Interleukin-17 Promotes Autoimmunity by Triggering a Positive-Feedback Loop via Interleukin-6 Induction

Hideki Ogura,1,4 Masaaki Murakami,1,4 Yuko Okuyama,1 Mineko Tsuruoka,1 Chika Kitabayashi,1 Minoru Kanamoto,1 Mika Nishihara,1 Yoichiro Iwakura,2 and Toshio Hirano1,3,*

1Laboratory of Developmental Immunology and the CREST Program of the Japan Science and Technology Agency, Graduate School of Frontier Biosciences, Graduate School of Medicine, and WPI Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan
2Center for Experimental Medicine and the CREST Program of the Japan Science and Technology Agency, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
3Laboratory of Cytokine Signaling, RIKEN Research Center for Allergy and Immunology, Yokohama 230-0045, Japan
4These authors contributed equally to this work
*Correspondence: hirano@molonc.med.osaka-u.ac.jp
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SUMMARY

Dysregulated cytokine expression and signaling are major contributors to a number of autoimmune diseases. Interleukin-17A (IL-17A) and IL-6 are important in many disorders characterized by immune self-recognition, and IL-6 is known to induce the differentiation of T helper 17 (Th17) cells. Here we described an IL-17A-triggered positive-feedback loop of IL-6 signaling, which involved the activation of the transcription factors nuclear factor (NF)-κB and signal transducer and activator of transcription 3 (STAT3) in fibroblasts. Importantly, enhancement of this loop caused by disruption of suppressor of cytokine signaling 3 (SOCS3)-dependent negative regulation of the IL-6 signal transducer gp130 contributed to the development of arthritis. Because this mechanism also enhanced experimental autoimmune encephalomyelitis (EAE) in wild-type mice, it may be a general etiologic process underlying other Th17 cell-mediated autoimmune diseases.

INTRODUCTION

Autoimmune diseases—a heterogeneous group of disorders that are controlled by many genetic and environmental factors—are most simply defined by the presence of mediators of autoimmunity, such as autoantibodies or autoreactive lymphocytes. Various complex molecular processes differentially interact in each of these disease states, which often manifest with similar symptoms, making it difficult to identify general etiologic mechanisms (Andersson and Holmdahl, 2005; Davidson and Diamond, 2001; Falcone and Sarvetnick, 1999; Marrack et al., 2001; Mathis and Benoist, 2004; Wandstrat and Wakeland, 2001). A common feature among them, however, is that disease expression depends on the activation status of the immune system. Thus, current research is focused on identifying the mechanisms that result in the causative dysregulation of the immune system. For example, it is believed that inflammation is critical for the induction of autoimmune diseases, because some autoimmune and autoimmune-related diseases, including rheumatoid arthritis, are characterized by robust immune responses that result in unresolved inflammation (Baccala et al., 2007; Hirano, 2002; O'Shea et al., 2002).

In the immune system, CD4+ T cells serve as an important source of proinflammatory cytokines. Interestingly, cytokines, and in particular such proinflammatory cytokines as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), are critically involved in autoimmune diseases (Hirano, 2002; Ioannou and Isenberg, 2000; O’Shea et al., 2002). In fact, anti-TNF-α and anti-IL-6 receptor therapies have been successfully employed for a number of chronic autoimmune diseases, including rheumatoid arthritis and Crohn’s disease (Andreakos et al., 2002; Nishimoto and Kishimoto, 2004). Although classification of CD4+ T cells into T helper 1 (Th1) and Th2 cells has provided a framework for understanding the roles of various CD4+ T cell subtypes in the development of autoimmune diseases (Glimcher and Murphy, 2000; Mosmann and Coffman, 1989; Zhu et al., 2006), recent studies have identified Th17 cells as a previously unknown arm of the CD4+ T cell effector response. These cells secrete several proinflammatory cytokines, including IL-17A (Bettelli et al., 2007; Cua et al., 2003; Harrington et al., 2005; Park et al., 2005; Veldhoen et al., 2006), deficiency of which in mice results in resistance to such autoimmune diseases as collagen-induced arthritis, experimental autoimmune encephalomyelitis (EAE), and the arthritis disorders that develop in SKG and IL-1 receptor antagonist-deficient mice (Afzali et al., 2007; Bettelli et al., 2007; Hirota et al., 2007; Iwakura and Ishigame, 2006). The development of Th17 cells requires a combination of T cell receptor (TCR) stimulation, TGF-β, and IL-6 followed by retinoic acid-related orphan receptor-γt expression via signal transducer and activator of transcription 3 (STAT3) activation (Bettelli et al., 2007; Ivanov et al., 2006; Nishihara et al., 2007; Veldhoen et al., 2006). Moreover, it was reported that T cells serve as a source of TGF-β during Th17 cell differentiation (Li et al., 2007). Among these mediators, the multifunctional cytokine IL-6 has been suggested to play a central role in a number of diseases (Hirano, 1998; Ishihara and Hirano, 2002). Indeed, IL-6 deficiency suppresses the development of
many of the aforementioned autoimmune diseases as well as the arthritis that develops in mice carrying a Y759F mutation in the IL-6 receptor subunit gp130 (F759 mice), which enhances the receptor signaling (Boe et al., 1998; Campbell et al., 1991; Hirota et al., 2007; Okuda et al., 1999; Sasai et al., 1999; Sawa et al., 2006). Considering the requirement of IL-6 for Th17 cell development, these results suggest that IL-6 acts upstream of IL-17A to induce the onset of various autoimmune diseases. How IL-17A contributes to autoimmune diseases and/or inflammation, however, has yet to be elucidated. Thus, identification of the downstream targets of IL-17A that directly contribute to the development of autoimmune diseases should advance our understanding of the mechanisms underlying IL-17A-mediated diseases.

