but also the development of IFN-γ-producing T cells and granuloma formation. Our data suggest that IL-17 is an important cytokine in the induction of optimal Th1 response and protective immunity against mycobacterial infection. Because IL-17 exerts beneficial effects on the development of protective cell-mediated immunity against mycobacteria, it is likely to be used as an immune adjuvant to enhance the efficacy of vaccination inducing the protective Th1 response.

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Disclosures

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Abnormal T cell activation caused by the imbalance of the IL-1/IL-1R antagonist system is responsible for the development of experimental autoimmune encephalomyelitis

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Abstract

IL-1 is a pro-inflammatory cytokine that plays an important role in inflammation and host responses to infection. We have previously shown that imbalances in the IL-1 and IL-1R antagonist (IL-1Ra) system cause the development of inflammatory diseases. To explore the role of the IL-1/IL-1Ra system in autoimmune disease, we analyzed myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) in mice bearing targeted disruptions of the IL-1a, IL-1b, IL-1a and IL-1b (IL-1) or IL-1Ra genes. IL-1a/b double-deficient (IL-1 $^{-/-}$) mice exhibited significant resistance to EAE induction with a significant reduction in disease severity, while IL-1a $^{-/-}$ or IL-1b $^{-/-}$ mice developed EAE in a manner similar to wild-type mice. IL-1Ra $^{-/-}$ mice also developed MOG-induced EAE normally with pertussis toxin (PTx) administration. In contrast to wild-type mice, however, these mice were highly susceptible to EAE induction in the absence of PTx administration. We found that both IFN-c and IL-17 production and proliferation were reduced in IL-1 $^{-/-}$ T cells upon stimulation with MOG, while IFN-c, IL-17 and tumor necrosis factor-a production and proliferation were enhanced in IL-1Ra $^{-/-}$ T cells. These observations suggest that the IL-1/IL-1Ra system is crucial for auto-antigen-specific T cell induction and contributes to the development of EAE.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease associated with the demyelination of the central nervous system (CNS). Approximately, one million individuals in the world are afflicted by MS (1). Although significant progress has been made elucidating the causes of MS and improving patient outcomes over the past decade (2), definitive therapies either reducing the number of attacks or slowing the progression of disease are not yet available.

Experimental autoimmune encephalomyelitis (EAE) is regarded as an animal model mimicking several aspects of the pathogenesis of human MS, which is clinically characterized by paralysis and lethargy (3). Immunization with self-neuronal antigens, such as MBP, myelin-associated glycoprotein, proteolipid protein or myelin oligodendrocyte glycoprotein (MOG) (3,4), results in inflammation within the CNS primarily mediated by $CD4^+$ T_h1 cells (1,2).

Systemic or local induction of cytokines is critical in the initiation, enhancement or perpetuation of CNS disease (5). T_h1 cell-derived IFN-c, which contributes to the etiology of a wide range of diseases, is markedly elevated within the CNS during EAE. IFN-c-deficient and IFN-cR $^{-/-}$ mice, however, remain highly susceptible to EAE (6–9). In fact, the overexpression of IFN-c in the CNS ameliorated the severity of EAE (10). Tumor necrosis factor (TNF)a, a potent pro-inflammatory

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cytokine, is produced by a variety of cell types, including T_h1 cells. Mice overexpressing TNFa within the CNS exhibit neuronal demyelination (11,12), while the development of EAE in TNFa $^{-/-}$ mice is partially suppressed (13,14). Other group reported, however, that in TNFa $^{-/-}$ mice, the course of EAE was exacerbated by the abnormal regression and expansion of myelin-specific T cells (15). Clinically, anti-TNF therapy resulted in more severe MS (16). Thus, the contribution of pro-inflammatory cytokines, such as IFN-c and TNFa, cannot fully explain the precise molecular mechanisms underlying EAE development.

IL-1 is produced by a variety of cells, including monocytes/macrophages, epithelial and endothelial cells and glial cells (17). Through the up-regulation of intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression, this cytokine plays a crucial role in leukocyte extravasation into inflammatory sites (18). Dysregulation of IL-1 function leads to autoimmune and abnormal immune responses, such as arthritis and aortitis in mouse models (19, 20). Furthermore, exogenous IL-1 administration exacerbated the course of EAE, while administration of soluble IL-1R type-I (IL-1RI) or IL-1R antagonist (IL-1Ra) significantly suppressed EAE in Lewis rats (21, 22). Consistent with these observations, mice deficient in IL-1RI or IL-1R-associated kinase 1, which is involved in IL-1-mediated signal transduction, fail to develop inflammatory lesions or any evidence of EAE (23). These observations suggest that IL-1 may initiate or promote local and/or systemic inflammation during EAE pathogenesis. In vitro, IL-1 can augment the activation of encephalitogenic T lymphocytes, contributing to the development of EAE induced by adoptive transfer (24). Thus, IL-1 likely contributes to the activation of auto-antigen-specific immune cells, including T cells. Indeed, IL-1 can influence antigen-specific T cell activation directly (25) or indirectly via modulation of dendritic cell (DC) function (26). The importance of IL-1 in DC function, including migration, activation and acquisition of T_h1-inducing ability, has been demonstrated previously (27, 28). The precise effect of IL-1 on DCs and/or T cells during the development of EAE, however, has yet to be elucidated.

In this report, we investigate the contribution of IL-1 to the development of EAE using IL-1 $^{-/-}$ and IL-1Ra $^{-/-}$ mice. We determined that IL-1 is responsible for the induction of autoreactive T cells. Our data provide evidence that the IL-1/IL-1Ra system is critical for the development of CNS autoimmune disease by modulating T cell-mediated immunity.

Methods

Mice

IL-1a $^{-/-}$, IL-1b $^{-/-}$, IL-1a/b $^{-/-}$ (IL-1 $^{-/-}$) and IL-1Ra $^{-/-}$ mice were generated as described (29). Mice were backcrossed to the C57BL/6 strain mice for eight generations. C57BL/6 mice (wild-type mice) were purchased from Clea (Tokyo, Japan). Age- and gender-matched wild-type mice were used as controls in each experiment. Mice were kept under specific pathogen-free conditions in an environmentally controlled clean room at the Center for Experimental Medicine, Institute of Medical Science, University of Tokyo. Animals were housed in an ambient temperature of 24 C on a daily cycle of 12 h of light and darkness (8:00 a.m. to 8:00 p.m.). All the experiments were performed according to the institutional ethical guidelines for animal experimentation.

MOG peptide

MOG 35-55 (MEVGWYRSPFSRVVHLYRNGK), corresponding to the murine sequence, was synthesized on a peptide synthesizer using fluorenylmethoxycarbonyl chemistry and purified by HPLC by Ohmi (Institute of Medical Science, University of Tokyo, Japan).

Induction and evaluation of EAE

Eight- to twelve-week-old mice were subcutaneously immunized with 100 lg MOG 35-55 emulsified in CFA (1:1) supplemented with 400 lg Mycobacterium tuberculosis H37RA (DIFCO Lab., Detroit, MI, USA) in both flanks. Pertussis toxin (PTx) (500 ng) (Alexis Corp., San Diego, CA, USA) was injected intravenously into animals on the day of immunization as well as 2 days later.

Mice were inspected daily for the clinical signs of EAE for up to 30 days after immunization. Scores were determined on a scale of 0-5: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, hind and fore limb paralysis and 5, moribund state. The mean clinical score was calculated by averaging the score of all of the mice in each group, including animals that did not develop EAE.

Tier for anti-MOG antibodies in serum

Detection of anti-MOG 35-55 antibodies was performed as described (25) with the following modifications. Briefly, MOG 35-55 peptide (0.5 lg per 96 well) was coated onto 96-well plates and incubated at 4 C overnight. After substantial washing and blocking, diluted sera (30 ll per well) were added to the wells for 2 h at room temperature. A series of serum dilutions were examined in preliminary experiments. After washing, alkaline phosphatase-conjugated goat anti-mouse Igs (Zymed, San Francisco, CA, USA) were added for 1 h at room temperature, followed by incubation in p-nitrophenyl phosphate substrate (Sigma-Aldrich, St Louis, MO, USA) as the substrate. The anti-MOG antibody tier is given as an OD_{415} value. Samples were measured in duplicate.

