

Fig. 8. Detection of a clone-specific TCR message of 35S clone in spleens by RT-PCR amplification and Southern blot analysis. After transfer of 1×10^7 clone cells to BALB/c nude mice, RNA was extracted from spleens at indicated days. PC, positive control (RNA from 35S clone diluted to 1%); NC, negative control (RNA from a 6-month-old non-treated BALB/c athymic nude mouse). The separate lanes represent individual mice.

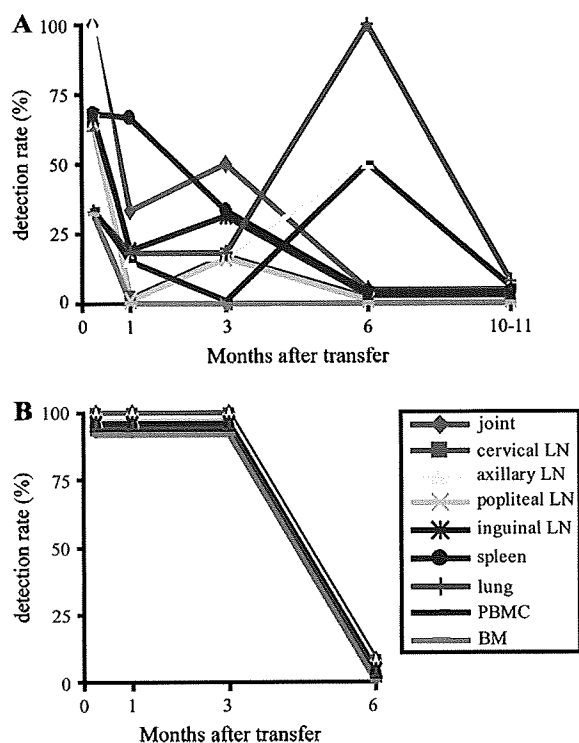


Fig. 9. Detection of TCR mRNA of the transferred clones in recipient BALB/c nude mice. 35S (A) or dengue 2F7 (B) in the recipients were detected by Southern blot analysis using primers and probes specific for TCR V and J region and CDR3 sequences of each clone. All mice with at least one positive signal out of four joints were considered to be positive. (A) $n = 3$ at 1 week; $n = 6$ at 1 month; $n = 6$ at 3 months; $n = 2$ at 6 months; $n = 2$ at 10–11 months. (B) $n = 2$ in every group. No signal was detected in control 6- or 11-month old BALB/c nude mice in each Southern blot analysis (data not shown).

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

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

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

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

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

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

We do not assert, however, that SKG arthritis and pneumonitis are solely mediated by T cells recognizing a ubiquitous common self-antigen. We have previously shown that SKG mice spontaneously produce IgG isotype auto-antibody specific for joint-rich type II collagen or IgG antibody cross-reactive with hsp-70 of Tuberculosis bacilli (14). This indicates that helper CD4⁺ T cells that specifically react with these self-antigens may also be induced in SKG mice either primarily or secondarily to joint damage. Moreover, we have recently shown that some self-reactive T cells in SKG mice may not be arthritogenic but can polyclonally stimulate antigen-presenting cells in the spleen and lymph nodes to secrete IL-6 and other cytokines, which in turn facilitate differentiation of potentially arthritogenic self-reactive T cells to T_H17 effector T cells that mediate synovitis (19). In addition to our current approach to the characterization of antigen specificity of SKG autoimmune T cells by preparing T cell clones, efforts are being made to further characterize infiltrating T cells *in situ* at a single-cell level by amplifying their TCR message.

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

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

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

Supplementary data

Supplementary figure is available at *International Immunology Online*.

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Disclosures

The authors declare no conflicting interests.

Abbreviations

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

References

- 1 Harris, E. D. 1997. *Rheumatoid Arthritis*. W.B. Saunders, Philadelphia, PA.
- 2 Firestein, G. S. 2003. Evolving concepts of rheumatoid arthritis. *Nature* 423:356.
- 3 Perez, T., Remy-Jardin, M. and Cortet, B. 1998. Airways involvement in rheumatoid arthritis: clinical, functional, and HRCT findings. *Am. J. Respir. Crit. Care Med.* 157:1658.
- 4 Demir, R., Bodur, H., Tokoglu, F., Olcay, I., Ucan, H. and Borman, P. 1999. High resolution computed tomography of the lungs in patients with rheumatoid arthritis. *Rheumatol. Int.* 19:19.
- 5 Gabbay, E., Tarala, R., Will, R. *et al.* 1997. Interstitial lung disease in recent onset rheumatoid arthritis. *Am. J. Respir. Crit. Care Med* 156:528.
- 6 McDonagh, J., Greaves, M., Wright, A. R., Heycock, C., Owen, J. P. and Kelly, C. 1994. High resolution computed tomography of the lungs in patients with rheumatoid arthritis and interstitial lung disease. *Br. J. Rheumatol.* 33:118.
- 7 Striebich, C. C., Falta, M. T., Wang, Y., Bill, J. and Kotzin, B. L. 1998. Selective accumulation of related CD4+ T cell clones in the synovial fluid of patients with rheumatoid arthritis. *J. Immunol.* 161:4428.
- 8 Fox, D. A. 1997. The role of T cells in the immunopathogenesis of rheumatoid arthritis: new perspectives. *Arthritis Rheum.* 40:598.
- 9 Panayi, G. S., Lanchbury, J. S. and Kingsley, G. H. 1992. The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. *Arthritis Rheum.* 35:729.
- 10 Nepom, G. T., Hansen, J. A. and Nepom, B. S. 1987. The molecular basis for HLA class II associations with rheumatoid arthritis. *J. Clin. Immunol.* 7:1.
- 11 Gao, X. J., Olsen, N. J., Pincus, T. and Stastny, P. 1990. HLA-DR alleles with naturally occurring amino acid substitutions and risk for development of rheumatoid arthritis. *Arthritis Rheum.* 33:939.
- 12 Firestein, G. S. and Zvaifler, N. J. 2002. How important are T cells in chronic rheumatoid synovitis? II. T cell-independent mechanisms from beginning to end. *Arthritis Rheum.* 46:298.
- 13 Sakaguchi, S. and Sakaguchi, N. 2005. Animal models of arthritis caused by systemic alteration of the immune system. *Curr. Opin. Immunol.* 17:589.
- 14 Sakaguchi, N., Takahashi, T., Hata, H. *et al.* 2003. Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. *Nature* 426:454.
- 15 Hata, H., Sakaguchi, N., Yoshitomi, H. *et al.* 2004. Distinct contribution of IL-6, TNF- α , IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. *J. Clin. Invest.* 114:582.
- 16 Yoshitomi, H., Sakaguchi, N., Kobayashi, K. *et al.* 2005. A role for fungal β -glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J. Exp. Med.* 201:949.
- 17 Chan, A. C., Iwashima, M., Turck, C. W. and Weiss, A. 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* 71:649.
- 18 Negishi, I., Motoyama, N., Nakayama, K. *et al.* 1995. Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature* 376:435.
- 19 Hirota, K., Hashimoto, M., Yoshitomi, H. *et al.* 2007. T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17+ Th cells that cause autoimmune arthritis. *J. Exp. Med.* 204:41.
- 20 Maini, R. N. and Feldmann, M. 2002. How does infliximab work in rheumatoid arthritis. *Arthritis Res.* 4:(Suppl. 2). S22.
- 21 Nishimoto, N., Yoshizaki, K., Miyasaka, N. *et al.* 2004. Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial. *Arthritis Rheum.* 50:1761.
- 22 Bottini, N., Vang, T., Cucca, F. and Mustelin, T. 2006. Role of PTPN22 in type 1 diabetes and other autoimmune diseases. *Semin. Immunol.* 18:207.
- 23 Begovich, A. B., Carlton, V. E., Honigberg, L. A. *et al.* 2004. A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. *Am. J. Hum. Genet.* 75:330.
- 24 Yoshida, R., Yoshioka, T., Yamane, S. *et al.* 2000. A new method for quantitative analysis of the mouse T-cell receptor V region repertoires: comparison of repertoires among strains. *Immunogenetics* 52:35.
- 25 Hori, S., Nomura, T. and Sakaguchi, S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057.
- 26 Arden, B., Clark, S. P., Kabelitz, D. and Mak, T. W. 1995. Mouse T-cell receptor variable gene segment families. *Immunogenetics* 42:501.
- 27 Koop, B. F., Rowen, L., Wang, K. *et al.* 1994. The human T-cell receptor TCRAC/TCRDC (C alpha/C delta) region: organization, sequence, and evolution of 97.6 kb of DNA. *Genomics* 19:478.
- 28 Malissen, M., Minard, K., Mjolsness, S. *et al.* 1984. Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the beta polypeptide. *Cell* 37:1101.
- 29 Gascoigne, N. R., Chien, Y., Becker, D. M., Kavaler, J. and Davis, M. M. 1984. Genomic organization and sequence of T-cell receptor beta-chain constant- and joining-region genes. *Nature* 310:387.
- 30 MacDonald, H. R., Lees, R. K., Sordat, B., Zaech, P., Maryanski, J. L. and Bron, C. 1981. Age-associated increase in expression of the T cell surface markers Thy-1, Lyt-1, and Lyt-2 in congenitally athymic (nu/nu) mice: analysis by flow microfluorometry. *J. Immunol.* 126:865.
- 31 MacDonald, H. R., Lees, R. K., Bron, C., Sordat, B. and Miescher, G. 1987. T cell antigen receptor expression in athymic (nu/nu) mice. Evidence for an oligoclonal beta chain repertoire. *J. Exp. Med.* 166:195.
- 32 Hodes, R. J., Sharrow, S. O. and Solomon, A. 1989. Failure of T cell receptor V beta negative selection in an athymic environment. *Science* 246:1041.
- 33 He, D., Wu, L., Kim, H. K., Li, H., Elmets, C. A. and Xu, H. 2006. CD8+ IL-17-producing T cells are important in effector functions for the elicitation of contact hypersensitivity responses. *J. Immunol.* 177:6852.
- 34 Ivanov, I. I., McKenzie, B. S., Zhou, L. *et al.* 2006. The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121.
- 35 Edwards, S. W. and Hallett, M. B. 1997. Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. *Immunol. Today* 18:320.
- 36 Jimenez-Boj, E., Redlich, K., Turk, B. *et al.* 2005. Interaction between synovial inflammatory tissue and bone marrow in rheumatoid arthritis. *J. Immunol.* 175:2579.
- 37 Keffer, J., Probert, L., Cazlaris, H. *et al.* 1991. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *EMBO J.* 10:4025.
- 38 Aidinis, V., Plows, D., Haralambous, S. *et al.* 2003. Functional analysis of an arthritogenic synovial fibroblast. *Arthritis Res. Ther.* 5:R140.
- 39 Niki, Y., Yamada, H., Seki, S. *et al.* 2001. Macrophage- and neutrophil-dominant arthritis in human IL-1 alpha transgenic mice. *J. Clin. Invest.* 107:1127.
- 40 Horai, R., Saijo, S., Tanioka, H. *et al.* 2000. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. *J. Exp. Med.* 191:313.
- 41 Atsumi, T., Ishihara, K., Kamimura, D. *et al.* 2002. A point mutation of Tyr-759 in interleukin 6 family cytokine receptor subunit gp130 causes autoimmune arthritis. *J. Exp. Med.* 196:979.
- 42 Huber, L. C., Distler, O., Tarnier, I., Gay, R. E., Gay, S. and Pap, T. 2006. Synovial fibroblasts: key players in rheumatoid arthritis. *Rheumatology (Oxford)* 45:669.
- 43 Strand, V., Kimberly, R. and Isaacs, J. D. 2007. Biologic therapies in rheumatology: lessons learned, future directions. *Nat. Rev. Drug Discov.* 6:75.
- 44 Vang, T., Congia, M., Macis, M. D. *et al.* 2005. Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat. Genet.* 37:1317.
- 45 Wu, J., Katrekar, A., Honigberg, L. A. *et al.* 2006. Identification of substrates of human protein-tyrosine phosphatase PTPN22. *J. Biol. Chem.* 281:11002.

