IL-1R Type 2 Suppresses Collagen-Induced Arthritis by Inhibiting IL-1 Signal on Macrophages

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IL-1α and IL-1β (in this article referred to as IL-1) play important roles in host defense against infection and inflammatory diseases. IL-1R1 is the receptor for IL-1, and IL-1R2 is suggested to be a decoy receptor, because it lacks the signal-transducing TIR domain in the cytoplasmic part. However, the roles of IL-1R2 in health and disease remain largely unknown. In this study, we generated EGFP-knock-in Il1r2−/− mice and showed that they were highly susceptible to collagen-induced arthritis, an animal model for rheumatoid arthritis in which the expression of IL-1R2 is augmented in inflammatory joints. Il1r2 was highly expressed in neutrophils but had only low expression in other cells, including monocyes and macrophages. Ab production and T cell responses against type II collagen were normal in Il1r2−/− mice. Despite the high expression in neutrophils, no effects of Il1r2 deficiency were observed; however, we found that production of inflammatory mediators in response to IL-1 was greatly enhanced in Il1r2−/− macrophages. These results suggest that IL-1R2 is an important regulator of arthritis by acting specifically on macrophages as a decoy receptor for IL-1.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMC, bone marrow cell; BMMP, bone marrow macrophage; CIA, collagen-induced arthritis; DC, dendritic cell; ES, embryonic stem; FLSC, fibroblast-like synovial cell; ICC, chicken type II collagen; LN, lymph node; RA, rheumatoid arthritis; ROS, reactive oxygen species; TGC, thyroglobulite; TGCMP, TGC-elicited peritoneal macrophage; WT, wild-type.

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characterized by chronic inflammation of synovial tissues in multiple joints that causes swelling of joints, pain, joint deformity, and loss of joint function. Studies using animal models demonstrated that IL-1 is involved in the development of RA (18, 19), and blockade of IL-1 signaling is effective in treating RA (20–23), although this cytokine is primarily important in the development of autoinflammatory diseases, such as Familial Mediterranean fever, cryopyrin-associated periodic syndrome, and Still’s disease (19, 24, 25). Because the concentration of IL-1R2 protein is increased in the synovial fluid (26) and plasma (27, 28) of RA patients, and the promoter region of the IL1R2 gene is hypo-methylated in PBMCs of RA patients (28), IL-1R2 is suggested to be involved in its pathogenesis.

In this study, we investigated the role of IL-1R2 in the pathogenesis of arthritis using Il1r2<sup>−/−</sup> mice. Our results demonstrate that IL-1R2 is an important negative regulator of collagen-induced arthritis (CIA) by suppressing IL-1 functions in macrophages as a decoy receptor.

Materials and Methods

Mice

C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan) and kept in mouse rooms of the Research Institute for Biomedical Sciences, Tokyo University of Science for ≥1 wk before use. Age-matched (7–10 wk-old) and sex-matched mice were used for all experiments. Il1r2<sup>−/−</sup> mice were obtained from the Central Institute for Experimental Animals. Il1r2<sup>−/−</sup> mice were generated, as previously described (5), and backcrossed to C57BL/6J mice for eight generations. Il1r2<sup>−/−</sup>Il1r2<sup>−/−</sup> mice were obtained by crossing Il1r2<sup>−/−</sup> mice and Il1r2<sup>−/−</sup>Il1r2<sup>−/−</sup> mice. Il1r2<sup>−/−</sup> mice were provided by Immunex (Amgen, Thousand Oaks, CA) (29). All mice were kept under specific pathogen–free conditions in environmentally controlled clean rooms in The Institute of Medical Science and The Graduate School of Agricultural and Life Science, The University of Tokyo, and the Research Institute for Biomedical Sciences, Tokyo University of Science. All experiments were approved by the institutional animal use committees and were conducted according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene-manipulation experiments.

Generation of Il1r2<sup>−/−</sup> mice

Il1r2<sup>−/−</sup> mice were generated by gene targeting using embryonic stem (ES) cells (Fig. 1). For the cloning of the homologous arms of the targeting vector, Il1r2 genomic clones were isolated from mouse 129 genomic phase libraries from Stratagene (129SvJ Mouse Genomic Library in the FIX II Vector; La Jolla, CA) using the cDNA probe amplified by the following PCR primers: forward, 5′-CCCCATTACATCGGAGAAGGCCCA-3′ and reverse, 5′-TCCATGCGACGTGGATATAC-3′. The 5.9-kb 5′ arm fragment was generated by ligating two DNA fragments; the 5′ side was cloned from a Il1r2 genomic clone digested with EcoRV and EcoRI, whereas the 3′ side was amplified using the following PCR primers: forward, 5′-GAATCTTACACAGATTTTCGAAAC-3′ and reverse, 5′-GAAGTGGTGTCGCGACCATCTCCTAC-3′. The 2.6-kb 3′ arm fragment was cloned from the Il1r2 genomic clone by digesting with EcoRV. Then, the homologous region of E14.1 ES cells containing the second, third, and fourth exons of the Il1r2 gene, which ranged from the initiation codon (ATG) to an EcoRV site just after the fourth exon, was replaced by the 2.5-kb DNA fragment containing the EFGP gene and the neomycin resistance gene (neo) under the phosphoglycerate kinase 1 promoter, which was flanked by lox P sequences using homologous recombination techniques (Fig. 1) (30). A diphtheria toxin A gene under the MC1 promoter was ligated to the 3′ end of the targeting vector for the negative selection.

