Table 3 *Immunohistochemical distribution of mutant AR in the non-neural tissues of patients with SBMA*

Region	Nuclear accumulation		Cytoplasmic accumulation
	Diffuse nuclear accumulation	NI	
Pituitary gland	–	–	–
Heart	–	–	–
Lung	–	–	–
Liver	+	+	+
Kidney	+ to ++	+	–
Pancreas	–	–	+ to ++
Intestine	–	–	–
Spleen	–	–	–
Thyroid	–	–	–
Adrenal gland	–	–	–
Testis	+ to ++	+	+ to ++
Prostate gland	+ to +++	+ to ++	+ to +++
Skeletal muscle	–	–	–
Scrotal skin	+ to +++	+	–
Skin	+ to ++	+	–

Frequency of cells expressing polyglutamine immunoreactivity: –, 0%; +, 0–3%; ++, 3–6%; +++, 6%.

accumulation exerts cytotoxicity in neural and non-neural tissues. Cytoplasmic mutant AR accumulation (Taylor *et al.*, 2003) as well as other mutant protein accumulations (Kegel *et al.*, 2000; Ravikumar *et al.*, 2002; Huynh *et al.*, 2003) involving an expanded polyQ tract in Golgi apparatus and lysosomes indeed has been found to induce cytotoxicity. Accumulation of mutant protein with expanded polyQ in the Golgi apparatus or lysosomes increases death of cultured cells through activation of apoptosis-related effectors such as caspase-3 (Ishisaka *et al.*, 1998; Kegel *et al.*, 2000; Huynh *et al.*, 2003). One should note that histologically or immunohistochemically evident mutant protein accumulation is not necessarily cytotoxic, while microaggregates at the molecular level that are histologically undetectable can also exert cytotoxicity. Indeed, excessive accumulation of mutant AR in aggresomes was found to protect cells from a cytotoxic form of mutant AR (Taylor *et al.*, 2003). However, our present study strongly suggests that these cytoplasmic mutant AR accumulations may be related to mutant AR-mediated cytotoxicity and eventual symptom manifestation. For instance, the pancreas showed only cytoplasmic mutant AR accumulation without obvious nuclear accumulation. Elevated serum glucose and impaired glucose tolerance were present in most of our patients, suggesting islet cell dysfunction in the pancreas. The frequency of cytoplasmic accumulations of mutant AR in pancreatic islet cells did not show a significant correlation with fasting blood glucose levels in the examined SBMA patients (data not shown), while certain symptoms and signs of SBMA apparently can be induced by cytoplasmic accumulation of mutant AR protein. Although further study of the significance of cytoplasmic mutant AR accumulation is needed, nuclear accumulation of the mutant AR protein appears to cause motor neuron dysfunction while cytoplasmic accumulation may underlie some visceral and possibly some neuronal dysfunction in SBMA. The pathological process is likely to differ between tissues, being more prominent in motor neuron nuclei, but mainly cytoplasmic in certain neuronal populations and visceral organs. We also need to clarify further which degradation process affecting mutant AR is most active in a given tissue, e.g. lysosomal in certain viscera versus via ubiquitination pathway in most neural tissues.

An important question here is why diffuse nuclear and possibly cytoplasmic accumulation of the mutant AR in the neuronal tissues beyond the major affected spinal and brainstem motor neurons has no apparent symptomatic involvement. First, the causative lesions for sensory impairment and essential-type tremor in SBMA patients have not yet been clearly substantiated. The novel lesion distribution of SBMA neurons shown in the present study, such as the posterior horn of the spinal cord, dorsal root ganglia, thalamus and cerebellum, might provide some explanations for these clinicopathological problems that have not been resolved. Since the cerebellothalamocortical pathway seems to be responsible for essential-type tremor (Pinto *et al.*, 2003), these lesions might contribute to mostly subclinical but definite sensory impairment and essential-type tremor in SBMA. Secondly, the occurrence of neuronal nuclear and cytoplasmic abnormalities in both clinically affected and non-affected neural regions in SBMA suggests that this alteration does not always induce neuronal cell dysfunction or death. The selective neuronal loss and dysfunction in neural lesions that are characteristic of SBMA might depend on additional factors that are specific to neurons in these systems. Recent studies have demonstrated that CREB-binding protein (CBP) is sequestered in AR-positive NIs, resulting in a decrease in CBP-dependent transcription (McCampbell *et al.*, 2000), and further histone acetylation is reduced in affected cells (McCampbell *et al.*, 2001; Steffan *et al.*, 2001; Minamiyama *et al.*, 2004). These reports suggest that CBP-dependent transcriptional dysregulations may cause symptomatic neuronal dysfunction. Since CBP-dependent transcriptional control differs among neurons, this difference may show the lack of their symptomatic involvement in certain polyQ-containing neurons. Alternatively, the population of neurons with nuclear accumulation of mutant AR in the regions beyond the commonly affected lesions may not be simply enough to manifest the responsible symptoms. A precise neuronal cell count assay combined with assessment of nuclear mutant AR accumulation will be needed to clarify these clinicopathological problems.

