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STAT3 and NF-κB Signal Pathway Is Required for IL-23-Mediated IL-17 Production in Spontaneous Arthritis Animal Model IL-1 Receptor Antagonist-Deficient Mice[1]

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IL-23 is a heterodimeric cytokine composed of a p19 subunit and the p40 subunit of IL-12. IL-23 has proinflammatory activity, inducing IL-17 secretion from activated $CD4^+$ T cells and stimulating the proliferation of memory $CD4^+$ T cells. We investigated the pathogenic role of IL-23 in $CD4^+$ T cells in mice lacking the IL-1R antagonist ($IL\text{-}1Ra^{-/-}$), an animal model of spontaneous arthritis. IL-23 was strongly expressed in the inflamed joints of $IL\text{-}1Ra^{-/-}$ mice. Recombinant adenovirus expressing mouse IL-23 (rAd/mIL-23) significantly accelerated this joint inflammation and joint destruction. IL-1β further increased the production of IL-23, which induced IL-17 production and OX40 expression in splenic $CD4^+$ T cells of $IL\text{-}1Ra^{-/-}$ mice. Blocking IL-23 with anti-p19 Ab abolished the IL-17 production induced by IL-1 in splenocyte cultures. The process of IL-23-induced IL-17 production in $CD4^+$ T cells was mediated via the activation of Jak2, PI3K/Akt, STAT3, and NF-κB, whereas p38 MAPK and AP-1 did not participate in the process. Our data suggest that IL-23 is a link between IL-1 and IL-17. IL-23 seems to be a central proinflammatory cytokine in the pathogenesis of this $IL\text{-}1Ra^{-/-}$ model of spontaneous arthritis. Its intracellular signaling pathway could be useful therapeutic targets in the treatment of autoimmune arthritis. *The Journal of Immunology,* **2006, 176: 5652–5661.**

Interleukin-23 plays a pivotal role in the establishment and maintenance of organ-specific inflammatory autoimmune diseases. IL-23-deficient ($IL\text{-}23p19^{-/-}$) mice are resistant to experimental autoimmune encephalomyelitis (1) and collagen-induced arthritis (2), highlighting the important role of this cytokine in autoimmune pathogenesis (3, 4). IL-12 and IL-23 share a common p40 subunit, but contain unique p35 and p19 subunits, respectively (5, 6). They are the critical cytokines that bridge innate and adaptive immunities, but they have different activities. IL-12 is a factor in the differentiation of naive T cells into IFN-γ-producing Th1 cells; it is essential for the antimicrobial response (7), and acts as a suppressor of human B cell tumors (8). IL-23 promotes a T cell population characterized by the production of IL-17, IL-17F, TNF, and IL-6 (4, 9–11). IL-23-driven T cells, a novel subset of T cells that produce IL-17 ($Th_{IL\text{-}17}$), are highly pathogenic, in contrast to Th1 cells, which do not induce experimental autoimmune encephalomyelitis. IL-23, but not IL-12, is essential for the induction and maintenance of inflammatory autoimmune diseases in the brain and joints (2, 12). The IL-23/IL-17 pathway is strongly regulated by the IL-12/IFN-γ axis of immune regulatory factors. Because both the Th1 and $Th_{IL\text{-}17}$ responses are often induced simultaneously, they can cross-regulate each other when the responses are highly elevated (4, 13). The role of IL-23 in stimulating the production of IL-17 is important in the pathogenesis of autoimmune arthritis. It has been reported recently that collagen-induced arthritis does not develop in $IL\text{-}23p19^{-/-}$ mice and, significantly, the synthesis of $Th_{IL\text{-}17}$ cells and IL-17 is markedly reduced (2).

IL-17 is mainly released by memory T cells, and is particularly associated with the severity of inflammation in the synovia of patients with rheumatoid arthritis (RA)[4] (14, 15). IL-17 activates NF-κB and stimulates the production of several inflammatory mediators, including IL-6, IL-8, GM-CSF, and PGE_2 in synoviocytes (16–20). IL-17 appears to be an essential mediator of T cells in fine-tuning the inflammatory response. It has been suggested that IL-17 is involved in the development of RA (17). The incidence of arthritis can be partly reduced by the administration of a fusion protein composed of the extracellular domain of the IL-17R and Fc (IL-17R:Fc), which inhibits IL-17–IL-17R binding in the elicitation phase of collagen-induced arthritis (21).

The IL-1R antagonist (IL-1Ra) is an endogenous inhibitor of IL-1 and is believed to regulate IL-1 activity. Polyarthritis develops in IL-1Ra-deficient ($IL\text{-}1Ra^{-/-}$) mice on the BALB/c background at age 5 wk, and by 12 wk of age, almost all mice are affected (22). The histopathology of the lesions closely resembles

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[4] Abbreviations used in this paper: RA, rheumatoid arthritis; rAd/mIL-23, recombinant adenovirus expressing mouse IL-23; PDTC, pyrrolidine dithiocarbamate; siRNA, small interfering RNA; eGFP, enhanced GFP; WT, wild type; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

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that of RA in humans, with marked synovial and periarticular inflammation and articular erosion caused by the invasion of granulation tissue (22). High levels of autoantibodies directed against Ig, type II collagen, and dsDNA are detectable in serum (22). In this model, excess IL-1 signaling due to a deficiency in the IL-1Ra causes T cell-mediated autoimmunity, resulting in joint-specific inflammation and bone destruction (22–24). IL-17 expression is greatly enhanced in IL-1Ra$^{-/-}$ mice, suggesting that IL-17 activity is involved in the pathogenesis of arthritis in these mice. Arthritis does not develop spontaneously in IL-1Ra$^{-/-}$ mice that are also deficient in IL-17 (24).

In this study, to clarify the pathogenesis of autoimmune arthritis in IL-1Ra$^{-/-}$ mice, we investigated the role of IL-23 in CD4$^+$ T cells from IL-1Ra$^{-/-}$ mice, which exhibit excess IL-1 signaling and are an animal model of spontaneous arthritis. We also examined the signal pathway by which IL-23 induces IL-17 production in the CD4$^+$ T cells of IL-1Ra$^{-/-}$ mice.

Materials and Methods

Animals

IL-1Ra$^{-/-}$ mice in the BALB/c background were provided by Y. Iwakura (University of Tokyo, Tokyo, Japan) and were maintained under specific pathogen-free conditions at the Institute of Medical Science, Catholic University of Korea, and fed standard mouse chow (Ralston Purina) and water ad libitum. All experimental procedures were examined and approved by the Animal Research Ethics Committee at the Catholic University of Korea.

Reagents

Recombinant Abs directed against IL-12, IL-23, IL-12p40, IL-17, IFN-γ, IL-1β, and IL-23p19 were purchased from R&D Systems. Pyrrolidine dithiocarbamate (PDTC) and LPS (*Escherichia coli* O111:B4) were obtained from Sigma-Aldrich. Anti-CD3 mAb was obtained from BD Biosciences. Cell signaling inhibitors AG490, LY294002, SB203580, SP600125, and PD98059 were obtained from Calbiochem.

Construction of replication-defective adenoviruses expressing IL-23

Recombinant replication-defective adenoviruses were generated according to AdEasy Vector System (Qbiogene) as previously described (25). Briefly, the bicistronic cDNA of IL-23 encoding p19 and p40 (26) or the enhanced GFP (eGFP) cDNA (BD Clontech) were subcloned into the *Sal*I/*Not*I and *Kpn*I/*Xba*I sites of the adenoviral shuttle vector (pShuttleCMV), respectively. pShuttleCMV/IL-23 and pShuttleCMV/eGFP constructs were cotransformed with adenoviral backbone vector, pAdEasy, into *E. coli* BJ5183 by electroporation to achieve homologous recombination. The recombinant obtained was transfected into 293 cells by the conventional calcium phosphate coprecipitation method. Recombinant adenovirus was isolated from a single plaque, expanded in 293 cells, and purified by double cesium chloride ultracentrifugation. Purified viruses were extensively dialyzed against 10 mM Tris, 5% sucrose, 2 mM $MgCl_2$ and stored in aliquots at −80°C. Titers of adenovirus were determined by $TCID_{50}$ and by plaque assays in 293 cells.