Cloned ES clones were screened by Southern blot hybridization analysis using the 3′ probe amplified by the following PCR primers: forward, 5′-TGGCAGGATATAACACATCA-3′ and reverse, 5′-ATTCCCCAGGCAGCTTAGTG-3′. Chimeric mice were generated by aggregating ES cells with C57BL/6J blastocysts (30), and the neo gene was deleted by injecting the Cre recombinase gene under the CAG promoter into fertilized eggs (31). Il1r2 mutant mice were backcrossed to C57BL/6J mice for 11 generations. The genotyping of Il1r2 mutant mice was carried out using the following PCR primers: primer 1, 5′-CCAACTGGAAGCTGAGATTCC-3′; primer 2, 5′-CACCTTGCGCCGTTCGATTCG-3′; and primer 3, 5′-CGCCGATATAGACGTTTGGG-3′. Primers 1 and 2 were used to detect the wild-type (WT) allele (0.2 kb), and primers 1 and 3 were used to detect the mutant allele (0.5 kb).

RT-PCR

Total RNA was isolated from bone marrow cells (BMCs) of WT and Il1r2<sup>−/−</sup> mice using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). Target gene cDNA was amplified by PCR with KOD-Fx Neo (TOYOBO, Osaka, Japan) and the following primers: for Il1r2, forward (exon 3; P1), 5′-TGGGTTAAGGGTGAACATACCTGGA-3′, forward (exon 5; P2), 5′-GGGCCGACATCTCTTGGTA-3′, and reverse (exon 9; P3): 5′-CCGTTTGATGCCAGCTGCAA-3′ and for Actb, forward, 5′-GATTCCACATCGTGCTGGAAAG-3′ and reverse, 5′-AAGTGTGACGTTGATCCG-3′.

Flow cytometry

Cells were treated with an anti-CD16/32 mAb (clone 93; BioLegend, San Jose, CA) for 30 min at 4˚C in HBSS containing 2% FBS and 0.01% sodium azide and then stained with Abs described in Table I for 30 min at 4˚C. For intracellular staining, cells were stained with 50 ng/ml PMA, 500 ng/ml ionomycin, and 2 µM monensin (all from Sigma, St. Louis, MO) for 5 h. After staining surface Abs, cells were fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer (eBioscience, San Diego, CA), according to the manufacturer’s instructions. The intracellular Abs were stained with the Abs described in Table I for 30 min at 4˚C, followed by analysis with a FACS Canto II (BD Biosciences, San Jose, CA) and FlowJo software (TreeStar, Ashland, OR). Biotin-conjugated anti-CD40 mAb was stained with Pacific Blue–conjugated streptavidin (Invitrogen, Carlsbad, CA). Dead cells were stained with 7-aminomethylcoumarin D (Sigma) or an Aqua Dead Cell Stain Kit (Molecular Probes, Eugene, OR).

Collagen-induced arthritis

CIA was achieved as previously described (32). CFA was prepared by mixing 1 mg heat-killed Mycobacterium tuberculosis (H37Ra; Difco Laboratories, Detroit, MI) with 1 ml IFA (Thermo Scientific, Waltham, MA). Chicken type II collagen (ICC; 4 mg; Sigma) was dissolved in 1 ml 10 mM acetic acid overnight at 4˚C. An emulsion was formed by combining CFA with an equal volume of ICC solution. Mice were immunized by s.c. injection with 200 µl emulsion at two different sites on each hind flank. Twenty days later, mice received the same immunization plus 10 µg LPS (Sigma) i.p. as a boost. Arthritis severity was scored for each limb with maximum possible score of 12: 0 = normal, 1 = slight swelling and/or erythema, 2 = extensive swelling and/or erythema, 3 = ankylosing change of the joint.

Histological assessment of arthritis

On day 40 of CIA, two hind limbs were fixed with 10% neutral-buffered formalin and decalcified with 10% EDTA. They were embedded in paraffin and cut into 6-µm sections. Hematoxylin and eosin staining was performed to count the number of infiltrating cells. Scoring of joint damage was performed by two independent raters. Histological parameters—synovial inflammation, pannus formation, cartilage damage, and bone damage—were scored with total score 6 for two hind legs for each parameters. Inflammation: 0 = normal, 1 = local infiltration of a few inflammatory cells, 2 = broad local infiltration, and 3 = broad infiltration invading the joint capsule. Pannus formation: 0 = normal, 1 = pannus formation at up to two sites, 2 = pannus formation at up to four sites, and 3 = pannus formation at more than four sites, one broad pannus formation counts as two sites. Cartilage damage: 0 = normal, 1 = small loss of articular chondrocytes, 2 = cartilage degradation in one region, and 3 = cartilage degradation in more than two regions. Bone damage: 0 = normal, 1 = rough surface of talus, 2 = shallow loss of talus, and 3 = deep loss of talus.

