notch-1 ICD (Figure 6C). Furthermore, stimulation of mouse FLS with DLL-1 induced Notch-2 ICD but not Notch-1 ICD (Figure 6D). In addition, the specific inhibition of Notch-2, but not that of Notch-1, in mouse FLS using siRNA resulted in a decrease in IL-6 and MMP-3 production, while cell viability was equivalent (Figure 6E). These results indi-

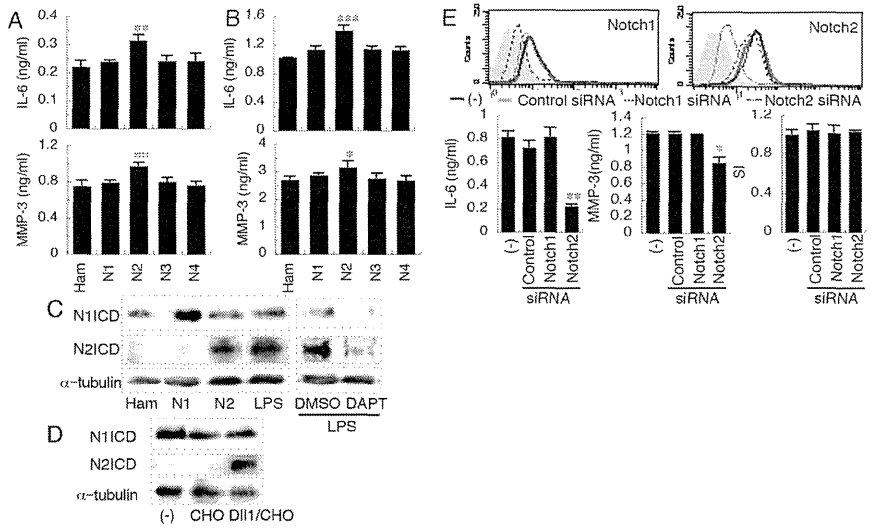


Figure 6. Notch-2 activation enhances IL-6 and MMP-3 production by fibroblast-like synoviocytes (FLS) from mice with CIA. **A** and **B,** Levels of IL-6 and MMP-3 in the supernatants of mouse FLS stimulated with 5 μg/ml of immobilized mAb (HMN1-12 [N1], HMN2-29 [N2], HMN3-133 [N3], or HMN4-14 [N4]) or control hamster IgG for 24 hours in the absence (**A**) or presence (**B**) of lipopolysaccharide (LPS). IL-6 and MMP-3 levels were measured by ELISA. Values are the mean ± SD from triplicate wells. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, versus control hamster IgG. **C** and **D,** Western blot analysis of the Notch-1 intracellular domain (N1ICD) and Notch-2 ICD in mouse FLS cultured with the indicated mAb or LPS in the presence of DAPT or DMSO control (**C**), or with paraformaldehyde-fixed delta-like protein 1 (DLL-1)–transfected Chinese hamster ovary (CHO) cells or control CHO cells (**D**) for 15 hours. The loading control was α-tubulin. Results are representative of 3 independent experiments. **E,** Levels of IL-6 and MMP-3 in mouse FLS after inhibition of Notch-1 and Notch-2. Notch-1 small interfering RNA (siRNA) or Notch-2 siRNA was transfected into mouse FLS. Expression of Notch-1 and Notch-2 was analyzed by flow cytometry to validate the reduction. Shaded histograms indicate control hamster IgG staining. Open histograms indicate Notch-1 or Notch-2 mAb staining. Transfected siRNA are indicated for each histogram. Mouse FLS transfected with the indicated siRNA were stimulated with LPS. IL-6 and MMP-3 were measured as described in **A**. Cell viability is indicated as the SI. * = $P < 0.05$; ** = $P < 0.01$, versus control. See Figure 2 for other definitions.

cate that Notch-2 signaling enhances the production of IL-6 and MMP-3 by mouse FLS.

DISCUSSION

We previously reported that DLL-1 blockade ameliorates the arthritis induced by K/BxN serum transfer in mice, and histologic examination indicated that DLL-1 blockade suppressed inflammation in the joint in addition to suppressing osteoclastogenesis (5). To examine how DLL-1 blockade suppressed joint inflammation, we investigated whether DLL-1 regulates the production of RA-related proinflammatory cytokines in the mouse joint.

In this study, we demonstrated that stimulation of mouse FLS with DLL-1 enhanced the production of IL-6 and MMP-3. Notch-2 signaling also enhanced the production of IL-6 and MMP-3 by mouse FLS, and DLL-1 stimulation activated Notch-2 signaling in mouse FLS. These results indicate that DLL-1 promotes the production of IL-6 and MMP-3 by mouse FLS via Notch-2. In these experiments, strong expression of DLL-1 on DLL-1–transfected CHO cells (15) activated Notch-2 vigorously, while the agonistic effect of HMN2-29 mAb to activate Notch-2 was weaker than the effect of the ligand. We also found that DLL-1 blockade suppressed the production of IL-6 and MMP-3 by joint cells from arthritic mice in vitro and in vivo. DLL-1 was expressed on joint macrophages, and the expression of DLL-1 on BMMs was up-regulated by IFNγ. This was consistent with our previous report that IFNγ induced DLL-1 expression on thioglycolate-elicited peritoneal macrophages (15). Of note, IFNγ did not induce the expression of DLL-1, or of DLL-4, Jagged-1, or Jagged-2, on mouse FLS (data not shown). IFNγ is produced by Th1 cells, CD8 T cells, and natural killer cells and is involved in arthritis. Collectively, DLL-1 on macrophages in the joint, which was up-regulated by IFNγ, could activate Notch-2 on mouse FLS, leading to the production of IL-6 and MMP-3. Therefore, DLL-1 blockade might ameliorate inflammation in the joint by suppressing the production of IL-6 and MMP-3 by mouse FLS.

In RA, blockade of IL-6 with biologic agents improves clinical outcomes, but K/BxN serum transfer resulted in comparable arthritis in IL-6–deficient mice and control mice (20), suggesting that suppression of IL-6 alone might not be sufficient to suppress K/BxN serum–induced arthritis. However, DLL-1 blockade also suppressed GM-CSF production. GM-CSF is important for the activation, differentiation, and survival of neutrophils and macrophages. Blockade of GM-CSF has been effective against RA in clinical trials (21). It has been reported that GM-CSF blockade ameliorated K/BxN serum–induced arthritis by regulating systemic and joint myeloid cell populations (22). Since DLL-1 was expressed on joint macrophages, macrophage–macrophage interactions may contribute to the GM-CSF production by Notch activation in the joint. Further studies are needed to address this possibility.

Notch-1 signaling was not involved in the production of IL-6 and MMP-3 by mouse FLS, while the expression was up-regulated by proinflammatory stimuli. DLL-1 stimulation did not activate Notch-1 signaling in mouse FLS, suggesting that Notch-1 on mouse FLS was not involved in the enhancement of arthritis by DLL-1. A preferential interaction of Notch-2 with DLL-1 has been reported in marginal zone B cell development (3) and osteoclastogenesis (5). Notch-1 was also expressed on these cells. Glycosylation of Notch extracellular domain by Fringe regulates DLL–Notch interactions (23–25). Therefore, a differential modification of Notch-1 and Notch-2 on mouse FLS by Fringe might be responsible for the preferential interaction of Notch-2–DLL-1. It would be interesting to elucidate whether Notch-1 on mouse FLS plays any role in arthritis.

Jagged-1 was the only Notch ligand that was expressed on mouse FLS, and Jagged-1 blockade, as well as Notch inhibitor, suppressed IL-6 and MMP-3 production by LPS-stimulated mouse FLS, indicating that Jagged-1 on mouse FLS stimulates Notch receptors on mouse FLS to produce these mediators. However, stimulation of mouse FLS with Jagged-1–transfected CHO cells did not enhance the production of these mediators, and the blockade of Jagged-1 did not suppress cytokine production by joint cells. Therefore, Jagged-1 could regulate IL-6 and MMP-3 production through mouse FLS–mouse FLS interactions but may play little role in total joint cells. The mechanism to explain this discrepancy remains to be determined.

The effect of DLL-1 blockade on LPS-stimulated mouse spleen cells suggests a systemic regulation of these cytokines by DLL-1 blockade. It is also intriguing to investigate whether IL-6, GM-CSF, and MMP-3 are direct target genes of Notch-2 ICD, but not Notch-1 ICD. The up-regulation of DLL-1 on macrophages by IFNγ seems to enhance the production of IL-6, MMP-3, and GM-CSF in the mouse joint, leading to augmentation of joint inflammation. The clinical relevance to human RA is now under investigation, but preliminary results demonstrated that IL-6 production by RA FLS was suppressed by DAPT, and RA FLS expressed Notch-2 and Jagged-1 but not Notch-1, as in mice (data not shown). Therefore, DLL-1 blockade may be a novel strategy to treat RA by suppressing not only osteoclastogenesis but also inflammatory cytokine production in the joint.

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. Sekine 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.

Study conception and design. Sekine, Yagita.
Acquisition of data. Sekine, Nanki.
Analysis and interpretation of data. Sekine, Yagita.

REFERENCES

1. Choy E. Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. Rheumatology (Oxford) 2012;51:v3–11.
2. Bartok B, Firestein G. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev 2010;233:233–55.
3. Radtke F, Fasnacht N, Macdonald HR. Notch signaling in the immune system. Immunity 2010;32:14–27.

4. Hori K, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. J Cell Sci 2013;126:2135–40.
5. Sekine C, Koyanagi A, Koyama N, Hozumi K, Chiba S, Yagita H. Differential regulation of osteoclastogenesis by Notch2/Delta-like 1 and Notch1/Jagged1 axes. Arthritis Res Ther 2012;14:R45.
6. Nakazawa M, Ishii H, Aono H, Takai M, Honda T, Aratani S, et al. Role of Notch-1 intracellular domain in activation of rheumatoid synoviocytes. Arthritis Rheum 2001;44:1545–54.
7. Gao W, Sweeney C, Connolly M, Kennedy A, Ng CT, McCormick J, et al. Notch-1 mediates hypoxia-induced angiogenesis in rheumatoid arthritis. Arthritis Rheum 2012;64:2104–13.
8. Gao W, Sweeney C, Walsh C, Rooney P, McCormick J, Veale D, et al. Notch signalling pathways mediate synovial angiogenesis in response to vascular endothelial growth factor and angiopoietin 2. Ann Rheum Dis 2013;72:1080–8.
9. Jiao Z, Wang W, Ma J, Wang S, Su Z, Xu H. Notch signaling mediates TNF-α-induced IL-6 production in cultured fibroblast-like synoviocytes from rheumatoid arthritis. Clin Dev Immunol 2012;2012:350209.
10. Wongchana W, Palaga T. Direct regulation of interleukin-6 expression by Notch signaling in macrophages. Cell Mol Immunol 2011;9:155–62.
11. Gentle M, Rose A, Bugeon L, Dallman M. Noncanonical Notch signaling modulates cytokine responses of dendritic cells to inflammatory stimuli. J Immunol 2012;189:1274–84.
12. Okamoto M, Matsuda H, Joetham A, Lucas J, Domenico J, Yasutomo K, et al. Jagged1 on dendritic cells and Notch on $CD4^+$ T cells initiate lung allergic responsiveness by inducing IL-4 production. J Immunol 2009;183:2995–3003.
13. Hardy R, Hulso C, Liu Y, Gasparini S, Fong-Yee C, Tu J, et al. Characterisation of fibroblast-like synoviocytes from a murine model of joint inflammation. Arthritis Res Ther 2013;15:R24.
14. Sekine C, Sugihara T, Miyake S, Hirai H, Yoshida M, Miyasaka N, et al. Successful treatment of animal models of rheumatoid arthritis with small-molecule cyclin-dependent kinase inhibitors. J Immunol 2008;180:1954–61.
15. Moriyama Y, Sekine C, Koyanagi A, Koyama N, Ogata H, Chiba S, et al. Delta-like 1 is essential for the maintenance of marginal zone B cells in normal mice but not in autoimmune mice. Int Immunol 2008;20:763–73.
16. Sekine C, Moriyama Y, Koyanagi A, Koyama N, Ogata H, Okumura K, et al. Differential regulation of splenic CD8- dendritic cells and marginal zone B cells by Notch ligands. Int Immunol 2009;21:295–301.
17. Kamata K, Kamijo S, Nakajima A, Koyanagi A, Kurosawa H, Yagita H, et al. Involvement of TNF-like weak inducer of apoptosis in the pathogenesis of collagen-induced arthritis. J Immunol 2006;177:6433–9.
18. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, et al. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity 1999;10:451–61.
19. Maccioni M, Zeder-Lutz G, Huang H, Ebel C, Gerber P, Hergueux J, et al. Arthritogenic monoclonal antibodies from K/BxN mice. J Exp Med 2002;195:1071–7.
20. Ji H, Rettit A, Ohmura K, Ortiz-Lopez A, Duchatelle V, Degott C, et al. Critical roles for interleukin 1 and tumor necrosis factor α in antibody-induced arthritis. J Exp Med 2002;196:77–85.
21. Burmester GR, Weinblatt ME, McInnes IB, Porter D, Barbarash O, Vatutin M, et al, for the EARTH Study Group. Efficacy and safety of mavrilimumab in subjects with rheumatoid arthritis. Ann Rheum Dis 2013;72:1445–52.
22. Cook AD, Turner AL, Braine EL, Pobjoy J, Lenzo JC, Hamilton JA. Regulation of systemic and local myeloid cell subpopulations by bone marrow cell–derived granulocyte–macrophage colony-stimulating factor in experimental inflammatory arthritis. Arthritis Rheum 2011;63:2340–51.
23. Hicks C, Johnston SH, diSibio G, Collazo A, Vogt TF, Weinmaster G. Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. Nat Cell Biol 2000;2:515–20.
24. Stanley P. Regulation of Notch signaling by glycosylation. Curr Opin Struct Biol 2007;17:530–5.
25. Tan JB, Xu K, Cretegny K, Visan I, Yuan JS, Egan SE, et al. Lunatic and Manic Fringe cooperatively enhance marginal zone B cell precursor competition for Delta-like 1 in splenic endothelial niches. Immunity 2009;30:254–63.

Yokoyama et al. Arthritis Research & Therapy 2014, 16:445
http://arthritis-research.com/content/16/5/445

arthritis research&therapy

RESEARCH ARTICLE Open Access

Abrogation of CC chemokine receptor 9 ameliorates collagen-induced arthritis of mice

Waka Yokoyama[1], Hitoshi Kohsaka[1], Kayoko Kaneko[1], Matthew Walters[2], Aiko Takayasu[1], Shin Fukuda[1], Chie Miyabe[1,3], Yoshishige Miyabe[1], Paul E Love[4], Nobuhiro Nakamoto[5], Takanori Kanai[5], Kaori Watanabe-Imai[1], Trevor T Charvat[2], Mark ET Penfold[2], Juan Jaen[2], Thomas J Schall[2], Masayoshi Harigai[1], Nobuyuki Miyasaka[1] and Toshihiro Nanki[1,6*]

Abstract

Introduction: Biological drugs are effective in patients with rheumatoid arthritis (RA), but increase severe infections. The CC chemokine receptor (CCR) 9 antagonist was effective for Crohn's disease without critical adverse effects including infections in clinical trials. The present study was carried out to explore the pathogenic roles of chemokine (C-C motif) ligand (CCL) 25 and its receptor, CCR9, in autoimmune arthritis and to study if the CCR9 antagonist could be a new treatment for RA.

Methods: CCL25 and CCR9 expression was examined with immunohistochemistry and Western blotting. Concentration of interleukin (IL)-6, matrix metalloproteinase (MMP)-3 and tumor necrosis factor (TNF)-α was measured with enzyme-linked immunosorbent assays. Effects of abrogating CCR9 on collagen-induced arthritis (CIA) was evaluated using CCR9-deficient mice or the CCR9 antagonist, CCX8037. Fluorescence labeled-$CD11b^+$ splenocytes from CIA mice were transferred to recipient CIA mice and those infiltrating into the synovial tissues of the recipient mice were counted.

Results: CCL25 and CCR9 proteins were found in the RA synovial tissues. CCR9 was expressed on macrophages, fibroblast-like synoviocytes (FLS) and dendritic cells in the synovial tissues. Stimulation with CCL25 increased IL-6 and MMP-3 production from RA FLS, and IL-6 and TNF-α production from peripheral blood monocytes. CIA was suppressed in CCR9-deficient mice. CCX8037 also inhibited CIA and the migration of transferred $CD11b^+$ splenocytes into the synovial tissues.

Conclusions: The interaction between CCL25 and CCR9 may play important roles in cell infiltration into the RA synovial tissues and inflammatory mediator production. Blocking CCL25 or CCR9 may represent a novel safe therapy for RA.

Introduction

Rheumatoid arthritis (RA) is characterized by persistent and erosive arthritis in multiple joints. The accumulation of a large number of T cells and macrophages [1-3], proliferation of fibroblast-like synoviocytes (FLS), production of inflammatory mediators and activation of osteoclasts are revealed in the affected joints and lead to destruction of the joints with pain and daily disability [4-8]. Biological drugs, such as tumor necrosis factor (TNF) blockers and interleukin (IL)-6 receptor antagonists, are effective in patients with RA [9-11]. Since the risk of severe infections is increased by biological drugs [12-14], safer therapies for RA should be developed.

