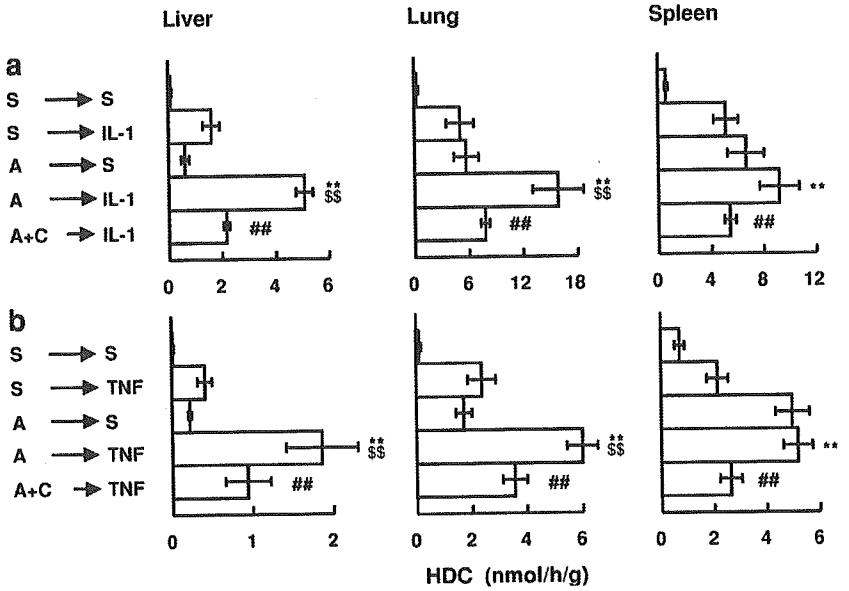

Fig. 2. HDC elevation by IL-1 (a) and TNF (b), and modulating effects of alendronate (A) and clodronate (C). Saline (S), A (40 μmol/kg), or A + C (mixture, each at 40 μmol/kg) was given (i.p.) to mice. Three days later, the mice were injected (i.p.) with S, IL-1β (10 μg/kg), or TNFα (200 μg/kg), then killed 3 h after the second injection. Each value is the mean ± SD from 4 mice. $^{**}P < 0.01$ vs. S → IL-1 or S → TNF. $^{\$\$}P < 0.01$ vs. A → S. $^{\#\#}P < 0.01$ vs. A → IL-1 or A → TNF.

the liver (Fig. 2a). These augmentations induced by alendronate were largely abolished when it was given in combination with clodronate (Fig. 2a). Essentially similar results were obtained in the liver and lungs when TNFα was used instead of IL-1 (Fig. 2b). However, in the spleen when the HDC elevation induced by IL-1 or TNF was examined, the augmentation was not clear (Figs. 2a, b). This may have been because a near-maximal HDC activity was induced in the spleen by alendronate alone. Be that as it may, the HDC elevation in the spleen induced by co-administration of alendronate and clodronate was markedly smaller than the elevations seen when alendronate alone was injected before IL-1 or TNF (Figs. 2a,b).

Alendronate-induced HDC elevation in LPS-pretreated mice and inhibitory effects of clodronate

Next, we examined the alendronate-induced HDC elevation in LPS-pretreated mice. In this experiment, alendronate was injected at 24 h after an LPS injection (0.1 mg/kg), and HDC activity was measured at 3 days after the injection of alendronate. As described above, LPS-induced HDC activity (in the liver, lungs, and spleen) peaks at 2–4 h after the LPS injection; it then declines to almost its basal level within 24 h. As shown in Fig. 3a, the HDC elevation induced by alendronate was markedly augmented by the pretreatment with LPS in all three tissues tested. When alendronate was co-administered with clodronate, the above augmentation effect was not observed (Fig. 3a). The time kinetics of the alendronate-induced HDC elevation seen in LPS-pretreated mice (Fig. 3b) were similar to those previously observed in mice given alendronate alone (Endo et al., 1993), suggesting that the cell types in which HDC is induced by alendronate in LPS-pretreated mice are similar to those in which it is induced in mice given alendronate alone.

Effects of alendronate in IL-1KO mice

We previously reported for N-BPs that their inflammatory actions (including HDC induction) are weak in IL-1KO mice, and that in such mice, N-BPs did not augment LPS-stimulated HDC induction (Yamaguchi et al., 2000). In the present study, we found that in IL-1KO mice, alendronate induced no significant HDC elevation even when the mice were pretreated with LPS (Fig. 4a), and that alendronate pretreatment did not augment the IL-1-induced HDC elevation (Fig. 4b).

Effects of alendronate on IL-1α and IL-1β production

As described above, the augmenting effect of alendronate on HDC induction, as well as the HDC-inducing activity of alendronate itself, depend on IL-1. However, alendronate itself did not induce any detectable elevation in the serum level of IL-1α, IL-1β, or HDC activity (data not shown), although LPS-stimulated IL-1 production is augmented in alendronate-treated mice (Sugawara et al., 1998; Yamaguchi et al., 2000; and Fig. 1 in this study). Hence, we tried to detect tissue levels of IL-1. To test the reliability of the methods we used to measure IL-1α and IL-1β in tissues, the following experiments were carried out. First, we compared the basal levels of IL-1α and IL-1β in the serum, liver, spleen, and lungs between control and IL-1KO mice. IL-1α and IL-1β were detected in all the tissues tested (particularly in the spleen) of control mice (Fig. 5). However, significant amounts of IL-1α and IL-1β were detected even in IL-1KO mice, several nanograms per gram in the tissues and several picograms per milliliter in the serum, although all levels were lower than the corresponding ones in control mice. These results indicate that the values for IL-1 obtained using the present method include IL-1 and also some non-IL-1 substances.

