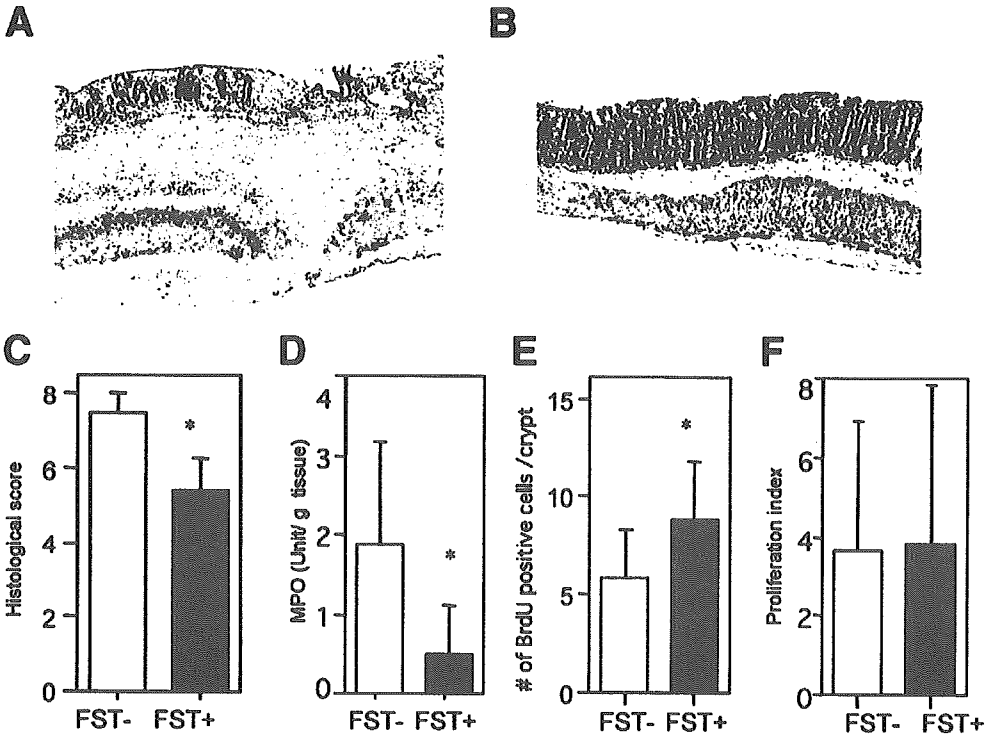

Figure 4. Effects of follistatin on established TNBS colitis. TNBS (48 μg/g) was administered on days 0 and 7, and then follistatin was injected or not on day 8 (*A* and *B*). H&E staining of frozen sections prepared from the colon of untreated (*A*) or follistatin-treated (*B*) mice with colitis. (*C*) Histologic score. (*D*) MPO assay for the total colon. FST-, control group; FST+, follistatin-treated group. (*E*) Numbers of BrdU-positive cells on day 10 are shown. The results shown are the average of 7 mice for each group with 1 standard deviation. (*F*) T-cell proliferation assay. *The differences between the groups with (*solid column*) or without (*open column*) follistatin were statistically significant ($P < .05$).

Follistatin Ameliorates Established Colitis

In our next experiments, we tested whether follistatin would affect established TNBS colitis. To do this, we injected follistatin IP on day 8 and then examined the tissue on day 10. Histologic scores revealed that the frequency of ulcer formation was lower in the follistatin-treated than in the control mice (Figure 4*C*), and the MPO activity was also lower in the former (Figure 4*D*). However, this treatment did not reduce the mucosal mononuclear cell infiltration, which clearly indicates a chronic inflammatory response (Figure 4*A* and *B*). On day 10, we examined the TNP-specific T-cell proliferative responses of sacral lymph nodes, which are the draining lymph nodes of the mouse colon. Unexpectedly, in the follistatin-treated mice, these responses were comparable with those seen in the control mouse group (Figure 4*F*). This finding indicates that treatment with follistatin suppressed ulcer formation and accumulation of neutrophils but did not decrease TNP-specific T-cell responses.

In this regimen, increased numbers of proliferating epithelial cells and well-preserved crypts were seen in the treated group (Figure 4*E*). These results clearly suggest that follistatin improves barrier functions through repair of epithelial cells damaged by infiltrating neutrophils; however, the repeated TNBS doses still induced TNBS-specific T-cell responses.

DSS colitis is a model for chronic colitis. Because damage to epithelial cells consistently occurs by the continuous ingestion of DSS in the drinking water, we next used this DSS model to evaluate the anti-colitis effects of follistatin. In our experiments, colitis was first established by allowing the mice to consume for 7 days DSS added to their drinking water. At this time point, follistatin was administered on days 7, 9, and 11 with continuous intake of DSS.

Administration of follistatin reduced the shortening of the colon (Figure 5*A* and Table 1), as well as the scores for stool consistency and rectal bleeding (Table 1). Colonic crypts were well preserved in the upper and middle colon in the mice treated with follistatin (Figure 5*B*), and proliferating BrdU-positive cells were more frequent (Figure 5*C*). The lower colon was the most affected in DSS colitis, and treatment with follistatin together with administration of DSS in the drinking water failed to improve the histologic score in the lower colon. None-