T cell and DC purification and proliferation assay

Mice were immunized subcutaneously with 100 lg MOG 35-55 emulsified CFA (1:1) with or without PTx. Ten days later, T cells were prepared from multiple lymph nodes (LNs) (axillary, inguinal, branchial, cervical and popliteal). Cells were washed, treated with anti-mouse Thy1.2 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and passed through a MACS column to collect Thy1.2$^+$ T cells.

DCs were prepared from the spleen. Spleens were collected, minced and digested with 1 mg ml^{-1} collagenase (Sigma-Aldrich) and 1 mg ml^{-1} DNase I (Sigma-Aldrich) in HBSS for 30 min at 37 C. Following the addition of EDTA (20 mM final concentration), cells were incubated for 5 min at room temperature, passed through a 70-lm nylon mesh, layered over RPMI 1640-10% FCS-14.5% metrizamide (Cedarlane Labs., Ontario, Canada) and centrifuged at room temperature for 30 min at 500 3 g. The low buoyant density cells at the

interface were collected and washed twice. Cells were then treated with anti-mouse CD11c magnetic beads (Miltenyi Biotec) and passed through a MACS column. The positively selected fraction was collected, washed and re-suspended for use.

Purified DCs (1 3 10^4 cells) in the presence or absence of T cells (1 3 10^5 cells) were plated on 96-well plates coated with MOG 35–55 in a final volume of 200 ll RPMI 1640–10% FCS. After 72 h of culture, cells were pulsed with [^{3}H]thymidine ([^{3}H]TdR) (0.25 lCi ml^{-1}; Amersham Biosciences, Tokyo, Japan) for 6 h. Cells were then harvested with a Micro 96 cell harvester (Skatron, Lier, Norway). The incorporated [^{3}H]TdR radioactivity was measured using a Micro Beta System (Amersham Biosciences, Piscataway, NJ, USA). Culture supernatants were collected prior to [^{3}H]TdR incorporation to measure cytokines levels.

ELISA of cytokine levels

The levels of IL-4, IL-17 and TNFa were measured as described (30,31). IFN-c levels were measured with OptEIA Set mouse IFN-c kit (BD PharMingen). All assays were done in duplicate.

Statistical analysis

All values were calculated as the average 6 SD. Comparisons were made using the Student's t-test, one-way analysis of variance (ANOVA), Fisher's protected least significant difference test and Mann–Whitney's U-test. Differences among the three groups were tested by Kruskal–Wallis one-way ANOVA.

Results

IL-1 / mice are resistant to EAE

To examine the role of the IL-1/IL-1Ra system in the development of EAE, we immunized C57BL/6 wild-type, IL-1 / and IL-1RI / mice with MOG 35–55 emulsified in CFA. Following the injection of PTx on days 0 and 2, the clinical signs of EAE were monitored daily and scored as described in Methods. IL-1RI / mice are known to demonstrate resistance to the development of EAE (23), suggesting that IL-1 is involved in EAE pathogenesis. We confirmed that IL-1 / mice exhibit significant resistance to EAE and that IL-1RI / mice demonstrate a reduction in disease severity (Fig. 1A and data not shown) (23). The onset of EAE in IL-1 / mice was also delayed from that of wild-type mice (Table 1). In contrast, mice deficient in either IL-1a or IL-1b developed EAE with a comparable severity and time course to wild-type mice (Fig. 1B). The incidence of disease, day of onset and maximal clinical score were not significantly different between wild-type, IL-1a / and IL-1b / mice (Table 1). All genotypes mice exhibited > 90% disease incidence. These observations suggest that, while IL-1 plays a principal role in the development of EAE, the presence of either IL-1a or IL-1b alone is sufficient to initiate development of the disease.

Development of EAE is exacerbated in IL-1Ra / mice without PTx administration

We immunized IL-1Ra / mice with MOG 35–55 emulsified in CFA. After an injection of PTx on days 0 and 2, IL-1Ra / mice developed EAE that was comparable in time of onset and

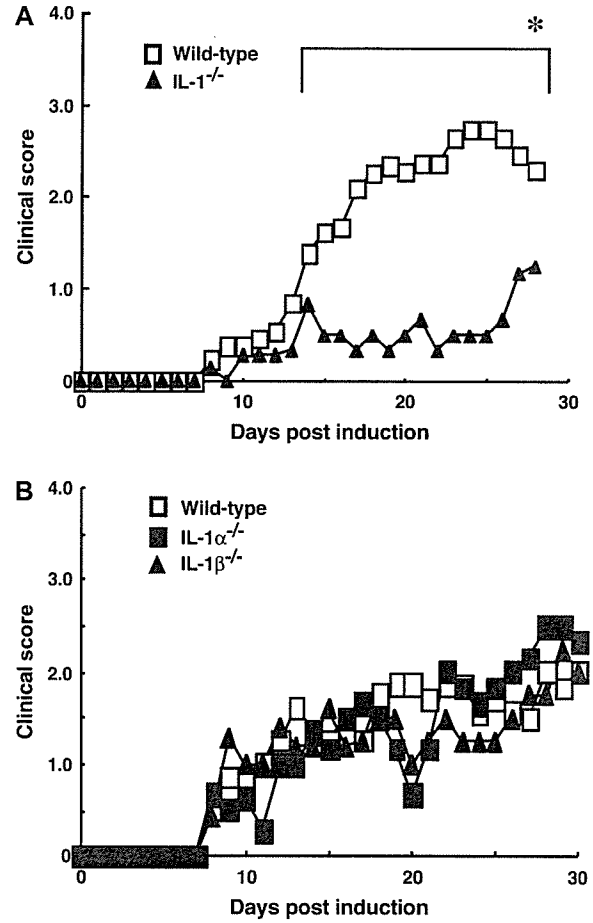

Fig. 1. Attenuated EAE induction in IL-1 / mice. The clinical scores after EAE induction were determined as described in Methods. The averages of clinical scores are shown from the day of MOG immunization (day 0) to day 28 post-immunization in (A) wild-type (open squares, n ¼ 9) and IL-1 / (filled triangles, n ¼ 7) mice and (B) wild-type (open squares, n ¼ 8), IL-1a / (filled squares, n ¼ 9) and IL-1b / (filled triangles, n ¼ 7) mice. The data indicate the averages of each group. Statistical significances were determined by Mann–Whitney's U-test (A) and Kruskal–Wallis one-way ANOVA (B). *P < 0.05 versus wild-type mice.

severity to the EAE course observed in wild-type mice (Fig. 2A and Table 1). PTx is routinely used to facilitate the induction of experimental autoimmune diseases in animals. Previous reports using IL-10 / and TNFa / mice suggested that co-administration of PTx veiled the effects of cytokines as an inflammatory factor in EAE (15,32,33). Therefore, to address the contribution of IL-1Ra to EAE without the complications of PTx co-administration, we examined the susceptibility of wild-type and IL-1Ra / mice to EAE in the absence of PTx. The severity of EAE was reduced in wild-type mice that were not treated with PTx (Fig. 2B). IL-1Ra / mice, however, developed severe EAE in both the absence and presence of PTx (Fig. 2B). Without PTx, IL-1Ra / mice developed more severe EAE at earlier time points than wild-type mice (Table 1). These results indicate that dysfunction of IL-1 signaling mediated by IL-1Ra deficiency contributes to EAE induction in the absence of PTx. In wild-type mice, PTx may be necessary to overcome the function of IL-1 in EAE induction.

