



The Attune™ NxT Flow Cytometer system

Let's get to the science

Find out more

invitrogen
by Thermo Fisher Scientific



IL-12p40 Homodimer Ameliorates Experimental Autoimmune Arthritis

This information is current as of July 9, 2019.

Seon-Yeong Lee, Young Ok Jung, Doo-Jin Kim, Chang-Min Kang, Young-Mee Moon, Yu-Jung Heo, Hye-Jwa Oh, Seong-Jeong Park, Se-Hwan Yang, Seung Ki Kwok, Ji-Hyeon Ju, Sung-Hwan Park, Young Chul Sung, Ho-Youn Kim and Mi-La Cho

J Immunol 2015; 195:3001-3010; Prepublished online 31 August 2015;
doi: 10.4049/jimmunol.1500400
<http://www.jimmunol.org/content/195/7/3001>

References This article **cites 44 articles**, 12 of which you can access for free at:
<http://www.jimmunol.org/content/195/7/3001.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Author Choice Freely available online through *The Journal of Immunology*
[Author Choice option](#)

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2015 by The American Association of
Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



IL-12p40 Homodimer Ameliorates Experimental Autoimmune Arthritis

Seon-Yeong Lee,^{*,†,1} Young Ok Jung,^{‡,1} Doo-Jin Kim,[§] Chang-Min Kang,^{*}
Young-Mee Moon,^{*,†} Yu-Jung Heo,^{*} Hye-Jwa Oh,^{*} Seong-Jeong Park,[§] Se-Hwan Yang,[§]
Seung Ki Kwok,^{*} Ji-Hyeon Ju,^{*} Sung-Hwan Park,^{*} Young Chul Sung,[§] Ho-Youn Kim,^{*,2}
and Mi-La Cho^{*,†,2}

IL-23 is the key cytokine that induces the expansion of Th17 cells. It is composed of p19 and p40 subunits of IL-12. The p40 subunit binds competitively to the receptor of IL-23 and blocks its activity. Our aim was to assess the preventive and therapeutic effect of the IL-12p40 homodimer (p40)₂ subunit in autoimmune arthritis animal models. In the current study, using IL-1R antagonist–knockout mice and a collagen-induced arthritis model, we investigated the suppressive effect of (p40)₂ on inflammatory arthritis. We demonstrated that the recombinant adenovirus-expressing mouse (p40)₂ model prevented the development of arthritis when given before the onset of arthritis. It also decreased the arthritis index and joint erosions in the mouse model if transferred after arthritis was established. (p40)₂ inhibited the production of inflammatory cytokines and Ag-specific T cell proliferation. It also induced CD4⁺CD25⁺Foxp3 regulatory T (Treg) cells in vitro and in vivo, whereas the generation of retinoic acid receptor–related organ receptor γ t and Th17 cells was suppressed. The induction of Treg cells and the suppression of Th17 cells were mediated via activated STAT5 and suppressed STAT3. Our data suggest that (p40)₂ suppressed inflammatory arthritis successfully. This could be a useful therapeutic approach in autoimmune arthritis to regulate the Th17/Treg balance and IL-23 signaling. *The Journal of Immunology*, 2015, 195: 3001–3010.

Th cells perform an essential role in the immune system by producing distinct cytokines. In addition to Th1 cells and Th2 cells, a subset of Th cells that produce IL-17 is known as Th17 cells (1). IL-17 is an inflammatory cytokine that strongly affects various stromal cells. IL-17 mediates inflammatory responses by recruiting inflammatory cells, inducing angiogenesis, and stimulating the production of proinflammatory mediators from

endothelial and epithelial tissues (2). IL-17 is known as a key cytokine in a diverse group of autoimmune diseases and immune-mediated diseases, including psoriasis, rheumatoid arthritis (RA), multiple sclerosis, inflammatory bowel disease, and asthma (3, 4). In RA, IL-17 enhances other proinflammatory cytokines, like IL-6, in fibroblast-like synoviocytes and shows synergistic effects with inflammatory cytokines, such as TNF- α and IL-1 (5, 6). It is now known as a key cytokine in the propagation of joint inflammation and destruction (7). Retinoic acid receptor-related organ receptor (ROR) γ t was recently identified as the master transcription factor guiding Th17 differentiation (8).

IL-23 is a key cytokine that induces expansion of Th17 cells (9, 10), and it is overexpressed in RA synovial tissues (9). It consists of the unique p19 and p40 subunits. p40 is also a subunit of IL-12, a heterodimeric cytokine of p40 and p35 (11). IL-12 and IL-23 also share a common subunit in their receptor complex due to the common p40 subunit (12). The IL-12p40 subunit, which contributes to both IL-12 and IL-23, is often considered an antagonist of these cytokines (13, 14). Recombinant murine IL-40 homodimer [(p40)₂] binds competitively to IL-12R β 1 and prevents IL-12–mediated immune responses (15, 16). Recombinant murine IL-12p40/p80 inhibited IL-23–mediated immune responses (17). Recently, (p40)₂ (or p80) was shown to be an inherently agonistic cytokine with an independent role. The most widely known function of (p40)₂ is competitive inhibition of IL-12 and IL-23; therefore, its primary role was assumed to be anti-inflammatory. However, proinflammatory properties for (p40)₂ were described in various reports. It acts as a chemoattractant for macrophages and pathogen-induced dendritic cells (18) and induces inflammation and fibrosis of the lung (19). Allograft rejection by inducing IFN- γ production by CD8⁺ T cells (20) and macrophage accumulation (21) were reported. Fathman and colleagues (22) demonstrated that local delivery of IL-12p40 by T cells inhibited

^{*}Rheumatism Research Center, Catholic Research Institutes of Medical Science, Catholic University of Korea, Seoul 137-701, Republic of Korea; [†]Laboratory of Immune Network, Conversant Research Consortium in Immunologic Disease, Catholic University of Korea, Seoul 137-701, Republic of Korea; [‡]Division of Rheumatology, Department of Internal Medicine, Hallym University Kang-Nam Sacred Heart Hospital, Seoul 150-950, Republic of Korea; and [§]Integrative Bioscience and Biotechnology, World Class University, Pohang University of Science and Technology Biotechnology Center, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea

¹S.-Y.L. and Y.O.J. contributed equally to this work.

²H.-Y.K. and M.-L.C. contributed equally to this work.

Received for publication February 19, 2015. Accepted for publication August 3, 2015.

This work was supported by the Research and Development Program of the Ministry of Knowledge and Economy/Korea Evaluation Institute of Industrial Technology (10028480, Development of Fc Fusion Protein for Autoimmune Disease Using IL-23 Antagonist) and a grant from the Korean Health Technology Research and Development Project, Ministry of Health and Welfare, Republic of Korea (H113C0016).

Address correspondence and reprint requests to Prof. Mi-La Cho and Prof. Ho-Youn Kim, Rheumatism Research Center, Catholic Research Institutes of Medical Science, Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, Republic of Korea. E-mail addresses: iammla@catholic.ac.kr (M.-L.C.) and ho0919@catholic.ac.kr (H.-Y.K.)

Abbreviations used in this article: CIA, collagen-induced arthritis; CII, type II collagen; IL-1RaKO, IL-1R antagonist–knockout; (p40)₂, IL-12p40 homodimer; RA, rheumatoid arthritis; ROR, retinoic acid receptor–related organ receptor; Treg, regulatory T cell.

This article is distributed under The American Association of Immunologists, Inc., [Reuse Terms and Conditions for Author Choice articles](#).

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/\$25.00

collagen-induced arthritis (CIA) by suppressing the autoimmune response. Recently, Kim et al. (23) reported that IL-12p40 homodimer attenuated autoimmune colitis by suppressing Th17 cells.

Regulatory T cells (Tregs) are a specialized subpopulation of T cells that suppress activation of the immune system and, thereby, maintain immune system homeostasis and tolerance to self-antigens. The best characterized Tregs are the CD4⁺, CD45RB^{low}, and CD25⁺ subsets (24). CD4⁺CD25⁺ Tregs express Foxp3, a unique transcription factor that is critically important in the development and function of these cells (25). Defects in Treg function are important in the pathogenesis of autoimmune diseases. Adoptive transfer of activated regulatory cells inhibits CIA (26), and induction of Tregs by immunomodulatory agents could ameliorate CIA and maintain immune tolerance (27).

The aim of this study was to investigate the potential therapeutic effect of the (p40)₂ subunit in an experimental animal model of RA. Administration of (p40)₂ demonstrated therapeutic effects in arthritis animal models. The therapeutic effect of (p40)₂ was exerted on multiple levels and was associated with the induction of CD4⁺CD25⁺Foxp3⁺ Tregs and with suppressive effects on Th17 cells.

Materials and Methods

Animals

Seven- to twelve-week-old male IL-1Ra^{-/-} mice were maintained in groups of two to four animals in polycarbonate cages in a semispecific pathogen-free environment. Six- to eight-week-old male DBA1/J mice (SLC, Shizuoka, Japan) were maintained in an semispecific pathogen-free environment. The Animal Research Ethics Committee at the Catholic University of Korea examined and approved all experimental procedures.