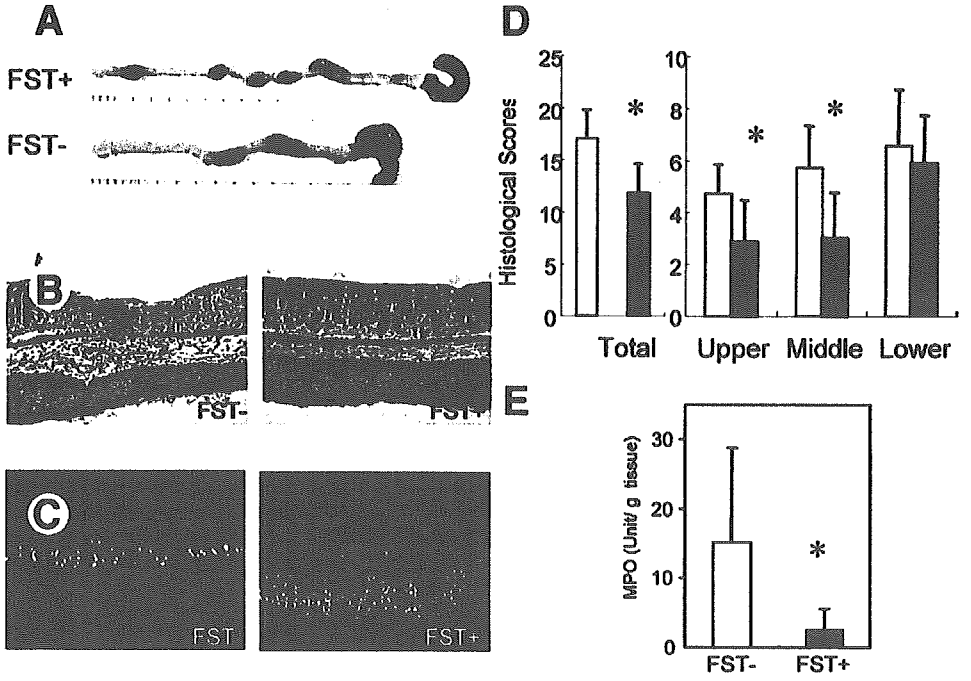

Figure 5. Follistatin treatment of established DSS colitis. (*A*) Macroscopic appearance of the colon on day 12 from mice with (FST+) or without (FST-) administration of follistatin. (*B*) Histologic features of the midcolon. (*C*) Typical staining with anti-BrdU Ab of the midcolon from mice treated with follistatin (right) or not (left). (*D*) Histologic scores of DSS colitis. Scores for upper, middle, and lower colon were determined separately. Total scores are the sum of those for all 3 parts. (*E*) MPO assay for the total colon. *The difference between the groups with (*solid column*) or without (*open column*) follistatin treatment was statistically significant ($P < .05$).

theless, a curative effect on the upper and middle colon was obvious after histologic inspection (Figure 5*B* and *D*). The MPO activity was also lower in the treated mice (Figure 5*E*).

Follistatin Promotes Colonic Epithelial Cell Division

To this point, our study revealed that neutralization of activin by follistatin efficiently suppresses colonic inflammation. However, evidence to suggest that activins are the actual inflammatory effector molecules remained to be established. To our knowledge, no past studies reporting up-regulation of activins have yet established a molecular mechanism involving them as a direct inflammatory mediator. Thus, the precise mechanism underlying the anti-inflammatory effect of follistatin has not yet been established. Although we found that LPS stimulation of peritoneal Mϕ in vitro induced a marked secretion of activin A (data not shown), the addition of exogenous activin A to resting peritoneal Mϕ or to colonic LPMCs did not alter their potential to produce inflammatory cytokines such as TNF-α (data not shown). Thus, we could not identify any direct proinflammatory effect of activins on Mϕ-type cells. On the other hand, previous studies showed that activin A inhibited DNA synthesis and enhanced differentiation of an epithelial cell line.[22]

Table 1. Treatment of DSS Colitis With Follistatin

Parameters assessed	Control (n = 7)	Treated with follistatin (n = 7)
Length of the colon (cm) at day 12	7.5 ± 0.4	8.3 ± 0.7[a]
Score for stool consistency at day 12	2.86 ± 1.07	1.14 ± 1.07[a]
Score for rectal bleeding at day 12	3.43 ± 0.98	2.00 ± 0.00[a]

NOTE. Values are mean ± standard deviation.
[a]The differences from the nontreated control are statistically significant ($P < .05$).

In the GI tract, rapid turnover of epithelial cells is essential for the maintenance of homeostasis. Because an increased number of proliferating cells accompanied the amelioration of colitis in the follistatin-treated group, we further investigated the effect of activin A and follistatin on epithelial cell proliferation. First, localization of activin receptor was examined by immunohistochemistry. Activin receptor IIB was detected in the colonic epithelium and vascular endothelial cells (Figure 6*A*). In the MCE301 mouse colonic cell line, addition of exogenous activin A tended to suppress cell growth in every repeated experiment; however, the difference was not always statistically significant (Figure 6*B*). Because we

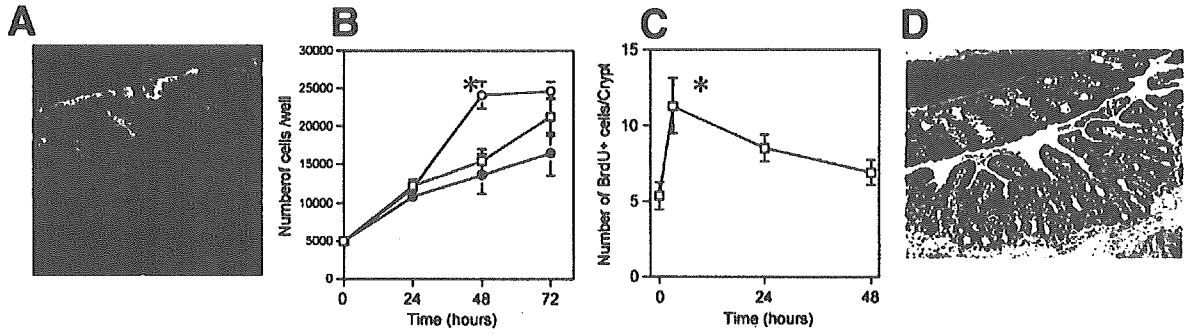

Figure 6. Follistatin promotes colonic epithelial cell division. (*A*) A frozen section prepared from the colon of a naïve mouse was stained with anti-activin receptor IIB antibody. (*B*) MCE301 cells (5000 cells/well) were placed in 96-well plates, and, after 24 hours, 50 ng/mL of activin A (*solid circles*) or 600 ng/mL of follistatin (*open circles*) was added. Numbers of living cells were counted by the dye exclusion technique at the indicated times after addition of the agents. *Open squares* indicate control cultures. Each datum point represents the average and standard deviation of values obtained from quadruplicate wells. These data are representative of 3 independent experiments that gave the same results. *The differences from controls (without treatment) were statistically significant ($P < .05$). (*C*) Five micrograms of follistatin was given i.p. to mice. After 3, 24, and 48 hours, the middle one-third of the colon was obtained, and frozen sections were then prepared. After staining with anti-BrdU antibody, the number of BrdU-positive cells per crypt was counted. Twenty crypts from 4 individual mice were examined for each time point. *The difference from time 0 (without follistatin) was statistically significant ($P < .05$). (*D*) Hyperplastic changes in the recovery phase of DSS colitis during treatment with follistatin. H&E stain of a section of middle colon is shown,

noted that treatment with activin A caused up-regulation of the mRNA for follistatin as well as for activin βA, activin βB, and activin receptors IIA and IIB in this cell line (data not shown), the effects of activin A may have been partially neutralized by the follistatin present. Treatment with exogenous follistatin did not affect the mRNA levels for activin βA, activin βB, follistatin, or activin receptors (data not shown). Therefore, to neutralize activins, we added 300 ng/mL follistatin to the cultures, which resulted in a significant increase in cell proliferation at 48 hours (Figure 6*B*).