Measurement of mRNA levels in inflammatory joints

On day 40 of CIA, two hind limbs were homogenized in Sepasol–RNA I Super, and RNA was purified according to the manufacturer’s instructions. The resulting RNAs were reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Quantitative PCR was performed with the SYBR Premix Ex Taq (Takara; Shiga, Japan) using an iCycler system (Bio-Rad, Hercules, CA). The content of mRNA was determined from the appropriate standard curve and normalized to the amount of Gspdh mRNA. The primer sets are shown in Table II.

Measurement of collagen-specific Ab concentrations

Sera were collected from the tail on days 0 and 40 of CIA. A total of 20 µg/ml ICB in PBS was coated on a 96-well plate at 4˚C overnight. Then the wells were blocked with 10% FBS in PBS at room temperature for 1 h. Next, diluted serum samples (5000-, 2500-, 500-, 50-, and 100-fold dilution for total IgG, IgG1, IgG2a, IgG2b, and IgG3, respectively) were
applied and incubated at room temperature for 1 h, followed by the addition of 0.8 μg/ml HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) or 0.4 μg/ml alkaline phosphatase–conjugated anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Santa Cruz, Dallas, TX). Then, 3,3′,5,5′-tetramethylbenzidine solutions (Dako, Carpinteria, CA) or p-nitrophenyl phosphate solutions (Sigma) were applied as the substrate, and 1 N HCl or 1 N NaOH was added to stop color development. The absorbancy at 450 or 415 nm was measured using a microplate reader (MTP-300; Corona Electric, Hitachinaka, Japan). The wells were washed with 0.05% Tween 20 in PBS between each step. The IgG Ab concentration was calculated using an anti-IIC polyclonal IgG standard with a known concentration. The anti-IIC polyclonal IgG was purified from pooled sera obtained from IIC-immunized mice. We used Protein G Sepharose for prepurification and NHS-activated Sepharose (both from GE Healthcare, Waukesha, WI) for affinity-based purification.

**IIC-specific proliferation assay and cytokine titration**

We immunized mice with IIC and CFA and harvested lymph nodes (LNs) at day 7 after immunization. Then, LN cells (5 × 10^5 cells/well in 96-well plate) were cultured in the absence or presence of 50 μg/ml heat-denatured IIC for 3 d and labeled with [3H]thymidine (0.25 μCi/ml; PerkinElmer, Boston, MA) for 6 h. RPMI 1640 (Wako, Osaka, Japan) containing 10% FBS, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Big Cabin, OK), so-called “R10 medium,” was used as culture medium. Then, cells were harvested with a Micro 96 cell harvester (Skatron, Tranby, Norway), and radioactivity was measured with Micro Beta (Pharmacia Biotech, Uppsala, Sweden).

To measure cytokine concentrations, we collected the culture supernatants from the culture for proliferation assay after 3 d and measured the concentration of IFN-γ, IL-17, and TNF with the mouse IFN-γ ELISA set, the mouse IL-17 ELISA set (both from R&D Systems, Minneapolis, MN), and the mouse TNF-α ELISA MAX (BioLegend), respectively.

**Myeloid cell preparation and culture**

Bone marrow macrophages (BMMPs) were prepared from BMCs, as previously described (33). In brief, we seeded BMCs obtained from femurs and tibiae at 1 × 10^6 cells/ml in a 100-mm nontreated dish using R10

Table II. Primer sets

<table>
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<th>Gene</th>
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<th>Reverse (5’ to 3’)</th>
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<td>Cxcl2</td>
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<tr>
<td>Gapdh</td>
<td>TTACACGAGAAGGAGCCAGCTT</td>
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<tr>
<td>Hprt</td>
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<td>Il1b</td>
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<td>Psig2</td>
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<td>GCCCTGAGTGTGAGGAGGAGT</td>
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<tr>
<td>Tnf</td>
<td>GCCTGCCTCTCAGGTACCTG</td>
<td>CACTGGGAGGTTAGTGTCTC</td>
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Table I. Abs to the following Ags

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<th>Label</th>
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<th>Source</th>
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<td>B220</td>
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<td>RA3-6B2</td>
<td>BioLegend</td>
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<tr>
<td>B220</td>
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</tr>
<tr>
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<tr>
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<td>PE</td>
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<td>3/23</td>
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<td>Armenian hamster</td>
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</table>

IgG isotype control

| Rat IgG2a isotype control | Allophycocyanin | RTK2758 | BioLegend |

Dried down and stored at −20°C. The IgG isotype control was purified from mouse IgG1 (Bethyl Laboratories, Montgomery, TX).