Delivery of adenoviral vector

IL-1Ra$^{-/-}$ mice were maintained until 8 wk of age. Mice were injected intra-articularly with recombinant adenovirus expressing mouse IL-23 (rAd/mIL-23) (1×10^8 PFU per joint for each vector), recombinant adenovirus encoding eGFP (rAd/eGFP), or PBS. Mice were maintained under isofluorane anesthesia, their knees were swabbed with 70% ethanol, and 20 μl of the treatment solution was injected into the synovial space with a 30-gauge needle. Seven days later, the knee joints were injected again with rAd/mIL-23, rAd/eGFP, or PBS. After two injections, the periarticular arthritic development of the hind paw and ankle joint was monitored for 4 wk, at which time the experiment was terminated and the mice were killed. Blood samples were collected from all treated and control mice and the sera were stored at −80°C until use.

Assessment of arthritis

Joint swelling was monitored by inspection and given an arthritic score as follows: 0, no joint swelling; 1, slight edema and erythema limited to the foot and/or ankle; 2, slight edema and erythema from the ankle to the tarsal bone; 3, moderate edema and erythema from the ankle to the tarsal bone; 4, edema and erythema extending from the ankle to the entire leg, with severe swelling of the wrist or ankle. The final arthritis score was calculated as the sum of scores from all four legs, assessed by three independent observers with no knowledge of the experimental groups.

Analysis of IgG Ab subtypes

Blood samples collected from IL-1Ra$^{-/-}$ and WT mice were used to investigate IgG Ab subtype concentrations using the mouse IgG1/IgG2a ELISA quantitation kit (Bethyl Laboratories). Levels of IgG1 and IgG2a were measured in mice sera diluted 50,000- to 400,000-fold.

Histological analysis

IL-1Ra$^{-/-}$ and wild-type (WT) mice were killed by cervical dislocation. Their knee joints were then dissected, fixed in 10% phosphate-buffered formalin for 1 day, decalcified in 10% EDTA for 8 h, and then embedded in paraffin. Tissue sections (7 μm) were prepared and stained with either H&E or safranin O.

Immunohistochemistry for IL-12p40, IL-23p19 subunit, IL-17, and IL-1β

Deparaffinized sections were incubated for 2 h at room temperature with specific Ab directed against murine IL-23p19, IL-12p40, IL-17, or IL-1β (R&D Systems), then incubated with an appropriate biotinylated secondary Abs and streptavidin-peroxidase conjugate, with *S*-(2-aminoethyl)-L-cysteine as substrate (Histostain-SP kit; Zymed Laboratories). Samples were counterstained with hematoxylin.

Cell preparation and culture

Spleens were collected for cell preparations from IL-1Ra$^{-/-}$ and WT mice. The spleens were meshed and the RBC lysed with 0.83% ammonium chloride. The cells were maintained in RPMI 1640 medium containing 10% FBS. To purify splenic CD4$^+$ T cells, splenocytes were incubated with CD4-coated magnetic beads (Miltenyi Biotec) and isolated on MACS separation columns (Miltenyi Biotec). The splenocytes, CD4$^+$ T cells or T cell depleted non-T cells were cultured with various stimuli, such as IL-23 (0.1–10 ng/ml), IL-12 (10 ng/ml), IL-12p40 (10 ng/ml), IL-1β (1–50 ng/ml), IL-17 (10 ng/ml), IFN-γ (10 ng/ml), LPS, anti-mouse CD3 mAb (5 μg/ml), and chemical cell signaling inhibitors such as PDTC (1 μM), SP600125 (1 μM) AG490 (50 μM), LY294002 (20 μM), PD98059 (20 μM), and SB203080 (5 μM) from 15 min to 120 h. The doses of chemical inhibitors were evaluated by MTT assay. The lacks of cytotoxicity were accessed by MTT assay

Detection by ELISA of cytokine production

Splenocytes, CD4$^+$ T cells, or T cell-depleted non-T cells were isolated and cultured at a density of 1×10^6 cells/ml in flat-bottom 48-well tissue culture plates (Corning). To determine the amount of IL-17 and IFN-γ in each supernatant, Abs directed against mouse IL-17, IFN-γ, and biotinylated anti-mouse IL-17, IFN-γ, and TNF-α Abs (R&D Systems) were used as the capture and detection Abs, respectively. The fluorescent substrate HRP-avidin (R&D Systems) was used for color development. The amounts of cytokines present in the test samples were determined from standard curves established with serial dilutions of recombinant murine IL-17 and IFN-γ (R&D Systems). The concentrations of IL-23 were determined using Ready-Set-Go Mouse IL-23(p19/40) ELISA (eBioscience) (27).

RT-PCR analysis of cytokine mRNA expression

mRNA was extracted using RNAzol B (Biotecx Laboratories) according to the manufacturer's instructions. Reverse transcription of 2 μg of total mRNA was conducted at 42°C using the Superscript Reverse Transcription system (Takara). PCR amplification of cDNA aliquots was performed by adding 2.5 mM dNTPs, 2.5 U *Taq* DNA polymerase (Takara), and 0.25 μM sense and antisense primers. The following sense and antisense primers for each molecules were used for: mice IL-23p19, 5′-TGCTGGATTG CAGAGCAGTAA-3′ (sense) and 5′-GCATGCAGAGATTCCGAGA GA-3′ (antisense); mice IL-12p40, 5′-GGAAGCACGGCAGCAGA ATA-3′ (sense) and 5′-AACTTGAGGGAGAAGTAGGAATGG-3′ (antisense); mice IL-17, 5′-GGTCAACCTCAAAGTCTTTAACTT-3′ (sense) and 5′-TTAAAAATGCAAGTAAGTTTG-3′ (antisense); mice IFN-γ, 5′-GAAAATCCTGCAGAGCCAGA-3′ (sense) and 5′-TGAGCTCATTGA ATGCTTGG-3′ (antisense); mice OX40, 5′-TACAAGCTTGGCCTTGA CTC-3′ (sense) and 5′-CAGGAGGCTTCTGTCCTCAC-3′ (antisense); mice CD40L, 5′-GGGTGTTTCATGTGCTGTTG-3′ (sense) and 5′-GGA

ATGAGGCATTTCCTTGA-3′ (antisense); mice STAT3, 5′-ACCAACA TCCTGGTGTCTCC-3′ (sense) and 5′-CATGTCAAACGTGAGCG ACT-3′ (antisense); and mice hypoxanthine-guanine phosphoribosyltransferase (HPRT), 5′-GTAATGATCAGTCAACGGGGGAC-3′ (sense) and 5′-CAGCAAGCTTGCAACCTTAACCA-3′ (antisense). Reactions were processed in a DNA thermal cycler (PerkinElmer Cetus) through 33 cycles of 94°C for 20 s, 58°C for 20 s, and 72°C for 20 s for IL-23p19 and IL-12p40; 32 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for IL-17, IFN-γ, OX40, CD40L, and STAT3; and 30 cycles of 94°C for 15 s, 53°C for 15 s, and 72°C for 30 s for HPRT. PCR products were run on a 1.5% agarose gel and stained with ethidium bromide. Results are expressed as the ratio of IL-23p19, IL-12p40, IL-17, IFN-γ, OX40, CD40L, and STAT3 PCR product to HPRT product.

Western blot analysis

Protein samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences). The membrane was preincubated with 5% skimmed milk in TBS for 2 h at room temperature. Primary Abs directed against IL-23p19 (R&D Systems), STAT3, phospho-STAT3 (BD Biosciences), Akt, IκB-α, and phosphorylated Akt and IκB-α (Cell Signaling Technology), diluted 1/1000 in 5% BSA/TBST (TBS with 0.1% Tween 20), were then added and the samples incubated overnight at 4°C. After the samples were washed four times with TBST, HRP-conjugated secondary Abs were added and incubated for 1 h at room temperature. After the samples were washed in TBST, the hybridized bands were detected with an ECL detection kit (Pierce) and Hyperfilm-ECL reagents (Agfa).

