Figure 5
Correlation between AREG and VEGF expression levels. (A) The VEGF mRNA expression levels in synovia from 10 RA patients and 6 OA patients were measured by real-time PCR, and are shown by box-plots (left). Significant differences from unstimulated cells are shown by asterisks (*P < 0.05). The correlation between the mRNA levels of VEGF and AREG is shown by a distribution chart (right). The linear regression coefficient (R^2) is 0.496 and the correlation coefficient (ρ) is 0.700 (P = 0.0067). (B) The concentrations of VEGF in synovial fluids from 7 RA patients and 6 OA patients were measured by ELISA, and are shown by box-plots (left). Significant differences from unstimulated cells are shown by asterisks (*P < 0.05). The correlation between the protein concentrations of VEGF and AREG is shown by a distribution chart (right), in which R^2 is 0.577 and ρ is 0.772 (P = 0.0075).

upregulated, similar to AREG and EREG whose augmented expressions were reported in our previous study. Although there was no significant difference between the levels of HB-EGF in the RA and OA samples, its high correlations with AREG and EREG (ρ = 0.788, P = 0.0008 and ρ = 0.823, P = 0.0005, respectively) imply that HB-EGF may be upregulated in RA. Although EGF and TGFα were also upregulated in RA, their expression levels showed no correlations with those of other members. These results suggest that AREG, EREG and HB-EGF may be regulated by a common expression-controlling system.

In RA-PBMCs, AREG and HB-EGF were significantly upregulated, and their expression levels were correlated with each other (ρ = 0.600, P = 0.0305). Among the seven EGF family members examined, only AREG expression was augmented in all three tissues tested in the present study. To confirm which lineage of blood cells expresses AREG, PBMCs were further separated into monocyte-, T lymphocyte- and B lymphocyte-rich fractions. None of these fractions was enriched in AREG-expressing cells in healthy controls or RA patients. In our recent report, we speculated that bone marrow-derived abnormal monocytes expressing AREG may migrate via the blood circula-

tion, and bring about disease in synovia and/or other tissues they infiltrated [25]. Although our present findings strongly support that hypothesis, the abnormal cells expressing AREG among RA-PBMCs were not restricted to monocytes. We conclude that they are mononuclear leukocytes and not of a particular lineage.

Herceptin, a specific inhibitor of ErbB2, was reported to suppress the proliferation of RA-FLS, but not OA-FLS, and augmented expression of ErbB2 was considered to be a major contributor to the autonomous proliferation of RA-FLS [16]. In the present study, EGFR and ErbB2 were found to be predominantly expressed in synovial tissues and cultured FLS, with no differences between their expression levels in RA and OA. Furthermore, the expression levels of ADAM10 and ADAM17, which are also important for the functions of EGF-like growth factors, showed no differences between RA and OA. On the other hand, the mRNA and protein levels of AREG were upregulated in RA synovial tissues. Furthermore, recombinant human AREG enhanced the proliferation of FLS in a dose-dependent manner. In our study, differences were detected for EGF-like growth factors between RA and OA synovia, but not for their receptors or sheddases. AREG induces tyrosine phosphorylation of EGFR and transduces a stronger signal when bound to EGFR/ErbB2 heterodimers [15]. It has been reported that synovitis with granulomatous hyperplasia occurs in AREG transgenic mice [35]. These findings suggest that overexpression of AREG may promote the proliferation of synoviocytes in affected joints of RA patients. We investigated whether recombinant AREG induced the expression of PDGF and bFGF, which are well-known growth factors for hyperplastic proliferation of RA-FLS. We found that AREG had no effects on the expression of these factors, suggesting that AREG did not stimulate RA-FLS to proliferate via these growth factors. However, AREG stimulated RA-FLS to express VEGF, an angiogenic factor involved in synovial hyperplasia.

A large number of reports have shown that RA-FLS produce proinflammatory cytokines when stimulated by various stimuli [36,37]. In addition, we previously reported that RA-FLS produce proinflammatory cytokines, such as IL-6, IL-8, GM-CSF, IL-1β and/or TNFα, when co-cultured with monocytes or lymphocytes [32,33]. Our analyses of cytokine production in the present study revealed that AREG enhanced the production of several proinflammatory cytokines (IL-6, IL-8 and GM-CSF) and VEGF in RA-FLS. Since they were suppressed by an EGFR tyrosine kinase inhibitor, the AREG-dependent induction of these cytokines seemed to occur via activation of EGFR/ErbB2. Interestingly, AREG downregulated the expression of ADAM10 and ADAM17 in a dose-dependent manner. These results suggest the presence of negative feedback

regulation of ADAMs via AREG/EGFR signaling. To assess the involvement of AREG in the elevated expression of VEGF in affected joints of RA patients, the correlations between their mRNA levels and protein levels in synovial fluid samples were analyzed, and good correlations were found for both the mRNA levels ($\rho = 0.700$, P = 0.0067) and protein levels ($\rho = 0.772$, P = 0.0075). There have been very few reports about cytokine induction by AREG to date. The present results suggest that the increased level of AREG may be involved in the upregulation of VEGF in RA joints, and demonstrate, for the first time, that AREG stimulates RA-FLS to produce proinflammatory cytokines, including angiogenic factors. IL-8 has been reported to be an angiogenic factor as well as a chemoattractant factor [38,39]. Although several studies have recently shown that AREG plays important roles in hyperplasia or angiogenesis of skin diseases or tumors [40-43], the role of AREG in RA pathology remains unknown. Ma et al. speculated on the involvement of AREG in the angiogenesis of tumors [40]. Although it is known that EGF and TGFα are potent angiogenic mediators [44,45], the proangiogenic activity of AREG has not been directly determined to date. Its induction of angiogenic factors, such as IL-8 and VEGF, strongly suggests that AREG may be involved in the angiogenesis of synovial hyperplasia in affected joints of RA patients.

Myeloid cells expressing abnormal cell surface markers have been observed in RA bone marrow [18,19] and reported to be correlated with the disease severity [20-23]. Consistent with these reports, the present study revealed that several EGF-like growth factors were upregulated in RA bone marrow cells, suggesting that the onset and/or progression of chronic synovitis may be influenced by alterations to the bone marrow in RA patients.

Conclusion

Among the seven EGF-like growth factors, AREG was upregulated in synovial tissues of RA patients. Recombinant human AREG stimulated RA-FLS to proliferate and produce several proinflammatory cytokines, including angiogenic factors. These results suggest that the elevated expression of AREG in synovial tissues may be involved in RA pathology containing synovial hyperplasia. AREG-expressing cells were observed in both the blood and bone marrow of RA patients as well as in RA synovial tissues. Abnormal leukocytes may lead to the upregulated expression of AREG in affected joints of RA patients.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions
SY designed the study, carried out the experiments, analyzed the data and drafted the manuscript. SI, KK and YH

carried out the RNA extractions and cDNA syntheses. RM and KT carried out the quantitative real-time PCR. NS performed the measurements of ³H-TdR incorporation into fibroblast-like synoviocytes. TM, TJ and TO participated in the study design and collection of clinical samples. NY, NF, TI and RS participated in the study design and coordination as well as editing of the manuscript. All authors have read and approved the final manuscript.

References

1. Hamilton JA: **Hypothesis: In vitro evidence for the invasive and tumor-like properties of the rheumatoid pannus.** *J Rheumatol* 1983, **10**:845-851.
2. Imamura F, Aono H, Hasunuma T, Sumida T, Tateishi H, Maruo S, Nishioka K: **Monoclonal expansion of synoviocytes in rheumatoid arthritis.** *Arthritis Rheum* 1998, **41**:1979-1986.
3. Aupperle KR, Boyle DL, Hendrix M, Seftor EA, Zvaifler NJ, Barbosa M, Firestein GS: **Regulation of synoviocyte proliferation, apoptosis, and invasion by the p53 tumor suppressor gene.** *Am J Pathol* 1998, **152**:1091-1098.
4. Pap T, Franz JK, Hummel KM, Jeisy E, Gay R, Gay S: **Activation of synovial fibroblasts in rheumatoid arthritis: lack of expression of the tumour suppressor PTEN at sites of invasive growth and destruction.** *Arthritis Res* 2000, **2**:59-64.
5. Schedel J, Distler O, Woenckhaus M, Gay RE, Simmen B, Michel BA, Muller-Ladner U, Gay S: **Discrepancy between mRNA and protein expression of tumour suppressor maspin in synovial tissue may contribute to synovial hyperplasia in rheumatoid arthritis.** *Ann Rheum Dis* 2004, **63**:1205-1211.
6. Williams WV, VonFeldt JM, Ramanujam T, Weiner DB: **Tyrosine kinase signal transduction in rheumatoid synovitis.** *Semin Arthritis Rheum* 1992, **21**:317-329.
7. Takeuchi T, Abe T: **Tyrosine phosphorylated proteins in synovial cells of rheumatoid arthritis.** *Intl Rev Immunol* 1998, **17**:365-381.
8. Migita K, Eguchi K, Tsukada T, Kawabe Y, Aoyagi T, Nagataki S: **The role of protein kinase in human synovial fibroblast growth.** *Biochem Biophys Res Commun* 1995, **210**:1066-1075.
9. Remmers EF, Lafyatis R, Kumkumian GK, Case JP, Roberts AB, Sporn MB, Wilder RL: **Cytokines and growth regulation of synoviocytes from patients with rheumatoid arthritis and rats with streptococcal cell wall arthritis.** *Growth Factors* 1990, **2**:179-188.
10. Butler DM, Leizer T, Hamilton JA: **Stimulation of human synovial fibroblast DNA synthesis by platelet-derived growth factor and fibroblast growth factor. Differences to the activation by IL-1.** *J Immunol* 1989, **142**:3098-3103.
11. Rubin K, Terracio L, Ronnstrand L, Heldin CH, Klareskog L: **Expression of platelet-derived growth factor receptors is induced on connective tissue cells during chronic synovial inflammation.** *Scand J Immunol* 1988, **27**:285-294.
12. Byrd V, Zhao XM, McKeehan WL, Miller GG, Thomas JW: **Expression and functional expansion of fibroblast growth factor receptor T cells in rheumatoid synovium and peripheral blood of patients with rheumatoid arthritis.** *Arthritis Rheum* 1996, **39**:914-922.
13. Sano H, Engleka K, Mathern P, Hla T, Crofford LJ, Remmers EF, Jelsema CL, Goldmuntz E, Maciag T, Wilder RL: **Coexpression of phosphotyrosine-containing proteins, platelet-derived growth factor-B, and fibroblast growth factor-1 in situ in synovial tissues of patients with rheumatoid arthritis and Lewis rats with adjuvant or streptococcal cell wall arthritis.** *J Clin Invest* 1993, **91**:553-565.
14. Watanabe N, Ando K, Yoshida S, Inuzuka S, Kobayashi M, Matsui N, Okamoto T: **Gene expression profile analysis of rheumatoid synovial fibroblast cultures revealing the overexpression of genes responsible for tumor-like growth of rheumatoid synovium.** *Biochem Biophys Res Commun* 2002, **294**:1121-1129.
15. Riese DJ 2nd, Stern DF: **Specificity within the EGF family/ErbB receptor family signaling network.** *Bioessays* 1998, **20**:41-48.
16. Satoh K, Kikuchi S, Sekimata M, Kabuyama Y, Homma MK, Homma Y: **Involvement of ErbB-2 in rheumatoid synovial cell growth.** *Arthritis Rheum* 2001, **44**:260-265.