**Gene Expression Analysis**

RPMI 1640 (Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS) (Life Technologies) and 1% Penicillin-Streptomycin (Life Technologies) was used for cell culture. The cells were cultured in 24-well plates at a density of 2 × 10^5 cells/well and incubated at 37°C in a 5% CO2 atmosphere. After 24 h, the medium was changed to fresh medium containing 50 μg/ml heat-denatured IIC. The cells were harvested at 24 h and immediately frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of total RNA using the SuperScript® III First-Strand Synthesis System for RT-PCR (Life Technologies). The mRNA expression levels of selected genes were determined using the TaqMan® Gene Expression Assays (Life Technologies) and the CFX96 Real-Time PCR Detection System (Bio-Rad). The expression levels of each gene were normalized to the housekeeping gene GAPDH, and fold changes were calculated using the 2^(-ΔΔCt) method.

**Statistical Analysis**

All data are presented as mean ± standard deviation (SD). The statistical significance was determined using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. Differences were considered statistically significant when *p* < 0.05. The data were analyzed using GraphPad Prism 8.0 software (GraphPad, San Diego, CA). The results are representative of at least three independent experiments.

**Supporting Information**

Table S1. List of antibodies used in this study.

**Acknowledgments**

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**References**


medium supplemented with 20 ng/ml recombinant mouse M-CSF (R&D Systems). On day 3, nonadherent cells were collected and recultured with another 10 ml fresh medium. At day 7, nonadherent cells were discarded, and adherent cells were collected by treating with 0.7 mM EDTA/PBS.

Thiglycollate (TGC)-elicited peritoneal macrophages (TGCMPs) were prepared by injecting mice i.p. with 1 ml 4% TGC (Nissui, Tokyo, Japan), followed by the collection of peritoneal cells; dish-adherent cells were used as TGCMPs after culture overnight. Neutrophils (Ly6G+Ly6C- and monocytes (Ly6G- Ly6Cint) were sorted from BMCs by flow cytometry on a FACSria (BD Biosciences) or MoFlo XDP IntelliSort II (Beckman Coulter, Brea, CA). Obtained myeloid cells were cultured in R10 medium containing the stimulants described below.

For cell survival assay, neutrophils were cultured with 10 ng/ml IL-1β, IL-4, or GM-CSF (all from PeproTech, Rocky Hill, NJ) for 48 h. Live cells (7AAD-AnnexinV- [BioLegend]) were counted by flow cytometry. Survival ratio was calculated by dividing the live cell number for each condition by the cell number without any cytokines. For the reactive oxygen species (ROS)-generation assay, neutrophils were cultured with 1 mM luminol (Santa Cruz) plus either 100 ng/ml IL-1β or 50 ng/ml C5a (R&D Systems). After stimulation, chemiluminescence was measured with an EnVision plate reader (PerkinElmer, Norwalk, CT) at the indicated time points. For the monocyte-activation assay, monocytes were stimulated with 100 ng/ml IL-1β or 1 μg/ml Pam3CSK4 (InvivoGen, San Diego, CA). Cells were collected 15 h later, and surface CD40 expression was examined by flow cytometry.

For the cytokine-production assay, neutrophils and monocytes were treated with either IL-1β or LPS (Sigma), whereas BMMPs and TGCMPs were cultured with 10 ng/ml IL-1α (PeproTech), 10 ng/ml IL-1β, or 1 ng/ml LPS. Culture supernatants were collected after 24 h, and the concentrations of TNF and IL-6 were measured with the Mouse TNF-α ELISA MAX and Mouse IL-6 ELISA MAX (both from BioLegend), respectively. For the measurement of mRNAs, cultured BMMPs and TGCMPs were stimulated with 10 ng/ml IL-1α, 10 ng/ml IL-1β, or 1 ng/ml LPS; 3 h later, total RNAs were purified using a GenElute Mammalian Total RNA Miniprep kit (Sigma). Similarly, mRNAs from monocytes were collected after stimulation with 100 ng/ml IL-1β or 1 μg/ml LPS. Quantitative PCR was performed, as described above, and the content of mRNA was normalized to the amount of Hprt mRNA.

For Western blotting analysis, BMMPs were cultured for 8 h in RPMI 1640 medium and stimulated with 5 ng/ml IL-1α plus 5 ng/ml IL-1β or 1 ng/ml LPS. Then, cells were lysed with sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% glycerol, 0.003% bromophenol blue, 5% 2-ME, 61.7 mM NaCl, 1.2 mM KCl, 4.5 mM Na2HPO4, and 0.8 mM KH2PO4) and electrophoresed on 12.5% polyacrylamide gels (Wako). After transfer of proteins onto a polyvinyl difluoride membrane (Bio-Rad Laboratories, Shingawa, Japan), the membrane was incubated with an Ab to JNK1/JNK2 (R&D Systems), phosphorylated JNK1/JNK2, phosphorylated p38, or 50 ng/ml C5a (R&D Systems), or PGE2 (Nacalai Tesque) for 20 h. Cultured supernatants were collected at 20 h, and the concentration of CCL2 and IL-6 was measured with the Mouse CCL2/JE/MCP-1 DuoSet (R&D Systems) and Mouse IL-6 ELISA MAX (BioLegend), respectively.

