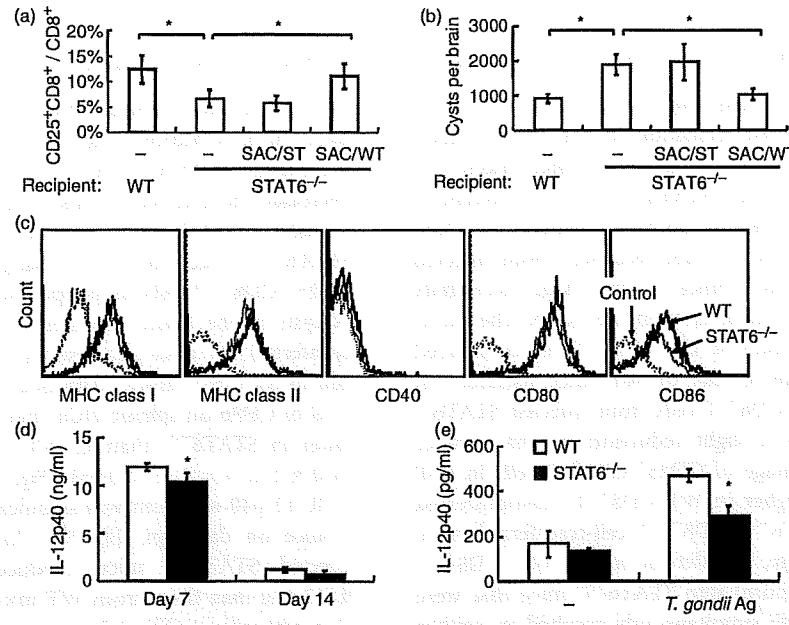

Figure 5. Effects of transfer of splenic adherent cells (SACs) on $CD8^+$ T-cell activation and cyst burden. (a) One million SACs derived from uninfected wild-type (WT) (SAC/WT) and signal transducer and activator of transcription (STAT) $6^{-/-}$ (SAC/ST) mice were injected into $STAT6^{-/-}$ mice. 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 ($P < 0{\cdot}05$). (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 ($P < 0{\cdot}05$).

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 immunogloblin 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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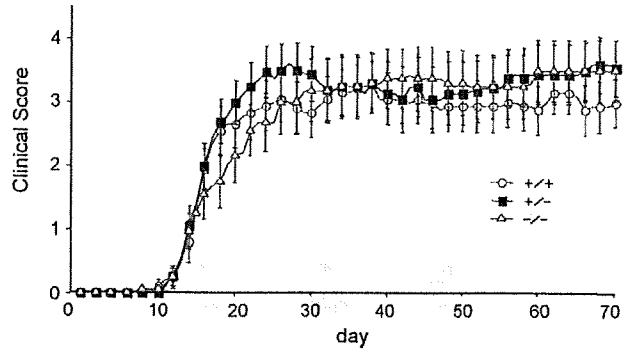

Fig. 1. Induction of EAE. EAE in AID$^{-/-}$, AID$^{+/-}$ and AID$^{+/+}$ ($n = 18$ in each group) was induced following immunization with MOG35–55, as described in Section 2. The severity of EAE is presented as mean clinical score ± S.E.M. in each group.

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$^{+/+}$

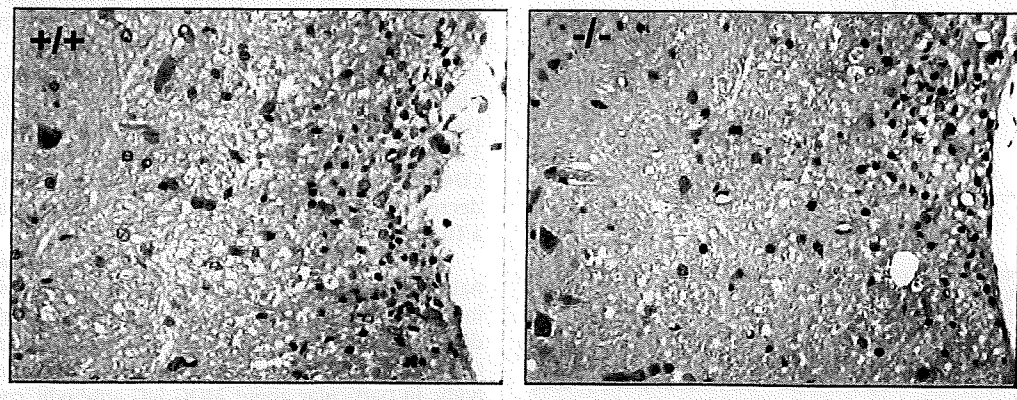

Fig. 2. Histology. Spinal cords were isolated from EAE mice randomly selected from AID$^{-/-}$ (clinical score: 2, 2 and 3), AID$^{+/-}$ (clinical score: 2, 2 and 3) and AID$^{+/+}$ (clinical score: 2, 2 and 2) mice on day 21. The numbers of demyelinating lesions and leukocytes in the lesions were similar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

