ated signaling, our findings, at the very least, provide a novel target for controlling the innate immune response.

## MATERIALS AND METHODS

**Reagents, mice, and cells.** LPS purified from *Escherichia coli* 0111:B4 (Sigma-Aldrich), poly(I:C) (Amersham Biosciences), CpG ODN (5′-TCCATGACGTTCCTGATGCT-3′; QIAGEN), and $Pam_3CSK_4$ (EMC microcollections) were purchased. The generation of Dok-1 or Dok-2 KO mice was described (14, 15), and these mice were backcrossed to C57BL/6 for at least eight generations. Mice were kept under specific pathogen-free conditions and subjected to experiments at 8–12 wk of age. Experiments and animal care were performed according to institutional guidelines. Peritoneal exudate cells (PECs) were collected with 2 mM EDTA/PBS 3 d after an i.p. injection of 0.5 ml of 3% thioglycollate (Nissui). Resident PECs were obtained by the same procedure without a thioglycollate injection. These cells were washed and resuspended in DMEM containing 15% FCS. After several hours of incubation in culture plates, adherent PECs and resident PECs were used as peritoneal macrophages and peritoneal resident macrophages, respectively. BM cells were cultured in DMEM containing 10 ng/ml of murine M-CSF (PeproTech) and 15% FCS. After 7 d of culture, adherent cells were maintained in the absence of M-CSF for 24 h and used as BM-derived macrophages. RAW 264.7 cells were cultured in DMEM containing 15% FCS.

**Flow cytometry.** A single cell suspension of peritoneal resident or RAW 264.7 macrophages was treated with 10 or 1.0 μg/ml LPS, respectively, and 2.0 μg/ml brefeldin A (Sigma-Aldrich) for 16 h, and then the former cells were stained with PE-conjugated mAbs to CD11b (BD Biosciences). Intracellular TNF-α was stained with a CytoStain kit (BD Biosciences), and flow cytometry was performed with a FACSCalibur (Becton Dickinson). Data representative of quintuplicate experiments are shown (refer to Fig. 1 A).

**NO production assay.** To evaluate NO production, cells were cultured for 24 h and the $NO_2^-$ concentration in the medium was measured with a $NO_2/NO_3$ Assay kit-CII (Dojindo).

**Immunoprecipitation and immunoblotting.** Cells treated with 1.0 or 2.0 μg/ml LPS, 10 μM CpG ODN, 100 μg/ml poly(I:C), or 100 ng/ml $Pam_3CSK_4$ were solubilized in 1.0% NP-40–based TNN buffer (18). For immunoprecipitation, cell lysates were cleared and incubated with antibodies to mouse Dok-1 (A3) or Dok-2 (M20; Santa Cruz Biotechnology, Inc.) followed by incubation with protein G–Sepharose (Amersham Biosciences). The immune complex was washed and collected as immunoprecipitates. For immunoblotting, immunoprecipitates or cleared cell lysates were separated by SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories), which was then incubated with antibodies to phospho-ERK (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho–IκB-α (Cell Signaling), phospho-JNK (Thr183/Tyr185), ERK, IκB-α, Dok-2 (H192), p120 rasGAP (Santa Cruz Biotechnology, Inc.), or phosphotyrosine (4G10; Upstate Biotechnology), followed by incubation with secondary horse radish peroxidase–labeled (Amersham Biosciences) or AP-labeled (Santa Cruz Biotechnology, Inc.) antibodies. The blots were visualized with the ECL system (Amersham Biosciences) or BCIP/NBT system (Promega). Data representative of triplicate experiments are shown.

**Gel mobility shift assay.** The nuclear extracts of cells treated with 1.0 μg/ml LPS were incubated with a specific probe for the NF-κB DNA binding site, electrophoresed, and visualized by autoradiography as described previously (29). Data representative of triplicate experiments are shown.

**Forced expression of Dok-1, Dok-1 YF, or Dok-2 in RAW 264.7 cells.** cDNA for mouse Dok-1, Dok-1 YF, or Dok-2 fused with the flag tag at the COOH terminus was generated by PCR. Each cDNA and the IRES-GFP fragment were appropriately inserted into the mammalian expression vector pA-puro (30). The expression plasmid was confirmed by sequencing and transfected into RAW 264.7 cells with FuGENE 6 (Roche). The puromycin-resistant clones were further selected for Dok-1 or Dok-2 expression.

**ELISA.** The serum TNF-α concentration of mice at 1 h after injection with LPS (25 mg per weight kg) to the peritoneal cavity or before it, was measured with an ELISA kit (Biosource International).

**Statistical analysis.** Statistical analysis was performed with Student's *t* test and analyzed using Microsoft Excel Software.

**Online Supplemental Material.** Fig. S1 shows normal expression of LPS receptors on macrophages from mice lacking Dok-1 or Dok-2. Fig. S2 shows normal TNF-α production upon stimulation of TLR9, TLR3, or TLR2 of these macrophages. Figs. S1 and S2 are available at http://www.jem.org/cgi/content/full/jem.20041817/DC1.

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**Note added in proof:** After acceptance of this manuscript, Niki et al. (Niki, M., A. Di Cristofano, M. Zhao, H. Honda, H. Hirai, L. Van Aelst, C. Cordon-Cardo, and P.P. Pandolfi. 2004. *J. Exp. Med.* 200:1689–1695) reported a role of Dok-1 and Dok-2 in leukemia suppression.

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# Involvement of Tumor Necrosis Factor-α in the Development of T Cell–Dependent Aortitis in Interleukin-1 Receptor Antagonist–Deficient Mice

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***Background*****—**Interleukin-1 receptor antagonist–deficient (IL-1Ra$^{-/-}$) mice on the BALB/c background spontaneously develop inflammatory arthropathy that resembles rheumatoid arthritis in humans. These mice also frequently develop aortitis at the root of the aorta, but the mechanism underlying the development of this disease has not been completely elucidated.

***Methods and Results*****—**Using IL-1Ra$^{-/-}$ mice (backcrossed 8 generations to the BALB/c background) and wild-type mice, we studied the histopathology and examined the immunologic mechanisms involved in the development of aortic inflammation by cell transplantation experiments. Half of the IL-1Ra$^{-/-}$ mice developed aortitis at the root of the aorta, with massive infiltration of macrophages and monocytes and loss of elastic lamellae in the aortic media. Left ventricular hypertrophy and mild aortic stenosis were also shown by transthoracic echocardiography. Transplantation of T cells from IL-1Ra$^{-/-}$ mice induced aortitis in recipient nu/nu mice. Bone marrow cell transplants from IL-1Ra$^{-/-}$ mice also induced aortitis in irradiated wild-type recipient mice. Furthermore, tumor necrosis factor (TNF)-α deficiency completely suppressed the development of aortitis in IL-1Ra$^{-/-}$ mice, whereas IL-6 deficiency did not affect pathology.

***Conclusions*****—**These observations suggest that IL-1Ra deficiency in T cells activates them excessively, resulting in the development of aortitis in IL-1Ra$^{-/-}$ mice in a TNF-α–dependent manner. **(*Circulation.* 2005;112:1323-1331.)**

**Key Words:** interleukins ■ inflammation ■ transplantation

Interleukin (IL)-1 is a major mediator of inflammation and plays important roles in host defense mechanisms through regulation of not only the immune system but also the neuronal and endocrine systems, which interface with the immune system.[1,2] IL-1 consists of 2 molecular species, IL-1α and IL-1β, both of which exert similar but not completely overlapping biological functions through the IL-1 type I receptor (IL-1RI). Another IL-1R, the type II receptor (IL-1RII), has also been identified, but it is not involved in signal transduction; rather, it plays a regulatory role as a decoy. The IL-1R antagonist (IL-1Ra), another member of the *IL-1* gene family, binds to IL-1Rs without exerting agonistic activity. IL-1Ra, IL-1RII, and the secreted forms of IL-1RI and IL-1RII are thought to be negative regulators of IL-1 signaling, participating in the complex regulation of IL-1 activity. Production of both IL-1 and IL-1Ra is induced by a number of other cytokines, bacterial and viral components, and mechanical stresses in a wide variety of cell types, including monocytes/macrophages, epithelial and endothelial cells, and glial cells.[3]

We previously reported that *IL-1Ra* gene–deficient (IL-1Ra$^{-/-}$) mice on the BALB/c background spontaneously developed chronic inflammatory arthropathy.[4] Histopathological analysis showed marked synovial and periarticular inflammation, with articular erosion caused by invasion of granulation tissues closely resembling rheumatoid arthritis in humans. Moreover, elevated levels of antibodies against IgG, type II collagen, and double-stranded DNA (dsDNA) were detected in the sera of these mice, suggesting the development of autoimmunity. Proinflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor (TNF)-α were overexpressed in the joints of these animals, indicating a regulatory role for IL-1Ra in the cytokine network. Therefore, it was suggested that IL-1Ra is crucial for homeostasis of the immune system.

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Classic primary vasculitis syndromes such as Takayasu arteritis and giant-cell (temporal) arteritis involve massive recruitment of lymphocytes and macrophages into the vascular wall, destruction of the medial layer with concurrent fibrosis, and proliferation of smooth muscle cells in the intima, leading to neointima formation.[5] Although a number of potential mechanisms, including microbial infection and autoimmune reactions, have been implicated in the development of inflammatory reactions in the vascular system, the precise mechanism underlying the development of vasculitis remains to be elucidated.

Nicklin et al[6] reported that IL-1Ra$^{-/-}$ mice developed aortic inflammation on the 129/Ola×MF1 background. Arterial inflammation with massive transmural infiltration of neutrophils, macrophages, and CD4$^{+}$ T cells was found at branch points and flexures of the aorta. IL-1$\beta$ expression was observed mainly in macrophages that were associated with CD4$^{+}$ cells deep within the vessel wall, suggesting the involvement of CD4$^{+}$ cells in enhancing IL-1$\beta$ production. Although the histological changes in the affected IL-1Ra$^{-/-}$ arteries were described in detail, the mechanism underlying the development of arteritis caused by IL-1Ra deficiency was not completely elucidated.

In this investigation, we examined the possibility that autoimmunity is involved in the development of spontaneous arterial inflammation in our IL-1Ra$^{-/-}$ mice on the BALB/c background by cell transplantation experiments. Furthermore, we investigated the role of the proinflammatory cytokines TNF-$\alpha$ and IL-6 in chronic arterial inflammation by generating cytokine-deficient IL-1Ra$^{-/-}$ mice.

## Methods

### Animals

IL-1Ra$^{-/-}$ mice were produced as described previously.[7] TNF-$\alpha^{-/-}$ and IL-6$^{-/-}$ mice were kindly provided by Dr K. Sekikawa (National Institute of Agrobiological Sciences, Tsukuba, Japan) and Dr M. Kopf (Swiss Federal Institute of Technology, Zurich, Switzerland), respectively. These mice were backcrossed to BALB/c or C57BL/6 mice for 8 generations and then intercrossed with IL-1Ra$^{-/-}$ mice to generate doubly deficient mice (IL-1Ra$^{-/-}$×TNF-$\alpha^{-/-}$ or IL-1Ra$^{-/-}$×IL-6$^{-/-}$ mice)[8]. BALB/c, C57BL/6, and BALB/c-nu/nu mice were purchased from Japan Clea (Tokyo, Japan). A group of wild-type mice of the same age and sex as the test mice was used as a control in each experiment. Mice were housed under specific pathogen-free conditions in an environmentally controlled clean room at the Center for Experimental Medicine, Institute of Medical Science, University of Tokyo. Mice were housed at an ambient temperature of 24°C and a daily light/dark cycle of 12 hours each (light from 8 AM to 8 PM). All experiments were carried out according to institutional ethics guidelines for animal experiments and safety guidelines for gene manipulation experiments.