EMSA for STAT3 binding

After 15 min of treatments, nuclear proteins were extracted from $\sim 1 \times 10^7$ T cells. The STAT3 binding sequence probe (5′-GTGCATTTCCCGTA AATCTTGTCTACA-3′) (28) were generated by 5′ end-labeling of the sense strand with [γ-^{32}P]ATP (Amersham Biosciences) using T4 polynucleotide kinase (TaKaRa Shuzo). The probe was purified with NucTrap probe purification columns (Stratagene). Nuclear extracts (5 μg of protein) were incubated with radiolabeled DNA probe (10 ng to 100,000 cpm) for 30 min at room temperature in 20 μl of binding buffer consisting of 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 1 mM DTT, 0.5 mM EDTA, 5% glycerol, 1 mg/ml BSA, 0.2% Nonidet P-40, and 50 ng/μl poly(deoxyinosinic-deoxycytidylic acid). Samples were electrophoresed on a nondenaturing 5% polyacrylamide gel in 0.5× TBE buffer (pH 8.0) at 100 V. Gels were dried under vacuum and exposed to Kodak X-OMAT film at −70°C with intensifying screens.

Small interfering RNA (siRNA) transfection

Mouse STAT3 siRNA was designed by Dharmacon. CD4$^+$ T cells were plated in 24-well plates and transfected with 100 nM siRNA or 100 nM negative control siRNA conjugated with Alexa Fluor 488 (Qiagen) to monitor transfection efficiency, using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocol.

Statistical analysis

Experimental values are presented as means ± SD. Statistical significance was determined by Student's *t* test using the SPSS program (version 10.0). A value of $p < 0.05$ was considered statistically significant.

Results

Increased expression of IL-23, IL-17, and IL-1β in IL-1Ra$^{-/-}$ mice

To verify the pathogenic role of IL-23, immunostaining with specific Abs directed against IL-23p19, IL-12p40, IL-17, and IL-1β was performed on the joints of 11-wk-old IL-1Ra$^{-/-}$ and WT mice. IL-23p19-, IL-17-, and IL-1β-positive cells were readily detectable in the inflamed joints of the IL-1Ra$^{-/-}$ mice, whereas few positive cells were observed in the joints of the WT mice. The expression of the IL-12p40 subunit was markedly enhanced in the IL-1Ra$^{-/-}$ mice, but was weak in the joints of the WT mice (Fig. 1*A*). When the expression of IL-23p19 and IL-12p40 subunit mRNAs was examined in splenic non-T cells, depleted of CD4$^+$ cells using MACS, the level of IL-23p19 mRNA was higher in IL-1Ra$^{-/-}$ mice than in WT mice. Although IL-1β induced IL-23p19 mRNA more weakly in WT cells, IL-1β further increased the expression of IL-23p19 mRNA in the cells of IL-1Ra$^{-/-}$ mice (Fig. 1*B*). In contrast, the IL-12p40 subunit was expressed constitutively in both WT and IL-1Ra$^{-/-}$ mice. To confirm the expression and secretion of IL-23 protein, non-T cells were stimulated with IL-1β (1 or 10 ng/ml) or LPS (100 ng/ml) for 48 h; the amounts of IL-23 protein in the cells and culture supernatants were measured by Western blot analysis (Fig. 1*C*) and ELISA (Fig. 1*D*), respectively. The expression and secretion of IL-23 protein were increased dose dependently by IL-1β. These results show that the expression of IL-23 is elevated in the inflamed joints of IL-1Ra$^{-/-}$ mice and that IL-1β induces IL-23 expression at both the mRNA and protein levels in splenocytes from IL-1Ra$^{-/-}$ mice, a model of spontaneous arthritis.

IL-23 acts on IL-1Ra$^{-/-}$ mice, accelerating arthritis development

In considering the role of IL-23 in the etiology of arthritis, it has been reported that arthritis does not develop in the IL-23p19-deficient mice on collagen-induced arthritis model (2). To confirm this more directly, rAd/mIL-23 was prepared and injected into the joints of IL-1Ra$^{-/-}$ mice. The IL-1Ra$^{-/-}$ mice injected with rAd/mIL-23 showed arthritic scores higher than those of mice injected with rAd/eGFP or PBS, or those of WT (BALB/c) mice, especially at early time points after injection (Fig. 2*A*). From 1 wk after the first injection, the arthritis scores of the IL-23-treated group were almost twice as high as those of the controls, and this increase lasted throughout the observation period. This suggests that the expression of IL-23 accelerated the development of arthritis in IL-1Ra$^{-/-}$ mice and maintained the arthritis. The concentration of total IgG2a, which reflects the Th1 response, and of IgG1, which reflects the Th2 response, was higher in the IL-1Ra$^{-/-}$ than in the WT mice and was also significantly elevated by injection with rAd/mIL-23 (Fig. 2*B*).

The severe pathology observed histochemically, which included articular cartilage erosion, synovial inflammation, and pannus formation, was more severe in IL-1Ra$^{-/-}$ mice injected with rAd/mIL-23 than in the control groups (Fig. 2*C*). These results imply that an increase in exogenous IL-23 accelerates the progression of arthritis, and thus IL-23 may play a very important role in the pathogenesis of spontaneous arthritis in IL-1Ra$^{-/-}$.

IL-17 and IFN-γ production induced by IL-23 and IL-12, respectively, was significantly higher in IL-1Ra$^{-/-}$ mice than in WT mice

Next, we examined whether IL-23 increases the production of IL-17 in the splenocytes and CD4$^+$ T cells of IL-1Ra$^{-/-}$ and WT mice in similar ways, at both the mRNA and protein levels (Fig. 3, *A* and *B*). The expression of IL-17 mRNA was significantly increased in the presence of IL-23 in both the splenocytes and CD4$^+$ T cells of IL-1Ra$^{-/-}$ and WT mice. However, the magnitude of the induction was significantly higher in the IL-1Ra$^{-/-}$ mice than in the WT mice. IL-12 induced the production of IFN-γ, but not of IL-17, in both IL-1Ra$^{-/-}$ and WT mice. IL-12-induced IFN-γ production was higher in the IL-1Ra$^{-/-}$ mice than in the WT mice. The increase in IL-17 production induced by IL-23 in CD4$^+$ T cells was dose- and time-dependent (Fig. 3*C*).

IL-17 production induced by IL-1 is dependent on IL-23

Although IL-17 plays an important role in the etiology of arthritis in IL-1Ra$^{-/-}$ mice, IL-1 does not induce the production of IL-17 directly (24). Therefore, we tested whether IL-17 production can be induced by IL-1 indirectly in long-term cultures of splenocytes from IL-1Ra$^{-/-}$ mice. The splenocytes were cultured in vitro with several different concentrations of IL-1, and the production of IL-17 was measured. The expression of IL-17 was significantly

