The administration of BPA resulted in an increase in the production of IL-4 by *L. major* antigen–stimulated splenocytes from *L. major*–infected BALB/c mice at week 8 in a dose-dependent manner. IL-4 levels were significantly higher in mice treated with 2.5 and 5 μmol of BPA than in untreated control mice. In addition, augmented production of IL-10 and IL-13 was observed in mice exposed to 5 μmol of BPA. However, no significant differences in levels of IFN-γ were observed between the untreated and BPA-treated groups (Figure 2). No significant differences in levels of production of T_H1/T_H2 cytokines were observed between untreated and BPA-treated C57BL/6 mice (data not shown).

Change in the percentage of CD4⁺CD25⁺ T cell in BPA-treated adult male mice. The percentages of $CD4^+CD25^+$ cells among $CD4^+$ T cells decreased significantly 1 week after treatment with 5 μmol of BPA in both BALB/c and C57BL/6 mice. Eight weeks after *L. major* infection, increased percentages of $CD4^+CD25^+$ cells were found in nonexposed susceptible BALB/c but not in resistant C57BL/6 mice. The percentages of $CD4^+CD25^+$ cells were significantly lower in BALB/c mice exposed to BPA at 2.5 and 5 μmol than in nonexposed mice. In contrast, no significant differences were seen between BPA-treated and nontreated C57BL/6 mice (Figure 3).

Effects of prenatal exposure to BPA on footpad swelling and production of IL-4 and IFN-γ in* L. major*–infected male offspring. Female BALB/c mice were given drinking water containing 1, 10, or 100 nM BPA for 2 weeks. They were then mated with male mice and given BPA-containing drinking water for another week. Male offspring were challenged with 5×10^6 stationary-phase promastigotes of *L. major* in the hind footpad at week 10 after birth. The footpad swelling increased rapidly in the nonexposed as well as all the BPA-exposed groups (Figure 4A). Offspring of mice exposed to 100 nM BPA developed significantly larger swelling than controls at weeks 6 and 8 after infection. Eight weeks after infection, footpad swelling was 1.50-fold larger in offspring born to dams exposed to 100 nM BPA than in controls.

Production of IL-4 by splenocytes was significantly increased in offspring from dams exposed to 10 and 100 nM BPA but not in those born to 1 nM BPA-treated females compared with the nonexposed control mice. Similar results were observed in IFN-γ production. Mice showing increased footpad swelling demonstrated increased production of both T_H1 and T_H2 cytokines (Figure 4B,C).

Change in the percentage of CD4⁺CD25⁺ T cell in male mice exposed prenatally to BPA. Before infection, a dose-dependent decrease in the percentages of $CD4^+CD25^+$ cells among $CD4^+$ T cells was observed in offspring of dams exposed to BPA. The percentages of $CD4^+CD25^+$ cells increased significantly after infection with *L. major*. The difference in the percentage of $CD4^+CD25^+$ cells became larger between offspring born to dams exposed to BPA and nonexposed mice (Figure 5).

Discussion

In the present article, we clearly demonstrate the effects of exposure to BPA on immune responses using mice infected with *L. major*. Mice exposed to BPA prenatally or in adulthood showed a dose-dependent increase in footpad swelling after being infected with *L. major*. BPA promoted the production of IL-4 and other cytokines in each case. Similar results were seen in adult mice infected with a nematode, *T. spiralis* (Tian et al. 2003). Especially, a smaller amount of BPA could affect the immune responses of the next generation. Promotion of cytokine production was associated with decreases in $CD4^+CD25^+$ Treg cells, indicating that BPA exerted its effects by reducing the number of Treg cells.

Exposure to BPA by subcutaneous injection in adulthood significantly promoted antigen-stimulated production of IL-4, IL-10, and IL-13 in T_H2-skewed BALB/c mice infected with *L. major*. However, oral administration with BPA resulted in an insignificant increase of T_H2 cytokine production and footpad swelling after infection with *L. major* (data not shown). Subcutaneous injection in the leg with BPA more effectively altered immune responses after *L. major* infection in the footpad than did oral administration.

BPA exposure comes from multiple sources. Although oral delivery appears to be most relevant for extrapolation to humans, other delivery routes may reveal effects of BPA (Richter et al. 2007). BPA has been reported to leach from hemodialyzers into the serum (Haishima et al. 2001). The concentration of BPA was much higher in sera of dialysis patients than in those of healthy subjects (Murakami et al. 2007). Our observation

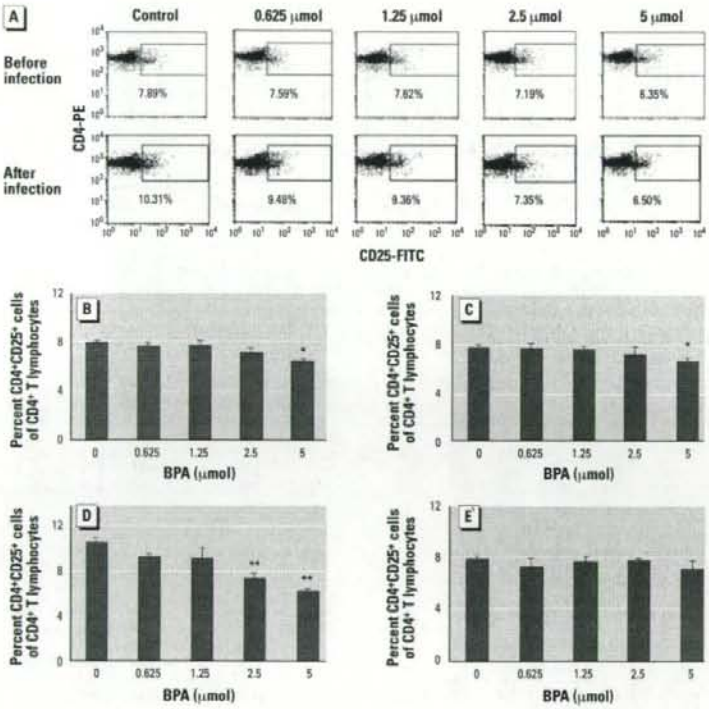

Figure 3. Effects of exposure to BPA on the percentage of $CD4^+CD25^+$ cells among $CD4^+$ T cells in adult male mice. (*A*) The representative FACS data of BALB/c mice before and after infection with *L. major*. (*B–E*) Splenocytes from BALB/c (*B, D*) and C57BL/6 (*C, E*) mice before (*B, C*) and 8 weeks after (*D, E*) infection. Values represent mean ± SE ($n = 3–4$).

*$p < 0.05$ and **$p < 0.01$ compared with the nonexposed control group.

gives a warning that immune responses in these patients would be affected by BPA.

In contrast to BALB/c mice, T_H1-skewed C57BL/6 mice showed no significant increase in cytokine production and footpad swelling, suggesting that exposure to BPA in adult mice did not influence T_H1 cells. This is consistent with the observation that BPA promotes *in vitro* IL-4 production by T_H2 cells from *L. major*–infected BALB/c mice, but not IFN-γ production by T_H1 cells from C57BL/6 and BALB/c mice (Tian et al. 2003). In resistant C57BL/6 mice, low levels of IL-4 are produced only transiently (Launois et al. 1997). Therefore, IL-4 production could not be promoted by BPA. These results indicate that BPA promotes T_H2 cytokine production but not a change in the balance of immune responses from T_H1 toward T_H2. Other EDCs, such as tributyltin and *p*-*n*-nonylphenol, have also been reported to induce T_H2 polarization (Iwata et al. 2004; Kato et al. 2004). T_H2 immune response might be easily enhanced by EDCs.

The effects of BPA on the developing immune system in embryos or fetuses have not been elucidated. BPA can leak from the placenta and accumulate in the fetus (Miyakoda et al. 1999; Takahashi and Oishi 2000; Zalko et al. 2003). Additionally, there is increasing evidence that the development of the fetal immune system is regulated by the maternal immune system (Warner 2004). BPA influences the immune responses in adult mice; it is therefore possible that maternal exposure to BPA may affect the immune function of the next generation. In this study, we investigated whether exposure to low doses of BPA during the early periods of immune development could induce immunotoxic effects. We showed that prenatal exposure to BPA increased the production of a T_H1 cytokine, IFN-γ, and a T_H2 cytokine, IL-4, after the offspring developed, suggesting that prenatal exposure to BPA can induce persistent immunologic effects lasting into adulthood. These results are consistent with a previous report that fetal exposure to BPA augmented T_H1 and T_H2 immune responses (Yoshino et al. 2004). Although prenatal exposure to BPA led to increased IFN-γ production, these offspring failed to control disease progression following challenge with *L. major*. This may be due to the antagonistic effects of IL-4. The higher IL-4 production inhibited the protective role of IFN-γ in the prenatally exposed mice. The present study showed that exposure to BPA promoted the production of T_H2 cytokines only in adult mice, but both T_H1 and T_H2 cytokines in mice exposed prenatally, although percentages of $CD4^+CD25^+$ cells decreased in either case. A possible explanation is that BPA might directly act on T_H2 cells to promote cytokine production in adult mice as demonstrated *in vitro*. This together with the decrease in Treg cells promoted the production of T_H2 cytokines alone. In contrast, not enough BPA existed in prenatally exposed mice to promote the production of T_H2 cytokines at the time of infection. Therefore, the decrease in Treg cells resulted in the promotion of T_H1 and T_H2 cytokine production. Further study is necessary to clarify this mechanism.

In recent years, attention has focused on the low-dose effects of EDCs. Xenoestrogens even at low levels were reported to exert estrogenic activity to affect the endocrine system (vom Saal and Hughes 2005). Our results showed that BPA at 2.5 and 5 μmol promoted T_H2 cytokine production and decreased the percentages of $CD4^+CD25^+$ cells in adult mice. Similar effects were induced in offspring of dams with significantly lower doses of BPA, showing that the immune system in developing mice is affected by lower doses of BPA than that in adult mice. Prenatal exposure to EDCs in laboratory animals may cause more severe effects on the immune system than exposure during

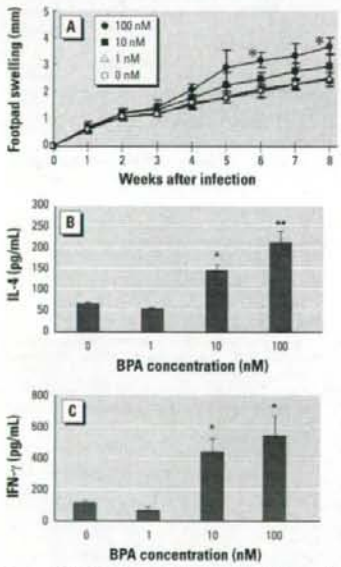

Figure 4. Effects of prenatal exposure to BPA on the course of *L. major* infection and T_H1- and T_H2-related cytokine production. (*A*) Footpad swelling after infection with *L. major*. (*B*, *C*) Splenocytes were obtained at 8 weeks after infection and cultured for 48 hr with *L. major* antigen. Concentrations of IL-4 (*B*) and IFN-γ (*C*) in culture supernatants were determined using CBA kits. Values represent the mean ± SE ($n = 4$).
*$p < 0.05$ and **$p < 0.01$ compared with the control group.