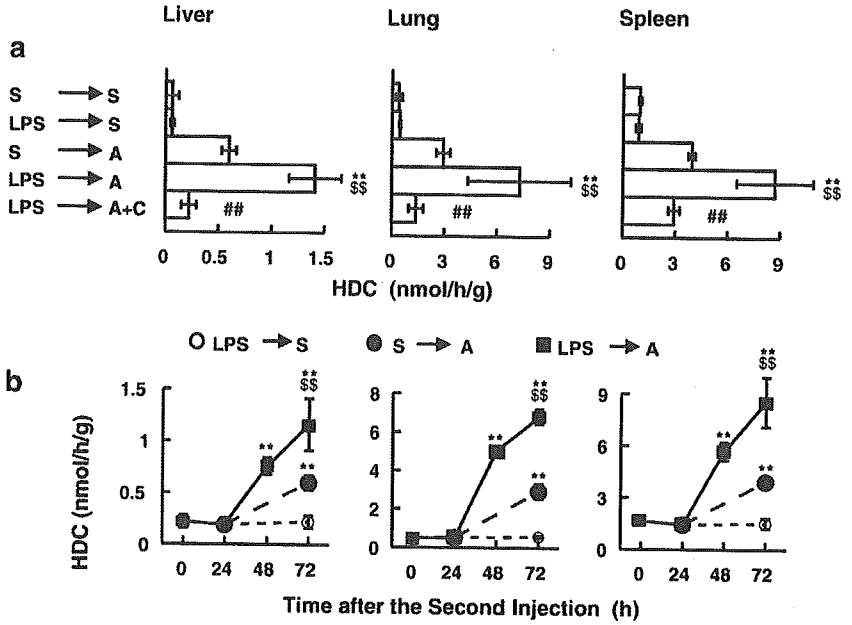

Fig. 3. (a) HDC elevation by alendronate (A) in LPS-pretreated mice, and effects of clodronate (C). Saline (S) or LPS (0.1 mg/kg) was given (i.p.) to mice. Twenty-four hours later, the mice were injected (i.p.) with S, A (40 μmol/kg), or A + C (mixture, each at 40 μmol/kg), and tissues were removed at 3 days after the second injection. Each value is the mean ± SD from 4 mice. $^{**}P < 0.01$ vs. LPS → S, $^{\$\$}P < 0.01$ vs. S → A, $^{\#\#}P < 0.05$ vs. LPS → A. (b) Time course of the HDC elevation induced by alendronate (A) in LPS-pretreated mice. Saline (S) or LPS (0.1 mg/kg) was given (i.p.) to mice, and 24 h later the mice were injected (i.p.) with S or A (40 μmol/kg). Tissues were removed at the indicated times after the second injection. Each value is the mean ± SD from 4 mice. $^{**}P < 0.01$ vs. time 0, $^{\$\$}P < 0.01$ vs. S → A.

As shown in Fig. 6, the IL-1β level increased in all three tissues tested (but not in the serum) following an alendronate injection. However, IL-1α showed an increase only in the liver (at 72 h after the alendronate injection, to about twice the basal level; data not shown). To confirm that the IL-1β elevations shown in Fig. 6 did indeed represent, or at least include, an elevation of IL-1β, we examined the effect of alendronate in IL-1KO mice. We could detect no significant elevation of IL-1α or IL-1β in the liver, lungs, and spleen of IL-1KO mice at 72 h after an injection of 40 μmol/kg alendronate (data not shown). These results strongly suggest that alendronate does indeed increase IL-1β in the tissues of control (wild-type) mice.

Next, we examined the levels of these cytokines at 48 and 72 h after an alendronate injection into mice pretreated with LPS 24 h before the alendronate injection. In this experiment,

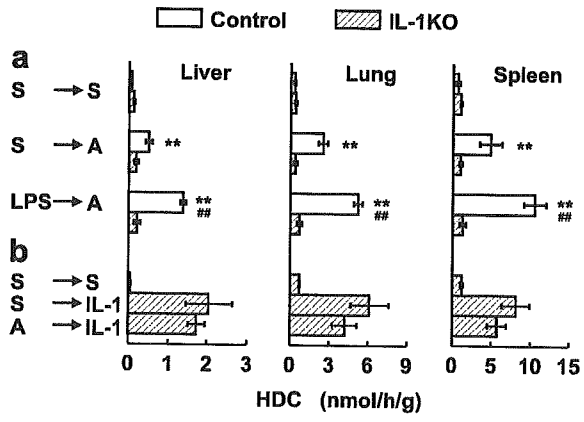

Fig. 4. HDC induction in IL-1KO mice. (a) Effects of alendronate (A) on HDC activity in IL-1KO mice pretreated with saline or LPS. Control and IL-1KO mice were injected (i.p.) with saline (S) or LPS (0.1 mg/kg). Twenty-four hours later, the mice were injected (i.p.) with S or A (40 μmol/kg), and tissues were removed at 3 days after the second injection. Each value is the mean ± SD from 4 mice. $^{**}P < 0.01$ vs. S → S in control (wild-type) mice, $^{\#\#}P < 0.01$ vs. S → A in control (wild-type) mice. (b) Effects of IL-1 on HDC activity in IL-1KO mice pretreated with S or A. First, S or A (40 μmol/kg) was injected (i.p.). Then, 3 days later, S or IL-1β (10 μg/kg) was injected (i.p.) into the some mice, and tissues were removed 3 h after the injection. Each value is the mean ± SD from 4 mice.