Clearly, motor neuron impairment with nuclear accumulation of mutant AR is the major problem in SBMA. Thus, for a therapeutic strategy against motor neuron dysfunction in SBMA, nuclear accumulation of mutant AR should be the main target, as we demonstrated in transgenic mice treated with leuprolerin. Cytoplasmic accumulation of mutant AR, on the other hand, should be considered a therapeutic target with respect to certain symptoms in SBMA patients.

Acknowledgements

We wish to thank Dr Yasushi Iwasaki and Mrs Sugiko Yokoi for technical assistance. This work was supported by a Center of Excellence (COE) grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and grants from the Ministry of Health, Labor, and Welfare of Japan.

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International Immunology, Vol. 18, No. 2, pp. 399–407
doi:10.1093/intimm/dxh379

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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Keywords: autoimmunity, cytokines, dendritic cells, knockout mouse, T cells

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-$1\alpha^{-/-}$ or IL-$1\beta^{-/-}$ 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-$\gamma R^{-/-}$ mice, however, remain highly susceptible to EAE (6–9). In fact, the overexpression 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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Transmitting editor: Okumura

Received 5 September 2005, *accepted* 30 November 2005

Advance Access publication 13 January 2006

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

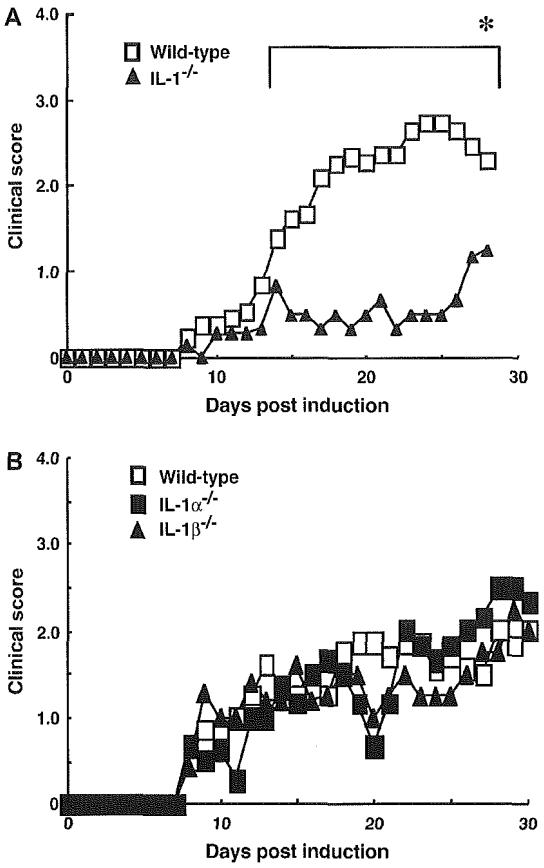

Fig. 1. Attenuated EAE induction in IL-1$^{-/-}$ mice. The clinical scores after EAE induction were determined as described in Methods. The averages of clinical scores are shown from the day of MOG immunization (day 0) to day 28 post-immunization in (A) wild-type (open squares, $n = 9$) and IL-1$^{-/-}$ (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).

