Several cell-biological questions are of interest concerning these receptors: (i) Are they internalized, and where do they deliver bound antigens? (ii) Are some receptors better at programming cross-presentation? (iii) Can individual receptors target functionally distinct DC subpopulations? (iv) Does antibody binding to a given receptor trigger DC maturation, and if so, does receptor activation help to control the balance between immunity and tolerance? In general, we must ask what the relationship is between the mode of antigen endocytosis and the function of antigen presentation to T cells (Fig. 1).

In recent work published in *Science*, Dudziak *et al.* have shown that two lectin receptors on mouse DCs, DEC-205 and DCIR (33D1), are differentially expressed by two different populations of DCs in the spleen: $CD8\alpha^+$ and $CD8\alpha^-$ DCs, respectively (*22*). Targeting antigens via DEC-205 to the $CD8\alpha^+$ subset was found to selectively prime MHCI-restricted responses by cross-presentation, whereas MHCII-restricted responses were more efficiently triggered by DCIR targeting to the $CD8\alpha^-$ subset. This finding is consistent with the idea that cross-presentation is, in general, more the purview of $CD8\alpha^+$ DCs (*23*). Reversing cell-type restriction of receptor expression did not appear to change this conclusion substantially, suggesting that specializations associated with $CD8\alpha^+$ DCs may favor their ability for cross-presentation.

At the same time, in vitro work suggested that another lectin (the mannose receptor) was also quite effective at inducing the formation of peptide-MHCI complexes from exogenous antigen in bone marrow cultures and macrophages, where subpopulation identities are less clear (*24*). The mannose receptor appeared to deliver bound antibody to early endocytic compartments, suggesting that cross-presentation may occur from here as opposed to in late endosomes and lysosomes, which was the case for loading onto MHCII. Although these results suggest that the receptor used and route of antigen entry may also help determine the resulting form of antigen presentation, the data relied only on low-resolution qualitative immunofluorescence to define the intracellular localization of delivered antigens—criteria too limited to support a firm conclusion. Moreover, the data did not account for the fact that DEC-205, which also efficiently mediates MHCI-restricted cross-presentation, has been extensively characterized as delivering its bound antigens to late endosomes and lysosomes as opposed to early compartments (*25*). In any event, these findings highlight a whole new problem set involving the relative contributions of endocytosis and DC subpopulations in determining the nature of the immune response.

Looking Forward by Looking Backward

There are many other problems that would benefit immediately from a more effective and bidirectional relationship between immunologists and cell biologists. For example, the dynamics and function of the immunological synapse remain incompletely understood, in part because these critically important structures have yet to be subjected to the type of rigorous analysis applied to "simpler" problems of cell adhesion. The relationship between autophagy and antigen presentation in viral immunity is also emerging as critical (*26*, *27*). Signaling in immune cells will provide a rich area to mine, and some direct interchange over what lipid rafts can and cannot do would be of great value in itself. Finally, there is the issue that immunologists have always appreciated far better than most molecular cell biologists: the in vivo or systems-level context. Immunology exists to study the way the immune system works as a whole to confer protection against disease. Broad and conceptually profound, it is understandably difficult for the field to devote equivalent attention to the cellular mechanisms involved. However, further progress will require such effort, and the best path forward will be to take steps to make the language, concepts, and culture of immunology more accessible to colleagues in cell biology to attract them in and to outsource what may be too diversionary to learn. One area that is particularly ripe for spectacular advance is in the area of in vivo or "intravital" imaging. Although still in a largely descriptive phase of development, immunologists are nicely demonstrating to cell biologists the conceptual value of this platform. When this area is combined with emerging technologies to permit interventional experiments using actuation switches and quantitative molecular reporters, we will have entered a new age of "systems cell biology," combining the best of both worlds.

Like Elyot and Amanda, immunology and cell biology were once intimate partners; we find ourselves again in close proximity, but this time with the chance to rekindle a beautiful relationship.

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10.1126/science.1142955

PERSPECTIVE

Emerging Challenges in Regulatory T Cell Function and Biology

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Much progress has been made in understanding how the immune system is regulated, with a great deal of recent interest in naturally occurring $CD4^+$ regulatory T cells that actively engage in the maintenance of immunological self-tolerance and immune homeostasis. The challenge ahead for immunologists is the further elucidation of the molecular and cellular processes that govern the development and function of these cells. From this, exciting possibilities are emerging for the manipulation of regulatory T cell pathways in treating immunological diseases and suppressing or augmenting physiological immune responses.

Walter B. Cannon, the originator of the concept of homeostasis, emphasized in his book *The Wisdom of the Body* that "when a factor is known which can shift a homeostatic state in one direction it is reasonable to look for a factor or factors having an opposing

effect." The immune system is not an exception to this. It harbors not only effector lymphocytes capable of attacking invading microbes but also an inhibitory population of T cells, called regulatory T (T_{reg}) cells. These lymphocytes are specialized in suppressing excessive or misguided immune responses that can be harmful to the host; for example, against normal self-constituents in autoimmune disease, innocuous environmental substances in allergy, or commensal microbes in certain inflammatory diseases (*1*, *2*). On the other hand, overzealous T_{reg} responses can impede host protective immunity in infectious disease and cancer. Recent advances in our understanding of the molecular mechanisms that control T_{reg} cell development have opened new avenues of investigation, but key questions concerning the antigen specificity of T_{reg} cells, their homeostasis, and mechanism of action remain. Here we discuss our current understanding of the biology and function of T_{reg} cells and how they might be clinically exploited to control physiological and pathological immune responses to self- and nonself-antigens.

Naturally occurring $CD4^+$ T_{reg} cells, which constitute approximately 10% of peripheral $CD4^+$ T cells in normal individuals, characteristically express CD25 [the interleukin-2 (IL-2) receptor α chain, which is a component of the high-affinity IL-2 receptor] (*1*, *2*). $CD4^+CD25^+$ T_{reg} cells play a nonredundant role in maintaining immunological self-tolerance and immune homeostasis. Their importance is made evident by the fact that the depletion of this population from normal rodents produces a variety of autoimmune inflammatory diseases, whereas reconstitution with $CD4^+$ $CD25^+$ T cells can inhibit disease development (*1*, *2*). They are produced by the normal thymus as a functionally distinct and mature population, although there is evidence that T cells with similar immune suppressive activity can be generated from naïve T cells in the periphery.

The identification of the transcription factor forkhead box p3 (Foxp3) as being specifically expressed by T_{reg} cells and crucial for their function has provided a molecular framework for dissecting T_{reg} function (*3*–*5*) (Fig. 1). Mutations in the gene encoding Foxp3 in humans and mice result in impaired development and function of $CD4^+CD25^+$ natural T_{reg} cells and lead to autoimmune inflammatory pathology. This is best exemplified by a human genetic disease called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, which is characterized by autoimmune disease (including type 1 diabetes and thyroiditis), allergy, and inflammatory bowel disease (IBD) (*6*). Further evidence for Foxp3 as a key controller of the development and suppressive function of natural T_{reg} cells comes from experiments in which transduction of the gene is sufficient to convert naïve T cells into T_{reg}-like cells (*3*–*5*). Notably, Foxp3 inhibits transcription of the gene encoding IL-2 and up-regulates the expression of CD25 and other T_{reg} cell–associated molecules (*3*, *4*). The resulting inability of $Foxp3^+$ T_{reg} cells to produce IL-2 appears to make them highly dependent on exogenous IL-2 for survival (*7*–*9*). Accordingly, mice genetically deficient in IL-2, CD25, or CD122 (the IL-2 receptor β chain) and humans with genetic deficiency of CD25 have both reduced numbers and impaired function of $Foxp3^+$ T_{reg} cells and succumb to severe autoimmune inflammatory disease (*8*, *10*).

A key question that has emerged from these findings is how Foxp3 orchestrates the cellular and molecular programs involved in T_{reg} function. Recent studies have shown that Foxp3 binds to other transcription factors such as NFAT (nuclear factor of activated T cells) and AML1 (acute leukemia-1)/Runx1 (runt-related transcription factor 1) and potentially interacts with activator protein 1 and nuclear factor κB (*11*–*13*). It is this Foxp3/NFAT/Runx1 complex, together with other coactivator or corepressor proteins, that is responsible for the observed repression of the IL-2 and other cytokine genes, as well as the activation of the genes for CD25, cytotoxic lymphocyte–associated antigen-4 (CTLA-4), and glucocorticoid-induced TNF receptor family–related protein (GITR) by binding to their respective promoters (*11*, *12*). MicroRNA genes also appear to be important in T_{reg} cell development; for example, T cell–specific depletion of Dicer, a ribonuclease enzyme required for processing double-stranded RNA, hampers thymic development of $Foxp3^+$ T cells and elicits IBD (*14*). In addition, it has been shown by genome-wide analysis combining chromatin immunoprecipitation with mouse genome tiling array profiling that Foxp3 directly or indirectly controls hundreds of genes, which include those that encode nuclear factors controlling gene expression and chromatin remodeling, membrane proteins, and signal transduction molecules (*15*, *16*). Assuming that the proteins encoded by Foxp3-controlled genes contribute to the suppressive activity of T_{reg} cells, it seems likely that further analysis of these pathways will provide insight into T_{reg} mechanisms of action.

In addition to the thymic production of natural $Foxp3^+$ T_{reg} cells, naïve T cells in the periphery acquire Foxp3 expression and T_{reg} function in several experimental settings, including in vitro antigenic stimulation in the presence of transforming growth factor–β (TGF-β) or after chronic antigen stimulation in vivo (*17*, *18*) (Fig. 1). Recent studies indicate that the intestine is a site of $Foxp3^+$ T_{reg} cell development and that specialized intestinal dendritic cells (DCs) promote Foxp3 expression via a mechanism that is dependent on local TGF-β and retinoic acid, a vitamin A metabolite (*19*–*21*). Peripheral development of $Foxp3^+$ T_{reg} cells may therefore represent a mechanism that helps broaden the T_{reg} repertoire in specialized anatomical sites. Recent studies have also revealed a reciprocal relationship between the development of $Foxp3^+$ T_{reg} and effector T cells, so that naïve $CD4^+$ T cells differentiate into $Foxp3^+$ T_{reg} cells in the presence of TGF-β or into T helper 17 (T_H17) cells (which secrete IL-17, a potent proinflammatory cytokine) in the presence of TGF-β and IL-6 (*22*, *23*). Therefore, TGF-β, which can be ubiquitously expressed in tissues, has the paradoxical effect of inducing distinct T cell subsets that appear to have opposing effects on immune responses. Moreover, IL-2 facilitates the differentiation of naïve $CD4^+$ T cells into T_{reg} cells but inhibits their differentiation into T_H17 cells, whereas IL-6 suppresses Foxp3 expression in T_{reg} cells in addition to enhancing T_H17 cell development (*23*, *24*). These results serve to illustrate the complexity of cytokine-mediated control of the differentiation of $Foxp3^+$ T_{reg} cells in the periphery, and further work is required to identify tissue-specific factors that influence the balance between T_{reg} and effector T cells in distinct tissue sites.

Although peripherally induced T_{reg} cells resemble thymically derived T_{reg} cells in phenotype and aspects of their function, future comparative studies of their functional and genetic stability, including the status of chromatin remodeling of the Foxp3 locus, need to be performed with the two populations (*25*). It should also be noted that, in contrast to mouse naïve T cells, in which it is difficult to induce Foxp3 by in vitro T cell receptor (TCR) stimulation, human naïve peripheral blood T cells readily express Foxp3 upon TCR stimulation although the expression level is generally much lower and more transient than in natural T_{reg} cells (*26*). Indeed, it is not yet established whether induced T_{reg} cells have identical functions to those of natural T_{reg} cells, to what extent they contribute to the pool of $Foxp3^+$ T_{reg} cells in the periphery, and whether this activation-induced Foxp3 expression in non-T_{reg} cells serves as a T cell–intrinsic brake on immune responses.

$Foxp3^+$ T_{reg} cells can both directly and indirectly suppress the activation and proliferation of many cell types, including T cells, B cells, DCs, natural killer (NK) cells, and NKT cells in vivo and/or in vitro (*27*, *28*). In vitro suppression of TCR-stimulated proliferation of other T cells is a commonly used assay for assessing T_{reg} cell suppressive activity; however, the mechanisms involved are incompletely understood. A number of different mechanisms have been linked to T_{reg} activity, including cell contact–dependent inhibition of the activation and proliferation of antigen-presenting cells (APCs) and T cells, the killing of either APCs or T cells or both, and suppression via cytokines such as IL-10 and TGF-β (*2*, *27*, *28*). These results suggest that $Foxp3^+$ T_{reg} cells do not suppress

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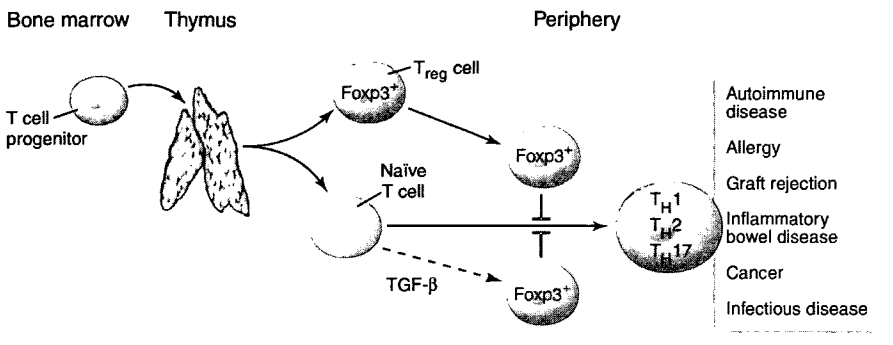

Fig. 1. Foxp3$^+$ natural T_{reg} cells produced by the normal thymus suppress the activation and expansion of naïve T cells and their differentiation to effector T cells, including T_H1, T_H2, and T_H17 cells, which mediate a variety of pathological and physiological immune responses. Foxp3$^+$ T_{reg} cells can also be generated from naïve T cells in the periphery, although the physiological significance of this T_{reg}-generative pathway remains to be determined.

immune responses by a single mechanism but use a variety of pathways in a context-dependent manner; for example, depending on cytokine milieu, the activation status of APCs, and the strength of antigen stimulation. A key challenge therefore is to validate putative mechanisms of T_{reg} activity in vivo and define the circumstances in which these operate. An important factor may be the site of action of T_{reg} cells. Elegant studies by intra-vital imaging with two-photon microscopy to examine the in vivo behavior of activated T_{reg} cells in lymph nodes suggest that they may hamper the access of effector T cells to DCs (*29*, *30*). There is also evidence that T_{reg} cells act in tissues to control established inflammation and that T_{reg} cell production of IL-10 plays a functional role (*2*). IL-10–secreting Foxp3$^+$ T cells are rare in the spleen but abundant in the inflamed intestine and also become detectable at the site of inflammation in autoimmune disease or chronic infection (*31*). This indicates that there is compartmentalization of the T_{reg} response and that mechanisms of suppression may be influenced by the anatomical location and dictated by the nature of the inflammatory response being regulated. It is also imperative to the host that appropriate effector responses can be activated after infection with pathogens. The production of IL-6 by activated DCs has been shown to overcome T_{reg}-mediated suppression in vitro (*32*). However, further information on the cellular and molecular pathways that control the delicate balance between effector and regulatory T cells in vivo is required.