As a new treatment, anti-chemokine therapy has been intensively studied for inflammatory diseases. Chemokines are a family of small secreted molecules that induce directed chemotaxis of responding cells and activation of inflammatory cells [15-17]. According to the results of a large phase II study, the CC chemokine receptor (CCR) 9 antagonist, CCX282-B was effective for Crohn's disease

* Correspondence: nanki@med.teikyo-u.ac.jp
[1]Department of Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yusima, Bunkyo-ku, Tokyo 110-8519, Japan
[6]Department of Clinical Research Medicine, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan
Full list of author information is available at the end of the article

without critical adverse effects [18,19]. Especially, this treatment did not increase the risk of infections for 12 months. CCR9, a unique receptor for chemokine (C-C motif) ligand (CCL) 25, is expressed on lymphocytes of intestinal lamina propria and intraepithelial and dendritic cells (DCs) in the small intestine and thymocytes [20,21]. CCL25 is expressed by the follicle-associated epithelium of Peyer's patches, the crypts of Lieberkühn in the small intestine and the thymus [21,22]. Physiologically, the interaction between CCL25 and CCR9 contribute to the T cell and DC migration into the small intestine and movement of T cells in the thymus.

It was reported that CCR9 expression on cell surface of peripheral blood monocytes from RA patients was higher than that from healthy donors [23]. CCR9 and CCL25 were expressed on macrophages in the RA synovial tissues [23]. These data suggest that interaction of CCL25 and CCR9 may contribute to the inflammatory cell migration into the RA synovial tissues. Although blockade of *CCL25* and *CCR9* interaction might also be applicable to RA, the pathogenic roles of these molecules in RA have been little known.

In this study, we examined the stimulatory effects of CCL25 on FLS and monocytes and effects of the abrogation of CCR9 on a murine model of RA.

Methods

Specimens

Synovial tissue samples were obtained from eleven RA patients who fulfilled the American College of Rheumatology classification criteria for RA [24] and seven patients with osteoarthritis (OA) who underwent total knee joint replacement. Nine RA patients were positive for rheumatoid factor (81%) and ten were positive for anti-citrullinated protein antibodies (91%). All subjects provided written informed consent. The experimental protocols were approved by the Ethics Committee of Tokyo Medical and Dental University.

Immunodetection

Mouse anti-CCR9 (248621: R&D Systems, Minneapolis, MN, USA), CCL25 (52513: R&D Systems), or β-actin (AC-15: Sigma-Aldrich, St Louis, MO, USA) monoclonal antibody (mAb) was used as a primary antibody for Western blotting [25]. Immunohistochemistry was conducted as described previously [25]. Frozen sections fixed with ice-cold acetone was blocked with Tris-buffered saline, 2% goat serum, 1% bovine serum albumin, 0.1% Triton X-100, and 0.05% Tween-20. Mouse anti-CCR9, CCL25 mAb (10 μg/ml: R&D Systems) or isotype control was used as a primary antibody. Alexa Fluor™ 546-conjugated goat anti-mouse IgG2a or IgG2b Ab (4 μg/ml: Invitrogen, Carlsbad, CA, USA) was used as a secondary antibody. For double immunohistochemistry, the sections were also stained with mouse anti-CD68 (10 μg/ml: KP1; Dako, Glostrup, Denmark), cadherin-11 (1 μg/ml: 16A; Acris Antibodies, Hiddenhausen, Germany), or dendritic cell lysosome-associated membrane glycoprotein (DC-LAMP) (10 μg/ml: 104.G4; Immunotec Inc., Quebec, Canada) mAb. They were then incubated with 4 μg/ml Alexa Fluor™ 488-conjugated goat anti-mouse IgG1 (Invitrogen). A nuclear stain was performed with 4', 6-diamidino-2-phenylindole. To determine the percentages of CCR9-expressed cells, the number of CCR9-positive cells in CD68-, cadherin-11-, or DC-LAMP-positive cells was counted in three randomly selected fields examined at x200 magnification under fluorescence microscope.

Cell culture

FLS was established from the RA and OA synovial tissues and used for experiments after five passages [26]. The cells did not express CD14 or human leukocyte antigen class II, suggesting that macrophages and DCs were not contained in the FLS [26]. Human peripheral blood $CD14^+$ monocytes from healthy donors were purified by magnetic-activated cell sorting microbeads coupled with mAb and magnetic cell separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of $CD14^+$ monocytes was more than 95%. CCR9 expression of RA FLS and purified human peripheral blood monocytes was evaluated with phycoerythrin-conjugated anti-CCR9 mAb (112509: R&D Systems) or the isotype control staining using an Accuri C6 flow cytometer (Accuri Cytometers, BD Biosciences, San Jose, CA, USA). RA FLS were cultured with 10% fetal calf serum (FCS) for 48 hours with or without recombinant human CCL25 (R&D Systems). Purified human peripheral blood monocytes were cultured with 10% FCS for 24 hours with or without CCL25. Concentrations of IL-6, matrix metalloproteinase (MMP)-3 and TNF-α in the culture supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits (DuoSet: R&D Systems).

Induction and treatment of collagen-induced arthritis (CIA)

To induce CIA, 10-week-old CCR9-deficient mice [27] and wild-type (WT) mice with a C57BL/6 J background were treated with chicken type II collagen (CII; Sigma-Aldrich) [28]. Eight-week-old DBA/1 J mice were treated with bovine CII (Collagen Research Center, Kiel, Germany) [29]. Disease severity was evaluated with the clinical arthritis score, incidence of arthritis, pathological score [29] and radiological score. Bone destruction was evaluated with bone erosion of the bilateral foot joints as follows: 0 = not obvious erosion, 1 = one erosion, 2 = two erosions, 3 = more than three erosions, and if a bone deformity was seen in the foot joint, one point was added (to a maximum of eight points).

DBA/1 J mice with CIA were injected subcutaneously with selective CCR9 antagonist, CCX8037 [30] or vehicle alone. Migration of $CD11b^+$ splenocytes into synovial tissues of CIA mice was evaluated as described previously [31,32]. CD11b-positive splenocytes from CIA mice were purified using magnetic-activated cell sorting microbeads coupled with mAb and magnetic cell separation columns (Miltenyi Biotec). The purified CD11b cells were labeled with CellTracker™ Orange 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTMR: Molecular Probes, Eugene, OR, USA) according to the protocol supplied by the manufacturer. The CMTMR-labeled 1×10^7 cells were intravenously injected into the tail vein of CIA mice at day 9. The recipient mice were treated with CCX8037 (10 mg/kg in 1% hydroxyprophyl methylcellulose) or vehicle 24 hours, 12 hours, and 30 minutes before the transfer, and 12 hours after the transfer. After 24 hours, ankle joints were harvested, embedded in glycol methacrylate, and sagittal 3-μm-thick microtome sections were prepared. *The numbers of CMTMR-labeled cells* that migrated into the synovium between tibiotalar and tarsometatarsal joints were counted under fluorescent microscopy. The experiment protocols were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Statistical analysis

To compare three or more groups, including results of concentration of inflammatory mediators, area under the curve (AUC) by arthritis score, histological and radiological scores by therapeutical treatment, a one-way analysis of variance (ANOVA) test was used. To compare arthritis score, a two-way ANOVA test was used. AUC by arthritis score, histological score and radiographic score by prophylactic treatment or experiments using CCR9-deficient mice, and number of migrated $CD11b^+$ splenocytes, Student's *t* test was also applied. A *P* value less than 0.05 was considered statistically significant.

Results

Expression of CCR9 and CCL25 in the RA synovial tissues

CCR9 expression in RA and OA synovial tissues was evaluated with Western blotting. The CCR9 protein was found more in the RA synovial tissues than in the OA synovial tissues (Figure 1A). Immunohistochemical analyses of the RA synovial tissues revealed that most $CD68^+$ macrophages expressed CCR9 (Figure 1B-D), which is consistent with previous reports [23]. To examine the proportion of CCR9-expressed cells, the number of CCR9-positive cells in 218.83 ± 24.11 $CD68^+$ cells (n =3), 206.33 ± 23.08 *cadherin-11*$^+$ *cells* (n =3) and 11.33 ± 0.53 DC-$LAMP^+$ cells (n =3) was counted. The percentage of CCR9-positive cells in $CD68^+$ macrophages was 76.1 ± 5.3%. In addition, cadherin-11^+ FLS and DC-$LAMP^+$ DCs also expressed CCR9 (67.1 ± 8.3%, 11.4 ± 15.2%, respectively) (Figure 1E- J).

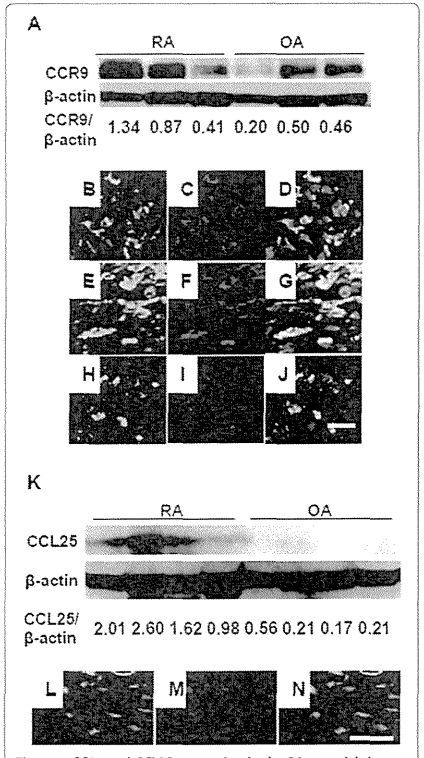


Figure 1 CCR9 and CCL25 expression in the RA synovial tissues. **(A)** Western blots of CCR9 protein expression in three RA and three OA synovial tissues. Ratio of CCR9/β-actin protein level is indicated below. **(B-J)** Sections of RA synovial tissue were double-stained with CCR9 and CD68, cadherin-11 and DC-LAMP (B, CD68; C, CCR9; D, merged B with C; E, cadherin-11; F, CCR9; G, merged E with F; H, DC-LAMP; I, CCR9; J, merged H with I). Bar, 20 μm. **(K)** Western blots of CCL25 protein expression in four RA and four OA synovial tissues. Ratio of CCL25/β-actin protein level is indicated below. **(L-N)** Sections of RA synovial tissue were double-stained with CCL25 and CD68 (L, CD68; M, CCL25; N, merged L with M). Bars, 20 μm. CCL, chemokine (C-C motif) ligand; CCR, CC chemokine receptor; *DC-LAMP, dendritic cell lysosome-associated membrane glycoprotein;* OA, osteoarthritis; RA, rheumatoid arthritis.

The CCL25 expression in RA and OA synovial tissues was analyzed with Western blotting. CCL25 was expressed more in the RA synovial tissues than in the OA synovial

tissues (Figure 1K). Double immunohistochemistry revealed that CD68$^+$ macrophages expressed CCL25 in the RA synovial tissues (Figure 1L-N), which is consistent with previous reports [23].

Stimulatory effects of CCL25 on RA FLS and human peripheral blood monocytes

Since FLS expressed CCR9 in the RA synovial tissues, we examined the stimulatory effects of CCL25 on the cultured RA FLS, which also expressed CCR9 (Figure 2A; mean fluorescence intensity (MFI) for CCR9: 157,963 ± 37,811, MFI for isotype control: 70,914 ± 3,163 (mean ± standard error of the mean (SEM)), n =3). We cultured RA FLS for 48 hours with various concentrations of CCL25. Stimulation with CCL25 increased IL-6 and MMP-3 levels in the culture supernatants in a dose-dependent manner (Figure 2B). Production of TNF-α was not detected by unstimulated or CCL25-stimulated RA FLS. We next examined the effect of CCL25 on IL-6 and MMP-3 production by OA FLS. IL-6 and MMP-3 were also secreted from unstimulated OA FLS, although the levels were lower than those from RA FLS (IL-6; RA: 1,393 ± 493 mg/dl (n =3), OA: 171 ± 83 mg/dl (n =2), P <0.05, MMP-3; RA: 2135 ± 644 mg/dl, OA: 296 ± 50 mg/dl, P <0.05). IL-6 secretion from OA FLS was slightly increased by CCL25, although it was not statistically significant (CCL25 5 ng/ml: 185 ± 93 mg/dl, 50 ng/ml: 206 ± 120 mg/dl, 500 ng/ml: 243 ± 123 mg/dl). Production of MMP-3 from OA FLS was not significantly altered by CCL25 (CCL25 5 ng/ml: 324 ± 115 mg/dl, 50 ng/ml: 324 ± 65 mg/dl, 500 ng/ml: 312 ± 112 mg/dl).

Instead of the synovial macrophages, which were not available on a large scale, we examined the effect of CCL25 on human peripheral blood monocytes. They also expressed CCR9 (Figure 3A; MFI for CCR9: 24,149 ± 13,536, MFI for isotype control: 2,038 ± 1,265, n =3) and were cultured for 24 hours with various concentrations of CCL25. This treatment promoted IL-6 and TNF-α production in a dose-dependent manner (Figure 3B).

Effects of CCR9 gene deletion on murine CIA

The above data prompted us to investigate the effect of the abrogation of CCR9 on murine CIA. We analyzed the development of CIA in CCR9-deficient mice. The clinical arthritis scores in the CCR9-deficient mice were significantly lower than those in the WT (Figure 4A). AUC by the arthritis score was calculated. The AUC tended to be smaller in CCR9-deficient mice compared to WT mice, although the difference was not statistical significant (WT: 13.40 ± 17.75, CCR9-deficient: 4.08 ± 10.34). Histological and radiographic examinations revealed that mononuclear cell infiltration and bone destruction were inhibited in the CCR9-deficient mice (Figure 4B and C).

Effects of the CCR9 antagonist on murine CIA

Next, we investigated the effect of the CCR9 antagonist, CCX8037, on murine CIA. To analyze its effects in a preventive protocol, CCX8037 (3 mg/kg or 10 mg/kg) or vehicle alone was injected subcutaneously twice daily from 7 days prior to the second immunization to 14 days after the immunization. This treatment significantly inhibited clinical arthritis score in a dose-dependent manner (Figure 5A). AUC by the arthritis score was also significantly smaller by CCX8037 (10 mg/kg)-treated mice compared with vehicle-treated mice (vehicle: 17.67 ± 11.78, CCX8037 3 mg/kg: 13.67 ± 11.79 (not significant, vs. vehicle), CCX8037 10 mg/kg: 6.79 ± 9.22 (P =0.01, vs. vehicle)). Moreover, CCX8037 significantly inhibited mononuclear cell infiltration and bone destruction (Figure 5B and C).

To investigate the effects of CCX8037 in a therapeutic protocol, we injected CIA mice with CCX8037 10 mg/kg or vehicle alone 5 days after the second immunization for 13 days. CCX8037 significantly inhibited arthritis, mononuclear cell infiltration and bone destruction (Figure 5D-F). AUC by the arthritis score was also significantly smaller in CCX8037-treated mice compared with vehicle-treated mice (vehicle: 30.50 ± 16.46, CCX8037 10 mg/kg: 14.46 ± 10.75 (P <0.01, vs. vehicle)).

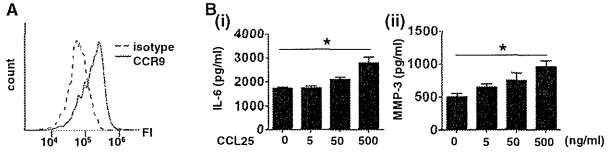


Figure 2 Effects of CCL25 on cultured RA FLS. (A) CCR9 expression on *in vitro* cultured RA FLS was determined by flow cytometry. Horizontal line indicates fluorescence intensity (FI). **(B)** RA FLS were cultured for 48 hours with various concentrations of CCL25. Concentrations of IL-6 (i) and MMP-3 (ii) in the culture supernatant were measured with ELISA. Values are the mean ± SEM of three independent experiments. *P <0.05. CCL, chemokine (C-C motif) ligand; CCR, CC chemokine receptor; ELISA, enzyme-linked immunosorbent assay; FLS, fibroblast-like synoviocytes; IL, interleukin; MMP, metalloproteinase; RA, rheumatoid arthritis; SEM, standard error of the mean.

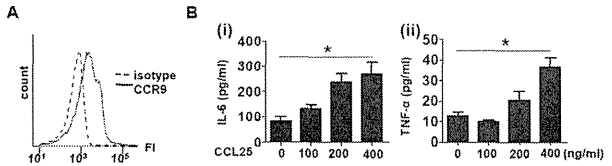


Figure 3 Effects of CCL25 on human peripheral blood monocytes. (A) CCR9 expression on peripheral blood CD14$^+$ monocytes was analyzed with flow cytometry. **(B)** Monocytes were cultured for 24 hours with various concentrations of CCL25. Concentrations of IL-6 (i) and TNF-α (ii) in the culture supernatant were measured with ELISA. Values are the mean ± SEM of three independent experiments. *P <0.05. CCL, chemokine (C-C motif) ligand; CCR, CC chemokine receptor; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; SEM, standard error of the mean; TNF, tumor necrosis factor.

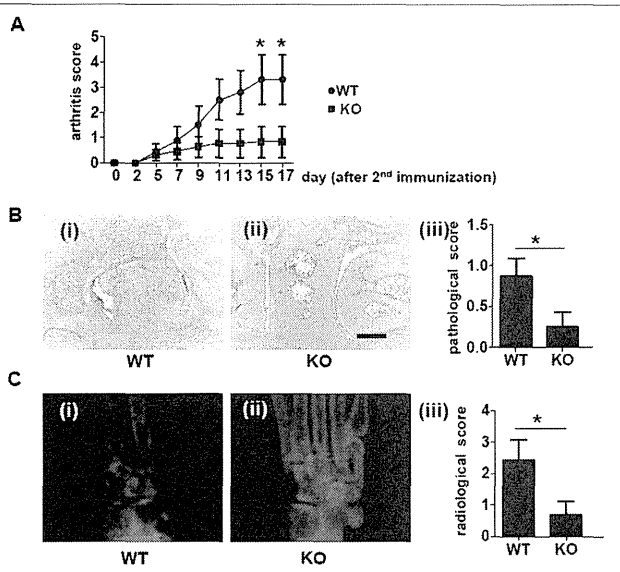


Figure 4 Suppressed CIA in CCR9-deficient mice. (A-C) CCR9-deficient mice (knockout (KO)) (n =13) and WT mice (n =16) were immunized with chicken CII on day −21 and 0. Day 0 means the day of the second immunization. Disease severity was recorded as the clinical arthritis score until day 18 (A). Ankle joints on day 18 from KO (B(i)) and WT mice (B(ii)) were stained with hematoxylin and eosin. Bar, 300 μm. Inflammatory cell infiltration in the right ankle joint was scored with a pathological score (B(iii)). Representative radiographs of the ankle joints of KO (C(i)) and WT mice (C(ii)). Bone erosion in the bilateral ankle joints was scored with radiological score (C(iii)). Representative photomicrographs are shown. Values are the mean ± SEM of each group. *P <0.05 versus WT. CCR, CC chemokine receptor; CIA, collagen-induced arthritis; CII, type II collagen; SEM, standard error of the mean; WT, wild-type.