17. Hallbeck AL, Walz TM, Briheim K, Wasteson A: **TGF-alpha and ErbB2 production in synovial joint tissue: increased expression in arthritic joints.** *Scand J Rheumatol* 2005, **34**:204-211.
18. Ochi T, Hakomori S, Adachi M, Owaki H, Okamura M, Ono Y, Yamasaki K, Fujimoto M, Wakitani S, Ono K: **The presence of a myeloid cell population showing strong reactivity with monoclonal antibody directed to difucosyl type 2 chain in epiphyseal bone marrow adjacent to joints affected with rheumatoid arthritis (RA) and its absence in the corresponding normal and non-RA bone marrow.** *J Rheumatol* 1988, **15**:1609-1615.
19. Kawanaka N, Yamamura M, Aita T, Morita Y, Okamoto A, Kawashima M, Iwahashi M, Ueno A, Ohmoto Y, Makino H: **CD14+, CD16+ blood monocytes and joint inflammation in rheumatoid arthritis.** *Arthritis Rheum* 2002, **46**:2578-2586.
20. Ohtsu S, Yagi H, Nakamura M, Ishii T, Kayaba S, Soga H, Gotoh T, Rikimaru A, Kokubun S, Itoh T: **Enhanced neutrophilic granulopoiesis in rheumatoid arthritis. Involvement of neutrophils in disease progression.** *J Rheumatol* 2000, **27**:1341-1351.
21. Berthelot JM, Bataille R, Maugars Y, Prost A: **Rheumatoid arthritis as a bone marrow disorder.** *Semin Arthritis Rheum* 1996, **26**:505-514.
22. Tomita T, Shimaoka Y, Kashiwagi N, Hashimoto H, Kawamura S, Lee SB, Nakagawa S, Shiho O, Hayashida K, Ochi T: **Enhanced expression of CD14 antigen on myeloid lineage cells derived from the bone marrow of patients with severe rheumatoid arthritis.** *J Rheumatol* 1997, **24**:465-469.
23. Hirohata S, Yanagida T, Itoh K, Nakamura H, Yoshino S, Tomita T, Ochi T: **Accelerated generation of CD14+ monocyte-lineage cells from the bone marrow of rheumatoid arthritis patients.** *Arthritis Rheum* 2005, **39**:836-843.
24. Hirohata S, Yanagida T, Nagai T, Sawada T, Nakamura H, Yoshino S, Tomita T, Ochi T: **Induction of fibroblast-like cells from CD34+ progenitor cells of the bone marrow in rheumatoid arthritis.** *J Leukocyte Biol* 2001, **70**:413-421.
25. Nakamura N, Shimaoka Y, Tougan T, Onda H, Okuzaki D, Zhao H, Fujimori A, Yabuta N, Nagamori I, Tanigawa A, Sato J, Oda T, Hayashida K, Suzuki R, Yukioka M, Nojima H, Ochi T: **Isolation and expression profiling of genes upregulated in bone marrow-derived mononuclear cells of rheumatoid arthritis patients.** *DNA Res* 2006, **13**:169-183.
26. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, Medsger TA Jr, Mitchell DM, Neustadt DH, Pinals RS, Schaller JG, Sharp JT, Wilder RL, Hunder GG: **The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.** *Arthritis Rheum* 1988, **31**:315-324.
27. Altman RD, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, Christy W, Cooke TD, Greenwald R, Hochberg M, Howell D, Kaplan D, Koopman W, Longley S III, Mankin H, McShane DJ, Medsger T Jr, Meenan R, Mikkelsen W, Moskowitz R, Murphy W, Rothschild B, Segal M, Sokoloff L, Wolfe F: **Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Development of criteria for the classification and reporting of osteoarthritis: classification of osteoarthritis of the knee.** *Arthritis Rheum* 1986, **29**:1039-1049.
28. Mograbi B, Rochet N, Imvert V, Bourget I, Bocciardi R, Emiliozzi C, Rossi B: **Human monocytes express amphiregulin and heregulin growth factors upon activation.** *Eur Cytokine Netw* 1997, **8**:73-81.
29. Johansson CC, Yndestad A, Enserink JM, Ree AH, Aukrust P, Tasken K: **The epidermal growth factor-like growth factor amphiregulin is strongly induced by the adenosine 3',5'-monophosphate pathway in various cell types.** *Endocrinology* 2004, **145**:5177-5184.
30. Sahin U, Weskamp G, Kelly K, Zhou HM, Higashiyama S, Peschon J, Hartmann D, Saftig P, Blobel CP: **Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands.** *J Cell Biol* 2004, **164**:769-779.
31. Horiuchi K, Le Gall S, Schulte M, Yamaguchi T, Reiss K, Murphy G, Toyama Y, Hartmann D, Saftiq P, Blobel CP: **Substrate selectivity of epidermal growth factor-receptor ligand sheddases and their regulation by phorbol esters and calcium influx.** *Mol Biol Cell* 2007, **18**:176-188.
32. Tanaka K, Mori T, Juji T, Suzuki S, Watanabe J, Goto A, Shiobara N, Yamane S, Fukui N, Suzuki R, Ochi T: **Production of interleukin-6 and interleukin-8 by nurse-like cells from rheumatoid arthri-**

tis patients after stimulation with monocytes. *Mod Rheumatol* 2005, 15:415-422.

33. Takeuchi E, Tomita T, Toyosaki-Maeda T, Kaneko M, Takano H, Hashimoto H, Sugamoto K, Suzuki R, Ochi T: **Establishment and characterization of nurse cell-like stromal cell lines from synovial tissues of patients with rheumatoid arthritis.** *Arthritis Rheum* 1999, 42:221-228.

34. Ikeda M, Hosoda Y, Hirose S, Okada Y, Ikeda E: **Expression of vascular endothelial growth factor isoforms and their receptors Flt-1, KDR, and neuropilin-1 in synovial tissues of rheumatoid arthritis.** *J Pathol* 2000, 191:426-433.

35. Cook PW, Brown JR, Cornell KA, Pittelkow MR: **Suprabasal expression of human amphiregulin in the epidermis of transgenic mice induces a severe, early-onset, psoriasis-like skin pathology: expression of amphiregulin in the basal epidermis is also associated with synovitis.** *Exp Dermatol* 2004, 13:347-356.

36. Jeong JG, Kim JM, Cho H, Hahn W, Yu SS, Kim S: **Effects of IL-1 beta on gene expression in human rheumatoid synovial fibroblasts.** *Biochem Biophys Res Commun* 2004, 324:3-7.

37. Ritchlin C: **Fibroblast biology: effector signals released by the synovial fibroblast in arthritis.** *Arthritis Res* 2000, 2:356-360.

38. Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM: **Interleukin-8 as a macrophage-derived mediator of angiogenesis.** *Science* 1992, 258:1798-1801.

39. Koch AE, Volin MV, Woods JM, Kunkel SL, Connors MA, Harlow LA, Woodruff DC, Burdick MD, Strieter RM: **Regulation of angiogenesis by the C-X-C chemokines interleukin-8 and epithelial neutrophil activating peptide 78 in the rheumatoid joint.** *Arthritis Rheum* 2001, 44:31-40.

40. Ma L, Gauville C, Berthois Y, Millot G, Johnson GR, Calvo F: **Antisense expression for amphiregulin suppress tumorigenicity of a transformed human breast epithelial cell line.** *Oncogene* 1999, 18:6513-6520.

41. Desruisseau S, Palmari J, Giusti C, Romain S, Martin PM, Berthois Y: **Clinical relevance of amphiregulin and VEGF in primary breast cancers.** *Int J Cancer* 2004, 111:733-740.

42. Riedel F, Gotte K, Li M, Hormann K, Grandis JR: **RGFR antisense treatment of human HNSCC cell lines down-regulates VEGF expression and endothelial cell migration.** *Int J Oncol* 2002, 21:11-16.

43. Bhagavathula N, Nerusu KC, Fisher GJ, Liu G, Thakur AB, Gemmell L, Kumar S, Xu ZH, Hinton P, Tsurushita N, Landolfi NF, Voorhees JJ, Warani J: **Amphiregulin and epidermal hyperplasia – amphiregulin is required to maintain the psoriatic phenotype of human skin grafts on severe combined immunodeficient mice.** *Am J Pathol* 2005, 166:1009-1016.

44. Schreiber AB, Winkler ME, Derynck R: **Transforming growth factor-alpha: a more potent angiogenic mediator than epidermal growth factor.** *Science* 1986, 232:1250-1253.

45. Detmar M, Brown LF, Claffey KP, Yeo KT, Kocher O, Jackman RW, Berse B, Dvorak HF, Van de Water L: **Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis.** *J Exp Med* 1994, 180:1141-1146.

Arthritis and pneumonitis produced by the same T cell clones from mice with spontaneous autoimmune arthritis

Chiaki Wakasa-Morimoto[1], Tomoko Toyosaki-Maeda[1], Takaji Matsutani[2], Ryu Yoshida[1], Shino Nakamura-Kikuoka[1], Miki Maeda-Tanimura[1], Hiroyuki Yoshitomi[3], Keiji Hirota[3], Motomu Hashimoto[3], Hideyuki Masaki[4], Yoshiki Fujii[5], Tsuneaki Sakata[1], Yuji Tsuruta[1], Ryuji Suzuki[6], Noriko Sakaguchi[3] and Shimon Sakaguchi[3]

[1]Discovery Research Laboratories, Shionogi & Co., Ltd, 2-5-1 Mishima Settsu-shi, Osaka 566-0022, Japan
[2]Department of Cell Biology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Sendai 980-8575, Japan
[3]Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, 53 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
[4]Department of Biochemistry, Kinki University School of Medicine, 377-2 Ohno-higashi, Osakasayama-shi, Osaka 589-8511, Japan
[5]Department of Virology 1, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[6]Clinical Research Center for Rheumatology and Allergy National Sagamihara Hospital, 18-1 Sakuradai, Sagamihara-shi, Kanagawa 228-8522, Japan

Abstract

SKG mice, a newly established model of rheumatoid arthritis (RA), spontaneously develop autoimmune arthritis accompanying extra-articular manifestations, such as interstitial pneumonitis. To examine possible roles of T cells for mediating this systemic autoimmunity, we generated T cell clones from arthritic joints of SKG mice. Two distinct CD8^{+} clones were established and both showed in vitro autoreactivity by killing syngeneic synovial cells and a variety of MHC-matched cell lines. Transfer of each clone to histocompatible athymic nude mice elicited joint swelling and histologically evident synovitis accompanying the destruction of adjacent cartilage and bone. Notably, the transfer also produced diffuse severe interstitial pneumonitis. Clone-specific TCR gene messages in the inflamed joints and lungs of the recipients gradually diminished, becoming hardly detectable in 6–11 months; yet, arthritis and pneumonitis continued to progress. Thus, the same CD8^{+} T cell clones from arthritic lesions of SKG mice can elicit both synovitis and pneumonitis, which chronically progress and apparently become less T cell dependent in a later phase. The results provide clues to our understanding of how self-reactive T cells cause both articular and extra-articular lesions in RA as a systemic autoimmune disease.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology that primarily affects the synovial membranes of multiple joints (1, 2). A cardinal feature of joint inflammation in RA is proliferative inflammation of the synovium, i.e. synovitis, which leads to the destruction of adjacent cartilage and bone. In addition, RA frequently accompanies extra-articular manifestations, for example the development of rheumatoid factors, rheumatic nodules, vasculitis and interstitial lung disease (ILD). Recent studies with high-resolution imaging have indeed revealed a high prevalence of ILD in patients with RA (3–6). RA is thus a systemic disease; yet, the immunological basis of this systemic autoimmunity is poorly understood.

T cells appear to play a key role in the development of RA as suggested by the infiltration of T cells, especially CD4^{+} T cells, into the synovial tissue of RA (7–9) and the association of genetic susceptibility to RA with particular alleles of HLA-DR (10, 11). On the other hand, there is evidence in humans and animal models that stimulated synoviocytes, composed of macrophage-like and fibroblast-like synovial cells, can

themselves mediate joint destruction in a T cell-independent manner (12, 13). A key issue in elucidating the pathogenetic mechanism of RA is, therefore, to determine how self-reactive T cells contribute to the initiation and progression of synovitis and possibly extra-articular lesions such as ILD.

The SKG strain of mice spontaneously develops T cell-mediated chronic autoimmune arthritis (14–16). The strain possesses a mutation in the gene encoding a Src homology 2 domain of the ζ-associated protein of 70 kDa (ZAP-70), a key signal transduction molecule in T cells (17, 18). Impaired signal transduction through SKG ZAP-70 results in thymic positive selection and failure in negative selection of highly self-reactive T cells that include potentially arthritogenic T cells (14). The SKG arthritis progresses chronically, starting from small joints of the digits and symmetrically progressing to larger joints, such as the wrists and ankles. Histologically, affected joints show hyperplasia of synoviocytes, inflammatory cell infiltration, pannus formation and destruction of cartilage and bone, eventually leading to joint deformity. As extra-articular lesions, they develop interstitial pneumonitis, dermatitis, necrobiotic nodules akin to rheumatic nodules in RA and systemic vasculitides. Serologically, they spontaneously develop IgM-type rheumatoid factors, auto-antibodies against type II collagen and antibodies cross-reactive with *Mycobacterium tuberculosis* heat shock protein (hsp) 70. IL-1, tumor necrosis factor (TNF)-α, IL-6 or IL-17 deficiency inhibits the development of arthritis in SKG mice (15, 19), similar to the effects of anti-cytokine therapies in RA (20, 21). Thus, autoimmune disease in SKG mice closely resembles RA in clinical and immunopathological characteristics. In addition, considering recent findings that genetic polymorphism of a signaling molecule at a TCR proximal step involving ZAP-70 significantly contributes to the susceptibility to RA and other autoimmune diseases (22, 23), SKG mice can be a suitable model for elucidating how a T cell-intrinsic anomaly contributes to the development of RA as a systemic autoimmune disease.

