TLR4 is present on the cell surface (22), we further examined the role of Dok-1 and Dok-2 upon the stimulation of macrophages with Pam_3CSK_4, which is an analogue of bacterial outer membrane lipoproteins and activates the MyD88-dependent pathway through a cell surface receptor, TLR2. However, Pam_3CSK_4 induced normal levels of MAP kinase activation and TNF-α production regardless of Dok-1 or Dok-2 mutation, indicating that both adaptors are dispensable to TLR2 signaling (Fig. 3 C and Fig. S2). Consistently, CpG-ODN, poly(I:C), or Pam_3CSK_4 treatment did not induce tyrosine phosphorylation of Dok-1 and Dok-2 or down-regulation of Dok-1, indicating that these adaptors are irrelevant to TLR9, TLR3, or TLR2 signaling (Fig. 3 D). Together, Dok-1 and Dok-2 are essential adaptors for the negative regulation of Erk specifically upon LPS treatment, likely because LPS, but not CpG ODN, poly(I:C), or Pam_3CSK_4, induces their tyrosine phosphorylation.

Mice lacking Dok-1 or Dok-2 are hypersensitive to LPS

Our in vitro and ex vivo findings suggest that mice lacking Dok-1 or Dok-2 are hypersensitive to LPS; therefore, we examined TNF-α production upon i.p. administration of LPS to such mutant mice. Because overproduction of TNF-α due to excessive inflammatory responses to LPS is a cause of endotoxin shock or lethality, we also examined the survival of LPS-injected mice. As expected, the serum concentration of TNF-α was increased three- to fourfold in mice lacking Dok-1 or Dok-2 as early as 1 h after injection as compared with the wild-type controls (Fig. 4 A). Consistently, the mutant mice displayed severe responses to LPS injection at a dose sublethal to the wild-type controls (Fig. 4 B). These results demonstrate that Dok-1 and Dok-2 are negative regulators of innate immunity, at least in the early inflammatory responses to LPS in vivo. Because the Dok-1 or Dok-2 deficiency did not influence TNF-α receptor–mediated activation of MAP kinases and NF-κB in peritoneal macrophages (unpublished data), such a mutation causes hypersensitivity to LPS, but not to TNF-α induced by LPS. It is of note that augmented production of NO, another cause of septic shock (23), was seen in macrophages lacking Dok-1 or Dok-2 (Fig. 1 B).

The recognition of microbial pathogens by cognate TLRs triggers the innate immune response. TLR-mediated signaling involves at least four crucial adaptors, MyD88, TRIF, TIRAP/Mal, and TRAM, having a Toll IL-1 receptor domain, which has the capability to bind an appropriate Toll IL-1 receptor domain in the cytoplasmic region of TLR(s) (24). Recent studies demonstrated that TIRAP and TRAM are essential for TLR4 to recruit MyD88 and TRIF, respectively (25–27). TLR2 also requires TIRAP to recruit MyD88. IRAK-M is a negative regulator of the MyD88-dependent pathway forming a complex with IRAK and IRAK4 to prevent phosphorylation of IRAK and its dissociation from the MyD88–TLR complex, thereby inhibiting NF-κB activation (8). MyD88s acts similarly by blocking the access of IRAK-4 to IRAK (28), and ST2 sequestrates MyD88 and TIRAP from TLR signaling (7). Although these negative regulators play important roles in LPS-mediated signaling in macrophages, there is an inevitable lag period for their induction upon LPS treatment of TLR4 as mentioned earlier. Here, we demonstrated that Dok-1 and Dok-2 are expressed at functional levels before LPS treatment and thus on standby to negatively regulate Erk immediately after the onset of TLR4 signaling. Interestingly, these adaptors are irrelevant to TLR2, TLR3, and TLR9, indicating the specificity of Dok-1 and Dok-2 to TLR4 signaling evoked by LPS, the most potent stimulator in innate immunity. Because TLR2 or TLR9 triggers the MyD88-dependent pathway and TLR3 triggers the TRIF-dependent pathway, each pathway does not suffice to induce the negative function of these adaptors. Although studies are underway to clarify the molecular basis for the Dok-1– and Dok-2–medi-

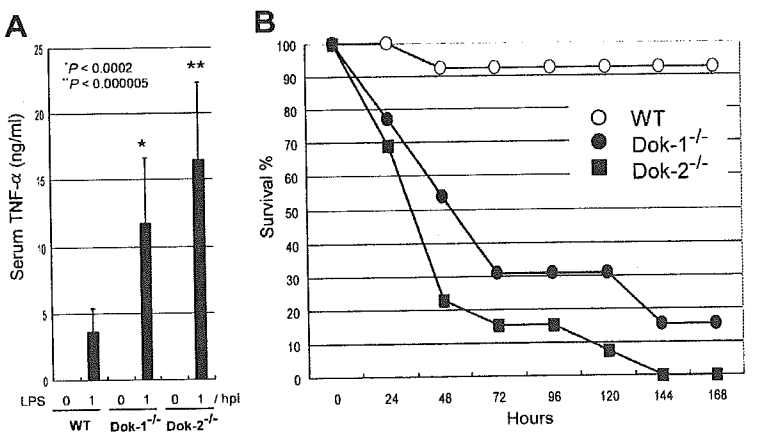

Figure 4. Mice lacking Dok-1 or Dok-2 are hypersensitive to LPS. (A) Serum concentration of TNF-α of 8-wk-old mice at 1 h after injection (1 hpi) with LPS to the peritoneal cavity or before it (0 hpi) was examined with ELISA and shown with SD (n = 7–13). The maximal p-value compared with the wild-type is indicated. (B) Mice at 8 wk of age (n = 13 for each) were injected with LPS as in A and monitored up to 7 d. Data representative of duplicate experiments are shown.

