(MMP)-3, MMP-9, and MMP-13, in streptococcal cell wall-induced arthritis (43). IL-17R-deficient (IL-17R$^{-/-}$) mice that were locally injected five times with streptococcal cell wall fragments into the knee joints showed a significant reduction of joint thickness and cartilage damage that was accompanied by reduced synovial expression of IL-1, IL-6, and the MMPs 3, 9, and 13 compared with arthritic wild-type mice. Therefore, these results indicate the critical role of IL-17R signaling during progression from an acute, macrophage-driven joint inflammation to a chronic, cartilage-destructive, T cell-mediated synovitis. There are four additional receptor-like molecules that share homology to IL-17R, i.e., IL-17Rh1 (also named IL-17RB or IL-17BR), IL-17RL (also named IL-17RC), IL-17RD, and IL-17RE. IL-17Rh1 was shown to bind to IL-17B, but with higher affinity to IL-17E (11, 12).

Although IL-17A transgenic mice have been reported to be embryonic lethal (39), we established BM-overexpressing mice that constitutively expressed IL-17A. The adequate control of the expression level was critically important. In our experiment, the serum concentration of IL-17A was elevated to ~600 pg/ml in IL-17A BM chimeric mice. This serum concentration of IL-17A was similar to those in patients with inflammatory diseases such as RA, inflammatory bowel diseases, familial Mediterranean fever, and the acute stage of Kawasaki disease (3, 44–46). Therefore, our BM chimeric mice approach may be useful to elucidate the physiological role of inflammatory cytokines that show lethal phenotypes in the conventional gene-transgenic technique.

In conclusion, we found that IL-17 family genes were up-regulated in association with their receptors in CIA. Each of the IL-17 family members clearly exacerbated the progression of CIA with the method of retrovirus-mediated BM chimeric mice. IL-17B and IL-17C have the capacity to exacerbate inflammatory arthritis in association with increased TNF-α and IL-6 productions from macrophages. Moreover, neutralization of IL-17B significantly suppressed the progression of arthritis and bone destruction in CIA mice. Therefore, our results suggest that not only IL-17A, but also the IL-17 family members IL-17B, IL-17C, and IL-17F play an important role in the pathogenesis of inflammatory arthritis and should be a new therapeutic target of arthritis.

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Disclosures

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References

1. Yao, Z., W. C. Fanslow, M. F. Seldin, A. M. Rousseau, S. L. Painter, M. R. Comeau, J. I. Cohen, and M. K. Spriggs. 1995. Herpesvirus Saimiri encodes a new cytokine: IL-17, which binds to a novel cytokine receptor. *Immunity* 3: 811–821.
2. Yao, Z., S. L. Painter, W. C. Fanslow, D. Ulrich, B. M. Macduff, M. K. Spriggs, and R. J. Armitage. 1995. Human IL-17: a novel cytokine derived from T cells. *J. Immunol.* 155: 5483–5486.
3. Ziolkowska, M., A. Koc, G. Luszczykiewicz, K. Ksiezopolska-Pietrzak, E. Klimczak, H. Chwalinska-Sadowska, and W. Maslinski. 2000. High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. *J. Immunol.* 164: 2832–2838.
4. Jovanovic, D. V., J. A. Di Battista, J. Martel-Pelletier, F. C. Jolicoeur, Y. He, M. Zhang, F. Mineau, and J. P. Pelletier. 1998. IL-17 stimulates the production and expression of proinflammatory cytokines IL-β and TNF-α, by human macrophages. *J. Immunol.* 160: 3513–3521.
5. Chabaud, M., F. Fossiez, J. L. Taupin, and P. Miossec. 1998. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *J. Immunol.* 161: 409–414.
6. Katz, Y., O. Nadiv, and Y. Beer. 2001. Interleukin-17 enhances tumor necrosis factor α-induced synthesis of interleukins 1, 6, and 8 in skin and synovial fibroblasts: a possible role as a "fine-tuning cytokine" in inflammation processes. *Arthritis Rheum.* 44: 2176–2184.
7. Lubberts, E., L. A. Joosten, B. Oppers, L. van den Bersselaar, C. J. Coenen-de Roo, J. K. Kolls, P. Schwarzenberger, F. A. van de Loo, and W. B. van den Berg. 2001. IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis. *J. Immunol.* 167: 1004–1013.
8. Lubberts, E., M. I. Koenders, B. Oppers-Walgreen, L. van den Bersselaar, C. J. Coenen-de Roo, L. A. Joosten, and W. B. van den Berg. 2004. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. *Arthritis Rheum.* 50: 650–659.
9. Nakae, S., S. Saijo, R. Horai, K. Sudo, S. Mori, and Y. Iwakura. 2003. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. *Proc. Natl. Acad. Sci. USA* 100: 5986–5990.
10. Koenders, M. I., E. Lubberts, F. A. van de Loo, B. Oppers-Walgreen, L. van den Bersselaar, M. M. Helsen, J. K. Kolls, F. E. Di Padova, L. A. Joosten, and W. B. van den Berg. 2006. Interleukin-17 acts independently of TNF-α under arthritic conditions. *J. Immunol.* 176: 6262–6269.
11. Moseley, T. A., D. R. Haudenschild, L. Rose, and A. H. Reddi. 2003. Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev.* 14: 155–174.
12. Kolls, J. K., and A. Linden. 2004. Interleukin-17 family members and inflammation. *Immunity* 21: 467–476.
13. Starnes, T., M. J. Robertson, G. Sledge, S. Kelich, H. Nakshatri, H. E. Broxmeyer, and R. Hromas. 2001. Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J. Immunol.* 167: 4137–4140.
14. Aggarwal, S., N. Ghilardi, M. H. Xie, F. J. de Sauvage, and A. L. Gurney. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* 278: 1910–1914.
15. Happel, K. I., P. J. Dubin, M. Zheng, N. Ghilardi, C. Lockhart, L. J. Quinton, A. R. Odden, J. E. Shellito, G. J. Bagby, S. Nelson, and J. K. Kolls. 2005. Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*. *J. Exp. Med.* 202: 761–769.
16. Shi, Y., S. J. Ullrich, J. Zhang, K. Connolly, K. J. Grzegorzewski, M. C. Barber, W. Wang, K. Wathen, V. Hodge, C. L. Fisher, et al. 2000. A novel cytokine receptor-ligand pair: identification, molecular characterization, and in vivo immunomodulatory activity. *J. Biol. Chem.* 275: 19167–19176.
17. Li, H., J. Chen, A. Huang, J. Stinson, S. Heldens, J. Foster, P. Dowd, A. L. Gurney, and W. I. Wood. 2000. Cloning and characterization of IL-17B and IL-17C, two new members of the IL-17 cytokine family. *Proc. Natl. Acad. Sci. USA* 97: 773–778.
18. Hurst, S. D., T. Muchamuel, D. M. Gorman, J. M. Gilbert, T. Clifford, S. Kwan, S. Menon, B. Seymour, C. Jackson, T. T. Kung, et al. 2002. New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. *J. Immunol.* 169: 443–453.
19. Chabaud, M., J. M. Durand, N. Buchs, F. Fossiez, G. Page, L. Frappart, and P. Miossec. 1999. Human interleukin-17: a T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum.* 42: 963–970.
20. Hymowitz, S. G., E. H. Filvaroff, J. P. Yin, J. Lee, L. Cai, P. Risser, M. Maruoka, W. Mao, J. Foster, R. F. Kelley, et al. 2001. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J.* 20: 5332–5341.
21. Hwang, S. Y., and H. Y. Kim. 2005. Expression of IL-17 homologs and their receptors in the synovial cells of rheumatoid arthritis patients. *Mol. Cell* 19: 180–184.
22. Nasu, K., H. Kohsaka, Y. Nonomura, Y. Terada, H. Ito, K. Hirokawa, and N. Miyasaka. 2000. Adenoviral transfer of cyclin-dependent kinase inhibitor genes suppresses collagen-induced arthritis in mice. *J. Immunol.* 165: 7246–7252.
23. Trentham, D. E., A. S. Townes, and A. H. Kang. 1977. Autoimmunity to type II collagen an experimental model of arthritis. *J. Exp. Med.* 146: 857–868.
24. Stuart, J. M., A. S. Townes, and A. H. Kang. 1982. Nature and specificity of the immune response to collagen in type II collagen-induced arthritis in mice. *J. Clin. Invest.* 69: 673–683.
25. Gerlag, D. M., L. Ransone, P. P. Tak, Z. Han, M. Palanki, M. S. Barbosa, D. Boyle, A. M. Manning, and G. S. Firestein. 2000. The effect of a T cell-specific NF-κB inhibitor on in vitro cytokine production and collagen-induced arthritis. *J. Immunol.* 165: 1652–1658.
26. Nanki, T., Y. Urasaki, T. Imai, M. Nishimura, K. Muramoto, T. Kubota, and N. Miyasaka. 2004. Inhibition of fractalkine ameliorates murine collagen-induced arthritis. *J. Immunol.* 173: 7010–7016.
27. Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* 139: 834–850.
28. Cheng, E. H., M. C. Wei, S. Weiler, R. A. Flavell, T. W. Mak, T. Lindsten, and S. J. Korsmeyer. 2001. BCL-2, Bcl-x_L sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol. Cell* 8: 705–711.
29. Morita, S., T. Kojima, and T. Kitamura. 2000. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* 7: 1063–1066.
30. Fujio, K., Y. Misaki, K. Setoguchi, S. Morita, K. Kawahata, I. Kato, T. Nosaka, K. Yamamoto, and T. Kitamura. 2000. Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the $\alpha\beta$ TCR complex. *J. Immunol.* 165: 528–532.
31. Fujio, K., A. Okamoto, H. Tahara, M. Abe, Y. Jiang, T. Kitamura, S. Hirose, and K. Yamamoto. 2004. Nucleosome-specific regulatory T cells engineered by triple gene transfer suppress a systemic autoimmune disease. *J. Immunol.* 173: 2118–2125.

32. McGaha, T. L., B. Sorrentino, and J. V. Ravetch. 2005. Restoration of tolerance in lupus by targeted inhibitory receptor expression. *Science* 307: 590–593.
33. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\delta\delta C_T)$ method. *Methods* 25: 402–408.
34. Ferreira, I. D., V. E. Rosario, and P. V. Cravo. 2006. Real-time quantitative PCR with SYBR Green I detection for estimating copy numbers of nine drug resistance candidate genes in *Plasmodium falciparum*. *Malar J.* 5: 1.
35. Corthay, A., A. Johansson, M. Vestberg, and R. Holmdahl. 1999. Collagen-induced arthritis development requires $\alpha\beta$ T cells but not $\gamma\delta$ T cells: studies with T cell-deficient (TCR mutant) mice. *Int. Immunol.* 11: 1065–1073.
36. Glansbeek, H. L., P. M. van der Kraan, F. P. Lafeber, E. L. Vitters, and W. B. van den Berg. 1997. Species-specific expression of type II TGF-β receptor isoforms by articular chondrocytes: effect of proteoglycan depletion and aging. *Cytokine* 9: 347–351.
37. Taniguchi, K., H. Kohsaka, N. Inoue, Y. Terada, H. Ito, K. Hirokawa, and N. Miyasaka. 1999. Induction of the p16INK4a senescence gene as a new therapeutic strategy for the treatment of rheumatoid arthritis. *Nat. Med.* 5: 760–767.
38. Honorati, M. C., R. Meliconi, L. Pulsatelli, S. Cane, L. Frizziero, and A. Facchini. 2001. High in vivo expression of interleukin-17 receptor in synovial endothelial cells and chondrocytes from arthritis patients. *Rheumatology* 40: 522–527.
39. Schwarzenberger, P., V. La Russa, A. Miller, P. Ye, W. Huang, A. Zieske, S. Nelson, G. J. Bagby, D. Stoltz, R. L. Mynatt, et al. 1998. IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *J. Immunol.* 161: 6383–6389.
40. Lubberts, E., L. van den Bersselaar, B. Oppers-Walgreen, P. Schwarzenberger, C. J. Coenen-de Roo, J. K. Kolls, L. A. Joosten, and W. B. van den Berg. 2003. IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-κB ligand/osteoprotegerin balance. *J. Immunol.* 170: 2655–2662.
41. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-β induces development of the T_H17 lineage. *Nature* 441: 231–234.
42. Hunter, C. A. 2005. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat. Rev. Immunol.* 5: 521–531.
43. Koenders, M. I., J. K. Kolls, B. Oppers-Walgreen, L. van den Bersselaar, L. A. Joosten, J. R. Schurr, P. Schwarzenberger, W. B. van den Berg, and E. Lubberts. 2005. Interleukin-17 receptor deficiency results in impaired synovial expression of interleukin-1 and matrix metalloproteinases 3, 9, and 13 and prevents cartilage destruction during chronic reactivated streptococcal cell wall-induced arthritis. *Arthritis Rheum.* 52: 3239–3247.
44. Fujino, S., A. Andoh, S. Bamba, A. Ogawa, K. Hata, Y. Araki, T. Bamba, and Y. Fujiyama. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 52: 65–70.
45. Haznedaroglu, S., M. A. Ozturk, B. Sancak, B. Goker, A. M. Onat, N. Bukan, I. Ertenli, S. Kiraz, and M. Calguneri. 2005. Serum interleukin 17 and interleukin 18 levels in familial Mediterranean fever. *Clin. Exp. Rheumatol.* 23: S77–S80.
46. Sohn, M. H., S. Y. Noh, W. Chang, K. M. Shin, and D. S. Kim. 2003. Circulating interleukin 17 is increased in the acute stage of Kawasaki disease. *Scand. J. Rheumatol.* 32: 364–366.

