a HiSpeed Plasmid Midi kit (Qiagen, Valencia, CA, USA). Jurkat cells (5×10^5), grown as described above, were transfected with 2.5 μg of the constructs and 0.5 μg of the pRL-TK vector (an internal control for transfection efficiency) using the Lipofectamin2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). After 24 h, the cells were collected and luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega) and an Ultra Sensitive Tube Luminometer, Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany). Each experiment was independently repeated three times and sextuplicate samples were assayed each time.

Statistical analysis

We used χ^2 contingency table tests to evaluate the significance of differences in allele frequency in the case–control subjects. We defined haplotype blocks using the Solid spine of LD definition of the Haploview v4.0. We performed a Mantel–Haenszel analysis to calculate the pooled *P*-value and odds ratio of two independent association studies. We calculated the power of each cohort for testing association by Quanto Software (http://hydra.usc.edu/gxe/). To adjust for the confounding effects such as gender, we performed a logistic regression analysis using the STATISTICA software (StatSoft). We calculated PARP using the following formula; $\mathrm{PARP} = f(\mathrm{OR}-1)/(1+f(\mathrm{OR}-1)) \times 100$, where f is the allele frequency in the control subjects and OR is the odds ratio. PARP is defined as the reduction of disease incidence that would be achieved if the population had been entirely unexposed. Luciferase assay data were analyzed by Student's *t*-test.

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Conflicts of Interest statement. None declared.

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REFERENCES

1. Hochberg, M.C. (1997) Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.*, **40**, 1725.
2. Kyogoku, C., Dijstelbloem, H.M., Tsuchiya, N., Hatta, Y., Kato, H., Yamaguchi, A., Fukazawa, T., Jansen, M.D., Hashimoto, H., van de Winkel, J.G. *et al.* (2002) Fcgamma receptor gene polymorphisms in Japanese patients with systemic lupus erythematosus: contribution of FCGR2B to genetic susceptibility. *Arthritis Rheum.*, **46**, 1242–1254.
3. Edberg, J.C., Langefeld, C.D., Wu, J., Moser, K.L., Kaufman, K.M., Kelly, J., Bansal, V., Brown, W.M., Salmon, J.E., Rich, S.S. *et al.* (2002) Genetic linkage and association of Fcgamma receptor IIIA (CD16A) on chromosome 1q23 with human systemic lupus erythematosus. *Arthritis Rheum.*, **46**, 2132–2140.
4. Kyogoku, C., Langefeld, C.D., Ortmann, W.A., Lee, A., Selby, S., Carlton, V.E., Chang, M., Ramos, P., Baechler, E.C., Batliwalla, F.M. *et al.* (2004) Genetic association of the R620W polymorphism of protein tyrosine phosphatase PTPN22 with human SLE. *Am. J. Hum. Genet.*, **75**, 504–507.
5. Sigurdsson, S., Nordmark, G., Goring, H.H., Lindroos, K., Wiman, A.C., Sturfelt, G., Jonsen, A., Rantapaa-Dahlqvist, S., Moller, B., Kere, J. *et al.* (2005) Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. *Am. J. Hum. Genet.*, **76**, 528–537.
6. Fernando, M.M., Stevens, C.R., Sabeti, P.C., Walsh, E.C., McWhinnie, A.J., Shah, A., Green, T., Rioux, J.D. and Vyse, T.J. (2007) Identification of two independent risk factors for lupus within the MHC in United Kingdom families. *PLoS Genet.*, **3**, e192.
7. Remmers, E.F., Plenge, R.M., Lee, A.T., Graham, R.R., Hom, G., Behrens, T.W., de Bakker, P.I., Le, J.M., Lee, H.S., Batliwalla, F. *et al.* (2007) STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. *N. Engl. J. Med.*, **357**, 977–986.
8. Graham, R.R., Cotsapas, C., Davies, L., Hackett, R., Lessard, C.J., Leon, J.M., Burtt, N.P., Guiducci, C., Parkin, M., Gates, C. *et al.* (2008) Genetic variants near TNFAIP3 on 6q23 are associated with systemic lupus erythematosus. *Nat. Genet.*, **40**, 1059–1061.
9. Kozyrev, S.V., Abelson, A.K., Wojcik, J., Zaghlool, A., Linga Reddy, M.V., Sanchez, E., Gunnarsson, I., Svenungsson, E., Sturfelt, G., Jonsen, A. *et al.* (2008) Functional variants in the B-cell gene BANK1 are associated with systemic lupus erythematosus. *Nat. Genet.*, **40**, 211–216.
10. Harley, J.B., Alarcon-Riquelme, M.E., Criswell, L.A., Jacob, C.O., Kimberly, R.P., Moser, K.L., Tsao, B.P., Vyse, T.J., Langefeld, C.D., Nath, S.K. *et al.* (2008) Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. *Nat. Genet.*, **40**, 204–210.
11. Hom, G., Graham, R.R., Modrek, B., Taylor, K.E., Ortmann, W., Garnier, S., Lee, A.T., Chung, S.A., Ferreira, R.C., Pant, P.V. *et al.* (2008) Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. *N. Engl. J. Med.*, **358**, 900–909.
12. Bottini, N., Musumeci, L., Alonso, A., Rahmouni, S., Nika, K., Rostamkhani, M., MacMurray, J., Meloni, G.F., Lucarelli, P., Pellecchia, M. *et al.* (2004) A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. *Nat. Genet.*, **36**, 337–338.
13. Plenge, R.M., Cotsapas, C., Davies, L., Price, A.L., de Bakker, P.I., Maller, J., Pe'er, I., Burtt, N.P., Blumenstiel, B., DeFelice, M. *et al.* (2007) Two independent alleles at 6q23 associated with risk of rheumatoid arthritis. *Nat. Genet.*, **39**, 1477–1482.
14. Shimane, K., Kochi, Y., Yamada, R., Okada, Y., Suzuki, A., Miyatake, A., Kubo, M., Nakamura, Y. and Yamamoto, K. (2009) A single nucleotide polymorphism in the IRF5 promoter region is associated with susceptibility to rheumatoid arthritis in the Japanese population. *Ann. Rheum. Dis.*, **68**, 377–383.
15. Gateva, V., Sandling, J.K., Hom, G., Taylor, K.E., Chung, S.A., Sun, X., Ortmann, W., Kosoy, R., Ferreira, R.C., Nordmark, G. *et al.* (2009) A large-scale replication study identifies TNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus. *Nat. Genet.*, **41**, 1228–1233.
16. Shao, H., Kono, D.H., Chen, L.Y., Rubin, E.M. and Kaye, J. (1997) Induction of the early growth response (Egr) family of transcription factors during thymic selection. *J. Exp. Med.*, **185**, 731–744.
17. Topilko, P., Schneider-Maunoury, S., Levi, G., Baron-Van Evercooren, A., Chennoufi, A.B., Seitanidou, T., Babinet, C. and Charnay, P. (1994) Krox-20 controls myelination in the peripheral nervous system. *Nature*, **371**, 796–799.
18. Decker, E.L., Skerka, C. and Zipfel, P.F. (1998) The early growth response protein (EGR-1) regulates interleukin-2 transcription by synergistic interaction with the nuclear factor of activated T cells. *J. Biol. Chem.*, **273**, 26923–26930.

19. Collins, S., Lutz, M.A., Zarek, P.E., Anders, R.A., Kersh, G.J. and Powell, J.D. (2008) Opposing regulation of T cell function by Egr-1/NAB2 and Egr-2/Egr-3. *Eur. J. Immunol.*, **38**, 528–536.
20. Zhu, B., Symonds, A.L., Martin, J.E., Kioussis, D., Wraith, D.C., Li, S. and Wang, P. (2008) Early growth response gene 2 (Egr-2) controls the self-tolerance of T cells and prevents the development of lupuslike autoimmune disease. *J. Exp. Med.*, **205**, 2295–2307.
21. Okamura, T., Fujio, K., Shibuya, M., Sumitomo, S., Shoda, H., Sakaguchi, S. and Yamamoto, K. (2009) CD4 + CD25-LAG3+ regulatory T cells controlled by the transcription factor Egr-2. *Proc. Natl Acad. Sci. USA*, **106**, 13974–13979.
22. Consortium, T.W.T.C.C. (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*, **447**, 661–678.
23. Rioux, J.D., Xavier, R.J., Taylor, K.D., Silverberg, M.S., Goyette, P., Huett, A., Green, T., Kuballa, P., Barmada, M.M., Datta, L.W. *et al.* (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat. Genet.*, **39**, 596–604.
24. Ina, K., Itoh, J., Fukushima, K., Kusugami, K., Yamaguchi, T., Kyokane, K., Imada, A., Binion, D.G., Musso, A., West, G.A. *et al.* (1999) Resistance of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax mucosal imbalance. *J. Immunol.*, **163**, 1081–1090.
25. Brand, S. (2009) Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. *Gut*, **58**, 1152–1167.
26. Newton, J.S., Li, J., Ning, Z.Q., Schoendorf, D.E., Norton, J.D. and Murphy, J.J. (1996) B cell early response gene expression coupled to B cell receptor, CD40 and interleukin-4 receptor co-stimulation: evidence for a role of the egr-2/krox 20 transcription factor in B cell proliferation. *Eur. J. Immunol.*, **26**, 811–816.
27. Mittelstadt, P.R. and Ashwell, J.D. (1999) Role of Egr-2 in up-regulation of Fas ligand in normal T cells and aberrant double-negative lpr and gld T cells. *J. Biol. Chem.*, **274**, 3222–3227.
28. Stranger, B.E., Nica, A.C., Forrest, M.S., Dimas, A., Bird, C.P., Beazley, C., Ingle, C.E., Dunning, M., Flicek, P., Koller, D. *et al.* (2007) Population genomics of human gene expression. *Nat. Genet.*, **39**, 1217–1224.
29. Suzuki, A., Yamada, R., Kochi, Y., Sawada, T., Okada, Y., Matsuda, K., Kamatani, Y., Mori, M., Shimane, K., Hirabayashi, Y. *et al.* (2008) Functional SNPs in CD244 increase the risk of rheumatoid arthritis in a Japanese population. *Nat. Genet.*, **40**, 1224–1229.
30. Begovich, A.B., Carlton, V.E., Honigberg, L.A., Schrodi, S.J., Chokkalingam, A.P., Alexander, H.C., Ardlie, K.G., Huang, Q., Smith, A.M., Spoerke, J.M. *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**, 330–337.
31. Vang, T., Congia, M., Macis, M.D., Musumeci, L., Orru, V., Zavattari, P., Nika, K., Tautz, L., Tasken, K., Cucca, F. *et al.* (2005) Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat. Genet.*, **37**, 1317–1319.
32. Hasegawa, K., Martin, F., Huang, G., Tumas, D., Diehl, L. and Chan, A.C. (2004) PEST domain-enriched tyrosine phosphatase (PEP) regulation of effector/memory T cells. *Science*, **303**, 685–689.
33. Lauritsen, J.P., Kurella, S., Lee, S.Y., Lefebvre, J.M., Rhodes, M., Alberola-Ila, J. and Wiest, D.L. (2008) Egr2 is required for Bcl-2 induction during positive selection. *J. Immunol.*, **181**, 7778–7785.
34. Strasser, A., Harris, A.W. and Cory, S. (1991) Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell*, **67**, 889–899.
35. Kamatani, N., Kitamura, M.K.Y., Harigai, M., Okumoto, T. and Sumio, Y. (2004) Establishment of B-cell lines derived from 996 Japanese individuals. *Tissue Culture Research Communications*, **23**, 71–80.
36. Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., Luthra, H.S. *et al.* (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.*, **31**, 315–324.
37. Andrews, N.C. and Faller, D.V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.*, **19**, 2499.

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REVIEW

The future of lupus therapy modulating autoantigen recognition

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The mainstay of the current treatment for systemic lupus erythematosus consists of steroids and immunosuppressants. However, these non-specific immunosuppressive therapies can cause infection and other serious adverse events. The regulation of the autoantigen-specific immune response is a promising therapeutic approach with maximal efficacy and minimal adverse effects. T cells are essential components of antigen-specificity in the immune system. At present, we do not have a sufficient strategy for manipulating the responses of antigen-specific T cells. In this review, we describe the efficacy of two therapeutic approaches involving the modulation of autoantigen recognition by T cells in lupus model mice: (1) therapy involving engineered autoantigen-specific regulatory T cells generated by the gene transfer of autoantigen-specific TCR genes and appropriate regulatory genes into self lymphocytes; (2) therapy involving selective depletion of autoantigen presenting phagocytes. These selective immunosuppressive approaches could be useful strategies for the treatment of systemic lupus erythematosus. *Lupus* (2010) **19**, 1474–1481.

Key words: antigen-specific T cells; autoantigen presentation; gene transfer; phagocytes; systemic lupus erythematosus; T-cell receptor

Introduction

Systemic lupus erythematosus (SLE) is one of the most common autoimmune diseases mainly affecting females of all ages. The survival of patients with SLE has improved significantly over the past 50 years,[1] but such patients still have an increased mortality compared with the general population. The current treatments for SLE rely mainly on the use of corticosteroids and immunosuppressive drugs. However, these non-specific immunosuppressive therapies can cause infections and other serious adverse effects.

