

- CD4⁺CD25⁺ regulatory T cells by TGF- β induction of transcription factor Foxp3. *J. Exp. Med.* 198, 1875–1886.
- Cobb, B.S., Hertweck, A., Smith, J., O'Connor, E., Graf, D., Cook, T., Smale, S.T., Sakaguchi, S., Livesey, F.J., Fisher, A.G., and Morkenslager, M.A. (2006). A role for Dicer in immune regulation. *J. Exp. Med.* 203, 2519–2527.
- Collison, L.W., Workman, C.J., Kuo, T.T., Boyd, K., Wang, Y., Vignali, K.M., Cross, R., Sehy, D., Blumberg, R.S., and Vignali, D.A. (2007). The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450, 566–569.
- Coombes, J.L., Siddiqui, K.R., Arancibia-Carcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103⁺ DCs induces Foxp3⁺ regulatory T cells via a TGF- β and retinoic acid-dependent mechanism. *J. Exp. Med.* 204, 1757–1764.
- Danke, N.A., Koelle, D.M., Yee, C., Beheray, S., and Kwok, W.W. (2004). Autoreactive T cells in healthy individuals. *J. Immunol.* 172, 5967–5972.
- Fehérvári, Z., and Sakaguchi, S. (2004). Control of Foxp3⁺ CD25⁺CD4⁺ regulatory cell activation and function by dendritic cells. *Int. Immunol.* 16, 1769–1780.
- Fisson, S., Darrasse-Jeze, G., Litvinova, E., Septier, F., Klatzmann, D., Liblau, R., and Salomon, B.L. (2003). Continuous activation of autoreactive CD4⁺CD25⁺ regulatory T cells in the steady state. *J. Exp. Med.* 198, 737–746.
- Floess, S., Freyer, J., Siewert, C., Baron, U., Olek, S., Polansky, J., Schlawe, K., Chang, H.D., Bopp, T., Schmitt, E., et al. (2007). Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol.* 5, e38. 10.1371/journal.pbio.0050038.
- Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat. Immunol.* 4, 330–336.
- Fontenot, J.D., Rasmussen, J.P., Gavin, M.A., and Rudensky, A.Y. (2005). A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 6, 1142–1151.
- Fowell, D., and Mason, D. (1993). Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4⁺ T cell subset that inhibits this autoimmune potential. *J. Exp. Med.* 177, 627–636.
- Gavin, M.A., Torgerson, T.R., Houston, E., DeRoos, P., Ho, W.Y., Stray-Pedersen, A., Ocheltree, E.L., Greenberg, P.D., Ochs, H.D., and Rudensky, A.Y. (2006). Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc. Natl. Acad. Sci. USA* 103, 6659–6664.
- Gavin, M.A., Rasmussen, J.P., Fontenot, J.D., Vasta, V., Manganiello, V.C., Beavo, J.A., and Rudensky, A.Y. (2007). Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 445, 771–775.
- Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E., and Roncarolo, M.G. (1997). A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389, 737–742.
- Haribhai, D., Lin, W., Reland, L.M., Truong, N., Williams, C.B., and Chatila, T.A. (2007). Regulatory T cells dynamically control the primary immune response to foreign antigen. *J. Immunol.* 178, 2961–2972.
- Hill, J.A., Feuerer, M., Tash, K., Haxhinasto, S., Perez, J., Melamed, R., Mathis, D., and Benoist, C. (2007). Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 27, 786–800.
- Hirota, K., Yoshitomi, H., Hashimoto, M., Maeda, S., Terada, S., Sugimoto, N., Yamaguchi, T., Nomura, T., Ito, H., Nakamura, T., et al. (2007). Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J. Exp. Med.* 204, 2803–2812.
- Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299, 1057–1061.
- Hsieh, C.S., Zheng, Y., Liang, Y., Fontenot, J.D., and Rudensky, A.Y. (2006). An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat. Immunol.* 7, 401–410.
- Huehn, J., and Hamann, A. (2005). Homing to suppress: address codes for Treg migration. *Trends Immunol.* 26, 632–636.
- Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F., and Sakaguchi, S. (1999). Thymus and autoimmunity: production of CD25⁺CD4⁺ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J. Immunol.* 162, 5317–5326.
- Jordan, M.S., Boesteanu, A., Reed, A.J., Petrone, A.L., Hohenbeck, A.E., Lerman, M.A., Najj, A., and Caton, A.J. (2001). Thymic selection of CD4⁺CD25⁺ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2, 301–306.
- Kajura, F., Sun, S., Nomura, T., Izumi, K., Ueno, T., Bando, Y., Kuroda, N., Han, H., Li, Y., Matsushima, A., et al. (2004). NF- κ B-inducing kinase establishes self-tolerance in a thymic stroma-dependent manner. *J. Immunol.* 172, 2067–2075.
- Kallikourdis, M., Andersen, K.G., Welch, K.A., and Betz, A.G. (2007). Alloantigen-enhanced accumulation of CCR5⁺ 'effector' regulatory T cells in the gravid uterus. *Proc. Natl. Acad. Sci. USA* 104, 594–599.
- Kawahata, K., Misaki, Y., Yamauchi, M., Tsunekawa, S., Setoguchi, K., Miyazaki, J., and Yamamoto, K. (2002). Generation of CD4⁺CD25⁺ regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression. *J. Immunol.* 168, 4399–4405.
- Khattri, R., Cox, T., Yasayko, S.A., and Ramsdell, F. (2003). An essential role for Scurfin in CD4⁺CD25⁺ T regulatory cells. *Nat. Immunol.* 4, 337–342.
- Kim, J.M., Rasmussen, J.P., and Rudensky, A.Y. (2007). Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat. Immunol.* 8, 191–197.
- Klein, L., Khazaie, K., and von Boehmer, H. (2003). In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc. Natl. Acad. Sci. USA* 100, 8886–8891.
- Koonpaew, S., Shen, S., Flowers, L., and Zhang, W. (2006). LAT-mediated signaling in CD4⁺CD25⁺ regulatory T cell development. *J. Exp. Med.* 203, 119–129.
- Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M.C., and von Boehmer, H. (2005). Inducing and expanding regulatory T cell populations by foreign antigen. *Nat. Immunol.* 6, 1219–1227.
- Lahl, K., Loddenkemper, C., Drouin, C., Freyer, J., Arnason, J., Eberl, G., Hamann, A., Wagner, H., Huehn, J., and Sparwasser, T. (2007). Selective depletion of Foxp3⁺ regulatory T cells induces a scurfy-like disease. *J. Exp. Med.* 204, 57–63.
- Laurence, A., Tato, C.M., Davidson, T.S., Kanno, Y., Chen, Z., Yao, Z., Blank, R.B., Meylan, F., Siegel, R., Hennighausen, L., et al. (2007). Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26, 371–381.
- Lee, I., Wang, L., Wells, A.D., Dorf, M.E., Ozkysnak, E., and Hancock, W.W. (2005). Recruitment of Foxp3⁺ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor. *J. Exp. Med.* 201, 1037–1044.
- Li, B., and Greene, M.I. (2007). FOXP3 actively represses transcription by recruiting the HAT/HDAC complex. *Cell Cycle* 6, 1432–1436.
- Lin, W., Haribhai, D., Reland, L.M., Truong, N., Carlson, M.R., Williams, C.B., and Chatila, T.A. (2007). Regulatory T cell development in the absence of functional Foxp3. *Nat. Immunol.* 8, 359–368.
- Malek, T.R., and Bayer, A.L. (2004). Tolerance, not immunity, crucially depends on IL-2. *Nat. Rev. Immunol.* 4, 685–674.
- Marson, A., Kretschmer, K., Frampton, G.M., Jacobsen, E.S., Polansky, J.K., MacIsaac, K.D., Levine, S.S., Fraenkel, E., von Boehmer, H., and Young, R.A. (2007). Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* 445, 931–935.

- Mathis, D., and Benoist, C. (2007). A decade of AIRE. *Nat. Rev. Immunol.* 7, 645–650.
- Maynard, C.L., Harrington, L.E., Janowski, K.M., Oliver, J.R., Zindl, C.L., Rudensky, A.Y., and Weaver, C.T. (2007). Regulatory T cells expressing interleukin 10 develop from Foxp3⁺ and Foxp3⁻ precursor cells in the absence of interleukin 10. *Nat. Immunol.* 8, 931–941.
- McHugh, R.S., Whitters, M.J., Piccirillo, C.A., Young, D.A., Shevach, E.M., Collins, M., and Byrne, M.C. (2002). CD4⁺CD25⁺ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16, 311–323.
- Miyara, M., and Sakaguchi, S. (2007). Natural regulatory T cells: mechanisms of suppression. *Trends Mol. Med.* 13, 108–116.
- Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M., and Cheroutre, H. (2007). Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 317, 256–260.
- Nishikawa, H., Jäger, E., Ritter, G., Old, L.J., and Grnjatic, S. (2005). CD4⁺CD25⁺ regulatory T cells control the induction of antigen-specific CD4⁺ helper T cell responses in cancer patients. *Blood* 106, 1008–1011.
- Ochs, H.D., Ziegler, S.F., and Torgerson, T.R. (2005). FOXP3 acts as a rheostat of the immune response. *Immunol. Rev.* 203, 156–164.
- Ono, M., Yaguchi, H., Ohkura, N., Kitabayashi, I., Nagamura, Y., Nomura, T., Miyachi, Y., Tsukada, T., and Sakaguchi, S. (2007). Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature* 446, 685–689.
- Pacholczyk, R., Kern, J., Singh, N., Iwashima, M., Kraj, P., and Ignatowicz, L. (2007). Nonself-antigens are the cognate specificities of Foxp3⁺ regulatory T cells. *Immunity* 27, 493–504.
- Pandiyar, P., Zheng, L., Ishihara, S., Reed, J., and Lenardo, M.J. (2007). CD4⁺CD25⁺Foxp3⁺ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4⁺ T cells. *Nat. Immunol.* 8, 1353–1362.
- Powrie, F., and Mason, D. (1990). OX-22⁺ CD4⁺ T cells induce wasting disease with multiple organ pathology: prevention by OX-22⁻ subset. *J. Exp. Med.* 172, 1701–1708.
- Ranger, A.M., Oukka, M., Rengarajan, J., and Glimcher, L.H. (1998). Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. *Immunity* 9, 627–635.
- Rao, A., Luo, C., and Hogan, P.G. (1997). Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* 15, 707–747.
- Read, S., Malmstrom, V., and Powrie, F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25⁺CD4⁺ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192, 295–302.
- Sakaguchi, S. (2000). Animal models of autoimmunity and their relevance to human diseases. *Curr. Opin. Immunol.* 12, 684–690.
- Sakaguchi, S. (2005). Naturally arising Foxp3-expressing CD25⁺CD4⁺ regulatory T cells in immunologic tolerance to self and non-self. *Nat. Immunol.* 6, 345–352.
- Sakaguchi, S., Takahashi, T., and Nishizuka, Y. (1982). Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J. Exp. Med.* 156, 1577–1586.
- Sakaguchi, S., Fukuma, K., Kuribayashi, K., and Masuda, T. (1985). Organ-specific autoimmune diseases induced in mice by elimination of T-cell subset. I. Evidence for the active participation of T cells in natural self-tolerance: deficit of a T-cell subset as a possible cause of autoimmune disease. *J. Exp. Med.* 161, 72–87.
- Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic tolerance maintained by activated T cells expressing IL-2 receptor α -chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155, 1151–1164.
- Salomon, B., Lenschow, D.J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A., and Bluestone, J.A. (2000). B7/CD28 costimulation is essential for the homeostasis of the CD4⁺CD25⁺ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12, 431–440.
- Samy, E.T., Parker, L.A., Sharp, C.P., and Tung, K.S. (2005). Continuous control of autoimmune disease by antigen-dependent polyclonal CD4⁺CD25⁺ regulatory T cells in the regional lymph node. *J. Exp. Med.* 202, 771–781.
- Sather, B.D., Treuting, P., Perdue, N., Miazgowski, M., Fontenot, J.D., Rudensky, A.Y., and Campbell, D.J. (2007). Altering the distribution of Foxp3⁺ regulatory T cells results in tissue-specific inflammatory disease. *J. Exp. Med.* 204, 1335–1347.
- Scheinecker, C., McHugh, R., Shevach, E.M., and Germain, R.N. (2002). Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. *J. Exp. Med.* 196, 1079–1090.
- Schneider, M.A., Meingassner, J.G., Lipp, M., Moore, H.D., and Rot, A. (2007). CCR7 is required for the in vivo function of CD4⁺CD25⁺ regulatory T cells. *J. Exp. Med.* 204, 735–745.
- Setoguchi, R., Hori, S., Takahashi, T., and Sakaguchi, S. (2005). Homeostatic maintenance of natural Foxp3⁺CD25⁺CD4⁺ regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 201, 723–735.
- Shevach, E.M. (2006). From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* 25, 195–201.
- Shevach, E.M., DiPaolo, R.A., Andersson, J., Zhao, D.M., Stephens, G.L., and Thornton, A.M. (2006). The lifestyle of naturally occurring CD4⁺CD25⁺Foxp3⁺ regulatory T cells. *Immunol. Rev.* 212, 60–73.
- Shimizu, J., Yamazaki, S., and Sakaguchi, S. (1999). Induction of tumor immunity by removing CD25⁺CD4⁺ T cells: a common basis between tumor immunity and autoimmunity. *J. Immunol.* 163, 5211–5218.
- Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y., and Sakaguchi, S. (2002). Stimulation of CD25⁺CD4⁺ regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* 3, 135–142.
- Singh, B., Read, S., Asseman, C., Malmstrom, V., Mottet, C., Stephens, L.A., Stepankova, R., Tsikalova, H., and Powrie, F. (2001). Control of intestinal inflammation by regulatory T cells. *Immunol. Rev.* 182, 190–200.
- Stephens, L.A., Gray, D., and Anderton, S.M. (2005). CD4⁺CD25⁺ regulatory T cells limit the risk of autoimmune disease arising from T cell receptor crossreactivity. *Proc. Natl. Acad. Sci. USA* 102, 17418–17423.
- Sugimoto, N., Oida, T., Hirota, K., Nakamura, K., Nomura, T., Uchiyama, T., and Sakaguchi, S. (2006). Foxp3-dependent and -independent molecules specific for CD25⁺CD4⁺ natural regulatory T cells revealed by DNA microarray analysis. *Int. Immunol.* 18, 1197–1209.
- Sun, C.M., Hall, J.A., Blank, R.B., Bouladoux, N., Oukka, M., Mora, J.R., and Belkaid, Y. (2007). Small intestine lamina propria dendritic cells promote de novo generation of Foxp3⁺ T reg cells via retinoic acid. *J. Exp. Med.* 204, 1775–1785.
- Sutmoller, R.P., Morgan, M.E., Netea, M.G., Grauer, O., and Adema, G.J. (2006). Toll-like receptors on regulatory T cells: expanding immune regulation. *Trends Immunol.* 27, 387–393.
- Szanya, V., Ermann, J., Taylor, C., Holness, C., and Fathman, C.G. (2002). The subpopulation of CD4⁺CD25⁺ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. *J. Immunol.* 169, 2461–2465.
- Tadokoro, C.E., Shakhar, G., Shen, S., Ding, Y., Lino, A.C., Maraver, A., Lafaille, J.J., and Dustin, M.L. (2006). Regulatory T cells inhibit stable contacts between CD4⁺ T cells and dendritic cells in vivo. *J. Exp. Med.* 203, 505–511.
- Tang, Q., and Bluestone, J.A. (2008). The Foxp3⁺ regulatory T cell: a jack of all trades, master of regulation. *Nat. Immunol.* 9, 239–244.
- Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J., and Sakaguchi, S. (1998). Immunologic self-tolerance maintained by CD25⁺CD4⁺ naturally anergic and suppressive T cells: induction of auto-