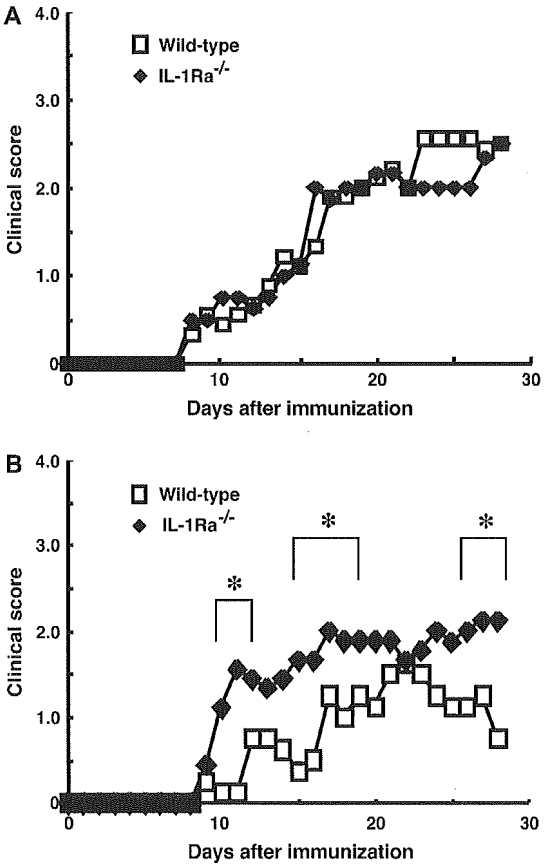

Fig. 2. Exacerbated EAE induction without PTx injection in IL-$1Ra^{-/-}$ mice. Clinical scores after MOG immunization in the (A) presence or (B) absence of PTx (500 ng) injection in wild-type [open squares, (A) $n = 13$ and (B) $n = 8$], and IL-$1Ra^{-/-}$ mice [filled diamonds, (A) $n = 12$ and (B)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

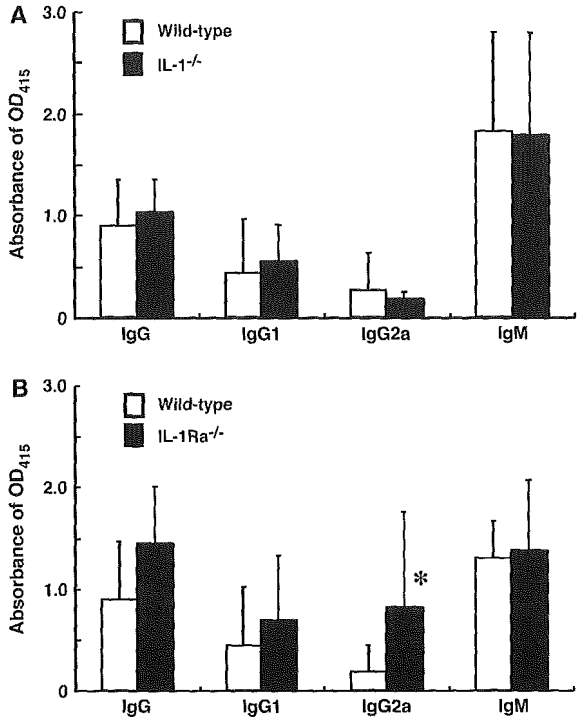

Fig. 3. Titer of anti-MOG antibodies in serum of IL-$1^{-/-}$ and IL-$1Ra^{-/-}$ mice. Thirty-seven days after EAE induction (A) with PTx injection (wild-type, open bars and IL-$1^{-/-}$ mice, filled bars) or (B) without PTx injection (wild-type, open bars and IL-$1Ra^{-/-}$ mice, filled bars), we collected 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

