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Distinct contribution of IL-6, TNF-α, IL-1, and IL-10 to T cell–mediated spontaneous autoimmune arthritis in mice

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Cytokines play key roles in spontaneous CD4+ T cell–mediated chronic autoimmune arthritis in SKG mice, a new model of rheumatoid arthritis. Genetic deficiency in IL-6 completely suppressed the development of arthritis in SKG mice, irrespective of the persistence of circulating rheumatoid factor. Either IL-1 or TNF-α deficiency retarded the onset of arthritis and substantially reduced its incidence and severity. IL-10 deficiency, on the other hand, exacerbated disease, whereas IL-4 or IFN-γ deficiency did not alter the disease course. Synovial fluid of arthritic SKG mice contained high amounts of IL-6, TNF-α, and IL-1, in accord with active transcription of these cytokine genes in the afflicted joints. Notably, immunohistochemistry revealed that distinct subsets of synovial cells produced different cytokines in the inflamed synovium: the superficial synovial lining cells mainly produced IL-1 and TNF-α, whereas scattered subsynovial cells produced IL-6. Thus, IL-6, IL-1, TNF-α, and IL-10 play distinct roles in the development of SKG arthritis; artiﬁgenic CD4+ T cells are not required to skew to either Th1 or Th2; and the appearance of rheumatoid factor is independent of joint inﬂammation. The results also indicate that targeting not only each cytokine but also each cell population secreting distinct cytokines could be an effective treatment of rheumatoid arthritis.

Introduction
Rheumatoid arthritis (RA) is a chronic systemic inﬂammatory disease of unknown etiology that primarily affects the synovial membranes of multiple joints (1). A cardinal feature of joint inﬂammation in RA is proliferative inﬂammation of synovial cells, i.e., synovitis, which results in the destruction of the adjacent cartilage and bone. Although CD4+ T cells are currently assumed to be the prime mediators of synovitis, it remains obscure how arthritogenic CD4+ T cells activate synovial cells to proliferate or, upon activation, how the autonomous proliferation of synovial cells is maintained, leading to the destruction of the joint (1). It has been well documented that cytokines play indispensable roles in these processes (2). TNF-α and IL-6, for example, contribute to joint inﬂammation in RA, as illustrated by the effects of neutralizing TNF-α or blocking IL-6 receptor to ameliorate RA (3, 4). IFN-γ, IL-4, and IL-10 formed by Th1 or Th2 CD4+ T cells may also participate in synovitis, as observed in other autoimmune diseases (1, 2). The way in which these cytokines contribute to the development of arthritis including RA, however, is a subject of controversy, as cytokines exert different effects in different models of arthritis.

For example, IL-6 deficiency variously results in exacerbation of, amelioration of, or no effects on arthritis, depending on the particular model (5–9). Neutralization of TNF-α has different effects in collagen-induced arthritis (CIA) and streptococcal cell wall–induced arthritis (10). IFN-γ may not only mediate Th1 responses in arthritis but also suppress the destruction of cartilage and bone by inhibiting the generation of osteoclasts (11). Furthermore, it is unclear how relevant the available arthritis models are to human RA. For example, CIA, one of the most widely used models of RA, is not accompanied by the appearance of rheumatoid factor (RF), which is present in about 70% of RA patients (1). In this report, we have analyzed the contribution of pro- and anti-inflammatory cytokines to the spontaneous development and chronic progression of CD4+ T cell–mediated autoimmune arthritis in a newly established mouse model of RA.

SKG mice spontaneously develop T cell–mediated chronic autoimmune arthritis as a consequence of a mutation of the gene encoding an Src homology 2 (SH2) domain of ζ-associated protein of 70 kDa (ZAP-70), a key signal transduction molecule in T cells (12). This mutation impairs positive and negative selection of T cells in the thymus, leading to thymic production of arthritogenic T cells. Clinically, joint swelling begins in small joints of the digits, progressing in a symmetrical fashion to larger joints including wrists and ankles. Histologically, the swollen joints show severe synovitis with formation of pannus invading and eroding adjacent cartilage and subchondral bone. SKG mice develop extra-articular lesions, such as interstitial pneumonitis, vasculitides, and subcuta-