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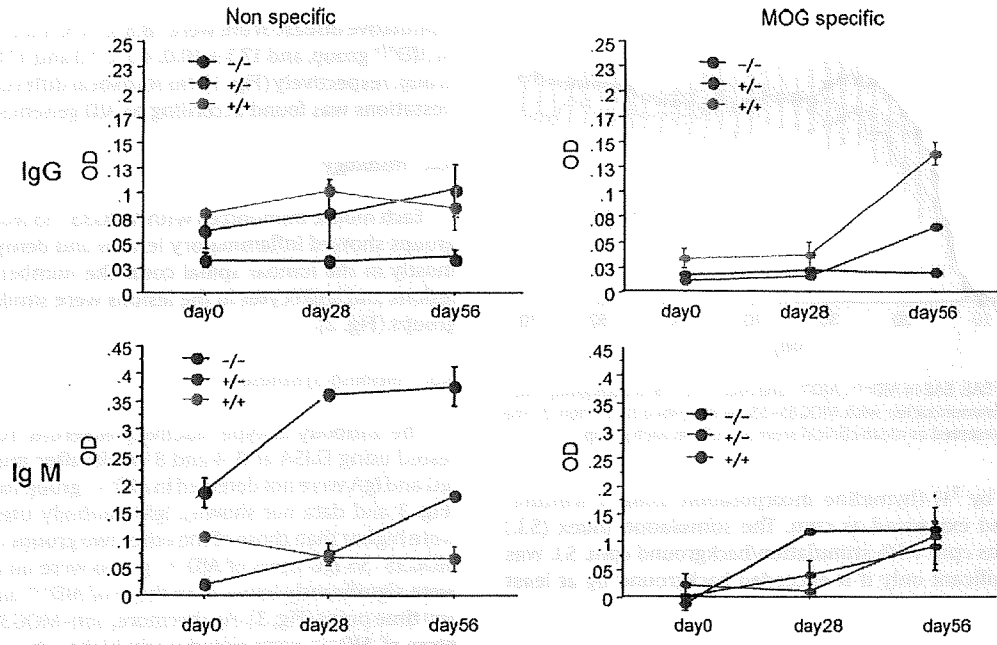

Fig. 3. Serum levels of antibodies. The mice in AID$^{-/-}$, AID$^{+/-}$ and AID$^{+/+}$ ($n=5$ in each) were immunized with MOGp35–55. Sera were collected on days 0, 28 and 56, and total IgG and IgM and anti-MOG IgG and anti-MOG IgM were measured by ELISA. The OD 450 is presented by the mean value and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

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.

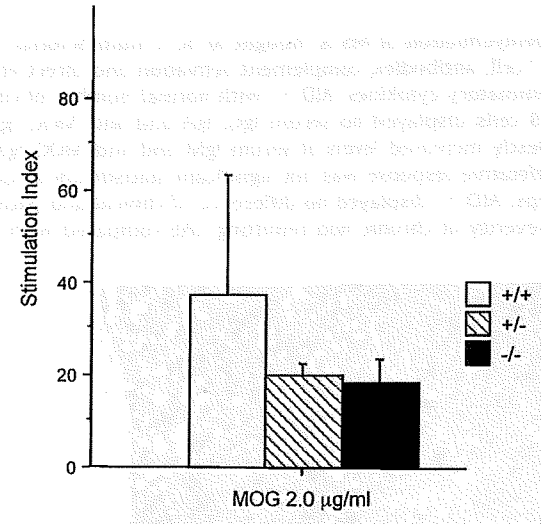

Fig. 4. MOG35–55-specific T cell proliferation. Cells were isolated from spleens of AID$^{-/-}$, AID$^{+/-}$ and AID$^{+/+}$ mice on day 14 after immunization. Cells (1×10^5) were cultured in 96-well flat bottom microculture plates in RPMI-1640 medium with 10% fetal calf serum. Triplicate cultures were 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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Bone marrow cell;
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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 neiointimal 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