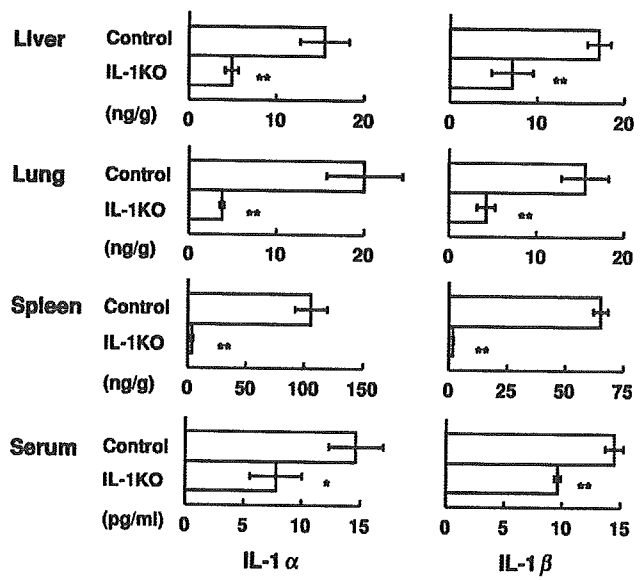

Fig. 5. Basal levels of IL-1α and IL-1β in the tissues of control and IL-1KO mice. Note the different scales among the various panels. Each value is the mean ± SD from 4 mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control (wild-type) mice.

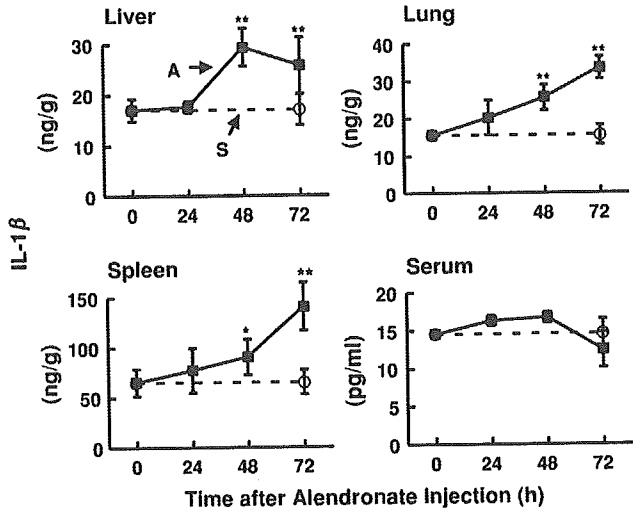

Fig. 6. IL-1β levels in the tissues and serum following alendronate injection. Alendronate (A, 40 μmol/kg) or saline (S) was injected (i.p.) into mice, and blood and tissues were taken at the indicated times. Each value is the mean ± SD from 4 mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. time 0.

too, we could detect no elevation of IL-1α or IL-1β in the serum. In addition, there was no significant elevation of IL-1α in the tissues (liver, lungs, or spleen) in mice given LPS before alendronate (data not shown). Although IL-1β was elevated in the tissues in mice given LPS + alendronate at both 48 h (Fig. 7a) and 72 h (Fig. 7b), these increases were no greater than those seen in mice given saline + alendronate. In mice given LPS alone (0.1 mg/kg) 3 or 4 days before sacrifice, no elevation of IL-1β was detected in these three tissues (data not shown). In this experiment, we also noted that co-administration of clodronate prevented or reduced the elevation of tissue IL-1β induced by alendronate in LPS-pretreated mice (Figs. 7a and b).

Discussion

Mutual augmentation between alendronate and immuno-stimulants (IL-1, TNF, and LPS)

IL-1 and TNF are important in diseases such as rheumatoid arthritis, sepsis, atherosclerosis, Alzheimer's disease, cancer, asthma, periodontitis, and gastrointestinal disorders (Dinarello, 1996; Iwakura, 2002; Hallegua and Weisman, 2002; Graves and Cochran, 2003). Here and previously, we demonstrated that alendronate pretreatment augments HDC induction by immuno-stimulants (LPS, IL-1, or TNF), and (conversely) LPS pretreatment augments HDC induction by alendronate. These results indicate that alendronate and those immuno-stimulants mutually augment HDC induction. These findings in murine models lead us to speculate that alendronate (or other N-BPs) might augment the inflammatory reactions induced in human patients by immuno-stimulants or bacterial infections, and that, conversely, the inflammatory actions of alendronate may be augmented in a patient who already has an infectious disease. We speculate that some of the human patients who exhibit an unusually high sensitivity to the inflammatory actions of N-BPs might have a preexisting bacterial infection. In recent years, a high incidence of osteonecrosis of the jaws has been reported in patients who have undergone N-BP therapy, and the involvement of oral bacteria has been suggested (Ruggiero et al., 2004; Bagan et al., 2005; Hellstein and Marek, 2005). Moreover, it should be noted that N-BPs bind strongly to bones and accumulate there upon repeated administration (Geddes et al., 1994; Mönkkönen et al., 1989). Thus, the present findings lead us to speculate that the serious effect of N-BPs on the jaw might be, at least in part, due to a mutual augmentation of inflammatory reactions between the accumulated N-BPs and oral bacteria or their products.