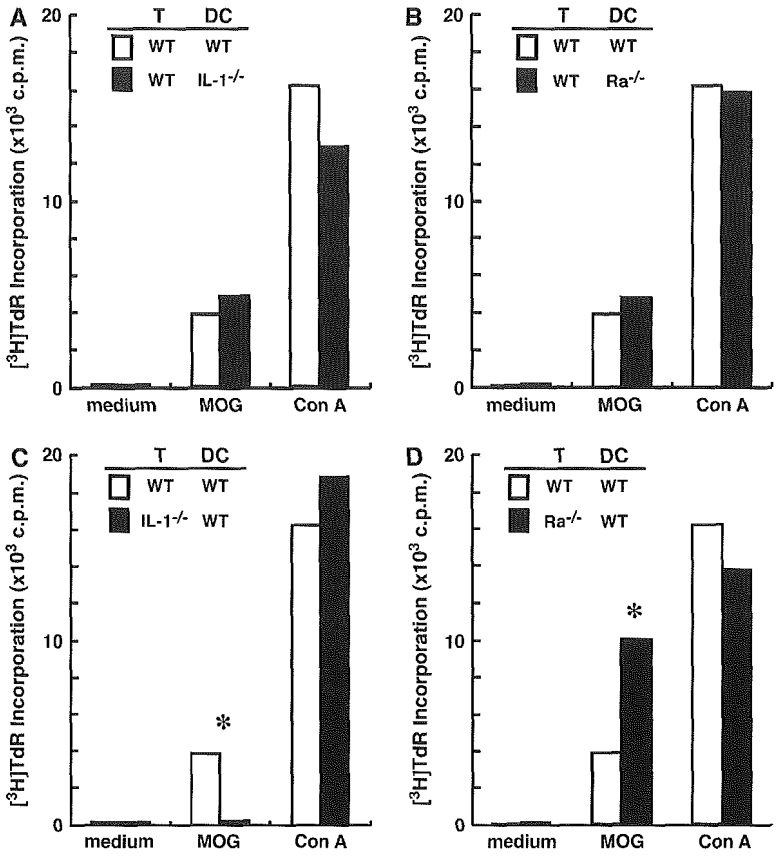

Fig. 4. Abnormal T cell activation in IL-1$^{-/-}$ and IL-1Ra$^{-/-}$ mice immunized with MOG. Wild-type, IL-1$^{-/-}$ and IL-1Ra$^{-/-}$ mice were immunized with a MOG 35–55/CFA emulsion without PTx co-administration. Ten days after MOG immunization, LN T cells were cultured with splenic DCs in the 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

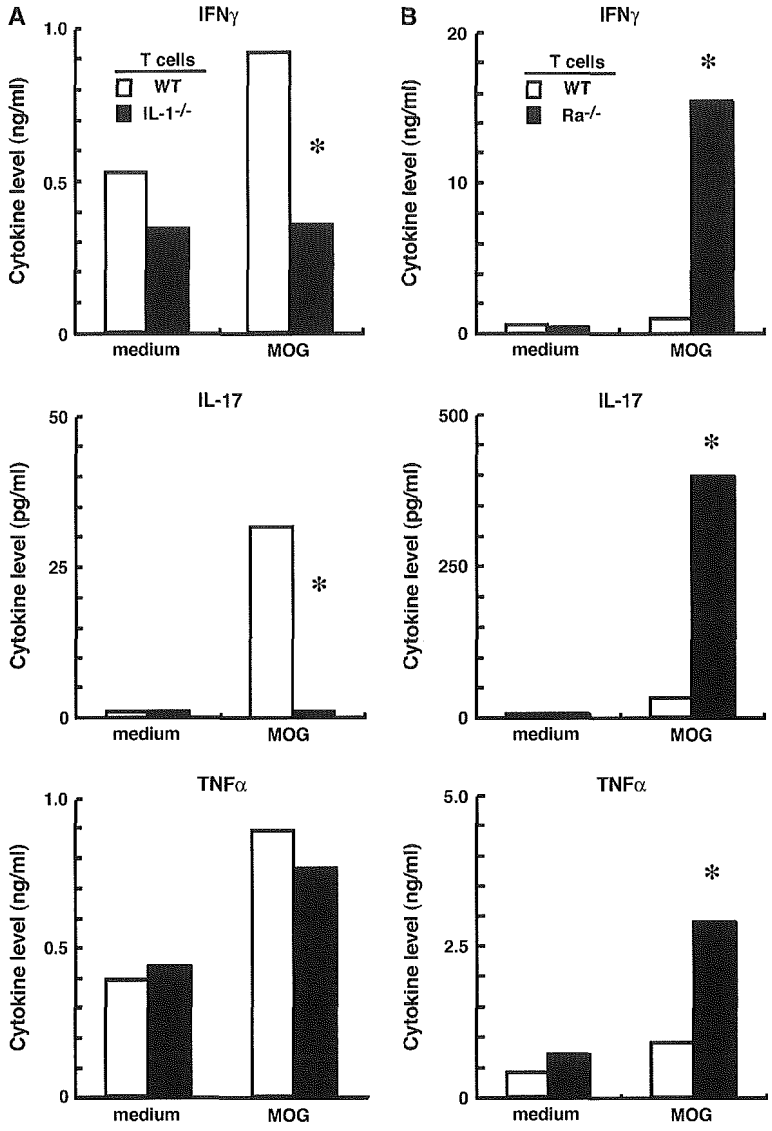

Fig. 5. Abnormal cytokine production from IL-$1^{-/-}$ T cells and IL-$1Ra^{-/-}$ T cells. MOG-sensitized T cells from (A) wild-type or IL-$1^{-/-}$ mice and (B) wild-type or IL-$1Ra^{-/-}$ mice were co-cultured with wild-type DCs in the absence (medium) or presence MOG of MOG 35–55, as shown in Fig. 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

