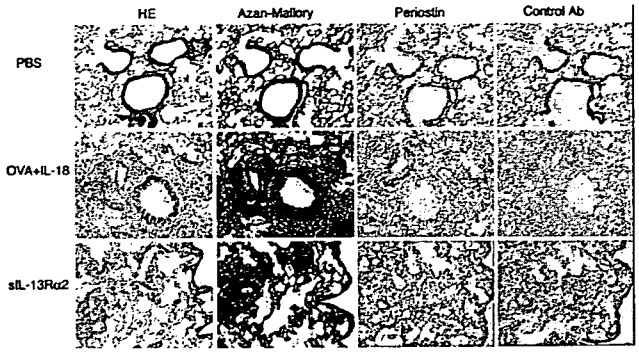

Fig. 7. Immunohistochemical staining of periostin in the lungs of Th1 mice after exposure to OVA and IL-18. Active Th1 mice were exposed daily to intranasally administered OVA plus IL-18 or OVA plus IL-18 plus sIL-13Rα2 as described in the legend of Fig. 1. At 24 h after final exposure, lungs from each group of mice were fixed and stained with H&E or Azan–Mallory. Immunohistochemical staining for periostin was as described in *Material and Methods*. (Original magnification, ×40.)

IL-18, both passive and active Th1 mice equally developed AHR and severe airway inflammation (Figs. 1–3). Histological examination revealed that additional IL-18 administration induced massive cell infiltrates composed of eosinophils, lymphocytes, and neutrophils (Fig. 3).

In our previous report, we tried to understand the mechanism by which Th1 cells induced AHR and airway inflammation when they were stimulated with Ag and IL-18 *in vivo*. We initially envisaged that IL-13 from Ag- plus IL-18-stimulated Th1 cells was a causative factor because IL-13 has been shown to induce AHR in Th2-cell-bearing mice (9–13). However, neutralization of IL-13 did not affect Th1-cell-induced AHR, although this treatment strongly diminished eosinophilic infiltration in the lungs (22) (Figs. 1 and 3). We could not reduce completely BALF levels of eosinophils to that in PBS-treated mice even if we performed daily administration of sIL-13Rα for neutralization of IL-13 (Fig. 1 *C* and *D*). Furthermore, the effects of this treatment on lung tissue eosinophils or neutrophils were less effective than those on BALF levels of these cells (Figs. 1 and 3). IL-13 neutralization in BALF might be more efficient than that in lung tissue.

In this study, we formally could exclude the contribution from IL-13 to AHR by showing the capacity of IL-4Rα$^{-/-}$ mice to normally develop AHR after OVA/CFA priming and subsequent OVA/IL-18 challenge (Fig. 4). Because Ag- plus IL-18-stimulated Th1 cells produced IFN-γ, IL-9, and IL-13 but not IL-5, we could assume that eosinophilic infiltration was induced by the action of IL-13. Of interest, compared with WT BALB/c mice, IL-4Rα$^{-/-}$ mice augmented neutrophilic infiltration in their lungs, suggesting that IL-13 down-regulates neutrophilic infiltration but up-regulates eosinophilic infiltration. We recently demonstrated that IL-13 recruits eosinophils in the lungs by the induction of eotaxin from lung epithelial cells (34).

We tried to determine the causative factor. Here, we could demonstrate that IFN-γ from Ag- plus IL-18-stimulatd Th1 cells was responsible for inducing AHR and airway inflammation (Figs. 1 and 3). Neutralization of IFN-γ inhibited AHR and neutrophilic accumulation in BALF and lung tissues. However, at present, we do not know how IFN-γ induces recruitment of neutrophils in the lungs. It is quite reasonable to speculate that IFN-γ induces production of some chemokines, which have the capacity to recruit neutrophils in the lungs.

Bronchial asthma is often induced by viral or bacterial infection (2, 31–33). We tested whether pathogen-associated molecular pattern (LPS) can induce IL-18 production from alveolar macrophage and/or lung epithelial cells. The lung epithelial cell line TGMBE02-3 cells (37) express Toll-like receptor 4 and produce IL-18 upon LPS stimulation *in vitro* (SI Fig. 9). Furthermore, OVA/CFA-immunized and OVA/LPS-challenged mice increased their serum levels of IL-18 (SI Fig. 10). IL-18 is also involved in human bronchial asthma. Serum levels of IL-18 are elevated in patients with bronchial asthma (SI Fig. 10), and a significant correlation between IL-18 serum levels and the disease severity of bronchial asthma has been reported (38).

In this study, we could demonstrate the unique capacity of OVA-specific Th1 cells to induce AHR after intranasal administration of OVA and LPS. Neutralization of IL-18 or IFN-γ attenuated AHR and decreased the number of neutrophils in BALF. Furthermore, OVA/CFA-immunized IL-18$^{-/-}$ or IFN-γ$^{-/-}$ mice failed to develop AHR upon challenge with OVA and LPS (Fig. 5). These results clearly indicated that endogenous IL-18 played a critical role in induction of this mouse model of bronchial asthma by activation of Th1 cells to produce IFN-γ.

We have shown that IL-13 induces lung fibrosis associated with periostin deposition (Figs. 6 and 7), which binds to extracellular matrix proteins to form a reticular structure (30). Indeed, neutralization of IL-13 inhibited lung fibrosis (Fig. 6), lung hydroxyproline content (Table 1), and periostin deposition (Fig. 7) without inhibiting AHR (Fig. 1). Thus, Ag- plus IL-18-stimulated Th1 cells exhibit two pathological effects on the development of bronchial asthma: one is IFN-γ-induced AHR, and the other is IL-13-induced lung fibrosis. Here, we also demonstrated that LPS induces Th1-cell-induced asthma by the induction of IL-18 production. It is well known that Th2 cell-induced asthma can be controlled by the treatment with anti-Th2 cytokines or anti-IgE (2, 39). However, there are no appropriate treatments for infection- or Th1-cell-induced bronchial asthma, in which super Th1 cells play very pathological roles. Our present results clearly indicated that we could regulate AHR and lung fibrosis by down-regulation of IL-18. Thus, IL-18 becomes rational target for the treatment of Th1-cell-induced bronchial asthma. Because difficult asthma or refractory asthma is often induced by bacterial or viral infection, anti-IL-18 therapy might be applicable for its treatment.

Materials and Methods

Animals and Reagents. Specific pathogen-free female BALB/c mice and IL-4Rα$^{-/-}$ mice (BALB/c background) were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c background IFN-γ$^{-/-}$ mice were kindly provided by Y. Iwakura (University of Tokyo, Tokyo, Japan). BALB/c background IL-1$^{-/-}$ mice were established in our laboratory (40) and used for experimentation. Mice transgenic for αβ T cell antigen receptor recognizing $OVA_{323-339}$ (DO11.10; BALB/c background) (41) were generously provided by D. Loh (Washington University, St. Louis, MO). All experiments were performed on the line heterozygous for the transgene. All mice were bred under specific pathogen-free conditions at the animal facilities of Hyogo College of Medicine (Nishinomiya, Japan) and were used at 6 to 10 weeks of age. Recombinant mouse IL-12 and IL-18 were purchased from R & D Systems (Minneapolis, MN) and MBL (Nagoya, Japan), respectively. LPS from *Salmonella minnesota* Re-595 was purchased from Sigma–Aldrich (St Louis, MO). Anti-CD3 (2C11) and anti-IL-4 (11B11) were prepared by Harlan Bioproducts for Science (Indianapolis, IN). Purified anti-mouse IL-18 rabbit serum was prepared in our laboratory.

Generation of Th1 Cells *in Vivo*. We induced Th1 cells by immunization of mice with OVA (50 μg) in CFA. Endotoxin contaminated in OVA was removed by END-X 15 (Seikagaku America, Cape Cod, MA). After removal, endotoxin level was <0.5 pg/ml in 1 mg/ml OVA. We used the resultant endotoxin-depleted OVA in all of our experiments.

***In Vivo* Intranasal Antigen Challenge.** The preparation of passive Th1 mice has been described previously (22, 42). Briefly, 1×10^7 Th1 cells, which we developed *in vitro* (22, 42), were injected i.v. into normal BALB/c mice (10–15 mice per group). At 4 weeks, or even later, after cell transfer, transplanted Th1 cells expressed the Ag-specific resting Th1 memory phenotype in host mice. Active Th1 mice were prepared by immunization of mice with OVA/CFA. Two weeks after OVA/CFA immunization or 4 weeks after Th1 cell transfer, both types of Th1 mice were exposed to daily intranasal administration of 50 μl of PBS or 50 μl of PBS containing OVA (100 μg) or OVA (100 μg) plus IL-18 (0.5 μg) for 3 days. In some experiments, Th1 mice were exposed to intranasal administration of LPS (5 μg) and OVA (100 μg). Mice were analyzed for their AHR and airway inflammation at 24 h after the final exposure. sIL-13Rα_2-Fc chimera (20 μg; R & D Systems) was administered daily intranasally for blocking of IL-13 *in vivo* as described previously (22). For the blockade of IFN-γ or IL-18 *in vivo*, 50 μg of anti-IFN-γ or 60 μg of anti-IL-18, as well as its corresponding control Ab (50 μg of rat IgG or 60 μg of rabbit IgG), was administered daily intranasally at the time of OVA challenge.

Noninvasive or Invasive Measurement of AHR. Noninvasive measurement of specific airway resistance has been described previously (22, 34). Briefly, we measured AHR to β-methacholine inhalation in mice by using Pulmos-I hardware and software (MIPS, Osaka, Japan). We placed a mouse in a chamber and first exposed it to aerosols of saline (baseline) and then to increased concentrations of β-methacholine (5, 10, 15, and 20 mg/ml). After each 2-min exposure, we measured enhanced pause, a dimensionless index that reflects changes in amplitude of pressure waveform and expiratory time (43), for 2 min. Invasive measurement of AHR was assessed as an increase in pulmonary resistance and a decrease in dynamic compliance (Cdyn) in response to aerosolized β-methacholine. Briefly, mechanical ventilation was achieved by using a MiniVent Model 845 ventilator (HSE, March–Hugstetten, Germany). Saline and increased concentrations of β-methacholine were aerosolized as noninvasive measurement. Pulmonary resistance and Cdyn were measured by Pulmos-II (MIPS) hardware and software (MIPS) (44).

Bronchoalveolar Lavage. Bronchoalveolar lavage was performed with three aliquots of 0.5 ml of PBS per mouse. Total cell counts were performed. Cytospin preparations of BALF were stained with Dif-Quik (Baxter Healthcare, Miami, FL), and differentials were performed based on morphology and staining characteristics.

Histology. Lungs were prepared for histological examination by perfusion of the animal via the right ventricle with 10 ml of PBS and then were fixed in 10% buffered formalin, cut into 3-mm sections, and stained with H&E for H&E stain or azocarmine G and aniline blue orange G for Azan–Mallory stain. Immunohistochemical staining for periostin has been described previously (30). Briefly, lung tissues were fixed with 10% formalin and incubated with primary Ab or normal IgG (control) overnight. The antigens were detected by the ENVISION+/HRP (DAB) system (Dako Cytomation, Glostrup, Denmark).

Hydroxyproline. Total hydroxyproline content was measured by HPLC (SRL, Tokyo, Japan).

We thank Dr. H. Tsutsui of Hyogo College of Medicine for critical reading of the manuscript; Dr. K. Yasuda, Dr. H. Tanaka, and Dr. M. Kuroda of Hyogo College of Medicine for enthusiastic discussion; and Ms. S. Yumikura-Futatsugi and Ms. M. Uemura for excellent technical assistance. This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas and Hitech Research Center from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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IL-27 Suppresses Th2 Cell Development and Th2 Cytokines Production from Polarized Th2 Cells: A Novel Therapeutic Way for Th2-Mediated Allergic Inflammation[1]

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IL-27 up-regulates Th1 but down-regulates Th2 responses. However, its molecular mechanism and regulatory effects on polarized Th2 cells remain unclear. In this study, we have revealed that IL-27 inhibits Th2 cell development as well as Th2 cytokines production from already polarized Th2 cells by down-regulation of GATA-3 and up-regulation of T-bet expression simultaneously. In vivo daily IL-27 treatment for 1 wk after *Leishmania major* infection protects BALB/c mice from footpad swelling by diminishing parasite burden via reciprocal regulation of Th1 and Th2 responses. Furthermore, IL-27 stimulation causes marked reduction in the capacity of host mouse to mount a Th2 response against *Strongyloides venezuelensis* infection. Thus, IL-27-treated mice failed to develop intestinal mastocytosis after *S. venezuelensis* infection and exhibited a marked delay in parasite expulsion. Finally, intranasal administration of IL-27 inhibits OVA-induced airway hyperresponsiveness and inflammation in OVA-sensitized animals. Thus, IL-27 could provide us with a novel therapeutic way for treating Th2-associated diseases such as bronchial asthma. *The Journal of Immunology*, 2007, 179: 4415–4423.

The differentiation of naive $CD4^+$ T cells into Th1 or Th2 cells is a critical process in the development of adaptive immune responses (1). Th1 cells mediate cellular immunity by production of IFN-γ, which is critical for eradication of intracellular pathogens. In contrast, Th2 cells produce IL-4, IL-5, and IL-13, which in combination induce humoral immunity, allergic inflammation, and promote host resistance, particularly to intestinal nematodes (2, 3). Although many factors influence the developmental process of $CD4^+$ T cells into Th1 or Th2 cells, cytokines have emerged as key determinants of the outcome (1). IL-12, a heterodimer of the p40 and p35 subunits, induces IFN-γ-producing Th1 cells through activation of Stat4 (4, 5). IFN-γ signal, transduced by Stat1, activates a downstream transcription factor, T-bet, that enhances the expression of genes specific to Th1 cells (6). Then, IL-18 acts on IL-18R-expressing Th1 cells to increase IFN-γ production (7, 8). In contrast, IL-4 induces Stat6 activation (9, 10), promoting the expression of GATA-3, the transcription factor essential for both IL-4 production and Th2 cell polarization (11).