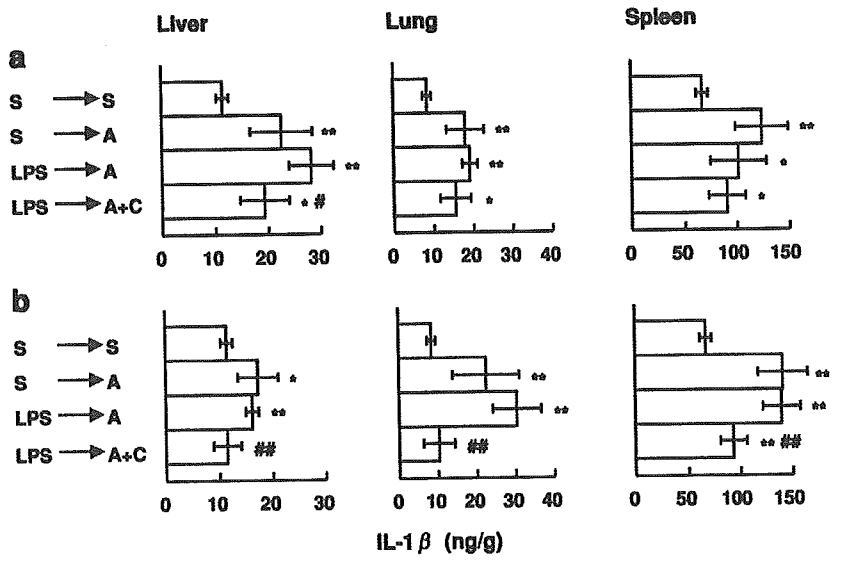

Fig. 7. Effects of LPS-pretreatment on the IL-1β elevation induced by alendronate (A), and the modulating effects of clodronate (C). Saline (S) or LPS (0.1 mg/kg) was given (i.p.) to mice. Twenty-four hours later, the mice were injected (i.p.) with S, A (40 μmol/kg), or A + C (mixture, each at 40 μmol/kg). Tissues were removed at 48 h (a) or 72 h (b) after the second injection. Each value is the mean ± SD from 4 mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. S → S. $^{\#}P < < 0.05$ and $^{\#\#}P < 0.01$ vs. LPS → A.

Involvement of tissue IL-1β in the inflammatory action of N-BPs

Our previous and present studies demonstrate that in alendronate-pretreated mice, LPS-induced elevations of serum IL-1α and IL-1β are augmented. The inflammatory actions (including HDC elevation) of N-BPs are largely abrogated in IL-1KO mice (Yamaguchi et al., 2000), and there was no mutual augmentation between alendronate and immuno-stimulants in IL-1KO mice. We detected IL-1β in all the tissues tested (liver, lungs, and spleen) after alendronate injection (Figs. 6 and 7). It is noteworthy that the increases in IL-1β in the tissues are at the level of nanogram per gram (Fig. 6). In mice, N-BPs increase macrophages and/or granulocytes in the peritoneal cavity and their precursor cells in the bone marrow (Endo et al., 1993; Nakamura et al., 1999). The augmentation of the LPS-induced production of IL-1β (and IL-1α) seen in alendronate-treated mice is markedly reduced by administration of clodronate-encapsulated liposomes (a macrophage-depleting agent), although a significant production of IL-1β still remains (Sugawara et al., 1998). These results support the idea that (1) IL-1 is a prerequisite for N-BPs to induce their inflammatory effects with IL-1β being more important than IL-1α, (2) the actions of IL-1β may be largely exerted within the tissue in which it is formed, and (3) macrophage-derived IL-1β plays a major role in mediating the inflammatory actions of alendronate.

Strangely, alendronate by itself did not induce any detectable increase in IL-1 in the serum, although alendronate increased IL-1β in the tissues (in particularly large amounts in the spleen). We investigated whether alendronate stimulates spleen cells to release IL-1α and IL-1β in vitro, but we failed to detect these cytokines (data not shown). At present, we are unable to explain why alendronate was ineffective at stimulating spleen cells to release IL-1 in vitro. The above results might be interpreted as suggesting that membrane-bound IL-1β, as observed on platelets (Howrylowicz et al., 1989), is responsible for the inflammatory actions of alendronate itself. In humans, IL-1 has not been detected in the serum after administration of N-BPs, either (Thiébaud et al., 1997). However, it is possible that IL-1 is increased in the tissues in humans, as it is in mice. Miyagawa et al. (2001) demonstrated that macrophages are essential for N-BPs to activate primary human γδ T cells, suggesting that the presentation of N-BPs to γδ T cells by macrophages is critical for the activation of the γδ T cells. During this reaction, it is likely that macrophages produce IL-1.

In alendronate-pretreated mice, the LPS-induced production of TNFα was suppressed (Fig. 1). However, TNFα was still produced in larger amounts than IL-1β (note that the scales in Fig. 1 for these cytokines are different). In humans, TNFα has been implicated in the inflammation induced by N-BPs (Schweitzer et al., 1995; Sauty et al., 1996; Thiébaud et al., 1997). Recent studies have demonstrated that N-BPs stimulate human γδ T cells in vitro to release TNFα and/or IFNγ (Kunzmann et al., 2000) via an inhibition of the mevalonate pathway (Thompson and Rogers, 2004). Takagi et al. (2005) reported that co-administration of pamidronate (an N-BP) and IFN-γ stimulates macrophages to increase the serum level of TNFα as early as 2 h after its injection into mice. However, we could not detect any increase in TNFα in the serum of mice at 24, 48, or 72 h after an injection of alendronate alone (data not shown). Since we detected no inflammatory reactions following alendronate injection in IL-1KO mice, the contribution of TNFα to the inflammatory effects of alendronate in mice may be smaller than that of IL-1 (or possibly TNFα may play roles different from those played by IL-1).

It has been shown that the inhibitory effects of N-BPs on bone resorption are due to their inhibitory effects on the formation of mevalonate-derived intermediates in the cholesterol biosynthesis (Rogers et al., 2000). The importance of this is that the above pathway is found widely in eukaryotic cells. Indeed, N-BPs act on rat liver (Amin et al., 1992), on osteocytes and osteoblasts (Plotkin et al., 1999), affect the synthesis of high- and low-density lipoprotein cholesterol (Adami et al., 2000), and deposit for a significant time in the spleen and liver in mice (Mönkkönen et al., 1989). N-BPs even exhibit weed-killer and anti-parasite activities (Martin et al., 1999). Moreover, N-BPs act on macrophage-like J774 cells (Amin et al., 1992, 1996) and γδT cells (Kunzmann et al., 1999, 2000) to stimulate the production of proinflammatory cytokines. Thus, N-BPs may influence the functions of a variety of cells once they are incorporated into these cells, and their inflammatory effects may be a reflection of one (or more) of these influences. However, at present, we have no data to indicate the involvement of the mevalonate pathway in the in vivo inflammatory actions of N-BPs. Incidentally, Töyräs et al. (2003) reported that inhibition of the mevalonate pathway is not involved in the secretion of proinflammatory cytokines from RAW 264 cells (a murine macrophage cell line) in vitro.