### Histological and Clinical Evaluation for Aortitis and Arthritis

For histological examination of aortitis, mice were anesthetized with pentobarbital and perfused with phosphate-buffered saline (PBS) followed by 10% formalin from an angiocatheter placed in the left ventricule (LV) of the heart. The aorta was fixed in 10% formalin for 48 hours and embedded in paraffin. Serial 10-$\mu$m sections of aorta were stained with hematoxylin/eosin for examination of cell infiltration. Masson's trichrome stain was used to evaluate connective tissue damage.[9,10] To detect calcification of the vessel, von Kossa staining, in which sections were treated with 3% $AgNO_3$ and exposed to bright light for 30 minutes, was used. Sections were counterstained with hematoxylin/eosin. Lesion sizes were measured with NIH Image 1.55 software (public domain software). The severity of aortitis was graded on a scale of 0 to 3 by the degree of inflammation near the aortic valve, as follows: grade 0=normal and no infiltration; grade 1=infiltration and loss of elastic lamellae over less than one third of the media of the aortic sinus; grade 2=loss in one third to two thirds of the aortic sinus; and grade 3=loss over more than two thirds of the aortic sinus (see Figure 1).

The incidence and severity of arthritis were judged macroscopically and histologically, as previously described.[4] In brief, each joint was examined weekly for swelling and redness, and severity was graded from 0 to 3 for each paw: grade 0=no special changes; grade 1-light swelling of the joint and/or redness of the foot pad; grade 2=obvious swelling of the joint; and grade 3=fixation of the joint. Severity score was calculated for the 4 legs for a total of 12 points for each mouse. For histological examination, joints were fixed with 10% phosphate-buffered formalin, decalcified in 10% EDTA-4Na, and embedded in paraffin. Sections (4 $\mu$m) were stained with hematoxylin/eosin.

### Echocardiography

To examine valve function, transthoracic echocardiography was performed with a Sonos 5500 unit (Phillips Co) equipped with 12-MHz and 15-MHz imaging transducers. Mice (female, 40 weeks old) were anesthetized with 2,2,2-tribromoethanol (250 mg/g IP), the chest was shaved, and ECG leads were attached to each limb with needle electrodes. Mice were imaged in a shallow left lateral decubitus position; short- and long-axis views of the LV were obtained by slight angulation and rotation of the transducer. Two-dimensional, targeted M-mode studies were generally taken from the short axis (at the level of the largest LV diameter).

Intraventricular septum thickness, end-diastolic LV internal diameter, end-systolic LV internal diameter, and LV posterior wall thickness were measured. Percent fractional shortening was calculated as [(end-diastolic LV internal diameter)−(end-systolic LV internal diameter)/(end-diastolic LV internal diameter×100)].[11]

Color flow Doppler measurements were used to identify areas of increased (aliased) velocities in the outflow tract from angulated parasternal long-axis views, and these were quantified by pulsed- and/or continuous-wave Doppler. Attempts were made to align the ultrasound beam as parallel as possible with the direction of flow and to record the highest velocities.[12] Then the peak pressure gradient through the LV outflow tract was estimated according to the simplified Bernoulli equation.[13]

### Blood Pressure and Heart Rate Measurements

To evaluate hemodynamics, blood pressure and heart rate were measured in nonanesthetized mice (female, 12 weeks old) by the tail-cuff method with a Softron BP-98A device (Softron Co) in the morning. Body and heart weights of these mice were also measured. Values were measured at least 3 times per mouse and were averaged for each individual.

### Plasma Cytokine Levels

Proinflammatory cytokine levels in the plasma from 8-week-old male IL-1Ra$^{-/-}$ and wild-type mice were measured by ELISA.[14] Hamster anti-mouse IL-1$\alpha$ monoclonal antibody, hamster anti-mouse IL-1$\beta$ monoclonal antibody, and polyclonal goat anti-mouse TNF-$\alpha$ antibody (all from Genzyme) were used as capture antibodies. Polyclonal rabbit anti-mouse IL-1$\alpha$, polyclonal rabbit anti-mouse IL-1$\beta$, and polyclonal biotinylated goat anti-mouse TNF-$\alpha$ antibodies (all from Genzyme) were used as secondary antibodies. Detection was performed with horseradish peroxidase–conjugated goat anti-rabbit IgG and horseradish peroxidase–streptavidin (Zymed). TMB substrate was purchased from Dako. IL-6 levels were measured with the OptEIASet mouse IL-6 kit (BD Pharmingen). All assays were performed in duplicate.

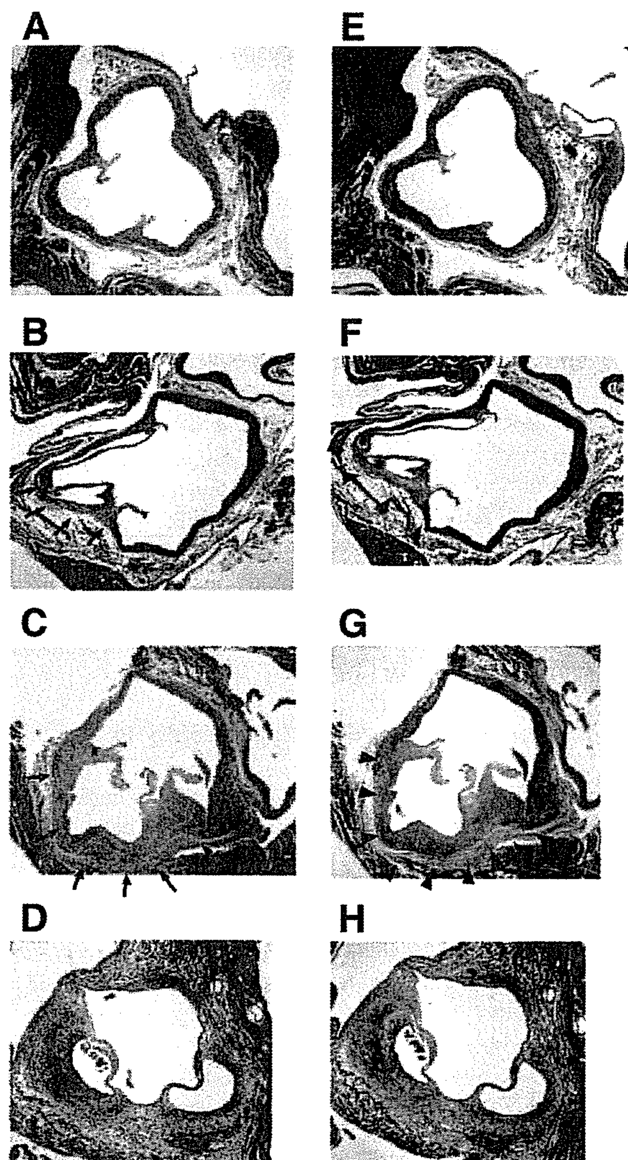


**Figure 1.** Arterial inflammation around the aortic sinus in IL-1Ra$^{-/-}$ mice. A and E, Normal sections of the aortic valve (score 0) from an 8-week-old, wild-type female mouse. B and F, Mild inflammatory cell infiltration and loss of elastic lamellae over less than one third of the media of the aortic sinus (score 1). Sections from a 4-week-old IL-1Ra$^{-/-}$ male mouse. C and G, Moderate inflammatory cell infiltration and loss of elastic lamellae over one third to two thirds of the media of the aortic sinus (score 2). Sections from an 8-week-old IL-1Ra$^{-/-}$ male mouse. D and H, Severe inflammatory cell infiltration and loss of elastic lamellae over more than two thirds of the media of the aortic sinus (score 3). Sections from an 8-week-old IL-1Ra$^{-/-}$ female mouse. The arrows show inflammatory infiltrates of monocytes and leukocytes, and the arrowheads point to the loss of elastic lamellae. Hematoxylin and eosin stains (A–D) and Masson's trichrome stains (E–H) of the aortic sinus. All images at original magnification of ×40.

## T Cell and Bone Marrow (BM) Cell Transplantation

To elucidate the role of T cells in the development of aortitis and arthritis, T-cell transplantation was performed.[12] In brief, cells were prepared from the spleen and lymph nodes of IL-1Ra$^{-/-}$ (n=10, female, 6 to 8 weeks old) and wild-type (n=10, female, 6 to 8 weeks old) mice, and then the cells were treated with hemolysis buffer (17 mmol/L Tris-HCl and 140 mmol/L $NH_4Cl$, pH 7.2) to remove red blood cells, washed, and passed through a nylon wool column. Then anti-mouse B220 and anti–Mac-1 magnetic bead (Miltenyi Biotec) –treated cells were passed through a MACS column (Miltenyi Biotec) to obtain T cells. The resulting purified T cells were resuspended in 0.2 mL PBS ($2\times10^7$ cells/mouse) and transplanted intravenously into BALB/c-nu/nu mice (n=20, female, 6 weeks old). The development of aortitis in recipient mice was analyzed 10 weeks later.

For BM cell transplantation, BM cells were taken from femurs, tibias, and pelves of IL-1Ra$^{-/-}$ (n=17, female, 5 to 6 weeks old) and wild-type (n=14, female, 5 to 6 weeks old) mice and were treated with hemolysis buffer. T cells were removed by treating the BM cells with anti-mouse Thy1.2 magnetic beads and passing the cells through an MACS column. Purified BM cells ($10^7$ cells/mouse) in 0.2 mL PBS were transplanted intravenously into lethally irradiated (750 rad) recipient mice at 4 weeks of age (IL-1Ra$^{-/-}$, n=12, female; wild-type mice, n=17, female). The recipient mice were histologically examined 12 and 24 weeks later.

**TABLE 1. Incidence of Aortitis in IL-1Ra$^{-/-}$ Mice**

| Age, wk | Incidence (Rate, %) | Median Score |
|---|---|---|
| 4 | 2/5 (40) | 1 |
| 8 | 3/6 (50) | 2 |
| 12 | 5/10 (50) | 2 |

The number of diseased mice among the total number of animals is shown. The number of male mice studied was 3, 3, and 4 and of female mice was 2, 3, and 6 at 4, 8, and 12 weeks, respectively. Severity of aortitis was graded on a scale of 0–3 by the degree of inflammation of the area near the aortic valve, as detailed in text.

## Statistical Analysis

All values were calculated as the mean±SD except where indicated. Fisher's exact test was used for evaluation of the incidence of aortitis between unpaired groups. To compare the values between 2 independent groups, we used the Student *t* test for echocardiographic and hemodynamic values, tissue weights, and cytokine levels. To compare discontinuous values between 2 independent groups, such as aortitis severity score, we used the Mann-Whitney *U* test. A value of $P<0.05$ was considered significant.

# Results

## Development of Aortitis in IL-1Ra$^{-/-}$ Mice

IL-1Ra$^{-/-}$ mice on the BALB/c background spontaneously developed arterial inflammation beginning at the age of 4 weeks, and ≈50% of them were affected by the age of 12 weeks (Table 1). Interestingly, on the C57BL/6J background, there were no signs of arterial inflammation (data not shown), suggesting the involvement of background genes in the development of aortitis; a similar observation has been made in the case of arthritis.[4] Inflammation developed at several sites in the artery, including the region of the coronary artery ostium near the aorta (Figure 1). Arterial inflammation in IL-1Ra$^{-/-}$ mice was not influenced by sex (incidence of 58% [7/12] in male mice and of 45% [5/11] in female mice at 10 to 14 weeks old; $P=0.42$ by Fisher's exact test). IL-1Ra$^{-/-}$ mice also developed mild myocarditis in the subepidermal pericardium at low incidence (data not shown).