To examine this effect in vivo, we gave follistatin i.p. to naïve mice and evaluated subsequent epithelial cell division. In the colon, an approximately 2-fold increase in the number of BrdU-positive epithelial cells was seen by 3 hours, and this effect was maintained up to 24 hours (Figure 6*C*). These effects of follistatin indicate the presence of biologically active endogenous activins in the colon that suppress colonic epithelial cell proliferation. The mRNA for follistatin did not parallel the up-regulation of activins (Figure 2*A*), and therefore excess biologically active activins during inflammatory conditions may have caused the delay in epithelial cell proliferation.

Because a cell division-promoting effect of follistatin was evident, this action may also affect normal tissue repair. To address this point, we examined whether follistatin would also affect the healing process in DSS colitis. Severe colitis was established by having the mice consume 5% DSS in their drinking water for 8 days. On days 7, 9, and 11, follistatin was given i.p., and histologic examination was then performed at day 14. At this time point, mice treated with follistatin showed hyperplasia of colonic crypts; however, this finding was not focal but diffuse, and no atypia of nuclei was seen (Figure 6*D*). The deformity of crypts in the follistatin-treated mice was to the same extent as that seen in untreated mice. We conclude that follistatin does not induce aberrant epithelial cell regeneration over the short term. Long-term effects of follistatin are currently under active investigation.

Treatment of Chronic Colitis in IL-10$^{-/-}$ Mice With Follistatin

Next, we tested the effect of follistatin on chronic spontaneous colitis in IL-10$^{-/-}$ mice. Eighteen-week-old IL-10$^{-/-}$ mice kept under conventional environmental conditions for 7 weeks manifested diarrhea but remained stable when the treatment was started. After 4 doses of follistatin, the body weight was not different between the 2 groups (Figure 7*A*); nevertheless, the overall wet weight of the colon was lower, although not significantly, in the follistatin-treated group (Figure 7*B*). When the thickness of the colonic mucosal layer was measured, it was much reduced after the follistatin treatment (Figure 7*C*). Both groups showed severe cell infiltration in the colon; however, we noted that crypt abscesses were more frequent in untreated mice than in the follistatin-treated ones (Figure 7*F* and *G*). Although the treated group had shorter crypts, their epithelial cells were well preserved. The mRNA levels for IFN-γ of the treated mice were significantly lower than those of the untreated mice (Figure 7*D*). Also, the sacral lymph nodes in the untreated mice were very large. In contrast, those in the treated mice were significantly smaller (Figure

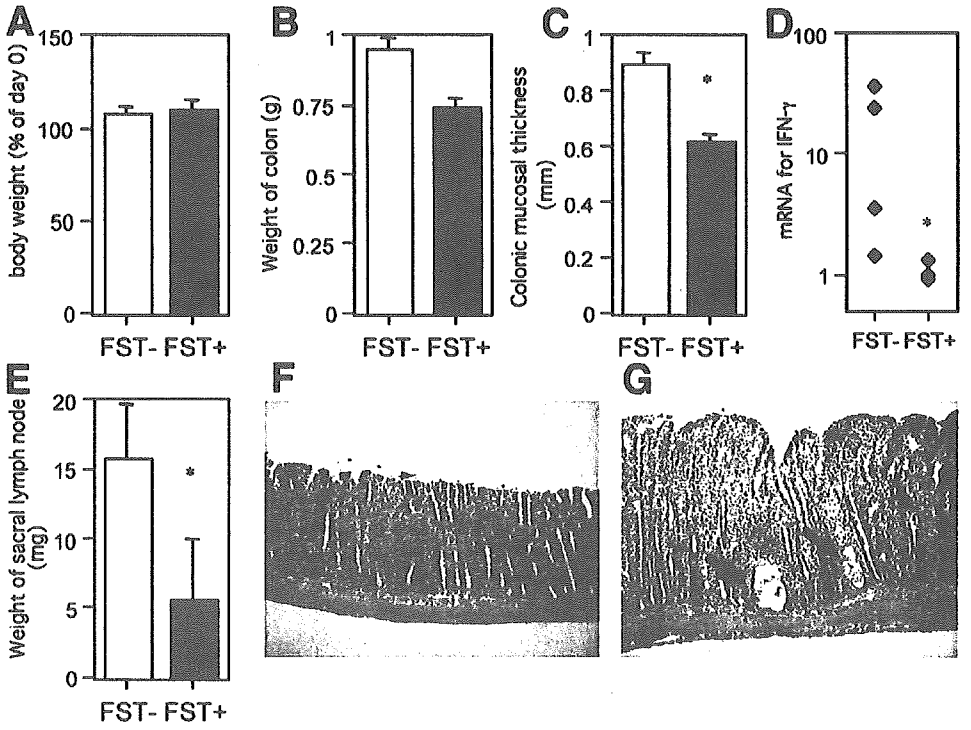

Figure 7. Effect of follistatin on colitis in IL-10$^{-/-}$ mice. IL-10$^{-/-}$ mice, which had shown signs of chronic colitis for 7 weeks, were treated with follistatin. (*A*) Changes in body weight of mice with (FST+, n = 4) or without (FST-, n = 4) administration of follistatin. (*B*) Wet weight of the colon. (*C*) Colonic mucosal thickness was measured with a micrometer under microscopic observation at 5 points of the middle and lower colon in each mouse. (*D*) Messenger RNA levels of IFN-γ. Total RNA was extracted from the colon and analyzed by quantitative RT-PCR. The results from 4 mice/group are shown as relative expression levels. (*E*) Wet weight of sacral lymph nodes. (*F*) Histologic features of the midcolon of follistatin-treated mice and untreated mice (*G*). *Arrows* indicate crypt abscesses. *The differences between the groups with or without follistatin were statistically significant ($P < .05$).