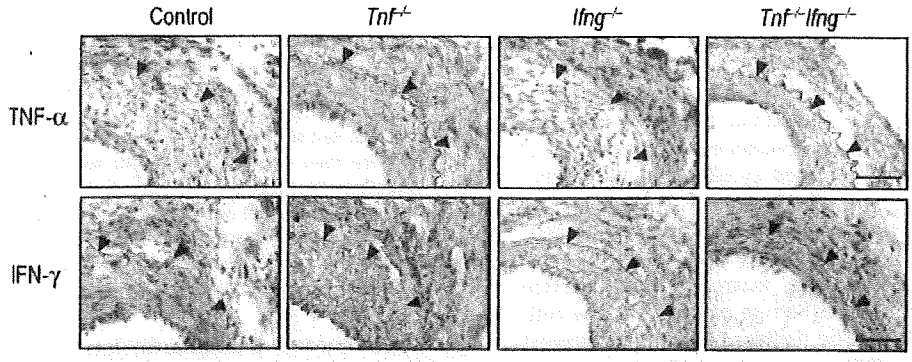

Figure 1 Expression of TNF-α and IFN-γ in neointimal lesion. Wire-mediated vascular injury was produced in the control, *Tnf*$^{+/+}$, *Ifng*$^{+/+}$, and *Tnf*$^{+/+}$ *Ifng*$^{+/+}$ mice. The femoral arteries were excised at 28 days after injury. Immunohistochemical staining for TNF-α and IFN-γ was performed. Representative photographs are shown ($n = 3$). The arrowheads indicate the internal elastic lamina. The bar represents 50 μm.

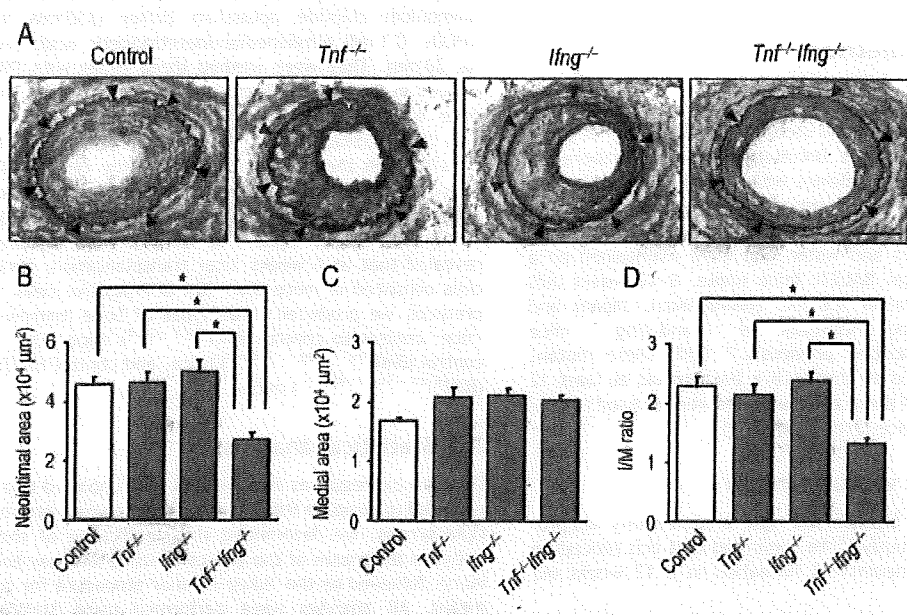

Figure 2 Effect of TNF-α and/or IFN-γ deficiency on neointimal formation. Wire-mediated vascular injury was produced in the control ($n = 10$), *Tnf*$^{+/+}$ ($n = 18$), *Ifng*$^{+/+}$ ($n = 18$), and *Tnf*$^{+/+}$ *Ifng*$^{+/+}$ ($n = 14$) mice. The femoral arteries were excised at 28 days after injury. The sample sections were stained with EVG, and neointimal formation was evaluated. (*A*) Representative photographs of EVG staining. The arrowheads indicate the internal elastic lamina. The bar represents 100 μm. (*B–D*) The bar graphs show the neointimal area (B), medial area (C), and I/M ratio (D) calculated using the NIH image software. Data are expressed as mean ± SEM. *$P < 0.05$.

days after vascular injury (*Figure 2A*). Although neointimal formation did not significantly differ between the control mice and *Tnf*$^{-/-}$ or *Ifng*$^{-/-}$ mice, that in the *Tnf*$^{-/-}$ *Ifng*$^{-/-}$ mice was significantly reduced. Quantitative analysis showed that the neointimal area and intima/media (I/M) ratio were reduced; however, no significant difference was observed in the medial area between *Tnf*$^{-/-}$ *Ifng*$^{-/-}$ mice and the other three types of mice (*Figure 2B–D*).