Infiltration of monocytes and occasionally neutrophils was observed in the aorta and valve, and a loss of elastic lamellae in the aortic media was observed on histological examination. Monocytes/macrophages and some neutrophils infiltrated the inflammatory sites in the aortic sinus (Figure 2A). Thus,

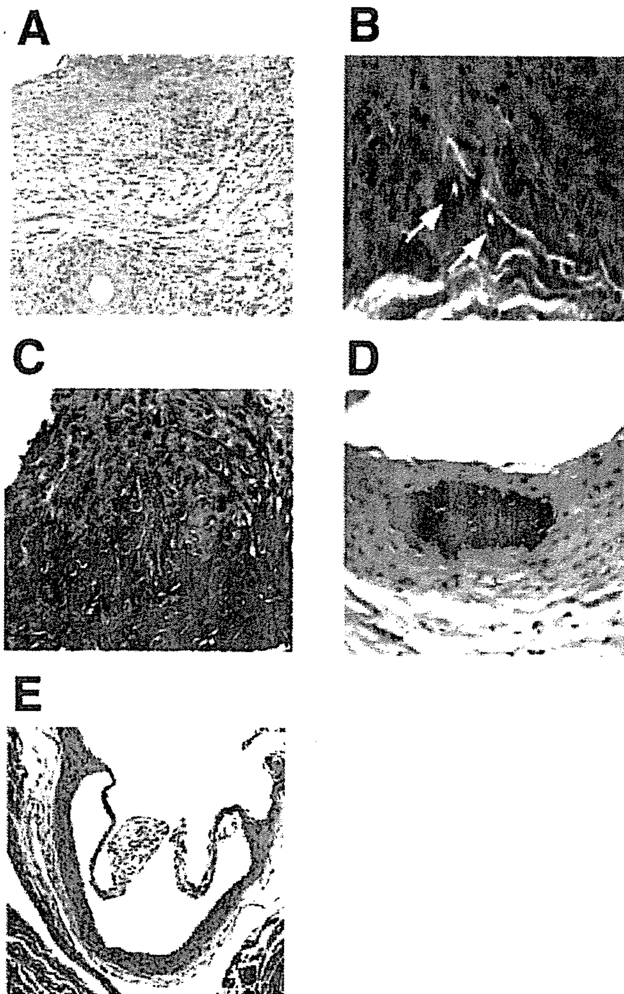


**Figure 2.** Characterization of arterial inflammation in IL-1Ra$^{-/-}$ mice. A, Inflammatory cell infiltration in the media and adventitia in a 12-week-old IL-1Ra$^{-/-}$ female mouse. B, Formation of microvessels (white arrows) in a section from an 8-week-old IL-1Ra$^{-/-}$ male mouse. C, Chondrocyte-like cells (arrowheads) and calcification (D) in the media in sections from an 8-week-old IL-1Ra$^{-/-}$ female mouse. E, Sections of aortic valve cusp from an 8-week-old IL-1Ra$^{-/-}$ female mouse. Hematoxylin and eosin staining (A, D–E) and Masson's trichrome staining (B and C). Magnification: A and D, ×100; B, ×150; C, ×400; E, ×50.

aortic inflammation may have characteristics of both the acute and chronic phases. We found numerous examples of neovascularization at sites of severe lesions (score 3; Figure 2B). Chondrocyte-like cells were observed in most of the aortas of IL-1Ra$^{-/-}$ mice, although no such cells were observed in the aortas of wild-type mice (Figure 2C). The chondrocyte-like cells were detected at sites of severe inflammation that also exhibited loss of elastic lamellae in the media. To determine whether calcification existed within arterial walls, the sections were stained with hematoxylin/eosin and von Kossa's technique after eosin staining. Calcification of the media of the aorta was observed in ≈30% of affected IL-1Ra$^{-/-}$ mice (Figure 2D and data not shown).

## Correlation Between Aortitis and Arthritis

As shown in Table 2, 53% of IL-1Ra$^{-/-}$ mice developed aortitis by 14 weeks of age, whereas 95% of these mice developed arthritis at 14 weeks of age. However, the mutant mice developed aortitis as early as 4 weeks of age, a time when they had not yet developed arthritis (Table 2). Although most of the mice that developed aortitis also developed arthritis, a few of them developed aortitis only without any sign of arthritis at 14 weeks of age (Table 2). This observation was confirmed by histological examination of the joints of IL-1Ra$^{-/-}$ mice that had developed aortitis (data not shown). Thus, the development of aortitis is not necessarily correlated with the development of arthritis.

## Development of Cardiac Hypertrophy in IL-1Ra$^{-/-}$ Mice

Because the aortic valve plays a crucial role in heart function and arterial inflammation in IL-1Ra$^{-/-}$ mice occurs specifically in the aortic sinus, we took echocardiograms of IL-1Ra$^{-/-}$ and wild-type mice to examine valve function under conditions of Avertin anesthesia (Table 3). The thickness of both the interventricular septum wall and the LV posterior wall was notably increased. In contrast, LV end-diastolic and end-systolic dimensions and fractional shortening, which are reported to be influenced by Avertin anesthesia,[15] were unchanged, suggesting that the effect of anesthesia was low, if at all. Pressure gradient and flow velocity were significantly increased in IL-1Ra$^{-/-}$ mice. These results suggest that LV function is normal, that the pressure gradient is affected by mild aortic stenosis, and that LV hypertrophy may be induced by pressure overload.

Furthermore, we measured blood pressure and heart rate in 4 IL-1Ra$^{-/-}$ mice and compared these values with these of 4 wild-type mice (Table 4). IL-1Ra$^{-/-}$ mice showed normal blood pressure, but they also showed a small but significant decrease in heart rate under nonanesthetized conditions. The heart weight of IL-1Ra$^{-/-}$ mice was similar to that of wild-type mice, as was their body weight (Table 4).

**TABLE 2. Correlation Between Aortitis and Arthritis in IL-1Ra$^{-/-}$ Mice**

| Aortitis | Arthritis | Incidence at 4 Weeks of Age (Rate, %) | Incidence at 6–8 Weeks of Age (Rate, %) | Incidence at 10–14 Weeks of Age (Rate, %) |
|---|---|---|---|---|
| − | − | 3/5 (60) | 0/9 (0) | 0/19 (0) |
| − | + | 0/5 (0) | 5/9 (56) | 9/19 (47.5) |
| + | − | 2/5 (40) | 0/9 (0) | 1/19 (5) |
| + | + | 0/5 (0) | 4/9 (44) | 9/19 (47.5) |

The number of diseased mice among the total number of animals is shown. Pathological examination of IL-1Ra$^{-/-}$ mice (male, n=3, 8, and 12; female, n=2, 3, and 7) was performed at 4, 6–8, and 10–14 weeks of age, respectively. Data for males and females of the same age were pooled, because no difference between males and females was observed.

**TABLE 3. Echocardiographic Measurements in IL-1Ra$^{-/-}$ and Wild-Type Mice**

| | Wild Type | IL-1Ra$^{-/-}$ | P |
|---|---|---|---|
| Interventricular septal wall thickness, mm | 0.74±0.11 | 1.20±0.22* | 0.0015 |
| Posterior wall thickness, mm | 0.75±0.11 | 1.14±0.14* | 0.0004 |
| End-diastolic diameter, mm | 0.19±0.04 | 0.21±0.04 | 0.2900 |
| End-systolic diameter, mm | 0.080±0.020 | 0.082±0.025 | 0.5300 |
| Fractional shortening, % | 57.1±3.7 | 60.6±5.6 | 0.1367 |
| Flow velocity, cm/s | 94±14 | 181±51* | 0.0028 |
| Pressure gradient, mm Hg | 3.6±1.1 | 14.0±7.1* | 0.0061 |

Values are mean±SD. Wild-type mice n=5; IL-1Ra$^{-/-}$ mice n=5 (female, 40 weeks old).
*$P<0.05$ vs wild-type mice (Student *t* test).

## Development of Aortitis in Mice That Received Transplants of IL-1Ra$^{-/-}$ T Cells or BM Cells

We have previously reported that IL-1Ra$^{-/-}$ mice showed increased levels of total IgG, IgG1, or IgE and autoantibodies against Igs, type II collagen, and dsDNA, suggesting involvement of an autoimmune mechanism in the development of disease in this mouse strain.[4] The observation of abundant $CD4^+$ T-cell infiltration at sites of arterial inflammation in IL-1Ra$^{-/-}$ mice also supports this notion.[6] Thus, we examined the role of T cells in the development of aortitis by peripheral T-cell transplantation. Transplantation of T cells from wild-type mice induced mild aortitis at a low incidence in nu/nu mice. In contrast, T cells from IL-1Ra$^{-/-}$ mice induced aortitis at a much higher incidence. The severity score was also significantly increased in this experimental group, indicating that T cells are involved in the development of aortitis in IL-1Ra$^{-/-}$ mice (Figure 3A and 3B and Table 5). To determine whether IL-1Ra deficiency in T cells itself or T-cell sensitization in IL-1Ra$^{-/-}$ mice was important for the development of aortitis, we performed IL-1Ra$^{-/-}$ BM cell transplantation into wild-type recipients. Irradiated control mice without BM cell transplantation died within 2 weeks. Wild-type mice that received wild-type BM cells did not develop any arterial inflammation. A high incidence (100% and 71% at 12 and 24 weeks after transplantation, respectively) of aortitis was observed in wild-type mice that received BM cells from IL-1Ra$^{-/-}$ mice (Figure 3C and 3D and Table 5). When wild-type BM cells were transplanted into IL-1Ra$^{-/-}$ mice, no protective effect on the development of aortitis was observed (incidence of 100% and 33% at 12 and 24 weeks after transplantation, respectively). These results demonstrate that IL-1Ra deficiency in T cells is responsible for the development of aortitis.

**TABLE 4. Hemodynamics and Weights in IL-1Ra$^{-/-}$ and Wild-Type Mice**

| | Wild Type | IL-1Ra$^{-/-}$ | *P* |
|---|---|---|---|
| Heart rate, bpm | 554.6±18.3 | 483.6±18.7* | 0.001 |
| Systolic pressure, mm Hg | 118.3±14.9 | 112.5±10.8 | 0.275 |
| Heart weight, mg | 151.7±8.5 | 134.0±15.0 | 0.915 |
| Body weight, g | 22.9±1.0 | 22.0±1.7 | 0.371 |

Values are mean±SD. Wild-type mice n=4; IL-1Ra$^{-/-}$ mice n=4 (female, 12 weeks old).
*$P<0.05$ vs wild-type mice (Student *t* test).

## Suppression of Aortitis in TNF-α–Deficient but Not IL-6–Deficient, IL-1Ra$^{-/-}$ Mice

It has been suggested that TNF-α and IL-6 are involved in the development of cardiovascular diseases.[16] Therefore, we studied the roles of TNF-α and IL-6 in the development of aortitis in IL-1Ra$^{-/-}$ mice by generating doubly gene-deficient mice. The aortic valves of TNF-α$^{-/-}$–IL-1Ra$^{-/-}$ or IL-6$^{-/-}$–IL-1Ra$^{-/-}$ mice were histologically analyzed at 14 or 8 weeks of age, respectively. Interestingly, TNF-α$^{-/-}$–IL-1Ra$^{-/-}$ mice showed no signs of arterial inflammation, whereas ≈50% of the IL-1Ra$^{-/-}$ mice developed aortitis (Figure 4 and Table 6). On the other hand, the incidence of aortitis was increased in IL-6$^{-/-}$–IL-1Ra$^{-/-}$ mice, although the difference was not statistically significant (by Fisher's exact test, $P=0.09$). The severity score was comparable to that in IL-1Ra$^{-/-}$ mice. These observations indicate that TNF-α is crucial for the development of aortitis in IL-1Ra$^{-/-}$ mice.