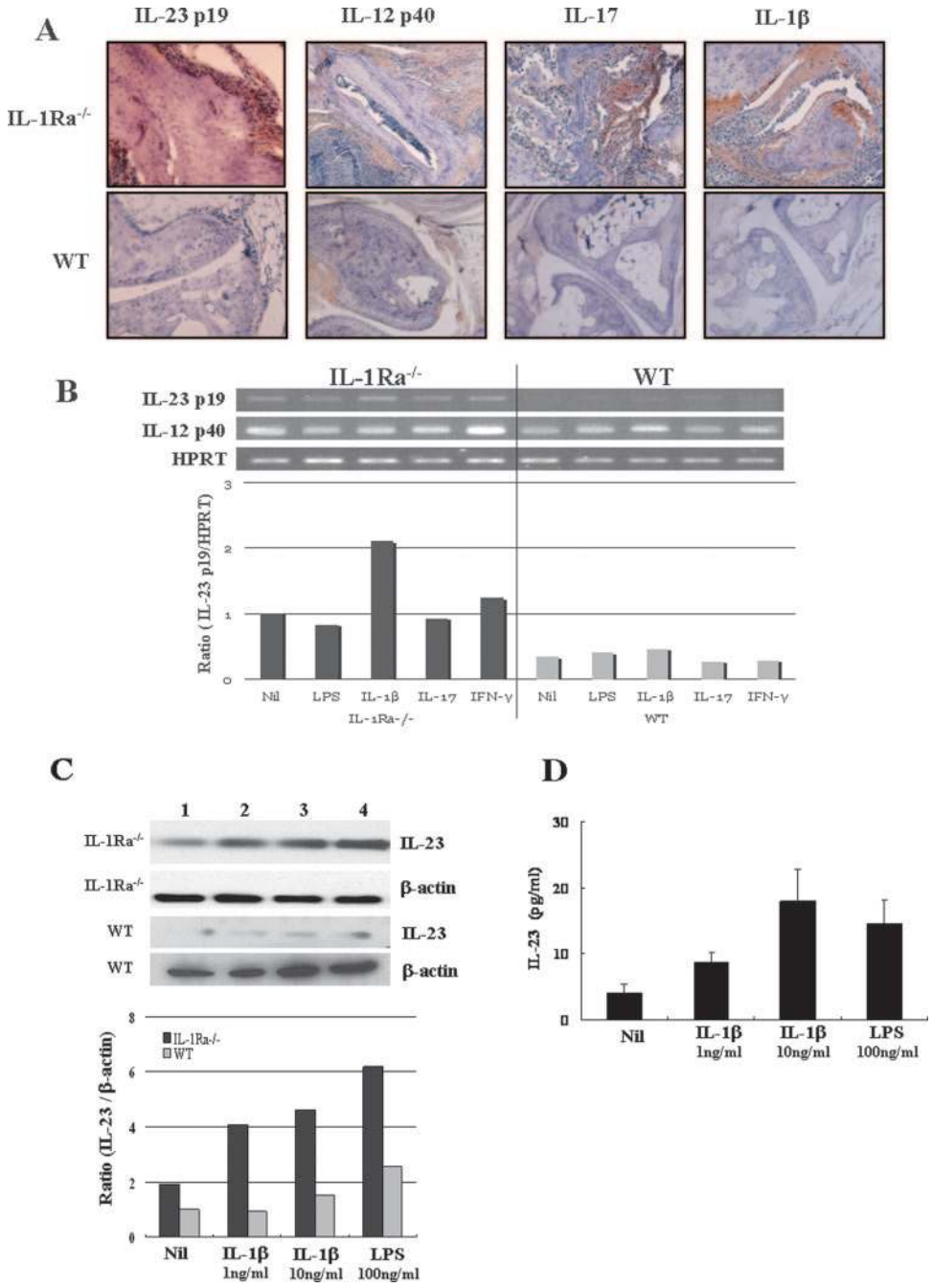

FIGURE 1. Increased IL-23, IL-17, and IL-1β expression in IL-1Ra$^{-/-}$ mice. *A*, The expression of IL-23p19, IL-12p40, IL-17, and IL-1β in the joints of 12-wk-old IL-1Ra$^{-/-}$ and WT mice. Immunostaining was performed using specific Abs. *B*, IL-23p19 and IL-12p40 mRNA expression in splenic non-T cells of IL-1Ra$^{-/-}$ and WT mice. Cells were cultured with LPS, IL-1β, IL-17, or IFN-γ for 12 h. Total RNA was extracted and subjected to semiquantitative RT-PCR. The expression of IL-23p19 was quantified densitometrically using LabWorks 4.0 software, and relative levels of expression were normalized to that of HPRT. The data represent the results of three similar experiments. *C*, Splenic non-T cells were cultured for 48 h with IL-1β or LPS and the induced IL-23p19 protein was analyzed by Western blot analysis using specific Abs. The optical densities of IL-23p19 bands were normalized to those of β-actin. The data represent one of three similar experiments. *D*, The secreted form of IL-23 was assessed by sandwich ELISA analysis from 48-h culture supernatants.

increased at later time points, such as after 3 or 5 days incubation with IL-1 (Fig. 4*A*). We hypothesized that IL-23 is a mediator of IL-1-induced IL-17 production at later time points. To test this, we added anti-IL-23p19 Ab to the splenocytes and measured IL-17 production at 5 days. As shown in Fig. 4*B*, IL-1-induced IL-17 production was inhibited by the presence of anti-IL-23p19 Ab but not by isotype Abs. These data strongly suggest that IL-23 is essential for the IL-17 production induced by treatment with IL-1. In other words, IL-23 is a mediator of the IL-1-induced IL-17 production in IL-1Ra$^{-/-}$ mice.

IL-23 enhances the expression of costimulatory molecules in CD4$^+$ T cells from IL-1Ra$^{-/-}$ mice

According to Iwakura et al. (24), the increase in costimulatory molecules, such as OX40, on the CD4$^+$ T cells of IL-1Ra$^{-/-}$ mice is associated with an increase in IL-17. Because we observed that the increase in IL-17 is mediated by IL-23, it was necessary to assess whether the enhanced expression of costimulatory molecules, such as OX40, is also mediated by IL-23 in IL-1Ra$^{-/-}$ mice. Splenic CD4$^+$ T cells obtained from IL-1Ra$^{-/-}$ mice and WT mice were cultured in vitro with IL-23, or with IL-12 as a control, and the increase in costimulatory molecules such as OX40 and CD40L was examined. A significant increase in the expression of OX40 costimulatory molecules induced by IL-23 was observed on IL-1Ra$^{-/-}$ cells compared with WT cells. IL-12 tended to increase OX40 production in both types of cells (Fig. 5). This finding indicates that IL-23 induces the expression of specific costimulatory molecules, such as OX40, on the CD4$^+$ T cells of IL-1Ra$^{-/-}$ mice and has a role in the immune activation that results in the development of arthritis in IL-1Ra$^{-/-}$ mice.