Our research has focused on IL-6 and the associated signaling pathways. We have established several knockin mouse lines expressing mutated variants of gp130, a transducer of IL-6 signaling (Ohtani et al., 2000), and showed that F759 mice spontaneously develop an rheumatoid arthritis-like disease that depends on mature lymphocytes (Atsumi et al., 2002). Because Y759 of gp130 is essential for the suppressor of cytokine signaling 3 (SOCS3)-dependent negative regulation of gp130 signaling (Ilangumaran et al., 2004; Yoshimura et al., 2007), IL-6- and gp130-mediated STAT3 activation is enhanced in F759 mice. In other words, SOCS3-mediated negative feedback is specifically defective in the gp130 signaling pathway of F759 mice. The development of disease in the F759 mice is at least partially dependent on IL-6 (Sawa et al., 2006). The disease severity in the F759 mice is also accelerated in a manner dependent on IL-6 by crossing the mice with human T cell leukemia virus 1 (HTLV-1) p40-Tax transgenic mice in which nuclear factor (NF)-κB signaling was enhanced (Ishihara et al., 2004). These results suggest that IL-6 is one of critical factors for the disease in F759 mice. Moreover, F759 mice develop arthritis in a manner dependent on the homeostatic proliferation of CD4+ T cells, although the F759 mutation is not required in T cells for the disease. Rather, the F759 mutation in nonhematopoietic cells is essential for IL-7-mediated CD4+ T cell homeostatic proliferation as well as disease development (Sawa et al., 2006).

Here, we showed that IL-6 not only functioned upstream of IL-17A but also acted as a critical downstream target of IL-17A and, unexpectedly, that IL-17A together with IL-6 triggered a positive-feedback loop of IL-6 expression through the activation of NF-κB and STAT3 in fibroblast cells. We showed that blockade of the IL-6 loop significantly suppresses the development of arthritis in F759 mice and EAE. Thus, we concluded that IL-17A promotes autoimmune diseases by triggering a positive-feedback loop via IL-6 induction.

RESULTS

An IL-17A-Triggered Positive-Feedback Loop of IL-6 Expression in Fibroblasts

Because many autoimmune diseases are dependent on both IL-17A and IL-6, and because IL-17A induces the expression of IL-6 in fibroblasts (Yao et al., 1995), we hypothesized that IL-6 functions not only as an inducer of Th17 cell differentiation but also as a downstream effector of IL-17A. Moreover, both IL-17A and IL-6 may function together during the effector phases of autoimmune diseases. We first investigated the roles of IL-17A and IL-6 signaling in fibroblasts because we previously used chimeric mice with transplanted bone-marrow cells to show that the F759 mutation in nonhematopoietic cells but not in hematopoietic cells results in spontaneous autoimmune arthritis (Sawa et al., 2006). We prepared mouse embryonic fibroblast (MEF) cells from WT mice. After the MEF cells were stimulated with IL-17A in the presence or absence of IL-6, we analyzed the expression of various target molecules of the IL-17A–NF-κB signaling pathway, including IL-6. The expression of each of the IL-17A–NF-κB targets tested (IL-6, keratinocyte-derived cytokine [KC], and MIP2) was synergistically enhanced by IL-17A and IL-6 (Figures 1A and 1B and Figure S1 available online). Depleting the MEF cells of molecules involved in either IL-17A or IL-6 signaling, such as NEMO or STAT3, respectively, showed that IL-6 expression is completely dependent on NF-κB signaling and that the synergistic effect of IL-17A and IL-6 on IL-6 expression was mediated by STAT3 signaling (Figures 1C and 1D).