We would like to thank Ohmi for providing the MOG peptide. We would also like to thank K. Habu and Y. Komiyama for their technical support and critical comments. We thank all the members of our laboratory for their kind discussion and help in animal care. This work was supported by grants from the Ministry of Education, Science, Sport and Culture of Japan, the Ministry of Health and Welfare of Japan, the Japan Society for the Promotion of Science and Pioneering Research Project in Biotechnology.

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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0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(12):5496–5502

doi: 10.1210/en.2005-0409

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

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

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

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

First Published Online September 1, 2005

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

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

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

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

Materials and Methods

Reagents

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

Animals

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

Hormone assays

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

In situ hybridization

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

Probe labeling

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

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

Western blot analysis

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

Statistical analysis

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

Results

HPAA response to IL-1α

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

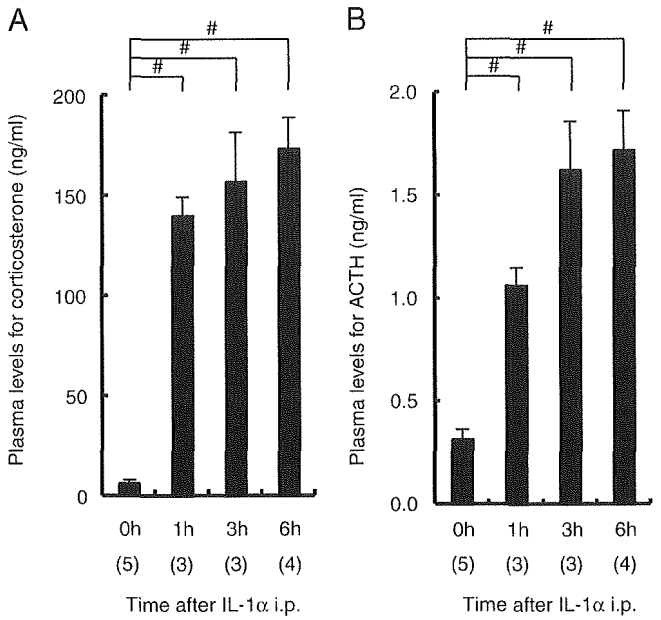

FIG. 1. Plasma corticosterone and ACTH levels after injection with IL-1α in WT mice. Blood samples were collected before treatment (0 h) or 1, 3, and 6 h after ip injection with rmIL-1α (20 μg/kg body weight), and corticosterone (A) and ACTH (B) levels in the plasma were measured. Same group of animals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

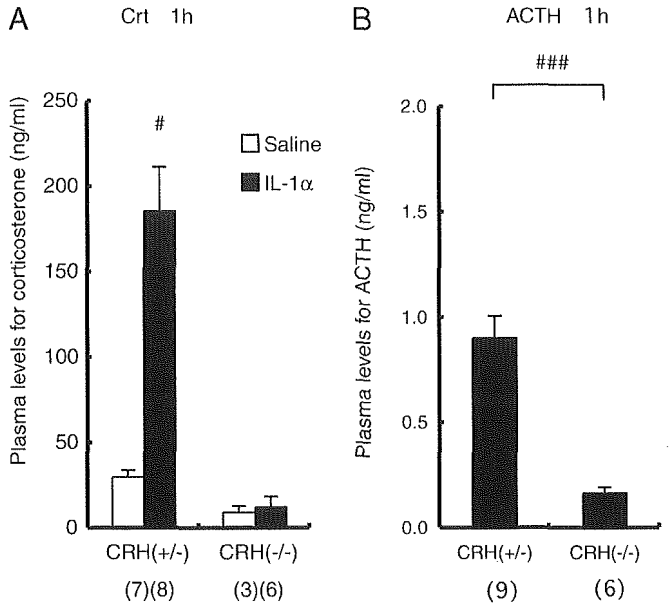

FIG. 2. Plasma corticosterone and ACTH levels after injection with IL-1α in *CRH* KO mice. Plasma corticosterone (A) and ACTH (B) levels were measured 1 h after ip injection of rmIL-1α (20 μg/kg body weight) or saline to *CRH* (+/−) and *CRH* (−/−)mice. Same group of animals were analyzed for corticosterone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