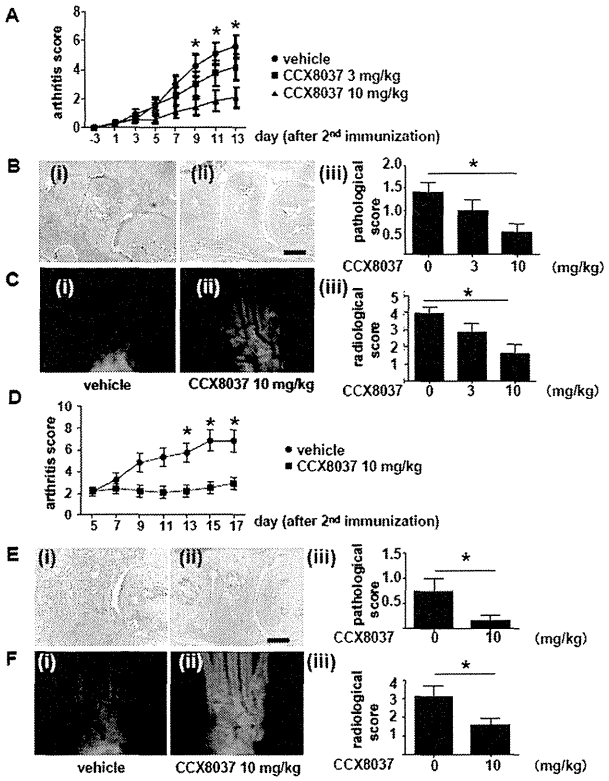


Figure 5 Effects of CCX8037 on CIA mice. (A-C) CCX8037 (3 mg/kg or 10 mg/kg) or vehicle (all n =12) was injected subcutaneously twice daily from day −7 to day 14. Disease severity was recorded as the clinical arthritis score until day 14 (A). Representative photographs showing hematoxylin and eosin staining of the ankle joints from mice with CIA treated with vehicle (B(i)) or CCX8037 (10 mg/kg) (B(ii)). Bar, 300 μm. Inflammatory cell infiltration in the right ankle joint was scored with a pathological score (B(iii)). Representative radiographs of the ankle joints of CIA mice treated with vehicle (C(i)) or CCX8037 (10 mg/kg) (C(ii)). Bone erosion in the bilateral ankle joints was scored with radiological score (C(iii)). Values are the mean ± SEM of each group. *P <0.05, CCX8037 (10 mg kg) versus vehicle. **(D-F)** To investigate the therapeutic effects of CCX8037, at day 5 after the second immunization, mice were divided into two groups with equal average arthritis score. CCX8037 (10 mg/kg) or vehicle (all n =12) was injected subcutaneously twice daily from day 5 to day 18. Disease severity was recorded as the clinical arthritis score (D). Representative hematoxylin and eosin staining of the ankle joints of CIA mice treated with vehicle (E(i)) or CCX8037 (10 mg/kg) (E(ii)). Bar, 300 μm. Pathological score (E(iii)). Representative radiographs of the ankle joints of CIA mice treated with vehicle (F(i)) or CCX8037 (10 mg/kg) (F(ii)). Radiological score (F(iii)). Values are the mean ± SEM of each group. *P <0.05 versus vehicle. CCX8037, CC chemokine receptor (CCR) 9 antagonist; CIA, collagen-induced arthritis; SEM, standard error of the mean.

The CCR9 antagonist inhibited migration of CD11b$^+$ splenocytes

In the RA synovial tissues, most macrophages expressed CCR9, and CCL25 was abundant (Figure 1B-D and 1K). It was reported that CCL25 induced migration of monocytes/macrophages *in vitro* [23,33,34], indicating that the interaction of CCL25 and CCR9 may have an important role in the migration of monocytes into the inflamed synovial tissues.

We then analyzed the effect of CCR9 blockade on inflammatory cell migration *in vivo*. We showed previously that CD11b$^+$ macrophages from CIA mice labeled and transferred to the recipient CIA mice were identified in the inflamed synovial tissues of the recipients [31,32]. CD11b$^+$ splenocytes express CCR9 [35]. To analyze the effect of CCX8037 on the macrophages migration, recipient mice were treated with CCX8037 or vehicle 24 hours, 12 hours, and 30 minutes before the cell transfer, and 12 hours after the transfer. Twenty-four hours after the transfer, the number of labeled cells in the synovial tissues was counted. Although, this short-term treatment did not alter the arthritis severity of the recipient mice, the treated mice had significantly reduced number of the migrated cells in the synovial tissues in contrast to the vehicle-treated group (Figure 6).

Discussion

In this study, we showed that the abrogation of CCR9 ameliorated arthritis in a murine model of RA. We also found that the *in vivo* migration of macrophages was suppressed by the administration of CCX8037. In addition, we showed stimulatory effects of CCL25 on the production of inflammatory mediators from RA FLS and human peripheral blood monocytes *in vitro*. The results suggest that CCR9 could be a therapeutic target for RA.

As was reviewed earlier, chemokines are apparent therapeutic targets in RA treatment. Especially, CCR1, CCR2 and CCR5 are abundantly expressed on RA synovial macrophages and the validity of CCR1, CCR2 and CCR5 antagonist for the animal model of arthritis has been studied. CCR1 antagonist was effective in a clinical trial [36], while blockade of CCL2, CCR2 or CCR5 was not [37-39]. In the trial of CCR2 or CCR5 antagonist, no significant reduction in numbers of macrophages in the synovial tissues was observed, suggesting that CCR2 and CCR5 may not play a critical role in the migration of monocytes. In addition, since CCR2 and CCR5 are expressed on regulatory T cells, their blockade might inhibit regulatory T cells that suppressed the disease.

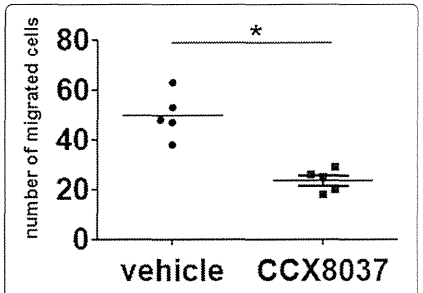


Figure 6 Effect of CCX8037 on the migration of CD11b$^+$ splenocytes into the joints. Cell Tracker Orange CMTMR-labeled CD11b$^+$ splenocytes from CIA mice (1×10^7) were adoptively transferred into each recipient CIA mouse on day 9 after the second immunization. The recipient mice were treated with CCX8037 (10 mg/kg in 1% hydroxyprophyl methylcellulose) or vehicle 24 hours, 12 hours, and 30 minutes before the transfer, and 12 hours after the transfer. Twenty-four hours after the transfer, ankle joints were harvested and examined for migrated cells under a fluorescent microscope. The total number of labeled cells in three fields of vision was counted in the synovial tissue between the tibiotalar and transmetatarsal joints (n =5) at x20 magnification. Horizontal bars indicate the mean of each group. *P <0.001 versus vehicle. CCX8037, CC chemokine receptor (CCR) 9 antagonist; CIA, collagen-induced arthritis; CMTMR; 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine.

CCR9 was expressed on FLS, macrophages and DCs in the RA synovial tissues. This should be driven by inflammatory cytokines in the synovial tissues, since stimulation with TNF-α increased CCR9 expression on THP monocytic cells [23]. CCR9 was also expressed on *in vitro* cultured RA FLS and peripheral blood monocytes from healthy donors. CCL25 stimulated them to produce inflammatory mediators that are important in the pathogenesis of RA. CCL25 should exacerbate arthritis via these effects in addition to the inflammatory cell recruitment. We could not measure CCL25 concentration in the RA synovial tissue. However, chemokines bind surface proteoglycans [40], and they could be sequestered and presented to target cells at high concentration in the local microenvironment.

In vivo macrophage migration was suppressed by CCR9 inhibition, which might have a great impact on inhibition of CIA. It was shown that CD3$^+$ T cells and CD20$^+$ B cells in the RA synovial tissues did not express CCR9 [23]. In addition, the serum concentration of anti-CII IgG1, IgG2a and IgG2b in the CCX8037-treated group was not lower than the vehicle group both in the preventive and therapeutic treatment experiments (data not shown). The effect of CCX8037 may not depend on the inhibition of T and B cells recruitment into the synovial tissues or reduction of antibody production. It was reported that deficiency of CCR9, as well as CCR1, CCR2 and CCR5, did not attenuate a murine model of serum transfer arthritis [41]. It is believed that innate immune system is important for serum transfer arthritis, while adaptive immune system is important for CIA. Macrophages and neutrophils are essential for serum transfer arthritis [41,42]. Reduction in the number of macrophages in the synovial tissues might not have enough of an impact to suppress the inflammation of arthritis in serum transfer arthritis, while that might suppress CIA. On the other hand, CCR9 was expressed on the DC-LAMP$^+$ cell in the synovial tissues, which is a mature DC [43] in the RA synovial tissues. We did not investigate the effect of the CCL25-CCR9 interaction on DCs in this study. Further studies are needed to investigate the regulation of CCR9 expression and effect of CCL25 stimulation on DCs.

Treatment with CCR9 antagonist did not accompany critical infections as an adverse effect in a large phase II study for Crohn's disease [18,19]. Although the reason is not clear, it might be also the case for treating RA.

Conclusions

In this manuscript, we showed that CCL25 and CCR9 were expressed in the RA synovial tissue. Stimulation with CCL25 enhanced production of inflammatory mediators from monocytes and RA FLS. Moreover, inhibition of CCR9 reduced arthritis and inflammatory cell migration *in mice*. Therefore, the interaction between CCL25 and CCR9 may play important roles in cell infiltration into the RA synovial tissues and inflammatory mediator production. CCR9 antagonist may become a novel, safe and effective treatment for RA.

Abbreviations

ANOVA: analysis of variance; AUC: area under the curve; CCL: chemokine (C-C motif) ligand; CCR: CC chemokine receptor; CIA: collagen-induced arthritis; CII: type II collagen; CMTMR: 5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine; DC: dendritic cell, DC-LAMP, dendritic cell lysosome-associated membrane glycoprotein; ELISA: enzyme-linked immunosorbent assays; FCS: fetal calf serum; FI: fluorescence intensity; FLS: fibroblast-like synoviocytes; IL: interleukin; KO: knockout; mAb: monoclonal antibody; MFI: mean fluorescence intensity; MMP: metalloproteinase; OA: osteoarthritis; RA: rheumatoid arthritis; SEM: standard error of the mean; TNF: tumor necrosis factor; WT: wild-type.

Competing interests

Drs. Walters, Charvat, Penfold, Jaen and Schall own stock or stock options in ChemoCentryx, Inc. The other authors declare that they have no competing interests.

Authors' contributions

WY participated in the design of the study, carried out the experiments and statistical analysis, and drafted the manuscript. KK, AT, SF, CM, YM and KW assisted in carrying out the experiments and in manuscript preparation. MW, TC, MP, JJ and TS provided the CCR9 antagonist, participated in study design, and assisted in manuscript preparation. PL, NN and TK provided CCR9-deficient mice and assisted in manuscript preparation. HK, MH, NM and TN conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Global Center of Excellence (GCOE) Program, International Research Center for *Molecular Science in Tooth and Bone Diseases at Tokyo Medical and* Dental University.
We thank Dr. Kazutaka Sugimoto for his critical advice and Megumi Yamaji for her excellent technical support.

Author details

[1]Department of Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yusima, Bunkyo-ku, Tokyo 110-8519, Japan. [2]ChemoCentryx, Inc, 850 Maude Avenue, Mountain View, CA 94043, USA. [3]Department of Dermatology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan. [4]National Institute of Child Health and Human Development, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA. [5]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. [6]Department of Clinical Research Medicine, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan.

Received: 7 May 2014 Accepted: 29 August 2014
Published online: 24 September 2014