3.3 Detection of endothelial cells, macrophages, and vascular smooth muscle cells

Since we previously demonstrated that early re-endothelialization following vascular injury inhibits neointimal formation,[14] immunohistochemical analysis of the endothelial marker CD31 was performed. No significant difference was observed in the re-endothelialization after injury among these mice (*Figure 3A* and *B*). Further, we performed immunohistochemical analysis to detect macrophages (F4/80) and VSMCs (α-SMA), and assessed the cellular contents of neointima in the mice. Consistent with previous reports,[13,16] our finding was that the neointimal lesion was composed of many VSMCs and some macrophages (*Figure 3A*). The number of macrophages and VSMCs per unit neointimal area did not differ among the mice (*Figure 3C* and *D*).

3.4 Proliferation activity in neointimal lesions

Neointimal lesions after vascular injury mainly comprise proliferative VSMCs; we determined their proliferation activity *in vivo* by immunohistochemical staining for PCNA. The number of PCNA-positive cells significantly decreased in the neointimal lesions in *Tnf*$^{-/-}$ *Ifng*$^{-/-}$ mice compared to that in the control mice (*Figure 4*).

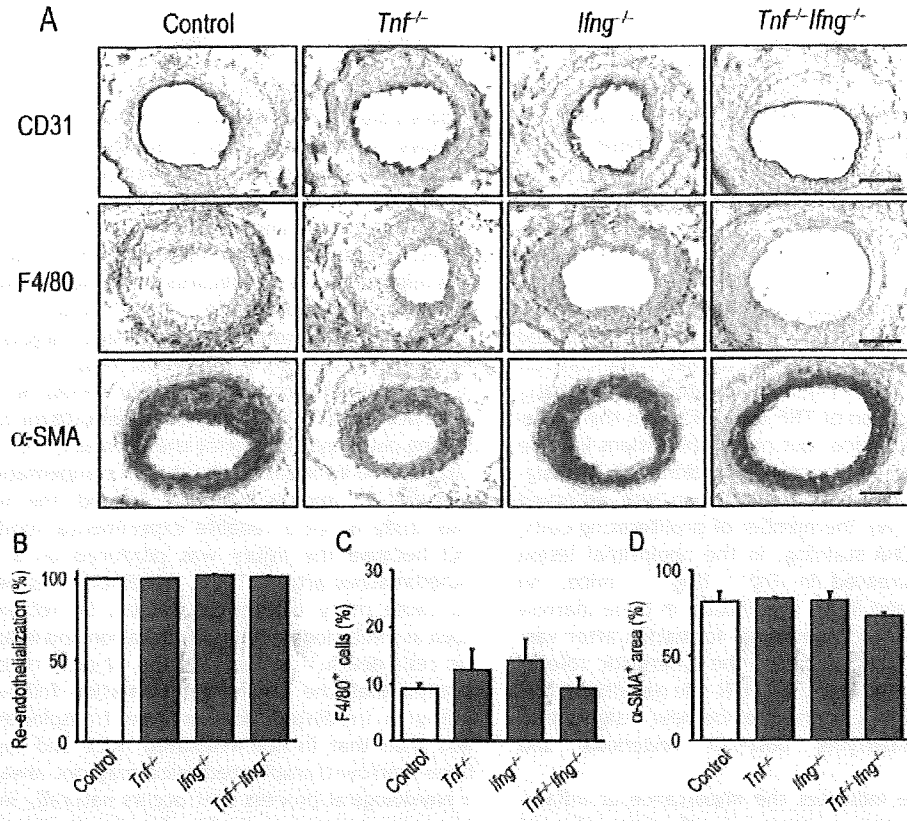

Figure 3 Detection of endothelial cells, macrophages, and VSMCs. Wire-mediated vascular injury was produced in the control, *Tnf*$^{-/-}$, *Ifng*$^{-/-}$, and *Tnf*$^{-/-}$*Ifng*$^{-/-}$ mice. The femoral arteries were excised at 28 days after injury. (*A*) Immunohistochemical staining for endothelial cells (CD31), macrophages (F4/80), and VSMCs (α-SMA) was performed. Representative photographs are shown. The bar represents 100 μm. (*B–D*) The bar graphs show the re-endothelialization (B), F4/80-positive cells (C), and α-SMA-positive area (D) in the neointimal lesions. Data are expressed as mean ± SEM ($n = 4$ for each).