Anti-TNF-α therapy is very successful in the treatment of rheumatoid arthritis. In SLE, some investigators have reported that IFN-α significantly contributes to the pathogenesis of SLE by promoting dendritic cell (DC) maturation and plasma cell differentiation.[2] Therapy with IFN-α in neoplastic disease and viral infections induces autoantibody formation in 4–19% of patients, and SLE symptoms have been reported in 0.2–0.7% of them.[3] About two-thirds of SLE patients exhibit a so-called 'IFN signature', that is, IFN-induced gene expression that correlates with disease activity, together with renal and CNS involvement.[4] A clinical trial of MEDI-545, a fully human mAb for IFN-α, began in 2007. The efficacy of anti-IFN-α therapy against SLE remains to be determined.

B cells are also emerging as targets of therapy for lupus. Although rituximab, a B-cell-depleting anti-CD20 monoclonal antibody, has demonstrated favorable results in several studies, two recent trials failed.[5] The EXPLORER study could not demonstrate that rituximab was superior to corticosteroids for the treatment of extra-renal SLE. In the LUNAR study, patients were allocated to receive placebo or rituximab added to mycophenolate mofetil and corticosteroids at study entry and six months later. No significant difference was found between the two groups in the rates of complete or partial renal remission after 52 weeks of treatment. LJP394 (Riquent™) was originally designed as a B cell tolerogen, which cross-links to the B cell receptor recognizing double-stranded DNA (dsDNA) to specifically reduce anti-dsDNA antibody levels. Unfortunately, the development of Riquent™ was recently stopped because it did not prevent the recurrence of lupus nephritis in remission in a phase III study. These results remind us of

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the heterogeneity of the disease and the importance of refining therapeutic strategy. In this regard, it is valuable to develop selective immunotherapy at the level of autoantigen recognition by T cells.

In autoimmune diseases, including SLE, many researchers have extensively attempted to identify the autoantigens recognized by autoreactive T cells and autoantibodies. These investigations are important not only for understanding the pathogeneses of autoimmune diseases but also for establishing antigen-specific immunotherapies. As a result, several target antigens have already been clarified in SLE. If we were able to prevent autoantigen-specific T cells from becoming activated and expanding before they differentiate into pathogenic T cells, we would be able to efficiently inhibit pathogenic responses. However, the feasibility of this approach is not guaranteed because most patients who require treatment have full-blown SLE.

'Epitope spreading' is generally accepted as a model of T-cell response in autoimmune diseases.[6,7] Firstly, a single epitope on an antigen activates limited numbers of T cells. These T cells may be cross-reactive to other epitopes on the same antigen or to epitopes on other molecules. In the late phase of the disease, the reactive epitopes spread, thereby activating T cells via the recognition of various epitopes on several different self-molecules.

Although this model may explain the autoimmune response induced by infection, it is not clear whether epitope spreading continues throughout the autoimmune process. The immune response to multiple self-antigens partially depends on individual HLA genotypes, stochastic events, and unknown factors. If epitope spreading is the only mechanism involved in autoimmunity, it would be difficult to predict the course of epitope spreading and to develop effective antigen-specific immunotherapies.

In this article, we propose that epitope spreading does not explain all aspects of the T-cell-mediated pathogenesis of autoimmune diseases and that clonal restriction of T cells occurs in the late phase of autoimmunity. Therefore, antigen-specific immunotherapy would be appropriate for established autoimmune diseases. T-cell receptor (TCR) gene transfer is a possible strategy for controlling SLE.

Also, antigen presentation to T cells is an important process in autoimmunity. We recently found that splenic phagocytes present autoantigens to T cells in lupus-prone mice.[8,9] The effect of phagocyte depletion in lupus-model mice will be discussed later.

Evaluation of antigen-specific T cells in autoimmune diseases

At first, we evaluated whether epitope spreading occurs throughout the entire course of autoimmune diseases. To investigate the behavior of antigen-specific T cells that have expanded in vivo, we focused on accumulated T-cell clones in inflamed lesions. We previously established a method for analyzing accumulated T-cell clones using RT-PCR and single-strand conformation polymorphism (SSCP) analysis of TCR signals.[7] In this method, a heterogeneous T-cell population shows a smear pattern representing amplified TCR messages on the non-denaturing polyacrylamide gel due to their heterogenous nucleotide sequences. Therefore, accumulations of particular T-cell clones appear as bands (Figure 1).

Using the method, the same T-cell clones were found to proliferate in different joints of a patient with rheumatoid arthritis.[7,10] These data suggested that the immune responses in rheumatoid arthritis are uniform throughout all arthritic lesions of the patient. In addition, we analyzed HTLV-1 env-pX transgenic mice,[11] which exhibit spontaneous arthritis similar to human rheumatoid arthritis.[12] In the early stage, T cells accumulated in the joints but the T-cell clonotypes were different among the lesions. In the middle stage, the accumulations composed of several identical clones were identified in different lesions. In the late stage, the majority of the accumulated clones in one lesion were observed in the other lesions, and the number of dominant clones decreased. These findings supported the assertion that autoimmune responses are relatively uniform throughout the course of autoimmune diseases.

We also analyzed T-cell clonality in various organs from lupus-prone mice using RT-PCR and SSCP analysis.[13] In young mice, no identical T-cell clones accumulated in the different organs. In nephritic mice, several identical $CD4^+$ T-cell clonotypes were expanded and accumulated in different organs (the bilateral kidneys, brain, lung, and intestine). The TCR Vβ usage of these clonotypes was restricted, and some amino acid motifs were conserved in CDR3 loops. FACS-sorted $CD4^+CD69^+$ cells from the kidney displayed clonotypes identical to those obtained from other organs in the same nephritic mouse. These findings suggest that activated and clonally expanded $CD4^+$ T cells accumulate in multiple organs of full-blown lupus mice. These clonotypes might recognize restricted T-cell

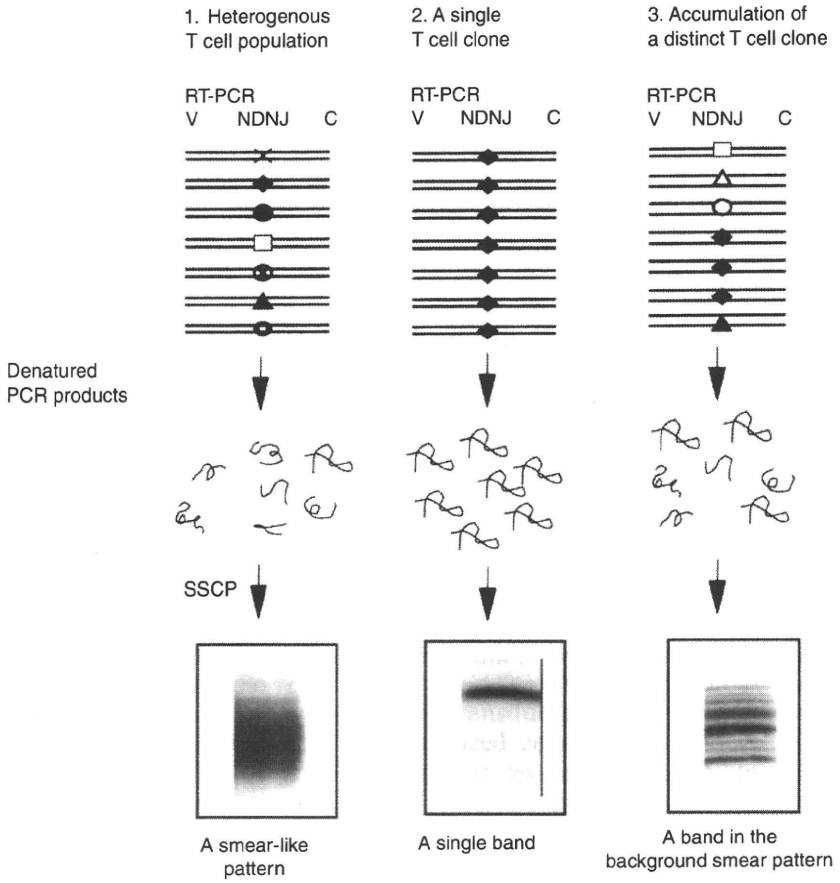

Figure 1 Single-strand conformation polymorphism analysis of T cell receptor (TCR) messenger signals. The products of a reverse transcriptase-polymerase chain reaction of TCR transcripts are heat-denatured into a single strand and then analyzed on non-denaturing polyacrylamide gel. The mobility of each strand reflects its conformation and reveals T cell clonality.[7]

epitopes on autoantigens and contribute to the pathogenesis of lupus.

Based on these observations, we speculate that epitope spreading does not necessarily occur in the late phase of autoimmune disorders. Instead, T cells reactive to certain self-antigens might be sustained, with clonal restriction of T cells continuing to occur throughout the course of autoimmune disease. A similar T-cell oligoclonality was reported as a 'driver clone' in experimental autoimmune encephalomyelitis.[14,15] In patients with polymyositis, several T-cell clones persisted for a number of years in blood T lymphocytes and consecutive muscle biopsy specimens.[16] From patients who had suffered with type 1 diabetes for a long period, oligoclonally expanded insulin-reactive T cells were found in pancreatic draining lymph nodes.[17] In addition, avidity maturation of a pathogenic T-cell population may play a role in the progression from benign inflammation to overt autoimmune disease.[18]

A recent study revealed that T-cell responses were dominated by a few clonotypes expressing a restricted set of TCR in immune responses to foreign antigens.[19] This clonal selection and dominance may depend on the competitive advantages of higher receptor affinity, the duration of the TCR-pMHC interaction, and the affinity threshold.[20] Also in response to viral infection, clonal T-cell 'immunodomination' was observed in $CD8^+$ T cells, probably due to proliferation advantages, differences in TCR affinity, and co-signal requirements.[21] Therefore, the clonal restriction of T cells is regarded as a universal T-cell response.

TCR gene transfer for controlling autoimmune diseases

From the analyses of antigen-specific T-cell clonality described above, we believe that antigen-specific

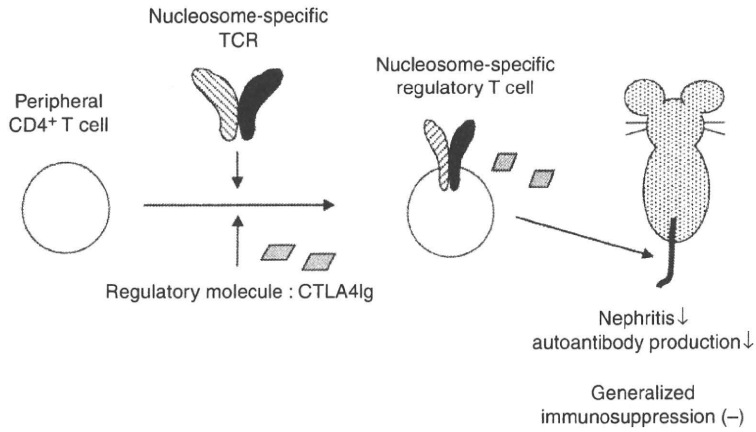

Figure 2 Gene therapy for murine lupus with autoantigen-specific regulatory T cells. Nucleosome-specific CTLA4Ig-producing T cells were retrovirally engineered and transferred into lupus-prone NZB/W F_1 mice. These regulatory cells prevented disease progression without generalized immunosuppression.

immunotherapy targeting T cells would be suitable for treating autoimmune diseases. In this context, we investigated whether autoantigen-specific regulatory T cells engineered by gene transfer are able to suppress lupus-like disease in NZB/W F_1 mice.[8] Anti-DNA antibody is believed to be a major pathogenic autoantibody for murine and human lupus nephritis. Datta and others have reported that nucleosomes are major immunogens in SLE.[22,23] Since a nucleosome consists of double stranded DNA (ds-DNA) and histones, nucleosome-reactive T cells may have the capacity to stimulate DNA-specific B cells to secrete anti-DNA antibody as a hapten-carrier model. We therefore attempted to generate nucleosome-specific T cells possessing an immunosuppressive function. A pair of nucleosome-specific TCR (AN3) recognizes the immunodominant nucleosomal epitope $H4_{71\text{-}94}$ in the context of $I\text{-}A^d$. $H4_{71\text{-}94}$ was also reported to be recognized by $CD4^+$ T cells from patients with lupus and has multiple HLA-DR binding motifs.[24] Retrovirally transduced AN3 was found to have specificity for nucleosomes on splenic $CD4^+$ T cells. CTLA4Ig was co-transduced with AN3 to investigate nucleosome-specific T cells with regulatory function (Figure 2). Approximately 10% of all CD4-positive cells expressed all three genes. These TCR and CTLA4Ig-transduced cells blocked the proliferation of polyclonal T cells. When these cells were co-cultured with DC, they secreted more CTLA4Ig than the mock and CTLA4Ig-transduced cells. To assess their therapeutic effect, a million TCR and CTLA4Ig-transduced cells were transferred into 10-week-old NZB/W F_1 mice. These mice were referred to as TCR-CTLA4Ig-treated mice. By week 22, all of the control mice that had received PBS, mock-transduced cells, TCR-transduced cells, or mock and CTLA4Ig-transduced cells started to develop nephritis with persistent severe proteinuria. In contrast, none of the TCR-CTLA4Ig-treated mice showed severe proteinuria by 30 weeks of age. The kidneys from the control mice displayed severe glomerulonephritis with membrano-proliferation, glomerular sclerosis, and tubular casts. The TCR-CTLA4Ig-treated mice demonstrated mild glomerular disease with reduced deposition of IgG and complement. The levels of anti-dsDNA and anti-histone antibodies were reduced at 22 weeks of age in the TCR-CTLA4Ig-treated mice, and the TCR-CTLA4Ig-treated mice, but not other experimental groups, had low but detectable levels of serum CTLA4Ig. The T-cell-dependent humoral response to active immunization of OVA was also equivalent between the control and TCR-CTLA4Ig-treated mice. These results suggested that autoantigen-specific regulatory cells efficiently deliver CTLA4Ig to the site where autoimmune responses are mediated by T cells. Local delivery of regulatory molecules would increase the therapeutic effects of treatment and minimize the possible systemic side effects. In this regard, antigen-specific T cells are suitable vehicles for targeted immunotherapies.