Regulatory T Cells and Immune Tolerance

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Regulatory T cells (Tregs) play an indispensable role in maintaining immunological unresponsiveness to self-antigens and in suppressing excessive immune responses deleterious to the host. Tregs are produced in the thymus as a functionally mature subpopulation of T cells and can also be induced from naive T cells in the periphery. Recent research reveals the cellular and molecular basis of Treg development and function and implicates dysregulation of Tregs in immunological disease.

Introduction

The mammalian immune system protects the host from a broad range of pathogenic microorganisms while avoiding misguided or excessive immune reactions that would be deleterious to the host. Both protective and harmful immune responses are principally mediated by T and B cells, which possess enormous diversity in antigen recognition, high antigen specificity, potent effector activity, and long-lasting immunologic memory. Because of this potency, serious damage to the host may ensue if aberrant immune responses, such as autoimmunity or allergy, are triggered. A major challenge in immunology and medicine is to determine how unresponsiveness of the adaptive immune system to self-antigens (that is, immunological self-tolerance) is established and maintained, and how the quality and magnitude of adaptive immune responses to non-self-antigens are controlled so as to avoid damage to the host. Understanding the mechanisms of immunological self-tolerance will also provide insights into how weak immune responses, such as those against tumor antigens in cancer patients or against microbial antigens in chronic infection, can be augmented, or conversely, how strong immune responses such as graft rejection can be restrained.

There are two types of mechanisms, "recessive" and "dominant," for achieving self-tolerance and immune homeostasis. In the recessive mechanisms of self-tolerance, the fate of antigen-exposed self-reactive lymphocytes is determined in a cell-intrinsic manner. For example, some lymphocytes are programmed to die by apoptosis when exposed to self-antigen at an immature stage of their development in the central generative organs (the thymus for T cells and the bone marrow for B cells). Other lymphocytes may replace self-reactive T cell or B cell receptors (TCRs and BCRs, respectively) with nonreactive ones, a process called receptor editing. Those that have escaped clonal deletion and receptor editing may further mature but can be rendered anergic (functionally inactivated) upon exposure to self-antigen. In addition, their activation thresholds may be raised by the expression of inhibitory receptors or negative signaling molecules or they may not survive long because of activation-induced cell death. The cell-intrinsic control of cell fate or activation threshold also contributes to the inhibition of excessive immune responses to non-self-antigens.

In the dominant or cell-extrinsic mechanism, certain T cells actively keep in check the activation and expansion of aberrant or overreactive lymphocytes, in particular other types of T cells. Until recently, the physiological significance, and even the existence of T cell-mediated immune suppression, has been highly contentious. Yet there is now firm evidence that the normal immune system produces a population of T cells, called regulatory T cells (Tregs), that are specialized for immune suppression. Disruption in the development or function of Tregs is a primary cause of autoimmune and inflammatory diseases in humans and animals. Moreover, it is now emerging that every adaptive immune response involves recruitment and activation of not only effector T and B cells but also Tregs, and that the balance between the two populations is critical for the proper control of the quality and magnitude of adaptive immune responses and for establishing or breaching tolerance to self- and non-self-antigens.

In this Review, we discuss the roles of Tregs in self-tolerance and immune homeostasis, the cellular and molecular basis of their development and function, and how they might be exploited at the cellular and molecular levels to control a wide spectrum of physiological and pathological immune responses.

Thymus-Derived Tregs

It has been known for nearly 40 years that simple manipulation of the thymus and T cells, but not self-antigen, can produce autoimmune disease in normal animals (reviewed in Sakaguchi, 2000). Neonatal thymectomy at a critical period (around day 3 after birth) of selected strains of normal mice results in autoimmune damage of various organs (such as the thyroid, stomach, ovaries, and testes) and the appearance of tissue-specific autoantibodies in the circulation. Adult thymectomy of selected strains of normal rats followed by several rounds of sublethal X-irradiation produces autoimmune thyroiditis and type I diabetes. Importantly, inoculation of normal T cells, in particular CD4⁺T cells or CD4⁺CD8⁻ thymocytes, from untreated syngeneic animals inhibits the development of autoimmunity (Sakaguchi et al., 1982; Fowell and Mason, 1993). These results indicated that normal animals harbor not only potentially pathogenic self-reactive T cells but also T cells that suppress autoimmunity, with

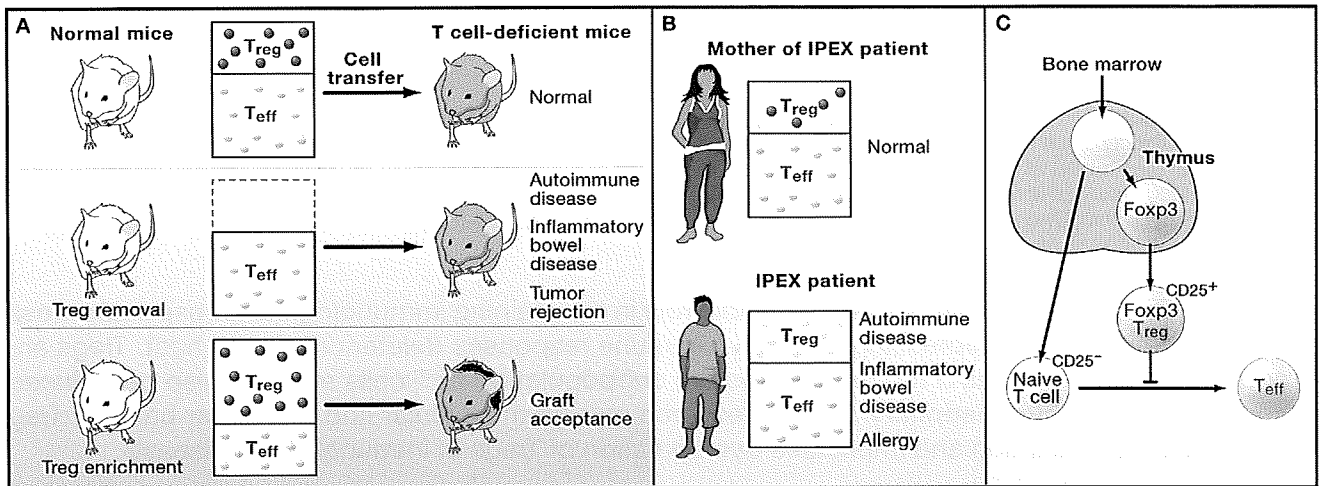


Figure 1. Effects of Treg Deficiency in Mice and Humans

(A) T cell suspensions prepared from normal mice can be depleted of CD25⁺CD4⁺ regulatory T cells (Treg) and transferred to syngeneic T cell-deficient mice (such as athymic nude mice). The recipient mice spontaneously develop autoimmune disease and inflammatory bowel disease and reject tumor cells. By contrast, when CD25⁺CD4⁺ Tregs are enriched from normal mice and transferred, the recipient nude mice accept allogeneic skin grafts.

(B) Male children are afflicted with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). Mothers bear heterozygous defects of the *FOXP3* gene and either have reduced numbers of normal Tregs or are mosaic for normal Tregs and functionally defective Tregs because of random inactivation of the X chromosome. Blue- and yellow-colored circles indicate intact Tregs and effector T cells (Teff), respectively. Dotted circles indicate dysfunctional Tregs (or the absence of Tregs).

(C) The normal thymus produces Foxp3-expressing natural Tregs. Tregs mainly suppress the development of effector T cells from naive T cells.

the latter dominantly controlling the former. A critical experiment to prove this interpretation was to identify autoimmune-suppressive T cells using a definitive phenotypic marker and to determine whether removal of the population from normal adult animals disrupts self-tolerance and leads to autoimmune disease. Indeed, when CD4⁺ T cell suspensions from the spleens of normal mice or rats were depleted of cells bearing a particular marker profile (CD5^{high} or CD45RC^{low}) *ex vivo*, and the remaining CD4⁺ T cells were transferred to syngeneic T cell-deficient athymic nude mice or rats, the recipients spontaneously developed autoimmune disease in multiple organs within a few months (Sakaguchi et al., 1985; Powrie and Mason, 1990) (Figure 1A). Reconstitution of the depleted CD4⁺ T cell subpopulation inhibited autoimmunity. Subsequent efforts to search for a more specific marker with which to delineate the putative autoimmune-preventive CD4⁺ T cells revealed the CD25 molecule (the IL-2 receptor α chain) as a candidate (Sakaguchi et al., 1995). Transfer of T cell suspensions depleted of CD25⁺ T cells indeed produces autoimmune disease in athymic nude mice, whereas cotransfer of a small number of CD25⁺CD4⁺ T cells clearly inhibits the development of autoimmunity.