The specialized immunological properties of Foxp3$^+$CD4$^+$ T_{reg} cells suggest that they might be clinically exploited to control a variety of physiological and pathological immune responses (*2*, *10*). These cells can recognize a broad repertoire of self- and nonself-antigens including pathogens (*33*), although their total repertoire is apparently more skewed to recognizing self-antigens (*34*, *35*). Phenotypically they appear in an "antigen-activated" state in the thymus, as illustrated by their high expression levels of various accessory molecules, including adhesion molecules (*10*). Thus, they are poised to exert suppressive function whenever exposed to relevant antigens and thus are suited for controlling autoimmunity. In addition, in contrast to their in vitro hyporesponsiveness to TCR stimulation, many natural T_{reg} cells are in a proliferative state in vivo, presumably as a consequence of the recognition of self-antigens and possibly commensal microbes, and can be stimulated to proliferate by antigenic stimulation (*10*). They are also functionally stable, retaining their suppressive activity after clonal expansion (*10*). By exploiting this stable and robust suppressive activity as well as proliferative capacity, strategies that clonally expand antigen-specific natural T_{reg} cells while inhibiting the activation and expansion of effector T cells may be useful to strengthen or reestablish self-tolerance in autoimmune disease or induce tolerance to nonself-antigens in organ transplantation, allergy, and IBD, or augment feto-maternal tolerance in pregnancy (Fig. 1). As a reciprocal approach, selective reductions in the number or function of natural T_{reg} cells while retaining or enhancing effector T cells may be a strategy for provoking and augmenting tumor immunity in cancer patients or microbial immunity in chronic infection. Biologicals and small molecules with such differential effects on T_{reg} cells and effector T cells may represent a next generation of therapeutic reagents for suppressing or enhancing immune responses with a high level of selectivity (*36*).

Besides Foxp3$^+$ T_{reg} cells, there are a number of Foxp3-nonexpressing T cells with immune suppressive activity that are in the scope of clinical use. These include CD4$^+$ cells producing IL-10 or TGF-β as well as CD8$^+$ T_{reg} cells with different modes of suppression (*28*, *37*). Although the physiological role of these populations in immune homeostasis is not known, they do offer the advantage for clinical use that antigen-specific T_{reg} cells can be prepared relatively easily.

It is now firmly established that Foxp3$^+$ T_{reg} cells, naturally arising or induced, constitute an indispensable component of the immune system. Further elucidation of the molecular and cellular basis of their development and function will facilitate our understanding of immune tolerance and homeostasis and provide ways to better control immune responses for the benefit of the host.

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38. S.S. is supported by the Japan Science and Technology Agency. F.P. is a Wellcome Trust Senior Fellow in basic biomedical science.

10.1126/science.1142331

Vol 446|5 April 2007|doi:10.1038/nature05673

LETTERS

Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1

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Naturally arising $CD25^+CD4^+$ regulatory T cells (T_R cells) are engaged in the maintenance of immunological self-tolerance and immune homeostasis by suppressing aberrant or excessive immune responses, such as autoimmune disease and allergy[1–3]. T_R cells specifically express the transcription factor Foxp3, a key regulator of T_R-cell development and function. Ectopic expression of Foxp3 in conventional T cells is indeed sufficient to confer suppressive activity, repress the production of cytokines such as interleukin-2 (IL-2) and interferon-gamma (IFN-γ), and upregulate T_R-cell-associated molecules such as CD25, cytotoxic T-lymphocyte-associated antigen-4, and glucocorticoid-induced TNF-receptor-family-related protein[4–7]. However, the method by which Foxp3 controls these molecular events has yet to be explained. Here we show that the transcription factor AML1 (acute myeloid leukaemia 1)/Runx1 (Runt-related transcription factor 1), which is crucially required for normal haematopoiesis including thymic T-cell development[8–11], activates *IL-2* and *IFN-γ* gene expression in conventional $CD4^+$ T cells through binding to their respective promoters. In natural T_R cells, Foxp3 interacts physically with AML1. Several lines of evidence support a model in which the interaction suppresses IL-2 and IFN-γ production, upregulates T_R-cell-associated molecules, and exerts suppressive activity. This transcriptional control of T_R-cell function by an interaction between Foxp3 and AML1 can be exploited to control physiological and pathological T-cell-mediated immune responses.

Functional studies of the *IL-2* promoter have ascertained that a minimal promoter region extending to 300 base pairs (bp) relative to the transcription start site of the *IL-2* gene, containing binding sites for nuclear factor of activated T cells (NFAT), Oct1, activator protein-1 (AP-1) and nuclear factor-κB (NF-κB)[12], is sufficient for inducible expression of the gene after stimulation of the T-cell antigen receptor (TCR) in reporter assays[13]. However, the upstream sequences beyond this region also enhance promoter activity[12]. By examining the 2.0-kb 5′ flanking region of the mouse *IL-2* gene, we have found three putative AML1–DNA binding consensus sites (5′-ACCACA-3′) at −370, −1,327 and −1,458 bp (relative to the transcription start site), denoted RE1, RE2 and RE3, respectively (Fig. 1a). In particular, a region containing the proximal RE1 site is highly conserved between humans, mice and other mammalian species (Supplementary Fig. S1) and is reported to be selectively and rapidly demethylated on T-cell activation[14].

To determine whether AML1 binds to the *IL-2* promoter and modifies *IL-2* gene expression, we performed several assays with mouse primary $CD4^+$ T cells and human Jurkat cells. In chromatin immunoprecipitation (ChIP) assays using an antibody against AML1, the *IL-2* promoter region adjacent to the RE1 site was co-precipitated in mouse primary $CD4^+$ T cells, whereas the 5′ and 3′ regions far from the start site were not (Fig. 1b). The human *IL-2* promoter region encompassing the AML1 site corresponding to the mouse RE1 site was also co-precipitated with the anti-AML1 antibody in Jurkat cells (Fig. 1b). Electrophoretic mobility-shift assays showed specific retardation of the complexes formed between the DNA-binding domain of AML1 and radiolabelled oligonucleotides corresponding to RE1, RE2 and RE3 (Fig. 1c). Moreover, mutating the RE1, RE2 and RE3 sites by altering two nucleotides at each site (mut RE1, mut RE2 and mut RE3; Supplementary Fig. S2) completely abolished AML1 binding (Fig. 1c).

To examine the functional responsiveness of the *IL-2* promoter to AML1, we made a reporter construct composed of the 1.6-kb *IL-2* promoter, containing the three AML1-binding sites, fused to a luciferase reporter gene. Stimulation of Jurkat cells with anti-CD3 and anti-CD28 activated the *IL-2* promoter in the construct[15], and transfection of AML1 enhanced this activation further (Fig. 1d). The use of a mutated *IL-2* promoter with mut RE1, mut RE2 and mut RE3 sites (*IL-2* promoter ΔAML-*luc*) resulted in a striking decrease in both stimulation-induced and AML1-enhanced promoter activities (Fig. 1e). Mutations that disrupted NFAT sites (*IL-2* promoter ΔNFAT-*luc*) led to a complete elimination of both stimulation-induced transcription activation and AML1-enhanced transactivation (Fig. 1f), suggesting that AML1-dependent activation of the *IL-2* promoter is reliant on the NFAT transcription factor complex.

Knockdown of endogenous *AML1* by RNA interference (RNAi) in Jurkat cells resulted in a marked decrease in IL-2 production after stimulation, indicating that AML1 is physiologically required for the induction of IL-2 expression in activated T cells (Fig. 1g). Moreover, in mouse primary T cells retrovirally transduced with *AML1*, IL-2 production in response to anti-CD3 and anti-CD28 stimulation was much higher than in empty-vector-transduced T cells (Fig. 1h, i). Conversely, IL-2 production was markedly reduced in mouse T cells transduced with a dominant-negative form of AML1 (AML1-DN, also called AML1a (ref. 16); see Fig. 2d), which lacks the C-terminal transcriptional activation and inhibition domains (Fig. 1h, i).

Taken together, the findings in Fig. 1 indicate that AML1 binds specifically to the AML1 sites present in the *IL-2* promoter region, thus enhancing *IL-2* gene expression.

Expression of AML1 protein in both conventional T cells and T_R cells (Supplementary Fig. S3) and strong repression of IL-2 expression by Foxp3 (Fig. 1h, i, refs 4–6) indicate that AML1 and Foxp3 may interact in T_R cells and may thereby control IL-2 expression. Indeed, an anti-FOXP3 antibody co-precipitated endogenous FOXP3 with AML1 from human peripheral blood mononuclear cells (PBMCs) (Fig. 2a). This indicates *in vivo* physiological interaction of

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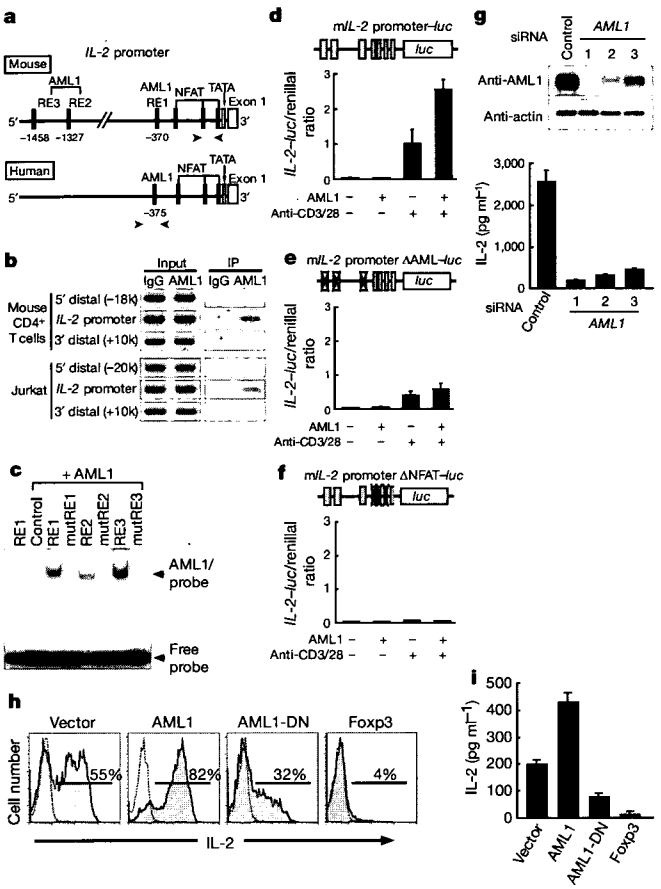

Figure 1 | AML1/Runx1 enhances IL-2 expression through binding to the *IL-2* promoter in activated T cells. **a**, Schematic representation of the mouse and human *IL-2* promoters. The putative AML1 binding sites, the NFAT sites and the TATA boxes are shown in red, blue and yellow, respectively. **b**, In ChIP assays performed with an antibody against AML1 or control rabbit IgG, aliquots of chromatin obtained before (Input) or after immunoprecipitation (IP) from mouse $CD4^+$ T cells or Jurkat cells were analysed by polymerase chain reaction with primers specific for the *IL-2* promoter. The positions of the ChIP primers are delineated in **a** by arrowheads. The primers for the 5′ and 3′ far distal regions were used as controls. **c**, In an electrophoretic mobility-shift assay, the DNA-binding domain of AML1 was incubated with radiolabelled oligonucleotides spanning the individual AML1 wild-type or mutated (mut) RE1, RE2 and RE3 sites on the *IL-2* promoter (sequences of the oligonucleotides are shown in Supplementary Fig. S2). **d–f**, Jurkat cells were simultaneously transfected with either an AML1-expressing or an empty vector, and with wild-type mouse *IL-2* (m*IL-2*) promoter–luciferase (*luc*) (**d**), a mutated promoter ΔAML–*luc* construct containing mut RE1, mut RE2 and mut RE3 (**e**), or a mutated promoter ΔNFAT–*luc* construct containing mutated NFAT consensus sites (**f**). Cells were either unstimulated or stimulated by plate-bound anti-CD3 and soluble anti-CD28 for 6 h, and luciferase activities were measured. Data shown are relative values of firefly luciferase normalized to *Renilla* luciferase and are expressed as means ± s.e.m. **g**, Jurkat cells were transfected with *AML1* siRNAs (1, 2 and 3) or control siRNA, and the expression of endogenous AML1 was evaluated by western blot analysis (top). Cells transfected with the indicated siRNAs were stimulated for 24 h with plate-bound anti-CD3 and soluble anti-CD28, and IL-2 levels in the supernatant were quantified by ELISA (bottom; means ± s.d.). **h**, Mouse $CD25^-CD4^+$ T cells were transduced with retroviral pMCsIg vectors encoding indicated proteins, IRES and GFP. GFP-positive cells were sorted and stimulated by plate-bound anti-CD3 and soluble anti-CD28 for 6 h and analysed for intracellular IL-2 expression by flow cytometry. Thin lines represent control staining with an isotype-matched antibody. **i**, Sorted GFP-positive cells were stimulated by soluble anti-CD3 and anti-CD28 with antigen-presenting cells for 24 h, and IL-2 levels in the supernatant were quantified by ELISA (means ± s.d.). Results represent three independent experiments.

endogenous FOXP3 and AML1 in natural T_R cells, because most $FOXP3^+$ T cells in PBMCs are naturally arising $CD4^+$ T_R cells[17–20]. Glutathione *S*-transferase (GST) pulldown experiments showed physical interaction of these two molecules *in vitro*: [^{35}S]methionine-labelled Foxp3 bound to the GST–AML1 fusion protein but not to the GST protein alone, and, reciprocally, labelled AML1 bound to GST–Foxp3 (Fig. 2b). In addition, under confocal microscopy, immunostained endogenous AML1 and Foxp3 were heterogeneously distributed in the nucleus and partly co-localized in mouse $CD25^+CD4^+$ T_R cells, suggesting that both proteins comprise part of the same molecular complex in natural T_R cells (Fig. 2c).