In this study, we have attempted to determine the role of T cells in SKG autoimmune disease by establishing T cell clones from their arthritic lesions. We have established two distinct CD8$^+$ clones and show that both of them have the potential to induce not only arthritis but also pneumonitis. This indicates that inflammation in both the joints and the lung can be mediated, at least in part, by common autoreactive T cell clones in SKG mice. In addition, by adoptively transferring these T cell clones to normal mice, we show that autoreactive T cells are able to initiate arthritis; yet, the arthritis can progress apparently in a T cell-independent manner in a later phase. These findings contribute to our understanding of how T cells cause chronic arthritis and ILD in RA.

Materials and methods

Mice

SKG and (SKG $\times$ BALB/c)F$_1$ mice (14) were maintained in the animal facility of Kyoto University under a microbially conventional condition. Female C.B-17 SCID mice (Clea Japan, Tokyo, Japan), DBA/1J, BALB/c and BALB/c-nu/nu mice (Charles River Japan, Kanagawa, Japan) were maintained under specific pathogen-free conditions at Kyoto University or Discovery Research Laboratories of Shionogi & Co., Ltd. All experiments were approved by the Animal Care and Use Committee at Kyoto University and Shionogi & Co., Ltd.

Culture medium

The culture medium for SKG T cell lines and clones was AIM-V supplemented with 20% RPMI-1640, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol (ME), 2 mM L-glutamine, ×1 penicillin/streptomysin (Gibco BRL, Gaithersburg, MD, USA), 10% heat-inactivated FCS (Hyclone, Logan, UT, USA), 10% rat T-STIM^{TM} with Con A (Becton Dickinson, Franklin Lakes, NJ, USA), 100 U/ml of recombinant mouse IL-2 (Genzyme, Cambridge, MA) and 5 µg/ml of Con A (Sigma, St Louis, MO, USA).

Establishment of T cell clones from arthritic joints of SKG mice

To establish T cell lines, severely swollen joints of SKG mice were aseptically excised, finely minced and cultured until clusters of mononuclear cells were confirmed in bulk culture. Outgrown T cells were cloned in 96-well microplates by using SKG synovial cells (1 × 10^3) as feeder cells. Synovial cells were prepared as previously described (16). Briefly, synovial tissues from wrist and ankle joints were digested with 400 Mandl U/ml of Liberase Brendzyme II (Roche) in RPMI-1640 medium for 1 h at 37°C; digested cells were filtrated through a nylon mesh to prepare single-cell suspensions. A typical composition of the synoviocyte preparation was ~10% CD11b$^+$ monocyte/macrophages, ~20% Gr-1$^+$ granulocytes, ~1% T cells and other cells. Several days later non-adherent cells were removed by washing the plates with culture medium. T cells that had outgrown from the bulk culture of synovial cells were dispensed at 1, 5, 20 or 50 cells per well and apparently single colonies were propagated in the culture medium described above. Clonality of each cell was confirmed by microplate hybridization assay (MHA) (24) and sequence analysis of TCR. Established T cell clones were maintained without feeder cells. Dengue 2F7 and 3F2 T cell clones, established by immunization of BALB/c mice with the NS3 peptide of dengue virus, were kindly provided by Dr H. Masaki (Kinki University). All cultures were performed in a humidified atmosphere of 7.5% CO$_2$ at 37°C.

Cytokine detection

Cytokine production by T cell clones were analyzed by ELISA. T cell clones were stimulated with 10 ng/ml of phorbol myristate acetate (PMA) (Wako Chemicals USA, Inc., Richmond, VA, USA) and 0.4 µg/ml of ionomycin (Calbiochem, Darmstadt, Germany) in culture medium at 1 × 10^6 cells/ml for 16 h. The supernatants were assayed for various cytokines using specific ELISA kits (Endogen, Woburn, MA, USA, and Axis-Shield, Oslo, Norway) according to the manufacturer's protocol. Cytokine mRNA levels in the joints and lungs of clone recipient mice were analyzed by quantitative PCR as described previously (25).

MHA for TCR AV and BV family and sequence analysis

MHA, cDNA synthesis and PCR amplifications of TCR of each T cell clone were performed as described previously (24). The

PCR products cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) were analyzed for TCR sequences using CEQ DTCS-Quick Start Kit according to the manufacturer's protocol (Beckman Coulter Inc., Fullerton, CA, USA).

^{51}Cr release cytotoxicity assay

BALB/3T3 fibroblast line (H-2^d), J774 macrophage line (H-2^d), p815 mastocytoma line (H-2^d), EL-4 lymphoma line (H-2^b), L929 fibroblast line (H-2^k) obtained from Dainippon Sumitomo Pharma (Osaka, Japan) and synovial cells of SKG mice (H-2^d) were used as target cells. Synovial cells (1×10^4) were seeded in 96-well flat-bottom plates with 40 U/well of IFN-γ for 2 days and radiolabeled with 2.5 μCi/well of Na$_2^{51}$CrO$_4$ (Daiichi Radioisotope Laboratories, Ltd, Tokyo, Japan) for 2 h. Other target cells (3×10^5) were radiolabeled with 20 μCi of Na$_2^{51}$CrO$_4$ for 2 h and seeded in 96-well round-bottom plates at 1×10^4 cells per well. Effector cells (4×10^5) were added in each well in triplicate and incubated for 8 h. Relative cytotoxicity was calculated as follows from the radioactivity released in the culture supernatant; percent specific lysis = 100(experimental − spontaneous)/(maximal − spontaneous) counts per minute. Maximal lysis and spontaneous release were determined from target cells incubated with surfactant ×7 (Flow Laboratories, ICN Biomedicals, Inc., Aurora, OH, USA) or without effector cells, respectively.

Adoptive transfer

Spleen T cells from SKG mice or (SKG × BALB/c)F$_1$ mice and each SKG T cell clones (1×10^7) were intravenously transferred to C.B-17 SCID mice (8 weeks) or BALB/c-nu/nu mice (6 weeks), respectively. Control dengue 2F7 and 3F2 clone were collected 10–14 days after *in vitro* stimulation with specific peptide-pulsed irradiated (33 Gray) BALB/c spleen cells and transferred as described above. Severity of arthritis was scored weekly as previously described (14).

Clinical assessment of arthritis

Joint swelling was monitored by inspection and scored as follows: 0, no joint swelling; 0.1, swelling of one finger joint; 0.5, mild swelling of wrist or ankle and 1.0, severe swelling of wrist or ankle. Scores for all fingers and toes, wrists and ankles were totalled for each mouse (14).

Histological assessment of interstitial pneumonitis

Interstitial pneumonitis was evaluated microscopically depending on diffusely affected area: −, normal histology; +, 10–30%; ++, 30–60%; +++, >60% of the sections of the lungs showed pneumonitis.

Histology and immunohistochemistry

Tissues were fixed in 10% neutral formalin, paraffin embedded and stained with Haematoxylin & Eosin (H&E). Joints were additionally decalcified for 3 weeks in 10% EDTA in PBS before staining. For immunohistochemistry of joints, deparaffinized sections were incubated with 20% normal rabbit serum (Dako, Hamburg, Germany) in PBS for 15 min to block non-specific binding, primary rat anti-Ly-6G mAb (Gr-1, RB6-8C5; BD PharMingen) with appropriate dilutions overnight at 4°C,

biotinylated polyclonal rabbit anti-rat antibody (Dako) and HRP-conjugated streptavidin (Dako). The slides were developed using diaminobenzidene (Elite Kit; Vector, Burlingame, CA, USA) and counterstained with Mayer's hematoxylin.

For immunohistochemistry of lungs, tissues were fixed in 4% phosphate-buffered PFA (pH 7.4) and embedded in Tissue-Tek OCT compound (Ted Pella, Inc., Redding, CA, USA). Cryostat sections were stained with rat mAbs to mouse CD4 (H129.19), CD8a (53-6.7), CD45R/B220 (RA3-6B2), Ly-6G (RB6-8C5) (BD PharMingen) and F4/80 (CI: A3-1) (CALTAG Laboratories, Burlingame, CA, USA) with appropriate dilutions followed by incubation with biotinylated secondary antibodies and HRP-conjugated streptavidin. The slides were developed as described above.

Southern blot analysis

The persistence of transferred clones in the recipients was assessed by Southern blot analysis. Two micrograms of total RNA of each tissue was treated with DNaseI and reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA, USA). Nested PCRs were performed as described previously (24) to amplify TCR β chain of 35S or dengue 2F7 with the primers specific for V, J and C region. Ten microliters of the PCR products were separated on 2% agarose gel, transferred onto Hybond-N+ membranes (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. The membranes were prehybridized overnight with PerfectHyb (TOYOBO CO., Ltd, Osaka, Japan) at 54°C and hybridized with the third complementarity-determining region (CDR3)-specific probes labeled with ^{32}P-deoxyadenosine triphosphate for 3 h at 54°C. The membranes were washed in ×2 standard saline citrate (SSC) and 0.1% SDS at room temperature and ×0.2 SSC and 0.1% SDS at 37°C. RNA extracts of 35S and dengue 2F7 clones, diluted to 1% of concentration with RNA of L9 cells, were used as positive controls. The detection limits of 35S and dengue 2F7 were compared using the serial dilution of positive controls and both systems detected the RNA extract corresponding to the amount of one cell.

The sequences of PCR primers and probes are as follows; 35S: first PCR (BV8S3-1: 5′-ATA TGG TGC TGG CAA CCT TC-3′ and MCB1: 5′-AGG ATT GTG CCA GAA GGT AG-3′), second PCR (BV8S3-2: 5′-ACC AGA ACA ACG CAA GAA GAC T-3′ and MCB2: 5′-TTG TAG GCC TGA GGG TCC-3′), third PCR (BV8S3-3: 5′-TTC CTC CTG CTG GAA TTG GC-3′ and BJ1.5: 5′-TAG AAC AGA GAT CGA GTC CC-3′) and probe (5′-AGT GGG ACA GGG GGC AAC CA-3′). Dengue 2F7: first PCR (BV8S1-1: 5′-CCC AAA GTC CAA GAA GCA AG-3′ and MCB1), second PCR (BV8S1-2: 5′-GTA CAA GGC CTC CAG ACC AA-3′ and MCB2), third PCR (BV8S1-3: 5′-TGG CTT CCC TTT CTC AGA CA-3′ and BJ2.7: 5′-AAG GAG ACC TTG GGT GGA GT-3′) and probe (5′-TGC CAC CAA CGA CAA CTC CT-3′).

Results

Induction of arthritis and interstitial pneumonitis in SCID mice by the transfer of SKG splenic T cells

In our conventional housing environment, SKG mice started to develop arthritis around 2 months of age and

histologically evident mild interstitial pneumonitis around 6 months of age (14). To determine the role of T cells in SKG mouse autoimmunity, we transferred splenic T cells from 3-month-old arthritic SKG mice (without histologically evident pneumonitis or colitis) to T/B-cell-deficient C.B-17 SCID mice, which are histocompatible with SKG mice on the BALB/c background (14). Within 2 months after transfer, the recipient developed arthritis (14) and mild but histologically evident interstitial pneumonitis (Table 1, Fig. 1); they also developed mild colitis (data not shown). Similar cell transfer from non-arthritic heterozygotes of the SKG mutation failed to induce such lesions in the recipients. Age-matched SCID mice similarly maintained in our facility did not develop these lesions histologically (data not shown). The results thus indicate that SKG T cells are able to adoptively transfer arthritis and also have a potential to induce interstitial pneumonitis and colitis when transferred to SCID mice.

Establishment of T cell clones from arthritic joints

To analyze the mechanism of such T cell-mediated inflammatory tissue damage in multiple organs, we attempted to establish T cell clones from arthritic joints of SKG mice, as described in Materials and methods. Two T cell clones, designated 35S and 73S, were established in separate experiments. The clones were maintained and expanded with culture medium containing IL-2 and Con A (see Materials and methods). CD8+ CTL clones specific for dengue virus NS3 protein were used as control.