Table 1. Clinical features of MOG 35–55-induced EAE in IL-1 $^{-/-}$ and IL-1Ra $^{-/-}$ mice

	Mice	Incidence (best)	Day of onset (average ± SD)	Maximal clinical score (average ± SD)
With PTx				
Experiment 1	Wild type	9/9 (2)	8.7 ± 0.9	3.5 ± 1.0
	IL-1 $^{-/-}$	6/7 (1)	12.4 ± 4.3*	1.6 ± 1.1*
Experiment 2	Wild type	8/8 (2)	8.3 ± 0.9	2.8 ± 1.2
	IL-1a $^{-/-}$	9/9 (3)	9.8 ± 1.3	2.6 ± 0.7
	IL-1b $^{-/-}$	6/7 (3)	8.5 ± 0.6	2.9 ± 1.7
Experiment 3	Wild type	13/13 (4)	9.2 ± 1.3	3.1 ± 1.0
	IL-1Ra $^{-/-}$	12/12 (4)	8.9 ± 1.2	3.1 ± 1.2
Without PTx				
Experiment 4	Wild type	8/8 (0)	12.8 ± 3.6	2.0 ± 0.8
	IL-1Ra $^{-/-}$	9/9 (1)	9.7 ± 0.7*	2.9 ± 0.6*

EAE was induced and scored as described in Methods. Incidence data represent the number of mice. *$P < 0.01$ versus wild-type mice of each experiment (by Student's t-test or one-way ANOVA).

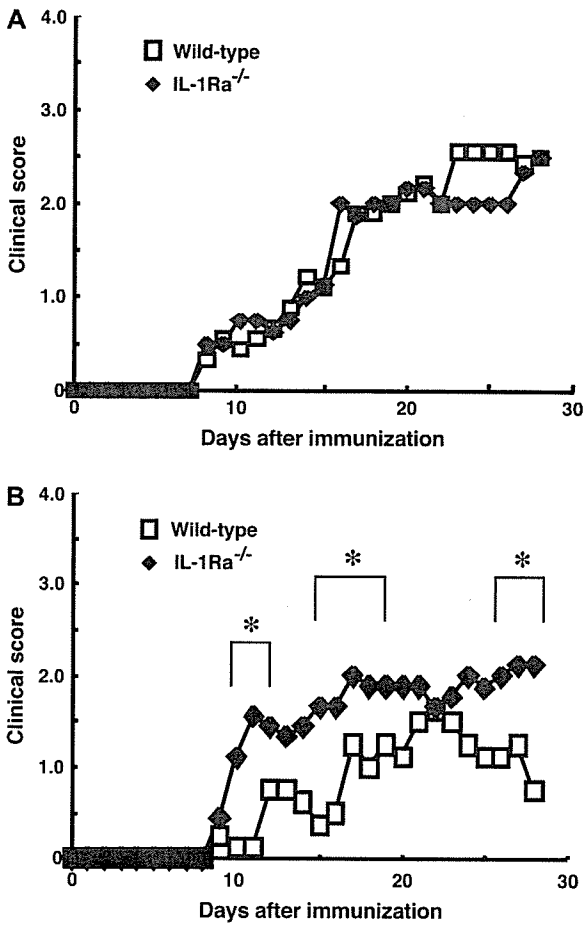

Fig. 2. Exacerbated EAE induction without PTx injection in IL-1Ra $^{-/-}$ mice. Clinical scores after MOG immunization in the (A) presence or (B) absence of PTx (500 ng) injection in wild-type (open squares, (A) n = 13 and (B) n = 8], and IL-1Ra $^{-/-}$ mice [filled diamonds, (A) n = 12 and (B) n = 9]. Data show the average from each group. Statistical significances were determined by Mann–Whitney's U-test. *$P < 0.05$ versus wild-type mice.

T_h1-type antibody production against MOG 35–55 is increased in sera of IL-1Ra $^{-/-}$ mice

Auto-antigen-specific Igs were detected in the sera of mice with EAE. At 37 days after immunization with MOG 35–55, blood samples were collected from mice for the measurement of MOG-specific auto-antibody levels in the sera. The levels of MOG-specific IgG and IgM classes and the IgG1 subclass in sera, as well as those of IgG2b and IgG3 (data not shown), were comparable among IL-1 $^{-/-}$ and wild-type mice given PTx and among IL-1Ra $^{-/-}$ and wild-type mice in the absence of PTx co-administration (Fig. 3A and B). In contrast, the levels of MOG-specific IgG2a, whose production depends on T_h1 cytokines, were significantly increased in sera from IL-1Ra $^{-/-}$ mice in comparison with those from wild-type and IL-1 $^{-/-}$ mice (Fig. 3A and B). These results suggest that IL-1 signaling promotes the polarization of T_h1 immune responses toward the production of high levels of auto-antigen-specific IgG2a, as seen in IL-1Ra $^{-/-}$ mice during the development of EAE.

IL-1 is involved in auto-antigen-specific T cell activation during EAE

EAE is considered to be a T cell-mediated autoimmune disease model (3). Abnormal EAE induction in IL-1 $^{-/-}$ and IL-1Ra $^{-/-}$ mice may be due to abnormal control of MOG-specific effector T cells. DCs also play a significant role in (auto)immune responses through the induction of T_h1 cell activation (26). In the EAE animal model, we examined if dysfunction of the IL-1/IL-1Ra system affected T cell or DC function using IL-1 $^{-/-}$ and IL-1Ra $^{-/-}$ mice. We examined in vitro the activation of T cells derived from wild-type, IL-1 $^{-/-}$ and IL-1Ra $^{-/-}$ mice immunized with MOG 35–55/CFA in the absence of PTx co-administration. Ten days after MOG 35–55 immunization, Thy1.2$^+$ T cells and CD11c$^+$ DCs were isolated from the draining LNs and spleen, respectively. LN T cells were then co-cultured with DCs in the presence of MOG 35–55. No proliferative responses were observed in DCs of gene-deficient and/or wild-type mice treated with MOG 35–55 in the absence of T cells (data not shown). Low proliferative responses of T cells were observed even without DCs in the absence or presence of MOG 35–55 (data not shown). When cultured with DCs in the presence of MOG 35–55, MOG-specific T cell proliferative responses were induced in a manner dependent on MOG 35–55 concentration (0, 10, 50 and 100 lg ml^{-1}) (data not shown). In these co-cultures, the MOG-specific proliferative responses of wild-type T cells were comparable among wild-type, IL-1Ra $^{-/-}$ and IL-1 $^{-/-}$ DCs (Fig. 4A and B), suggesting that IL-1 or IL-1Ra deficiency

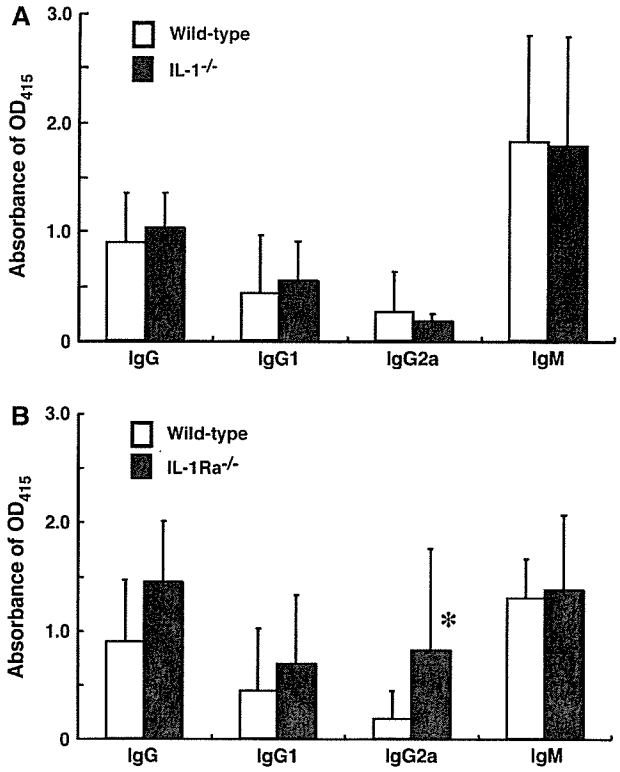

Fig. 3. Titer of anti-MOG antibodies in serum of IL-1 $^{-/-}$ and IL-1Ra $^{-/-}$ mice. Thirty-seven days after EAE induction (A) with PTx injection (wild-type, open bars and IL-1 $^{-/-}$ mice, filled bars) or (B) without PTx injection (wild-type, open bars and IL-1Ra $^{-/-}$ mice, filled bars), we collected serum samples. The levels of IgG, IgG1, IgG2a and IgM specific for the MOG 35–55 peptide are shown as OD values. Data show the average 6 SD from each group. Statistical significances were determined by Student's t-test. *$P < 0.05$ versus wild-type mice.