Nonstandard abbreviations used: ζ-associated protein of 70 kDa (ZAP-70); collagen-induced arthritis (CIA); rheumatoid arthritis (RA); rheumatoid factor (RF); Src homology 2 (SH2).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J. Clin. Invest. 114:582–588 (2004). doi:10.1172/JCI200421795.
neous necrobiotic nodules not unlike rheumatoid nodules in RA. Serologically, they develop high titers of RF and autoantibodies specific for type II collagen. Furthermore, CD4+ T cells can adaptively transfer arthritis in SKG mice, which have a BALB/c genetic background, to T cell–deficient BALB/c nude or T cell/B cell–deficient SCID mice, which indicates that the disease is a T cell–mediated autoimmune disease. In addition to the causative gene, the polymorphism of the MHC gene also contributes to the occurrence of SKG arthritis depending on environmental conditions. Thus, this spontaneous autoimmune arthritis in mice resembles human RA in clinical and histological characteristics of articular and extra-articular lesions, in serological characteristics, and in the key role of CD4+ T cells in initiating arthritis (12).

In contrast to other organ-specific autoimmune diseases in which self-reactive T cells destroy the target cells (e.g., insulin-secreting β cells in type 1 diabetes mellitus), a key feature of SKG autoimmune arthritis, and human RA for that matter, is that T cells do not destroy but stimulate synoviocytes to proliferate and invade the surrounding cartilage and bone. The selective development of arthritis in SKG mice, despite their general alteration in the T cell repertoire, could be attributed at least in part to a high sensitivity of synoviocytes to immunological stimuli, including T cell self-reactivity, due to their immunologically unique features. The synoviocytes are, for example, intrinsically capable of producing proinflammatory cytokines and matrix metalloproteinases; are composed of type A macrophage-like and type B fibroblast-like synoviocytes, both of which are highly sensitive to various immunological stimuli including cytokines; and are devoid of basement membrane and tight junctions, allowing their easy invasion to the surrounding tissue (1). We have therefore analyzed in this report how the synoviocytes stimulated by arthritogenic CD4+ T cells mediate arthritis in SKG mice and how those cytokines produced by the stimulated synoviocytes or arthritogenic CD4+ T cells contribute to the development and progression of arthritis.

**Results**

**Clinical and histological features of SKG arthritis.** In SKG mice, joint swelling began to develop in a few digits around 2 months of age, progressing to other digits and to larger joints (wrists, ankles, and knees, as do humans with RA (1)).

Immunohistochemistry of SKG synovitis. Immunohistochemical staining of synovial tissues of 4-month-old SKG mice with various antibodies revealed the following. A number of CD4+ T cells, but few CD8+ T cells, infiltrated the sublining tissue, where B cells also formed aggregates (Figure 1, A–C). Granulocytes (Figure 1D) and macrophages (Figure 1E) were abundant in the joint cavity and also subsynovial region. The distribution of macrophages corresponded to that of class II MHC–expressing cells (Figure 1F). Many cells expressing CD49d (also known as very late antigen 4; VLA-4) infiltrated the subsynovia (Figure 1G), and its ligand VCAM-1 was expressed in vascular endothelial cells and interstitial cells (Figure 1H), which indicates recruitment of lymphocytes to the inflamed synovial tissue (Figure 1I) (14). Toluidine blue staining revealed few mast cells in the inflamed tissue (data not shown).

Cytokine production in arthritic joints. Ankle joints of the majority of 24-week-old SKG mice with severe joint swelling exhibited high-level expression of IL-1β and IL-6 mRNA, as assessed by real-time RT-PCR; half of the mice also actively transcribed TNF-α mRNA (Figure 2A). In contrast, 8-week-old SKG mice without apparent joint swelling showed no IL-6 transcription and only a slight expression of IL-1β, although TNF-α mRNA was detected in some young SKG mice. IL-2, IL-4, IL-10, and IFN-γ mRNA messages were not detected even in 24-week-old SKG mice (data not shown).