Cytofluorometric analyses revealed that the 35S and 73S clones were CD8+. Both expressed α and β chains of the TCR, and the expression level of the TCR on 35S was slightly lower than normal (Fig. 2). In response to in vitro PMA and ionomycin stimulation, 35S and 73S produced IFN-γ but no detectable amount of TNF-α, IL-4, IL-5, IL-6, IL-10 or IL-17 by ELISA (Table 2).

Clonality of each T cell line was confirmed by MHA (24) (data not shown) and sequence analysis of the TCR α and β chains with determination of the amino acid sequences of the TCRs (Table 3). Interestingly, these T cell clones shared in common the BV8S3 TCR Vβ subfamily; yet, the CDR3 sequences of the TCR β chains were different (26–29).

Autoreactivity of T cell clones

In ^{51}Cr release cytotoxicity assay to determine cytotoxic activity of the SKG clones against syngeneic synovial cells, 35S and 73S lysed SKG synovial cells prepared by crude collagenase digestion of inflamed synovium (44.0 and 16.3% of specific lysis, respectively, at a high 40:1 ratio), while control dengue 2F7 clone did not (Fig. 3A). 35S lysed not only syngeneic synovial cells but also MHC-matched cell lines, such as BALB/c-derived 3T3 cells, macrophage-like J774 cells and DBA/2 (H-2^d)-derived P815 cells, whereas the clone failed to lyse allogenic EL-4 (H-2^b) lymphoid or L929 (H-2^k) fibroblast cell line (Fig. 3B). Thus, 35S appears to recognize a ubiquitous self-peptide in an MHC-restricted manner. These
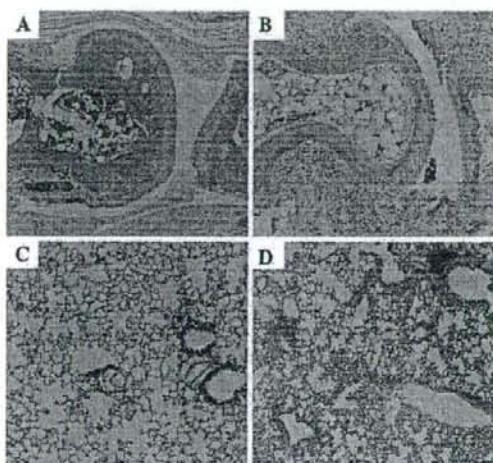
Fig. 1. Arthritis and pneumonitis in SCID mice transferred with T cells from SKG mice. Histology of a joint (A) and lung (C) of a SCID mouse T cell transferred from (SKG × BALB/c)F$_1$ mouse. Arthritis (B) and interstitial pneumonitis (D) in a SCID mouse T cell transferred from a SKG mouse. H&E staining (A and B, ×100; C and D, ×50).

Table 1. Induction of arthritis, interstitial pneumonitis and colitis in SCID mice by the transfer of SKG splenic T cells

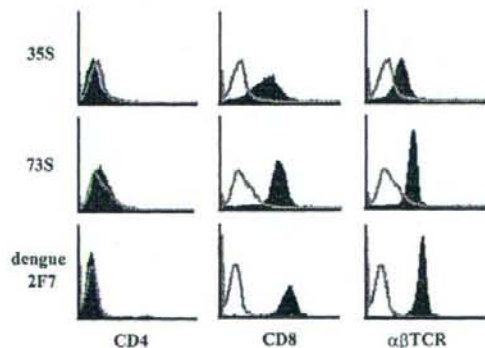| Spleen cell donor	Recipients	Arthritis	Interstitial pneumonitis	Colitis
SKG	1	++ (4.6)	++	+
	2	++ (4.0)	++	+
	3	++ (4.0)	+	+
	4	++ (3.0)	+	–
(SKG × BALB/c)F$_1$	1	–	–	–
	2	–	–	–
	3	–	–	–
	4	–	–	–

Cells (1 × 10^7) of T cells prepared from spleens of indicated mice were intravenously transferred to 8-week-old SCID mice. The severity of arthritis, interstital pneumonitis and colitis in these mice was histologically assessed 2 months later.

Fig. 2. Expression levels of CD4, CD8 and $\alpha\beta$ TCR on 35S, 73S and dengue 2F7 clones.

functional characteristics, together with cell surface and cyto-kine-secreting profiles, indicate that 35S and 73S are CTL and that they bear self-reactive specificity.

Induction of synovitis in BALB/c nude mice by adoptive transfer of T cell clones

To examine possible arthritogenicity of the T cell clones, they were transferred to BALB/c nude mice once, and the degree of joint swelling of each recipient mouse was assessed once a week for 12 months (Fig. 4). Transfer of 35S and 73S

Table 2. Cytokine production (ng/ml) of T cell clones derived from SKG joints and control clones

	TNF-α	IFN-γ	IL-4	IL-5	IL-10	IL-6	IL-17
35S	0.02	180	0.03	<0.02	<0.04	0.2	<0.01
73S	0.02	80	0.03	<0.02	1.2	<0.05	<0.01
Dengue 2F7	0.2	10	ND	ND	<0.04	<0.05	<0.01
Dengue 3F2	0.02	20	ND	ND	<0.04	<0.05	<0.01

Culture supernatant of activated cells by PMA and ionomycin for 16 h were assayed by ELISA. ND, not done.

Table 3. CDR3 sequences of the TCR α and β chain used by the SKG T cell clones

TCR α chains

	AV	V	N	J	AJ
35S	3S6	C A V T	S D	S G T Y Q R F	13
73S	3S1	C A A S M	R R	N S G T Y Q R F	13
Dengue 2F7	2S2/7	C A A		N Q G G R A L I F	15
Dengue 3F2	2S2/7	C A A	S G R D	Y A N K M I F	47

TCR β chains

	BV	V	N-D-N	J	BJ
35S	8S3	C A S S G	T G G	N Q A P L F	1.5
73S	8S3	C A S S G	W G D	A E Q F F	2.1
Dengue 2F7	8S1	C A T	N D N	S Y E Q Y E	2.7
Dengue 3F2	8S2	C A S E	T R	E Q Y F	2.6

The amino acid sequences of the V, D and J regions of the TCR were determined according to the nucleotide sequences. AV and BV gene families were assigned according to Arden *et al.* (26). AJ genes were numbered according to Koop *et al.* (27). BJ genes were assigned according to Malissen *et al.* (28) and Gascoigne *et al.* (29).

clones induced joint swelling with incidences of 57.1% (4 out of 7 mice) and 42.9% (3 out of 7 mice), respectively, during the observation period; synovitis was histologically evident in 71.4% (5 out of 7 mice) in each transfer (Table 4, Fig. 5). Once joint swelling started in one joint following cell transfer, it slowly progressed with remissions and exacerbations, leading to swelling of other joints in a symmetrical fashion (Figs 4 and 5A–D). Two mice showed progressive debilitation to death without an apparent cause, although one of them showed dermatitis; with debilitation, joint swelling somehow remitted in these mice.

Histologically, swollen joints showed marked synovial and peri-articular inflammation when examined 6–12 months after cell transfer (Fig. 5E). The inflammation accompanied a marked proliferation of synovial lining cells, infiltration of inflammatory cells into subsynovial tissue and joint cavity and active angiogenesis; pannus eroded the adjacent cartilage and bone (Fig. 5F). Gr-1-positive neutrophils were abundant among the infiltrating cells, as observed in the arthritic lesions of SKG mice (14, 15), whereas few T cells infiltrated into the inflammation sites (Fig. 5G and H).

In accordance with the appearance of multinuclear cells at the interface between proliferating synoviocytes and bone, many tartrate-resistant acid phosphatase-positive osteoclasts were observed in the inflamed joints (Fig. 6A–D). Safranin-O staining revealed a decrease in proteoglycan in the articular cartilage matrix of severely affected joints (Fig. 6E and F). Notably, Gr-1-positive cells, mainly neutrophils, also increased in the bone marrow (BM) of the affected recipients (Fig. 6G and H).

A high level of circulating rheumatoid factors was detected in one mouse out of seven recipients of the 35S clone and in none of the recipients of other clones (data not shown).

Some of the swollen joints following transfer of 35S CD8+ clones exhibited higher expression levels of IL-17 mRNA assessed by quantitative reverse transcription (RT)–PCR than those from mice transferred with control CD8+ clones (Supplementary Figure 1A, available at *International Immunology* Online), despite that 35S failed to produce IL-17 upon *in vitro* stimulation.

Taken together, the CD8+ T cell clones prepared from arthritic lesions of SKG mice were able to induce arthritis in athymic nude recipients, leading to the destruction of the surrounding cartilage and the bone.

Fig. 3. *In vitro* self-reactivity of SKG T cell clones. (A) CTL activity of SKG T cell clones against SKG synovial cells. CTL clones specific for dengue virus NS3 protein, dengue 2F7, was used as control. IFN-γ-treated target cells were ^{51}Cr labeled in adherent condition and incubated with effector cells for 8 h (E:T ratio = 40). (B) CTL activity of SKG T cell clones against various types of cell lines (E:T ratio = 40). CTL activity of dengue 2F7 clone was also analyzed against H-2^d cells pulsed with a specific peptide (E:T ratio = 10). All assays were conducted in triplicate with 8 h of incubation. The mean and standard deviation of three independent experiments are shown in each bar.

Induction of interstitial pneumonitis in BALB/c nude mice by the transfer of T cell clones

Notably, histologically evident severe alveolitis and diffuse interstitial pneumonitis also developed in all the recipients of 35S and 73S but not in those recipients of dengue 2F7 and 3F2 clones (Table 4 and Fig. 7A–D). Some recipients of 35S and 73S developed only pneumonitis without histologically evident synovitis. No histologically apparent inflammation was observed in other tissues/organs including the liver and the colon in any of these recipient mice (data not shown). The diffuse pulmonary lesions (Fig. 7A and B) comprised thickening of the alveolar walls, and perivascular and peribronchiolar infiltration by inflammatory cells (Fig. 7C and D). Immunohistochemical analysis of the 73S recipients 6 months after cell transfer revealed the infiltration of a large number of granulocytes as Gr-1^{+} cells (Fig. 7E), macrophages as F4/80^{+} cells
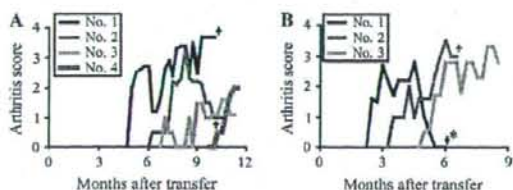
(Fig. 7F) and B cells as B220^{+} cells (Fig. 7G) into the alveolar walls and spaces and also the perivascular and peribronchiolar area where only a small number of CD8^{+} T cells were detected, which might be transferred to CD8^{+} clones or derived from nude mice (30) (Fig. 7H). CD4^{+} T cells were occasionally found in the lesions and could be those derived from endogenous T cells that might develop extrathymically in aged nude mice (Fig. 7I) (30).

The pulmonary tissues with severe interstitial pneumonitis following CD8^{+} clone transfer exhibited higher expression levels of IL-17 mRNA by quantitative RT–PCR compared with the mice transferred with control CD8^{+} clones (Supplementary Figure 1B, available at *International Immunology* Online).

Thus, the SKG arthritogenic T cell clones are able to induce interstitial pneumonitis when transferred to athymic nude mice.

Detection of transferred clones in recipient mice

Since T cells were hardly detected by immunohistochemistry at the site of synovitis or pneumonitis 6 months after clone transfer (data not shown and see above), the persistence of transferred clones in the recipients was assessed by RT–PCR amplification of TCR β chain gene and Southern blot analysis of the products with a CDR3 sequence-specific probe. We adopted this method to avoid detecting nude mouse-derived oligoclonal endogenous T cells that may expand with aging (see above) (30–32). For example, a clone-specific TCR message of the 35S clone was detected in the majority of recipient spleens 1 month after transfer but not in the spleens examined 6 months later (Fig. 8). As shown in Fig. 9, the messages were

Fig. 4. Time course of joint swelling in the recipient mice of SKG T cell clones, 35S (A) and 73S (B). Score for all paws were totalized for each mouse. +, Sacrificed at the indicated time points; *, the mouse developed dermatitis at 5 months after transfer.