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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Interleukin-1-deficient mice exhibit high sensitivity to gut-derived sepsis caused by *Pseudomonas aeruginosa*

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Abstract

Background: The role of interleukin (IL)-1 in infectious diseases is controversial; some investigators indicated an enhancing effect of IL-1 on host resistance whereas others demonstrated the protective role of IL-1 receptor antagonist in infection. We evaluated the role of endogenous IL-1 in gut-derived sepsis caused by *Pseudomonas aeruginosa*, by comparing IL-1-deficient mice and wild-type (WT) mice.

Methods: Gut-derived sepsis was induced by intraperitoneal injection of cyclophosphamide after colonization of *P. aeruginosa* strain D4 in the intestine.

Results: The survival rate of IL-1-deficient mice was significantly lower than that of WT mice ($P < 0.01$). Bacterial counts in the liver, mesenteric lymph node and blood were significantly higher in IL-1-deficient mice than in WT mice. Tumor necrosis factor alpha and IL-6 in the liver were significantly higher in IL-1-deficient mice than in WT mice. In vitro, phagocytosis and cytokine production by macrophages were impaired in IL-1-deficient mice compared with WT mice.

Conclusion: Our results indicate a critical role for IL-1 during gut-derived *P. aeruginosa* sepsis. The results also suggest that both impairment of cytokine production and phagocytosis by macrophages are caused by IL-1 deficiency and lead to impaired host response.

Keywords: Interleukin-1; *Pseudomonas aeruginosa*; Sepsis; Bacterial translocation

1. Introduction

In spite of advances in antimicrobial therapy and supportive care of critically injured patients, sepsis develops in 750,000 individuals per year in the United States [1], with a mortality rate as high as 30–70% [2]. In this regard, sepsis due to Gram-negative bacteria is especially associated with higher mortality rate compared with sepsis caused by other type of pathogens.

Activation of inflammatory pathway, including the cytokine network, is considered to play a major role in the pathogenesis of sepsis [3]. Inhibition of interleukin (IL)-1 by IL-1 receptor antagonist (IL-1ra) improved mortality from endotoxin shock [4], whereas administration

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of recombinant IL-1 (rIL-1) increased the host resistance against infection caused by *Staphylococcus aureus* [5] and *Pseudomonas aeruginosa* [6–8]. These studies suggest that the role of IL-1 varies according to the experimental conditions.

P. aeruginosa is an important pathogen of nosocomial infections, especially in the immunocompromised host [9]. The mortality rate of *P. aeruginosa* bacteremia is still high, especially in patients with neutropenia [10–12]. Whereas clinical studies have shown that sepsis could be due to one or more microorganisms [13,14], the exact focus of sepsis could not be identified in 14–30% of patients [11,12,14]. Bacterial translocation, defined as the passage of microbes and microbial products through the intestinal barrier, also leads to sepsis, and is of clinical importance, especially in critically ill patients [15,16]. One of the important factors that promote bacterial translocation is immunosuppression induced by immunosuppressive chemotherapeutic agents [17].

We previously demonstrated that gut-derived *P. aeruginosa* sepsis could be induced by administering cyclophosphamide to mice fed *P. aeruginosa* [18–20]. In this model, bacterial translocation from the gastrointestinal tract is induced by administration of cyclophosphamide. In this model, mice ultimately develop *P. aeruginosa* septicemia. Tadros et al. [21] demonstrated that rIL-1 reduced bacterial translocation induced by burn and inoculation of lipopolysaccharide (LPS), but it was not clear whether IL-1 deficiency influences cyclophosphamide-induced bacterial translocation. The aim of this study was to determine the influence of endogenous IL-1 on host defense mechanism during gut-derived sepsis, using IL-1-deficient mice.

2. Results

2.1. Increased mortality of IL-1-deficient mice following gut-derived sepsis caused by P. aeruginosa

We evaluated the role of IL-1 on the survival of mice with gut-derived sepsis. Fig. 1 shows the survival kinetics of IL-1-deficient mice and wild-type mice. We found that IL-1-deficient mice were significantly more susceptible than wild-type mice in this model ($P < 0.01$). These results indicate that IL-1 plays a critical role during gut-derived sepsis due to *P. aeruginosa*.

2.2. Viable bacteria counts in body organs

Fig. 2 shows the number of viable bacteria in the blood, livers and MLNs of mice on the second day after the final cyclophosphamide treatment. The mean numbers of viable bacteria in body organs of IL-1-deficient mice were all significantly higher than those of wild-type mice. The results of bacterial counts in MLNs suggest the enhancement of bacterial translocation form the gastrointestinal tract of IL-1-deficient mice. Furthermore, the results of bacterial counts in the liver suggest that bacterial clearance was impaired in IL-1-deficient mice.