of DCs did not result in any defects in antigen presentation or cytokine production that would influence the induction of MOG-specific T cell recall responses in vitro. Interestingly, the proliferative responses of MOG-specific IL-1Ra $^{-/-}$ T cells were significantly hyperactive following co-cultured with either wild-type (Fig. 4D) or IL-1Ra $^{-/-}$ (data not shown) DCs in comparison with wild-type T cells. In contrast, the responses of IL-1 $^{-/-}$ T cells after co-culture with either wild-type (Fig. 4C) or IL-1 $^{-/-}$ (data not shown) DCs were profoundly impaired, despite comparable non-specific proliferative responses of T cells against mitogenic stimuli (Con A 1 lg ml^{-1}) (Fig. 4). These results indicate that intrinsic IL-1 is responsible for the activation of auto-antigen-specific T cells during the priming process in vivo.

T cells from IL-1Ra $^{-/-}$ mice produce high levels of pro-inflammatory cytokines

We measured cytokine production by MOG-specific T cells by assaying the supernatants of proliferative response cultures. The levels of IFN-c and TNFa in supernatants from wild-type T cells co-cultured with either IL-1 $^{-/-}$ or IL-1Ra $^{-/-}$ DCs were similar to those cultured with wild-type DCs. In contrast, the levels of IFN-c and IL-17, but not TNFa, in the supernatants of IL-1 $^{-/-}$ T cells co-cultures with wild-type DCs was reduced from the levels seen in wild-type T cells co-cultures with wild-type DCs (Fig. 5A and data not shown). In correlation with MOG-specific T cell proliferative responses, IFN-c, IL-17 and TNFa levels measured in the supernatants of IL-1Ra $^{-/-}$ T cells co-cultures with wild-type or IL-1Ra $^{-/-}$ DCs were significantly increased in comparison with those from wild-type or IL-1 $^{-/-}$ T cells co-cultured with wild-type DCs (Fig. 5B and data not shown). The levels of IL-4, a T_h2-skewing cytokine, were below the limits of detection in the supernatants from any of the culture conditions (data not shown). These results suggest that excess IL-1 signaling breaks tolerance for auto-antigens in peripheral lymphoid tissues, resulting in hyperresponsive effector T cell activation and auto-antigen-specific T cell proliferation and inflammatory cytokine production as seen in IL-1Ra $^{-/-}$ mice during EAE pathogenesis.

Discussion

Using IL-1a $^{-/-}$, IL-1b $^{-/-}$, IL-1 $^{-/-}$ and IL-1Ra $^{-/-}$ mice, we demonstate that IL-1 is responsible for the development of EAE. Either IL-1a or IL-1b alone was sufficient to induce EAE; excess IL-1 signaling resulting from the lack of IL-1Ra augmented EAE severity in the absence of PTx injection. These findings suggested that the adjuvant effect of PTx exerts a related function as IL-1 in the induction of EAE. We clearly demonstrated that, while IL-1 controls optimal antigen-specific T cell activation, dysfunction of the IL-1/IL-1Ra system leads to excess T cell activation by breaking peripheral tolerance for auto-antigens during the pathogenesis of EAE.

In a series of inflammatory response models, we have previously shown that antigen-presenting cell (APC)-derived IL-1 was required for (auto)antigen-specific T cell activation. We previously showed that IL-1 plays an important role in the interaction between T cells and APCs in priming process through inducing CD40L (CD154) and OX40 (CD134) on T cells (25). CD40L and OX40 expressions were enhanced in T cells stimulated with antigen-bearing IL-1Ra $^{-/-}$ APCs compared with wild-type APCs (25). Thus, upon interaction with antigens, APCs produce IL-1, and IL-1 activates T cells, resulting in the induction of CD40L (34, 35). Then, CD40L–CD40 interaction activates APCs to produce TNFa (34). This TNFa induces OX40 on T cells (36), that leads to enhancement of cytokine production, especially IL-17 (37). With these mechanisms, APCs-derived IL-1 contributes to the development of allergic and/or autoimmune diseases in mice (28, 36, 38). IL-1RI $^{-/-}$ DCs demonstate impaired cytokine production, leading to insufficient CD4^{+} T cell activation (26). Thus, IL-1 can modulate T cell function both directly and indirectly by influencing DC activation. These findings suggest that IL-1 may play a role in the induction and/or activation of auto-reactive T cells in EAE. Despite comparable non-specific T cell proliferation upon stimulation with mitogen Con A among wild-type, IL-1 $^{-/-}$ and IL-1Ra $^{-/-}$ mice (Fig. 4C and D), the proliferation of MOG-specific IL-1 $^{-/-}$ T cells co-cultured with wild-type DCs, which can produce IL-1, was markedly impaired. The proliferation of IL-1Ra $^{-/-}$ T cells co-cultured with wild-type DCs, which could produce IL-1Ra, was greatly enhanced (Fig. 4C and D). The proliferation of MOG-specific wild-type T cells co-cultured with IL-1 $^{-/-}$ DCs was similar to that observed with wild-type DCs (Fig. 4A), indicating that DC-derived IL-1 is not essential for the activation of MOG-specific