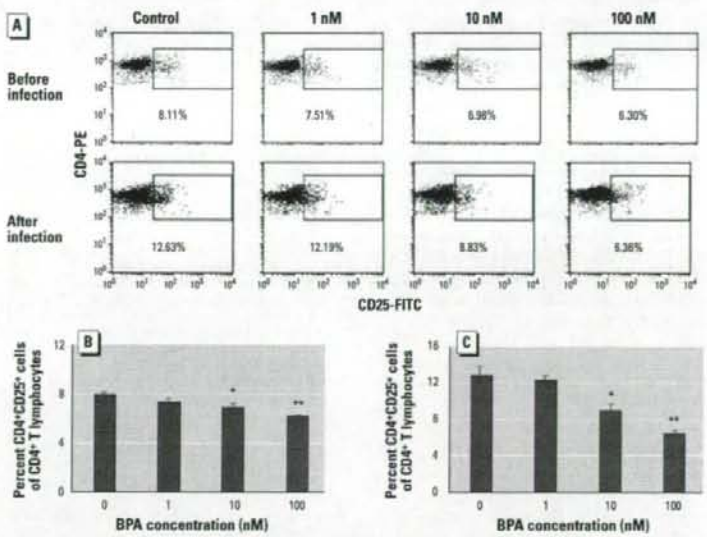

Figure 5. Effects of prenatal exposure to BPA on the percentage of $CD4^+CD25^+$ cells among $CD4^+$ T cells. (*A*) The representative FACS data of offspring on day 22 of birth (before infection) and after *L. major* infection. (*B*, *C*) Splenocytes from offspring of dams exposed to the indicated dose of BPA were obtained on day 22 of birth (*B*) and 8 weeks after *L. major* infection (*C*), and stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD25 mAbs. $CD4^+$ lymphocytes were gated and percentages of $CD25^+$ cells were determined. Values represent mean ± SE ($n = 3$–4).
*$p < 0.05$ and **$p < 0.01$ compared with the nonexposed control group.

adult life. It is important to note that BPA exerted its effects at a dose of 10 nM (equivalent to 0.3 μg/kg bw/day) after prenatal administration, which is 100 times lower than 50 μg/kg bw/day (a permissible dose of BPA authorized by the U.S. Food and Drug Administration). The present results indicate that EDCs at concentrations even below the safety limit might affect our immune system.

The precise mechanism underlying the immunomodulatory effects of EDCs, especially BPA, has not been clarified, and multiple mechanisms are considered. In this study, we made a potentially important discovery, that the alteration of cytokine production induced by BPA might be mediated through a decrease in numbers of $CD4^+CD25^+$ Treg cells. Treg cells, which constitute 5–10% of peripheral $CD4^+$ T cells in normal rodents and humans, are known to regulate immune responses. We found that exposure to BPA resulted in decreased percentages of $CD4^+CD25^+$ Treg cells in a dose-dependent manner in both adult and offspring mice. Because $CD4^+CD25^+$ Treg cells play a negative role in proliferation and the production of cytokines in T_H1 and T_H2 cells (Xu et al. 2003), decreasing numbers of $CD4^+CD25^+$ Treg cells might result in the activation of T cells. As a result, BPA exerted a stimulatory effect on the production of cytokines by the activated T cells. The decrease of $CD4^+CD25^+$ cells before infection did not result in the increased production of IL-4 and other cytokines in C57BL/6 mice. The course of infection also was not changed. This observation agrees with the result that depletion of $CD4^+CD25^+$ cells in C57BL/6 mice before infection with *L. major* did not alter the course of infection (Aseffa et al. 2002). After infection with *L. major*, the difference in percentage of $CD4^+CD25^+$ cells induced by BPA was more notable between BPA-exposed and nonexposed groups in BALB/c mice, but not in C57BL/6 mice. IL-4 may play a role in this process. The generation of peripheral $CD4^+CD25^+$ cells was induced by IL-4 (Pace et al. 2005; Skapenko et al. 2005). IL-4 production is low and only transient in C57BL/6 mice but dominant in BALB/c mice. Therefore, peripheral $CD4^+CD25^+$ cells might be decreased solely in BALB/c mice by exposure to BPA.

In conclusion, our results clearly demonstrate that the production of T_H2 cytokines is promoted by BPA in adult mice and in offspring during developmental exposure. This suggests the possibility that BPA might cause allergy and asthma. Epidemiologic studies have shown that allergic diseases have markedly increased over the last several decades. Environmental factors such as pollutants and food additives are suspected of playing an important role. Several environmental pollutants have been reported to increase allergic responses (Bommel et al. 2000; Kato et al. 2004). BPA and other EDCs may have similar effects on allergic diseases. Furthermore, the enhanced cytokine production reported in this article was mediated through a decrease in the number of $CD4^+CD25^+$ Treg cells. Human naturally occurring Treg cells are prominent in young adults and decrease with age (Valmori et al. 2005). The decrease of Treg cells would predispose to immune dysfunction in aged individuals, explaining their higher risk of immune-mediated diseases, cancer, and infections. Our data also suggest the possibility that BPA might cause these diseases. Thus, avoiding exposure to or promoting the excretion of BPA and other EDCs would help in preventing diseases and adverse health effects.

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IMMUNOLOGY ORIGINAL ARTICLE

STAT6 signalling is important in CD8$^+$ T-cell activation and defence against *Toxoplasma gondii* infection in the brain

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Summary

Signal transducer and activator of transcription (STAT) 6 is a molecule involved in interleukin (IL)-4 and -13 signalling. We investigated the role of STAT6 signalling in *Toxoplasma gondii*-infected mice using STAT6-deficient (STAT6$^{-/-}$) and wild-type (WT) mice. A significantly larger number of cysts were recovered from the brain in STAT6$^{-/-}$ than in WT mice on days 28 and 56 post-infection. CD8$^+$ T cells in cerebrospinal fluid and spleen stimulated with *T. gondii* antigen produced higher levels of interferon (IFN)-γ in WT than in STAT6$^{-/-}$ mice. CD8$^+$ T-cell function, estimated by expression of CD25 and cytotoxic activity, was lower in STAT6$^{-/-}$ than in WT mice. Transfer of CD8$^+$ but not CD4$^+$ T cells, purified from infected WT mice, into STAT6$^{-/-}$ mice successfully prevented formation of cysts in the brain. However, transfer of naïve CD8$^+$ T cells from WT into STAT6$^{-/-}$ mice did not show either activation of CD8$^+$ T cells or a decrease in the number of cysts in the brain. Transfer of splenic adherent cells from WT into STAT6$^{-/-}$ mice induced activation of CD8$^+$ T cells and decreased the number of cysts in the brain. Expression of CD86 on splenic dendritic cells and IL-12 p40 production were weaker in STAT6$^{-/-}$ than in WT mice after *T. gondii* infection. These results indicate that STAT6 signalling is important in CD8$^+$ T-cell activation, possibly through regulation of antigen-presenting cells, which could suppress *T. gondii* infection in the brain.

Keywords: CD8$^+$ T cell; signal transducer and activator of transcription 6; *Toxoplasma gondii*

Introduction

Toxoplasma gondii is an intracellular protozoan parasite that infects humans and other mammals. After peroral infection, *T. gondii* affects multiple organs including the spleen, liver, heart, lung and brain. The ensuing immune responses eliminate the pathogen from most organs, but not from the brain, where the parasite persists with development of chronic toxoplasmic encephalitis (TE).[1] This intracerebral parasite is regulated by interferon-γ (IFN-γ)-producing CD4$^+$ and CD8$^+$ T cells, which are recruited into the brain.[2] CD8$^+$ T cells are the major lymphocyte subpopulation involved in the protective immune response to TE.[3,4] CD8$^+$ T cell- or IFN-γ-deprived mice are unable to control both acute and chronic toxoplasmosis.[5,6]

In contrast to the important role of IFN-γ, the role of interleukin-4 (IL-4) in *T. gondii* infection is still unclear. Significantly greater acute focal inflammation with tachyzoites and a larger number of cysts in the brain were observed in IL-4-deficient (IL-4$^{-/-}$) than in wild-type (WT) mice on days 28 and 56 post-infection (pi). Mortality was also higher in IL-4$^{-/-}$ mice compared with WT mice during the late stage of infection. These results indicate that IL-4 is protective against TE by preventing formation of cysts and proliferation of tachyzoites in the brain.[7] In contrast, another group reported that IL-4$^{-/-}$ mice were resistant to *T. gondii* infection, showing a

higher survival rate than WT mice during the early acute phases of infection. Pathology in the small intestine was less severe in IL-4$^{-/-}$ mice although conversely liver pathology was greater than in WT mice.[8]

Intracellular signalling mechanisms provide the link between binding of a cytokine with its receptor and the effect of the cytokine on cellular function. The janus kinase (JAK) and signal transducer and activator of transcription (STAT) family plays a critical role in the signalling of many cytokine receptors. The IL-4 receptor (IL-4R) is associated with JAK1-3 and STAT6. STAT6-deficient (STAT6$^{-/-}$) mice are unable to process IL-4R-induced signals.[9,10] Furthermore, IL-13, which is closely related to IL-4 in biological function, shares receptor components and signalling through the STAT6 pathway with IL-4.[11]

In this study, the role of STAT6 signalling in cyst formation, TE in the brain and the immune response following *T. gondii* infection was investigated.

Materials and methods

Animals

STAT6$^{-/-}$ mice were donated by Dr S. Akira (Osaka University, Suita City, Japan)[10] and backcrossed to C57BL/6 (B6) mice at least 10 times. Six-week-old WT B6 mice were purchased from Clea Japan (Tokyo, Japan). Animals were housed in polycarbonate cages and fed with a commercial diet (Funabashi Farm, Chiba, Japan) in the Shinshu University Animal House. All mice were maintained under a 12 : 12 hr light/dark cycle (lights on at 9:00 AM) at 24 ± 2° and 55 ± 10% relative humidity. The Animal Ethics Committee of Shinshu University approved all protocols used in this study.

Monoclonal and polyclonal antibodies

Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) against murine CD4 (RM4-5), CD8 (53-6·7), B220 (RA3-6B2), CD11b (M1/70), CD11c (HL3), CD25 (7D4), CD44 (IM7), CD62L (MEL-14), H-2D^b (KH95) and IFN-γ (XMG1·2) were purchased from BD Biosciences (San Jose, CA). Phycoerythrin (PE)-conjugated mAbs against CD4 (RM4-5), CD8 (53-6·7), CD11c (HL3), I-A^b (25-9-7), CD40 (3/23), CD80 (16-10A1) and CD86 (GL1) and PE-Cy5-conjugated anti-CD4 (RM4-5) mAb were also purchased from BD Biosciences. Rat immunoglobulin G (IgG) was purchased from Sigma-Aldrich (St Louis, MO).