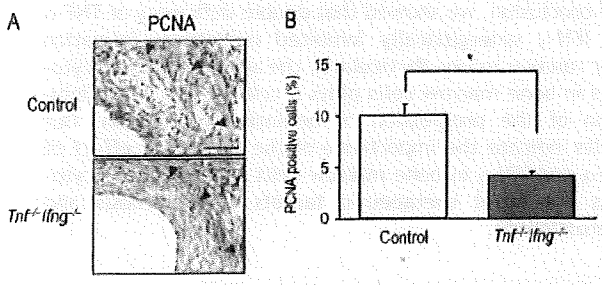

Figure 4 Proliferation activity in neointimal lesion. Wire-mediated vascular injury was produced in the wild-type and *Tnf*$^{-/-}$ *Ifng*$^{-/-}$ mice. The femoral arteries were excised at 28 days after injury. Immunohistochemical staining for PCNA was performed. (*A*) Representative photographs of PCNA staining are shown. The arrowheads indicate the internal elastic lamina. The bar represents 50 μm. (*B*) The bar graph shows the number of PCNA-positive cells in the neointimal lesion. Data are expressed as mean ± SEM ($n = 4$ for each). $^{*}P < 0.05$.

3.5 Role of bone marrow cell-derived tumour necrosis factor-α and interferon-γ in neointimal formation

To assess the role of bone marrow cell-derived TNF-α and IFN-γ in neointimal formation after vascular injury, we produced three types of bone marrow-transplanted mice ($BMT^{Cont \rightarrow Cont}$ mice, $BMT^{Tnf\,-/-\,Ifng\,-/- \rightarrow Cont}$ mice, and $BMT^{Cont \rightarrow Tnf\,-/-\,Ifng\,-/-}$ mice) and evaluated neointimal formation following injury. The formation of neointima

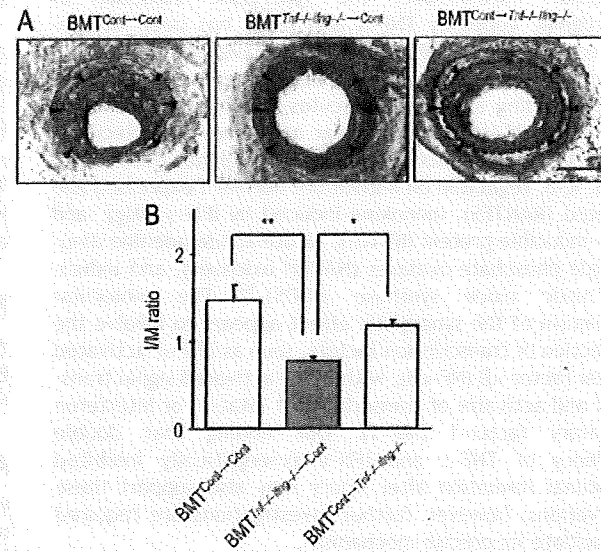

Figure 5 Contribution of bone marrow cells to neointimal formation. Bone marrow-transplanted mice [$BMT^{Cont \rightarrow Cont}$ ($n = 7$), $BMT^{Tnf\,-/-\,Ifng\,-/- \rightarrow Cont}$ ($n = 6$), and $BMT^{Cont \rightarrow Tnf\,-/-\,Ifng\,-/-}$ mice ($n = 6$)] were developed, and wire-mediated vascular injury was produced in them 8 weeks after BMT. The femoral arteries were excised at 28 days after injury. The sample sections were stained with EVG and neointimal formation was evaluated. (*A*) Representative photographs of EVG staining. The arrowheads indicate the internal elastic lamina. The bar represents 100 μm. (*B* and *C*) The bar graphs show the neointimal area (B), medial area (C), and I/M ratio (*D*). Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

following vascular injury in $BMT^{Cont\ \to Cont}$ mice tended to be reduced when compared with that in the wild-type mice (no irradiation) (*Figure 5A* and *B*). Moreover, neointimal formation in $BMT^{Tnf^{-/-}Ifng^{-/-}\to Cont}$ was significantly decreased when compared with that in $BMT^{Cont\ \to Cont}$ (I/M ratio, $P < 0.01$) and $BMT^{Cont\ \to Tnf^{-/-}Ifng^{-/-}}$ ($P < 0.05$). These results indicate that TNF-α and IFN-γ in bone marrow cells are critical for neointimal formation following vascular injury.