Isolation of CD4⁺ T cells

Cell pellets were prepared from the spleens of CIA mice. Anti-mouse CD4 MicroBeads were used, as recommended by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells were resuspended in 100 μ l MACS buffer (1% BSA, 5 mM EDTA, and 0.01% sodium azide). CD4 MicroBeads (1 \times 10⁷ cells/10 μ l) were added and incubated for 10 min at 4°C. Cells were diluted in 10 μ l MACS buffer, pelleted, resuspended in 500 μ l, and separated magnetically in an AutoMACS magnet fitted with a MACS MS column (Miltenyi Biotec).

Cell culture

Isolated CD4⁺ T cells (5 \times 10⁵) from CIA mice were incubated with IL-23 (0.1, 1, 10 ng/ml) and IL-23 (10 ng/ml) plus IL-23p19 Ab (0.1, 1, 10 μ g/ml), IL-12p40 Ab (0.1, 1, 10 μ g/ml), soluble IL-23R (0.01, 0.05, 0.1 μ g/ml), or (p40)₂ (0.01, 0.05, 0.1 μ g/ml) for 3 d. To investigate the suppressive effect of (p40)₂ on IL-17 and IFN- γ production induced by IL-23, isolated CD4⁺ T cells were cultured with IL-23 (0.1, 1, 10 ng/ml), IL-12p70 (0.1, 1, 10 ng/ml), and IL-23 (10 ng/ml) plus (p40)₂ (0.1, 1, 5, 10, 20, 100 ng/ml) for 3 d.

Generation of recombinant adenoviruses

The recombinant replication-defective adenovirus expressing mouse (p40)₂ was generated using the AdEasy Vector System (Qbiogene, Carlsbad, CA). Briefly, IL-12(p40)₂ cDNA was subcloned into the pShuttle-CMV vector (Qbiogene) using the BglIII and XhoI/XbaI restriction sites. The pShuttle-CMV construct was cotransfected with pAdEasy into *Escherichia coli* BJ5183 via electroporation. The recombinant construct was transfected into 293 cells using the calcium phosphate method, and the generated recombinant adenovirus was expanded and purified by cesium-gradient ultracentrifugation. The adenovirus containing EGFP was produced in a similar manner. The titer of each purified virus was determined by TCID₅₀ assay.

(p40)₂ for prevention and therapeutic effect in IL-1Ra^{-/-} mice

For the preventative effect of (p40)₂, 7-wk-old male mice ($n = 10$) were injected intra-articularly with 1 \times 10⁶/PFU (p40)₂ vector or mock vector. Three days later, the mice were reinjected intra-articularly with 1 \times 10⁶/PFU the (p40)₂ vector or mock vector. To examine the therapeutic effect of (p40)₂, 11-wk-old male mice (spontaneous arthritis induction) were injected intra-articularly with 1 \times 10⁶/PFU the (p40)₂ vector or mock

vector. Three days later, the mice were reinjected intra-articularly with 1 \times 10⁶/PFU the (p40)₂ vector or mock vector.

Induction of arthritis and (p40)₂ for prevention and therapeutic effect in CIA mice

Seven-week-old male DBA1/J mice (Orient, Seongnam-si, Korea) ($n = 10$) were immunized intradermally at the base of the tail with 100 μ g bovine type II collagen (CII; Chondrex, Seattle, WA) emulsified in CFA (Arthrogen-CIA; Chondrex) (1:1, w/v, day 0). Two weeks later, the mice were boosted by intradermal injection with 100 μ g bovine CII in IFA (Chondrex) (1:1, v/v, day 14). (p40)₂ was injected intra-articularly three times at intervals of 9 d from day -2 before CIA induction. To examine the therapeutic effect of (p40)₂, p40 was injected intra-articularly three times at intervals of 9 d after the booster (day 14). The severity of arthritis was recorded using the mean arthritis index (scale of 0–4), as reported previously (28).

Immunohistochemistry

Mouse joint tissues ($n = 10$) were fixed in 4% paraformaldehyde, decalcified in EDTA bone decalcifier, and embedded in paraffin. Sections (7 μ m) were prepared and stained with H&E and Safranin O to detect proteoglycans. The sections were dewaxed using xylene and dehydrated in a gradient of alcohols. Endogenous peroxidase activity was quenched with 3% methanol H₂O₂. Immunohistochemistry was performed using a VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, CA). Tissues were incubated with the first IL-12p70, IL-17, IL-23, IFN- γ , TNF- α , and IL-6 Abs (R&D Systems), and the IL-1 β Ab (Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C in a biotinylated secondary linking Ab, and then in a streptavidin-peroxidase complex for 1 h. The final color product was developed using a DAB Chromogen Kit (Dako, Carpinteria, CA). The sections were counterstained with hematoxylin and photographed with an Olympus photomicroscope (Tokyo, Japan).

Immunohistochemistry scoring

The H&E-stained sections were scored for inflammation and bone erosion. Inflammation was scored according to the following criteria: 0 = no inflammation; 1 = slight thickening of the lining layer or some infiltrating cells in the underlying layer; 2 = slight thickening of the lining layer plus some infiltrating cells in the underlying layer; 3 = thickening of the lining layer, an influx of cells in the underlying layer, and the presence of cells in the synovial space; and 4 = synovium highly infiltrated with many inflammatory cells. Cartilage damage was determined using Safranin O staining and toluidine blue, and the extent of cartilage damage was scored according to the following criteria: 0 = no destruction; 1 = minimal erosion limited to single spots; 2 = slight to moderate erosion in a limited area; 3 = more extensive erosion; and 4 = general destruction (28).

TRAP staining

Decalcified ankle joints were processed for paraffin embedding, and 7- μ m-thick tissue sections were prepared. These sections were stained for TRAP with the leukocyte acid phosphatase kit (Sigma), according to the manufacturer's protocol. TRAP⁺ cells with three or more nuclei were deemed to be osteoclasts and were counted.

Ig measurement

The mice were bled from the eye after immunization, and individual sera were analyzed for IgG, IgG1, and IgG2a (Bethyl, Montgomery, TX). IgG, IgG1, and IgG2 were measured using an ELISA kit (Bethyl).

Real-time PCR

Expression of IL-23p19, IL-12, IL-1 β , TNF- α , IL-6, IL-17, IFN- γ , TGF- β , Foxp3, and ROR γ t mRNA was determined by real-time PCR with SYBR Green I (Roche Diagnostic, Mannheim, Germany). Reaction mixtures were amplified in a LightCycler (Roche Diagnostic). Fluorescence curves were analyzed with LightCycler software v.3.0. The expression levels were calculated and corrected for the values of the endogenously expressed housekeeping gene (β -actin) controls.

Intracellular cytokine staining and flow cytometry

IL-23-treated or IL-23 plus (p40)₂-treated mouse spleen cells were stained with anti-mouse CD4-PerCP mAb (eBioscience, San Diego, CA) and anti-mouse CD25-FITC mAb (Miltenyi Biotec, San Diego, CA). After staining, the cells were permeabilized and fixed with Cytotfix/Cytoperm (BD Pharmingen, San Diego, CA). Cells were stained with anti-mouse

Foxp3-PE mAb (eBioscience) and subjected to flow cytometric analysis using a FACSCalibur (Becton Dickinson).

Confocal stain

Tissue specimens were snap-frozen in liquid nitrogen and stored at -80°C . Tissue sections ($7\ \mu\text{m}$) of spleens were preserved in 4% paraformaldehyde and stained using directly labeled Abs to anti-mouse Foxp3-FITC mAb (eBioscience), anti-mouse CD25-allophycocyanin Ab (BioLegend, San Diego, CA), and anti-mouse CD4-biotin mAb (BD Biosciences, San Jose, CA). Streptavidin Cy-3 (BD Biosciences) was added to PBS (pH 7.5) and incubated overnight at 4°C . For STAT analysis, the tissues were stained with anti-mouse p-STAT3 705, p-STAT3 727, p-STAT5, and anti-mouse CD4-FITC mAb. Stained sections were analyzed using a confocal microscopy system (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany).

MLR

Irradiated APCs (1×10^5 cells) were used as stimulators, and CD4^+ T cells (1×10^5 cells) were used as responders. Plates were incubated for 72 h at 37°C . All wells were pulsed with $0.5\ \mu\text{Ci}$ [^3H]thymidine in $20\ \mu\text{l}$ RPMI 1640 for 16 h. Thymidine incorporation was measured using the beta-counter system (Packard Instrument Company, Meridan, CA).