Monoclonal Abs against murine CD4 (GK1·5) and CD8 (53-6·7) were purified as described previously.[12] Mice were injected intraperitoneally with anti-CD4 and anti-CD8 mAbs (0·5 mg/week) to deplete CD4$^+$ and CD8$^+$ T cells, respectively. Control mice received rat IgG. Depletion was confirmed by flow cytometric analysis.

Infection, cyst count and antigen preparation of T. gondii

An avirulent Fukaya strain and a virulent RH strain of *T. gondii* were donated by Dr N. Watanabe (Jikei Medical School, Tokyo, Japan). The Fukaya strain was maintained in B6 mice. Brains of infected mice were gently homogenized with a tissue homogenizer (UltraTurax; IKA-WERK, Staufen, Germany). Cysts were enriched by centrifugation of the homogenate. They were counted under a microscope in portions of precipitates that were smeared on cover-slipped glass slides. Mice were orally inoculated with brain homogenate containing 10 cysts using a stomach tube with a 1-ml syringe.[13]

Tachyzoites of the RH strain were collected from the peritoneal cavity of B6 mice injected 4 days previously. *Toxoplasma gondii* crude antigen was prepared by sonication of the tachyzoites followed by centrifugation.[14] The supernatant was stored as *T. gondii* antigen at −30° until use.

Histopathological evaluation

Mice were killed on days 14, 28 and 56 pi and their heads were fixed in 10% neutral buffered formalin solution. The cranium was then decalcified by immersion in K-CX (Fujisawa Co., Osaka, Japan) for 16 hr, washed with tap water for 12 hr and embedded in paraffin. Sections of 5 μm thickness were cut. Serial sections at 500-μm intervals were stained with haematoxylin and eosin. The histopathological changes in the brain were evaluated under a microscope.[13]

Collection of cerebrospinal fluid (CSF)

Mice were anaesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg; Dainippon Sumitomo Pharma, Osaka, Japan) and perfused through the heart with phosphate-buffered saline (PBS) to remove contaminating intravascular leucocytes. CSF was harvested by suboccipital puncture as follows.[13] The dura mater above the cisterna magna was exposed and cut. Then, 10–15 μl of CSF per mouse was aspirated with a micropipette. Total CSF cells were stained with Turk solution and counted under a microscope. The percentages of CD4$^+$, CD8$^+$, B220$^+$ and CD11b$^+$ cells were determined by flow cytometric analysis on cells stained with FITC-conjugated respective mAbs.

Culture of CSF cells and splenocytes

CSF cells (2×10^4 cells/well) in 200 μl or a single suspension of splenocytes (4×10^6 cells/well) in 1 ml of RPMI-1640 medium containing 10% fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 μg/ml),

and amphotericin B (0·25 mg/ml) were incubated with *T. gondii* antigen (12 μg/ml) at 37° in a humidified atmosphere of 5% CO_2 and 95% air for 48 hr. In order to block activation of CD4$^+$ and/or CD8$^+$ T cells in culture conditions, mAbs against murine CD4 (GK1·5) and/or CD8 (53–6·7) were added to the culture medium at 5 μg/ml before *T. gondii* antigen stimulation.[12] Supernatants were then collected and stored at −30° until use.

Flow cytometric analysis

Single nucleated cell suspensions of spleen, CSF and peripheral blood were stained with fluorescence-labelled mAbs for 30 min on ice in the dark after lysing erythrocytes with ammonium chloride solution. Cell populations were analysed by FACSCalibur (BD Biosciences).

Determination of cytokines using cytometric bead array (CBA)

The cytokine concentrations in CSF and culture supernatants of splenocytes and CSF cells were determined using a CBA mouse inflammation kit (BD Biosciences) according to the manufacturer's instructions.[15] The concentration of IFN-γ was also determined using the CBA Flex set (BD Biosciences). IL-12 p40 was determined using an OptEIA enzyme-linked immunosorbent assay (ELISA) set (BD Biosciences).

Intracellular IFN-γ staining

Splenocytes and CSF cells were incubated with *T. gondii* antigen for 24 hr as described above. Brefeldin A (10 μg/ml) was added during the last 4 hr of incubation. Cells were harvested, and stained with PE-Cy5-labelled anti-CD4 mAb and PE-labelled anti-CD8 mAb, followed by fixation with paraformaldehyde. After washing, cells were permeabilized with saponin and stained with FITC-labelled anti-IFN-γ mAb. Double-stained cells were analysed by flow cytometric analysis.

Separation and transfer of CD8$^+$ T cells, splenic adherent cells (SACs) and dendritic cells (DCs)

CD8$^+$ T cells were purified from splenocytes using a nylon wool column and magnetic beads. Briefly, 1×10^8 splenocytes were added to an autoclaved nylon wool column and incubated at 37° for 1 hr. The non-adherent T cells were collected by washing the column. Then, CD8$^+$ T cells were separated by indirect negative selection using Dynabeads M-450 (Dynal, Oslo, Norway), which bind to sheep anti-rat IgG after treatment of the cells with anti-CD4 mAb (GK1·5). The purity of CD8$^+$ T cells was > 95% as determined by flow cytometric analysis. Then, 5×10^6 CD8$^+$ T cells were injected into the tail vein of STAT6$^{-/-}$ mice at the indicated time.

SACs were separated from splenocytes by attachment to a polystyrene dish. Bone marrow (BM)-derived DCs were generated as described previously.[16] Briefly, BM cells were harvested from the femur of mice, and cultured at 1×10^5 cells/ml in the presence of 10 ng/ml recombinant murine granulocyte–macrophage colony-stimulating factor (GM-CSF; Endogen, Woburn, MA). Every 2 days, non-adherent cells were discarded and the remaining cells were fed with fresh medium containing 10 ng/ml GM-CSF. On day 6, loosely adherent cells were harvested by gentle pipetting. The purity of the cell population was determined to be more than 80% CD11c$^+$ by flow cytometry. SACs (1×10^6 cells/mouse) and DCs (1×10^6 cells/mouse) were injected into the tail vein of STAT6$^{-/-}$ mice before *T. gondii* infection.

Cytotoxic analysis

Cytotoxic activity of CD8$^+$ T cells was analysed as described previously with some modifications.[17] Briefly, peritoneal macrophages were harvested by lavage from untreated B6 mice. They were washed three times with PBS and labelled with PKH67 (Sigma-Aldrich). The cells were incubated with an optimal concentration of *T. gondii* antigen in 5% CO_2 and 95% air at 37° for 1 hr. They were then washed three times and incubated with purified CD8$^+$ T cells at various effector to target cell (E:T) ratios at 37° for 3·5 hr. Dead cells were determined by flow cytometry following labelling with 7-amino-actinomycin D (7-AAD) (BD Biosciences).

Statistical analysis

Statistical analysis of the data was performed using Student's *t*-test. A value of $P < 0·05$ was accepted as indicating significance.

Results

Histopathological evaluation and cyst burden in the brain

STAT6$^{-/-}$ and WT mice were orally infected with 10 cysts of an avirulent Fukaya strain of *T. gondii*. Less than 10% of infected mice died between days 10 and 14 pi. The number of cysts in the brain was greater in STAT6$^{-/-}$ than in WT mice on days 28 and 56 pi (Fig. 1a). Before day 14 pi, little evident inflammatory change was observed in the brains of STAT6$^{-/-}$ and WT mice (data not shown). However, on day 28 pi, cellular infiltrate was observed in the subarachnoid space and cerebral cortex in both STAT6$^{-/-}$ and WT mice (Fig. 1b). It was relatively mild in STAT6$^{-/-}$ compared with WT mice, in contrast

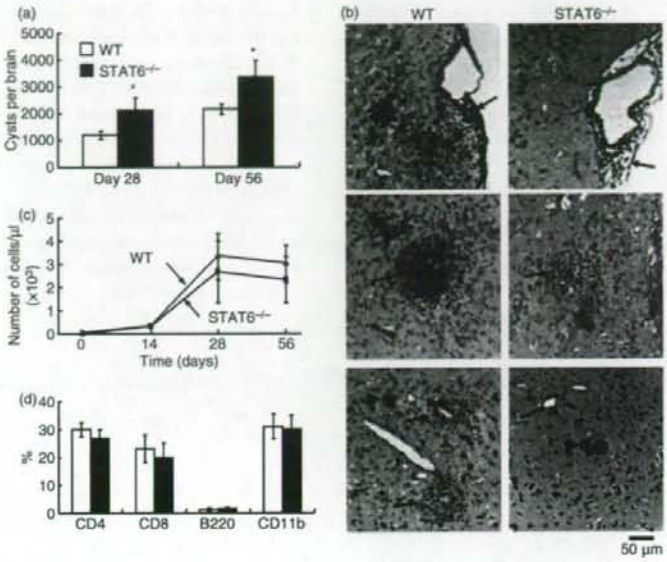

Figure 1. Cyst burden, histopathological findings and immune responses in the brain. (a) Brains were removed on days 28 and 56 pi, and the number of cysts was counted under a microscope. Data are expressed as mean ± standard deviation (SD) ($n = 5$). Similar results were obtained from five replicate experiments. (b) Representative photographs of the brain on day 28 post-infection (pi) are shown. Sections were fixed and stained with haematoxylin and eosin. Arrows indicate cellular infiltration in the subarachnoid space and cerebral cortex. Arrow heads indicate *Toxoplasma gondii* cysts. Note that the area of inflammation is wider in wild-type (WT) than in STAT6$^{-/-}$ mice. (c) Cerebrospinal fluid (CSF) was obtained from uninfected and infected mice on days 14, 28 and 56 pi. Nucleated cells were stained with Turk solution, and the total number of cells was counted. (d) Classification of cells in CSF was determined by flow cytometric analysis. Data are expressed as mean ± SD ($n = 5$). Similar results were obtained from three replicate experiments. *Significantly different from WT mice ($P < 0.05$).

to cyst burden. Cysts were not surrounded by inflammatory cells. The number of CSF cells increased after infection and peaked on day 28 pi, and then decreased in both STAT6$^{-/-}$ and WT mice (Fig. 1c). The total number of CSF cells in STAT6$^{-/-}$ mice was comparable to that in WT mice. Monocytes and CD4$^+$ and CD8$^+$ T cells were present in CSF, and there was no difference in the the percentage of each population between STAT6$^{-/-}$ and WT mice (Fig. 1d).