4. Discussion

The major findings of this study are as follows: (i) neointimal formation following vascular injury was significantly reduced in the $Tnf^{-/-}Ifng^{-/-}$ mice compared to that in the control, $Tnf^{-/-}$, and $Ifng^{-/-}$ mice. (ii) Immunohistochemical analysis revealed the expression of TNF-α and IFN-γ in the neointimal lesions in control mice, but not in the lesions in mice with deficiency of the corresponding cytokine. (iii) No significant difference in re-endothelialization was observed among these groups. (iv) The number of proliferating cells, as determined by PCNA staining, in the neointimal lesion was significantly decreased in $Tnf^{-/-}Ifng^{-/-}$ mice. (v) Deficiency of TNF-α and IFN-γ specifically in bone marrow cells significantly inhibited neointimal formation after vascular injury. These findings indicate the synergistic role of bone marrow cell-derived TNF-α and IFN-γ in neointimal formation after vascular injury and provide new insights into the mechanism underlying post-PCI restenosis and atherosclerosis.

Increasing evidence indicates the importance of inflammatory responses in the pathogenesis of restenosis and atherosclerosis. Although TNF-α and IFN-γ are inflammatory cytokines and have been shown to be involved in this pathogenetic process, the precise role of these cytokines is controversial. Furthermore, the synergistic induction of atherogenic genes by TNF-α and IFN-γ has been demonstrated;[10] Indeed, TNF-α and IFN-γ have been shown to synergistically induce many atherogenic genes, such as those encoding adhesion molecules [intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)], inflammatory cytokines/chemokines [interleukin (IL)-6, IL-8, regulated on activation, normal T expressed and secreted (RANTES), monokine-induced by IFN-γ (Mig), and IFN-γ-inducible protein (IP-10)], nicotinamide adenine dinucleotide phosphate oxidases (NADPH oxidases), and inducible nitric oxide synthase (iNOS).[10] The molecular mechanism of the synergistic effect appears to involve the interaction of transcription factors, such as TNF-α-activated nuclear factor-κB (NF-κB), with IFN-γ-activated signal transducer and activator of transcription 1 (Stat-1) or interferon regulatory factor-1 (IRF-1). Our finding that double deficiency of TNF-α and IFN-γ synergistically inhibited neointimal formation after injury may also support these observations; however, further investigations are required to elucidate its precise mechanism.

We clearly demonstrated that bone marrow cell-derived TNF-α and IFN-γ are critical for neointimal formation after vascular injury. Previously, several investigators showed that the expression of TNF-α and IFN-γ is upregulated in the injured arteries at the early phase of vascular injury.[5,6,9] Furthermore, recent investigations have also demonstrated the contribution of bone marrow cells to neointimal formation after injury;[11,16] however, the precise role of bone marrow cells has not yet been fully understood. Our data suggest the critical role of bone marrow cells as a cellular source of TNF-α and IFN-γ. On the basis of our findings, we postulated the mechanism responsible for neointimal formation after injury as follows: (i) vascular injury directs bone marrow-derived cells such as monocytes and lymphocytes to the injury site in the artery; (ii) the accumulated bone marrow-derived cells secrete inflammatory cytokines such as TNF-α and IFN-γ; and (iii) secreted TNF-α and IFN-γ cytokines initiate the migration and proliferation of VSMCs, thereby resulting in neointimal formation. Thus, bone marrow-derived TNF-α and IFN-γ could be therapeutic targets for the prevention of restenosis and atherosclerosis.

Several limitations of this study should be noted. First, gene disruption in mice might compensate for the loss of signalling pathways by altering the expression of other proteins although no information about such compensation in $Tnf^{-/-}$ and $Ifng^{-/-}$ mice is available. Second, the model used in this study is not a reliable experimental model of human PCI because the injury was produced on a normal non-atheromatous artery. Third, irradiation has been reported to cause many deleterious effects on recipient animals, such as inhibiting cellular proliferation and inducing apoptotic cells death.[17] In fact, Tanaka *et al.*[18] recently demonstrated that the neointimal formation following vascular injury in irradiated bone marrow-transplanted mice was less than that in non-irradiated mice and suggested that bone marrow-transplanted mice may not always represent a physiological process that occurs naturally in response to injury in non-irradiated mice. Consistent with their findings, we also observed reduced neointimal formation after injury in $BMT^{Cont\ \to Cont}$ mice, suggesting that other models (e.g. parabiosis model[18]) might be useful for exploring the precise role of bone marrow-derived cells.