We next attempted to determine the Foxp3-interacting domain of AML1 and the AML1-interacting domain of Foxp3. In reciprocal co-immunoprecipitation experiments using lysates of human embryonic kidney 293T cells co-transfected with constructs encoding tagged AML1 and Foxp3 proteins, deletion mutants of AML1 lacking

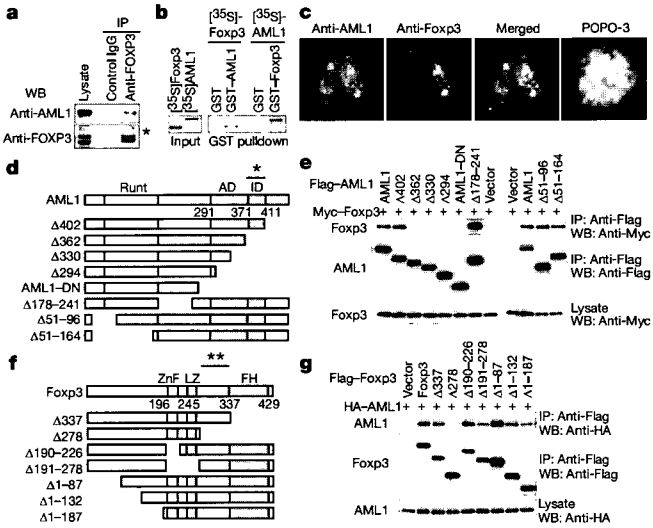

Figure 2 | Foxp3 interacts physically with AML1. **a**, Endogenous interaction between AML1 and FOXP3 in human PBMCs. PBMCs were lysed, subjected to immunoprecipitation (IP) with anti-FOXP3 antibody or control IgG, and western blotted (WB) with anti-AML1 and anti-FOXP3 antibodies. Asterisk indicates immunoglobulin heavy chain. **b**, *In vitro* interaction between Foxp3 and AML1 was analysed by GST pulldown assays. *In vitro* transcribed/translated [^{35}S]methionine-labelled FoxP3 or AML1 was incubated with bacterially expressed GST–AML1 or GST–Foxp3, respectively. GST alone was included as a negative control. Input consisted of 10% of the [^{35}S]methionine-labelled products. **c**, The intracellular localizations of endogenous AML1 and Foxp3 in mouse T_R cells were analysed by confocal microscopy with antibodies directed against AML1 and Foxp3. A representative image of a single T_R cell is shown. Images of AML1 (green) and Foxp3 (red) were merged to show regions of co-localization (yellow). The nucleus was revealed with POPO-3 staining (blue). **d**, Schematic diagram of wild-type AML1 (shown on top) and the deletion constructs. The Runt, activation and inhibition domains are indicated as Runt, AD and ID, respectively. **e**, Myc-tagged Foxp3 was co-transfected into 293T cells with the indicated Flag-tagged AML1 constructs and immunoprecipitated with anti-Flag M2 agarose; protein blots were probed with anti-Myc (top) or anti-Flag (middle) antibodies. The expression of Foxp3 in the lysates was monitored by immunoblotting with an anti-Myc antibody (bottom). **f**, Schematic diagram of wild-type Foxp3 (top) and the deletion constructs. The zinc finger, leucine zipper and forkhead domains are indicated as ZnF, LZ and FH, respectively. **g**, Haemagglutinin (HA)-tagged AML1 was co-transfected into 293T cells with the indicated Flag-tagged Foxp3 constructs and immunoprecipitated with anti-Flag M2 agarose, and protein blots were probed with anti-HA (top) or anti-Flag (middle) antibodies. The expression of AML1 in the lysates was monitored by immunoblotting with an anti-HA antibody (bottom). Figures represent three independent experiments.

the carboxy-terminal region (amino acids 362–402; asterisk in Fig. 2d) failed to bind to Foxp3, indicating a requirement of this region for interaction with Foxp3 (Fig. 2d, e). This region of AML1 corresponds to the domain that was shown to have inhibitory activity on transcription[21]. Similar experiments using deletion mutants of Foxp3 indicated that the AML1-interacting domain of the Foxp3 protein was located between the forkhead domain and the leucine zipper motif (amino acids 278–336; asterisks in Fig. 2f) (Fig. 2f, g).

ChIP assays showed that Foxp3 bound to the *IL-2* promoter (Supplementary Fig. S4), as reported recently[22]. To establish the functional significance of the interaction between Foxp3 and AML1, we used the same *IL-2* reporter construct described in Fig. 1d to examine whether Foxp3 affects the transactivation activity of AML1 in Jurkat cells (Fig. 3a). Foxp3 did indeed repress both stimulation-induced transcriptional activation and AML1-dependent transcriptional enhancement of the *IL-2* promoter. When the mutated *IL-2* promoter ΔAML1-*luc* (depicted in Fig. 1e) was employed, *IL-2* promoter activity was substantially attenuated and Foxp3 failed to repress the transcription further, suggesting that the repression of *IL-2* transcription by Foxp3 is dependent on AML1 (Fig. 3b).

Next, to determine whether Foxp3 is also able to repress AML1-mediated IL-2 production in mouse primary T cells, we co-transduced $CD25^{-}CD4^{+}$ conventional T cells with retroviral constructs of Foxp3–internal ribosome entry site (IRES)–nerve growth factor receptor (NGFR) and AML1–IRES–green fluorescent protein (GFP), and analysed IL-2 production in those T cells co-expressing NGFR and GFP (Fig. 3c, d). Consistent with the results of the luciferase reporter assays was our observation that Foxp3 repressed AML1-driven IL-2 production in the co-transduced T cells (Fig. 3c, d, and Supplementary Fig. S5a). In addition, retroviral gene transduction of AML1 failed to elicit IL-2 production in Foxp3-expressing natural $CD25^{+}CD4^{+}$ T_R cells (Fig. 3e, and Supplementary Fig. S5b).

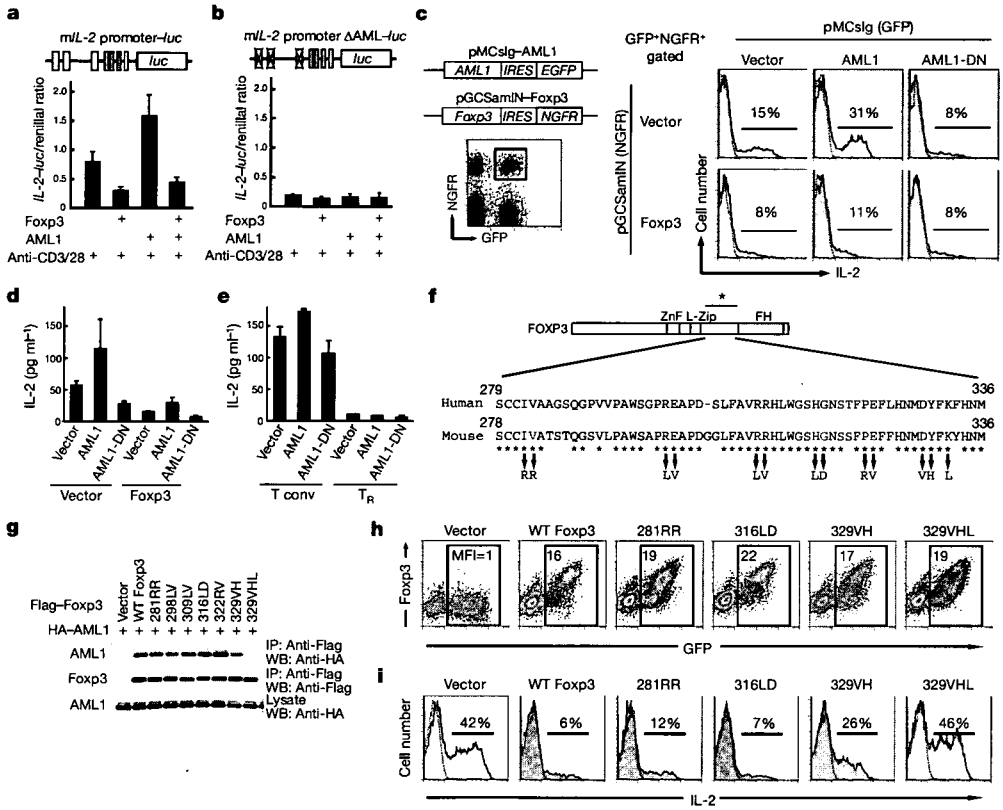

Figure 3 | Foxp3 represses AML1-induced IL-2 expression by interacting with AML1. **a**, **b**, Jurkat cells were co-transfected with expression vectors for the indicated proteins and wild-type *IL-2* promoter (**a**) or mutated *IL-2* promoter ΔAML–*luc* constructs (**b**), stimulated as in Fig. 1d–f, and luciferase activities were measured. **c**, Schematic representation of the retroviral vectors, including pMCsIg–AML1 (*AML1–IRES–GFP*) and pGCSamIN–Foxp3 (*Foxp3–IRES–NGFR*), which were used to express AML1 and Foxp3, respectively, in this study. Mouse $CD4^{+}$ T cells were co-transduced with the pMCsIg and pGCSamIN vectors to express the indicated proteins and were activated by soluble anti-CD3 and anti-CD28 for 6 h. Cells were stained with a biotinylated anti-NGFR antibody and allophycocyanin-conjugated streptavidin, and subsequently fixed and stained with phycoerythrin-conjugated anti-IL-2, and analysed by flow cytometry for intracellular IL-2 in GFP^{+}/$NFGR^{+}$ double-positive cells (rectangles). **d**, Co-transduced mouse $CD4^{+}$ T cells sorted by gating GFP^{+}/$NFGR^{+}$ double-positive cells were stimulated as in Fig. 1i, and IL-2 levels in the supernatant were quantified by ELISA. **e**, Mouse $CD25^{-}CD4^{+}$ conventional T cells (T conv) or $CD25^{+}CD4^{+}$ T cells (T_R) were transduced with the pMCsIg vectors to express the indicated proteins, sorted by gating GFP^{+} cells and stimulated as in Fig. 1i; IL-2 levels in the supernatant were quantified by ELISA. Expression of transduced genes in **d** and **e** was confirmed by western blot analysis with sorted $GFP^{+}NGFR^{+}$ (or GFP^{+}) cells (Supplementary Fig. S5). **f**, Sequence alignment of the AML1-binding region in human and mouse FOXP3. Substitutions were introduced at conserved residues of human/mouse FOXP3 (asterisks) as indicated. **g**, Haemagglutinin (HA)-tagged AML1 was co-transfected into 293T cells with the indicated Flag-tagged Foxp3 mutants and immunoprecipitated with anti-Flag M2 agarose, and protein blots were probed with anti-HA (top) or anti-Flag (middle) antibodies. The expression of AML1 in the lysates was monitored by immunoblotting using an anti-HA antibody (bottom). WT, wild type. Abbreviations of mutants are as follows: 281RR: I281R and V282R; 298LV: R298L and E299V; 309LV: R309L and R310V; 316LD: H316L and G317D; 322RV: P322R and E323V; 329VH: D329V and Y330H; 329VHL: D329V, Y330H and K332L. **h**, Mouse $CD4^{+}$ T cells transduced with pMCsIg vectors encoding wild-type and mutant Foxp3 proteins, IRES and GFP were stained intracellularly with phycoerythrin-conjugated anti-Foxp3, and analysed by flow cytometry. Numbers in dot plots indicate mean fluorescence intensity (MFI) of Foxp3 in the rectangle gates. **i**, Gene-transduced T cells were stimulated as in Fig. 1h, and $CD4^{+}$ cells were analysed for intracellular IL-2 by flow cytometry. Error bars represent s.e.m.; all experiments were repeated at least three times with similar results.

To examine further whether IL-2 repression by Foxp3 is dependent on the interaction of Foxp3 with AML1, we made Foxp3 mutants with two or three amino acid substitutions in the region required for interaction with AML1 (Fig. 3f). In co-immunoprecipitation experiments using these Foxp3 mutants and wild-type AML1, mutant 329VHL, which has three amino acid changes in the region, scarcely bound to AML1, and mutant 329VH exhibited less AML1 binding than other mutants with two amino acid substitutions or than wild-type Foxp3 (Fig. 3g). After retroviral gene transduction of mutated or wild-type Foxp3 into conventional $CD4^+$ T cells, the expression levels of the Foxp3 proteins were equivalent (Fig. 3h). However, the AML1-non-binding 329VHL mutant was unable to suppress IL-2 production when transduced to conventional $CD4^+$ T cells, and the 329VH mutant with reduced AML1 binding was less suppressive than other mutants or wild-type expressers (Fig. 3i). 329VHL is not a simple loss-of-function mutant, because it retained the ability to interact with NFAT (Supplementary Fig. S6) and regulate some Foxp3-regulated genes in a DNA microarray analysis (Supplementary Fig. S7). Thus, in addition to the NFAT–Foxp3 interaction[22], the AML1–Foxp3 interaction is critical on its own for suppressing IL-2.

We next examined whether AML1 is involved in the regulation of the genes encoding T_R-cell-associated cell surface molecules, such as CD25, cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF-receptor-family-related protein (GITR). ChIP assays showed that AML1 bound to intron 1 of *CD25*, to the promoter of *CTLA-4*, and to the promoter and intron 1 of *GITR* (Supplementary Fig. S8a, b). ChIP assays using the same primer pairs as in Fig. S8a showed that Foxp3 bound to *CD25* intron 1 and *GITR* intron 1 (Supplementary Fig. S9), which was consistent with recent reports[22]. Retroviral transduction of AML1 in mouse primary $CD4^+$ T cells downregulated the expression of CD25, CTLA-4 and, in particular, GITR (Supplementary Fig. S8c), whereas similar transduction of wild-type Foxp3 upregulated the expression of these molecules (Fig. 4a). Notably, the 329VHL Foxp3 mutant failed to upregulate them, and the 329VH mutant was less efficient in the upregulation than other mutants or wild-type Foxp3 (Fig. 4a). Taken together, these findings suggest that AML1 and the Foxp3–AML1 complex control these genes in conventional T cells and natural T_R cells, respectively.