References

1. Kinne RW, Bräuer R, Stuhlmüller B, Palombo-Kinne E, Burmester GR: **Macrophages in rheumatoid arthritis.** *Arthritis Res* 2000, **2**:189–202.
2. Goronzy JJ, Weyand CM: **Rheumatoid arthritis.** *Immunol Rev* 2005, **204**:55–73.
3. Ritchlin C: **Fibroblast biology. Effector signals released by the synovial fibroblast in arthritis.** *Arthritis Res* 2000, **2**:356–360.
4. Huber LC, Distler O, Tarner I, Gay RE, Gay S, Pap T: **Synovial fibroblasts: key players in rheumatoid arthritis.** *Rheumatology (Oxford)* 2006, **45**:669–675.
5. Bartok B, Firestein GS: **Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis.** *Immunol Rev* 2010, **233**:233–255.
6. Nakano K, Okada Y, Saito K, Tanaka Y: **Induction of RANKL expression and osteoclast maturation by the binding of fibroblast growth factor 2 to heparan sulfate proteoglycan on rheumatoid synovial fibroblasts.** *Arthritis Rheum* 2004, **50**:2450–2458.
7. Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, Koshihara Y, Oda H, Nakamura K, Tanaka S: **Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis.** *Arthritis Rheum* 2000, **43**:259–269.
8. Gravallese EM: **Bone destruction in arthritis.** *Ann Rheum Dis* 2002, **61**:ii84–ii86.
9. Smolen JS, Landewé R, Breedveld FC, Dougados M, Emery P, Gaujoux-Viala C, Gorter S, Knevel R, Nam J, Schoels M, Aletaha D, Buch M, Gossec L, Huizinga T, Bijlsma JW, Burmester G, Combe B, Cutolo M, Gabay C, Gomez-Reino J, Kouloumas M, Kvien TK, Martin-Mola E, McInnes I, Pavelka K, van Riel P, Scholte M, Scott DL, Sokka T, Valesini G, *et al*: **EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs.** *Ann Rheum Dis* 2010, **69**:964–975.
10. Feldmann M: **Development of anti-TNF therapy for rheumatoid arthritis.** *Nat Rev Immunol* 2002, **2**:364–371.
11. Nishimoto N, Yoshizaki K, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, Hashimoto J, Azuma J, Kishimoto T: **Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial.** *Arthritis Rheum* 2004, **50**:1761–1769.
12. Crawford M, Curtis JR: **Tumor necrosis factor inhibitors and infection complications.** *Curr Rheumatol Rep* 2008, **10**:383–389.
13. Komano Y, Tanaka M, Nanki T, Koike R, Sakai R, Kameda H, Nakajima A, Saito K, Takeno M, Atsumi T, Tohma S, Ito S, Tamura N, Fujii T, Sawada T, Ida H, Hashiramoto A, Koike T, Ishigatsubo Y, Eguchi K, Tanaka Y, Takeuchi T, Miyasaka N, Harigai M, REAL Study Group: **Incidence and risk factors for serious infection in patients with rheumatoid arthritis treated with tumor necrosis factor inhibitors: a report from the Registry of Japanese Rheumatoid Arthritis Patients for Longterm Safety.** *J Rheumatol* 2011, **38**:1258–1264.
14. Nishimoto N, Ito K, Takagi N: **Safety and efficacy profiles of tocilizumab monotherapy in Japanese patients with rheumatoid arthritis: meta-analysis of six initial trials and five long-term extensions.** *Mod Rheumatol* 2010, **20**:222–232.
15. Szekanecz Z, Strieter RM, Kunkel SL, Koch AE: **Chemokines in rheumatoid arthritis.** *Springer Semin Immunopathol* 1998, **20**:115–132.
16. Koch AE: **Chemokines and their receptors in rheumatoid arthritis: future targets?** *Arthritis Rheum* 2005, **52**:710–721.
17. Vergunst CE, Tak PP: **Chemokines: their role in rheumatoid arthritis.** *Curr Rheumatol Rep* 2005, **7**:382–388.
18. Eksteen B, Adams DH: **GSK-1605786, a selective small-molecule antagonist of the CCR9 chemokine receptor for the treatment of Crohn's disease.** *IDrugs* 2010, **13**:472–781.
19. Keshav S, Vaňásek T, Niv Y, Petryka R, Howaldt S, Bafutto M, Rácz I, Hetzel D, Nielsen OH, Vermeire S, Reinisch W, Karlén P, Schreiber S, Schall TJ, Bekker P, Prospective Randomized Oral-Therapy Evaluation in Crohn's Disease Trial-1 (PROTECT-1) Study Group: **A randomized controlled trial of the efficacy and safety of CCX282-B, an orally-administered blocker of chemokine receptor CCR9, for patients with Crohn's disease.** *PLoS One* 2013, **8**:e60094.
20. Zabel BA, Agace WW, Campbell JJ, Heath HM, Parent D, Roberts AI, Ebert EC, Kassam N, Qin S, Zovko M, LaRosa GJ, Yang LL, Soler D, Butcher EC, Ponath PD, Parker CM, Andrew DP: **Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis.** *J Exp Med* 1999, **190**:1241–1256.
21. Kunkel EJ, Campbell JJ, Haraldsen G, Pan J, Boisvert J, Roberts AI, Ebert EC, Vierra MA, Goodman SB, Genovese MC, Wardlaw AJ, Greenberg HB, Parker CM, Butcher EC, Andrew DP, Agace WW: **Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity.** *J Exp Med* 2000, **192**:761–768.
22. Wurbel MA, Philippe JM, Nguyen C, Victorero G, Freeman T, Wooding P, Miazek A, Mattei MG, Malissen M, Jordan BR, Malissen B, Carrier A, Naquet P: **The chemokine TECK is expressed by thymic and intestinal epithelial cells and attracts double- and single-positive thymocytes expressing the TECK receptor CCR9.** *Eur J Immunol* 2000, **30**:262–271.
23. Schmutz C, Cartwright A, Williams H, Haworth O, Williams JH, Filer A, Salmon M, Buckley CD, Middleton J: **Monocytes/macrophages express chemokine receptor CCR9 in rheumatoid arthritis and CCL25 stimulates their differentiation.** *Arthritis Res Ther* 2010, **12**:R161.
24. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JM, Hobbs K, Huizinga TW, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Ménard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, *et al*: **2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative.** *Ann Rheum Dis* 2010, **69**:1580–1588.
25. Kaneko K, Miyabe Y, Takayasu A, Fukuda S, Miyabe C, Ebisawa M, Yokoyama W, Watanabe K, Imai T, Muramoto K, Terashima Y, Sugihara T, Matsushima K, Miyasaka N, Nanki T: **Chemerin activates fibroblast-like synoviocytes in patients with rheumatoid arthritis.** *Arthritis Res Ther* 2011, **13**:R158.
26. Nanki T, Nagasaka K, Hayashida K, Saita Y, Miyasaka N: **Chemokines regulate IL-6 and IL-8 production by fibroblast-like synoviocytes from patients with rheumatoid arthritis.** *J Immunol* 2001, **167**:5381–5385.
27. Uehara S, Grinberg A, Farber JM, Love PE: **A role for CCR9 in T lymphocyte development and migration.** *J Immunol* 2002, **168**:2811–2819.
28. Inglis JJ, Simelyte E, McCann FE, Criado G, Williams RO: **Protocol for the induction of arthritis in C57BL/6 mice.** *Nat Protoc* 2008, **3**:612–618.
29. Sato A, Watanabe K, Kaneko K, Murakami Y, Ishido M, Miyasaka N, Nanki T: **The effect of synthetic retinoid, Am80, on T helper cell development and antibody production in murine collagen-induced arthritis.** *Mod Rheumatol* 2010, **20**:244–251.
30. *Tubo NJ, Wurbel MA, Charvat TT, Schall TJ, Walters MJ, Campbell JJ:* **A systemically-administered small molecule antagonist of CCR9 acts as a tissue-selective inhibitor of lymphocyte trafficking.** *PLoS One* 2012, **7**:e50498.
31. Nanki T, Urasaki Y, Imai T, Nishimura M, Muramoto K, Kubota T, Miyasaka N: **Inhibition of fractalkine ameliorates murine collagen-induced arthritis.** *J Immunol* 2004, **173**:7010–7016.
32. Miyabe Y, Miyabe C, Iwai Y, Takayasu A, Fukuda S, Yokoyama W, Nagai J, Jona M, Tokuhara Y, Ohkawa R, Albers HM, Ovaa H, Aoki J, Chun J, Yatomi Y, Ueda H, Miyasaka M, Miyasaka N, Nanki T: **Necessity of lysophosphatidic acid receptor 1 for development of arthritis.** *Arthritis Rheum* 2013, **65**:2037–2047.
33. Vicari AP, Figueroa DJ, Hedrick JA, Foster JS, Singh KP, Menon S, Copeland NG, Gilbert DJ, Jenkins NA, Bacon KB, Zlotnik A: **TECK: a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development.** *Immunity* 1997, **7**:291–301.
34. Chu PS, Nakamoto N, Ebinuma H, Usui S, Saeki K, Matsumoto A, Mikami Y, Sugiyama K, Tomita K, Kanai T, Saito H, Hibi T: **C-C motif chemokine receptor 9 positive macrophages activate hepatic stellate cells and promote liver fibrosis in mice.** *Hepatology* 2013, **58**:337–350.
35. Nakamoto N, Ebinuma H, Kanai T, Chu PS, Ono Y, Mikami Y, Ojiro K, Lipp M, Love PE, Saito H, Hibi T: **CCR9+ macrophages are required for acute liver inflammation in mouse models of hepatitis.** *Gastroenterology* 2012, **142**:366–376.
36. Tak PP, Balanescu A, Tseluyko V, Bojin S, Drescher E, Dairaghi D, Miao S, Marchesin V, Jaen J, Schall TJ, Bekker P: **Chemokine receptor CCR1 antagonist CCX354-C treatment for rheumatoid arthritis: CARAT-2, a randomised, placebo controlled clinical trial.** *Ann Rheum Dis* 2013, **72**:337–344.
37. Haringman JJ, Gerlag DM, Smeets TJ, Baeten D, van den Bosch F, Bresnihan B, Breedveld FC, Dinant HJ, Legay F, Gram H, Loetscher P, Schmouder R, Woodworth T, Tak PP: **A randomized controlled trial with an anti-CCL2 (anti-monocyte chemotactic protein 1) monoclonal antibody in patients with rheumatoid arthritis.** *Arthritis Rheum* 2006, **54**:2387–2392.
38. Vergunst CE, Gerlag DM, Lopatinskaya L, Klareskog L, Smith MD, van den Bosch F, Dinant HJ, Lee Y, Wyant T, Jacobson EW, Baeten D, Tak PP: **Modulation of CCR2 in rheumatoid arthritis: a double-blind, randomized, placebo-controlled clinical trial.** *Arthritis Rheum* 2008, **58**:1931–1939.
39. van Kuijk AW, Vergunst CE, Gerlag DM, Bresnihan B, Gomez-Reino JJ, Rouzier R, Verschueren PC, van de Leij C, Maas M, Kraan MC, Tak PP: **CCR5 blockade in rheumatoid arthritis: a randomised, double-blind, placebo-controlled clinical trial.** *Ann Rheum Dis* 2010, **69**:2013–2016.
40. Tanaka Y, Adams DH, Hubscher S, Hirano H, Siebenlist U, Shaw S: **T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta.** *Nature* 1993, **361**:79–82.
41. Wipke BT, Allen PM: **Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis.** *J Immunol* 2001, **167**:1601–1608.
42. Solomon S, Rajasekaran N, Jeisy-Walder E, Snapper SB, Illges H: **A crucial role for macrophages in the pathology of K/B x N serum-induced arthritis.** *Eur J Immunol* 2005, **35**:3064–3073.
43. de Saint-Vis B, Vincent J, Vandenabeele S, Vanbervliet B, Pin JJ, Ait-Yahia S, Patel S, Mattei MG, Banchereau J, Zurawski S, Davoust J, Caux C, Lebecque S: **A novel lysosome-associated membrane glycoprotein, DC-LAMP, induced upon DC maturation, is transiently expressed in MHC class II compartment.** *Immunity* 1998, **9**:325–336.

doi:10.1186/s13075-014-0445-9
Cite this article as: Yokoyama *et al.*: Abrogation of CC chemokine receptor 9 ameliorates collagen-induced arthritis of mice. *Arthritis Research & Therapy* 2014 **16**:445.

BMC Musculoskeletal Disorders

RESEARCH ARTICLE Open Access

Cannabinoid receptor 2 as a potential therapeutic target in rheumatoid arthritis

Shin Fukuda[1], Hitoshi Kohsaka[1], Aiko Takayasu[1], Waka Yokoyama[1], Chie Miyabe[1,2], Yoshishige Miyabe[1], Masayoshi Harigai[1,3], Nobuyuki Miyasaka[1] and Toshihiro Nanki[1,3,4*]

Abstract

Background: Some of cannabinoids, which are chemical compounds contained in marijuana, are immunosuppressive. One of the receptors, CB receptor 1 (CB_1), is expressed predominantly by the cells in the central nervous system, whereas CB receptor 2 (CB_2) is expressed primarily by immune cells. Theoretically, selective CB_2 agonists should be devoid of psychoactive effects. In this study, we investigated therapeutic effects of a selective CB_2 agonist on arthritis.

Methods: The expression of CB_2 was analyzed with immunohistochemistry and Western blotting. Interleukin (IL)-6, matrix metalloproteinase-3 (MMP-3), and chemokine (C-C motif) ligand 2 (CCL2) were quantified with enzyme-linked immunosorbent assays (ELISA). Osteoclastogenesis was assessed with tartrate-resistant acid phosphatase staining and the resorption of coated-calcium phosphate. Effect of JWH133, a selective CB_2 agonist, on murine collagen type II (CII)-induced arthritis (CIA) was evaluated with arthritis score, and histological and radiographic changes. IFN-γ and IL-17 production by CII-stimulated splenocytes and serum anti-CII Ab were analyzed by ELISA.

Results: Immunohistochemistry showed that CB_2 was expressed more in the synovial tissues from the rheumatoid joints than in those from the osteoarthritis joints. CB_2 expression on RA FLS was confirmed with Western blot analysis. JWH133 inhibited IL-6, MMP-3, and CCL2 production from tumor necrosis factor-α-stimulated fibroblast-like synoviocytes (FLS) derived from the rheumatoid joints, and osteoclastogenesis of peripheral blood monocytes. Administration of JWH133 to CIA mice reduced the arthritis score, inflammatory cell infiltration, bone destruction, and anti-CII IgG1 production.

Conclusion: The present study suggests that a selective CB_2 agonist could be a new therapy for RA that inhibits production of inflammatory mediators from FLS, and osteoclastogenesis.

Keywords: Cannabinoid, Cannabinoid receptor 2 (CB_2), Rheumatoid arthritis, JWH133, Fibroblast-like synoviocyte, Monocyte

Background

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown etiology. It is associated with chronic inflammation, bone destruction in multiple joints, and various extra-articular manifestations. Unless treated properly, it is generally progressive with functional decline, significant morbidity, premature mortality, and socioeconomic costs [1]. Recently, biological agents, represented by anti-tumor necrosis factor (TNF) monoclonal antibodies (mAb), have been used widely to improve arthritis and to inhibit bone destruction. However, there remain patients who do not respond satisfactorily. While pain control is a significant issue for the patients, disease-modifying antirheumatic drugs (DMARDs) do not have immediate effects for pain relief. The patients have to depend on corticosteroids or nonsteroidal anti-inflammatory drugs (NSAIDs).

Cannabinoids are pharmacologically active components of *Cannabis sativa*. The endogenous ligands for cannabinoid receptors represented by anandamide and 2-arachidonoylglycerol also occur as endocannabinoids. This system regulates various physiological processes such as appetite control, pain perception and immune responses. Cannabinoids transmit signals through cannabinoid (CB) receptors, CB receptor 1 (CB_1) and CB receptor 2 (CB_2) [2,3]. CB_1 is expressed predominantly by the cells in the central nervous system (CNS), whereas CB_2 is expressed primarily by immune cells [2,3] and by peripheral nerve terminals [4]. Recently, G-coupled receptor 55 (GPR55) was proved to be a receptor of many endocannabinoids [5,6]. It is expressed ubiquitously in many organ systems, including CNS [7]. Furthermore, endocannabinoids act as agonists of transient receptor potential vanilloid type-1 and type-4 (TRPV-1, -4) [8,9], and nuclear peroxisome proliferator-activated receptors (PPARs) [10]. Because of the potential effects on a wide variety of the receptors in the various organs, cannabinoids have not been accepted as therapeutic agents. Especially, the major concerns are their psychoactive effects such as catalepsy and hypolocomotion. In this regards, selective CB_2 agonists should be devoid of psychoactive activities.

Immune cells sensitive to cannabinoids are macrophages, natural killer cells, T cells, and B cells [11]. Some cannabinoids were applied to treat collagen-induced arthritis (CIA), which is a murine model of RA. HU320, a synthetic cannabinoid ameliorated established CIA [12]. Since HU320 has no affinity for CB_1 or CB_2, its therapeutic effect on CIA may derive from actions on other receptors. Blockade of fatty acid amide hydrolase (FAAH), which is the primary degradative enzyme of anandamide, and acts as an agonist for CB_1, CB_2 and other receptors, reduced the severity of CIA [13]. However, therapeutic effect of selective CB_2 agonists on animal models of RA has not been investigated.

It was reported that local administration of JWH133, which is a selective CB_2 agonist with 200-fold selectivity for CB_2 over CB_1 (Ki values are 3.4 and 677 nM respectively) [14], inhibited pain reaction of mice with carrageenan-injected paws [15]. NSAIDs and corticosteroids have several toxicities especially in long-term treatment or at high doses. In many cases, opioid analgesics would be better choice for avoiding the toxicities. Add-on therapy for RA patients with tramadol, which is a weak opioid analgesic and acetaminophen combination tablet, significantly improved joint pain without severe adverse effects [16]. Thus, selective CB_2 agonists would be expected not only to relief pain, but also to suppress arthritis.

In the present study, we investigated the expression of CB_2 in the RA synovial tissues, *in vitro* effects of JWH133 on RA fibroblast-like synoviocytes (FLS) and human monocytes, and its *in vivo* therapeutic effects on CIA.

Methods

Specimens

Synovial tissue samples were obtained from seven RA patients, who fulfilled the ACR classification criteria [17] and three osteoarthritis (OA) patients undergoing total knee joint replacement. The RA patients were 62 (38-75) years old [median (range)], with a disease duration of 9 (3-15) years and C-reactive protein level of 4.6 (0.3-81) mg/l. Informed consent was obtained from all the patients. All experimental protocols were approved by the Ethics Committee of Tokyo Medical and Dental University.

Immunohistochemistry

Immunohistochemical analysis was conducted on formalin-fixed paraffin-embedded sections of synovial tissues. The sections were incubated overnight at 4°C with 2 μg/ml rabbit anti-CB_2 polyclonal antibody (pAb) (abcam, Cambridge MA) or normal rabbit IgG as a control. Subsequently, the samples were incubated with 2 μg/ml biotinylated goat anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX) for 30 min at room temperature, and then incubated for 30 min with streptavidin–horseradish peroxidase (Dako, Glostrup, Denmark). Diaminobenzidine (Dako) was used for visualization. The sections were counterstained with hematoxylin. The CB_2 staining of three RA and three OA samples was semi-quantitatively evaluated by randomly selected three fields with scored as follows: 0 = none, 1 = focal, and 2 = diffuse. The maximum score was six for each sample.

For immunofluorescence double-staining with CD68, CD4, CD8, CD21 or vimentin, and CB_2, the sections were incubated overnight at 4°C with 2 μg/ml rabbit anti-CB_2 pAb or normal rabbit IgG together with 1 μg/ml mouse anti-CD68 mAb (KP1; Dako), 1 μg/ml mouse anti-CD4 mAb (RPA-T4; eBioscience, San Diego, CA), 1 μg/ml mouse anti-CD8 mAb (HIT8a; BD Bioscience, San Diego, CA), 1 μg/ml mouse anti-CD21 mAb (1 F8; Dako) or 1 μg/ml mouse anti-vimentin mAb (V9; Dako). Subsequently, the samples were incubated with 2 μg/ml Alexa Fluor 488-conjugated goat anti-mouse IgG1 (Invitrogen, Grand Island, NY) and Alexa Fluor 568-conjugated goat anti-rabbit IgG1 (Invitrogen) for 30 min at room temperature. A nuclear stain was performed with 4', 6-diamidino-2-phenylindole.

Protein detection in cultured FLS

FLS from the RA synovial tissues was cultured as was reported previously [18]. RA FLS was lysed with radioimmunoprecipitation assay buffer (Millipore, Billerica, MA, USA) for 30 min at 4°C. A total of 20 μg of protein were boiled in the presence of sodium dodecyl sulfate (SDS) sample buffer and separated on a 10% SDS-polyacrylamide gel (ATTO, Tokyo, Japan). Proteins were then electrotransferred onto a polyvinylidene fluoride microporous membrane (Millipore) in a semidry system. The membrane was blocked with Block Ace (Snow Brand Milk Products, Tokyo, Japan) for 1 h at room temperature, and then the immunoblots were incubated overnight with 1 μg/ml rabbit anti-CB_2 pAb in Can Get Signal

* Correspondence: nanki@med.teikyo-u.ac.jp
[1]Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yushima, 113-8519 Bunkyo-ku, Tokyo, Japan
[3]Department of Pharmacovigilance, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yushima, 113-8519 Bunkyo-ku, Tokyo, Japan
Full list of author information is available at the end of the article

Immunoreaction Enhancer Solution (Toyobo, Osaka, Japan) at 4°C. After washing, the immunoblots were incubated with 2 μg/ml biotinylated goat anti-rabbit IgG for 30 min at room temperature, and then incubated for 30 min with streptavidin–horseradish peroxidase. ECL Prime detection reagent and the ImageQuant LAS 4000 Mini Biomolecular Imager (both from GE Healthcare) were used to detect the bands.

RA FLS (1×10^5 cells/ml) was cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich) + 10% fetal bovine serum (FBS) (Sigma-Aldrich) and stimulated with 5 ng/ml recombinant TNF-α (R&D Systems, Minneapolis, MN) for 24 h in the presence or absence of JWH133 (Tocris bioscience, Ellisville, MO) [14]. The concentrations of Interleukin (IL)-6, metalloproteinase-3 (MMP-3) and chemokine (C-C motif) ligand 2 (CCL2) in the culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits (DuoSet; R&D Systems).