Table 4. Development of arthritis and interstitial pneumonitis in BALB/c nude mice transferred with T cell clones

Clone	Individual recipients	Macroscopically evident arthritis			Histological analysis	
		Onset (months)	Sacrifice (months)	Clinical score[a]	Synovitis[b]	Interstitial pneumonitis[c]
35S	1	5	10	3.7	++	+
	2	6	11	3.2	++	++
	3	7	11	1.6	++	+++
	4	10	12	2.0	++	+++
	5	–	9	0	+	±
	6	–	12	0	–	+++
	7	–	12	0	–	+++
73S	1	2.5	6	2.8	++	++
	2	3.5	6	3.5	++	+
	3	5	9	3.3	++	++
	4	–	8	0	+	++
	5	–	9	0	+	+++
	6	–	12	0	–	++
	7	–	12	0	–	++
Dengue 2F7	1	–	9	0	–	–
	2	–	9	0	–	–
	3	–	9	0	–	–
	4	–	9	0	–	–
	5	–	12	0	–	–
	6	–	12	0	–	–
	7	–	12	0	–	–
Dengue 3F2	1	–	12	0	–	–
	2	–	12	0	–	–

Six-week-old BALB/c nude mice were intravenously injected with 1×10^7 cells of each clone. The incidence of joint swelling of the recipient mice was examined weekly. Mice were sacrificed 6–12 months after cell transfer.
[a]Maximum clinical score of arthritis.
[b]–, Without change; +, microscopically observed synovitis without joint swelling; ++, macroscopically obvious joint swelling.
[c]–, Normal histology; +, 10–30%; ++, 30–60%; +++, >60% of the sections of the lungs showed pneumonitis (Fig. 7).
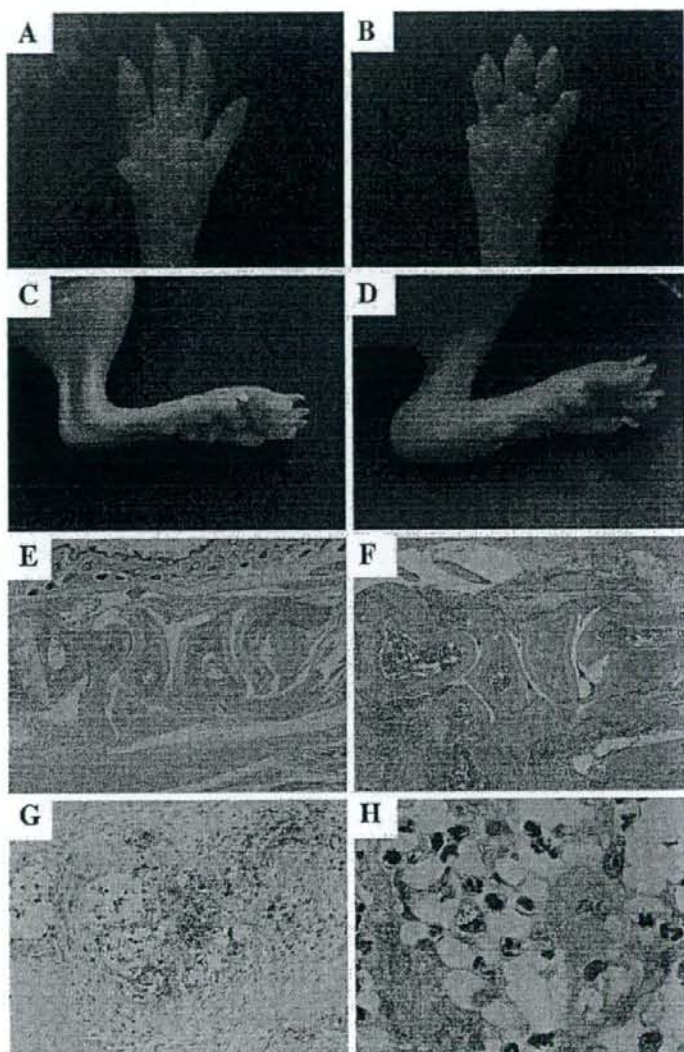
Fig. 5. Arthritis in athymic nude mice transferred with SKG T cell clones. (A–D) Macroscopic views of a forepaw (A) and a hind paw (C) of a recipient of control dengue 2F7 and a forepaw (B) and a hindpaw (D) of a recipient of 35S. (E–H) Histology of the joints of recipients of control dengue 2F7 (E) or 35S (F). Proliferation of the synovial lining cells, erosive destruction of cartilage and bone and infiltration of inflammatory cells is noted in a joint of a 35S recipient (F) (H&E staining, ×40). (G) Gr-1-positive cells were abundant among the infiltrating cells in a joint of 35S recipient mouse (×200). High-magnification view (×1000) of the synovial lesion in 35S transferred mouse, showing that most of the infiltrating cells are granulocytes or monocytes (H) (H&E staining). (A, C and E) 12 months after transfer. (B, D and F–H) 10 months after transfer.

detected in every tested tissue with high frequency for the first 3 months after cell transfer; the detection rate became lower with time; clone-specific TCR signals were not detected in most tissues examined at 6–11 months after transfer, irrespective of the swelling of the joints and the presence of interstitial pneumonitis by histological examination. These findings collectively indicate that the T cell clones initiate arthritis but the progression and persistence of the disease may not require the expansion of the clones even if a small number of them might persist in the joints and the lung.
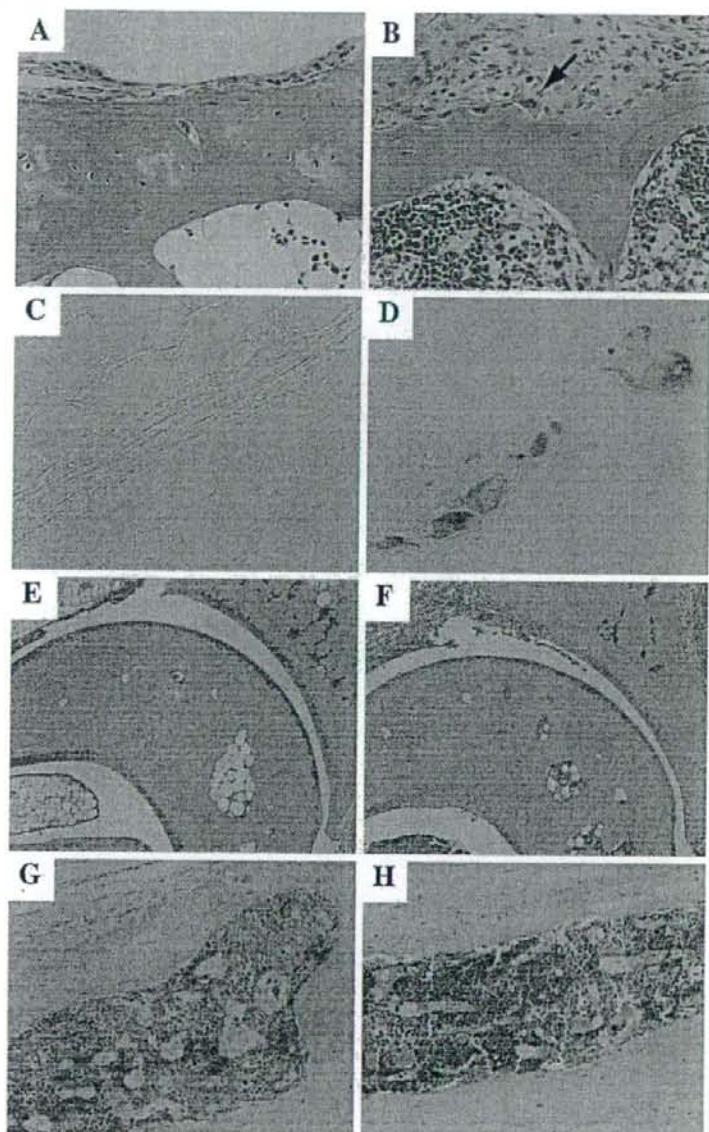
Fig. 6. Bone and cartilage destruction in athymic nude mice transferred with SKG T cell clones. High magnification of H&E-stained sections of a nude mouse recipient of dengue 2F7 (A) or 35S (B), showing bone erosion by pannus and BM activation (×400). Multinuclear cells (osteoclasts) (arrow) are also observed. Tartrate-resistant acid phosphatase-positive cells (osteoclasts) are detected in a 35S recipient (D) but not in a 2F7 recipient (C) (×400). By Safranin-O staining, proteoglycan stained red decreases in the articular cartilage matrix of a recipient of 35S (F) but not in a recipient of 2F7 (E) (×100). By immunohistochemistry, Gr-1-positive cells increase in the BM of a 35S recipient (H) but not in a 2F7 recipient (G) (×200). (A, C, E and G) 12 months after transfer; (B, D, F and H) 10 months after transfer.

Discussion

In this study, we have established two distinct CD8+ T cell clones from arthritic lesions of SKG mice. Interestingly, both exhibited *in vitro* autoreactivity against not only synoviocytes but also a variety of MHC-matched cell lines and elicited both arthritis and interstitial pneumonitis when transferred to
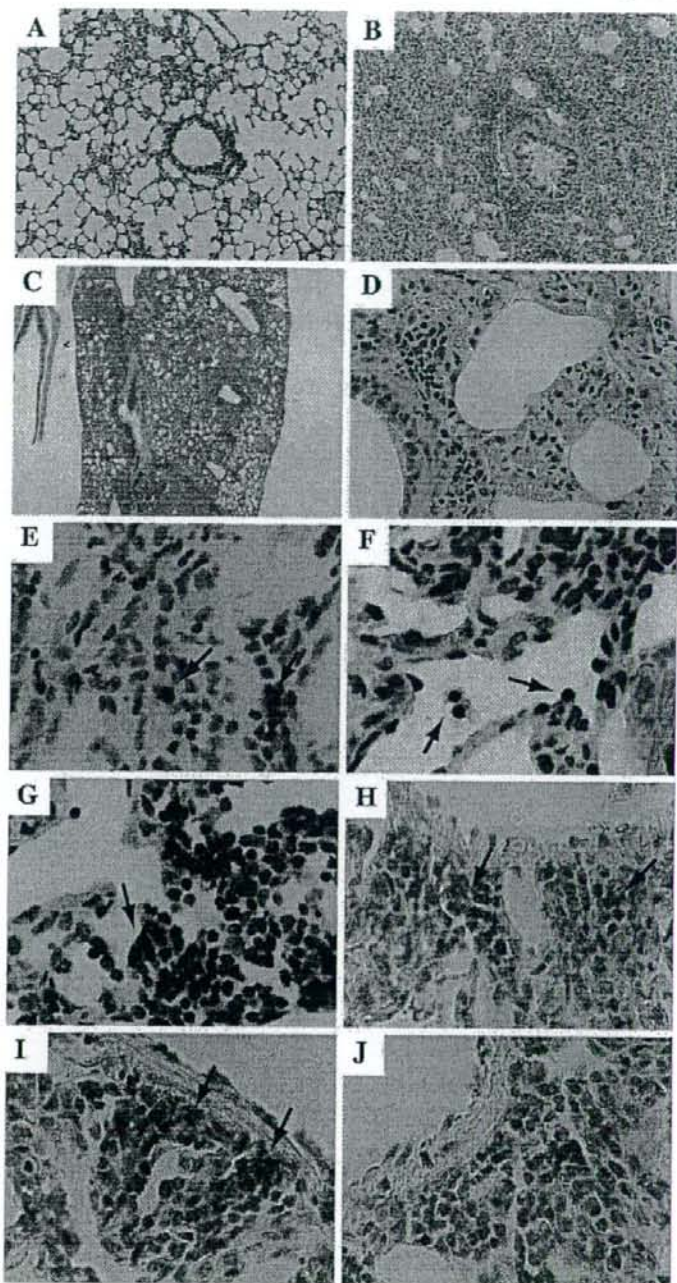
Fig. 7. Interstitial pneumonitis induced by the transfer of SKG T cell clones. (A–D) H&E-stained sections of the lungs of the recipients of control dengue 2F7 clone (A) or 73S clone (B–D) (A–B, ×100). Lower (C, ×10) and higher (D, ×400) magnification of the lung of 73S clone recipient show thickening of alveolar walls diffusely in the lung. (E–J) Serial sections of a lung of a 73S recipient mouse were stained for Ly-6G (Gr-1) (E), F4/80 (F), B220/CD45R (G), CD8a (H) or CD4 (I), with staining control (J) (×400). Typically positive cells in these stainings are arrowed. (A–J) 6 months after transfer.
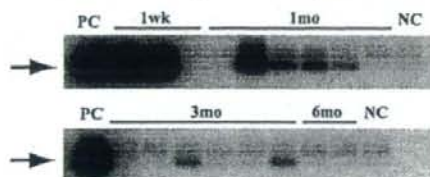
Fig. 8. Detection of a clone-specific TCR message of 35S clone in spleens by RT–PCR amplification and Southern blot analysis. After transfer of 1 × 10⁷ clone cells to BALB/c nude mice, RNA was extracted from spleens at indicated days. PC, positive control (RNA from 35S clone diluted to 1%); NC, negative control (RNA from a 6-month-old non-treated BALB/c athymic nude mouse). The separate lanes represent individual mice.
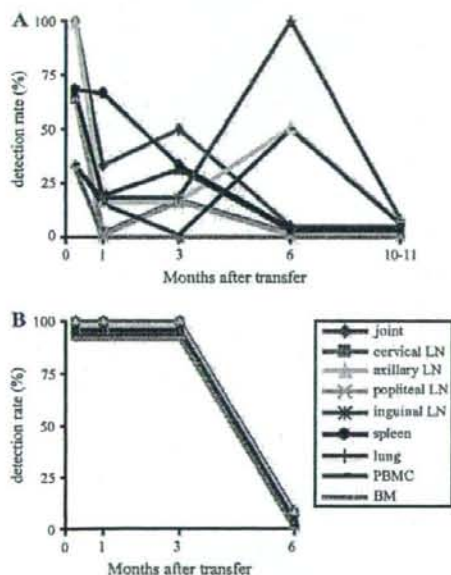
Fig. 9. Detection of TCR mRNA of the transferred clones in recipient BALB/c nude mice. 35S (A) or dengue 2F7 (B) in the recipients were detected by Southern blot analysis using primers and probes specific for TCR V and J region and CDR3 sequences of each clone. All mice with at least one positive signal out of four joints were considered to be positive. (A) n = 3 at 1 week; n = 6 at 1 month; n = 6 at 3 months; n = 2 at 6 months; n = 2 at 10–11 months. (B) n = 2 in every group. No signal was detected in control 6- or 11-month old BALB/c nude mice in each Southern blot analysis (data not shown).