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

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

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

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

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

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

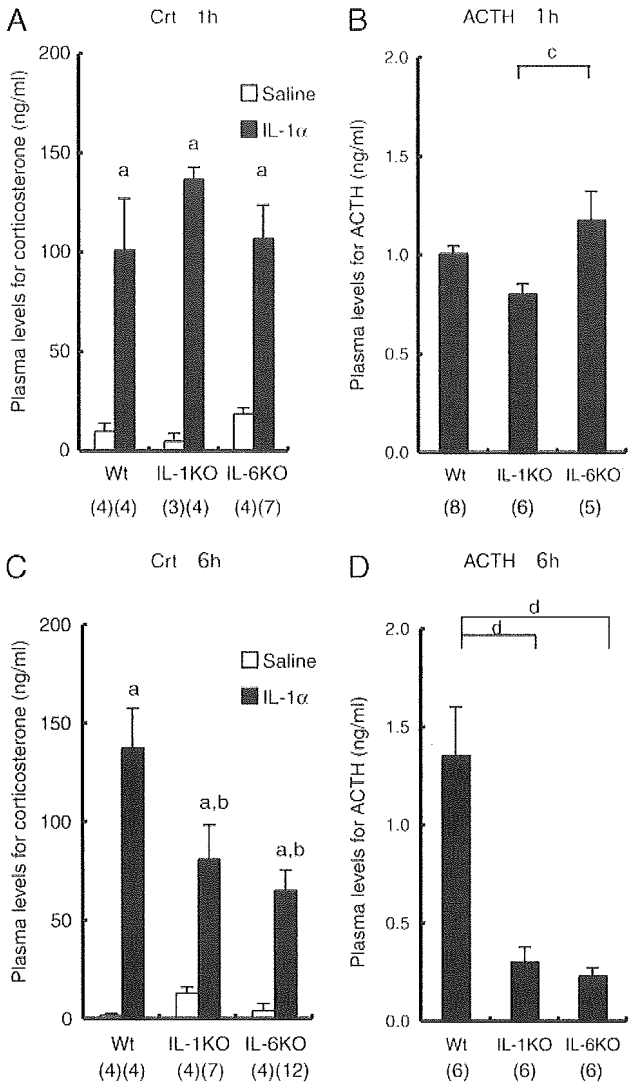

FIG. 3. Plasma corticosterone (A and C) and ACTH (B and D) levels after injection with IL-1α in WT, *IL-1α/β* KO, and *IL-6* KO male mice. (A and B) Plasma corticosterone (A and C) and ACTH (B and D) levels were measured 1 h (A and B) or 6 h (C and D) after ip injection of rmIL-1α (20 μg/kg body weight)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

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

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

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

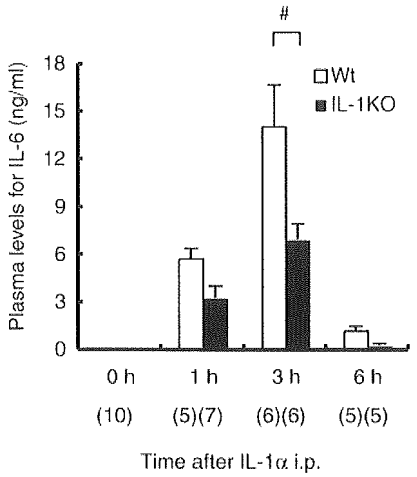

FIG. 4. Plasma IL-6 levels after injection with IL-1α into *IL-1α/β* KO mice or WT mice. Blood samples were collected before treatment (0 h) or 1, 3, and 6 h after ip injection of rmIL-1α (20 μg/kg body weight) in WT and in *IL-1α/β* KO mice, and the plasma IL-6 levels were measured. *Numbers beneath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