- immune disease by breaking their anergic/suppressive state. *Int. Immunol.* **10**, 1969–1980.
- Takahashi, T., Tagami, T., Yamazaki, S., Ueda, T., Shimizu, J., Sakaguchi, N., Mak, T.W., and Sakaguchi, S. (2000). Immunologic self-tolerance maintained by CD25⁺CD4⁺ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* **192**, 303–310.
- Tang, Q., Adams, J.Y., Tooley, A.J., Bi, M., Fife, B.T., Serra, P., Santamaria, P., Locksley, R.M., Krummel, M.F., and Bluestone, J.A. (2006). Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat. Immunol.* **7**, 83–92.
- Taniuchi, I., Osato, M., Egawa, T., Sunshine, M.J., Bae, S.C., Komori, T., Ito, Y., and Littman, D.R. (2002). Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* **111**, 621–633.
- Thornton, A.M., and Shevach, E.M. (1998). CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* **188**, 287–296.
- Uhlir, H.H., Coombes, J., Mottet, C., Izcue, A., Thompson, C., Fanger, A., Tannapfel, A., Fontenot, J.D., Ramsdell, F., and Powrie, F. (2006). Characterization of Foxp3⁺CD4⁺CD25⁺ and IL-10-secreting CD4⁺CD25⁺ T cells during cure of colitis. *J. Immunol.* **177**, 5852–5860.
- van Santen, H.M., Benoist, C., and Mathis, D. (2004). Number of T_{reg} cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. *J. Exp. Med.* **200**, 1221–1230.
- Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M., and Stockinger, B. (2006). TGF- β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* **24**, 179–189.
- Vieira, P.L., Christensen, J.R., Minaae, S., O'Neill, E.J., Barrat, F.J., Boonstra, A., Barthlott, T., Stockinger, B., Wraith, D.C., and O'Garra, A. (2004). IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4⁺CD25⁺ regulatory T cells. *J. Immunol.* **172**, 5988–5993.
- von Boehmer, H. (2005). Mechanisms of suppression by suppressor T cells. *Nat. Immunol.* **6**, 338–344.
- Waldmann, H., Adams, E., Fairchild, P., and Cobbold, S. (2006). Infectious tolerance and the long-term acceptance of transplanted tissue. *Immunol. Rev.* **212**, 301–313.
- Wan, Y.Y., and Flavell, R.A. (2007). Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* **445**, 766–770.
- Wang, H.Y., and Wang, R.F. (2007). Regulatory T cells and cancer. *Curr. Opin. Immunol.* **19**, 217–223.
- Watanabe, N., Wang, Y.H., Lee, H.K., Ito, T., Wang, Y.H., Cao, W., and Liu, Y.J. (2005). Hassall's corpuscles instruct dendritic cells to induce CD4⁺CD25⁺ regulatory T cells in human thymus. *Nature* **436**, 1181–1185.
- Wellcome Trust Case Control Consortium (2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* **447**, 661–678.
- Wu, Y., Borda, M., Heissmeyer, V., Feuerer, M., Lapan, A.D., Stroud, J.C., Bates, D.L., Guo, L., Han, A., Ziegler, S.F., et al. (2006). FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* **126**, 375–387.
- Yagi, H., Nomura, T., Nakamura, K., Yamazaki, S., Kitawaki, T., Hori, S., Maeda, M., Onodera, M., Uchiyama, T., Fujii, S., and Sakaguchi, S. (2004). Crucial role of FOXP3 in the development and function of human CD25⁺CD4⁺ regulatory T cells. *Int. Immunol.* **16**, 1843–1856.
- Yamaguchi, T., Hirota, K., Nagahama, K., Ohkawa, K., Takahashi, T., Nomura, T., and Sakaguchi, S. (2007). Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. *Immunity* **27**, 145–159.
- Yamanouchi, J., Rainbow, D., Serra, P., Howlett, S., Hunter, K., Garner, V.E., Gonzalez-Munoz, A., Clark, J., Vejjola, R., Cubbon, R., et al. (2007). Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nat. Genet.* **39**, 329–337.
- Yamazaki, S., Iyoda, T., Tarbell, K., Olson, K., Velinzon, K., Inaba, K., and Steinman, R.M. (2003). Direct expansion of functional CD25⁺CD4⁺ regulatory T cells by antigen-processing dendritic cells. *J. Exp. Med.* **198**, 235–247.
- Yao, Z., Kanno, Y., Kerenyi, M., Stephens, G., Durant, L., Watford, W.T., Laurence, A., Robinson, G.W., Shevach, E.M., Moriggl, R., et al. (2007). Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* **109**, 4368–4375.
- Yurchenko, E., Tritt, M., Hay, V., Shevach, E.M., Belkaid, Y., and Piccirillo, C.A. (2006). CCR5-dependent homing of naturally occurring CD4⁺ regulatory T cells to sites of Leishmania major infection favors pathogen persistence. *J. Exp. Med.* **203**, 2451–2460.
- Zheng, Y., Josefowicz, S.Z., Kas, A., Chu, T.T., Gavin, M.A., and Rudenski, A.Y. (2007). Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* **445**, 936–940.

Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model

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This report shows that interleukin (IL) 17-producing T helper type 17 (Th17) cells predominantly express CC chemokine receptor (CCR) 6 in an animal model of rheumatoid arthritis (RA). Th17 cells induced *in vivo* in normal mice via homeostatic proliferation similarly express CCR6, whereas those inducible *in vitro* by transforming growth factor β and IL-6 additionally need IL-1 and neutralization of interferon (IFN) γ and IL-4 for CCR6 expression. Forced expression of ROR γ t, a key transcription factor for Th17 cell differentiation, induces not only IL-17 but also CCR6 in naive T cells. Furthermore, Th17 cells produce CCL20, the known ligand for CCR6. Synoviocytes from arthritic joints of mice and humans also produce a large amount of CCL20, with a significant correlation ($P = 0.014$) between the amounts of IL-17 and CCL20 in RA joints. The CCL20 production by synoviocytes is augmented *in vitro* by IL-1 β , IL-17, or tumor necrosis factor α , and is suppressed by IFN- γ or IL-4. Administration of blocking anti-CCR6 monoclonal antibody substantially inhibits mouse arthritis. Thus, the joint cytokine milieu formed by T cells and synovial cells controls the production of CCL20 and, consequently, the recruitment of CCR6⁺ arthritogenic Th17 cells to the inflamed joints. These results indicate that CCR6 expression contributes to Th17 cell function in autoimmune disease, especially in autoimmune arthritis such as RA.

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Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that primarily affects multiple joints. Autoimmune CD4⁺ T cells are required for the progression of RA, especially in an initial phase of the disease (1). It is obscure, however, how such arthritogenic CD4⁺ T cells are produced in the immune system, become activated, and mediate RA. Recent studies with animal models have suggested that CD4⁺ T cells secreting IL-17 (IL-17A), called Th17 cells, may play a key role in the progression of RA as well as multiple sclerosis (2–6). Hindering the development of Th17 cells or blocking IL-17 activity indeed inhibits autoimmune pathology in these models (2–6). A particular cytokine milieu contributes to this preferential differentiation of naive self-reactive T cells to Th17

effector cells (2–6). In addition, the transcription factor ROR γ t specifically controls Th17 cell differentiation, indicating that Th17 cells form a T cell lineage distinct from Th1 or Th2 cells (7). To further analyze the roles of Th17 cells in autoimmune disease, we have searched for cell-surface molecules that are specifically expressed in Th17 cells and are crucial for their functions, such as their migration to inflamed joints in RA.

The SKG strain of mice, a mutant on the BALB/c background, spontaneously develops T cell-mediated autoimmune arthritis, which clinically and immunologically resembles human RA (8–10). The strain harbors a recessive mutation of the gene encoding an Src homology 2 domain of ζ -associated protein 70, a key signaling molecule in T cells. Impaired signal transduction through SKG ζ -associated protein 70 results in thymic positive selection and failure

K. Hirota and H. Yoshitomi contributed equally to this work.
The online version of this article contains supplemental material.

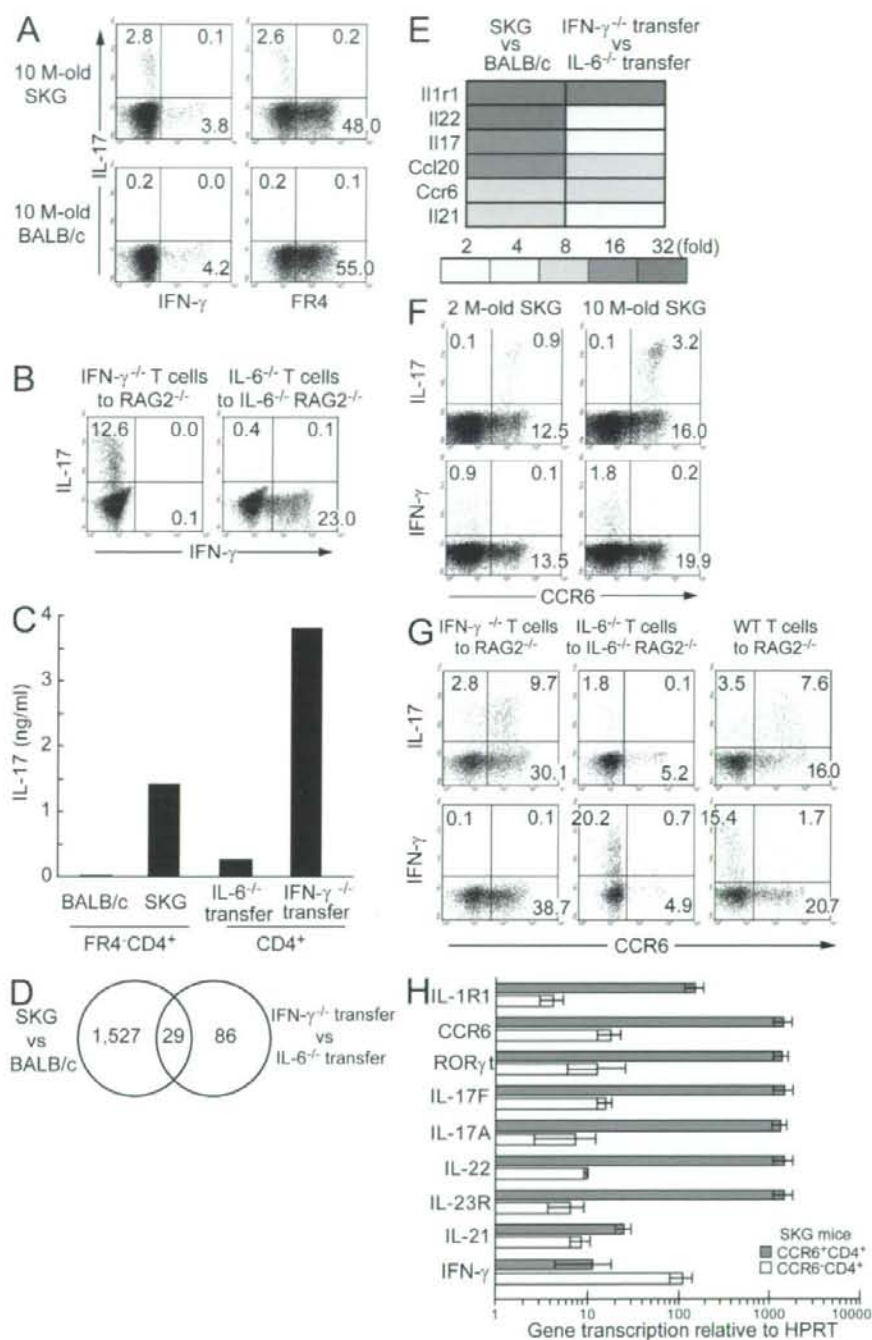


Figure 1. Gene microarray analysis of Th17 cells and their predominant expression of CCR6. (A) LN CD4⁺ T cells from 10-mo-old BALB/c or SKG mice were stained for intracellular IL-17 and IFN- γ , or these cytokines and cell-surface FR4. (B) CD4⁺ T cells from IFN- γ ^{-/-} or IL-6^{-/-} mice were transferred to RAG2^{-/-} mice or IL-6^{-/-}RAG2^{-/-} mice, respectively. Intracellular IL-17 and IFN- γ in recipient splenic CD4⁺ T cells were stained on day 7. (C) Purified FR4-CD4⁺ T cells from BALB/c or SKG mice or CD4⁺ T cells after homeostatic proliferation, as shown in A and B, were stimulated with PMA/ionomycin for 3 h,

in the negative selection of highly self-reactive T cells that include potentially arthritogenic CD4⁺ T cells (8). Our previous report showed that SKG CD4⁺ T cells spontaneously differentiated to arthritogenic Th17 cells, which were nonredundant in mediating SKG arthritis (6). The study also showed that not only self-reactive CD4⁺ T cells in SKG mice but also naive T cells in normal mice were able to differentiate to Th17 cells when they were subjected to homeostatic proliferation in a T cell-deficient environment (6). In both systems, IL-6 deficiency inhibited Th17 cell differentiation, whereas IFN- γ deficiency augmented it (6). Use of these two *in vivo* Th17 cell induction systems allowed us to determine the genes commonly up-regulated in Th17 cells. We show in this report that Th17 cells predominantly express CC chemokine receptor (CCR) 6 and produce its ligand, CCL20. Inflamed synovial cells in both SKG arthritis and human RA also produce CCL20, facilitating the migration of arthritogenic Th17 cells to inflamed joints. Thus, CCR6 is an important functional marker for Th17 cells and contributes to their preferential migration to a particular inflammation site.