IFN-γ concentration in CSF and *in vitro* production by CD8$^+$ T cells were reduced in *T. gondii*-infected STAT6$^{-/-}$ mice

Inflammatory cytokine levels in CSF were determined on days 14, 28 and 56 pi. On day 14 pi, no significant difference was observed in levels of IL-6, IL-10, monocyte chemotactic protein (MCP)-1, IFN-γ, and tumour necrosis factor (TNF), which were very low in STAT6$^{-/-}$ and WT mice. However, on days 28 and 56 pi, their concentrations in CSF were markedly increased, and the IFN-γ level was significantly lower in STAT6$^{-/-}$ than in WT mice (Fig. 2a). After *in vitro* cultivation of CSF cells with *T. gondii* antigen, IFN-γ production by CD8$^+$ but not CD4$^+$ T cells and the percentage of IFN-γ-producing cells in CD8$^+$ T cells were greater in WT than in STAT6$^{-/-}$ mice (Fig. 2b and 2d). Splenocytes recovered on day 28 pi were cultured with *T. gondii* antigen *in vitro*. IFN-γ production by CD4$^+$ T cells in STAT6$^{-/-}$ mice was comparable to that in WT mice. The percentage of IFN-γ-producing CD4$^+$ T cells in STAT6$^{-/-}$ mice was not different from that in WT mice, whereas IFN-γ production by CD8$^+$ T cells was significantly lower in STAT6$^{-/-}$ mice than in WT mice. The percentage of IFN-γ-producing CD8$^+$ T cells was also significantly lower in STAT6$^{-/-}$ mice than in WT mice (Fig. 2c and 2e). These results indicate that IFN-γ production by CD8$^+$ T cells, but not by CD4$^+$ T cells, was systemically reduced in *T. gondii*-infected STAT6$^{-/-}$ mice.

Activated CD8$^+$ T cells were decreased in STAT6$^{-/-}$ mice

We further investigated CD8$^+$ T cell activation and function during *T. gondii* infection in STAT6$^{-/-}$ and WT mice. CD25$^+$, CD62L^{low} and CD44$^+$ CD8$^+$ T cells

increased in CSF, spleen and peripheral blood after infection. The percentage of CD25$^+$ CD8$^+$ and CD62L^{low} CD8$^+$ T cells in CD8$^+$ T cells was significantly lower in STAT6$^{-/-}$ than in WT mice in the CSF and spleen (Fig. 3a). The percentage of CD25$^+$ CD8$^+$ T cells in CD8$^+$ T cells peaked on day 10 pi, and was significantly lower in STAT6$^{-/-}$ mice than in WT mice in peripheral blood (Fig. 3b). In addition, cytotoxic activity of CD8$^+$ T cells in infected mice was significantly lower in STAT6$^{-/-}$ than in WT mice (Fig. 3c). The role of CD8$^+$ T cells in protection against *T. gondii* cyst formation in the brain was then studied. As shown in Fig. 1c, infiltration of T cells into the brain started on day 14 pi. Therefore, we injected anti-CD8 mAb on days 14 and 21 pi to deplete CD8$^+$ T cells. Injection of the mAb before day 14 pi induced the death of mice. A significantly larger number of cysts formed in the brains of the anti-CD8 mAb-treated group than in the rat IgG-treated control group in STAT6$^{-/-}$ and WT mice, respectively (Fig. 3d). Depletion of CD4$^+$ T cells had little influence on the number of cysts. No significant difference in number of cysts was observed between CD8$^+$ T cell-depleted STAT6$^{-/-}$ and WT mice.

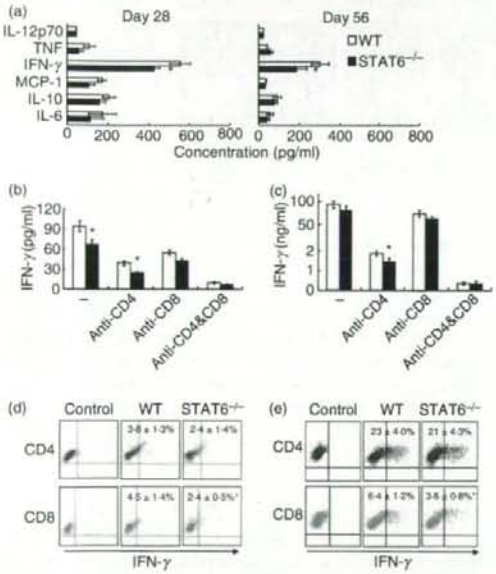

Figure 2. Production of interferon (IFN)-γ by T cells in the cerebrospinal fluid (CSF) and spleen. (a) CSF was taken from signal transducer and activator of transcription (STAT) 6$^{-/-}$ and wild-type (WT) mice on days 28 and 56 post-infection (pi). Concentrations of cytokines in CSF were determined using a cytometric bead array (CBA) kit. IFN-γ level was significantly lower in STAT6$^{-/-}$ as compared with WT mice. Data are mean ± standard deviation (SD) (n = 5). CSF cells (b) and splenocytes (c) were taken from STAT6$^{-/-}$ and WT mice on day 28 pi, and were incubated with *Toxoplasma gondii* antigen. Anti-CD4 and/or anti-CD8 monoclonal antibodies (mAbs) (5 μg/ml) were added to the culture to block activation of CD4$^+$ and/or CD8$^+$ T cells. The IFN-γ concentration in culture supernatants was determined using a CBA kit. Data are mean ± SD (n = 5). CSF cells (d) and splenocytes (e) were incubated with *T. gondii* antigen, and stained for T-cell markers and IFN-γ. Cells without antigen stimulation were used as a control. Numbers indicate the percentage of IFN-γ-producing cells in CD4$^+$ and CD8$^+$ T cells. Data are mean ± SD (n = 5). Experiments were carried out three times with similar results. *Significantly different from WT mice (P < 0·05).

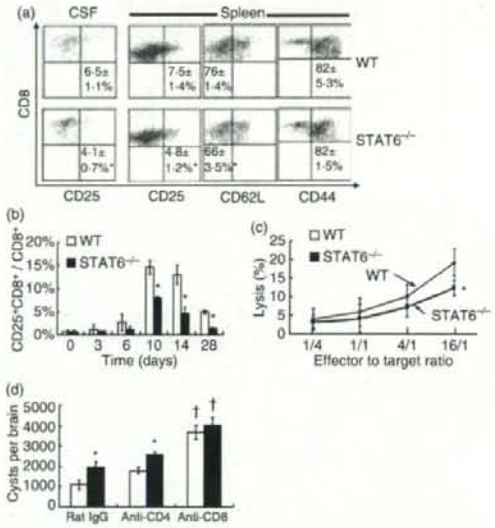

Figure 3. Activation of CD8$^+$ T cells after *Toxoplasma gondii* infection. (a) Cerebrospinal fluid (CSF) cells and splenocytes were taken from mice on day 28 post-infection (pi), and stained with fluorescein isothiocyanate (FITC)-anti-CD25, FITC-anti-CD62L, FITC-anti-CD44 and phycoerythrin (PE)-anti-CD8 monoclonal antibodies (mAbs) and then analysed by flow cytometry. Numbers indicate the percentage of CD25$^+$ CD8$^+$, CD62L^{low} CD8$^+$ and CD44$^+$ CD8$^+$ T cells in CD8$^+$ T cells in the CSF and spleen. Data are mean ± standard deviation (SD) (n = 4). (b) The percentage of CD25$^+$ CD8$^+$ T cells in CD8$^+$ T cells in peripheral blood is shown. Data are mean ± SD (n = 5). (c) Purified CD8$^+$ T cells and peritoneal macrophages labelled with PKH67 were co-cultured at various effector to target cell (E:T) ratios. Dead cells were determined by flow cytometry after staining with 7-amino-actinomycin D (7-AAD). Data are mean ± SD (n = 4). (d) *Toxoplasma gondii*-infected mice were intraperitoneally injected with 0·5 mg of anti-CD4 mAb, anti-CD8 mAb or rat immunoglobulin G (IgG) on days 14 and 21 pi. Cysts were counted on day 28 pi. Data are mean ± SD. (n = 5). Experiments were carried out three times with similar results. *Significantly different from WT mice (P < 0·05). †Significantly different from rat IgG-treated mice (P < 0·05).

Transfer of immune CD8$^+$ T cells was effective in reducing cyst number in the brain of STAT6$^{-/-}$ mice

Activation of CD8$^+$ T cells was considered to be important for preventing cyst formation in the brain. Therefore, we transferred CD8$^+$ T cells from either infected or uninfected WT mice into STAT6$^{-/-}$ mice to determine the effects of cyst burden in the brain. Transfer of CD8$^+$ T cells but not of CD4$^+$ T cells, recovered from infected WT mice, into STAT6$^{-/-}$ mice on day 14 pi successfully decreased the formation of cysts in the brain. The number of cysts in the brain of WT CD8$^+$ T cell-transferred mice was comparable to that of WT mice without cell transfer. Transfer of CD8$^+$ T cells from infected STAT6$^{-/-}$ mice showed only a slight reduction in cyst number (Fig. 4a). The percentage of CD25$^+$ CD8$^+$ T cells in CSF CD8$^+$ T cells was higher in WT CD8$^+$ T cell-transferred mice than in STAT6$^{-/-}$ CD8$^+$ T cell-transferred mice (Fig. 4b). Interestingly, transfer of native WT CD8$^+$ T cells (5×10^6 cells/mouse) into STAT6$^{-/-}$ mice that were infected with *T. gondii* simultaneously resulted in neither an increase in the percentage of CD25$^+$ CD8$^+$ T cells in peripheral blood nor a decrease in the number of cysts in the brain (Fig. 4c and 4d). These results indicate that STAT6 signalling in CD8$^+$ T cells is not important in their activation and that activated CD8$^+$ T cells predominantly suppress cyst formation in the brain.

Activation of CD8$^+$ T cells by antigen-presenting cells (APCs) may be impaired in STAT6$^{-/-}$ mice

Transfer of SACs from uninfected WT mice into STAT6$^{-/-}$ mice 10 days before infection successfully increased the percentage of CD25$^+$ CD8$^+$ T cells in peripheral blood, and decreased the number of cysts in the brain (Fig. 5a and 5b). Transfer of SACs from uninfected STAT6$^{-/-}$ mice into STAT6$^{-/-}$ mice did not change the percentage of CD25$^+$ CD8$^+$ T cells in peripheral blood or the number of cysts in the brain. Transfer of BM-derived DCs from uninfected WT mice also resulted in a decreased cyst burden in STAT6$^{-/-}$ mice (data not shown). The expression level of CD86 on splenic DCs after *T. gondii* infection was lower in STAT6$^{-/-}$ than in WT mice ($17{\cdot}5 \pm 1{\cdot}3$ versus $21{\cdot}0 \pm 1{\cdot}1$, $n = 4$, $P < 0{\cdot}05$) (Fig. 5c). The concentration of IL-12 p40 in serum was significantly lower in STAT6$^{-/-}$ mice on day 7 pi (Fig. 5d). In addition, SACs from infected STAT6$^{-/-}$ mice produced significantly lower IL-12 p40 than those from WT mice after incubation with *T. gondii* antigen (Fig. 5e).