Notably, depletion of naturally arising Tregs not only elicits autoimmunity but also augments immune responses to non-self-antigens. Treg depletion produces inflammatory bowel disease, which likely results from excessive immune responses to commensal bacteria in the intestine (Singh et al., 2001). Removal or reduction of CD25⁺CD4⁺ Tregs also provokes effective tumor immunity in otherwise nonresponding animals and augments microbial immunity in chronic infection, leading to eradication of tumors or microbes, respectively (reviewed in Wang and Wang, 2007; Belkaid and Rouse, 2005). Conversely, CD25⁺CD4⁺ T cells enriched from normal

mice suppress allergy, establish tolerance to organ grafts, prevent graft-versus-host disease after bone marrow transplantation, and promote fetomaternal tolerance (reviewed in Sakaguchi, 2005) (Figure 1A).

CD25⁺CD4⁺ Tregs are also present in the periphery of humans (reviewed in Baecher-Allan et al., 2004). T cells that are reactive with a particular self-antigen targeted in autoimmune disease (such as glutamic acid decarboxylase in type I diabetes) or a tumor-associated antigen can be easily expanded *in vitro* from peripheral blood of normal healthy individuals when T cells are stimulated with self or tumor antigen after depletion of CD25⁺CD4⁺ Tregs (Danke et al., 2004).

With these findings, it is possible to formulate several key notions concerning dominant self-tolerance and immune regulation. First, the normal immune system generates CD25⁺CD4⁺ Tregs that are engaged in suppressing immune responses toward self, quasi-self (such as autologous tumor cells), and non-self (such as microbes and allografts). Second, the normal thymus produces potentially pathogenic self-reactive T cells as well as functionally mature Tregs; mature Tregs persist in the periphery and exert dominant control over the self-reactive T cells. Third, Treg deficiency in the periphery is sufficient to evoke chronic T cell-mediated autoimmunity and immunopathology.

Foxp3 Controls Treg Development and Function

Naturally occurring Tregs specifically express the transcription factor Foxp3 (forkhead box P3), a member of the forkhead/winged-helix family of transcription factors. Foxp3 is a master regulator of Treg development and function. The *Foxp3* gene was first identified as the defective gene in the

mouse strain Scurfy. Surf is an X-linked recessive mutant that is lethal in hemizygous males within a month after birth, exhibiting hyperactivation of CD4⁺ T cells and overproduction of proinflammatory cytokines (Brunkow et al., 2001). Mutations of the human gene *FOXP3* are the cause of the genetic disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is the human counterpart of Scurfy (reviewed in Ochs et al., 2005; Figure 1B).

Immunological and clinical similarities between IPEX in humans and autoimmunity/inflammation produced in rodents by Treg depletion prompted researchers to determine the possible role of Foxp3 in Treg development and function (Hori et al., 2003; Khattry et al., 2003; Fontenot et al., 2003). The studies revealed that CD25⁺CD4⁺ peripheral T cells and CD25⁺CD4⁺CD8⁻ thymocytes in normal mice express Foxp3, whereas other thymocytes/T cells, either in a resting or activated state, do not. Ectopic retroviral transduction of the *Foxp3* gene in CD25⁻CD4⁺ T cells can convert them to CD25⁺CD4⁺ Treg-like cells that are able to suppress proliferation of other T cells in vitro and inhibit the development of autoimmune disease and inflammatory bowel disease in vivo (Hori et al., 2003). *Foxp3* transduction in naive T cells also upregulates the expression of CD25 and other Treg-associated cell-surface molecules, such as cytotoxic T cell-associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related gene/protein (GITR), whereas it represses the production of IL-2, IFN- γ , and IL-4. Both Foxp3-deficient mice and Scurfy mice, whose Foxp3 protein lacks the forkhead domain, harbor few CD25⁺CD4⁺ Tregs, and inoculation of CD25⁺CD4⁺ T cells from normal mice prevents severe systemic inflammation in Scurfy mice (Fontenot et al., 2003). In bone marrow chimera with a mixture of cells from wild-type and *Foxp3*-deficient mice, *Foxp3*-deficient bone marrow cells failed to give rise to CD25⁺CD4⁺ Tregs, whereas *Foxp3*-intact bone marrow cells generated Tregs that suppressed disease development. Conversely, in transgenic mice that overexpress Foxp3, the number of CD25⁺CD4⁺ T cells is enhanced; CD25⁻CD4⁺ T cells and CD8⁺ T cells expressed high levels of *Foxp3* and exerted suppression in vitro (Khattry et al., 2003). Recent analyses reveal that Foxp3-expressing T cells appear shortly after birth and show that the development of autoimmune/inflammatory disease follows their depletion (Kim et al., 2007; Lahl et al., 2007).

Thus, the transcription factor Foxp3 is critical for α/β TCR-positive T cells to differentiate to Tregs in the thymus (Figure 1C). High-level expression of Foxp3 is sufficient to confer suppressive activity to normal non-Treg cells. Further, genetic disruption of Foxp3 in humans and mice provides unequivocal evidence that dominant self-tolerance is operating in both species (Figure 1B). Foxp3 is currently the most reliable molecular marker for natural Tregs and provides clues with which to decipher the molecular and genetic basis of Treg development and function.

Interleukin 2 Maintains Foxp3⁺ Tregs

Interleukin 2 (IL-2) is another molecule critical for the function of Tregs. The Treg marker CD25 is a component of the high-affinity IL-2 receptor (IL-2R) and is functionally essential for

Treg development. IL-2 has long been thought to be a major cytokine for T cell proliferation and differentiation based on its effects on T cell growth in vitro. It has therefore been puzzling that mice lacking IL-2 do not exhibit serious defects in T cell differentiation and function. Instead, these mice spontaneously develop T cell-mediated fatal lymphoproliferative/inflammatory disease with autoimmune components (such as hemolytic anemia and lymphocytic infiltration into multiple organs) and hyperreactivity to commensal microbes (reviewed in Malek and Bayer, 2004). Mice deficient in CD25 or CD122 (another component of the IL-2R) also succumb to a similar set of ailments, generally called IL-2 deficiency syndrome. In humans, CD25 deficiency, which accompanies severe autoimmunity and allergy, is indistinguishable from IPEX (Caudy et al., 2007). Evidence suggests that the syndrome is due to deficiency or dysfunction of Foxp3⁺ Tregs. First, the number of Foxp3⁺ Tregs is reduced in mice lacking either CD25 or IL-2 (Antony et al., 2006), and autoimmunity in CD25-deficient mice can be prevented by inoculating them with wild-type CD25⁺CD4⁺ T cells (reviewed in Malek and Bayer, 2004). Second, T cell-specific deficiency of STAT5a and b, which mediate signaling from the IL-2R β chain to the nucleus, abrogates the development of Foxp3⁺ Tregs, producing autoimmune/inflammatory disease (Burchill et al., 2007; Yao et al., 2007). Third, administration of a high dose of neutralizing anti-IL-2 monoclonal antibody to normal neonatal mice substantially reduces the number of Foxp3⁺CD25⁺CD4⁺ T cells for a limited period and elicits autoimmune diseases similar to those produced by Treg depletion (Setoguchi et al., 2005). In addition, IL-2 is required for sustained expression of Foxp3 and CD25 in natural Tregs and enhances their suppressive function, at least in vitro (Fontenot et al., 2005; Shevach et al., 2006). Although other γ_c cytokines also contribute to Treg development and maintenance, they are unable to fully replace the roles of IL-2 or prevent IL-2 deficiency syndrome. For example, in contrast with mild reduction of Foxp3⁺ cells in mice lacking either IL-2 or CD25, deficiency of CD122 (shared by IL-2R and IL-15R) or CD132 (shared by IL-2R, IL-4R, IL-7R, IL-9R, IL-21R, and IL-15R), or double deficiency of IL-2 and IL-15 lead to a profound reduction in the number of Tregs (Fontenot et al., 2005; Burchill et al., 2007). Yet, deficiency of IL-4, IL-7, or IL-15 alone does not alter the number of Foxp3⁺ cells or produce autoimmunity. Thus, IL-2 is vital and irreplaceable for the development, survival, and function of Foxp3⁺ natural Tregs.

It is well known that IL-2 has multiple targets, including CD4⁺ and CD8⁺ T cells, B cells, and natural killer cells, and exerts pleiotropic functions with apparently contradictory effects on immune responses. It facilitates differentiation of CD4⁺ T cells to Th1 and Th2 cells and expands CD8⁺ memory T cells and natural killer cells. On the other hand, IL-2 promotes apoptosis in antigen-activated T cells. IL-2 also maintains Foxp3⁺ natural Tregs, expands them at high doses, and facilitates TGF- β -dependent differentiation of naive T cells to Foxp3⁺ Tregs, but inhibits TGF- β /IL-6-dependent differentiation of naive T cells to inflammatory Th17 cells (Laurence et al., 2007). Thus, assuming that the main source of IL-2 is activated T cells, there is a negative feedback control of immune responses via IL-2; that is, IL-2 produced by activated nonregulatory T cells contributes

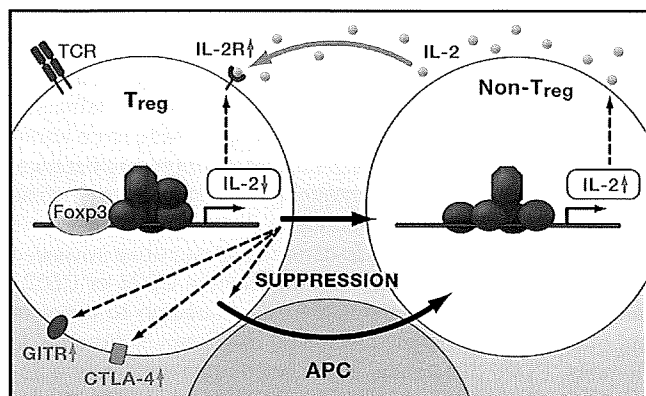


Figure 2. Key Roles of IL-2 in Immune Homeostasis

Interactions among Tregs, non-Treg cells, and antigen-presenting cells (APC) and feedback control of Treg function via IL-2. Foxp3, together with other transcription factors and coactivators/corepressors, represses the transcription of IL-2 in Tregs, rendering them highly dependent on exogenous IL-2 (mainly produced by activated non-Treg cells) for their maintenance and function. Foxp3 also activates the genes encoding Treg-associated molecules such as CD25, CTLA-4, and GITR and confers suppressive activity to Tregs, which directly suppress non-Treg cells or modulate the function of APC to activate non-Treg cells.

to the maintenance, expansion, and activation of natural Tregs, which in turn limits the expansion of nonregulatory T cells (Figure 2). Disruption of this IL-2-mediated feedback loop at any stage promotes the development of autoimmune/inflammatory disease. Further, manipulation of this feedback loop is instrumental in tuning the intensity of Treg-mediated suppression, hence the strength of a variety of immune responses.