The joint fluid taken from swollen ankle joints of 32-week-old SKG mice contained high amounts of IL-1β, IL-6, and TNF-α (Figure 2B). Serum levels of these cytokines were below detectable levels by ELISA in the majority of mice, although 20% of them bore low but significant amounts of IL-6 in their sera (data not shown).

Immunohistochemical staining of inflamed synovial tissue of SKG mice revealed distinct staining patterns of TNF-α and IL-1β versus IL-6. Cells expressing IL-1β and TNF-α were detected almost exclusively in one or two layers of hypertrophic superficial lining cells facing the joint cavity (Figure 2, C and D). In contrast, cells expressing IL-6 were smaller than the superficial
Figure 2
Expression of cytokines at mRNA and protein levels. (A) Quantitative RT-PCR for indicated genes was performed with ankle joints from 8- or 24-week-old SKG or BALB/c mice. Bars show the means ± SD. See Methods for the definition of units. (B) Concentrations of indicated cytokines, assessed by ELISA, in the joint fluid of 32-week-old SKG and BALB/c mice (n = 10 each). Bars show the means ± SD. (C–F) Immunohistochemical staining of the synovial tissue of a finger joint of a 4-month-old SKG mouse for IL-1β (C), TNF-α (D), or IL-6 (E), with staining control (F) (original magnification, ×40). Insets show higher magnifications of a part of synovium.

Figure 3

The table and the figure illustrate the effects of deficiencies in various cytokines on the development and progression of SKG arthritis. The data show that deficiency in specific cytokines can influence the severity and incidence of arthritis in SKG mice. For example, IL-10–− SKG mice showed a higher incidence of disease compared with wild-type controls. Similarly, IFN-γ–− SKG mice displayed progressive weight loss and died earlier, indicating that IFN-γ is a key factor in the development ofSKG arthritis.

The researchers concluded that the inflamed synovial tissue of SKG mice actively produces IL-1β, TNF-α, and IL-6; the cells forming TNF-α and IL-1β are apparently the same and constitute the superficial lining layer; and the cells mainly forming IL-6 are apparently different from those forming IL-1β and TNF-α and localize differently.

Effect of deficiency in particular cytokines on the development and progression of SKG arthritis. The study shows that cytokines like IL-1β, IL-1α, IFN-γ, and IL-6 play a crucial role in the development of SKG arthritis. Homozygously cytokine gene−deficient (−/−), heterozygously cytokine gene−deficient (+/−), or cytokine gene−intact (+/+ female SKG mice were prepared and maintained in our conventional animal facility, where the majority of cytokine−intact female SKG mice began to exhibit joint swelling at 11 to 12 weeks of age. Severity of arthritis in these mice varied from swelling of some joints to marked swelling of large joints (the average score was 3.6 ± 1.5; see Methods for clinical assessment of arthritis) at 32 weeks of age. Histology of swollen joints of 32-week-old SKG mice revealed severe synovitis and destruction of cartilage and bone (Figure 3B). Notably, IL-6−− SKG mice showed no joint swelling macroscopically, no histologically evident synovitis, and no destruction of cartilage and bone (Figure 3C). Some IL-1α/β−− SKG mice started to develop arthritis around 15 weeks of age with a significantly lower incidence than IL-1α/β+/− SKG mice. Although the incidence in IL-1−− mice was 60% at 32 weeks of age, severity was very low, with joint swelling restricted to only 1 or 2 joints of the digits. These swollen joints showed histologically evident synovitis but destruction of cartilage and bone was generally less severe than in cytokine−intact SKG mice (data not shown). These results were similar in the TNF-α−− SKG mice: the onset was delayed, incidence lowered to 20% of controls, and severity substantially reduced to swelling of a single joint of a digit (see legend to Figure 3A for statistical analyses of the data). Interstitial pneumonitis — which was observed in SKG mice older than 8 months (12) — among these cytokine−deficient mice was too mild in severity and low in incidence at 32 weeks of age for comparisons to be made (data not shown).