RESULTS AND DISCUSSION

Gene microarray analysis of Th17 cells

Th17 cells increase with age in SKG mice, constituting ~3–10% of LN CD4⁺ T cells at 10 mo of age compared with ~0.2–0.7% in age-matched BALB/c mice (Fig. 1 A). Notably, they scarcely express the folate receptor 4 (FR4), which is highly expressed by CD25⁺CD4⁺ natural regulatory T cells and central memory-like CD4⁺ T cells but not by effector or effector memory-like CD4⁺ T cells (Fig. 1 A) (11–12). Th17 cells can therefore be enriched by sorting FR4⁺CD4⁺ T cells from SKG mice. A large number of Th17 cells also developed spontaneously when CD4⁺ T cells from IFN- γ -deficient (IFN- γ ^{-/-}) BALB/c mice were transferred to T cell-deficient RAG2-deficient (RAG2^{-/-}) mice and subjected to homeostatic proliferation, whereas they failed to develop in a similar transfer of IL-6-deficient (IL-6^{-/-}) CD4⁺ T cells to IL-6^{-/-} RAG2^{-/-} mice (Fig. 1 B) (6). The numbers of Th17 cells assessed by cytofluorometric analysis were well correlated with the amounts of the IL-17 protein secreted after *in vitro* stimulation with PMA/ionomycin (Fig. 1 C).

To explore the functional molecules specifically expressed by Th17 cells, we conducted gene microarray analysis between 10-mo-old SKG FR4⁺CD4⁺ cells and age-matched

BALB/c FR4⁺CD4⁺ cells, and between IFN- γ ^{-/-}CD4⁺ cells and IL-6^{-/-}CD4⁺ T cells transferred to RAG2^{-/-} mice, as described in the previous paragraph. The analysis revealed that 1,556 and 115 genes were up-regulated in 10-mo-old SKG FR4⁺CD4⁺ and IFN- γ ^{-/-}CD4⁺ T cells after homeostatic proliferation, respectively, with 29 genes shared by the two groups of genes (Fig. 1 D). The 29 genes included those encoding cytokines, chemokines, and their receptors, such as IL-1R1, IL-17, IL-22, IL-21, CCR6, and CCL20 (Fig. 1 E; the rest of the genes are shown in Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20071397>).

Expression of CCR6 by Th17 cells

With our interest in Th17 cell-specific cell-surface molecules, we analyzed CCR6 expressed by Th17 cells in young (2-mo-old) or aged (10-mo-old) SKG mice, or BALB/c Th17 cells induced via homeostatic proliferation (Fig. 1 B). The majority of Th17 cells in SKG mice expressed CCR6 whether the mice were young or aged, whereas SKG Th1 cells did not (Fig. 1 F). After cell transfer to RAG2^{-/-} mice, the majority of IL-17⁺CD4⁺ T cells that had differentiated from IFN- γ ^{-/-} or WT T cells were CCR6⁺, whereas IFN- γ ⁺CD4⁺ T cells derived from WT CD4⁺ T cells were CCR6⁻ (Fig. 1 G). Furthermore, IL-6^{-/-}CD4⁺ T cells not only failed to give rise to IL-17⁺CCR6⁺ cells but also preferentially differentiated to IFN- γ ⁺CCR6⁻ cells (Fig. 1 G).

It was also noted that CCR6⁺ cells, either IL-17⁺ or IL-17⁻, increased in number among CD4⁺ T cells in aged SKG mice compared with aged BALB/c or young SKG mice (Fig. 1 F and Fig. S2 A, available at <http://www.jem.org/cgi/content/full/jem.20071397>). Both IL-17⁺CCR6⁺ and IL-17⁻CCR6⁺ populations similarly increased in RAG2^{-/-} mice transferred with IFN- γ ^{-/-} cells and, to a lesser extent, in those transferred with WT T cells, but not in IL-6^{-/-} cell-transferred RAG2^{-/-} mice (Fig. 1 G and Fig. S2 B). In addition, CCR6⁺CD4⁺ T cells in BALB/c mice gave rise to IL-17⁺CCR6⁺CD4⁺ T cells via homeostatic proliferation when transferred to RAG2^{-/-} mice (Fig. S2 C).

We also examined a possible correlation between CCR6 expression and transcription of the other genes depicted by the microarray analysis in Fig. 1 E or those genes reported to be functional in Th17 cells (e.g., IL-17, IL-17F, IL-21, IL-22, IL-1R1, IL-23R, and ROR γ t; Fig. 1 H) (7, 13, 14). When CCR6⁺ or CCR6⁻CD4⁺ T cells purified from aged SKG mice

and the amounts of IL-17 in culture supernatants were measured by ELISA. (D and E) Total RNA extracted from activated cells, as shown in C, was subjected to gene microarray for SKG versus BALB/c FR4⁺CD4⁺ T cells or IFN- γ ^{-/-} versus IL-6^{-/-}CD4⁺ T cells after homeostatic proliferation. The expression of each gene was averaged from three independent experiments and analyzed by GeneSpring software. The Venn diagram shown in D represents a group of up-regulated genes in SKG FR4⁺CD4⁺ T cells and IFN- γ ^{-/-}CD4⁺ T cells after homeostatic proliferation. Genes for cytokines, chemokines, or their receptors commonly up-regulated in the two groups of genes are shown in E. Graded colors represent relative expression levels. (F) LN CD4⁺ T cells from 2- or 10-mo-old SKG mice were stained for CCR6 and intracellular IL-17 or IFN- γ . (G) CD4⁺ T cells from IFN- γ ^{-/-}, IL-6^{-/-}, or WT mice were stained for CCR6 and intracellular IL-17 or IFN- γ after homeostatic proliferation, as shown in B. (H) Total RNA extracted from purified SKG CCR6⁺ or CCR6⁻CD4⁺ T cells stimulated with PMA/ionomycin for 3 h was subjected to quantitative RT-PCR for the indicated genes and normalized for hypoxanthine-guanine phosphoribosyltransferase (HPRT) messenger RNA expression, as previously described (reference 7). Data are shown as the mean \pm SD of three independent experiments. Results in A–C, F, and G represent three to five independent experiments. The percentage of cells in each quadrant is shown in A, B, F, and G.

were activated *in vitro* with PMA/ionomycin and expression levels of individual genes were analyzed by quantitative RT-PCR. CCR6⁺CD4⁺ SKG T cells indeed transcribed most of the genes much more actively than CCR6⁻CD4⁺ T cells, in contrast with much less transcription of the IFN- γ gene. As both aged SKG CD4⁺ T cells and BALB/c IFN- γ ^{-/-}CD4⁺ T cells highly expressed the *Il1r1* gene after homeostatic proliferation (Fig. 1 E), IL-17⁺CD4⁺ T cells in aged SKG mice expressed the IL-1R1 protein higher than IL-17⁻CD4⁺ T cells by cytofluorometric analysis (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20071397>).

Previous studies with mice have shown that CCR6 is mainly expressed on memory T cells, some natural CD25⁺CD4⁺ regulatory T cells, B cells, some DCs, and Langerhans cells, and suggested that CCR6 may be required for the trafficking of these cells via CCL20 (15–20). The present results show that, in addition to these cell populations, Th17 cells also express CCR6 and that CCR6 expression can distinguish Th17 cells from Th1 cells. The condition that facilitates *in vivo* Th17 cell differentiation might also promote differentiation of naive CD4⁺ T cell to CCR6⁺IL-17⁺IFN- γ ⁻ cells,

which are FR4 high and, therefore, phenotypically similar to natural CD25⁺CD4⁺ regulatory T cells and central memory-like T cells, as recently shown (12), and in accord with the results by others (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20071397>).

Induction of CCR6 in Th17 cells *in vitro*

ROR γ t crucially controls Th17 cell differentiation from naive CD4⁺ T cells (7). To test whether forced expression of ROR γ t directs the differentiation of CD4⁺ T cells to CCR6⁺ Th17 cells, ROR γ t was transduced in CCR6⁻CD4⁺ T cells by a bicistronic retroviral vector expressing ROR γ t and GFP (7). Notably, GFP-high (i.e., ROR γ t-high) cells expressed CCR6 as well as IL-17, whereas GFP-low cells did not (Fig. 2, A and B). Th17 cells can also be induced *in vitro* from naive CD4⁺ T cells by TCR stimulation in the presence of IL-6 and TGF- β , as reported by others (21–23). Interestingly, this *in vitro* induction of a large number of Th17 cells did not accompany CCR6 expression (Fig. 2 C). Yet, further addition of IL-1, based on the high expression of IL-1R by IL-17⁺ cells (Fig. 1 E and Fig. S3), elicited CCR6 expression in a

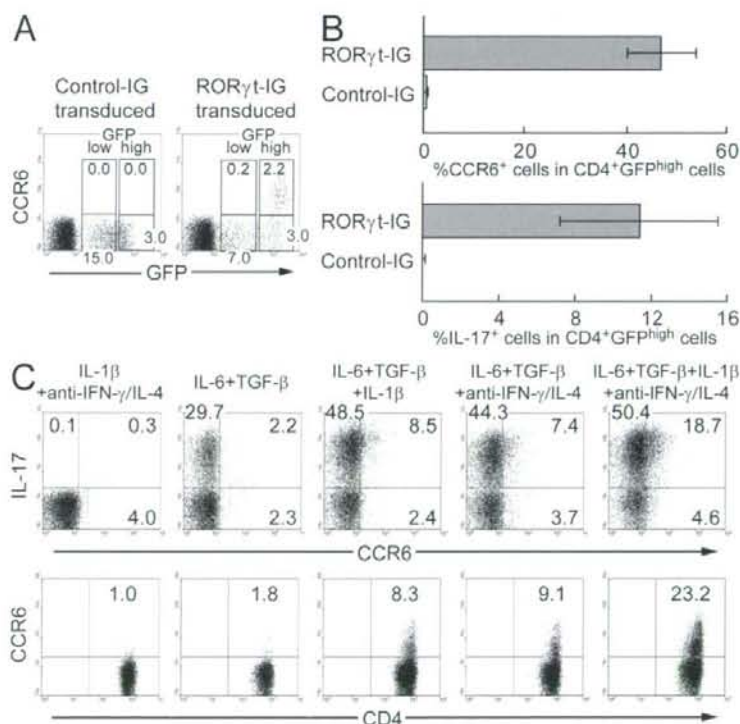


Figure 2. Induction of CCR6 on Th17 cells *in vitro*. (A) Retroviral vector, pMx-ROR γ t-IRES-GFP (ROR γ t-IG), or control pMx-IRES-GFP (Control-IG), was transduced into BALB/c CCR6⁻CD4⁺ T cells, as previously described (reference 7), and the cells expressing GFP were stained for CCR6 on day 4 after transduction. (B) The mean percentages \pm SD of CCR6⁺ or IL-17⁺ cells among CD4⁺GFP^{high} cells (as gated in A) in three independent experiments are shown. (C) CD4⁺CD25⁻CCR6⁻ T cells were *in vitro* driven to differentiate to Th17 cells in the indicated conditions, as previously described (reference 21). CCR6 and intracellular IL-17 gated on CD4⁺ T cells were stained on day 5. Results in A and C represent three independent experiments, and the percentage of cells in each quadrant is shown.