We, and others, have shown previously that natural T_R cells and Foxp3-transduced T cells are anergic (that is, non-proliferative) on stimulation of the TCR *in vitro*, whereas stimulation of the TCR together with high-dose IL-2 and anti-CD28 stimulation can abrogate the anergic state[4,5,23,24]. As shown in Fig. 4b, c, 329VHL-transduced T cells were slightly less anergic than T cells transduced with other mutants or wild-type Foxp3, and the addition of high-dose IL-2 and anti-CD28 antibody more easily abrogated their anergic state. Furthermore, whereas natural T_R cells and Foxp3-transduced T cells suppress the proliferation of co-cultured naive T cells on stimulation of the TCR *in vitro*[4,5,23,24], the 329VHL-transduced cells were much less suppressive than T cells transduced with other mutants (Fig. 4d). In addition, knockdown of AML1 in human $CD25^{high}CD4^+$ T cells abrogated their anergic state and attenuated their suppressive activity (Fig. 4e–g, and Supplementary Fig. S10). Taken together, these results support a model in which the formation of the Foxp3–AML1 complex controls anergy and the suppressive function of natural T_R cells.

AML1 and the Foxp3–AML1 complex may control the expression of a broad range of genes in addition to those encoding the IL-2 and T_R-cell-associated molecules described above (Supplementary Fig. S7). For example, AML1 and Foxp3 controlled IFN-γ production in a similar manner to that of IL-2: AML1 activated *IFN-γ* gene transcription by binding to its promoter, and Foxp3 suppressed AML1-induced IFN-γ production (Supplementary Information and Supplementary Fig. S11). Although the precise molecular mechanism of how AML1 and the Foxp3–AML1 complex control their

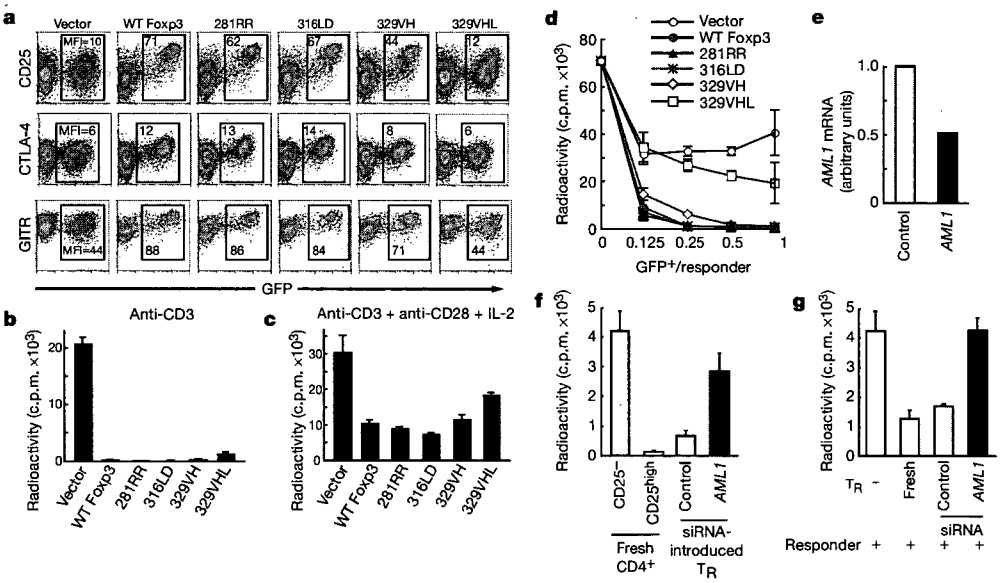

Figure 4 | Foxp3 requires interaction with AML1 to confer T_R-cell phenotype and function on conventional T cells. **a**, Mouse conventional T cells transduced with pMCsIg vector encoding wild-type (WT) or mutant Foxp3 shown in Fig. 3g–j were stained with anti-CD25 (top), anti-CTLA-4 (intracellular, middle) or anti-GITR (bottom), and analysed by flow cytometry. MFI, mean fluorescence intensity. **b**, **c**, Proliferation of sorted GFP^+ cells in the presence of soluble anti-CD3 and antigen-presenting cells (APCs) without (**b**) or with (**c**) IL-2 and anti-CD28, measured by $[^3H]$thymidine incorporation; results are means ± s.d. for triplicate cultures. **d**, Suppressive activity of T cells transduced with wild-type or mutant Foxp3. Graded doses of sorted GFP^+ cells were cultured with freshly prepared $CD25^-CD4^+$ cells for 72 h with soluble anti-CD3 and APCs, and $[^3H]$thymidine incorporation was measured as in **b**. **e**, Knockdown of AML1 results in repression of *AML1* mRNA in human primary $CD25^{high}CD4^+$ T_R cells. Relative expression of AML1 was quantified by real-time polymerase chain reaction, using HPRT as an internal control. **f**, Proliferation of control or *AML1* siRNA (shown in Fig. 1g as AML1 no. 1)-introduced human primary $CD25^{high}CD4^+$ T_R cells in the presence of soluble anti-CD3 and APCs. **g**, Suppressive activity of human primary $CD25^{high}CD4^+$ T_R cells transduced with control or *AML1* siRNA. Fresh or siRNA-transduced $CD25^{high}CD4^+$ T_R cells were mixed with freshly prepared $CD25^-CD4^+$ responder T cells, and stimulated by soluble anti-CD3 in the presence of APCs. Proliferation of cells was assessed by $[^3H]$thymidine incorporation and is shown as mean ± s.d. for triplicate cultures. All experiments were repeated at least three times with similar results.

downstream genes remains to be established, the present study provides several models for transcriptional controls by AML1 and Foxp3 (Supplementary Discussion). In addition, Foxp3 could bind to two other members of the AML/Runx protein family, AML2 (Runx3) and AML3 (Runx2) (Supplementary Discussion and Supplementary Fig. S12). There are recent reports that polymorphisms of AML1 and AML1-binding sites are associated with the susceptibility to several autoimmune diseases in humans[25–28]. Such genetic polymorphisms might affect T_R-cell-mediated maintenance of immunological self-tolerance (Supplementary Discussion). The interaction between AML1 and Foxp3 may be a potential therapeutic target for controlling physiological and pathological immune responses.

METHODS

RNA interference. Human PBMCs were freshly separated from whole blood and purified to $CD4^+$ T cells. Sorted cells were subsequently transfected with the Stealth RNAi duplex oligonucleotides against *AML1* (HSS141472; Invitrogen). Stealth RNAi negative control GC high was used as a control short interfering RNA (siRNA). At 16 h after transfection, 20 U ml^{-1} human recombinant IL-2 (eBiosciences) was added to the culture. At 40 h after transfection, $CD25^{high}CD4^+$ T_R cells (defined as the high 2% fraction of $CD4^+$ T cells) were sorted. At this time point, control and *AML1* siRNA-introduced T_R cells expressed CD25, FOXP3 and CTLA-4 at equivalent levels (Supplementary Fig. S10). $CD25^-CD4^+$ T cells were similarly sorted and used as responders. $CD3^-$ cells were irradiated (25 Gy) and used as APCs. A total of 10,000 $CD25^{high}CD4^+$ T_R cells were mixed with 20,000 APCs with or without 10,000 responder cells. Cells were stimulated with 20 ng ml^{-1} anti-CD3 (HIT3a; BD Pharmingen) for 4 days. [^{3}H]Thymidine incorporation for the last 8 h of cell culture was measured as an indicator of cell proliferation and is expressed as the mean ± s.d. for triplicate cultures.

Retroviral transduction. For co-transduction experiments, cells were first infected with pMCsIg-AML1 and kept for a further 5 h at 32 °C with 5% CO_2 and were subsequently infected with pGCSamIN–Foxp3. Gene transduction into $CD25^-CD4^+$ conventional T cells and $CD25^+CD4^+$ T_R cells was performed by stimulating cells with plate-bound anti-CD3 (10 μg ml^{-1}) and soluble anti-CD28 (1 μg ml^{-1}) with 100 U ml^{-1} mouse recombinant IL-2 for 16 h. Activated T cells were infected by resuspending cells with viral supernatants supplemented with 100 U ml^{-1} IL-2 and 5 μg ml^{-1} Polybrene, followed by centrifugation for 1 h at 3,200 r.p.m. Cells were cultured at 37 °C with 5% CO_2 for a further 24 h and sorted by Moflo (DakoCytomation) for ELISA analyses.

Received 12 December 2006; accepted 9 February 2007.
Published online 21 March 2007.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank M. Kakino, R. Ishii and M. Yoshida for technical assistance; F. Rawle for valuable comments on the manuscript; T. Kitamura for the pMCsIg retroviral vector; M. Onodera for the pGCSamIN retroviral vector; and F. Macian for the NFAT-CA construct.

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Foxp3 and Aire in thymus-generated T_{reg} cells: a link in self-tolerance

Takashi Nomura and Shimon Sakaguchi

The transcriptional regulators Foxp3 and Aire have key functions in self-tolerance. New studies emphasize potential links between Aire-expressing thymic stromal cells and the development of Foxp3-expressing regulatory T cells.

The thymus accomplishes two tasks essential for the maintenance of immunological self-tolerance: negative selection (for example, clonal deletion) of potentially hazardous self-reactive T cells, and the production of $CD25^+CD4^+$ regulatory T cells (T_{reg} cells), which act in the periphery to control self-reactive T cells that escaped thymic negative selection. The transcription factors Aire (autoimmune regulator) and Foxp3 (forkhead box p3) have key functions in clonal deletion and T_{reg} cell selection, respectively, and have been the focus of research aiming to decipher the molecular basis of self-tolerance. Three recent reports, including two in this issue of *Nature Immunology*, emphasize potential new conceptual links between Aire expression, Foxp3 upregulation and T_{reg} cell selection[1–3].

Aire is expressed in thymic medullary epithelial cells (mTECs) and to a lesser degree in dendritic cells (DCs). Mutations in *AIRE* in humans result in the autoimmune disease 'APECED' (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy) or 'APS-1' (polyglandular syndrome type 1). Evidence suggests that Aire deficiency affects the thymic negative selection of self-reactive T cells. Foxp3 controls the development or function or both of $CD25^+CD4^+$ 'natural' T_{reg} cells. Genetic anomalies in *FOXP3* result in deficiency in or dysfunction of T_{reg} cells and the occurrence of the severe autoimmune disorder 'IPEX syndrome' (immune dysregulation, polyendocrinopathy, X-linked) in humans. Compared with the thymic selection of conventional T cells, the selection of T_{reg} cells requires agonistic interactions of higher avidity between T cell receptors (TCRs) on developing thymocytes and self peptide–major histocompatibility complex (MHC) on thymic stromal cells; however, the avidity must not be so high as to lead to deletion.

Those observations, considered together, raise key questions regarding the functions of Foxp3 in thymocytes and Aire in thymic stromal cells during the production of T_{reg} cells. Is Foxp3 expression 'turned on' in developing thymocytes after their interaction with thymic stromal cells? If so, does Foxp3 upregulation influence the commitment of thymocytes to the T_{reg} cell lineage? Do thymic stromal cells, particularly Aire-expressing mTECs, in addition to promoting negative selection of self-reactive T cells, also elicit Foxp3 upregulation and T_{reg} cell production?

Lin *et al.*[1] and Gavin *et al.*[2] have attempted to determine whether Foxp3 is necessary and sufficient for commitment to the T_{reg} cell lineage in the thymus. They made mice with a *Foxp3* allele directing expression of enhanced green fluorescent protein (EGFP) without Foxp3 production or encoding a fusion protein of EGFP and nonfunctional Foxp3[1,2]. As *Foxp3* is located on the X chromosome, male mice expressing the altered *Foxp3* alleles demonstrated T_{reg} cell deficiency and succumbed to severe autoimmunity and inflammation; in contrast, heterozygous female mice were completely normal as a result of random X chromosome inactivation, which ensures expression of wild-type *Foxp3* alleles in approximately half of the T_{reg} cell (or T_{reg} cell precursor) population.

Notably, male and female mice with the altered *Foxp3* alleles had a sizable population $EGFP^+$ cells that resembled true T_{reg} cells in terms of surface phenotype (expression of CD25, CTLA-4 and GITR), dependence on paracrine interleukin 2 (IL-2) for survival, and expression of a T_{reg} cell–like 'genetic signature', as shown by DNA microarray analysis. These $EGFP^+$ cells, however, failed to suppress conventional T cell–driven immune responses *in vivo* and *in vitro*. The $EGFP^+$ cohort was produced in the thymus but was not substantially induced from peripheral naive T cells.

These data indicate that thymocytes can differentiate into T_{reg} cell–like precursors even in the absence of Foxp3 and that once thymocytes become committed to the T_{reg} cell lineage, Foxp3 expression is 'turned on', resulting in stabilization and maintenance of the T_{reg} cell phenotype and T_{reg} cell survival. However, the data show that Foxp3 is required for induction of the suppressive property of T_{reg} cells. This observation is in agreement with the published finding that new expression of Foxp3 can convert mature naive conventional T cells into T_{reg} cell–like cells with full suppressive function that can inhibit autoimmunity and immunopathology *in vivo*[4–6].