Expert Opinion

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Antigen-specific immunotherapy for autoimmune diseases

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The status of autoimmune disease therapies is not satisfactory. Antigen-specific immunotherapy has potential as a future therapy that could deliver maximal efficacy with minimal adverse effects. Several trials of antigen-specific immunotherapy have been performed, but so far no clear directions have been established. With regard to antigen-specificity in the immune system, T cells are essential components. However, at present, we do not have a sufficient range of strategies for manipulating antigen-specific T cells. In this review, the authors propose that T cell receptor gene transfer could be used for antigen-specific immunotherapy. In the proposed technique, important disease-related and, thus, antigen-specific T cells in patients would first be identified, and then a pair of cDNAs encoding α and β T cell receptors would be isolated from these single T cells. These genes would then be transferred into self lymphocytes. These engineered antigen-specific cells can also be manipulated to express appropriate functional genes that could then be applied to specific immunotherapy.

Keywords: autoimmune diseases, antigen-specific T cells, gene transfer, T cell receptor

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1. Introduction

Autoimmune diseases are fairly common disorders affecting ~ 5% of the population, predominantly women [1]. Existing treatment of autoimmune diseases is based mainly on the use of immunosuppressive drugs such as corticosteroids and cytotoxic reagents. These treatments reduce mortality and significantly lengthen the life expectations of patients in some diseases. However, because these drugs suppress overall immune reactions, they have several serious adverse effects. In this regard, selective immunotherapies are considered more promising. As cytokines are known to play a pivotal role in regulating immune reactions, the application of cytokines to control autoimmune diseases has been extensively studied. Systemic administration of suppressive cytokines, such as transforming growth factor-β, interleukin (IL)-4 and IL-10, has served as an effective therapy in models of autoimmune diseases. Some of these protocols seem to work by shifting the balance of cytokines; however, systemic cytokine therapy potentially leads to deleterious side effects, as in the case of recombinant human IL-4 [2]. Therapies neutralizing a certain cytokine have been successful and will serve as important strategies in many autoimmune diseases in the near future; however, because such strategies still have important drawbacks [3], it is also necessary to explore other specific immunotherapies.

In a variety of autoimmune diseases, trials to identify molecules recognized by T cells and autoantibodies have been extensively performed. These efforts are essential not only for understanding the pathogeneses of autoimmune diseases, but also for establishing antigen-specific immunotherapies. Although knowledge of target antigens in several autoimmune diseases has greatly increased, our ability to selectively silence particular pathogenic immune responses has not. One of the

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feasible approaches to treat autoimmunity is suppression of the activation and expansion of antigen-specific T cells before they differentiate into pathogenic T cells. This approach could be useful because once the pathogenic responses are established, intervention appears to be less effective on activated T cells subsets. However, the majority of patients who require clinical treatment have full-blown autoimmune disease and this approach would not be useful in such cases.

It is generally believed that in autoimmune diseases, an immune response to a single epitope on a self antigen at the start of the disorder can trigger immune responses to neighboring epitopes on the same molecule or to other epitopes on related molecules. This is termed 'epitope spreading'. Although it is not clear whether the epitope spreading occurs throughout the autoimmune process, some researchers argue against antigen-specific immunotherapy because of the difficulties of predicting such expanding autoimmune reactions. In this article, the authors discuss the behavior of antigen-specific T cells in autoimmune diseases. The authors propose that epitope spreading is not the sole mechanism of the T cell related pathogenesis of autoimmune diseases and that clonal restriction of T cells occurs in the late phase of autoimmunity. Therefore, antigen-specific immunotherapy would be feasible even for established autoimmune diseases. T cell receptor (TCR) gene transfer could be one of the possible strategies.

2. Immunotherapies and targeted immunotherapy in autoimmune diseases

Several autoimmune diseases, and particularly systemic types of autoimmune diseases, such as systemic lupus erythematosus and systemic vasculitis syndromes, are treated with corticosteroids and immunosuppressive drugs. The latter include cytotoxic drugs such as alkylating agents and purine analogues, and calcineurin inhibitors, such as cyclosporin and tacrolimus. At present, these drugs offer the best chance of suppressing – or sometimes inducing the remission of – these diseases. However, they have potentially life-threatening side effects due to their severe depression of immune function. Furthermore, a serious complication among patients undergoing immunosuppressive therapy would be the risk of developing cancer [4]. Similarly, corticosteroids are commonly used to treat several autoimmune diseases. Corticosteroids inhibit prostaglandin synthesis, block cytokine secretion and T cell activation, and are one of the most effective therapies against autoimmune diseases. However, they also have several effects on physiological systems, and if used over the long term, they can cause profound immunosuppression with increased risk of infection, cancer, osteoporosis, hypertension and endocrine abnormalities.

Due to improvements in our understanding of immune reactions and advances in molecular biology, new biological agents – especially monoclonal antibodies – are now available for specific blockade of effector molecules such as inflammatory mediators. Administration of monoclonal antibody against tumor necrosis factor (TNF) (infliximab) or a soluble recombinant TNF receptor–immunoglobulin fusion protein (etanercept) led to the suppression of inflammation and a remarkable improvement of function of patients with rheumatoid arthritis (RA) [5]. However, there are several limitations to the use of anti-TNF therapy. In fact, some patients do not respond to these TNF inhibitors. Furthermore, the blockade in pro-inflammatory cytokines can put some individuals at increased risk of tuberculosis or other infections [6,7].

Apart from anticytokine therapies, several molecules have been investigated as possible targets of selective immunotherapy. For example, activated lymphocytes bind to specific receptors along the vessel walls to initiate the process of penetrating the target organ. Therefore, clinical trails with antibodies against such homing receptors appear to be promising. In fact, a humanized, monoclonal antibody, an α_4 integrin antagonist, has shown some degree of success in a placebo-controlled trial; however, there was an increased rate of infection in the treated patients [8]. A depletion of 95% of circulating lymphocytes in patients with multiple sclerosis (MS) by a monoclonal antibody against CD52 suppressed the disease activity of MS, but a third of patients developed autoimmune thyroid disease [9]. Cytotoxic T lymphocyte antigen (CTLA)-4Ig and a high-affinity mutant form, LEA29Y, are now in clinical trials in patients with autoimmunity [10]. These agents appear to work by blocking CD28 costimulation, leading to an inhibition of pathogenic T cell activation. However, it should be emphasized that a subset of regulatory T cells also depend on CD28/B7 interaction for their development and function. Furthermore, in the majority of autoimmune situations, effector T cells have already been established and are less dependent on costimulation for their activity effectiveness [11,12]. Thus, the situation is more complex. Recently, an approach for depleting B cells with antibodies against CD20 (rituximab) proved successful in several autoimmune disorders [13]. Further candidates for immunotherapy would include complement inhibitors [14] and Toll-like receptor modulations [15].

3. Trials of antigen-specific suppression in autoimmune diseases

Immunologists have tried to develop methods to treat autoimmune diseases by identifying and applying self-antigens, which are the target of autoimmune processes. In this regard, stimulation of T cells with the target antigen is an attractive direction. The spectrum of possible approaches involving T cell stimulation includes ablation of antigen-specific T cells, achieving specific T cell anergy, induction of regulatory T cells, and induction of a shift in the predominant phenotype of the antiself response from T helper (Th)1 to Th2 type. In fact, specific antigen

vaccination by administration of a target antigen in aqueous solution has been shown to significantly decrease the disease severity in several animal models. However, there is a high degree of anaphylactic sensitization in this method, making it difficult to directly apply it to human disorders [16,17]. Vaccination of antigen-coding DNA plasmids could bypass the immunological sensitization in protein administration. DNA-based immunotherapy, composed of unmethylated CpG repeats, is capable of inducing a shift in the cytokine profile and immune response that favours the Th1 arm. This observation makes DNA-based immunotherapy a promising candidate for the treatment of allergic diseases, which are known to be mediated by Th2-based responses [18]. DNA vaccination combined with a Th2 inducing costimulation is also effective in the treatment of several animal models of autoimmune diseases [19,20]. Although DNA vaccination appeared to have great potential as a safe and efficacious type of antigen-specific immunotherapy, the route of DNA administration and the combination of costimulation should be carefully examined before clinical application.

Oral antigen administration suppresses animal models of autoimmune diseases, including experimental autoimmune encephalitis, uveitis, arthritis and diabetes in the non-obese diabetic mouse [21]. Oral antigen induces antigen-specific Th2 and $CD4^+CD25^+$ regulatory cells. Based on the success in these animal experiments, oral tolerance has been examined in human autoimmune diseases, including MS, arthritis, uveitis and diabetes. Although positive results have been observed in Phase II trials, no effects were observed in Phase III trials of CII in RA or oral myelin and glatiramer acetate in MS [21,22]. Recently, oral insulin has prevented progression of immune-mediated (Type 1) diabetes [23]; however, further analysis of the immunological basis of oral tolerance is required for the effective therapy in human autoimmune diseases.

Altered peptide ligand (APL) is an antigenic peptide with amino acid modifications and is expected to block antigen-specific T cell responses by acting as a partial agonist or TCR antagonist, or by inducing regulatory T cell populations. In MS, myelin basic protein (MBP)-specific T cells are considered to be essential in the pathogenesis and MBP_{83-99} are estimated to be one candidate epitope. One APL has already been designed and submitted to a Phase II clinical trial; however, the treatment was poorly tolerated at the dose tested and the trial was halted. Some patients developed exacerbations of the disease due to the expansion of T cells specific for MBP_{83-99} by this APL [24]. A more sophisticated approach of antigen-specific T cells redirected against autoreactive T lymphocytes was reported [25]. However, it is not clear whether such methods could be used in the clinic.

Dendritic cells (DCs) are professional antigen-presenting cells with the potential to either stimulate or inhibit immune responses. DCs loaded with antigen can be used as a DC vaccine. Although most DC vaccines have been used to stimulate immune responses in patients with cancer [26], an increasing number of preclinical studies are focusing on the capacity of immature DCs to induce antigen-specific non-responsiveness [27-29]. DCs in the steady-state are immature and can induce tolerance in an antigen-specific manner. Immature DCs incubated with an agent, such as dexamethasone [30], vitamin D [31] or the Rel-B inhibitor [32], can induce tolerance. However, as discussed in cancer therapy, a careful study design incorporating standardized and quality-controlled clinical and immunological criteria is needed [33]. At present, we do not know exactly how robust the immunostimulatory DCs and tolerogenic DCs are.