Mechanisms underlying the HDC-augmenting effect of N-BPs

We have suggested elsewhere that the major cells in the body in which HDC is induced in response to IL-1, TNFα, or LPS may be vascular endothelial cells and granulocytic cells (Endo et al., 1995; Wu et al., 2004). As described above, N-BPs increase macrophages and/or granulocytes in the peritoneal cavity and also their precursor cells in the spleen and bone marrow, and moreover, a variety of cells (including these cells) are known to respond to IL-1 and TNF (Dinarello, 1984, 1996). As shown in Fig. 7, the increase in IL-1β in the tissues of mice given LPS and then alendronate was no larger than that seen in mice given saline and then alendronate. Thus, the mutual augmentations of HDC elevation described above may mainly be due to an IL-1-dependent increase in the number of HDC-inducing cells. It is also possible that the cells capable of inducing HDC acquire the potential to induce more HDC.

Antagonism between clodronate and N-BPs

The N-BP-induced inflammatory actions examined by us can be largely suppressed by clodronate (Endo et al., 1999 and the present study). For its anti-bone-resorptive activity, clodronate requires a dose several hundred times larger than

that of alendronate in milligrams per kilogram terms (Geddes et al., 1994). However, in our model, the inflammatory actions of alendronate were strongly inhibited by clodronate at a dose equivalent to the dose of alendronate. Incidentally, clodronate (even at 500 μmol/kg), when injected at 10 min, 30 min, or 3 days before an LPS injection, does not inhibit the LPS-induced production of IL-1α and IL-1β or the LPS-induced elevation of HDC activity (Endo et al., 1999), indicating that the antagonism by clodronate is specific against N-BPs. Antagonism between clodronate and N-BPs has recently been observed in vitro in osteoclasts, J774 cells, and human peripheral blood γδ T cells (Frith and Rogers, 2003; Thompson and Rogers, 2004). Those authors supposed that clodronate might compete with N-BPs for cellular uptake via a membrane-bound transport protein, although no such protein has yet been identified. In that hypothesis, clodronate is also supposed to enter the cells. However, the in vitro competitive effect of clodronate is very weak, reaching significance only after a long period of culture (8 h or more) in the presence of as much as a 40-fold greater amount of clodronate than of the N-BP (Frith and Rogers, 2003). In mice in vivo, clodronate is largely excreted via the kidney within 30 min of its intravenous injection, although a small proportion is retained for a very long time within bone (Mönkkönen et al., 1989). In contrast, a significant amount of N-BPs is retained within the liver and spleen for 10 h or more (Mönkkönen et al., 1989). These results suggest that N-BPs may enter cells in these tissues, but that clodronate may hardly enter the cells at all. Thus, we think that the above hypothesis may not be an adequate explanation for the antagonizing effect of clodronate, at least in our murine in vivo model. We speculate that clodronate may inhibit the binding of alendronate (or other N-BPs) to specific sites (on macrophages, granulocytes, and/or vascular endothelial cells) that are involved either in the inflammatory effects of N-BPs or in the cellular uptake of N-BPs. As clodronate does not inhibit the action of LPS, the sites may be different from Toll-like receptor 4 (TLR4), a pattern recognition receptor for LPS (Akira, 2003). If our hypothesis is correct, co-administration of clodronate and an N-BP may be a convenient way of restricting the effect of the N-BP to osteoclasts alone (osteoclasts being the cells that take up the N-BPs bound to bones during the course of bone resorption; Sato et al., 1991).

Conclusion

The present results indicate that in their ability to induce HDC, mutual augmentations exist between alendronate and immuno-stimulants (IL-1, TNF, and LPS), and that tissue IL-1β is important in alendronate-stimulated HDC induction. If they can be extrapolated to humans, our findings suggest that when an N-BP-treated patient catches an infectious disease, the infection-related inflammatory reactions might be augmented, and that, conversely, the inflammatory effects of alendronate itself might be augmented in a patient who already has an inflammatory or infectious disease. Our results also suggest that the use of clodronate plus an N-BP might represent a useful strategy for preventing or reducing both the inflammatory side effects of the N-BP itself and the mutual augmentation of inflammation between the N-BP and immuno-stimulants, while preserving the powerful anti-bone-resorptive activity of the N-BP. However, some issues remain to be clarified, namely, the precise nature of the mechanisms underlying the inflammatory actions of N-BPs, as well as those underlying their antagonism by clodronate.