Discussion

Our current study has demonstrated that STAT6 signalling is important in activation of CD8$^+$ T cells, resulting in a decrease in cyst number in the brain of *T. gondii*-

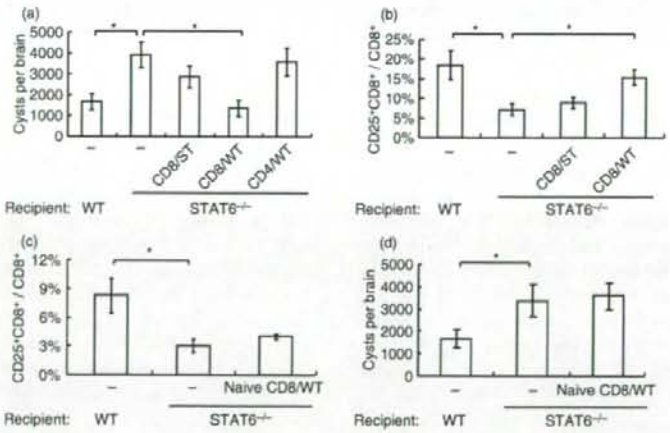

Figure 4. Effects of transfer of CD8$^+$ T cells on cyst burden in the brain. (a) Wild-type (WT) CD8$^+$ T cells (CD8/WT), WT CD4$^+$ T cells (CD4/WT) and signal transducer and activator of transcription (STAT) 6$^{-/-}$ CD8$^+$ T cells (CD8/ST) were purified from infected mice on day 14 post-infection (pi). Then 5×10^6 of these cells were injected intravenously into STAT6$^{-/-}$ mice that had been infected 14 days previously. The cyst burden in the brain was then determined on day 28 pi. (b) The percentage of CD25$^+$ CD8$^+$ T cells in CD8$^+$ T cells in cerebrospinal fluid (CSF) was determined on day 28 pi. Data are mean ± standard deviation (SD) ($n = 5$). (c) Five million CD8$^+$ T cells from uninfected WT mice (naïve CD8/WT) were transferred into STAT6$^{-/-}$ mice which were simultaneously infected with *Toxoplasma gondii*. The percentage of CD25$^+$ CD8$^+$ T cells in CD8$^+$ T cells in peripheral blood was determined on day 14 pi. (d) Cysts in the brains of these mice were determined on day 28 pi. Data are mean ± SD ($n = 5$). Experiments were carried out three times, with similar results. *Significantly different ($P < 0{\cdot}05$).

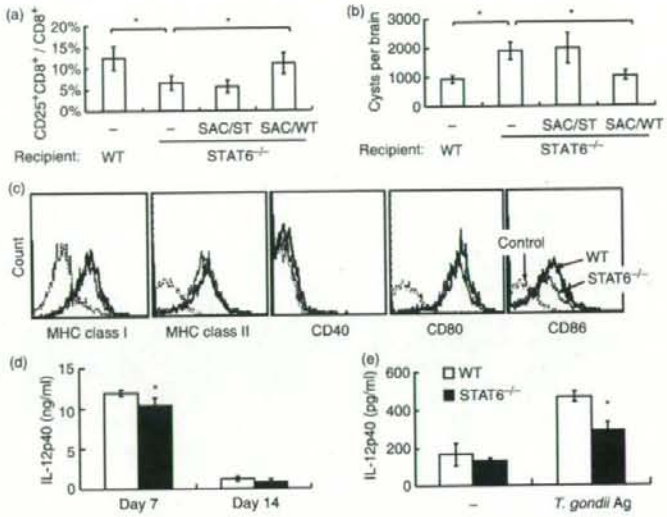

Figure 5. Effects of transfer of splenic adherent cells (SACs) on CD8⁺ T-cell activation and cyst burden. (a) One million SACs derived from uninfected wild-type (WT) (SAC/WT) and signal transducer and activator of transcription (STAT) $6^{-/-}$ (SAC/ST) mice were injected into $STAT6^{-/-}$ mice. Ten days after injection, mice were infected with *Toxoplasma gondii*. The percentage of $CD25^+$ $CD8^+$ T cells in $CD8^+$ T cells in peripheral blood was determined on day 14 post-infection (pi). *Significantly different ($P < 0.05$). (b) Cysts in the brains of these mice were determined on day 28 pi. Data are mean ± standard deviation (SD) ($n = 5$). (c) On day 28 pi, splenocytes were prepared and stained with anti-CD11c and the indicated monoclonal antibodies (mAbs). $CD11c^+$ cells were gated and analysed by flow cytometry. Expression of CD86 was reduced in $STAT6^{-/-}$ as compared with WT mice. Unstained cells were used as controls. The mean fluorescence intensity (MFI) for expression of the molecules in $STAT6^{-/-}$ and WT mice were 154 ± 23 and 190 ± 36 [major histocompatibility complex (MHC) class I], 72 ± 10 and 83 ± 7 (MHC class II), 2·1 ± 0·3 and 1·9 ± 0·1 (CD40), 56 ± 3 and 58 ± 4 (CD80), and 17 ± 1 and 21 ± 1 (CD86), respectively ($n = 4$). (d) The interleukin (IL)-12 p40 concentration in serum was determined by enzyme-linked immunosorbent assay (ELISA) on days 7 and 14 pi. Data are mean ± SD ($n = 5$). (e) The IL-12 p40 concentration in culture supernatants of splenocytes was determined by ELISA on day 7 pi. Data are mean ± SD ($n = 5$). Experiments were carried out three times, with similar results. *Significantly different from WT mice ($P < 0.05$).

infected mice. $CD8^+$ T-cell activation by APCs is possibly impaired in a STAT6-deficient environment.

Recent *in vivo* studies have shown that IL-4 is critical for the development of protective $CD8^+$ T-cell memory responses against tumours and infections with protozoan parasites such as *Leishmania* and *Plasmodium*.[18] Cytotoxic T lymphocyte-mediated immune responses against mammary and colon carcinoma were abrogated or did not develop in the absence of IL-4.[19] More recently, vaccination studies demonstrated that the development of $CD8^+$ T cell-mediated protective immune responses against *Leishmania donovani* in mice was fully dependent on IL-4.[20] Studies using parasite-specific T cell receptor-transgenic $CD8^+$ T cells revealed a critical role of IL-4 in the generation of memory $CD8^+$ T-cell responses against the liver stages of the rodent malaria parasite *Plasmodium yoelii*.[21] In the present study, we also observed that STAT6 signalling was important in $CD8^+$ T-cell activation in *T. gondii*-infected mice. However, the underlying mechanism of STAT6 signalling in $CD8^+$ T-cell activation is still unclear.

Two possible mechanisms have been considered for the role of STAT6 signalling in $CD8^+$ T-cell responses. One is that STAT6 signalling might be involved in maturation of APCs such as DCs, and contribute to $CD8^+$ T-cell activation. DCs are the most potent APCs that can activate T cells to induce a primary immune response.[22] Recently, IL-4R/IL-13R-associated STAT6 signalling in DC maturation has been well studied and has been shown to have important roles in IL-12 production and activation marker expression by DCs.[23–25] STAT6 signalling is constitutively activated in primary immature DCs and progressively declines as the cells differentiate into mature DCs.[26] These results suggest that STAT6 signalling may be important in DC maturation. Our current results also suggested an important role of STAT6 signalling in DC maturation and in $CD8^+$ T-cell activation. Expression of an activation marker, CD86, on spleen DCs was lower in $STAT6^{-/-}$ than in WT mice. IL-12 p40 production was lower in $STAT6^{-/-}$ mice. In addition, activation of $CD8^+$ T cells in $STAT6^{-/-}$ mice was impaired, and was restored after transfer of SACs or BM-derived DCs from WT mice.

Another possible mechanism is that STAT6 may directly influence $CD8^+$ T cells. As reported by Marsland *et al.*, $CD8^+$ T cells possess the IL-4 receptor, and STAT6 signalling in these cells is stimulated by IL-4.[27] In our experiments, $STAT6^{-/-}$ $CD8^+$ T cells were activated by transfer of WT SACs into $STAT6^{-/-}$ mice. However, naïve WT $CD8^+$ T cells transferred into $STAT6^{-/-}$ mice were not activated. These results indicate that STAT6 signalling not in $CD8^+$ T cells but in APCs is important in the activation of $CD8^+$ T cells in *T. gondii*-infected mice.

Our results agree with those previous reported by Suzuki *et al.*[7] They demonstrated that cyst number in the brain was greater in $IL\text{-}4^{-/-}$ mice than in WT mice in the chronic stage of infection. Their results also showed that IFN-γ production by splenocytes from $IL\text{-}4^{-/-}$ mice was comparable to that in WT mice in spite of the expected up-regulation of T helper type 1 (Th1) responses in $IL\text{-}4^{-/-}$ mice. However, they did not report IFN-γ production by $CD8^+$ T cells. Mortality was significantly higher, with more severe TE and heavier cyst burden in $IL\text{-}4^{-/-}$ than in WT mice. In contrast, Roberts *et al.* reported that $IL\text{-}4^{-/-}$ mice showed significantly higher mortality, with less severe TE and decreased cyst burden compared with WT (129/Sv × B6)F2 mice.[28] The same group reported that $IL\text{-}4^{-/-}$ mice on a B6 background showed a higher survival rate in spite of a heavier cyst burden and more severe histopathological changes in the liver than WT mice.[8] Brain inflammatory responses were less severe in $STAT6^{-/-}$ mice than in WT mice, with a similar mortality rate to that in our study. Taken together, these findings suggest that the virulence of the *T. gondii* strain and the susceptibility of the mouse strain might influence mortality and pathology. Reciprocal effects of IL-4 on the production of IFN-γ might also be involved in this discrepancy.

$CD8^+$ T cells, once activated, functioned also in $STAT6^{-/-}$ mice, because transfer of $CD8^+$ T cells recovered from infected WT mice into $STAT6^{-/-}$ mice on day 14 pi resulted in a decrease in the number of cysts in the brain, whereas transfer of $CD8^+$ T cells from uninfected WT mice did not show any effect on the formation of cysts in the brain. These results imply that $CD8^+$ T cells, once activated in infected WT mice, regulate the formation of cysts in the brain. Activation of $CD8^+$ T cells is important in the acute phase of *T. gondii* infection.[3,4] Therefore, $STAT6^{-/-}$ mice were considered to be less resistant than WT mice in the acute phase. Surprisingly, anti-CD8 mAb treatment on days 14 and 21 pi resulted in a similar cyst burden in the brain in $STAT6^{-/-}$ and WT mice on day 28 pi, indicating the possibility of no difference in parasite burden in the brain on day 14 pi. This needs to be further clarified.

Our results clearly demonstrate that STAT6 signalling is important in $CD8^+$ T-cell activation, possibly through regulation of APCs, which could suppress *T. gondii* infection in the brain.