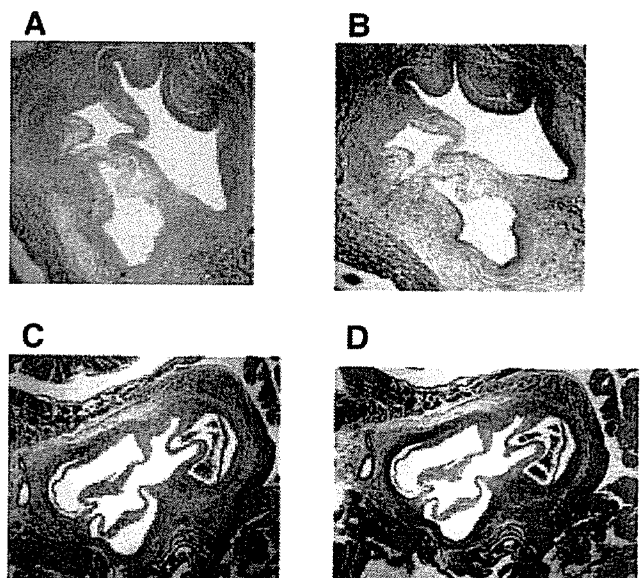


**Figure 3.** Induction of aortitis by transplantation of IL-1Ra$^{-/-}$ peripheral T cells and BM cells. A and B, Sections from nu/nu female mice 10 weeks after transplantation of T cells from IL-1Ra$^{-/-}$ mice (score 3). C and D, Sections from a wild-type, female mice 12 weeks after transplantation of BM-derived cells from IL-1Ra$^{-/-}$ mice (score 3). Hematoxylin and eosin staining (A and C) or Masson's trichrome staining (B and D). Original magnification ×40.

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**TABLE 5. Transplantation of T Cells and BM Cells**

| Donor Mice→Recipient Mice | Incidence (Rate, %) | Median Score |
|---|---|---|
| T cell transplantation | | |
| IL-1Ra$^{-/-}$→nu/nu | 12/13 (92)* | 2† |
| Wild type→nu/nu | 2/6 (33) | 1 |
| BM cell transplantation | | |
| 12 Weeks later | | |
| IL-1Ra$^{-/-}$→wild type | 6/6 (100) | 2 |
| Wild type→IL-1Ra$^{-/-}$ | 6/6 (100) | 2 |
| Wild type→wild-type | 0/2 (0) | NA |
| 24 Weeks later | | |
| IL-1Ra$^{-/-}$→wild type | 5/7 (71) | 2 |
| Wild type→IL-1Ra$^{-/-}$ | 2/6 (33.3) | 1 |
| Wild type→wild type | 0/2 (0) | NA |

NA indicates not applicable. The number of diseased mice among the total number of animals is shown.

*$P$=0.017, vs wild-type mice by Fisher exact test.

†$U$ value was significant ($P$<0.05) vs wild-type mice by the Mann-Whitney $U$ test.

In IL-1Ra$^{-/-}$ mice, TNF-$\alpha$ protein levels in the blood were slightly higher than in wild-type mice, whereas the levels of IL-1$\alpha$, IL-1$\beta$, and IL-6 were normal compared with wild-type mice (Table 7).

## Discussion

In this report, we have demonstrated that T cells play a crucial role in the pathogenesis of aortitis in IL-1Ra$^{-/-}$ mice on the BALB/c background and that TNF-$\alpha$ is essential for development of the disease. Inflammation of the cardiovascular system was preferentially observed at the aortic root of IL-1Ra$^{-/-}$ mice. As a result, these mice developed mild aortic stenosis and hyperplasia of both the interventricular septum wall and the LV posterior wall. However, the severity of these phenotypes seemed to be much milder on the BALB/c background than on the 129/Ola×MF1 background,[6] in which not only the aortic root but also the main arteries were affected at a high incidence, especially at branch points. It is possible, however, that this apparent difference may reflect not that due to genetic backgrounds but to the ages of the mice, because exact ages of the mice were not known in the preceding report.[6] Shepherd et al[17] also recently reported that IL-1Ra$^{-/-}$ mice on the BALB/c background spontaneously develop aortitis. These authors reported that these mice also spontaneously develop cutaneous inflammation, and we also observed similar signs in our IL-1Ra$^{-/-}$ mice (authors' unpublished observations). Shepherd et al reported that aortic inflammation was normally observed in IL-1Ra$^{-/-}$ (BALB/c×C57BL/6) $F_2$ hybrid mice as in IL-1Ra$^{-/-}$–BALB/c mice, whereas arthritis was rarely seen in the hybrid mice, suggesting that different background genes are involved in the development of aortitis and arthritis.

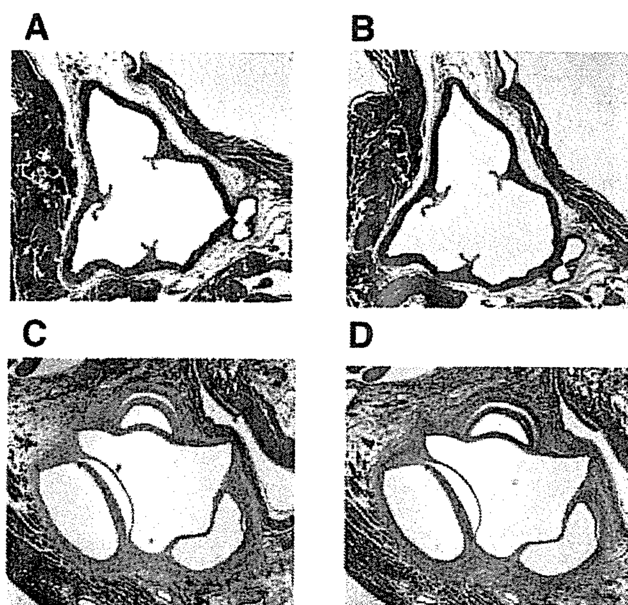


**Figure 4.** Complete suppression of the development of aortitis in TNF-$\alpha$–deficient, IL-1Ra$^{-/-}$ mice. Sections of the aortic valves from TNF-$\alpha^{-/-}$–IL-1Ra$^{-/-}$ female mice (14 weeks old, score 0) (A and B) and IL-6$^{-/-}$–IL-1Ra$^{-/-}$ female mice (8 weeks old, score 3) (C and D). Hematoxylin and eosin staining (A and C) or Masson's trichrome staining (B and D). Original magnification ×40.

**TABLE 6. Incidence of Aortitis in IL-1Ra$^{-/-}$ Female Mice Crossed With IL-6$^{-/-}$, TNF-$\alpha^{-/-}$ Mice**

| Group (Age, wk) | Incidence (Rate, %) | Median Score |
|---|---|---|
| IL-6$^{+/+}$, IL-1Ra$^{-/-}$ (8) | 3/6 (50) | 1 |
| IL-6$^{-/-}$, IL-1Ra$^{-/-}$ (8) | 6/6 (100)* | 3 |
| TNF-$\alpha^{+/+}$, IL-1Ra$^{-/-}$ (14) | 5/9 (55) | 2 |
| TNF-$\alpha^{-/-}$, IL-1Ra$^{-/-}$ (14) | 0/7 (0)† | NA |

NA indicates not applicable. The numbers of diseased mice among the total number of animals is shown. Severity scores were not significantly different vs control mice by the Mann-Whitney $U$ test.

*$P$=0.090, †$P$=0.028 vs control mice by Fisher exact test.

At the aortic root of IL-1Ra$^{-/-}$ mice, infiltration of monocytes and macrophages was observed frequently, but accumulation of foam cells, which are derived from macrophages and cause atherosclerosis, was not observed. Occasional infiltration of neutrophils was observed. Loss of elastic lamellae in the aortic media and occasional calcification of the media, signs of degenerative processes that mainly reflect degradation of smooth muscle cells,[18,19] were observed in these mice. Neovascularization was also frequently observed, reflecting inflammation. These pathological findings resemble some aspects of Takayasu arteritis or polyarteritis nodosa in humans, in agreement with a previous report.[6]

**TABLE 7. Plasma Levels of Proinflammatory Cytokines in IL-1Ra$^{-/-}$ and Wild-Type Mice**

| Cytokine Level, pg/mL | Wild Type | IL-1Ra$^{-/-}$ | P |
|---|---|---|---|
| IL-1$\alpha$ | 15.9±7.7 | 14.9±4.0 | 0.891 |
| IL-1$\beta$ | 28.9±17.3 | 47.6±27.6 | 0.230 |
| TNF-$\alpha$ | 111.1±6.2 | 208.4±18.0* | 0.001 |
| IL-6 | 46.7±35.3 | 72.5±18.4 | 0.753 |

Values are mean±SD. Wild-type mice n=6; IL-1Ra$^{-/-}$ mice n=5 (male, 8 weeks old).

*$P$<0.05 vs wild-type mice (Student $t$ test).

We have demonstrated that peripheral T cells from IL-1Ra$^{-/-}$ mice can cause aortitis in nu/nu mice, suggesting that activated and/or memory T cells are generated and involved in the development of aortitis. Because IL-1Ra deficiency in BM cells could induce aortitis in wild-type recipient mice, it was suggested that T-cell intrinsic disjunction rather than abnormality of positive-negative selection of T cells in the thymus was responsible for the development of aortitis. With regard to this concept, we have shown that the development of arthritis in IL-1Ra$^{-/-}$ mice was also dependent on T cells.[8] We showed that IL-1 signaling activates T cells by enhancing CD40L and OX40 expression on T cells and causes the development of autoimmunity.[4,20,21] Furthermore, we showed that IL-1Ra is produced by CD4$^+$ T cells and regulates the action of IL-1 in an autocrine manner.[8] Thus, we suggest that IL-1Ra–deficient T cells are excessively activated even by physiological levels of IL-1 and may lose tolerance for aortic endothelial cell components, resulting in the development of autoimmunity and inflammation.

It is known that a small portion of mainstream aortic flow is intercepted during systole by the sinus ridge, or the downstream corner of the sinus of Valsalva; this fluid curls back toward the ventricle to form a large eddy, or vortex, that spins within the sinus cavity and generates turbulence.[22] Hemodynamic force may affect structural and metabolic aspects of vascular endothelial cells,[23] and high shear forces on the leaflet may lead to increased cell damage or turnover,[24] resulting in production of IL-1 from these cells. Indeed, it is known that IL-1 release is increased at the aortic root or at the branch point of the aorta where cells are exposed to mechanical stress caused by blood flow.[25] Therefore, in the absence of IL-1Ra, T cells near the areas where cells are exposed to mechanical stress may be excessively activated.

In IL-1Ra$^{-/-}$ mice, serum levels of myeloperoxidase anti-neutrophil cytoplasmic antibodies, which increase in some types of systemic vasculitis in humans, were not increased (data not shown), although the levels of other autoantibodies such as anti-IgG and anti–type II collagen were increased.[4] These pathologies closely resemble human systemic vasculitis that is typically not associated with anti-neutrophil cytoplasmic antibodies (polyarteritis nodosa, Takayasu arteritis, and giant-cell arteritis). The pathogenic antigens in the aorta in this model remain to be elucidated.

We have shown that most of the mice that developed aortitis also developed arthritis, suggesting that these 2 diseases have a similar pathogenesis (or mechanism). Indeed, we have shown that both diseases are caused by a T cell–dependent mechanism. However, considering the facts that aortitis begins to develop earlier than arthritis and that a large proportion of mice develop only 1 of the diseases, either aortitis (5%) or arthritis (47%), at 14 weeks of age, the pathogenic processes underlying these diseases may be different in part.