Regulatory T cells are now recognized as one of the most central mechanisms of immune regulation. $CD4^+CD25^+$ T cells, in particular, have been shown to develop in the thymus or in the periphery to maintain the homeostatic equilibrium of immunity and tolerance [34]. Thus, researchers are now trying to expand these regulatory T cells and use them for the treatment of autoimmune diseases. However, this is not effective in some cases. For example, in the case of non-obese diabetic mice, which spontaneously develop diabetes, the suppression was relatively inefficient when heterogeneous regulatory T cells were simply expanded and adoptively transferred [35-37]. It appears that effective, regulatory T cell activity depends on both an appropriate phenotype and a high frequency of autoantigen specificity. Therefore, management to expand regulatory T cells in an antigen-specific manner would be required [34].

As discussed, regulatory cytokines and cytokine antagonists have been considered for the treatment of autoimmune diseases. However, systemic administration could potentially lead to deleterious side effects. Thus, local delivery of such molecules would be more efficient and eliminate the possible systemic side effects. In this regard, antigen-specific T cells or T cell hybridomas are believed to be suitable vehicles for targeted immunotherapies. In fact, the authors, as well as others, have reported T cell-mediated gene therapy for autoimmune animal models [38-41]. T cell-mediated, adoptive, cellular gene therapy is based on site-specific homing and retention of the vehicle, and local effects of the delivered effector molecules.

4. Evaluation of antigen-specific T cells in autoimmune diseases

With respect to the modes of autoimmune reactions, the idea of epitope spreading or determinant spreading has been widely accepted [42,43]. At the T cell level, this is a diversification of specificity from the initial, limited epitope-specific immune response to a hierarchical cascade of autoreactive T cell specificities. This mechanism may explain, for example, the pathway of infection induced autoimmunity. According to this idea, the initial phase of the autoimmune reaction might be carried out by a few activated T cells against limited numbers of epitopes. These T cells may be crossreactive T cells that recognize both microbial epitopes and self epitopes. However,

in the late phase of the disorders, the reactive epitopes might spread, and T cells recognizing a variety of different epitopes on several different self-molecules would be activated. However, if epitope spreading is the only mechanism involved in the T cell immune responses in autoimmune disorders, development of effective antigen-specific immunotherapies will be difficult, as target epitopes and molecules will always have the potential to spread and it would be difficult to define the pattern of spreading in a chronic human autoimmune disorder. The hierarchy of immune response to multiple tissue antigens would depend partially on individual HLA genotypes, but also on unknown factors and probably stochastic events.

In order to determine whether the phenomenon of epitope spreading is operative throughout the entire autoimmune process, it is important to detect how specific T cells behave within the lymphocyte population in the pathological lesions. Antigen-specific T cells should proliferate and form accumulated T cell clones in the heterogeneous lymphocyte population to exert their function. Therefore, evaluation of accumulated T cell clones in the pathological lesions would be informative. Several years ago, the authors' group established a system to analyze accumulated T cell clones in the lymphocyte population using reverse transcriptase polymerase chain reaction (RT-PCR) and single-strand conformation polymorphism (SSCP) on TCR messages [44]. In this system, a heterogeneous T cell population exhibits a smear pattern of amplified TCR messages. If there is an accumulation of certain T cell clones in the heterogeneous lymphocyte population, bands corresponding to each clone are observed in the background smear. Identification of accumulated T cell clones in different samples can be easily compared because the same clone exhibits the same electrophoretic mobility. Separation using cell surface phenotypes can be used to further identify disease- related important clones.

Using this system, the authors' group analyzed several synovial tissue samples of an RA patient. As a result, the same clones were found to exist in different joints [44,45]. These data clearly suggested that immune responses in RA were uniform throughout all of the arthritic lesions of the patient. In order to compare this finding in human samples with murine models, the authors' group analyzed several cases of spontaneous autoimmune models. For example, human T cell leukemia virus-1 env-pX transgenic mice exhibit spontaneous symmetrical arthritis similar to human RA [46]. In one study, the T cell clonality among different arthritic lesions in each stage was compared [47]. In the early stage, there were vigorous accumulations of T cells in the joints, but they were different among the different lesions. In the middle stage, several identical clones were found to be accumulated in the different lesions. In the late stage, the majority of the accumulated clones in one lesion were found to exist in the other lesions, suggesting that the autoimmune responses in the pathological lesions were rather uniform in the mouse. The number of dominant clones did not necessarily increase. The finding in the late stage was similar to what was observed in human RA synovial samples.

From the analyses of several spontaneous autoimmune animal models [48,49], it is now speculated that epitope spreading does not necessarily work in the late phase of the whole disease progression and it is possible that some form of clonal restriction of T cells occurs in autoimmune disorders. During the progression of autoimmune disease, immune response might become rather restricted to certain targets. Some restricted T cell clones directed towards such target self-antigens might be sustained. A similar, limited T cell oligoclonality as a 'driver clone' in autoimmunity was described in experimental autoimmune encephalomyelitis [50,51]. Oligoclonally expanded insulin-reactive T cells were also identified in the pancreatic draining lymph nodes of Type 1 diabetes patients with prolonged disease durations, [52]. It was reported that avidity maturation of a pathogenic T cell population may be the key event in the progression of benign inflammation to overt disease in autoimmunity [53] Therefore, an ideal way to control the disorder would be to suppress such sustained pathologic responses without globally interfering with the immunity of the host.

With respect to general immune responses to foreign antigens, T cell responses are reported to be dominated by few clonotypes that express a restricted set of TCRs [54]. This clonal selection and dominance may be due to the competitive advantages of the higher-affinity receptor, the duration of TCR–pMHC interaction or the affinity threshold [55]. In addition, in response to viruses, clonal T cell 'immunodomination' appears to occur in $CD8^+$ T cells, probably due to proliferation advantages, differences of TCR affinity or cosignal requirements [56]. Therefore, the clonal restriction of T cells found in autoimmune disorders is not behaviour specific to these diseases, but can be considered as a usual T cell response.

5. TCR gene transfer for controlling autoimmune diseases

From the results of the evaluation of antigen-specific T cell clonality, continuous expansion of immune response by acquisition of self-reactive epitopes does not appear to occur in the advanced stages of autoimmune disorders. Thus, the authors now believe that antigen-specific, immunotherapy targeting T cells would be feasible in autoimmune diseases. In this regard, extensive attempts have been made to try and establish antigen-specific T cell clones or lines by *in vitro* culture; however, there are several difficulties with this process. Usually, the culture should be performed without the information of appropriate autoantigens. A candidate autoantigen in cloning culture of autoantigen-specific T cells has to be selected based on the limited information. Moreover, there is no guarantee that *in vitro* established T cell clones represent real disease-associated T cells, mainly because *in vivo*-activated T cells are more easily rendered in

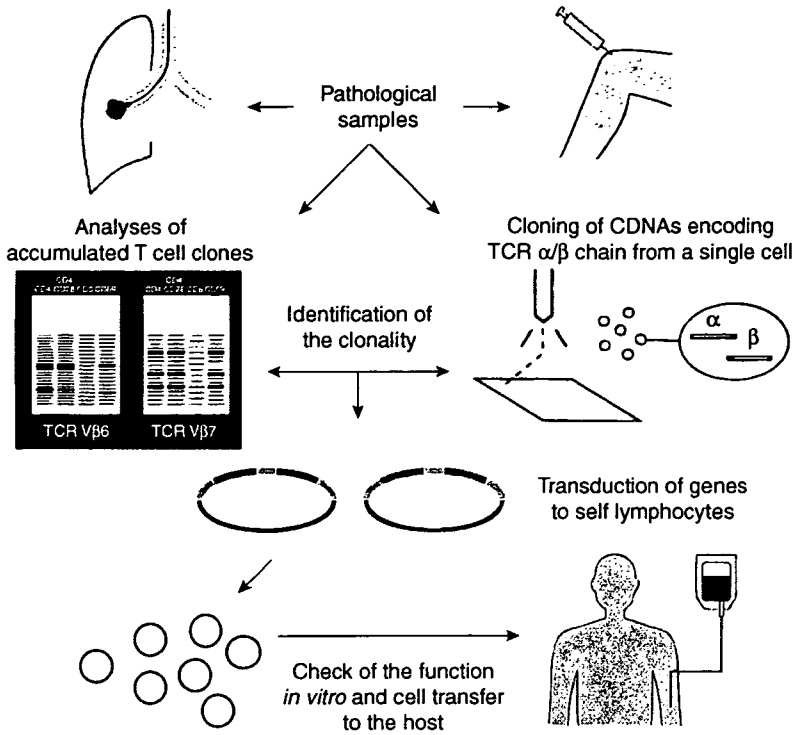

Figure 1. Proposed antigen-specific immunotherapy. Clonal analyses of TCRs in the pathological lesions could be performed as indicated on the upper left hand of the panel. This information could be combined with the *in vitro* reconstitution of the TCR function by cloning TCR cDNAs from a single cell and transferring them into self lymphocytes as in the right-hand panel. These engineered cells could be applied for antigen-specific immunotherapy.
TCR: T cell receptor.

activation-induced cell death. Therefore, the authors' group is now trying to produce autoimmune-associated T cells by gene transfer of TCR obtained from *in vivo*, as discussed below.

TCRs of the accumulated T cell clones in the established autoimmune lesions can be visualized by RT-PCR/SSCP analysis. If we obtain a pair of full-length cDNAs encoding α and β chains of TCR expressed in a single cell in the lesion, and if we express them efficiently by gene transfer to self lymphocytes, TCR function can be reconstructed using the information obtained *in vivo*. A schematic of the authors' system is given in **Figure 1**. For the gene transfer to lymphocytes, the authors' group have established a highly efficient retrovirus vector system with PLAT-E and pMX. PLAT-E is a packaging cell transfected with *gag–pol* and *env* segments separately. Two independent, monocistronic retrovirus vectors harbouring α and β TCR cDNAs were generated. For the first study, the class II MHC-restricted *α* and *β TCR* genes specific for chicken ovalbumin (OVA) were used. These *TCR* genes were cloned from TCR transgenic mice designated DO11.10. These TCRs were transduced to splenocytes from BALB/c mice. The results indicated that *α* and *β TCR* gene transfer into peripheral T cells reconstituted the antigen-specific immunity [57]. The amount of TCR expression and both the *in vitro* and *in vivo* antigen-specific functions were comparable with those obtained with splenocytes from DO11.10 transgenic mice.

The authors' group next attempted to use this TCR gene transfer to control autoimmune disorders [58]. The target was lupus nephritis. NZB/W F1 mice spontaneously develop a lupus-like syndrome and nephritis. Anti-DNA antibodies are believed to be one of the major pathogenic autoantibodies for nephritis. Datta and co-workers [59,60] have pointed out that nucleosome is a major immunogen in systemic lupus erythematosus. As DNA and the nucleosome are physically linked, it is speculated that nucleosome reactive T cells help the activation of anti-DNA specific B cells as the hapten-carrier model. Therefore, the authors tried to generate nucleosome-specific T cells with an immunosuppressive function. The authors selected CTLA-4Ig as a suppressive molecule. TCR cDNAs were engineered based on the published sequence of nucleosome-specific TCR by fusing a TCR V region sequence with a synthesized CDR3 sequence and a TCR J-C region sequence [59]. The V regions used were V α 13 and V β 4. This TCR recognizes the immunodominant I-A^d-restricted nucleosomal epitope.