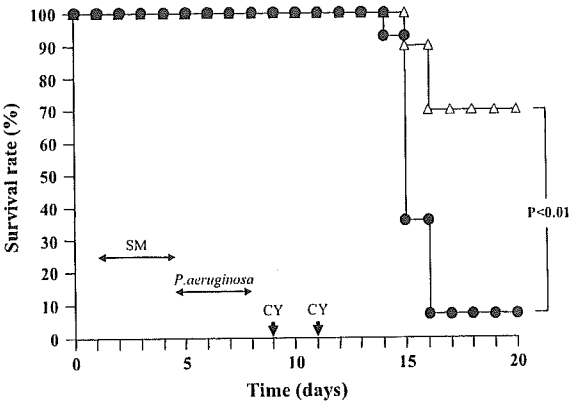

Fig. 1. Survival of mice with gut-derived sepsis caused by *P. aeruginosa*. IL-1-deficient mice ($n = 14$) and wild-type mice ($n = 10$) were treated with cyclophosphamide intraperitoneally on the indicated days (arrow). There was a significant difference between the groups ($P < 0.01$). Closed circles, IL-1-deficient mice; open triangles, wild-type mice. SM, streptomycin; CY, cyclophosphamide treatment.

2.3. Cytokines production during gut-derived sepsis

Fig. 3 shows cytokine levels in liver homogenates. TNF-α and IL-6 levels in the liver homogenates of IL-1-deficient mice were significantly higher than those of wild-type mice ($P < 0.01$). IL-10 levels in the liver of IL-1-deficient mice were also higher than those of wild-type mice, although the difference was not significant. The results suggest that the increase in inflammatory cytokines in IL-1-deficient mice reflected the severity of infection, compared with wild-type mice.

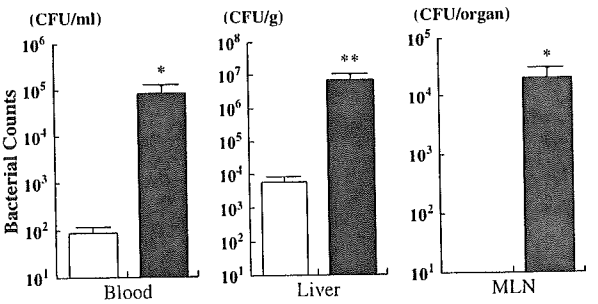

Fig. 2. Viable numbers of *P. aeruginosa* strain D4 in blood, liver and mesenteric lymph node (MLN). There was a significant difference between IL-1-deficient mice (closed columns) and wild-type mice (open columns). Data are mean ± SEM values of eight mice. Symbols: $*P < 0.05$; $**P < 0.01$.

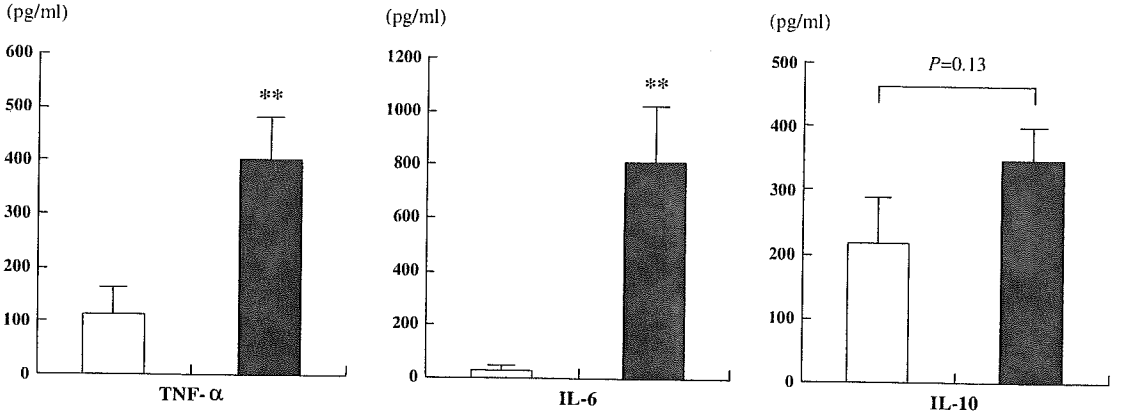

Fig. 3. Production of TNF-α, IL-6 and IL-10 in the liver. Livers were removed 2 days after the second cyclophosphamide administration. There were significant differences between IL-1-deficient mice (closed columns) and control mice (open columns) in TNF-α and IL-6. Data are mean ± SEM values of eight mice. Symbols: $^{**}P < 0.01$.

2.4. Effect of cyclophosphamide on peripheral blood leukocyte count

Leukocyte count is critical in evaluating the prognosis of gut-derived sepsis, especially in mice with cyclophosphamide-induced leukopenia. Leukocyte counts before cyclophosphamide treatment in IL-1-deficient mice and wild-type mice were 2280 ± 167 and 2117 ± 195/mm^3, respectively ($P = 0.59$). Cyclophosphamide administration resulted in a similar level of leukopenia in both group of animals (IL-1-deficient mice: 1064 ± 53, wild-type mice: 992 ± 119/mm^3, $P = 0.55$).

2.5. Role of TNF-α on survival of IL-1-deficient mice

TNF-α is another proinflammatory cytokine that plays an important role in the pathophysiology of gut-derived sepsis [19]. In the next step, we examined the role of TNF-α in IL-1-deficient mice by using anti-TNF-α MAb. In this experimental model, mice were given only 75 mg of cyclophosphamide per kg of body weight by intraperitoneal injection on days 9 and 11. Survival kinetics demonstrated that neutralization of TNF-α significantly worsened the mortality rate of IL-1-deficient mice (Fig. 4, $P < 0.01$). These results indicate that TNF-α has a crucial role in IL-1-deficient mice on gut-derived sepsis.