Control of Treg Function by Foxp3

How does Foxp3 orchestrate the cellular and molecular programs involved in Treg function? It has been shown that Foxp3, which appears to operate as a homo-oligomer, interacts with the transcription factors NFAT (nuclear factor of activated T cells), AML1 (acute myeloid leukemia-1)/Runx1 (runt-related transcription factor 1), the histone acetyl transferase (HAT)/histone deacetyl transferase (HDAC) complex, and possibly NF- κ B (Figure 3A).

The activity of NFAT is controlled by calcium and the calcium-dependent protein phosphatase calcineurin. Upon activation, NFAT forms a complex with AP-1 and NF- κ B and promotes the expression of *Il2*, *Il4*, *Ctla4*, and other genes in conventional T cells, thus contributing to the activation of T cells and their differentiation to effector T cells (reviewed in Rao et al., 1997). A recent study has shown that the interaction between Foxp3 and NFAT is dependent upon their cooperative binding to DNA (Wu et al., 2006). Amino acid substitutions in the forkhead domain of Foxp3 that disrupt the Foxp3-NFAT interaction impair the ability of Foxp3 to repress *Il2*, activate *Ctla4* and *Cd25*, and confer suppressive activity when expressed in normal T cells. Although four known types of NFAT may function redundantly, mice deficient in both NFATc2 and NFATc3 spontaneously develop severe lymphadenopathy and selective activation of Th2 cells accompanying hyperproduction of IgE, suggesting that the deficiency might disrupt the function of Foxp3 and consequently impair Treg function (Ranger et al., 1998).

AML1/Runx1 is crucial for normal hematopoiesis including thymic T cell development (Taniuchi et al., 2002). It is expressed in conventional T cells and to a slightly lesser degree in natural Tregs (Ono et al., 2007). In contrast with NFAT, which is dephosphorylated by calcineurin and translocates from the cytoplasm to the nucleus upon T cell activation, AML1/Runx1 may constitutively bind to the *Il2* promoter at the site upstream to the binding sites of NFAT, AP-1, and NF- κ B. Upon T cell activation, it may act as an organizing factor that facilitates the assembly of transcriptional activation complexes containing NFAT on the *Il2* promoter. Alternatively, AML1/Runx1, alone or together with its interacting partners such as p300 and CREB-binding protein, may cooperate with the NFAT transcription complex to activate the *Il2* promoter. In contrast, in Tregs, AML1/Runx1 physiologically binds to Foxp3 at its N-terminal region between the leucine zipper domain and the forkhead box (Figure 3A). Disrupting the binding between Foxp3 and AML1/Runx1 impairs the Foxp3-dependent suppression of IL-2 production and attenuates suppressive activity without affecting the binding of Foxp3 to NFAT. Knockdown of AML1/Runx1 in natural Tregs abrogates their suppressive activity. Further, Treg-specific disruption of the AML1/Runx1 gene in mice affects Foxp3 function and causes autoimmune disease similar to that produced by Treg depletion (A. Kitoh, M.O., and S.S., unpublished data).

Transcriptional control exerted by Foxp3 also involves HATs, such as TIP60, and HDACs, such as HDAC7 and HDAC9 (Li and Greene, 2007) (Figure 3A). Acetylation of Foxp3 by TIP60 enhances the binding of Foxp3 to the *Il2* promoter and augments the repression of *Il2* (Chen et al., 2006). It remains to be determined how the interaction of Foxp3 with coactivators and corepressors modifies the function of NFAT and AML1/Runx1 and controls the expression of Foxp3 target genes including *Il2*, *Cd25*, *Ctla4*, and *Gitr*.

Another approach to understanding how Foxp3 controls Treg function is to identify Foxp3 target genes genomewide. Transcriptome analysis has revealed several genes, such as those encoding G protein-coupled receptor 83 (Gpr83) or Extracellular matrix 1 (Ecm1), that are predominantly expressed in natural Tregs and Foxp3-transduced T cells. Other genes, such as those encoding Granzyme B and the transcription factor Helios, are highly specific to Tregs but are apparently expressed independently of Foxp3 (Fontenot et al., 2005; Sugimoto et al., 2006; Hill et al., 2007). Retroviral transduction of either group of genes in CD25-CD4⁺ T cells failed to confer suppressive activity in vitro (Sugimoto et al., 2006).

Recent searches for Foxp3 target genes have shown that Foxp3 directly or indirectly controls hundreds (~700) of genes and binds directly to ~10% of them (Marson et al., 2007; Zheng et al., 2007). Foxp3-binding genes include those encoding signal transduction molecules (such as *Zap70* and *Ptpn22*), transcription factors (such as *Crem*), cytokines (e.g., *Il2*), cell-surface molecules (such as *Il2ra*, *Ctla4*, and *FasL*), enzymes for cell metabolism (such as *Pde3b*), and intergenic microRNAs (such as *miR-155*). microRNAs do indeed appear to play an important role in Treg development. T cell-specific

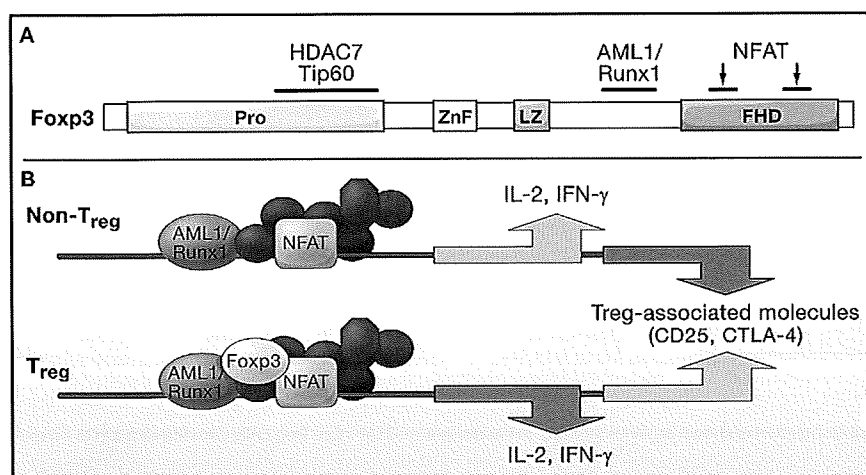


Figure 3. Control of Treg Function by Foxp3

(A) The structure of the transcription factor Foxp3. Bars indicate the binding sites for other transcription factors or chromatin-remodeling enzymes. Pro: Proline-rich region, ZnF: zinc finger domain, LZ: leucine zipper domain, FHD: forkhead box.

(B) The transcriptional complexes involving NFAT and AML1/Runx1 activate or repress the genes encoding cytokines and several cell-surface molecules in Treg and non-Tregs, depending on the presence of Foxp3.

thymocytes (Fontenot et al., 2005) (Figure 4A). Only a small fraction of Foxp3⁺ thymocytes develop in mice deficient in the class I and II (MHC) histocompatibility complex, indicating that Foxp3⁺ thymocytes or their Foxp3⁻ precursors

are subjected to positive and negative selection in the thymus through interactions with self-peptide/MHC ligands expressed by thymic stromal cells.

How then do thymocytes differentiate into functional Foxp3⁺ Tregs through the thymic selection process? Evidence suggests that natural Tregs may possess TCRs with higher affinity for thymic MHC/self-peptide ligands than those of other T cells, and that such highly self-reactive T cells would be recruited to the Treg cell lineage in the course of thymic T cell selection. First, in double-transgenic mice expressing a transgene-encoded peptide (e.g., an ovalbumin peptide) in the thymic stromal cells at a high level, the majority of T cells expressing transgenic α and β chains specific for the peptide differentiate to CD25⁺CD4⁺ Tregs (Jordan et al., 2001; Kawahata et al., 2002). In contrast, Tregs fail to develop when double-transgenic mice express either a low-affinity transgenic TCR or a high concentration of the peptide, presumably due to insufficient positive selection or strong negative selection, respectively. This apparently high self-reactivity of Foxp3⁺ cells could be a consequence of positive selection of highly self-reactive T cells to the Treg lineage or a resistance of differentiated Foxp3⁺ cells to negative selection (van Santen et al., 2004). Second, in TCR transgenic mice, which spontaneously develop CD25⁺CD4⁺ Tregs in an equivalent number as in nontransgenic mice, Tregs predominantly express endogenous TCR α chains paired with the transgenic β chain. In contrast, the majority of CD25⁺CD4⁺ T cells express the TCR solely composed of transgenic α and β chains (Itoh et al., 1999). RAG deficiency, which blocks gene arrangement at the endogenous TCR α chain locus, abrogates the development of CD25⁺CD4⁺ Tregs in TCR transgenic mice, indicating that self-reactive TCRs utilizing endogenous α chains may contribute to the positive selection of thymocytes to Tregs. The lack of Tregs due to RAG deficiency exacerbates autoimmune disease in mice expressing autoimmune transgenic TCRs. Third, a mutation in the gene encoding LAT (linker of activated T cell), a TCR proximal signal transduction molecule, abolishes the generation of Foxp3⁺ Tregs in the thymus and periphery while allowing the development of other T cells. This indicates that Treg development requires a strong signal

depletion of Dicer, an RNase enzyme required for processing double-stranded RNA, hampers thymic development of Foxp3⁺ T cells and elicits inflammatory bowel disease (Cobb et al., 2006). Altogether, these genomewide studies clearly show that Foxp3 functions as an activator as well as a repressor of transcription depending on the target.

Taken together, the characterization of Foxp3 interacting partners and Foxp3 target genes has revealed that Foxp3 interacts with transcription factors (such as NFAT and AML1/Runx1) that otherwise facilitate the activation of non-Treg cells and their differentiation into effector T cells. This suggests a model of Foxp3 function, in which Foxp3 overrides or hijacks the transcription machinery for effector T cells, thereby functionally converting them to Tregs (Figure 3B). Supporting this model, in mice genetically engineered to express a substantially lower amount of Foxp3 than normal, natural Tregs not only lose suppressive activity but also spontaneously differentiate to effector T cells that secrete IL-2, IFN- γ , and IL-4 (Wan and Flavell, 2007). Furthermore, Foxp3 may interact with ROR γ t, a key transcription factor for the differentiation of naive T cells to Th17 cells, and thereby inhibit Th17 differentiation (Bettelli et al., 2006, and see below). It remains an open question as to how the Foxp3 complex containing NFAT and AML1/Runx1 (together with HATs and HDACs) controls the genes that mediate suppression. In addition, the existence of genes that are highly Treg specific but Foxp3 independent suggests that there might be a higher level of transcriptional regulation upstream of Foxp3 or coregulation of Treg differentiation and function with other transcription factors.