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Statistical analysis

The *p* values were calculated using the Mann–Whitney *U* test for clinical and histological scores of CIA, the χ² test for incidence of CIA, and the two-tailed unpaired Student *t* test for all other experiments. The *p* values < 0.05 were considered significant.

Results

Generation of II1r2<sup>−/−</sup> mice

We generated II1r2<sup>−/−</sup> mice by replacing exons 2, 3, and 4 of the II1r2 gene with the EGFP gene and the neomycin-resistant gene using homologous-recombination techniques (Fig. 1A). Homologous recombination was confirmed by genomic Southern blot hybridization analysis (Fig. 1B). II1r2 deficiency was verified by RT-PCR using the RNA from bone marrow. Consistent with this, we did not detect the PCR product from exons 3–9. The PCR product from exons 5–9 also was not detected, indicating that there was no truncated mRNA from the targeted gene (Fig. 1C).

When II1r2<sup>+/−</sup> mice were intercrossed, II1r2<sup>−/−</sup> mice were born at the expected Mendelian ratio. They were fertile and showed no obvious phenotypic abnormalities under specific pathogen–free conditions (data not shown). The content of T cells, B cells, plasmacytoid DCs, conventional DCs, migratory DCs, monocytes, and neutrophils in the LNs and bone marrow of II1r2<sup>−/−</sup> mice was normal, as analyzed by flow cytometry (Fig. 1D, 1E).

We analyzed the expression of II1r2 through the expression of EGFP in II1r2<sup>+/−</sup> mice, which express EGFP in place of II1r2. High EGFP expression was observed in neutrophils (Fig. 2A). Low levels of EGFP expression were detected in macrophages and monocytes from TGC-treated mice, although the expression was very low under physiological conditions. Expression was not detected in T cells or B cells. The EGFP expression level was consistent with the II1r2 mRNA level determined by quantitative PCR (Fig. 2B). Similarly, II1rn and II1rap mRNAs were highly expressed in neutrophils and modestly expressed in other types of cells. In contrast, high levels of II1r1 mRNA expression were observed in FLSCs, but its expression was low in other cells.

II1r2<sup>−/−</sup> mice show increased susceptibility to CIA

Because it was suggested that IL-1R2 is involved in the pathogenesis or progression of RA (26–28), we examined the susceptibility of II1r2<sup>−/−</sup> mice to CIA. II1r2<sup>−/−</sup> mice showed higher clinical scores and incidence of CIA (Fig. 3A, 3B). Histological severity scores, as evaluated by the infiltration of polymorphonuclear leukocytes, pannus formation, erosion of cartilage, and bone destruction, were higher in arthritic joints of II1r2<sup>−/−</sup> mice than in WT mice (Fig. 3C–G). These results suggest that IL-1R2 negatively controls the development of arthritis.
Inflammatory mediator production, but not Ab production or T cell proliferative responses, is enhanced in Il1r2−/− mice after CIA induction

It is well known that both humoral and cell-mediated immunity are involved in the development of CIA (35, 36). To elucidate the mechanism for the exacerbation of CIA in Il1r2−/− mice, we first analyzed anti-IIC IgG concentration in sera. No significant differences were found between WT mice and Il1r2−/− mice (Fig. 4A). The levels of anti-IIC IgGs in each IgG subclass also did not differ between WT and Il1r2−/− mice (Fig. 4B). Next, we examined IIC-specific recall T cell responses; inguinal LN cells were harvested 7 d after immunization, and the cells were incubated or not with IIC for 3 d. The proliferative response against IIC of Il1r2−/− LN cells was similar to WT cells (Fig. 4C). Consistent with the proliferative response, cytokine production, such as IFN-γ, IL-17, and TNF, was normal in LN cells from Il1r2−/− mice upon stimulation with IIC (Fig. 4D–F). Furthermore, the numbers of total LN cells, B cells, CD4+ and CD8+ T cells, IFN-γ+ CD4+ and CD8+ T cells, IL-17+ CD4+ T cells, and Foxp3+ CD4+ T cells were normal in Il1r2−/− mice at day 7 after immunization (Fig. 4G). Interestingly, we found that the expression of Il6, Cxcl2, Nos2, and Il1b mRNAs, which are produced in macrophages and fibroblasts (37–40), was strongly up-regulated in inflammatory joints from Il1r2−/− mice (Fig. 4H). Il1r2−/− neutrophils show normal phenotype

IIC-specific T cells and Abs are important for the development of arthritis by recruiting neutrophils and monocytes/macrophages, which enhance inflammation by producing cytokines, chemokines, NO, and...
chemical mediators. In addition, joint-resident FLSCs are involved in
the development of inflammation (41). Because Ab production and
T cell priming were normal in \( \text{Il1r}^{-2/2} \) mice, we next examined the
effect of IL-1R2 deficiency on these cells.