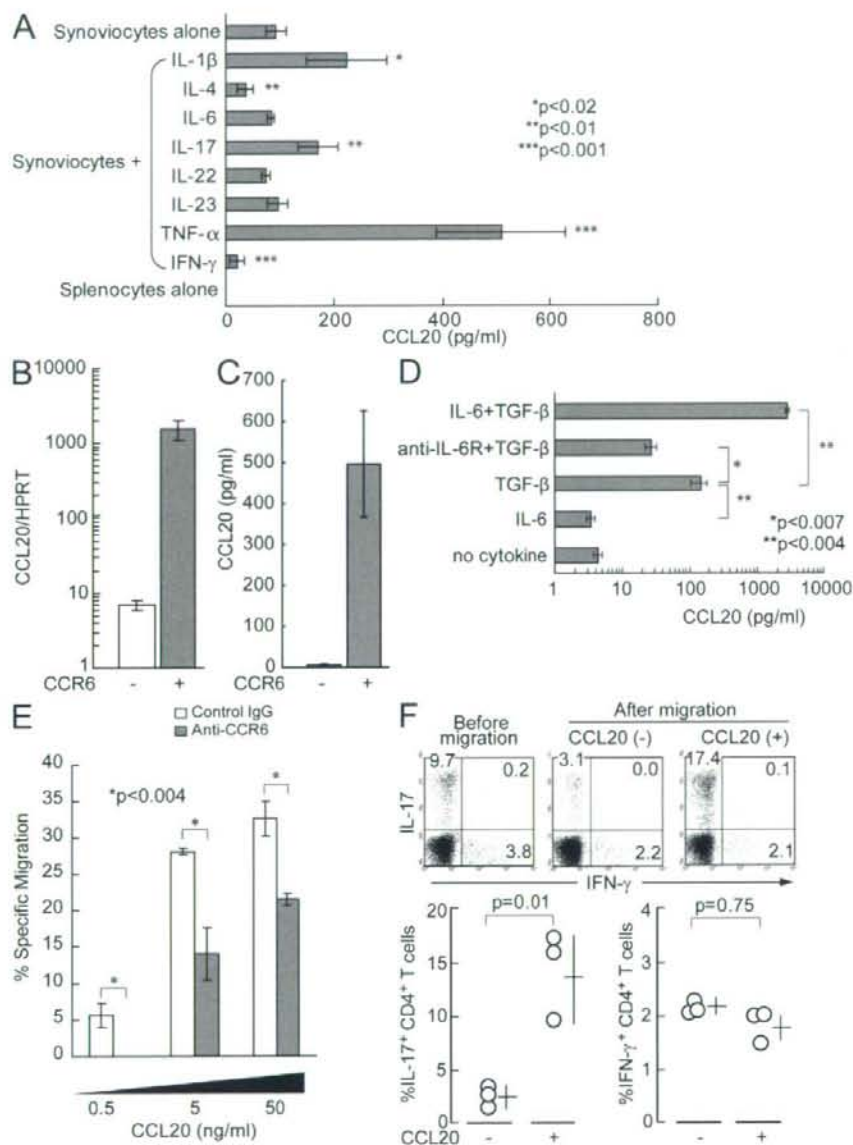


Figure 3. Cross-regulation of synoviocyte CCL20 production by cytokines and preferential migration of Th17 cells in response to CCL20. (A) 2.5×10^4 adherent synoviocytes, prepared as previously described (reference 10), were cultured with 10 ng/ml of the indicated cytokine for 24 h. The amounts of CCL20 in culture supernatants were measured by ELISA. Data are shown as the mean \pm SD of triplicate wells. (B and C) Purified CCR6 $^{-}$ or CCR6 $^{+}$ CD4 $^{+}$ T cells from SKG mice were stimulated with PMA/ionomycin for 3 h; CCL20 messenger RNA was assessed by quantitative RT-PCR (B), and CCL20 protein was measured by ELISA (C). Data are shown as the mean \pm SD of three independent experiments. (D) CD4 $^{+}$ T cells were in vitro driven to differentiate to Th17 cells in the presence of IL-6 and TGF- β , as previously described (reference 21). Cells were restimulated with PMA/ionomycin for 3 h on day 4, and the amounts of CCL20 were measured by ELISA. (E) SKG LN cells were placed with 100 μ g/ml anti-CCR6 antibody or control IgG on the upper well of a Transwell system. The migration assay was performed in the presence of designated concentrations of CCL20 added to the bottom well. See Materials and methods for details of the experiments. Data are shown as the mean \pm SD of triplicate wells. (F) The migration assay was performed in the absence or the presence of 50 ng/ml CCL20, as in E. CD4 $^{+}$ cells before being added to the upper well and CD4 $^{+}$ T cells that had migrated to the lower well were stained for intracellular IL-17 and IFN- γ (top). Percentages of IL-17 $^{+}$ or IFN- γ $^{+}$ cells among CD4 $^{+}$ cells in the lower wells in three independent migration assays with (+) or without (-) CCL20 are shown (bottom). Vertical bars represent the means \pm SD. Results in A, D, and E represent three independent experiments.

small (~15%) fraction of IL-17⁺ cells, whereas addition of IL-23, TNF- α , or IL-21 did not; the percentage further increased to ~30% by neutralization of both IFN- γ and IL-4 (Fig. 2 C).

Collectively, these results indicate that, although IL-6 and TGF- β together can induce ROR γ t at an amount sufficient for directing IL-17 production in vitro (7), a higher amount of ROR γ t or additional cytokines including IL-1 may be required for CCR6 expression. Our results also suggest that IFN- γ and IL-4, both of which inhibit the differentiation of naive T cells to Th17 cells (3), may also suppress CCR6 expression in Th17 cells.

CCL20 production by inflamed synoviocytes and Th17 cells

CCL20 is so far known to be the sole ligand for CCR6 and able to direct the migration of CCR6⁺ cells (15–19). To assess the possible production of CCL20 in inflamed synovial tissue to attract arthritogenic Th17 cells, collagenase-digested synovial tissues from swollen ankle or wrist joints of SKG mice were in vitro cultured, as previously described (10), and the amounts of CCL20 in culture supernatants were measured by ELISA. The dispersed granulocytes and monocytes from the inflamed synovial tissue, or the splenocytes of arthritic mice, did not produce CCL20 without stimulation (Fig. 3 A and not depicted). Notably, adherent synovial cells, mainly fibroblast-like synoviocytes, spontaneously produced CCL20 (Fig. 3 A). Adding recombinant IL-1 β , IL-17, or TNF- α to the culture at the

doses used for in vitro Th17 cell induction (21) augmented CCL20 production by the adherent synoviocytes, whereas the addition of recombinant IFN- γ or IL-4 inhibited production (Fig. 3 A). Other Th17 cell-associated cytokines, such as IL-6, IL-22, and IL-23, had no significant effect on the production at the doses used for controlling Th17 cell differentiation (Fig. 3 A) (21–23).

In addition to synoviocytes, purified CCR6⁺CD4⁺ SKG T cells actively transcribed CCL20 messenger RNA and secreted a large amount of the CCL20 protein compared with very low transcription or secretion by CCR6⁺CD4⁺ SKG T cells (Fig. 3, B and C). This concurred with the result of the microarray analysis, which showed a high expression of CCL20 by SKG FR4⁺CD4⁺ T cells and BALB/c IFN- γ ^{-/-}CD4⁺ T cells after homeostatic proliferation (Fig. 1 E). In addition, Th17 cells that were induced in vitro from normal BALB/c CD4⁺ T cells by TCR stimulation in the presence of IL-6 and TGF- β produced a large amount of CCL20 (Fig. 3 D).

Functionally, in vitro migration assays showed that CCR6⁺CD4⁺ SKG T cells migrated in response to CCL20 in a dose-dependent manner and that the addition of an anti-CCR6 mAb, which blocks the binding of CCL20 to CCR6, effectively inhibited the migration at such low doses of CCL20 as those secreted by cultured synoviocytes in Fig. 3 A (Fig. 3 E). Furthermore, CD4⁺ SKG T cells that had migrated in the presence of CCL20 were significantly enriched for IL-17⁺ CD4⁺ T cells, but not for IFN- γ ⁺ CD4⁺ T cells,

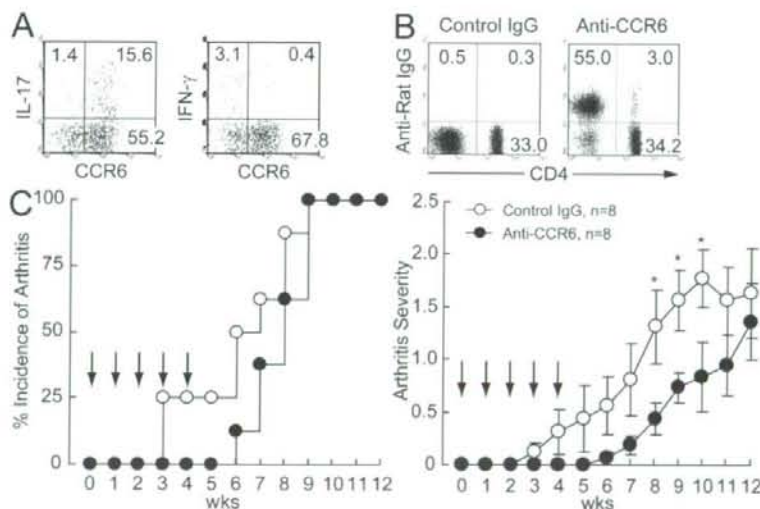


Figure 4. Contribution of CCR6/CCL20 to trafficking of Th17 cells in SKG arthritis. (A) CD4⁺ T cells infiltrating arthritic joints were prepared from collagenase-digested ankle joints of SKG mice, and stained for CCR6 and intracellular IL-17 or IFN- γ . (B) SKG mice received an i.v. injection of 100 μ g anti-CCR6 antibody or control IgG; LN cells were stained by anti-rat IgG and CD4 48 h after injection. The percentage of cells in each quadrant is shown in A and B. (C) SCID mice received an i.v. injection of 10⁶ SKG CD4⁺ T cells, and then injections of 100 μ g anti-CCR6 mAb or control IgG once a week for 6 wk. The arrows indicate i.v. injections of anti-CCR6 mAb. The incidence and severity of arthritis was scored every week, as previously described (reference 8). Vertical bars represent the means \pm SEM. The disease curves of arthritis scores are significantly different between the two groups [$P < 0.005$ according to the analysis of covariance test]. The differences in scores are statistically significant (according to the Mann-Whitney U test) in the 8th (*, $P = 0.04$), 9th (*, $P = 0.02$), and 10th (*, $P = 0.04$) wk. Results in A and B represent three independent experiments.

indicating that Th17 cells preferentially migrated in response to CCL20, whereas Th1 cells did not (Fig. 3 F).

Collectively, these results indicate that both activated synoviocytes and CCR6⁺Th17 cells themselves secrete CCL20 and further recruit other CCR6-expressing Th17 cells to the site of Th17 cell-mediated joint inflammation.

The effect of CCR6 blockade on the initial phase of Th17 cell-mediated arthritis

We previously reported that Th17 cells predominantly infiltrated into the arthritic joints of SKG mice (6). These infiltrating

Th17 cells indeed expressed CCR6, in contrast with infiltrating Th1 cells, which were CCR6⁻ (Fig. 4 A). To investigate the role of CCR6 on SKG autoimmune arthritis, we initially examined the effect of in vivo anti-CCR6 mAb treatment on lymphocytes. The anti-CCR6 mAb, which was used for in vitro CCR6 blockade (Fig. 3 E), was not cell depleting in vivo: after one injection into SKG mice, it bound to CCR6 on the cell surface of B cells and a population of CD4⁺ T cells, and the binding persisted for at least 8 d (Fig. 4 B and Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20071397>). To assess the effect of this CCR6 blockade

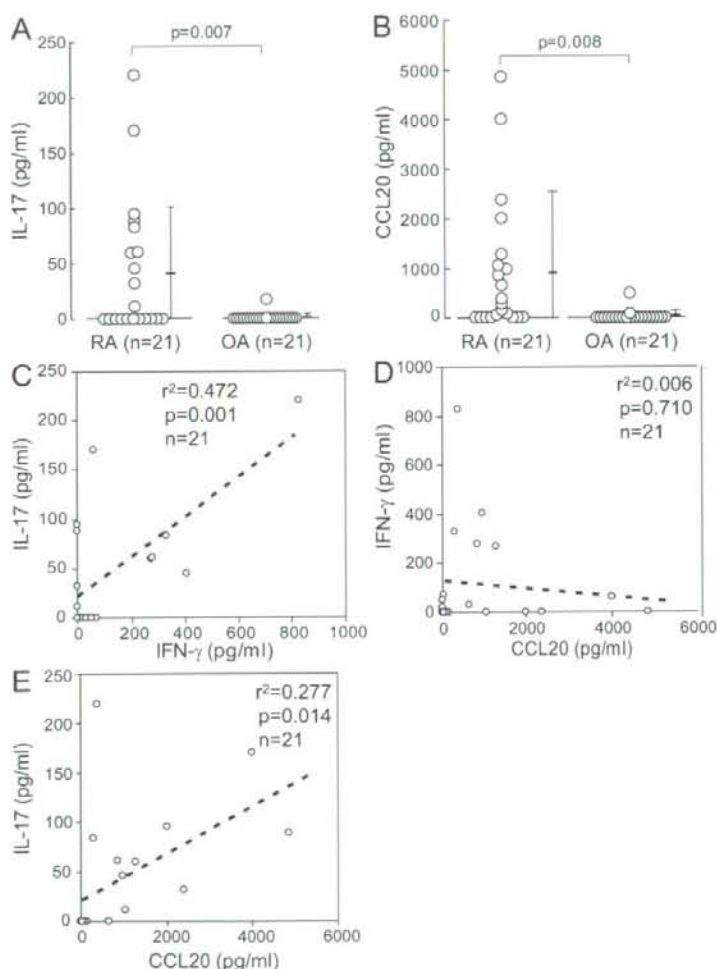


Figure 5. Production of IL-17 and CCL20 in RA joints. (A and B) The concentrations of IL-17 and CCL20 in synovial fluid from RA or OA patients were measured by ELISA. Vertical bars represent the means \pm SD. (C-E) Scatter plots show the correlation between IL-17 and IFN- γ (C), CCL20 and IFN- γ (D), or CCL20 and IL-17 (E) in the synovial fluid of RA patients. RA and OA patients are 66.6 ± 11.6 and 74 ± 7.4 yr old, respectively. r^2 values of Pearson's product-moment correlation and p-values of their null hypothesis are shown. Similar analyses performed only on the samples with the amounts of IL-17, IFN- γ , or CCL20 detectable by ELISA yielded the following statistics: IL-17 versus CCL20, $r^2 = 0.147$ and $P = 0.177$ ($n = 14$); IFN- γ versus CCL20, $r^2 = 0.0034$ and $P = 0.481$ ($n = 17$); IL-17 versus IFN- γ , $r^2 = 0.359$ and $P = 0.003$ ($n = 13$).

on the development of arthritis, we transferred SKG CD4⁺ T cells to syngeneic SCID mice and i.v. injected mAb once a week for 5 wk after cell transfer (Fig. 4 C). The treatment significantly suppressed the onset and severity of arthritis in an early phase: 3 wk after cell transfer, when control antibody-treated mice started to show joint swelling, much smaller number of T cells infiltrated into the joints of anti-CCR6-treated mice than control antibody-treated mice (Fig. S6). Thus, the blockade of CCL20 binding to CCR6 could suppress the development of Th17 cell-mediated autoimmune arthritis, at least in the initial phase of disease progression, presumably by interfering with the trafficking of Th17 cells (6).

Production of IL-17 and CCL20 in arthritic joints of human RA

We extended our analysis of IL-17 and CCR6/CCL20 in SKG arthritis to human RA. Synovial fluid of RA patients contained significantly higher amounts of IL-17 and CCL20 compared with osteoarthritis (OA) patients, in accord with other reports (Fig. 5, A and B) (24–26). A significant correlation was observed between the amounts of IL-17 and IFN- γ (Fig. 5 C). Notably, however, the level of CCL20 in RA joints was well correlated with that of IL-17 but not of IFN- γ (Fig. 5, D and E). As in mice, intracellular staining of IL-17 and IFN- γ or IL-4 of peripheral CD4⁺ T cells in normal healthy individuals showed that human Th17 cells were distinct from Th1 or Th2 cells and expressed CCR6, whereas CD4⁺ T cells producing IFN- γ or IL-4 were CCR6⁻ (Fig. S7, available at <http://www.jem.org/cgi/content/full/jem.20071397>), similar to the result recently reported on human Th17 cells in individuals with infectious diseases (27). Collectively, these findings suggest that Th17 cell trafficking via CCR6/CCL20 may contribute to RA pathology.