7*E*). Twelve days after the start of treatment, transient or continuous anal prolapse was seen in all untreated mice but was absent in 3 of the 4 follistatin-treated mice. Thus, although inflammation essentially continued in both groups of IL-10$^{-/-}$ mice, the treatment with follistatin showed some beneficial effects on the chronic colitis. Another set of experiments using 8-week-old mice (n = 4 for each group) that had been kept in the conventional environment for 2 weeks gave similar results, although the inflammatory changes were much milder in both control and follistatin-treated groups.

Colonic Epithelial Cells Produce Inflammatory Chemokines in the Presence of Activin A

To investigate further activin A as an inflammatory mediator, we next tested its effect on the production of chemokines by epithelial cells. The addition of a high concentration of activin A induced basal secretion of MCP-1 in the MCE301 cell line (Figure 8*A*); however, apical secretion of MCP-1 by this cell line was not affected by the addition of the activin (not shown). In the case of human colonic cell line HT29, the addition of activin A induced secretion of IL-8 into the culture supernatant in a dose-dependent manner (Figure 8*B*). This effect was inhibited by the presence of follistatin (Figure 8*C*).

Discussion

The rapid and dynamic turnover of intestinal epithelial cells is one of the most important events for maintaining homeostasis in the GI tract. In this study, we found that during colitis, both local and systemic up-regulation of activin occurred. Furthermore, neutralization of activin by follistatin effectively suppressed the associated inflammatory response. We also showed that activins acted by suppressing epithelial cell division and enhancing the production of proinflammatory chemokines.

The activin/follistatin system is known to be involved in tissue regenerative processes.[12,23] Neutralization of activin in rats by follistatin promoted regeneration of hepatocytes after partial hepatectomy[21,25] and tubular

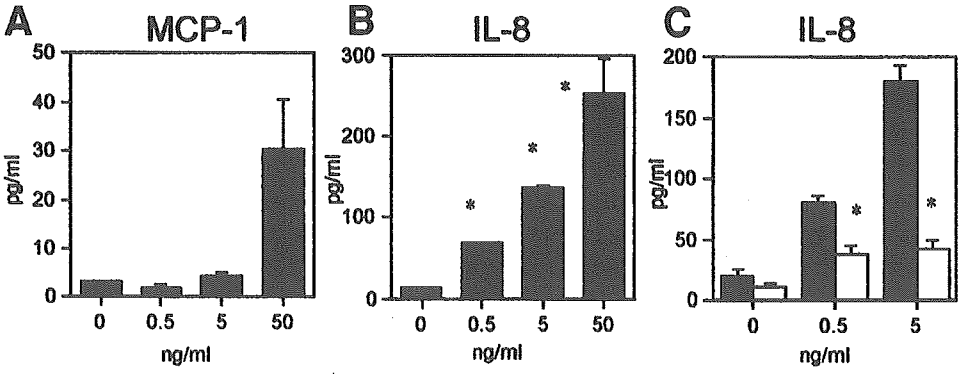

Figure 8. High concentrations of activin A enhanced production of chemokines from colonic epithelial cell lines. (*A*) The mouse colonic epithelial cell line MCE301 was cultured on a collagen-coated membrane insert set in each well of a 24-well plate and then allowed to form a monolayer and become polarized. At that point, the indicated concentrations of activin A were added to the basal side of the cell monolayers. After 24 hours, MCP-1 released into the basal side of the culture medium was measured. (*B*) Human colonic cell line HT 29 was cultured in 48-well plates, and activin A was subsequently added. IL-8 released into the culture supernatant was then measured after 24 hours. *The difference from control (without activin A) was statistically significant ($P < .05$). (*C*) HT 29 cells were cultured with the indicated concentrations of activin A in the presence (*open column*) or absence (*solid column*) of 100 ng/mL of follistatin. IL-8 released into the culture supernatant was then measured after 24 hours. *The difference from the control (without follistatin) was statistically significant ($P < .05$).

epithelial regeneration after renal ischemia.[6] However, the functional significance of activin in the intestinal epithelium had not been studied in detail until now. A recent report based on an in vitro study showed that activin A inhibited cell proliferation in the rat intestinal cell line IEC-6.[10] We also found presently that autocrine activins produced by a mouse colonic cell line in vitro suppressed cell growth, as evidenced by the neutralizing effect of follistatin. Furthermore, our current results clearly show that endogenous activins in vivo indeed regulated colonic epithelial cell turnover by suppressing proliferation under both steady state and inflammatory conditions. It should be noted that LPMCs, as well as epithelial cells, are a source of activin A in the colon. Messenger RNA for activins βA and βB were detected in LPMCs, and the adherent cell population of the LPMCs secreted a considerable amount of activin A in the steady state (Figure 2*D*). In previous studies on human specimens, activin mRNA and its protein were only detected in inflamed tissues.[10,11] This finding may indicate a different action of activins in the steady state between mice and humans. Thus, in humans, activin A may be expressed in an inflammation-specific manner. Alternatively, the methods used in the previous study may not have allowed detection of activin during the steady state. On the other hand, up-regulation of activins is a common feature of the inflamed GI tissues of both humans and mice. For example, production of activins was dramatically up-regulated in the acute phase of TNBS colitis in mice. By in situ hybridization, we noted high expression of activin βA in epithelial cells, infiltrating cells, and endothelial cells. RT-PCR analysis of separated LPMCs showed that mRNA levels were up-regulated in Mϕ-type cells. We also found that the addition of TNF-α or activin A to the colonic cell line MCE301 resulted in up-regulation of the mRNAs for activin βA, activin βB, and activin receptor IIB. Furthermore, when peritoneal and splenic Mϕ were stimulated with LPS, marked secretion of activin A was found (data not shown).

As shown previously, TNBS colitis in mice was characterized by increased levels of inflammatory cytokines and erosion of the epithelium.[16,17] This could lead to bacteremia in the acute phase of colitis. Thus, there appears to be an overall up-regulation system for the production of activins by epithelial cells and Mϕ-type cells of the inflamed colon, both locally as well as systemically. That is, that activins are produced not only by inflammatory Mϕ recruited to the colon but also by peritoneal and circulating Mϕ that had been activated by bacterial components that had passed through the disrupted mucosal barrier during inflammation. This rapid series of events resulted in the presence of high levels of activin A in the colon by day 1 and in the plasma by day 3.