Recently, a novel member of the IL-12 family, IL-27 has been identified (12). IL-27, a heterodimeric cytokine produced by activated APCs, is composed of IL-12 p40-related protein, EBV-induced gene 3 (EBI3),[3] and newly discovered IL-12 p35-related protein, p28 (12, 13). IL-27R consists of IL-27Rα chain (WSX-1/TCCR), homologous to IL-12Rβ2, and gp130, a common subunit of the IL-6R family (14, 15). IL-27 induces naive $CD4^+$ T cells to proliferate and to express T-bet and subsequently exhibit IL-12Rβ2 (16). Thus, IL-27 renders naive $CD4^+$T cells to the Th1-polarizing effects of IL-12 (12, 16–18).

Previous studies of IL-27Rα-deficient ($IL\text{-}27R\alpha^{-/-}$) mice revealed that they are susceptible to infection with *Leishmania major* and *Listeria monocytogenes* due to impaired Th1 response (14, 15, 19). However, subsequent studies have demonstrated the regulatory effects of IL-27 on progressive $CD4^+$ T cell-dependent inflammatory responses. Indeed, $IL\text{-}27R\alpha^{-/-}$ mice infected with *Toxoplasma gondii* or *Trypanosoma cruzi* develop lethal inflammatory responses, illustrating its regulatory effects (20). In contrast, $IL\text{-}27R\alpha^{-/-}$ mice are resistant to infection with *Trichuris muris*, which is a well-characterized helminth expelled by Th2-mediated immune responses, suggesting the inhibitory effect of IL-27 on Th2 cytokine production (21). $IL\text{-}27R\alpha^{-/-}$ mice also exhibited enhanced asthmatic phenotypes, including airway hyperresponsiveness (AHR), eosinophilic infiltration, and mucus overproduction in response to Ag challenge (22). Furthermore, IL-27 directly inhibits GATA-3 expression through the Stat1-dependent pathway (23). Therefore, IL-27 may play a critical role in suppression of Th2 responses. Very recent studies have noticed another important function of IL-27, that IL-27 inhibits development of IL-17-producing helper T (Th-17) cells (24, 25). Therefore, IL-27 is a critical cytokine possibly involved in regulation of human immunological diseases induced by Th1, Th2, or Th-17 cells (13, 26).

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Received for publication April 20, 2007. Accepted for publication July 23, 2007.

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[1] This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas and Hitech Research Center Grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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[3] Abbreviations used in this paper: EBI3, EBV-induced gene 3; AHR, airway hyperresponsiveness; Tg, transgenic; L3, third-stage larvae; mMCP-1, mouse mast cell protease 1; Th-17, IL-17-porducing helper T.

In this study, we investigated the molecular mechanism and regulatory effects of IL-27 on Th2 cells. We showed that mice treated with IL-27 or IL-27- transgenic (Tg) mice are resistant to *L. major* infection but simultaneously develop a disability to promptly expel gastrointestinal nematode *Strongyloides venezuelensis* infection. Finally, we have shown that intranasal administration of a mixture of IL-27 and OVA into OVA-immunized animals can inhibit OVA-induced allergic airway inflammation, suggesting its therapeutic usage for the treatment of Th2 cell-mediated allergic diseases.

Materials and Methods

Mice

Specific pathogen-free female BALB/c and C57BL/6 mice, 8 wk of age, were purchased from The Jackson Laboratory. Mice transgenic for $\alpha\beta$ TCR recognizing $OVA_{323-339}$ (DO11.10; BALB/c genetic background) were provided by D. Loh (Washington University, St. Louis, MO). IL-27 Tg mice were generated as described bellow. The cDNA for 3xFLAG-tagged single-chain mouse IL-27, in which EBI3 is flexibly linking to p28 with the $(Gly_4Ser)_3$ linker, in p3xFLAG-CMV-9 (Sigma-Aldrich) vector (27), was amplified using standard PCR methods and inserted into the pLG1-SAP vector that contains the liver-specific human serum amyloid P component promoter and the rabbit β-globin gene (28). IL-27 Tg mouse lines were generated by pronuclear microinjection of the cDNA into fertilized eggs obtained from C57BL/6 mice. All mice were bred under specific pathogen-free conditions at the animal facilities of the Hyogo College of Medicine (Nishinomiya, Japan) and were used at 8–12 wk of age.

Reagents

Recombinant mouse IL-12 and IL-18 were purchased from Genetics Institute (Cambridge, MA) and MBL, respectively. Recombinant mouse IL-23 and IL-27 ware prepared in our laboratory as described before (29, 30). Endotoxin level was <8 pg/1 μg of rIL-27 or rIL-23 as determined by the *Limulus* amebocyte lysate method (Wako Pure Chemical). Recombinant mouse IL-4 was purified as described before (8). Purified Abs (anti-mouse CD28 (37.51), anti-mouse CD3 (2C11), anti-mouse IL-4 (11B11), anti-mouse IL-12p40 (C17.8), and anti-mouse IFN-γ (R4-6A2)) were prepared in our laboratory. PE-anti-mouse CD4 (GK1.5), FITC-anti-mouse CD62L (MEL-14), FITC-anti-mouse CD11C, PE- anti-mouse I-A/I-E (M5/114.15.2), CyChrome-anti-CD4, and biotin-anti-mouse IgE (R35118) were purchased from BD Biosciences. Anti-mouse IgE (23G3) was purchased from Southern Biotechnology Associates. Mouse mAbs against GATA-3 (HG3-31), T-bet (39D), and actin (H-196) were purchased from Santa Cruz Biotechnology. Anti-Stat1 (1/Stat1) and anti-phospho-Stat1 (Y701) were obtained from BD Biosciences and Upstate Biotechnology, respectively. Anti-Stat3 and anti-phospho-Stat3 (Tyr705) were purchased from Cell Signaling Technology.

Generation of Th1 or Th2 cells

Naive splenic $CD4^+CD62L^+$ T cells (1×10^5/ml) from DO11.10 mice were stimulated with IL-2 (100 pM) and $OVA_{323-339}$ (1 μM) in the presence of 2×10^4/ml APCs (irradiated splenic dendritic cells prepared as described in Ref. 31) in 24-well plate in a total 1-ml volume of RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin for 7 days. For induction of Th1 or Th2 cells, IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml) or IL-4 (1000 U/ml), anti-IL-12p40 (20 μg/ml), and anti-IFN-γ (20 μg/ml) were further added to the culture, respectively. Twenty micrograms of IL-18, IL-23, and IL-27 per milliliter, either by itself or in various combinations, was added from the beginning of the culture.

In vitro cytokines production

Polarized Th2 cells in vitro were recultured at 1×10^5/0.2 ml per well with 100 pM IL-2, 1 μM $OVA_{323-339}$, and 1×10^5/0.2 ml of APCs (irradiated T cell-depleted BALB/c splenocytes) in the presence of IL-27 (0–100 μg/ml). Popliteal or mesenteric lymph nodes cells from mice infected with *L. major* or *S. venezuelensis* third-stage larvae (L3) were cultured at 2×10^5/0.2 ml per well under stimulation with immobilized anti-CD3 and anti-CD28 (each 5 μg/ml) for 48 h and tested for IFN-γ, IL-4, IL-5, and IL-13 production using ELISA.

Flow cytometry

For preparation of $CD4^+CD62L^+$ resting T cells, splenic $CD4^+$ T cells from DO11.10 mice were purified by MicroBeads (anti-mouse CD4; clone RM4-5; Miltenyi Biotec). The enriched $CD4^+$ T cells were first treated with 10 μg/ml anti-FcγRII/III for 30 min at 4°C, followed by treatment with PE-anti-CD4 and FITC-anti-CD62L for 30 min at 4°C in staining buffer (PBS/1% FCS). Stained samples were separated into $CD4^+CD62L^+$ T cells by FACSAria (BD Biosciences). Purity of sorted cells was >98.5% after reanalysis. For intracellular cytokine staining, polarized Th2 cells (1×10^6/ml) were restimulated with 50 ng/ml PMA plus 0.5 μM ionomycin for 4 h with a pulse of 1 μg/ml brefeldin A (BD Biosciences) during the final 2 h to inhibit cytokine secretion. Cells were stained with CyChrome-anti-CD4 and followed by fixation with 4% (w/v) paraformaldehyde in PBS and permeabilization of cell membrane with ice-cold PBS containing 1% FCS plus 0.1% saponin. Resultant cells were further stained with 0.5 μg of PE-rat anti-mouse IL-4 plus 0.5 μg of FITC-rat anti-mouse IFN-γ or isotype-matched control Abs (BD Biosciences) and analyzed for their proportion of cytoplasmic $IL\text{-}4^+$ or $IFN\text{-}\gamma^+$ cells by FACSCalibur (BD Biosciences).

L. major *infection*

L. major (WHO strain MHOM/SU/73-5-ASKH) were maintained in vivo and grown in vitro as described in our previous report (32). For infection, mice were inoculated with s.c. injections of 5×10^6 stationary phase promastigotes into the hind footpad. The footpad lesions were measured weekly with a dial gauge caliper and compared with the thickness of uninfected footpads. Parasites burdens in the popliteal lymph node draining the site of infection were determined by limiting dilution cultures as described previously (32).

S. venezuelensis *infection*

S. venezuelensis has been maintained as described in our previous report (33). Animals were s.c. inoculated with 5000 *S. venezuelensis* L3 to initiate a complete infection. The degree of infection of individual mice was monitored by counting the number of eggs excreted daily (eggs per gram of feces).

Generation and analysis of OVA-specific bronchial asthma

BALB/c mice were immunized i.p. with 50 μg of OVA complexed with aluminum potassium sulfate on day 1 and challenged intranasally with 50 μg of OVA in 50 μl of PBS on days 7–9. Control mice were immunized with OVA and exposed to PBS alone. Mice were analyzed as described below at 24 h after the final exposure to PBS or OVA. For measurement of AHR to β-methacholine inhalation in mice, we used Pulmos-I (MIPS) hardware and software as described in our previous reports (34, 35). Bronchoalveolar lavage was performed with three aliquots of 1.0 ml of PBS/mouse. Total cell counts were performed. Cytospin preparations of bronchoalveolar lavage fluid were stained with Diff-Quik (Baxter Healthcare), and differentials were performed based on morphology and staining characteristics. Histological analysis of lungs was performed as described previously (34, 35). For detection of IL-13 production, lungs were removed at 24 h after the final exposure to PBS or OVA and homogenized and centrifuged as described in our previous report (34). The obtained supernatants were tested for IL-13 contents using ELISA.

In vivo treatment

For host defense experiments for *L. major* or *S. venezuelensis* infection, infected mice were daily i.p. injected with PBS, IL-27 (1 μg/day), or a combination of IL-12 (10 ng/day) and IL-18 (1 μg/day) for 7 days. For experiments of airway inflammation, OVA-immunized mice were intranasally challenged with 50 μg of OVA with or without IL-27 (0.2, 1, 5 μg) in 50 μl of PBS on days 7–9.

ELISA

ELISA kits for IL-4, IL-5, IL-13, and IFN-γ (R&D Systems) were used. Serum levels of mouse mast cell protease-1 (mMCP-1) and IgE were measured by ELISA as described in our previous reports (36). Serum level of FLAG-tagged IL-27 protein was determined by sandwich ELISA using anti-FLAG (M2; Sigma-Aldrich) as capture Ab and biotin-anti-IL-27p28 (R&D Systems) as detection Ab. rIL-27 prepared as a FLAG-tagged single chain protein by flexibly linking EBI3 to p28 as described before (30) was used as a standard.