We have thus shown that CCR6 and CCL20 are expressed by Th17 cells and are required for the migration of Th17 cells to initiate self-destructive immune reactions in the joints, leading to the development of autoimmune arthritis such as RA. Once synovial inflammation occurs, synovio- cytes may further recruit Th17 cells through CCL20 production, which is enhanced by proinflammatory cytokines produced by activated synovio- cytes, such as IL-17, IL-1 β , and TNF- α (9), and dampened by IFN- γ or IL-4. Thus, Th1, Th2, and Th17 cell-produced cytokines, collectively with those produced by synovio- cytes, form a cytokine milieu to cross-regulate not only the development of Th17 cells but also the trafficking of CCR6⁺ Th17 cells via controlling the production of CCL20. It has been shown that joint infiltration of CCR6⁺ T cells is associated with human RA, and high expression of CCL20 in the central nervous system is observed in an animal model of multiple sclerosis (25, 26, 28–30). It remains to be determined whether intervention in Th17 cell trafficking via CCR6/CCL20 is useful to treat and prevent Th17 cell-mediated autoimmune diseases, including RA and multiple sclerosis. Such treatments include blocking CCR6 on Th17 cells at both the initial and chronic phases of disease

progression and reducing the production of CCL20 by changing the cytokine milieu at inflammation sites.

MATERIALS AND METHODS

Mice. BALB/c and SCID mice were purchased from Japan Clea. BALB/c IFN- γ ^{-/-} mice were purchased from the Jackson Laboratory. IL-6^{-/-} mice were backcrossed to BALB/c mice more than eight times. RAG2^{-/-} BALB/c mice were a gift from Y. Shinkai (Kyoto University, Kyoto, Japan) and were crossed to IL-6^{-/-} mice to generate IL-6^{-/-} RAG2^{-/-} BALB/c mice. These mice were maintained in our animal facility under specific pathogen-free conditions and treated in accordance with the institutional guidelines for animal care at the Institute for Frontier Medical Sciences of Kyoto University. The animal experiments were approved by the animal ethics committee of the Institute for Frontier Medical Sciences.

Antibodies. The following reagents were purchased from BD Biosciences: anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD16/CD32 (2.4G2), anti-TCR $\alpha\beta$ (H57-597), anti-CCR6 (140706), anti-IL-4 (11B11), anti-IFN- γ (XMG1.2), anti-IL-17 (TC11-18H10.1), PE-labeled goat anti-rat Ig, and isotype control IgG. Purified anti-IL-6R (MR16-1) was a gift from N. Nishimoto (Osaka University, Osaka, Japan). Purified anti-CCR6 (140706) was a gift from BD Biosciences. Anti-FR4 (Th6), which is of the rat IgG2a isotype, were purified from the culture supernatant of the hybridoma and labeled in our laboratory, as previously described (12).

Intracellular cytokine staining. LN or spleen cells were stimulated with 20 ng/ml PMA and 1 μ M ionomycin in the presence of GolgiStop (BD Biosciences) for 5 h, stained for surface antigens, fixed, and permeabilized using Cytofix/Cytoperm (BD Biosciences), followed by anti-IL-17 and anti-IFN- γ or IL-4 staining.

Measurement of cytokines and chemokines. Mouse IL-17 and CCL20 were measured by ELISA using Quantikine M (R&D Systems), with a detection limit of 11 pg/ml and 3.9 pg/ml, respectively. Human IL-17, IFN- γ (both from eBioscience), and CCL20 (R&D Systems) in synovial fluid were measured by ELISA, with a detection limit of 4, 8, and 8 pg/ml, respectively.

Gene microarray analysis. Total RNA was extracted using the RNeasy Micro Kit (QIAGEN) and was subjected to gene microarray (GeneChip Mouse Genome 430 2.0 Array; Affymetrix). Analysis of gene expression was performed by GeneSpring software (Agilent Technologies). Microarray data are available from the National Center for Biotechnology Information Gene Expression Omnibus under accession no. GSE9316.

Retroviral constructs and transduction. Complementary DNA (cDNA) encoding full-length mouse *Rargt* was amplified by RT-PCR from the cDNA of SKG CD4⁺ T cells and cloned into the pMxS-IRES-GFP vector. Retroviral transduction was performed as previously described (7). The pMxS-IRES-GFP vector was a gift from T. Kitamura (The University of Tokyo, Tokyo, Japan).

Preparation of synovio- cytes. Synovial tissues from inflamed ankle joints were digested with 400 Mandl U/ml of Liberase Blendzyme II (Roche) in plain RPMI 1640 medium for 1 h at 37°C to prepare single-cell suspensions. Synovial cells were cultured in RPMI 1640 medium containing 20% FBS, and synovio- cytes were prepared from the adherent cells.

Chemotaxis assay. Cell migration was evaluated using the 24-well, 5- μ m pore size Transwell system (Costar). 10⁶ LN cells were placed on the top of the Transwell in RPMI 1640 containing 10 mM Hepes buffer. CCL20 was added to the bottom of the Transwell system in RPMI 1640 containing 10 mM Hepes buffer and 1% FBS. After 4 h of incubation at 37°C, the number of cells that had migrated into the lower well was analyzed by counting CCR6⁺ CD4⁺ cells for 3 min using a flow cytometer (FACSCalibur; BD Biosciences).

Analysis of synovial fluid. Synovial fluid was collected from RA patients, fulfilling the revised classification criteria of the American College of Rheumatology for RA, or OA patients during orthopedic operation under written informed consent. The experiments were approved by the ethics committee of the Kyoto University Graduate School and Faculty of Medicine.

Statistical analysis. The Student's *t* test was used for statistical analyses, unless indicated otherwise. $P \leq 0.05$ was considered significant.

Online supplemental material. Fig. S1 presents the 23 genes, among 29 genes commonly up-regulated in the two sets of analyses in Fig. 1 D, that are not encoding cytokines, chemokines, or their receptors. Fig. S2 shows the expression of CCR6 on BALB/c or SKG CD4⁺ T cells and the induction of CCR6 after homeostatic proliferation. Fig. S3 exhibits the expression of IL-1R1 on Th17 cells. Fig. S4 shows that CCR6⁺CD4⁺ T cells include not only IL-17-producing cells but also TNF- α -producing cells and Foxp3⁺ cells. Fig. S5 shows that anti-CCR6 is not a cell-depleting antibody. Fig. S6 shows that the treatment of anti-CCR6 antibody inhibits the migration of CCR6⁺CD4⁺ cells into the joints of the SCID mice that received cell transfer. Fig. S7 demonstrates the expression of CCR6 on human Th17 cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20071397>.

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REFERENCES

- Harris, E.D. 1997. Rheumatoid Arthritis. W.B. Saunders Co., Philadelphia, 417 pp.
- Langrish, C.L., Y. Chen, W.M. Blumenschein, J. Mattson, B. Basham, J.D. Sedgwick, T. McClanahan, R.A. Kastelein, and D.J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201:233-240.
- Harrington, L.E., R.D. Hatton, P.R. Mangan, H. Turner, T.L. Murphy, K.M. Murphy, and C.T. Weaver. 2005. Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6:1123-1132.
- Park, H., Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6:1133-1141.
- 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.
- Hirota, K., M. Hashimoto, H. Yoshitomi, S. Tanaka, T. Nomura, T. Yamaguchi, Y. Iwakura, N. Sakaguchi, and S. Sakaguchi. 2007. T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17⁺ Th cells that cause autoimmune arthritis. *J. Exp. Med.* 204: 41-47.
- Ivanov, I.I., B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelletier, J.J. Lafaille, D.J. Cua, and D.R. Littman. 2006. The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17⁺ T helper cells. *Cell.* 126:1121-1133.
- Sakaguchi, N., T. Takahashi, H. Hata, T. Nomura, T. Tagami, S. Yamazaki, T. Sakihama, T. Matsutani, I. Negishi, S. Nakatsuru, and S. Sakaguchi. 2003. Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. *Nature.* 426:454-460.
- Hata, H., N. Sakaguchi, H. Yoshitomi, Y. Iwakura, K. Sekikawa, Y. Azuma, C. Kanai, E. Moriizumi, T. Nomura, T. Nakamura, and S. Sakaguchi. 2004. Distinct contribution of IL-6, TNF- α , IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. *J. Clin. Invest.* 114:582-588.
- Yoshitomi, H., N. Sakaguchi, K. Kobayashi, G.D. Brown, T. Tagami, T. Sakihama, K. Hirota, S. Tanaka, T. Nomura, I. Miki, et al. 2005. A role for fungal β -glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J. Exp. Med.* 201:949-960.
- Sakaguchi, S. 2005. Naturally arising Foxp3-expressing CD25⁺CD4⁺ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 6:345-352.
- Yamaguchi, T., K. Hirota, K. Nagahama, K. Ohkawa, T. Takahashi, T. Nomura, and S. Sakaguchi. 2007. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. *Immunity.* 27:145-159.
- Sutton, C., C. Breteron, B. Keogh, K.H. Mills, and E.C. Lavelle. 2006. A crucial role for interleukin (IL) 1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J. Exp. Med.* 203: 1685-1691.
- Zheng, Y., D.M. Danilenko, P. Valdez, I. Kasman, J. Eastham-Anderson, J. Wu, and W. Ouyang. 2007. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature.* 445:648-651.
- Liao, F., R.L. Rabin, C.S. Smith, G. Sharma, T.B. Nutman, and J.M. Farber. 1999. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 α . *J. Immunol.* 162:186-194.
- Charbonnier, A.S., N. Kohrgruber, E. Kriehuber, G. Stügel, A. Rot, and D. Maurer. 1999. Macrophage inflammatory protein 3 α is involved in the constitutive trafficking of epidermal Langerhans cells. *J. Exp. Med.* 190:1755-1768.
- Dieu-Nosjean, M.C., C. Massacrier, B. Homey, B. Vanbervliet, J.J. Pin, A. Vicari, S. Lebecque, C. Dezutter-Dambuyant, D. Schmitt, A. Zlotnik, and C. Caux. 2000. Macrophage inflammatory protein 3 α is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J. Exp. Med.* 192:705-718.
- Iwasaki, A., and B.L. Kelsall. 2000. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3 α , MIP-3 β , and secondary lymphoid organ chemokine. *J. Exp. Med.* 191:1381-1394.
- Le Borgne, M., N. Etchart, A. Goubier, S.A. Lira, J.C. Sirard, N. van Rooijen, C. Caux, S. Ait-Yahia, A. Vicari, D. Kaiserlian, and B. Dubois. 2006. Dendritic cells rapidly recruited into epithelial tissues via CCR6/CCL20 are responsible for CD8⁺ T cell crosspriming in vivo. *Immunity.* 24:191-201.
- Kleinewietfeld, M., F. Puentes, G. Borsellino, L. Battistini, O. Rotzschke, and K. Falk. 2005. CCR6 expression defines regulatory effector/memory-like cells within the CD25⁺CD4⁺ T-cell subset. *Blood.* 105:2877-2886.
- Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley, and B. Stockinger. 2006. TGF β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity.* 24:179-189.
- Betelli, E., Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, and V.K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector Th17 and regulatory T cells. *Nature.* 441:235-238.
- 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(H)17 lineage. *Nature.* 441:231-234.
- Kotake, S., N. Udagawa, N. Takahashi, K. Matsuzaki, K. Itoh, S. Ishiyama, S. Saito, K. Inoue, N. Kamatani, M.T. Gillespie, et al. 1999. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J. Clin. Invest.* 103:1345-1352.
- Matsui, T., T. Akahoshi, R. Namai, A. Hashimoto, Y. Kurihara, M. Rana, A. Nishimura, H. Endo, H. Kitasato, S. Kawai, et al. 2001. Selective

- recruitment of CCR6-expressing cells by increased production of MIP-3 alpha in rheumatoid arthritis. *Clin. Exp. Immunol.* 125:155-161.
26. Ruth, J.H., S. Shahrara, C.C. Park, J.C. Morel, P. Kumar, S. Qin, and A.E. Koch. 2003. Role of macrophage inflammatory protein-3alpha and its ligand CCR6 in rheumatoid arthritis. *Lab. Invest.* 83:579-588.
27. Acosta-Rodriguez, E.V., L. Rivino, J. Geginat, D. Jarrosway, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* 8:639-646.
28. Serafini, B., S. Columba-Cabezas, F. Di Rosa, and F. Aloisi. 2000. Intracerebral recruitment and maturation of dendritic cells in the onset and progression of experimental autoimmune encephalomyelitis. *Am. J. Pathol.* 157:1991-2002.
29. Ambrosini, E., S. Columba-Cabezas, B. Serafini, A. Muscella, and F. Aloisi. 2003. Astrocytes are the major intracerebral source of macrophage inflammatory protein-3alpha/CCL20 in relapsing experimental autoimmune encephalomyelitis and in vitro. *Glia.* 41:290-300.
30. Kohler, R.E., A.C. Caon, D.O. Willenborg, I. Clark-Lewis, and S.R. McColl. 2003. A role for macrophage inflammatory protein-3 alpha/CC chemokine ligand 20 in immune priming during T cell-mediated inflammation of the central nervous system. *J. Immunol.* 170: 6298-6306.

IL-17B and IL-17C Are Associated with TNF- α Production and Contribute to the Exacerbation of Inflammatory Arthritis¹

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IL-17A is a T cell-derived proinflammatory cytokine that contributes to the pathogenesis of rheumatoid arthritis. Recently, six related molecules have been identified to form the IL-17 family, as follows: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. Whereas IL-17A and IL-17F up-regulate IL-6 in synovial fibroblasts, IL-17B and IL-17C are reported to stimulate the release of TNF- α and IL-1 β from the monocytic cell line, THP-1 cell. However, their detailed function remains to be elucidated. We report in this study the effects of IL-17 family on the collagen-induced arthritis (CIA) progression by T cell gene transfer and bone marrow chimeric mice. The mRNA expressions of IL-17 family (IL-17A, IL-17B, IL-17C, and IL-17F) and their receptor (IL-17R and IL-17Rh1) genes in the arthritic paws of CIA mice were elevated compared with controls. Although IL-17A and IL-17F were expressed in CD4⁺ T cells, IL-17B and IL-17C were expressed in the cartilage and in various cell populations in the CIA arthritic paws, respectively. In vitro, IL-17A, IL-17B, IL-17C, and IL-17F induced TNF- α production in mouse peritoneal exudate cells. In vivo, adoptive transfer of IL-17B- and IL-17C-transduced CD4⁺ T cells evidently exacerbated arthritis. Bone marrow chimeric mice of IL-17B and IL-17C exhibited elevated serum TNF- α concentration and the high arthritis score upon CIA induction. Moreover, neutralization of IL-17B significantly suppressed the progression of arthritis and bone destruction in CIA mice. Therefore, not only IL-17A, but also IL-17B and IL-17C play an important role in the pathogenesis of inflammatory arthritis. *The Journal of Immunology*, 2007, 179: 7128–7136.