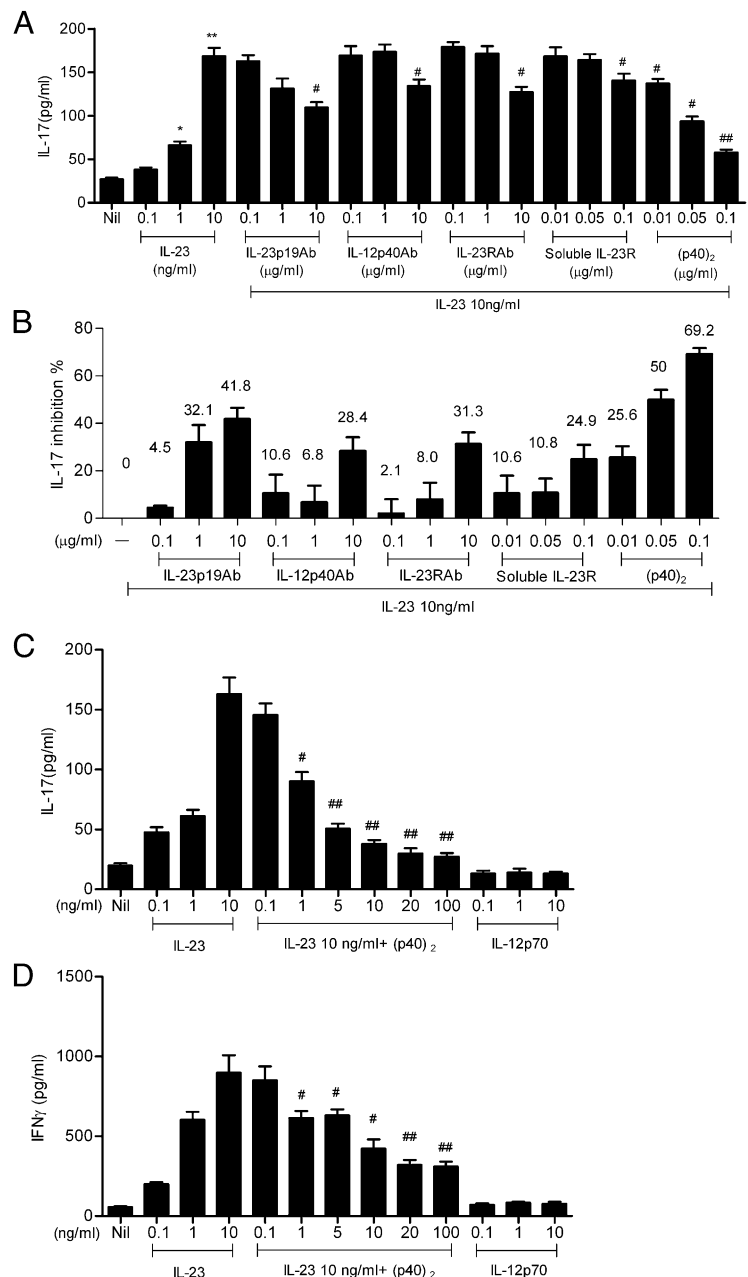
Western blot analysis

Cells were cultured for 3 d in the presence of IL-23 or IL-23 plus $(\text{p40})_2$. Then whole-cell lysates were prepared by homogenization in the lysis buffer. Protein samples were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). For Western blot hybridization, the membrane was preincubated with 0.1% skimmed milk in 0.1% Tween-20 in TBS at room temperature for 2 h. Abs to Foxp3, p-STAT3, STAT3, p-STAT4, STAT4, p-STAT5, and STAT5 (Cell Signaling Technology, Danvers, MA) were added to the membrane and incubated overnight at 4°C . After washing with 0.1% Tween-20 in TBS, horseradish peroxidase-conjugated secondary Abs were added and incubated for 1 h at room temperature. Hybridized bands were detected using the ECL detection kit and Hyperfilm ECL reagents (Amersham Pharmacia Biotech).

Statistical analysis

All data are expressed as the mean \pm SD. Statistical analysis was performed using SPSS 10.0 for Windows (SPSS, Chicago, IL). The differences between groups were analyzed using an unpaired Student *t* test, assuming equal variances. The *p* values <0.05 were considered significant.

FIGURE 1. $(\text{p40})_2$ inhibits IL-23-induced IL-17 production in CD4^+ T cells in CIA model. **(A)** IL-17 production was analyzed by ELISA from the culture supernatants of spleen CD4^+ T cells in CIA mice. Spleen CD4^+ T cells were cultured with IL-23 (0.1–10 ng/ml) or IL-23 (10 ng/ml) plus IL-23p19 mAb (0.1–10 $\mu\text{g/ml}$), IL-12p40 mAb (0.1–10 $\mu\text{g/ml}$), IL-23R mAb (0.1–10 $\mu\text{g/ml}$), soluble IL-23R (0.01–0.1 $\mu\text{g/ml}$), and IL-12p40 (0.01–0.1 $\mu\text{g/ml}$). **(B)** Results in (A) as an indicated inhibition percentage. **(C and D)** Spleen CD4^+ T cells from CIA mice were cultured with IL-23 (0.1–10 ng/ml) or IL-12p70 (10 ng/ml) and IL-23 (10 ng/ml) plus IL-12p40 (indicated dose). Production of IL-17 and IFN- γ was analyzed by ELISA. #versus IL-23 (10 ng/ml). Data are mean \pm SD and are representative of three independent experiments. ***p* < 0.01, **p* < 0.05 and ##*p* < 0.01, **p* and #*p* < 0.05.



Results

(p40)₂ inhibited IL-23–induced IL-17 production

We investigated the suppressive effect of (p40)₂ on IL-17 production induced by IL-23 with CD4⁺ T cells from CIA mice. The

concentration of IL-17 was increased in a dose-dependent manner in the presence of IL-23. IL-23p19 Ab, IL-12p40 Ab, IL-23R Ab, soluble IL-23R, and (p40)₂ all decreased the IL-17 level (Fig. 1A). However, the percentage inhibition of IL-17 was the largest with (p40)₂, and 0.1 μg/ml of (p40)₂ inhibited IL-17 production up to