The Antigen Recognition Repertoire of Tregs

A cardinal feature of Foxp3⁺ natural Tregs is that unlike the majority of thymus-produced naive T cells, they are already functionally mature (that is, competent to prevent autoimmune disease and exert suppression *in vitro*) and "antigen-primed" in the thymus, before encountering antigen in the periphery (Sakaguchi et al., 1982; Itoh et al., 1999). Foxp3⁺ thymocytes are detectable from a late CD4⁺CD8⁺ stage to the CD4⁻ or CD8⁻ single positive stage, constituting ~5% of mature CD4⁺CD8⁻ thymocytes and less than 1% of CD4⁻CD8⁺

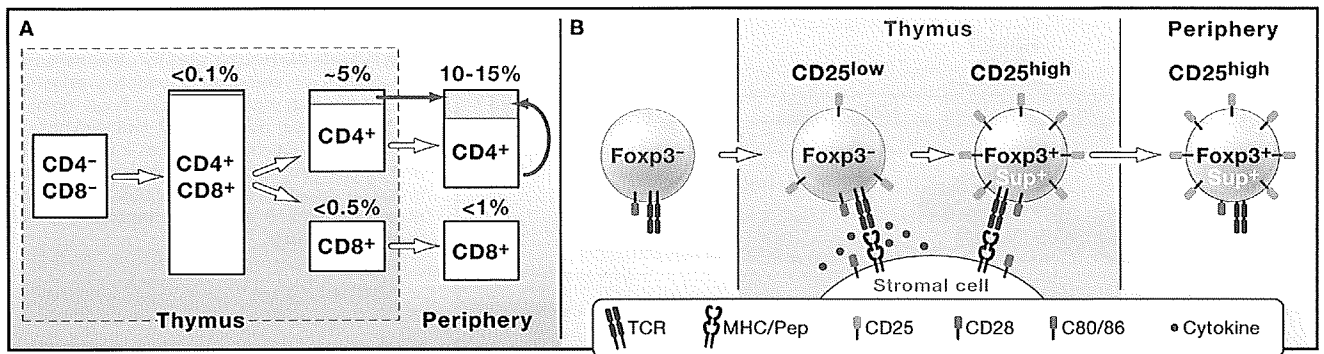


Figure 4. Thymic Development of Foxp3⁺ Cells

(A) The composition of Foxp3⁺ cells in each thymocyte subpopulation is shown as a percentage. In the periphery, CD4⁺ non-Treg cells can differentiate to Foxp3⁺ Tregs under certain conditions.

(B) Development of Foxp3⁺ Tregs in the thymus involves interaction with thymic stromal cells via various molecules. Foxp3⁻ thymocytes at a late CD4⁺CD8⁺ or an early CD4⁻CD8⁻ stage turn on a Treg differentiation program when they receive signals produced by the interaction between their TCRs and MHC/self-peptide complexes on thymic stromal cells, between their accessory molecules (e.g., CD28) and their ligands (e.g., CD80 and CD86), and/or via stromal cell-derived humoral factors (e.g., cytokines). Foxp3 expression following cell fate determination confers suppressive activity and stabilizes Treg function and phenotype (e.g., CD25 expression). Blue indicates that the thymocyte has a suppressive function (Sup⁺).

via the TCR in the thymus (Koonpaew et al., 2006). Furthermore, in TCR β -fixed transgenic mice with a single copy of the TCR α locus or a "mini-TCR α " transgenic locus (which inhibits the appearance of T cells expressing double α chains), sequence analyses of TCR V α segments of a particular V α subfamily in CD25⁺ or CD25⁻CD4⁺ T cells have revealed distinct repertoires of the two populations, although the degree of overlap needs to be assessed more precisely (Hsieh et al., 2006; Pacholczyk et al., 2007).

In addition to the affinity of the interaction between the TCR and self-peptide/MHC, the intensity of the interaction between T cell accessory molecules and their ligands on thymic stromal cells contributes to the generation of natural Tregs. For example, deficiency of CD28, CD40, CD11a/CD18 (LFA1), or CD80 and CD86 (B7) results in a substantial reduction of CD25⁺CD4⁺ Tregs in the thymus and periphery (reviewed in Sakaguchi, 2005).

At the cellular level, both medullary thymic epithelial cells (mTECs) and dendritic cells in the thymus contribute to Treg generation as well as negative selection of naive T cells. Deficiency in the tumor necrosis factor receptor-associated factor 6 (TRAF6) or NF- κ B-inducing kinase results in the absence of mature mTECs, hampering the development of Tregs (Akiyama et al., 2005; Kajiura et al., 2004). In humans, the cytokine TSLP (thymic stromal lymphopoietin) secreted by Hassal's corpuscles, which are derived from mTECs, appears to act on thymic dendritic cells to promote the differentiation of thymocytes to Tregs (Watanabe et al., 2005). A population of mTECs ectopically express a set of tissue-specific antigens (TSAs), such as insulin, under direct or indirect control of the gene *Aire* (autoimmune regulator) (reviewed in Mathis and Benoist, 2007). Interestingly, *Aire*-deficient mice spontaneously develop autoimmune diseases similar to those produced by Treg depletion. A recent study has suggested that *Aire*⁺ mTECs expressing TSAs may induce or facilitate the development of TSA-specific Tregs in normal thymus, and that a paucity of TSA-specific Tregs may contribute to

the occurrence of autoimmune disease in *Aire* deficiency (Aschenbrenner et al., 2007).

Thus, the avidity of interaction between thymocytes and stromal cells is critical in determining the fate of developing thymocytes to the Treg lineage. Interestingly, recent studies suggest that Foxp3 per se is not required for this initial cell fate determination in the thymus (Lin et al., 2007; Gavin et al., 2007). In mice in which the Foxp3 encoding region is replaced by a green fluorescent protein (GFP) reporter, GFP-positive thymocytes, which do not express the Foxp3 protein, exhibit some elements of the Treg phenotype (including the expression of Treg-associated cell-surface molecules, such as CD25), although they are not suppressive in vitro. This indicates that the interaction of developing thymocytes with thymic stromal cells turns on a program of transcriptional regulation in parallel with or upstream of Foxp3, and that once the *Foxp3* gene is switched on, Foxp3 may stabilize and sustain the Treg phenotype and confer suppressive activity. Future efforts will examine how spatiotemporal interactions between thymocytes and stromal cells turn on the Treg differentiation program in developing thymocytes. Specifically, it needs to be determined whether the intensity of signaling via the TCR and accessory molecules alone is sufficient for cell fate determination to the Treg lineage or whether stromal cell-derived humoral factor(s) including cytokines are also required. It is also unclear how the interaction evokes Foxp3 expression in Treg-committed thymocytes (Figure 4B).

Peripheral Generation of Tregs from Naive T Cells

Naive T cells in the periphery can also acquire Foxp3 expression and consequently Treg function in several experimental settings, for example, in vitro antigenic stimulation of naive T cells in the presence of TGF- β (Chen et al., 2003; Apostolou and von Boehmer, 2004; Kretschmer et al., 2005). In TGF- β -dependent in vitro Treg induction, IL-6 hampers the differentiation of naive T cells to Foxp3⁺ cells (Bettelli et al.,

2006). Interestingly, TGF- β stimulation in the presence of IL-6 facilitates T cell differentiation to Th17 cells in mice (Veldhoen et al., 2006; Bettelli et al., 2006). IL-2 facilitates the differentiation of naive CD4⁺ T cells into Foxp3⁺ Tregs but inhibits their differentiation into Th17 cells (Laurence et al., 2007). Additionally, retinoic acid, which is secreted by a particular subset of dendritic cells in the gut-associated lymphoid tissue, inhibits IL-6-driven induction of Th17 cells. In the presence of TGF- β , retinoic acid facilitates the differentiation of naive T cells to Foxp3⁺ Tregs (Benson et al., 2007; Mucida et al., 2007; Sun et al., 2007; Coombes et al., 2007). Hence, orally administered protein antigens presented by retinoic acid-producing dendritic cells in the gut-associated lymphoid tissue may induce Foxp3⁺ Tregs. This could be a plausible mechanism of oral tolerance (Figure 5).

Yet, it remains to be determined whether Tregs induced from naive T cells in the periphery are functionally stable *in vivo* and to what extent they contribute to the peripheral pool of Foxp3⁺ Tregs (Figure 4A). For example, in mice the regulatory regions of the *Foxp3* gene are more widely demethylated in natural Tregs than in TGF- β -induced Tregs, suggesting functional instability of the latter (Floess et al., 2007). In addition, unlike mice, naive T cells in humans readily express Foxp3 upon TCR stimulation, although the expression is generally much lower and more transient than in natural Tregs (Yagi et al., 2004; Gavin et al., 2006). Assuming that Foxp3 can suppress possible effector programs in T cells, it is an intriguing question whether Foxp3 not only confers suppressive function to natural Tregs but also may serve as an intrinsic brake to the further activation of antigen-stimulated effector T cells in humans.

Besides Foxp3⁺ Tregs, there are other types of Treg cells that can be induced from naive T cells in the periphery; for instance, CD4⁺ T cells secreting IL-10 and TGF- β , called Tr1 cells, are produced *in vitro* by antigenic stimulation of naive T cells in the presence of IL-10 (Chen et al., 1994; Groux et al., 1997). Antigen-specific TGF- β -secreting T cells, called Th3 cells, were originally propagated from animals that became tolerant to orally administered protein antigen. At least some Th3 cells appear to be TGF- β -induced Foxp3⁺ Tregs. Tr1 cells do not express Foxp3, yet their properties *in vitro* are very similar to those of Foxp3⁺ Tregs. For example, they exhibit diminished proliferation in response to antigenic stimulation, exert cell contact-dependent suppression, scarcely produce IL-2, and display an activated cell-surface phenotype (Vieira et al., 2004). It is likely that Tr1 and natural Foxp3⁺ Tregs may share some common suppressive mechanisms *in vitro*, although long-term stability of Tr1 and Th3 cells needs to be further assessed *in vivo*.