histocompatible T cell-deficient mice. Furthermore, the arthritic and pulmonary lesions chronically progressed irrespective of the decline in the number of transferred T cell clones to hardly detectable levels in either lesion.

Our previous study showed that bulk CD4⁺ T cells alone from arthritic SKG mice were able to transfer the disease to athymic nude mice, whereas bulk CD8⁺ T cells alone were not and that abundant CD4⁺ T cells and only a small number of CD8⁺ T cells were found by immunohistochemistry in the

arthritic subsynovial tissue of arthritic SKG mice (14). These apparently opposing results with CD8⁺ T cell clones versus bulk CD8⁺ T cells indicate that potentially arthritogenic CD8⁺ T cells are present in SKG mice and may usually need CD4⁺ T cell help for induction of arthritis; yet, they are potentially able to mediate arthritis without CD4⁺ T cell help if they are strongly activated, clonally expanded to a large number or possibly selected for stronger self-reactivity during *in vitro* culture. It remains to be determined how CD8⁺ clones elicit proliferative synovitis rather than cytotoxic killing of certain cellular elements in the joint. One possibility is that these CD8⁺ clones, which exert *in vitro* killing activity at a high T cell/target cell ratio, might also be able to stimulate synoviocytes through secreting cytokines. It is of interest in this regard that the joints and the lungs with severe pneumonitis in some recipients of the CD8⁺ clones showed active transcription of IL-17 mRNA (Supplementary Figure 1, available at *International Immunology* Online). Although the CD8⁺ clones did not produce detectable amounts of IL-17 by *in vitro* stimulation, they might produce the cytokine in the joints or interact with nude mouse-derived α/β or γ/δ T cells and stimulate them to secrete IL-17 (33, 34). It is of note that a large number of Gr-1⁺ mature neutrophils exuded into the joint fluid and infiltrated into the subsynovial tissue of the recipient nude mice, as in the arthritic lesions of SKG mice (14). BM of the clone recipients also showed an increase in the number of Gr-1⁺ mature neutrophils. It remains to be determined how CD8⁺ T cells mediate arthritis and pneumonitis in SKG mice by recruiting other cellular elements including neutrophils, how they increase neutrophils in the BM and whether IL-17, which is capable of recruiting neutrophils, is involved in these processes (35, 36).

It also needs further investigation whether IFN-γ secreted by the transferred CD8⁺ clones or their killing activity could contribute to the development of synovitis. IFN-γ may activate synoviocytes directly or indirectly through activating macrophages, facilitating synoviocyte proliferation. It might up-regulate the expression of MHC class I in synovial cells, rendering them susceptible to cytotoxic activity of CD8⁺ T cells. With these apparently opposing activities of arthritogenic CD8⁺ T cells (i.e. killing versus proliferation of synoviocytes), they mediate proliferative synovitis rather than synoviocyte destruction presumably because synoviocytes might be more sensitive *in vivo* to the stimulatory effect than the cytotoxicity (see Discussion below).

The CD8⁺ clones exhibited *in vitro* cytotoxic activity against not only syngeneic synovial cells but also a variety of MHC-matched lymphoid and non-lymphoid cell lines. Although their precise antigen specificities need to be determined, this finding suggests that these clones may recognize a ubiquitous self-antigen (for example, ubiquitous cellular protein such as hsp complexed with MHC or the MHC molecule itself) expressed in the joint and lung and other tissues, rather than a common self-antigen exclusively expressed in the joint and lung. If this is the case, how are the joint and the lung selectively affected by these T cell clones? For the following reasons, one could attribute this to unique characteristics of the synoviocytes, and possibly the alveolar macrophage, as the target of this autoimmunity. Compared with other tissue cells, the synoviocytes are

highly sensitive to pro-inflammatory cytokines, for example systemic overproduction of transgenic TNF-α or IL-1 almost exclusively produces chronic arthritis even in mice deficient of both T and B cells (37–39); similarly, systemic deficiency of the IL-1R antagonist, and resulting overproduction of IL-1, or systemic alteration of signal transduction via IL-6 receptor results in predominant development of arthritis with no inflammatory damage to other tissues (40, 41). These findings collectively indicate that synoviocytes are much more sensitive to the SKG self-reactive T cell clones (at least to those secreting pro-inflammatory cytokines) than other tissue cells, even if the common self-antigens recognized by the clones are ubiquitously expressed. In addition, synoviocytes are unique in that they are the target cells and also the mediators of autoimmunity, i.e. upon stimulation (e.g. by cytokines or via cell contact stimulation by self-reactive T cells), they proliferate and secrete pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNF-α) and other inflammatory substances (matrix metalloproteinases and prostaglandins), mediating inflammation and tissue damage (42). It is likely that the cells composing the alveolar walls, in particular the alveolar macrophages, are sensitive and responsive to T cell self-reactivity in a similar manner as synoviocytes and that excessively and chronically activated macrophages might mediate alveolitis and interstitial inflammation. A similar mechanism might also be responsible for the development of colitis in SKG mice (Table 1).

We do not assert, however, that SKG arthritis and pneumonitis are solely mediated by T cells recognizing a ubiquitous common self-antigen. We have previously shown that SKG mice spontaneously produce IgG isotype auto-antibody specific for joint-rich type II collagen or IgG antibody cross-reactive with hsp-70 of Tuberculosis bacilli (14). This indicates that helper CD4^{+} T cells that specifically react with these self-antigens may also be induced in SKG mice either primarily or secondarily to joint damage. Moreover, we have recently shown that some self-reactive T cells in SKG mice may not be arthritogenic but can polyclonally stimulate antigen-presenting cells in the spleen and lymph nodes to secrete IL-6 and other cytokines, which in turn facilitate differentiation of potentially arthritogenic self-reactive T cells to T_h17 effector T cells that mediate synovitis (19). In addition to our current approach to the characterization of antigen specificity of SKG autoimmune T cells by preparing T cell clones, efforts are being made to further characterize infiltrating T cells *in situ* at a single-cell level by amplifying their TCR message.

Tracing the fate of transferred T cell clones revealed that clone-specific TCR gene messages gradually diminished not only in the inflamed joints and the lungs but also in the regional lymph nodes and spleens of the recipients, becoming hardly detectable in 6–11 months; yet, inflammation in the joints and the lung continued to progress and severe arthritis and pneumonitis were apparent even 12 months after clone transfer. Thus, initial triggering of synovitis requires arthritogenic T cells; yet, synovitis apparently becomes less T cell dependent in a later phase, albeit it chronically progresses with the formation of pannus destroying adjacent cartilage and bone, as in human RA (2). This may correlate with the findings in humans that T cell-targeted mAb therapy

is not much efficacious in the treatment of RA at a chronic stage (43). Further characterization of each stage of disease development in SKG mice will contribute to our understanding of the cellular and molecular basis of the T cell-dependent and -independent phases of disease progression in the joints and also in the lung in RA.

In conclusion, we have shown that CD8^{+} T cell clones established from arthritogenic lesions of SKG mice are capable of mediating not only arthritis but also interstitial pneumonitis immunopathologically resembling ILD in RA. This provides a possible common pathogenetic basis between arthritis and ILD in RA. The etiology of RA is largely obscure at present (1, 2). Yet, there are recent findings that genetic polymorphism of the PTPN22-encoded lymphoid tyrosine phosphatase, which alters signal transduction at a TCR proximal step involving ZAP-70, contributes significantly (second only to MHC polymorphism) to the susceptibility to RA and other autoimmune diseases (22, 23, 44, 45). The polymorphism might be responsible for thymic generation of arthritogenic and other self-reactive T cells. Further elucidation of the mechanism by which such autoreactive T cells are generated and activated in SKG mice, and characterization of putative ubiquitous self-antigen recognized by self-reactive T cells capable of mediating arthritis and pneumonitis, would facilitate our understanding of the etiology and the pathogenetic mechanism of RA as a systemic autoimmune disease. This should help devising preventive or curative measures for the disease.

Supplementary data

Supplementary figure is available at *International Immunology* Online.

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Disclosures

The authors declare no conflicting interests.

Abbreviations

BM	bone marrow
CDR3	the third complementarity-determining region
H&E	haematoxylin & eosin
hsp	heat shock protein
ILD	interstitial lung disease
MHA	microplate hybridization assay
PMA	phorbol myristate acetate
RA	rheumatoid arthritis
RT	reverse transcription
SSC	standard saline citrate
TNF	tumor necrosis factor
ZAP-70	ζ-associated protein of 70 kDa

References

1 Harris, E. D. 1997. *Rheumatoid Arthritis*. W.B. Sounders, Philadelphia, PA.

2 Firestein, G. S. 2003. Evolving concepts of rheumatoid arthritis. *Nature* 423:356.

3 Perez, T., Remy-Jardin, M. and Cortet, B. 1998. Airways involvement in rheumatoid arthritis: clinical, functional, and HRCT findings. *Am. J. Respir. Crit. Care Med.* 157:1658.

4 Demir, R., Bodur, H., Tokoglu, F., Olcay, I., Ucan, H. and Borman, P. 1999. High resolution computed tomography of the lungs in patients with rheumatoid arthritis. *Rheumatol. Int.* 19:19.

5 Gabbay, E., Tarala, R., Will, R. *et al.* 1997. Interstitial lung disease in recent onset rheumatoid arthritis. *Am. J. Respir. Crit. Care Med.* 156:528.

6 McDonagh, J., Greaves, M., Wright, A. R., Heycock, C., Owen, J. P. and Kelly, C. 1994. High resolution computed tomography of the lungs in patients with rheumatoid arthritis and interstitial lung disease. *Br. J. Rheumatol.* 33:118.

7 Striebich, C. C., Falta, M. T., Wang, Y., Bill, J. and Kotzin, B. L. 1998. Selective accumulation of related CD4+ T cell clones in the synovial fluid of patients with rheumatoid arthritis. *J. Immunol.* 161:4428.

8 Fox, D. A. 1997. The role of T cells in the immunopathogenesis of rheumatoid arthritis: new perspectives. *Arthritis Rheum.* 40:598.

9 Panayi, G. S., Lanchbury, J. S. and Kingsley, G. H. 1992. The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. *Arthritis Rheum.* 35:729.

10 Nepom, G. T., Hansen, J. A. and Nepom, B. S. 1987. The molecular basis for HLA class II associations with rheumatoid arthritis. *J. Clin. Immunol.* 7:1.