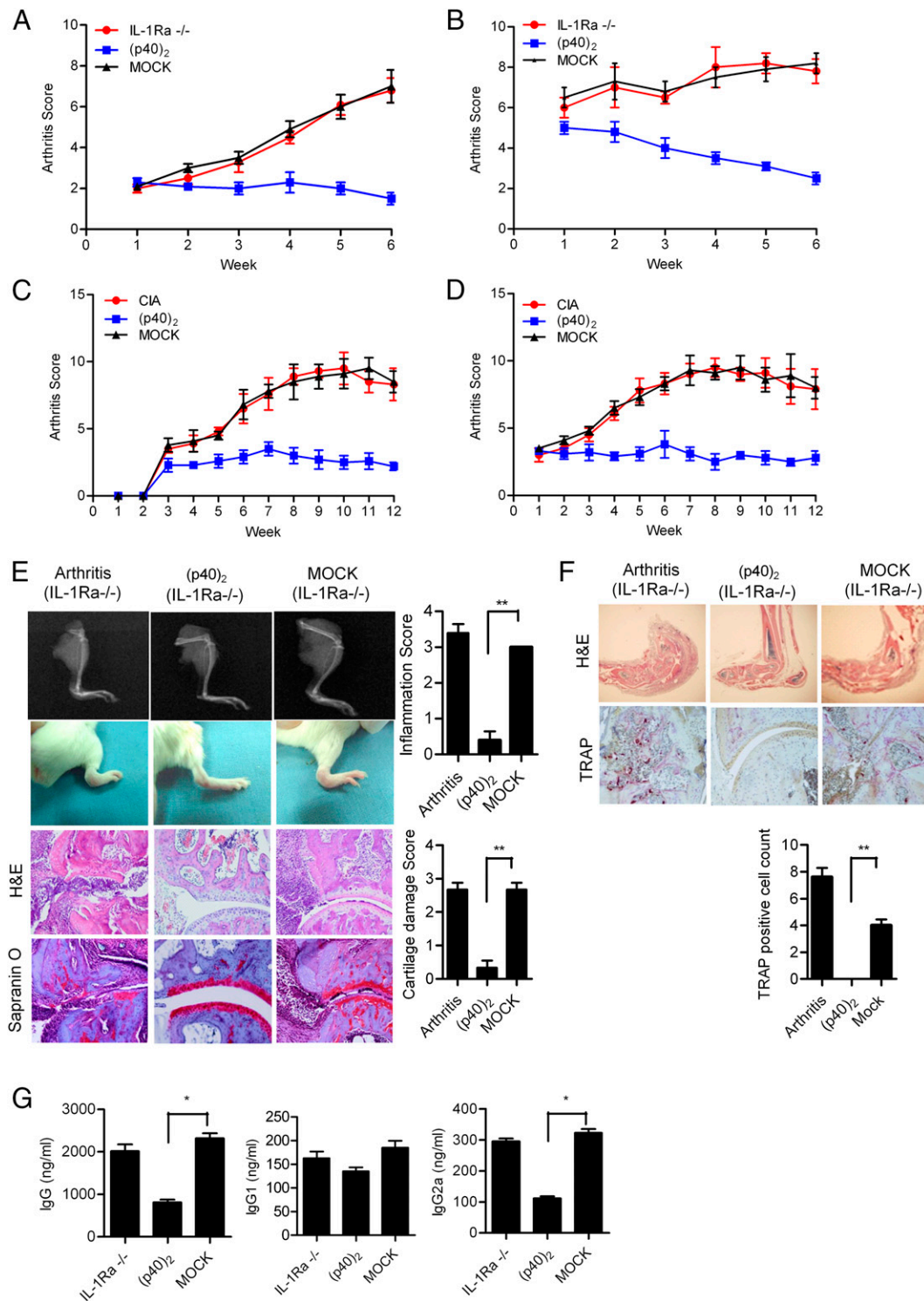


FIGURE 2. (p40)₂ treatment inhibits arthritis in IL-1RaKO and CIA mice. (**A** and **B**) Seven- or twelve-week-old IL-1RaKO mice ($n = 10$) were injected intra-articularly with a 1×10^6 /PFU (p40)₂ vector two times at an interval of 3 d. (**C** and **D**) Six-week-old DBA1/J mice were injected with a 1×10^6 /PFU (p40)₂ vector before or after CII immunization. Average clinical scores of IL-1RaKO and CIA mice are shown after injection ($n = 5$ for each group). (**E** and **F**) All tissues were obtained from therapeutically treated in IL-1RaKO mice group [original magnification $\times 10$ (**E**) and $\times 200$ (**F**)]. The joint tissue of IL-1RaKO mice as represented by x-rays and photographs. The embedding paraffin was stained with H&E, Safranin O. Ankle joints were stained with TRAP. (**G**) Each mouse group (therapeutically treated in the arthritis model) was bled from the eye after immunization, and individual sera were analyzed for IgG, IgG1, and IgG2a using ELISA, respectively. Data are mean \pm SD and are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

69.2% (Fig. 1B). In the presence of 1 ng/ml of (p40)₂, the concentration of IL-17 was decreased from 155 ± 27 pg/ml to 88 ± 21 pg/ml (Fig. 1C). On the contrary, the concentration of INF-γ induced by IL-23 was decreased with a higher concentration of (p40)₂ (Fig. 1D). The half-maximal (50%) inhibitory concentration of (p40)₂ for INF-γ was ~10 ng/ml, which was 10-fold higher than the concentration for IL-17 (Fig. 1D).

(p40)₂ inhibited the development of arthritis in arthritis animal models

We observed the effect of (p40)₂ in vivo in the IL-1R antagonist-knockout (IL-1RaKO) and CIA models. To determine the preventive effect of (p40)₂, we transferred the recombinant replication-defective adenovirus expressing mouse (p40)₂ before the onset of arthritis (Fig. 2A, 2C). To see the therapeutic effect, we transferred the (p40)₂-adenovirus vector after the onset of arthritis (Fig. 2B, 2D). The arthritis indexes were measured for 6 wk for IL-1RaKO mice and 12 wk for CIA mice. The mean arthritis index was significantly lower in (p40)₂-transferred mice than in control mice throughout the observational period. The therapeutic effect of (p40)₂ in these arthritis models also was observed (Fig. 2B, 2D).

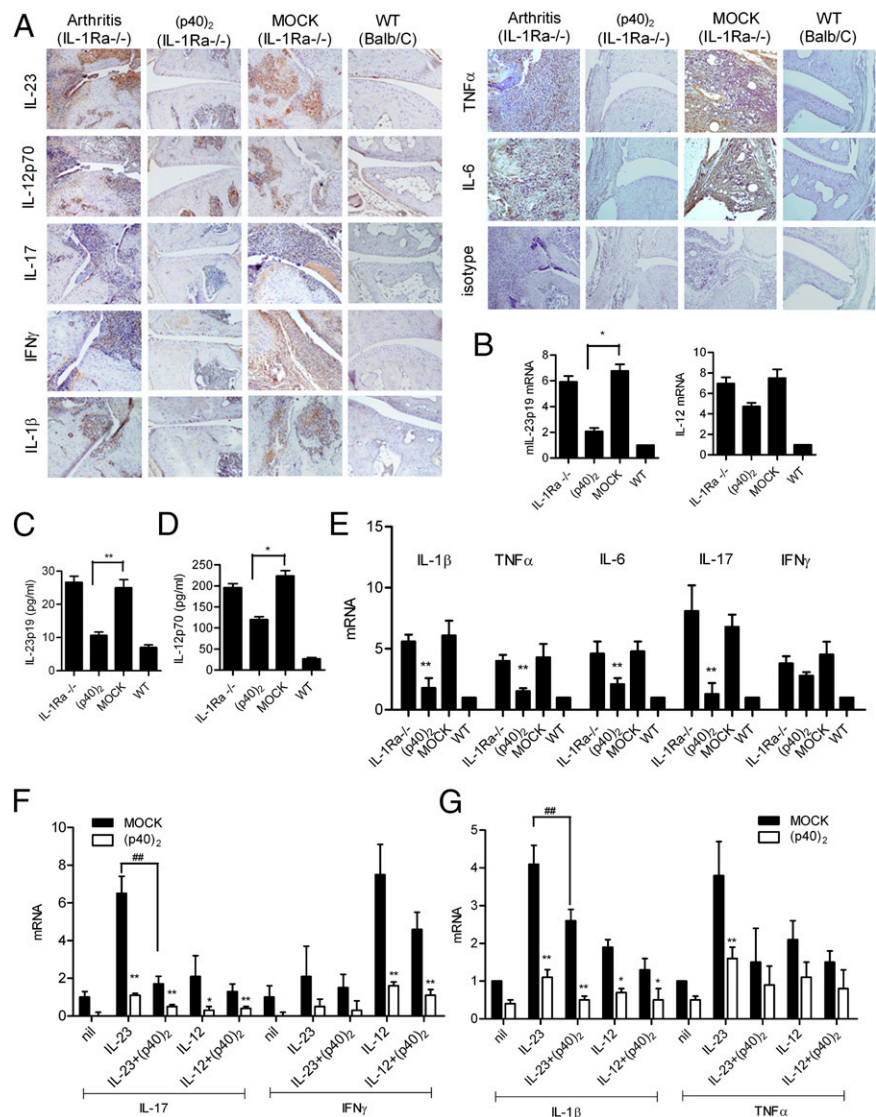
Histopathologic study of the hind leg joints showed normal joint tissue and well-preserved joint space in (p40)₂ therapeutically

treated mice compared with the extensive infiltration of inflammatory cells and loss of joint integrity in IL-1RaKO mice and mock-treated mice (Fig. 2E). TRAP staining showed a differentiation of osteoclasts in synovial tissues from therapeutically treated mice in the arthritis model. The synovial tissues from (p40)₂-transferred mice were TRAP⁻ (Fig. 2F). IgG in the mice sera also decreased in (p40)₂-transferred mice (Fig. 2G). The subtype of the decreased IgG was IgG2a, which is known to be related to the Th1-immune response. The level of IgG1 related to the Th2-type response was similar to that of IL-1RaKO mice.

(p40)₂ inhibited inflammatory cytokines in IL-1RaKO mice

We performed immunohistochemical staining for various cytokines in joints tissues from mock-treated and (p40)₂ therapeutically treated IL-1RaKO mice. IL-23, IL-12p70, IL-17, INF-γ, IL-1β, TNF-α, and IL-6 were expressed strongly in joint tissues from IL-1RaKO mice (Fig. 3A). Expression of those cytokines was suppressed dramatically in joint tissues from (p40)₂-transferred mice. The protein and mRNA expression levels of cytokines were checked in the serum and splenic cells. mRNA expression of IL-23p19 and IL-12p70 was decreased markedly in splenic cells from (p40)₂-transferred mice (*p* < 0.01) (Fig. 3B–D). Next, we evaluated the cytokine levels in joint cells. Expression of the proinflammatory cytokines IL-1β, TNF-α, IL-6, and IL-17 was

FIGURE 3. (p40)₂ inhibits inflammatory cytokine expression in IL-1RaKO mice. **(A–G)** All tissue and cells were obtained from therapeutically treated IL-1RaKO mice. **(A)** IL-1RaKO mice joint tissue was stained with mAb species for IL-23, IL-12p70, IL-17, IFN-γ, IL-1β, TNF-α, and IL-6 (obtained at 6 wk). Brown represents positive staining for IL-23, IL-12p70, IL-17, IFN-γ, IL-1β, TNF-α, and IL-6 (original magnification ×200). Data shown are representative of three independent experiments. **(B)** Spleen cells of wild-type (WT), mock vector, (p40)₂ vector, and PBS mice were harvested at the peak of disease (obtained at 6 wk). mRNA expression of IL-23p19 and IL-12 was analyzed by real-time PCR. **(C and D)** IL-23p19 and IL-12 production was analyzed using ELISA from the culture supernatants of each group. **(E)** mRNA expression of IL-1β, TNF-α, IL-6, IL-17, and IFN-γ was analyzed by real-time PCR in joint cells. **(F and G)** Joint cells of the (p40)₂ injection group and control group were cultured with IL-23 and IL-12, with or without (p40)₂, for 3 d. mRNA expression of IL-17, IFN-γ, IL-1β, and TNF-α was assessed by real-time PCR. Data are mean ± SD and are representative of three independent experiments. **p* < 0.05, ***p* < 0.01, and ##*p* < 0.01.



significantly lower in (p40)₂-transferred mice ($p < 0.01$). The level of INF- γ was lower in (p40)₂-transferred mice than in IL-1RaKO mice, but the difference did not reach statistical significance (Fig. 3E).