A variety of other T cell subpopulations including CD8⁺, CD4⁺CD8⁻, and $\gamma\delta$ T cells have also been reported to exhibit an immunosuppressive activity (reviewed in Shevach, 2006). There is so far little evidence that they play crucial roles in natural self-tolerance. CD8⁺ suppressor T cells were widely and intensively studied in the 1970s but have defied precise molecular characterization. These cells may now need to be recharacterized with currently available molecular tools. It is noteworthy that antigen immunization with strong adjuvant

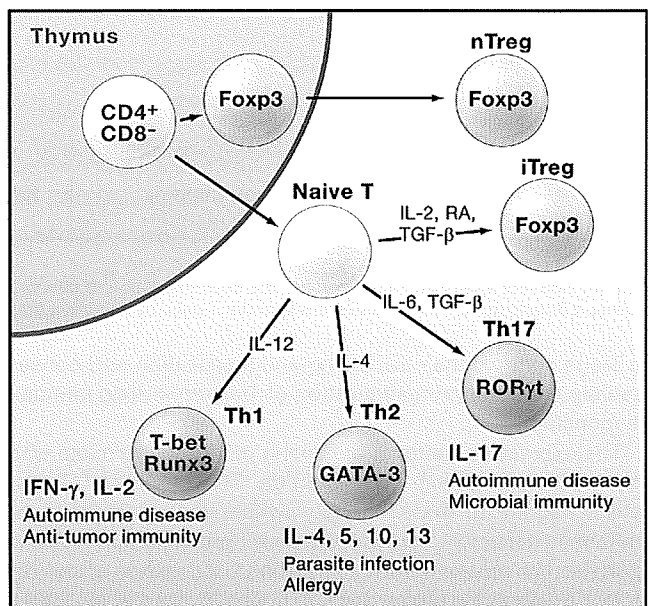


Figure 5. Differentiation of Naive CD4⁺ T Cells into Tregs or Effector T Cells

Cytokines and transcription factors that promote the differentiation of naive T cells into Tregs or effector T cells are shown. The transcription factors T-bet and Runx3, GATA3, or ROR γ t are required for the differentiation of naive T cells into Th1, Th2, or Th17 cells, respectively. nTreg, natural Treg; iTreg, induced Treg; RA, retinoic acid.

expands not only CD4⁺Foxp3⁺ T cells but also CD8⁺Foxp3⁺ T cells from a number that is negligible in unimmunized mice (Haribhai et al., 2007). These cells could plausibly correspond to some of the antigen-induced CD8⁺ suppressor T cells previously described (Cantor et al., 1976).

Trafficking and Localization of Tregs

Where are Foxp3⁺ natural Tregs found in the body? Dendritic cells in the draining lymph nodes present tissue-specific self-antigens (Scheinecker et al., 2002), and Foxp3⁺ Tregs specific for tissue self-antigens are enriched in the regional lymph nodes (Samy et al., 2005). This indicates that antigen-specific Tregs migrate to and become activated in regional lymph nodes where tissue-specific self-antigens or microbial antigens are presented. They also migrate into inflamed tissues, infectious sites, and tumors (Belkaid et al., 2002). In addition to TCRs that recognize specific antigens, Foxp3⁺ Tregs express a spectrum of homing receptors, including adhesion molecules and chemokine receptors (Huehn and Hamann, 2005). In the peripheral lymph nodes, the majority (~80%) of Foxp3⁺ Tregs express CCR7 at high levels. Foxp3⁺ Tregs are slightly higher than Foxp3⁻ T cells in the expression of CCR4, CCR6, CXCR4, and CXCR5. A fraction (~30%) of Foxp3⁺ Tregs express CD103 (integrin $\alpha_E\beta_7$), which interacts with its ligand E-cadherin expressed by epithelial cells; a substantial fraction (~50%) highly express CD62L (L-selection), which interacts with the vascular addressins CD34, GlyCAM-1, and MAdCAM-1 expressed by the endothelium in lymph nodes and mucosal lymphoid tissue (reviewed in Huehn and Hamann, 2005). The expression of these homing receptors

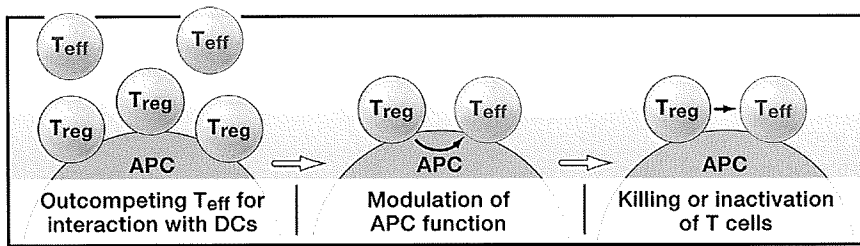


Figure 6. Possible Mechanisms of Treg-Mediated Suppression

More than one mechanism of Treg-mediated suppression may operate for the control of a particular immune response in a synergistic and sequential manner. Antigen-activated Tregs are recruited to antigen-presenting cells (APCs), especially dendritic cells (DCs), and out-compete antigen-specific naive T cells regarding interactions with dendritic cells mainly because of the high expression of adhesion molecules (such as LFA-1) by Tregs. Tregs then modulate dendritic

cell function. For example, Tregs promote the downregulation of dendritic cell CD80 and CD86 by a CTLA-4-dependent mechanism. Some Tregs may further differentiate to kill or inactivate responder T cells by secreting granzyme/perforin or immunosuppressive cytokines (such as IL-10).

in Tregs controls their trafficking and localization and consequently the compartmentalization of Treg-controlled immune responses.

The expression patterns of homing receptors correlate with the functional heterogeneity of Tregs. $CCR7^+CD62L^{high}$ Tregs, which migrate to the secondary lymphoid tissues, have an enhanced capacity to prevent the development of autoimmune diabetes in a mouse model, suggesting suppression of self-reactive T cells in the regional lymph nodes (Szanya et al., 2002). $Foxp3^+$ Tregs that express CD103 are memory-like and more suppressive than CD103⁻ Tregs in vitro and in vivo. In a murine colitis model, $Foxp3^+$ Tregs that express CD103 are able to control colitis in the colonic mucosa (Uhlir et al., 2006). Similarly, $CCR5^+$ Tregs preferentially migrate to cutaneous lesions of *Leishmania major* infection (Yurchenko et al., 2006).

The expression patterns of homing receptors are basically similar to those of effector T cells migrating to the same tissue site. $CCR4^+$ Tregs as well as $CCR4^+$ effector T cells accumulate in inflamed skin following local antigen inoculation (Sather et al., 2007). $CCR6^+$ Tregs and $CCR6^+$ Th17 cells are both elevated in inflamed joints in an animal model of rheumatoid arthritis (Hirota et al., 2007). Additionally, retinoic acid secreted by intestinal CD103⁺ dendritic cells induces gut-homing integrin $\alpha_4\beta_7$ in Tregs and non-Treg cells.

Treg-specific deficiency of a particular homing receptor or its blockade compromises Treg function, for example, in the control of tissue-localized inflammation, local maintenance of allograft tolerance, and fetomaternal tolerance (Lee et al., 2005; Yurchenko et al., 2006; Schneider et al., 2007; Sather et al., 2007; Kallikourdis et al., 2007). Thus, migration receptors on Tregs are highly informative in assessing Treg behavior in vivo and can be exploited to control immune responses through altering Treg trafficking and localization.

Treg Activation, Proliferation, and Differentiation

Upon antigen exposure in the regional lymph nodes, $Foxp3^+$ Tregs become activated and exert suppression at a much lower concentration of antigen than naive T cells. In TCR transgenic mice, the concentration of peptide required to activate the peptide-specific Tregs to exert suppression in vitro has been estimated to be 10- to 100-fold lower than the concentration needed for activating naive T cells with the same antigen specificity (Takahashi et al., 1998). This finding suggests that natural Tregs can be activated even by immature dendritic cells whose expression levels of CD80/86 and self-peptide/MHC are too low to activate naive self-reactive T cells. This might enable natural

Tregs to exert dominant and tonic suppression of self-reactive T cells. It could also contribute to the prevention of autoimmunity stemming from molecular mimicry (the antigenic crossreaction between a self-molecule and a microbial substance) because natural Tregs could be more easily activated than self-reactive T cells by such a substance (Stephens et al., 2005).

Notably, unlike their hypoproliferation upon antigenic stimulation in vitro, a sizable fraction of $Foxp3^+$ natural Tregs are continuously proliferating in vivo presumably through the recognition of self-antigen and commensal microbes (Fisson et al., 2003; Setoguchi et al., 2005). Natural Tregs can also expand clonally in vivo and in vitro following antigenic stimulation and retain their suppressive function after expansion (Klein et al., 2003; Yamazaki et al., 2003; Fehérvári and Sakaguchi, 2004; Yamaguchi et al., 2007).

Various stimuli via accessory molecules expressed by $Foxp3^+$ Tregs also lead to their expansion in vivo and in vitro. Stimulation of GITR, which is constitutively expressed in Tregs at a higher level than in other T cells, facilitates vigorous proliferation of Tregs in the presence of IL-2 in vitro (McHugh et al., 2002; Shimizu et al., 2002). In addition, Tregs can expand following stimulation via Toll-like receptors (TLRs), independent of TCR recognition of a specific antigen. Tregs respond to ligands for TLR2, 4, 5, and 8, including bacterial flagellin that acts through TLR5 (reviewed in Suttmüller et al., 2006). A similar effect is observed with 60 kDa heat shock protein, a self-molecule, with Pam3Cys via mouse TLR2, or with lipopolysaccharide via TLR4.

Irrespective of active proliferation the number of $Foxp3^+$ natural Tregs is fairly constant in normal animals (10%–15% of $CD4^+$ T cells), indicating that cell death helps to maintain Treg homeostasis. Indeed, following antigenic stimulation Tregs downregulate Bcl-2, an antiapoptotic protein (Yamaguchi et al., 2007). Further, among $Foxp3^+CD4^+$ T cells in humans, antigen-stimulated naive $Foxp3^{low}$ cells differentiate into effector/memory-like $Foxp3^{high}$ cells, which vigorously proliferate and then die (M. Miyara and S.S., unpublished data).

Thus, $Foxp3^+$ Tregs that have migrated to lymphoid and nonlymphoid tissues become activated, proliferate, exert suppressive activity, and then die. However, it remains to be determined whether some Tregs further differentiate into long-lived memory-type cells.