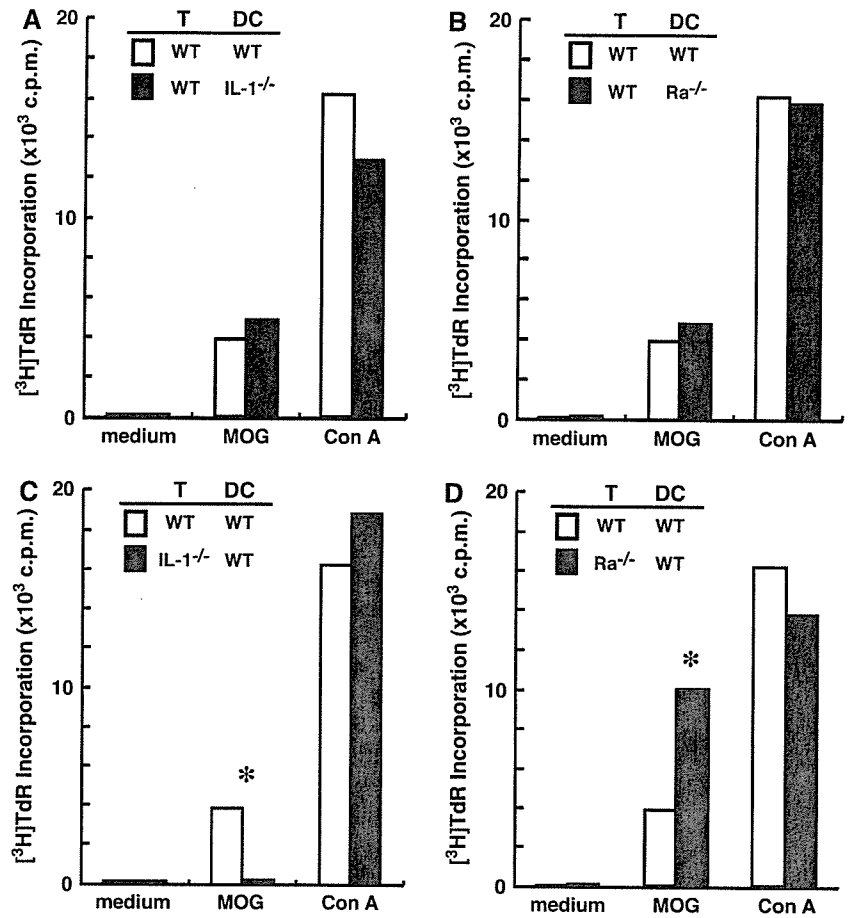

Fig. 4. Abnormal T cell activation in IL-1 $^{-/-}$ and IL-1Ra $^{-/-}$ mice immunized with MOG. Wild-type, IL-1 $^{-/-}$ and IL-1Ra $^{-/-}$ mice were immunized with a MOG 35–55/CFA emulsion without PTx co-administration. Ten days after MOG immunization, LN T cells were cultured with splenic DCs in the absence (medium) or presence MOG of MOG 35–55 (100 lg ml $^{-1}$) or Con A (1 lg ml $^{-1}$) for 72 h. MOG-sensitized T cells from wild-type mice were co-cultured with DCs from (A) wild-type or IL-1 $^{-/-}$ mice and (B) wild-type or IL-1Ra $^{-/-}$ mice. MOG-sensitized T cells from (C) wild-type or IL-1 $^{-/-}$ mice and (D) wild-type or IL-1Ra $^{-/-}$ mice were co-cultured with DCs from wild-type mice. The genotypes of the T cells (T) and DCs (DC) are indicated as WT: wild-type mice, IL-1 $^{-/-}$: IL-1 $^{-/-}$ mice and Ra $^{-/-}$: IL-1Ra $^{-/-}$ mice. Data indicate the averages. These data were reproducible in three independent experiments. Statistical significances were determined by one-way ANOVA and Fisher's protected least significant difference test. *$P < 0.01$ versus wild-type mice.

memory T cells. Instead, IL-1 is likely involved in the induction of MOG-specific memory T cells in vivo. Thus, insufficient induction of MOG-specific T cells resulting from IL-1 deficiency may lead to the attenuated development of EAE as observed in IL-1 $^{-/-}$ mice. In contrast, excess MOG-specific T cell activation observed in IL-1Ra-deficient mice may explain the exacerbation of EAE in IL-1Ra $^{-/-}$ mice.

Despite the normal development of EAE following PTx injection, IL-1Ra $^{-/-}$ mice exhibited more severe MOG-induced EAE in the absence of PTx injection than wild-type mice. Similarly, the development of EAE in TNFa $^{-/-}$ mice was completely suppressed in the presence of low doses of PTx, although susceptibility to the disease in TNFa $^{-/-}$ mice was normal at high doses of PTx (33). PTx is widely used to enhance T_h1-mediated organ-specific autoimmune disease through inhibition of the G i/o protein signaling pathways that negatively regulate IL-12 production (39) and induction of pro-inflammatory cytokines, MHC class II, CD80, CD86 and CD40 on APCs (40, 41). These observations imply that PTx exerts a similar function as the pro-inflammatory cytokines IL-1 and TNFa. We, as well as others, previously observed that the function of IL-1 in ovalbumin-induced airway hypersensitivity responses could be substituted for by a potent adjuvant, aluminum potassium sulfate (42, 43). Therefore, the physiological function of IL-1 (and TNFa) may be masked by the excessive adjuvant-dependent artificial activation of the immune system observed in MOG-EAE with PTx injection and ovalbumin-induced airway hypersensitivity responses with aluminum potassium sulfate.

IFN-c, TNFa and IL-17, T cell-derived inflammatory cytokines, play critical roles in multiple pathological inflammatory responses. TNFa has a similar biological activity to IL-1 as a potent pro-inflammatory cytokine. As seen in studies using TNFa $^{-/-}$ mice, TNFa is also involved in the development of EAE (13,14). Interestingly, TNFa production is normal in IL-1 $^{-/-}$ mice after MOG/CFA immunization (Fig. 5A), despite the profound suppression of EAE development in IL-1 $^{-/-}$ mice (Fig. 1 and Table 1). In contrast, IL-1Ra $^{-/-}$ mice exhibited elevated TNFa production (Fig. 5B) and exacerbated development of EAE (Fig. 2 and Table 1). Thus, TNFa is not essential for, but contributes to, the development of EAE (15,23,44). Excess TNFa production resulting from excessive

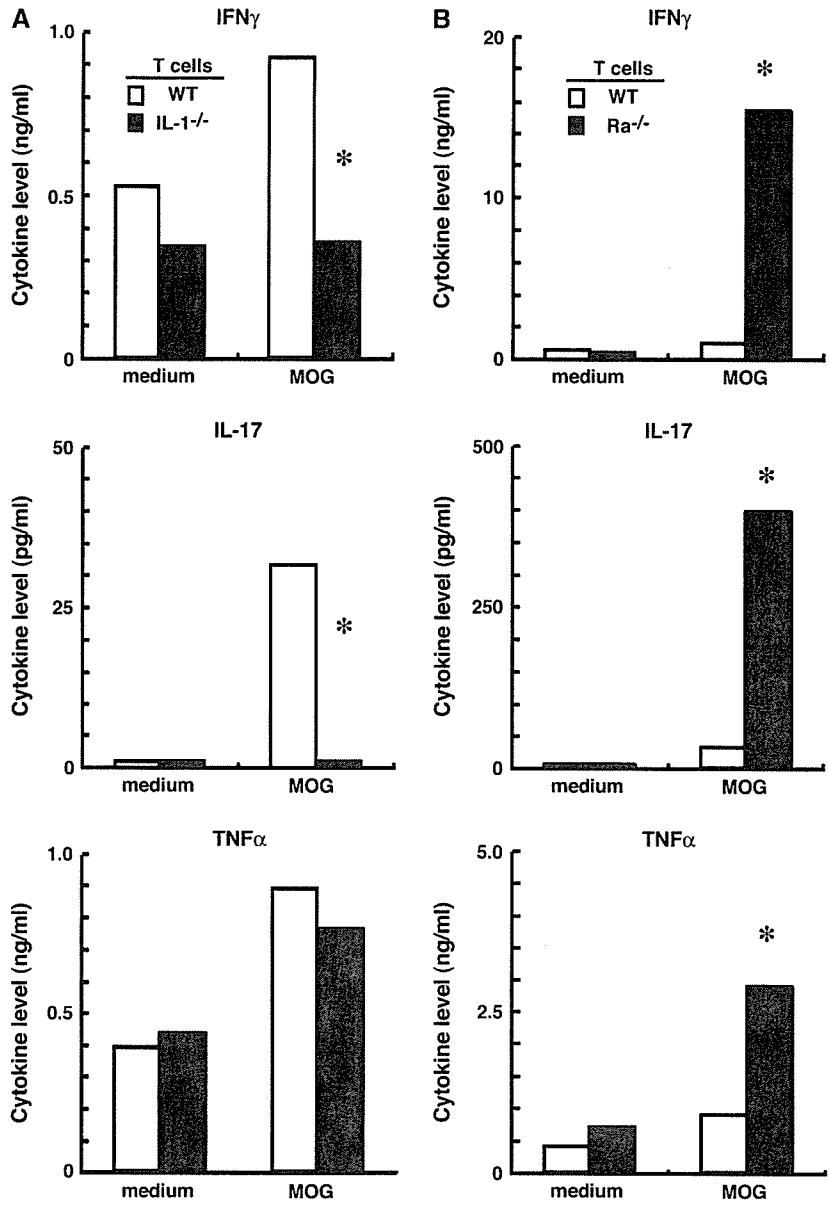

Fig. 5. Abnormal cytokine production from IL-1 $^{-/-}$ T cells and IL-1Ra $^{-/-}$ T cells. MOG-sensitized T cells from (A) wild-type or IL-1 $^{-/-}$ mice and (B) wild-type or IL-1Ra $^{-/-}$ mice were co-cultured with wild-type DCs in the absence (medium) or presence MOG of MOG 35–55, as shown in Fig. 4. IFN-c, IL-17 and TNFa levels in culture supernatants were determined by ELISA. The genotypes of the T cells are indicated as WT: wild-type mice, IL-1 $^{-/-}$: IL-1 $^{-/-}$ mice and Ra $^{-/-}$: IL-1Ra $^{-/-}$ mice. Data indicate the averages. These data were reproducible in two independent experiments. Statistical significances were determined by one-way ANOVA and Fisher's protected least significant difference test. *$P < 0.01$ versus wild-type mice.