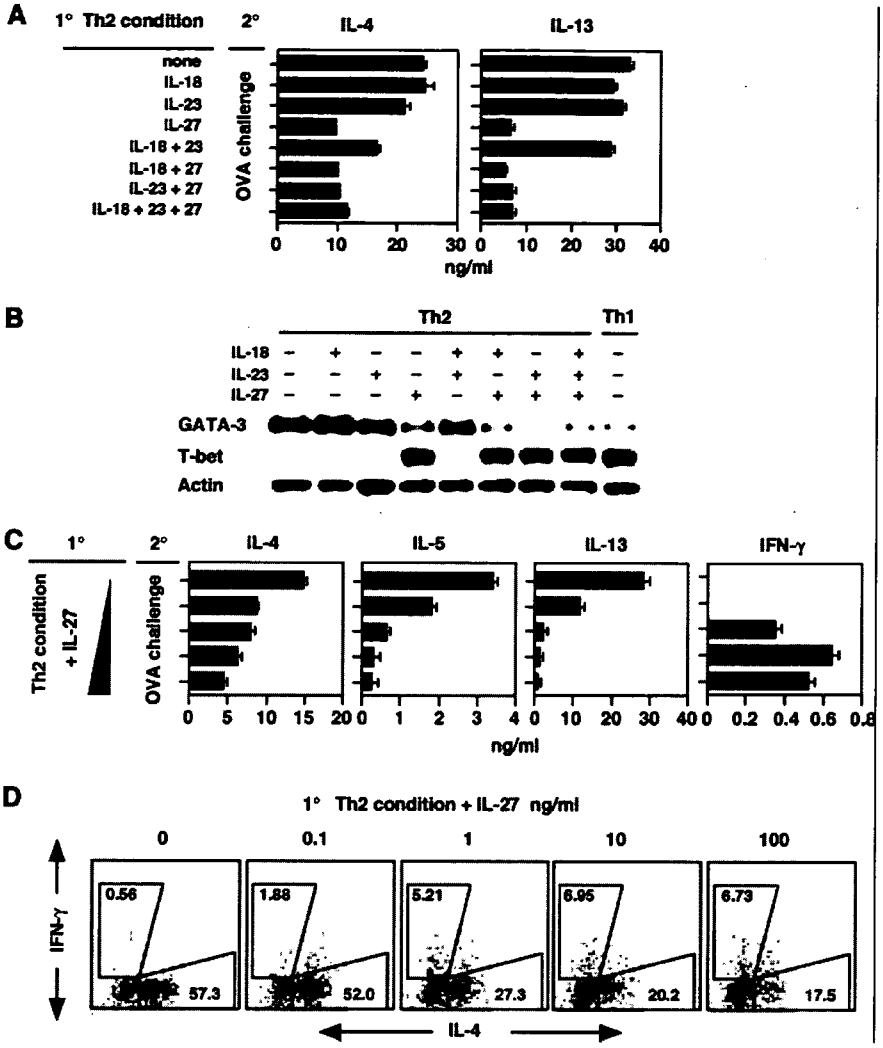

FIGURE 1. IL-27 inhibits Th2 cell polarization. Naive splenic $CD4^+CD62L^+$ T cells (10^5/ml) from DO11.10 mice were cultured with 100 pM IL-2, 1 μM $OVA_{323-339}$, and 2×10^4/ml irradiated BALB/c splenic dendritic cells in Th2 condition (1000 U/ml IL-4 plus 20 μg/ml anti-IL-12p40/anti-IFN-γ) or Th1 condition (10 ng/ml IL-12 and 10 μg/ml anti-IL-4) in 24-well plates in a total 1-ml volume for 7 days. Twenty micrograms of IL-18, IL-23, and IL-27 per milliliter, either by itself or in various concentration (*A* and *B*) or various concentrations of IL-27 (0–100 ng/ml; *C* and *D*) were added from the beginning of the culture. After initial priming, cells were washed and recultured with 100 pM IL-2, 1 μM $OVA_{323-339}$, plus irradiated T cell-depleted BALB/c splenocytes for 48 h for induction of cytokine secretion (*A* and *C*), or recultured with PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 4 h and analyzed by FACS for cytosolic IL-4 and IFN-γ (*D*). Supernatants were harvested and tested for IL-4, IL-5, IL-13, and IFN-γ production by ELISA (*A* and *C*). Numbers represent the percentage of $IL\text{-}4^+$ or $IFN\text{-}\gamma^+$ cells gated on $CD4^+$ T cells (*D*). After 5 days of initial priming and washing cells, nuclei or total cell lysates were prepared and subjected to Western blotting using anti-GATA-3 or anti-T-bet, respectively (*B*). Results are geometric means + SEM. Results are representative of three independent experiments.

Western blotting

Protein levels of GATA-3 and T-bet were determined using Western blot techniques. To detect GATA-3 expression, cells were washed once with PBS and resuspended in buffer A (10 mM HEPES (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2 μg/ml aprotinin, 0.5 mM PMSF, and 0.5% Nonidet P-40). Nuclei were pelleted, and the cytoplasmic proteins were carefully removed. The nuclei were then resuspended in buffer C (50 mM HEPES (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 2 μg/ml aprotinin, and 0.5 mM PMSF) as described elsewhere (37). After vortexing and stirring for 30 min at 4°C, the samples were centrifuged, and the nuclear proteins in the supernatant were transferred to a fresh vial. Nuclear extracts were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were probed with murine mAbs against GATA-3 (HG3–31, dilution 1/200). HRP-conjugated goat anti-mouse IgG Ab (dilution 1/2000; Chemicon International) was used for visualization. To detect the expression of T-bet and tyrosine phosphorylation of Stat1 and 3, whole cell lysates were subjected to Western blot analysis as described previously (38).

To detect circulating FALG-tagged IL-27 in IL-27 Tg mice, sera obtained form each mice were precleared and incubated with anti-FLAG conjugated to protein G-Sepharose (Amersham Pharmacia Biotech). After washing the beads, the complexes were separated on an SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes (Millipore). The membrane was blocked, probed with anti-FLAG and then with anti-mouse IgG conjugated to HRP, and visualized with the ECL detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

RT-PCR

Total RNA was extracted by using a guanidine thiocyanate procedure, cDNA was prepared using an oligo(dT) primer and SuperScript RT (Invitrogen Life Technologies), and RT-PCR was performed using TaqDNA polymerase as described elsewhere (39). Cycle conditions were 94°C for 40 s, 60°C for 20 s, and 72°C for 40 s. The following primers were used to detect FLAG-tagged IL-27: sense primer, 5′-GATCCTGAGAACTTCAGGCTC-3′; antisense primer and 5′-GTA ACCAGGTAGCTTGTCATCGTCATCC-3′. Primers used for HPRT were described previously (40).

Statistics

Statistical comparisons between experimental groups were determined by the paired Student *t* test. All analysis was performed by GraphPad Instat Software. A value of $p < 0.05$ was considered to be significantly different.

Results

IL-27 inhibits Th2 cell differentiation

We first examined the capacity of IL-27 to inhibit Th2 cell differentiation in vitro. We established Th2 cells by culturing naive $CD4^+$ T cells obtained from OVA-specific TCR Tg BALB/c (DO11.10) mice with OVA peptide, IL-4, and anti-IL-12 plus anti-IFN-γ for 1 wk (35). The resultant Th2 cells challenged with OVA produced significant amounts of IL-4, IL-5, and IL-13 but not

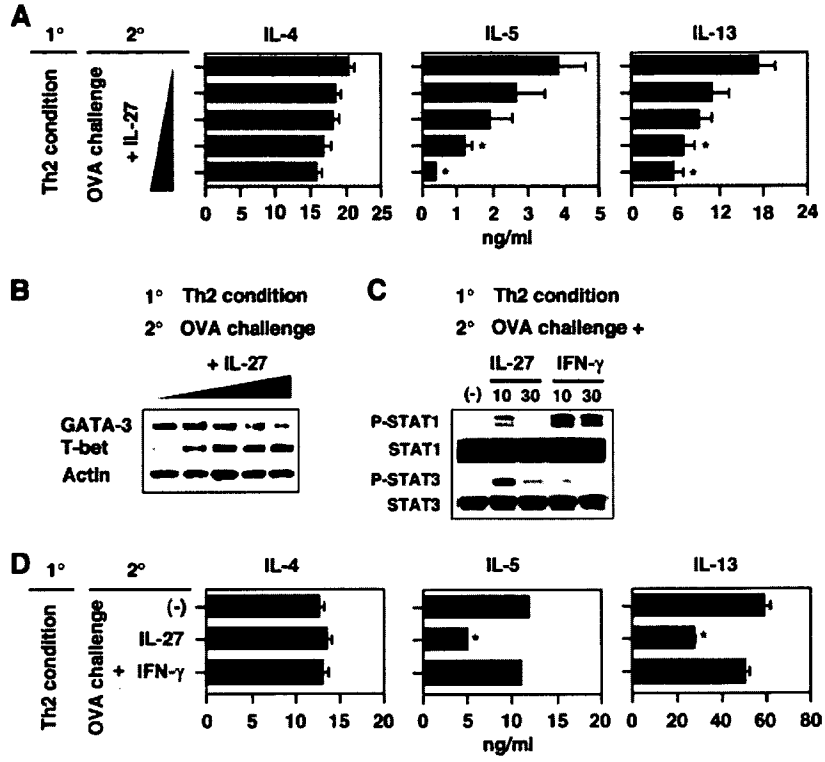

FIGURE 2. IL-27 suppresses cytokine production from polarized Th2 cells. After initial priming of Th2 conditions as indicated in Fig. 1, cells were washed and recultured with 100 pM IL-2, 1 μM $OVA_{323-339}$ plus irradiated T cell-depleted BALB/c splenocytes in the presence of IL-27 (0 – 100 ng/ml) for 48 h for induction of cytokine secretion (*A*) and expression of GATA-3 and T-bet (*B*). Results are geometric means + SEM. Results are representative of three independent experiments. *C*, Polarized Th2 cells were stimulated with IL-27 (10 ng/ml) or IFN-γ (20 ng/ml) for 10 and 30 min. Total cell lysates were then prepared and subjected to Western blotting with Abs specific for indicated phosphorylated Stat proteins. To control for equal protein loading, blots were stripped and reprobed with Abs specific for the indicated total Stat proteins. Experiments were repeated twice with similar results. *D*, Polarized Th2 cells were recultured with 100 pM IL-2, 1 μM $OVA_{323-339}$ plus irradiated T cell-depleted BALB/c splenocytes with IL-27 (10 ng/ml) or IFN-γ (20 ng/ml) for 48 h for induction of cytokine secretion. Results are geometric means + SEM. Results are representative of two independent experiments. *, $p < 0.05$ vs corresponding value for Th2 cells recultured with $OVA_{323-339}$ alone.

IFN-γ (Fig. 1, *A* and *C*). Addition of IL-27 directly and dose-dependently inhibited Th2 cell differentiation and conversely induced Th1 cell differentiation even in the absence of IFN-γ and IL-12 (Fig. 1, *A* and *C*). We also examined the mechanism by which IL-27 inhibits Th2 development but simultaneously induces Th1 development and found that IL-27 suppressed GATA-3 expression but induced T-bet expression (Fig. 1*B*). Since IL-12 and IL-18 synergistically inhibit Th2 responses (7), we simultaneously examined the potential of IL-18 to enhance the action of IL-27 and found that IL-18 has no such function (Fig. 1, *A* and *B*). Another member of the IL-12 family, IL-23 (26, 41), failed to replace IL-27 in inhibition of Th2 cell development (Fig. 1, *A* and *B*). We also examined the effects of IL-27 on Th2 polarization by FACS analysis. We found that 57 and 0.56% of $CD4^+$ T cells cultured under Th2-inducing condition were positive for cytoplasmic IL-4 and IFN-γ, respectively. Addition of IL-27 dose-dependently decreased the proportion of cytoplasmic IL-4-positive cells (to 17.5%) but increased the proportion of cytoplasmic IFN-γ-positive cells (to 6.73); Fig. 1*D*).

IL-27 inhibits Th2 cytokines production from already polarized Th2 cells

It is important to examine the inhibitory effect of IL-27 on the function of already differentiated Th2 cells. Polarized Th2 cells produce Th2 cytokines (Fig. 2 *A*). We found that IL-27 dose-dependently inhibited IL-5 and IL-13 but not IL-4 production from polarized Th2 cells (Fig. 2 *A*). Surprisingly, IL-27 diminished GATA-3 expression and conversely induced T-bet expression even in highly polarized Th2 cells (Fig. 2*B*). As reported previously (16–18, 23, 30, 38, 42), both IL-27 and IFN-γ activated Stat1 (Fig. 2*C*). However, only IL-27 stimulation strongly activated Stat3 (Fig. 2*C*). Furthermore, IL-27 stimulation but not IFN-γ stimulation suppressed IL-5 and IL-13 production from polarized Th2 cells (Fig. 2*D*). Further studies are definitively needed to understand the role of Stat1 and Stat3 in regulation of Th2 cytokine production.

Administration of IL-27 protects BALB/c mice from leishmaniasis

BALB/c mice infected with *L. major* developed progressive disease, as accessed by footpad swelling, while C57BL/6 mice are highly resistant to *L. major* (Fig. 3*A*). The resistance and susceptibility of inbred strains of mice to infection are intimately associated with the capacity to produce IFN-γ and IL-4, respectively. Susceptible BALB/c mice develop a Th2 response and resistant C57BL/6 mice develop a Th1 response following *L. major* infection (43, 44). As reported elsewhere, popliteal lymph nodes cells from *L. major*–infected BALB/c mice strongly produced IL-4 and little IFN-γ, while those from *L. major*-infected C57BL/6 mice strongly produced IFN-γ and little IL-4 (Fig. 3*B*). Consistent with our previous report (32), administration of a mixture of IL-12 (10 ng/day) and IL-18 (1 μg/day) for the first week of infection protected BALB/c mice from footpad swelling (Fig. 3*A*) and diminished parasite burden (Fig. 3*C*) by down-regulation and up-regulation of Th2 and Th1 responses, respectively (Fig. 3*B*), whereas administration of IL-18 alone did not show such protective effects (data not shown) (32). Next, to address the inhibitory effect of IL-27 on the generation of Th2 cells in vivo, we daily administered IL-27 (1 μg/day) into *L. major*-infected BALB/c mice for 1 wk. This administration partially but significantly protected mice from footpad swelling (Fig. 3*A*) and considerably diminished parasite burden (Fig. 3*C*). In addition to 1-wk treatment with IL-27, we administered IL-27 for 2 wk into *L. major*-infected BALB/c mice and found that both group showed similar footpad swelling. Importantly, IL-27-treated *L. major*-infected BALB/c mice all survived at 12- 16 wk after infection, whereas control PBS-treated *L. major*-infected BALB/c mice died of disseminated infection

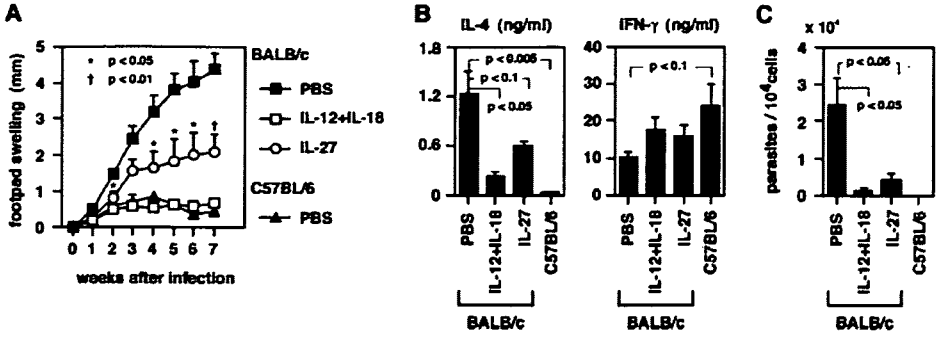

FIGURE 3. IL-27 protects BALB/c mice from leishmaniasis. *A*, Mice were s.c. inoculated with stationary-phase promastigotes on the first day of experiment. BALB/c mice (five per group) were daily i.p. injected with PBS, IL-12 (10 ng/day) plus IL-18 (1 μg/day) or IL-27 (1 μg/day) during the first 7 days after infection. Weekly footpad measurements represent the average footpad swelling + SEM. *, $p < 0.05$; †, $p < 0.01$ vs corresponding value for infected mice treated with PBS. *B*, Draining popliteal lymph node cells from each group of mice (five per group) were harvested at 7 wk after infection. Cell suspensions were cultured at 2×10^5/0.2 ml per well under stimulation with immobilized anti-CD3 and anti-CD28 (each 5 μg/ml). After 48h of culture, supernatants were harvested and tested for IFN-γ and IL-4 production by ELISA. *C*, At 7 wk after infection, parasite burdens in 1×10^3 cells of the popliteal lymph node draining the site of infection were determined. Statistical differences between samples were determined using Student's *t* test. Results are representative of three independent experiments.

around these periods (data not shown). We simultaneously measured the capacity of popliteal lymph nodes cells to produce IL-4 and IFN-γ upon stimulation with plate-bound anti-CD3 plus anti-CD28 in vitro. Lymph nodes cells from *L. major*-infected and IL-27-administered BALB/c mice diminished IL-4 production but increased IFN-γ production (Fig. 3*B*). These results taken together indicated that administration of IL-27 into *L. major*-infected BALB/c mice protected them from progressive leishmaniasis by direct inhibition of Th2 response and simultaneous induction Th1 response. We suspect that IL-27-stimulated T cells come to express IL-12Rβ2 and develop into Th1 cells under the stimulation of endogenous IL-12 in vivo.