Acknowledgments

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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-1α, IL-1β, IL-1α and IL-1β (IL-1) or IL-1Ra genes. IL-1α/β double-deficient (IL-1$^{-/-}$) mice exhibited significant resistance to EAE induction with a significant reduction in disease severity, while IL-1α$^{-/-}$ or IL-1β$^{-/-}$ 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-γ and IL-17 production and proliferation were reduced in IL-1$^{-/-}$ T cells upon stimulation with MOG, while IFN-γ, IL-17 and tumor necrosis factor-α 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-γ, which contributes to the etiology of a wide range of diseases, is markedly elevated within the CNS during EAE. IFN-γ-deficient and IFN-γR$^{-/-}$ mice, however, remain highly susceptible to EAE (6–9). In fact, the over-expression of IFN-γ in the CNS ameliorated the severity of EAE (10). Tumor necrosis factor (TNF)α, a potent pro-inflammatory

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cytokine, is produced by a variety of cell types, including T_h1 cells. Mice over-expressing TNFα within the CNS exhibit neuronal demyelination (11, 12), while the development of EAE in $TNF\alpha^{-/-}$ mice is partially suppressed (13, 14). Other group reported, however, that in $TNF\alpha^{-/-}$ 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-γ and TNFα, 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 $IL\text{-}1^{-/-}$ and $IL\text{-}1Ra^{-/-}$ 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

$IL\text{-}1\alpha^{-/-}$, $IL\text{-}1\beta^{-/-}$, $IL\text{-}1\alpha/\beta^{-/-}$ ($IL\text{-}1^{-/-}$) and $IL\text{-}1Ra^{-/-}$ 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 μg MOG 35–55 emulsified in CFA (1 : 1) supplemented with 400 μg *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.

Titer 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 μg per 96 well) was coated onto 96-well plates and incubated at 4°C overnight. After substantial washing and blocking, diluted sera (30 μl 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 titer 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 μg 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 poplitial). 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-μm 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 × *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 (1×10^4 cells) in the presence or absence of T cells (1×10^5 cells) were plated on 96-well plates coated with MOG 35–55 in a final volume of 200 μl RPMI 1640–10% FCS. After 72 h of culture, cells were pulsed with [^{3}H]thymidine ([^{3}H]TdR) (0.25 μCi 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 TNFα were measured as described (30, 31). IFN-γ levels were measured with OptEIA® Set mouse IFN-γ kit (BD PharMingen). All assays were done in duplicate.

Statistical analysis

All values were calculated as the average ± 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-1α or IL-1β 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-1α$^{-/-}$ and IL-1β$^{-/-}$ 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-1α or IL-1β 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

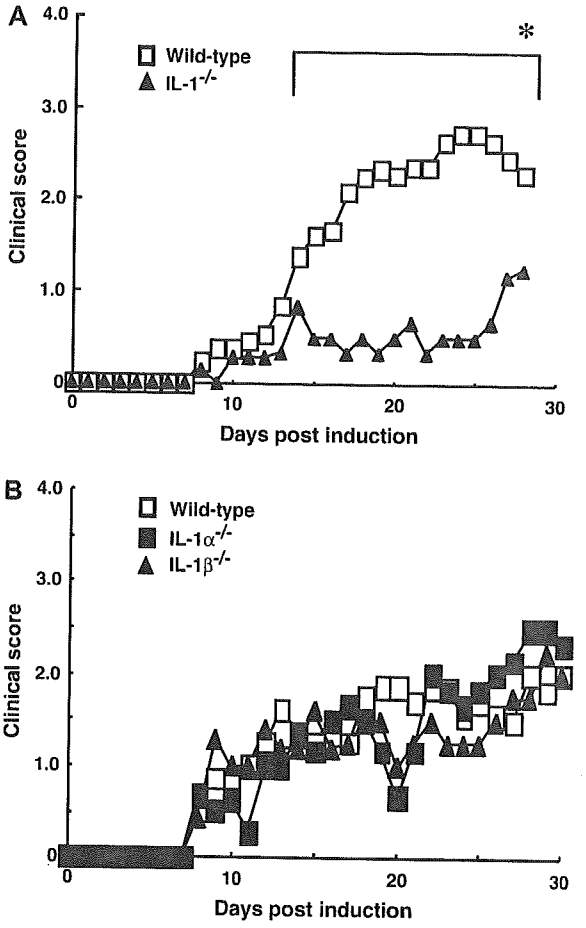

Fig. 1. Attenuated EAE induction in IL-1$^{-/-}$ mice. The clinical scores after EAE induction were determined as described in Methods. The averages of clinical scores are shown from the day of MOG immunization (day 0) to day 28 post-immunization in (A) wild-type (open squares, $n = 9$) and IL-1$^{-/-}$ (filled triangles, $n = 7$) mice and (B) wild-type (open squares, $n = 8$), IL-1α$^{-/-}$ (filled squares, $n = 9$) and IL-1β$^{-/-}$ (filled triangles, $n = 7$) 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 TNFα$^{-/-}$ 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 (lost)	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-$1\alpha^{-/-}$	9/9 (3)	9.8 ± 1.3	2.6 ± 0.7
	IL-$1\beta^{-/-}$	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).

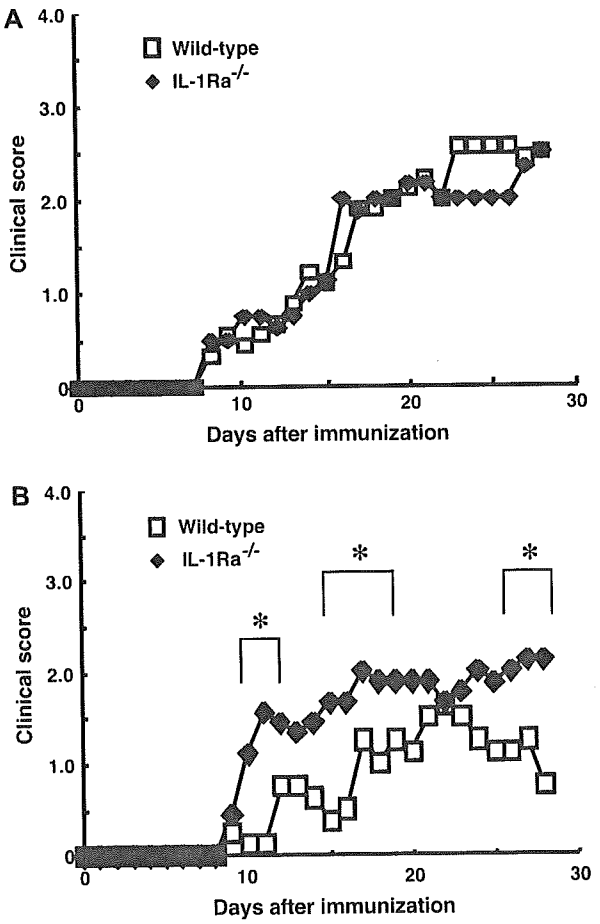

Fig. 2. Exacerbated EAE induction without PTx injection in IL-$1Ra^{-/-}$ mice. Clinical scores after MOG immunization in the (A) presence or (B) absence of PTx (500 ng) injection in wild-type [open squares, (A) $n = 13$ and (B) $n = 8$], and IL-$1Ra^{-/-}$ mice [filled diamonds, (A) $n = 12$ and (B) $n = 9$]. 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 μg 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