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Antibodies to myelin oligodendrocyte glycoprotein are not involved in the severity of chronic non-remitting experimental autoimmune encephalomyelitis

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ABSTRACT

To elucidate the role of antibodies in development of chronic non-remitting experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice, which is a well-established Th1-mediated autoimmune disease, and the involvement of activation-induced cytidine deaminase (AID) in Th1-mediated function, we have investigated the myelin oligodendrocyte glycoprotein (MOG)-induced EAE in mice deficient of AID, which is absolutely required for class switching and somatic hypermutation. Following immunization with MOG, $AID^{-/-}$ had completely same levels of clinical and pathological severity of EAE when compared with $AID^{+/-}$ and $AID^{+/+}$, although $AID^{-/-}$ did not produce IgG and anti-MOG IgG. Similar levels of T cell proliferation and a modest increase of anti-MOG IgM synthesis were found in spleen cells of $AID^{-/-}$ stimulated with MOG. These results indicate that antibodies are not involved in development of EAE in C57BL/6 mice.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is an immunologically mediated disease of central nervous system (CNS) that provides an animal model for acute or chronic human demyelinating disorders: multiple sclerosis (MS) and acute disseminated encephalomyelitis [1]. EAE is induced in a variety of animals by sensitization of myelin basic protein (MBP), proteolipid protein, myelin oligodendrocyte glycoprotein (MOG) or their derived peptide [2]. Among these, the injection of MOG or its encephalitogenic peptide MOG35–55 in C57BL/6 mice produces a paralytic neurological disease with extensive plaque-like demyelination. The mice develop a chronic non-remitting neurological disease 12–16 days postimmunization lasting entire 45 days of observation and the antibody reactivity to MOG35–55 is detected in mice 4 weeks postimmunization [3]. A number of studies have reported that anti-MOG antibodies are able to induce demyelination in vivo or in vitro [4–7]. However, the role of anti-MOG35–55 antibodies in acute/chronic EAE induced by sensitization of MOG35–55 C57BL/6 mice remains to be elucidated. EAE is considered a Th1-mediated autoimmune disease. Recent study has shown that in B6 mice MOG-reactive $CD8^+$ cells are pathogenic [8]. It is believed that the presence of pro-inflammatory cytokines such as INF-γ and TNF-α lead to damage of myelin [9]. Furthermore, the idea that Th17 cells have an important role in EAE has emerged in light of the following observations. Deficiency in either p40 or p19, which form IL-23, results in a decreased number of Th17 cells and protection from EAE, although deficiency in the IL-12p35 subunit, which is specific for IL-12, does not alter the progression of EAE [10,11]. In addition, transfer of myelin-reactive IL-17-producing T cells expanded with IL-23 in vitro induced severe EAE. Although IL-17-deficient mice, nevertheless, develop attenuated EAE, Th17 cells are considered potent inducers of autoimmunity [12].

The role of B cells and myelin-specific Abs in mediating myelin loss is controversial. While the pathology of multiple sclerosis implicates a role for B cells and antibodies in the disease process [13], results from animal models have yielded conflicting results. B cell-deficient mice do not develop EAE when immunized with MOG [14] or developed disease with a reduced disease severity [15]. Conversely, no difference is observed in the onset or severity of disease in the absence of mature B cells by deletion of their mu chain transmembrane region, suggesting that B cells and Abs are not necessary for primary demyelination in MOG-induced EAE

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in mice [16–18]. Recently, Fillatreau et al. demonstrate that B cell-derived IL-10 plays a key role in controlling the pro-inflammatory type 1 immune response and EAE [19]. In addition, IL-17 and IL-23 play a crucial role in the development of EAE [20,21]. In a specific condition, it is shown that myelin-specific Abs contribute to the EAE onset. Transgenic mice engineered to produce high titers of autoantibodies against MOG both accelerate and exacerbate EAE [22,23]. $Lyn^{-/-}$ which show IgM hyperglobulinemia and elevation in serum anti-IgM MOG antibodies levels have higher clinical and pathological severity of MOG-induced EAE when compared with wild type [24]. In system of EAE which used normal mice, it remains unclear whether an anti-myelin antibody contributes to the onset of EAE.

The B cells in the immune system use multiple genetic mechanisms such as gene rearrangement, class switching and somatic hypermutation to drive the generation of antibody diversity. Recently, activation-induced cytidine deaminase (AID), which is involved in regulation of the DNA modification step of both class switching and somatic hypermutation, has been identified [25,26]. Examining the profiles of expressed genes revealed expression of the AID gene to be largely restricted to germinal center B cells, memory B cells and activated B cells, but not other cells including T cells, suggesting that AID is not involved in T cell function [27,28]. $AID^{-/-}$ with circulating B cells produced IgM which may be low affinity antibodies due to the lack of somatic hypermutation allowing the generation of antibodies with improved affinity for antigen, but not IgG, IgA and IgE [25]. Accordingly, $AID^{-/-}$ is suitable for in vivo examination to evaluate the effects of MOG-specific antibodies on the development of EAE.

To clarify the effect of antibodies on the development of chronic non-remitting EAE and to conform whether AID is not involved in Th1-mediated immune response, we studied severity of EAE in $AID^{-/-}$ which have normal levels of circulation B cells, and defect of IgG, IgA and high affinity IgM.

2. Materials and methods

2.1. Animals and reagents

Established $AID^{-/-}$, $AID^{+/-}$ and $AID^{+/+}$ eighth backcrossed from (CBA × C57BL/6) × C57BL/6 to C57BL/6 were maintained in the animal facility of Shinshu University and used between 8- and 10-week old. Mice were housed and cared for in a nationally approved facility in accordance with the National Institutes of Health guidelines of the United States. The synthetic mouse MOG peptides 35–55 (Met–Glu–Val–Gly–Trp–Tyr–Arg–Ser–Pro–Phe–Ser–Arg–Val–Val–His–Leu–Try–Arg–Asn–Gly–Lys), obtained from Sawady Technology Co., Ltd. (Tokyo, Japan), were synthesized using standard F-moc chemistry; purity (>90% pure) was determined using reverse phase HPLC.

2.2. Immunization

$AID^{-/-}$, $AID^{+/-}$ and $AID^{+/+}$ were injected with MOG35–55 peptide (100 μg) dissolved in saline and emulsified with an equal volume of complete Freund's adjuvant (CFA), supplemented with 4 mg/ml of *Mycobacterium tuberculosis* (Difco, H37Ra) or CFA alone in hip (50 μl) and in the nape of the neck (50 μl) as previously described [29,30]. Immediately before and 2 days after injection of the emulsion, the mice received an intravenous injection of 300 ng pertussis vaccine in 100 μl of phosphate-buffered saline (PBS). The mice were assessed daily for clinical signs of disease; signs of neurological dysfunction were graded using the following system: normal, 0; limp tail, 1; hind leg weakness, 2; hind leg paralysis, 3; fore leg paralysis, 4; moribund or death, 5. The batches of the peptide and adjuvant used were identical throughout the present studies.

2.3. Serum collection

Serum samples were prepared from the peripheral blood obtained by tail vein puncture every week up to 10 weeks after immunization, and were stored at −30 °C until use.

2.4. Histological examination of tissues

Mice were anesthetized with an i.p. injection of pentobarbital and exsanguinated by transcardial perfusion with fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) on day 21 after immunization. Whole CNS tissues, including an entire portion of the spinal cord and optic nerves, were carefully dissected free from the cranium and spinal canal, then immersed overnight in the same fixative solution at room temperature. The CNS was longitudinally and coronally sectioned along the rostrocaudal axis. The tissue slices were dehydrated in a graded series of ethanol, cleared in Hemo-D and embedded in paraffin. Serial sections of 5 μm thickness were mounted on silane-coated glass slides. For light microscopic examination, sections were stained using hematoxylin–eosin and Klüver–Barrera (luxol fast blue–cresyl violet) procedures.

2.5. ELISA

Ninety-six-well microtiter plates (Nunc-Immunoplate, Denmark), pretreated with 0.2% glutaraldehyde, were coated (50 μl/well) with MOG35–55 peptide diluted to a concentration of 1 μg/ml in carbonate buffer (pH 9.6) as previously described [30,31] for MOG-specific-antibody titration. For immunoglobulin titration, microtiter plates were coated (50 μl/well) with rabbit anti-mouse IgG (rabbit anti-mouse IgG (H + L), Wako pure chemical Industry, Osaka, JP) or rat anti-mouse IgM (LO-MM9, Monosan, NL) or rat anti-mouse IgA (LO-MA7, Monosan, NL) diluted to a concentration of 0.2 μg/ml in carbonate buffer (pH 9.6). After incubation at 37 °C for 3 h, the plates were washed three times with PBS containing 0.1% Tween-20 and blocked with 2% BSA–PBS at 4 °C overnight. The plates were then incubated with 50 μl of 1/1000 dilutions of mouse serum for 1 h at room temperature.

For MOG-specific-antibody titration, mouse standard serum (Nordic Immunological Lab., Ltd., The Netherlands) was used as negative control. After washing three times, 1/2000 diluted alkaline phosphatase labeled goat anti-mouse IgG (KPL Inc., MD), rat anti-mouse IgA (Zymed, San Francisco, CA) or rat anti-mouse IgM (Zymed) was added to the plates and incubated for 1 h at room temperature. The reaction products were visualized using *p*-nitrophenyl phosphate as substrate. The plates were then read at 405 nm with a microplate reader (Tosoh Co., Yamaguchi, Japan), and OD 405 level of each sample was compared.

2.6. T cell proliferation assay

At 2 weeks after immunization, mice were sacrificed. Spleen cells were harvested from three arbitrarily selected mice in each group. Cells (1×10^5) were cultured in 96-well flat bottom microculture plates in RPMI-1640 medium with 10% fetal calf serum. Triplicate cultures were stimulated with 2.0 μg/ml of MOG35–55, 0.5 μg/ml phytohemagglutinin (GIBCO, Grand Island, NY) or none at 37 °C in a 5% CO_2 incubator. During the last 12 h of the 4 days of culture, 1 μCi of ^{3}H-thymidine was added and the cell proliferation

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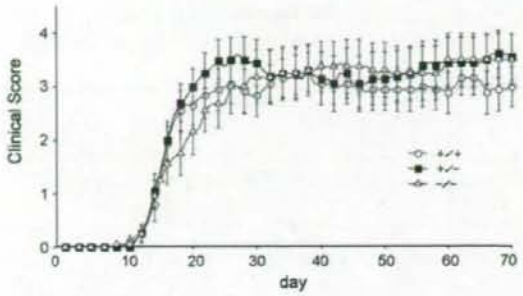

Fig. 1. Induction of EAE. EAE in AID$^{-/-}$, AID$^{+/-}$ and AID$^{+/+}$ ($n = 18$ in each group) was induced following immunization with MOG35–55, as described in Section 2. The severity of EAE is presented as mean clinical score ± S.E.M. in each group.

was estimated by ^{3}H-thymidine incorporation using a scintillation counter and expressed as cpm. The stimulation index (S.I.) was calculated as cpm with stimulation/background cpm. S.I. was considered significant only if it exceeded background by at least threefold.