Interleukin-17A is a T cell-derived proinflammatory cytokine that is involved in the development of rheumatoid arthritis (RA).³ IL-17A was originally named CTLA-8 after being cloned from activated T cells, and shares 57% homology to the protein encoded by the open reading frame 13 gene of the T lymphotropic herpesvirus saimiri (1). IL-17A is present at significant levels in the synovium and synovial fluid of patients with RA (2, 3). IL-17A is a potent inducer of various cytokines such as IL-1, TNF- α , and IL-6. T cell IL-17A stimulates the production of IL-1 and TNF- α from human PBMC-derived macrophages in vitro (4). IL-17A also enhances IL-1-mediated IL-6 production by RA synovial cells in vitro as well as TNF- α -induced synthesis of IL-1, IL-6, and IL-8 (5, 6). These results indicate that IL-17A synergizes with IL-1 and TNF- α and contributes to inflammation of RA.

In vivo studies, systemic as well as local overexpression of IL-17A in collagen-induced arthritis (CIA) has been shown to accelerate the onset of CIA and to aggravate the joint pathology (7). Moreover, treatment with anti-IL-17A Abs after the onset of CIA reduces the joint inflammation and histologic destruction of cartilage (8). IL-17A deficiency protects IL-1R antagonist-deficient mice from spontaneous development of destructive arthritis (9). Therefore, IL-17A plays a crucial role in the pathogenesis of arthritis through synergistic effects with IL-1 and TNF- α . However, IL-17A can directly induce joint destruction in an IL-1-independent manner and can bypass TNF-dependent arthritis (7, 10). This suggests that there is an IL-17A-dependent pathway to the destructive arthritis and anti-IL-17A cytokine therapy is an additional new antirheumatic strategy for RA besides anti-TNF/anti-IL-1 therapy.

Recently, the IL-17 family was determined to consist of six related molecules, as follows: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. These molecules have a molecular mass of 20–30 kDa and consist of 163–202 aa that bear 20–50% homology to IL-17A, especially within the C-terminal region. They share four conserved cysteine residues that may participate in the formation of intermolecular disulfide linkages (11, 12). The different IL-17 family members seem to have very distinct expression patterns, suggesting distinct biological roles.

Interestingly, IL-17F has the highest homology with IL-17A and is also expressed by activated T cells in response to IL-23 stimulation (13–15). However, the precise effect of IL-17F on arthritis has not been clarified. In contrast to the restricted expression of IL-17A and IL-17F, IL-17B mRNA can be detected in a wide range of tissues, including the spinal cord, testis, stomach, small intestine, pancreas, prostate, and ovary (16, 17). It has been recently reported that IL-17B is highly expressed in chondrocytes

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³ Abbreviations used in this paper: RA, rheumatoid arthritis; BCII, bovine type II collagen; BM, bone marrow; CIA, collagen-induced arthritis; mIL, murine IL; MMP, matrix metalloproteinase; PEC, peritoneal exudate cell; pMIG, murine stem cell virus internal ribosome entry site/GFP.

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that are located at the mid and deep zones of normal bovine articular cartilage (11). In contrast, IL-17C expression has been confined only to rare expression sequence tags in adult prostate and fetal kidney libraries (17). However, the cell sources of IL-17B and IL-17C have not been identified in the development of inflammatory arthritis.

A common feature of IL-17 family members is the induction of neutrophil migration. IL-17A and IL-17F both mobilize neutrophils partly through granulopoiesis and CXC chemokine induction (12). Intranasal administration of adenovirus expressing IL-17A, IL-17C, or IL-17F resulted in neutrophilia in the bronchoalveolar lavage (18). Moreover, i.p. injection of human rIL-17B caused marked neutrophil migration in normal mice (17). In contrast, the members can be divided into two groups according to the induction of cytokine production. Although IL-17A and IL-17F up-regulate IL-6 and IL-8 in human fibroblasts (19, 20), IL-17B and IL-17C are reported to stimulate the release of TNF- α and IL-1 β from the monocytic cell line THP-1 (17). Taken together, these results indicate that IL-17 family members induce inflammatory cytokines not only through activated T cells, but also through activated monocytes/macrophages.

Based on the structural and functional similarities among IL-17 family members, we speculated that not only IL-17A, but also other IL-17 family members are involved in the pathogenesis of many inflammatory and autoimmune disorders, especially in the development of RA. We focused on IL-17A, IL-17B, IL-17C, and IL-17F, which can affect inflammatory cytokine production of fibroblasts and macrophages. Recently, IL-17C expression in synovial fluid mononuclear cells and PBMCs of RA patients was reported (21). However, the biological effect of IL-17 family members in arthritis has not been analyzed.

In the present study, we investigated the expression and effect of IL-17 family members in arthritis. In vitro, not only IL-17A, but also IL-17B and IL-17C induced the mRNA expression of inflammatory cytokines such as IL-1 β , IL-6, and IL-23 in the 3T3 cell line and peritoneal exudate cells (PECs). The supernatant of the PECs stimulated with each IL-17 family member all increased TNF- α production significantly compared with controls. In vivo, CD4⁺ T cells transduced with each of IL-17B, IL-17C, or IL-17F exacerbated CIA in mice to the same degree as CD4⁺ T cells transduced with IL-17A. Mice reconstituted with bone marrow (BM) cells transduced with each of IL-17B, IL-17C, or IL-17F suffered from severe CIA. Moreover, neutralization of IL-17B significantly suppressed the progression of arthritis and bone destruction in CIA mice. Our results suggest that not only IL-17A, but also the other IL-17 family members (IL-17B, IL-17C, and IL-17F) are associated with inflammatory cytokines such as IL-1 and TNF- α and contribute to the exacerbation of autoimmune arthritis.

Materials and Methods

Animals

DBA/1J mice were purchased from Japan SLC. All mice were used at 6–8 wk of age. All animal experiments were conducted in accordance with the institutional and national guidelines.

Collagen-induced arthritis

CIA was induced, as described previously (22–24). In brief, bovine type II collagen (BCII) (Chondrex) was emulsified with an equal volume of CFA (Chondrex). DBA/1J mice were immunized intradermally at the base of the tail with 100 μ g of BCII emulsified with CFA. On day 21, the mice were boosted by intradermal injection with 100 μ g of BCII emulsified with IFA (Difco). The arthritis score was determined by erythema, swelling, or ankylosis per paw, as described previously (25, 26). The clinical arthritis score was defined as the sum of the scores of all four paws of each mouse.

Cytokines and cell lines

Recombinant murine IL (mIL)-17A, mIL-17B, mIL-17C, and mIL-17F were obtained from R&D Systems. The mouse fibroblast cell line 3T3 was obtained from American Type Culture Collection. This cell line was cultured with RPMI 1640 (Invitrogen Life Technologies) medium supplemented with 10% FCS, 2 mM γ -glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 5×10^{-5} M 2-ME. Ba/F3 cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM γ -glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1 ng/ml mIL-3 (R&D Systems).

Murine PECs

Murine PECs were isolated after i.p. injection of 3 ml of 5% sterile fluid Brewer's thioglycolate broth (Sigma-Aldrich) into 8-wk-old DBA/1J mice (27). After culture of the PECs in a 6-well plate for 2 h, floating cells were removed by extensive washing, and attached cells were maintained in the medium described above for 3 days. More than 80% of the cultured cells were macrophages as determined by flow cytometric analysis of CD11b-positive cells. The following recombinant murine cytokines were added to the culture medium and incubated for 24 h: 50 ng/ml mIL-17A, mIL-17B, mIL-17C, or mIL-17F.

Preparation of retroviral constructs of mIL-17 family cDNAs

mIL-17A, mIL-17B, mIL-17C, and mIL-17F were isolated from the murine T lymphocyte cDNA library according to the reported nucleotide sequence from National Center for Biotechnology Information (mIL-17A NM_010552; mIL-17B NM_019508; mIL-17C NM_145834; mIL-17F NM_145856). The full-length fragments were subcloned into retrovirus vector murine stem cell virus/internal ribosome entry site/GFP (pMIG), as described previously (28).

Production of retroviral supernatants and retroviral transduction

Retroviral supernatants were obtained by transfection of pMIG carrying each of the IL-17 family genes into PLAT-E packaging cell lines using FuGENE 6 transfection reagent (Roche Diagnostic System), as described previously (29). For the detection of GFP-positive cells, we used an EPICS XL flow cytometer (Beckman Coulter).

Gene transduction to mouse splenocytes and adoptive transfer

Total splenocytes were cultured for 48 h in the presence of Con A (10 μ g/ml) (Sigma-Aldrich) and mIL-2 (50 ng/ml) (R&D Systems). Retroviral gene transduction was performed, as described previously (30, 31). A CD4⁺ T cell population was prepared by negative selection by MACS with anti-CD19 mAb, anti-CD11c mAb, and anti-CD8a mAb (BD Pharmingen). The gene-transduced CD4⁺ T cells were suspended in PBS and injected i.v. (1×10^7) at 23 days after the first immunization of BCII.

BM precursor cell isolation, infection, and transfer

BM precursor cell isolation, retrovirus infection, and transfer were performed, as described previously (32). In brief, DBA/1J mice were treated with 5 mg/body 5-fluorouracil (Sigma-Aldrich) dissolved in PBS. After 5 days, BM cells were harvested and cultured with 50 ng/ml mIL-3, mIL-6, and murine stem cell factor (R&D Systems) for 48 h. Then the BM cells were spin infected with the retrovirus supernatants with 16 μ g/ml polybrene (Sigma-Aldrich) for 90 min at 2400 rpm and 25°C. Recipient mice were treated by 700 rad of whole-body radiation and were injected with 1×10^6 of the BM cells i.v. Recipient mice were maintained for 6 wk until analysis or immunization.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

RNA of the cells was extracted using an RNeasy Micro Kit and RNeasy Mini Kit (Qiagen). RNA from the tissues was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method using ISOGEN (Nippon Gene). RNA was reverse transcribed to cDNA with random primers (Invitrogen Life Technologies) and Superscript III, according to the manufacturer's protocol (Invitrogen Life Technologies). To determine the cellular expression of each protein, quantitative real-time PCR analysis was performed using an iCycler (Bio-Rad). The PCR mixture consisted of 25 μ l of SYBR Green Master Mix (Qiagen), 15 pmol of forward and reverse primers, and the cDNA samples, in a total volume of 50 μ l. We calculated the quantitative PCR data with δ cycle threshold method, and relative RNA abundance was determined based on control β -actin abundance. To measure the relative efficiency,

amplifications were performed on the serial diluted cDNA samples using primers for the target and the reference (β -actin) genes. We made plots of the log cDNA dilution vs δ cycle threshold, and confirmed that the efficiencies of the target and the reference genes were similar because the absolute value of the slope was close to zero (data not shown) (33, 34). The primer pairs used in the quantitative real-time PCR were as follows: mouse IL-17A, sense 5'-GCTCCAGAAGGCCCTCAGA-3', antisense 5'-AGCTTCCCTCCGATTGA-3'; mouse IL-17B, sense 5'-CGGTGCTATGTTGGGTTGC-3', antisense 5'-GGTTG GTGGTGGCTCAGAA-3'; mouse IL-17C, sense 5'-CACAGATGAG AACCGCTACCC-3', antisense 5'-GCGGATGAACCTGGTGGGA A-3'; mouse IL-17F, sense 5'-CAACGCTGCATACAAAATCA-3', antisense 5'-TTAAGTGAGGCATTGGGAACA-3'; mouse IL-17R, sense 5'-CCACTCTGTAGACCCCAATG-3', antisense 5'-CCTGGA GATGTAGCCCTGGTC-3'; mouse IL-17Rh1, sense 5'-GCAAGGAA GGAGACGAAGAC-3', antisense 5'-CTCGGCGATTTCTTTTCT G-3'; mouse TNF- α , sense 5'-CATCTTCTCAAAATTCGAGTGACA A-3', antisense 5'-TGGGAGTAGACAAGGTACAACCC-3'; mouse IL-1 β , sense 5'-CAACCAACAAGTATATTCTCCATG-3', antisense 5'-GATCCACACTCTCCAGTCA-3'; mouse IL-6, sense 5'-CACT TCACAAGTCGGAGGCTTA-3', antisense 5'-GCAAGTGCATCATC GTTGTTC-3'; mouse IL-23, sense 5'-TGGCATCGAGAACTGTGAG A-3', antisense 5'-TCAGTTCGATTGGTAGTCTGTTA-3'; and mouse β -actin, sense AGAGGGAATCGTGCGTGAC-3', antisense 5'-CAATAGTGATGACCTGGCCGT-3'.

Immunoassays of cytokines and anti-type II collagen Ab

The concentrations of mIL-6, mTNF- α , and mIL-17A in mouse sera and culture supernatants were measured by sandwich ELISA, according to the manufacturer's protocol (BD Pharmingen). An automatic microplate reader (Bio-Rad 550) was used to measure the OD. Mouse serum IgG anti-type II collagen Ab titer was measured, as previously described (35).

Isolation of cartilage

Murine articular cartilage was isolated from patellae, as described previously (36). In brief, patellae were decalcified in 3.5% EDTA for 4 h at 4°C, when the whole cartilage layer was stripped off. Because old cartilage is more calcified, decalcification of the patellae of old mice (>3 mo) was performed overnight at 4°C.

Cell purification

Briefly, the arthritic paws of the CIA mice were cut into pieces, digested with collagenase type IV (Sigma-Aldrich), and stained with mAbs (Fc blocking with anti-mouse CD16/CD32 mAb, and staining with anti-mouse CD3-PE mAb, anti-mouse CD4-allophycocyanin mAb, anti-mouse CD11b-FITC mAb, anti-mouse CD11c-FITC mAb, anti-mouse CD19-FITC mAb, biotinylated anti-mouse I-A/I-E (MHC class II) mAb, and streptavidin PE Ab that were obtained from BD Pharmingen). Cell sorting of a specific cell population was performed with a FACSVantage flow cytometer (BD Biosciences).