(p40)₂ decreased IL-23- or IL-12-induced inflammatory cytokine production

We evaluated the effect of (p40)₂ on cytokine production induced by IL-23 or IL-12 in vitro. The splenic cells obtained from mock-treated IL-1RaKO mice and (p40)₂ therapeutically treated mice were cultured with IL-23, IL-23 plus (p40)₂, IL-12, or IL-12 plus (p40)₂ for 3 d. We observed a significant decrease in IL-23-induced IL-17, IL-1 β , and TNF- α expression and IL-12-induced INF- γ expression by (p40)₂ in splenic cells from mock-transferred mice (Fig. 3F, 3G, $^{##}p < 0.01$). mRNA expression levels of measured cytokines were significantly lower in splenic cells from (p40)₂-transferred mice than in cells from mock-transferred mice.

(p40)₂ inhibited Ag-specific T cell proliferation and cytokine production in CIA mice

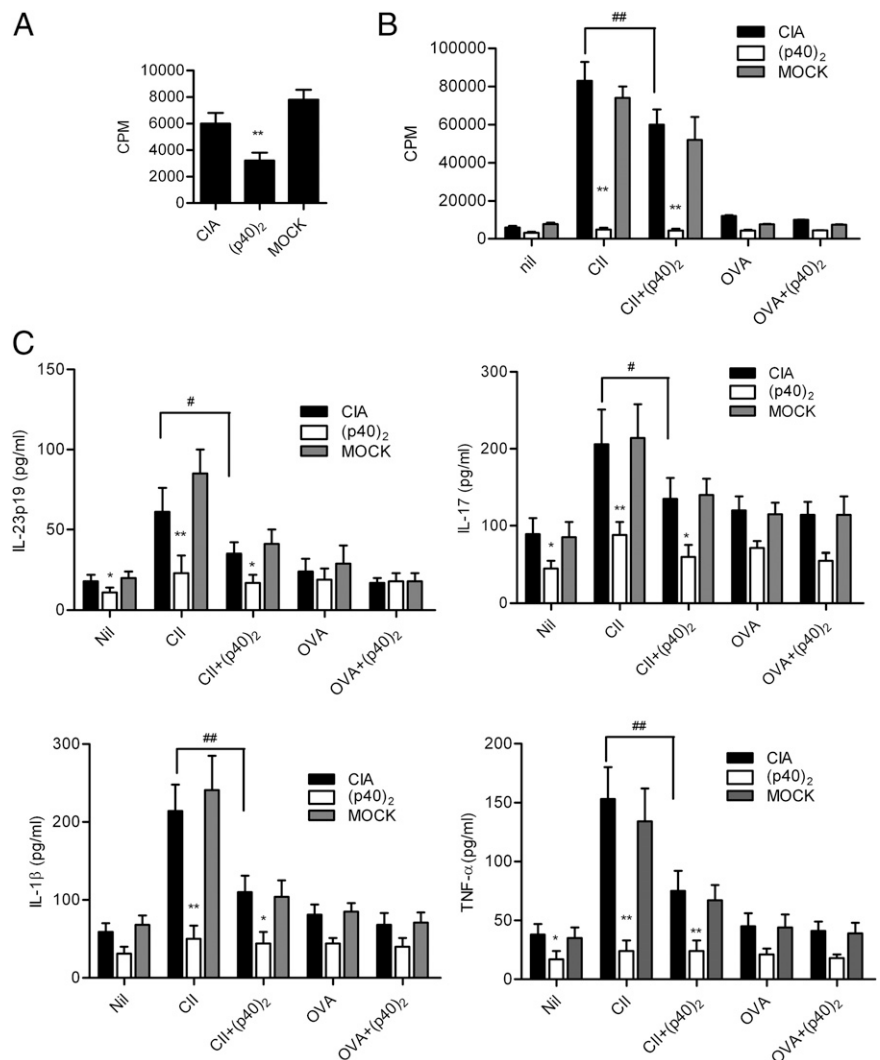
We evaluated the effect of (p40)₂ on the T cell-proliferation response of CD4⁺ T cells from the splenic cells of CIA mice in the therapeutic model 5 wk after the induction of arthritis. The T cell-proliferative response was decreased markedly in splenic cells from (p40)₂ therapeutically treated CIA mice (Fig. 4A, $^{**}p < 0.01$). T cell proliferation was measured in CD4⁺ T cells and APCs for 3 d

after adding CII, CII plus (p40)₂, OVA, or OVA plus (p40)₂ (Fig. 4B). T cell proliferation increased significantly in splenic cells from CIA mice and mock-treated mice in the presence of CII, which suggests that the proliferation is CII specific. The change in T cell proliferation in the presence of CII was not obvious in splenic cells from (p40)₂-transferred mice (Fig. 4B). Furthermore, we observed that (p40)₂ suppressed CII-specific T cell proliferation in vitro (Fig. 4B, $^{##}p < 0.01$). Inflammatory cytokines were measured in the culture supernatant of CD4⁺ T cells and APCs for 3 d after adding CII, CII plus (p40)₂, OVA, and OVA plus (p40)₂ (Fig. 4C). CII significantly increased the levels of IL-23, IL-17, IL-1 β , and TNF- α in T cell-APC cocultures from CIA and mock-transferred mice but not (p40)₂-transferred mice. (p40)₂ in vitro significantly suppressed the increase in inflammatory cytokines ($^{*}p < 0.05$, $^{##}p < 0.01$).

(p40)₂ induced CD4⁺CD25⁺ Tregs in vivo and in vitro

Next, we verified the proportion of CD4⁺CD25⁺Foxp3⁺ Tregs in the spleens of (p40)₂-treated and mock-treated mice using confocal microscopy. Tregs were increased in the spleens from the (p40)₂-transferred mice (Fig. 5A).

We confirmed the effect of the Foxp3⁺ Treg induction of (p40)₂ in vitro. CIA splenic cells were cultured for 72 h with IL-23 or IL-23 plus (p40)₂ in vitro. The levels of Foxp3 protein, as measured by Western blotting, increased significantly after 3 d of culture with IL-23 plus (p40)₂ (Fig. 5B). In addition, Foxp3⁺ Tregs were