In the authors' usual experimental protocol, the proportion of clonotypic TCR expression cells with two transferred TCR genes was estimated to be ~ 25% in $CD4^+$ T cells. The

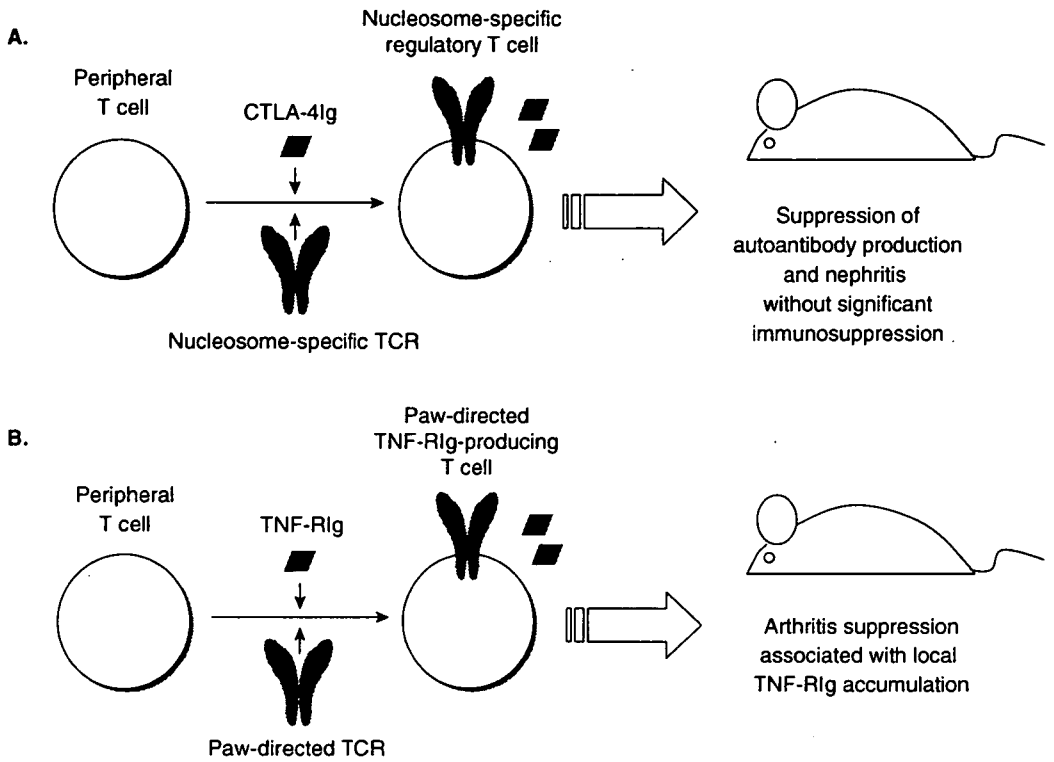

Figure 2. Experimental outlines of TCR gene transfer for controlling autoimmune diseases. A and **B** illustrate images of triple gene transfer to generate nucleosome-specific regulatory T cells and paw-directed TNF-R Ig-producing T cells.
CTLA: Cytotoxic T lymphocyte antigen; TCR: T cell receptor; TNF-R: Tumor necrosis factor receptor.

introduction of TCR was found to reconstitute the specificity for the nucleosome. Triple gene transfer was then performed together with CTLA-4Ig to generate regulatory T cells (Figure 2A). Calculations showed that ~ 10% of the total $CD4^+$ cells expressed all three genes. The CTLA-4Ig secreted from transduced T cells blocked the proliferation of the polyclonal T cell population. The TCR and CTLA-4Ig transduced cells showed the increase of CTLA-4Ig secretion on T cell activation in the presence of DCs. A million of the nucleosome-specific regulatory T cells engineered by the triple genes were then transferred into 10-week-old NZB/W F1 mice. The mice were monitored for proteinuria. By week 22, all of the control mice that received phosphate-buffered saline, cells transferred with mock vectors, TCR alone and CTLA-4Ig started to develop severe nephritis diagnosed by persistent proteinuria of > 300 mg/dl. By 30 weeks of age, the majority of these control mice showed severe proteinuria; however, none of the mice treated with cells transferred by the TCR and CTLA-4Ig showed excess proteinuria. The kidneys of the control mice showed severe glomerulonephritis with membrano-proliferation, glomerular sclerosis and tubular casts. The treated mice had mild glomerular disease with less deposition of IgG and complement, especially in the capillary loop. The autoantibodies usually found in NZB/W F1 mice were measured in the sera from different groups. The elevations of anti-dsDNA and antihistone antibodies were suppressed at the age of 22 weeks in the TCR and CTLA-4Ig-treated mice. The T cell-dependent humoral response to active immunization of OVA was also analyzed. The level of anti-OVA IgG antibody titre was not significantly different from those of the control mice, indicating there was not an overt systemic immunosuppression of the triple gene-treated mice.

In order to obtain the whole TCR information from pathological lesions, the authors' group next tried to clone a pair of full-length TCR cDNAs from a single cell accumulated in the inflamed joints of DBA/1 mice with collagen-induced arthritis [61]. Cloning of full-length cDNA encoding TCR was already established [62]. Single-cell sorting with $CD4^+$ and Vβ 8.1/8.2-positive cells was performed and TCR messages were amplified with three-step nested PCR using a fixed Vβ primer and multiple Vα primers. The authors then compared the clones obtained from the single cells with accumulated clones observed in the arthritis joints using the RT-PCR/SSCP method. Some TCRs from sorted single cells were actually identical to major clones accumulated in the joints. These TCR cDNAs were converted into full-length cDNAs and transferred to DBA/1 splenocytes. Interestingly, some of the pairs of TCR were found to be nonspecific to immunized type II collagen, but specific to self-antigen because

TCR-transferred cells proliferated in the culture with DCs from normal and arthritic mice. The carboxyfluorescein diiacetate succinimidyl ester (CSFE) labelling experiments showed that such TCR-transduced cells accumulated and proliferated in the arthritic joints. The authors' group next performed experimental therapy using the triple-gene engineered T cells. In this experiment, a soluble fusion protein consisting of TNF receptor p75 and Fc domain of IgG2a (TNF-RIg), was used as a regulatory molecule. Control cells were transduced with either TCR alone or TNF-RIg alone. With regard to the arthritis score and the percentage of severe arthritis, only TCR plus TNF-RIg-transduced cells significantly suppressed the arthritis (**Figure 2B**). Interestingly, the serum concentration of TNF-RIg was not the main determinant of arthritis suppression in the TCR plus TNF-RIg group, as the serum concentrations of TNF-RIg protein in the TCR plus TNF-RIg group were equivalent to those in the TNF-RIg group. In contrast, the amount of TNF-RIg in the paws of the TCR plus TNF-RIg group was significantly higher than that in the paws of the TNF-RIg group. Therefore, local accumulation of the TNF-RIg transcript suppressed arthritis in the TCR plus TNF-RIg group. Therefore, biological agents producing T cells may have an advantage over the conventional biological agents that depends on serum concentration. A reduced serum concentration may be associated with less systemic immunosuppression. Taking these results together, the system illustrated in the **Figure 2** was shown to be feasible for use in experimental animals.

Recently, the clinical appreciation of retroviral TCR gene transfer was reported in the treatment of melanoma patients. T cells transduced with melanoma antigen-specific TCRs suppressed disease progression in patients with advanced melanoma [63]. This result showed the essential efficacy and safety of TCR gene transfer in human. Therefore, autoimmune disease can be a suitable target for TCR gene transfer.

6. Expert opinion and conclusion

T cells are one of the most decisive components in immune responses, especially in terms of antigen specificity. TCR determines the specificity. However, the *TCR* genes are rearranged in each cell to obtain a variety of antigenic specificities, and so T cells are enormously heterogeneous. A small number of T cells in the total lymphocyte population participate in an antigen-specific immune response. Therefore, this limited population should be the main target in antigen-specific immunotherapy, without affecting systemic immunity. However, it is rather difficult to evaluate and further manipulate such specific T cells. The authors believe that much effort should be required for the analysis and manipulation of antigen-specific T cells in the future research. The authors propose that TCR gene cloning using the information of TCR clonal analysis, and reconstitution of the TCR function by gene transfer would be a promising strategy for antigen-specific immunotherapy in autoimmune disorders.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. JACOBSON DL, GANGE SJ, ROSE NR, GRAHAM NM: Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin. Immunol. Immunopathol.* (1997) 84:223-243.
2. LEACH MW, SNYDER EA, SINHA DP, ROSENBLUM IY: Safetay evaluation of recombinant human interleukine-4 I Preclinical studies. *Clin. Immunol. Immunopathol.* (1997) 83:8-11.
3. OLSEN NJ, STEIN CM: New drugs for rheumatoid arthritis. *N. Engl. J. Med.* (2004) 350:2167-2179.

•• **Comprehensive review of new drugs for rheumatoid arthritis.**

4. EUVRARD S, KANITAKIS J, CLAUDY A: Skin cancers in organ transplant recipients. *N. Engl. J. Med.* (2003) 348:1681-1691.
5. FELDMAN M, MAINI R: TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. *Nat. Med.* (2003) 9:1245-1250.

•• **Theory and clinical application of anti-TNF therapy.**

6. KEANE J, GERSHON S, WISE RP *et al.*: Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N. Engl. J. Med.* (2001) 345:1098-1104.
7. HYRICH KL, SILMAN AJ, WATSON KD, SYMMONS DP: Anti-tumour necrosis factor alpha therapy in rheumatoid arthritis: an update on safety. *Ann. Rheum. Dis.* (2004) 63:1538-1543.
8. MILLER DH, KHAN OA, SHERERNATA WA *et al.*: A controlled trial of Natalizumab for relapsing multiple sclerosis. *N. Engl. J. Med.* (2003) 348:15-23.
9. COLES AJ, WING M, SMITH S *et al.*: Pulsed monoclonal antibody treatment and autoimmune thyroid disease in multiple sclerosis. *Lancet* (1999) 354:1691-1695.
10. KREMER JM, WESTHOVENS R, LEON M *et al.*: Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *N. Engl. J. Med.* (2003) 349:1907-1915.
11. CROFT M, BRADLEY LM, SWAIN SL: Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J. Immunol.* (1994) 152:2675-2685.
12. GARCIA S, DISANTO J, STOCKINGER B: Following the development of a CD4 T cell response *in vivo*: from activation to memory formation. *Immunity* (1999) 11:163-171.
13. DE VITA S, ZAJA F, SACCO S *et al.*: Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis: evidence for a pathogenic role of B cells. *Arthritis Rheum.* (2002) 46:2029-2033.
14. HOLERS VM: The complement system as a therapeutic target in autoimmunity. *Clin. Immunol.* (2003) 107:140-151.