IL-1β- and TLR ligand-triggered IL-6 expression has been reported to depend on IκB-ζ, another target of NF-κB (Yamamoto et al., 2004). Similarly, the expression of IκB-ζ was synergistically enhanced by IL-17A plus IL-6 in MEF cells, and this effect was ameliorated by knocking down STAT3 expression (Figure 1E). Furthermore, deleting IκB-ζ completely suppressed IL-6 expression even after treatment with IL-17A and IL-6 (Figure 1F). Consistent with these results, IL-17A overexpression increased the serum IL-6 concentration in WT animals (Figure 1G). Taken together, the data showed that fibroblasts contain an IL-17A-triggered positive-feedback loop of IL-6 expression, which is mediated through NF-κB and STAT3.

Enhancement of the IL-17A-Triggered Positive-Feedback Loop of IL-6 Expression in F759 Mice

We next hypothesized that excess IL-6 signaling resulting from an enhancement of this positive-feedback loop in fibroblasts is involved in autoimmune diseases. To test this hypothesis, we used F759 mice as an animal model; the IL-6–gp130-STAT3 signaling pathway is specifically enhanced in these mice because of the lack of SOCS3-dependent negative feedback. As expected, compared to the amounts observed in WT cells, F759 MEF cells expressed higher IL-6 and the NF-κB target KC in response to treatment with IL-17A plus IL-6 (Figures 2A and 2B), indicating that the IL-17A-triggered positive-feedback loop of IL-6 expression is enhanced in F759 MEF cells. Importantly, overexpression of IL-17A resulted in a larger increase in the serum IL-6 concentration in young F759 mice (2 months old and arthritis free) compared with that observed in age-matched WT animals (Figures 2C). These results demonstrated that the IL-17A-triggered positive-feedback loop of IL-6 expression is enhanced in F759 mice. This conclusion was further supported by the significantly higher serum IL-17A concentrations in older F759 mice compared to young F759 or age-matched WT mice (Figure 2D), the increased numbers of Th17 cells in older F759 mice compared with control mice (Figure 2E), and the elevated serum IL-6 concentration observed in older F759 mice (Figure 2F). Thus, F759 mice especially in age had an enhanced IL-17A-triggered positive-feedback loop of IL-6 expression.

Soluble IL-6 receptor (IL-6R) has been detected in the serum of autoimmune-prone mice as MRL/lpr, BWF1, and BXSB mice (Suzuki et al., 1993). We examined whether the amount of
soluble IL-6R is increased in F759 mice and found that the serum concentration of soluble IL-6R age-dependently increased in F759 mice (Figure S2A), although young F759 and age-matched WT mice show similar amounts (data not shown). We also showed that soluble IL-6R enhanced IL-6 production in MEF cells in the presence of IL-6 and IL-17A (Figure S2B). Thus, soluble IL-6R may play a role to IL-17A-triggered positive-feedback loop of IL-6 expression in F759 mice with age.

IL-6 Downstream of IL-17A Is Involved in the Development of Arthritis

We then looked for a link between the enhanced IL-17A-triggered positive-feedback loop of IL-6 expression and the development of autoimmune arthritis in the F759 mice. We first established double mutant animals by crossing mice carrying the Y759F mutation in gp130 with IL-17A-deficient (Il17a<sup>-/-</sup>) mice. Depleting F759 mice of IL-17A significantly suppressed the development of arthritis (Figure 3A), indicating that the arthritis progression is dependent on IL-17A. WT mice show similar amounts (data not shown). We also showed that soluble IL-6R enhanced IL-6 production in MEF cells in the presence of IL-6 and IL-17A (Figure S2B). Thus, soluble IL-6R may play a role to IL-17A-triggered positive-feedback loop of IL-6 expression in F759 mice with age.