Interestingly, we found that TNF-α deficiency suppressed the development of aortitis in IL-1Ra$^{-/-}$ mice. In contrast, IL-6 deficiency in IL-1Ra$^{-/-}$ mice showed pathological findings of aortitis. These results indicate that TNF-α plays an important role in the development of aortitis. TNF-α deficiency but not IL-6 deficiency also suppressed the development of arthritis in IL-1Ra$^{-/-}$ mice.[8] Consistent with these observations, circulating levels of TNF-α but not of IL-6 were increased in IL-1Ra$^{-/-}$ mice. In this context, it is known that activation of antigen-presenting cells by activated T cells through interaction with CD40/CD40L induces TNF-α.[26] Thus, TNF-α production may be enhanced in antigen-presenting cells through interaction with activated T cells in IL-1Ra$^{-/-}$ mice. Furthermore, we previously reported that TNF-α production was induced in T cells by IL-1 stimulation[27] and that T cell–derived TNF-α played an important role in the pathogenesis of contact hypersensitivity[27] and arthritis.[8] TNF-α production by CD4$^+$ T cells is also induced on stimulation with anti-CD3 monoclonal antibody, and IL-1Ra$^{-/-}$ T cells produce significantly higher levels of TNF-α together with IL-4 and interferon-γ than do wild-type T cells in culture supernatants.[8] Other investigators have also reported the production of TNF-α in T cells[28,29] and the presence of TNF receptors in aortic smooth muscle and endothelial cells.[30] Thus, excess TNF-α produced by IL-1Ra$^{-/-}$ T cells and antigen-presenting cells may activate endothelial cells to produce excessive amounts of various inflammatory cytokines and chemokines, resulting in the development of inflammation.[31] It is also known that TNF-α induces the expression of vascular cell adhesion molecule-1 in endothelial cells, which promotes early adhesion of mononuclear leukocytes to the arterial endothelium at sites of inflammation.[32] Although transfer of TNF-α$^{-/-}$–IL-1Ra$^{-/-}$ T cells into nu/nu mice will help evaluate the contribution of T cell–derived TNF-α to the development of aortitis separately from that of antigen-presenting cells, we were unable to address this question because of the difference in the major histocompatibility locus between TNF-α$^{-/-}$ mice (H-2 locus b/b) and BALB/c-nu/nu mice (H-2 locus d/d), even after 8 generations of backcrossing to BALB/c strain.

Taken together, our observations suggest that excessively activated T cells are responsible for the development of aortitis and that TNF-α mediates the inflammatory process. Autoimmune responses against specific antigens on vessel walls may thus be induced, as in the case of arthritis in these mice. However, further analysis is necessary to confirm this finding, because it is also possible that excessively activated T cells directly induce inflammation by producing inflammatory cytokines without the involvement of autoimmunity. Nonetheless, it is possible that both aortitis in IL-1Ra$^{-/-}$ mice and anti-neutrophil cytoplasmic antibody–associated systemic vasculitis in humans share a similar pathogenic process involving TNF-α. Consistent with this notion, it was recently reported that infliximab, an anti–TNF-α antibody, improved endothelial dysfunction in anti-neutrophil cytoplasmic antibody–associated systemic vasculitis in humans.[33] These observations provide new insights into the pathogenesis of vasculitis, and the IL-1Ra$^{-/-}$ mouse should be a useful model to study the pathogenic mechanisms of vasculitis.

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## CLINICAL PERSPECTIVE

Vasculitis syndromes such as Takayasu arteritis and giant-cell arteritis involve massive recruitment of lymphocytes and macrophages into the vascular wall, destruction of the medial layer with concurrent fibrosis, and proliferation of smooth muscle cells in the intima, leading to neointima formation. Although a number of potential mechanisms, including microbial infection and autoimmune reactions, have been implicated in the development of inflammatory reactions in the vasculature, the precise mechanism underlying the development of vasculitis remains to be elucidated. In this issue, we showed that IL-1Ra$^{-/-}$ mice, in which excess IL-1 signaling is induced under physiological conditions owing to deficiency of the antagonist, spontaneously develop aortitis at the root of the aorta, with massive infiltration of macrophages and monocytes and loss of elastic lamellae in the aortic media. LV hypertrophy and mild aortic stenosis were also shown by transthoracic echocardiography. These pathological findings resemble some aspects of Takayasu arteritis or polyarteritis nodosa in humans, indicating that IL-1Ra$^{-/-}$ mice are a good model for these vascular diseases. Interestingly, transplantation of T cells from IL-1Ra$^{-/-}$ mice induced aortitis in recipient nu/nu mice, suggesting involvement of T cells in pathogenesis. Furthermore, TNF-α deficiency completely suppressed the development of aortitis in IL-1Ra$^{-/-}$ mice, whereas IL-6 deficiency did not. These observations indicate that both IL-1 and TNF-α play crucial roles in the development of aortitis in IL-1Ra$^{-/-}$ mice. Therefore, control of either IL-1 or TNF-α activity may be beneficial for the treatment of vasculitis in humans.

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# Involvement of Corticotropin-Releasing Hormone- and Interleukin (IL)-6-Dependent Proopiomelanocortin Induction in the Anterior Pituitary during Hypothalamic-Pituitary-Adrenal Axis Activation by IL-1$\alpha$

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**IL-1$\alpha/\beta$ and IL-6 are endogenous modulator of hypothalamo-pituitary-adrenal axis (HPAA) and are thought to play key roles in immune-neuroendocrine interactions during inflammation. Here, we show IL-1$\alpha$ induced a normal HPAA activation in *IL-1$\alpha/\beta$* knockout (KO) and *IL-6* KO mice at 1 h; however, at 6 h HPAA activation was reduced relative to wild-type mice, indicating a role for endogenous IL-1$\alpha/\beta$ and IL-6 in prolonged HPAA activation. We found that the induction of *proopiomelanocortin (POMC)* transcript in the anterior pituitary (AP) at 6 h in response to IL-1$\alpha$ was reduced in *IL-1$\alpha/\beta$* KO and *IL-6* KO mice, as well as in *CRH* KO mice, suggesting IL-1$\alpha/\beta$, IL-6, and CRH are all required for *POMC* induction. The induction of *CRH* transcript in the paraventricular nucleus at 6 h and plasma IL-6 levels, in response to IL-1$\alpha$, were reduced in *IL-1$\alpha/\beta$* KO mice. Because IL-1$\alpha$-induced activation of signal transducer and activator of transcription 3 in the AP was also suppressed in *IL-6* KO mice, we suggest that plasma IL-6 is first induced by IL-1$\alpha$, and IL-6 activates signal transducer and activator of transcription 3 in the AP, leading to the induction of *POMC* in concert with *CRH*. Our results suggest a role for IL-1$\alpha/\beta$ in the induction of *POMC* in the AP through the induction of two independent pathways, *CRH* and IL-6. (*Endocrinology* 146: 5496–5502, 2005)**

ACTIVATION OF THE hypothalamic-pituitary-adrenal axis (HPAA) is a key host response to stress and inflammation. The resulting increase in adrenal glucocorticoid secretion prevents overshoot of immune/inflammatory responses, limiting the host defense response without the potentially deleterious effects of a hyperactive immune system (*e.g.* autoimmunity) (1). The secretion of glucocorticoids is stimulated by ACTH that is synthesized and secreted by the anterior pituitary (AP) gland. CRH is synthesized in the hypothalamic paraventricular nucleus (PVN) and secreted into the hypophysial portal circulation. In response to variety of stresses, secretion of ACTH and glucocorticoid occurs as a result of the increased activity of CRH-secreting neurons in the PVN (2). CRH signaling in the corticotroph increases both the transcription of the *proopiomelanocortin* (*POMC*) gene and the secretion of mature ACTH peptide (3). Induction of *POMC* gene expression by CRH is also observed in primary pituitary cultures and in the mouse corticotroph cell line AtT-20 (3, 4).

Proinflammatory cytokines, released during systemic and localized inflammation, elicit a number of responses in the host, including fever and anorexia. The landmark studies by Besedovsky *et al.* and Blalock and colleagues (5, 6) indicated that IL-1$\alpha/\beta$ and IL-6 could be the extrahypothalamic CRH released by injured tissue. Several reports have shown that IL-1$\alpha/\beta$ stimulates HPAA mainly through the hypothalamus, and its action depends on CRH release (7). We have previously demonstrated that IL-1$\alpha/\beta$ not only induce CRH release, but also induces expression of CRH in the PVN and *POMC* in the AP, which is a precursor of ACTH (8). Moreover, we showed the importance of IL-1$\alpha/\beta$ in *in vivo* HPAA activation induced by turpentine; the corticosterone response in *IL-1$\alpha/\beta$* knockout (KO) mice was completely abolished 8 h after injection of turpentine, whereas it was normal 2 h after injection of turpentine (9). Although IL-1$\alpha/\beta$ is known to be important for the activation of HPAA, the molecular mechanism by which these cytokines induce HPAA activation is poorly understood. IL-6 is another proinflammatory cytokine whose effects on the HPAA have been investigated extensively (10–12). Its levels in the circulation are increased during physical, psychological, and inflammatory stresses (2). Peripheral IL-6 administration in rodents induces ACTH and glucocorticoid secretion. Because peripheral injection of IL-1$\alpha$ or IL-1$\beta$ induces plasma IL-6 (13), some of the actions of IL-1$\alpha/\beta$ on the neuroendocrine network is thought to be mediated by the action of IL-6 (14, 15). Although a synergism between gp130 family cytokines and CRH on HPAA activation and *POMC* gene expression *in vitro*

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Abbreviations: AP, Anterior pituitary; HPAA, hypothalamic-pituitary-adrenal axis; KO, knockout; LPS, lipopolysaccharide; *POMC*, *proopiomelanocortin*; PVN, paraventricular nucleus; rm, recombinant murine; ROD, relative OD; STAT3, signal transducer and activator of transcription 3; WT, wild type.

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was reported by several studies (10, 16, 17), *in vivo* significance of each pathway remains to be elucidated.

In this report, we have examined the HPAA response in *CRH* KO mice, *IL-1α/β* KO mice, and *IL-6* KO mice to elucidate the signaling cascade induced by periphery-administered IL-1α. We demonstrate that IL-1α induces *POMC* in the AP, and both IL-1α/β and IL-6 are involved in the prolonged activation of HPAA and the induction of *POMC*. It is suggested that IL-1α activates HPAA through the induction of *CRH* in the PVN and IL-6 in the plasma.

## Materials and Methods

### *Reagents*

Recombinant murine IL-1α (rmIL-1α) was obtained from Pepro Tech EC LTD (London, UK). The lyophilized protein was dissolved in 0.9% NaCl (saline) containing 0.1% BSA (A9306; Sigma, St. Louis, MO).

### *Animals*

*IL-1α/β* double-KO mice were produced as described (9) and *IL-6* KO mice were kindly provided by Dr. Manfred Kopf (18). These mice were back-crossed to C57BL/6J mice for eight generations, and C57BL/6J (SLC Inc., Shizuoka, Japan) mice were used as controls. *CRH* KO mice generated by targeted mutation in embryonic stem cells were used in this study (19). Mice were housed individually after weaning at 4 wk of age, and age-matched (8–12 wk of age) male mice were used for each experiment. Mice were kept under specific pathogen-free conditions in an environmentally controlled clean room at the Center for Experimental Medicine, the Institute of Medical Science, the University of Tokyo. Mice were housed at an ambient temperature of 24 C and a daily cycle of 12 h light (0800–2000 h) and 12 h darkness. All experiments were carried out according to the institutional ethical guidelines for animal experiments and according to the safety guidelines for gene manipulation experiments.

### *Hormone assays*

*IL-1α/β* double-KO, *IL-6* KO, *CRH* KO, and wild-type (WT) mice were injected with IL-1α (20 μg per kg of body weight, ip) or saline at 1000 h and killed 1, 3, or 6 h after administration. Mice were rapidly anesthetized with diethyl-ether, and blood samples were collected from the heart. Plasma corticosterone levels were determined by RIA (detection limit: 0.6 ng/ml; Amersham Biosciences, Buckinghamshire, UK). The intra- and interassay assay coefficients of variation were 5.0% and 5.9%, respectively. Plasma ACTH concentration was determined by immunoradiometric assay (detection limit: 5 pg/ml; Mitsubishi, Tokyo, Japan). The intra- and interassay assay coefficients of variation were 3.5% and 5.0%, respectively. Plasma IL-6 levels were measured by ELISA (detection limit: 10 ng/ml; PharMingen, San Diego, CA) according to the manufacturer's instructions.