15. LAWTON JA, GHOSH P: Novel therapeutic strategies based on toll-like receptor signaling. *Curr. Opin. Chem. Biol.* (2003) 7:446-451.
16. MCDEVITT H: Specific antigen vaccination to treat autoimmune disease. *Proc. Natl. Acad. Sci. USA* (2004) 101:1462-1463.
17. MONNEAUX F, MULLER S: Peptide-based immunotherapy of systemic lupus erythematosus. *Autoimmun. Rev.* (2004) 3:16-24.
18. TSALIK EL: DNA-based immunotherapy to treat atopic disease. *Ann. Allergy Asthma Immunol.* (2005) 95:403-410.
19. QUINTANA FJ, CARMI P, MOR F, COHEN IR: Inhibition of adjuvant arthritis by a DNA vaccine encoding human heat shock protein 60. *J. Immunol.* (2002) 169:3422-3428.
20. GARREN H, RUIZ PJ, WATKINS TA *et al.*: Combination of gene delivery and DNA vaccination to protect from and reverse Th1 autoimmune disease via deviation to the Th2 pathway. *Immunity* (2001) 15:15-22.
21. FARIA AM, WEINER HL: Oral tolerance. *Immunol. Rev.* (2005) 206:232-259.
22. CHOY EH, SCOTT DL, KINGSLEY GH *et al.*: Control of rheumatoid arthritis by oral tolerance. *Arthritis Rheum.* (2001) 44:1993-1997.
23. ERGUN-LONGMIRE B, MARKER J, ZEIDLER A *et al.*: Oral insulin therapy to prevent progression of immune-mediated (type 1) diabetes. *Ann. NY Acad. Sci.* (2004) 1029:260-277.
24. BIELEKOVA B, GOODWIN B, RICHERT N *et al.*: Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a Phase II clinical trial with an altered peptide ligand. *Nat. Med.* (2000) 6:1167-1175.
25. JYOTHI MD, FLAVELL RF GEIGER TL: Target autoantigen-specific T cells and suppression of autoimmune encephalomyelitis with receptor-modified T lymphocytes. *Nat. Med.* (2002) 20:1215-1220.
- **A sophisticated approach to target autoantigen-specific T cells.**
26. SRIVASTAVA PK: Therapeutic cancer vaccines. *Curr. Opin. Immunol.* (2006) 18:201-205.
27. MILLER JF, KURTS C, ALLISON J *et al.*: Induction of peripheral $CD8^+$ T-cell tolerance by cross-presentation of self antigens. *Immunol. Rev.* (1998) 165:267-277.
28. LO J, CLARE-SALZLER MJ: Dendritic cell subsets and Type I diabetes: focus upon DC-based therapy. *Autoimmun. Rev.* (2006) 5:419-423.
29. NOURI-SHIRAZI M, THOMSON AW: Dendritic cells as promoters of transplant tolerance. *Expert Opin. Biol. Ther.* (2006) 6:325-339.
30. REA D, VAN KOOTEN C, VAN MEIJGAARRDEN KE *et al.*: Glucocorticoids transfer CD40-triggering of dendritic cells into an alternative activation pathway resulting in antigen-presenting cells that secrete IL-10. *Blood* (2000) 95:3162-3167.
31. ADORINI L, PENNA G, GIARRATANAN N, USKOKOVICM: Tolerogenic dendritic cells induced by vitamin D receptor ligand enhance regulatory T cells inhibiting allograft rejection and autoimmune diseases. *J. Cell. Biochem.* (2003) 88:227-233.
32. MARTIN E, O'SULLIVAN B, LOW P, THOMAS R: Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10. *Immunity* (2003) 18:155-157.
33. FIGDOR CG, DE VRIES IJ, LESTERHUIS WJ, MELIEF CJ: Dendritic cell immunotherapy: mapping the way. *Nat. Med.* (2004) 10:475-480.
- **Perspective review on DC immunotherapy.**
34. SAKAGUCHI S: Naturally arising Foxp3-expresing $CD25^+CD4^+$ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* (2005) 6:345-352.
- **Review on naturally occurring regulatory T cells, especially related to several disorders.**
35. BLUESTONE JA, TANG Q: Therapeutic vaccination using $CD4^+CD25^+$ antigen-specific regulatory T cells. *Proc. Natl. Acad. Sci. USA* (2004) 101:14622-14626.
36. 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.
37. TARBELL KV, YAMAZAKI S, OLSON K, TOY P, STEINMAN RM: $CD25^+CD4^+$ T cells, expanded with dendritic cells presenting an single autoantigenic peptide, suppress autoimmune diabetes. *J. Exp. Med.* (2004) 199:1467-1477.
38. SHAW MK, LORENS JB, DHAWAN A *et al.*: Local delivery of interleukin 4 by retrovirus-transduced T lymphocytes ameliorates experimental autoimmune encephalomyelitis. *J. Exp. Med.*. (1997) 185:1711-1714.
39. MATHISEN PM, YU M, JOHNSON JM, DRAZB JA, TUOHY VK: Treatment of experimental autoimmune encephalomyelitis with genetically modified memory T cells. *J. Exp. Med.* (1997) 186:159-164.
40. NAKAJIMA A, SEROOGY CM, MATTHEW SR *et al.*: Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis. *J. Clin. Invest.* (2001) 107:1293-1301.
41. SETOGUCHI K, MISAKI Y, ARAKI Y *et al.*: Antigen-specific T cells transduced with IL-10 ameliorate experimentally induced arthritis without impairing the systemic immune response to the antigen. *J. Immunol.* (2000) 165:5980-5986.
42. LEHMANN PV, FORSTHUBER T, MILLER A, SERCARZ EE: Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* (1992) 358:155-157.
43. VANDERLUGT CL, MILLER SD: Epitope spreading in immune-mediated diseses:implications for immunotherapy *Nat. Rev. Immunol.* (2002) 2:85-95.
- **Current idea on epitope spreading.**
44. 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.
45. 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.
46. IWAKURA Y, TOSU M, YOSHIDA E *et al.*: Induction of inflammatory arthropathy resembling rheumatoid arthritis in mice transgenic for HTLV-1. *Science* (1991) 253:1026-1028.

47. KOBARI Y, MISAKI Y, SETOGUCHI K *et al.*: T cell accumulating in the inflamed joints of spontaneous murine model of rheumatoid arthritis become restricted to common clonotypes during disease progression. *Int. Immunol.* (2004) 16:131-138.

48. KOMAGATA Y, MASUKO K, TASHIRO F *et al.*: Clonal prevalence of T cells infiltrating into the pancreas of prediabetic non-obese diabetic mice. *Int. Immunol.* (1996) 8:807-814.

49. 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.

50. VAN DEN ELZEN P, MENEZES JS, AMETANI A *et al.*: Limited clonality in autoimmunity: drivers and regulators. *Autoimmun. Rev.* (2004) 3:524-529.

51. 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.

52. 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.

• **First evidence for accumulation of insulin-specific T cell clones in human diabetes.**

53. AMRANI A, VERDGUER J, SERRA P, TAFURO S, TAN R, SANTAMARIA P: Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. *Nature* (2000) 406:739-742.

54. KEDZIERSKA K, TURNER SJ, DOHERTY P: 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.

55. 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.

56. FACCHINETTI A, SANTA SD, 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.

57. FUJIO K, MISAKI Y, SETOGUCHI K *et al.*: Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the alpha beta TCR complex. *J. Immunol.* (2000) 165:528-532.

58. 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.

59. KALIYAPERULAL A, MOHAN C, WU W, DATTA SK: Nucleomosomal peptide epitope for nephritis-inducing T helper cells of murine lupus. *J. Exp. Med.* (1996) 183:2459-2469.

60. MOHAN C, ADEM S, STANIK V, DATTA SK: Nucleosome: a major immunogen for pathogenic autoantigen-inducing T cells of lupus. *J. Exp. Med.* (1993) 177:1367-1381.

61. 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.

62. TAHARA H, FUJIO K, ARAKI Y *et al.*: Reconstitution of CD8+ T cells by retroviral transfer of the TCR alpha beta-chain genes isolated from a clonally expanded P815-infiltrating lymphocyte. *J. Immunol.* (2003) 171:2154-2160.

63. MORGAN RA, DUDLEY ME, WUNDERLICH JR *et al.*: Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* (2006) 314:126-129.

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IgE- and FcεRI-mediated migration of human basophils

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Keywords: allergy, antigen, chemotaxis, non-releaser

Abstract

Local accumulation of basophils at inflammatory sites is observed in experimental antigen challenge and in allergic diseases. It is not fully known what factor(s) regulates local basophil influx in tissues, and it has not been determined whether antigens belong in a panel of basophil chemoattractants. This study was designed to elucidate whether IgE- and high-affinity receptor for IgE (FcεRI)-mediated stimulation can induce human basophil migration. The migration-inducing potency of an anti-FcεRI α-chain mAb, CRA-1, was examined on human basophils. CRA-1 mAb elicited significant migration of basophils. The migration-inducing potency of this mAb was maximal at 100 ng ml^{-1}, and CRA-1 mAb at 100 ng ml^{-1} attracted ~10% of total inoculated basophils above baseline levels after incubation for 2.5 h. Checkerboard analysis indicated that basophil migration induced by this mAb was mainly chemotactic and partially chemokinetic. An antigen, Der f 2, also induced migration of basophils from Der f-sensitive subjects. Basophils mixed with 1 ng ml^{-1} of CRA-1 mAb showed an exaggerated migration response to eotaxin, indicating that FcεRI cross-linkage enhances basophil migration to other chemoattractants. Induction of basophil migration by IgE- and FcεRI-cross-linking stimulation may, at least in part, explain the pathogenesis of local basophil accumulation clinically observed in allergic diseases such as asthma.

Introduction

Basophils are the least abundant leukocytes in peripheral blood, but they possess biogenic amines such as histamine in their cytoplasmic granules. When these cells encounter specific antigens, they are stimulated and release various chemical mediators including histamine. Basophils are thus thought to be an active participant in the pathogenesis of immediate-type hypersensitivity reactions, as an important cellular source of pro-inflammatory mediators (1, 2).

In experimental antigen challenge models of the airways and skin, basophil accumulation has been demonstrated at the sites of inflammation (3, 4). Significantly increased numbers of basophils are also reported in local tissues of allergic patients such as asthmatics (5). These facts clearly show that local mechanisms exist for attracting basophils from the circulation. Influx of basophils to tissues is generally thought to be composed of three steps: adhesion, transendothelial migration and migration. Both adhesion molecules and cytokines play regulatory roles in adhesion (6) and transendothelial migration (7). Migration of basophils is mainly regulated by the interaction of soluble molecules and their receptors on the cell surface. Various factors including complement C5a (8), cytokines IL-3 and granulocyte macrophage colony-stimulating factor (9), chemokines (10) and other agents have been demonstrated to be able to induce basophil migration. However, it is not fully known which chemoattractant(s) plays a central or partial role at local inflammation sites *in vivo*.

Abundant expression of high-affinity receptor for IgE (FcεRI) is one major characteristic of basophils as well as mast cells. Cross-linkage of FcεRI by allergens and specific IgE induces

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cell activation, leading to the release of both granule-associated and newly synthesized mediators (1, 2). In addition, it has recently been increasingly understood that IgE and FcεRI can generate intracellular signals affecting various biological aspects of cells other than release functions. In terms of cell motility, rodent mast cells are reportedly able to migrate toward specific antigens, and aggregation of IgE and FcεRI initiates this migration (11, 12). Considering that migration is an essential step for blood basophils to act at local tissue sites, it is of great interest whether IgE and FcεRI mediate the migration of mature human basophils. In this study, we assessed IgE- and FcεRI-mediated basophil migration, and demonstrate that cross-linking stimulation of FcεRI induces direct migration, and enhances chemokine-induced migration, of human basophils.

Methods

Reagents

The following reagents were purchased as indicated: Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), PBS and FCS (GIBCO, Grand Island, NY, USA), prostaglandin D_2 (PGD_2) (Cayman Chemical, Ann Arbor, MI, USA), human eotaxin/CCL11 (R&D, Minneapolis, MN, USA), human recombinant C5a and piperazine-*N,N'*-bis-2-ethanesulfonic acid (PIPES) (Sigma Chemical Co., St Louis, MO, USA), human monocyte chemoattractant protein-1 (MCP-1)/CCL2 (PeproTech Inc., Rocky Hill, NJ, USA), recombinant Der f 2 (Asahi Brewery, Tokyo, Japan) and ionophore A23187 (Calbiochem-Behring, La Jolla, CA, USA). Human recombinant IL-3 was kindly donated by Kirin Brewery (Tokyo, Japan). A mouse IgG2b anti-human FcεRI α-chain mAb, CRA-1, was used; this antibody can bind to the FcεRI α-chain regardless of whether or not it is occupied by IgE (13). The following antibodies were purchased as indicated: mouse IgG2b mAb with irrelevant specificity (MOPC195; Cappel, Aurora, OH, USA), FITC-conjugated goat anti-human IgE antibody (Biosource International, Camarillo, CA, USA), PE-conjugated anti-CD11b mAb (mouse IgG1, clone Bear1) and FITC- or PE-conjugated mouse IgG1 (clone 679.1Mc7) (Coulter Immunotech, Marseille, France). FITC-conjugated anti-CCR3 mAb (IgG1, clone 444) was prepared as described previously (14).

Cell preparation

Leukocytes were isolated from venous blood obtained from consenting volunteers with no history of atopic diseases. In some experiments, peripheral blood was drawn from consenting subjects with mite-sensitive allergic asthma, fulfilling the criteria for diagnosis of bronchial asthma (15). Basophils were semi-purified by density centrifugation using Percoll solutions of different densities (1.080 and 1.070 g ml^{-1}). The purity of these Percoll-separated basophil preparations was ~12.3%. For some experiments, the Percoll-separated basophils were further purified by negative selection with MACS beads (Basophil Isolation Kit; Miltenyi BioTech, Belgisch-Gladbach, Germany) according to the manufacturer's instructions.