Regulatory T (Treg) cells are potent suppressors of excessive immune responses. Mice depleted of $CD4^+CD25^+$ Treg cells developed autoimmune disease, and some of them produced anti-dsDNA Ab.[25] $CD4^+CD25^+$ T cells develop in the thymus or the periphery, and their role is to maintain the

homeostatic balance of immunity and tolerance.[26] Thus, investigators are now trying to expand them for the treatment of autoimmune diseases. Several groups have reported that patients with SLE have fewer Treg cells than healthy individuals and that these Treg cells are more susceptible to Fas-mediated apoptosis.[27] Moreover, Treg cells from active SLE patients may have a functional defect.[28] Therefore, Treg cell therapy could be a rational approach for the treatment of lupus. However, adoptive transfer of heterogeneous Treg cells did not fully suppress diabetes in NOD mice.[29–31] It appears that regulatory T-cell function depends on the appropriate phenotype and autoantigen specificity. Therefore, a method is required for expanding Treg cells in an antigen-specific manner.[26]

Gene transfer approaches using antigen-specific TCR and intracellular suppressive molecules are able to generate antigen-specific regulatory T cells. Recently, we succeeded in performing gene therapy for collagen induced arthritis (CIA) using Foxp3-expressing T cells, which accumulated in the arthritic paw.[32] Several pairs of full-length cDNA encoding TCRs were cloned from each single cell. These TCR pairs were found to be identical to major accumulated clones in the inflamed joints of DBA/1 mice. Some of these TCR pairs were reactive to self-antigen presenting cells. One of the TCR pairs, B47, was selected for CIA treatment. B47-transduced $CD4^+$ T cells accumulated and proliferated in the arthritic joints. Paw-directed regulatory T cells were generated by retroviral gene transfer of B47 and Foxp3. Adoptive transfer of B47 and Foxp3-transduced cells significantly suppressed the development of arthritis accompanied by decreases in inflammatory cytokine gene expression and bone destruction. In contrast, adoptive transfer of Foxp3 alone-transduced cells only marginally suppressed arthritis. However, T cells co-transduced with the B47 and Foxp3 genes suppressed the progression of established CIA. Autoantigen specificity is important for the migration and expansion of regulatory T cells.[31,33] TCR and Foxp3 gene transfer is a suitable approach to obtain antigen-specific regulatory T cells.

A clinical evaluation of retroviral TCR gene transfer was reported in the treatment of human metastatic melanoma. Adoptive transfer of T cells transduced with melanoma antigen-specific TCR resulted in the regression of metastatic melanoma lesions.[34] This result showed the fundamental efficacy and safety of TCR gene transfer in humans. Therefore, TCR gene transfer has the potential to treat patients with SLE.

Immunotherapy by suppression of autoantigen-presentation to T cells

In human and murine SLE, intrinsic abnormalities in antigen-presenting cells (APCs) have been reported. APCs play several important roles in the immune system (e.g. clearance of antigens, cytokine production, and antigen presentation to T cells). As shown in MFG-$E8^{-/-}$ mice[35] and Mer^{kd} mice,[36] defective clearance of apoptotic cells by macrophages predisposes mice to lupus-like disease. In contrast, $CD14^{-/-}$ mice did not display autoimmunity in spite of apoptotic cells accumulation.[37] Therefore, impaired clearance of apoptotic cells may not simply lead to autoimmunity. Myeloid DCs from lupus patients revealed an aberrant phenotype characterized by accelerated differentiation, maturation, and secretion of proinflammatory cytokines. Lupus DCs also promote allogeneic T-cell proliferation and activation.[38] Although autoantigen presentation to T cells is crucial for the development of lupus, the precise mode of autoantigen presentation by APCs is poorly understood.

We previously found that reconstituted nucleosome-specific T cells proliferated significantly in the spleens of prenephritic NZB/W F_1 mice, but not in the thymus or lymph nodes.[8,9] Moreover, we observed that splenic phagocytes, including $F4/80^+$ macrophages, presented nucleosomes to T cells efficiently in vitro. In contrast, B cells did not stimulate nucleosome-specific T cells. Based on these results, we depleted splenic phagocytes transiently with Cl_2MDP (dichloromethylene diphosphonate)-liposomes to selectively suppress autoimmunity (Figure 3). Cl_2MDP-liposomes are artificial lipid vesicles that encapsulate Cl_2MDP solution as previously described.[39] Splenic macrophages are depleted dominantly by i.v. administration of Cl_2MDP-liposomes. Treatment with Cl_2MDP-liposomes at 20 and 22 weeks of age suppressed autoantibody production and proteinuria progression compared with treatment with PBS-liposomes or without treatment. The number of autoantibody-secreting cells was decreased in the spleens of the Cl_2MDP-liposome-treated mice. However, the immune response to peripheral immunization was preserved in Cl_2MDP-liposome-treated mice, consistent with its marginal effects on phagocytes in the lymph node.

Our results suggest that splenic phagocytes, including macrophages, are responsible for pathogenic autoantigen presentation. Transient depletion of splenic phagocytes efficiently suppresses autoantigen presentation, autoantibody production, and disease progression. We also observed that

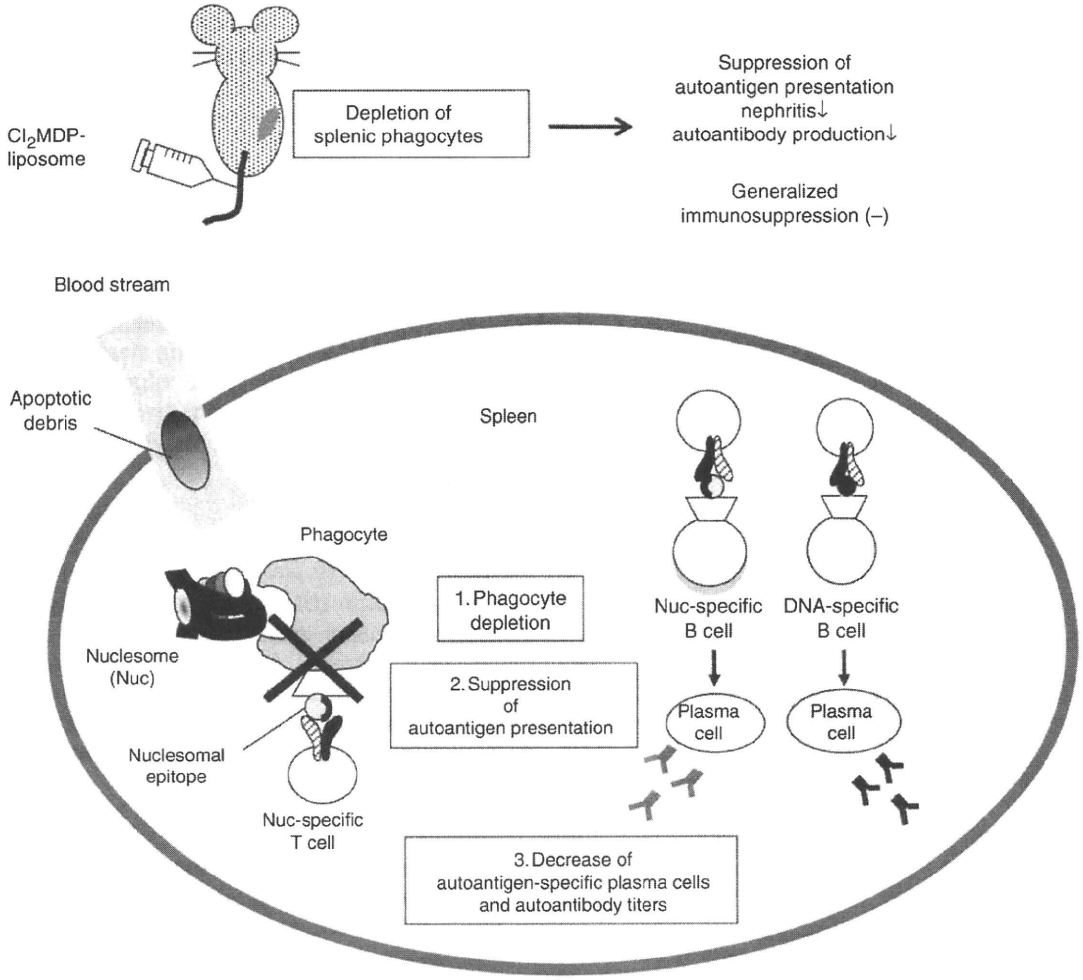

Figure 3 Proposed model for the mechanism of therapy involving transient depletion of autoantigen presenting phagocytes in murine lupus. Splenic phagocytes were depleted by i.v. administration of Cl_2MDP-liposomes. Depletion of splenic phagocytes suppressed autoantigen presentation to T cells and prevented autoantibody production and disease progression without generalized immunosuppression.

nucleosome-specific T cells were retained and activated in the splenic red pulp, where $F4/80^+$ macrophages are located. Initial activation of T cells in the red pulp may be important for autoantigen priming. We speculate that splenic phagocytes ingest apoptotic debris in the circulation and present nucleosomes specifically to T cells because splenic macrophages play an important role in trapping particulate antigens in the circulation.[40,41] Moreover, we found that the expression of MHC class II was increased in NZB/W $F4/80^+$ macrophages. This phenomenon may be associated with pathogenic autoantigen presentation of $F4/80^+$ macrophages.

Recently, autoantigen presentation by human splenic macrophages was reported in patients with immune thrombocytopenic purpura. Splenic macrophages that have ingested opsonized platelets via FcgammaRI are major APCs for cryptic GPIIb/IIIa peptides, activate autoreactive $CD4^+$ T cells, and contribute to the maintenance of antiplatelet autoantibody production.[42] This observation is consistent with our findings. Another group found that peritoneal macrophages from lupus-prone mice showed aberrant cytokine expression following an encounter with apoptotic cells.[43] Such macrophages display increased adhesion capacity and spread dendritic morphology.[44] Autoantigen presentation by phagocytes should be addressed further in association with inflammation, antigen processing, and cell signaling. Our data revealed an apparent paradoxical role of phagocytes in lupus. Impaired clearance of apoptotic cells by macrophages is considered to play a significant

role in the development of autoimmunity.[35,36] The balance between the clearance of apoptotic cells and autoantigen presentation by phagocytes should be considered when developing targeted immunotherapy for lupus.

Another notable observation in our experiment was that the depletion of splenic phagocytes was associated with a reduction in the number of autoantigen-specific plasma cells. Splenic phagocyte depletion may affect survival niches for autoantigen-specific plasma cells directly or indirectly, because plasmablasts and plasma cells migrate into the splenic red pulp after antigen-specific differentiation.[41,45] Further analyses of the pathogenic roles of phagocytes are required in SLE.

Conclusion

T cells are responsible for antigen specificity in immune responses. They are heterogeneous in nature because TCR genes are rearranged in each cell to obtain a variety of antigenic specificities. We found that restricted T-cell clones in the lymphocyte population were involved in antigen-specific immune responses. Therefore, these restricted clones should be the targets of antigen-specific immunotherapy. TCR gene cloning using information from TCR clonal analysis and reconstitution of TCR function by gene transfer would be powerful methods for the analysis and manipulation of antigen-specific T cells. Gene transfer of autoantigen-specific TCR with a soluble or intracellular regulatory molecule is a rational approach for selective immunosuppression. On the other hand, we found that transient depletion of splenic phagocytes including $F4/80^{+}$ macrophages suppressed autoantigen presentation and the development of lupus. Although impaired clearance of apoptotic cells is considered to lead to autoimmunity, pathogenic autoantigen presentation by phagocytes may significantly contribute to lupus pathogenesis. In summary, both genetically engineered autoantigen-specific regulatory T cells and the suppression of autoantigen presentation by phagocytes would be promising selective therapeutic strategies for SLE.