Because neutrophils expressed high amounts of \( \text{Il1r}^{-2/2} \), and a
previous report suggested involvement of IL-1R2 in neutrophil
survival (13), we analyzed the effects of IL-1 on neutrophils. We
found that neutrophil survival did not change upon stimulation

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Inflammatory mediator production, but not Ab production or T cell–proliferative responses, is enhanced in \( \text{Il1r}^{-2/2} \) mice upon induction of CIA. (A and B) Anti-IIC Ab titer in sera. Sera of WT and \( \text{Il1r}^{-2/2} \) mice (8 each) were collected after induction of CIA, and anti-IIC IgG levels were measured by ELISA. (C–F) Inguinal LNs were isolated on day 7 from IIC-immunized WT mice (4) and \( \text{Il1r}^{-2/2} \) mice (3). Single cells were prepared and incubated for 72 h in the absence or presence of IIC. (C) T cell–proliferative response was determined by [\( ^{3} \text{H} \)] thymidine (TdT) incorporation. Concentrations of IFN-\( \gamma \) (D), IL-17 (E), and TNF (F) in the culture supernatant were measured by ELISA. (G) Cell numbers of the indicated T cell and B cell subsets in inguinal LNs were determined by flow cytometry on day 7 after immunization in WT and \( \text{Il1r}^{-2/2} \) mice (5 each). Each circle represents an individual mouse. (H) mRNA levels of inflammatory mediators. RNA was purified from paws of WT mice (8) and \( \text{Il1r}^{-2/2} \) mice (9) on day 40 after CIA induction, and mRNA levels were determined by quantitative PCR. Data are mean ± SD (A–F) or mean ± SEM (H) of each group and are representative of two (A, G, and H) or five (C–F) independent experiments. *\( p < 0.05 \) two-tailed unpaired Student \( t \) test.
with IL-1β, even in Il1r2−/− neutrophils (Fig. 5A). IL-6 production in WT or Il1r2−/− neutrophils also did not change as a result of treatment with IL-1β (Fig. 5B). In contrast, GM-CSF and LPS prolonged neutrophil survival and induced cytokine production in both WT and Il1r2−/− neutrophils. Although IL-4 is suggested to induce Il1r1 and Il1r2 mRNA in neutrophils (13), IL-4 or IL-1β plus IL-4 did not affect the survival of WT or Il1r2−/− neutrophils (Fig. 5A). IL-1β also failed to induce ROS generation from both WT and Il1r2−/− neutrophils under the conditions in which C5a efficiently induced ROS generation (Fig. 5C).

We also examined the effect of IL-1R2 deficiency on neutrophil survival in vivo. Peritonitis was induced by i.p. injection of IL-1α to

![Graph A](image1)

**Figure 5.** The response of Il1r2−/− neutrophils to IL-1 is normal. (A) Neutrophils from WT and Il1r2−/− mice were treated with the indicated cytokines (10 ng/ml), and the survival ratio was measured by flow cytometry after a 48-h culture. (B) IL-6 production was measured by ELISA at 20 h after treatment with IL-1β or LPS. (C) ROS generation was measured by chemiluminescence with a luminometer at the indicated time points after IL-1β or C5a stimulation. (D and E) A mixture of CD45.1+ WT and CD45.2+ Il1r2−/− BMCs were transferred into irradiated Rag2−/− mice, and these bone marrow chimeras were injected with IL-1α to induce peritonitis. (D) After 6 h, neutrophil and monocyte infiltration into the peritoneal cavity was examined. (E) The content of neutrophils (Neut), monocytes (Mono), macrophages (Mac), and B cells of each genotype was determined by allelic forms of CD45 Ag in the peritoneal cavity and bone marrow. Data are mean ± SD of triplicate cultures (A–C) or four mice (E) and are representative of two (A, C, and E) or four (B) independent experiments.
CD45.1+/WT and CD45.2+/Il1r2−/− bone marrow chimeras; infiltration of neutrophils and monocytes into the peritoneal cavity was examined 6 h later. As shown in Fig. 5D, neutrophil and monocyte infiltration was clearly observed. However, the WT/Il1r2−/− ratio did not change in the peritoneal cavity or bone marrow, suggesting comparable survival between WT and Il1r2−/− neutrophils in these mice (Fig. 5E).

Excess cytokine production in Il1r2−/− macrophages was observed upon stimulation with IL-1

Next, we examined inflammatory mediator production from FLSCs upon treatment with IL-1, TNF, IL-17A, or PGE2. FLSCs were prepared as described in Materials and Methods. The percentage of contaminated macrophages in the FLSC preparation was <1%, as determined by flow cytometry using anti-CD45 mAb (Supplemental Fig. 1A). mRNAs for proteoglycan 4 (Prg4) or lubricin, which is a component of synovial fluid, and α1 type 1 collagen (Col1a1), which is the major component of type 1 collagen, were highly expressed in FLSCs (Supplemental Fig. 1B). We found that the production of IL-6 and CCL2 was normal in Il1r2−/− FLSCs (Fig. 6).

In contrast, monocytes from both WT and Il1r2−/− mice did not respond to IL-1 to produce TNF- or IL-6–like neutrophils, although these cells produced those cytokines at similar levels in response to LPS (Fig. 7A, 7B). Although we detected a slight upregulation of Tnf, Il1b, and Cxcl2 mRNA upon IL-1 stimulation, no difference was observed between WT and Il1r2−/− monocytes (Fig. 7C–E). Similarly, cell surface expression of CD40 was marginally upregulated by treatment with IL-1, but its expression levels were comparable between WT and Il1r2−/− monocytes (Fig. 7F, 7G).