Migration

Basophil migration was analyzed using 24-well culture plates (IWAKI, Tokyo, Japan) and chemotaxicell (Kurabo, Osaka, Japan) with a 5-μm pore size. A total of 100 μl of PIPES buffer containing 25 mM PIPES, 119 mM NaCl, 5 mM KCl, 2 mM Ca^{2+}, 0.5 mM Mg^{2+}, 0.03% human serum albumin and 2×10^4 basophils was added to the upper chamber, and 300 μl of test reagent was placed in the lower chamber. After incubation for 2.5 h at 37°C, migrated cells in the lower chamber were collected and stained with 10 μg ml^{-1} of FITC-conjugated goat anti-human IgE for 30 min at 4°C. Cells were then analyzed using an EPICS XL System II (Coulter, Miami, FL, USA) (7). Migrated basophils were identified as cells strongly positive for IgE. The number of migrated cells might be potentially under-represented in our multistep experimental procedures using flow cytometer; to attain the data reliability, we usually spent longer time period for flow cytometric analysis of each sample under this method (7, 14). Migration was expressed as a percentage of the inoculated basophils after subtracting the spontaneous migration (5.1 ± 0.7%, $n = 23$) unless otherwise specified. All the experiments in this study were performed at least in duplicate.

Flow cytometry

CD11b expression experiments were performed using Percoll-separated basophils. Following stimulation, cells were incubated with 10 μg ml^{-1} of either PE-conjugated anti-CD11b mAb or PE-conjugated control mouse IgG1 and then stained with FITC-conjugated anti-human IgE antibody at 10 μg ml^{-1}. Stained cells were analyzed by flow cytometry. The median values of fluorescence intensity of basophils were converted to the numbers of molecules of equivalent soluble fluorochrome units (MESFs), as previously described (16). Surface receptor levels were semi-quantified using the following formula: ΔMESF = (MESF of cells stained with anti-CD11b mAb) – (MESF of cells stained with control IgG).

CCR3 expression experiments were performed using MACS-separated basophils. Following stimulation, cells were stained for 30 min with 10 μg ml^{-1} of either FITC-conjugated anti-CCR3 mAb or FITC-conjugated control mouse IgG1 before flow cytometric analysis.

Degranulation

Basophil degranulation was examined using Percoll-separated basophils. Briefly, cells were re-suspended in PIPES buffer containing 2 mM Ca^{2+}, 0.5 mM Mg^{2+} and 0.03% human serum albumin, and then stimulated at 37°C for 45 min in polystyrene tubes. The supernatants were stored at 4°C until histamine assay by an automated fluorometric technique (17). Histamine release was expressed as a percentage of the total cellular histamine after subtracting the spontaneous release (2.8 ± 0.8%, $n = 9$).

Statistics

All data are expressed as the mean ± SEM. Differences between values were analyzed by the one-way analysis of variance test. When this test indicated a significant difference,

Fisher's protected least significant difference test was used to compare individual groups.

Results

Anti-FcεRI α-chain mAb, CRA-1, induces basophil migration

We first examined whether FcεRI cross-linkage induces human basophil migration, by using an anti-FcεRI α-chain mAb, CRA-1. As shown in Fig. 1(A), there was clear basophil migration toward CRA-1 mAb at 100 ng ml^{-1} (10.1 ± 1.9%, $n = 7$). The migration-inducing potency of CRA-1 mAb was statistically significant ($P < 0.05$). Basophil migration toward various known chemoattractants such as eotaxin, MCP-1, PGD_2 and C5a is also included in Fig. 1(A). In clear contrast to the control IgG2b mAb which completely failed to increase the number of migrated basophils, CRA-1 mAb demonstrated induction of basophil migration at concentrations as low as 10 ng ml^{-1}, and maximal migration was seen at 100–1000 ng ml^{-1} of this mAb (Fig. 1B). We next tested the migratory responses using semi-purified basophils (purity 12.3 ± 2.9%) and MACS-separated basophils (purity 99.0 ± 0.6%) simultaneously. CRA-1 at 100 ng ml^{-1} induced significant migration in both basophil preparations; no statistical difference was seen in the magnitude of migratory responses between the two preparations ($P = 0.27$) (Fig. 1C), indicating that CRA-1 mAb can exert the migration-inducing capacity via its direct action on basophils.

It has been reported that ~2 h of incubation is sufficient to detect apparent migration of basophils toward eotaxin or other known chemoattractants (9, 10). In contrast, CRA-1-induced basophil migration revealed a slower process: after incubation for 2 h, we observed only a small number of migrated basophils, but their number continued to increase until up to 7 h of incubation (Fig. 1D). At the 7-h time point, the percentage of basophils which had migrated toward CRA-1 at 10 ng ml^{-1} was comparable to that toward eotaxin at 50 nM.

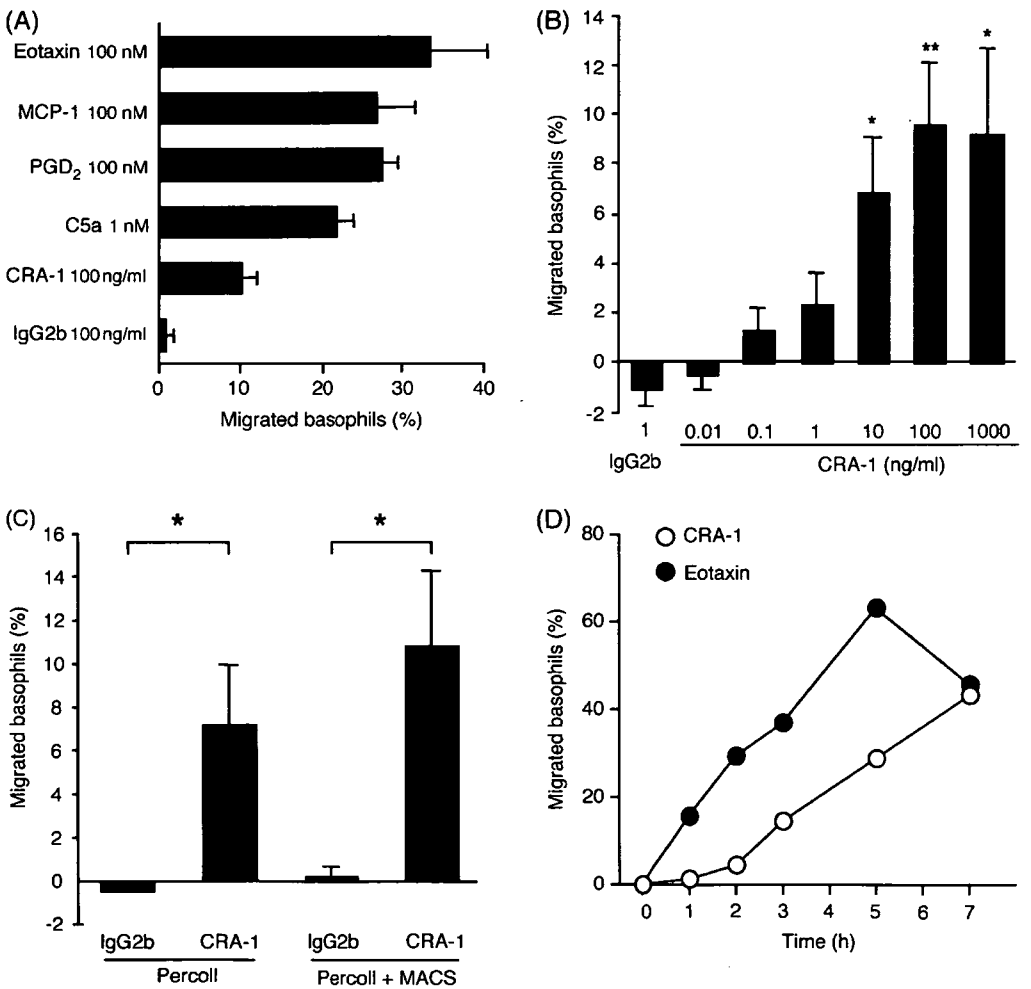

Fig. 1. Anti-FcεRI α-chain mAb, CRA-1, induces basophil migration. (A) Percoll-separated basophils were tested for migration toward lower chambers containing various known chemoattractants or CRA-1 at 100 ng ml^{-1} ($n = 7$). (B) Concentration-dependent basophil migration induced by CRA-1. Bars represent the SEM ($n = 4$). *$P < 0.01$, **$P < 0.001$, versus migration toward control mouse IgG2b. (C) Both Percoll- and MACS-separated basophils were tested for migration toward CRA-1 or control mouse IgG2b at 100 ng ml^{-1} ($n = 4$). Bars represent the SEM. *$P \leq 0.01$. (D) Time course of basophil migration induced by eotaxin (50 nM) and CRA-1 (10 ng ml^{-1}). Data are representative of two separate experiments. The percentages of background migration in the absence of any reagent were 1.4, 3.2, 4.3, 8.7 and 5.5% at 1, 2, 3, 5 and 7 h, respectively.

It should be noted that basophils from some donors demonstrated apparent spontaneous migration after 3 h or longer incubation in the absence of any chemoattractant. Accordingly, we decided that routinely the 2.5-h time point is appropriate for clearly distinguishing active attraction of basophils by CRA-1 from spontaneous migration.

Checkerboard analysis

Checkerboard analysis was performed by adding 1–100 ng ml^{-1} of CRA-1 mAb to the upper and/or lower wells. The most potent migration of basophils was observed when the optimal concentration of CRA-1 mAb was included only in the lower chambers, whereas weak migration was also seen when CRA-1 mAb was added to both chambers (Table 1). These results suggest that CRA-1-induced basophil migration was mainly chemotactic and partially chemokinetic.

Comparison of the effects of CRA-1 on various biological functions of basophils

It has already been established that cross-linkage of basophil surface FcεRI results in degranulation and up-regulation of surface CD11b expression (6). We next compared the concentration-dependent effects of CRA-1 on basophil migration, CD11b expression and degranulation. To adjust the experimental design for cell treatment procedures among these three indices, migration of basophils was analyzed using cell preparations mixed with CRA-1 mAb before inoculation to the upper wells, and non-directional migration was thus measured. As shown in Fig. 2, all three indices of basophil activation demonstrated CRA-1 dose dependency within the tested concentration range. Maximal migration of basophils was observed at 100 ng ml^{-1} of CRA-1, but the extent of histamine release and up-regulation of surface CD11b expression plateaued at 1000 ng ml^{-1} of CRA-1. When the effective dose of 50% (ED_{50}) concentration of CRA-1 was compared, half-maximal induction of basophil migration and CD11b up-regulation (ED_{50}: within 1–10 ng ml^{-1}) occurred at a slightly lower concentration of CRA-1 than that of histamine release (ED_{50}: within 10–100 ng ml^{-1}).

Basophils migrate toward antigen

We next tested whether human basophils can migrate toward a specific antigen. Basophils were obtained from subjects with mild allergic asthma showing positive radioalloergosorbent test (RAST) for Der f. These basophils not only released histamine, but also migrated toward Der f 2 at sub-nanogram per milliliter doses (Table 2). In contrast, basophils from non-allergic subjects with negative RAST for Der f failed to be degranulated by, or migrate toward, Der f 2. These results indicate that sensitized basophils can migrate to a specific antigen.

Table 1. Checkerboard analysis of FcεRI-mediated basophil migration

CRA-1 mAb (ng ml^{-1}) in lower chamber	CRA-1 mAb (ng ml^{-1}) in upper chamber			
	0	1	10	100
0	7.8 ± 3.3	9.0 ± 2.4	13.9 ± 2.2	13.5 ± 2.2
1	10.2 ± 0.6	9.4 ± 3.6		
10	14.8 ± 1.4		12.0 ± 2.2	
100	18.5 ± 3.1			11.0 ± 2.8

Data are the mean ± SEM of three separate experiments performed on basophils from three different donors. Actual percentages of migrated basophils are shown.

Non-releasing basophils do not migrate toward CRA-1

It is known that basophils from 10 to 20% of normal subjects do not degranulate in response to IgE- or FcεRI-dependent stimulation (18). Using basophils from such non-releasers, we studied CRA-1-induced migration. As shown in Table 3, non-releaser basophils failed to migrate toward CRA-1 but did migrate toward an FcεRI-independent chemoattractant, eotaxin. These results imply that an FcεRI-dependent early signal transduction component essential for degranulation, presumably Syk (19, 20), is critically involved in FcεRI-dependent migration of basophils.