2.6. Impaired phagocytic activity of murine peritoneal macrophages of IL-1-deficient mice

We hypothesized that macrophage function was impaired in IL-1-deficient mice. To test this, we evaluated the phagocytic function of macrophages in vitro. The results revealed that the number of *P. aeruginosa* adhered to peritoneal macrophages of IL-1-deficient mice was significantly lower than that found in wild-type mice (Fig. 5a). Furthermore, the numbers of viable bacteria in the supernatant of macrophages culture were significantly higher in IL-1-deficient mice than in wild-type mice (Fig. 5b, $P < 0.01$). These results suggest that peritoneal macrophages of IL-1-deficient mice may show impaired phagocytosis and bactericidal activity against *P. aeruginosa*.

2.7. Downregulation of cytokine production by peritoneal macrophages after bacterial stimulation in IL-1-deficient mice

Cytokine levels were measured in the supernatant of culture media after incubation of peritoneal macrophages with bacteria in vitro. As shown Fig. 6, TNF-α,

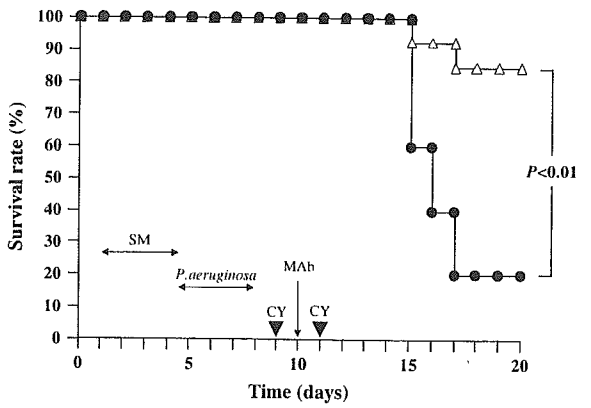

Fig. 4. Effects of anti-TNF-α MAb on gut-derived sepsis caused by *P. aeruginosa* in IL-1-deficient mice. Anti-TNF-α MAb-treated mice ($n = 10$) and control mice ($n = 13$) were administered cyclophosphamide (75 mg/kg, arrowhead) and anti-TNF-α MAb or bovine serum albumin (200 μg/mouse, arrow) intraperitoneally on the indicated days. Mice treated with TNF-α MAb were significantly more susceptible than control mice ($P < 0.01$). Closed circles; control IL-1-deficient mice, open circles; anti-TNF MAb-treated IL-1-deficient mice. SM, streptomycin; CY, cyclophosphamide treatment; MAb, anti-TNF MAb, or bovine serum albumin.

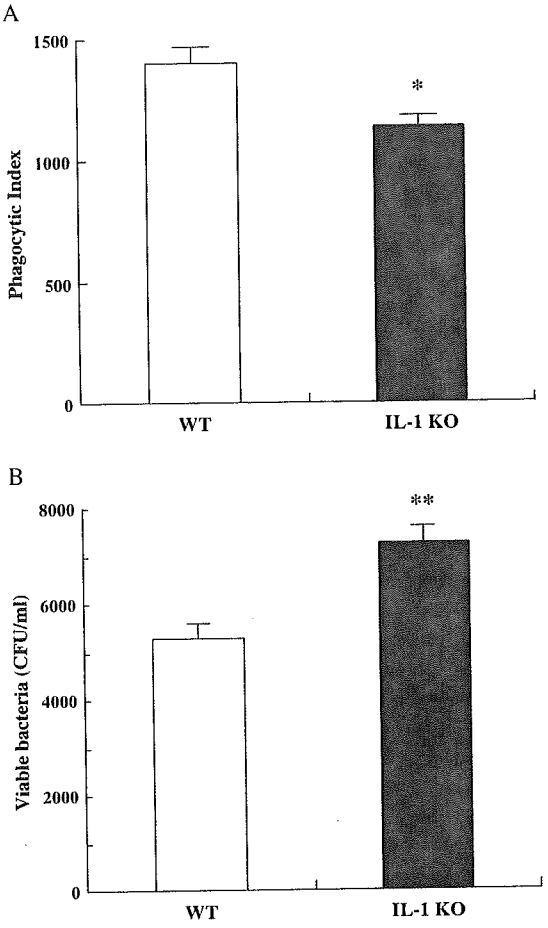

Fig. 5. (A) Number of *P. aeruginosa* D4 phagocytosed by peritoneal macrophages. Phagocytic index represents the number of bacteria per 100 macrophages. There was a significant difference between IL-1-deficient mice (closed columns) and wild-type mice (open columns). Data are mean ± SEM values of four wells. Symbols: $*P < 0.05$. (B) Viable bacteria counts in the cell culture supernatant 3 h after bacterial inoculation. There was a significant difference between IL-1-deficient mice (closed columns) and wild-type mice (open columns). Data are mean ± SEM values of four wells. Symbols: $**P < 0.01$.

IL-6 and IL-10 levels in the culture supernatant were significantly lower in IL-1-deficient mice than in wild-type mice ($P < 0.01$). Sudlow et al. demonstrated significant release of TNF from human peripheral blood mononuclear cells after stimulation with recombinant human IL-1β [22]. These results suggest impaired cytokine production by peritoneal macrophages of IL-1-deficient mice.