### *In situ hybridization*

Mice were deeply anesthetized and perfused transcardially with 4% neutralized paraformaldehyde. Frozen sections (30 μm) were cut on a sliding microtome, mounted onto silane-coated slides (Matsunami, Tokyo, Japan), and air-dried. The hybridization protocol was similar to that previously described (20). Before hybridization, sections were dried overnight under vacuum, digested with proteinase K (10 μg/ml, 37 C, 15–20 min), acetylated, and dehydrated. After vacuum drying, 100 μl of the hybridization mixture ($10^6$ cpm/ml, with 10 mM dithiothreitol) was spotted onto each slide, sealed under a coverslip, and incubated at 65 C overnight. The coverslips were then removed and the slides were rinsed in 4× SSC [1× SSC = 15 mM trisodium citrate buffer (pH 7.0)/0.15 M NaCl] at room temperature. The sections were digested with ribonuclease A (20 μg/ml, 37 C, 30 min) and washed in 0.1× SSC for 30 min at 65 C. These sections were then exposed to double-sided x-ray film (XAR-5; Eastman Kodak, Rochester, NY) at 4 C for periods of 7–14 d (depending upon the nature of the probes used), dipped in NTB2 nuclear emulsion (1:1 with water) (Kodak), exposed for 14–30 d, and developed. The slides were counterstained with thionin. An adjoining series of sections were stained with thionin to provide better cytoarchitectonic definition for analysis. All samples from a single experiment were assayed simultaneously.

### *Probe labeling*

A pGEM-4 plasmid containing rat *CRH* cDNA (1.2 kb, a gift from Dr. K. Mayo, Northwestern University, Chicago, IL) was linearized with *Hin*dIII. Mouse *POMC* cDNA (923 bp, a gift from Dr. Douglass, Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland, OR), subcloned into *Psp*65O, was linearized with *Hin*dIII. The *Eco*RI fragment of rat c-*fos* cDNA (2.0 kb, Dr. I. Verma, Salk Institute, San Diego, CA) was subcloned into p-Bluescript SK-I and linearized with *Bam*HI. Radioactive antisense cRNA copies were synthesized by incubating 0.1 μg linearized plasmid with SP6 (Roche Molecular, Indianapolis, IN) for *CRH* and *POMC* probes or T7 (Roche Molecular) for c-*fos* probe, in a reaction mixture containing 6 mM $MgCl_2$, 2 mM spermidine, 8 mM dithiothreitol, 25 mM ATP/GTP/CTP, 5 mM unlabeled uridine triphosphate, ($\alpha$-$^{35}$S)-uridine triphosphate (370 MBr/ml, Amersham Biosciences), 1 U RNAsin (Promega, Madison, WI), 36 mM Tris (pH 7.5), for 60 min at 37 C. All probes were purified on resin columns (Nensorb 20; NEN Life Science Products, Wilmington, MA). The specific activity of each probe was approximately $1.0 \times 10^8$ cpm/μl.

The densities of *CRH* and c-*fos* mRNA in the PVN or *POMC* mRNA in the AP were semiquantified from the film autoradiograms using an MCID image analysis system (Imaging Research, St. Catherines, Canada) (21). The levels obtained were converted to relative ODs (RODs) using the formula: ROD = $\log_{10}$ (256/levels). Using the mouse brain atlas of Paxinos and Watson (22) as an anatomical guide, we enclosed the area of the medial parvocellular PVN by a rectangle (300 × 520 μm), forming a fixed window. The ROD within the window was measured and the background was assessed by measuring the ROD when the window was placed over another area of the brain where no specific hybridization for CRH was detected. The OD of the PVN was measured bilaterally for each subject.

### *Western blot analysis*

*IL-6* KO and WT mice were injected with IL-1α (20 μg per kg of body weight, ip) at 1000 h and decapitated at 1, 3, or 6 h after administration. Whole cell lysate from the pituitary was prepared and separated on a 7.5% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA), probed with monoclonal antiphospho-STAT3 (signal transducer and activator of transcription 3) (Tyr705) antibodies (no. 9131; Cell Signaling, Beverly, MA), and visualized by ECL (RPN 2131; Amersham Biosciences). Blots were stripped and reprobed with the anti-STAT3 antibody (no. 9132; Cell Signaling).

### *Statistical analysis*

All values were calculated as means ± SEM. Comparisons of two groups was analyzed by the Student's *t* test; for the comparisons of more than two groups, one- or two-way ANOVA was performed followed by Fisher's protected least significant difference, Dunnett's or Tukey's tests were used to analyze statistical differences in each group. In all analyses, a two-tailed probability of less than 5% (*i.e.* $P < 0.05$) was considered statistically significant, and significance was confirmed in at least two independent experiments.

## Results

### *HPAA response to IL-1α*

To elucidate the roles of *CRH*, *IL-1α/β*, or *IL-6* in the activation of HPAA in response to exogenous IL-1α, we injected recombinant murine IL-1α into *CRH* KO, *IL-1α/β* KO, and *IL-6* KO mice, respectively, and measured the plasma corticosterone, ACTH, and IL-6 levels. Firstly, we analyzed the time course of the HPAA activation after IL-1α stimulation. When IL-1α was administered to WT mice (20 μg/kg of body weight, ip), corticosterone levels (Fig. 1A) and ACTH



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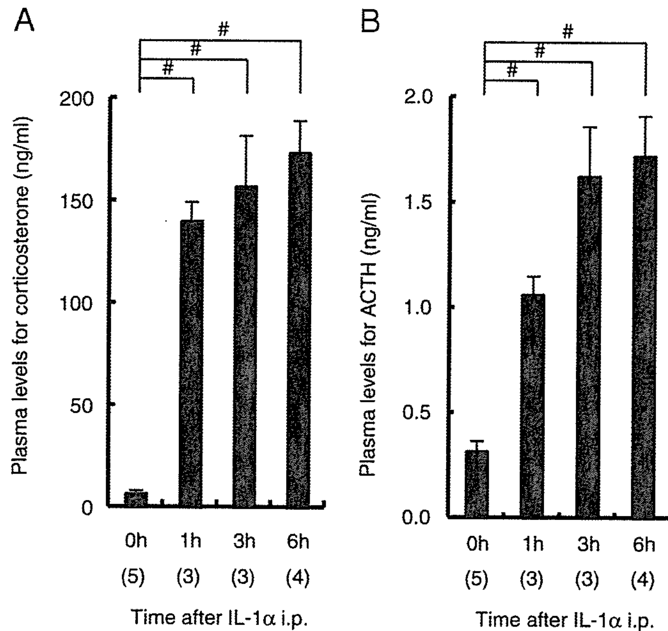


FIG. 1. Plasma corticosterone and ACTH levels after injection with IL-1α in WT mice. Blood samples were collected before treatment (0 h) or 1, 3, and 6 h after ip injection with rmIL-1α (20 μg/kg body weight), and corticosterone (A) and ACTH (B) levels in the plasma were measured. Same group of animals were analyzed for corticosterone level and ACTH levels. *Numbers beneath each column* show the number of animals per group. #, Statistical difference ($P < 0.05$) compared with untreated animals (Dunnett's test). Similar results were obtained in two independent experiments.

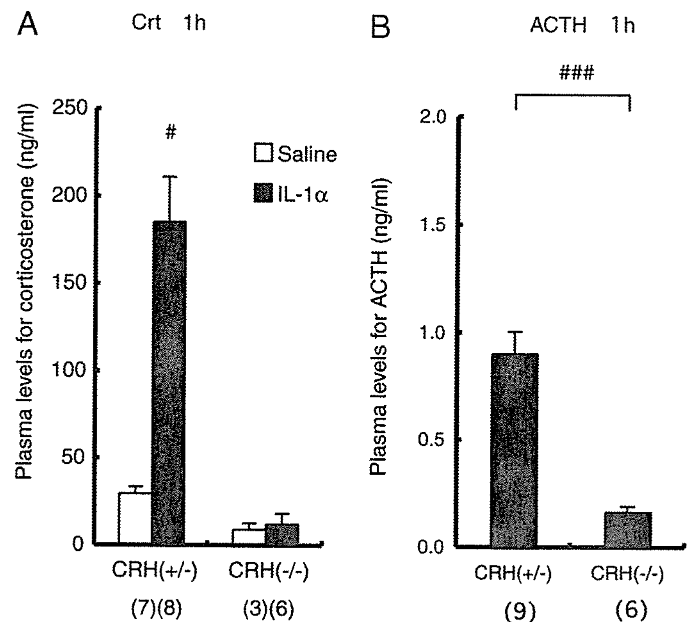


FIG. 2. Plasma corticosterone and ACTH levels after injection with IL-1α in *CRH* KO mice. Plasma corticosterone (A) and ACTH (B) levels were measured 1 h after ip injection of rmIL-1α (20 μg/kg body weight) or saline to *CRH* (+/−) and *CRH* (−/−)mice. Same group of animals were analyzed for corticosterone level and ACTH levels, but one sample for corticosterone measurement was lost in the IL-1α-injected CRH (+/−) group. *Numbers beneath each column* show the number of animals per group. Statistical difference between the other three groups determined by one-way ANOVA and Tukey's test (A) or Student's *t* test (B). #, $P < 0.05$; ###, $P < 0.001$ Similar results were obtained in two independent experiments. Crt, Corticosterone.

levels (Fig. 1B) were significantly elevated at 1, 3, and 6 h after injection.

To examine the effect of *CRH* deficiency on HPAA activation in response to IL-1α , we measured plasma corticosterone and plasma ACTH levels at 1 h after peripheral injection of IL-1α in *CRH* KO mice. *CRH* KO mice failed to induce corticosterone (Fig. 2A) and ACTH in the plasma (Fig. 2B), indicating that endogenous *CRH* is required for the activation of HPAA at 1 h.

Because exogenously administered IL-1α-induced endogenous IL-1α and IL-1β in the periphery as well as in the brain (9, 23–25), we next analyzed the role of these endogenously induced IL-1α/β components in *IL-1α/β* KO mice. We measured plasma corticosterone (Fig. 3, A and C) and plasma ACTH levels (Fig. 3, B and D) in *IL-1α/β* KO mice at 1 and 6 h after ip injection of IL-1α. *IL-1α/β* KO mice showed similar corticosterone and ACTH levels to these of WT mice at 1 h after injection (Fig. 3, A and B), indicating that endogenous *IL-1α/β* expression is not required for HPAA activation at 1 h. In contrast, *IL-1α/β* KO mice showed reduced plasma corticosterone and ACTH levels at 6 h after injection (Fig. 3, C and D) relative to WT mice, suggesting that endogenous *IL-1α/β* is required for a prolonged HPAA response at 6 h.

It is well known that IL-1α/β induces IL-6. Furthermore, it was suggested that IL-6 plays an important role in turpentine- or lipopolysaccharide (LPS)-induced HPAA activation (10, 11). Then we examined the effect of IL-6 deficiency on HPAA activation. Significant reduction of corticosterone and ACTH levels was observed in IL-6 KO mice at 6 h after injection (Fig. 3, C and D), whereas the levels of these hormones were not reduced at 1 h after injection consistently with a previous report (26) (Fig. 3, A and B). Consistently with previous observations, plasma IL-6 levels were significantly elevated upon IL-1α stimulation in WT mice (Fig. 4). However, plasma IL-6 levels were reduced in *IL-1α/β* KO mice at 3 h compared with WT mice (Fig. 4), indicating that endogenous IL-1α/β is also necessary for the induction of IL-6. These results indicate that endogenous IL-6 is required for the prolonged HPAA activation at 6 h but is not required for the HPAA response at 1 h.