Funding

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References

1 Cervera R, Khamashta MA, Font J, *et al.* Morbidity and mortality in systemic lupus erythematosus during a 10-year period: a comparison of early and late manifestations in a cohort of 1,000 patients. *Medicine (Baltimore)* 2003; 82: 299–308.
2 Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science* 2001; 294: 1540–1543.
3 Stewart TA. Neutralizing interferon alpha as a therapeutic approach to autoimmune diseases. *Cytokine Growth Factor Rev* 2003; 14: 139–154.
4 Baechler EC, Gregersen PK, Behrens TW. The emerging role of interferon in human systemic lupus erythematosus. *Curr Opin Immunol* 2004; 16: 801–807.
5 Sanz I. Connective tissue diseases: The conundrum of B cell depletion in SLE. *Nat Rev Rheumatol* 2009; 5: 304–305.
6 Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol* 2002; 2: 85–95.
7 Yamamoto K, Masuko-Hongo K, Tanaka A, *et al.* Establishment and application of a novel T cell clonality analysis using single-strand conformation polymorphism of T cell receptor messenger signals. *Hum Immunol* 1996; 48: 23–31.
8 Fujio K, Okamoto A, Tahara H, *et al.* Nucleosome-specific regulatory T cells engineered by triple gene transfer suppress a systemic autoimmune disease. *J Immunol* 2004; 173: 2118–2125.
9 Okamoto A, Fujio K, van Rooijen N, *et al.* Splenic phagocytes promote responses to nucleosomes in (NZB x NZW) F1 mice. *J Immunol* 2008; 181: 5264–5271.
10 Yamamoto K, Sakoda H, Nakajima T, *et al.* Accumulation of multiple T cell clonotypes in the synovial lesions of patients with rheumatoid arthritis revealed by a novel clonality analysis. *Int Immunol* 1992; 4: 1219–1223.
11 Iwakura Y, Tosu M, Yoshida E, *et al.* Induction of inflammatory arthropathy resembling rheumatoid arthritis in mice transgenic for HTLV-I. *Science* 1991; 253: 1026–1028.
12 Kobari Y, Misaki Y, Setoguchi K, *et al.* T cells accumulating in the inflamed joints of a spontaneous murine model of rheumatoid arthritis become restricted to common clonotypes during disease progression. *Int Immunol* 2004; 16: 131–138.
13 Zhou G, Fujio K, Sadakata A, *et al.* Identification of systemically expanded activated T cell clones in MRL/lpr and NZB/W F1 lupus model mice. *Clin Exp Immunol* 2004; 136: 448–455.
14 Van den, Elzen P, Menezes JS, Ametani A, *et al.* Limited clonality in autoimmunity: drivers and regulators. *Autoimmun Rev* 2004; 3: 524–529.
15 Huang JC, Ober RJ, Ward ES. The central residues of a T cell receptor sequence motif are key determinants of autoantigen recognition in murine experimental autoimmune encephalomyelitis. *Eur J Immunol* 2005; 35: 299–304.
16 Hofbauer M, Wiesener S, Babbe H, *et al.* Clonal tracking of autoaggressive T cells in polymyositis by combining laser microdissection, single-cell PCR, and CDR3-spectratype analysis. *Proc Natl Acad Sci USA* 2003; 100: 4090–4095.
17 Kent SC, Chen Y, Bregoli L, *et al.* Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope. *Nature* 2005; 435: 224–228.
18 Amrani A, Verdaguer J, Serra P, *et al.* Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. *Nature* 2000; 406: 739–742.
19 Kedzierska K, Turner SJ, Doherty PC. Conserved T cell receptor usage in primary and recall responses to an immunodominant influenza virus nucleoprotein epitope. *Proc Natl Acad Sci USA* 2004; 101: 4942–4947.
20 Malherbe L, Hausl C, Teyton L, McHeyzer-Williams MG. Clonal selection of helper T cells is determined by an affinity threshold

with no further skewing of TCR binding properties. *Immunity* 2004; 21: 669–679.
21 Facchinetti A, Dalla Santa S, Mezzalira S, Rosato A, Biasi G. A large number of T lymphocytes recognize Moloney-murine leukemia virus-induced antigens, but a few mediate long-lasting tumor immunosurveillance. *J Immunol* 2005; 174: 5398–5406.
22 Mohan C, Adams S, Stanik V, Datta SK. Nucleosome – a major immunogen for pathogenic autoantibody-inducing T-cells of lupus. *J Exp Med* 1993; 177: 1367–1381.
23 Kaliyaperumal A, Mohan C, Wu W, Datta SK. Nucleosomal peptide epitopes for nephritis-inducing T helper cells of murine lupus. *J Exp Med* 1996; 183: 2459–2469.
24 Lu L, Kaliyaperumal A, Boumpas DT, Datta SK. Major peptide autoepitopes for nucleosome-specific T cells of human lupus. *J Clin Invest* 1999; 104: 345–355.
25 Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; 155: 1151–1164.
26 Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; 6: 345–352.
27 Miyara M, Amoura Z, Parizot C, *et al.* Global natural regulatory T cell depletion in active systemic lupus erythematosus. *J Immunol* 2005; 175: 8392–8400.
28 Valencia X, Yarboro C, Illei G, Lipsky PE. Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. *J Immunol* 2007; 178: 2579–2588.
29 Bluestone JA, Tang Q. Therapeutic vaccination using CD4+CD25+ antigen-specific regulatory T cells. *Proc Natl Acad Sci USA* 2004; 101: 14622–14626.
30 Tang Q, Henriksen KJ, Bi M, *et al.* In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 2004; 199: 1455–1465.
31 Tarbell KV, Yamazaki S, Olson K, Toy P, Steinman RM. CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J Exp Med* 2004; 199: 1467–1477.
32 Fujio K, Okamoto A, Araki Y, *et al.* Gene therapy of arthritis with TCR isolated from the inflamed paw. *J Immunol* 2006; 177: 8140–8147.
33 Van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. *Arthritis Rheum* 2004; 50: 2775–2785.
34 Morgan RA, Dudley ME, Wunderlich JR, *et al.* Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006; 314: 126–129.
35 Hanayama R, Tanaka M, Miyasaka K, *et al.* Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* 2004; 304: 1147–1150.
36 Scott RS, McMahon EJ, Pop SM, *et al.* Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature* 2001; 411: 207–211.
37 Devitt A, Parker KG, Ogden CA, *et al.* Persistence of apoptotic cells without autoimmune disease or inflammation in CD14-/- mice. *J Cell Biol* 2004; 167: 1161–1170.
38 Ding D, Mehta H, McCune WJ, Kaplan MJ. Aberrant phenotype and function of myeloid dendritic cells in systemic lupus erythematosus. *J Immunol* 2006; 177: 5878–5889.
39 Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 1994; 174: 83–93.
40 Aichele P, Zinke J, Grode L, *et al.* Macrophages of the splenic marginal zone are essential for trapping of blood-borne particulate antigen but dispensable for induction of specific T cell responses. *J Immunol* 2003; 171: 1148–1155.
41 Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol* 2005; 5: 606–616.
42 Kuwana M, Okazaki Y, Ikeda Y. Splenic macrophages maintain the anti-platelet autoimmune response via uptake of opsonized platelets in patients with immune thrombocytopenic purpura. *J Thromb Haemost* 2009; 7: 322–329.
43 Koh JS, Wang Z, Levine JS. Cytokine dysregulation induced by apoptotic cells is a shared characteristic of murine lupus. *J Immunol* 2000; 165: 4190–4201.
44 Fan H, Patel VA, Longacre A, Levine JS. Abnormal regulation of the cytoskeletal regulator Rho typifies macrophages of the major murine models of spontaneous autoimmunity. *J Leukoc Biol* 2006; 79: 155–165.
45 Sze DM, Toellner KM, Garcia de Vinuesa C, Taylor DR, MacLennan IC. Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival. *J Exp Med* 2000; 192: 813–821.

Discontinuation of infliximab after attaining low disease activity in patients with rheumatoid arthritis: RRR (remission induction by Remicade in RA) study

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ABSTRACT

Background Tumour necrosis factor (TNF) inhibitors enable tight control of disease activity in patients with rheumatoid arthritis (RA). Discontinuation of TNF inhibitors after acquisition of low disease activity (LDA) is important for safety and economic reasons.

Objective To determine whether infliximab might be discontinued after achievement of LDA in patients with RA and to evaluate progression of articular destruction during the discontinuation.

Methods 114 patients with RA who had received infliximab treatment, and whose Disease Activity Score, including a 28-joint count (DAS28) was <3.2 (LDA) for 24 weeks, were studied.

Results The mean disease duration of the 114 patients was 5.9 years, mean DAS28 5.5 and mean modified total Sharp score (mTSS) 63.3. After maintaining LDA for >24 weeks by infliximab treatment, the drug was discontinued and DAS28 in 102 patients was evaluated at year 1. Fifty-six patients (55%) continued to have DAS28<3.2 and 43% reached DAS<2.6 at 1 year after discontinuing infliximab. For 46 patients remission induction by Remicade in RA (RRR) failed: disease in 29 patients flared within 1 year and DAS28 was >3.2 at year 1 in 17 patients. Yearly progression of mTSS (ΔTSS) remained <0.5 in 67% and 44% of the RRR-achieved and RRR-failed groups, respectively. The estimated ΔmTSS was 0.3 and 1.6 and Health Assessment Questionnaire-Disability Index was 0.174 and 0.614 in the RRR-achieved and RRR-failed groups, respectively, 1 year after the discontinuation.

Conclusion After attaining LDA by infliximab, 56 (55%) of the 102 patients with RA were able to discontinue infliximab for >1 year without progression of radiological articular destruction.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease that causes significant morbidity and mortality. The combined use of biological agents targeting tumour necrosis factor (TNF) and methotrexate (MTX) has produced significant improvements in clinical, radiographic and functional outcomes that were not previously seen and has revolutionised the treatment goal of RA to clinical remission, structural remission and functional remission.[1–5] The next goal should be remission without the use of biological agents and subsequent drug-free remission. Although global evidence of the efficacy and safety of TNF inhibitors such as infliximab has accumulated, including the ATTRACT study, ASPIRE study, our RECONFIRM studies and many others,[5–10] there is no well-established firm evidence for remission free from the use of biological agents.

The initial report of the potential for remission without the use of biological agents in patients with RA was reported by a British group (TNF20 study).[11] The combination of infliximab and MTX in patients with early RA who had fewer than 12 months of symptoms provided tight control of the disease activity and a significant reduction in MRI evidence of synovitis and erosions at 1 year. At 2 years, functional and quality of life benefits were sustained, despite withdrawal of infliximab treatment. On the other hand, the Behandelstrategieën (BeSt) study was conducted to observe clinical and radiological outcomes of patients with early RA treated with initial infliximab and MTX who discontinued infliximab after achieving a sustained Disease Activity Score (DAS) ≤2.4. Five years after receiving infliximab and MTX as initial treatment for RA, 58% of 120 patients discontinued infliximab because of a continuous DAS ≤2.4 and 19% of patients have stopped all antirheumatic drugs and remain in clinical remission, with minimal joint damage progression. These findings indicate that treatment using infliximab and MTX, guided by DAS, is an effective and tight control to maintain low disease activity (LDA) and may alter the course of early RA.[12–16]

Discontinuation of TNF inhibitors after acquisition of LDA is important for reasons of safety and economy. For instance, the problem of the incidence of haematological malignancy owing to the long-term use of TNF inhibitors remains unresolved. In Japan a large majority of patients have to pay 30% of their medical costs and all wish to know for how long biological agents must be continued, but we have no answer. We successfully discontinued infliximab after attaining DAS-guided remission for >24 weeks,[10] but evidence based on multicentre studies is needed. Reports published to date on this topic are confined to those from the BeSt study and TNF20 study involving only patients at an early stage of RA.[11–16]

Thus, this multicentre study was undertaken to seek the possibility of discontinuing infliximab after attaining DAS-guided remission and maintaining LDA without infliximab, in patients with RA, including patients with long-established disease, and to evaluate progression of articular destruction and functional disabilities during the discontinuation.

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PATIENTS AND METHODS

Patients

Data and information on patients with RA fulfilling the diagnostic criteria of the American College of Rheumatology were collected from 26 centres of remission induction by Remicade in RA (RRR) investigator groups in Japan.[17] Disease activity of individual patients was assessed by Disease Activity Score, including a 28-joint count (DAS28)-erythrocyte sedimentation rate (ESR) or DAS28-C reactive protein (CRP) that was calculated according to the authorised formula (http://www.das-score.nl/, accessed 15 February 2010).[18] Since none of the patients, except for one, achieved LDA measured by DAS28 despite MTX or a combination of MTX and other disease-modifying antirheumatic drugs for at least 3 months, infliximab treatment (3 mg kg, every 8 weeks) was administered in the investigators' institutions, according to the treatment guideline proposed by the Japan College of Rheumatology.[17] Joint damage was assessed by the van der Heijde-modified total Sharp score (mTSS)[19] and for 102 patients, x-ray images of the hands and feet at baseline, RRR-study entry and 1 year after the study were available; these were evaluable for 49 patients owing to loss of the radiographs and/or low quality of the x-ray images.[20 21] Two blinded expert readers independently scored articular damage and progression according to the mTSS scoring method. The difference between the two readers' scores for each patient's radiographs was <1% of the maximum mTSS score—that is, 448.[9 20 21] To confirm that the x-ray results of the 49 patients represented the outcomes of the whole group, we compared multiple background characteristics and changes of each characteristic from baseline to RRR-study entry between 49 patients with evaluable x-ray images and 53 patients without them and no significant difference was seen between the two groups.