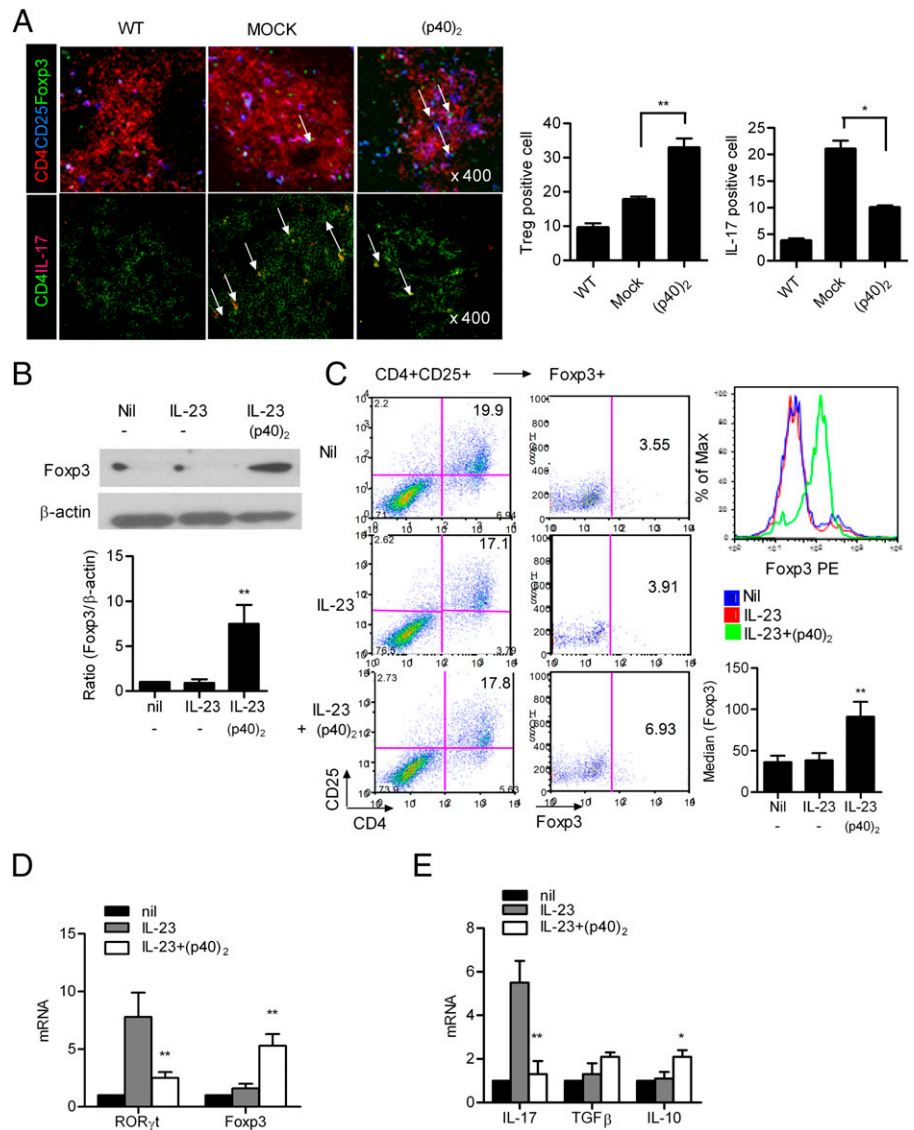


FIGURE 5. (p40)₂ induces CD4⁺CD25⁺ Foxp⁺ Tregs in vivo and in vitro. **(A and B)** Spleen and joint tissue from (p40)₂-injected CIA and control mice were stained with anti-mouse CD4-PE, anti-mouse CD25-allophycocyanin, and anti-mouse Foxp3-FITC. Stained spleen tissue was analyzed using a confocal microscope (original magnification ×400). Arrowheads indicate Treg or Th17 cells. Tregs are purple. Data shown are representative of three independent experiments. **(B and C)** Spleen cells were isolated from CIA mice. The cells were cultured with IL-23 (10 ng/ml) and IL-23 plus (p40)₂ (10 ng/ml) for 3 d. **(B)** Foxp3 protein was measured in cell lysates by Western blot analysis using the Foxp3-specific Ab. **(C)** Cultured cells were stained with anti-mouse CD4-PerCP, anti-mouse CD25-FITC, and anti-mouse Foxp3-PE. CD4⁺CD25⁺Foxp3⁺ Tregs were analyzed using FlowJo software. **(D)** RORγt and Foxp3 mRNA expression was measured in spleen cells by real-time PCR. **(E)** IL-17, TGF-β, and IL-10 mRNA expression was measured in spleen cells by real-time PCR. Data are mean ± SD are representative of three independent experiments. **p* < 0.05, ***p* < 0.01.

increased in the presence of (p40)₂ plus IL-23 (6.93%) compared with IL-23 alone (3.91%). The fluorescent intensity of the Foxp3 signal was increased significantly in the presence of (p40)₂ (Fig. 5C, ***p* < 0.01). The expression of RORγt and IL-17 was significantly decreased in cultures containing IL-23 plus (p40)₂ compared with IL-23 alone (Fig. 5D, 5E, ***p* < 0.01). In contrast, the expression of Foxp3 and IL-10 was significantly increased in cultures carried out in the presence of IL-23 plus (p40)₂ compared with IL-23 alone (***p* < 0.01). The level of TGF-β tended to be higher in cultures performed in the presence of IL-12p40; however, this difference was not significant (Fig. 5E).

We observed the expression of CD4⁺CD25⁺Foxp3⁺ Tregs in the spleens of mice using confocal microscopy. Tregs were increased in the spleens of (p40)₂-transferred mice (Fig. 5).

(p40)₂ induced CD4⁺CD25⁺ Tregs through regulation of STAT molecules

To evaluate the signal molecule of T cell regulation, we observed the expression of p-STAT3 705, p-STAT3 727, and p-STAT5 in spleens of wild-type, mock-treated, and (p40)₂-treated mice using confocal microscopy. p-STAT3 705 and p-STAT3 727 were decreased in the spleens of IL-12p40-transferred mice. In contrast, p-STAT5 was potentially increased in (p40)₂-treated mice compared

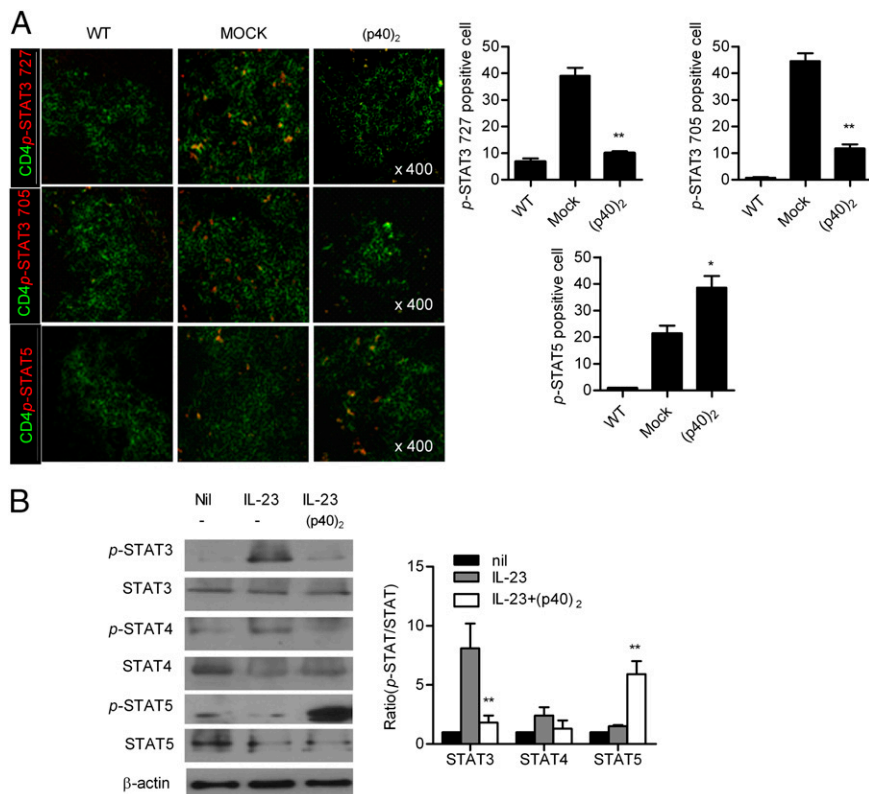
with mock control mice (Fig. 6A). Next, we measured STAT3, STAT4, and STAT5 in the spleen cells of CIA mice. CIA spleen cells were cultured with IL-23 or IL-23 plus (p40)₂ for 72 h. The ratio of p-STAT/STAT was calculated. p-STAT3, which is important in Th17 cell regulation, was increased with IL-23 and decreased with IL-12p40 (Fig. 6B, ***p* < 0.01). STAT4, which is involved in Th1 cell regulation, was a little higher with IL-23 alone than with IL-23 plus IL-12p40. STAT5, which is related to Treg regulation, was highly expressed only with IL-12p40 (Fig. 6).

Discussion

We observed the preventive and therapeutic effect of the (p40)₂ subunit in autoimmune arthritis animal models. In this study, the inhibitory effect on IL-17 production was strongest with (p40)₂ compared with IL-23p19 Ab, IL-12p40 Ab, IL-23R Ab, and soluble IL-23R. We found that (p40)₂ blocked IL-17 effectively at a much lower concentration compared with IL-12p40. (p40)₂ suppressed production of inflammatory cytokines, osteoclastogenesis, and the Ag-specific T cell-proliferation response, and it modulated Th17/Treg balance via STAT3 and STAT5.