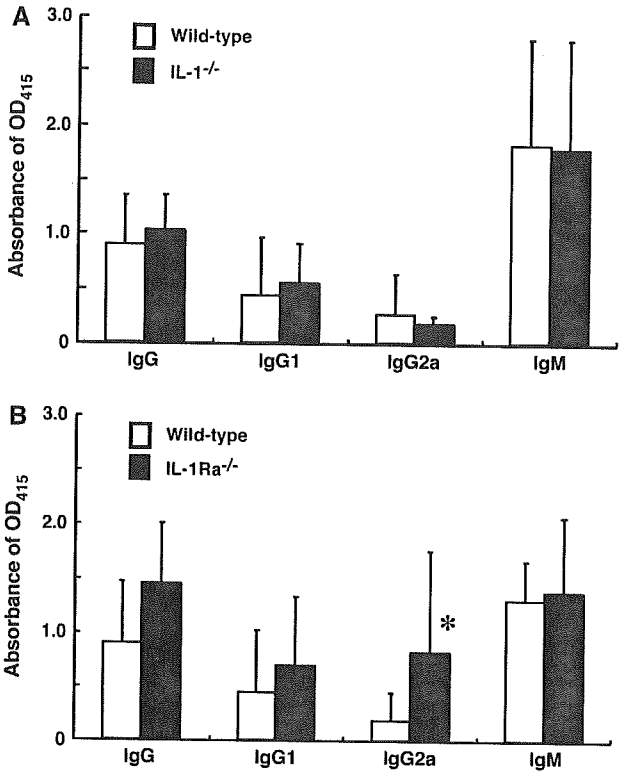

Fig. 3. Titer of anti-MOG antibodies in serum of IL-$1^{-/-}$ and IL-$1Ra^{-/-}$ mice. Thirty-seven days after EAE induction (A) with PTx injection (wild-type, open bars and IL-$1^{-/-}$ mice, filled bars) or (B) without PTx injection (wild-type, open bars and IL-$1Ra^{-/-}$ mice, filled bars), we collected 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 ± 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 μg 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-γ and TNFα 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-γ and IL-17, but not TNFα, 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-γ, IL-17 and TNFα 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-$1\alpha^{-/-}$, IL-$1\beta^{-/-}$, IL-$1^{-/-}$ and IL-$1Ra^{-/-}$ mice, we demonstrate that IL-1 is responsible for the development of EAE. Either IL-1α or IL-1β 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 TNFα (34). This TNFα 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 demonstrate 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