IL-1 activity may explain the synergistic exacerbation of EAE development in IL-1Ra $^{-/-}$ mice. These results suggest, however, that TNFa alone is not sufficient to induce adequate responses in the absence of IL-1, as observed in IL-1 $^{-/-}$ mice.

While IFN-c-producing T_h1 cells are crucial for the induction of autoimmune diseases, IFN-c $^{-/-}$ and/or IFN-cR $^{-/-}$ mice develop autoimmune diseases, such as EAE and collagen-induced arthritis (6–9, 45). Currently, T cell-derived IL-17, rather than IFN-c, is suspected to be critical in the pathogenesis of EAE. In support of this hypothesis, increased levels of IL-17 were observed in the lesions of MS patients (46). Otherwise, IL-12 has been well characterized as a potent activator of IFN-c-producing T_h1 cells, while IL-23, a member of the IL-12 family consisting of IL-23 p19 and IL-12 p40, can induce IL-17 production by T cells (47). IL-23, but not IL-12, is crucial for the development of EAE (48). As seen with IFN-c $^{-/-}$ and IL-12 $^{-/-}$ mice, IL-12Rb2 $^{-/-}$ mice exhibited exacerbated EAE development and increased IL-17 production (49). IL-12 administration, however, led to the inhibition of IL-17 mRNA expression during EAE pathogenesis (50). Currently, the contribution of IL-17 to the pathogenesis of EAE was suggested in mice treated with anti-IL-17-neutralizing antibody (51). We

determined that, in MOG-stimulated T cells, IL-17 production was reduced in IL-1 $^{-/-}$ mice and increased in IL-1Ra $^{-/-}$ mice (Fig. 5A and B). Thus, our data suggest that IL-1 plays an important role in the activation of both IFN-c-producing T_h1 and IL-17-producing $CD4^+$ T cells, contributing to the development of EAE.

In conclusion, our findings suggest that dysregulation of the IL-1/IL-1Ra balance leads to the failure of peripheral lymphoid tolerance for self-antigens, resulting in the severe inflammation seen in EAE. These observations may provide a clue to develop new therapeutics against MS.

Acknowledgements

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Abbreviations

ANOVA	analysis of variance
APC	antigen-presenting cell
CNS	central nervous system
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
IL-1Ra	IL-1R antagonist
IL-1RI	IL-1R type-I
LN	lymph node
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
PTx	pertussis toxin
[^{3}H]TdR	[^{3}H]thymidine
TNF	tumor necrosis factor

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8 The Role of TNFα and IL-17 in the Development of Excess IL-1 Signaling-Induced Inflammatory Diseases in IL-1 Receptor Antagonist-Deficient Mice

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Abstract. IL-1 receptor antagonist (IL-1Ra)-deficient mice spontaneously develop several inflammatory diseases, resembling rheumatoid arthritis, aortitis, and psoriasis in humans. As adoptive T cell transplantation could induce arthritis and aortitis in recipient mice, it was suggested that an autoimmune process is

involved in the development of diseases. In contrast, as dermatitis developed in *scid/scid*-IL-1Ra-deficient mice and could not be induced by T cell transfer, a T cell-independent mechanism was suggested. The expression of proinflammatory cytokines was augmented at the inflammatory sites. The development of arthritis and aortitis was significantly suppressed by the deficiency of TNFα or IL-17. The development of dermatitis was also inhibited by the deficiency of TNFα. These observations suggest that TNFα and IL-17 play a crucial role in the development of autoimmunity downstream of IL-1 signaling, and excess IL-1 signaling-induced TNFα also induces skin inflammation in a T cell-independent manner.

8.1 Introduction

IL-1 is a proinflammatory cytokine functioning in inflammation and host responses to infection (for reviews, see Durum and Oppenheim 1993; Dinarello 1996; Tocci and Schmidt 1997; Nakae et al. 2003a). Originally identified as an endogenous pyrogen, the alternate names for IL-1 of lymphocyte-activating factor, hemopoietin-1, and osteoclast activating factor serve to demonstrate its pleiotropic activity (Dinarello 1991). IL-1, produced by a variety of cells including macrophages, monocytes, keratinocytes, and synovial lining cells, induces inflammation via the activation of synovial cells, endothelial cells, lymphocytes, and macrophages. Upon activation, these cells produce a variety of additional chemokines, cytokines, and inflammatory mediators (Feldmann et al. 1996), including IL-1 itself, IL-17, TNFα, IL-6, IL-8, and cyclooxygenase (COX)-2; these molecules ultimately cause infiltration of leukocytes into inflammatory sites, increase the permeability of blood vessels, and induce fever (Davis and MacIntyre 1992; Dinarello 1996; Tocci and Schmidt 1997; Nakae et al. 2003d).

Two molecular species of IL-1, IL-1α and IL-1β, are derived from two distinct genes on chromosome 2 (mouse, human). Despite a minimal 25% amino acid sequence identity between the molecules (Dinarello 1991), these species exert similar, but not completely overlapping, biological activities through binding to the IL-1 type I receptor (IL-1RI) (Sims et al. 1993; Nakae et al. 2001a, 2001c). Recently, it was reported that the IL-1α precursor, but not IL-1β, moves to the nucleus and activates the transcription of cytokine genes via activation of NF-κB and

AP-1 in an IL-1R-independent mechanism (Werman et al. 2004). While an IL-1 type II receptor (IL-1RII) also exists, this receptor does not appear to function in signal transduction (Colotta et al. 1993).

The IL-1 receptor antagonist (Ra), an additional member of the IL-1 gene family, binds IL-1 receptors without exerting agonist activity (Carter et al. 1990; Hannum et al. 1990). This is because IL-1Ra cannot recruit the IL-1R-accessory protein, a necessary component of an active receptor complex (Greenfeder et al. 1995). Since IL-1Ra competes with IL-1α and IL-1β for the binding of IL-1 receptors (Carter et al. 1990; Hannum et al. 1990), IL-1Ra is considered to be a negative regulator of IL-1 signal.

We have generated IL-1Ra-deficient (IL-1Ra$^{-/-}$) mice (Horai et al. 1998) in which all the three isoforms of IL-1Ra (Muzio et al. 1995) are deleted and demonstrated that these mice spontaneously developed chronic inflammatory arthropathy (Horai et al. 2000). Mice deficient in the IL-1Ra gene have also been reported by Hirsch et al. (1996) and Nicklin et al. (2000); these animals exhibited early mortality and arteritis, respectively. It was recently reported that these mice also spontaneously develop inflammatory dermatitis (Shepherd et al. 2004). However, the pathogenesis of these diseases has not been elucidated completely. Hence, it is remarkable that expression of a variety of inflammatory cytokines, including TNFα, is augmented in these mice.

TNFα, a proinflammatory cytokine, was originally identified as an endotoxin-induced serum factor that causes tumor necrosis (Carswell et al. 1975). TNFα is produced by multiple cell types, including monocytes, macrophages, keratinocytes, and activated T cells. Upon activation with soluble bacterial components or by direct contact with activated T cells at inflammatory sites, TNFα is synthesized in these cells as a membrane-bound precursor. Cleavage by metalloproteinase, TACE/ADAM-17, results in the secretion of a soluble, mature form (Fowlkes and Winkler 2002). While the soluble form may be more potent, both forms of TNFα are biologically active. TNFα can bind to two different cell surface receptors, TNFRI (p55) and TNFRII (p75), on the target cells, including T cells, NK cells, keratinocytes, osteoclasts, and endothelial cells.