Exogenous IL-27 in wild-type or endogenous IL-27 in IL-27 Tg mice inhibits S. venezuelensis *-induced Th2 responses*

Mice infected with L3 of the gastrointestinal nematodes *S. venezuelensis* increased serum IgE levels, number of intestinal mucosal mast cells, and serum mMCP-1 through primary Th2 responses (33, 45). The expulsion of parasites is tightly associated with the level of intestinal mastocytosis, which correlates well with the serum mMCP-1 level (33, 46). Infected mice completed parasite expulsion within 2 wk. Because IL-27 reciprocally regulates Th1 and Th2 responses (Figs. 1–3), we tested whether in vivo administration of IL-27 into mice inoculated with *S. venezuelensis* L3 inhibits Th2-driven IgE and intestinal mastocytosis. This treatment considerably inhibited IgE and mMCP-1 levels and parasite expulsion (Fig. 4, *A–C*). Furthermore, this IL-27 administration markedly increased Th1 cells but diminished Th2 cells by reciprocal regulation of Th1 and Th2 responses in *S. venezuelensis* L3-infected C57BL/6 mice (Fig. 4*D*).

To substantiate further this conclusion, we generated two IL-27 Tg mouse lines, 1 and 6, as described in *Materials and Methods*. Circulating FLAG-tagged IL-27 in each Tg mice was determined by ELISA and further analyzed by Western blot analysis using anti-FLAG Ab (Fig. 5, *A* and *B*). Expression of FLAG-tagged IL-27 mRNA in liver was detected by RT-PCR analysis (Fig. 5*C*). Liver-selective expression of FLAG-tagged IL-27 was also determined by RT-PCR analysis using RNA obtained form various tissues (Fig. 5*D*). The mean concentrations of FLAG-tagged IL-27 in sera of IL-27 Tg1 and Tg6 mice at 6–8 wk of age were 0.962 ± 0.543 ng/ml ($n = 17$) and 0.569 ± 0.338 ng/ml ($n = 19$), respectively. Although the IL-27 Tg mice appeared normal at birth, approximately one-half of IL-27 Tg1 mice and 10% of IL-27 Tg6 mice died by 10 mo of age. More shortened survival rate in IL-27 Tg1 mice than that in IL-27 Tg6 mice could be due to more increased expression of FLAG-tagged IL-27 (Fig. 5, *A–C*). IL-27 Tg

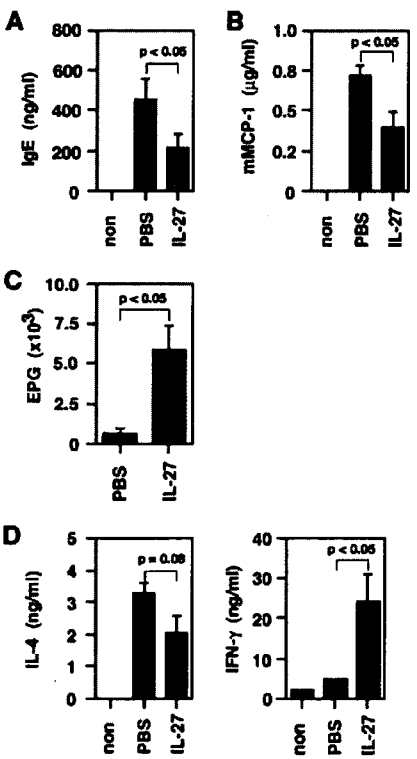

FIGURE 4. IL-27 inhibits *S. venezuelensis*-induced Th2 responses. C57BL/6 mice were inoculated with 5000 *S. venezuelensis* L3 on the first day of experiment. Mice (five per group) were daily i.p. injected with PBS or IL-27 (1 μg/day) during the first 7 days after infection. Serum levels of IgE (*A*) and mMCP-1 (*B*) and the number of eggs per gram of feces (*C*) at day 10 after infection were measured. *D*, Draining mesenteric lymph node cells from each group of mice were harvested at day 11 after infection. Cell suspensions were cultured at 1×10^5/0.2 ml per well under stimulation with immobilized anti-CD3 and anti-CD28 (each 5 μg/ml). After 48 h of culture, supernatants were harvested and tested for IFN-γ and IL-4 production by ELISA. Results are geometric means + SEM of five animals per group and are representative of three independent experiments. Statistical differences between samples were determined using Student's *t* test.

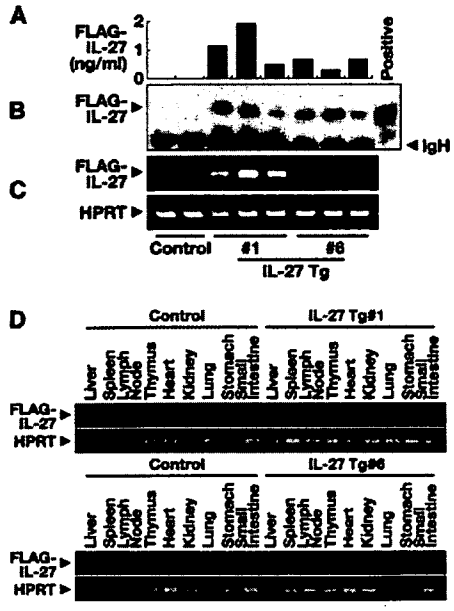

FIGURE 5. Establishment of IL-27 Tg mice. Two IL-27 Tg mouse lines #1 and #6 using a liver-specific promoter were established. Expression of FLAG-tagged IL-27 in sera (n = 3 each) was determined by specific ELISA (*A*) and immunoprecipitation followed by Western blotting using anti-FLAG (*B*). Purified recombinant FLAG-tagged IL-27 protein was used as a positive control for Western blot analysis. *C*, mRNA expression in liver was detected by RT-PCR analysis. *D*, Liver-selective expression of FLAG-tagged IL-27 was also detected by RT-PCR analysis using RNA obtained from various tissues.

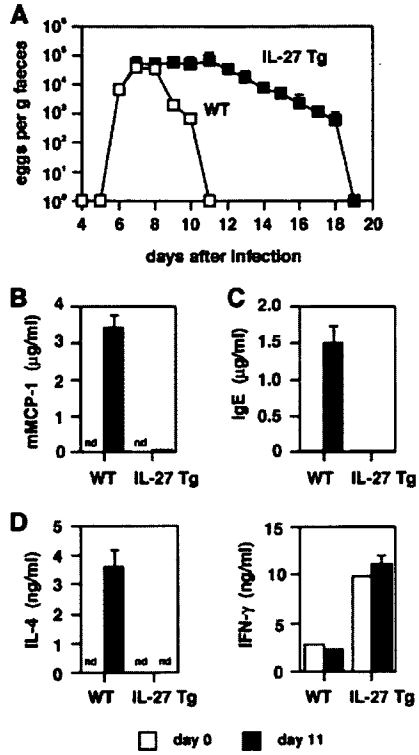

FIGURE 6. IL-27 Tg mice inhibit *S. venezuelensis*-induced Th2 responses. Kinetics of the number of eggs per gram of feces (*A*) and serum levels of mMCP-1 (*B*) and IgE (*C*) from C57BL/6 wild-type (WT) and IL-27 Tg mice (five per group) inoculated with 5000 *S. venezuelensis* L3. *D*, Draining mesenteric lymph node cells from each group of mice (five per group) were harvested at day 11 after infection. Cell suspensions were cultured at 2×10^5/0.2 ml per well under stimulation with immobilized anti-CD3 and anti-CD28 (each 5 μg/ml). After 48 h of culture, supernatants were harvested and tested for IFN-γ and IL-4 contents using ELISA. nd; Not detected. Results are geometric means + SEM of five animals per group and are representative of three independent experiments.

mice could live at least by 6 mo of age. Histological analysis at 8–12 wk of age revealed extramedullary hemopoiesis in the spleens of IL-27 Tg mice compared with wild-type mice. However, their livers had no obvious morphological abnormalities. Hematological examination of peripheral blood revealed no significant difference in the number of leukocytes, erythrocytes, and platelets. Furthermore, IL-27 Tg mice had no immunological defects and percentages of $CD4^+$ and $CD8^+$ T cells in their thymus and secondary lymphoid organs were comparable to those in wild-type mice. Finally, both T cells and B cells were not activated when examined using FACS analysis (our unpublished observation). We suspected that IL-27 Tg mice died of severe anemia at 10 mo of age.

We inoculated wild-type C57BL/6 mice and C57BL/6 background IL-27 Tg mice with *S. venezuelensis* L3 and daily counted the fecal egg number (eggs per gram of feces). We used line #1 IL-27 Tg mice for these experiments. Inoculated wild-type mice completed parasite expulsion by day 11, whereas IL-27 Tg mice exhibited significantly prolonged production of the parasite eggs (Fig. 6*A*), suggesting that IL-27 inhibited Th2 response and simultaneously augmented Th1 response. Expectedly, wild-type mice inoculated with *S. venezuelensis* L3 had increased serum levels of mMCP-1 and IgE. In contrast, IL-27 Tg mice failed to exhibit these responses (Fig. 6, *B* and *C*). Lymph node cells from *S. venezuelensis*-inoculated wild-type mice produced IL-4 strongly but IFN-γ poorly upon stimulation with plate-bound anti-CD3 plus anti-CD28 (Fig. 6*D*), indicating that *S. venezuelensis* induces a Th2 response in wild-type mice. In contrast, there were no IL-4-producing T cells in the lymph nodes of *S. venezuelensis*-inoculated IL-27 Tg mice (Fig. 6*D*), substantiating further their disability to normally expel gastrointestinal nematodes. Interestingly, IL-27 Tg mice before and after infection only developed Th1 cells in their lymph nodes (Fig. 6*D*), indicating that excessive IL-27 production preferentially induced Th1 cell possibly by up-regulation of IL-12Rβ2 in the presence of endogenous IL-12 (12, 16–18).