Analysis of osteoclastogenesis

Peripheral blood mononuclear cells from healthy donors were collected using Ficoll-Conray (Imuuno-Biological Laboratories, Gunma, Japan) gradient centrifugation. Positive selection of $CD14^+$ monocytes was performed using CD14 MicroBeads (Miltenyi Biotec, Auburn, CA). The purified peripheral blood $CD14^+$ monocytes (1×10^6 cells/well) were incubated in 96-well plates in α-Minimum Essential Medium (Sigma-Aldrich) with 10% FBS, and incubated with 25 ng/ml macrophage colony-stimulated factor (M-CSF) (R&D systems) + 40 ng/ml receptor activator of nuclear factor kappa-B ligand (RANKL) (Peprotech, Rocky Hill, NJ). These cells incubated in the presence or absence of JWH133. The medium was replaced with fresh medium 3 days later, and after incubation for 7 days the cells were stained for tartrate-resistant acid phosphatase (TRAP) expression using a commercial kit (Hokudo, Sapporo, Japan). The number of TRAP-positive multinucleated cells (MNC: more than 3 nuclear) in a randomly selected field examined at ×40 magnification was counted under light microscopy. The $CD14^+$ monocytes were seeded onto plates coated with calcium phosphate thin films (Osteo Assay Plate, Corning, NY, USA) and were incubated with 25 ng/ml M-CSF + 40 ng/ml RANKL for 7 days in the presence or absence of JWH133. The cells were then lysed in bleach solution (6% NaOCl, 5.2% NaCl). The resorption lacunae were examined under light microscopy. The viability of the cells treated with JWH133 (up to 50 μM) was more than 95% relative to the vehicle-treated cells.

Induction of collagen-induced arthritis (CIA)

Male 8-week-old DBA/1 J mice were purchased from Oriental Yeast (Tokyo, Japan) and were kept in the temperature of 23.5 ± 2 degrees Celsius with 40-50% humidity. Bovine collagen type II (CII; Collagen Research Center, Tokyo, Japan) was dissolved in 0.05 M acetic acid at 4 mg/ml and emulsified in equal volume of complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI). Mice were immunized with 100 μl of the emulsion injected intracutaneously at the base of the tail (day 1). After 21 days (day 22), the same amount of bovine CII emulsified in CFA was injected intracutaneously at the base of the tail as a booster immunization [19].

Treatment of collagen-induced arthritis (CIA) mice with JWH133

Twelve mice with CIA per group were twice daily injected intraperitoneally with JWH133, 1 mg/kg/day or 4 mg/kg/day in total volume of 200 μl/day of 20% dimethyl sulphoxide or vehicle alone from day 15 to day 35. To determine the therapeutic effects of JWH133, we also treated mice with 4 mg/kg JWH133 from day 28, after the development of arthritis, to day 35 and observed the mice for signs of arthritis. Disease severity for each limb was recorded as follows: 0 = normal, 1 = erythema and swelling of one digit, 2 = erythema and swelling of two digits or erythema and swelling of ankle joint, 3 = erythema and swelling of more than three digits or swelling of two digits and ankle joint, and 4 = erythema and severe swelling of the ankle, foot, and digits with deformity. The clinical arthritis score was defined as the sum of the scores for all 4 paws of each mouse. Thickness of each paw was measured using a pair of digital slide calipers. On day 36, the ankle joints were harvested and examined radiographically and histologically. The bilateral second-to-fourth metatarsophalangeal (MTP) joints were assessed radiographically as follows: 0 = not obvious, 1 = marginal osteoporosis, and 2 = erosion. This system yields a possible score between 0 and 4 per animal. The hind paw of each mouse was dissected and examined histologically after hematoxylin and eosin staining. The severity of arthritis was evaluated according to synovial inflammation, as follows: 0 = no inflammation, 1 = focal inflammatory infiltration, and 2 = severe and diffuse inflammatory infiltration. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

The harvested splenocytes (1×10^6 cells) on day 36 were cultured in 48-well plates in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich) with 10% FBS supplemented with 50 μg/ml denatured (100°C, 10 min) CII. After 72 h, the concentrations of interferon (IFN)-γ and IL-17 in the culture supernatant were measured using ELISA kits (DuoSet; R&D Systems).

Serum samples were obtained on day 36 for measurement of IgG1 anti-CII antibody by ELISA (normal, n = 4; vehicle, n = 12; 4 mg/kg JWH133, n = 12) as described previously [19].

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Immunohistological score was analyzed by student's t-test. Concentration of inflammatory mediators and osteoclastogenesis were analyzed by Kruskal-Wallis test and Dunnett's test. Overtime analysis of arthritis score and paw thickness was performed by 2 way-ANOVA, and point-by-point analysis was followed by student's t-test. Histological and radiographic score, and concentration of IgG1 were analyzed by Kruskal-Wallis test and student's t-test.

Results

CB_2 expression in the RA synovial tissues and cells

The RA and OA synovial tissues were examined for CB_2 expression with immunohistochemical staining. We found positive staining in the lining, and sub-lining layer (Figure 1A), and follicle-like aggregates (Figure 1B). In contrast, minimal staining was observed in the OA synovial tissues (Figure 1C). No signal was observed on specimens stained with an isotype-matched IgG control of irrelevant specificity (Figure 1D). The CB_2 staining of 3 RA and 3 OA samples was evaluated with immunohistological score. The scores of CB_2 staining of RA samples was significantly higher than that of OA samples (Figure 1E).

In consistence with the fact that macrophages and lymphocytes express CB_2 [20,21], immunofluorescence double staining of the RA synovial tissues revealed CB_2 expression on the synovial $CD68^+$ macrophages, $CD4^+$ T cells, $CD8^+$ T cells, $CD21^+$ B cells and $vimentin^+$ fibroblast-like appearance cells (Figure 1F).

Inhibition of inflammatory mediator production from RA FLS with a selective CB_2 agonist

FLS produce various inflammatory mediators that play important roles in development of RA. CB_2 expression on *in vitro* cultured RA FLS was confirmed with Western blot analysis (Figure 2A), which agreed with previous observation [22]. We evaluated effects of JWH133, a selective CB_2 agonist, on the production of IL-6, MMP-3, and CCL2 from FLS stimulated with TNF-α. TNF-α treatment enhanced production of these mediators from RA FLS, which was suppressed by JWH133 dose-dependently (Figure 2B-D). Treatment with JWH133 did not inhibit the proliferation of FLS evaluated with the cell counting kit (Dojindo, Kumamoto, Japan) using WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (data not shown).

Inhibition of osteoclastogenesis of peripheral blood monocytes with a selective CB2 agonist

In the following experiments, we used human peripheral blood $CD14^+$ monocytes, which have been shown to express CB_2 [23], since it is hard to prepare a large number of synovial macrophages. Incubation of peripheral blood $CD14^+$ monocytes with M-CSF and RANKL promoted development of TRAP-positive multinucleated cells. Co-incubation with JWH133 suppressed osteoclast formation dose-dependently (Figure 3A and B). M-CSF and RANKL treatment induced resorption of coated calcium. This was inhibited by the co-incubation with JWH133 dose-dependently (Figure 3C and D). These data showed that JWH133 inhibited the formation and function of human osteoclasts.

Treatment of murine CIA with JWH133

The inhibitory effects of JWH133 on RA FLS and human osteoclasts prompted us to examine the compound for the inhibitory effects on murine CIA, an animal model of RA. Intraperitoneal administration of JWH133 (1 mg/kg/day or 4 mg/kg/day) or vehicle twice daily was initiated two weeks after the first immunization and continued for 21 days. Treatment with JWH133 (4 mg/kg/day) ameliorated clinical severity of the arthritis (Figure 4A and B). However, the incidence of arthritis was 100% in all groups. On day 36, the ankle joints were harvested and examined histologically and radiographically. Cell infiltration in the synovial tissues and radiographical bone destruction were observed in vehicle-treated CIA mice, and reduced significantly in the JWH133-treated mice (Figure 4C,D,E and F).

Splenocytes from the mice at day 36 were stimulated with CII and the production of IFN-γ and IL-17 was measured. IFN-γ and IL-17 production was upregulated by the CII stimulation. The treatment with JWH133 did not alter the cytokine production (Figure 4G and H). To determine the effect of JWH133 on anti-CII antibody production, we measured serum anti-CII IgG1 antibody titer by ELISA on day 36. While anti-CII IgG1 antibody was not detected in normal mice, it was detected in CIA mice. The treatment with JWH133 significantly lowered anti-CII IgG1 antibody level (Figure 4I). Although IgG2a and IgG2b anti-CII antibodies were also detected in CIA mice, treatment with JWH133 did not significantly alter the levels of antibodies (data not shown).

To examine the therapeutic effects of JWH133 after the onset of the arthritis, we treated the CIA mice with 4 mg/kg JWH133 from day 28 to day 35, and observed inhibition of the arthritis (Figure 5A and B).

Discussion

The present studies demonstrated that the selective CB_2 agonist, JWH133 inhibited production of IL-6, MMP-3 and CCL2 from FLS and M-CSF and RANKL-induced osteoclastogenesis of monocytes/macrophages. Administration of JWH133 ameliorated arthritis severity and bone destruction, and decreased anti-CII IgG1 antibody in a murine model of RA. This effectiveness could

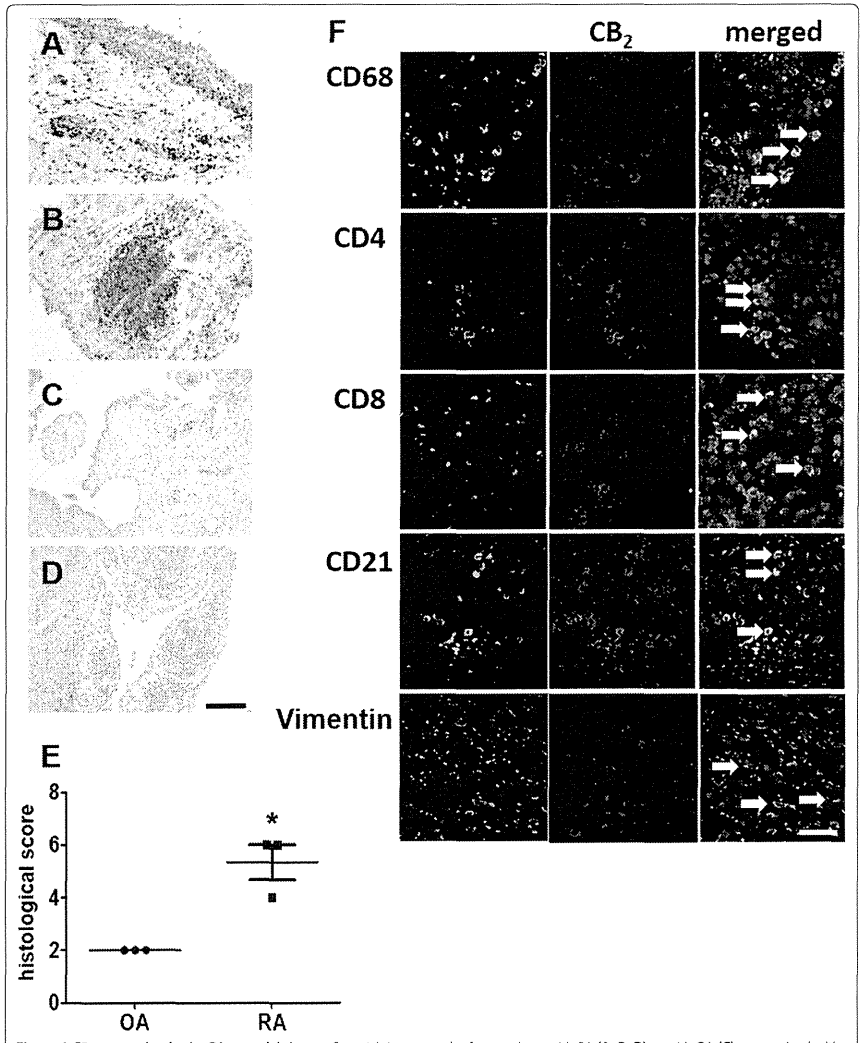


Figure 1 CB_2 expression in the RA synovial tissues. Synovial tissue samples from patients with RA **(A, B, D)** or with OA **(C)** were stained with anti-CB_2 pAb **(A B, and C)**, or normal rabbit IgG **(D)**. All sections were counterstained with hematoxylin. Bar, 400 μm. The representative figures of three RA and three OA synovial tissues are depicted. The severity of the CB_2 staining of samples was shown **(E)**. Double immunofluorescence staining of the RA synovial tissues with CB_2 and CD68, CD4, CD8, CD21 or vimentin, and DAPI are shown **(F)**. Arrows indicate double-positive cells in the merged image. Bar, 40 μm. *p < 0.05.

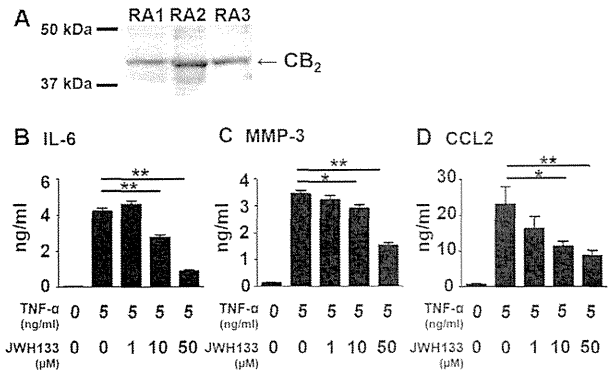


Figure 2 Inhibition of IL-6, MMP-3, and CCL2 production from FLS by the selective CB_2 agonist JWH133. CB_2 expression on *in vitro cultured FLS from three RA patients was detected with Western blotting analysis* **(A)**. Bars indicate 50 and 37 kDa. Predicted molecular weight of CB_2 is 45 kDa. RA FLS were treated with various concentrations of JWH133, from 30 minutes before the stimulation with TNF-α for 24 h. Concentrations of IL-6 **(B)**, MMP-3 **(C)**, and CCL2 **(D)** in the cultured supernatants were measured with ELISA. Data is presented as means ± SEM of one of three independent experiments analyzed in quadruplicate. *p < 0.05, **p < 0.01.

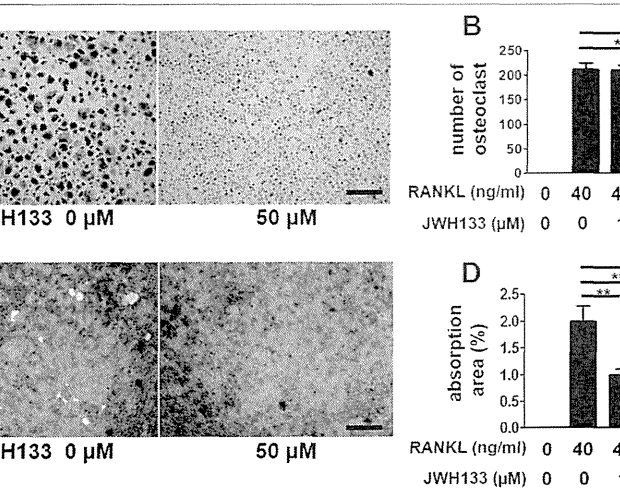


Figure 3 Effects of the selective CB_2 agonist JWH133 on peripheral blood monocytes. Peripheral blood monocytes were treated with various concentrations of JWH133, from 30 minutes before the stimulation with M-CSF and RANKL. Cells were stained with TRAP **(A)**. TRAP-positive *multinucleated cells in a randomly selected field examined at ×40 magnification were counted* **(B)**. *Representative data (mean ± SEM) from one of* three independent experiments analyzed in triplicate are shown. The osteoclasts were incubated on calcium phosphate-coated plates **(C)**. The area of resorption lacunae was examined under light microscopy **(D)**. Representative data (mean ± SEM) from one of three independent experiments analyzed in triplicate are shown. Bar, 400 μm. *p < 0.05, **p < 0.01.

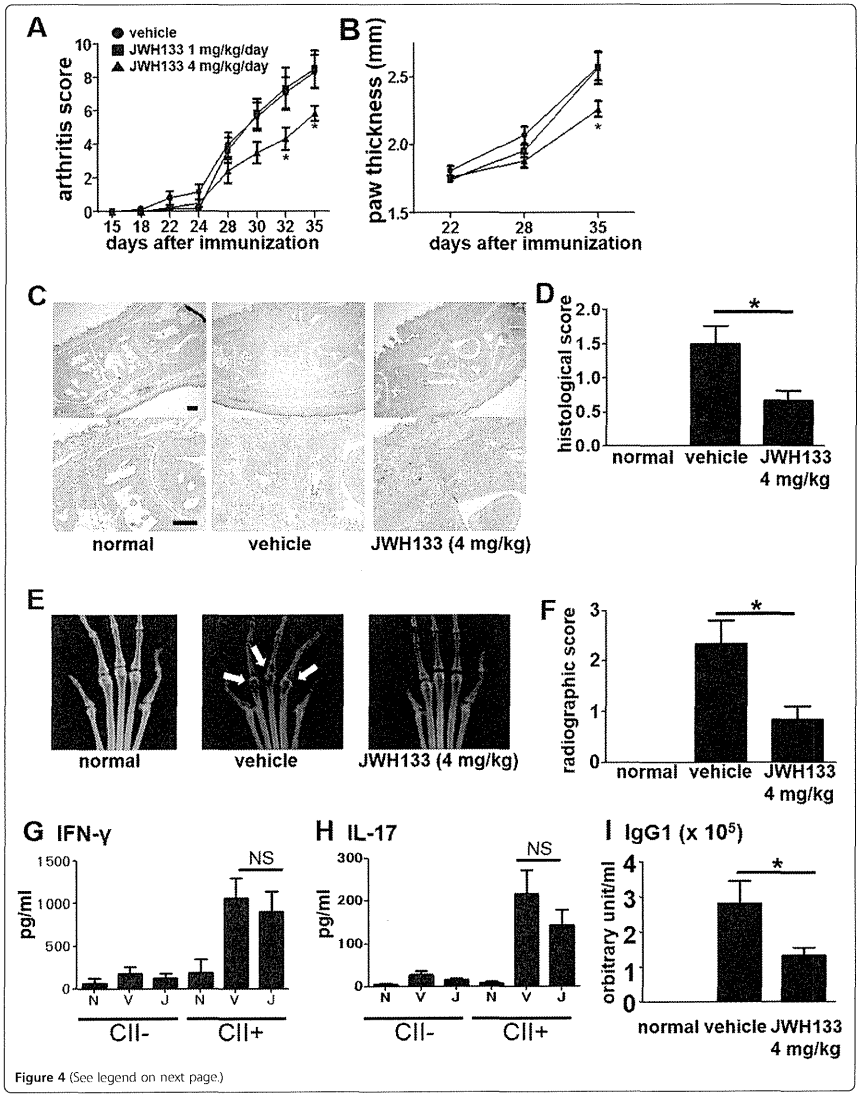


Figure 4 (See legend on next page.)