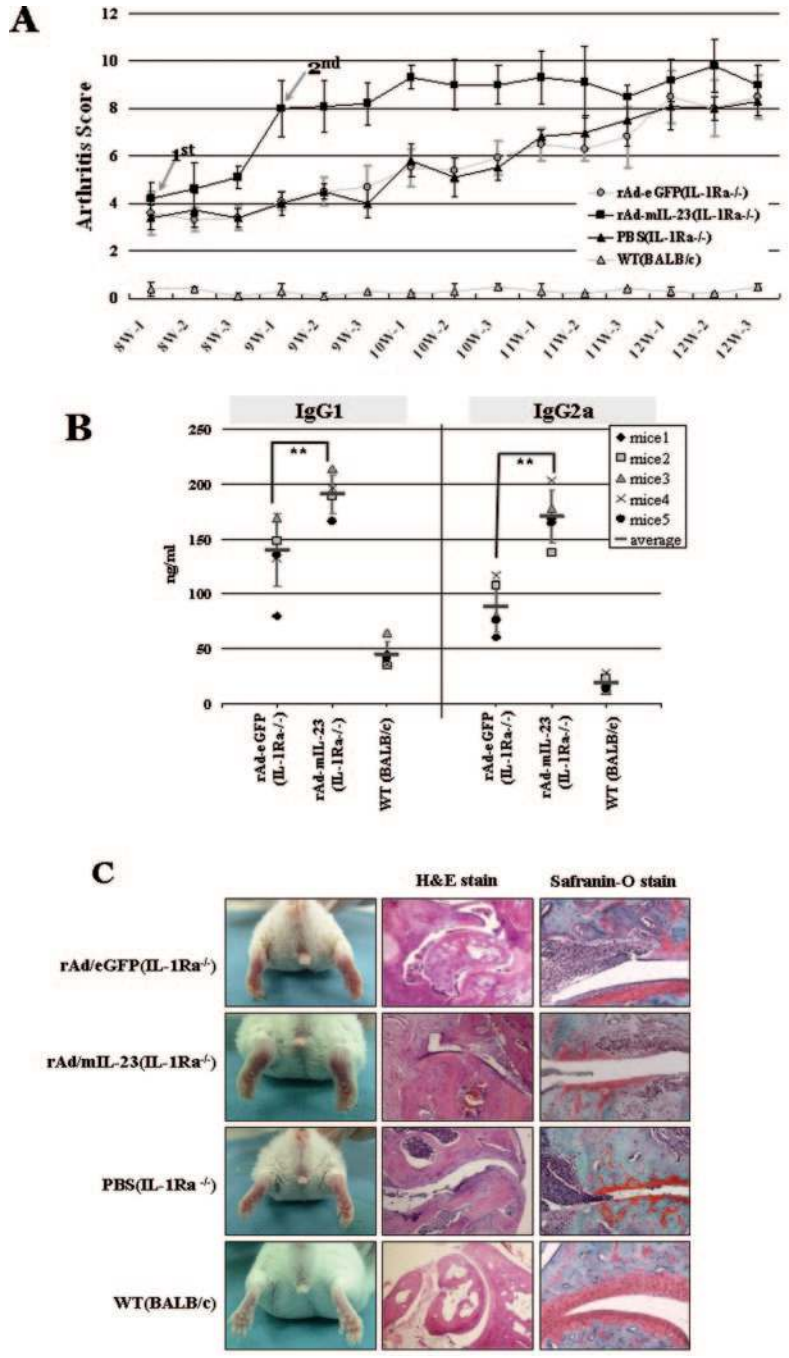

FIGURE 2. rAd/mIL-23 acts on IL-1Ra$^{-/-}$ mice to accelerate arthritis development. *A*, The progress of arthritis in IL-1Ra$^{-/-}$ mice after adenoviral gene delivery. The arthritic scores of IL-1Ra$^{-/-}$ mice injected with IL-23-expressing adenovirus, rAd/mIL-23 (■), adenovirus encoding eGFP (rAd/eGFP) (gray circle), or PBS (▲) were compared with those of WT mice (gray triangle) throughout the examination period (8–12 wk). The data represent the average values of five individual mice in each group. *B*, IgG1 and IgG2a subtype concentrations in sera from IL-1Ra$^{-/-}$ mice treated with rAd/mIL-23 or rAd/eGFP, and sera from WT mice. The data represent individual values and average values from five individual mice in each group. **, $p < 0.01$. *C*, Histological sections of mouse knee joints from IL-1Ra$^{-/-}$ mice treated with rAd/mIL-23, rAd/eGFP, or PBS and from WT mice, collected at 12 wk. H&E staining and safranin O staining of the hind joints is shown.

STAT3 and NF-κB signaling pathway is required for IL-23-stimulated IL-17 production in CD4$^+$ T cells from IL-1Ra $^{-/-}$ mice

It has been reported that IL-23 signal transduction is activated through the Jak-STAT pathway (5, 29). Based on this finding, we examined the signaling pathway used by IL-23 to accelerate IL-17 production. Splenic CD4$^+$ T cells isolated from IL-1Ra$^{-/-}$ mice were pretreated for 2 h with signal pathway inhibitors, such as PDTC (NF-κB inhibitor), SP600125 (AP-1 inhibitor), AG490 (Jak2 inhibitor), SB203580 (p38 inhibitor), PD98059 (MEK inhibitor), and LY294002 (PI3K/Akt inhibitor), and cultured with IL-23 (10 ng/ml). The production of IL-17 was determined by RT-PCR at 12 h (Fig. 6*A*) and by ELISA at 3 days (Fig. 6*B*) after treatment with IL-23. The PI3K inhibitor LY294002, the NF-κB inhibitor PDTC, and the Jak2 inhibitor AG490 suppressed IL-23-induced IL-17 expression in CD4$^+$ T cells. However, the AP-1 inhibitor SP600125, the MEK inhibitor PD98059, and the p38 inhibitor SB203580 had no significant effect on the induction of IL-17 (Fig. 6). Therefore, the expression of IL-17, mediated by IL-23, is controlled by Jak2, PI3K/Akt, and NF-κB.

To confirm these results, we examined whether the phosphorylation of IκB-α, PI3K, and STAT3 proteins was altered by IL-23. CD4$^+$ T cells were isolated from IL-1Ra$^{-/-}$ mice, pretreated for 30 min with PDTC, LY294002, or AG490, and cultured for 1 h with IL-23. As shown in Fig. 7*A*, the phosphorylation of STAT3 was increased by IL-23, and this phosphorylation was clearly suppressed by pretreatment with AG490 (Jak2 inhibitor) or LY294002 (PI3K/Akt inhibitor) (Fig. 7*A*). To confirm that STAT3 activity is related to its phosphorylation, we also performed a gel-shift assay. As shown in Fig. 7*B*, STAT3 activity was increased by IL-23