After patients had achieved DAS28 (ESR)<3.2 (LDA) for >24 weeks, informed consent to discontinue infliximab was obtained from 126 patients. Other criteria were that patients were controlled with <5 mg/day of oral prednisolone (PSL) and were >18 years old. Concomitant use of MTX was started in all patients, and the dose of MTX was determined by each attending doctor. Twelve patients dropped out at the screening period, and 114 patients were enrolled in the study and discontinued infliximab (figure 1). The demographic indicators and baseline disease characteristics of the 114 patients enrolled are summarised in table 1.

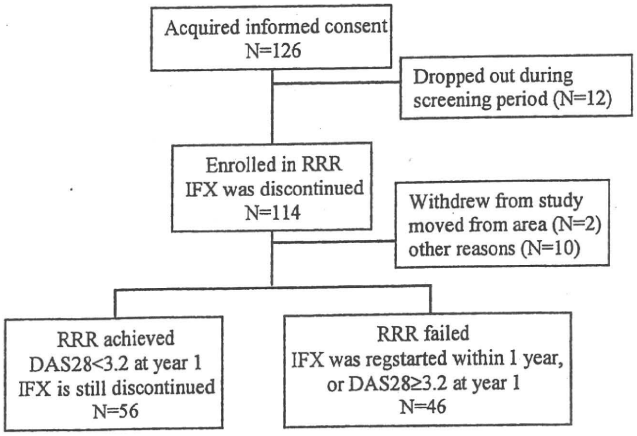

Figure 1 Study design and profile. DAS28, Disease Activity Score, including a 28-joint count; IFX, infliximab; RRR, remission induction by Remicade in rheumatoid arthritis.

Table 1 Demographic indicators and baseline disease characteristics

	Enrolled patients (N=114)	RRR-achieved (N=56)	RRR-failed (N=46)	p (probability >χ^2)
Women	87 (76%)	42 (75%)	38 (83%)	0.4691
Age (years)	51.4 (20.0–73.0)	49.5±12.6	56.1±12.2	*0.0053*
Disease duration (years)	5.9 (0.1–38.0)	4.8±5.9	7.8±7.7	*0.0238*
Tender joint count (0–28)	8.2±6.7	8.6±7.0	7.5±5.8	0.5798
Swollen joint count (0–28)	9.0±7.2	10.1±7.7	7.6±5.8	0.1674
PaGA (0–100 mm, VAS)	50.0±23.0	50.0±24.2	49.3±23.1	0.9520
CRP (mg/dl)	2.5±3.0	2.6±2.6	2.7±3.7	0.5531
ESR (mm/h)	46.2±26.9	43.1±24.2	54.1±30.1	0.1555
DAS28 (ESR) score	5.5±1.2	5.5±1.4	5.6±1.1	0.9112
DAS28 (CRP) score	4.9±1.2	5.1±1.3	4.8±1.3	0.5486
HAQ-DI	1.0±0.7	0.9±0.6	1.2±0.7	0.1112
mTSS*	63.3 (1.0–314.0)	46.9±46.5	97.2±86.9	*0.0207*
RF (U/ml)	201.9±496.5 (68.5%)	225.7±583.3	197.9±427.8	0.5190
MTX (mg/week)	7.7±2.3	7.9±1.9	7.8±2.8	0.3232
PSL (mg/day)	2.5±3.4 (45.6%)	2.4±3.5	2.8±3.5	0.5223

Data are number of patients (%) for categorical data and the means for continuous data. Statistical difference was assessed by non-parametric Wilcoxon t test and p (probability >χ^2) values are shown. Values in italic indicate a significant difference ($p<0.05$).
*Data supplied for 33 patients who achieved RRR and 16 patients for whom RRR failed.
CRP, C-reactive protein; DAS28, Disease Activity Score, including a 28-joint count; ESR, erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire-Disability Index; mTSS, modified total Sharp score; MTX, methotrexate; PaGA, patient global assessment of disease activity; PSL, prednisolone; RF, rheumatoid factor; RRR, remission induction by Remicade in rheumatoid arthritis; VAS, visual analogue scale.

Procedures

Study protocol was a simple observation after discontinuation of infliximab. The follow-up observation was monitored by symptoms, signs and DAS28 (ESR) every 4–13 weeks for 2 years. The dose of concomitant MTX was basically consistent, but tapering of non-steroidal anti-inflammatory drugs and glucocorticoid was allowed during the study period. The primary end points were that after discontinuing infliximab, DAS28 remains <3.2 (LDA) for 1 year and (B) yearly progression of mTSS remains <0.5 (structural remission) for 1 year. Secondary end points were DAS28 remains <2.6 (clinical remission) for 1 year, DAS28 remains <3.2 for 2 years, yearly progression of mTSS remains <0.5 for 2 years and no rescue with infliximab for 1 or 2 years is needed, after discontinuing infliximab. When a flare-up occurred in patients after the discontinuation, restart of infliximab was allowed and patients were categorised into the 'RRR-failed' group. For the restart of infliximab, the same dose (3 mg/kg) and the same premedication as used before the study entry were used.

Statistical analysis

Baseline characteristics of patients are summarised in table 1 using the mean values for continuous variables. All multivariate analyses were conducted using the variables gender, age, duration of disease, DAS28 (ESR) score, DAS28 (CRP) score, tender joint count (0–28), swollen joint count (0–28), patient global assessment of disease activity (PaGA, 0–100 mm, visual analogue scale), ESR, CRP, Health Assessment Questionnaire-Disability Index (HAQ-DI), rheumatoid factor (RF), MTX dose and PSL dose at baseline. Spearman correlation analyses were performed to evaluate the

association between multivariables at RRR-study entry and DAS28 at the primary end point (last observation carried forward) of 102 patients. Logistic regression analysis was carried out to estimate DAS28 at the primary end point as dependent variables (probability) by DAS28 at RRR entry as independent variables. A receiver operating characteristic (ROC) curve was developed based on the logistic analysis and the significant cut-off point was determined from the curve. For categorical response parameters, group comparisons were made using a non-parametric Wilcoxon t test. Statistical analyses were performed using JMP software version 7 (SAS Institute, Cary, North Carolina, USA). All reported p values are two sided and p values <0.05 were considered significant.

RESULTS

Study end points

The demographic indicators and baseline characteristics of the 114 patients enrolled were as follows: mean age 51.4 years, mean disease duration 5.9 years and mean mTSS 63.3, indicating that the population included patients with long-established disease, and the mean DAS28 (ESR) score was 5.5, implying that most patients had highly active disease (table 1). Figure 1 shows the study profile. After maintaining DAS28<3.2 (LDA) for >24 weeks by infliximab treatment, infliximab was discontinued in 114 patients. Twelve patients withdrew because they moved from area (n=2) and for other reasons (n=10), and thus DAS28 could be evaluated in 102 patients at year 1.

Of the 102 patients, 56 patients achieved the primary end point having a DAS28<3.2 and remaining without infliximab for 1 year after the discontinuation (figure 2A). Thus, 55% of the enrolled patients met the primary end point that LDA was maintained for 1 year after discontinuing infliximab. Furthermore, 44 patients (43%) reached DAS<2.6 after the discontinuation. On the other hand, 29 patients flared within 1 year (mean duration was 6.4 months) after the discontinuation and in 17 patients DAS28 was >3.2 at year 1 and thus RRR failed for 46 patients (45%) at year 1. Re-treatment with infliximab in 32 patients was effective and the majority of patients reached DAS28<3.2 within 24 weeks (figure 2B). Minimal adverse reactions at infusion of the agent were seen in five patients only at the first or second infusion.

To clarify the background factors related to the RRR-achievement, multiple clinical parameters at baseline were compared between patients for whom RRR was achieved and those for whom it failed. Patients for whom RRR was achieved were younger (49.5 vs 56.1), their disease duration was shorter (4.8 vs 7.8) and mTSS was lower (46.9 vs 97.2) than for those for whom RRR failed. Among 56 patients who achieved RRR, 10 patients had early RA (disease duration <1.0 year) and eight long-established disease (>10 years). Of 46 patients for whom RRR failed, eight had early RA and 12 established disease. These results imply that infliximab can be discontinued in patients with long-established RA. In contrast, no significant difference was seen in gender, DAS including DAS28, tender or swollen joint count, ESR and CRP, HAQ-DI, RF and the dose of MTX and PSL. Since these factors interact with one another, we analysed the relationship between RRR-achievement and a series of clinical parameters at baseline using multivariate analysis after adjusting for confounding variables. No significant relations

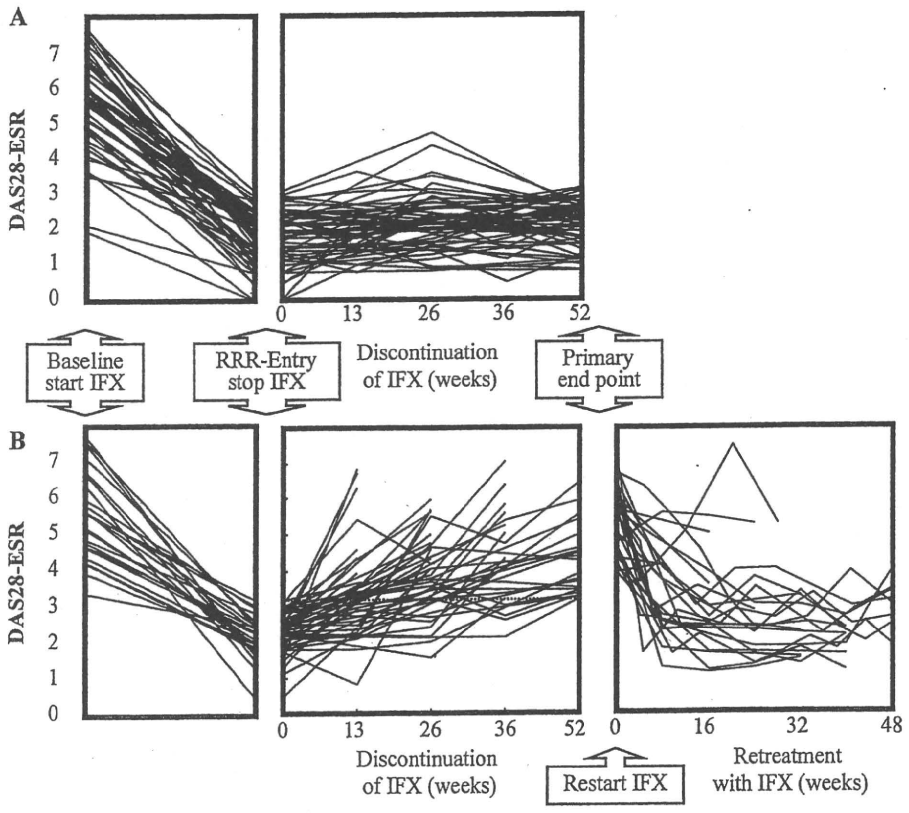

Figure 2 Changes of Disease Activity Score, including a 28-joint count (DAS28) in patients with remission induction by Remicade in rheumatoid arthritis-achieved (RRR-achieved) and patients for whom RRR failed (RRR-failed). (A) Changes of Disease Activity Score, including a 28-joint count (DAS28) at baseline when infliximab (IFX) was administered, at RRR-study entry when infliximab was discontinued and at the primary end point at week 52 after discontinuing IFX in 56 patients who were still satisfied with DAS28 (erythrocyte sedimentation rate (ESR)) <3.2 at week 52, RRR-achieved'. (B) Changes of DAS28 at baseline, at RRR entry and the end point in 46 patients whose disease activity flared after the discontinuation of IFX or DAS28 >3.2 at week 52, 'RRR-failed'. The lower right panel shows changes of DAS28 after the restarting IFX in 32 patients for whom RRR failed.

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between RRR-achievement and age, gender, DAS28 (ESR) score, PaGA and CRP were found, whereas a significant correlation was found with disease duration (p=0.0019) and serum levels of RF (p=0.0128) in RRR-achievement.