In conclusion, we showed that double deficiency of TNF-α and IFN-γ synergistically inhibited neointimal formation after vascular injury. Particularly, the absence of these cytokines in bone marrow cells plays a critical role in the inhibition of the progression of neointimal formation. Our results indicate the importance of the synergistic effect of TNF-α and IFN-γ in bone marrow cells and that these cytokines are novel therapeutic targets for restenosis and atherosclerosis.

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IFN-γ deficiency worsen *Pneumocystis* pneumonia with Th17 development in nude mice

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ABSTRACT

Pneumocystis pneumonia (PCP) occurs frequently in patients with immunodeficiency syndromes, especially AIDS. In order to investigate the role of IFN-γ on PCP, nude mice deficient in IFN-γ (GKO nude) and their wild-type ones (WT nude) were infected with murine *Pneumocystis*. Nine weeks later they were sacrificed, and cytokines in BALF and lung histopathology were compared between them. Cyst burden was greater in GKO than in WT nude mice. Histopathology in the lung was severer and granulomatous lesions were observed more frequently in GKO nude mice. Levels of IL-17 were higher in BALF of GKO than in that of WT nude mice. Greater number of $CD4^+$ T cells from lungs of infected GKO nude mice produced IL-17 than those from WT ones. These results suggest that deficiency in IFN-γ induces the differentiation of Th17 and that IL-17 is responsible for inflammatory response in PCP.

1. Introduction

Pneumocystis pneumonia (PCP) is one of the most important opportunistic mycoses in immunocompromised individuals, especially AIDS patients. The major host defense system against *Pneumocystis* is the adaptive immunity, in which $CD4^+$ T cells are the most important [1]. The risk of PCP in AIDS patients was greatly increased in those with $CD4^+$ cell counts at base line of 200 per microlitter or less [2]. In addition, many studies have clearly demonstrated that $CD4^+$ T cell-deprived animals are susceptible to *Pneumocystis* [1,3]. Animals immunosuppressed by administration with corticosteroid were also susceptible to *Pneumocystis* [4]. $CD4^+$ T cells do not exist or affected by corticosteroid in these animal models, therefore, it is difficult to assess the role of $CD4^+$ T cells in PCP.

Transfer of $CD4^+$ T cells from immunized mice to *Pneumocystis*-infected SCID or $RAG^{-/-}$ mice resulted in severe pulmonary inflammation with mononuclear cells [5,6]. Transfer of $CD25^-CD4^+$ T cells without $CD25^+CD4^+$ regulatory T (Treg) cells led to lethal pneumonia. However, $CD25^+CD4^+$ population prevented the development of disease induced by $CD25^-CD4^+$ cells [6]. In addition, immune reconstitution inflammatory syndrome (IRIS) in response to a number of microorganisms including *Pneumocystis jirovecii* has been described in patients immunosuppressed by HIV infection and by other mechanisms, including chemotherapy [7–9]. In addition, corticosteroid adjunctive therapy could prevent death in immunocompromised patients with severe PCP [10]. Together, it is important to evaluate $CD4^+$ T cells in the course of PCP.

Congenitally athymic nude mice are known as "T cell-deficient" animals, and susceptible to *Pneumocystis*. However, $CD4^+$ and $CD8^+$ T cells age-dependently develop in nude mice [11] and significant production of cytokines and immune responses are observed in them [12]. In this paper, we newly produced IFN-γ deficient nude mice, and tried to evaluate the effects of IFN-γ on lung histopathology of PCP.

2. Materials and methods

2.1. Mice

BALB/cA and BALB/cA-nu/nu mice were purchased from Clea Japan (Tokyo Japan). The generation of BALB/cA-background IFN-γ-deficient (GKO) mice and PCR typing of *ifng* gene were done as described previously [13,14]. Female offspring of GKO females and a male nude mouse were backcrossed to nude mice to obtain IFN-$\gamma^{+/-}$ male nude and IFN-$\gamma^{+/-}$ female nu/+ mice. GKO nude mice were first obtained by mating of IFN-$\gamma^{+/-}$ male nude and IFN-$\gamma^{+/-}$ female nu/+ mice. Nude offspring of GKO male nude and GKO female nu/+ mice were used as GKO nude mice in the experiments. They were supplied with autoclaved bedding, sterile food and water

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containing tetracycline and nystatin (Wako, Osaka, Japan) during the experiment. The Animal Ethics Committee of Shinshu University approved all protocols used in this study.