Intranasal administration of IL-27 suppresses allergic airway inflammation

Because IL-27 has a striking capacity to inhibit Th2 cells to produce IL-5 and IL-13 (Fig. 2*A*), we examined its therapeutic potential on allergic diseases. For this purpose, we established an experimental allergic asthma model. BALB/c mice, immunized with OVA 1 wk before and challenged with intranasal OVA administration for 3 days developed AHR in response to β-methacholine exposure (Fig. 7*A*). Furthermore, they showed peribronchial accumulation with eosinophils and neutrophils (Fig. 7*B*) and goblet cell metaplasia in their airways (Fig. 7*C*). Daily i.p. injections of IL-27 (1 μg/day) for the first 7 days following immunization with OVA modestly diminished AHR to that of β-methacholine (data not shown). However, intranasal administration of IL-27 at the time of OVA challenge dose-dependently diminished AHR (Fig. 7*A*), airway eosinophilic inflammation (Fig. 7*B*), and goblet cell metaplasia (Fig. 7*C*). Among the Th2-related cytokines, IL-13 is thought to be most bronchogenic (34, 47). Thus, we nasally administered various doses of IL-27. We

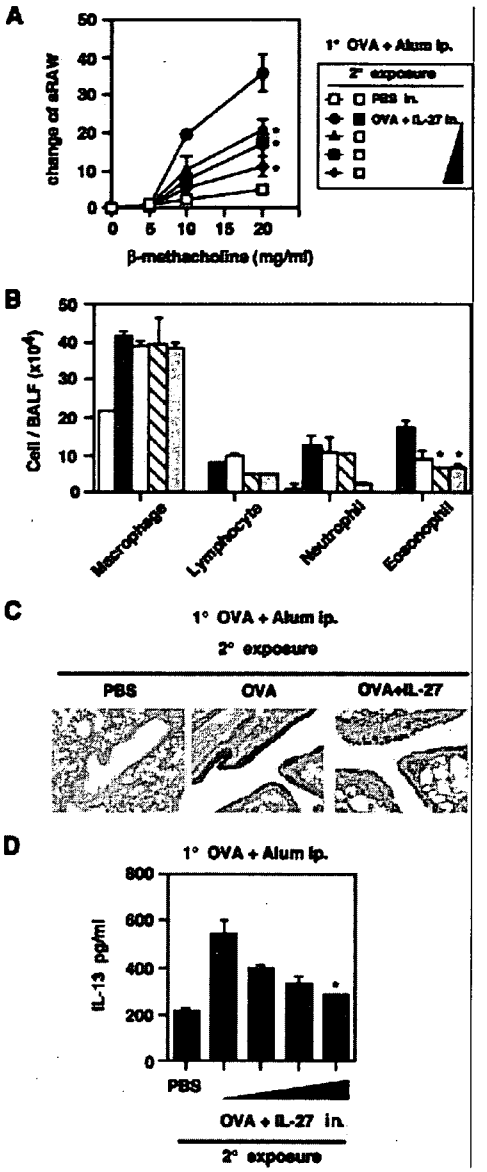

FIGURE 7. IL-27 protects against allergic airway inflammation. BALB/c mice (five mice per group) were immunized i.p. with 50 μg of OVA with aluminum potassium sulfate (alum) on day 1 and challenged intranasally with 50 μg of OVA with or without IL-27 (0.2, 1, 5 μg) in 50 μl of PBS on days 7–9. Control mice were immunized with OVA and exposed to PBS alone. The allergic phenotype was assessed 24 h after the final exposure to Ag. *A*, AHR in response to increased concentrations of inhaled β-methacholine was measured in a whole-body plethysmograph. *B*, Inflammatory cell composition of bronchoalveolar lavage from each group of mice. Cell differential percentages were determined by light microscopic evaluation of cytospin preparations. Data are expressed as absolute numbers of cells. *C*, Lungs were prepared for histology by perfusing the animal via the right ventricle with 10 ml of PBS, then fixed in 10% buffered formalin, cut into 3-μm sections, and stained with predigested periodic acid-Schiff. Original magnification, ×100. *D*, Lungs were homogenized and centrifuged as described in *Materials and Methods*. The obtained supernatants were tested for IL-13 contents by ELISA. in, Intranasal. Results are geometric means ± SEM of five animals per group and are representative of three independent experiments. *, $p < 0.05$ vs corresponding value for OVA-sensitized and OVA-administered mice.

found that IL-27 dose-dependently diminished the IL-13 level in the lung (Fig. 7*D*). These results clearly indicated that intranasal administration of IL-27 into OVA-immunized animals inhibits IL-13 production from OVA-stimulated Th2-polarized cells, providing promising a therapeutic way for the treatment of allergic asthma.

Discussion

Yoshida and his colleagues (22) reported that IL-27R$\alpha^{-/-}$ mice immunized with OVA exhibited enhanced asthmatic phenotypes in response to OVA challenge. In addition, Hunter and colleagues (21) reported that IL-27R$\alpha^{-/-}$ mice infected with gastrointestinal nematode *T. muris* displayed increased Th2 cell-dependent intestinal goblet cell hyperplasia, mastocytosis, and Th2 cytokine production. Therefore, IL-27 seems to inhibit Th2 responses in vivo. Furthermore, IL-27 inhibits $CD4^+$ T cell expression of GATA-3 by activation of Stat1 (23). However, the direct evidence and molecular mechanism by which IL-27 exerts an inhibitory effect on Th2 responses remains unclear. To address these issues, we examined the capacity of rIL-27 to inhibit Th2 polarization in vitro and found that IL-27 inhibits Th2 cell development, even in the presence of anti-IFN-γ Ab and anti-IL-12 Ab (Fig. 1*A*). Furthermore, IL-27 induced Th1 cell differentiation under Th2-inducing conditions (Fig. 1, *C* and *D*). Thus, IL-27 can directly inhibit Th2 cell development and conversely induce them to develop into Th1 cells that produce IFN-γ. In line with this, in vivo treatment with IL-27 for the first week after *L. major* infection significantly diminished footpad swelling and parasite burden by induction of Th1 cell development and inhibition of Th2 cell development simultaneously (Fig. 3).

It is well known that expulsion of some types of helminths depends on the action of activated mast cells (45, 46, 48). Recently, we reported that administration of a mixture of IL-2 and IL-18 induces intestinal mastocytosis by induction of mast cell growth factors such as IL-3 and IL-9 and that such IL-2 and IL-18-pretreated mice rapidly expel implanted adult worms of *S. venezuelensis* (33). However, as we reported here, IL-27-treated *S. venezuelensis*-infected C57BL/6 mice failed to do so and sustained infection. They exhibited significantly reduced levels of IgE and mMCP-1 in their sera and decreased Th2 response but increased Th1 response simultaneously (Fig. 4). IL-27 Tg mice, that we have newly established, could not mount the Th2 response in response to infection with *S. venezuelensis*. Accumulated IL-27 in IL-27 Tg mice strongly induces Th1 cells before infection with *S. venezuelensis*. Thus, infected IL-27 Tg mice could not increase mMCP-1 and IgE levels in their sera (Fig. 6, *B* and *C*), resulting in sustained infection (Fig. 6*A*). The molecular mechanism by which IL-27 inhibits Th2 cell differentiation could be explained by down-regulation of GATA-3 and simultaneous up-regulation of T-bet (Fig. 1*B*).

The most remarkable inhibitory effect of IL-27 on Th2 cells presented here is direct suppression of IL-5 and IL-13 production from already polarized Th2 cells (Fig. 2*A*). It is well known that IFN-γ counteracts Th2 development (49). However, IFN-γ failed to suppress IL-5 and IL-13 production from polarized Th2 cells (Fig. 2*D*), even though IFN-γ could induce Stat1 activation in polarized Th2 cells (Fig. 2*C*). Furthermore, it was recently demonstrated that IL-27 more efficiently inhibits Th-17 cell development than IFN-γ (24). These results taken together indicate the importance of IL-27 for the treatment of diseases induced by Th2 cells or Th-17 cells.

It is well known that IFN-γ increases the expression of T-bet through Stat1 activation (50). However, IL-27 induced Stat1 activation and T-bet expression in the absence of IFN-γ and IL-12 (Fig. 1). These results suggest that IL-27 directly induces T-bet via Stat1 activation independently of IFN-γ and IL-12. Usui et al. (51)

have revealed that administration of retroviral T-bet into established Th2 clones inhibits IL-5 production without affecting IL-4 production by down-regulation of GATA-3 expression. Furthermore, Zhu et al. (52) have shown that deletion of GATA-3 in established Th2 cells completely abolished their capacity to produce IL-5 and IL-13 but not IL-4. Functional GATA-3 binding sites were found in both *il5* and *il13* promoters but not in *il4* promoter (53, 54), suggesting the involvement of GATA-3 as an essential transcription factor in directly activating *il5* and *il13* promoters. Thus, IL-27 can inhibit production of IL-5 and IL-13 by down-regulation of GATA-3 and up-regulation of T-bet through Stat1 activation (Fig. 2, *B* and *C*), substantiating further these previous reports. Moreover, IL-27 can activate both Stat1 and Stat3 in polarized Th2 cells, while IFN-γ only activates Stat1 (Fig. 2*C*). Further studies are necessary to elucidate the precise molecular mechanisms by which IL-27 inhibits the Th2 differentiation and Th2 cytokine production from polarized Th2 cells through activation of both Stat1 and Stat3.

In vivo treatment with high doses of IL-12 alone or low doses of IL-12 plus IL-18 prevents the development of Th2 cells (55), which results in down-regulation of allergic inflammation including bronchial asthma (49). In this report, we showed that IL-27 significantly suppressed IL-5 and IL-13 production from already differentiated Th2 cells by down-regulating GATA-3 and up-regulation of T-bet simultaneously (Fig. 2, *A* and *B*). According to this result, intranasal administration of IL-27 with OVA into OVA-sensitized animals dose-dependently diminished AHR to β-methacholine exposure, airway eosinophilic inflammation, and goblet cell metaplasia with mucous overproduction by suppressing IL-13 production (Fig. 7). IL-13 is suggested to play a critical role in induction of AHR, airway eosinophilia, and mucus oversecretion. Indeed, our group and others (34, 47) have reported that blockade of IL-13 markedly inhibits allergen-induced AHR, eosinophilic inflammation, and goblet cell metaplasia in animal models. We have elucidated that IL-27 treatment has no apparent adverse effects such as inflammatory bowel disease and liver injury with elevated serum glutamic-oxaloacetic transaminase and alanine aminotransferase activities, which are often seen with high doses of IL-12 treatment (27, 56). These results taken together strongly suggest that intranasal IL-27 administration might become an important therapeutic approach for the establishment of the treatment for bronchial asthma.

Acknowledgments

We thank Dr. W. E. Paul for his critical reading of this manuscript and Dr. Yuriko Ishikawa for her excellent experimental assistance.

Disclosures

The authors have no financial conflict of interest.

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Protective Effect of IL-18 on Kainate- and IL-1β-Induced Cerebellar Ataxia in Mice[1]

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The pathogenesis of sporadic cerebellar ataxia remains unknown. In this study, we demonstrate that proinflammatory cytokines, IL-18 and IL-1β, reciprocally regulate kainate-induced cerebellar ataxia in mice. We show that systemic administration of kainate activated IL-1β and IL-18 predominantly in the cerebellum of mice, which was accompanied with ataxia. Mice deficient in caspase-1, IL-1R type I, or MyD88 were resistant to kainate-induced ataxia, while IL-18- or IL-18R α-deficient mice displayed significant delay of recovery from ataxia. A direct intracerebellar injection of IL-1β-induced ataxia and intracerebellar coinjection of IL-18 counteracted the effect of IL-1β. Our data firstly show that IL-18 and IL-1β display differential direct regulation in kainate-induced ataxia in mice. Our results might contribute toward the development of a new therapeutic strategy for cerebellar ataxia in humans. ***The Journal of Immunology,*** **2008, 180: 2322–2328.**

Kainate, an excitatory amino acid extracted from seaweed, has significantly contributed to understanding epileptogenesis (1). Previously, the effects of kainate on hippocampal neurons have been studied for delineating the mechanism of kainate-induced ataxia (1, 2). It is reported that L-glutamate, the major excitatory neurotransmitter in the brain, acts on three classes of ionotropic glutamate receptors: N-methyl-D-aspartate, α-amino-hydroxy-5-methyl-4-isoxazole propionic acid, and kainate receptors (3, 4). Kainate receptors consist of a set of genes (GluR5–7, KA-1, and KA-2), are widely distributed throughout the brain (5–10), and are implicated in epileptogenesis and neuronal cell death (11).

IL-1β and IL-18 are proinflammatory cytokines that are produced as a precursor form and proteolytically activated by caspase-1 (12). They are expressed in various tissues including the CNS (13–15). IL-1β is shown to exert neuroendocrine as well as neurodegenerative effects on animals (14, 15). It has been reported that convulsant stimuli increase the production of IL-1β and its receptor in rodent CNS within hours of seizure induction (16–18). Recently, Vezzani et al. reported that IL-1β prolongs hippocampal seizures in a N-methyl-D-aspartate receptor-dependent manner, and the action was inhibited by IL-1 receptor antagonist (IL-1ra)[3] (19, 20). Concerning IL-18, a crucial role for IL-18 in mediating neuroinflammation and neurodegeneration in the CNS under pathological conditions has been indicated (21).

Cerebellar ataxia, dysfunction of the cerebellum, causes problems such as loss of balance and motor coordination. Some types of cerebellar ataxia can be caused by several genetic mutations, including a group of autosomal dominant spinocerebellar ataxias (22) and autosomal recessive Ataxia telangiectasia (23); however, a large number of patients remain undiagnosed (sporadic cerebellar ataxias). In this study, we examined the roles of IL-1β and IL-18 in kainate-induced ataxia in mice. We demonstrated that IL-1β is activated specifically in the cerebellum by the systemic administration of kainate and is involved in kainate-induced ataxia in mice. Furthermore, we show that IL-18 in the cerebellum is involved in the recovery phase of kainate-induced ataxia by counteracting the function of IL-1β in the cerebellum. Our results show the possible anti-ataxic effect of IL-18 and may suggest new therapeutic strategies for cerebellar ataxia in humans.

Materials and Methods

Antibodies

Abs to GluR-5, GluR-6, IL-1β, IL-18, IL-1RI, calbindin, and glial fibrillary acidic protein were purchased from Santa Cruz Biotech; Abs to IL-18R and ST2L were purchased from R&D Systems; and an Ab to IL-33 were purchased from Alexa Biochem. Alexa488- or and Alexa564-conjugated anti-IgG were purchased from Molecular Probes.

Mice

Six- to 10-wk-old male mice were used in this study. BALB/c mice and C57BL/6 mice were purchased from Sankyo Laboratories. IL-1RI$^{-/-}$ mice with a C57BL/6 × 129 background and IL-18$^{-/-}$ mice with a C57BL/6 background were purchased from The Jackson Laboratory, and caspase-1$^{-/-}$ mice (24) with a BALB/c background, IL-18Rα$^{-/-}$ mice (25) with a C57BL/6 background, and MyD88$^{-/-}$ mice with a C57BL/6 background were provided by Dr. K. Kuida (Vertex Pharmaceuticals, Cambridge, MA) (24), Dr. T. Hoshino (Kurume University School of Medicine, Fukuoka, Japan) (25), and S. Akira (WPI Immunology Frontier Research Center, Osaka, Japan), respectively. These mice were maintained in our animal facility. Mice were housed under controlled temperature (23–25°C) and light (lights on from 08:00 h to 20:00 h) conditions. Food and water were freely available. The procedures for these animal experiments were reviewed and approved by the Committee for Animal Experiments at the University of Toyama.

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Received for publication September 11, 2007. Accepted for publication December 4, 2007.