In this study, we demonstrated that (p40)₂ suppressed inflammatory arthritis via reciprocal regulation of Th17 and Tregs. We

FIGURE 6. (p40)₂ induces CD4⁺CD25⁺Foxp3⁺ Tregs through STAT molecule regulation. **(A)** Spleen tissue from IL-12(p40)₂-injected CIA and control mice was stained with anti-mouse p-STAT3 705-PE, anti-mouse p-STAT3 727-PE, p-STAT5-PE, and CD4-FITC. Stained spleen tissue was analyzed using a confocal microscope (original magnification ×400). STAT⁺ T cells are yellow. Data shown are representative of three independent experiments. **(B)** Spleen cells from CIA mice were stimulated with IL-23 (10 ng/ml) and IL-23 (10 ng/ml) plus IL-12p40 (10 ng/ml). Protein levels of STAT3, STAT4, STAT5, and the phosphorylated forms of STAT were measured in cell lysates by Western blot analysis using a specific Ab for STAT (left panel). Data are shown as the mean ± SD (right panel). Data are representative of three independent experiments. **p* < 0.05, ***p* < 0.01.



reported previously that simultaneous regulation of Th17 and Tregs is important in the treatment of RA, because an imbalance in Th17/Treg contributed to the development and progression of RA (29–34). We observed that (p40)₂ upregulated the generation of CD4⁺CD25⁺Foxp3⁺ Tregs in an in vitro culture with spleen cells from mice and in vivo with the splenic tissues from (p40)₂-transferred mice. CD4⁺CD25⁺ Tregs are known to play a key role in self-tolerance and in the prevention of autoimmune diseases (25, 35). We observed that (p40)₂ increased the expression of Foxp3 and decreased the expression of RORγt. Th17 cells were shown to be differentiated from CD4⁺ T cells by cytokine-mediated regulation and transcriptional programming. RORγt is as a key regulator of Th17 cell differentiation (8). IL-21 and IL-23 induced RORγt, which, in synergy with STAT3, promoted IL-17 expression (36). STAT3 is a crucial component of Th17 cell differentiation, whereas STAT5 functions as a downregulator of Th17 cells (37). STAT3 and STAT5 signal balance, rather than the absolute magnitude of these molecules, is more important to the generation of Th17 cells (38). We postulated that (p40)₂ stimulated STAT5 and suppressed STAT3 simultaneously, so (p40)₂ also regulated the upregulation of Foxp3+Treg and the suppression of RORγt+Th17 (Fig. 6). We suggest that Fig. 6 explains the mechanism of the antiarthritic property of (p40)₂.

IL-23 is known to be essential to the in vivo function of Th17 cells (36). However, the precise effect of IL-23 on Th17 cell differentiation is not known, although it is important for the proliferation of Th17 cells and for Th17 cell-mediated immune diseases. TGF-β and IL-6 are essential cytokines for inducing Th17 cells. We reported previously that IL-23 is a link between IL-1 and IL-17 and that it is a central proinflammatory cytokine in the pathogenesis of the IL-1Ra^(-/-) model of spontaneous arthritis (39). The suppressive effect of (p40)₂ on arthritis in the IL-1Ra^(-/-) model may occur, in part, through antagonizing IL-23. Although (p40)₂ is known to inhibit both IL-12 and IL-23, the potency of inhibition of the cytokines may be different. We observed that the concentration

of INF-γ induced by IL-12 was decreased with a higher concentration of (p40)₂. Kim et al. (23) also observed that the suppressive effect of (p40)₂ is stronger for IL-23 than for IL-12. They suggested that the affinity of (p40)₂ for receptors of each cytokine may be different. In their study, IFN-γ production from mesenteric lymph node cells was even increased. In our in vitro study, the half-maximal (50%) inhibitory concentration of (p40)₂ for IFN-γ was 10-fold higher than the concentration for IL-17. The differential effect of (p40)₂ on IFN-γ in synovial cells and mesenteric lymph node cells needs to be investigated further. A difference in the concentration of IL-12Rs may be one possible explanation.

Brentano et al. (40) reported that p19, but not p40, was abundantly expressed in RA synovium. They detected heterodimeric IL-23 in only 5 of 28 patients. The differential expression of p19 and p40 in RA synovium may suggest that expression of p40 is independent involvement of p19 in the pathogenesis of RA. We did not observe expression of p19 and p40 in joint tissues of mice. If most CIA animals only express p19, and not p40, the antiarthritic efficacy of (p40)₂ may be primarily through inhibition of IL-12. The expression of heterodimeric proteins of IL-23 and IL-12, as well as the treatment efficacy of (p40)₂ according to the differential expression of the subunits, needs further investigation.

TRAP⁺ osteoclasts were found at the site of active bone erosions in joint tissues in arthritis and are responsible for bone erosions in inflammatory arthritis (41). Inhibition of osteoclastogenesis is important for preventing bone erosion and joint destruction in the treatment of RA. In our experiment, (p40)₂-transferred mice showed intact joint histology with negative TRAP staining, which means that osteoclastogenesis was suppressed by (p40)₂ in vivo. IL-17 and IL-23 are important cytokines that activate osteoclastogenesis in arthritis (42, 43). The preventive effect of the bone erosion by IL-17 neutralization using the soluble IL-17R:Fc fusion protein in experimental arthritis was reported (44). Decreased production of IL-17 and antagonizing IL-23 by (p40)₂ may result in inhibition of osteoclastogenesis in (p40)₂-transferred mice.

RA is a chronic autoimmune disease in which inflammatory cytokines play essential roles in its pathogenesis. TNF- α is considered a key cytokine in the pathogenesis of RA, and the efficacy of TNF- α blockade in the treatment of RA is well documented. The additional role of IL-23 and IL-17 in RA pathogenesis was suggested recently. Unexpectedly, blockade of TNF increased the expression of IL-23p40 and Th17 cells in CIA mice (45). This observation may explain the nonresponse of some RA patients to TNF- α blockade. The therapeutic effect of IL-17 blockade has been assessed in many studies in murine models of arthritis, and clinical trials blocking IL-17 in patients with RA are ongoing. However, the effect is still unknown and needs to be studied as the most physiologically relevant target of IL-17. Our results suggest that blocking IL-23 and IL-17 by (p40)₂ may be a useful preventive and therapeutic method in inflammatory arthritis.

In summary, we demonstrated the powerful inhibitory effects of (p40)₂ on the development of inflammatory arthritis in an animal model of RA. The results showed that (p40)₂ inhibited the generation of Th17 cells, whereas it stimulated the generation of Tregs via the STAT3 and STAT5 pathways.

Acknowledgments

We thank the Institutional Animal Care and Use Committee, School of Medicine, Catholic University of Korea, for help with animal care and study.

Disclosures

The authors have no financial conflicts of interest.