3. Discussion

The main mechanisms involved in promoting bacterial translocation are bacterial overgrowth, disruption of the gut mucosal barrier and impairment of host defense mechanisms [16]. Our gut-derived sepsis model, which is caused by impairment of host defense due to cyclophosphamide, resembles the clinical pathophysiology of sepsis. Therefore, in this study, we used this model to examine the role of IL-1 against *P. aeruginosa* infection.

A number of investigators have examined the role of IL-1 in bacterial infection, using IL-1 receptor-deficient mice. Hultgren et al. [23] showed that IL-1 receptor-deficient mice were more susceptible to bacterial infection and had impaired bacterial clearance during *S. aureus* bacteremia. We also found that IL-1-deficient mice were more susceptible during the gut-derived sepsis caused by *P. aeruginosa*. However, in contrast to these results, Schultz et al. [24] found significantly fewer bacteria in the lung of IL-1 receptor-deficient mice compared with those of wild-type mice, suggesting that IL-1 deficiency was associated with impaired bacterial clearance during *P. aeruginosa*-induced pneumonia. The discrepancy between the results of Schultz et al. [24] and ours may be due to differences in the site of infection and/or immunological condition of the host. First, while they studied a model of pulmonary infection, we used a model of systemic bacteremia. Second, we studied cyclophosphamide-treated immunosuppressed mice whereas Schultz et al. did not use immunosuppressive agents. In our study, we treated mice with the immunosuppressive and antimicrobial agents, such as cyclophosphamide and streptomycin, which suggest that the immunological conditions and microbial flora of the host were a long way from the normal host. Therefore, our results may indicate that IL-1 plays an important role especially in the immunocompromised host.

It is important to understand the role of each cytokine during sepsis. However, the large network of cytokines makes it difficult to understand the role of these compounds because cytokines exhibit synergistic and/or suppressive effects with and on each other. For example, we previously demonstrated that TNF and IL-1 acted synergistically to reduce the level of infection [20]. A number of investigators showed that exogenous IL-1β reduced TNF-α production [25,26], whereas others showed inhibition of IL-1 reduced [27,28], increased [25,29] or did not affect TNF-α production [30]. Our results showed that TNF-α levels in IL-1-deficient mice were significantly higher than those of control mice. Therefore, we think that the level of TNF-α might be increased in compensation in IL-1-deficient mice, or that IL-1-deficient mice might suffer a more severe sepsis than wild-type mice.

Because exogenous TNF-α causes shock and tissue injury [31], we hypothesized that elevation of TNF-α levels in IL-1-deficient mice enhanced susceptibility of mice to gut-derived sepsis. To investigate this hypothesis, we evaluated the influence of an anti-TNF-α MAb in this model and found that IL-1-deficient mice treated with anti-TNF-α MAb were more susceptible

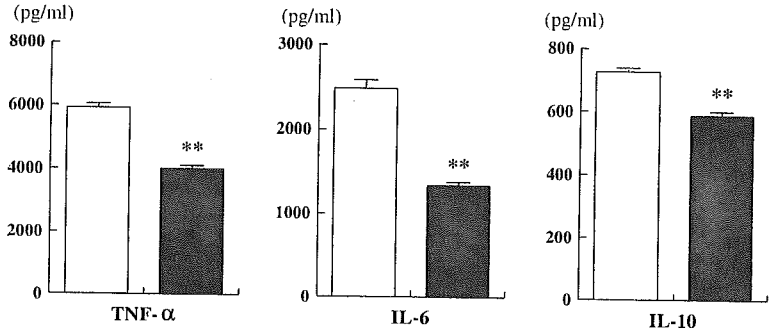

Fig. 6. Cytokines production by peritoneal macrophage 24 h after bacterial stimulation. The concentrations of TNF-α, IL-6 and IL-10 produced by peritoneal macrophages of IL-1-deficient mice (closed columns) were significantly lower than those of wild-type mice (open columns). Data are mean ± SEM values of four wells. $^{**}P < 0.01$.

than those treated with bovine serum albumin. In our preliminary study, we compared the survival rates of mice treated either with bovine serum albumin or rat IgG after the induction of gut-derived sepsis. The results revealed that there is no apparent difference in the survival between these two groups (data not shown).

Rijneveld et al. [32] demonstrated that administration of anti-TNF-α MAb in IL-1 receptor-deficient mice resulted in worsening of mortality rate during pneumococcal pneumonia. These results indicated that the high level of TNF-α play a beneficial role in IL-1-deficient mice in both Gram-positive and -negative bacterial infections.

IL-6 levels are increased in plasma, liver and intestinal mucosa in endotoxemic mice [33], and this cytokine has both pro- and anti-inflammatory properties. IL-6 activates the production of acute-phase reactants while IL-6 suppresses IL-1 and TNF-α production [34]. In addition, high IL-6 levels correlated with the severity of infectious disease and higher mortality [35–38]. Therefore, the high level of IL-6 in our study suggests that the condition of IL-1-deficient mice was more severe than wild-type mice.

Anti-inflammatory cytokines have important roles in severe infection. Souza et al. [25] reported the presence of low IL-10 levels in mice treated with inhibition of IL-1, and exogenous IL-10 had a protective effect against reperfusion-associated injury and lethality. We also showed that administration of IL-10 had a protective effect on gut-derived sepsis [18]. Therefore, we hypothesized that IL-10 levels might be decreased in IL-1-deficient mice. Contrary to our expectation, the levels of IL-10 in the liver homogenates of IL-1-deficient mice were higher than those of wild-type mice although the difference was not significant. Marchant et al. [39] demonstrated continued production of IL-10 during severe sepsis. Therefore, increased level of IL-10 may reflect a more severe condition in IL-1 deficient mice.