IL-10−− SKG mice developed arthritis with no statistically significant differences in severity and incidence from IFN-γ+/+ or IFN-γ+/− mice during 24 weeks of observation, although IFN-γ−− SKG mice displayed progressive weight loss and died earlier as compared with wild-type controls maintained under the same conditions (12). We did not observe significant difference in histology between IFN-γ+/− and IFN-γ+/+ or IFN-γ−− mice, although the IFN-γ−− mice, when they became debilitated, generally showed lesser degrees of arthritis compared with the IFN-γ+/− or IFN-γ+/+ mice (data not shown). IL-4−− SKG mice, on the other hand, survived well to 32 weeks of age; the time of onset, incidence and severity of arthritis were not significantly different among IL-4+/−, IL-4−−, or IL-4+/+ mice. Thus, IFN-γ and IL-4 are apparently dispensable for the development of SKG arthritis.

IL-10−− SKG mice showed a consistently higher incidence of disease and mean arthritis score than IL-10−− or IL-10+/− SKG mice, although the susceptibility to colitis of IL-10−− mice restricted the period of observation to 24 weeks (15). Histological examination of IL-10−− mice that had survived to this age showed severe synovitis accompanying joint destruction (Figure 3D). Thus, IL-10 is suppressive for the development of SKG arthritis.

In these experiments, SKG mice with heterozygous deficiency in IL-1, TNF-α, IL-6, or IL-10 showed incidences and severities of arthritis that were intermediate between those of homozygously cytokine−deficient and cytokine−intact mice. This result indicates that the disease-enhancing or -protective effects of the cytokines are dose dependent.
RF and joint inflammation. The IL-6-, TNF-α-, or IL-1–deficient mice developed high titers of IgM-type RF even in the absence of joint inflammation (Figure 4A). This indicates that the development of IgM-RF is independent of joint inflammation. The result contrasted with the development of IgG anti–type II collagen autoantibody in arthritic mice but not in nonarthritic IL-6–deficient mice, which suggests the possible roles of anti–type II collagen antibody as an active mediator of joint inflammation or a product of joint destruction (Figure 4B).

Discussion
This study showed that proliferative synovitis with pannus formation and resultant joint destruction in SKG mice are immunopathologically similar to those observed in human RA and that, despite the pleiotropy, redundancy, and cross-regulation in cytokine functions, deficiency in either IL-6, IL-1, or TNF-α can inhibit the development and progression of SKG arthritis, which is apparently independent of the development of RF.

It is well documented in human RA and in animal models that IL-1, IL-6, and TNF-α synergistically mediate synovitis and destruction of cartilage and bone (1–3, 5, 6, 16–19), that IL-1 and TNF-α trigger the secretion of IL-6 by synovial cells (2), and that anti–TNF-α treatment can lower IL-6 formation (20, 21). It is still unclear, however, how the formation and action of these cytokines are controlled at molecular and cellular levels (22–24). Here we showed that IL-6 is apparently produced by subsynovial stromal cells (macrophages and DCs; Figure 1) and presumably some CD4+ T cells, whereas IL-1 and TNF-α are mainly produced by superficial lining cells facing the joint cavity and producing matrix metalloproteinases as also shown in other arthritis models and RA (25–28).

This difference in cellular sources between IL-6 and IL-1/TNF-α indicates that not only neutralization of these cytokines or blockade of their actions at a molecular level, but also reduction in the number of the stromal cells producing each cytokine or modifying their functions may ameliorate arthritis.

TNF-α deficiency inhibited arthritis effectively but not completely in SKG mice; i.e., the onset was delayed, incidence lowered...
to 20% of controls, and severity substantially reduced to swelling of a single joint of a digit. It can be argued that the MHC haplotype of TNF-α-deficient SKG mice could influence the development of arthritis, since TNF-α-deficient SKG mice bear an H-2b haplotype in contrast with SKG mice, which bear an H-2d haplotype. This is because the founder strain of TNF-α deficiency was of H-2b haplotype, and the TNF-α locus, which is closely linked with the MHC locus, was cosegregated with the MHC in establishing the congenic TNF-α-deficient SKG mice (29). We previously showed that the MHC haplotype of SKG mice can influence the incidence and severity of arthritis (e.g., H-2d haplotype confers susceptibility to arthritis in SKG mice [ref. 12]). It is unlikely, however, that the H-2b haplotype of TNF-α-deficient SKG mice alters the incidence and severity of arthritis, because SKG mice on BALB.B (H-2b) background showed an incidence and severity of arthritis similar to those of SKG mice (our unpublished data). Thus, TNF-α deficiency per se can inhibit the development of arthritis. On the other hand, the failure of TNF-α deficiency to completely inhibit arthritis suggests that proinflammatory cytokine pathways that spare TNF-α may also be operative in SKG arthritis. This substantial but incomplete inhibition of SKG arthritis by TNF-α deficiency may have a common basis with the clinical result that only about 30–40% of RA patients show dramatic responses to TNF inhibitors (30).