Figure 2. The IL-17A-Triggered Positive-Feedback Loop of IL-6 Expression Is Enhanced in F759 Mice

(A and B) MEF cells were prepared from F759 mice and then stimulated with human IL-6 + soluble IL-6R and/or IL-17A for 24 hr. The culture supernatant was collected and assessed with ELISAs specific for (A) IL-6 or (B) keratinocyte-derived cytokine (KC).

(C) F759 mice (2 months old) were intravenously injected with mock or IL-17A expression vector. Serum concentrations of IL-6 were determined with ELISAs.

(D–F) Young F759 (2 months old), older F759 (10–12 months old), or control mice were sacrificed and T cells isolated from their lymph nodes and spleens were subjected to intracellular labeling of IL-17A. (E) The actual number of CD4<sup>+</sup>CD44<sup>hi</sup>IL-17A<sup>+</sup> T cells was calculated. Serum concentrations of IL-17A (D) and IL-6 (F) were determined with ELISAs.

All graphs show mean ± SD of more than three independent experiments. p values were calculated with Student’s t tests. *p < 0.05; **p < 0.01; ***p < 0.005.
in F759 mice is dependent in IL-17A. To confirm this result, we overexpressed IL-17A by using the hydrodynamics-based transfection method (Zhang et al., 1999) in young F759 mice (2 months old and arthritis free) and used the mice by the time they reached 4–5 months of age, because we have found that the clinical scores of F759 mice normally show a significant difference over 8–10 days, and assessed for signs of arthritis.

Data represent the mean ± SEM of the scores. p values were calculated with Student’s t tests. *p < 0.05; **p < 0.01; ***p < 0.005.

IL-6–STAT3 Signaling in Fibroblasts Plays a Role for the Development of Arthritis in F759 Mice

To examine whether or not IL-6 is a critical downstream target of IL-17A for the development of arthritis in F759 mice, we overexpressed IL-6 via the hydrodynamics-based transfection method in young F759 mice (2 months old and arthritis free) and used the mice by the time they reached 4–5 months of age. IL-6 overexpression resulted in the development of arthritis in the young F759 mice but not in control C57BL/6 mice (Figure 3C and Figure S3B) as was observed for IL-17A overexpression (Figure 3B and Figure S3A), although IL-6 overexpression did not markedly increase the serum concentrations of IL-17A in the F759 mice (data not shown). These results indicated that the enhanced IL-6 signaling contributes to arthritis in F759 mice and suggested that IL-6 is a critical downstream target of IL-17A during this process.

To examine the relative positions of IL-17A and IL-6 in this signaling hierarchy directly, we used the two double mutant mouse lines: IL-17a−/− F759 and IL-6−/− F759 mice. We then overexpressed IL-17A and IL-6 in the IL-6−/− F759 and IL-17a−/− F759 mice, respectively; IL-6 expression induced arthritis in the IL-17a−/− F759 mice, whereas IL-17A did not result in the development of arthritis in the IL-6−/− F759 mice (Figures 4A and 4B, and Figure S3A and S3B). These data showed that IL-6 is a downstream target of IL-17A in the IL-17A-dependent arthritis that develops in F759 mice. Taken together, the results show that excess IL-6 expression, which is induced by an IL-17A-triggered positive-feedback loop, leads to an autoimmune arthritis in F759 mice.

IL-6–STAT3 Signaling in Fibroblasts Plays a Role for the Development of Arthritis in F759 Mice