We investigated in vitro phagocytosis and cytokine production by using macrophages, because the function of macrophages are relatively maintained even after cyclophosphamide treatment. We found that cytokine production was impaired in macrophages from IL-1-deficient mice than control mice. Hultgren et al. [23] also demonstrated that spleen cells in IL-1 receptor-deficient mice produced less TNF and IL-6 than control mice. In addition, increased IL-6 and IL-10 productions after stimulation of IL-1 were observed in various types of cells [40–42]. Therefore, our results suggest that IL-1 may be one of the key mediators of these kinds of cytokines. We also found that phagocytosis and bacterial killing were impaired in macrophages of IL-1-deficient mice. Therefore, suppression of production of proinflammatory cytokines and impaired phagocytosis and adherence by macrophages are caused by IL-1 deficiency and lead to impaired host response.

4. Materials and methods

4.1. Animals

IL-1-deficient mice weighing 21–32 g were used in the experiments. Specific pathogen-free, IL-1-deficient mice on a BALB/c background, and corresponding control BALB/c mice (Charles River, Kanagawa, Japan, Inc.) were used. IL-1-deficient mice were produced together with mice deficient in either the IL-1α, or IL-1β genes. The IL-1-deficient mice were supported at Laboratory Animal Research Center Institute of Medical Science, University of Tokyo. The mice were born healthy, and their growth was normal [43]. The animals were housed in sterile cages and received sterile water, except during the period of oral administration of antibiotics or bacteria.

4.2. Bacterial strain

P. aeruginosa strain D4 isolated from the blood of a neutropenic mouse with bacteremia [44] was used.

The strain was maintained frozen at −80 °C in heart–brain broth containing 15% glycerol.

4.3. Reagents

Streptomycin (Meiji Seika Kaisha, Tokyo, Japan) and cyclophosphamide (Shionogi & Co., Tokyo) were purchased. Hybridoma cell lines secreting monoclonal antibody (MAb) against mouse TNF-α (MP6-XT22.11; rat immunoglobulin G1) were used [45]. MP6-XT22.11 cells were kindly provided by J.S. Abrams, DNAX Research Institute of Cellular and Molecular Biology, Palo Alto, CA. Anti-TNF-α MAb found in the ascites fluid were partially purified by 50% $(NH_4)_2SO_4$ precipitation.

4.4. Murine gut-derived sepsis with P. aeruginosa

To aid the colonization of *P. aeruginosa*, the normal intestinal flora of mice was disturbed by oral administration of streptomycin in drinking water (1 mg/ml) from days 1 to 4. *P. aeruginosa* were grown on nutrient agar (Eiken Chemical Co., Tokyo) overnight at 37 °C and suspended in sterilized 0.45% saline. Mice were given this bacterial suspension in drinking water from days 4 to 7. Mice were then given 100 mg of cyclophosphamide per kg of body weight by intraperitoneal injection on days 9 and 11. The animals were scored for mortality every 24 h for up to 14 days after the first cyclophosphamide administration.

To confirm the role of TNF-α on the survival of IL-1-deficient mice, 200 μg of anti-TNF-α MAb was injected intraperitoneally in each mouse on the next day of first administration of cyclophosphamide. Control mice received 200 μg/mouse of bovine serum albumin (Sigma Chemical Co., St. Louis, MO). Rat IgG (Sigma) was also used as the control.

The experimental protocols were approved by the Institutional Animal Care and Use Committee at the Toho University School of Medicine.

4.5. Determination of viable bacteria in organs

Mice from each group were sacrificed by inhalation of ether on the second day after the second cyclophosphamide treatment. Cardiac blood, liver and mesenteric lymph node (define MLN) samples were obtained aseptically. The liver and MLN were homogenized using tissue homogenizer (Yamato Scientific Co., Tokyo) in sterile saline. Portions of blood samples, liver and MLN were plated onto nutrient agar and cultured at 37 °C for 24 h for detection of the challenge strain of *P. aeruginosa*. The remaining blood samples were allowed to clot at 4 °C in sterile glass tubes, and then centrifuged at 10,000 × *g* for 5 min. Serum samples and the remaining liver supernatant samples were preserved at −80 °C until measurement of cytokine levels.

4.6. Role of IL-1 on phagocytic activity of murine peritoneal macrophages

The role of IL-1 on phagocytosis of *P. aeruginosa* D4 by murine peritoneal macrophages was assessed according to the previously described method [46,47]. Briefly, 5-ml of RPMI 1640 medium (Gibco Laboratories, Tokyo) were injected into the peritoneal cavity of each euthanized mice, and then the abdomen was massaged. Resident peritoneal macrophages were harvested by peritoneal lavage and resuspended at 3×10^5 cells/ml in RPMI 1640 medium containing 5% normal mouse serum in an 8-well tissue culture plate (Falcon 3047; Becton Dickinson & Co., Tokyo). After overnight culture, peritoneal macrophages were incubated with *P. aeruginosa* strain D4 (1.85×10^7 CFU/ml) at 37 °C in 5% CO_2. After 1 h incubation, the cells were washed three times with saline and were stained by the diff-quick method (diff-quick solution, International Reagents Corporation, Kobe, Japan). Numbers of bacteria that adhered to macrophages were determined by counting the bacteria at 20 macrophages in each well under light microscopy. The results were expressed as the phagocytic index (PI), which meant the numbers of the bacteria adherent to 100 macrophages. The numbers of bacteria in the supernatant of cell culture media were determined with the culture of supernatant fluids after 3 h incubation with *P. aeruginosa* (3.0×10^3 CFU/ml).