2.7. Statistical analysis

Clinical scores were analyzed using the repeated measure ANOVA (StatView, Abacus Concepts, Berkeley, CA). Other results were statistically evaluated using an unpaired Student's *t*-test. All values are expressed as the mean ± S.D. and $P < 0.05$ was taken as indicating statistical significance.

3. Results

3.1. Clinical manifestation

Following immunization with MOG35–55, all 18 mice of each AID$^{-/-}$, AID$^{+/-}$ or AID$^{+/+}$ exhibited same disease courses of the typical MOG peptide-induced EAE. In AID$^{-/-}$ group, mean onset day was 16.6 ± 6.3, and the maximum disease score was 3.8 ± 1.4 and cumulative disease score from days 0 to 70 was 157.7 ± 104.2. The first symptoms of the disease, weight loss and limp tail, were observed 8–33 days after immunization with the mean onset day of 16.6 ± 6.3 in the AID$^{-/-}$ (Fig. 1). In AID$^{+/-}$ and AID$^{+/+}$ groups, the disease severities were same compared with AID$^{-/-}$ indicated by the same mean onset day, the same mean maximum clinical score and cumulative disease score. The mean onset day, the maximum disease score and cumulative disease score were 18.6 ± 11.5, 4.0 ± 0.8 and 165.5 ± 78.6 in AID$^{+/+}$ group, and 17.3 ± 10.0, 4.2 ± 1.0 and 171.7 ± 106.6 in AID$^{+/-}$ group, respectively (Fig. 1). No statistical difference in clinical manifestations was found according to AID genetics.

3.2. Histology

Each mouse immunized with MOG35–55 from the three genetic groups showed inflammatory lesions and demyelination localized mostly in the lumbar spinal cord. The numbers of demyelinating lesions and leukocytes in the lesions were similar among the three groups (Fig. 2).

3.3. Antibody synthesis

The antibody isotype reactivity of serum from each group was tested using ELISA at 0, 4 and 8 weeks after immunization. Serum IgG and IgA were not detected in AID$^{-/-}$ group throughout the study (Fig. 3 and data not shown). IgM antibody titers in AID$^{-/-}$ group were higher than those of the other two groups (Fig. 3). Serum anti-MOG35–55 IgG titers of AID$^{-/-}$ group were on baseline value, and were significantly lower than those of AID$^{+/+}$ and AID$^{+/-}$ groups at any time point (Fig. 3). Furthermore, anti-MOG35–55 IgM antibody titers of AID$^{-/-}$ were significantly higher than those of other two groups at 4 weeks (Fig. 3). The production of IgA against MOG35–55 was not observed in any mice (data not shown).

3.4. MOG35–55-specific T cell proliferation

To determine the effect of disruption of the AID gene on T cell function after MOG immunization, we examined T cell proliferative response of spleen cells following stimulation by MOG35–55. Spleen cells of AID$^{-/-}$, AID$^{+/-}$ and AID$^{+/+}$ showed positive response to MOG35–55 stimulation. AID$^{+/+}$ group showed tendencies of higher proliferation with MOG35–55 stimulation, but was not statistically significant (Fig. 4).

4. Discussion

Demyelination of MS is thought to be a multifactorial including T cell, antibodies, complement activation and direct effect of inflammatory cytokines. AID$^{-/-}$ with normal number of circulating B cells displayed no serum IgG, IgA and anti-MOG IgG, but modestly increased levels of serum IgM and anti-MOG IgM. The proliferative response was not significant statistically among the groups. AID$^{-/-}$ displayed no difference of clinical and pathological severity of chronic non-remitting EAE compared with AID$^{+/+}$

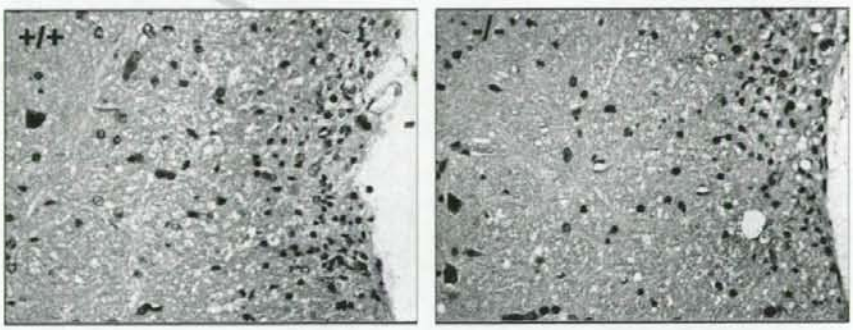

Fig. 2. Histology. Spinal cords were isolated from EAE mice randomly selected from AID$^{-/-}$ (clinical score: 2, 2 and 3), AID$^{+/-}$ (clinical score: 2, 2 and 3) and AID$^{+/+}$ (clinical score: 2, 2 and 2) mice on day 21. The numbers of demyelinating lesions and leukocytes in the lesions were similar among the three groups.

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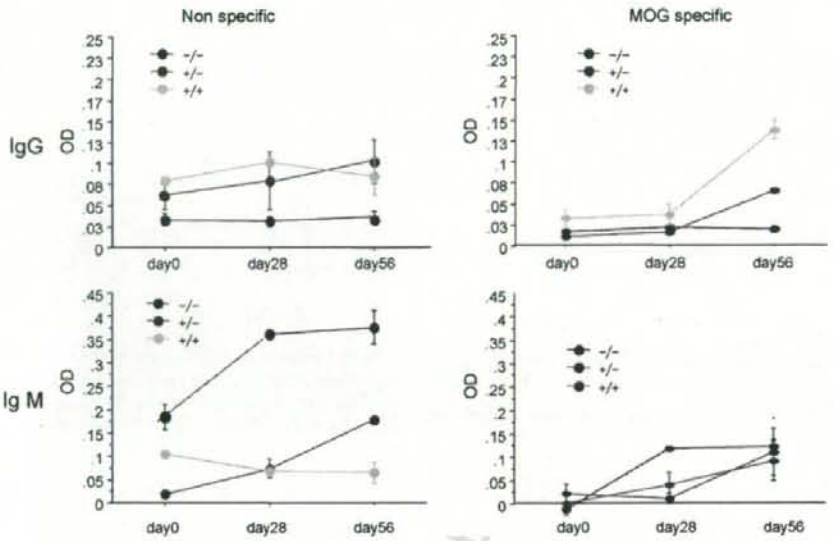

Fig. 3. Serum levels of antibodies. The mice in AID$^{-/-}$, AID$^{+/-}$ and AID$^{+/+}$ ($n = 5$ in each) were immunized with MOGp35–55. Sera were collected on days 0, 28 and 56, and total IgG and IgM and anti-MOG IgG and anti-MOG IgM were measured by ELISA. The OD 450 is presented by the mean value and S.D. of three separate measurements of each sample. *$P = 0.10$ and **$P = 0.08$. Q1

and AID$^{-/-}$. MS is a chronic neurodegenerative disease characterized by central nervous system inflammation and demyelination. The clinical heterogeneity of MS is well described and numerous animal models: EAE has been developed in different species of animals induced by different autoantigens [2]. Each represents certain aspects of MS. Immunization of MOG35–55 in C57BL/6 mice develops a chronic non-remitting type of demyelinating disease which resembles chronic MS. Thus, our studies by mice chronic MS model demonstrated that antibodies did not contribute to the regulation of the development of chronic non-remitting EAE in C57BL/6 mice.

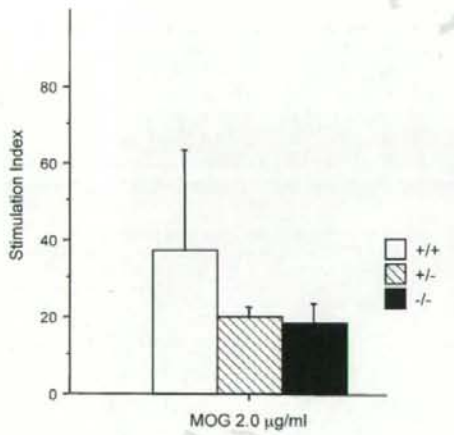

Fig. 4. MOG35–55-specific T cell proliferation. Cells were isolated from spleens of AID$^{-/-}$, AID$^{+/-}$ and AID$^{+/+}$ mice on day 14 after immunization. Cells (1×10^5) were cultured in 96-well flat bottom microculture plates in RPMI-1640 medium with 10% fetal calf serum. Triplicate cultures were stimulated with 2.0 μg/ml of MOG35–55 at 37 °C in a 5% CO_2 incubator for 4 days. During the last 12 h of the culture, 1 μCi of ^{3}H-thymidine was added and the cell proliferation was estimated by ^{3}H-thymidine incorporation using a scintillation counter. Mean stimulation index (S.I.) ± S.D. in each group was shown.

Although EAE is considered a prototypic Th1-mediated autoimmune disease, a number of studies have shown a role for B cells in the development of EAE [32]. There is much evidence to implicate B cells, plasma cells and their products in the pathogenesis of MS [19,32]. Despite unequivocal evidence that EAE, the animal model for MS, is initiated by myelin-specific T cells, there is accumulating evidence of a role for B cells, plasma cells and their products in EAE pathogenesis [24]. In the earlier studies, depletion of B cells lead to the resistance of MBP-induced EAE [33,34]. In MOG Ag-induced EAE, Lyons et al. [14] suggested that B cells were required for development of EAE induced following immunization with whole human MOG protein, but not for the EAE induced following immunization with MOGp35–55 [16,17]. MOG is expressed on the cell surface, and therefore is a potential target for autoantibodies [35]. EAE is accelerated with increased severity when high levels of MOG-specific Ab are produced genetically in mice. In transgenic mice that were engineered to produce MOG-specific autoantibodies, severity of EAE was increased, suggesting an important role of Ag-specific antibodies in EAE [22]. In the marmoset, the administration of MOG antigens can increase concentrations of pathogenic autoantibodies and in some circumstances cause a severe worsening of EAE after recovery from the acute attack of EAE [36]. Lyn$^{-/-}$ with IgM hyperglobulinemia and elevation in serum anti-IgM MOG Ab levels have higher clinical and pathological severity of MOG-induced EAE [24]. However, as for lyn$^{-/-}$ system, possibilities that lyn contributes to T cell function remain. Disruption of lyn gene may interfere with CD40 signaling in antigen presenting cells [37], and consequently decrease IL-12 production and indirectly affect the development of a Th1 response.

Studies on mechanism of autoantibody IgG-mediated demyelination in EAE indicate that these autoantibodies enhance inflammation in the CNS via activation of complement [38] or directly through Ab-dependent cell-mediated cytotoxicity [39]. We chose AID$^{-/-}$ by three reasons to examine whether an MOG antibody, in particular about MOG IgG antibody, contributed to the onset of EAE equally. First, AID is involved in regulation or catalysis

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of the DNA/RNA modification step of both class switching recombination and somatic hypermutation, subsequently AID$^{-/-}$ do not produce IgG and IgA, and IgM synthesized by AID$^{-/-}$ is low affinity antibody, indicating that the Ag-specific IgM may not act sufficiently [25,40]. Second, there is not manifestation of AID expression in T cells and AID may not contribute T cell function. The patients with the autosomal recessive form of hyper-IgM syndrome (Hyper-IgM syndrome type II), which is caused by mutations of AID genes and have less sever clinical courses, have normal numbers and normal functions of circulating T cells, indicating that AID is restricted to immunoglobulin editing [41]. Third, normal numbers of B cells exist in AID$^{-/-}$. Therefore, we can exclude the influence of B cell itself.