In contrast, significantly higher amounts of TNF and IL-6 were produced in Il1r2−/− BMMPs and TGCMPs compared with WT cells in response to IL-1α or IL-1β but not LPS (Fig. 8A, 8B). Quantitative PCR revealed that mRNA expression of Il1a, Il1b, Cxcl2, Nos2 (encoding iNOS), and Ptgs2 (encoding COX-2) also was enhanced in Il1r2−/− BMMPs and TGCMPs (Fig. 8C, 8D).

Excess cytokine production in Il1r2−/− macrophages was observed upon stimulation with IL-1

Next, we prepared cell lysates from IL-1– or LPS-stimulated BMMPs and examined the phosphorylation of MAPKs and degradation of IκBα by Western blotting. The phosphorylation of JNK and p38, as well as the degradation of IκBα, was enhanced in Il1r2−/− BMMPs upon stimulation with IL-1 but not LPS (Fig. 8E). These results suggest that IL-1R2 negatively regulates IL-1 signaling in macrophages but not in other cells, including neutrophils, fibroblasts, and monocytes.

Discussion

IL-1R2 is considered a decoy receptor for IL-1α and IL-1β, and many reports support this concept in vitro (13–16). The negative-regulatory function of IL-1R2 against IL-1 signaling is also suggested in Il1r2-transgenic mice, which are designed to express IL-1R2 in keratinocytes (17). However, the physiological and pathological roles of endogenous IL-1R2 have not been elucidated completely. In this study, we have shown that IL-1R2 is functional in macrophages as a negative regulator of IL-1 and suppresses the development of CIA.

Il1r2−/− mice were born healthy and breed normally; however, we found that the development of CIA was enhanced. The severity score and incidence of arthritis were increased. Anti-IIC concentrations in the serum, IIC-specific T cell proliferative responses, and cytokine production in LN cells upon stimulation with IIC were normal in Il1r2−/− mice, suggesting that Il1r2 is not involved in the control of T cell priming and production of Ag-specific Abs. However, we showed that the expression of mRNAs for inflammatory mediators, such as IL-6, CXCL2, Nos2, and IL-1β, which are important for the development of arthritis (42–45), was upregulated in the joints of Il1r2−/− mice. Furthermore, we found that, upon stimulation with IL-1α and IL-1β, cytokine and inflammatory mediator production, including IL-6, CXCL2, Nos2, and IL-1β, was greatly enhanced in macrophages, but not in FLSCs or monocytes, from Il1r2−/− mice compared with WT mice, suggesting that increased inflammatory mediators in Il1r2−/− mouse joints are derived from macrophages. Consistent

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** Normal inflammatory mediator production in Il1r2−/− FLSCs. FLSCs from WT and Il1r2−/− mice were stimulated with cytokines, as indicated. The concentrations of IL-6 (A) and CCL2 (B) in the culture supernatant were measured by ELISA. No significant difference was observed. Data are mean ± SD of triplicate cultures and are representative of three independent experiments.
with this notion, it was reported that macrophage depletion results in the suppression of CIA associated with downregulation of these inflammatory mediators (46). Thus, these observations suggest that IL-1R2 regulates the development of arthritis by suppressing IL-1 activity on macrophages.

We found that IL-1R2 is expressed most prominently in neutrophils among T cells, B cells, monocytes, BMMPs, and FLSCs, consistent with a recent report (47). However, in contrast to macrophages, we did not detect any functional abnormality of $Ilr2^{−/−}$ neutrophils. The neutrophil content in the bone marrow was similar between WT and $Ilr2^{−/−}$ mice. Survival after treatment with IL-1β, IL-4, or both was not different between WT and $Ilr2^{−/−}$ neutrophils. IL-6 and ROS production after treatment with IL-1α or IL-1β also did not change. Furthermore, the content