(See figure on previous page.)

Figure 4 Effects of the selective CB_2 agonist, JWH133 on CIA mice. JWH133 (1 mg/kg/day or 4 mg/kg/day) or vehicle (n = 12 each) was injected intraperitoneally twice daily from day 15 to day 35. The arthritis severity was recorded as the arthritis score **(A)** and paw thickness **(B)**. Overtime analysis of arthritis score and paw thickness by 2-way ANOVA revealed significant difference between vehicle group and 4 mg/kg/day grope ($p < 0.01$). Representative hematoxylin and eosin staining of ankle joints from normal mice and from mice with CIA after the treatment with vehicle or JWH133 (4 mg/kg/day) are shown **(C)**. Bar, 200 μm. Inflammatory cell infiltration in the ankle joints was evaluated with the histological score **(D)**. Representative radiographs of the ankle joints of normal mice and CIA mice treated with vehicle or JWH133 (4 mg/kg/day) **(E)**. Arrows *indicate bone erosion. Bone erosion in the bilateral MTP joints was evaluated with the bone destruction score* **(F)**. *Splenocytes from normal mice* and CIA mice treated with vehicle or JWH133 (4 mg/kg/day) were cultured with CII for 72 h. Concentrations of IFN-γ **(G)** and IL-17 **(H)** in the cultured supernatant were measured by ELISA. N: normal, V: vehicle, J: JWH133, NS: not significant. Serum samples were obtained at day 36 from normal mice and CIA mice treated with vehicle or JWH133 (4 mg/kg/day), and anti-CII IgG1 antibody level was measured by ELISA **(I)**. Values are the mean ± SEM. *$p < 0.05$ versus vehicle.

be attributable to the suppression of inflammatory mediator secretion from *FLS* and osteoclastogenesis and autoantibody production. Thus, selective CB_2 agonists could be a therapeutic agent for RA.

While medical use of cannabinoids such as sedation and analgesia was recorded in 19th century [24], narcotic addiction was always concerned. Recent identification of cannabinoid receptors revealed two distinctive receptors, CB_1 and CB_2, and putative third receptor, GPR55. Some cannabinoids also act as agonists of TRPV-1, -4 and PPARs. It is expected that selective CB_2 agonists act as therapeutic agents that modulate immune functions without any psychoactive effects. JWH133 was applied to our study since it has high selectivity for CB_2 against CB_1 and has no affinity for GPR55, TRPV-1, -4 or PPARs.

In RA patients, FLS expresses hyperplastic, inflammatory, cartilage- and bone-destructive phenotypes. Cytokines, chemokines and MMPs are secreted by FLS. It was reported that MMP-3 production from FLS stimulated with TNF-α or IL-1β was suppressed by ajulemic acid, a synthetic cannabinoid [22]. However, the receptors of this compound have not been identified. In this study, we showed that the selective CB_2 agonist, JWH133 significantly reduced the production of IL-6, MMP-3, and CCL2 from FLS stimulated with TNF-α.

IL-6 has a wide range of functions on immune cells, and plays an important role in RA [25]. Blockade of IL-6 signaling by anti-IL-6 or -IL-6 receptor mAbs is effective treatment [26,27]. Among various chemokines, only CCL2 gene expression is reportedly higher in RA FLS than in OA FLS [28]. Monocyte migration induced by RA stromal cell line supernatants was blocked with anti-CCL2 mAbs [29]. MMP-3 was highly expressed in the RA pannus tissues [30]. In the cytoplasm, CB_2 stimulation leads to inhibition of adenylyl cyclase and subsequent decrease of the intracellular cAMP level. It results in decreased activity of protein kinase A and transcription factors such as NF-κB and NFAT [31,32]. Thus, the treatment of JWH133 could suppress other inflammatory mediators than IL-6, CCL2 and MMP-3, which are involved in pathology of the arthritis.

JWH133 may affect other types of cells involved in the arthritis. RANTES/CCL5-induced chemotaxis of macrophages was inhibited with delta-9-tetrahydrocannabinol, the major component in *marijuana*, acting through CB_2 [33]. Anandamide, one of endocannabinoids, suppressed IL-17 production from Th17 cells primarily via CB_2 [34]. *JWH015*, another selective CB_2 agonist, inhibited CXCL12-induced chemotaxis of T cells [35]. We found that CB_2 was expressed broadly in the RA synovial cells including macrophages, T cells, and B cells.

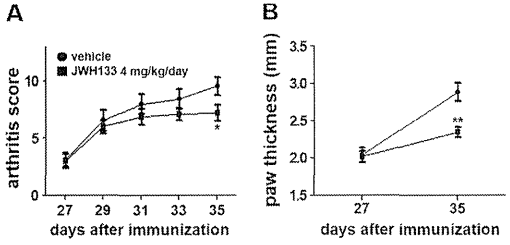


Figure 5 Therapeutic effect of JWH133 on CIA. CIA mice were treated with intraperitoneal injection of JWH133 (4 mg/kg/day) or vehicle (n=12 each) twice daily from day 28 to day 35, after the onset of the disease. The arthritis severity was recorded as the arthritis score **(A)** and paw thickness **(B)**. Overtime analysis of arthritis score and paw thickness by 2-way ANOVA revealed significant difference between vehicle group and 4 mg/kg/day grope ($p < 0.01$). Values are the mean ± SEM. *$p < 0.05$, **$P < 0.01$ versus vehicle.

Suppression of these cells could contribute to suppression of the arthritis.

It was reported that CB_2-deficient mice develop osteoporosis with age [36]. HU308, another selective CB_2 agonist, inhibited osteoclast formation of RANKL-stimulated RAW264.7 cells as well as bone marrow cells from normal mice but not from CB_2-deficient mice [36]. In agreement with this observation, we demonstrated that JWH133 inhibited osteoclastogenesis of human peripheral blood monocytes and bone destruction of CIA mice. Since bone destruction is often a serious issue in RA patients as well as chronic pain and can result in functional decline, inhibitory effect of selective CB_2 agonists for osteoclastogenesis could be another feature in RA treatment.

In this study, we observed amelioration of CIA with JWH133. Since T helper cell differentiation influences the development of CIA [37,38], we measured the production of IFN-γ and IL-17 by CII-stimulated splenocytes from the CIA mice. No significant difference among the groups was revealed in our study. It is suggested that the treatment with JWH133 did not affect Th1 and Th17 differentiation, which may not be attributable to the amelioration of CIA. On the other hand, the treatment with JWH133 decreased the level of serum anti-CII IgG1 antibody. In the previous study, CB_2 mRNA was detected in peripheral B cells [20]. We determined CB_2 expression on B cells in RA synovial tissue. Although the effect of CB_2 for immunoglobulin production has not been reported, the administration of JWH133 directly or indirectly may affect B cells to suppress anti-CII IgG1 antibody production of CIA mice and contribute to the amelioration of the arthritis.

This is the first report of therapeutic effect of a selective CB_2 agonist on CIA. Although the effect was mild, optimization of dosage and/or treatment protocol might enhance the effect. Perhaps, more potent selective CB_2 agonists might solve this problem.

Conclusions

We demonstrated that JWH133, the selective CB_2 agonist, provides clinical effectiveness against CIA mice probably through the immunosuppressive effects for FLS and monocytes and inhibition of anti-CII Ab production. Addition to the analgesic effect as previously reported, selective CB_2 agonists could be a new therapy for RA.

Abbreviations

CB: Cannabinoid; CB_1: Cannabinoid receptor 1; CB_2: Cannabinoid receptor 2; CCL2: CC chemokine ligand 2; CFA: Complete Freund's adjuvant; CIA: Collagen-induced arthritis; CII: Collagen type II; FLS: Fibroblast-like synoviocytes; M-CSF: Macrophage colony-stimulated factor; MMP: Matrix metalloproteinase; MNC: Multinucleated cells; OA: Osteoarthritis; RA: Rheumatoid arthritis; RANKL: Receptor activator of nuclear factor kappa-B ligand; TRAP: Tartrate-resistant acid phosphatase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SF participated in the design of the study, carried out the experiments and statistical analysis, and drafted the manuscript. AT, WY, CM and YM assisted carrying out the experiments and manuscript preparation. HK, MH, NM and TN conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements and Funding

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and by Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases at Tokyo Medical and Dental University.

Author details

[1]Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yushima, 113-8519 Bunkyo-ku, Tokyo, Japan. [2]Department of Dermatology, Tokyo Medical University, 6-1-1 Shinjuku, 160-8402 Shinjuku-ku, Tokyo, Japan. [3]Department of Pharmacovigilance, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yushima, 113-8519 Bunkyo-ku, Tokyo, Japan. [4]Department of Clinical Research Medicine, Teikyo University, 2-11-1 Kaga, 173-8605 Itabashi-ku, Tokyo, Japan.

Received: 12 December 2013 Accepted: 7 August 2014
Published: 12 August 2014

References

1. McInnes IB, Schett G: **The pathogenesis of rheumatoid arthritis.** *N Engl J Med* 2011, **365**(23):2205–2219.
2. Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI: **Structure of a cannabinoid receptor and functional expression of the cloned cDNA.** *Nature* 1990, **346**(6284):561–564.
3. Munro S, Thomas KL, Abu-Shaar M: **Molecular characterization of a peripheral receptor for cannabinoids.** *Nature* 1993, **365**(6441):61–65.
4. Griffin G, Fernando SR, Ross RA, McKay NG, Ashford ML, Shire D, Huffman JW, Yu S, Lainton JA, Pertwee RG: **Evidence for the presence of CB2-like cannabinoid receptors on peripheral nerve terminals.** *Eur J Pharmacol* 1997, **339**(1):53–61.
5. Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley PJ: **The orphan receptor GPR55 is a novel cannabinoid receptor.** *Br J Pharmacol* 2007, **152**(7):1092–1101.
6. Gasperi V, Dainese E, Oddi S, Sabatucci A, Maccarrone M: **GPR55 and its interaction with membrane lipids: comparison with other endocannabinoid-binding receptors.** *Curr Med Chem* 2013, **20**(1):64–78.
7. Henstridge CM, Balenga NA, Kargl J, Andradas C, Brown AJ, Irving A, Sanchez C, Waldhoer M: **Minireview: recent developments in the physiology and pathology of the lysophosphatidylinositol-sensitive receptor GPR55.** *Mol Endocrinol* 2011, **25**(11):1835–1848.
8. McVey DC, Schmid PC, Schmid HH, Vigna SR: **Endocannabinoids induce ileitis in rats via the capsaicin receptor (VR1).** *J Pharmacol Exp Ther* 2003, **304**(2):713–722.
9. *Nilius B, Vriens J, Prenen J, Droogmans G, Voets T:* **TRPV4 calcium entry channel: a paradigm for gating diversity.** *Am J Physiol Cell Physiol* 2004, **286**(2):C195–C205.
10. O'Sullivan SE: **Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors.** *Br J Pharmacol* 2007, **152**(5):576–582.
11. *Croxford JL, Yamamura T:* **Cannabinoids and the immune system: potential for the treatment of inflammatory diseases?** *J Neuroimmunol* 2005, **166**(1–2):3–18.
12. Sumariwalla PF, Gallily R, Tchilibon S, Fride E, Mechoulam R, Feldmann M: **A novel synthetic, nonpsychoactive cannabinoid acid (HU-320) with antiinflammatory properties in murine collagen-induced arthritis.** *Arthritis Rheum* 2004, **50**(3):985–998.
13. Kinsey SG, Naidu PS, Cravatt BF, Dudley DT, Lichtman AH: **Fatty acid amide hydrolase blockade attenuates the development of collagen-induced arthritis and related thermal hyperalgesia in mice.** *Pharmacol Biochem Behav* 2011, **99**(4):718–725.

14. Huffman JW, Liddle J, Yu S, Aung MM, Abood ME, Wiley JL, Martin BR: **3-(1',1'-Dimethylbutyl)-1-deoxy-delta8-THC and related compounds: synthesis of selective ligands for the CB2 receptor.** *Bioorg Med Chem* 1999, **7**(12):2905–2914.
15. Elmes SJ, Jhaveri MD, Smart D, Kendall DA, Chapman V: **Cannabinoid CB2 receptor activation inhibits mechanically evoked responses of wide dynamic range dorsal horn neurons in naïve rats and in rat models of inflammatory and neuropathic pain.** *Eur J Neurosci* 2004, **20**(9):2311–2320.
16. Lee EY, Lee EB, Park BJ, Lee CK, Yoo B, Lim MK, Shim SC, Sheen DH, Seo YI, Kim HA, Baek HJ, Song YW: **Tramadol 37.5-mg/acetaminophen 325-mg combination tablets added to regular therapy for rheumatoid arthritis pain: a 1-week, randomized, double-blind, placebo-controlled trial.** *Clin Ther* 2006, **28**(12):2052–2060.
17. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JM, Hobbs K, Huizinga TW, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Ménard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, *et al.*: **2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative.** *Arthritis Rheum* 2010, **62**(9):2569–2581.
18. Kaneko K, Miyabe Y, Takayasu A, Fukuda S, Miyabe C, Ebisawa M, Yokoyama W, Watanabe K, Imai T, Muramoto K, Terashima Y, Sugihara T, Matsushima K, Miyasaka N, Nanki T: **Chemerin activates fibroblast-like synoviocytes in patients with rheumatoid arthritis.** *Arthritis Res Ther* 2011, **13**(5):R158.
19. *Sato A, Watanabe K, Kaneko K, Murakami Y, Ishido M, Miyasaka N, Nanki T:* **The effect of synthetic retinoid, Am80, on T helper cell development and antibody production in murine collagen-induced arthritis.** *Mod Rheumatol* 2010, **20**(3):244–251.
20. Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P: **Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations.** *Eur J Biochem* 1995, **232**(1):54–61.
21. Miller AM, Stella N: **CB2 receptor-mediated migration of immune cells: it can go either way.** *Br J Pharmacol* 2008, **153**(2):299–308.
22. Johnson DR, Stebulis JA, Rossetti RG, Burstein SH, Zurier RB: **Suppression of fibroblast metalloproteinases by ajulemic acid, a nonpsychoactive cannabinoid acid.** *J Cell Biochem* 2007, **100**(1):184–190.
23. Montecucco F, Burger F, Mach F, Steffens S: **CB2 cannabinoid receptor agonist JWH-015 modulates human monocyte migration through defined intracellular signaling pathways.** *Am J Physiol Heart Circ Physiol* 2008, **294**(3):H1145–H1155.
24. Kalant H: **Medicinal use of cannabis: history and current status.** *Pain Res Manag* 2001, **6**(2):80–91.
25. Tanaka T, Narazaki M, Kishimoto T: **Therapeutic targeting of the interleukin-6 receptor.** *Annu Rev Pharmacol Toxicol* 2012, **52**:199–219.
26. Maini RN, Taylor PC, Szechinski J, Pavelka K, Bröll J, Balint G, Emery P, Raemen F, Petersen J, Smolen J, Thomson D, Kishimoto T, CHARISMA Study Group: **Double-blind randomized controlled clinical trial of the interleukin-6 receptor antagonist, tocilizumab, in European patients with rheumatoid arthritis who had an incomplete response to methotrexate.** *Arthritis Rheum* 2006, **54**(9):2817–2829.
27. Mease P, Strand V, Shalamberidze L, Dimic A, Raskina T, Xu LA, Liu Y, Smith J: **A phase II, double-blind, randomised, placebo-controlled study of BMS945429 (ALD518) in patients with rheumatoid arthritis with an inadequate response to methotrexate.** *Ann Rheum Dis* 2012, **71**(7):1183–1189.
28. Cagnard N, Letourneur F, Essabbani A, Devauchelle V, Mistou S, Rapinat A, Decraene C, Fournier C, Chiocchia G: **Interleukin-32, CCL2, PF4F1 and GFD10 are the only cytokine/chemokine genes differentially expressed by in vitro cultured rheumatoid and osteoarthritis fibroblast-like synoviocytes.** *Eur Cytokine Netw* 2005, **16**(4):289–292.
29. Hayashida K, Nanki T, Girschick H, Yavuz S, Ochi T, Lipsky PE: **Synovial stromal cells from rheumatoid arthritis patients attract monocytes by producing MCP-1 and IL-8.** *Arthritis Res* 2001, **3**(2):118–126.
30. Ainola MM, Mandelin JA, Liljeström MP, Li TF, Hukkanen MV, Konttinen YT: **Pannus invasion and cartilage degradation in rheumatoid arthritis: involvement of MMP-3 and interleukin-1beta.** *Clin Exp Rheumatol* 2005, **23**(5):644–650.
31. Condie R, Herring A, Koh WS, Lee M, Kaminski NE: **Cannabinoid inhibition of adenylate cyclase-mediated signal transduction and interleukin 2 (IL-2) expression in the murine T-cell line, EL4.IL-2.** *J Biol Chem* 1996, **271**(22):13175–13183.
32. Borner C, Smida M, Höllt V, Schraven B, Kraus J: **Cannabinoid receptor type 1- and 2-mediated increase in cyclic AMP inhibits T cell receptor-triggered signaling.** *J Biol Chem* 2009, **284**(51):35450–35460.
33. Raborn ES, Marciano-Cabral F, Buckley NE, Martin BR, Cabral GA: **The cannabinoid delta-9-tetrahydrocannabinol mediates inhibition of macrophage chemotaxis to RANTES/CCL5: linkage to the CB2 receptor.** *J Neuroimmune Pharmacol* 2008, **3**(2):117–129.
34. Cencioni MT, Chiurchiù V, Catanzaro G, Borsellino G, Bernardi G, Battistini L, Maccarrone M: **Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB2 receptors.** *PLoS One* 2010, **5**(1):e8688.
35. Ghosh S, Preet A, Groopman JE, Ganju RK: **Cannabinoid receptor CB2 modulates the CXCL12/CXCR4-mediated chemotaxis of T lymphocytes.** *Mol Immunol* 2006, **43**(14):2169–2179.
36. Ofek O, Karsak M, Leclerc N, Fogel M, Frenkel B, Wright K, Tam J, Attar-Namdar M, Kram V, Shohami E, Mechoulam R, Zimmer A, Bab I: **Peripheral cannabinoid receptor, CB2, regulates bone mass.** *Proc Natl Acad Sci U S A* 2006, **103**(3):696–701.
37. Nakae S, Nambu A, Sudo K, Iwakura Y: **Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice.** *J Immunol* 2003, **171**(11):6173–6177.
38. Hirota K, Hashimoto M, Yoshitomi H, Tanaka S, Nomura T, Yamaguchi T, Iwakura Y, Sakaguchi N, Sakaguchi S: **T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17+ Th cells that cause autoimmune arthritis.** *J Exp Med* 2007, **204**(1):41–47.

doi:10.1186/1471-2474-15-275
Cite this article as: Fukuda *et al.*: Cannabinoid receptor 2 as a potential therapeutic target in rheumatoid arthritis. *BMC Musculoskeletal Disorders* 2014, **15**:275.