To determine the correlation of DAS28 at the primary end point with clinical parameters at RRR-study entry, univariate analysis of multiple variables was carried out. No significant correlations between DAS28 (ESR) at the primary end point and a series of clinical parameters were found, whereas DAS28 (ESR) and DAS28 (CRP) at RRR-entry were significantly correlated with DAS28 (ESR) at the primary end point. Subsequently, logistic regression analysis to estimate the probability of DAS28<3.2 at the primary end point as dependent variable by DAS28 at RRR-entry as independent variable was assessed. A significant logistic regression curve was drawn between the dependent and independent variables (p=0.0005) (figure 3A). Thus, DAS28 at RRR-study entry had the most marked correlation with the maintenance of LDA for 1 year after the discontinuation. By reciprocal statistics, DAS28 at RRR-study entry was estimated as 2.22, to attain DAS28<3.2 at the end point in 50% of the 102 patients (figure 3A). Furthermore, 71.4% of patients whose DAS28 at study entry was <2.225, a cut-off point calculated from the ROC curve, continued to have DAS28<3.2 for 1 year, whereas only 32.6% of patients whose DAS28 at RRR-entry was 2.225–3.2 continued to have DAS28<3.2 (figure 3B), indicating that 'deep remission' was required to maintain lower disease activity for 1 year after discontinuation of infliximab.

Structural and functional changes

From the 102 patients enrolled in the study, 49 patients were selected in whom both hand and feet x-ray data were available and evaluable; experts examined the structural damage before and after the infliximab treatment. When the baseline characteristics of the 49 patients in the study were compared with the rest of the patients in the study with insufficient x-ray data (n=53), no significant difference was seen. Next, the baseline characteristics of the 33 patients who achieved RRR and 16 patients for whom RRR failed were compared. As described in table 1, disease duration was shorter and mTSS was lower in patients who achieved RRR than in patients for whom RRR failed, but yearly progression of mTSS (ΔmTSS) was comparable between two groups (table 2). ΔmTSS at RRR entry was also comparable between two groups. However, means (0.3 vs 1.6) and medians (0.0 vs 1.5) of ΔmTSS were lower in the RRR-achieved group than in the RRR-failed group and more patients in the RRR-achieved group (67%) achieved ΔmTSS<0.5, radiographic remission, than patients in the RRR-failed group (44%). Thus, another primary end point for structural remission was achieved for 1 year after the discontinuation. Furthermore, HAQ-DI at baseline and RRR entry was comparable between patients for whom RRR was achieved and those for whom it failed, whereas HAQ-DI at the primary end point in patients who achieved RRR was significantly lower than that in patients for whom RRR failed (0.174 vs 0.614) (figure 4).

DISCUSSION

This multicentre study was undertaken to determine the possibility of discontinuing infliximab treatment in patients with RA after acquiring DAS-guided LDA, including those with long-established disease. Among 102 patients who could be evaluated at year 1, 56 patients (55%) satisfied the primary end point by maintaining DAS28<3.2 (LDA) and 44 patients (43%) reached DAS<2.6 (remission), remaining without infliximab at year 1 after the discontinuation. Of the 102 patients, 83 (81.4%) were in clinical remission at study entry and after discontinuing infliximab, 39/83 patients (47%) remained in remission and 10/83 patients (12%) progressed to LDA at the primary end point.

These data are similar to those of the BeSt study. However, the greatest difference between the patient populations enrolled in the two studies is mean disease duration—0.4 years in the BeSt study versus 5.9 years in our RRR study.[12–16] Joint destruction also differed between the two studies—mean mTSS 7.0 in the BeSt study versus 63.3 in our RRR study—suggesting that discontinuation of infliximab after reaching LDA is possible in patients with early RA and also in patients with long-established disease.[13–15] On the other hand, among multiple clinical parameters at baseline, disease duration was statistically related to RRR-achievement by multivariate analysis and disease duration was shorter (4.8 vs 7.8) and mTSS was lower (46.9 vs 97.2) in patients who achieved

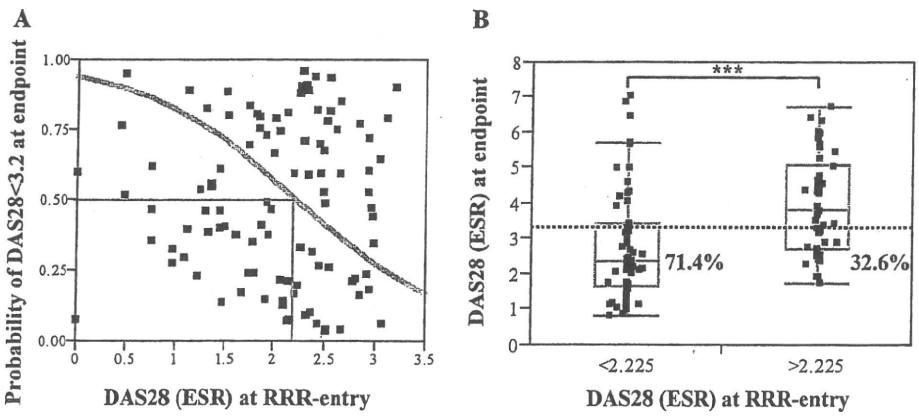

Figure 3 Logistic analysis of probability of Disease Activity Score, including a 28-joint count (DAS28) was <3.2 at primary end point by DAS28 at remission induction by Remicade in rheumatoid arthritis entry (RRR entry). (A) Logistic regression analysis to estimate DAS28 at primary end point as dependent variables by DAS28 at RRR entry as independent variables. The y-axis shows the probability of DAS28 <3.2 at the primary end point after the 52 weeks discontinuation of infliximab and a scatter diagram of an individual patient and logistic regression curve (solid line) are shown. To attain DAS28 <3.2 at the end point in 50% of the 102 patients, DAS28 at RRR study entry was estimated by reciprocal statistics. (B) From the receiver operating characteristic curve based on the logistic regression analysis above, the cut-off point of DAS28 at RRR-study entry was 2.225. Subsequently, one-way analysis of DAS28 at the primary end point by DAS28 at study entry, <2.225 versus between 2.225 and 3.2, was performed and the statistical difference of the two groups was sought by non-parametric Wilcoxon t test (***p<0.001). ESR, erythrocyte sedimentation rate.

Table 2 Radiographic indicators and baseline disease characteristics

	RRR-achieved (N=33)	RRR-failed (N=16)	p (probability >χ^2)
Baseline			
Disease duration (years)	4.7 (0.5–14.0)	8.6 (0.5–25.0)	*0.0280*
DAS28 (ESR) score	5.5 (1.9–7.6)	5.7 (4.2–6.8)	0.6976
HAQ-DI	1.0 (0.0–2.3)	1.1 (0.0–1.8)	0.6271
mTSS	46.9 (1.0–216.5)	97.2 (6.0–314.0)	*0.0207*
Bone erosion score	23.7 (0.0–127.5)	55.5 (1.5–192.5)	*0.0119*
Joint space narrowing score	23.2 (1.0–89.0)	41.6 (4.5–121.5)	*0.0621*
Yearly progression of mTSS	13.1 (0.8–51.3)	15.0 (1.0–47.8)	0.5794
RRR-entry			
Yearly progression of mTSS	1.0 (−2.9 to 10.5)	0.7 (−2.0 to 6.7)	0.5788
Primary end point			
Yearly progression of mTSS	0.3 (−3.6 to 8.5)	1.6 (−3.6 to 7.0)	0.1087
Median of yearly progression of mTSS	0.0	1.5	–
Yearly progression of mTSS <0.5 (%)	67	44	0.2161

Data are number of patients (%) for categorical data and the means for continuous data. Statistical difference was assessed by non-parametric Wilcoxon t test and p (probability >χ^2) values were shown. Values in italic indicate a significant difference ($p<0.05$). DAS28, Disease Activity Score based on assessments of 28 tender and 28 swollen joints; ESR, erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire-Disability Index; mTSS, modified total Sharp score; RRR, remission induction by Remicade in rheumatoid arthritis.

RRR than in those for whom RRR failed. These results imply that remission free from biological agents can be more easily obtained in patients with shorter disease duration than in those with more established disease, but discontinuation of infliximab is still possible even in patients with long-established RA, because eight patients whose disease duration was >10 years successfully remained without infliximab for 1 year.

Among 102 evaluated patients, disease in 29 patients flared within 1 year, 17 patients had DAS28≥3.2 at year 1 after discontinuing infliximab and 32 patients had already been re-treated with infliximab. One of the major concerns of restarting infliximab is the possibility of an infusion reaction after the long-term discontinuation, partly owing to human anti-chimeric antibodies.[22] However, minimal adverse reactions at infusion of the agent were seen only in five patients at the first or second infusion. Another concern is the progress of joint damage after discontinuation of infliximab. However, although the yearly progression of mTSS at RRR-study entry was also comparable between two groups, means (0.3 vs 1.6) and medians (0.0 vs 1.5) of ΔmTSS were shorter in the RRR-achieved group than in the RRR-failed group. Furthermore, at year 1 after the discontinuation of infliximab, more patients in the RRR-achieved group (67%) tended to satisfy ΔmTSS<0.5—that is, structural remission, than those in the RRR-failed group (44%) and HAQ-DI in patients who achieved RRR was significantly lower than that in patients for whom RRR failed (0.174 vs 0.614). These results indicate that both structural remission and functional remission were maintained for 1 year in patients with LDA even after discontinuing infliximab.

This study also shows the significance of DAS-guided tight control of RA in order to maintain remission free from biological agents. There was a significant correlation between DAS28 (ESR) or DAS28 (CRP) at RRR entry and DAS28 (ESR) at year 1 after the discontinuation of infliximab by univariate analysis of multiple variables and a logistic regression analysis. Thus, DAS28 at RRR-study entry had the greatest correlation with maintenance of LDA for 1 year after discontinuation. Also, DAS28 at study entry was mainly influenced by PaGA and ESR among the composite measures. By reciprocal statistics, the estimated DAS28 (ESR) at RRR-study entry was 2.22 (1.85–2.70), to attain DAS28<3.2 at the primary end point in 50% of the 102 patients studied. Also, 71.4% of patients whose DAS28 at study entry was <2.225, a cut-off point calculated from ROC curve, remained DAS28<3.2 for 1 year, whereas only 32.6% of patients whose DAS28 at RRR-entry was 2.225–3.2 remained DAS28<3.2. These results indicate that 'deep remission' appears to be required to maintain lower disease activity for 1 year after discontinuation of infliximab.

About 55% of the 102 patients, who were in an LDA state for >24 weeks with infliximab and MTX treatment, could discontinue infliximab for >1 year without progression of radiological articular destruction or functional disturbance. These data may have significant implications for the optimal use of expensive biological treatments: (*a*) re-treatment with infliximab is efficient and tolerable in the patients for whom RRR failed; (*b*) DAS-guided monitoring is valuable to keep tight control; (*c*) 'deep

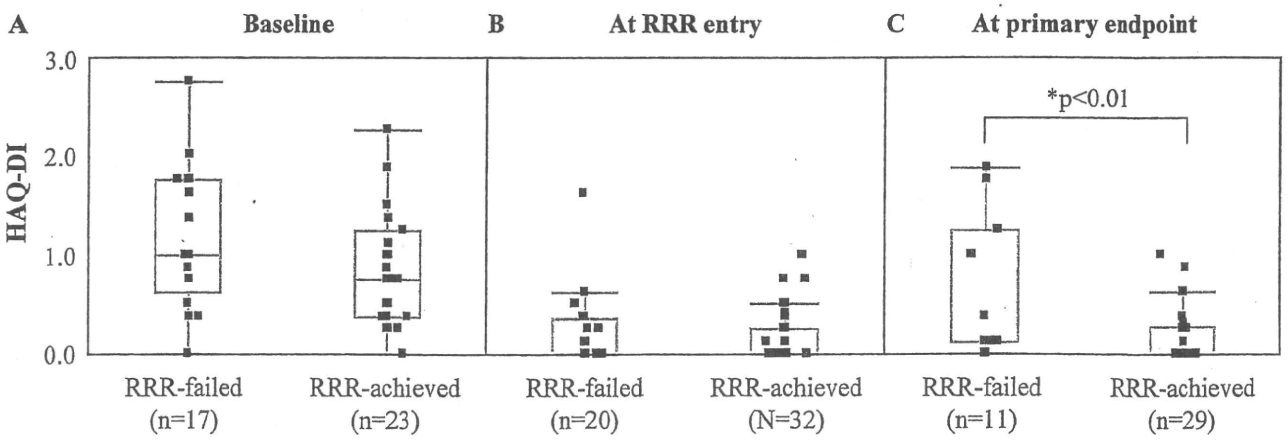

Figure 4 Health Assessment Questionnaire-Disability Index (HAQ-DI) in patients for whom remission induction by Remicade in rheumatoid arthritis failed (RRR-failed)' and in patients for whom 'RRR was achieved (RRR-achieved)' at (A) baseline, (B) RRR entry and (C) the primary end point. The line in the box represents the median value and the upper and lower ends of the box indicate the 25th and 75th centiles of the population. Statistical difference was assessed by non-parametric Wilcoxon t test.

remission' by tight control is required to maintain discontinuation of infliximab; (*d*) remission free from biological agents may be easier to attain in patients with early RA, but is possible for patients with long-established disease; (*e*) treatment aimed at reaching a target of LDA is pivotal to the approach to remission free from biological agents. Finally, TNFα is not a cause of RA, but if appropriate treatment with infliximab can lead to drug-free remission, TNF inhibitors may shut down pathological processes and may change or modify the disease course in RA. Thus, a clinical and basic research approach to the 'process-driven disease' of RA is warranted.