*Induction of POMC in the AP in response to IL-1α in IL-1α/β KO mice*

To know the reason for the requirement of endogenous *IL-1α/β* and *IL-6* in prolonged activation of the HPAA in response to IL-1α, we analyzed the expression of *POMC*, the precursor of ACTH, in the AP. After ip administration of IL-1α, *POMC* expression was significantly induced in the AP after 6 h, but not at 1 h, in WT mice, as examined by *in situ* hybridization (Fig. 5A). In contrast, *POMC* induction was not observed after IL-1α injection after 6 h in *IL-1α/β* KO mice (Fig. 5B) and *IL-6* KO mice (Fig. 5C). The suppression of *POMC* induction in *IL-1α/β* KO mice was confirmed in another experiment using 6 mice (see supplemental Fig. 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). These results suggest a possibility that blunted induction of *POMC* is responsible for the reduced activation of the HPAA in *IL-1α/β*



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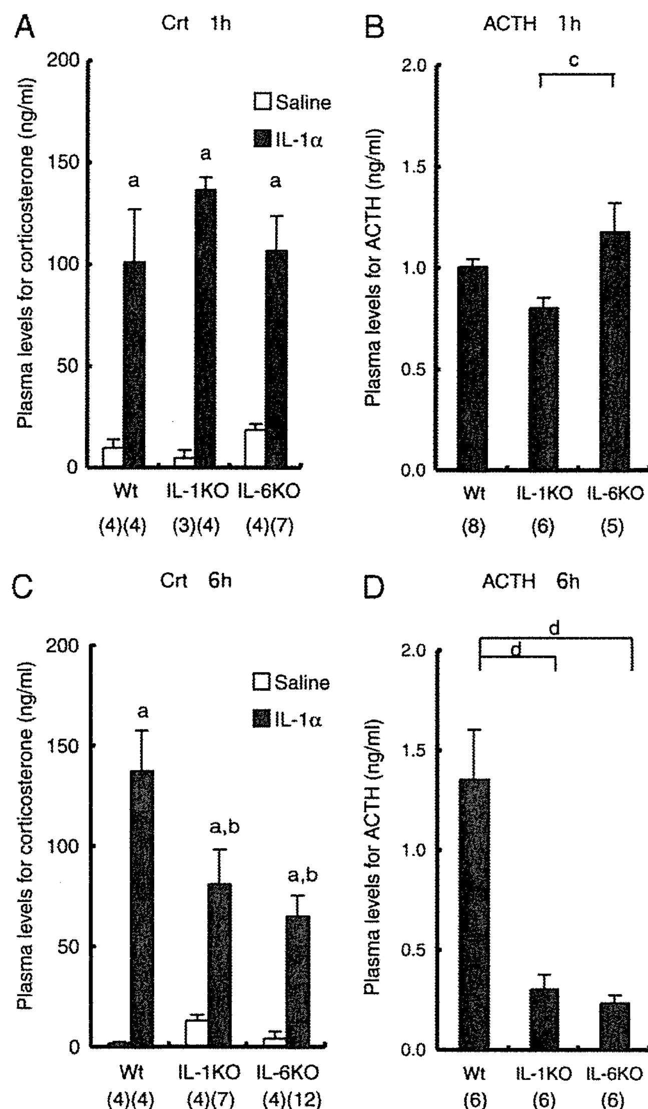


FIG. 3. Plasma corticosterone (A and C) and ACTH (B and D) levels after injection with IL-1α in WT, *IL-1α/β* KO, and *IL-6* KO male mice. (A and B) Plasma corticosterone (A and C) and ACTH (B and D) levels were measured 1 h (A and B) or 6 h (C and D) after ip injection of rmIL-1α (20 μg/kg body weight) or saline to WT, *IL-1α/β* KO, and *IL-6* KO mice. Separate groups of the animal were used for each measurement, and *numbers beneath each column* show the number of animals per group. Statistical significance was determined by one-way ANOVA and Tukey's test (A and C) or Fisher's protected least significant difference test (B and D). a: Statistical difference ($P < 0.05$) between saline- and IL-1α-injected mice of the same genotype (A and C). b,–d, Statistical difference (b, $P < 0.05$; c, $P < 0.01$; d, $P < 0.001$) between WT mice and *IL-1α/β* KO or *IL-6* KO mice injected with IL-1α (B–D). Similar results were obtained in four independent experiments. Crt, Corticosterone.

KO and *IL-6* KO mice. We found that IL-1α also failed to induce *POMC* expression at 6 h after injection in *CRH* KO mice (Fig. 5D).

*Induction of CRH in the PVN in response to IL-1α in IL-1α/β KO and IL-6 KO mice*

Because *POMC* expression is dependent on the expression of *CRH*, we next analyzed the induction of *CRH* in the PVN

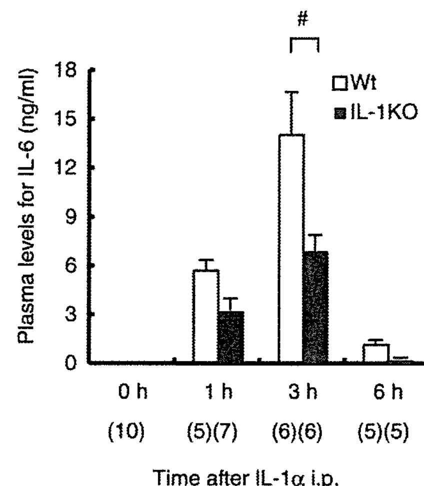


FIG. 4. Plasma IL-6 levels after injection with IL-1α into *IL-1α/β* KO mice or WT mice. Blood samples were collected before treatment (0 h) or 1, 3, and 6 h after ip injection of rmIL-1α (20 μg/kg body weight) in WT and in *IL-1α/β* KO mice, and the plasma IL-6 levels were measured. *Numbers beneath each column* indicate the number of animals per group. Statistical significance was determined by two-way ANOVA and Tukey's test. #, $P < 0.05$. Similar results were obtained in two independent experiments.

in WT, *IL-1α/β* KO, and *IL-6* KO mice. *CRH* transcript was clearly induced in the PVN of both WT and *IL-6* KO mice at 6 h after IL-1α injection (Fig. 5, E and G). However, the induction of *CRH* was not observed in *IL-1α/β* KO mice (Fig. 5F). These results indicate that IL-1α/β, but not IL-6, is necessary for the induction of *CRH* in the PVN.

*Activation of STAT3 in the pituitary in response to IL-1α is dependent on IL-6*

Because IL-6 activates STAT3, and STAT3 is suggested to play an important role in the expression of *POMC* in the pituitary (27, 28), we next analyzed the activation of STAT3 in the pituitary. Activation of STAT3 was observed 1–3 h after injection of IL-1α in WT mice (Fig. 6A), whereas it was blunted at 1 h after injection of IL-1α in *IL-6* KO mice, clearly correlated with the levels of IL-6 in the plasma (Fig. 6B). Low-level activations of STAT3 were observed at later time points (data not shown). These results indicate that activation of STAT3 in response to IL-1α depends on plasma IL-6.

## Discussion

In this study, we have analyzed the HPAA activation mechanisms in response to peripheral injection of IL-1α and demonstrated that the HPAA activation mechanism at 6 h is different from that at 1 h after stimulation; the activation depends only on CRH, but not IL-α/β or IL-6, at 1 h, whereas it depends on both IL-1α/β and IL-6 expression at 6 h. We found that *POMC* was induced at 6 h in the AP in a IL-1α/β-, IL-6-, and CRH-dependent manner, although it was not induced at 1 h. In agreement with our observations, Melmed and colleagues (27, 28) suggested that two independent pathways, *CRH* and gp130-STAT3, are important for the regulation of *POMC* gene expression in the AP.

We showed recently that, upon induction of fever by peripheral IL-1α injection, endogenous *IL-1α/β* expression is



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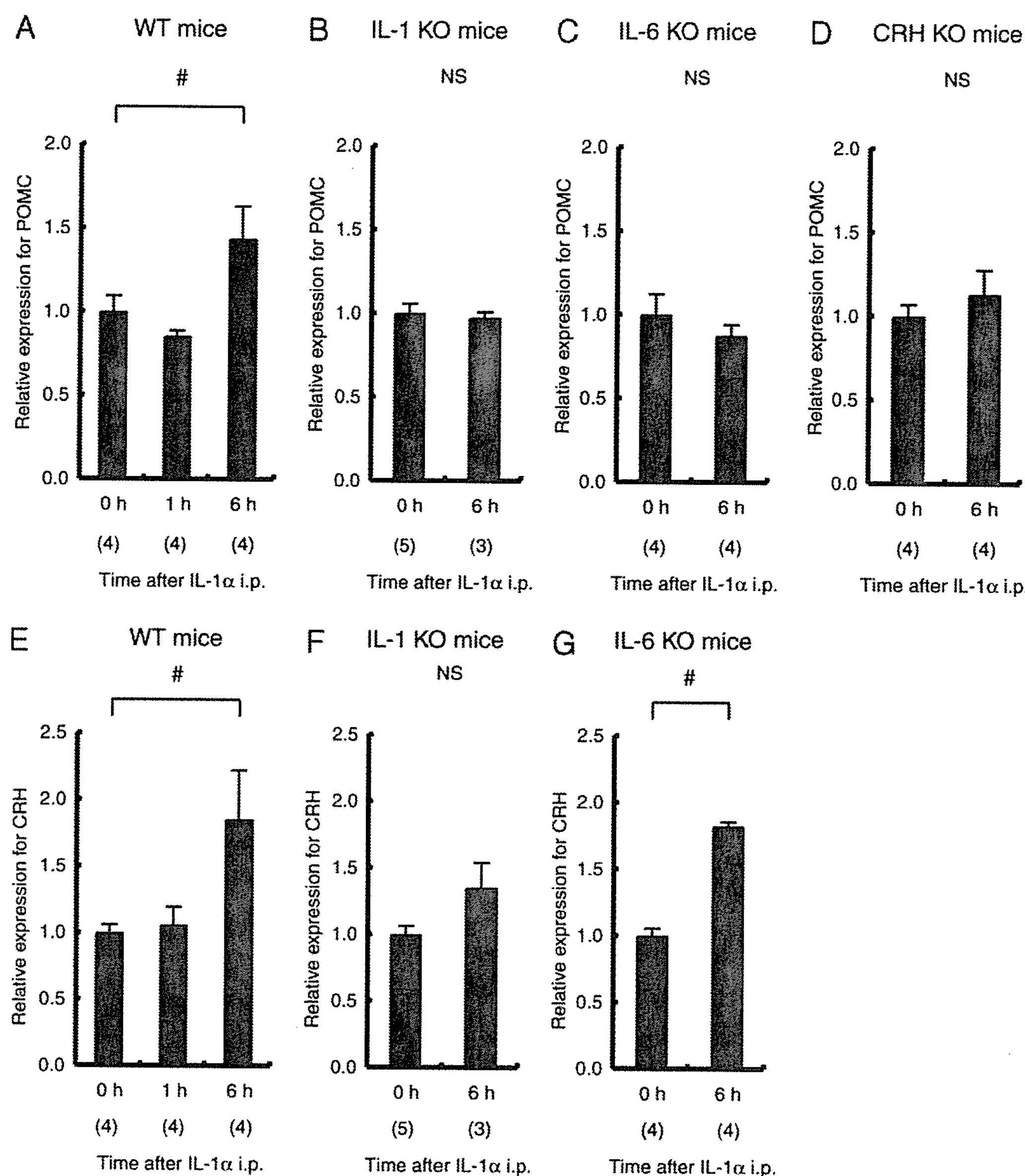


FIG. 5. Induction of *POMC* transcript (A–D) in the AP and *CRH* transcript in PVN (E–G) in response to peripheral injection of IL-1α. A, *POMC* mRNA levels in the AP were estimated by *in situ* hybridization after ip injection of rmIL-1α (20 μg/kg body weight) to WT mice. B–D, *POMC* mRNA levels in the AP were measured after ip injection of rmIL-1α to *IL-1α/β* KO mice (B), *IL-6* KO mice (C), or *CRH* KO mice (D). E, *CRH* mRNA levels in the PVN were estimated by *in situ* hybridization after ip injection of rmIL-1α to WT mice. F and G, *CRH* mRNA levels in the PVN were measured after ip injection of rmIL-1α to *IL-1α/β* KO mice (F), or *IL-6* KO mice (G). Levels relative to these of untreated mice (0 h) of each genotype were shown. Same groups of the animal were used for *POMC* and *CRH* measurements and *numbers beneath each column* show the number of animals per group. Statistical significance was determined by Dunnett's test (A and E) or the Student's *t* test (B, C, D, F, and G). #, $P < 0.05$. Similar results were obtained in two independent experiments. NS, Not significant.

not necessary and IL-1α-induced PGE2 and IL-6 in the brain play important roles (25). However, it is not known whether endogenous *IL-1α/β* expression is required for the HPAA activation or CRH neuron activation in the PVN (29). In this report, we showed that the endogenous expression of *IL-1α/β* is not necessary for the activation of HPAA at 1 h after injection with IL-1α. In contrast to the febrile response, however, an important role for endogenous *IL-1α/β* was suggested in the prolonged activation of HPAA at 6 h. The difference between the febrile response may be explained by the fact that HPAA, in contrast to fever, is regulated at the level of the pituitary, which is considered to be the peripheral part of the neuroendocrine system.