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

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

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

Discussion

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

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

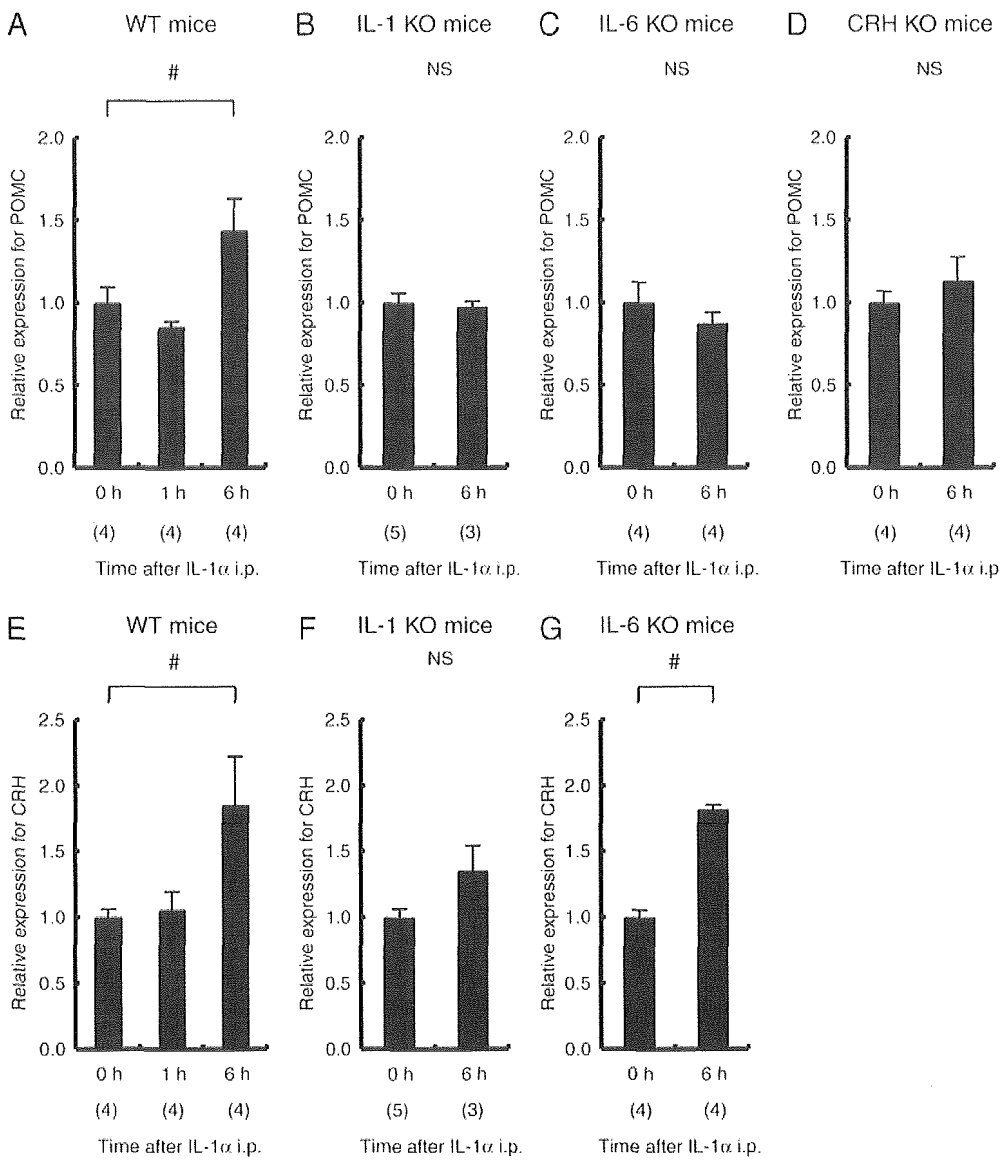

FIG. 5. Induction of *POMC* transcript (A–D) in the AP and *CRH* transcript in PVN (E–G) in response to peripheral injection of IL-1α. A, *POMC* mRNA levels in the AP were estimated by *in situ* hybridization after ip injection of rmIL-1α (20 μg/kg body weight) to WT mice. B–D, *POMC* mRNA levels in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