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[1] This work was supported by Grants in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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[3] Abbreviations used in this paper: IL-1ra, IL-1 receptor antagonist; IL-1RI, IL-1 receptor type I.

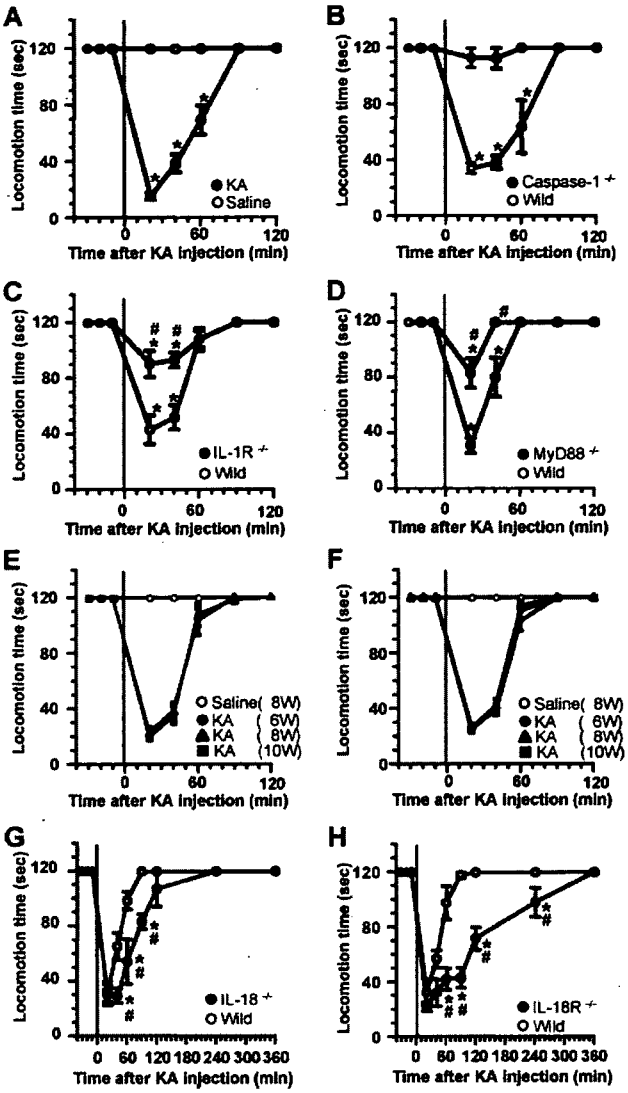

FIGURE 1. Deficiency with caspase-1, IL-1 receptor type I, or MyD88 attenuates the effect of kainate on inducing ataxia, but deficiency with IL-18 or its receptor delay the recovery from kainate-induced ataxia. *A*, Effect of kainate on locomotion activity in normal BALB/c mice was examined. Before kainate administration, a rotarod test was performed every 10 min (three times) and then kainate (KA) (20 mg/kg: ●) or saline (○) was i.p. injected into BALB/c mice at time 0. A rotarod test was performed at 20, 40, 60, 90, and 120 min after kainate injection. *, $p < 0.05$ when compared with average data before kainate injection ($n = 5$). *B–D*, *G*, and *H*, Effect of kainate on locomotion activity in wild-type mice vs gene-targeted mice was examined. A rotarod test was performed using (*B*) caspase-$1^{-/-}$ (●) or wild-type (○) mice, (*C*) IL-$1RI^{-/-}$ (●) or wild-type (○) mice, (*D*) $MyD88^{-/-}$ (●) or wild-type (○) mice, (*G*) IL-$18^{-/-}$ (●) or wild-type (○) mice, and (*H*) IL-$18R^{-/-}$ mice or wild-type (○) mice as mentioned above. *E* and *F*, Effect of kainate on locomotion activity in mice with different ages and genetic backgrounds was examined. A rotarod test was performed using either C57BL/6 mice (*E*) or BALB/c mice (*F*) 6- to 10-wk old. At time 0, kainate was injected. *, $p < 0.05$ when compared with the average data before kainate injection ($n = 5$). #, $p < 0.05$ when compared with wild-type mice ($n = 5$). Data are represented as the mean ± SEM.

Intracerebellar injection

Mice were lightly anesthetized with ethyl alcohol (wake up time: <20 s). The solution (0.5 μl) was injected into the center of the cerebellum using a 27G needle with a stopper held ~2 mm from the top of the needle and a microsyringe pump system.

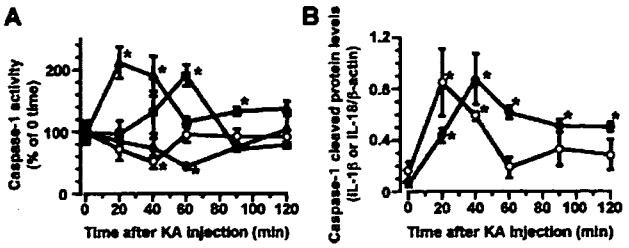

FIGURE 2. Systemic administration of kainate activates caspase-1, IL-1β, and IL-18 predominantly in cerebellum. Kainate (KA, 20 mg/kg) was i.p. injected into normal BALB/c mice and activity of caspase-1, IL-1β, and IL-18 was assessed. *A*, Activity of caspase-1 in cerebral cortex (○), cerebellum (△), hippocampus (■), and spinal cord (♦) was examined at various times after kainate-injection. Y-axis shows the percentage of caspase-1 activity compared with that at time 0. *, $p < 0.05$ when compared with the data at time 0 in each brain sample ($n = 4$). *B*, Caspase-1-processed protein levels of IL-1β and IL-18 in cerebellum were analyzed after systemic administration of kainate. Extracts from various parts of brain were separated on SDS-PAGE, and activated IL-1β (○) and IL-18 (●) were detected with immunoblotting. Y-axis shows the relative protein levels of activated IL-1β and IL-18 that are normalized with the protein level of β-actin ($n = 3$). Because significant levels of activated IL-1β and IL-18 were not detected in the cerebral cortex, hippocampus, and spinal cord, their data are omitted. Data are represented as the mean ± SEM.

Behavioral experiment

To estimate the effect of kainate and the other reagents on behavioral activity, a rotarod test was performed. To this end, Rota-Rod Treadmill (Ugo Basile) that consists of a gritted plastic rod flanked by two large round plates was used. Before performing the test, mice were trained on the rotarod until they reached a stable performance in this test (>120 s on rotarod) for one or two days before experiments. The test session was performed in accordance with the training session. Mice were brought to the experimental room at least 1 h before the experiment, and then placed on the accelerating rotarod apparatus (Ugo Basile Accelerating Rota-Rod "Jones & Roberts" for Rats 7750) with an initial speed of four rotations per

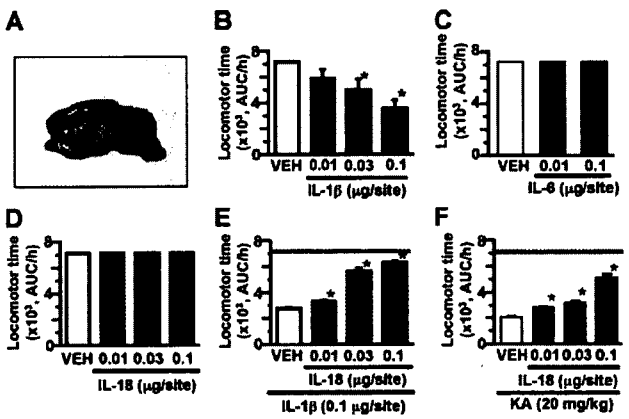

FIGURE 3. Intracerebellar injection of IL-1β induces ataxia, but intracerebellar injection of IL-18 together with IL-1β counteracted the effect of IL-1β in normal mice. *A*, Evans blue (0.5 μl) was intracerebellarly injected and its distribution was examined. *B–F*, Effect of intracerebellar injection of IL-1β, IL-18, or IL-6 on locomotion activity was examined. Various doses of IL-1β (*B*), IL-6 (*C*), IL-18 (*D*), or IL-18 with IL-1β (0.1 nmol/site) (*E*) were intracerebellarly injected and rotarod test was performed as mentioned in Fig. 1. *F*, Five min after intracerebellar injection of various doses of IL-18, kainate (KA, 20 mg/kg) was i.p. administered, and a rotarod test was performed. Y-axis shows the area under the curve (AUC) for 1 h after injection. *, $p <$ 0.05 when compared with vehicle (VEH: saline)-treated group ($n = 5$). Dashed line shows the data from vehicle-treated group (*E*, without IL-1β; *F*, without kainate). Data are represented as the mean ± SEM.

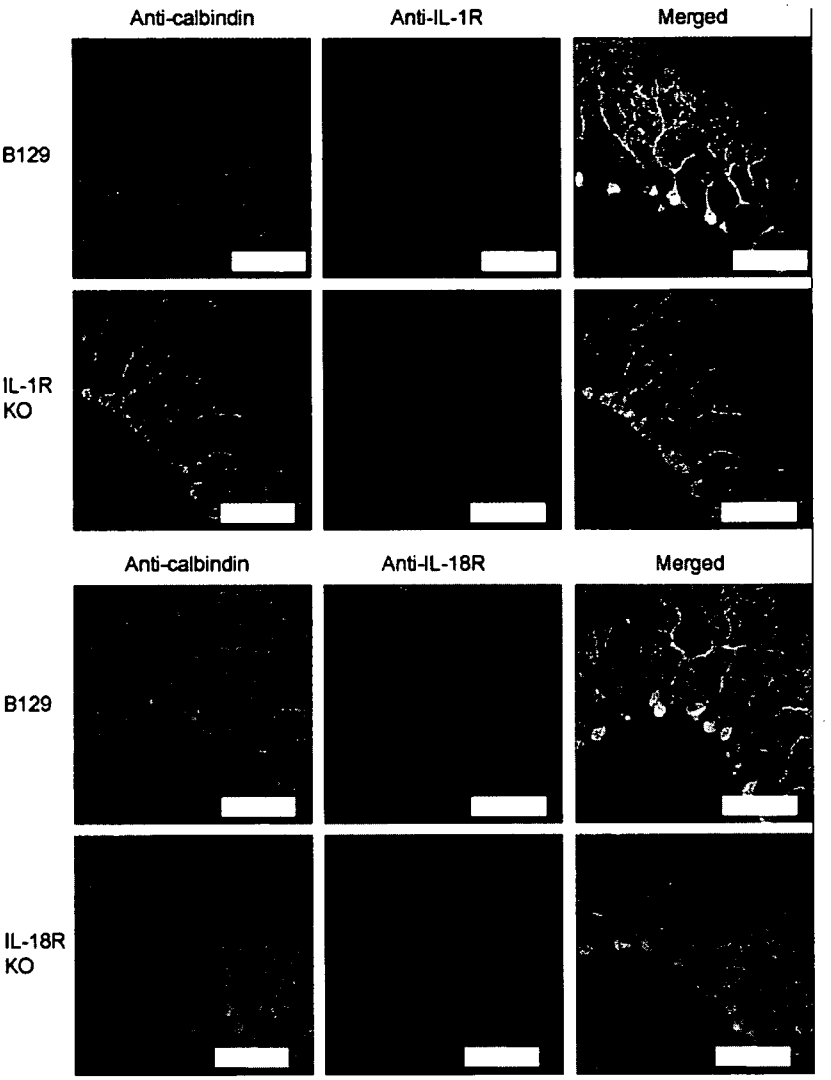

FIGURE 4. Immunohistochemical analysis of IL-1R and IL-18R in cerebellum is performed after the isolation of cerebellum from either normal mice, IL-1R$^{-/-}$ mice, or IL-18R$^{-/-}$ mice. The tissue section was stained with Ab to either IL-1RI or IL-18R, and then reacted with Alexa564-conjugated anti-rabbit or anti-goat IgG. To identify Purkinje cells, tissue sections were stained with Ab to calbindin, followed by Alexa488-conjugated anti-rabbit or anti-goat IgG. Fluorescent signals were detected using a confocal microscope. Scale bar, 100 μm.

minute; thereafter, the speed gradually increased to 60 rotations per minute. The time that the mouse remained on the rod was measured. A maximum of 120 s was allowed to test each animal. In the rotarod test, mice were pretreated with reagents or the vehicle, and the experiment was started 20 min after treatment.

Caspase-1 activity

Cell lysates were prepared by homogenizing the tissues (cerebral cortex, cerebellum, hippocampus, or spinal cord) in lysis buffer, centrifuged at 10,000 × *g* for 5 min at 4°C, and the supernatant was harvested. After determining the protein concentration, the cell lysates were used to measure the activity of caspase-1 using *N*-acetyl-Tyr-Val-Ala-Asp-*P*-nitroanilide as a substrate (Medical and Biological Laboratories). The reaction mixture was incubated for 2 h at 37°C. Caspase-1 activity was monitored with the absorbance at 420 nm, reacting chromophore *P*-nitroanilide.

Western blotting

Tissue samples (cerebral cortex, cerebellum, hippocampus, spinal cord) were homogenized in cell lysis buffer containing 137 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, and 1 μg/ml leupeptin. The total protein concentration was determined using a Bio-Rad protein assay kit. Proteins were separated by electrophoresis using a 10 or 16% SDS-polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane. The membrane was preincubated with a 5% skim milk solution for 1 h at room temperature, then incubated with primary Ab to either IL-1β or IL-18 at 4°C overnight, and subsequently incubated with a HRP-linked Ab against either mouse, rabbit, or goat IgG for 1 h. Membrane-bound HRP-labeled protein bands were reacted with a chemiluminescence detection solution (Amersham Biosciences). Chemiluminescent signals were detected using x-ray film. The amount of active IL-1β and IL-18 was evaluated by measuring the density of the active form cleaved by caspase-1 (17kD and 18 kD, respectively) using the National Institutes of Health Image program. The data were normalized with β-actin.