It is well documented in arthritis that pro- or anti-inflammatory cytokines facilitate or inhibit activation of various types of cells, including macrophages/monocytes, neutrophils, and T cells, which are involved in local inflammation (16). Another function of cytokines may be to alter the degree of T cell-mediated control of autoimmune T cells, thereby allowing arthritogenic T cells to chronically mediate arthritis. For example, IL-10 not only suppresses the formation of TNF-α and IL-6 by macrophages, synoviocytes, and T cells, as shown in experimental models (31–33), but also may mediate or augment CD25+CD4+ T cell-mediated immunoregulation, as shown in various autoimmune/inflammatory diseases (34, 35). IL-10 deficiency may therefore attenuate the T cell–mediated suppression on arthritogenic T cells, thus contributing to the exacerbation of arthritis in SKG mice. Furthermore, in addition to its local and systemic proinflammatory effects (20, 36), IL-6 may render arthritogenic T cells refractory to the suppressive control exerted by naturally arising CD25+CD4+ regulatory T cells (37). CD25+CD4+ T cells, derived from normal BALB/c mice, indeed prevented SKG arthritis effectively when the animals were inoculated before onset of the disease, as observed in CIA (ref. 38 and our unpublished data). We are currently investigating whether IL-6 deficiency rectifies the susceptibility of arthritogenic T cells to the CD25+CD4+ T cell–mediated suppression and thereby contributes to inhibiting the development of SKG arthritis. Whether TNF-α or IL-1 can affect T cell–mediated immunoregulation by altering the functions of APCs or regulatory T cells also remains to be determined (39).

Although CD4+ T cells play pivotal roles in SKG arthritis, they show impaired activation and proliferation upon T cell receptor stimulation because of a structurally altered ZAP-70 and resulting alteration in T cell signal transduction in SKG T cells (12). Despite this defect, SKG T cells are competent in secreting various cytokines, including IL-2, IL-4, IL-10, TNF-α, and IFN-γ, upon PMA/ionomycin stimulation, in a pattern of cytokine production and kinetics similar to that of BALB/c T cells (Supplemental Figure 5A). CD4+ T cells from young SKG mice even produced slightly higher amounts of IFN-γ, IL-4, and IL-10 and smaller amounts of TNF-α than BALB/c CD4+ T cells (Supplemental Figure 5B). Moreover, there was no significant alteration of the disease course in SKG mice with IFN-γ or IL-4 deficiency, which is in contrast with other models (40, 41). These findings taken together indicate that SKG CD4+ T cells are not committed to Th1 or Th2 cells, or TNF-α production, due to the altered signal transduction through ZAP-70; skewing of CD4+ arthritogenic T cells to either Th1 or Th2 is not absolutely required for the development and/or progression of arthritis; and cross-regulation, if any, by Th1 or Th2 cells is not significant in inhibiting arthritis in this model. It is possible that T cells in SKG mice trigger the activation and proliferation of synoviocytes in a cell-contact manner or via other cytokines (42). Notably, a large amount of mRNA for IL-17, which is secreted by activated T cells and able to stimulate synovial cells (43), can be detected in the joints of arthritic SKG mice (our unpublished data).