4.7. Cytokine analysis

TNF-α, IL-6 and IL-10 levels in the serum and supernatants of liver homogenates were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN). Twenty-four hours after incubation of 4.8×10^5 cells/ml of peritoneal macrophages with 3.0×10^3 CFU/ml of *P. aeruginosa*, the supernatant of the culture were collected. TNF-α, IL-6 and IL-10 levels in the supernatants were determined with ELISA assay.

4.8. Statistical analysis

Differences in the survival rates of mice groups were evaluated by the chi-square test. Viable bacterial counts in different organs, cytokine concentrations, and number of the bacteria in macrophages were compared by the Mann–Whitney *U* test. A probability level of 5% was considered significant.

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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/O1a×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-1β 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-1β 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-α 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-μm 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 μm) 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-1α monoclonal antibody, hamster anti-mouse IL-1β monoclonal antibody, and polyclonal goat anti-mouse TNF-α antibody (all from Genzyme) were used as capture antibodies. Polyclonal rabbit anti-mouse IL-1α, polyclonal rabbit anti-mouse IL-1β, and polyclonal biotinylated goat anti-mouse TNF-α 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.

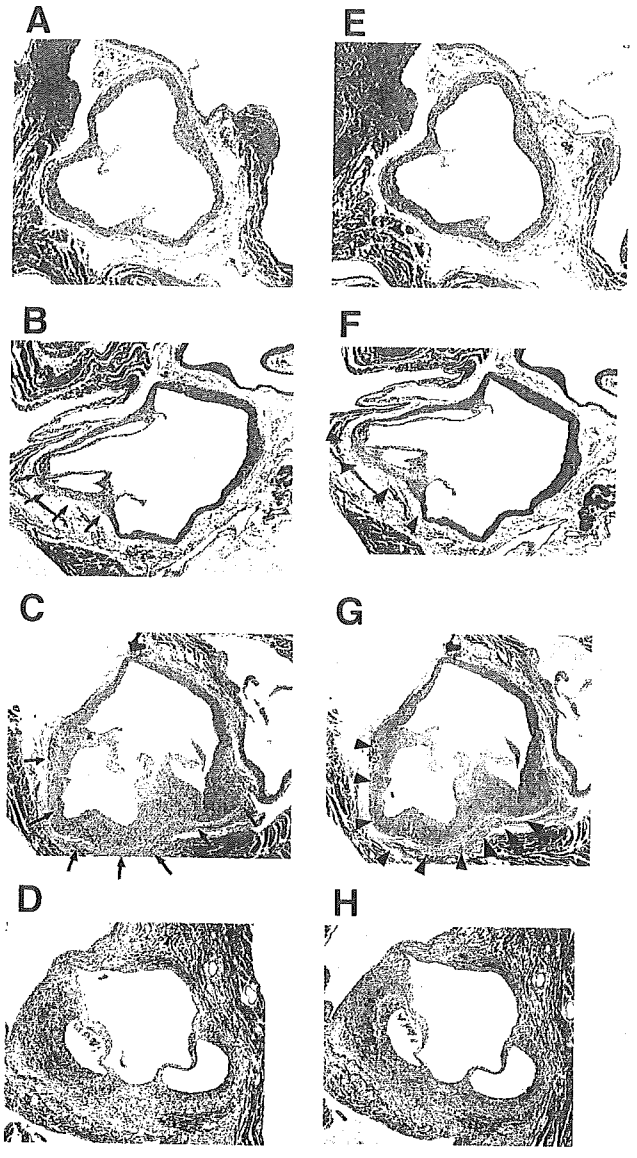

Figure 1. Arterial inflammation around the aortic sinus in IL-1Ra$^{-/-}$ mice. A and E, Normal sections of the aortic valve (score 0) from an 8-week-old, wild-type female mouse. B and F, Mild inflammatory cell infiltration and loss of elastic lamellae over less than one third of the media of the aortic 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 (2×10^7 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,