Japan College of Rheumatology
MODERN RHEUMATOLOGY

http://informahealthcare.com/mor
ISSN 1439-7595 (print), 1439-7609 (online)

Mod Rheumatol, 2014; 24(4): 685–687
© 2014 Japan College of Rheumatology
DOI: 10.3109/14397595.2013.874734

informa
healthcare

CASE REPORT

Successful treatment of eosinophilic granulomatosis with polyangiitis (EGPA; formerly Churg–Strauss syndrome) with rituximab in a case refractory to glucocorticoids, cyclophosphamide, and IVIG

Natsuka Umezawa[1], Hitoshi Kohsaka[1], Toshihiro Nanki[1,2], Kaori Watanabe[1,2], Michi Tanaka[1,2], Peter Y. Shane[1], and Nobuyuki Miyasaka[1]

[1]*Department of Medicine and Rheumatology, Graduate School of Medicine and Dental Science, Tokyo Medical and Dental University, Tokyo, Japan and* [2]*Department of Pharmacovigilance, Tokyo Medical and Dental University, Tokyo, Japan*

Abstract

A 44-year old woman with eosinophilic granulomatosis with polyangiitis (EGPA) developed sequential paralysis of different cranial nerves despite treatments including methylprednisolone pulse therapy, intravenous immunoglobulins (IVIG), and cyclophosphamide. Infusions of rituximab ameliorated her neurological symptoms and serological inflammatory findings. Rituximab, a specific B cell-targeting therapy, might offer an alternative for refractory EGPA with possible advantages of cost and ease of use compared to IVIG, which also targets (at least in part) B lymphocytes and immunoglobulin production.

Keywords

Eosinophilic granulomatosis with polyangiitis, IVIG, Rituximab

History

Received 21 September 2012
Accepted 28 November 2012
Published online 12 December 2012

Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA) (formerly Churg–Strauss syndrome) is a systemic granulomatous vasculitis with eosinophilia in a patient with a history of allergic disease. The involvement of small to medium vessels is characteristic of EGPA, as is the presence of antineutrophil cytoplasmic antibody (ANCA) in the serum. Both of these features qualify EGPA for inclusion as an ANCA-associated vasculitis (AAV).

Although high-dose glucocorticoids (GC) with cyclophosphamide (CPA) has been the main treatment applied in severe cases of EGPA, about 10 % of these cases have been found to be treatment resistant [1]. Further therapeutic options have been sought for such cases, and successful induction of remission has been reported with the use of both intravenous immunoglobulins (IVIG) and rituximab (RTX) [2]. However, repeated administration and/or combination therapies are sometimes required to achieve maximum benefit [3].

Rituximab (RTX) is a chimeric anti-CD20 monoclonal antibody that has been approved for use in cases of lymphoid malignancy, rheumatoid arthritis, and, more recently, microscopic polyangiitis (MPA) and granulomatosis with polyangiitis (GPA). Although it has proven to be efficacious for AAV in two randomized controlled studies [4, 5], its efficacy in EGPA cases has remained unclear.

We hereby present a case of EGPA resistant to both CPA and IVIG, which was successfully treated with RTX.

Correspondence to: N. Miyasaka, Department of Medicine and Rheumatology, Graduate School of Medicine and Dental Science, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. Tel: +81-3-5803-4773. Fax: +81-5803-5998. E-mail: miya.rheu@tmd.ac.jp

Case report

A 44-year-old woman with a history of bronchial asthma was admitted to a local hospital in June 2011 for fever with numbness and weakness of her extremities. Laboratory data showed peripheral eosinophilia (6579/μl) and elevated CRP (34.5 mg/l), as well as the presence of MPO–ANCA (180 EU/l). Nerve conduction test revealed low amplitude in the right median, the left ulnar, and the right sural nerves, suggestive of mononeuritis multiplex. She was diagnosed with EGPA, and treatment was initiated with intravenous pulses of methylprednisolone.

Peripheral blood eosinophil count and MPO–ANCA normalized within six weeks during the oral prednisolone (PSL) taper. However, she remained febrile, her neurological symptoms persisted, and her CRP remained elevated (Fig. 1). A trial of IVIG (400 mg/kg/day over five days) was ineffective, with a new left facial nerve paralysis developing during the second course. One intravenous dose of CPA (500 mg) was administered along with the initiation of trimethoprim-sulfamethoxazole for preventing pneumocystis pneumonia, during which time the facial nerve paralysis worsened. There was no evidence of meningitis or hypertrophic pachymeningitis by cerebrospinal fluid analysis and brain MRI.

At this point (in August), the patient was transferred to our hospital, and she still presented febrile. The physical examination revealed that the cranial nerves other than the left facial nerve were intact and that paresthesia and weakness of her extremities had persisted. Peripheral blood count showed an absence of eosinophils, with total white blood cells 17,200/μl, hemoglobin level 8.2 g/dl, and platelets 469,000/μl. Serum CRP (159 mg/l) and eosinophil cationic protein (ECP; 36 μg/dl) were elevated, while MPO–ANCA remained negative. Serum IgG concentration was 1517 mg/ml (normal range: 868–1780 mg/ml). Methylprednisolone pulses improved the facial nerve palsy and the patient became

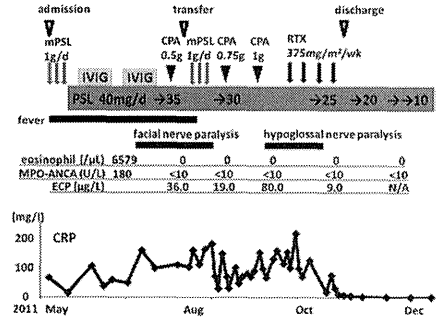


Fig. 1 Clinical course. This EGPA patient was resistant to corticosteroid, IVIG, and CPA. Treatment with RTX ameliorated cranial nerve involvement and reduced serum ECP and CRP levels. She remains in remission with B-cell depletion six months after RTX therapy. *mPSL* methylprednisolone, *PSL* prednisolone, *IVIG* intravenous immunoglobulin, *CPA* cyclophosphamide, *RTX* rituximab, *ECP* eosinophil cationic protein

afebrile, although her CRP remained high. Two additional intravenous doses of CPA (750 mg, followed by 1000 mg) also failed to decrease the CRP. Moreover, the patient started to complain of difficulty swallowing, and paralysis of the right hypoglossal nerve was demonstrated clinically. Serum ECP rose to 80 μg/dl while serum IgG decreased to 801 mg/ml.

Subsequently, RTX was administered at a dose of 480 mg (375 mg/m^2) once a week for four weeks under written informed consent. Three weeks after the first dose, the hypoglossal nerve paralysis had disappeared completely, with decreased peripheral B-cell counts of 2 cells/μl as compared to 164 cells/μl before the RTX administration. Serum CRP and ECP diminished to within normal ranges by the time of the fourth dose, although the peripheral neuropathy at the extremities did not improve. There was no occurrence of adverse events despite the depletion of peripheral B cells and the low serum IgG concentration (760 mg/ml). At six months after completing treatment with RTX, the patient remained in remission on PSL 10 mg/day without peripheral B-cell recovery.

Discussion

Rituximab showed dramatic efficacy in our patient who had EGPA that was resistant to both conventional treatment and IVIG. The success obtained with RTX in the present case is consistent not only with the demonstrated efficacy of RTX in cases of MPA and GPA [4, 5], but also with the results achieved when it was used in eleven previously reported cases (Table 1) [6–11]. Among these 12 cases (including the case herein), three were resistant to previous IVIG, and at least five achieved remission without concomitant immunosuppressants or high-dose corticosteroids, suggesting a possible advantage of RTX in remission induction. As IVIG is presumed to exert its efficacy partly by providing a negative feedback signal on B cells mediated through FcγRIIB [12], the depletion of B cells using RTX may be a more potent and direct mechanism.

The efficacy of IVIG in EGPA has been previously demonstrated in two nonrandomized interventional studies. Tsurikisawa et al. [13] reported that IVIG showed significantly greater improvement of muscle weakness, dysesthesia, and cardiac output in 22 EGPA patients refractory to conventional treatment compared to 24 patients who did not receive IVIG. In a separate report, Danieli et al. [14] found that repeated IVIG combined with plasmapheresis in addition to conventional treatment achieved a significantly higher remission rate (100 %) than conventional treatment alone (44 %) in newly diagnosed EGPA. Based on these findings, IVIG can be recommended in particular for cases with persistent neurological deficits and/or cardiac dysfunction, as well as for difficult-to-treat cases. The efficacy of IVIG monotherapy for inducing remission remains unproven.

Although the potential clinical superiority of RTX needs to be demonstrated in larger studies, its economic and logistical advantages are clear. The administration of IVIG requires hospitalization, which sometimes needs to be repeated. Furthermore, the clinical status of the patient may dictate the need for concomitant plasma exchange, significantly increasing the cost of treatment. Logistically, the availability of IVIG has been problematic globally, and the use of alternative treatments, when possible, has

Table 1. Cases of EGPA successfully treated with RTX

Patient no. [reference]	Age, sex	Involved organs	ANCA	Previous treatments (other than GC)	Concomitant treatments with RTX	Observation period after RTX administration/relapse	Additional RTX use/its indication
1 [6]	49, M	Kidney, skin	PR3	IVCY, AZA	PSL 30 mg/day	3 months/not relapsed	None
2 [7]	37, F	Myocarditis	(–)	IVCY, IVIG, MMF, alemtuzumab	PSL 15 mg/day	9 months/not relapsed	At 6 months/ prophylaxis
3 [7]	37, F	PNS, skin	(–)	CPA, AZA, MMF, alemtuzumab	PSL 10 mg/day	12 months/relapsed at 6 months	At 6 months/ relapse
4 [8]	40, M	Lung	PR3	IVCY, PE	PSL[a] + CPA	9 months/not relapsed	None
5 [8]	66, M	PNS	MPO	IVIG, IVCY, PE	Low-dose PSL[a]	3 months/not relapsed	None
6 [9]	46, F	CNS	MPO	IVCY, MMF	PSL 5 mg/day + MMF	4 months/not relapsed	None
7 [10]	50, M	PNS, skin	(–)	MTX, CyA, AZA IFX, anakinra	PSL[a]	12 months/not relapsed	At 6 and 12 months/ prophylaxis
8 [10]	35, F	Lung	(–)	AZA	PSL[a]	6 months/not relapsed	At 6 months/ prophylaxis
9 [11]	54, F	Kidney	MPO	IVCY, MTX	PSL 1 mg/kg/day	12 months/relapsed at 6 months	At 6 months/ relapse
10 [11]	54, F	Kidney, PNS	MPO	(–)	PSL 1 mg/kg/day	12 months/not relapsed	None
11 [11]	65, M	Kidney, PNS	MPO	(–)	PSL 1 mg/kg/day	12 months/not relapsed	None
12 (present case)	44, F	CNS, PNS	MPO	IVIG, IVCY	PSL 25 mg/day	6 months/not relapsed	None

PNS peripheral nervous system, *CNS* central nervous system, *IVCY* intravenous CPA pulse therapy, *IVIG* intravenous immunoglobulin, *AZA* azathioprine, *MMF* mycophenolate mofetil, *CPA* oral cyclophosphamide, *PE* plasma exchange, *MTX* methotrexate, *CyA* cyclosporine A, *IFX* infliximab
[a]The dosage of PSL was not available

been recommended. Compared with IVIG, one course of RTX may provide sustained efficacy for approximately six months or longer, with at least some of the infusions possible in an outpatient setting.

As pharmacotherapeutic decisions need to be made by weighing up the balance of efficacy and safety, the risk of progressive multifocal leukoencephalopathy (PML) needs to be considered specifically for RTX. Fortunately, these cases are rare; most of them are associated with either previous or concomitant exposure to other immunosuppressive agents. Still, when compared to the safety of IVIG, the risk of developing serious infections should be an important consideration with RTX.

RTX could be an alternative for remission induction in EGPA cases. However, it remains unclear whether the scheduled RTX treatment or treatments with other oral immunosuppressants should be followed as maintenance therapy. In the present case, azathioprine was used successfully, in accordance with the EULAR recommendation for maintenance therapy after the conventional remission induction treatments [2]. An ongoing randomized controlled trial of RTX versus azathioprine as maintenance therapy (MAINRITSAN) will provide us with important information.

In general, peripheral eosinophil counts as well as serum ECP levels serve as biomarkers of the disease activity of EGPA [15, 16]. In the present case, the ECP level reflected the disease activity better than the eosinophil count. Together with another report that described a discrepancy between the levels of these two biomarkers [17], our observation suggests that serum ECP might be the more sensitive biomarker.

In conclusion, RTX is a potent alternative therapy for refractory EGPA. Further clinical investigations, especially with larger numbers of patients, are needed to confirm its efficacy and safety, as well as its most appropriate position in the therapeutic armamentarium.

Conflict of interest

PYS is currently employed by UCB Japan, Co., Ltd. All other authors have declared no conflict of interest.

References

1. Cohen P, Pagnoux C, Mahr A, Arene JP, Mouthon L, Le Guern V, et al. Churg–Strauss syndrome with poor-prognosis factors: a prospective multicenter trial comparing glucocorticoids and six or twelve cyclophosphamide pulses in forty-eight patients. Arthritis Rheum. 2007;57:686–93.
2. Mukhtyar C, Guillevin L, Cid MC, Dasgupta B, de Groot K, Gross W, et al. EULAR recommendations for the management of primary small and medium vessel vasculitis. Ann Rheum Dis. 2009;68:310–17.
3. Taniguchi M, Tsurikisawa N, Higashi N, Saito H, Mita H, Mori A, et al. Treatment for Churg–Strauss syndrome: induction of remission and efficacy of intravenous immunoglobulin therapy. Allergol Int. 2007;56:97–103.
4. Jones RB, Tervaert JW, Hauser T, Luqmani R, Morgan MD, Peh CA, et al. Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis. J N Eng Med. 2010;363:211–20.
5. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, et al. Rituximab versus cyclophosphamide for ANCA-associated vasculitis. N Eng J Med. 2010;363:221–32.
6. Kaushik VV, Reddy HV, Bucknall RC. Successful use of rituximab in a patient with recalcitrant Churg–Strauss syndrome. Ann Rheum Dis. 2006;65:1116–7.
7. Koukoulaki M, Smith KG, Jayne DR. Rituximab in Churg–Strauss syndrome. Ann Rheum Dis. 2006;65:557–9.
8. Pepper RJ, Fabre MA, Pavesio C, Gaskin G, Jones RB, Jayne D, et al. Rituximab is effective in the treatment of refractory Churg–Strauss syndrome and is associated with diminished T-cell interleukin-5 production. Rheumatology (Oxford). 2008;47:1104–5.
9. Saech J, Owczarczyk K, Rosgen S, Petereit H, Hallek M, Rubbert-Roth A. Successful use of rituximab in a patient with Churg–Strauss syndrome and refractory central nervous system involvement. Ann Rheum Dis. 2010;69:1254–5.
10. Donvik KK, Omdal R. Churg–Strauss syndrome successfully treated with rituximab. Rheumatol Int. 2011;31:89–91.
11. Cartin-Ceba R, Keogh KA, Specks U, Sethi S, Fervenza FC. Rituximab for the treatment of Churg–Strauss syndrome with renal involvement. Nephrol Dial Transplant. 2011;26:2865–71.
12. Sewell WA, Jolles S. Immunomodulatory action of intravenous immunoglobulin. Immunology. 2002;107:387–93.
13. Tsurikisawa N, Saito H, Oshikata C, Tsuburai T, Akiyama K. High-dose intravenous immunoglobulin treatment increases regulatory T cells in patients with eosinophilic granulomatosis with polyangiitis. J Rheumatol. 2012;39:1019–25.
14. Danieli MG, Cappelli M, Malcangi G, Logullo F, Salvi A, Danieli G. Long term effectiveness of intravenous immunoglobulin in Churg–Strauss syndrome. Ann Rheum Dis. 2004;63:1649–54.
15. Herrmann K, Gross WL, Moosig F. Extended follow-up after stopping mepolizumab in relapsing/refractory Churg–Strauss syndrome. Clin Exp Rheumatol. 2012;30:S62–5.
16. Guilpain P, Auclair JF, Tamby MC, Servettaz A, Mahr A, Weill B, et al. Serum eosinophil cationic protein: a marker of disease activity in Churg–Strauss syndrome. Ann N Y Acad Sci. 2007;1107:392–9.
17. Hurst S, Chizzolini C, Dayer JM, Olivieri J, Roux-Lombard P. Usefulness of serum eosinophil cationic protein (ECP) in predicting relapse of Churg and Strauss vasculitis. Clin Exp Rheumatol. 2000;18:784–5.

