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.

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...
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-17+/+) and–deficient (IL-17−/−) SKG mice, prepared by genetic backcrossing from IL-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-17+/+ CD4+ T cells developed arthritis with high arthritis scores within 3 mo, whereas none of those transferred with IL-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-17+ cells in SKG, but not in BALB/c mice (Fig. 1 G). Thus, naïve CD4+ T cells in SKG mice are spontaneously activated and differentiate to Th17 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.
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 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 and having a similar function (6), is dispensable for the disease.

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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 × 106) 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 mAbs. 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.
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-17+ CD4+ T cells, whereas TNF-α−, IL-1−, or IFN-γ−deficient SKG mice harbored equivalent numbers of IL-17+ CD4+ T cells as cytokine-intact SKG mice (Fig. 4 A). When IL-6−/− SKG or BALB/c CD4+ T cells devoid of Th17 cells were transferred to IL-6−/− or 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-6−/− T cells to IL-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).

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-γ−/− SKG mice differentiated more efficiently to Th17 cells than IFN-γ−/+ 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-γ−/−, or IL-17−/− mice (Fig. 5 C). The blockade inhibited the differentiation/expansion of both normal and IFN-γ−/− 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/expansion 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.

**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-γ−/− 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-α−/− mice were backcrossed to BALB/c more than eight times and crossed to SKG mice to make cytokine-deficient SKG mice (3). RAG2 backcrossed to BALB/c more than eight times and crossed to SKG mice to generate IL-6−/− or IFN-γ−/− cells were described previously (10). IL-1−, IL-6−, and IL-7 pg/ml for TNF-α were measured by BD Pharmingen’s bead-based assay (BD Biosciences), with the detection limits of 2 pg/ml for IL-6 and TNF-α. In vitro cytokine secretion was measured by Bio-Plex Multi-plex Bead Array (BD Biosciences), with a cytokine detection limit of 3 pg/ml for IL-6 and TNF-α.

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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-17+ or IFN-γ+ cells in individual RAG−/− mice transferred with CD4+ T cells treated and with anti–TGF-β mAb. Figs. S1 and S2 are available at http://www.jem.org/cgi/content/full/jem.20062259/DC1.

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