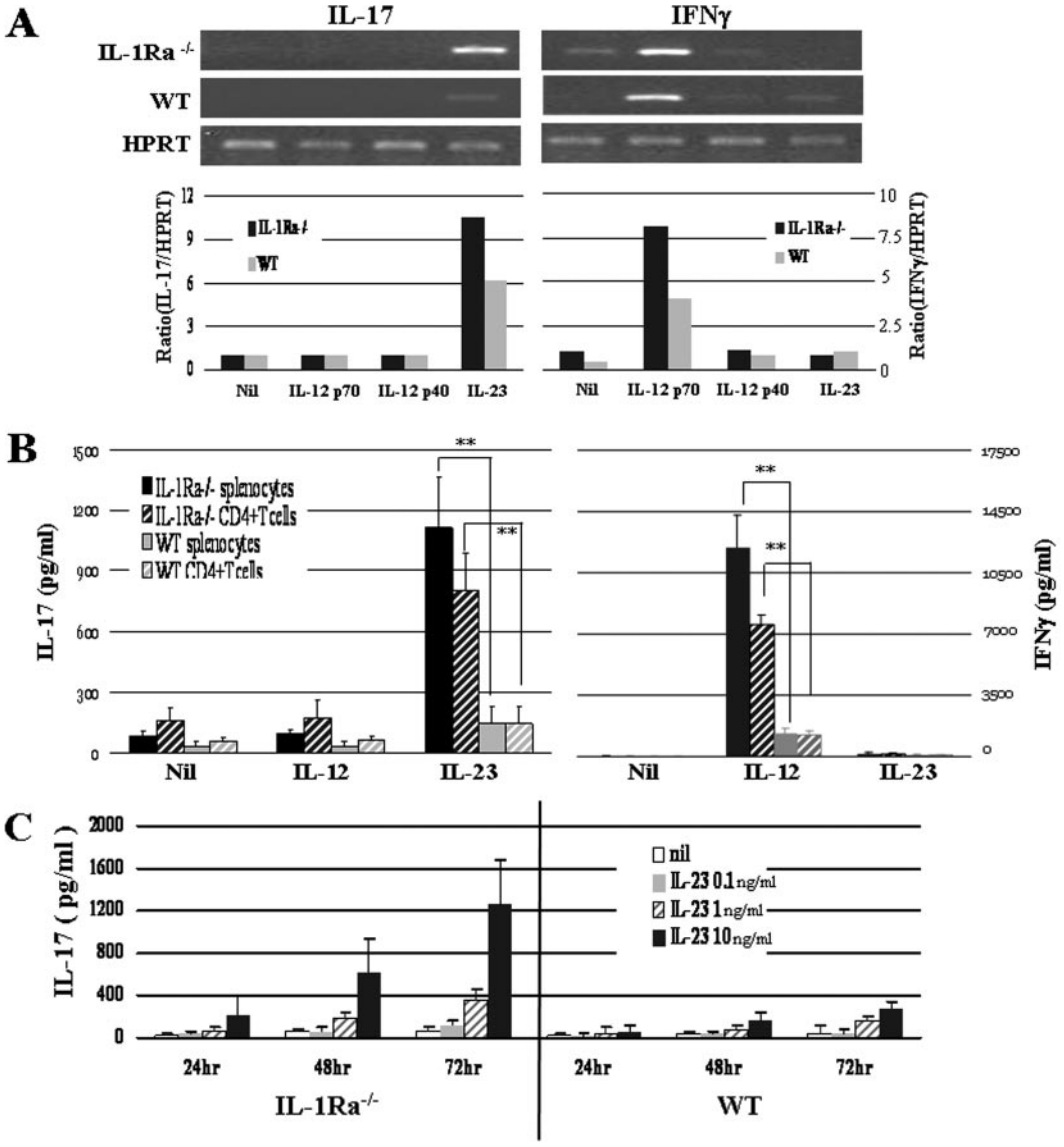

FIGURE 3. IL-17 and IFN-γ production induced by IL-23 and IL-12, respectively, was significantly higher in IL-1Ra$^{-/-}$ mice than in WT mice. *A*, IL-17 and IFN-γ mRNA expression induced by IL-23 and IL-12, respectively. CD4$^+$ T cells were cultured with IL-12p70 (10 ng/ml), IL-12p40 (10 ng/ml), or IL-23 (10 ng/ml) for 12 h. The expression of IL-17 mRNA and IFN-γ mRNA was evaluated by RT-PCR. Optical densities were normalized to the band for HPRT. *B*, Secretion of IL-17 and IFN-γ induced by IL-23 and IL-12, respectively, from CD4$^+$ T cells. Splenic CD4$^+$ T cell were cultured with IL-12p70 (10 ng/ml) or IL-23 (10 ng/ml) for 72 h. IL-17 and IFN-γ expression was assessed at 72 h by sandwich ELISA analysis from culture supernatants. The data represent average values from three independent experiments. **, $p < 0.01$. *C*, Secretion of IL-17 after IL-23 treatment at different concentrations and time points. CD4$^+$ T cells were cultured with IL-23 at the indicated concentrations (0.1–10 ng/ml) for the indicated periods (24–72 h). The data represent the average values for three independent experiments.

treatment, but AG490 abolished the effect of IL-23. To examine whether STAT3 is indeed a signal transduction molecule that directly regulates the expression of IL-17, T cells from IL-1Ra$^{-/-}$ mice were treated with STAT3 siRNA and cultured. The expression of the STAT3 gene was suppressed by STAT3 siRNA (Fig. 7*C*), and the IL-23-induced expression of IL-17 mRNA was suppressed only in cells treated with STAT3 siRNA (Fig. 7*C*). We also confirmed by ELISA that IL-17 production was reduced in the culture supernatants of siRNA-treated cells (Fig. 7*D*).

IL-23 also increased the phosphorylation of PI3K/Akt, whereas LY294002 and AG490 suppressed the IL-23-stimulated phosphorylation of PI3K/Akt. In contrast, the NF-κB inhibitor PDTC did not affect the IL-23-stimulated phosphorylation of PI3K/Akt (Fig. 7*E*). IL-23 also increased the phosphorylation of IκB-α, whereas PDTC suppressed the IL-23-stimulated phosphorylation of IκB-α. However, LY294002 did not affect the IL-23-stimulated phosphorylation of IκB-α. The signal transduction associated with NF-κB is generally known in terms of PI3K/Akt (30, 31). However, our results imply that the activation of PI3K/Akt caused by IL-23 is not directly associated with NF-κB activation, but that the activation of PI3K/Akt may activate signal transduction molecules such as AP-1 and STAT3 (32, 33). Furthermore, we confirmed that AG490 suppresses the IL-23-stimulated phosphorylation of IκB-α (Fig. 7*F*), which is consistent with previous reports that Jak2 leads to the phosphorylation of the inhibitor of NF-κB (IκB-α) in neurons (34, 35). These data suggest that when Jak2 binds to the IL-23R, the phosphorylation of STAT3, PI3K/Akt, and IκB-α is induced, and that PI3K/Akt may be involved in the phosphorylation of STAT3.

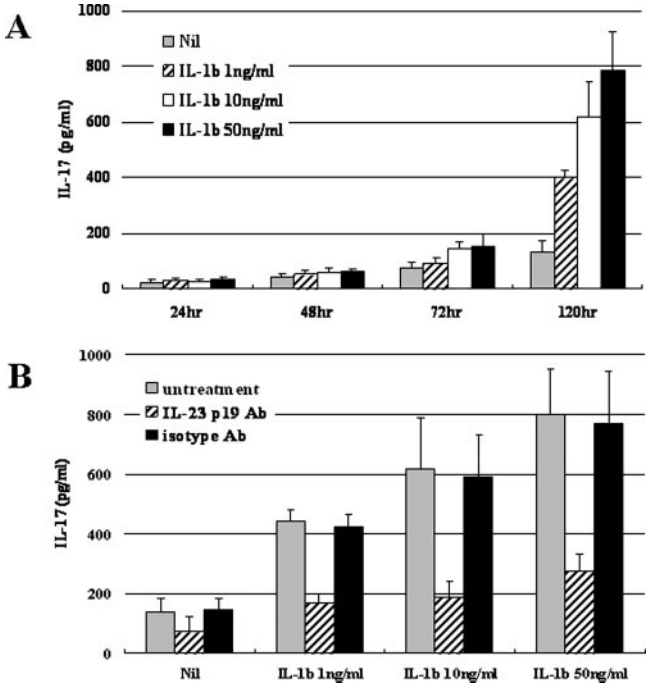

FIGURE 4. IL-17 production induced by IL-1β is dependent on IL-23. *A*, IL-17 production after IL-1 treatment in splenocytes from IL-1Ra$^{-/-}$ mice. Splenocytes were cultured with IL-1β at the indicated concentration (1–50 ng/ml) for the indicated periods (24–120 h). *B*, The blocking effect of anti-IL-23p19 Ab. Splenocytes were cultured in the presence of IL-1β (1–50 ng/ml) and neutralizing Ab directed against IL-23p19 or the appropriate isotype-matched Ab for 120 h. IL-17 was assessed by sandwich ELISA analysis.

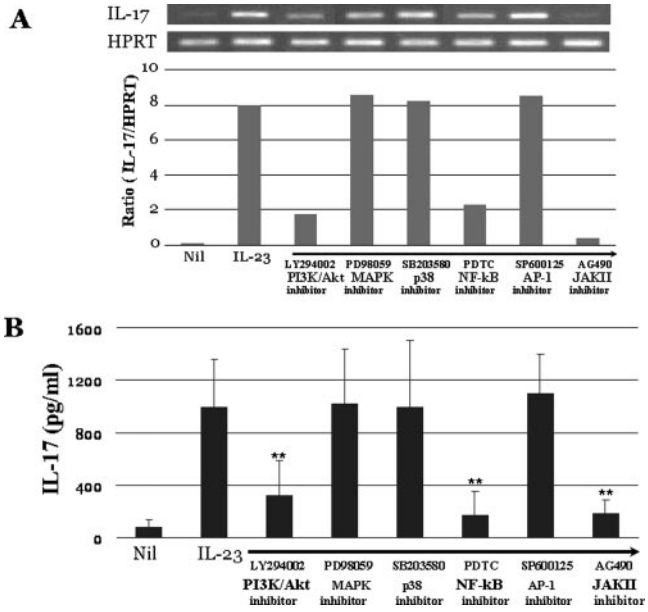

FIGURE 6. IL-23-mediated IL-17 protein and mRNA expression were inhibited by PI3K/Akt, NF-κB, and Jak2 inhibitors in CD4$^+$ T cells from IL-1Ra$^{-/-}$ mice. *A*, The effect of cell-signaling inhibitors on IL-23-induced IL-17 mRNA expression. IL-17 mRNA expression was evaluated by RT-PCR at 12 h after IL-23 treatment. The cells were pretreated with inhibitors for 2 h. The OD of each band was normalized to that of HPRT. *B*, The effect of cell-signaling inhibitors on IL-23-induced IL-17 protein secretion. Culture supernatants were assayed for IL-17 by sandwich ELISA after treatment with IL-23 and inhibitors for 72 h. Each value represents the mean and SD of three independent experiments. **, $p < 0.01$.