The finding that T_{reg} cell lineage commitment occurs independently of Foxp3 suggests that interaction between developing thymocytes and thymic stromal cells may somehow drive a population of thymocytes into the T_{reg} cell lineage and then trigger *Foxp3* expression. It has been shown that costimulatory or accessory molecules expressed on stromal cells and thymocytes influence the development of T_{reg} cells in the thymus (**Fig. 1**). For example, deficiency in CD28, CD40, LFA-1 or B7 results in a substantial reduction in T_{reg} cell numbers in the thymus[7]. Given that $Foxp3^+$ thymocytes are detectable in the 'late' $CD4^+CD8^+$ population, how and where thymocyte–stromal cell interactions permitting costimulatory and

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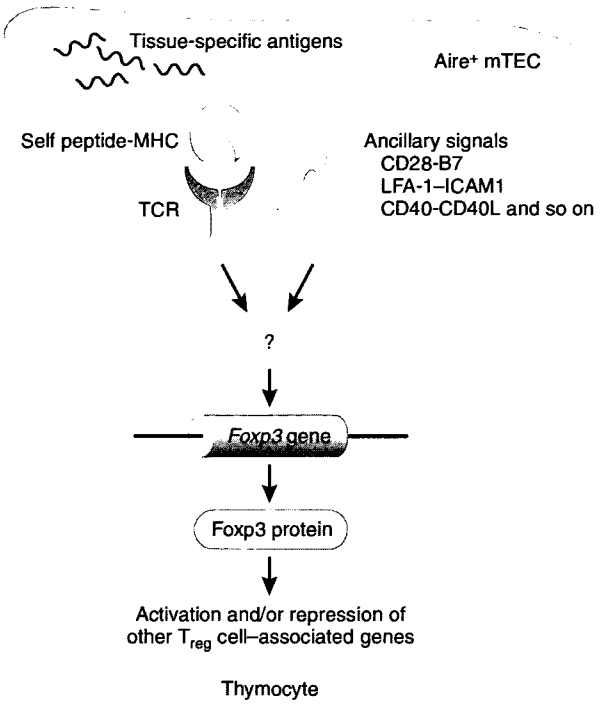

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Figure 1 Interactions between developing thymocytes and thymic stromal cells, in particular Aire-expressing mTECs, may drive thymocytes into the T_{reg} cell lineage. Signaling via the TCR and accessory molecules, which interact with their ligands expressed by stromal cells, somehow activates a T_{reg} cell genetic program (not understood at present), thereby driving thymocytes into the T_{reg} cell lineage and subsequently activating expression of Foxp3, which subsequently controls the expression of many other genes needed to confer and stabilize the phenotype and suppressive activity of T_{reg} cells.

TCR–self peptide–MHC binding occur needs to be determined.

Aire triggers the ectopic expression of genes encoding a set of tissue-specific antigens (TSAs), such as insulin, in a small population of mTECs[8]. Such 'promiscuous' expression of peripheral TSAs in the thymic medulla is believed to induce effective negative selection of TSA-specific self-reactive T cells. Aire-deficient mice indeed develop an autoimmune disease that affects a spectrum of organs similar to that affected in the autoimmune disorder in wild-type mice depleted of T_{reg} cells; however, Aire-deficient mice do not have an obvious abnormality in the number or the general suppressive function of $Foxp3^+$ T_{reg} cells[8].

Aschenbrenner *et al.* have asked whether Aire-expressing mTECs are involved in the generation of TSA-specific $Foxp3^+$ T_{reg} cells[3]. They made transgenic mice that express hemagglutinin in mTECs and DCs under faithful control of the *Aire* promoter and crossed those transgenic mice with mice expressing a transgenic TCR that recognizes a hemagglutinin peptide. The resultant 'double-transgenic' mice developed a large number of $Foxp3^+CD25^+CD4^+CD8^-$ transgenic thymocytes with hemagglutinin-specific suppressive activity *in vitro*.

In a series of elegant chimera experiments, the authors show that antigen presentation on mTECs is sufficient for the generation of hemagglutinin-specific T_{reg} cells and that antigen presentation on DCs (which could acquire and cross-present mTEC-derived antigen) is dispensable for hemagglutinin-specific T_{reg} cell differentiation. In addition, the authors show that hemagglutinin-expressing $Aire^+$ mTECs, which have abundant expression of MHC class II and B7 molecules, activate hemagglutinin-specific T cells *in vitro* more efficiently than thymic DCs do. Their results collectively indicate that $Aire^+$ mTECs expressing TSAs induce or facilitate the development of TSA-specific T_{reg} cells in the thymus and suggest that a paucity of TSA-specific T_{reg} cells may contribute to the occurrence of autoimmune disease in Aire-deficient mice and human patients with APECED (**Fig. 1**). It now seems that Aire is involved in both clonal deletion and T_{reg} cell differentiation.

That and other studies indicate that both thymic mTECs and DCs are involved in negative selection[3,9,10] and the study by Aschenbrenner *et al.*[3] in particular does not exclude the possibility that these two thymic stromal cell populations may cooperate during T_{reg} cell production in the thymus. For example, thymic stromal lymphopoietin secreted by Hassal's corpuscles, structures derived from medullary epithelial cells, can stimulate thymic DCs to promote the differentiation of thymocytes to T_{reg} cells *in vitro*[10]. How thymic DCs and mTECs manage to elicit negative selection as well as T_{reg} cell production remains to be determined.

Thymic DCs that cross-present mTEC-derived self antigens negatively select both $CD4^+$ and $CD8^+$ T cells, whereas mTECs efficiently delete $CD8^+$ but not $CD4^+$ T cells[9]. Immature DCs are apparently capable of generating T_{reg} cells from naive T cells, whereas mature DCs can efficiently cause the proliferation of antigen-specific mature T_{reg} cells. Given the contribution of $Aire^+$ mTECs to the generation of T_{reg} cells in the thymus, these functional differences of mTECs and DCs could be generally attributed to the efficiency of antigen presentation, the expression of particular costimulatory molecules and/or the secretion of specific cytokines (such as transforming growth factor-β). Further comparative analysis at the molecular level of $Aire^+$ mTECs versus other mTECs, and DCs treated with thymic stromal lymphopoietin or left untreated, may shed light on these issues.

Monogenic autoimmune diseases such as IPEX and APECED provide the empirical basis from which the plausibility of hypotheses concerning the etiology of T cell–mediated organ-specific autoimmune disease can be judged. Studies of the genes underlying IPEX and APECED (*Foxp3* and *Aire*, respectively) and the functions of the proteins they encode, have advanced understanding of the dominant (regulatory) and recessive (deletional) mechanisms of self-tolerance. Further studies are needed to show to what extent the two mechanisms operate in coordinated and complementary way to maintain stable self-tolerance.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

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T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17$^+$ Th cells that cause autoimmune arthritis

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This report shows that highly self-reactive T cells produced in mice as a result of genetically altered thymic T cell selection spontaneously differentiate into interleukin (IL)-17–secreting CD4$^+$ helper T (Th) cells (Th17 cells), which mediate an autoimmune arthritis that clinically and immunologically resembles rheumatoid arthritis (RA). The thymus-produced self-reactive T cells, which become activated in the periphery via recognition of major histocompatibility complex/self-peptide complexes, stimulate antigen-presenting cells (APCs) to secrete IL-6. APC-derived IL-6, together with T cell–derived IL-6, drives naive self-reactive T cells to differentiate into arthritogenic Th17 cells. Deficiency of either IL-17 or IL-6 completely inhibits arthritis development, whereas interferon (IFN)-γ deficiency exacerbates it. The generation, differentiation, and persistence of arthritogenic Th17 cells per se are, however, insufficient for producing overt autoimmune arthritis. Yet overt disease is precipitated by further expansion and activation of autoimmune Th17 cells, for example, via IFN-γ deficiency, homeostatic proliferation, or stimulation of innate immunity by microbial products. Thus, a genetically determined T cell self-reactivity forms a cytokine milieu that facilitates preferential differentiation of self-reactive T cells into Th17 cells. Extrinsic or intrinsic stimuli further expand these cells, thereby triggering autoimmune disease. Intervention in these events at cellular and molecular levels is useful to treat and prevent autoimmune disease, in particular RA.

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A key question for understanding the mechanism of autoimmune disease is how hazardous self-reactive T cells are produced by the thymus, become activated in the periphery, and differentiate to effector T cells that destroy the target organ, or how genetic and environmental factors contribute to this process. Autoimmune disease due to a defect of a single gene is instrumental in addressing these questions, especially when the disease is clinically and immunologically similar to common autoimmune diseases that are supposed to be multifactorial (1).

The SKG strain of mice, a mutant on the BALB/c background, spontaneously develops T cell–mediated autoimmune arthritis, which clinically and immunologically resembles rheumatoid arthritis (RA) in humans (2, 3). The strain harbors a recessive mutation of the gene encoding an SH2 domain of ζ-associated protein 70 (ZAP-70), a key signaling molecule in T cells (4). Impaired signal transduction through SKG ZAP-70 results in thymic positive selection and failure in negative selection of highly self-reactive T cells that include potentially arthritogenic CD4$^+$ T cells (2). SKG mice spontaneously develop severe arthritis in a conventional environment, whereas they fail to develop the disease in microbially clean environments, for example, under a specific pathogen-free (SPF) condition (5). Yet arthritis can be elicited in an SPF environment through antigen-nonspecific activation of innate immunity, for example, by injection of zymosan, a crude fungal extract containing β-glucans, or purified β-glucans such as laminarin (5). The disease can also be triggered by provoking

The online version of this article contains supplemental material.

JEM © The Rockefeller University Press $15.00
Vol. 204, No. 1, January 22, 2007 41–47 www.jem.org/cgi/doi/10.1084/jem.20062259

Supplemental Material can be found at:
http://www.jem.org/cgi/content/full/jem.20062259/DC1

homeostatic proliferation of self-reactive T cells (5). The strain is therefore a suitable model for elucidating how self-reactive T cells develop and differentiate to arthritogenic effector T cells, and how autoimmune arthritis can be triggered by environmental insults in the presence of genetic predisposition.

In this report, we show that autoimmune arthritis in SKG mice is highly dependent on the development of $CD4^+$ T cells secreting IL-17, a proinflammatory cytokine capable of recruiting and activating neutrophils and other inflammatory cells (6). We have analyzed how self-reactive $CD4^+$ T cells produced by the thymus differentiate to arthritogenic Th17 cells through internally forming a particular cytokine milieu by interacting with APCs, and how they become activated to cause autoimmune arthritis.

RESULTS AND DISCUSSION

Spontaneous development of arthritogenic Th17 cells in SKG mice and its augmentation by zymosan or β-glucan administration

In vitro PMA/ionomycin treatment for 5 h, which activates a signal transduction step down-stream of ZAP-70 and therefore equally activates SKG and BALB/c T cells, revealed that a significant fraction of LN $CD4^+$ T cells from nonarthritic SKG mice in an SPF environment were producing IL-17A (hereafter IL-17), whereas SKG or BALB/c CD4-SP thymocytes or BALB/c $CD4^+$ T cells were not (Fig. 1 A and Fig. S1, which is available at http://www.jem.org/cgi/content/full/jem.20062259/DC1). Such IL-17–producing SKG $CD4^+$ T cells also produced at a single cell level TNF-α and IL-2, but not IFN-γ, IL-4, or IL-10, a profile distinct from Th1 or Th2 cells and similar to that of Th17 cells (Fig. 1 A; references 7–9). $CD4^+$ T cells freshly prepared from nonarthritic SKG mice also actively transcribed IL-17 and IL-23R mRNA (Fig. 1 B). In arthritic SKG mice raised in a conventional environment, arthritic joints actively transcribed IL-17 mRNA, whereas nonarthritic ones did not (Fig. 1 C). Correspondingly, $CD4^+$ T cells producing IL-17 and not IFN-γ infiltrated into the arthritic joints as revealed by intracellular cytokine staining of $CD4^+$ T cells dispersed from the inflamed synovial tissue (Fig. 1 D; reference 5). Both IL-17–intact ($IL\text{-}17^{+/+}$) and –deficient ($IL\text{-}17^{-/-}$) SKG mice, prepared by genetic backcrossing from $IL\text{-}17^{-/-}$ BALB/c mice (10), did not develop arthritis under our SPF conditions, although the former harbored IL-17–producing $CD4^+$ T cells (Fig. 1 A). When $CD4^+$ T cell suspensions prepared from each strain were transferred to $RAG2^{-/-}$ BALB/c mice, however, all the recipients of $IL\text{-}17^{+/+}$ $CD4^+$ T cells developed arthritis with high arthritis scores within 3 mo, whereas none of those transferred with $IL\text{-}17^{-/-}$ $CD4^+$ T cells showed joint swelling (Fig. 1 E). The former exhibited histologically severe synovitis and destruction of cartilage and bone (Fig. 1 F). Furthermore, injection of zymosan or laminarin, which can trigger arthritis in SPF SKG mice (5), increased three- to fourfold the number of $IL\text{-}17^+$ cells in SKG, but not in BALB/c mice (Fig. 1 G). Thus, naive $CD4^+$ T cells in SKG mice are spontaneously activated and differentiate to Th17

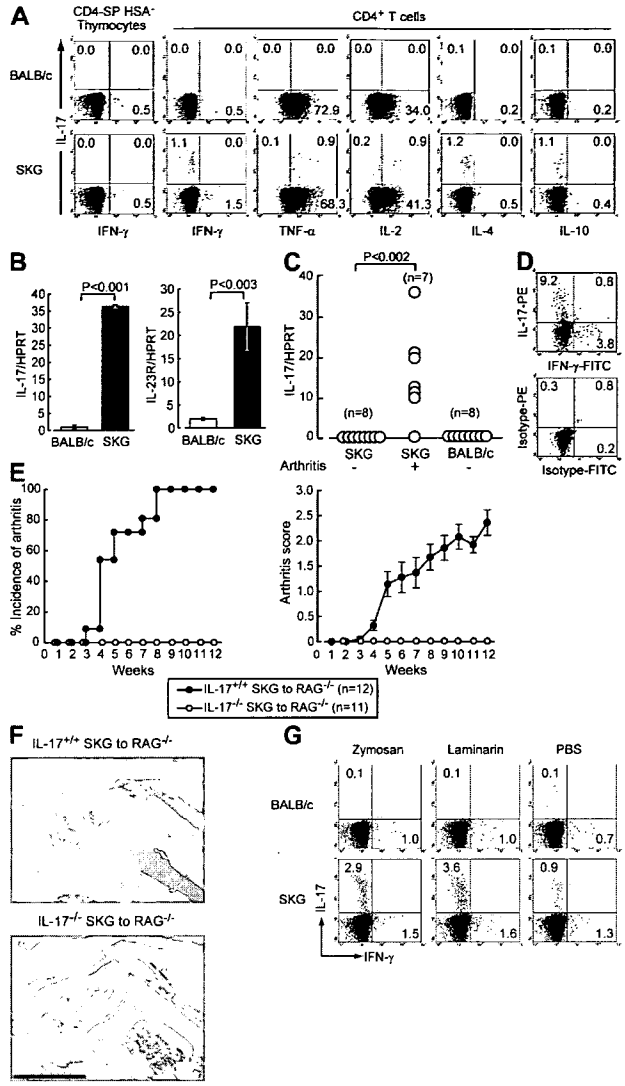

Figure 1. Spontaneous development of arthritogenic Th17 cells in SKG mice. (A) HSA^- CD4-SP thymocytes or LN $CD4^+$ T cells were stained for intracellular cytokines. (B) Quantitative RT-PCR for IL-17 and IL-23R mRNA in $CD4^+$ T cells. Data are shown as the mean ± SD of three independent experiments. (C) Total RNA extracted from the ankle joints of individual mice with or without arthritis was subjected to quantitative RT-PCR for IL-17 mRNA. (D) $CD4^+$ T cells infiltrating arthritic joints were stained as in A. (E) 10^6 $CD4^-$ T cells from $IL\text{-}17^{+/+}$ or $IL\text{-}17^{-/-}$ SKG mice were transferred to $RAG2^{-/-}$ mice. Incidence and severity of arthritis were scored every week as described previously (reference 2). Vertical bars represent the means ± SEM. (F) Histology of an ankle joint of a $RAG2^{-/-}$ mouse transferred with $IL\text{-}17^{-/-}$ or $IL\text{-}17^{+/+}$ SKG $CD4^+$ T cells (bar, 1 mm; hematoxylin and eosin staining). (G) Mice received a single i.p. injection of 2 mg zymosan or 30 mg laminarin. LN $CD4^+$ T cells were stained for intracellular IL-17 and IFN-γ 2 wk later. Results in A, D, and G represent three to five independent experiments.