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Competing interests YT has received consultant fees from Mitsubishi-Tanabe Pharma, Pfizer Inc; lecture fees from Mitsubishi-Tanabe Pharma, Takeda Pharmaceutical Co Ltd, Abbott, Eisai Pharma, Chugai Pharma. TT has received consultant fees from Mitsubishi-Tanabe Pharma, Wyeth Japan, Abbott, Eisai Pharma, Janssen Pharma, Chugai Pharma, Bristol-Myers-Squibb, Novartis; lecture fees from Mitsubishi-Tanabe Pharma, Takeda Pharmaceutical Co Ltd, Abbott, Eisai Pharma, Chugai Pharma. HK has received lecture fees from Mitsubishi-Tanabe Pharma, Centocor, Wyeth Japan, Takeda Pharmaceutical Co Ltd, Abbott, Eisai Pharma, Chugai Pharma. NM has received consultant fees from Mitsubishi-Tanabe Pharma; Abbott, Eisai Pharma, Janssen Pharma, Chugai Pharma, Bristol-Myers-Squibb; lecture fees from Mitsubishi-Tanabe Pharma, Takeda Pharmaceutical Co Ltd, Wyeth Japan, Abbott, Eisai Pharma, Chugai Pharma. TK has received consultant fees from Bristol-Myers-Squibb, Abbott; lecture fees from Mitsubishi-Tanabe Pharma, Takeda Pharmaceutical Co Ltd, Wyeth Japan, Abbott, Eisai Pharma, Chugai Pharma.

Patient consent Obtained.

Ethics approval This study is an observational study and is registered with the University Hospital Medical Information Network-Clinical trials Registry, number R000002571. Also, ethics committees of the participating centres approved the study protocol.

Provenance and peer review Not commissioned; externally peer reviewed.

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REFERENCES

1. **Klareskog L**, Catrina AI, Paget S. Rheumatoid arthritis. *Lancet* 2009;**373**:659–72.
2. **Emery P**, Breedveld FC, Hall S, *et al.* Comparison of methotrexate monotherapy with a combination of methotrexate and etanercept in active, early, moderate to severe rheumatoid arthritis (COMET): a randomised, double-blind, parallel treatment trial. *Lancet* 2008;**372**:375–82.
3. **Breedveld FC**, Weisman MH, Kavanaugh AF, *et al.* The PREMIER study: a multicenter, randomized, double-blind clinical trial of combination therapy with adalimumab plus methotrexate versus methotrexate alone or adalimumab alone in patients with early, aggressive rheumatoid arthritis who had not had previous methotrexate treatment. *Arthritis Rheum* 2006;**54**:26–37.
4. **Klareskog L**, van der Heijde D, de Jager JP, *et al*. Therapeutic effect of the combination of etanercept and methotrexate compared with each treatment alone in patients with rheumatoid arthritis: double-blind randomised controlled trial. *Lancet* 2004;**363**:675–81.
5. **St Clair EW**, van der Heijde DM, Smolen JS, *et al.* For the Active-Controlled Study of Patients Receiving Infliximab for the Treatment of Rheumatoid Arthritis of Early Onset Study Group. Combination of infliximab and methotrexate therapy for early rheumatoid arthritis: a randomized, controlled trial. *Arthritis Rheum* 2004;**50**:3432–43.
6. **Lipsky PE**, van der Heijde DM, St Clair EW, *et al*. For the Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. Infliximab and methotrexate in the treatment of rheumatoid arthritis. *N Engl J Med* 2000;**343**:1594–602.
7. **Yamanaka H**, Tanaka Y, Sekiguchi N, *et al*. Retrospective clinical study on the notable efficacy and related factors of infliximab therapy in a rheumatoid arthritis management group in Japan (RECONFIRM). *Mod Rheumatol* 2007;**17**:28–32.
8. **Tanaka Y**, Takeuchi T, Inoue E, *et al*. Retrospective clinical study on the notable efficacy and related factors of infliximab therapy in a rheumatoid arthritis management group in Japan: 1-year clinical and ragiographic outcomes (RECONFIRM-II). *Mod Rheumatol* 2008;**18**:146–52.
9. **Takeuchi T**, Yamanaka H, Inoue E, *et al*. Retrospective clinical study on the notable efficacy and related factors of infliximab therapy in a rheumatoid arthritis management group in Japan: one-year outcome of joint destruction (RECONFIRM-2J). *Mod Rheumatol* 2008;**18**:447–54.
10. **Nawata M**, Saito K, Nakayamada S, *et al*. Discontinuation of infliximab in rheumatoid arthritis patients in clinical remission. *Mod Rheumatol* 2008;**18**:460–4.
11. **Quinn MA**, Conaghan PG, O'Connor PJ, *et al*. Very early treatment with infliximab in addition to methotrexate in early, poor-prognosis rheumatoid arthritis reduces magnetic resonance imaging evidence of synovitis and damage, with sustained benefit after infliximab withdrawal: results from a twelve-month randomized, double-blind, placebo-controlled trial. *Arthritis Rheum* 2005;**52**:27–35.
12. **Goekoop-Ruiterman YP**, Vries-Bouwstra JK, Allaart CF, *et al*. Comparison of treatment strategies in early rheumatoid arthritis: a randomized controlled trial. *Ann Intern Med* 2007;**146**:406–15.
13. **van der Kooij SM**, le Cessie S, Goekoop-Ruiterman YP, *et al*. Clinical and radiological efficacy of initial vs delayed treatment with infliximab plus methotrexate in patients with early rheumatoid arthritis. *Ann Rheum Dis* 2009;**68**:1153–8.
14. **Goekoop-Ruiterman YP**, de Vries-Bouwstra JK, Allaart CF, *et al*. Clinical and radiographic outcomes of four different treatment strategies in patients with early rheumatoid arthritis (the BeSt study): a randomized, controlled trial. *Arthritis Rheum* 2008;**58**:S126–35.
15. **van der Bijl AE**, Goekoop-Ruiterman YP, de Vries-Bouwstra JK, *et al*. Infliximab and methotrexate as induction therapy in patients with early rheumatoid arthritis. *Arthritis Rheum* 2007;**56**:2129–34.
16. **Goekoop-Ruiterman YP**, de Vries-Bouwstra JK, Kerstens PJ, *et al*. DAS-driven therapy versus routine care in patients with recent-onset active rheumatoid arthritis. *Ann Rheum Dis* 2010;**69**:65–9.
17. **Miyasaka N**, Takeuchi T, Eguchi K. Proposed (corrected) Japanese guidelines for the use of infliximab for rheumatoid arthritis. *Mod Rheumatol* 2005;**15**:4–8.
18. **Arnett FC**, Edworthy SM, Bloch DA, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;**31**:315–24.
19. **van der Heijde DMPM**, van 't Hof MA, van Riel PLCM, *et al*. A comparison of validity of single variables and composed indices for measuring disease activity in rheumatoid arthritis. *Ann Rheum Dis* 1992;**51**:177–81.
20. **van der Heijde D**. Radiographic progression in rheumatoid arthritis: does it reflect outcome? Does it reflect treatment? *Ann Rheum Dis* 2001;**60**(Suppl 3):iii47–50.
21. **van der Heijde D**. How to read radiographs according to the Sharp/van der Heijde method. *J Rheumatol* 2000;**27**:261–3.
22. **Takeuchi T**, Tatsuki Y, Nogami Y, *et al*. Postmarketing surveillance of the safety profile of infliximab in 5000 Japanese patients with rheumatoid arthritis. *Ann Rheum Dis* 2008;**67**:189–94.

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Mast Cell–Derived Tryptase Inhibits Apoptosis of Human Rheumatoid Synovial Fibroblasts via Rho-Mediated Signaling

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Objective. **An abundance of mast cells are found in the synovium of patients with rheumatoid arthritis (RA). However, the role of mast cells in the pathogenesis of RA remains unclear. This study was undertaken to elucidate a role for mast cells in RA by investigating the antiapoptotic effects of tryptase, a major product of mast cells, on RA synovial fibroblasts (RASFs).**

Methods. **RA synovial tissue was obtained from RA patients during joint replacement surgery, and histologic changes in the tissue were examined. The expression of cell surface molecules and apoptotic markers on RASFs were detected by flow cytometry. Rho activation was determined using a pull-down assay.**

Results. **Mast cells, bearing both c-Kit and tryptase, accumulated in the sublining area of proliferating synovial tissue from RA patients. Protease-activated receptor 2 (PAR-2), a receptor for tryptase, was expressed on RASFs in the lining area, close to tryptase-positive mast cells in the RA synovium. Fas-mediated apoptosis of RASFs was significantly inhibited, in a dose-dependent manner, by the addition of tryptase, and this effect correlated with increased activation of Rho kinase. Furthermore, Y27632, a Rho kinase inhibitor, reduced the antiapoptotic effect of tryptase on RASFs, suggesting that Rho was responsible for the antiapoptotic effects of tryptase.**

Conclusion. **These results demonstrate that tryptase has a strong antiapoptotic effect on RASFs through the activation of Rho. Thus, we propose that the release of tryptase by mast cells leads to the binding of tryptase to PAR-2 on RASFs and inhibits the apoptosis of RASFs via the activation of Rho. Such mechanisms could play a pivotal role in the marked proliferation of RASFs and hyperplasia of synovial tissue seen in RA synovium.**

Rheumatoid arthritis (RA) is an inflammatory disease that is characterized by persistent joint inflammation, eventually leading to destruction of the joints, which results in significant impairment of daily activity. In addition to decreased mobility, joint destruction causes tenderness and pain, and the quality of life and life expectancy of RA patients is drastically reduced compared with that of healthy subjects (1). The recent emergence of biologic drugs that target inflammatory cytokines, including tumor necrosis factor α (TNFα), has greatly improved the treatment of RA. Despite such advances, the RA remission rate still remains low, at only 30–40%. Thus, further understanding of the pathogenesis of RA in order to yield new perspectives on RA treatment is necessary in those cases in which the current therapeutic strategy is insufficient.

In the joints of RA patients, a proliferating mass in the synovium, known as pannus, covers the RA joint cartilage and contributes to joint erosion and fibrous ankylosis. Pannus consists of granulation tissue and proliferating synovial fibroblasts (SFs), accompanied by neoangiogenesis and inflammatory cell infiltrates (2). A main contributor to joint destruction is the RASF, which

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multiplies in the same manner as neoplastic cells and infiltrates into cartilage and bone, resulting in tissue destruction. However, it is puzzling that RASFs proliferate excessively in vivo despite a high level of expression of Fas and sensitivity to Fas-mediated killing in vitro (2,3). Thus, it is possible that RA joint cartilage contains a mechanism for the suppression of apoptosis of SFs, which leads to inappropriate SF hyperplasia.

We have previously reported that mast cells may be an effective therapeutic target in the treatment of chronic inflammation in RA (4). Such notions stem from the observation that an abundance of mast cells is present in the synovial tissue of RA patients compared with those with other joint diseases (5). Mast cells appear to contribute to RA pathology in mouse studies, as was shown in a mouse model of autoantibody-induced arthritis in which mast cell–deficient mice exhibited attenuated joint inflammation (6). Moreover, mast cells produce cytokines that are of great relevance in RA, including TNFα and interleukin-1 (7). Thus, although the specific details remain unclear (8), it is conceivable that mast cells also play an important role in the pathogenesis of human RA.

In the present study, we hypothesized that mast cells may contribute to the pathogenesis of RA by inhibiting the apoptosis of RASFs. The results demonstrate that mast cells are found in close proximity to RASFs in the synovium of RA patients. Furthermore, RASFs express the receptor for mast cell tryptase, known as protease-activated receptor 2 (PAR-2), and are protected from Fas-mediated apoptosis by tryptase in a Rho GTPase–dependent manner. We propose that such mechanisms could play a pivotal role in the marked proliferation of RASFs and hyperplasia seen in RA synovium.

PATIENTS AND METHODS

Human studies. The study protocol was approved by the Human Ethics Review Committee of the University of Occupational and Environmental Health in Japan. Signed informed consent was obtained from each subject involved in this study.

Synovial tissue and culture of SFs. Synovial tissue was obtained from 5 women (ages 47–60 years) with active RA, whose disease had been diagnosed according to the criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (9) and who had undergone joint replacement surgery. All enrolled patients had >6 swollen joints, >3 tender joints, and an erythrocyte sedimentation rate (Westergren) of >28 mm/hour.

Synovial tissue samples were dissected under sterile conditions in phosphate buffered saline, and fibroblast-like synovial cells were isolated and cultured. Briefly, the tissue samples were minced into small pieces and digested with collagenase (Sigma-Aldrich, Tokyo, Japan) in serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY). The cells were filtered through a nylon mesh, washed extensively, and suspended in DMEM supplemented with 10% fetal calf serum (FCS; Bio-Pro, Karlsruhe, Germany) and streptomycin/penicillin (10 units/ml; Sigma-Aldrich). Finally, isolated cells were seeded in tissue culture flasks (Falcon, Lincoln Park, NJ), and nonadherent cells were removed. The medium was changed biweekly, and the cells were used after 5 passages.