Japan College of Rheumatology
MODERN RHEUMATOLOGY

http://informahealthcare.com/mor
ISSN 1439-7595 (print), 1439-7609 (online)

Mod Rheumatol, 2014; 24(4): 572–579

DOI: 10.3109/14397595.2013.860695

informa
healthcare

ORIGINAL ARTICLE

A comparison of incidence and risk factors for serious adverse events in rheumatoid arthritis patients with etanercept or adalimumab in Korea and Japan

Soo-Kyung Cho[1,2,3]*, Ryoko Sakai[1,4]*, Toshihiro Nanki[1,4], Ryuji Koike[1,4,5], Kaori Watanabe[1,4], Hayato Yamazaki[1,4], Hayato Nagasawa[6], Yoshiya Tanaka[7], Atsuo Nakajima[8], Shinsuke Yasuda[9], Atsushi Ihata[10], Kazuhiko Ezawa[11], Soyoung Won[3], Chan-Bum Choi[2,3], Yoon-Kyoung Sung[2,3], Tae-Hwan Kim[2,3], Jae-Bum Jun[2,3], Dae-Hyun Yoo[2,3], Nobuyuki Miyasaka[4,12], Sang-Cheol Bae[2,3], Masayoshi Harigai[1,4] for the RESEARCH investigators[2,3], and the REAL Study Group

[1]*Department of Pharmacovigilance, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan,* [2]*Hanyang University Hospital for Rheumatic Diseases, Seoul, South Korea,* [3]*Clinical Research Center for Rheumatoid Arthritis (CRCRA), Seoul, South Korea,* [4]*Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan,* [5]*Clinical Research Center, Tokyo Medical and Dental University Hospital, Tokyo, Japan,* [6]*Department of Rheumatology/Clinical Immunology, Saitama Medical Center, Saitama Medical University, Saitama, Japan,* [7]*The First Department of Internal Medicine, University of Occupational and Environmental Health, Japan, Fukuoka, Japan,* [8]*Department of Rheumatology, Tokyo Metropolitan Police Hospital, Tokyo, Japan,* [9]*Department of Internal Medicine II, Hokkaido University, Graduate School of Medicine, Sapporo, Japan,* [10]*Department of Internal Medicine and Clinical Immunology, Yokohama City University Graduate School of Medicine, Yokohama, Japan,* [11]*Department of Internal medicine, Kurashiki Kousai Hospital, Kurashiki, Japan, and* [12]*Global Center of Excellence (GCOE) Program; International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan*

Abstract

Objective. To compare the incidence and risk factors of serious adverse events (SAEs) in rheumatoid arthritis (RA) patients treated with etanercept (ETN) or adalimumab (ADA) between Korean and Japanese registries.

Methods. We recruited 416 RA patients [505.2 patient-years (PYs)] who started ETN or ADA from Korean registry and 537 RA patients (762.0 PY) from Japanese registry. The patient background, incidence rate (IR) of SAE in 2 years, and risk factors for SAEs were compared.

Results. Korean patients were younger and used more nonbiologic DMARDs, higher doses of methotrexate, and lower doses of prednisolone (PSL). The IR of SAEs (/100 PY) was higher in the Japanese registry compared to the Korean [13.65 vs. 6.73]. In both registries, infection was the most frequently reported SAE. The only significant risk factor for SAEs in Korean registry was age by decade [1.45]. In Japanese registry, age by decade [1.54], previous use of nonbiologic DMARDs ≥ 4 [1.93], and concomitant use of oral PSL ≥ 5 mg/day [2.20] were identified as risk factors for SAEs.

Conclusions. The IR of SAE in Japan, especially infection, was higher than that of Korea, which was attributed to the difference of demographic and clinical characteristics of RA patients and treatment profiles.

Keywords

Epidemiology, Registry, Rheumatoid arthritis, Safety

History

Received 12 August 2013
Accepted 27 October 2013
Published online 9 December 2013

Introduction

The introduction of biologic disease-modifying antirheumatic drugs (biologic DMARDs) in the past decade has revolutionized treatment of rheumatoid arthritis (RA). Efficacy and safety of treatment with biologic DMARDs have been demonstrated in a number of clinical trials, but cost and long-term effectiveness of treatment with biologic DMARDs and safety in older patients or those with comorbidities, who are generally excluded from clinical trials, have been of concern [1]. To complement the evidence obtained from clinical trials, observational cohorts for RA patients treated with biologic DMARDs have been established in many countries, and have provided indispensable evidence for the safety and effectiveness of biologic DMARDs in clinical practice. However, some cohorts have reported results with differing magnitudes or even discordance of risk for the same adverse events [2]. For example, the incidence of serious infections in European RA registries was comparable [3,4], whereas in the US, lower rates have been reported in some studies [5]. These discrepant results arise from methodological differences, such as case definition for adverse events, length of follow-up, or selection and structure of a comparator group. Difference in treatment profile and ethnics may also account for the discrepancy. Therefore, a careful comparison of registries from various point of views including methodology is

*Dr. Cho and Dr. Sakai contributed equally to this work.
Correspondence to: Masayoshi Harigai, MD, PhD, Department of Pharmacovigilance, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, Japan. Tel: +81-3-5803-4677. Fax: +81-3-5803-4694. E-mail: mharigai.mpha@tmd.ac.jp

imperative to understand similarities and differences in the results obtained from each registry [6].

Through international collaborations among countries, the comparison of data from RA patients treated with biologic DMARDs will allow us to investigate the impact of differences in patients' characteristics and health care systems on efficacy and safety of the treatment. Curtis et al. [2] have conducted the qualitative comparison of RA biologics registries in US and Europe and reported that different patients' demographics, patterns of comorbidities, and sociodemographic characteristics provide valuable information to address the comparative safety of treatments for RA. However, no international collaborative studies have yet been reported to investigate the same outcomes using harmonized methodologies. In Korea, the effectiveness and safety of biologic DMARDs in clinical practice have been reported using a retrospective biologic DMARDs registry (REtrospective study for Safety and Effectiveness of Anti-RA treatment with biologiCs, RESEARCh) [7]. In Japan, the REgistry of Japanese rheumatoid Arthritis patients on Biologics for Long-term safety (REAL) has provided evidence about safety of biologic DMARDs in Japanese RA patients [8,9]. Taking advantage of these established cohorts for patients with RA, we conducted the first epidemiological study to compare data from two countries where biologic DMARDs are widely used for treatment of RA.

For this study, we carefully scrutinized features of Korean and Japanese registries and considered standardization of methodological approaches. We conducted this study to reveal the factors influencing safety of adalimumab (ADA) or etanercept (ETN) by comparing RA patients treated with these drugs from Korean and Japanese registries in terms of retention rates and reasons for discontinuation of biological DMARDs, incidence rates (IR) of serious adverse events (SAEs), and factors influencing their development.

Patients and methods

Database and patients

RESEARCh

The retrospective registry of Korean patients with RA, the RESEARCh, was established to evaluate the safety and effectiveness of biologic DMARDs by Clinical Research Center of Rheumatoid Arthritis (CRCRA) funded by Ministry of Health and Welfare, Republic of Korea [7]. All patients meeting the 1987 American College of Rheumatology criteria for RA who had ever been treated with biologic DMARDs from December 2000 to June 2011were identified from the medical records of Hanyang University Hospital for Rheumatic Diseases. The RESEARCh study was approved by the ethics committees of the Hanyang University Hospital, and informed consent was not required because the data was deidentified and collected retrospectively.

Comprehensive chart reviews for all patients were undertaken by well-trained health professionals; and demographics, disease activity, comorbidities, medications, and laboratory data during the use of biologic DMARDs and their SAEs were collected. For the patients who were in use of biologic DMARDs at the time of data collection, the observational period was defined from starting point of current agent to assessment date. For the other patients who had stopped biologic DMARDs before data collection, the agent with longest use for each patient was included in this database. Demographic features of RA patients and the persistence of TNF inhibitors in the RESEARCh database were quite similar to those of a previously reported study using nation-wide claims database of Korea; mean age (50.5 ± 13.2 in the RESEARCh vs. 50.6 ± 14.9 in the nation-wide database), proportion of female (86.1% vs. 84.9%), and persistence of TNF inhibitors during one year (74% vs. 73%) [7,10].

REAL

REAL is a prospective cohort established to investigate the long-term safety of biologic DMARDs in RA patients. Twenty-seven institutions participate, including 16 university hospitals and 11 referring hospitals. Details of the REAL have been previously described [9,11]. Briefly, the criteria for patient enrollment in the REAL include meeting the 1987 American College of Rheumatology criteria for RA, written informed consent, and starting or switching treatment with biologic DMARDs or starting, adding, or switching nonbiologic DMARDs at the time of enrollment in the REAL. Demography, disease activity, comorbidities, treatments, and laboratory data at the time of enrollment in the REAL were recorded. A follow-up form was submitted every 6 months by participating physicians to the REAL Data Center at the Department of Pharmacovigilance of Tokyo Medical and Dental University to report the occurrence of SAEs, current RA disease activity, treatments, and clinical laboratory data. Each patient is followed for 5 years. Enrollment in the REAL database was started in June 2005 and closed in January 2012. Data were retrieved from the REAL database on August 24, 2011 for this study. The REAL study was approved by the ethics committees of the Tokyo Medical and Dental University Hospital and other participating institutions. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983.

Patients and follow-up

We first identified 416 Korean RA patients whose registered biologic DMARDs in the RESEARCh database were ADA or ETN and 537 Japanese patients with RA who used ADA or ETN as the first biologic DMARDs in the REAL database, and enrolled themselves in this study. The reason for selecting ADA and ETN for this study is that these two biologics were approved within two calendar years in both countries. The observation period for this study started at the first dose of one of these biologic DMARDs. Observation of each patient was stopped either 2 years after the start of the observation period, or on the date of discontinuation of these biologic DMARDs, switching to other biologic DMARD, death, loss-to-follow up, or enrollment in clinical trials, whichever came first. We defined discontinuation of treatment with ADA or ETN as stopping administration of these agents for more than 90 days. Reasons for discontinuation of these biologic DMARDs were retrieved from medical records and classified into adverse events (AEs), Lack of efficacy (LOE), or miscellaneous. When a patient had two or more reasons for drug discontinuation, site investigators assigned precedence and the primary reason contributing to drug discontinuation for the patient was used.

Definition for comorbidity

For qualitative comparison, comorbidity was defined as cardiovascular and cerebrovascular diseases, including angina, myocardial infarction, heart failure, and strokes; pulmonary diseases, including interstitial lung diseases, chronic obstructive pulmonary diseases, and asthma; or liver diseases, including abnormalities in liver function tests, liver cirrhosis, hepatitis B, and hepatitis C. Renal dysfunction was defined using the estimated glomerular filtration rate (eGFR). We used a modification of diet in renal disease (MDRD) formula to calculate eGFR and categorized according to the stage of chronic kidney disease (CKD) [12]. Anemia was defined using the WHO criteria (hemoglobin level < 13 g/dl for men and < 12 g/dl for women) [13].

Definition of SAEs

Our definition of a SAE, including serious infection (SI), was based on the report by the International Conference on Harmonization. In addition, bacterial infections that required intravenous administration of antibiotics, as well as opportunistic infections, were also regarded as SAEs. SAEs were classified using the System Organ Class (SOC) of the medical dictionary for regulatory activities (MedDRA version 11.1). SAEs were attributed to ETN or ADA when they developed during treatment with these biologics and no risk window was applied.

Statistical analysis

The chi-square test was used for comparison of categorical variables and the Mann–Whitney test for continuous variables. Drug retention rates were compared using the Kaplan–Meier method and the log-rank test. Crude IRs per 100 PY and crude incidence rate ratios (IRRs) with their 95% confidence intervals (CI) comparing Japan to Korea were calculated for all SAEs occurring from the first dose of ADA or ETN to the end of the observation period. For multivariate analysis, the Cox regression model with the forced entry method was employed. These statistical analyses were performed using SPSS (version 20.0, SPSS Inc., Chicago, IL USA). All p values were 2-tailed and $p < 0.05$ was considered statistically significant.

Results

Demographic and clinical baseline characteristics of patients from the two registries

We first compared baseline demographic and clinical characteristics of RA patients who used ADA or ETN in each registry (Table 1 and Figure 1). Patients in the RESEARCh were younger (47.5 ± 15.8 vs. 58.9 ± 13.3 years-old, $p < 0.001$) and had shorter disease duration (8.5 ± 6.7 vs. 9.9 ± 9.0, years $p = 0.009$) than those in the REAL. The proportions of patients without previous exposure to biologic DMARDs (i.e., biologic DMARD-naïve patients) did not differ between the two registries, while 60.0% of the patients in the RESEARCh, but only 29.4% in the REAL, experienced four or more nonbiologic DMARDs ($p < 0.001$). The mean numbers of previous nonbiologic DMARDs were 4.1 in the RESEARCh and 2.6 in the REAL; the distribution is shown in Figure 1A. The mean Disease Activity Score calculated based on three variables including 28-swollen and tender joints count and C-reactive protein at starting biologic DMARDs did not differ between the registries. Patients in the RESEARCh used concomitant methotrexate (MTX) more frequently and at higher dosage than those in the REAL (75.9% and 13.3 ± 3.2 mg/week vs. 54.2% and 7.7 ± 2.4 mg/week, $p < 0.001$ for both) (Table 1 and Figure 1B). On the other hand, patients in the REAL used concomitant corticosteroids (CSs) more frequently and at higher dosage than those in the RESEARCh (PSL-equivalent dose, 6.0 ± 3.5 mg/day vs. 4.5 ± 3.1 mg/day, $p < 0.001$) (Table 1 and Figure 1C).

The rates for comorbidities differ significantly between the two registries. The rates for patients with peptic ulcer (6.0% for the RESEARCh vs. 0.7% for the REAL), liver disease (10.8% vs. 6.7%), hypertension (21.9% vs. 15.8%), and anemia (73.8% vs. 60.5%) were significantly higher in the RESEARCh compared to the REAL. However, the rates for pulmonary disease (5.3% vs. 20.3%) and diabetes mellitus (9.4% vs.13.6%) were significantly higher in the REAL than in the RESEARCh (Table 1).

Table 1. Demographic and clinical characteristics of patients with RA treated with ETN or ADA from Korean (RESEARCh) and Japanese (REAL) registries.

	RESEARCh ($n = 416$)	REAL ($n = 537$)	p value
Age (years-old) mean ± SD	47.5 ± 15.8	58.9 ± 13.3	< 0.001
> 65, n (%)	57 (13.7)	201 (37.4)	< 0.001
Gender (female), %	84.3	79.0	0.067
Disease duration (years), mean ± SD	8.5 ± 6.7	9.9 ± 9.0	0.009
DAS28(3)/CRP*, mean ± SD	4.4 ± 0.9	4.5 ± 1.3	0.973
Unexposed to biological DMARDs, (%)	333 (80.0)	440 (81.9)	0.505
Number of previous nonbiologic DMARDs† ≥ 4, (%)	249 (60.0)	158 (29.4)	< 0.001
MTX, mg/week (%)	13.3 ± 3.2 (75.9)	7.7 ± 2.4 (54.2)	< 0.001
Corticosteroid**, mg/week (%)	4.5 ± 3.1 (72.1)	6.0 ± 3.5 (67.6)	< 0.001
Cardiovascular disease, n (%)	11 (2.6)	30 (5.6)	0.026
Pulmonary disease‡, n (%)	22 (5.3)	109 (20.3)	< 0.001
Liver disease, n (%)	45 (10.8)	36 (6.7)	0.024
Peptic ulcer, n (%)	25 (6.0)	4 (0.7)	< 0.001
Diabetes mellitus, n (%)	39 (9.4)	73 (13.6)	0.045
Hypertension, n (%)	91 (21.9)	85 (15.8)	0.017
Anemia¶, n (%)	307 (73.8)	325 (60.5)	< 0.001
Renal dysfunction§, n (%)			
Advanced staged CKDs (CKD3, 4 or 5), n (%)§	23 (5.5%)	14 (2.6%)	0.021

SD, standard deviation; DAS28, disease activity score including 28-joint count; CRP, C-reactive protein; DMARDs, disease-modifying antirheumatic drugs; MTX, methotrexate; PSL, prednisolone; CKD, chronic kidney disease; GFR, glomerular filtration rate

*DAS 28(3)/CRP was calculated based on three variables; swollen and tender joint counts and CRP.

**The oral corticosteroid dose was converted to the equivalent PSL dosage.

†Nonbiologic DMARDs included MTX, hydroxycholoroquine, sulfasalazine, leflunomide, bucillamine, mizoribine, tacrolimus, azathioprine, cyclosporin.

‡Pulmonary disease included interstitial lung disease, chronic obstructive pulmonary disease, and asthma.

¶Anemia was defined using WHO criteria.

§Renal dysfunction was defined using GFR calculated by modification of diet in renal disease. GFR was categorized according to the staging of CKD.

MTX and corticosteroid doses are shown as the mean ± SD among users of these drugs.