From the results of the present study and previous ones, it is clear that activins and follistatin are up-regulated following stimulation by various inflammatory stimuli.[11,12] For example, activin βA secretion was induced after in vivo LPS challenge in sheep[26] and in human monocytes and Mϕ stimulated with LPS.[27] In the inflammatory state, activin was present in local infiltrating cells in IBD[14] and in cells associated with pulmonary fibrosis.[28] Thus, it is logical to have assumed that neutralization of activin with follistatin would have an anti-

inflammatory effect. However, the biologic impact of up-regulated activin during inflammation had never been investigated until presently. Previous studies showed that activin has both proinflammatory and anti-inflammatory effects. For example, in rat bone marrow-derived Mϕ, high levels of activin promoted proinflammatory responses such as the induction of prostanoid, nitric oxide, and cytokines.[29] On the other hand, activin A inhibited the production of IL-1β, a potent proinflammatory cytokine in studies using human monocytic cell lines THP-1 and U-937.[30] Activin A also exhibited the potential to antagonize the multiple effects of IL-6[31]: In experiments using thymocytes, activin A modulated the action of IL-6; however, its effect was divergent and dependent on the experimental conditions used. When we examined the effects of exogenous activin A on resting peritoneal Mϕ, treatment with activin A alone did not affect the production of TNF-α, and IL-6 secretion was only modestly enhanced. Thus, we could not detect major effects of activins on Mϕ. These results suggest that Mϕ themselves are a source of activin during inflammation; however, this cell type is not likely to be the major target of activin A. Instead, epithelial cells appear to be the most important target of activins. In fact, by immunohistologic analysis, activin receptor IIB was found mainly on colonic epithelial and endothelial cells. Furthermore, we found that activin A acted on epithelial cells by inhibiting cell division and enhancing the secretion of the proinflammatory chemokines MCP-1 and IL-8.

Previous studies, after in vivo challenge with LPS, had shown that up-regulation of activins occurred first, followed by that of follistatin.[26] In our study, enhanced activin production was seen during the acute phase of TNBS colitis, and levels of follistatin did not parallel the changes in activins. This finding indicates that excess amounts of biologically active activins are present in the inflamed colon and explains why follistatin is so effective in reducing colitis. The amelioration of colitis through administration of follistatin was accompanied by suppression of plasma levels of amyloid A and IL-6. We consider that rapid mucosal repair through promotion of epithelial cell division prevented the erosive type of mucosal lesion and translocation of bacteria and their components during TNBS colitis. Follistatin was also effective in treating ongoing epithelial damage. In DSS colitis, follistatin was administered after the establishment of colitis, and the DSS was continually given during treatment. Cell infiltration and depletion of the crypts are typical histologic findings in DSS colitis. In mice treated with follistatin, cellular infiltration was observed; however, the epithelial cells were less damaged when compared with those in the untreated group. In the case of TNBS colitis, treatment with follistatin ameliorated the inflammatory responses but did not affect TNBS-specific T-cell responses in the sacral lymph nodes, obtained 3 days after the second dose of TNBS had been administered. This finding also indicates that the major target of the activin/follistatin system was the colonic epithelium.

In our studies on IL-$10^{-/-}$ mice, the administration of follistatin 4 times, once every 3 days, reduced the frequency of anal prolapse and the size of the sacral lymph nodes. Crypt abscesses were less frequent in the follistatin-treated group than in the controls. We interpret these results to indicate that improved barrier function of the epithelium reduced exposure to nonspecific luminal bacteria and their translocation into lymph nodes and then reduced the responses in the sacral lymph nodes.

In addition to these findings, the administration of follistatin may have a systemic effect to improve the conditions surrounding colitis. Increases in plasma activin levels were related to a wasting disease and a cachexia-like syndrome in inhibin-deficient mice.[32,33] Furthermore, follistatin improved this cachexia-like syndrome. In addition, activins were found to be expressed in the hypothalamus, and injection of activin A into the third ventricle resulted in decreased food and water intake.[31] Although it is not known whether activins and follistatin penetrate the blood-brain barrier, their functions as hormones or as neurotransmitters may be involved in the anticolitis effects of follistatin in vivo as well.

In summary, inhibition of activin by follistatin efficiently prevented hapten-induced colitis. Furthermore, follistatin was effective in treating already established colitis in the DSS colitis model, a system that emphasizes epithelial cell damage, and colitis in IL-$10^{-/-}$ mice, which is mediated by T cells. Our results also indicate that promotion of epithelial cell regeneration can be a significant strategy for treating intestinal inflammation. Because follistatin is a natural inhibitor of activin, we speculate that it may be applied to human diseases including IBD.

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Modeling on Social Spread from Immunity

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SUMMARY: We are now planning to make a transmission model of infectious diseases in the scale of a city. People live in the city contacting other persons with daily life. The model regards a contact as a source of infection. A person will be simulated as a simple system of differential equations. As a candidate of differential equations, we are now investigating Marchuk's simple model. We adopt Marchuk's simple model because it has formation time, i.e., latent time. As Dr. Takeuchi showed, latent time is very important. There remain problems of choosing parameters for special diseases. We are now planning to use Marquardt method to minimize residuals form clinical data to estimate parameters. As for contacts, there are many approaches. The approach of the MIDAS project is very intensive. Our approach is simple. There are about 30,000 Japanese every fifteen minutes daily life data, sleeping, eating, work, study, house keeping, etc. Our approach is to make virtual families, husband, wife, children in a city and assign actions from the every fifteen minutes data statistically and estimate their contacts in the companies or schools, etc.

We simulated the spread of infectious disease based on the contact model of Japanese people. We also simulated an immune response of a person to get the parameters for the model of contacts of people. When a cold or influenza prevails in winter, schools are shut down in Japan. We investigated the effect of this strategy by simulations of our model.