The resulting synovial cells were spindle-shaped and grew in a cobblestone pattern. Flow cytometric analysis of these cells indicated that they lacked macrophage markers, such as class II major histocompatibility complex, CD14, and CD11b (results not shown). Thus, the RA synovial cells obtained appeared to represent type B synovial fibroblast-like cells.

Reagents. Human β-tryptase was purchased from Promega (Madison, WI). The following monoclonal antibodies (mAb) were used: fluorescein isothiocyanate–conjugated control mAb anti-Thy1.2 (Becton Dickinson, San Jose, CA) and anti-human PAR-2 mAb (R&D Systems, Minneapolis, MN). A Rho activation kit containing glutathione S-transferase–Rhotekin-Rho binding domain (GST-RBD) beads was purchased from Cytoskeleton (Denver, CO).

Immunohistochemistry. Synovial tissue was stained as previously described (10). Briefly, sections (6 μm) were fixed in ice-cold acetone, and endogenous peroxidase was quenched with 3% H_2O_2/methanol. Sections were incubated with blocking buffer and then with the SAM-11 mAb (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 μg/ml for 12 hours at 4°C. SAM-11 is a previously characterized (11) highly specific mAb to human PAR-2 that binds the membrane-bound part of the receptor, in both cleaved and uncleaved states. Endogenous biotin was blocked using an avidin–biotin kit (Vector, Peterborough, UK), and biotinylated secondary antibody (Autogen Bioclear, Wiltshire, UK) was then applied, followed by the addition of peroxidase-conjugated streptavidin. Antigen–antibody complexes were visualized utilizing 3,3'-diaminobenzidine. Sections were also probed with an antibody specific for mast cell tryptase (Dako, Ely, UK).

Flow cytometry. Staining and flow cytometric analyses of RASFs were performed using a FACScan (BD PharMingen, San Diego, CA) and standard procedures as described elsewhere (12). The RASFs (2×10^5 cells) were incubated with a negative control antibody (mAb anti-Thy1.2; Becton Dickinson) or phycoerythrin-conjugated anti–PAR-2 mAb (Mouse-Mono 344222; R&D Systems) in fluorescence-activated cell sorting (FACS) medium consisting of Hanks' balanced salt solution (Nissui, Tokyo, Japan), 0.5% human serum albumin (Yoshitomi, Osaka, Japan), and 0.2% NaN_3 (Sigma, St. Louis, MO) for 30 minutes at 4°C. After washing the cells 3 times with FACS medium, the fluorescence intensity was detected using a FACScan.

Apoptosis assay. Apoptosis was evaluated by flow cytometry utilizing annexin V binding (Annexin V–Fluorescein Isothiocyanate Apoptosis Detection Kit I; Becton Dickinson). Briefly, RASFs were cultured under starved conditions for 24 hours with 1% DMEM, and were then incubated with or

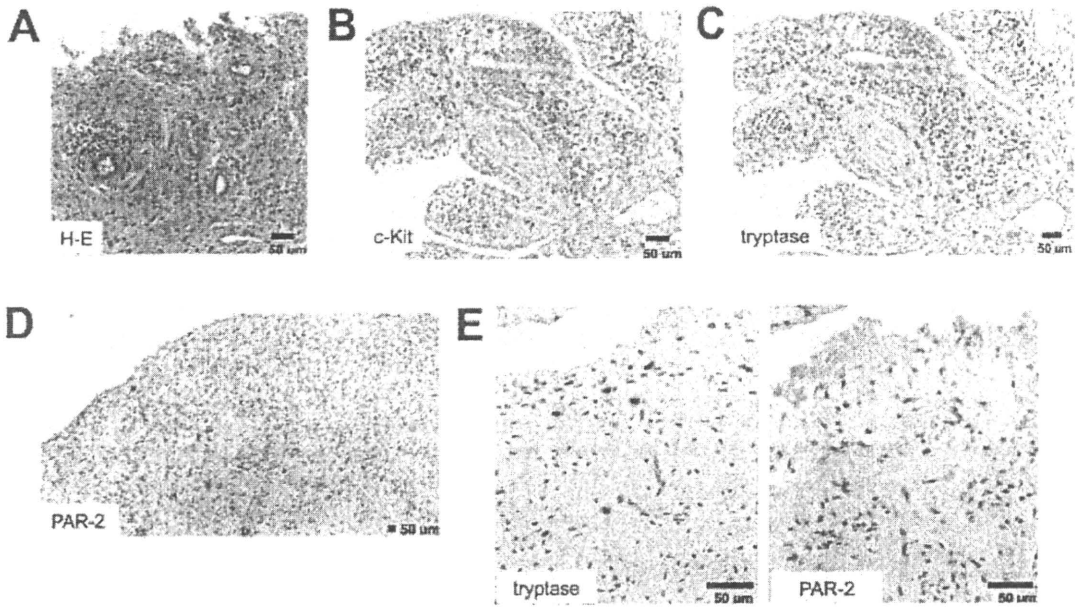

Figure 1. Detection of mast cells and protease-activated receptor 2 (PAR-2)–expressing synovial fibroblasts in rheumatoid arthritis (RA) synovial tissue. **A–D,** Synovial tissue specimens (surgically removed basal layer) from a patient with RA were stained with hematoxylin and eosin (H&E) **(A)** or subjected to immunohistochemical staining using an anti–c-Kit antibody **(B)**, antitryptase antibody **(C)**, or anti–PAR-2 antibody **(D)**. **E,** In synovial tissue from the same patient, immunohistochemical staining was performed on serial sections using antitryptase antibody **(left)** and anti–PAR-2 antibody **(right)**. Representative results from 1 of 5 RA patients are shown.

without CH11 (1 μg/ml), tryptase (1–4 μg/ml), and Y27632 (0.1–10 μ*M*; Calbiochem, La Jolla, CA) for 12 hours in DMEM containing 1% FCS. In some experiments, E11 fibroblasts (immortalized RASF cell line [13]) were treated with CH11 (1 μg/ml) with or without tryptase (2 μg/ml) and/or nafamostat mesylate (1 n*M*; Tocris Biosciences, Ellsville, MO) for 12 hours in DMEM containing 10% FCS. Cells were then stained with annexin V and propidium iodide (PI), according to the manufacturer's instructions, and analyzed using a FACScan flow cytometer (Becton Dickinson). All PI-positive cells were considered dead. PI-negative and annexin V–positive cells were considered early apoptotic cells, and the remaining double-negative cells were considered viable.

Rho activation assay. Rho activation was determined with the use of a pull-down assay with GST-RBD beads (14,15). RASFs were stimulated with 0.1–2 μg/ml tryptase, quickly washed with ice-cold Tris buffered saline, and lysed in 500 μl of lysis buffer (50 m*M* Tris, pH 7.5, 10 m*M* $MgCl_2$, 0.5*M* NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 500 μg/ml tosyl arginine methyl ester, and 10 μg/ml each of leupeptin and aprotinin). Cell lysates were immediately centrifuged at 8,000 revolutions per minute at 4°C for 5 minutes, and equal volumes of lysates were incubated with 30 μg GST-RBD beads for 1 hour at 4°C. The beads were washed twice with wash buffer (25 m*M* Tris, pH 7.5, 30 m*M* $MgCl_2$, 40 m*M* NaCl), and bound Rho was eluted by boiling each sample in Laemmli sample buffer. Eluted samples from the beads and total cell lysate were then electrophoresed on 12% SDS–polyacrylamide electrophoresis gels, transferred to nitrocellulose, blocked with 5% nonfat milk, and analyzed by Western blotting using a polyclonal anti-Rho antibody.

Statistical analysis. Results are expressed as the mean ± SD. Differences in comparison with the control group were examined for statistical significance by the Mann-Whitney U test. *P* values less than 0.01 were considered statistically significant.

RESULTS

Detection of mast cells in close proximity to PAR-2–expressing SFs in RA synovial tissue. Synovial tissue specimens were surgically removed from the joints of patients with RA, and the samples were used to investigate the localization of mast cells and SFs in the synovial tissue. Histologic examination of the tissue by hematoxylin and eosin staining revealed the presence of pannus, represented by detection of SFs, inflammatory cell infiltrates, and finer vessels (Figure 1A). Moreover, immunohistochemical staining of the tissue samples demonstrated numerous c-Kit–positive cells (Figure 1B)

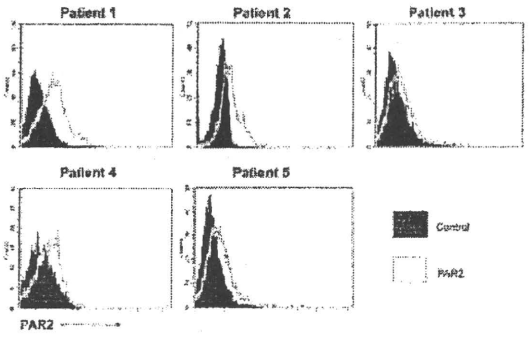

Figure 2. Expression of protease-activated receptor 2 (PAR-2) by isolated rheumatoid arthritis synovial fibroblasts (RASFs) from the synovial tissue of 5 patients with RA. RASFs were labeled with an antibody against PAR-2, in comparison with an isotype control, and the fluorescence intensity in 5 separate RA patient samples was measured by flow cytometry.

and tryptase-positive cells (Figure 1C), which are characteristic of mast cells, in the pannus and sublining area. This suggested that large numbers of mast cells are present in hyperplastic synovial tissue.

Since we hypothesized that tryptase, a mast cell–specific protease, may be involved in the pathogenesis of RA, we next determined which cells could respond to tryptase, by examining the expression of PAR-2, the receptor for tryptase. PAR-2 was expressed in spindle-shaped cells, most likely representing RASFs, which were present in the lining area (Figure 1D). Notably, in serial sections, PAR-2–expressing cells were found in close proximity to the area in which tryptase was expressed (Figure 1E). Similar results were obtained in synovial tissue samples from 5 other patients with RA (results not shown).

To verify that PAR-2 is expressed on RA fibroblasts, SFs were isolated from the RA synovial tissue samples and PAR-2 expression was detected with the use of flow cytometry. Consistent with the findings on immunohistochemical analysis, flow cytometry revealed the expression of PAR-2 on isolated RASFs obtained from 5 separate RA patients (Figure 2), thus confirming that PAR-2 is expressed on RASFs.

Inhibition of anti-Fas antibody–induced cell death by tryptase in RASFs. We previously reported that RASFs express Fas and are susceptible to Fas-induced cell death (3). Nevertheless, in the synovial tissue of RA patients, RASFs proliferate, rather than undergo apoptosis, suggesting that there might be a mechanism that prevents RASFs from undergoing apoptosis in situ. Given that mast cells lie in close proximity to RASFs and that RASFs express PAR-2, we questioned whether a mast cell–specific PAR-2 activator such as tryptase would suppress apoptosis induction in RASFs. To test this notion, RASFs were treated with or without anti-Fas antibody (CH11) in the presence or absence of tryptase, under starved conditions. As expected, cell death was morphologically apparent and increased 12 hours after incubation with CH11, as compared with that in cultures with untreated cells (Figure 3). In contrast, the addition of tryptase significantly inhibited such morphologic changes in the RASFs (Figure 3).

To enumerate the proportion of live cells remaining in each well, the cells were removed from the wells with the use of trypsin, and live cells were counted using trypan blue exclusion. Compared with untreated cells, a significant decrease in the proportion of live cells was found in cells treated with CH11, which was reversed by the addition of tryptase (Figure 3). Of note, cell death was also observed in ~50% of the untreated cells, which was attributable to the starved culture conditions necessary to make the cells more sensitive to CH11-induced apoptosis.

To confirm the results obtained by trypan blue exclusion, we next used a flow cytometric approach involving annexin V and PI staining to detect apoptotic cells. The fraction of PI^{low}annexin V^{high} cells (early apoptotic) and PI^{high}annexin V^{high} cells (late apoptotic) increased after treatment of RASFs with CH11 (Figure

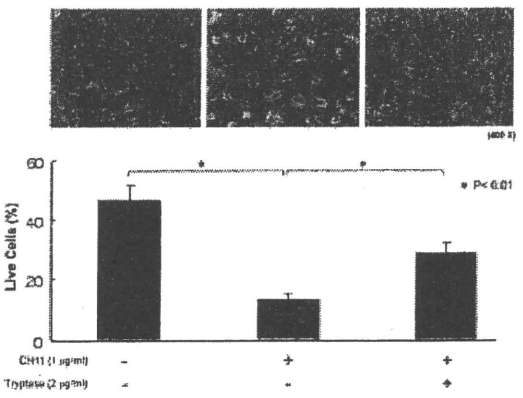

Figure 3. Inhibition of anti-Fas antibody–mediated apoptosis of rheumatoid arthritis synovial fibroblasts (RASFs) by tryptase. **Top,** RASFs were left untreated (**left**) or treated with anti-Fas antibody in the absence (**middle**) or presence (**right**) of tryptase. Cells were observed using an inverted light microscope 12 hours after treatment (original magnification × 400). **Bottom,** The total number of viable cells was determined by counting the number of live cells on trypan blue staining. Bars show the mean and SD percentage of live cells.