Therefore, in CD4$^+$ T cells, the binding of IL-23 to the IL-23R activates Jak2 protein, and sequentially activates the phosphorylation of STAT3, PI3K/Akt, and IκB-α, and the activation of PI3K/Akt activates STAT3. Taken together, these data suggest that both NF-κB and STAT3 form the major intracellular pathway involved in IL-23-induced IL-17 expression in the CD4$^+$ T cells of IL-1Ra$^{-/-}$ mice.

Discussion

We have found that the expression of IL-23p19, IL-12p40, IL-17, and IL-1β is augmented in IL-1Ra$^{-/-}$ mouse joints compared with that in the joints of WT mice. Excess IL-1β signal induced the increased production of IL-23 in IL-1Ra$^{-/-}$ mice compared with its effects in WT mice. Iwakura and colleagues (22–24) have reported that IL-1Ra$^{-/-}$ mice that are also deficient in IL-17 or TNF-α do not develop spontaneous arthritis. Interestingly, they found that IL-1 does not directly induce the production of IL-17 or TNF-α by CD4$^+$ T cells. However, they reported that the expression of the costimulatory molecule OX40 by T cells increased in the presence of IL-1β, suggesting that a certain mediator of the IL-1 signal is associated with the production of IL-17 and its activation in CD4$^+$ T cells. The mediator of the IL-1 signal that stimulates the production of IL-17 had not been characterized before this study. A clue to the mediator of the IL-1β signal was found in a study of myofibroblasts (36), which secrete IL-23 in the presence of IL-1β. In our study, the expression of IL-23 mRNA was significantly and dose dependently elevated by IL-1β in the splenic non-T cells of IL-1Ra$^{-/-}$ mice. Furthermore, IL-23 was also increased by LPS stimulation, suggesting that both the IL-1 signal and the TLR pathway are involved in the production of IL-23.

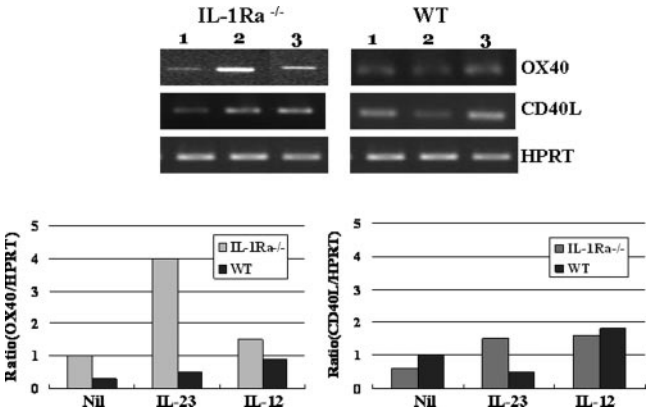

FIGURE 5. IL-23 induced the expression of costimulatory molecules on CD4$^+$ T cells from IL-1Ra$^{-/-}$ mice. CD4$^+$ T cells were cultured with IL-23 or IL-12 for 12 h. The expression of OX40 and CD40L mRNAs was evaluated by RT-PCR. The OD of each band was normalized to that for HPRT. The data are representative of three similar experiments.

Several elegant studies have suggested that IL-23 can activate new Th_{IL-17} cells and stimulate the production of IL-17 in animal models of arthritis, as well as in animal models of experimental autoimmune encephalomyelitis (1, 2, 4, 37). Therefore, it has been speculated that the interaction between IL-23-producing APCs and Th_{IL-17} cells might be important in the pathogenesis of autoimmune diseases (2, 38, 39). We have shown that IL-23 is strongly expressed in inflamed joints, and that IL-23 induces IL-17 production in IL-1Ra$^{-/-}$ mice. However, the level of induction was surprisingly high in IL-1Ra$^{-/-}$ mice compared with levels in WT mice. Based on the results of Jovanovic et al. (40), showing that IL-17 can induce IL-1 production, and on our results, we infer that