11 Gao, X. J., Olsen, N. J., Pincus, T. and Stastny, P. 1990. HLA-DR alleles with naturally occurring amino acid substitutions and risk for development of rheumatoid arthritis. *Arthritis Rheum.* 33:939.

12 Firestein, G. S. and Zvaifler, N. J. 2002. How important are T cells in chronic rheumatoid synovitis? II. T cell-independent mechanisms from beginning to end. *Arthritis Rheum.* 46:298.

13 Sakaguchi, S. and Sakaguchi, N. 2005. Animal models of arthritis caused by systemic alteration of the immune system. *Curr. Opin. Immunol.* 17:589.

14 Sakaguchi, N., Takahashi, T., Hata, H. *et al.* 2003. Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. *Nature* 426:454.

15 Hata, H., Sakaguchi, N., Yoshitomi, H. *et al.* 2004. Distinct contribution of IL-6, TNF-alpha, IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. *J. Clin. Invest.* 114:582.

16 Yoshitomi, H., Sakaguchi, N., Kobayashi, K. *et al.* 2005. A role for fungal {beta}-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J. Exp. Med.* 201:949.

17 Chan, A. C., Iwashima, M., Turck, C. W. and Weiss, A. 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* 71:649.

18 Negishi, I., Motoyama, N., Nakayama, K. *et al.* 1995. Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature* 376:435.

19 Hirota, K., Hashimoto, M., Yoshitomi, H. *et al.* 2007. T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17+ Th cells that cause autoimmune arthritis. *J. Exp. Med.* 204:41.

20 Maini, R. N. and Feldmann, M. 2002. How does infliximab work in rheumatoid arthritis. *Arthritis Res.* 4:(Suppl. 2). S22.

21 Nishimoto, N., Yoshizaki, K., Miyasaka, N. *et al.* 2004. Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial. *Arthritis Rheum.* 50:1761.

22 Bottini, N., Vang, T., Cucca, F. and Mustelin, T. 2006. Role of PTPN22 in type 1 diabetes and other autoimmune diseases. *Semin. Immunol.* 18:207.

23 Begovich, A. B., Carlton, V. E., Honigberg, L. A. *et al.* 2004. A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. *Am. J. Hum. Genet.* 75:330.

24 Yoshida, R., Yoshioka, T., Yamane, S. *et al.* 2000. A new method for quantitative analysis of the mouse T-cell receptor V region repertoires: comparison of repertoires among strains. *Immunogenetics* 52:35.

25 Hori, S., Nomura, T. and Sakaguchi, S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057.

26 Arden, B., Clark, S. P., Kabelitz, D. and Mak, T. W. 1995. Mouse T-cell receptor variable gene segment families. *Immunogenetics* 42:501.

27 Koop, B. F., Rowen, L., Wang, K. *et al.* 1994. The human T-cell receptor TCRAC/TCRDC (C alpha/C delta) region: organization, sequence, and evolution of 97.6 kb of DNA. *Genomics* 19:478.

28 Malissen, M., Minard, K., Mjolsness, S. *et al.* 1984. Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the beta polypeptide. *Cell* 37:1101.

29 Gascoigne, N. R., Chien, Y., Becker, D. M., Kavaler, J. and Davis, M. M. 1984. Genomic organization and sequence of T-cell receptor beta-chain constant- and joining-region genes. *Nature* 310:387.

30 MacDonald, H. R., Lees, R. K., Sordat, B., Zaech, P., Maryanski, J. L. and Bron, C. 1981. Age-associated increase in expression of the T cell surface markers Thy-1, Lyt-1, and Lyt-2 in congenitally athymic (nu/nu) mice: analysis by flow microfluorometry. *J. Immunol.* 126:865.

31 MacDonald, H. R., Lees, R. K., Bron, C., Sordat, B. and Miescher, G. 1987. T cell antigen receptor expression in athymic (nu/nu) mice. Evidence for an oligoclonal beta chain repertoire. *J. Exp. Med.* 166:195.

32 Hodes, R. J., Sharrow, S. O. and Solomon, A. 1989. Failure of T cell receptor V beta negative selection in an athymic environment. *Science* 246:1041.

33 He, D., Wu, L., Kim, H. K., Li, H., Elmets, C. A. and Xu, H. 2006. CD8+ IL-17-producing T cells are important in effector functions for the elicitation of contact hypersensitivity responses. *J. Immunol.* 177:6852.

34 Ivanov, I. I., McKenzie, B. S., Zhou, L. *et al.* 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121.

35 Edwards, S. W. and Hallett, M. B. 1997. Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. *Immunol. Today* 18:320.

36 Jimenez-Boj, E., Redlich, K., Turk, B. *et al.* 2005. Interaction between synovial inflammatory tissue and bone marrow in rheumatoid arthritis. *J. Immunol.* 175:2579.

37 Keffer, J., Probert, L., Cazlaris, H. *et al.* 1991. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *EMBO J.* 10:4025.

38 Aidinis, V., Plows, D., Haralambous, S. *et al.* 2003. Functional analysis of an arthritogenic synovial fibroblast. *Arthritis Res. Ther.* 5:R140.

39 Niki, Y., Yamada, H., Seki, S. *et al.* 2001. Macrophage- and neutrophil-dominant arthritis in human IL-1 alpha transgenic mice. *J. Clin. Invest.* 107:1127.

40 Horai, R., Saijo, S., Tanioka, H. *et al.* 2000. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. *J. Exp. Med.* 191:313.

41 Atsumi, T., Ishihara, K., Kamimura, D. *et al.* 2002. A point mutation of Tyr-759 in interleukin 6 family cytokine receptor subunit gp130 causes autoimmune arthritis. *J. Exp. Med.* 196:979.

42 Huber, L. C., Distler, O., Tarner, I., Gay, R. E., Gay, S. and Pap, T. 2006. Synovial fibroblasts: key players in rheumatoid arthritis. *Rheumatology (Oxford)* 45:669.

43 Strand, V., Kimberly, R. and Isaacs, J. D. 2007. Biologic therapies in rheumatology: lessons learned, future directions. *Nat. Rev. Drug Discov.* 6:75.

44 Vang, T., Congia, M., Macis, M. D. *et al.* 2005. Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat. Genet.* 37:1317.

45 Wu, J., Katrekar, A., Honigberg, L. A. *et al.* 2006. Identification of substrates of human protein-tyrosine phosphatase PTPN22. *J. Biol. Chem.* 281:11002.

The interaction of monocytes with rheumatoid synovial cells is a key step in LIGHT-mediated inflammatory bone destruction

Satoru Ishida,[1,2] Shoji Yamane,[1,2] Saori Nakano,[1] Toru Yanagimoto,[2] Yukie Hanamoto,[2] Miki Maeda-Tanimura,[2] Tomoko Toyosaki-Maeda,[2] Jun Ishizaki,[2] Yoshiyuki Matsuo,[2] Naoshi Fukui,[1] Tsunetoshi Itoh,[3] Takahiro Ochi[1] and Ryuji Suzuki[1]

[1]Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Sagamihara, Kanagawa, Japan, [2]Discovery Research Laboratories, Shionogi & Co., Ltd., Toyonaka, Osaka, Japan, and [3]Department of Immunology and Embryology, Tohoku University School of Medicine, Aoba-ku, Sendai, Japan

Summary

Formation of osteoclasts and consequent joint destruction are hallmarks of rheumatoid arthritis (RA). Here we show that LIGHT, a member of the tumour necrosis factor (TNF) superfamily, induced the differentiation into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) of CD14^{+} monocytes cocultured with nurse-like cells isolated from RA synovium, but not of freshly isolated CD14^{+} monocytes. Receptor activator of nuclear factor-κB ligand (RANKL) enhanced this LIGHT-induced generation of TRAP-positive MNCs. The MNCs showed the phenotypical and functional characteristics of osteoclasts; they showed the expression of osteoclast markers such as cathepsin K, actin-ring formation, and the ability to resorb bone. Moreover, the MNCs expressed both matrix metalloproteinase 9 (MMP-9) and MMP-12, but the latter was not expressed in osteoclasts induced from CD14^{+} monocytes by RANKL. Immunohistochemical analysis showed that the MMP-12-producing MNCs were present in the erosive areas of joints in RA, but not in the affected joints of osteoarthritic patients. These findings suggested that LIGHT might be involved in the progression of inflammatory bone destruction in RA, and that osteoclast progenitors might become competent for LIGHT-mediated osteoclastogenesis via interactions with synoviocyte-like nurse-like cells.

Keywords: differentiation; LIGHT/TNFSF14; monocyte; osteoclast; rheumatoid arthritis

Introduction

Osteoclasts are large, tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). Receptor activator of nuclear factor-κB ligand (RANKL) is a key regulator of osteoclast differentiation from haematopoietic precursors of the monocyte/macrophage lineage.[1–3] Although osteoclasts have an essential role in physiological bone remodelling, increases in their number and activity, would lead to diseases accompanied by local bone destruction. Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by arthritis affecting multiple joints and the progressive destruction of cartilage and bone.[4] Osteoclasts are important contributors to the joint destruction in RA. Inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1), which are upregulated in RA synovial tissues, are known to induce the differentiation and activation of

Abbreviations: CTX-I, type I collagen C-telopeptide; HVEM, herpes virus entry mediator; LTβR, lymphotoxin β receptor; MMP, matrix metalloproteinase; MNCs, multinucleated cells; NLCs, nurse-like cells; OA, osteoarthritis; RA, rheumatoid arthritis; RANKL, receptor activator of nuclear factor-κB ligand; TRAP, tartrate-resistant acid phosphatase.

osteoclasts.[5,6] Invasive synovial tissue at sites of bone destruction, also termed pannus, plays important roles in osteoclastic bone resorption.[7–9]

We previously established nurse-like cells (NLCs) from the synovial tissues of RA patients.[10] Although having the same appearance as fibroblast-like synoviocytes, NLCs have a number of distinct activities that could contribute to rheumatoid inflammation.[10–14] Among these are their ability to promote antibody production by B cells, the capacity to protect lymphocytes from apoptosis, and the ability to secrete large amounts of cytokines and chemokines such as IL-6 and IL-8 that could promote the accumulation and activation of lymphocytes and monocytes. However, fibroblast-like synoviocytes from patients with osteoarthritis (OA) hardly show any such activities.[10,15] Therefore, to distinguish them from general fibroblast-like synoviocytes, we have defined synovial NLCs as those that go through the active cell population from the RA synovium. The NLCs promote the survival of peripheral blood monocytes via macrophage colony-stimulating factor (M-CSF) production.[16,17] Monocytes cocultured with NLCs for 4 weeks possessed TRAP activity and differentiated into osteoclasts in response to some cytokines, including RANKL.[17] These reports have suggested that NLCs might be involved in RA-induced bone destruction by maintaining osteoclast precursors in areas of progressive synovial expansion.

LIGHT, which is homologous to lymphotoxin, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes, was recently identified as a type 2 transmembrane glycoprotein of the TNF ligand superfamily (TNFSF14).[18] LIGHT is expressed on activated T lymphocytes,[18,19] monocytes,[20] granulocytes[20] and immature dendritic cells.[21] LIGHT signalling is transduced via two members of the TNFR family, herpes virus entry mediator (HVEM, TNFRSF14) and lymphotoxin β receptor (LTβR, TNFRSF3). The HVEM is expressed prominently on monocytes, dendritic cells and lymphocytes,[19,22–24] whereas LTβR is expressed on many cell types with the exception of lymphocytes.[18,20,25] LIGHT has been shown to regulate cell proliferation[21,26,27] and apoptosis,[20,28] to induce the secretion of various cytokines, and to augment the expression of adhesion molecules.[26,29–31] Recently, Kim *et al.* reported that LIGHT was overexpressed in the synovial tissue of RA patients and that it induced the production of chemokines, cytokines and matrix metalloproteinase 9 (MMP-9) from macrophages in synovial fluid.[32] Moreover, LIGHT contributes to the survival and activation of synovial fibroblasts in RA.[33,34] These studies have suggested that LIGHT may be an important inflammatory cytokine in the development of RA. However, the roles of LIGHT in the bone destruction in RA have not yet been elucidated.

In this study, we compared the abilities to differentiate into osteoclasts in response to LIGHT, between fresh CD14+ monocytes and CD14+ monocytes cocultured with NLCs. We found that LIGHT induced osteoclast differentiation from CD14+ monocytes cocultured with NLCs, but not from freshly isolated CD14+ monocytes. Furthermore, LIGHT-induced osteoclasts express MMP-12, which was not expressed in osteoclasts induced by RANKL, and the MMP-12-expressing osteoclasts were observed at the erosive areas in the subchondral bones of RA patients, but not in those of OA patients. These findings suggest that CD14+ monocytes gain the ability to differentiate into osteoclasts in response to LIGHT through their interactions with NLCs, and that LIGHT plays a critical role in the inflammatory joint destruction in RA.