To investigate whether the F759 mutation in nonhematopoietic cells is required for the development of the spontaneous arthritis,
CD4+ T cells expressing high amounts of CD44 as well as those day 11 (data not shown). The percentages and numbers of 6B and 6C). These results showed that IL-6–STAT3 signaling in suppression of EAE after the Th17 cells were transferred (Figures 6B and 6C). The transfer of Th17 nonhematopoietic cells (type I collagen + cells) contributes to the development of EAE. We showed that con- moles in WT control mice by using pathogenic Th17 cells from other WT mice (Figure 6 A). This result was consistent with the of CD4+CD25+ cells among the CD4+CD44hi T cells were also similar in these mice (Figures S4A–S4D). Importantly, the expression of IL-17F and IFN-γ were also comparable between Il17a−/− and WT T cells cultured in vitro (Figures S4F and S4G), whereas IL-17A was not detected in cultured CD4+ T cells from Il17a−/− mice (Figure S4E). These results suggested that IL-17A deficiency does not markedly affect Th17 cell development. Then, we transferred the CD4+ T cells into WT mice and investigated the development of EAE. We showed that a lack of IL-17A expres- level of IL-17A-triggered positive-feedback loop of IL-6 expres- we deleted STAT3 from the type I collagen+ fibroblasts of F759 mice by using the Cre-loxP system. The development of arthritis was significantly suppressed in the type I collagen-Cre-STAT3flx/flox F759 mice compared with controls (Figure 5). These results showed that IL-6–STAT3 signaling, a component of the IL-17A-triggered positive-feedback loop of IL-6 expression, in type I collagen+ fibroblasts is important for the develop- means ± SEM of the scores. p values were calculated with Student’s t tests. *p < 0.05; **p < 0.005.

The IL-17A-Mediated Positive-Feedback Loop of IL-6 Expression Contributes to Enhanced Autoimmunity in EAE

To determine whether the IL-17A-mediated positive-feedback loop of IL-6 expression promotes and/or enhances autoimmunity in WT mice, we performed several in vivo transfer experiments in WT mice by using pathogenic Th17 cells from mice that had challenged EAE. We cultured CD4+ T cells from EAE mice with bone marrow-derived dendritic cells in the presence of MOG peptide plus IL-23, resulting in IL-17A-producing MOG-specific CD4+ T cells (Figure S4E). The transfer of Th17 cells induced EAE and increased the serum IL-6 concentration in WT mice (Figure 6A). This result was consistent with the IL-17A-mediated IL-6 expression contributing to EAE in WT mice.

To further examine this possibility, we transferred the Th17 cells into Il6−/−, type I collagen-Cre-STAT3flx/flox, or control WT mice to examine the development of EAE. We showed that control animals developed EAE disease. Importantly, both Il6−/− and type I collagen-Cre-STAT3flx/flox mice showed at least partial suppression of EAE after the Th17 cells were transferred (Figures 6B and 6C). These results showed that IL-6–STAT3 signaling in nonhematopoietic cells (type I collagen+ cells) contributes to Th17 cell-mediated EAE in WT mice. Additionally, we induced Th17 cell development from Il17a−/− and control mice immunized with MOG peptide by the same method. The percentages and numbers of CD4+ T cells in the inguinal lymph nodes were similar in Il17a−/− and control mice immunized with MOG peptide on day 11 (data not shown). The percentages and numbers of CD4+ T cells expressing high amounts of CD44 as well as those

**DISCUSSION**

We found here that IL-17A together with IL-6 synergistically increased IL-6 expression in MEF cells through NF-κB and STAT3 signaling. Indeed, in vivo expression of IL-17A increased...
the serum IL-6 concentration. These results demonstrate that an IL-17A-triggered positive-feedback loop of IL-6 expression is present in WT fibroblasts. IL-17A and IL-6 also synergistically induced the expression of various NF-κB target genes, including KC, MIP2, and IL-6. IL-17A mediated the expression of these genes in a manner that was IL-6 dose dependent. Thus, these results suggested that STAT3 may directly or indirectly interact with NF-κB, as reported previously (Battle and Frank, 2002; Hagihara et al., 2005; Yang et al., 2007).

We hypothesized that dysregulation of this feedback loop may contribute to the development of various IL-17A- and IL-6-mediated autoimmune diseases. We first selected F759 mice as an animal model to examine the dysregulation of the loop and demonstrated that the spontaneously developing arthritis in F759 mice is dependent on IL-17A: F759 mice lacking IL-17A showed significantly reduced clinical arthritis scores, and overexpression of IL-17A in young arthritis-free F759 mice induced the development of arthritis. Importantly, overexpression of IL-17A did not trigger arthritis in Il6−/− F759 mice, whereas overexpression of IL-6 induced the disorder in Il17a−/− F759 mice, indicating that IL-6 is a critical downstream target of IL-17A. Consistent with this result, IL-17A expression greatly increased the expression of IL-6 in F759 mice.