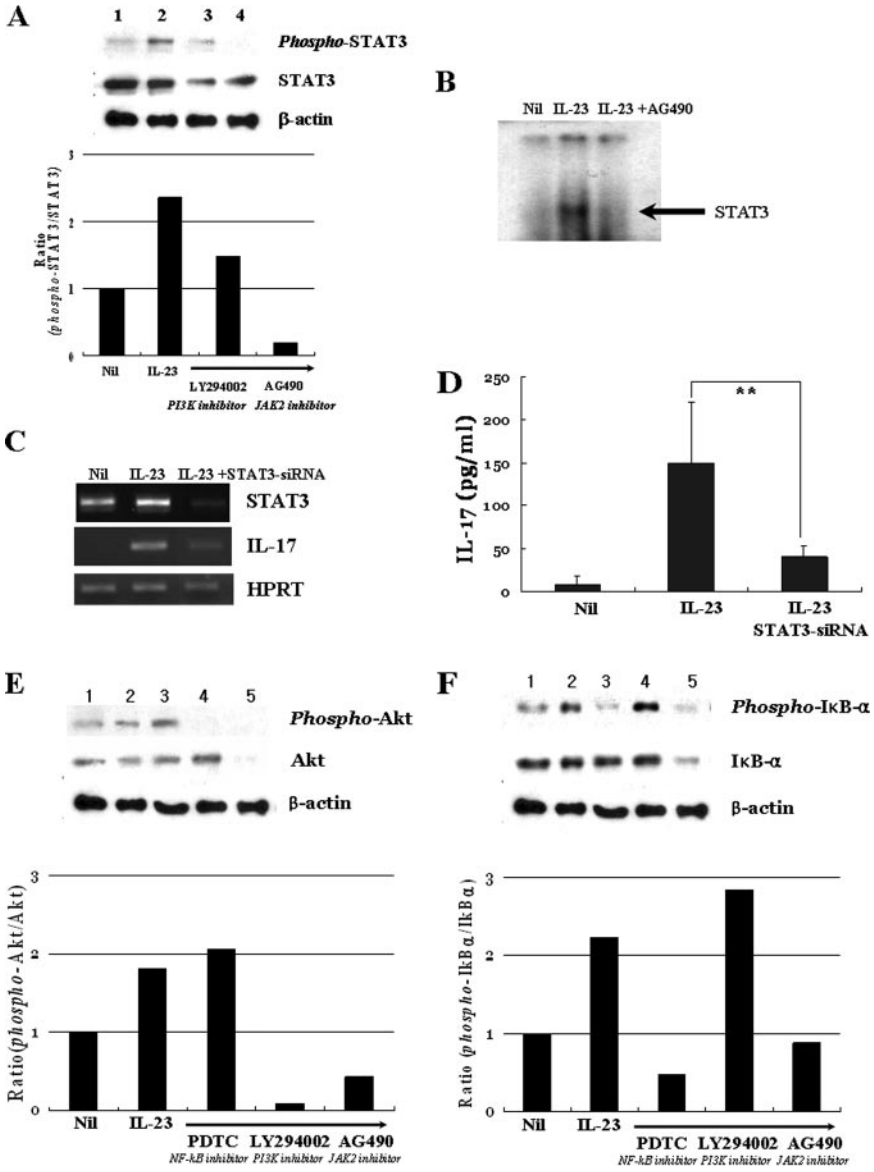

FIGURE 7. IL-23-mediated IL-17 production involves STAT3, PI3K/Akt, and IκB-α phosphorylation signaling in $CD4^+$ T cells from IL-$1Ra^{-/-}$ mice. $CD4^+$ T cells were pretreated for 30 min with PDTC (1 μM), LY294002 (20 μM), or AG490 (50 μM) before exposure to IL-23 (10 ng/ml) for 1 h. Cell lysates were analyzed for STAT3 (*A*), Akt (*D*), and IκB-α (*E*) activation by Western blot analysis of total and phosphorylated STAT3, phosphorylated IκB-α, and phosphorylated Akt using specific Abs. Levels of phosphorylated STAT3, phosphorylated IκB-α, and phosphorylated Akt were normalized to those of the corresponding total protein in the same sample. A representative example of three separate experiments is shown. *B*, Gel-shift assay for STAT3. STAT3 binding activity induced by IL-23 in $CD4^+$ T cells was assayed by EMSA. $CD4^+$ T cells were pretreated for 30 min with AG490 (50 μM) before exposure to IL-23 (10 ng/ml) for 15 min. STAT3 binding activity (*lane 2*) was enhanced by IL-23 (10 ng/ml). The specific Jak2 inhibitor AG490 abrogated the STAT3 binding activity induced by IL-23 (*lane 3*). The inhibition by STAT3 siRNA of IL-23-induced IL-17 expression was evaluated by RT-PCR (*C*) and ELISA (*D*). $CD4^+$ T cells were pretreated for 30 min with STAT3 siRNA before exposure to IL-23 (10 ng/ml) for 24 h. STAT3 and IL-17 mRNAs were analyzed by RT-PCR (*C*). The OD of each band was normalized to that of HPRT. Each value represents the mean and SD of three independent experiments. **, $p < 0.01$. *E* and *F*, IL-23 phosphorylation of PI3K/Akt an IκB-α.

the absence of IL-1Ra may cause a cytokine loop: IL-1 to IL-23, IL-23 to IL-17, and IL-17 to IL-1. This loop may contribute the higher levels of IL-1, IL-23, and IL-17 observed in IL-$1Ra^{-/-}$ mice. Blocking IL-23 with Abs blocks this loop, resulting in a decrease in the IL-17 production induced by IL-1. Considering these data together, we propose a new inflammation loop from IL-1 to IL-17 via IL-23.

To examine the biological effects of IL-23 on the development and progression of arthritis, we prepared rAd/mIL-23 and injected it into the joints of IL-$1Ra^{-/-}$ mice. The introduction of IL-23 quickly exaggerated the arthritis of IL-$1Ra^{-/-}$ mice compared with that of the control group, as shown by the measurement of arthritic scores, the amounts of anti-type II collagen IgG, and an immunohistochemical study. Our study suggests that the increase in arthritic scores of mice injected with rAd/IL-23 was mainly due to the biological effects of IL-23 on the development of arthritis. Our data support previous studies of IL-23p19 knockout mice, in which arthritis was not induced (2), and confirm the fact that IL-23 is involved in the etiology of arthritis. Based on the suggestions that IL-17-producing $CD4^+$ cells are a distinct lineage (41) and that IL-23 promotes a T cell population characterized by the production of IL-17, IL-17F, TNF, and IL-6 (4, 9–11), the higher level of IL-23 in IL-$1Ra^{-/-}$ mice may facilitate the development of $Th_{IL\text{-}17}$, thus regulating tissue inflammation.

Like other cytokine receptors, IL-12R and IL-23R subunits lack intrinsic enzymatic activity. Instead, IL-12Rβ1 binds the Jak family member Tyk2, whereas IL-12Rβ2 and IL-23R associate with Jak2 (5, 29). IL-23 activates the same Jak-STAT signaling molecules as IL-12: Jak2, Tyk2, and STAT1, STAT3, STAT4, and STAT5. However, STAT4 activation is substantially weaker and different DNA-binding STAT complexes form in response to IL-23 compared with those that form in response to IL-12 (29). IL-23 activates the Jak-STAT3 signal transduction pathway through the IL-23R (29), and this signaling pathway of IL-23 is quite different from the STAT4 pathway of IL-12 (5, 42–44). We have shown that IL-23-induced IL-17 production by $CD4^+$ T cells in IL-$1Ra^{-/-}$ mice is mediated via the activation of PI3K/Akt, NF-κB, and STAT3, whereas p38 MAPK and AP-1 do not participate in this process. Interestingly, when we examined the active forms of the signal molecules, Jak2 binding to the IL-23R activated not only STAT3, but also PI3K and NF-κB. However, PI3K did not activate NF-κB, but directly activated STAT3 (Fig. 7). Jak2 is a receptor-binding protein associated with STAT3 (45). However, it has recently been reported that Jak2 directly activates

PI3K or phosphorylates IκB-α (34, 35). Furthermore, PI3K is a signal pathway molecule that has been investigated thoroughly in association with cell proliferation (46). For instance, numerous reports of the activation of NF-κB and AP-1 are available (47–49), and our previous research showed that TCR-induced IL-17 expression is dependent on PI3K and NF-κB in human $CD4^+$ T cells (50). Based on these data, we concluded that the activation of PI3K by IL-23 may directly activate STAT3 and that NF-κB can be activated by IL-12 without the activation of PI3K. To confirm the direct effect of STAT3 on the IL-23-induced expression of IL-17, we examined the production of IL-17 after treatment with STAT3 siRNA in T cells. The expression of STAT3 was down-regulated by siRNA and the IL-23-induced expression of IL-17 was reduced. Conclusively, IL-23 activates the IL-23R binding protein, Jak2, and further stimulates the activation of STAT3, PI3K, and phospho-IκB-α in $CD4^+$ T cells, resulting in the induction of IL-17. In other words, the IL-23-induced production of IL-17 is mediated not only by the STAT3 pathway via PI3K, but also by NF-κB through the phosphorylation of IκB-α (Fig. 8).

In summary, the production of IL-23 is significantly enhanced under conditions of excess IL-1β. The increase in IL-23 undoubtedly stimulates the production of IL-17, with concomitant overexpression of OX40, in $CD4^+$ T cells. The biological activity of IL-23 apparently exacerbates the joint inflammation of IL-$1Ra^{-/-}$ mice. Our study suggests that the IL-1β-induced increase in IL-23 has an essential role in the pathogenesis of spontaneous arthritis. Furthermore, the STAT3 and NF-κB pathways are critically involved in the biological function of IL-23 in IL-$1Ra^{-/-}$ mice. Taken together, our data suggest that a newly identified inflammatory loop involving IL-1β, IL-23, and IL-17, and the intracellular signaling pathway of IL-23 should provide clues to the development of new therapies for autoimmune arthritis.

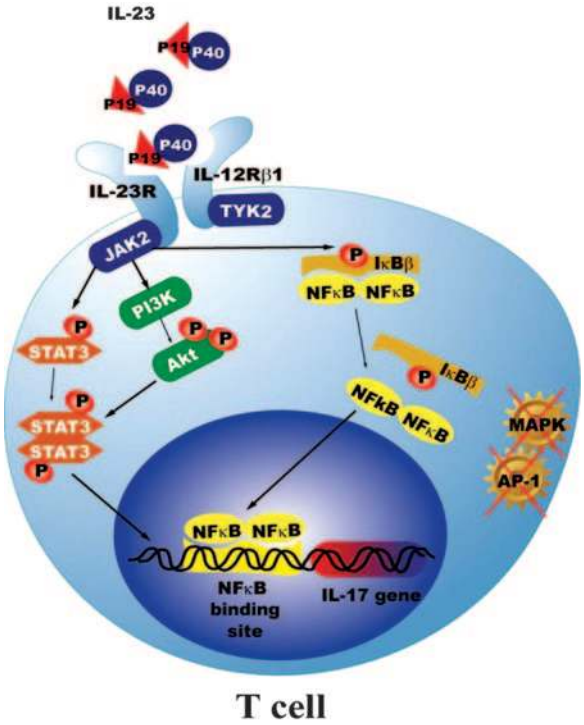

FIGURE 8. Schematic illustration of IL-17 expression in T cells via IL-23 signaling.

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Disclosures

The authors have no financial conflict of interest.

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