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

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

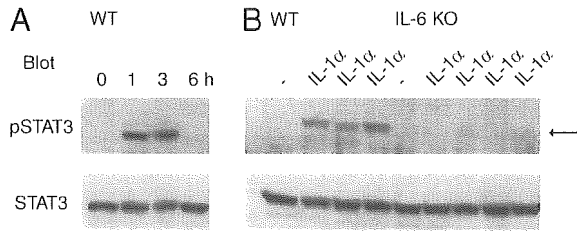

FIG. 6. Activation of STAT3 in the pituitary in response to peripheral injection of IL-1α. A, Whole cell lysates of the pituitary were prepared before (0 h), or 1, 3, and 6 h after ip injection of rmIL-1α (20 μg/kg body weight) into WT mice, and STAT3 and phosphorylated STAT3 (pSTAT3) were detected using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

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

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

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

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

Acknowledgments

We thank Dr. Manfred Kopf for *IL-6* KO mice. We thank all the members of our laboratory for their kind discussion and help with animal care.

Received April 8, 2005. Accepted August 15, 2005.

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This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Health, Labor and Welfare of Japan.

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Early increase in mRNA levels of pro-inflammatory cytokines and their interactions in the mouse hippocampus after transient global ischemia

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Received 9 June 2005; received in revised form 15 August 2005; accepted 24 August 2005

Abstract

There is convincing evidence that cytokines are involved in the inflammatory response following cerebral ischemia, but the interactions among the pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in the early stage of ischemic reperfusion are not yet completely understood. In this study, we examined the early mRNA expressions of pro-inflammatory cytokines in the ischemic hippocampus after 30 min of bilateral common carotid artery occlusion in C57BL/6J wild-type (WT) and TNF-α, IL-1α/β or IL-6 gene knockout (KO) mice utilizing real-time polymerase chain reaction. The mRNA expressions of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β were significantly induced in ischemic WT mice compared with in the sham-operated mice. These increases peaked at 3 to 24 h for TNF-α, at 12 h for IL-1β, and at 6 to 24 h for IL-6 after ischemia. The pattern of temporal expression of the cytokine mRNAs in ischemic gene KO mice, however, differed from that in WT mice. The TNF-α mRNA expression showed a similar temporal expression pattern in IL-6 KO mice compared to in WT mice following ischemic reperfusion, and the levels at all time points were lower than in WT mice. The IL-1β mRNA level was very low in ischemic TNF-α KO mice and IL-6 KO mice in spite of a small peak observed in both at 24 h. The IL-6 mRNA level was significantly upregulated at all time points in both ischemic WT and TNF-α KO mice; however, the peak was delayed by 12-h in IL-1α/β KO mice. In conclusion, the present study indicates that the rapid increases in cytokine levels are interdependent, interactive, and possibly modulate each other in the mouse hippocampus after transient global ischemia.

Keywords: Cytokine; Cerebral ischemia; Knockout mouse; Bilateral common carotid artery occlusion; Central nervous system

Inflammatory processes are known to be associated with acute neurodegenerative conditions (e.g. ischemic brain damage, stroke, and cerebral hemorrhage). These conditions include the activation of brain resident cells such as microglia localized within the ischemic region, and the rapid synthesis of cytokines and chemokines [2]. Cytokines have been classified as either pro-inflammatory agents such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 or anti-inflammatory agents such as interleukin-1 receptor antagonist (IL-1ra), IL-10 and transforming growth factor (TGF)-β [1]. There is ample evidence that an increase in the levels of pro-inflammatory cytokines is an early feature of acute brain injury induced by clinical and experimental cerebral ischemia [5,23].

Although rats and gerbils have been widely used to investigate the alteration of cytokines and the molecular mechanisms of selective neuronal death following transient cerebral ischemia [20,21], recent studies have demonstrated that the mouse strain C57BL/6 is most susceptible to cerebral ischemia following bilateral common carotid artery occlusion (BCCAO) and most likely to develop selective neuronal death in the hippocampus [22], and that neuronal damage depends upon ischemic duration [8,22]. This implies that investigation into the early response of pro-inflammatory cytokines and their interactions in the hippocampus following transient cerebral ischemia may help elucidate the role of cytokines in neuronal damage using C57BL/6J mice.

In this study, we examined the early mRNA production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in the vulnerable hippocampus following transient global ischemia, and documented the interactions among the pro-inflammatory

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0304-3940/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2005.08.072