To detail the relationship between the enhancement of the IL-17A-triggered positive-feedback loop of IL-6 signaling and arthritis in F759 mice, we have performed several in vivo and in vitro experiments, which led to the following findings: (1) the serum IL-6 and IL-17A concentrations markedly increased in an age-dependent manner in F759 mice compared to the concentrations observed in WT mice; (2) IL-17A overexpression produced a larger increase in the serum IL-6 concentration in F759 mice than that in WT mice; and (3) IL-6 expression via the IL-17A-triggered positive-feedback loop was enhanced in F759 MEF compared to WT MEF cells. Collectively, the data underscore the central role of the enhanced signaling mediated through the IL-17A-triggered positive-feedback loop of IL-6 expression in fibroblasts in the development of arthritis in F759 mice. In fact, specific deletion of the essential loop component STAT3 in type I collagen+ fibroblasts suppressed the development of arthritis in F759 mice. Because F759 mice lack SOCS3-mediated negative feedback only in the gp130 signaling axis, it is reasonable to speculate that specifically deleting SOCS3 in nonhematopoietic cells would also increase the risk of autoimmune and/or inflammatory diseases. Consistent with this notion, SOCS3 deficiency in liver cells increased the degree of liver fibrosis (Ogata et al., 2006), which likely mirrors NF-κB activation in fibroblasts. In other words, dysregulated NF-κB activation in type I collagen+ fibroblasts may trigger the feedback loop to increase IL-6 expression in F759 mice, in which IL-6-mediated STAT3 activation is already dysregulated. Consistent with this scenario, we previously showed that transgenic expression of HTLV-1 Tax, which activates NF-κB, enhanced the IL-6-dependent development of arthritis in F759 mice (Ishihara et al., 2004). Moreover, viral IL-17A from herpesvirus saimiri was shown to activate NF-κB and induce IL-6 production in fibroblasts (Yao et al., 1995). These results suggest that viral infections, which stimulate the NF-κB signaling pathway in fibroblasts, may result in an increased risk for the development of autoimmune diseases by triggering the positive-feedback loop controlling IL-6 expression.

Considering the requirement of IL-6 for Th17 cell development (Bettelli et al., 2007; Nishihara et al., 2007), upregulation of IL-6 expression may expand the Th17 cell population on one hand, while also amplifying the IL-17A-triggered positive-feedback loop. This hypothesis is supported by results showing that both the number of Th17 cells and the serum IL-17A concentration were elevated in older F759 mice compared with WT control mice. Therefore, we propose that two cell populations, type I collagen+ fibroblasts and Th17 cells, are components of an IL-6-signal amplifying loop in vivo. Under normal conditions, the IL-6 amplifier is controlled by several negative-feedback mechanisms, which maintain homeostasis of IL-6 and IL-17A signaling. Under pathological conditions, in which Th17 cells trigger an autoimmune disease, the IL-6 amplifier abnormally enhances signaling; the dysregulated enhancement mediated by the amplifier may be induced by dysregulated activation of NF-κB and/or STAT3 in fibroblasts via a variety of stochastic environmental and/or genetic factors.

IL-17A is a member of cytokine subfamily and is highly homologous to IL-17F, a cytokine known to stimulate the NF-κB pathway. Because IL-17A deficiency resulted in a lower but recognizable arthritis in F759 mice, we hypothesized that IL-17F in addition to IL-17A may contribute to disease development. We found that IL-17F was barely detectable in F759 mice and IL6−/− F759 mice regardless of age, whereas it was present in Il17a−/− F759 mice (data not shown). These results suggested that, under specific conditions, IL-17F may be involved in the development of autoimmune arthritis.

Numerous studies support strategies that target TNF-α for the treatment of rheumatoid arthritis as well as other chronic autoimmune diseases (Feldmann and Maini, 2001). Moreover, TNF-α acts in concert with IL-17A to induce IL-6 production (Awane et al., 1999; Chen et al., 2003; Ruddy et al., 2004). Therefore, TNF-α may also enhance the IL-6 amplifier proposed here. Consistent with this idea, we observed that depletion of TNF-α in F759 mice partially but significantly inhibited the development of arthritis (data not shown). In addition, TLR-mediated signaling is known to induce IL-6 expression (Akira et al., 2001; Medzhitov et al., 1997); for example, spontaneous arthritis develops in SKG mice in response to pathogens, whereas the mice remain healthy under specific pathogen-free conditions (Sakaguchi et al., 2003). This pathogen response can be mimicked via zymosan, which triggers IL-6 release (Hata et al., 2004). It is possible that IL-6 expression induced by TLR-mediated signaling triggers the IL-6 amplifier especially in the presence of activation of Th17 cells via the pathogens. Thus, a number of factors, including such cytokines as TNF-α and other IL-6 family cytokines, TLR-mediated signaling, and soluble IL-6R, may act upstream of the IL-6 amplifier, which may explain why targeting IL-6 signaling shows therapeutic efficacy in humans with rheumatoid arthritis (Nishimoto and Kishimoto, 2004).