Immunohistochemistry

Mice were anesthetized with ethyl carbamate (1.5g/kg, i.p.) and perfused with 4% paraformaldehyde following PBS (pH 7.4). The mouse cerebellum was then isolated. The tissue was soaked in 4% paraformaldehyde for 4 h and then in 30% sucrose solution overnight at 4°C. A section (30 μm) was prepared using a cryostat. After washing with PBS, the section was soaked in PBS containing 0.3% Triton X-100 for 30 min and then in PBS containing 0.1% FBS for 30 min. The tissue section was incubated with the first Ab to either GluR-5, GluR-6, IL-1, IL-18, IL-1RI, IL-18R, calbindin, or glial fibrillary acidic protein overnight at 4°C, then washed with PBS, and reacted with Alexa488- or Alexa564-conjugated anti-IgG for 2 h. After washing with PBS, the sections were mounted with PBS/glycerol containing 0.05% triethylenediamine. Fluorescent signals were detected using a confocal microscope (Bio-Rad).

Data processing

All data are represented as the mean ± SEM. Statistical significance was analyzed using one-way ANOVA followed by Dunnett's multiple comparisons or Student's *t* test; $p < 0.05$ was considered significant.

Results

Involvement of caspase-1 and IL-1β signaling in kainate-induced ataxia

The effect of kainate on motor coordination in mice was examined with the rotarod test (26), as judged by a decrease in latency before falling from a rotating rod. An i.p. injection of kainate (20 mg/kg), but not saline as a vehicle, induced ataxic gait (Fig. 1*A*). The retention time on the rotating rod was decreased at 20 min and the effect was almost abolished by 90 min after injection (Fig. 1*A*). Because IL-1β is indicated to play a critical role in seizures (16–20) and kainate is reported to increase in IL-1β transcripts in several regions of the brain (27), we considered whether kainate-induced ataxia might be mediated by IL-1β. To verify this possibility, we first examined the effect of kainate on caspase-$1^{-/-}$ mice in which IL-1β cannot be activated. Interestingly enough, kainate showed almost no effect on motor coordination in caspase-$1^{-/-}$ mice (Fig. 1*B*), suggesting that IL-1β is involved in kainate-induced ataxia. We then examined the effect of kainate injection in IL-1 receptor type I (IL-1RI)$^{-/-}$ mice. We found that kainate showed little effect, if any, on the motor coordination of IL-1RI$^{-/-}$ mice (Fig. 1*C*). Because MyD88 is reported to play a central role in IL-1β-mediated signal transduction (28), we then examined the effect of kainate in mice deficient in MyD88. We found that the effect of kainate was limited in MyD88$^{-/-}$ mice as seen in IL-1RI$^{-/-}$ mice (Fig. 1*D*). Next, we examined whether the difference of ages and genetic backgrounds showed the different susceptibility to kainate-induced ataxia using various ages (six to 10 wk old) of C57BL/6 (Fig. 1*E*) or BALB/c mice (Fig. 1*F*), because we used caspase-$1^{-/-}$ mice on a BALB/c background and IL-1RI$^{-/-}$ or MyD88$^{-/-}$ mice on a C57BL/6 background of 6- to 10-wk-old. The result showed that the time courses of ataxia-induction and its recovery after systemic administration of kainate were corresponding in different ages of BALB/c or C57BL/6 mice. Taken together, these results indicate that i.p. injection of kainate induces ataxia gait in mice through the activation of caspase-1 and IL-1β.

Activation of caspase-1 and IL-1β in cerebellum with systemic administration of kainate

Next, we examined which region in the brain caspase-1 and IL-1β is activated. Caspase-1 activity was measured in various regions of the brain after kainate administration, and specifically increased in the cerebellum as well as hippocampus, but not in the cerebral cortex or spinal cord (Fig. 2*A*). Kinetic study showed that activity in the cerebellum peaked within 20 min and returned to the basal level by 60–90 min, while activity in the hippocampus peaked within 60 min and fell to the basal level by 90 min (Fig. 2*A*). Because ataxia was induced within 20 min after kainate administration, it is conceivable that kainate-induced activation of caspase-1 in the cerebellum evoked IL-1β activation and gait disturbance in mice. We then examined the expression of activated IL-1β at the protein level in various regions of the brain by Western blotting (Fig. 2*B*). As the precursor form of IL-1β is processed with caspase-1, their active form can be detected by the decrease of their m.w. IL-1β was activated within 20 min after kainate injection in the cerebellum (Fig. 2*B*), but was not detected in the cerebral cortex, hippocampus, or spinal cord (data not shown). Although it has been reported that kainate induced the increase in IL-1β mRNA in the cerebral cortex, thalamus, and hypothalamus (27), it remains unclear whether IL-1β is activated at the protein level in these loci. Our results show that systemic administration of kainate activates IL-1β predominantly in the cerebellum and indicates that this activation may play a critical role in kainate-induced ataxia.

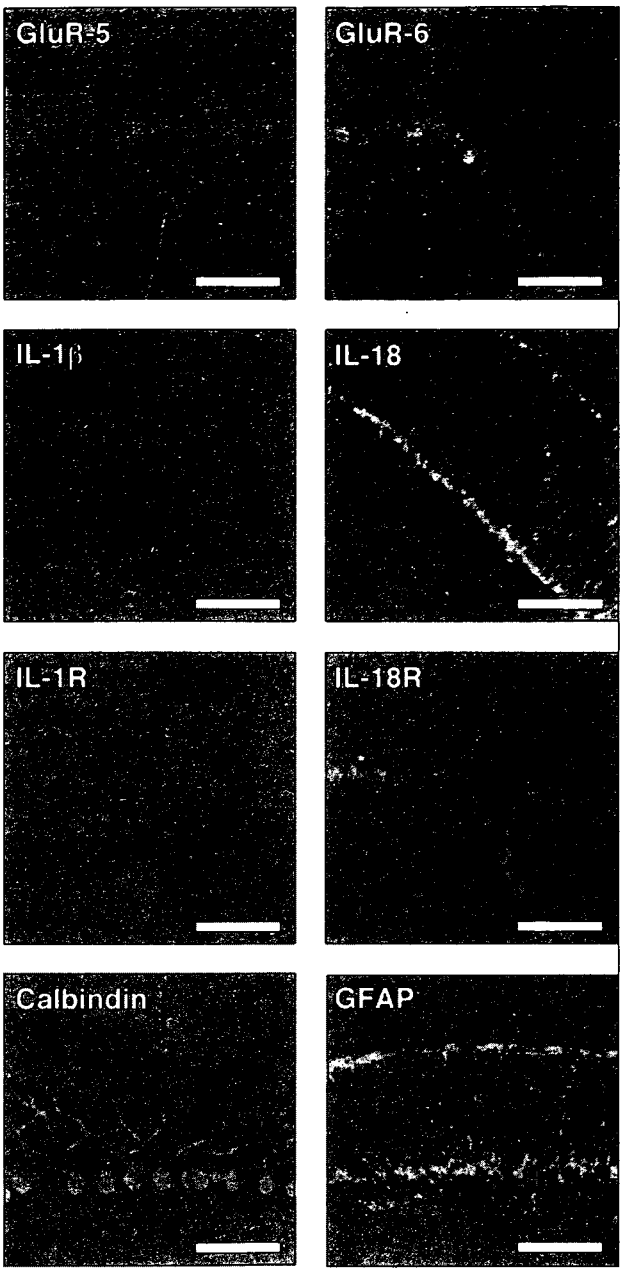

FIGURE 5. Immunohistochemical analysis of kainate receptors, IL-1, IL-18, IL-1R, and IL-18R in cerebellum was performed after isolation of cerebellum from normal mice. The tissue section was prepared and stained with Ab to either GluR5, GluR6, IL-1β, IL-18, IL-1RI, IL-18Rα, calbindin, or glial fibrillary acid protein (GFAP), then followed with FITC-conjugated anti-IgG for 1 h. Fluorescent signals were detected using a confocal microscope. Scale bar, 100 μm.

Direct injection of IL-1β to cerebellum induces ataxia

To verify the above possibility, we directly injected recombinant IL-1β into the cerebellum and examined the motor coordination. To confirm whether the intracerebellar injection was accurate, Evans blue solution (0.5 μl of 1% solution) was injected. As shown in Fig. 3*A*, Evans blue solution was spread only in the field of the cerebellum. An intracerebellar injection of IL-1β (0.01–0.1 μg/site) induced ataxia in a dose-dependent manner (Fig. 3*B*). As a negative control, we injected IL-6 (0.01 and 0.1 μg/site) intracerebellarly and observed no induction of ataxia (Fig. 3*C*), which confirmed the IL-1β-specific ataxia induction.

IL-18 is involved in the recovery phase of kainate-induced ataxia

Because casapse-1 also activates IL-18 (29), we examined the activation of IL-18 in the cerebellum after kainate i.p. injection. Like IL-1β, IL-18 was also activated in the cerebellum (Fig. 2*B*). The level of activated IL-18 in the cerebellum peaked within 40 min and activated IL-18 was still observed until 2 h after kainate injection. We then tested whether kainate induces ataxia in IL-18$^{-/-}$ or IL-18Rα$^{-/-}$ mice (Fig. 1, *G* and *H*). Systemic administration of kainate induced ataxia in these mice within 20 min, as seen in wild-type mice. To our surprise, recovery from the kainate-induced ataxia was significantly delayed in these mice. Ataxic gait was still observed after 2 h in IL-18$^{-/-}$ mice, and after 4 h in IL-18Rα$^{-/-}$ mice. The results posed the possibility that IL-18 may enhance the recovery phase of kainate-induced ataxia.

Intracerebellar injection of IL-18 counteracts the effect of IL-1β and kainate

To assess the possibility described above, we examined the effect of intracerebellar injection of IL-18 with or without IL-1β. As shown in Fig. 3*D*, an intracerebellar injection of only IL-18 (0.01–0.1 μg/site) did not induce ataxia. Interestingly enough, intracerebellar injection of IL-18 (0.01–0.1 μg/site) together with IL-1β dose-dependently inhibited IL-1β (0.1 μg/site)-induced ataxia (Fig. 3*E*). Furthermore, pretreatment with IL-18 (0.01–0.1 μg/site, intracerebellarly, at −10 min) inhibited kainate-induced ataxia in a dose-dependent manner (Fig. 3*F*). These results strongly indicate that IL-18 in the cerebellum may play an important role in the recovery phase of kainate-induced ataxia in mice by counteracting with IL-1β.

Expression of IL-1R and IL-18R on Purkinje cells

Finally, we investigated which cells in the cerebellum express IL-1R and IL-18R by immunohistochemistry. Fig. 4 shows that both IL-1R and IL-18R were expressed in Purkinje cells. Their specific expression was confirmed by staining tissue sections of either IL-1RI$^{-/-}$ mice or IL-18Rα$^{-/-}$ mice. We also examined the expression of kainate receptors, IL-1β, and IL-18 in the cerebellum by immunohistochemistry (Fig. 5). GluR5 was expressed in astrocytes (glial fibrillary acid protein-positive) as well as Purkinje cells (calbindin-positive), while GluR6 was mainly expressed in Purkinje cells. IL-1β was expressed mainly in Purkinje cells and IL-18 was expressed in both Purkinje cells and astrocytes. We could not clearly detect the expression of IL-1β or IL-18 in other cells, including microglias or granular cells.

Discussion

Kainate receptors are reported to be expressed in various regions in the brain, including the cerebellum, in rodents (5), and it has been demonstrated that kainate induced the expression of IL-1β transcripts in various regions in the brain (27). In the present study, we showed the kainate-induced processing of IL-1β and IL-18 in the cerebellum, and demonstrated that IL-1β plays an important role in inducing kainate-triggered ataxia and that IL-18 has a positive regulatory role in recovery from kainate-induced ataxia.

This effect of kainate was really induced via a kainate receptor in the cerebellum since i.p. administration of 2-amino-5-hydroxy-5-methyl-4-isoxazolepropion acid/kainate-receptor antagonists, 6,7-dinitroquinoxaline-2,3-dione (0.3–3 mg/kg), and 6-cyano-7-nitroquinoxaline-2,3-dione (0.3–3 mg/kg), 30 min before systemic administration of kainate (20 mg/kg) injection specifically and dose-dependently inhibited kainate-induced ataxia (data not shown). Furthermore, intracerebellar injection of 6,7-dinitroquinoxaline-2,3-dione (0.25–0.75 μg/site) dose-dependently inhibited kainate-induced ataxia. These results indicate that the ataxia was induced specifically via 2-amino-5-hydroxy-5-methyl-4-isoxazolepropion acid/kainate receptors.

Resistance to kainate-induced ataxia of caspase-1-, IL-1R-, and MyD88-deficient mice (Fig. 1) suggested an important role of IL-1β in kainate-induced ataxia. Time course of caspase-1-activation and the resultant IL-1β-processing in the cerebellum was corresponding to that of kainate-induced ataxia (Fig. 1 and 2), and direct intracerebellar injection of IL-1β elicited the ataxia (Fig. 3). These results indicate the involvement of IL-1β in the cerebellum in kainate-induced ataxia. Regarding the relationship between IL-1β in the cerebellum and ataxia, it was reported that two ataxic mutant mice, staggerer mice with retinoic acid receptor-related orphan receptor (nuclear hormone receptor superfamily) deficiency (30) and lurcher mice with δ2-glutamate receptor deficiency (31) showed abnormal IL-1β expression in the cerebellum (32). In these mice, the neurodegenerative effect of IL-1β was attributed to the cerebellar ataxia. Concerning kainate, it was reported that kainate-induced ataxia was due to its neurodegenerative effect (2, 11). In this study, we examined apoptosis in the cerebellum at various times (30 min to 24 h) after kainate injection using TUNEL methods; however, we could not observe any apoptotic cells in the cerebellum (data not shown). The result indicates that although kainate affects various regions in the brain to induce neurodegenerative effects, the neurodegenerative effect may not be the direct cause in kainate-induced and IL-1β-mediated cerebellar ataxia in mice. The other possible effect of IL-1β on kainate-induced ataxia is its regulatory effect on neurotransmitter systems. IL-1 was reported to be involved in the regulation of inhibitory as well as excitatory neurotransmitter systems (33), while Purkinje cells were reported to express γ-amino-butyric acid, an inhibitory neurotransmitter (34). Of note, γ-amino-butyric acid was indicated to be involved in fast cerebellar oscillation associated with ataxia in a mouse model of Angelman syndrome (35). Taken together, it is assumed that IL-1 might induce the release of γ-amino-butyric acid from Purkinje cells and inhibits neuronal activity, including that of Purkinje cells. In this context, we are electrophysiologically examining the effect of kainate, IL-1β or IL-18, or both on Purkinje cells in the cerebellum.