cells, which are indispensable for the development of this autoimmune disease. Such potentially arthritogenic Th17 cells appear to persist in the periphery and begin mediating arthritis

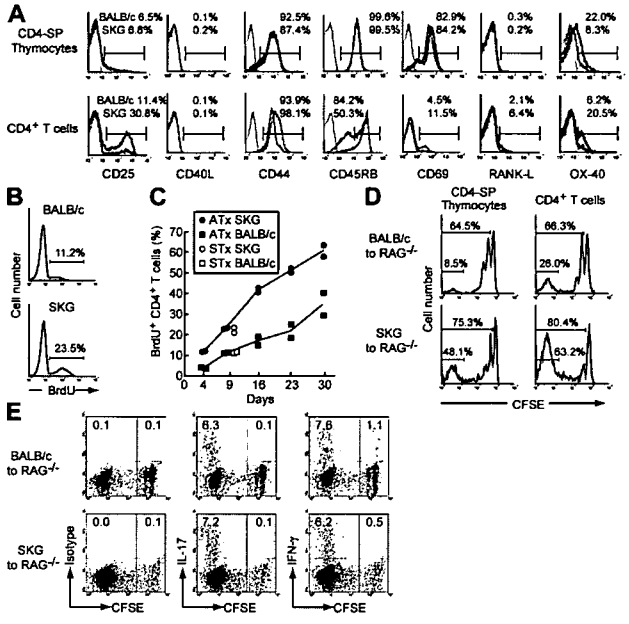

Figure 2. T cell self-reactivity and in vivo spontaneous differentiation of Th17 cells. (A) CD4-SP thymocytes and LN CD4+ T cells from 6-wk-old BALB/c or SKG mice were stained with designated mAbs. (B) Mice were given BrdU for 9 d, and LN CD4+ T cells were stained with anti-BrdU. (C) Mice that had been thymectomized at 4 wk of age were administered with BrdU for the indicated days from 6 wk of age, and percentages of BrdU-stained cells among CD4+ T cells are shown. ATx, adult thymectomy; STx, sham thymectomy. (D and E) HSA− CD4-SP thymocytes or CD4+ T cells (3×10^6) were labeled by CFSE and transferred to RAG2$^{-/-}$ mice. 5 d later, recipient splenic CD4+ T cells were assessed for CFSE profile and intracellular IL-17 and IFN-γ. Results in A, B, D, and E represent three to five independent experiments.

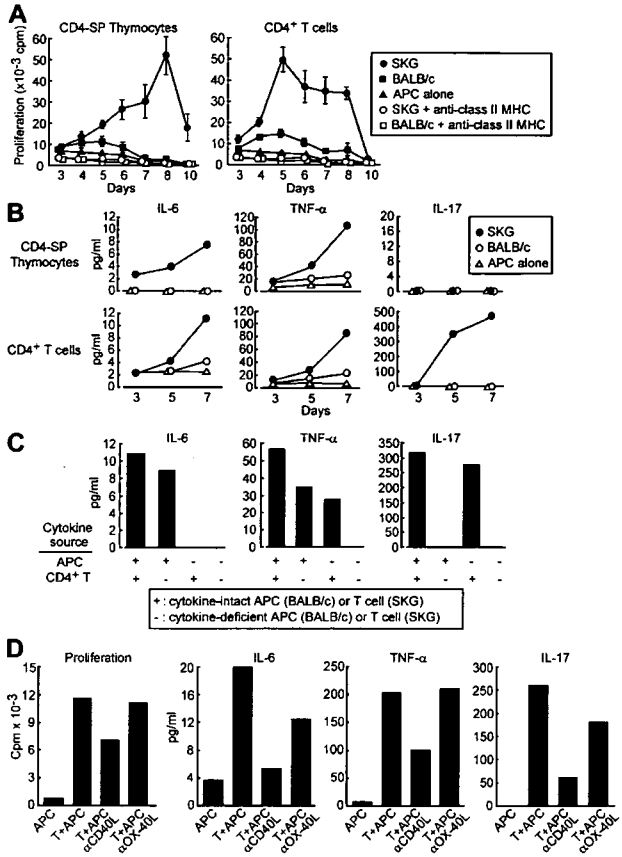

Figure 3. T cell self-reactivity and in vitro cytokine production. (A) HSA− CD4-SP thymocytes or CD4− T cells were cultured with autologous APCs in the presence or absence of anti-class II MHC blocking mAb. Proliferation was measured by [3H]thymidine incorporation. Vertical bars signify SD. (B) Culture supernatants in the AMLR shown in A were collected and assessed for IL-6, TNF-α, and IL-17 production. (C) CD4+ T cells from IL-6$^{-/-}$, TNF-α$^{-/-}$, IL-17$^{-/-}$, or cytokine-intact SKG mice were cultured with cytokine-deficient or -intact APCs, and culture supernatants were collected on day 7 for cytokine assessment as in B. (D) Anti-CD40L or anti-OX40L blocking mAb (100 μg/ml) was added to the culture, and proliferation or cytokine production was assessed as shown in A and B. Results in A–D represent three independent experiments.

when stimulated, for example, by their transfer to a T cell–deficient environment and resulting homeostatic proliferation (see also below), or by exposure to microbial products, such as fungal or bacterial β-glucans, which further facilitate expansion/differentiation of Th17 cells, presumably by stimulating APCs (5). In addition, complete inhibition of disease development by the deficiency of IL-17 alone indicates that IL-17F, another IL-17 family member secreted by CD4+ T cells and having a similar function (6), is dispensable for the disease.

In vivo differentiation of self-reactive T cells to Th17 cells in SKG mice

SKG mice harbored phenotypically activated CD4+ T cells whether they had developed arthritis or not, whereas SKG CD4+CD8− (CD4–single-positive [SP]) thymocytes, CD8-SP thymocytes, and CD8+ T cells were of a naive surface phenotype and similar to their BALB/c counterparts (Fig. 2 A and not depicted). Regardless of hyporesponsiveness to TCR stimulation because of the ZAP-70 anomaly (2), SKG CD4+ T cells were twice as proliferative as BALB/c CD4+ T cells in the physiological state, as shown with in vivo BrdU incorporation (Fig. 2 B). Divided cells constituted 50% of SKG CD4+ T cells within 3 wk compared with 20% of BALB/c CD4+ T cells (Fig. 2 C). Thymectomy in adults did not affect the proliferation, indicating that the proliferating T cells are not recent thymic emigrants, but peripheral T cells (Fig. 2 C). When heat-stable antigen–negative (HSA−) CD4-SP mature thymocytes or splenic CD4+ T cells labeled by CFSE were transferred to RAG2$^{-/-}$ mice, transferred SKG CD4+ T cells or thymocytes gave rise to higher percentages of CFSE-diluted (i.e., proliferating) cells, in particular highly proliferating CFSE^{low} cells, than their BALB/c counterparts (Fig. 2 D). Notably, BALB/c CD4+ T cells, which scarcely produced IL-17 before transfer (Fig. 1 A), also differentiated spontaneously to Th17 cells to a similar extent as SKG CD4+

T cells (Fig. 2 E). The differentiation required cell division: BALB/c $CD4^+$ T cells produced IL-17 or IFN-γ only after several cell divisions.

Collectively, these results indicate that SKG thymus produces highly self-reactive T cells, which are constantly activated in the periphery, proliferate, and differentiate to Th17 cells (Fig. 1 A). Both SKG and BALB/c T cells can equally differentiate to Th17 as well as Th1 cells after homeostatic proliferation; however, BALB/c T cells fail to produce arthritis in this setting, presumably because of their lack or insufficiency of relevant arthritogenic self-reactivity.

In vitro self-reactivity of SKG $CD4^+$ T cells and their stimulation of APCs to secrete cytokines

Supporting the in vivo high proliferative activity of SKG T cells, SKG CD4-SP mature thymocytes and $CD4^+$ T cells exhibited vigorous in vitro proliferative responses to autologous APCs, and the responses were completely inhibited by adding anti–class II MHC mAb to the culture (Fig. 3 A). They produced large amounts of IL-6 and TNF-α in this autologous MLR (AMLR), whereas only peripheral $CD4^+$ T cells produced a detectable amount of IL-17 (Fig. 3 B). Use of cytokine-deficient BALB/c APCs or SKG $CD4^+$ T cells in various combinations revealed that IL-6 was predominantly derived from APCs, TNF-α from both SKG $CD4^+$ T cells and BALB/c APCs, and IL-17 solely from SKG $CD4^+$ T cells (Fig. 3 C). Moreover, blockade of CD40L substantially reduced cell proliferation and production of IL-6, TNF-α, and IL-17. OX40L blockade exerted similar effects, although to lesser extents (Fig. 3 D). Collectively, SKG $CD4^+$ thymocytes and T cells strongly respond to class II MHC/self-peptide complexes expressed by autologous APCs, and reciprocally stimulate APCs to secrete IL-6 and TNF-α. In addition, CD40–CD40L and to a lesser extent OX40–OX40L interactions contribute to this T cell–APC interaction and, consequently, to the formation of IL-17 by T cells, IL-6 by APCs, and TNF-α by both.

Critical role of IL-6 for the development of arthritogenic Th17 cells in SKG mice

We then examined with cytokine-deficient SKG mice how a cytokine milieu affects in vivo spontaneous development of Th17 cells in SKG mice. IL-6–deficient SKG mice were completely devoid of $IL\text{-}17^+$ $CD4^+$ T cells, whereas TNF-α–, IL-1–, or IFN-γ–deficient SKG mice harbored equivalent numbers of $IL\text{-}17^+$ $CD4^+$ T cells as cytokine-intact SKG mice (Fig. 4 A). When $IL\text{-}6^{-/-}$ SKG or BALB/c $CD4^+$ T cells devoid of Th17 cells were transferred to $IL\text{-}6^{+/+}$ $RAG2^{-/-}$ mice, they gave rise to Th17 cells within a week after homeostatic proliferation (Fig. 4 B). This in vivo Th17 differentiation did not happen in the transfer of SKG or BALB/c $IL\text{-}6^{-/-}$ T cells to $IL\text{-}6^{-/-}$ $RAG2^{-/-}$ mice and occurred to a small degree when either the T cell donors or the recipients were IL-6 deficient. Of note in these cell transfers is that the degree of Th17 development from SKG $CD4^+$ T cells was well correlated with the incidence and severity of arthritides in the recipients (Fig. 4 C).

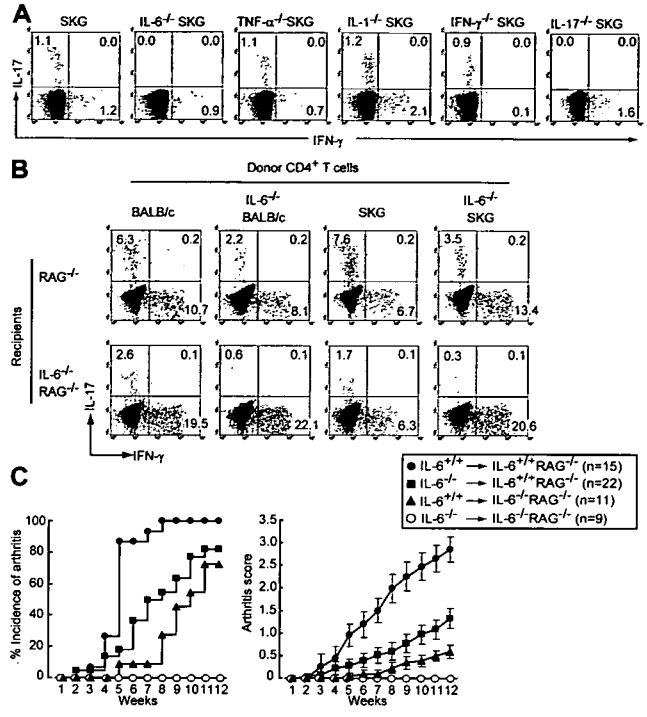

Figure 4. The role of IL-6 for the development of arthritogenic Th17 cells in SKG mice. (A) LN $CD4^+$ T cells from cytokine-deficient SKG mice were stained for intracellular IL-17 and IFN-γ. (B) 2×10^6 $CD4^+$ T cells from the indicated donor mice were transferred to $IL\text{-}6^{-/-}$ or intact $RAG2^{-/-}$ mice. Intracellular IL-17 and IFN-γ in recipient splenic $CD4^+$ T cells were stained on day 7. (C) 10^6 $CD4^+$ T cells from $IL\text{-}6^{+/+}$ or $IL\text{-}6^{-/-}$ SKG mice were transferred to $IL\text{-}6^{+/+}$ or $IL\text{-}6^{-/-}$ $RAG2^{-/-}$ mice. Incidence and severity of arthritis in four groups of mice were assessed every week. Vertical bars represent the means ± SEM of scores. In comparison of four groups (●, $IL\text{-}6^{+/+} \rightarrow IL\text{-}6^{+/+}RAG^{-/-}$; ■, $IL\text{-}6^{-/-} \rightarrow IL\text{-}6^{+/+}RAG^{-/-}$; ▲, $IL\text{-}6^{+/+} \rightarrow IL\text{-}6^{-/-}RAG^{-/-}$; ○, $IL\text{-}6^{-/-} \rightarrow IL\text{-}6^{-/-}RAG^{-/-}$), statistically significant ($P < 0.05$) differences in scores are: ● versus ■, 5–12 wk; ● versus ▲, 5–12 wk; ● versus ○, 5–12 wk; ■ versus ○, 9–12 wk; ▲ versus ○, 9–12 wk; ■ versus ▲, at 12 wk. Results in A and B represent three independent experiments.