The disease in SKG mice is a chronic systemic inflammatory disease like human RA (1, 12). For example, SKG mice spontaneously develop RA as a systemic manifestation of the disease. An intriguing finding in cytokine-deficient SKG mice is that they developed high

**Figure 4**
Autoantibodies in cytokine-deficient SKG mice. RF (A) or anti-type II collagen (anti-CII) autoantibody titers (B) in 32-week-old SKG mice homozygously or heterozygotically deficient in indicated cytokines, with wild-type SKG mice and age-matched BALB/c mice as controls. Filled circles indicate mice with joint swelling (arthritis score ≥ 1.0); open circles indicate mice without joint swelling (arthritis score < 1.0). First 10 mice that had reached 32 weeks of age in each group shown in Figure 3 were analyzed.
titers of RF irrespective of whether arthritis was inhibited or not. For example, IL-6-deficient mice with no arthritis developed titers of RF equivalent to those of SKG mice. This cannot be attributed to hypergammaglobulinemia in SKG mice, because SKG mice without arthritis did not develop anti-DNA autoantibodies or anti-type II collagen autoantibodies, which may mediate joint inflammation or develop as a consequence of joint destruction (Figure 4) (12). Furthermore, transfer of thymocytes alone from SKG mice produced severe arthritis in SCID mice, which suggests that B cells may not be an absolute requirement for elicitation and progression of arthritis in this model (12). These results taken together indicate that RF is independent of joint inflammation per se in SKG mice. It is known, on the other hand, that RA patients with RF undergo a more aggressive and destructive course of arthritis (1). Considering that RF also develops in various other inflammatory diseases including microbial infections, it is likely that RF may not be a primary mediator of arthritis but may secondarily enhance joint inflammation, for example, by forming immune complexes that deposit in the joint (44, 45).

In conclusion, cytokines play essential roles in SKG arthritis in a manner similar to that in human RA. SKG mice can be instrumental for further studying the contribution of pro- and anti-inflammatory cytokines to the development and progression of RA, for elucidating how arthritogenic T cells stimulate synoviocytes to cause arthritis, and for devising effective treatment of the disease.

Methods

Mice. IL-1β-, TNF-α-, or IL-6-deficient mice (IL-1−/−, TNF-α−/−, and IL-6−/−, respectively) were backcrossed to BALB/c mice more than 8 times (29). BALB/c IL-10-deficient (IL-10−/−) mice were kindly provided by D. Rennick (DNAX Institute, Palo Alto, California, USA) (15). BALB/c IFN-γ−/− and IL-4−/− mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) (46, 47). These cytokine-deficient BALB/c mice were backcrossed three times to SKG mice, which have the BALB/c genetic background (12). Such SKg-homozygous but cytokine gene–heterozygous mice were intercrossed, and resulting female SKg/Skg littermates with homozygously or heterozygously cytokine-deficient or cytokine-intact female mice were used in the present study. All mice were maintained in our animal facility under conventional microbial conditions. All experiments were conducted according to the institutional guidelines for animal welfare of the Institute for Frontier Medical Sciences at Kyoto University.

Clinical assessment of SKG arthritis. Joint swelling was monitored by inspection and scored as follows: 0, no joint swelling; 0.5, swelling of one finger joint; 0.5, mild swelling of wrist or ankle; 1.0, severe swelling of wrist or ankle. Scores for all digits, wrists, and ankles were totaled for each mouse (12).

Antibodies. The following antibodies were used: anti-CD4 (H129.19), anti-CD8a (53-6.7), anti-CD11b (M1/70), anti-CD45RB/B220 (RA3-6B2), anti-CD49d (9C10), anti-CD106 (429), and anti-I-A/I-E (M5/114.15.2), purchased from BD Biosciences — Pharmingen (San Diego, California, USA) (46, 47). These cytokine-deficient BALB/c mice were backcrossed to SKG mice more than 8 times (29) and used in the present study. All mice were maintained in our animal facility under conventional microbial conditions. All experiments were conducted according to the institutional guidelines for animal welfare of the Institute for Frontier Medical Sciences at Kyoto University.

Statistical analyses. Student’s t test was used for statistical analyses.

Acknowledgments

This work was supported by grants-in-aid from the Ministry of Education, Science, Sports, and Culture, the Ministry of Human Welfare, and the Japan Science and Technology Agency. We thank Zoltan Fehervari for critical reading of the manuscript.

Received for publication April 5, 2004, and accepted in revised form June 29, 2004.

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