IL-17 is another proinflammatory cytokine, originally named cytotoxic T lymphocyte associated serine esterase (CTLA-8) (Rouvier et al.

1993). IL-17 is produced by TCRα/β$^+$CD4$^-$CD8$^-$ thymocytes, as well as activated CD4$^+$ and CD4$^+$CD45RO$^+$ memory T cells (Yao et al. 1995b; Kennedy et al. 1996). In humans, activated CD8$^+$ and CD8$^+$ CD45RO$^+$ memory T cells may also produce IL-17 upon activation with PMA/ionomycin (Shin et al. 1999). While Aarvak et al. reported that IL-17 is produced by Th1/Th0 clone cells, but not by Th2 cells found in the joints of rheumatoid arthritis (RA) patients, Albanesi et al. reported that both Th1 and Th2 cell clones from human skin-derived nickel-specific T cells could produce IL-17 (Aarvak et al. 1999; Albanesi et al. 2000). In patients with lyme arthritis and in mice with microbial infection, however, IL-17 is produced by CD4$^+$ T cells expressing TNFα, but not by Th1 or Th2 cells (Infante-Duarte et al. 2000). In addition to CD4$^+$ and CD8$^+$ T cells, neutrophils also produce IL-17 upon lipopolysaccharide-induced airway neutrophilia (Ferretti et al. 2003). Therefore, a wide variety of cells of the immune system are capable of producing IL-17 under different conditions.

IL-17 exerts pleiotropic activities through activation of IL-17R, which exhibits a ubiquitous tissue distribution (Yao et al. 1995a). The activities include the induction of TNFα, IL-1β, IL-6, IL-8, G-CSF, and MCP-1 on various cell types, the upregulation of ICAM-1 and HLA-DR on keratinocytes, the induction of iNOS and COX-2 on chondrocytes, the stimulation of osteoclast differentiation factor (ODF) production on osteoblasts, and the promotion of SCF- and G-CSF-mediated granulopoiesis (Aggarwal and Gurney 2002; Moseley et al. 2003). IL-17 is detectable in the sera and the diseased organs and tissues of various patients, suggesting involvement in the development of human diseases such as RA, osteoarthritis, multiple sclerosis, systemic lupus erythematosus, and asthma (Aggarwal and Gurney 2002; Kolls and Linden 2004). Furthermore, the use of IL-17R$^{-/-}$ mice has implicated IL-17 in the host defense mechanisms against *Klebsiella pneumoniae* infection (Ye et al. 2001). Recently, we have shown that IL-17 is involved in contact, delayed-type, and airway hypersensitivity responses as well as T-dependent antibody production, but not in acute graft-versus-host reaction, using IL-17$^{-/-}$ mice (Nakae et al. 2002). Furthermore, it was suggested that impaired responses were caused by the defects of allergen-specific T cell activation. Thus, IL-17 plays an important

role in activating T cells in allergen-specific T cell-mediated immune responses.

The pathologic roles of these cytokines and the functional interrelationship among these cytokines in the development of arthritis, aortitis, and dermatitis that develop in IL-1Ra$^{-/-}$ mice, however, remain to be elucidated. In this paper, we will describe the roles for TNFα and IL-17 in the development of diseases resulting from excess IL-1 signaling.

8.2 The Roles of TNFα and IL-17 in the Development of Arthritis

8.2.1 Development of Autoimmune Arthritis in IL-1Ra$^{-/-}$ Mice

IL-1Ra$^{-/-}$ mice on the BALB/c background developed arthritis spontaneously; arthritis began to develop at 5 weeks of age and almost all of the animals suffered from arthritis at 12 weeks of age (Horai et al. 2000). The incidence of arthritis in IL-1Ra$^{-/-}$ mice differed among different genetic backgrounds; the incidence was high on the BALB/c background, but low on the C57BL/6 background, suggesting involvement of BALB/c-specific host genes. The histopathology of the lesions demonstrated a marked synovial and periarticular inflammation with articular erosion caused by invasion of granulation tissues, closely resembling the phenotype of RA in humans (Fig. 1). Osteoclast activation was obvious at the pannus, while inflammatory cell infiltration, consisting mostly of neutrophils, and fibrin clots were detectable in the synovial spaces.

Both total immunoglobulin levels and the levels of autoantibodies specific for immunoglobulin, type II collagen, and dsDNA were elevated in IL-1Ra$^{-/-}$ mice (Horai et al. 2000). The development of arthritis was completely suppressed in *scid/scid*-IL-1Ra$^{-/-}$ mice and adoptive transfer of IL-1Ra$^{-/-}$ T cells induced arthritis in *nu/nu* mice, suggesting a critical role for T cells in the pathogenesis of arthritis in this animal model (Horai et al. 2004). Arthritogenic activated and/or memory T cells were generated in IL-1Ra$^{-/-}$ mice, because T cells from arthritic IL-1Ra$^{-/-}$ mice could transfer disease more efficiently than those from nonarthritic mice. Since IL-1Ra mRNA expression was observed even in unstimulated T cells at low levels and the expression was enhanced

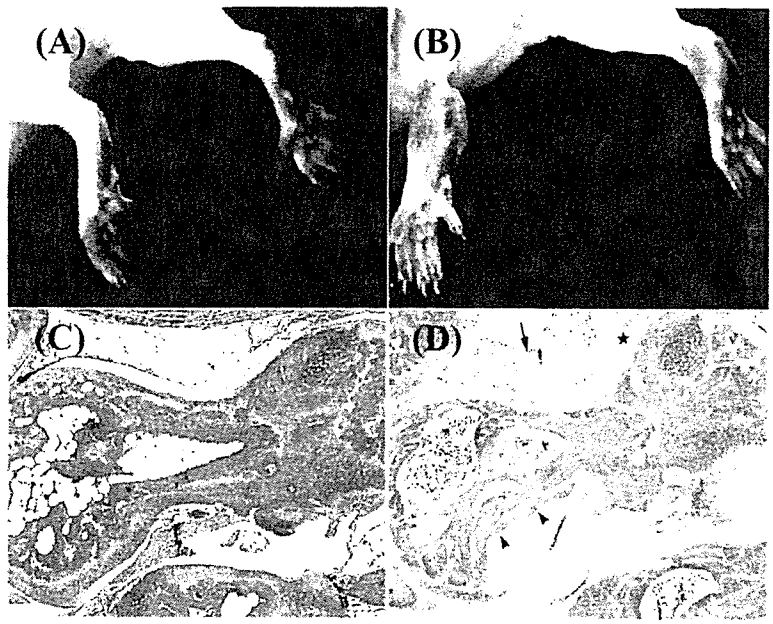

Fig. 1. Histopathology of ankle joints from BALB/c-IL-1Ra$^{-/-}$ mice. The ankles of a normal IL-1Ra$^{+/+}$ mouse (**A**) and an affected IL-1Ra$^{-/-}$ mouse (**B**) were examined at 16 weeks of age. Swelling and redness of the joints were observed in the IL-1Ra$^{-/-}$ mouse. Microscopic observation of the joints of IL-1Ra$^{+/+}$ (**C**) and IL-1Ra$^{-/-}$ (**D**) mice demonstrated the erosive destruction of bone in the IL-1Ra$^{-/-}$ mouse (*arrowheads*). The infiltration of inflammatory cells (*arrows*) and the proliferation of the synovial membrane lining cells (*asterisk*) were remarkable

in activated T cells, these data suggest that, although IL-1Ra is produced by cells of various types including monocytes and macrophages in the synovial lining layer, T cell-derived IL-1Ra likely regulates T cell activity in an autocrine manner.