Collectively, IL-6 produced by either T cells or non–T cells is indispensable for in vivo development and/or expansion of Th17 cells and consequently the occurrence of autoimmune arthritis. IL-6 produced by either cell source is synergistic in promoting this T cell differentiation and autoimmune development. Although IL-23 is capable of amplifying and sustaining Th17 cells (11), it is unable to replace the function of IL-6 to induce Th17 cells. In addition, not only SKG $CD4^+$ T cells but also $CD4^+$ T cells in normal BALB/c mice are similarly dependent on IL-6 in this setting of Th17 differentiation.

Spontaneous development of arthritis in IFN-γ–deficient SKG mice due to enhanced Th17 differentiation

Notably, IFN-γ–deficient SKG mice spontaneously developed histologically severe arthritides even under SPF conditions (Fig. 5, A and B). After homeostatic proliferation in

$RAG2^{-/-}$ mice, $CD4^+$ T cells from IFN-$\gamma^{-/-}$ SKG mice differentiated more efficiently to Th17 cells than IFN-$\gamma^{+/+}$ SKG $CD4^+$ T cells, suggesting that IFN-γ may suppress the differentiation/expansion of Th17 cells (Fig. 5 C). To examine the relationship between IL-6 and IFN-γ in this Th17 differentiation, we blocked IL-6R by administering anti–IL-6R mAb to $RAG2^{-/-}$ mice transferred with $CD4^+$ T cells from wild-type, IFN-$\gamma^{-/-}$, or IL-$17^{-/-}$ mice (Fig. 5 C). The blockade inhibited the differentiation/expansion of both normal and IFN-$\gamma^{-/-}$ $CD4^+$ T cells to Th17 cells, indicating that IL-6 can directly promote Th17 differentiation, and not via the reduction of IFN-γ. In addition, IL-$17^{-/-}$ $CD4^+$ T cells more efficiently differentiated/expanded to IFN-γ–producing cells than wild-type $CD4^+$ T cells, and IL-6R blockade facilitated this differentiation/expansion of both wild-type and IL-$17^{-/-}$ $CD4^+$ T cells.

Thus, these findings, together with efficient development of IFN-γ–producing cells under IL-6 deficiency (Fig. 4 B) and the known capacity of IL-6 to directly inhibit Th1 cell differentiation (12), indicate that IL-6 and IL-17 suppress Th1 differentiation and IFN-γ production, and, reciprocally, IFN-γ suppresses Th17 differentiation. This in vivo cross-regulation between IL-17/IL-6 and IFN-γ plays a critical role in the maintenance of immunological self-tolerance, as IFN-γ deficiency can break self-tolerance in SPF SKG mice by facilitating the differentiation/expansion of arthritogenic Th17 cells.

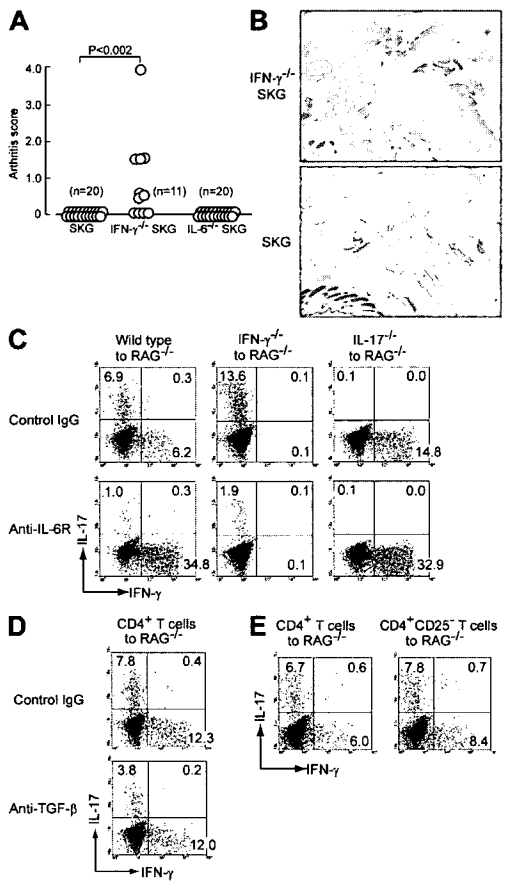

Figure 5. Spontaneous development of autoimmune arthritis in IFN-γ–deficient SKG mice and IL-6–dependent cross-regulation between Th17 and Th1 cells. (A) Arthritis score in 6-mo-old cytokine-deficient SKG mice under SPF conditions. (B) Histology of an ankle joint of a 6-mo-old SKG or IFN-$\gamma^{-/-}$ SKG mouse in A. (C) $CD4^+$ T cells from wild-type, IFN-$\gamma^{-/-}$, or IL-$17^{-/-}$ mice were transferred to $RAG2^{-/-}$ mice, which were i.v. injected with 1 mg anti-IL-6R mAb or control rat IgG twice (on the same day and day 3). Intracellular IL-17 and IFN-γ in recipient splenic $CD4^+$ T cells were stained on day 7. (D) $CD4^+$ T cells from BALB/c mice were transferred to $RAG2^{-/-}$ mice, which were i.v. treated with 1 mg anti-TGF-β and assessed as in C. (E) BALB/c $CD4^+$ T cells nondepleted or depleted of $CD4^+CD25^+$ T cells were transferred to $RAG2^{-/-}$ mice and assessed as in C. Results in C–E represent three independent experiments.

In vivo contribution of TGF-β and natural regulatory T (T reg) cells to the development of Th17 cells

There is recent in vitro evidence that IL-6 and TGF-β together promote the differentiation of naive $CD4^+$ T cells to Th17 cells and IFN-γ inhibits it (8, 9, 13–15). In our in vivo induction of Th17 cells from BALB/c or SKG $CD4^+$ T cells via homeostatic proliferation, i.v. administration of neutralizing anti–TGF-β mAb at in vivo–saturating doses reduced the number of IL-17^+ cells to a half of control mice without reduction of IFN-γ^+ cells (Fig. 5 D and Fig. S2, which is available at http://www.jem.org/cgi/content/full/jem.20062259/DC1). $CD25^+CD4^+$ natural T reg cells were suggested as a possible source of TGF-β (13). Th17 cells, however, equally developed from $CD25^+$ cell-depleted or nondepleted BALB/c T cells after homeostatic proliferation (Fig. 5 E; reference 16). Furthermore, T reg cell depletion exacerbated SKG arthritis, whereas inoculation of natural T reg cells from normal BALB/c mice suppressed disease development (unpublished data). Thus, TGF-β physiologically produced by various tissues may promote in vivo Th17 differentiation in the presence of IL-6. How natural T reg cells are involved in this process remains to be determined.

The SKG thymus produces self-reactive T cells with a variety of antigen specificities as illustrated by polyclonal activation of self-reactive thymocytes and T cells in AMLR. Some self-reactive T cells may recognize joint self-antigens as indicated by their helper function for the development of IgG autoantibodies against type II collagen and other constituents of the joint (2). Others may stimulate APCs to secrete cytokines, especially IL-6, and, together with T cell–derived IL-6, form a cytokine milieu for the preferential differentiation of joint-specific self-reactive T cells to Th17 cells. Other cytokines, including IFN-γ, TGF-β, TNF-α, IL-1, and IL-23, may also positively or negatively contribute to forming the cytokine milieu for Th17 development (3, 13, 17, 18). With this generation and persistence of potentially arthritogenic autoimmune Th17 cells in apparently nonarthritic animals, various extrinsic or intrinsic stimuli (e.g., exposure to physical, chemical, or biological agents that activate APCs,

cause T lymphocytopenia, or alter cytokine milieu) may precipitate arthritis by further facilitating expansion/differentiation of arthritogenic Th17 cells.

The etiology of RA is largely obscure at present (19). Yet a genetically determined T cell anomaly might play a role in its pathogenesis in some RA patients, as suggested by recent findings that genetic polymorphism of a signaling molecule at a TCR proximal step significantly contributes to the susceptibility to RA (20, 21). The polymorphism might contribute to thymic generation of potentially arthritogenic self-reactive T cells and their differentiation to arthritogenic Th17 cells, as shown here with a mouse model of RA.

MATERIALS AND METHODS

Mice. BALB/c and BALB/c IFN-$\gamma^{-/-}$ mice were purchased from Japan Clea and The Jackson Laboratory, respectively. BALB/c IL-$17^{-/-}$ mice were described previously (10). IL-$1^{-/-}$, IL-$6^{-/-}$, or TNF-$\alpha^{-/-}$ mice were backcrossed to BALB/c more than eight times and crossed to SKG mice to make cytokine-deficient SKG mice (3). RAG$2^{-/-}$ BALB/c mice were crossed to IL-$6^{-/-}$ mice to generate IL-$6^{-/-}$ RAG$2^{-/-}$ BALB/c mice. These mice were maintained in our animal facility and treated in accordance with the guidelines of Kyoto University.

Antibody. The following reagents were purchased from BD Biosciences: anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD16/CD32 (2.4G2), anti-CD25 (PC61), anti-CD40L (MR1), anti-CD45RB (16A), anti-BrdU (3D4), anti-TCR-αβ (H57-597), anti–IL-4 (11B11), anti–IL-10 (JESS-16E3), anti–IFN-γ (XMG1.2), anti–TNF-α (MP6-XT22), anti–IL-17 (TC11-18H10.1), and isotype control IgG. The following reagents were purchased from eBioscience: anti-CD44 (IM7), anti-CD69 (H1.2F3), anti–RANK-L (1K22/5), anti-OX40 (OX-86), anti-OX40L (RM134L), and anti–IL-2 (JES6-5H4). Anti–class II MHC (CA4) and anti–TGF-β (1D11) were purified in our laboratory. Purified anti–IL-6R (MR16-1) was provided by N. Nishimoto (Osaka University, Osaka, Japan).

Intracellular cytokine staining. LN or spleen cells were stimulated with 20 ng/ml PMA and 1 μM ionomycin in the presence of GolgiStop (BD Biosciences) for 5 h, and then stained with anti-CD4 or anti–TCR-αβ and fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences), followed by anti–IL-17 and anti–IFN-γ, TNF-α, IL-2, IL-4, or IL-10 staining.

In vivo BrdU labeling. Mice were i.p. injected with 1.0 mg BrdU (Sigma-Aldrich) every 12 h twice and given 0.8 mg/ml BrdU in drinking water until cytofluorometric analysis.

Lymphocyte labeling with CFSE. HSA^- CD4-SP thymocytes or $CD4^+$ T cells were labeled with 3 μM CFSE (Dojindo).

AMLR. 2×10^4 HSA^- CD4-SP thymocytes or $CD4^+$ T cells were cultured with 10^5 BALB/c splenic APCs, which were prepared by depleting $Thy1.2^+$ cells by MACS (Miltenyi Biotec) in a 96-well round-bottom plate in complete RPMI medium. [^{3}H]thymidine (1 μCi/well; Du Pont/New England Nuclear) was added during the last 12 h of culture.

Measurement of cytokines. IL-6 and TNF-α were measured by Cytometric Bead Array (BD Biosciences), with the detection limits of 2 pg/ml for IL-6 and 7 pg/ml for TNF-α. IL-17 was measured by ELISA (R&D Systems), with the detection limit of 11 pg/ml.

Statistical analysis. Student's *t* test was used for statistical analyses. All p-values ≤0.05 were considered significant.

Online supplemental material. Fig. S1 shows IL-17 expression in BALB/c and SKG thymocytes assessed by RT-PCR and intracellular IL-17 staining. Fig. S2 shows percentages of $IL\text{-}17^+$ or $IFN\text{-}\gamma^+$ cells in individual $RAG^{-/-}$ mice transferred with $CD4^+$ T cells and treated with anti–TGF-β mAb. Figs. S1 and S2 are available at http://www.jem.org/cgi/content/full/jem.20062259/DC1.

The authors thank Z. Fehervari for critically reading the manuscript and the members of our laboratories for valuable comments. We thank T. Matsushita for histology and N. Nishimoto for mAb.

This work was supported by grants-in-aid from the Ministry of Education, Sports and Culture, and the Japan Science and Technology Agency.

The authors have no conflicting financial interests.