Intracellular cytokine staining and flow cytometry

IL-17 family expressions of Ba/F3 cells transduced with each of IL-17 family members were examined using intracellular cytokine staining. Ba/F3 cells were infected with the retroviral supernatants in the presence of 10 μ g/ml polybrene (Sigma-Aldrich) for 120 min. These cells were stained with anti-mouse IL-17A mAb conjugated to PE (BD Pharmingen), biotinylated anti-mouse IL-17B polyclonal Ab (R&D Systems), anti-mouse IL-17C polyclonal Ab (R&D Systems), and anti-mouse IL-17F mAb (R&D Systems), respectively. Bovine anti-goat IgG-PE (Santa Cruz Biotechnology) and F(ab')₂ goat anti-rat IgG PE (Serotec) were used as secondary reagents for IL-17C and IL-17F staining, respectively. Cell fixation and permeabilization were performed using Cytofix/Cytoperm reagent (BD Pharmingen), according to the manufacturer's protocol (BD Pharmingen), and analyzed by flow cytometry. Splenocytes isolated from BM chimeric mice of IL-17A were also stained with anti-mouse IL-17A mAb in the same way.

Anti-IL-17B Ab treatment in CIA mice

CIA was induced in DBA/1J mice, as described above. Mice exhibited the first clinical signs of arthritis (arthritis score between 1 and 2) and were injected i.p. with 100 μ g of polyclonal anti-mouse IL-17B Abs (R&D Systems). PBS was i.p. injected as a control. Arthritis was assessed using a scoring system, as described above. Mice were sacrificed at 10 days after the onset of arthritis, and the paws were removed. Joint pathology was evaluated on decalcified H&E-stained sections.

Histopathology

The tarsal joints of sacrificed CIA mice were embedded in paraffin wax after 10% formaldehyde fixation and decalcification. The sections were stained with H&E. Synovial tissues were graded by mononuclear cell infiltration and pannus invasion, as described previously (37).

Statistical analysis

Data are expressed as the means \pm SD. All results were obtained by at least three independent experiments. Statistical significance was determined by the Mann-Whitney *U* test and unpaired Student's *t* tests. A value of *p* < 0.05 was considered statistically significant.

Results

IL-17 family genes (IL-17A, IL-17B, IL-17C, and IL-17F) were highly expressed in the arthritic paws of CIA mice

First, we examined the expressions of IL-17 family members and IL-17Rs in the arthritic paws of CIA mice by quantitative PCR. The mRNA expressions of all IL-17 family genes examined (IL-17A, IL-17B, IL-17C, and IL-17F) were highly elevated in the arthritic paws compared with the controls. In accordance with previous report of high *in vivo* expression of IL-17R in RA (38), mRNA expressions of IL-17Rs (IL-17R and IL-17Rh1) were also elevated (Fig. 1A). As expected, the mRNA expressions of inflammatory cytokines (TNF- α , IL-1 β , IL-6, and IL-23) were also elevated in the arthritic paws compared with controls (Fig. 1B).

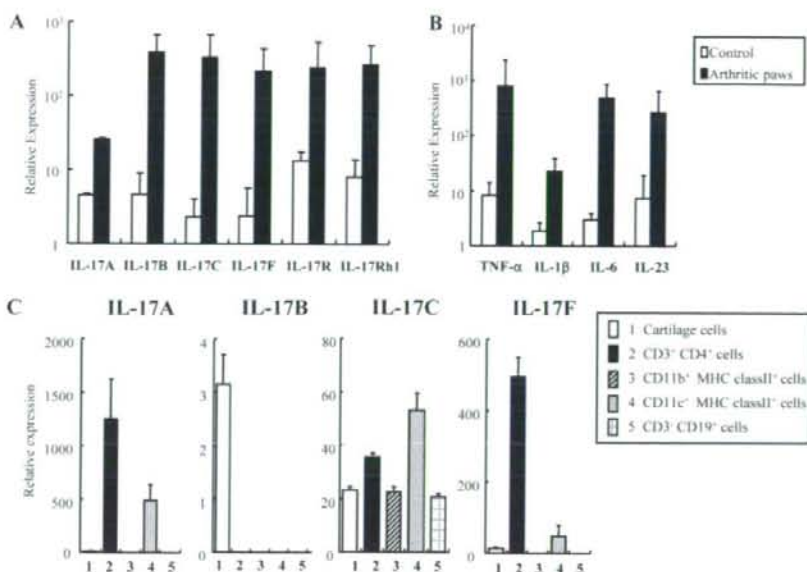
We next examined cell populations in the arthritic paws of CIA mice that express IL-17 family members. Subpopulations of the cells were sorted with various cell surface markers using a flow cytometer. As expected, CD4⁺ T cells expressed IL-17A and IL-17F significantly. IL-17B was expressed exclusively in the inflammatory cartilage of CIA mice. In contrast, IL-17C was expressed in a broad range of cells, i.e., CD4⁺ T cells, CD11b⁺ MHC class II⁺ macrophages, and CD11c⁺ MHC class II⁺ dendritic cells (Fig. 1C). These results suggested that CD4⁺ T cells mainly express IL-17 family members, especially IL-17A, IL-17C, and IL-17F, at the inflammatory site.

IL-17 family induced several proinflammatory cytokines

We next investigated whether IL-17 family members have an influence on mouse fibroblast cell lines and mouse peritoneal macrophages. Cells of the mouse fibroblast line 3T3 were cultured with each of the IL-17 family members (50 ng/ml), and cytokine expression was examined after 24 h of incubation. IL-17A induced IL-1 β and IL-6 expressions, as previously reported (2). Moreover, IL-17B, IL-17C, and IL-17F also induced IL-1 β expression in 3T3 (Fig. 2A).

To examine the effects of IL-17 family members on mouse macrophages, thioglycolate-elicited PECs were isolated and cultured with each of the IL-17 family members (50 ng/ml). IL-17A induced IL-1 β , IL-6, and IL-23 expressions in PECs. Interestingly, IL-17B also induced IL-1 β , IL-6, and IL-23 expressions. Moreover, IL-17C induced IL-1 β and IL-23 expressions in PECs (Fig. 2B). In addition, PECs stimulated with every IL-17 family member produced significantly increased amount of TNF- α protein compared with the control, and PECs stimulated with IL-17A and IL-17B produced significantly increased amount of IL-6 protein (Fig. 2C). These results suggested that IL-17A, IL-17B, IL-17C, and IL-17F stimulate fibroblasts and macrophages to produce inflammatory cytokines.

FIGURE 1. The expression of IL-17 family members and IL-17R genes in the arthritic paws of CIA mice. **A**, The expressions of IL-17 family genes and IL-17R genes were examined in the arthritic paws of CIA mice (■; $n = 3$) and in control mice (□; $n = 3$) by quantitative PCR. **B**, The expressions of inflammatory cytokines. **C**, The expressions of IL-17 family members in the sorted cell populations of the arthritic paws of CIA mice. The data are representative of three independent experiments.



Exacerbation of CIA by transfer of IL-17 family-transduced CD4⁺ T cells

Because IL-17B and IL-17C induce the expression of inflammatory cytokines in fibroblasts and macrophages, we hypothesized that IL-17B and IL-17C have an effect on the process of arthritis. We subcloned cDNA fragment of mIL-17A, mIL-17B, mIL-17C, or mIL-17F to pMIG retrovirus vector. These vectors were retrovirally transduced to Ba/F3 cells, and protein expressions of IL-17 family members were confirmed with intracellular staining of each IL-17 family cytokine (Fig. 3A).

To examine the proinflammatory effects of the IL-17 family *in vivo*, we retrovirally transduced the IL-17 family genes to CD4⁺ T cells. The transduction efficiencies were ~30% on average (Fig. 3B). These IL-17 family-transduced CD4⁺ T cells were adoptively transferred to BCII-immunized DBA1 mice before the onset of arthritis. They exacerbated the progression of arthritis, as observed by the arthritis score (Fig. 3, C and D). The IL-17 family member-transduced CD4⁺ T cells had no significant effect on the serum levels of anti-BCII IgG Abs at 14 days after the onset of CIA (data not shown). These results

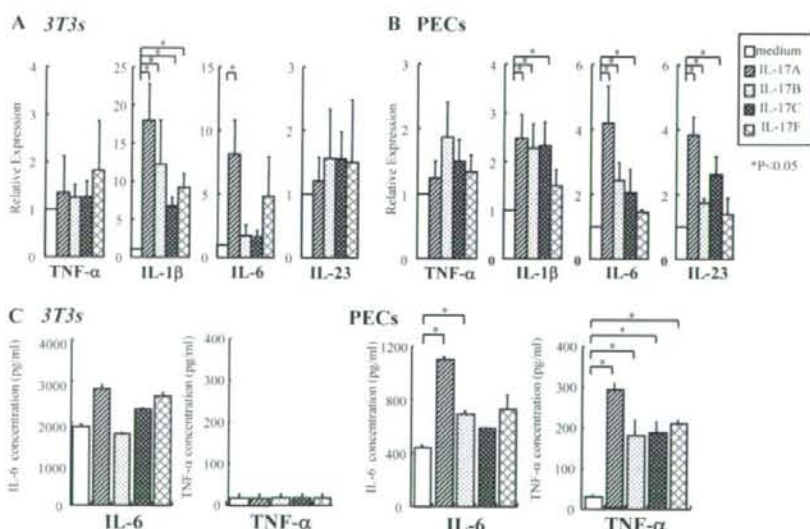
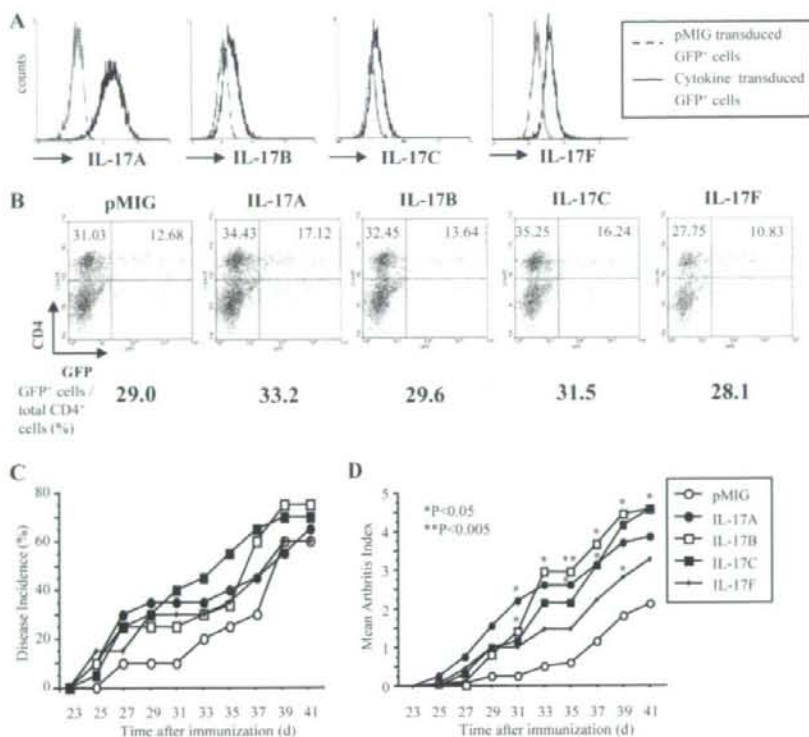


FIGURE 2. The proinflammatory effects of IL-17 family members on mouse fibroblasts and macrophages. **A**, Relative expression of the cytokine genes in 3T3 cell. The mouse fibroblast cell line 3T3 was cultured with each of mIL-17A, mIL-17B, mIL-17C, or mIL-17F for 24 h, and the expressions of inflammatory cytokines were measured by quantitative PCR. **B**, Relative expression of the cytokine genes in mouse thioglycolate-elicited PECs. PECs were cultured with each of mIL-17A, mIL-17B, mIL-17C, or mIL-17F for 24 h, and the expressions of inflammatory cytokines were measured by quantitative PCR. **C**, The secreted IL-6 and TNF- α levels in the supernatants of 3T3 and PECs were measured by ELISA. Error bars indicate \pm SD. The data are representative of three independent experiments. Significance of differences between control (medium) and each IL-17 family was determined; *, $p < 0.05$.

FIGURE 3. The effects of transfer of IL-17 family-transduced CD4⁺ T cells on CIA. **A**, Intracellular IL-17 family expressions in Ba/F3 cells retrovirally transduced with each IL-17 family member. GFP-gated IL-17 family-transduced (mIL-17A, mIL-17B, mIL-17C, or mIL-17F) Ba/F3 cells were analyzed for IL-17A, IL-17B, IL-17C, or IL-17F expression compared with GFP-gated empty vector (pMIG)-transduced Ba/F3 cells. **B**, Representative FACS analysis of IL-17 family-transduced CD4⁺ T cells was shown. Numbers in dot plots indicate the percentage of GFP⁺ CD4⁺ and GFP⁻ CD4⁺ cells, and the percentages of the GFP⁺ cells within total CD4⁺ cells were shown below. **C** and **D**, CD4⁺ T cells transduced with each of IL-17 family genes were transferred to collagen-immunized mice before the onset of arthritis (day 23). The incidence of arthritis (**C**) and the progression of arthritis scores (**D**) are shown. Values are the mean of arthritis score ($n = 20$ mice per group). Significance of differences between control (pMIG) and each IL-17 family-transduced mice was determined; **, $p < 0.005$; *, $p < 0.05$.



indicated that the effect of IL-17 family members on the progression of arthritis was not associated with the elevations of anti-BCII Abs.

IL-17 family BM chimeric mice exhibited high arthritis scores upon CIA induction

To examine the proinflammatory effect of constitutively expressed IL-17 family members, we established IL-17 family BM chimeric mice by transfer of gene-transduced BM cells to lethally irradiated mice. In a previous study, the attempt to generate IL-17A-overexpressing mice with a conventional transgenic approach was unsuccessful because these mice were embryonic lethal (39). In accordance with the previous report, mice that expressed IL-17A with high efficiency (i.e., for which the percentage of GFP⁺ cells in the spleen was >50%) became gaunt and died within 1 mo after BM transplantation (data not shown). When the percentage of GFP⁺ cells in the spleen was 5–15%, the mice appeared to be healthy for several months. We therefore used BM chimeric mice that expressed IL-17 family genes in ~5–15% of spleen cells. Eight weeks after the BM transplantation, mIL-17A was readily detected by intracellular cytokine staining (Fig. 4A). Moreover, the serum concentration of mIL-17A was significantly elevated in these mice (Fig. 4B). Therefore, the BM chimeric mice were successfully allowed to express the transduced cytokines systemically. Then we immunized these mice with BCII 8 wk after BM transplantation. BM chimeric mice of IL-17A and IL-17F exhibited early onset and high arthritis scores upon CIA induction (Fig. 5, A and B). BM chimeric mice of IL-17B and IL-17C clearly exacerbated arthritis, as assessed by the arthritis score. In contrast, BM chimeric mice of IL-17B and IL-17C did not result in significant differences in the onset of disease (Fig. 5, C and D). BM ex-

pression of IL-17 family member did not affect the anti-BCII Ab responses at 14 days after the onset of CIA (data not shown). These results indicated that the effect of IL-17 family members on the exacerbation of arthritis was not associated with the responses of anti-BCII Abs.