References

- Furuzawa-Carballeda, J., M. I. Vargas-Rojas, and A. R. Cabral. 2007. Autoimmune inflammation from the Th17 perspective. *Autoimmun. Rev.* 6: 169–175.
- Ouyang, W., J. K. Kolls, and Y. Zheng. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 28: 454–467.
- Tesmer, L. A., S. K. Lundy, S. Sarkar, and D. A. Fox. 2008. Th17 cells in human disease. *Immunol. Rev.* 223: 87–113.
- Shahrara, S., Q. Huang, A. M. Mandelin, II, and R. M. Pope. 2008. TH-17 cells in rheumatoid arthritis. *Arthritis Res. Ther.* 10: R93.
- Cho, M. L., Y. O. Jung, K. W. Kim, M. K. Park, H. J. Oh, J. H. Ju, Y. G. Cho, J. K. Min, S. I. Kim, S. H. Park, and H. Y. Kim. 2008. IL-17 induces the production of IL-16 in rheumatoid arthritis. *Exp. Mol. Med.* 40: 237–245.
- Hwang, S. Y., J. Y. Kim, K. W. Kim, M. K. Park, Y. Moon, W. U. Kim, and H. Y. Kim. 2004. IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF- κ B- and PI3-kinase/Akt-dependent pathways. *Arthritis Res. Ther.* 6: R120–R128.
- Koenders, M. I., L. A. Joosten, and W. B. van den Berg. 2006. Potential new targets in arthritis therapy: interleukin (IL)-17 and its relation to tumour necrosis factor and IL-1 in experimental arthritis. *Ann. Rheum. Dis.* 65(Suppl 3): iii29–iii33.
- Ivanov, I. I., B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelletier, J. J. Lafaille, D. J. Cua, and D. R. Littman. 2006. The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126: 1121–1133.
- Kim, H. R., H. S. Kim, M. K. Park, M. L. Cho, S. H. Lee, and H. Y. Kim. 2007. The clinical role of IL-23p19 in patients with rheumatoid arthritis. *Scand. J. Rheumatol.* 36: 259–264.
- Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201: 233–240.
- Oppmann, B., R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, et al. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13: 715–725.
- Tan, Z. Y., K. W. Bealgey, Y. Fang, Y. M. Gong, and S. Bao. 2009. Interleukin-23: immunological roles and clinical implications. *Int. J. Biochem. Cell Biol.* 41: 733–735.
- Crotti, L., C. Spazzolini, P. J. Schwartz, W. Shimizu, I. Denjoy, E. Schulze-Bahr, E. V. Zaklyazminskaya, H. Swan, M. J. Ackerman, A. J. Moss, et al. 2007. The common long-QT syndrome mutation KCNQ1/A341V causes unusually severe clinical manifestations in patients with different ethnic backgrounds: toward a mutation-specific risk stratification. *Circulation* 116: 2366–2375.
- Vignali, D. A., and V. K. Kuchroo. 2012. IL-12 family cytokines: immunological playmakers. *Nat. Immunol.* 13: 722–728.
- Gillessen, S., D. Carvajal, P. Ling, F. J. Podlaski, D. L. Stremlo, P. C. Familletti, U. Gubler, D. H. Presky, A. S. Stern, and M. K. Gately. 1995. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *Eur. J. Immunol.* 25: 200–206.
- Mattner, F., S. Fischer, S. Guckes, S. Jin, H. Kaulen, E. Schmitt, E. Rude, and T. Germann. 1993. The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. *Eur. J. Immunol.* 23: 2202–2208.
- Abdi, K. 2002. IL-12: the role of p40 versus p75. *Scand. J. Immunol.* 56: 1–11.
- Khader, S. A., S. Partida-Sanchez, G. Bell, D. M. Jelley-Gibbs, S. Swain, J. E. Pearl, N. Ghilardi, F. J. Desautave, F. E. Lund, and A. M. Cooper. 2006. Interleukin 12p40 is required for dendritic cell migration and T cell priming after *Mycobacterium tuberculosis* infection. *J. Exp. Med.* 203: 1805–1815.
- Huax, F., M. Arras, D. Tomasi, V. Barbarin, M. Delos, J. P. Coutelier, A. Vink, S. H. Phan, J. C. Renauld, and D. Lison. 2002. A profibrotic function of IL-12p40 in experimental pulmonary fibrosis. *J. Immunol.* 169: 2653–2661.
- Piccotti, J. R., S. Y. Chan, K. Li, E. J. Eichwald, and D. K. Bishop. 1997. Differential effects of IL-12 receptor blockade with IL-12 p40 homodimer on the induction of CD4+ and CD8+ IFN- γ -producing cells. *J. Immunol.* 158: 643–648.
- Mikols, C. L., L. Yan, J. Y. Norris, T. D. Russell, A. P. Khalifah, R. R. Hachem, M. M. Chakinala, R. D. Yusem, M. Castro, E. Kuo, et al. 2006. IL-12 p80 is an innate epithelial cell effector that mediates chronic allograft dysfunction. *Am. J. Respir. Crit. Care Med.* 174: 461–470.
- Nakajima, A., C. M. Seroogy, M. R. Sandora, I. H. Turner, G. L. Costa, C. Taylor-Edwards, M. H. Bachmann, C. H. Contag, and C. G. Fathman. 2001. Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis. *J. Clin. Invest.* 107: 1293–1301.
- Kim, D. J., K. S. Kim, M. Y. Song, S. H. Seo, S. J. Kim, B. G. Yang, M. H. Jang, and Y. C. Sung. 2012. Delivery of IL-12p40 ameliorates DSS-induced colitis by suppressing IL-17A expression and inflammation in the intestinal mucosa. *Clin. Immunol.* 144: 190–199.
- Shevach, E. M. 2002. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2: 389–400.
- Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
- Kelchtermans, H., L. Geboes, T. Mitera, D. Huskens, G. Leclercq, and P. Matthys. 2009. Activated CD4+CD25+ regulatory T cells inhibit osteoclastogenesis and collagen-induced arthritis. *Ann. Rheum. Dis.* 68: 744–750.
- Gonzalez-Rey, E., A. Chorny, N. Varela, F. O'Valle, and M. Delgado. 2007. Therapeutic effect of urocortin on collagen-induced arthritis by down-regulation of inflammatory and Th1 responses and induction of regulatory T cells. *Arthritis Rheum.* 56: 531–543.
- Camps, M., T. Rückle, H. Ji, V. Ardissonne, F. Rintelen, J. Shaw, C. Ferrandi, C. Chabert, C. Gillieron, B. Françon, et al. 2005. Blockade of PI3K γ suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. *Nat. Med.* 11: 936–943.
- Son, H. J., J. Lee, S. Y. Lee, E. K. Kim, M. J. Park, K. W. Kim, S. H. Park, and M. L. Cho. 2014. Metformin attenuates experimental autoimmune arthritis through reciprocal regulation of Th17/Treg balance and osteoclastogenesis. *Mediators Inflamm.* 2014: 973986.
- Jhun, J., J. Lee, J. K. Byun, E. K. Kim, J. W. Woo, J. H. Lee, S. K. Kwok, J. H. Ju, K. S. Park, H. Y. Kim, et al. 2014. Red ginseng extract ameliorates autoimmune arthritis via regulation of STAT3 pathway, Th17/Treg balance, and osteoclastogenesis in mice and human. *Mediators Inflamm.* 2014: 351856.
- Moon, S. J., M. A. Lim, J. S. Park, J. K. Byun, S. M. Kim, M. K. Park, E. K. Kim, Y. M. Moon, J. K. Min, S. M. Ahn, et al. 2014. Dual-specificity phosphatase 5 attenuates autoimmune arthritis via reciprocal regulation of Th17/Treg balance and inhibition of osteoclastogenesis. *Arthritis Rheumatol.* 66: 3083–3095.
- Moon, S. J., J. S. Park, Y. J. Woo, M. A. Lim, S. M. Kim, S. Y. Lee, E. K. Kim, H. J. Lee, W. S. Lee, S. H. Park, et al. 2014. Rebamipide suppresses collagen-induced arthritis through reciprocal regulation of th17/treg cell differentiation and heme oxygenase 1 induction. *Arthritis Rheumatol.* 66: 874–885.
- Yang, E. J., J. Lee, S. Y. Lee, E. K. Kim, Y. M. Moon, Y. O. Jung, S. H. Park, and M. L. Cho. 2014. EGCG attenuates autoimmune arthritis by inhibition of STAT3 and HIF-1 α with Th17/Treg control. *PLoS One* 9: e86062.
- Lim, J. Y., M. J. Park, K. I. Im, N. Kim, E. J. Jeon, E. J. Kim, M. L. Cho, and S. G. Cho. 2014. Combination cell therapy using mesenchymal stem cells and regulatory T-cells provides a synergistic immunomodulatory effect associated with reciprocal regulation of TH1/TH2 and th17/treg cells in a murine acute graft-versus-host disease model. *Cell Transplant.* 23: 703–714.
- Sakaguchi, S., M. Ono, R. Setoguchi, H. Yagi, S. Hori, Z. Fehervari, J. Shimizu, T. Takahashi, and T. Nomura. 2006. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol. Rev.* 212: 8–27.
- Zhou, L., I. I. Ivanov, R. Spolski, R. Min, K. Shenderov, T. Egawa, D. E. Levy, W. J. Leonard, and D. R. Littman. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* 8: 967–974.
- Dong, C. 2008. TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nat. Rev. Immunol.* 8: 337–348.
- Yang, X. P., K. Ghoreschi, S. M. Steward-Tharp, J. Rodriguez-Canales, J. Zhu, J. R. Grainger, K. Hirahara, H. W. Sun, L. Wei, G. Vahedi, et al. 2011. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat. Immunol.* 12: 247–254.
- Cho, M. L., J. W. Kang, Y. M. Moon, H. J. Nam, J. Y. Jhun, S. B. Heo, H. T. Jin, S. Y. Min, J. H. Ju, K. S. Park, et al. 2006. STAT3 and NF- κ B signal

- pathway is required for IL-23-mediated IL-17 production in spontaneous arthritis animal model IL-1 receptor antagonist-deficient mice. *J. Immunol.* 176: 5652–5661.
40. Brentano, F., C. Ospelt, J. Stanczyk, R. E. Gay, S. Gay, and D. Kyburz. 2009. Abundant expression of the interleukin (IL)23 subunit p19, but low levels of bioactive IL23 in the rheumatoid synovium: differential expression and Toll-like receptor-(TLR) dependent regulation of the IL23 subunits, p19 and p40, in rheumatoid arthritis. *Ann. Rheum. Dis.* 68: 143–150.
 41. Wu, Y., J. Liu, X. Feng, P. Yang, X. Xu, H. C. Hsu, and J. D. Mountz. 2005. Synovial fibroblasts promote osteoclast formation by RANKL in a novel model of spontaneous erosive arthritis. *Arthritis Rheum.* 52: 3257–3268.
 42. Lubberts, E. 2003. The role of IL-17 and family members in the pathogenesis of arthritis. *Curr. Opin. Investig. Drugs* 4: 572–577.
 43. Ju, J. H., M. L. Cho, Y. M. Moon, H. J. Oh, J. S. Park, J. Y. Jhun, S. Y. Min, Y. G. Cho, K. S. Park, C. H. Yoon, et al. 2008. IL-23 induces receptor activator of NF-kappaB ligand expression on CD4+ T cells and promotes osteoclastogenesis in an autoimmune arthritis model. *J. Immunol.* 181: 1507–1518.
 44. Bush, K. A., K. M. Farmer, J. S. Walker, and B. W. Kirkham. 2002. Reduction of joint inflammation and bone erosion in rat adjuvant arthritis by treatment with interleukin-17 receptor IgG1 Fc fusion protein. *Arthritis Rheum.* 46: 802–805.
 45. Notley, C. A., J. J. Inglis, S. Alzabin, F. E. McCann, K. E. McNamee, and R. O. Williams. 2008. Blockade of tumor necrosis factor in collagen-induced arthritis reveals a novel immunoregulatory pathway for Th1 and Th17 cells. *J. Exp. Med.* 205: 2491–2497.