Among the molecules that affect kainate-induced ataxia, caspase-1 deficiency showed the most complete abrogation of kainate-induced ataxia, while deficiency of either IL-1RI or MyD88 showed partial effect (Fig. 1). It is reported that more of the IL-1 cytokine family may be activated with caspase-1 (36). Recently, IL-1-like cytokine, IL-33, that was activated with caspase-1 and its receptor, ST2, was demonstrated to exert its effect on the induction of Th2-associated cytokines (37). With this regard, we detected the expression of IL-33 and ST2 in cerebellum by RT-PCR and immunohistochemical method (data not shown). Further investigation is necessary to clarify a role of ST2/IL-33 signaling including other caspase-1-activated IL-1-like cytokine signaling in kainate-induced ataxia in a future study.

Concerning MyD88-deficient mice, partial resistance to the effect of kainate suggests that signaling molecules other than MyD88 could be involved in kainate-induced caspase-1-dependent ataxia. In this respect, signaling of the IL-1R/TLR family is finely and sophisticatedly tuned with multiple signaling molecules (38). For example, Toll/IL-1R domain-containing adaptor inducing IFN-β-related adaptor molecule was reported to be involved in the TLR 4-mediated MyD88-independent signaling pathway, although MyD88 is involved in TLR 4 signaling (39).

It was reported that human purinergic receptor ($P2X_7$) modulates IL-1β and IL-18 processing and their release in response to

ATP in caspase-1-independent fashion (40). To examine a possibility whether $P2X_7$ is involved in kainate-induced ataxia, we examined the effect of oxidized ATP, one of $P2X_7$ receptor antagonists (41), on kainate-induced ataxia. An intracerebellar injection of oxidized ATP did not inhibit the kainate-induced ataxia during at least 60 min after kainate-injection (data not shown). Therefore, the result suggested that the participation of $P2X_7$ is little on the induction of kainate-induced ataxia. In accordance with our results, Nicklas et al. (42) showed that kainate decreased the content (or release) of ATP in cerebellar slices.

The most interesting finding in this study is that IL-18, another caspase-1-activated proinflammatory cytokine (12), counteracted the effect of IL-1β in the induction of ataxia with kainate. IL-18- and IL-18R-deficient mice showed the delay of recovery from kainate-induced ataxia (Fig. 1), and intracerebellar injection of IL-18 together with IL-1β inhibited the IL-1β-induced ataxia (Fig. 3). We assume three possible explanations for these results: firstly, IL-18 may induce the expression of IL-1ra that inhibits the binding of IL-1β to its receptor; secondly, IL-1β and IL-18 may exert their effects on different cells in the neuronal network in the cerebellum; thirdly, IL-1β and IL-18 may exert their effects on the same target cells but induce counteracting signals. As for the first possibility, we examined the effect of intracerebellar injection of IL-18 on IL-1ra mRNA expression. IL-18 did not affect the expression of IL-1ra mRNA expression significantly during 60 min after intracerebellar injection, compared with intracerebellar injection of vehicle only (data not shown). Because intracerebellar injection of IL-18 showed a counteracting effect on kainate-induced or IL-1β-induced ataxia within 60 min (Fig. 3), the result suggests that IL-1ra is not involved in the anti-ataxia effect of IL-18 in cerebellum. Regarding the second possibility, immunohistochemical analysis showed that both IL-1RI and IL-18Rα were expressed on Purkinje cells in the cerebellum (Fig. 4). The analysis indicated the expression of IL-18R but not IL-1RI on astrocytes (Fig. 5). Thus, IL-18 may counteract IL-1β by exerting its effect through different target cells. Concerning the third possibility, it has been so far reported that receptors for IL-1 and IL-18 use the same signaling module, MyD88 and IL-1-receptor associated kinases (43). However, because the receptors for both cytokines are expressed on Purkinje cells, it is possible that the receptors for these cytokines use a different signaling pathway in Purkinje cells. With this possibility, ST2, one of the IL-1 receptor family, has been described as a negative regulator for TLR-IL-1R signaling (44), in line with an earlier report that ST2 was unable to activate the IL-1-activated transcription factor NF-κB (45, 46). The detailed mechanisms on the interaction of IL-1β and IL-18 in the induction and recovery of ataxia remain unknown. The electrophysiological examination might reveal the effect of kainate, IL-1β or IL-18, or both on neural network in the cerebellum.

Concerning the genetic background of knockout mice, we had used knockout mice of the same genetic backgrounds, i.e., C57BL/6 background for IL-1RI$^{-/-}$, MyD88$^{-/-}$, IL-18$^{-/-}$, and IL-18R$^{-/-}$ mice, but used a BALB/c background for caspase-I$^{-/-}$ mice. Regarding the differences in the development of inflammatory disease of the IL-1ra$^{-/-}$ mice, Horai et al. (47) showed that the IL-1ra-deficient mice on a BALB/c background, but not those on a C57BL/6J background, spontaneously developed chronic inflammatory polyarthropathy. Their results suggested that genes other than IL-1ra are involved in the development of spontaneously developed arthritis. They also observed that IL-1ra$^{-/-}$ mice on the C57BL/6 background developed arthritis at a high incidence when these mice were immunized with type II collagen. Their result demonstrated that IL-1ra$^{-/-}$ mice on C57BL/6 background as well as BALB/c background showed the corresponding susceptibility to experimentally induced arthritis. In our study, we observed the effect of kainate on the acutely induced ataxia (induction within 20 min after the kainate injection and the following subsidence within 60–120 min). Our data showed that the mice deficient with IL-1RI, or MyD88 on the same C57BL/6 background were resistant to kainate-induced ataxia, while IL-18- or IL-18Rα-deficient mice on the same C57BL/6 background displayed significant delay of the recovery from ataxia. In these experiments, we had compared the responses with the wild-type mice on the same C57BL/6 background. We have also compared the locomotion time of normal BALB/c mice and C57BL/6 mice after systemic administration of kainate, and observed the similar responses in normal BALB/c mice and C57BL/6 mice (Fig. 1, *E* and *F*). Our data suggest that the effect of the genetic background of BALB/c mice and C57BL/6 mice on kainate-induced ataxia in the acute phase is less important. Taken together, our data clearly showed that IL-1RI or MyD88 was involved in the induction of ataxia, and IL-18 and its receptor in the recovery of the kainate-induced ataxia in the C57BL/6 genetic background.

Recently Zhang et al. (48) showed that IL-18-deficient mice were more sensitive to kainate administration in the induction of neurodegeneration compared with the normal animals. The observation that IL-18 deficiency aggravated kainate-induced hippocampal neurodegeneration seems similar to our observation that the mice with IL-18 deficiency showed a delay of the recovery from kainate-induced ataxia. However, they showed that the exogenous administration of IL-18 aggravated the kainate-induced neurodegeneration. They concluded that IL-18 had a disease-promoting role in kainate-induced excitotoxicity but that the roles of IL-18 in excitotoxic injury in IL-18-deficient mice might be overcompensated by an increase of other microglia-derived disease-promoting factors, such as IL-12. In contrast, we demonstrated that the exogenous administration of IL-18 showed the counteracting effect on kainate-induced ataxia (Fig. 3*F*). Our results suggest that although IL-18 and IL-1β are both proinflammatory cytokines, IL-18 showed the counteracting effect in kainate-induced ataxia.

In this study, we provided evidence that caspase-1 and IL-1RI in the cerebellum are essential for kainate-induced ataxia and that IL-18 and IL-18R are positive regulators for the recovery phase of kainate-induced ataxia in mice. Earlier reports on the effect of kainate have demonstrated its effect mainly in the hippocampus, and kainate is reported to show a neurodegenerative effect (2, 49). In this regard, the present study showed that caspase-1 was activated not only in the cerebellum but also in the hippocampus (Fig. 2). The activation of caspase-1 in the hippocampus peaked at 60 min after kainate administration, although ataxia was induced within 20 min and then gradually subsided within 60 to 90 min after kainate stimulation. In our studies, we could not detect either activated IL-1β or activated IL-18 at the protein level in the hippocampus, while we could clearly detect them in the cerebellum. Our result indicates that the activation of caspase-1 in the hippocampus may not be directly attributed to kainate-induced ataxia, but may lead to a neurodegenerative effect of kainate. Further analysis of the molecular as well as physiological basis of IL-1β-induced ataxia and its inhibition by IL-18 in the cerebellum may contribute to clarify the mechanism of kainate-induced ataxia, and might contribute toward the development of a new strategy for the therapy of cerebellar ataxia in humans.

Acknowledgments

We thank K. Kuida, K. Hoshino, and S. Akira for providing us with caspase-1$^{-/-}$, IL-18R$\alpha^{-/-}$, and MyD88$^{-/-}$ mice, respectively. We are also grateful to S. Hirota for taking care of the knock out mice.

Disclosures

The authors have no financial conflict of interest.

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ICS-07150; No of Pages 8

International Congress Series xx (2007) xxx–xxx

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The role of tryptophan and its derivatives for development of malaria parasite in vector mosquito 2 3

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Abstract. Xanthurenic acid (XA), a lateral reaction product of tryptophan metabolism in the omochrome pathway of eye pigment synthesis in insects, induces gametogenesis of malaria parasites. We have succeeded in measuring XA contents in the mosquito tissues using a high performance liquid chromatography with electrochemical detection system. XA content is not enough for activating gametocytes in the midgut where blood meal is taken. We have found that the salivary gland of mosquito contains a sufficient amount of XA for activating gametocytes and mosquito ingests saliva into the midgut during blood feeding. Taken together, it is likely that XA is discharged from salivary gland during blood feeding and is swallowed to the midgut where it affects malaria gametocytes. In the present study, we compared young mosquitoes (2–3 day-old after emergence) with old mosquitoes (12–14 day-old after emergence) in terms of transmission efficacy. XA contents in whole body and the salivary gland were larger in the young group. Numbers of oocyst developed on the midgut after taking blood from the same mouse with malaria were also higher in the young group. When both groups fed a blood meal with cultured ookinetes of malaria parasites, the numbers of oocyst were similar in both groups, suggesting that conditions for development from ookinetes to oocysts were similar in young and old mosquitoes. Taking these results together , we conclude that transmission efficacy is controlled by the amount of XA in the salivary gland. © 2007 Published by Elsevier B.V. 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

Keywords: Gametogenesis; HPLC; Malaria; Mosquito; Salivary gland; Tryptophan; Xanthurenic acid 26

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0531-5131/ © 2007 Published by Elsevier B.V.
doi:10.1016/j.ics.2007.07.033

Please cite this article as: H. Matsuoka et al., The role of tryptophan and its derivatives for development of malaria parasite in vector mosquito, Int. Congr. Ser. (2007), doi:10.1016/j.ics.2007.07.033

1. Introduction

Malaria parasites are transmitted with mosquitoes. Only the anopheline mosquito can transmit mammalian malaria parasite. The mosquito's head is known to contain a large amount of malaria gametocyte activating factor (GAF) [1]. When the mosquito takes blood from a malaria patient, GAF activates male gametocytes of malaria parasite and exflagellation starts in the mosquito's midgut. If the head extract is added to the blood infected with malaria parasites *in vitro*, the same phenomenon is observed [2]. However, the midgut does not contain a sufficient amount of GFP to start exflagellation. This was a scientific mystery as to how the midgut increased GAF when mosquito took blood.

We solved the mystery. We found that mosquitoes, in particular anopheline mosquitoes, drank their saliva when they took blood, and the salivary gland (SG) of the mosquito contained a sufficient amount of GAF for exflagellation [3–5]. Male gametocytes meet GAF from the SG in the midgut and they start exflagellation. On the other hand, other groups have found that actual substance of GFP is xanthurenic acid (XA) [6,7], which is one of tryptophan derivatives and is a lateral reaction product of ommochrome pathway of eye pigment synthesis in insects. Therefore we expected that the SG should contain a sufficient amount of XA for starting exflagellation.

Since a method to measure XA in the tissue extracts using a high performance liquid chromatography with electric chemical detector (HPLC-ECD) was developed [8], we can now measure XA content in mosquito's organs. In the present report, we demonstrate that XA amount in the SG co-relates the transmission efficacy of malaria parasite when mosquitoes take blood from malaria-infected animals.

2. Materials and methods

2.1. Malaria parasites

A rodent malaria parasite, *Plasmodium berghei* ANKA strain, was used.

2.2. Mosquitoes

Anopheles stephensi mosquitoes (SDA 500 strain) were maintained [9], and used in all experiments.

2.3. Mice

BALB/c mice were used for the experiments.

2.4. Maintaining malaria parasite

Infective mosquitoes, which keep malaria parasites in the SG are allowed to feed on naïve mice for 30–60 min. Five to seven days after the mosquito biting, parasites appear in the blood stream of the mice. Usually parasite density increases 10-fold a

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