In conclusion, our studies show that AID does not play an important role in the development of chronic non-remitting EAE in C57BL/6 mice. Although there is a tendency to compartmentalize autoimmune diseases into those that are T cell mediated and those that are B cell dependent (antibody mediated), these boundaries have not been clear-cut. The role of autoantibodies, anti-MOG IgG and unmutated anti-MOG IgM, is not crucial to mediate chronic non-remitting EAE in C57BL/6 mice, at least in the AID$^{-/-}$ system lacking IgG and mutated IgM. Also, since EAE is Th1-mediated disease, our data confirm that AID is not involved in the function of Th1 cells. Our studies can contribute not only to the understanding of EAE but also the development of therapeutic strategies of MS.

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Rapid Communication

Deficiency of tumour necrosis factor-α and interferon-γ in bone marrow cells synergistically inhibits neointimal formation following vascular injury

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Aims Neointimal formation after percutaneous coronary intervention (PCI), termed restenosis, limits therapeutic revascularization. Since it is now known that vascular injury involves an inflammatory response, we examined the role of tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in the neointimal formation after injury.

Methods and results Control (BALB/c), TNF-α-deficient ($Tnf^{-/-}$), IFN-γ-deficient ($Ifng^{-/-}$), or double-deficient ($Tnf^{-/-}$ $Ifng^{-/-}$) mice were subjected to wire-mediated vascular injury of the right femoral artery. Neointimal formation after injury was significantly reduced after the injury in the $Tnf^{-/-}$ $Ifng^{-/-}$ mice, compared to that in the control, $Tnf^{-/-}$, and $Ifng^{-/-}$ mice. Immunohistochemical analysis showed that TNF-α and IFN-γ were expressed in neointimal lesions in the control mice, but not in mice with deficiency of the corresponding cytokine. No significant difference in re-endothelialization was observed among these groups. The number of proliferating cell nuclear antigen in the neointimal lesions was significantly decreased in the $Tnf^{-/-}$ $Ifng^{-/-}$ mice. Bone marrow transplantation experiments revealed that deficiency of TNF-α and IFN-γ specifically in bone marrow cells significantly inhibited neointimal formation after vascular injury.

Conclusion The absence of TNF-α and IFN-γ in bone marrow cells synergistically inhibits neointimal formation following vascular injury, and thus, may provide new insights into the mechanisms underlying restenosis after PCI.

1. Introduction

Neointimal formation after vascular injury is the pathological basis of atherosclerosis and restenosis following a percutaneous coronary intervention (PCI), such as angioplasty and stenting. Although the pathogenic mechanisms have not been completely elucidated, an accumulating body of evidence suggests that inflammatory response plays a key role in these processes. Tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are inflammatory cytokines that mediate a wide range of immune and inflammatory responses and have been found to be involved in the development of post-PCI restenosis and atherosclerosis. It has been reported that TNF-α stimulates the expression of adhesion molecules and the proliferation and migration of vascular smooth muscle cells (VSMCs), and that is up-regulated at the site of vascular injury and in atherosclerotic plaque specimens.[1,2] On the other hand, IFN-γ has also been shown to be expressed in vascular lesions and to regulate the expression of platelet-derived growth factor receptor-β that promotes the proliferation of VSMCs.[3,4] However, the *in vivo* role of these cytokines remains controversial. For instance, inhibition of TNF-α by gene disruption resulted in reduced neointimal formation in a murine model of carotid artery injury.[5] In contrast, the inhibition of TNF-α by neutralizing antibody had no effect on neointimal formation following vascular injury in rabbits.[6] With regard to the role of IFN-γ, the administration of recombinant IFN-γ inhibited VSMC proliferation and neointimal formation after vascular injury.[7,8] On the other hand, the inhibition of IFN-γ pathway due to the overexpression of a soluble mutant of IFN-γ receptor inhibited only neointimal formation after injury.[9] In addition, several

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studies have demonstrated that TNF-α and IFN-γ synergistically regulate many biological functions, including gene induction and cellular proliferation.[10]

In the present study, we investigated the effect of TNF-α and/or IFN-γ deficiency on neointimal formation following vascular injury in mice. Recent evidence indicates a critical role of bone marrow cell-derived cytokines in the pathogenesis of restenosis and atherosclerosis;[11] however, no information is available on the role of bone marrow cell-derived TNF-α and IFN-γ in vascular injury. Therefore, we prepared the irradiation/bone marrow transplantation (BMT) model and determined the role of bone marrow cell-derived TNF-α and IFN-γ. The findings of our study suggest the synergistic role of TNF-α and IFN-γ derived from bone marrow cells in neointimal formation after injury and provide new insights into the mechanism underlying restenosis and atherosclerosis.

2. Materials and methods

2.1 Animals

The animal experimental protocol used in this study was reviewed and approved by the Shinshu University Guide for Laboratory Animals. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). The animals were fed a standard diet and water, and were maintained on a 12 h light and dark cycle. BALB/c mice (male, 8–12 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan) and used as control throughout this study. *Tnf*$^{-/-}$ and *Ifng*$^{-/-}$ mice were generated as described previously.[12] Both these mutant animals were backcrossed to the BALB/c background at least 12 times. Mice with either of these two mutations were crossed to generate double-knockout mice (*Tfn*$^{-/-}$*Infg*$^{-/-}$).

2.2 Wire-mediated vascular injury

A wire-mediated vascular injury of the right femoral artery was produced as described previously.[13] We confirmed that this procedure induced a reproducible neointimal formation in 8–12 weeks old mice.[14,15]

2.3 Histology and immunohistochemistry

Histological and immunohistochemical analyses were performed as described previously.[11] Mice were euthanized after brief irrigation with saline to ensure that the blood was completely washed out of the femoral artery. The femoral arteries were excised from each mouse, embedded in the optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Laboratories, IN, USA), and frozen in liquid nitrogen. Neointimal formation in the femoral arteries was evaluated at five locations at 100 μm intervals, with the most distal site located at the origin of branch through which a wire was inserted. The sections were stained with elastica van Gieson (EVG). To measure the intimal and medial areas, each image was digitized and analysed under a microscope (BX-51; Olympus, Tokyo, Japan) by using NIH image software ver. 1.63. The values at the five locations in each artery were averaged. All the measurements were performed in a double-blind manner by two different researchers.

For immunohistochemical analysis, arterial sections were incubated with primary antibodies against TNF-α (ab6671; Abcam, Cambridge, MA, USA), IFN-γ (BioSource International Inc., Camarillo, CA, USA), CD31 (clone MEC13.3; BD Biosciences, San Jose, CA, USA), F4/80 (clone A3-1; RDI, Flanders, NJ, USA), α-smooth muscle actin (α-SMA, clone 1A4, alkaline phosphatase (AP)-conjugated; Sigma, Saint Louis, MO, USA), and proliferating cell nuclear antigen (PCNA, Dako Cytomation, Glostrup, Denmark). This was followed by incubation with biotin-conjugated secondary antibodies. Next, the sections were washed and treated with avidin-peroxidase (ABC kit; Vector Laboratories, Burlingame, CA, USA). The reaction was developed using the DAB Substrate Kit (Vector Laboratories). Vector Red AP substrate kit was used for α-SMA staining. The sections were then counterstained with haematoxylin. No signals were detected when species- and isotype-matched immunoglobulin (Ig)G (Vector Laboratories) was used instead of the primary antibody as a negative control. Quantitative staining for CD31, F4/80, and α-SMA was quantified independently performed in a double-blind manner by at least two researchers.

2.4 Bone marrow transplantation

Bone marrow-transplanted mice were developed as described previously.[11,14] Whole bone marrow cells from the control and *Tfn*$^{-/-}$*Infg*$^{-/-}$ mice were harvested by flushing their femurs with phosphate-buffered saline (PBS). Red blood cells were lysed with ammonium chloride potassium buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylenediaminetetraacetic acid; pH 7.2) at 4°C for 20 min. They were washed three times with PBS and resuspended in 0.5 mL PBS. Recipient mice (control and *Tfn*$^{-/-}$*Infg*$^{-/-}$ mice, 6–8 weeks old) were lethally irradiated with a total dose of 9 Gy (MBR-155R2, Hitachi, Japan) and injected with bone marrow cells through the tail vein. To verify the reconstitution of bone marrow after transplantation by this protocol, we used green fluorescent protein (GFP)-transgenic mice (kindly provided by Professor M. Okabe, Osaka, Japan) as donors. Flow cytometry analysis revealed that at 6 weeks after transplantation, peripheral blood cells consisted of more than 90% GFP-positive cells. By using this protocol, we produced three types of bone marrow-transplanted mice: control to control ($BMT^{Cont.\rightarrow Cont.}$) mice, *Tfn*$^{-/-}$*Infg*$^{-/-}$ to control ($BMT^{Tnf^{-/-}Ifng^{-/-}\rightarrow Cont.}$) mice, and control to *Tfn*$^{-/-}$*Infg*$^{-/-}$ ($BMT^{Cont.\rightarrow Tnf^{-/-}Ifng^{-/-}}$) mice.

2.5 Statistical analysis

Data are expressed as mean ± SEM. The unpaired two-tailed *t*-test was used to compare the two groups. For comparisons between multiple groups, we determined the significance of the differences between the means of the groups by using one-way analysis of variance, followed by the Tukey–Kramer procedure for comparison of means. All analyses were performed using StatView software (Abacus Concepts Inc., Berkeley, CA, USA). Differences with *P*-values of <0.05 were considered to be statistically significant.

3. Results

3.1 Expression of tumour necrosis factor-α and interferon-γ in neointimal lesions

We next examined whether TNF-α and IFN-γ were expressed in the neointimal lesion after injury. The expression of TNF-α and IFN-γ was clearly visualized in the neointimal lesion at 28 days after injury by using immunohistochemical analysis (*Figure 1*). As expected, TNF-α was not expressed in *Tnf*$^{-/-}$ and *Tnf*$^{-/-}$*Ifng*$^{-/-}$ mice, and IFN-γ was not expressed in *Ifng*$^{-/-}$ and *Tnf*$^{-/-}$*Ifng*$^{-/-}$ mice.

3.2 Effects of tumour necrosis factor-α and/or interferon-γ deficiency on neointimal formation following injury

We first investigated whether TNF-α and/or IFN-γ deficiency influenced the development of neointimal formation after wire-mediated vascular injury. EVG staining showed marked neointimal formation in the control mice at 28