Immune response

We simulated an immune response of a person as a system of differential equations using Marchuk's simple model (1) shown below. We adopt Marchuk's simple model, because it has formation time, i.e., latent time, one of key factor of infection.

$$\frac{d}{dt}\begin{pmatrix} V \\ C \\ F \\ m \end{pmatrix} = \begin{pmatrix} (\beta - \gamma F)V \\ \xi(m)\alpha V(t-\tau)F(t-\tau) - \mu_c(C - C^*) \\ \rho C - (\mu_f + \eta\gamma V)F \\ \sigma V - \mu_m m \end{pmatrix}$$

where V(t): concentration of pathogenic organ, F(t): concentration of antibodies, C(t): concentration of plasma cells, m(t): relative characteristic of affected organ, β: multiplication, γ: neutralized, τ: formation time, α: antigen-antibody collision *: constant level, μ: life time, ρ: production, η: efficiency, ξ: function of m.

We set 3 days as the parameter of latent time after our experience. The remains of parameters are decided referring a study of pneumonia (2). We used Runge-Kutta method to simulate this delayed differential equations. Period of infection is also an important parameter to make a model. We decided the parameter by the simulation of an immune response. We set 7 days as the period of infection based on the simulations and this coincides with our experiences.

Contact model

People live their lives contacting other persons and infectious diseases spread by contacts. As for infection by contacts, there are many approaches (3-6). The MIDAS project employed a very intensive agent technology to model contacts (7). We used a statistics of Japanese daily life. The statistics is about every 15 minutes actions of daily life of 30,000 Japanese, such as sleeping, eating, work, study, house keeping, etc. (8). Our approach is to make virtual families, i.e., husbands, wives, children in a city, assign their behavior by Monte Carlo method according to the statistics and estimate their contacts in the companies or schools, etc.

Simulation

We assumed 1,000 persons living in a small city and infection spread from a specified family consists of three persons. As for infection, latent time is 3 days and people get infected for 7days and recovered. Infection is not severe, so infected people do not change their behaviors. Infection rate of simulation is assumed as 1%, 10%, 20% and 50%.

In the case of 1% infection prevailed for the longest and the total number of infected people is not so small compared

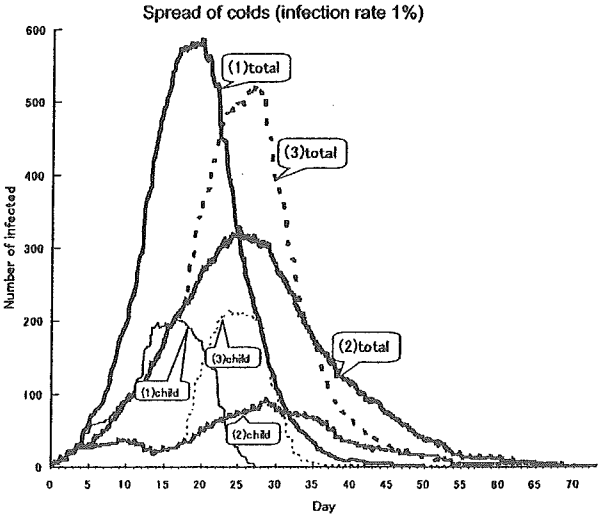

Fig. 1. Number of infected persons in case of infection rate is 1%. (1) Original case, (2) Schools were shut down after 4 days, (3) Schools were shut down after 4 days and opened after 13 days from shutdown. Total numbers of case of (1), (2), (3) are 216,408, 190,488, 217,128 and total numbers of case of (1) child, (2) child (3) child are 63,528, 52,968, 63,528.

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to other cases.

When schools were shut down after 4 days from the outbreak, the peak of infected is decreased but the period of infection is prolonged.

As for children, in case of infection rate is 1%, the total number decreased 17% compared to the original case.

We also simulated the case that schools are opened again after 13 days from school shutdown in infection rate 1%. As for children, the total number does not decrease compared to the original case. The peak of the graph does not decrease also, it only shifted later. This is shown in Fig. 1, graph (1) means original case, graph (2) means the case that schools are shut down after 4 days from outbreak, graph (3) means the case that schools are shut down after 4 days from outbreak and opened again after 13 days from school shutdown.

According to our simulations, strategy of (3) did not improve the situation and the results of (2) showed that the effect of school shut down is not so big.

Our model is simple, but validation is not done well. Professor Koopman cautioned simple models as follows (9). Do not ignore detail and realistic aspect of data. But we can do many parameter runs not only in moderate case but also in extreme cases because of simplicity of the model. Robustness will be gained comparing the results.

ACKNOWLEDGMENTS

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International Symposium on Infectious Agent Transmission Model Building – Focusing on Assessment of Risk to Communities

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SUMMARY: The Susceptible Infected and Recovery (SIR) Model proposed by Robert May in the UK is the basis of the present mathematical model building of infectious disease epidemics. Need for model building incorporating more social and other relevant factors has been recognized. An important example is the introduction of idea of the scale-free distribution of links among the people. More refined models by taking into account the nature of a pathogen, geo-sociological factors, lifestyles of the people, etc., have been developed. For example, Koopman proposed a model for prediction of epidemic expansion based on actual epidemiological data. Eubank proposed a model assessing the bio-terror attack using a model city where every day activity is going on. The present workshop, participated by experts from the US, the UK and Japan, is the first meeting of the proposed series of conference on this issue.

Why mathematical model?

Outbreak of infectious diseases is mostly unpredictable. Continued surveillance and early detection of emerging epidemic are basics of its prevention. Nations have to be prepared for any emergent epidemics. For this, it is essential to know possible consequence of coming plagues. Epidemiological studies of the past cases are useful. However, such studies have limitations. For example, we have experienced no bio-terror attacks in the modern cities. We cannot make assessment by experiments of bio-terrorism for obvious reasons. A mathematical modeling is an alternative approach. Recent progress in computer science has made such an approach more realistic.

Mathematical models developed so far

The Susceptible Infected and Recovery (SIR) model proposed by Robert May (1) is now generally supported and used as a prototype of the mathematical models (Fig. 1). The model is based on the assumption of direct transmission of a pathogen from man to man. However, more parameters, such as geo-sociological elements, lifestyles of different people, climate, transportation system, water supply, etc., have to be incorporated. Several models incorporating such diverse factors have been developed.

Koopman proposed an approach that could be more realistic by incorporating medical and epidemiological data (2). More recently, Eubank (3) proposed a model assessing the bio-terror attack in a model city by using parameters of geo-demography of the city, people's household, people's twenty-four-hour activities, etc.