Multiple Modes of Treg-Mediated Suppression?

A key issue of current research on Tregs is to understand the mechanism of Treg-mediated suppression. $Foxp3^+CD25^+CD4^+$ natural Tregs suppress the proliferation of naive T cells and

their differentiation to effector T cells *in vivo*. They can also suppress effector activities of differentiated CD4⁺ and CD8⁺ T cells and the function of natural killer cells, natural killer T cells, B cells, macrophages, osteoclasts, and dendritic cells (reviewed in von Boehmer, 2005; Shevach, 2006; Miyara and Sakaguchi, 2007; Tang and Bluestone, 2008). *In vitro* Tregs suppress the proliferation and cytokine production (in particular of IL-2) of responder T cells when the two populations are cocultured and stimulated by antigen in the presence of antigen-presenting cells (APC) (Takahashi et al., 1998; Thornton and Shevach, 1998). Once activated by a particular antigen, Tregs can suppress responder T cells irrespective of whether they share antigen specificity with the Treg (Takahashi et al., 1998).

Several mechanisms of Treg-mediated suppression have been proposed, and these include secretion by the Treg of immunosuppressive cytokines, cell-contact-dependent suppression, and functional modification or killing of APC (Figure 6). For example, IL-10 and TGF- β contribute to suppression of inflammatory bowel disease induced in mice by Treg depletion (Read et al., 2000). Tregs coexpressing Foxp3 and IL-10 at a single-cell level are indeed found in lamina propria of the intestine but not in the spleen (Uhlir et al., 2006; Maynard et al., 2007). TGF- β may act as a direct mediator of suppression, condition responder T cells to be sensitive to suppression, and/or maintain Foxp3 expression and suppressive activity in Tregs (reviewed in von Boehmer, 2005). A recent study has shown that Foxp3⁺ natural Tregs predominantly produce immunosuppressive IL-35, a new member of the IL-12 family (Collison et al., 2007).

Alternatively, absorption of cytokines by Tregs may induce apoptosis in responder T cells (Pandiyani et al., 2007). Tregs might also kill responder T cells or APC through cell-to-cell contact by a granzyme- or perforin-dependent mechanism, or through delivery of a negative signal to responder T cells. Possible negative signals include upregulation of intracellular cyclic AMP, which leads to inhibition of T cell proliferation and IL-2 production, or the generation of pericellular adenosine catalyzed by CD39 (ectonucleoside triphosphate diphosphohydrolase 1) and CD73 (ecto-5'-nucleotidase) expressed by Tregs (reviewed in Tang and Bluestone, 2008). Activated Tregs may also downmodulate CD80/86 expression on APC or stimulate dendritic cells to form the enzyme indoleamine 2, 3-dioxygenase, which catabolizes the essential amino acid tryptophan to kynurenines that are toxic to T cells; both appear to be dependent on the expression of CTLA-4 by Tregs.

Taken together, these findings suggest a model of Treg-mediated suppression: (1) upon antigenic stimulation, antigen-specific Tregs, which are highly mobile, are swiftly recruited via chemokines to dendritic cells presenting the antigen and out-compete antigen-specific naive T cells in aggregating around the dendritic cells; (2) antigen-activated Tregs contacting dendritic cells then downmodulate dendritic cell function, thereby hindering the activation of other T cells that are recruited to the dendritic cells; (3) Tregs may then further differentiate to secrete granzyme/perforin, IL-10, or other immunosuppressive cytokines (such as IL-35) depending on the strength and duration of antigenic stimulation and

the local milieu of cytokines and other substances (Figure 6). Consistent with this model, intravital imaging of Tregs and responder T cells in a lymph node by two-photon microscopy suggests that activated Tregs hamper stable contacts between responder T cells and antigen-presenting dendritic cells (Tang et al., 2006; Tadokoro et al., 2006). Assuming that *in vivo* activation of a naive T cell is a process that requires contact with an antigen-presenting dendritic cell for several hours (Celli et al., 2007), Treg-mediated interference may suffice to render abortive the activation of responder T cells, thereby suppressing an immune response.

Deficiencies of some Treg-expressing molecules (such as LAG3, granzymes, and IL-35) can impair Treg suppression *in vitro* by affecting a particular mode of suppression but fail to cause autoimmunity *in vivo* because other modes may effectively compensate for the deficiencies. Among the various molecules involved in suppression *in vivo* or *in vitro*, CTLA-4 is critically important for the following reasons. Foxp3⁺ Tregs constitutively express CTLA-4, Foxp3 directly controls the expression of CTLA-4, and CTLA-4 blockade abrogates suppression. Further, not only germline deletion but also Treg-specific conditional deficiency of CTLA-4 induces fatal autoimmunity/inflammation in mice (Salomon et al., 2000; Read et al., 2000; Takahashi et al., 2000; K. Wing and S.S., unpublished data). Additional study is required to elucidate the molecular basis of suppression mediated by Tregs. The fate of responder T cells that are suppressed by Tregs is also unclear, that is, whether they remain nonactivated, die by apoptosis, or become anergic.

Tregs: A Clinical Perspective

To what extent are Treg anomalies responsible for immunological diseases in humans and how might Tregs be exploited to control physiological and pathological immune responses?

Polymorphisms of several genes including *Ctla4*, *Ii2*, *Cd25*, and *Ptpn22* significantly contribute to genetic susceptibility to common autoimmune diseases, such as type 1 diabetes (Wellcome Trust Case Control Consortium, 2007). Deficiency of these genes, in particular *Ctla4*, *Ii2*, and *Cd25*, produces severe autoimmunity in mice presumably through an effect on Treg development and function. Similarly, CTLA-4 blockade or IL-2 neutralization for a limited period elicits T cell-mediated autoimmune disease in otherwise normal mice. It is therefore possible that the polymorphisms of these genes may alter Treg development or function and thereby render the host susceptible to autoimmune disease (Yamanouchi et al., 2007). Environmental factors might also affect Tregs and thereby contribute to the development of autoimmunity. Tregs display greater proliferation and higher metabolic activity than non-Tregs under physiological conditions. As a consequence, they are more sensitive to ionizing radiation, radiomimetic drugs (such as cyclophosphamide), and deficiency of certain vitamins such as folic acid (Fisson et al., 2003; Brode et al., 2006; Setoguchi et al., 2005; Yamaguchi et al., 2007).

Although Treg depletion can evoke autoimmunity, it can also provoke and enhance tumor immunity in rodents (Shimizu et al., 1999) (Figure 1). *In vitro* T cell responses against tumor-associated antigens are enhanced by stimulating T

cells from cancer patients (or even normal individuals) with tumor antigen after the depletion of natural Tregs (Danke et al., 2004; Nishikawa et al., 2005). In addition, Foxp3⁺ Tregs are abundant in tumors. Thus, natural Tregs that promote self-tolerance may act to impede immune surveillance against cancers in normal individuals and suppress potential responsiveness to autologous tumors in cancer patients. Targeting Tregs is a promising approach for cancer immunotherapy. Such approaches could include local depletion of Tregs in the tumor mass, attenuation of Treg function at the time of therapeutic vaccination with tumor antigen, and ex vivo expansion of tumor-infiltrating lymphocytes after the depletion of Tregs.

Similar to tumor immunity, depletion or reduction of natural Tregs enhances immune responses to pathogenic microbes (reviewed by Belkaid and Rouse, 2005). Yet, complete eradication of microbes via Treg depletion might hamper the development of effective immunological memory for the microbes, thus impairing the secondary immune responses upon reinfection (Belkaid et al., 2002). Hence, Tregs control the quality and tune the magnitude of antimicrobial immune responses to protect the host from pathogenic microbes while avoiding collateral immunopathology or inappropriate responses to commensal microbes.

Foxp3⁺ natural Tregs retain their suppressive function after expansion in vivo and in vitro. By exploiting this stable suppressive activity and proliferative capacity, strategies that clonally expand antigen-specific natural Tregs while inhibiting the activation and expansion of effector T cells will help to induce transplantation tolerance and suppress graft rejection. Furthermore, in the presence of Tregs that actively maintain graft tolerance, naive T cells could be newly recruited to the graft site and could differentiate into graft-specific Tregs, thereby augmenting graft tolerance (Waldmann et al., 2006). The same principle could be applied to the treatment of autoimmune disease, as well as allergy, and inflammatory diseases and might be used to augment fetomaternal tolerance in pregnancy.

For the purpose of attenuating or enhancing Treg-mediated immune suppression in various clinical settings, it is necessary to find a specific molecular marker that can selectively and reliably differentiate between Tregs and effector T cells. Biologicals, such as monoclonal antibodies, and small molecules that have differential effects on Tregs and effector T cells may represent the next generation of therapeutic reagents to selectively suppress or enhance immune responses by controlling the balance between Tregs and effector T cells. Further elucidation of the cellular and molecular processes underlying the development and function of Tregs will help to establish new strategies for the treatment and prevention of immunological diseases and for the control of a wide spectrum of physiological immune responses.