We have previously shown that antigen-presenting cell (APC)-derived IL-1 can promote T cell activation through the induction of CD40L and OX40 on T cells, suggesting that IL-1 is an important regulator of acquired immune responses (Nakae et al. 2001b). In support of this notion, antibody production against sheep red blood cells increased in

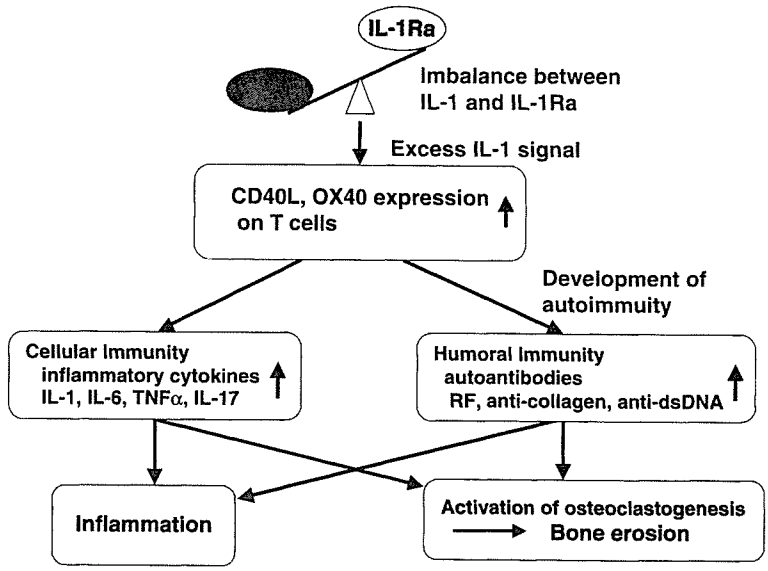

Fig. 2. Excess IL-1 signaling causes autoimmune arthritis

IL-1Ra$^{-/-}$ mice and decreased in IL-1$^{-/-}$ mice (Nakae et al. 2001a). Thus, it is suggested that, in the absence of IL-1Ra, even physiological levels of IL-1, which is constitutively expressed in the joints, can activate naive T cells excessively, resulting in the development of autoimmunity. These observations indicate that the balance between IL-1 and IL-1Ra is critical for homeostasis of the immune system (Fig. 2).

8.2.2 The Role of TNFα in the Development of Arthritis

The expression of numerous inflammatory cytokines, including TNFα and IL-17, was augmented in the joints of IL-1Ra$^{-/-}$ mice. To elucidate the roles of these cytokines in arthritis development, we examined the effect of cytokine deficiency on disease initiation and progression. We determined that the development of arthritis was markedly suppressed in TNFα$^{-/-}$ mice, indicating the crucial role for TNFα in pathogenesis (Horai et al. 2004). A dominant role for TNFα in the development of

RA has been demonstrated by recent clinical trials using therapeutic anti-TNFα antibody (Feldmann and Maini 2001). Studies in the type II collagen-induced arthritis (CIA) mouse model also support this notion (Thorbecke et al. 1992; Joosten et al. 1999; Feldmann and Maini 2001).

While bone marrow (BM) cell transplantation from TNFα$^{+/+}$-IL-1Ra$^{-/-}$ mice into γ-ray-irradiated wild-type (WT) recipient mice induced arthritis, TNFα$^{-/-}$-IL-1Ra$^{-/-}$ BM cells could not induce arthritis. These results indicate that BM-derived cells are responsible for the production of TNFα (Horai et al. 2004). It is interesting, however, that TNFα$^{-/-}$-IL-1Ra$^{-/-}$ BM cells could induce arthritis when inoculated into IL-1Ra$^{-/-}$, but not WT recipient mice. Since TNFα expression is augmented in the joints of IL-1Ra$^{-/-}$ mice, this TNFα may compensate for the deficiency in the BM-derived cells. T cells are sensitive to irradiation, while synovial lining cells are relatively resistant to irradiation. Some of the synovial lining cells, such as type A cells, are derived from the BM origin and are eventually replaced by donor cells after BM cell transplantation. In recipient IL-1Ra$^{-/-}$ mice, TNFα is likely produced by these synovial lining cells, which may donate the arthritogenic milieu observed in IL-1Ra$^{-/-}$ mice in BM cell transfer experiments.

Furthermore, we found that the transfer of TNFα$^{-/-}$-IL-1Ra$^{-/-}$ T cells into *scid/scid* mice did not promote the development of arthritis as robustly as TNFα$^{+/+}$-IL-1Ra$^{-/-}$ T cells, suggesting that T cells are not efficiently sensitized in TNFα$^{-/-}$ mice (Table 1). Thus, these results

Table 1. Inefficiency of the development of arthritis in *scid/scid* mice transferred with T cells from TNFα$^{-/-}$-IL-1Ra$^{-/-}$ mice

Donor	Arthritis	
	Incidence	Score
TNFα$^{+/+}$-IL-1Ra$^{-/-}$	4/5	5.0
TNFα$^{-/-}$-IL-1Ra$^{-/-}$	1/11*	7.5
TNFα$^{+/+}$-IL-1Ra$^{+/+}$	0/5**	0.0

Splenic CD4^{+}T cells (5×10^7) were transferred into BALB.B-*scid/scid* mice, and the development of arthritis was inspected after 12 weeks

* $p < 0.05$ vs TNFα$^{+/+}$-IL-1Ra$^{-/-}$ mice by χ^2 test

** $p < 0.01$ vs TNFα$^{+/+}$-IL-1Ra$^{-/-}$ mice by χ^2 test

suggest that TNFα derived from both T cells and synovial lining cells is involved in the development of arthritis.

We previously showed that IL-1 plays an important role in the enhancement of T cell–APC interactions through the induction of CD40L and OX40 on T cells (Nakae et al. 2001b). Consistently with this observation, CD40L and OX40 expression were enhanced in T cells stimulated with antigen-bearing IL-1Ra$^{-/-}$ APCs in comparison to WT APCs. On the other hand, ligation of CD40 on APCs by CD40L induces OX40L expression and TNFα production by APCs (van Kooten and Banchereau 2000; Weinberg 2002). Furthermore, we have demonstrated that TNFα induces OX40 expression on T cells (Horai et al. 2004). Thus, upon interaction with antigens, APCs produce IL-1. IL-1, in turn, activates T cells, resulting in the induction of CD40L. The CD40L-CD40 interaction induces APCs to produce OX40L and TNFα, resulting in the induction of OX40 on T cells. Therefore, it is suggested that TNFα plays an important role in the sensitization of T cells and contributes to the development of autoimmunity.

It is known that TNFα$^{-/-}$ mice lack splenic primary B cell follicles and cannot form either organized follicular dendritic cell (DC) networks or germinal centers in the spleen and peripheral lymphatic organs (Pasparakis et al. 1996). Prolonged antibody responses are generally impaired in TNFα$^{-/-}$ mice, although Ig class-switching is not completely deficient. It is therefore reasonable to suppose that these functions of TNFα in the humoral immune responses may also contribute to the development of autoimmunity in IL-1Ra$^{-/-}$ mice. However, since we could not induce arthritis by transferring IL-1Ra$^{-/-}$ mouse serum into WT mice, only a weak contribution of humoral immune responses is suggested in this animal model (Horai and Nakajima, unpublished results).

On the other hand, it is known that TNFα elicits inflammation by activating and recruiting inflammatory cells and inducing proinflammatory cytokines and chemokines, such as IL-1, IL-6, and CXCLIO (Pang et al. 1994; Nakae et al. 2003b). In this context, mouse models that exhibit higher amounts of TNFα protein, such as transgenic (Tg) mice carrying the TNFα gene or mice deficient for the TNFα AU-rich element (TNF$^{\Delta ARE}$), spontaneously develop arthritis (Keffer et al. 1991; Kontoyiannis et al. 1999). It was suggested that innate and/or stromal