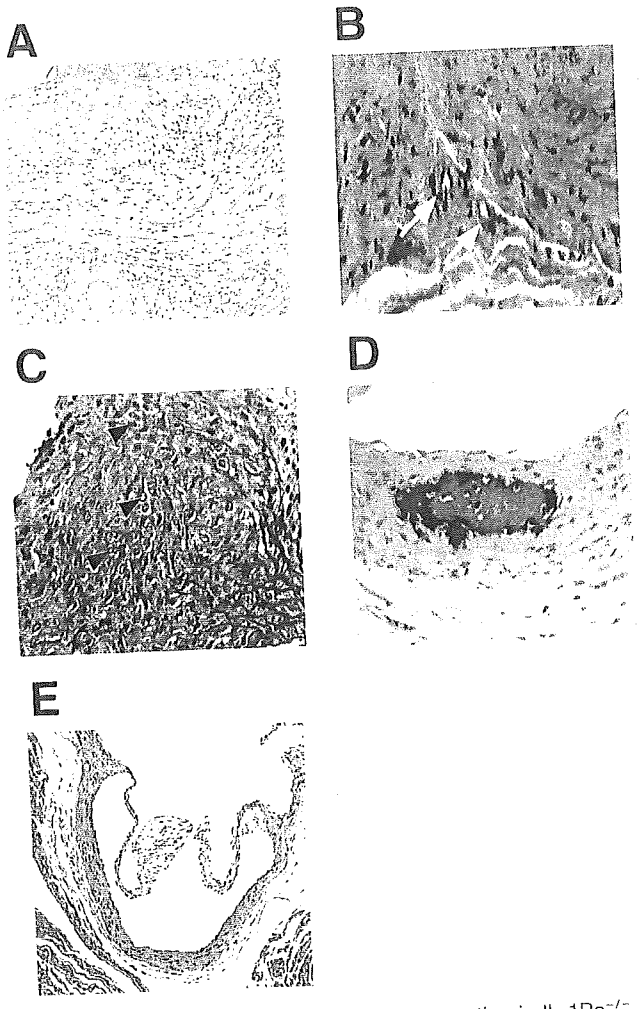

Figure 2. Characterization of arterial inflammation in IL-1Ra$^{-/-}$ mice. A, Inflammatory cell infiltration in the media and adventitia in a 12-week-old IL-1Ra$^{-/-}$ female mouse. B, Formation of microvessels (white arrows) in a section from an 8-week-old IL-1Ra$^{-/-}$ male mouse. C, Chondrocyte-like 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.

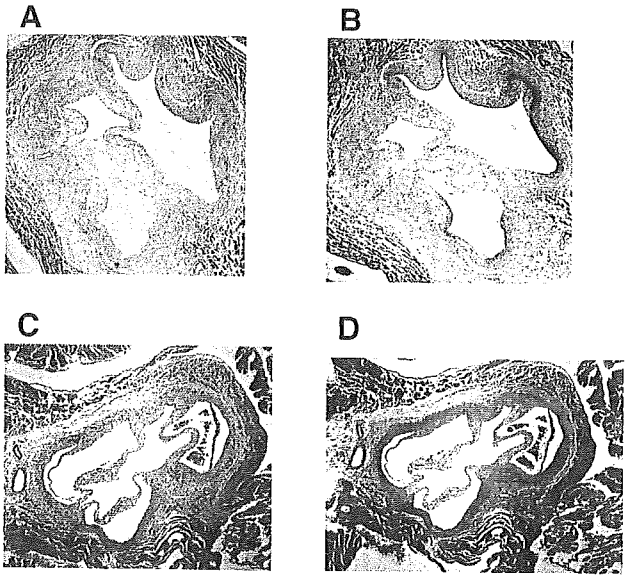

Figure 3. Induction of aortitis by transplantation of IL-1Ra$^{-/-}$ peripheral T cells and BM cells. A and B, Sections from nu/nu female mice 10 weeks after transplantation of T cells from IL-1Ra$^{-/-}$ mice (score 3). C and D, Sections from a wild-type, female mice 12 weeks after transplantation of 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.

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-α protein levels in the blood were slightly higher than in wild-type mice, whereas the levels of IL-1α, IL-1β, 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-α 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/O1a×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.

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]

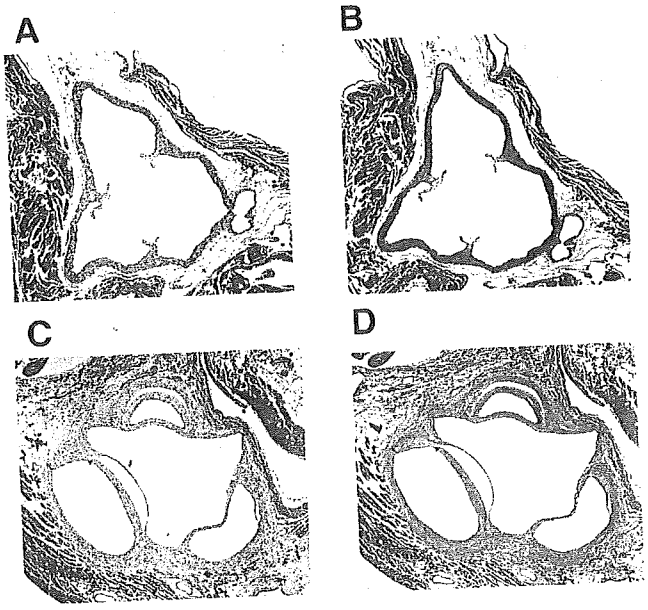

Figure 4. Complete suppression of the development of aortitis in TNF-α–deficient, IL-1Ra$^{-/-}$ mice. Sections of the aortic valves from TNF-α$^{-/-}$–IL-1Ra$^{-/-}$ female mice (14 weeks old, score 0) (A and B) and IL-6$^{-/-}$–IL-1Ra$^{-/-}$ female mice (8 weeks old, score 3) (C and D). Hematoxylin 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-α$^{-/-}$ 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-α$^{+/+}$, IL-1Ra$^{-/-}$ (14)	5/9 (55)	2
TNF-α$^{-/-}$, 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.

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

Cytokine Level, pg/mL	Wild Type	IL-1Ra$^{-/-}$	P
IL-1α	15.9±7.7	14.9±4.0	0.891
IL-1β	28.9±17.3	47.6±27.6	0.230
TNF-α	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.