It should be noted, however, that other mechanisms can induce autoimmune diseases, because not all rheumatoid arthritis patients respond to anti-IL-6R therapy and depletion of IL-6 does not universally suppress the development of autoimmune diseases in experimental models. Indeed, recent data has highlighted a critical role for IL-23 in generating pathogenic Th17 cells, whereas IL-6 alone generates T cells that produce both IL-10 and IL-17A (McGeachy et al., 2007), suggesting that...
IL-6-independent mechanisms can induce Th17-mediated autoimmune diseases. Thus, the mechanisms underlying the development of autoimmune diseases are complex. Here we have shown that dysregulation of an IL-17A-triggered positive-feedback loop of IL-6 expression in fibroblasts, at least in part, drives the IL-17A-mediated autoimmune arthritis observed in F759 mice.

It is important to show that this IL-17A-triggered positive-feedback loop of IL-6 expression in type I collagen+ fibroblasts mediates other autoimmune diseases. Because it was reported that a neuroinflammation induced activation of fibroblasts followed by type I collagen expression in spinal cord (Okada et al., 2007), we used EAE as another experimental model. In addition, to exclude a possible involvement of IL-6 in Th17 cell development in vivo, we employed a Th17 cell-transfer system to show that the feedback loop may play a role in EAE in WT mice. Injection of established MOG-specific Th17 cells into WT mice increased IL-6 expression, which was followed by encephalomyelitis, and IL-6 deficiency in the recipient mice attenuated the development of EAE after Th17 cells were injected. Moreover, specific depletion of STAT3 in type I collagen+ fibroblasts attenuated EAE development. Finally, depletion of IL-17A from the injected MOG-specific Th17 cells at least partially suppressed the development of EAE. Together, these results strongly support a role for an IL-17A-triggered positive-feedback loop of IL-6 expression in nonhematopoietic cells in the development of EAE in WT mice. Therefore, we hypothesize that the IL-17A-triggered positive-feedback loop of IL-6 expression in nonhematopoietic cells may provide a general etiologic mechanism for various autoimmune diseases.

**EXPERIMENTAL PROCEDURES**

**Mouse Strains**
C57BL/6 mice were purchased from Japan SLC. The F759 mouse line carrying a human version of gp130 (ST10L) was established previously (Atsumi et al., 2002; Ishihara et al., 2004; Sawada et al., 2006). IL-6-deficient mice were provided by M. Kopf (Max-Planck-Institute of Immunobiology, Germany), backcrossed with C57BL/6 mice more than 10 times, and crossed with F759 mice. Type I collagen-Cre mice were provided by G. Karsenty (Baylor College of Medicine, Houston, TX) and crossed with STAT3fl/fl mice that were provided by M. Kopf (Max-Planck-Institute of Immunobiology, Germany), backcrossed with C57BL/6 mice more than 10 times, and crossed with F759 mice. All mice were maintained under specific pathogen-free conditions according to the protocols of the Osaka University Medical School. All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committees of the Graduate School of Frontier Bioscience and Graduate School of Medicine, Osaka University.

**Clinical Assessment of Arthritis and Histological Analysis**
Mice were inspected and assessed for signs of arthritis as described previously (Huseby et al., 2001). Surface markers for CD4+ T cells after culture (data not shown), Cytokine concentrations in supernatants of cultured Th17 cells from IL-17A-deficient and WT control mice were determined with ELISA kits (BD Biosciences, eBiosciences, or R&D).