Synergistic effect of CRA-1 on chemoattractant-induced basophil migration

Finally, we investigated whether CRA-1 affects basophil migration induced by other chemoattractants. Treatment with CRA-1 at 1 ng ml^{-1} did not alter the level of surface CCR3 expression by basophils (Fig. 3A). However, it did greatly enhance basophil migration toward eotaxin although the low concentration of CRA-1 induced little or no migration in the absence of eotaxin in the lower chamber (Fig. 3B). CRA-1 at 0.1 ng ml^{-1} enhanced eotaxin-induced basophil migration

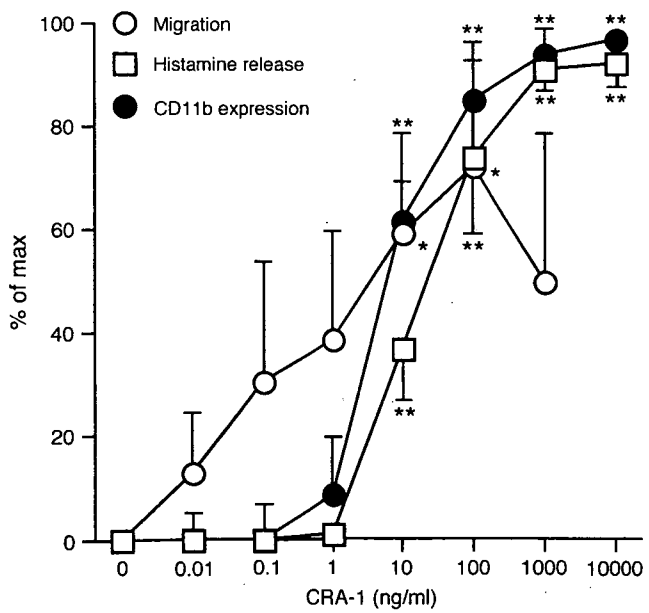

Fig. 2. Effects of CRA-1 on various functions of basophils. Percoll-separated basophils were used, and the effects of CRA-1 on basophil migration, histamine release and CD11b expression were assessed. Data are the mean ± SEM ($n = 4$), calculated based on percentages of the maximum for each donor. *$P < 0.01$, **$P < 0.001$, versus the baseline in the absence of CRA-1. The actual percentage of maximal migration in the presence of CRA-1 was 14.6 ± 3.8%. The maximal level of surface CD11b expression in CRA-1-treated cells was 86.6 ± 10.6% above the baseline value.

Table 2. Degranulation and migration of sensitized basophils by Der f 2

Reagents		Histamine release (%)		Migration (%)	
		RAST (−)	RAST (+)	RAST (−)	RAST (+)
Der f 2 (ng ml^{-1})	0.001	0 ± 0	0.7 ± 0.6	0 ± 0	0.9 ± 0.8
	0.01	0 ± 0	5.3 ± 4.1	0 ± 0	2.7 ± 1.9
	0.1	0 ± 0	28.0 ± 16.6*	0 ± 0	5.5 ± 1.9*
	1	0 ± 0	49.3 ± 6.8**	0 ± 0	5.4 ± 2.6*
	10	0 ± 0	29.5 ± 8.4**	0 ± 0	1.0 ± 1.7
Ca ionophore (μg ml^{-1})	0.2	52.0 ± 7.3**	33.3 ± 4.1**	n.d.	n.d.
Eotaxin (nM)	50	n.d.	n.d.	19.7 ± 8.4**	17.4 ± 2.5**

Percoll-separated basophils were obtained from three asthmatic subjects with positive RAST for Der f 2 and three healthy subjects with negative RAST for Der f 2. Migration was assessed by adding Der f 2 or eotaxin to the lower chamber. Data are the mean ± SEM of three separate experiments performed on basophils from three different donors. *$P \leq 0.05$, **$P \leq 0.01$, versus corresponding value without stimulation. n.d.: not done.

Table 3. Non-releasing basophils fail to show FcεRI-mediated migration

Reagents		Histamine release (%)		Migration (%)	
		Releaser	Non-releaser	Releaser	Non-releaser
CRA-1 (ng ml^{-1})	0.1	0	0	2.5	0.7
	1	0.2	1.3	3.7	0
	10	6.3	0.4	6.7	0
	100	15.3	0	13.6	0
	1000	20	0.5	14.9	0
Ca ionophore (μg ml^{-1})	0.2	58.1	55.0	n.d.	n.d.
Eotaxin (nM)	50	n.d.	n.d.	30.8	24.4

Basophil migration was assessed by adding CRA-1 or eotaxin to the lower chamber. Data are the averages of separate experiments performed on basophils from two releasers and two non-releasers. n.d.: not done.

moderately compared with its 1-ng ml^{-1} dose. Slight and variable enhancement of eotaxin-induced migration was also observed in basophils treated with 10 ng ml^{-1} or higher concentrations of CRA-1. However, such high concentrations of CRA-1 affected basophil functions in a complicated manner, such as up-regulation of random migration and down-regulation of surface FcεRI and CCR3 levels (data not shown). These results indicate that nanogram per milliliter doses of CRA-1 synergistically enhance eotaxin-induced basophil migration without up-regulating the surface expression of CCR3. We also investigated this synergistic effect using basophils from two separate non-releasers. However, the enhancement of eotaxin-induced migration was not seen with these basophils (data not shown), indicating that not only FcεRI-mediated direct migration but also enhancement of eotaxin-induced migration of basophils is hampered in the non-releasing phenotype.

Discussion

In this study, we have demonstrated that human peripheral basophil preparations can migrate in response to IgE- and FcεRI-cross-linking stimulation. Most of our experiments were performed using an anti-FcεRI α-chain mAb, CRA-1. This antibody is highly selective for the FcεRI α-chain, with very low non-specific binding to FcεRI-negative cells (13), suggesting that CRA-1 mAb interacts with basophils solely via its Fab portion. In addition, the basophil migration induced by this mAb was considered not to be an indirect event mediated by contaminating cells since it was observed even when using highly pure basophil preparations. Moreover, the Der f 2 antigen attracted basophils obtained from Der f-sensitive subjects. These results collectively imply that human basophils do migrate in response to stimulation via IgE and FcεRI.

The intracellular mechanism(s) by which FcεRI-dependent signals direct basophil migration is of special interest. We found that basophils from non-releasers, which do not degranulate in response to IgE-mediated stimulation, failed to migrate toward CRA-1 mAb, although binding of CRA-1 on these basophils' surface was confirmed by flow cytometry. Furthermore, up-regulation of eotaxin-induced basophil migration was not observed in non-releasers. Although the overall signal defect(s) in non-releaser basophils might not be fully clarified, one important point is the Syk deficiency (19, 20). This early signal tyrosine kinase is probably critically involved in cell motility changes following FcεRI cross-linkage, as well as in degranulation.

It is known that IgE and FcεRI are important molecules for the initiation of antigen-induced immediate-type hypersensitivity reactions. Recent progress in basic and clinical research has revealed that IgE and FcεRI on the surface of mast cells and basophils may be more profoundly involved in the pathogenesis of allergy than had been previously conceived. Others and we have shown that IgE can regulate the surface expression of FcεRI in various types of FcεRI-positive cells such

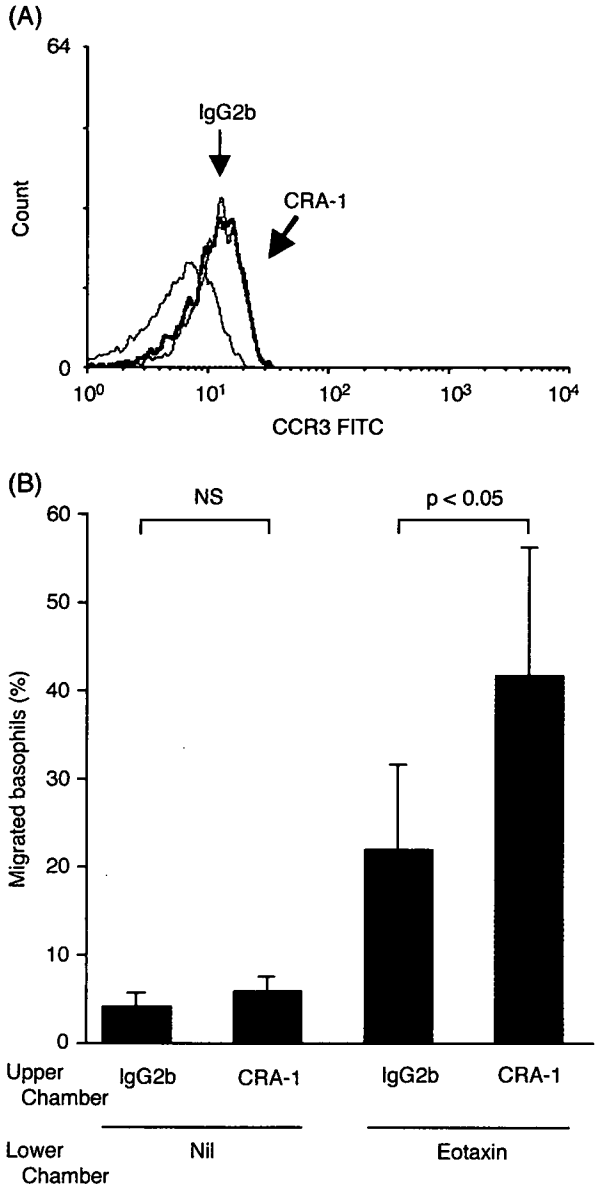

Fig. 3. CRA-1 enhances basophil migration induced by eotaxin. (A) After stimulation with either CRA-1 or control IgG2b at 1 ng ml^{-1} for 2.5 h, surface CCR3 expression on basophils was assessed by flow cytometry. Basophils stained with FITC–control mouse IgG1, in place of FITC–anti-CCR3 mAb, are shown as shaded area. Data are representative of two separate experiments, showing similar results. (B) Basophils were mixed with either CRA-1 or control IgG2b (1 ng ml^{-1}) and then placed in the upper chamber. Eotaxin at 10 nM was added to the lower chamber. Spontaneous migration of untreated basophils in the absence of eotaxin in the lower chamber was 4.8 ± 0.8%. The bars represent the SEM ($n = 4$).

as mast cells and basophils (16, 21, 22). Recent reports have shown that IgE itself can regulate apoptosis of mouse mast cells (23, 24). With regard to cell motility, IgE aggregation has been demonstrated to induce rodent mast cell migration (11, 12). Ishizuka *et al.* (12) recently reported that sensitized mouse mast cell line MC/9 cells and bone marrow-derived cultured mast cells migrate toward a specific antigen and that the migration is chemotactic. Our results presented herein are basically similar to their mast cell findings. And, importantly, we found that eotaxin-induced migration of basophils is augmented by treatment of the cells with CRA-1 mAb at a concentration as low as 1 ng ml^{-1}, although this concentration is unable to evoke significant degranulation. It is noteworthy that such weak stimulation can affect basophils; our results coincide with a previous report that concentrations of stimulus lower than those required for histamine release enhance basophil adherence to vascular endothelium (25). Since treatment with CRA-1 failed to increase the level of basophil surface CCR3 expression, the intracellular signal pathway following eotaxin and CCR3 interaction may be up-regulated. Such a migration-enhancing action arising from FcεRI cross-linkage might be similar to that known in mast cells (26). Thus, previous reports and the present study collectively imply that the effect of IgE- and FcεRI-dependent stimulation on cell locomotion, in both direct and indirect (enhancing) ways, might be a phenomenon common to both FcεRI-abundant basophils and mast cells.

Local influx of basophils at inflammatory sites is an important aspect of allergen-induced late-phase reactions as well as allergic diseases such as asthma (3–5). In normal conditions, basophils reside only in circulating blood; thus, there must be some mechanism(s) that induces basophil migration into local tissues during allergic reactions. Since the first description of *in vitro* basophil chemotaxis by Kay and Austen (27), various agents have been identified as basophil chemoattractants, including complement (8), bacteria-derived peptides (9, 28), cytokines (9), chemokines (10), enzymes such as urokinase (28) and, in this study, specific antigens. Our results showing that allergens can induce basophil migration may need to be taken into account when we try to identify potential chemoattractant(s) in clinical allergy. Moreover, our findings that eotaxin-induced migration is up-regulated in basophils treated with low levels of CRA-1 mAb might explain, at least in part, the pathogenesis of basophil accumulation at inflammatory sites in allergic diseases, where prolonged antigen exposure and various pro-inflammatory mediators co-exist (29).