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REFERENCES

- Akiyama, T., Maeda, S., Yamane, S., Ogino, K., Kasai, M., Kajiwara, F., Matsumoto, M., and Inoue, J. (2005). Dependence of self-tolerance on TRAF6-directed development of thymic stroma. *Science* 308, 248–251.
- Antony, P.A., Paulos, C.M., Ahmadzadeh, M., Akpınarli, A., Palmer, D.C., Sato, N., Kaiser, A., Hinrichs, C.S., Klebanoff, C.A., Tagaya, Y., and Restifo, N.P. (2006). Interleukin-2-dependent mechanisms of tolerance and immunity in vivo. *J. Immunol.* 176, 5255–5266.
- Apostolou, I., and von Boehmer, H. (2004). In vivo instruction of suppressor commitment in naive T cells. *J. Exp. Med.* 199, 1401–1408.
- Aschenbrenner, K., D'Cruz, L.M., Vollmann, E.H., Hinterberger, M., Emmerich, J., Swee, L.K., Rolink, A., and Klein, L. (2007). Selection of Foxp3⁺ regulatory T cells specific for self antigen expressed and presented by Aire⁺ medullary thymic epithelial cells. *Nat. Immunol.* 8, 351–358.
- Baecher-Allan, C., Viglietta, V., and Hafler, D.A. (2004). Human CD4⁺CD25⁺ regulatory T cells. *Semin. Immunol.* 16, 89–98.
- Belkaid, Y., and Rouse, B.T. (2005). Natural regulatory T cells in infectious disease. *Nat. Immunol.* 6, 353–360.
- Belkaid, Y., Piccirillo, C.A., Mendez, S., Shevach, E.M., and Sacks, D.L. (2002). CD4⁺CD25⁺ regulatory T cells control *Leishmania* major persistence and immunity. *Nature* 420, 502–507.
- Benson, M.J., Pino-Lagos, K., Roseblatt, M., and Noelle, R.J. (2007). All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J. Exp. Med.* 204, 1765–1774.
- Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., and Kuchroo, V.K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441, 235–238.
- Brode, S., Raine, T., Zaccane, P., and Cooke, A. (2006). Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4⁺CD25⁺Foxp3⁺ regulatory T cells. *J. Immunol.* 177, 6603–6612.
- Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paepfer, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27, 68–73.
- Burchill, M.A., Yang, J., Vogtenhuber, C., Blazar, B.R., and Farrar, M.A. (2007). IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3⁺ regulatory T cells. *J. Immunol.* 178, 280–290.
- Cantor, H., Shen, F.W., and Boyse, E.A. (1976). Separation of helper T cells from suppressor T cells expressing different Ly components. II. Activation by antigen: after immunization, antigen-specific suppressor and helper activities are mediated by distinct T-cell subclasses. *J. Exp. Med.* 143, 1391–1400.
- Caudy, A.A., Reddy, S.T., Chatila, T., Atkinson, J.P., and Verbsky, J.W. (2007). CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. *J. Allergy Clin. Immunol.* 119, 482–487.
- Celli, S., Lemaître, F., and Bousso, P. (2007). Real-time manipulation of T cell-dendritic cell interactions in vivo reveals the importance of prolonged contacts for CD4⁺ T cell activation. *Immunity* 27, 625–634.
- Chen, C., Rowell, E.A., Thomas, R.M., Hancock, W.W., and Wells, A.D. (2006). Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation. *J. Biol. Chem.* 281, 36828–36834.
- Chen, Y., Kuchroo, V.K., Inobe, J., Hafler, D.A., and Weiner, H.L. (1994). Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalitis. *Science* 265, 1237–1240.
- Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G., and Wahl, S.M. (2003). Conversion of peripheral CD4⁺CD25[−] naive T cells to

- CD4⁺CD25⁺ regulatory T cells by TGF-beta 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 Mermerschlager, 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.
- Coomes, 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-beta 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., Relland, 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., Teradaira, 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., Naji, 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.
- Kajiura, F., Sun, S., Nomura, T., Izumi, K., Ueno, T., Bando, Y., Kuroda, N., Han, H., Li, Y., Matsushima, A., et al. (2004). NF-kappa 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., Ozkaynak, E., and Hancock, W.W. (2005). Recruitment of Foxp3⁺ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor. *J. Exp. Med.* 207, 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., Relland, 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, 665–674.
- 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 Gnjatich, 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.
- Pandiyani, 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^{high} CD4⁺ T cells induce wasting disease with multiple organ pathology: prevention by OX-22^{low} 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 immunological 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., Miazgowiec, 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., Tlaskalova, 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., Uede, 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., Minaee, 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**, 5986–5993.
- 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., Borde, 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**, 1643–1656.
- 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., Veijola, 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., Kerényi, M., Stephens, G., Durant, L., Watford, W.T., Laurence, A., Robinson, G.W., Shevach, E.M., Moriggi, 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 Rudensky, 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.
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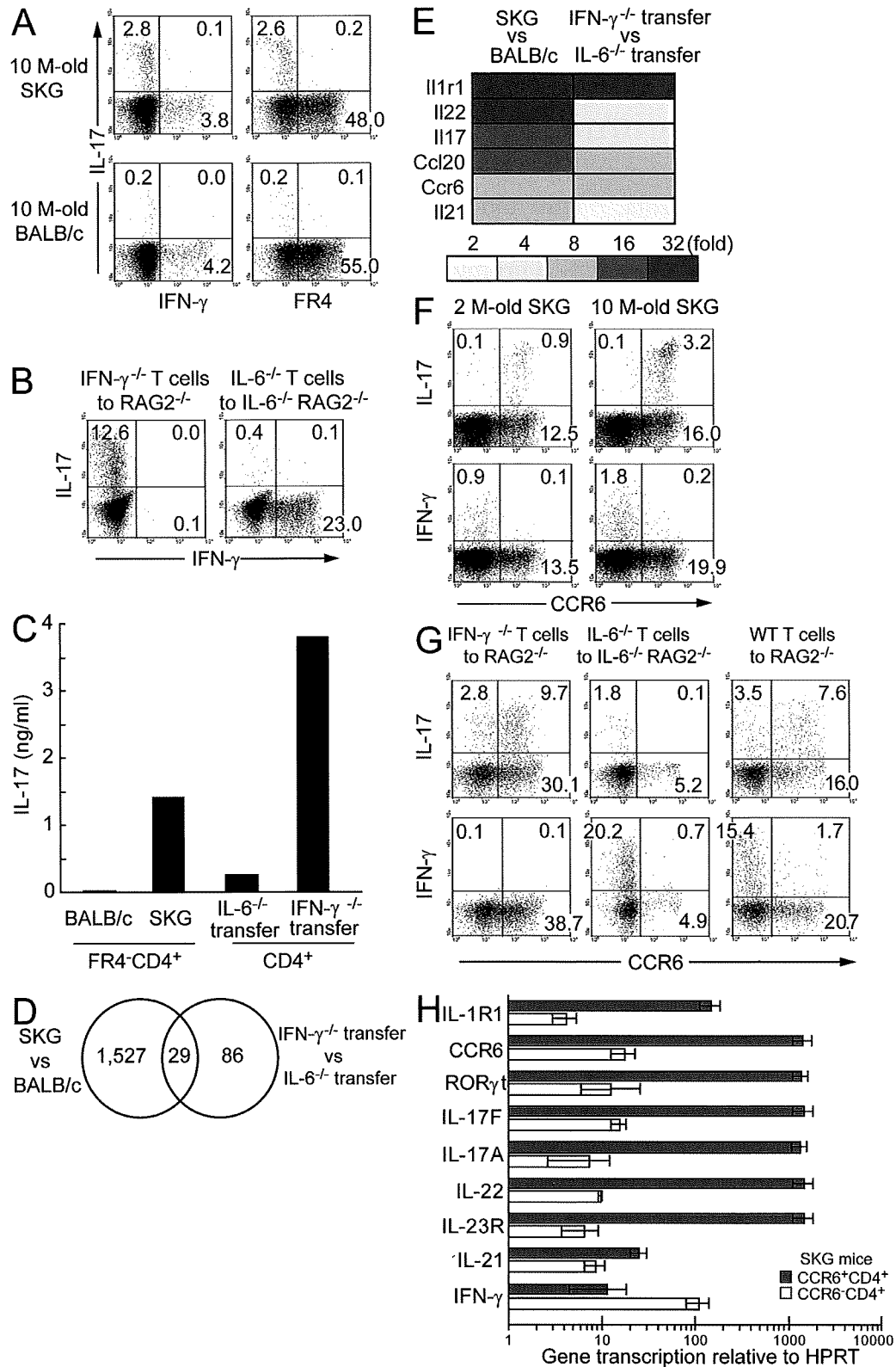


Figure 1. Gene microarray analysis of Th17 cells and their predominant expression of CCR6. (A) LN CD4⁺ T cells from 10-mo-old BALB/c or SKG mice were stained for intracellular IL-17 and IFN- γ , or these cytokines and cell-surface FR4. (B) CD4⁺ T cells from IFN- γ ^{-/-} or IL-6^{-/-} mice were transferred 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

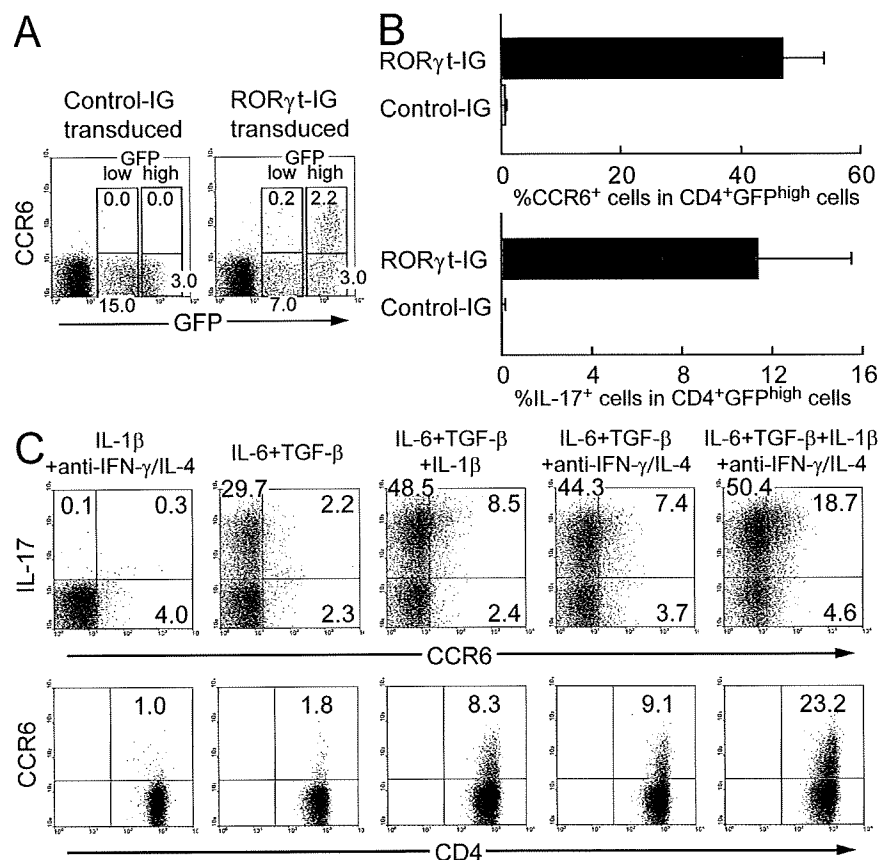


Figure 2. Induction of CCR6 on Th17 cells in vitro. (A) Retroviral vector, pMxs-ROR γ t-IRES-GFP (ROR γ t-IG), or control pMxs-IRES-GFP (Control-IG), was transduced into BALB/c CCR6⁻CD4⁺ T cells, as previously described (reference 7), and the cells expressing GFP were stained for CCR6 on day 4 after 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.