Several lines of studies, including ours, have demonstrated that a variety of stresses that induces *IL-1α/β* in the brain (9, 30), such as hypertonic saline injection (31), insulin-induced hypoglycemia (32), foot shock stress (33), and restraint stress (34), as well as peripheral injection of IL-1α or β, induces *CRH* transcripts in the PVN (8). Furthermore, it was demonstrated that intracerebroventricular infusion of IL-1Ra attenuates the corticosterone response 24 h after tail shock stress (35) and that continuous intracerebroventricular



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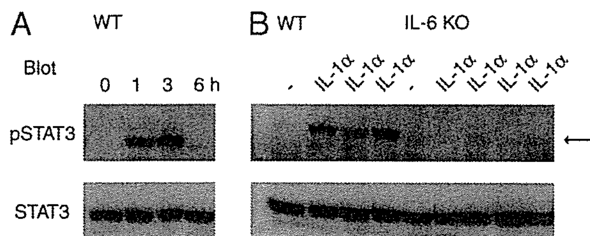


FIG. 6. Activation of STAT3 in the pituitary in response to peripheral injection of IL-1α. A, Whole cell lysates of the pituitary were prepared before (0 h), or 1, 3, and 6 h after ip injection of rmIL-1α (20 μg/kg body weight) into WT mice, and STAT3 and phosphorylated STAT3 (pSTAT3) were detected using specific antibodies on the Western blots. B, STAT3 activation in the pituitary 1 h after ip injection of rmIL-1α in WT mice and *IL-6* KO mice was examined by Western blot analysis. Each lane represents independent sample from different mice. Similar results were obtained in three independent experiments.

infusion of IL-1Ra completely prevents the rise of *CRH* mRNA in PVN observed 8 h after administration of LPS (36). These results suggest that *IL-1α/β* in the brain plays an important role in the activation of HPAA by inducing *CRH* mRNA in the PVN. Consistently with this notion, induction of *CRH* by peripherally injected IL-1α was abolished in *IL-1α/β* KO mice (Fig. 5F), indicating that endogenous brain *IL-1α/β*, which is induced by IL-1α is important for the induction of *CRH*. On the other hand, IL-1α induced *CRH* in *IL-6* KO mice, consistently with our previous observation that *IL-1α/β* is normally induced in the brain by the peripheral injection of IL-1α in *IL-6* KO mice (25). These results indicate that endogenous expression of *IL-1α/β*, but not *IL-6*, is required for the induction of *CRH* in response to IL-1α. It should be noted here that *IL-1α/β* KO mice have normal responsiveness to exogenously administered IL-1α because the induction of *c-fos* in the PVN in response to IL-1α in *IL-1α/β* KO mice was similar to that in WT mice (see supplemental Fig. 2).

We found that STAT3 activation in the pituitary after administration of IL-1α correlated with the induction of *POMC*. It is still controversial whether or not IL-1α/β directly acts on the pituitary (37, 38). On the other hand, it was suggested that IL-6 can directly stimulate the pituitary because IL-6 receptor is expressed on the pituitary, plasma corticosterone levels after bacterial LPS injection in *IL-6* KO mice are significantly lower than in WT mice, and administration of IL-6 induces ACTH release (11). Consistently with this idea, we found that peripheral injections of IL-1α induced STAT3 activation in the pituitary, which was abolished in *IL-6* KO mice (Fig. 6). Furthermore, we found that *POMC* expression in the AP was reduced in *IL-6* KO mice (Fig. 5G). Thus, it was suggested that IL-1α -induced IL-6 directly induces *POMC* in the AP through activation of STAT3. Although it was reported that *LIF* expression in the AP is important for the induction of *POMC* in response to IL-1β (39), we could not detect significant change of *LIF* expression under our experimental conditions (data not shown).

Consistently with our notion, Venihaki *et al.* (12) demonstrated that, upon turpentine injection, immunoneutralization of ACTH abolished corticosterone rise in *CRH* KO mice despite the concomitant very high circulating IL-6 levels, suggesting that ACTH, which is induced in the AP by circulating IL-6, is the major mediator for HPAA activation. However, because Bethin *et al.* (11) demonstrated that IL-6 receptor is expressed on the adrenal glands, it is possible that IL-6 directly activates adrenal glands to secrete corticosterone. Actually, we detected STAT3 activation in the adrenal glands of WT mice in response to IL-1α (Chida, D., Y. Iwakura, unpublished results). The direct effect of IL-6 on the adrenal glands may be examined in the absence of *CRH*, in which ACTH and *POMC* are not induced (12) (Fig. 5D). However, as the zona fasciculate of the adrenal gland of *CRH* KO mice is atrophic due to chronic CRH deficiency (19), the lack of corticosterone response to IL-1α in CRH KO mice does not necessarily mean that IL-1α-induced IL-6 cannot induce corticosterone response in the adrenal gland. Direct effect of IL-6 on adrenal gland might be observed if CRH were acutely deficient or adrenal size of *CRH* KO mice was restored by previous CRH or ACTH administration (40). Analysis of *ACTH receptor* (melanocortin receptor type II) KO mice should be useful to discriminate whether the effect of IL-6 on the HPAA depends on ACTH activity or not (*i.e.* direct effects of IL-6 on the adrenal glands).

Taken together, we demonstrated that endogenous IL-1α/β induction is important for prolonged activation of HPAA in response to IL-1α, and that IL-1α induces *CRH* in the PVN and also induces IL-6, both of which are independently important for the *POMC* induction in the AP.

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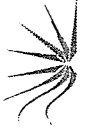

# Distinct contribution of IL-6, TNF-α, IL-1, and IL-10 to T cell–mediated spontaneous autoimmune arthritis in mice

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

## Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of unknown etiology that primarily affects the synovial membranes of multiple joints (1). A cardinal feature of joint inflammation in RA is proliferative inflammation of synovial cells, i.e., synovitis, which results in the destruction of the adjacent cartilage and bone. Although CD4$^+$ T cells are currently assumed to be the prime mediators of synovitis, it remains obscure how arthritogenic CD4$^+$ T cells activate synovial cells to proliferate or, upon activation, how the autonomous proliferation of synovial cells is maintained, leading to the destruction of the joint (1). It has been well documented that cytokines play indispensable roles in these processes (2). TNF-α and IL-6, for example, contribute to joint inflammation in RA, as illustrated by the effects of neutralizing TNF-α or blocking IL-6 receptor to ameliorate RA (3, 4). IFN-γ, IL-4, and IL-10 formed by Th1 or Th2 CD4$^+$ T cells may also participate in synovitis, as observed in other autoimmune diseases (1, 2). The way in which these cytokines contribute to the development of arthritides including RA, however, is a subject of controversy, as cytokines exert different effects in different models of arthritis. For example, IL-6 deficiency variously results in exacerbation of, amelioration of, or no effects on arthritis, depending on the particular model (5–9). Neutralization of TNF-α has different effects in collagen-induced arthritis (CIA) and streptococcal cell wall–induced arthritis (10). IFN-γ may not only mediate Th1 responses in arthritis but also suppress the destruction of cartilage and bone by inhibiting the generation of osteoclasts (11). Furthermore, it is unclear how relevant the available arthritis models are to human RA. For example, CIA, one of the most widely used models of RA, is not accompanied by the appearance of rheumatoid factor (RF), which is present in about 70% of RA patients (1). In this report, we have analyzed the contribution of pro- and anti-inflammatory cytokines to the spontaneous development and chronic progression of CD4$^+$ T cell–mediated autoimmune arthritis in a newly established mouse model of RA.

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

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

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

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

## Results

*Clinical and histological features of SKG arthritis.* In SKG mice, joint swelling began to develop in a few digits around 2 months of age, progressing to other digits and to larger joints (wrists and ankles) in a symmetrical fashion (Supplemental Figure 1; supplemental material available at http://www.jci.org/cgi/content/full/114/4/582/DC1). Histology of swollen joints showed vigorous proliferation of synovial cells, resulting in pannus formation and infiltration of mononuclear cells and neutrophils to the subsynovial region (Supplemental Figure 2). Pannus-destroyed cartilage and bone showed the appearance of multinuclear osteoclasts at the interface between invading synovial tissue and the adjacent cartilage and subchondral bone. Synoviocytes that formed a few layers of superficial lining of pannus were characteristically tall and plump and bore a large cytoplasm, as shown by H&E staining. Electron microscopy revealed that the majority of these superficial lining cells were type B fibroblast-like synoviocytes with occasional type A macrophage-like synoviocytes (Supplemental Figure 3). It also showed infiltration of lymphocytes and neutrophils to the sublining region and some synoviocytes apparently in apoptosis, as has been observed in human RA (13). In 8-month-old SKG mice, microcomputerized tomography revealed erosion of the cartilage and subchondral bone in knee joints (Supplemental Figure 4). Thus, SKG mice develop severe proliferative synovitis accompanying destruction of cartilage and subchondral bone of digits, wrists, ankles, and knees, as do humans with RA (1).

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

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

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

Immunohistochemical staining of inflamed synovial tissue of SKG mice revealed distinct staining patterns of TNF-α and IL-1β versus IL-6. Cells expressing IL-1β and TNF-α were detected almost exclusively in one or two layers of hypertrophic superficial lining cells facing the joint cavity (Figure 2, C and D). In contrast, cells expressing IL-6 were smaller than the superficial

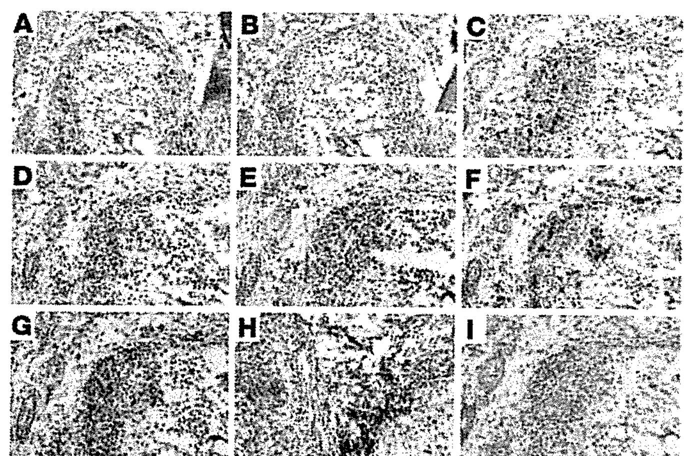


**Figure 1**
Immunohistology of synovitis. Serial sections of a finger joint of a 5-month-old SKG mouse were stained for CD4 (**A**), CD8 (**B**), B220 (**C**), Gr-1 (**D**), CD11b (**E**), I-A/I-E (**F**), CD49d (**G**), or VCAM-1 (**H**) with staining control (**I**). Arrow indicates vasculature. Original magnification, ×20.