**FIGURE 7.** The response of $Ilr2^{−/−}$ monocytes to IL-1β is normal. (A and B) Monocytes from WT and $Ilr2^{−/−}$ mice were stimulated with IL-1β or LPS, and concentrations of TNF and IL-6 were measured by ELISA. (C-E) Monocytes from WT and $Ilr2^{−/−}$ mice were stimulated with IL-1β or LPS, and the mRNA levels of Tnf, Il1b, and Cxc22 were determined by quantitative PCR. (F and G) Monocytes from WT and $Ilr2^{−/−}$ mice were stimulated with IL-1β or Pam3CSK4 (Pam3), and cell surface expression of CD40 was analyzed by flow cytometry. Representative graphs (F) and mean fluorescence intensity levels (G) of CD40. Data are mean ± SD of triplicate cultures (A-E and G) and are representative of three (A and B) or two (E and F) independent experiments.
of Il1r2−/− neutrophils, monocytes, macrophages, and B cells in peritoneal cavity, blood, and bone marrow was similar to that of WT after induction of peritonitis in mixed bone marrow chimera mice. Consistent with our notion, Prince et al. (48) demonstrated that IL-1 is not involved in neutrophil survival, cell adhesion molecule expression, or cytokine production. Although it was reported that IL-1 prolongs neutrophil survival (13), Prince et al. (48) showed that neutrophils purified by the Percoll gradient method, which was used in the previous report (13), were contaminated with other types of cells, and neutrophils were indirectly activated by IL-1 through these contaminated cells. Regarding the reason why Il1r2−/− neutrophils are refractory to IL-1 stimulation, we first thought that this is because IL-1R antagonist (IL-1Ra) is highly expressed in neutrophils (Fig. 2B).
However, we found that this is not the case, because neutrophils from Il1rn and Il1r2 double-deficient mice still did not respond to IL-1 (Supplemental Fig. 2). Therefore, it is conceivable that IL-1R1 expression on the cell surface is too low to respond to IL-1, some signaling molecules are missing, or some negative regulators of signal transduction are highly expressed in neutrophils. Consistent with the first possibility, IL-1R1 expression was not detected on neutrophils, although the mRNAs for IL-1R1 and IL-1RACp were clearly detected. Unfortunately, we also failed to detect expression of IL-1R1 on macrophages for which we clearly observed the effects of IL-1. IL-1R1 expression on macrophages and neutrophils was not detected, even after amplification using biotin-conjugated anti-PE Ab and after stimulation with GM-CSF, IFN-γ, and IL-23 (Supplemental Fig. 3). Because we could easily detect cell surface IL-1R1 expression on peritoneal CD62L^−/γδ T cells from WT mice, but not from Il1r1^−/− mice, as described previously (36), the expression levels of IL-1R1 on neutrophils and macrophages are much lower than that on peritoneal CD62L^−/γδ T cells.

Nonetheless, high expression of IL-1R2 in neutrophils may play some physiological and/or pathological roles. In fact, it was demonstrated that neutrophils scavenge IL-1β through IL-1R2 (49), and both human and mouse neutrophils cleave IL-1R2 to release the extracellular domain of IL-1R2 (47, 50). Because an IL-1R2–expressing T cell line can neutralize IL-1 activity through binding of soluble IL-1R2 to IL-1β, but less efficiently through binding of soluble IL-1R2 to IL-2 (51), we examined the IL-1–neutralizing capacity of neutrophils in two models. Coculture of WT or Il1r2^−/− neutrophils with Il1r2^−/− macrophages did not affect IL-1β–induced IL-6 production in macrophages (Supplemental Fig. 4A). Furthermore, preincubation of 2 × 10^6 neutrophils with 5 pg/ml IL-1β failed to inactivate IL-1β activity, although 10^5 IL-1R2–transfected T cells neutralized 10 pg/ml IL-1β in the previous study (51) (Supplemental Fig. 4B). Therefore, we conclude that the IL-1R2 expression levels in neutrophils are not enough to neutralize IL-1 activity in trans. Neutrophil-specific Il1r2^−/−-deficient mice may help us to elucidate the roles of IL-1R2 in neutrophils.

We reported previously that Il1rn^−/− mice spontaneously develop autoimmune arthritis on the BALB/cA background (52). In contrast, Il1r2^−/− mice did not develop autoimmunity on the BALB/cA or C57BL/6 background. This difference between Il1rn^−/− and Il1r2^−/− mouse phenotypes could be explained by the difference in target cells; IL-1Ra can inhibit IL-1R1 on all cell types, whereas IL-1R2 can only compete with IL-1R1 on macrophages. Mice deficient for other type IL-1R signal regulators, such as ST2 and SIGIRR, also do not develop arthritis (53, 54). These observations suggest that IL-1Ra is the most potent and cell type–independent regulator among endogenous IL-1 inhibitors.

We found that Il1rn^−/−/Il1r2^−/− neutrophils, produced higher levels of TNF than did WT neutrophils when cells were treated with LPS (Supplemental Fig. 2). Interestingly, Il1rn^−/−/Il1r2^−/− mice developed more severe skin inflammation and emaciation compared with Il1rn^−/− mice (T. Ikarashi and S. Kakuta, manuscript in preparation). Therefore, Il1rn^−/−/Il1r2^−/− neutrophils seem to be constitutively activated as a result of chronic inflammation of the skin, and they produce TNF easily upon stimulation with LPS. Alternatively, IL-1R2 suppresses IL-1 signaling in neutrophils to induce TNF in collaboration with TLR4 signaling (55). Clearly, further investigations are needed to distinguish these possibilities and to elucidate the roles of IL-1R2 during skin inflammation and emaciation.

In summary, we showed that IL-1R2 plays a regulatory role in the progression of CIA through the inhibition of IL-1 action on macrophages. This regulatory function of IL-1R2, unlike IL-1Ra, is strictly cell type specific. These observations may provide a clue for the use of IL-1R2 as a treatment for inflammatory diseases.

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Disclosures

The authors have no financial conflicts of interest.

References

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