**Induction of Th17 Cell Differentiation and EAE Development**
WT or IL-17A-deficient mice were injected with MOG(33-55) peptide (synthesized by Sigma) in complete Freund’s adjuvant (Sigma-Aldrich) at the base of the tail on day 0 followed by intravenous injection of pertussis toxin (List Biological Laboratories) on days 0, 2, and 7. On day 11, CD4+ T cells were then sorted with anti-CD4 microbeads (BD Biosciences). The resulting CD4+ T cell-enriched population (2 × 10^6 cells) was cocultured with rIL-23 (20 ng/ml; R&D) in the presence of MOG peptide-pulsed bone marrow-derived dendritic cells (5 × 10^5 cells) for 3 days. Cells (5 × 10^6 cells) were then injected intravenously into sublethally irradiated (5 gray) recipients on day 0 followed by intravenous injection of pertussis toxin on days 0, 2, and 7. The clinical score used is described previously (Huseby et al., 2001). Surface markers for CD4+ T cells, such as TCR, CD4, CD44, and CD25, were examined with the Th17 cells. For cell-surface labeling, 10^6 T cells were incubated with fluorescence-conjugated antibodies for 30 min on ice. The cells then were analyzed with CyAn flow cytometers (BeckmanCoulter) and the collected data were analyzed with Summit software (BeckmanCoulter) and/or Flowjo software (Tree Star).

**Antibodies and Reagents**
APC-conjugated anti-CD4 (BioLegend); FITC-conjugated anti-CD44 (eBiosciences); PE-conjugated anti-CD25 (eBiosciences), anti-IL-17A (eBiosciences) or control-IgG2a (eBiosciences); PE-Cy5-conjugated anti-CD4 (BioLegend), and biotin-conjugated anti-CD8 (eBiosciences), anti-CD19 (eBiosciences), anti-I-A/I-E (BioLegend), or anti-NK.1.1 (eBiosciences) antibodies as well as streptavidin-conjugated Pacific Blue (Invitrogen) were used for cell staining.

**Flow Cytometry**
For cell-surface labeling, 10^6 cells were incubated with fluorescence-conjugated antibodies for 30 min on ice. The cells then were analyzed with CyAn flow cytometers (BeckmanCoulter) and the collected data were analyzed with Summit software (BeckmanCoulter) and/or Flowjo software (Tree Star).

**Cell Preparation and Stimulation**
MEF cells from WT and F759 fetuses (14–15 days postcoitus) were prepared as described previously (Sawa et al., 2006). NEMO-deficient MEF cells were kindly provided by M. Pasparakis (University of Cologne), M. Safto, and S. Akira (Osaka University, Japan). IL-12B-deficient MEF cells were kindly provided by M. Yamamoto and S. Akira (Osaka University, Japan). MEF cells were plated in 6-well plates (2 × 10^5 cells/well) and stimulated with IL-6 plus soluble IL-6R (20 ng/ml each) and/or IL-17A (50 ng/ml) for 24 hr after 2 hr of serum starvation. Human IL-6 (Toray), human soluble IL-6R (R&D Systems), and mouse IL-17A (R&D Systems) were used for the experiments. For knockdown of STAT3, MEF cells were transfected with specific siRNA (Sigma-Aldrich) with GenePORTER transfection reagent (Genlantis) 48 hr before the cells were stimulated with the cytokines. Cells were harvested, total RNA was prepared for real-time PCRs, and cell-culture supernatant was collected for ELISA assays.

**Real-Time PCRs**
A GeneAmp 5700 sequence detection system (ABI, Warrington, USA) and SYBER green PCR Master Mix (Sigma-Aldrich) were used to quantify the levels of IL-6 mRNA, IL-6 receptor mRNA, and HPRT mRNA. Total RNA was prepared from MEF cells with a GenElute Mammalian Total RNA kit (Sigma-Aldrich) and DNase I (Sigma-Aldrich). The PCR primer pairs used for real-time
PCRs were as follows: mouse HPRT primers, 5'-GATTACGATGATGACAAGGTT-3' and 5'-CTTCCATCTCCTCTAGACA-3'; mouse interleukin-6 receptor primers, 5'-ACAGTTGGAAGAACTGTCCT-3' and 5'-CCTGTAACCTCTTGTGACCAT-3'. The conditions for the real-time PCRs were 40 cycles of 94°C for 15 s followed by 60°C for 60 s. The relative mRNA expression levels were normalized to the levels of HPRT mRNA.

Statistical Analysis

Student’s t tests (two-tailed) were used for statistical testing between two groups.

SUPPLEMENTAL DATA

Supplemental Data include four figures and can be found with this article online at http://www.immunity.com/cgi/content/full/29/4/628/DC1/.

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