S.I.R. model **(Susceptible–*I*nfected–*R*ecovered)**

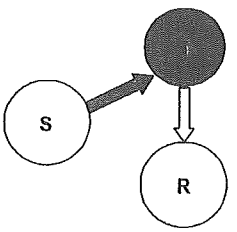

Fig. 1. Traditional modeling

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Present meeting

With the above background, the present symposium was held at the National Institute of Infectious Diseases (NIID), Japan in February 2005 aiming at the review of the ongoing activities in the related field and discussion on the future perspectives (Table 1).

Eubank, Virginia Bioinformatics Institute and Modeling Infectious Disease Agent Study (MIDAS), USA, presented a model of propagation of small pox in a city. Koopman, University of Michigan, USA, proposed an influenza virus spread model, which incorporated the parameters, such as social structures and vaccination options. Yasuda, Josai University, Japan, and Suzuki, NIID, presented a simulation of spread

Table 1. International symposium on trends in transmission models for infectious diseases - 2005: modeling biology focusing to social risk assessment

Program

Opening remarks: Kazuo Suzuki, National Institute of Infectious Diseases, Japan

1. Models for a Science of Infection Transmission
   James S. Koopman, University of Michigan, USA
2. Mathematical Models of the Evolution and Spread of Infections
   Angela McLean, Zoology Department, Oxford University, UK
3. Network Based Models of Infectious Disease Spread
   Stephen Eubank, Virginia Bioinformatics Institute and MIDAS, USA
4. Modeling on Social Spread from Immunity
   Hidenori Yasuda, Josai University, Japan
5. Sensing and Network
   Mami Furukubo, Hitachi Software Engineering, Japan
6. Effectiveness of Vaccination Strategies for Infectious Diseases According to Human Contact Networks
   Fumihiko Takeuchi, Juntendo University, Japan
7. Social Interaction Models
   Takashi Iba, Keio University, Japan
8. Simulation of Human Network
   Kenji Yamamoto, International Medical Center of Japan, Japan

Closing remarks: Takeshi Kurata, National Institute of Infectious Diseases, Japan

Commentators: Hiroshi Yoshikura, National Institute of Infectious Diseases, Japan, and others

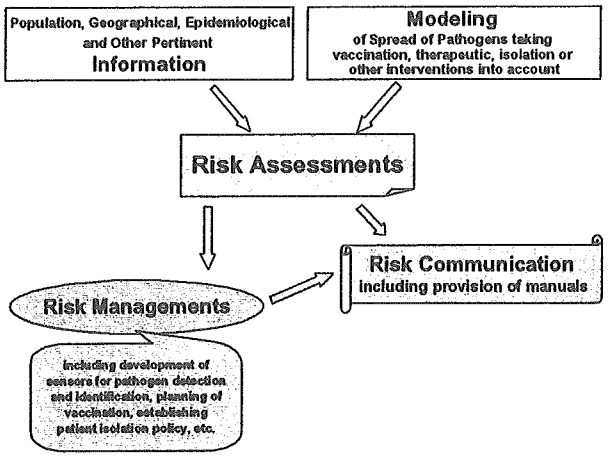

Fig. 2. Transmission models for risk assessment.

of influenza virus incorporating the parameters of immune state of the population and examined the effect of classroom closure on the persistence and spread in a community. Takeuchi presented a mathematical analysis of propagation in a community where man-to-man linking number follows a scale-free distribution. Yamamoto, International Medical Center of Japan, presented a data of hospital infection, frequency of which could be predicted by a mathematical model. McLean presented a model of HIV transmission that is under the effect of frequent mutation. Iba, Keio University, Japan, presented a social interaction model, which could strongly affect man to man spread of pathogens. Kokubo presented a recently developed a pathogen sensor device coupled with reporting and data collection for sensing outbreaks. Commentators were Kurata, Director-General of NIID and Yoshikura, Emeritus member of NIID. The latter commentator discussed influence of social link structure among the homosexuals on the exponential spread of HIV in Japan. Based on discussion in this meeting, reduction of risks of infectious diseases by transmission modeling for infectious agent will be proposed (Fig. 2).

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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.

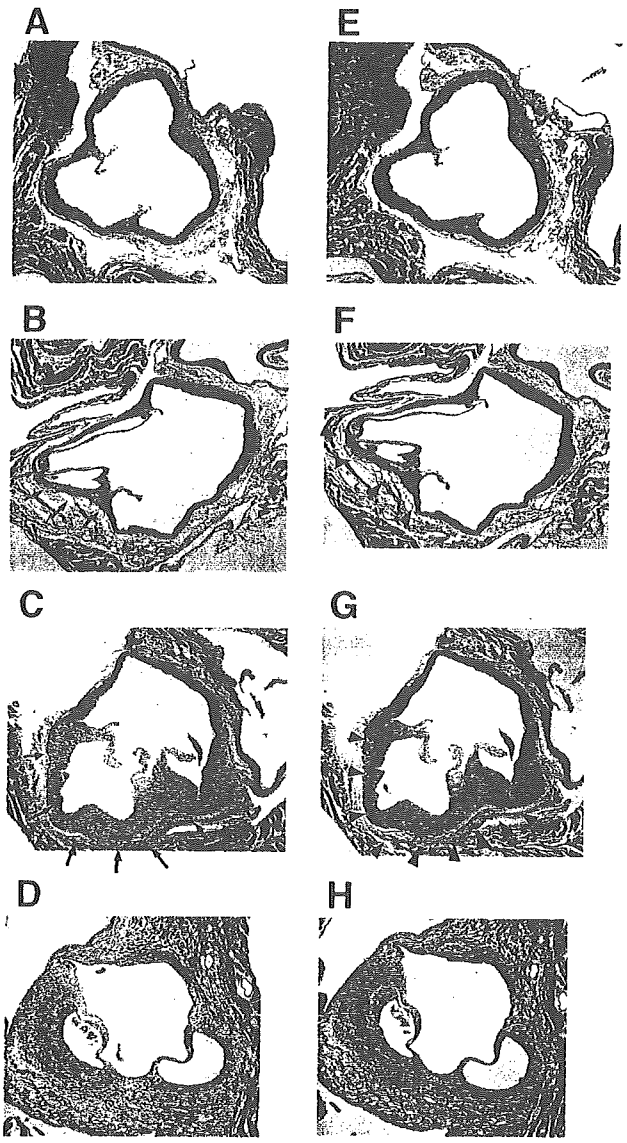

Figure 1. Arterial inflammation around the aortic sinus in IL-1Ra$^{-/-}$ mice. A and E, Normal sections of the aortic valve (score 0) from an 8-week-old, wild-type female mouse. B and F, Mild inflammatory cell infiltration and loss of elastic lamellae over less than one third of the media of the aortic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