Materials and methods

Cells and cultures

Nurse-like cells were established from synovial tissues obtained from RA patients, as described previously.[10] NLCs were cultured in Dulbecco's modified Eagle's minimum essential medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS). The NLCs from passages 4–9 were used for each experiment. Mononuclear cells were collected from the venous blood of healthy volunteers and CD14+ monocytes were prepared by further separation using anti-CD14 antibody-coated beads, as described previously.[16] CD14+ monocytes (2.0×10^6 cells/well) were cocultured with NLCs (2.0×10^5 cells/well) in six-well plates. Half of the medium was replaced every 3 days with fresh medium. After coculture for 4 weeks, floating or weakly adherent monocytes were harvested as NLC-supported CD14+ monocytes (NCD14+ monocytes) by gently washing the culture with fresh medium. Over 97% of NCD14+ monocytes were TRAP positive, and their purity was confirmed cytochemically, as reported previously.[16] All human specimens were obtained with written informed consent according to the study protocol, which was approved by the review board of the Sagamihara National Hospital.

Osteoclast formation assay

In the presence of 25 ng/ml recombinant human M-CSF (R&D Systems, Minneapolis, MN), freshly isolated CD14+ monocytes (1.0×10^5 cells/well) and NCD14+ monocytes (2.0×10^4 cells/well) were cultured in 96-well plates in α-minimum essential medium (Invitrogen) supplemented with 10% FCS. As indicated, the cells were further stimulated with 40 ng/ml recombinant human RANKL (Peprotech, London, UK) and/or various concentrations of recombinant human LIGHT (R&D Systems). After vari-

ous periods of time, as indicated in the Results, cells were fixed and stained for TRAP using a TRAP staining kit (Wako, Osaka, Japan). Osteoclasts were identified as TRAP-positive MNCs (more than five nuclei). AlexaFluor546-conjugated phalloidin was used to stain for F-actin (Invitrogen).

Bone resorption assay

In the presence of 25 ng/ml M-CSF, NCD14^{+} monocytes were cultured on cortical bone slices in α-minimum essential medium supplemented with 10% FCS and further stimulated with 40 ng/ml RANKL and/or 100 ng/ml LIGHT. After 21 days, the bone slices were stained with Mayer's haematoxylin solution to detect resorption pits. The concentration of the type I collagen C-telopeptide (CTX-I) in the culture supernatant was quantified using the CrossLaps for Culture kit (Nordic Biosciences Diagnostics, Herlev, Denmark), according to the manufacturer's instructions.

Quantitative polymerase chain reaction analysis

Total RNA was prepared using an RNeasy Micro kit (Qiagen, Tokyo, Japan) and complementary DNA (cDNA) was generated from the RNA using Omniscript Reverse Transcriptase (Qiagen) following the manufacturer's instructions. The cDNA was used as a template for real-time quantitative polymerase chain reaction (PCR) in a LightCycler (Roche Diagnostics, Tokyo, Japan). The PCR was performed using SYBR Premix Ex Taq (Takara, Kyoto, Japan). The PCR primers used in this study were as follows: for NFATc1, 5'-TACCAGGTGCACCGCATCA-3' and 5'-TTTCAGGATTCCGGCACAGTC-3'; for TRAP, 5'-TGCA GATCCTGGGTGCAGAC-3' and 5'-GAGTATGCAATC TGGGCAGAGACA-3'; for cathepsin K, 5'-AGCT GCAATAGCGATAATCTGAACC-3' and 5'-CGTTGTTC TTATTTCGAGCCATGA-3'; for carbonic anhydrase II, 5'-GCGACCATGTCCCATCACTG-3' and 5'-TGGCTGTAT GAGTGTCGATGTCAA-3'; for gyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-GCACCGTCAAGGCTGAG AAC-3' and 5'-ATGGTGGTGAAGACGCCAGT-3'; for LIGHT, 5'-TCACGAGGTCAACCCAGCAG-3' and 5'-CC CAGCTGCACCTTGGAGTAG-3'; for HVEM, 5'-TTTG CTCCACAGTTGGCCTAATC-3' and 5'-CAATGACTGT GGCCTCACCTTC-3'; for LTβR, 5'-ATGCTGATGCTG-GCCGTTC-3' and 5'-AGGCTCCCAGCTTCCAGCTA-3'; for RANK, 5'-TTGTGCCGCCTAAGTGGA-3' and 5'-ACC ACCTTGATCTGGGTAGCACATA-3'; for MMP-9, 5'-AC CTCGAACTTTGACAGCGACA-3' and 5'-GATGCCATTC ACGTCGTCCTTA-3'; for MMP-12, 5'-TTGATGGCAAA GGTGGAATCCTA-3' and 5'-AGGAATGGCCAATCTCGT GAAC-3'. The PCR was performed under the following conditions: initial denaturation at 95° for 10 seconds, then 40 cycles of 95° for 5 seconds and 60° for 20

seconds. SYBR green dye was used to detect amplified products and melting curves were routinely recorded to verify the singularity of the PCR product. In each sample, the level of cDNA was normalized based on the expression level of GAPDH.

Immunohistochemical and TRAP staining of tissue samples

Affected knee joints were resected during joint replacement surgery from five RA and three OA patients who had given written informed consent. Serial sections of the decalcified and paraffin-embedded subchondral bone were dewaxed and reacted with anti-human MMP-12 monoclonal antibody (clone 4D2, R&D Systems). Sections were then reacted with anti-mouse immunoglobulin G–horseradish peroxidase conjugate, chromogenic substrate and hydrogen peroxide. The neighbouring sections of those stained with anti-MMP-12 were subjected to staining with second antibody alone as a negative control or with TRAP staining as described above.

Statistical analysis

All data are expressed as means ± SD. A non-paired Student's t-test was used for comparison, using the STATVIEW program (Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered to be statistically significant.

Results

LIGHT induces the differentiation of NCD14^{+} monocytes into TRAP-positive MNCs

To investigate whether or not LIGHT is involved in local bone destruction, we examined the effects of LIGHT on osteoclastogenesis using established osteoclast precursors (NCD14^{+} monocytes) in addition to freshly prepared CD14^{+} monocytes, as described in the *Materials and methods* section.

In the presence of M-CSF, CD14^{+} or NCD14^{+} monocytes were cultured for 6 days with RANKL and/or LIGHT. As shown in Fig. 1(a,b), M-CSF alone did not induce TRAP-positive MNCs from either type of monocytes. CD14^{+} monocytes were differentiated into TRAP-positive MNCs by RANKL, but not by LIGHT. The combination of RANKL and LIGHT had little effect on MNC formation. Conversely, NCD14^{+} monocytes were strongly differentiated into TRAP-positive MNCs when treated with LIGHT. Although RANKL had only a slight effect on NCD14^{+} monocytes, the combination with LIGHT enhanced the formation of TRAP-positive MNCs more than LIGHT alone (Fig. 1b). The LIGHT-induced formation of MNCs was time dependent (4–8 days, Fig. 1c), and dose dependent (1–100 ng/ml, data not shown). Freshly isolated
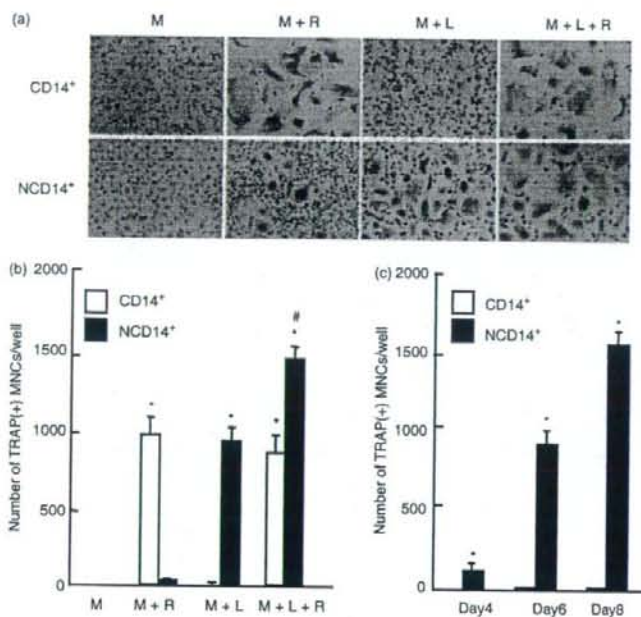
(a)

(b)

(c)

Figure 1. LIGHT induces the differentiation of NCD14$^+$ monocytes into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). (a, b) In the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF; M), CD14$^+$ or NCD14$^+$ monocytes were cultured for 6 days with 40 ng/ml receptor activator of nuclear factor-κB ligand (RANKL; M + R), 100 ng/ml LIGHT (M + L), or 40 ng/ml RANKL plus 100 ng/ml LIGHT (M + L + R). *$P < 0.01$ versus M-CSF alone. #$P < 0.01$ versus NCD14$^+$ monocytes stimulated with M-CSF plus LIGHT. (c) CD14$^+$ or NCD14$^+$ monocytes were cultured for the indicated periods in the presence of 25 ng/ml M-CSF plus 100 ng/ml LIGHT. Cultured cells were fixed and stained for TRAP. The number of TRAP-positive MNCs was counted. *$P < 0.01$ versus CD14$^+$ monocytes. Representative results of at least three independent sets of similar experiments are shown as means ± SD of triplicate experiments.

CD14$^+$ monocytes, however, did not differentiate into TRAP-positive MNCs, even after stimulation with 100 ng/ml LIGHT for 14 days (data not shown). When cultured with M-CSF for 4 weeks, CD14$^+$ monocytes could not differentiate into TRAP-positive MNCs in the presence of RANKL or LIGHT (data not shown).

Increased HVEM messenger RNA expression in NCD14$^+$ monocytes

Next, to clarify the reason for the difference in the efficiency of LIGHT-induced TRAP-positive MNC formation between NCD14$^+$ and CD14$^+$ monocytes, we analysed the messenger RNA (mRNA) expression of the LIGHT receptors, HVEM and LTβR, in both groups of monocytes. Quantitative real-time PCR analysis revealed that while the mRNA expression level of LTβR was not different between groups of monocytes, the level of HVEM mRNA was significantly higher in NCD14$^+$ monocytes than in CD14$^+$ monocytes (Fig. 2). Unexpectedly, the level of RANK mRNA in NCD14$^+$ monocytes was higher than that in CD14$^+$ monocytes (Fig. 2).

Analysis of the molecular phenotype of LIGHT-induced TRAP-positive MNCs derived from NCD14$^+$ monocytes

Furthermore, we investigated the mRNA expression of major osteoclast markers, such as nuclear factor of activated T cells (NFATc1), TRAP, cathepsin K (CTSK) and
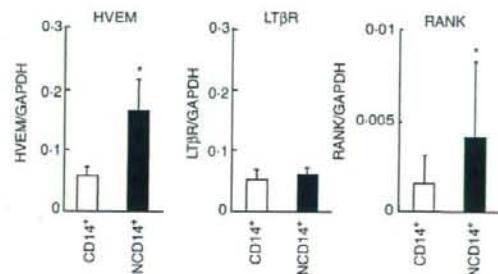

Figure 2. Expression of herpes virus entry mediator (HVEM), lymphotoxin β receptor (LTβR) and receptor activator of nuclear factor-κB (RANK) messenger RNA (mRNA) on CD14$^+$ and NCD14$^+$ monocytes. Total RNA was extracted from CD14$^+$ and NCD14$^+$ monocytes and the mRNA expression levels of HVEM, LTβR and RANK were analysed by quantitative real-time polymerase chain reaction. Representative results of at least three independent sets of similar experiments are shown as means ± SD of triplicate experiments. *$P < 0.01$ versus CD14$^+$ monocytes.

carbonic anhydrase II (CAII), in LIGHT-induced TRAP-positive MNCs derived from NCD14$^+$ monocytes, using quantitative real-time PCR analysis. In comparison with the control (M-CSF alone), the expression levels of all four genes were upregulated in TRAP-positive MNCs induced to differentiate by LIGHT for 6 days (Fig. 3a). The combination of LIGHT and RANKL stimulated their expression to a similar or slightly larger extent than LIGHT alone. When NCD14$^+$ monocytes are stimulated