Submitted: 23 October 2006
Accepted: 7 December 2006

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ARTHRITIS & RHEUMATISM
Vol. 56, No. 4, April 2007, pp 1295–1303
DOI 10.1002/art.22506

Clinical and Immunogenetic Features of Patients With Autoantibodies to Asparaginyl–Transfer RNA Synthetase

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Objective. **We have previously described anti-KS autoantibodies and provided evidence that they are directed against asparaginyl–transfer RNA (tRNA) synthetase (AsnRS). The aim of the present study was to identify patients with anti-AsnRS autoantibodies and elucidate the clinical significance of this sixth antisynthetase antibody. In particular, we studied whether it was associated with the syndrome of myositis (polymyositis or dermatomyositis [DM]), interstitial lung disease (ILD), arthritis, and other features that had been previously associated with the 5 other anti–aminoacyl–tRNA synthetase autoantibodies.**

Methods. **More than 2,500 sera from patients with connective tissue disease (including myositis and ILD) and controls were examined for anti-AsnRS autoantibodies by immunoprecipitation (IP). Positive and control sera were tested for the ability to inhibit AsnRS by preincubation of the enzyme source with the serum. The HLA class II (DRB1, DQA1, DQB1, DPB1) alleles were identified from restriction fragment length polymorphism of polymerase chain reaction–amplified genomic DNA.**

Results. **Anti-AsnRS antibodies were identified in the sera of 8 patients (5 Japanese, 1 American, 1 German, and 1 Korean) by IP of the same distinctive set of tRNA and protein that differed from those precipitated by the other 5 antisynthetases, and these antibodies showed specific inhibition of AsnRS activity. Two of these patients had DM, but 7 of 8 (88%) had ILD. Four patients (50%) had arthritis, and 1 had Raynaud's phenomenon. This antisynthetase was very rare among myositis patients (present in 0% of Japanese myositis patients), but it was found in 3% of Japanese ILD patients. Thus, most patients with anti-AsnRS had chronic ILD with or without features of connective tissue disease. Interestingly, all 4 Japanese patients tested had DR2 (DRB1*1501/1502), compared with 33% of healthy controls.**

Conclusion. **These results indicate that anti-AsnRS autoantibodies, like anti–alanyl–tRNA synthetase autoantibodies, have a stronger association with ILD than with myositis and may be associated with the DR2 phenotype.**

The aminoacyl–transfer RNA (aminoacyl-tRNA) synthetases are a family of cytoplasmic enzymes that catalyze the formation of aminoacyl-tRNA from a specific amino acid and its cognate tRNA and play a crucial role in protein synthesis. Autoantibodies to certain of these synthetases (histidyl–, threonyl–, alanyl–, isoleucyl–, and glycyl-tRNA synthetases) have been identified in patients with inflammatory myopathies (1–6). Among these "antisynthetase autoantibodies," the most common is anti–Jo-1 (anti–histidyl–tRNA synthetase [anti-HisRS]), found in 20% of patients with polymyositis/dermatomyositis (PM/DM) (7–11). Anti–PL-7 (anti–threonyl–tRNA synthetase [anti-ThrRS])

Supported in part by grants from the Japanese Ministry of Education, Science, Culture, Sports, and Technology, the Japanese Ministry of Health, Labor, and Welfare, and the Keio University School of Medicine. Dr. Targoff's work was supported in part by the US Department of Veterans Affairs.

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Submitted for publication September 25, 2005; accepted in revised form January 4, 2007.

and anti–PL-12 (anti–alanyl–tRNA synthetase [anti-AlaRS]) autoantibodies are less common, found in 3–4% of all patients with PM/DM (4,5,11–13), while anti-OJ (anti–isoleucyl–tRNA synthetase [anti-IleRS]) and anti-EJ (anti–glycyl–tRNA synthetase [anti-GlyRS]) autoantibodies are the least common, occurring in <2% (6,14,15), although the frequency may vary in different populations (16).

Characteristic clinical features have been found in patients with anti-HisRS and other antisynthetase autoantibodies (1,9,10). These features include myositis, interstitial lung disease (ILD), arthritis, Raynaud's phenomenon, fever with exacerbations, and the skin lesion of the fingers referred to as mechanic's hands, and they appear to form a distinct syndrome referred to as the "antisynthetase syndrome" (8–11). Although the similarity of the clinical features associated with different antisynthetases is impressive (17,18), certain differences have been noted, which must be considered preliminary due to the small reported number of patients with non-HisRS antisynthetases (1,9,19). Patients with anti-AlaRS appear to be more likely than those with anti-HisRS to have ILD and/or arthritis either without myositis or with little evidence of muscle disease. Absence of significant myositis over the full disease course in patients with anti-HisRS is rare (<5%), although it may occur. Clinically significant myositis was seen in 60% of US patients with anti-AlaRS (13), whereas none of 6 Japanese patients with anti-AlaRS autoantibodies fulfilled criteria for myositis (20). Among patients with anti-IleRS, 2 of 10 had ILD without evidence of myositis, and 1 had ILD with subclinical myositis (14). In addition, antisynthetases may occur in either PM or DM, but PM is usually more common with anti-HisRS (10,16,21), and DM is usually more common with other antisynthetases, especially anti-GlyRS (15,22).

We recently described anti-KS autoantibodies and provided evidence that the KS antigen is asparaginyl–tRNA synthetase (AsnRS) (23). This sixth antisynthetase was found in sera from 3 patients with ILD and/or inflammatory arthritis without evidence of myositis. It immunoprecipitated a 65-kd protein and a unique tRNA that was distinct from that precipitated by any previously described antisynthetase or other reported tRNA-related antibody. Each of the 3 sera and their IgG fractions showed significant inhibition of AsnRS activity, but did not inhibit any of the other 19 aminoacyl–tRNA synthetase activities.

In this report, we describe the clinical and immunogenetic features of 5 additional patients with anti-AsnRS autoantibodies, most of whom had the syndrome of ILD with arthritis and/or myositis. Immunoprecipitation (IP) and aminoacylation inhibition studies with sera from these patients provide additional evidence that anti-KS (anti-AsnRS) reacts with asparaginyl–tRNA synthetase.

PATIENTS AND METHODS

Sera. Serum samples from a collection of sera from ~800 patients seen at the current or previous collaborating centers of the authors (Keio University, Tokyo, Japan; Kyoto University, Kyoto, Japan; Seoul National University, Seoul, Korea; Clinic and Research Institute for Rheumatic Diseases Aachen, Aachen, Germany; University of Oklahoma Health Sciences Center, Oklahoma City; National Institutes of Health, Bethesda, MD) or sera referred there for testing were stored at −20°C and were tested for the presence of anti-AsnRS autoantibodies. Sera from the following patients were included: 1) patients with PM or DM according to the criteria described by Bohan and Peter (24,25); 2) patients with a condition suggesting the clinical diagnosis of myositis; 3) patients with ILD who had no evidence of myositis and did not meet criteria for other connective tissue diseases; and 4) patients with serum anticytoplasmic antibodies, regardless of diagnosis. Approximately 1,700 other sera have also been tested, including sera from patients with other conditions including systemic lupus erythematosus, systemic sclerosis, and rheumatoid arthritis, as well as sera from normal subjects. Many of the sera were tested in studies of other autoantibodies. All samples were obtained after the patients gave their informed consent, as approved by the corresponding institutional review boards. Stored sera known to contain autoantibodies against synthetases for histidine, threonine, alanine, glycine, and isoleucine were used as controls.

ILD was considered to be present if an interstitial infiltrate was observed on chest radiography. DM was considered to be present if a heliotrope rash and/or Gottron's papules were observed.

IP. IP from HeLa cell extracts was performed as previously described (6,10). Ten microliters of patient sera was mixed with 2 mg of protein A–Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) in 500 μl of IP buffer (10 m*M* Tris HCl at pH 7.5, 500 m*M* NaCl, 0.1% Nonidet P40 [NP40]) and incubated with end-over-end rotation (Labquake shaker; Lab Industries, Berkeley, CA) for 2 hours at 4°C. The IgG-coated Sepharose was washed 4 times in 500 μl of IP buffer using 10-second spins in a microfuge tube, and resuspended in 400 μl of NET-2 buffer (50 m*M* Tris HCl at pH 7.5, 150 m*M* NaCl, 0.05% NP40).

For analysis of RNAs, this suspension was incubated with 100 μl of extracts, derived from 6×10^6 cells, on the rotator for 2 hours at 4°C. The antigen-bound Sepharose was then collected with a 10-second centrifugation in the microfuge, washed 4 times with NET-2 buffer, and resuspended in 300 μl of NET-2 buffer. To extract bound RNAs, 30 μl of 3.0*M* sodium acetate, 30 μl of 10% sodium dodecyl sulfate (SDS), and 300 μl of phenol/chloroform/isoamyl alcohol (50:50:1; containing 0.1% 8-hydroxyquinoline) were added to the Sepharose beads. After agitation in a Vortex mixer and

spinning for 1 minute, RNAs were recovered in the aqueous phase after ethanol precipitation and dissolved in 20 μl of electrophoresis sample buffer, composed of 10*M* urea, 0.025% bromphenol blue, and 0.025% xylene cyanol FF (Bio-Rad, Hercules, CA) in Tris–borate–EDTA buffer (90 m*M* Tris HCl at pH 8.6, 90 m*M* boric acid, and 1 m*M* EDTA). The RNA samples were denatured at 65°C for 5 minutes and then resolved by 7*M* urea–10% polyacrylamide gel electrophoresis (PAGE), with silver staining (Bio-Rad).

For protein studies, antibody-coated Sepharose was mixed with 400 μl of ^{35}S-methionine–labeled HeLa extract derived from 2×10^5 cells and rotated at 4°C for 2 hours. After 4 washes with IP buffer, the Sepharose was resuspended in SDS sample buffer (2% SDS, 10% glycerol, 62.5 m*M* Tris HCl at pH 6.8, 0.005% bromphenol blue). After heating at 90°C for 5 minutes, the proteins were fractionated by 10% SDS-PAGE, enhanced with 0.5*M* sodium salicylate, and dried. Labeled proteins were analyzed by autoradiography.

Aminoacylation. Aminoacylation inhibition reactions were performed as described previously, with minor modification (6,26). Six microliters of HeLa cell extract diluted 1:10 in Tris buffered saline was incubated with 3 μl of a 1:10 dilution of serum for 2 hours at 4°C. This was combined with 17 μl of reaction solution (50 m*M* Tris HCl at pH 7.5, 0.02*M* NaCl, 0.01*M* $MgSO_4$, 1 m*M* dithiothreitol) containing 8 units of yeast tRNA, 3 μl of ^{14}C-asparagine or other ^{3}H-labeled amino acid, and 1 μl of 200 m*M* cold amino acid. Ten-microliter aliquots were tested at 10 minutes and 20 minutes, spotted onto filter paper treated with 5% trichloroacetic acid (TCA), washed 5 times with 5% TCA, then with ethanol, then dried for counting. Results of inhibition testing were expressed as the percent inhibition of the average activity seen with the normal serum included in that experiment, as follows: % inhibition = [(average counts per minute with normal serum) − (cpm with test serum)] × 100/(average cpm with normal serum). Inhibition of >50% compared with the activity with normal serum was considered significant. In previous studies, although nonspecific effects on aminoacylation reactions by serum were common, nonspecific inhibition was usually <25%, and inhibition >50% reliably reflected specific antibody effects (6,7,12,13,26).

DNA typing of the HLA class II (DRB1, DQA1, DQB1, DPB1) alleles by polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP). Genomic DNA was isolated by phenol extraction of SDS-lysed and proteinase K–treated peripheral blood leukocytes, and then amplified by the PCR procedure using an automated PCR thermal cycler (PerkinElmer Cetus, Norwalk, CT). The primers used for specific amplification of the polymorphic exon 2 domains of the DRB1, DQA1, DQB1, and DPB1 genes were previously described (27). Amplified DNA was digested by all-specific restriction endonucleases and subjected to electrophoresis using a 12% polyacrylamide gel. Digested fragments were detected by staining with ethidium bromide, and HLA genotypes were determined on the basis of the RFLP patterns generated as previously described (27).

Other. Ouchterlony double immunodiffusion was performed as described previously, using HeLa cell extract as antigen (10).

Cases. *Patient 1.* The patient, a 61-year-old Japanese woman, noticed chest pain, followed 3 months later by dyspnea on mild exertion. Chest radiography and computed tomography (CT) scanning showed bilateral basilar infiltrates. The patient had hypoxemia, with a restrictive pattern on pulmonary function tests. No muscle weakness was observed, and the creatine kinase (CK) level was normal (67 IU/liter). A lung biopsy specimen obtained by video-assisted thoracic surgery showed mild interstitial chronic inflammation and interstitial fibrosis lacking a temporal heterogeneity pattern, and a diagnosis of fibrotic nonspecific interstitial pneumonia was made.

Patient 2. The patient, a 51-year-old German woman, developed a nonproductive cough and dyspnea on exertion. Chest radiography showed bibasilar interstitial fibrosis, and pulmonary function tests showed a restrictive pattern with decreased diffusing capacity for carbon monoxide (DLco). A diagnosis of ILD was made, and the patient's pulmonary function remained stable throughout her disease course. She had polyarthralgia and developed erythema and keratosis of the palms and fingers consistent with mechanic's hands, but no cutaneous scleroderma, Raynaud's phenomenon, or DM rash (Gottron's papules or heliotrope rash) was observed. No muscle weakness was found, and the CK level was normal (56 IU/liter at the first visit) each time it was measured. When the patient was age 58 years, ovarian carcinoma was found, and surgery with subsequent irradiation was performed. She died of metastatic ovarian carcinoma at age 63 years.

Patient 3. The patient, a 72-year-old American woman, developed an itchy red eczematous rash that was thought to be due to a medication for hypertension. The rash was soon accompanied by progressive weakness, myalgias, mild dyspnea, and difficulty swallowing. She was started on prednisone and methotrexate, and 6 months after the rash had first appeared, she was referred to the Arthritis and Rheumatism Branch of the National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health. There was a widespread maculopapular rash of the trunk, extremities, and head, and Gottron's papules were observed. Proximal muscle weakness was present, and her CK level was 358 IU/liter. Magnetic resonance imaging of the thighs showed both atrophy and probable inflammation on the STIR images. A biopsy of the deltoid muscle showed changes of an active inflammatory myopathy. No malignancy was identified. She was treated with pulse methylprednisolone. However, her muscle weakness and rash were not significantly improved, and infectious complications limited the therapeutic options. Her disease course was subsequently complicated by herpes zoster and the Ramsay-Hunt syndrome as well as by skin infections and cellulitis, mastoiditis, heart failure, and a cerebrovascular accident.

Patient 4. The patient, a 53-year-old Korean woman with intermittent episodes of productive cough due to bronchiectasis, noticed easy fatigability and myalgia in 1994 and later developed muscle weakness and was admitted to Seoul National University Hospital in February 1995. Proximal muscle weakness in her extremities and a dark pigmentation over the extensor surface of both knees were observed. The CK level was elevated at 3,808 IU/liter. The findings on electromyogram and muscle biopsy were consistent with inflammatory myopathy. A diagnosis of DM associated with ILD was made, and she was treated with prednisolone (60 mg/day). Her muscle enzyme levels gradually normalized, and her muscle weakness improved. Her chest radiograph and high-resolution