Recent studies have shown that FcεRI-positive cells include not only mast cells and basophils but also eosinophils, macrophages, dendritic cells, neutrophils and platelets in humans (30–34). In this context, it will be of great interest to assess whether IgE- and FcεRI-mediated migrations occur in all of these $FcεRI^+$ cells, and, if so, to analyze to what extent this mechanism can account for the clinical efficacy of the IgE-targeting approach to treatment of allergic diseases.

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Abbreviations

ED_{50}	effective dose of 50%
FcεRI	high-affinity receptor for IgE
MCP-1	monocyte chemoattractant protein-1
MESF	molecules of equivalent soluble fluorochrome unit
PGD_2	prostaglandin D_2

PIPES piperazine-*N,N'*-bis-2-ethanesulfonic acid
RAST radioalloergosorbent test

References

1 Ishizaka, T. and Ishizaka, K. 1984. Activation of mast cells for mediator release through IgE receptors. *Prog. Allergy* 34:188.
2 Costa, J. J., Weller, P. F. and Galli, S. J. 1997. The cells of the allergic response: mast cells, basophils, and eosinophils. *JAMA* 278:1815.
3 Bascom, R., Wachs, M., Naclerio, R. M., Pipkorn, U., Galli, S. J. and Lichtenstein, L. M. 1988. Basophil influx occurs after nasal antigen challenge: effects of topical corticosteroid pretreatment. *J. Allergy Clin. Immunol.* 81:580.
4 Charlesworth, E. N., Hood, A. F., Soter, N. A., Kagey-Sobotka, A., Norman, P. S. and Lichtenstein, L. M. 1989. Cutaneous late-phase response to allergen. Mediator release and inflammatory cell infiltration. *J. Clin. Invest.* 83:1519.
5 Koshino, T., Teshima, S., Fukushima, N. *et al.* 1993. Identification of basophils by immunohistochemistry in the airways of post-mortem cases of fatal asthma. *Clin. Exp. Allergy* 23:919.
6 Bochner, B. S., McKelvey, A. A., Sterbinsky, S. A. *et al.* 1990. IL-3 augments adhesiveness for endothelium and CD11b expression in human basophils but not neutrophils. *J. Immunol.* 145:1832.
7 Iikura, M., Ebisawa, M., Yamaguchi, M. *et al.* 2004. Trans-endothelial migration of human basophils. *J. Immunol.* 173:5189.
8 Lett-Brown, M. A., Boetcher, D. A. and Leonard, E. J. 1976. Chemotactic responses of normal human basophils to C5a and to lymphocyte-derived chemotactic factor. *J. Immunol.* 117:246.
9 Yamaguchi, M., Hirai, K., Shoji, S. *et al.* 1992. Haemopoietic growth factors induce human basophil migration *in vitro*. *Clin. Exp. Allergy* 22:379.
10 Yamada, H., Hirai, K., Miyamasu, M. *et al.* 1997. Eotaxin is a potent chemotaxin for human basophils. *Biochem. Biophys. Res. Commun.* 231:365.
11 Orida, N., Feldman, J. D., Katz, D. H. and Liu, F. T. 1983. IgE-mediated chemotaxis of rat basophilic leukemia cells towards specific antigen. *J. Exp. Med.* 157:2166.
12 Ishizuka, T., Okajima, F., Ishiwara, M. *et al.* 2001. Sensitized mast cells migrate toward the antigen: a response regulated by p38 mitogen-activated protein kinase and Rho-associated coiled-coil-forming protein kinase. *J. Immunol.* 167:2298.
13 Yamaguchi, M., Sayama, K., Yano, K. *et al.* 1999. IgE enhances FcεRI expression and IgE-dependent release of histamine and lipid mediators from human umbilical cord blood-derived mast cells: synergistic effect of IL-4 and IgE on human mast cell FcεRI expression and mediator release. *J. Immunol.* 162:5455.
14 Iikura, M., Miyamasu, M., Yamaguchi, M. *et al.* 2001. Chemokine receptors in human basophils: inducible expression of functional CXCR4. *J. Leukoc. Biol.* 70:113.
15 National Asthma Education and Prevention Program. 1997. *Expert Panel Report II. Guidelines for the Diagnosis and Management of Asthma.* Publication no. 97-4051. National Institutes of Health, Bethesda, MD, USA.
16 Yamaguchi, M., Lantz, C. S., Oettgen, H. C. *et al.* 1997. IgE enhances mouse mast cell FcεRI expression *in vitro* and *in vivo*: evidence for a novel amplification mechanism in IgE-dependent reactions. *J. Exp. Med.* 185:663.
17 Komiya, A., Hirai, K., Iikura, M. *et al.* 2003. Induction of basophil desensitization in physiological medium: enhancement after IgE-dependent upregulation of surface IgE binding on basophils. *Int. Arch. Allergy Immunol.* 130:40.
18 Yamaguchi, M., Hirai, K., Ohta, K. *et al.* 1996. Nonreleasing basophils convert to releasing basophils by culturing with IL-3. *J. Allergy Clin. Immunol.* 97:1279.
19 Lavens-Phillips, S. E. and MacGlashan, D. W., Jr. 2000. The tyrosine kinases p53/56lyn and p72syk are differentially expressed at the protein level but not at the messenger RNA level in nonreleasing human basophils. *Am. J. Respir. Cell Mol. Biol.* 23:566.
20 Kepley, C. L., Youssef, L., Andrews, R. P., Wilson, B. S. and Oliver, J. M. 1999. Syk deficiency in nonreleaser basophils. *J. Allergy Clin. Immunol.* 104:279.
21 MacGlashan, D. W., Jr, Bochner, B. S., Adelman, D. C. *et al.* 1997. Down-regulation of FcεRI expression on human basophils during *in vivo* treatment of atopic patients with anti-IgE antibody. *J. Immunol.* 158:1438.
22 Lantz, C. S., Yamaguchi, M., Oettgen, H. C. *et al.* 1997. IgE regulates mouse basophil FcεRI expression *in vivo*. *J. Immunol.* 158:2517.
23 Asai, K., Kitaura, J., Kawakami, Y. *et al.* 2001. Regulation of mast cell survival by IgE. *Immunity* 14:791.
24 Kalesnikoff, J., Huber, M., Lam, V. *et al.* 2001. Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity* 14:801.
25 Bochner, B. S., MacGlashan, D. W., Jr, Marcotte, G. V. and Schleimer, R. P. 1989. IgE-dependent regulation of human basophil adherence to vascular endothelium. *J. Immunol.* 142:3180.
26 Taub, D., Dastych, J., Inamura, N. *et al.* 1995. Bone marrow-derived murine mast cells migrate, but do not degranulate, in response to chemokines. *J. Immunol.* 154:2393.
27 Kay, A. B. and Austen, K. F. 1972. Chemotaxis of human basophil leucocytes. *Clin. Exp. Immunol.* 11:557.
28 de Paulis, A., Montuori, N., Prevete, N. *et al.* 2004. Urokinase induces basophil chemotaxis through a urokinase receptor epitope that is an endogenous ligand for formyl peptide receptor-like 1 and -like 2. *J. Immunol.* 173:5739.
29 Yamada, H., Yamaguchi, M., Yamamoto, K. *et al.* 2000. Eotaxin in induced sputum of asthmatics: relationship with eosinophils and eosinophil cationic protein in sputum. *Allergy* 55:392.
30 Gounni, A. S., Lamkhioued, B., Ochiai, K. *et al.* 1994. High-affinity IgE receptor on eosinophils is involved in defence against parasites. *Nature* 367:183.
31 Iikura, M., Yamaguchi, M., Hirai, K. *et al.* 2001. Regulation of surface FcεRI expression on human eosinophils by IL-4 and IgE. *Int. Arch. Allergy Immunol.* 124:470.
32 Maurer, D., Fiebiger, S., Ebner, C. *et al.* 1996. Peripheral blood dendritic cells express FcεRI as a complex composed of FcεRI α- and FcεRI γ-chains and can use this receptor for IgE-mediated allergen presentation. *J. Immunol.* 157:607.
33 Gounni, A. S., Lamkhioued, B., Koussih, L., Ra, C., Renzi, P. M. and Hamid, Q. 2001. Human neutrophils express the high-affinity receptor for immunoglobulin E (FcεRI): role in asthma. *FASEB J.* 15:940.
34 Hasegawa, S., Pawankar, R., Suzuki, K. *et al.* 1999. Functional expression of the high affinity receptor for IgE (FcεRI) in human platelets and its' intracellular expression in human megakaryocytes. *Blood* 93:2543.

Citrullination by Peptidylarginine Deiminase in Rheumatoid Arthritis

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ABSTRACT: **Rheumatoid arthritis (RA) is a complex, multifactorial disease with genetic and immunological aspects. Because RA is an autoimmune condition, dysregulation of the immune system is implied. Many linkage and association studies have also indicated that multiple genetic factors are associated with RA. Although the contribution of each genetic factor is small, the combination of these factors affects RA development. Previous studies have suggested that genetic changes affect the internal immunological environment, which results in autoimmune diseases. More recent genetic studies indicate that the HLA-DRB gene is the predominant cause of RA and that other non-HLA genes are also involved. We reported that peptidylarginine deiminase (gene name abbreviated to PADI, protein name abbreviated to PAD) type 4 is the one of the non-HLA genetic factors involved in RA via citrullination. Antibodies against citrullinated proteins/peptides are highly specific to RA, but the physiological roles of PADI gene, PAD proteins as their products and citrullinated proteins/peptides are obscure. However, levels of anticitrullinated protein antibodies are apparently also increased and were involved in the pathogenesis of autoimmune arthritis in mice with collagen-induced arthritis (CIA). These data suggested that citrullinated protein and anticitrullinated protein antibodies play important roles in the development of RA. This review summarizes the relationship between RA and citrullination, as well as the role of PADI4 genetics.**

KEYWORDS: **rheumatoid arthritis (RA); peptidylarginine deiminase (PADI); anti citrullinated peptide antibody; single nucleotide polymorphism (SNP)**

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by the destruction of many joints accompanied by multiple organ involvement. The disorder is an autoimmune disease and many autoantibodies that react against various autoantigens are detectable in the serum of RA patients. However, the etiology of RA remains unknown. Autoantibodies that recognize citrullinated self-proteins (anticitrullinated peptide antibodies [ACPA]) have recently been established as highly specific autoantibodies in RA,[1] and PADI4, a gene that encodes an enzyme producing citrullinated proteins is associated with RA.[2] These findings suggest that protein citrullination by peptidylarginine deiminase (PAD; gene name abbreviate to PADI, protein name abbreviate to PAD) is essential for the development of RA and thus citrullinated proteins and ACPA should play a pathogenic role in the autoimmunity associated with RA. We review recent findings of citrulline, proteins citrullinated by PAD enzymes, and ACPA from genetic, biochemical, histological, immunological, and clinical aspects of RA.

CITRULLINE AND CITRULLINATION

Citrulline

Citrulline is a noncoding, native, deiminated form of arginine (FIG. 1) that in mammals assumes free amino acid and peptidyl forms with independent metabolic pathways. Citrulline is part of the citric acid and ornithine cycles, and its metabolism is tightly regulated. Hypercitrullinemia is an innate metabolic disorder that results from the abnormal metabolism of free citrulline.

Citrulline might have a pathological function in inflammatory diseases because it induces nitric oxide (NO), and serum nitrite and citrulline, in addition to urinary citrulline levels are higher in patients with systemic lupus erythematosus (SLE) than in controls.[3] Citrulline also has a ureide group, which is reactive because of a highly electrophilic carbon atom.

Citrullination

Peptidyl-citrulline residues in proteins are produced only through post-translational modification of arginine residues catalyzed by PAD, which is encoded by the PADI gene, because the tRNA for citrulline is unknown. This enzymatic reaction is called citrullination or deimination. The PADI 1, 2, 3, 4/5 (human PADI5 is orthologous to mouse PADI4 and has been renamed human PADI4), and 6 isozymes with highly conserved peptide sequences have been identified in several mammals. Although the chemical reactivity of peptidyl-citrulline and free citrulline differs, amino acid substitution from arginine