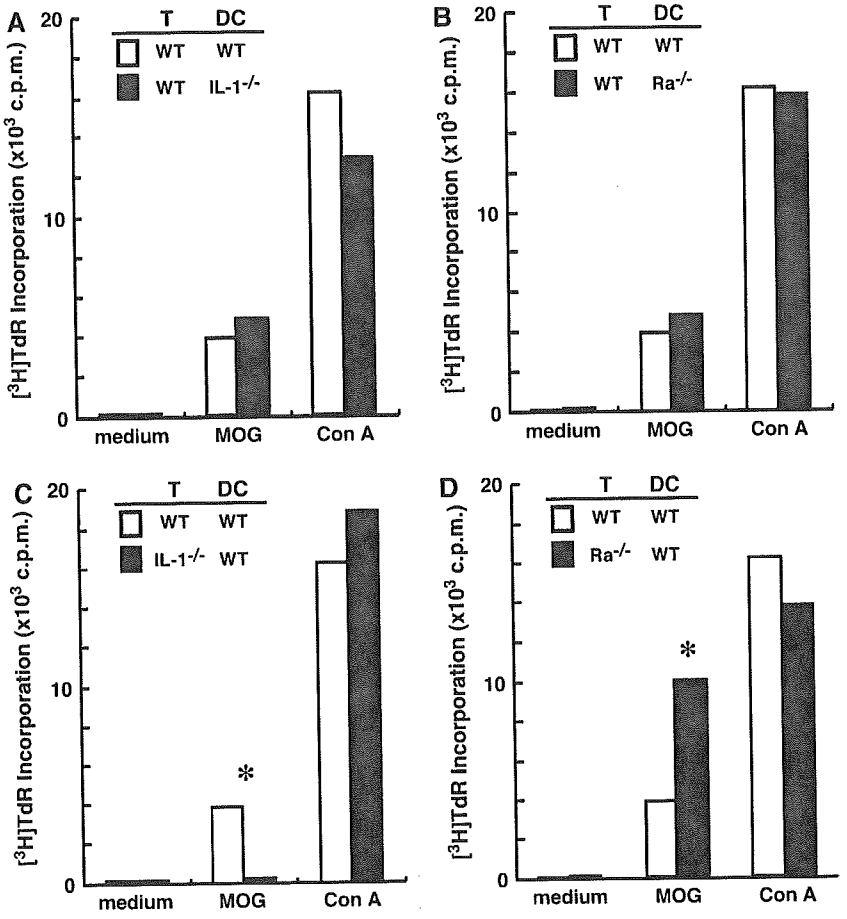

Fig. 4. Abnormal T cell activation in IL-$1^{-/-}$ and IL-$1Ra^{-/-}$ mice immunized with MOG. Wild-type, IL-$1^{-/-}$ and IL-$1Ra^{-/-}$ mice were immunized with a MOG 35–55/CFA emulsion without PTx co-administration. Ten days after MOG immunization, LN T cells were cultured with splenic DCs in the absence (medium) or presence MOG of MOG 35–55 (100 μg ml^{-1}) or Con A (1 μg 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 TNFα$^{-/-}$ mice was completely suppressed in the presence of low doses of PTx, although susceptibility to the disease in TNFα$^{-/-}$ 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 Gi/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 TNFα. 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 TNFα) 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-γ, TNFα and IL-17, T cell-derived inflammatory cytokines, play critical roles in multiple pathological inflammatory responses. TNFα has a similar biological activity to IL-1 as a potent pro-inflammatory cytokine. As seen in studies using TNFα$^{-/-}$ mice, TNFα is also involved in the development of EAE (13, 14). Interestingly, TNFα 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 TNFα production (Fig. 5B) and exacerbated development of EAE (Fig. 2 and Table 1). Thus, TNFα is not essential for, but contributes to, the development of EAE (15, 23, 44). Excess TNFα production resulting from excessive

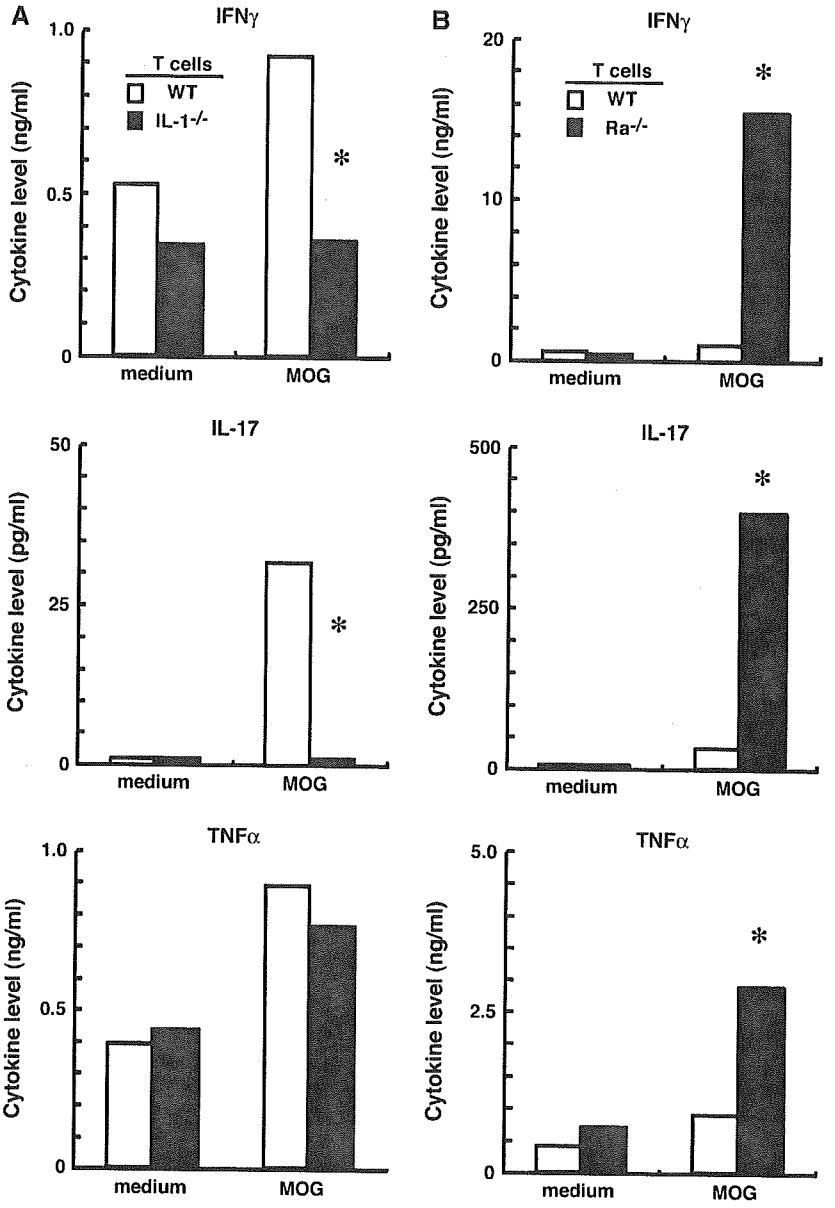

Fig. 5. Abnormal cytokine production from IL-$1^{-/-}$ T cells and IL-$1Ra^{-/-}$ T cells. MOG-sensitized T cells from (A) wild-type or IL-$1^{-/-}$ mice and (B) wild-type or IL-$1Ra^{-/-}$ mice were co-cultured with wild-type DCs in the absence (medium) or presence MOG of MOG 35–55, as shown in Fig. 4. IFN-γ, IL-17 and TNFα 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 TNFα alone is not sufficient to induce adequate responses in the absence of IL-1, as observed in IL-$1^{-/-}$ mice.

While IFN-γ-producing T_h1 cells are crucial for the induction of autoimmune diseases, IFN-$\gamma^{-/-}$ and/or IFN-$\gamma R^{-/-}$ mice develop autoimmune diseases, such as EAE and collagen-induced arthritis (6–9, 45). Currently, T cell-derived IL-17, rather than IFN-γ, 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-γ-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-$\gamma^{-/-}$ and IL-$12^{-/-}$ mice, IL-12Rβ$2^{-/-}$ 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-γ-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