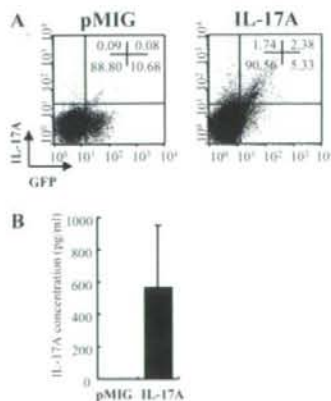
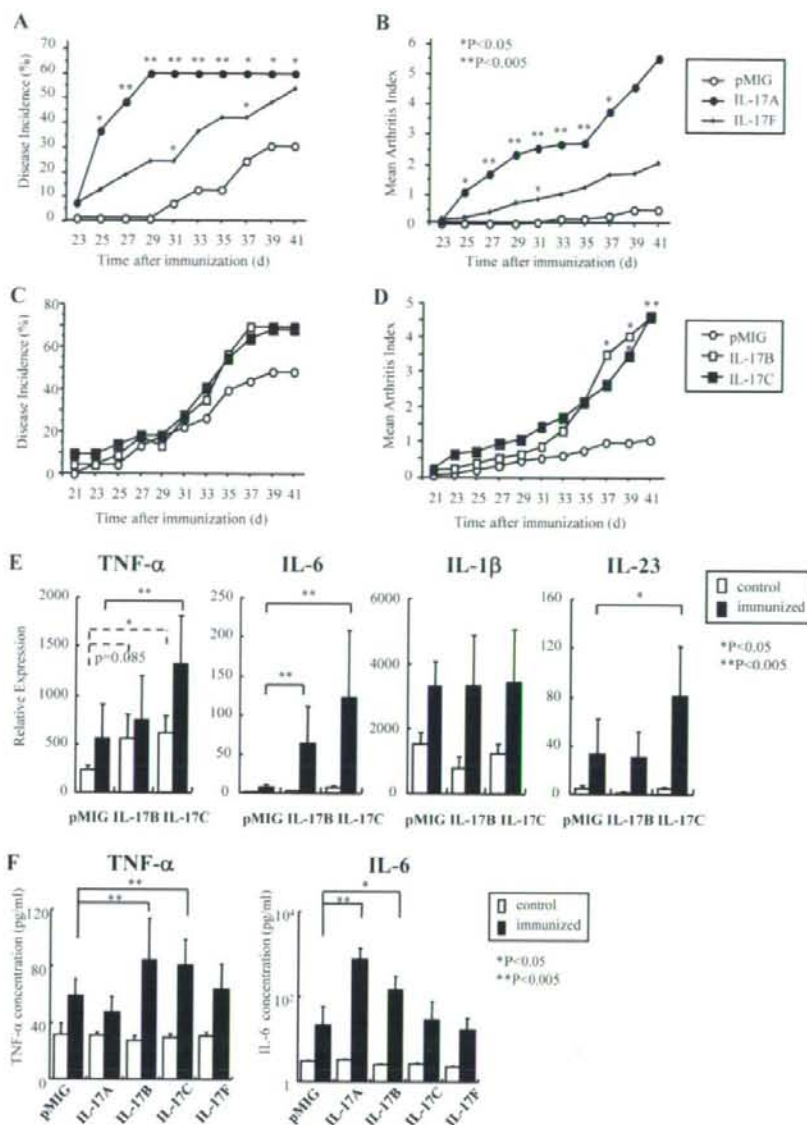


FIGURE 4. Generation of IL-17 family chimeric mice by BM transplantation of gene-transduced BM cells. Each of IL-17 family genes was transduced to BM cells with retrovirus vector and transferred to lethally irradiated mice. **A**, The intracellular expression of IL-17A protein in the spleen of IL-17A BM chimeric mice 8 wk after BM transplantation. The percentage of GFP⁺ cells expressing IL-17A is indicated. The data are representative of three independent experiments. **B**, The concentration of IL-17A protein in the serum of IL-17A BM chimeric mice ($n = 6$) and control mice (pMIG BM chimeric mice) ($n = 6$). The levels of IL-17A were measured by ELISA.

FIGURE 5. Incidence of CIA and arthritis scores in IL-17 family BM chimeric mice. Incidence of CIA and arthritis scores in IL-17A and IL-17F BM chimeric mice (A and B), and in IL-17B and IL-17C BM chimeric mice (C and D). Mice were immunized with BCII 8 wk after the BM transplantation. Values are the mean of experiments for IL-17A and IL-17F BM chimeric mice ($n = 20$ per group) and experiments for IL-17B and IL-17C BM chimeric mice ($n = 30$ per group). Significance of differences between control (pMIG) and each IL-17 family BM chimeric mice was determined; **, $p < 0.005$; *, $p < 0.05$. E. The mRNA expression of inflammatory cytokines in the spleen of BM chimeric mice of IL-17B and IL-17C, which were immunized with BCII (■, $n = 15$ per group) or nonimmunized controls (□, $n = 6$ per group). Significance of differences between control (pMIG) and each IL-17 family BM chimeric mice was determined; **, $p < 0.005$; *, $p < 0.05$. F. The secreted TNF- α and IL-6 levels in the sera of IL-17 family BM chimeric mice that were immunized with BCII (■, $n = 15$) or nonimmunized controls (□, $n = 6$). Significance of differences between control (pMIG) and each IL-17 family BM chimeric mice was determined; **, $p < 0.005$; *, $p < 0.05$.



We next examined the alterations of inflammatory cytokine production in these BM chimeric mice. Interestingly, nonimmunized IL-17C BM chimeric mice showed increased mRNA expression of TNF- α in the spleen compared with controls (Fig. 5E). Moreover, in the spleen of BCII-immunized IL-17C BM chimeric mice, the mRNA expressions of TNF- α , IL-6, and IL-23 were up-regulated. In contrast, BCII-immunized IL-17B BM chimeric mice showed increased mRNA expression of IL-6 in the spleen compared with controls (Fig. 5E). When we examined the concentrations of TNF- α and IL-6 protein in the sera of IL-17 family BM chimeric mice, the BCII-immunized IL-17B and IL-17C BM chimeric mice showed increased TNF- α concentration in the sera. And the BCII-immunized IL-17A and IL-17B BM chimeric mice showed increased IL-6 production in the sera (Fig. 5F). These results suggested that IL-

17B and IL-17C enhanced inflammation in this mouse model of arthritis by increased inflammatory cytokine production.

Neutralization of IL-17B significantly suppressed the progression of arthritis

As shown in Fig. 5, we found that IL-17B exacerbated the progression of CIA as well as IL-17A with the method of retrovirus-mediated BM chimeric mice. Regarding IL-17A, neutralizing Abs against IL-17A have been previously shown to be effective in the treatment of CIA (8). We examined the effect of IL-17B blockade in CIA mice. CIA mice were systemically treated with polyclonal anti-mouse IL-17B Abs immediately after the first signs of arthritis. Neutralization of IL-17B significantly suppressed the progression of CIA compared with the controls (Fig. 6A). Moreover, histological analysis revealed significant reduction of cell infiltration

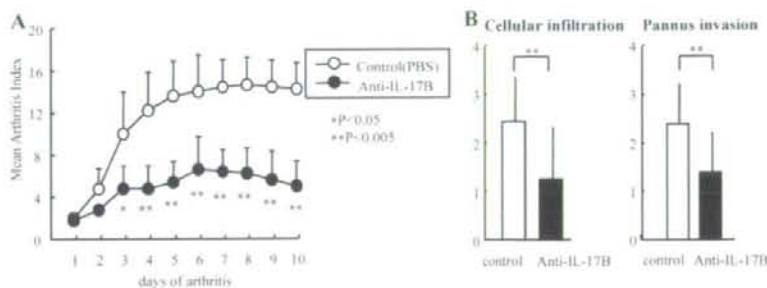


FIGURE 6. Effect of anti-IL-17B Ab treatment in CIA mice. *A*, CIA mice received i.p. injection of anti-mouse IL-17B Abs after the first clinical signs of arthritis (arthritis score between 1 and 2). As a control, PBS was injected. The arthritis score was shown. *B*, Histological score of the inflammatory joints of CIA mice treated with anti-IL-17B Abs was evaluated at 10 days after the onset of arthritis. Cellular infiltration and pannus invasion were graded in all four paws of the mice. Values are the mean of arthritis scores for anti-IL-17B Ab-treated mice and control mice ($n = 5$ per group). Significance of differences between control and anti-IL-17B Ab-treated mice was shown.

and pannus invasion in the anti-IL-17B Ab-treated mice (Fig. 6B). These results indicated that IL-17B was associated with the progression of arthritis in CIA mice.

Discussion

RA is considered to be an autoimmune disease, and is characterized by sustained inflammation of the joints and destruction of cartilage and bone. Several inflammatory cytokines are known to mediate the pathogenesis of arthritis, and TNF- α and IL-6 are the most important cytokines in the pathogenesis of RA. IL-17A, IL-17B, IL-17C, and IL-17F have the capacity to induce TNF- α production in PECs *in vitro*. *In vivo*, the mRNA expression of TNF- α was spontaneously increased in the spleen of IL-17C BM chimeric mice. Moreover, TNF- α productions in the sera of BCII-immunized IL-17B and IL-17C BM chimeric mice were up-regulated. Although IL-17A induced TNF- α production in PECs, IL-17A BM chimeric mice did not show up-regulated production of TNF- α . This result is consistent with previous observation in THP-1 cell line that IL-17B and IL-17C stimulated the release of TNF- α , whereas IL-17A has only a weak effect on TNF- α (17). In contrast to IL-17B and IL-17C, IL-17A may not be directly associated with TNF- α production *in vivo*. Moreover, the mRNA expression in the spleen and serum concentration of IL-6 were significantly up-regulated in IL-17B BM chimeric mice that were immunized with BCII. These results showed the close association of IL-17B and IL-17C with TNF- α and IL-6 *in vivo* and clearly suggested the importance of IL-17B and IL-17C in the pathogenesis of RA.

To date, the cell sources of IL-17B and IL-17C have not been identified. In this study, we showed that IL-17B was expressed in the inflammatory cartilage of CIA mice, whereas IL-17C was expressed in a broad range of cells, i.e., CD4⁺ T cells, CD11b⁺ MHC class II⁺ macrophages, and CD11c⁺ MHC class II⁺ dendritic cells. IL-17A and IL-17F were expressed in CD4⁺ T cells, as expected. These results suggested that CD4⁺ T cells are involved in the expression of IL-17 family members, especially IL-17A, IL-17C, and IL-17F, at the inflammatory site. Although we did not detect a unique cell source of IL-17C, the arthritis-promoting effect of IL-17C-transduced CD4⁺ T cells suggests the importance of IL-17C expressed in CD4⁺ T cells.

In our *in vivo* analysis, we observed arthritis-promoting effects of the IL-17 family members. As shown in Fig. 3, the transfer of mIL-17A-, mIL-17B-, mIL-17C-, and mIL-17F-transduced CD4⁺ T cells evidently exacerbated arthritis as assessed by the arthritis score. This effect was also confirmed in the CIA of the mIL-17A, mIL-17B, mIL-17C, and mIL-17F BM chimeric mice. The arthri-

tis-promoting effect of IL-17A was previously reported in a study using adenovirus vector (5, 40). In contrast to IL-17A, which hastened the onset of arthritis, IL-17B and IL-17C did not affect the onset of arthritis evidently. This fact suggests that IL-17B and IL-17C affect arthritis rather in the effector phase. To our knowledge, this is the first observation of an *in vivo* arthritis-promoting effect of IL-17B and IL-17C.

Blockade of IL-17A has recently been shown to be effective in the treatment of CIA (8). In the present study, we have demonstrated the therapeutic potential of IL-17B blockade after the onset of CIA. Because blockade of TNF- α or IL-1 β is not always effective in RA patients, blockade of additional cytokine might be a useful therapeutic option. Therefore, our data strongly suggest that IL-17B as well as IL-17A could be an important target for the treatment of inflammatory arthritis.

In a recent study, the combination of IL-6 and TGF- β was reported to strongly induce IL-17A production in Th17 cells (41). Moreover, it was recently recognized that IL-23 contributes to the expansion of autoreactive IL-17A-producing T cells and promotes chronic inflammation dominated by IL-17A, IL-6, IL-8, and TNF- α (14, 42). Thus, IL-17B and IL-17C may exacerbate arthritis via IL-6- and IL-23-mediated promotion of IL-17A production. However, the possibility that IL-17B and IL-17C exert a cooperative proinflammatory response together with IL-17A and IL-17F in arthritis by regulating the release of cytokines such as IL-6, IL-1 β , and IL-23 still remains to be examined.

IL-17F has the highest homology with IL-17A and, like IL-17A, is produced by activated T cells. IL-17F appears to have an effect similar to that of IL-17A on cartilage proteoglycan release and inhibition of new cartilage matrix synthesis (11). Although IL-17F is thought to contribute to the pathology of inflammatory disorders such as RA, the *in vivo* effect of IL-17F on arthritis was not elucidated. In this study, we found that transduction of BM-expressed IL-17F resulted in both an earlier onset and a subsequent aggravation of arthritis.

We also found that the mRNA expression of all IL-17 family and IL-17R genes examined (mIL-17A, mIL-17B, mIL-17C, mIL-17F, mIL-17R, and mIL-17Rh1) was elevated in the arthritic paws of CIA mice compared with the paws of the control mice. The receptor for IL-17A is IL-17R (also named IL-17AR), which is extensively expressed in various tissues or cells tested, in contrast to the exclusive expression of IL-17A in activated T cells. Recently, IL-17R signaling has been suggested to play a crucial role in driving the synovial expression of proinflammatory and catabolic mediators, such as IL-1, IL-6, matrix metalloproteinase