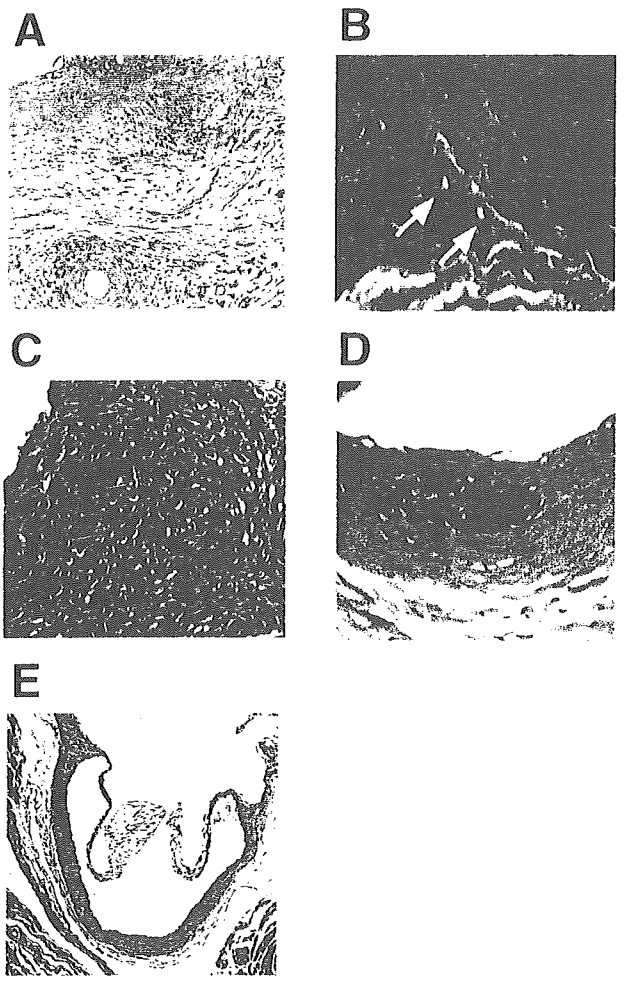

Figure 2. Characterization of arterial inflammation in IL-1Ra$^{-/-}$ mice. A, Inflammatory cell infiltration in the media and adventitia in a 12-week-old IL-1Ra$^{-/-}$ female mouse. B, Formation of microvessels (white arrows) in a section from an 8-week-old IL-1Ra$^{-/-}$ male mouse. C, Chondrocyte-like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

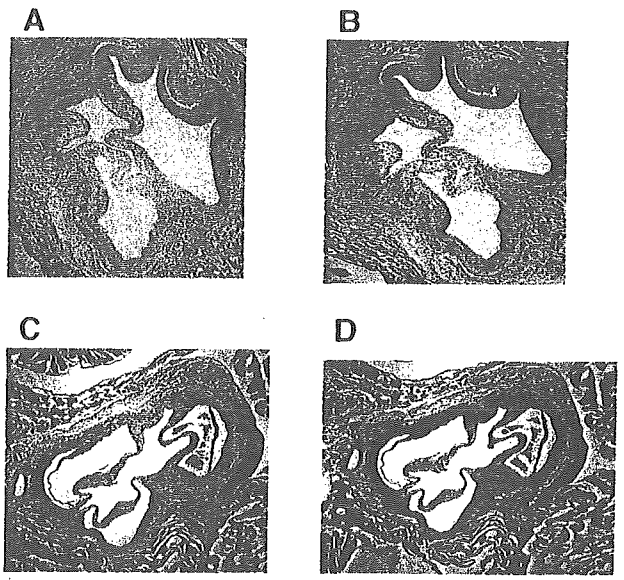

Figure 3. Induction of aortitis by transplantation of IL-1Ra$^{-/-}$ peripheral T cells and BM cells. A and B, Sections from nu/nu female mice 10 weeks after transplantation of T cells from IL-1Ra$^{-/-}$ mice (score 3). C and D, Sections from a wild-type, female mice 12 weeks after transplantation of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

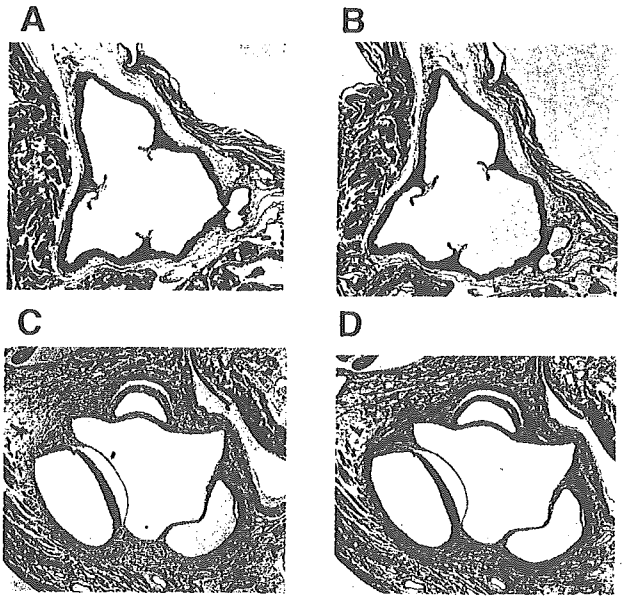

Figure 4. Complete suppression of the development of aortitis in TNF-α–deficient, IL-1Ra$^{-/-}$ mice. Sections of the aortic valves from TNF-α$^{-/-}$–IL-1Ra$^{-/-}$ female mice (14 weeks old, score 0) (A and B) and IL-6$^{-/-}$–IL-1Ra$^{-/-}$ female mice (8 weeks old, score 3) (C and D). Hematoxylin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

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-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.

Acknowledgments

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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.