2.2. Infection with Pneumocystis

Muine *Pneumocystis* was maintained in BALB/c-scid mice (Clea Japan) as described previously [3]. Female wild-type (WT) nude and GKO nude mice were infected with muine *Pneumocystis* by co-housing with infected scid mice. Nine weeks later, they were sacrificed and their lungs were homogenized under aseptic condition. Aliquots of the homogenates were diluted, cytocentrifuged onto glass slides, fixed in methanol, and stained with Fungi-Fluor™ kit (Polysciences, Warrington, PA). The number of cysts in 30 fields was counted by a single investigator under a fluorescent microscope (ECLIPSE E800, Nikon, Tokyo, Japan) as described previously [3]. The lower limit for the detection of cysts was 1.0×10^3/mouse.

2.3. Lung histopathology

Mice were sacrificed 9 weeks after infection by exsanguination following anesthesia with 3.6% chloral hydrate. Lungs were removed and fixed with neutral buffered formalin solution. Paraffin-embedded tissue blocks were then sectioned to 3 μm thickness and stained with hematoxylin and eosin.

2.4. Recovery of bronchoalveolar lavage fuluid (BALF)

Lung airways were lavaged three times with 0.5 ml PBS at 30–37 °C through an intratracheal cannula. BALF were centrifuged at $150 \times g$ for 5 min and the supernatants of the first lavage were stored at −30 °C for determination of cytokines. Cells in BALF were collected by centrifugation and used for intracellular staining.

2.5. In vitro culture of lung cells

Lungs from the infected mice were minced and passed through stainless steel mesh as described previously [15]. After centrifugation collected lung cells were incubated in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% FBS (BioWest, Nuaillé, France) and 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Grand Island, NY), and anti-CD3 mAb at 37 °C for 48 h in an atmosphere of 5% CO_2 and 95% air.

2.6. Determination of cytokines by flow cytometric analysis

Concentrations of cytokines in BALF and culture supernatants were determined using FlowCytomix (Bender Medsystems, Burlingame, CA) and CBA Flex set (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. They were analyzed by FACSCalibur (BD Biosciences).

2.7. Intracellular staining

BALF cells were incubated with PMA and ionomycin in the presence of BD GoldiStop for 5 h. Cells were harvested, and stained with PE-Cy5-labeled anti-CD4 and FITC-labeled anti-CD8 mAbs (BD Biosciences), followed by fixation and permialization with BD Cytofix/Cytoperm. After washing with BD Perm/Wash buffer, cells were stained with PE-labeled anti-IL17 mAb (BD Biosciences). Double-stained cells were analyzed by flow cytometric analysis.

2.8. Statistics

Data were evaluated with the Student's *t*-test for two independent groups. $P < 0.05$ was accepted as indicating significance.

3. Results

3.1. The changes in the body weight and the number of Pneumocystis

WT nude and GKO nude mice were co-housed with *Pneumocystis*-infected SCID mice and weighed every week for evaluating symptoms. Both mice started to lose body weight 7 weeks after infection. WT nude displayed quicker loss of body weight than GKO nude mice. The reduction in body weight of WT nude mice was significantly larger than that in GKO nude mice 8 weeks after infection (Fig. 1A). In contrast, the number of cysts in the lung was significantly smaller in WT nude than in GKO nude mice 9 weeks after infection (Fig. 1B).

3.2. Histopathology in the lung

WT nude mice exhibited pulmonary injury accompanying the infiltration of macrophages, neutrophils and eosinophils into alveoli, hemorrhage and clusters of lymphocytes around the bronchioles (Fig. 2A and B). In GKO nude mice, more hypersensitive pneumonia was observed (Fig. 2C and D). Granulomatous lesions were seen more frequently in GKO nude mice (Fig. 2C). Large numbers of multinucleated giant cells were also observed in them.

3.3. Increased IL-17 production by $CD4^+$ T cells in the lung

In order to determine which cytokines are owing to severe inflammatory responses in the lungs of GKO nude mice, levels of cytokines in the BALF were compared between GKO nude and WT nude mice 9 weeks after infection. TNF-α, IL-6 and IL-10 were in BALF of WT nude but scarcely in that of GKO nude mice. In contrast, IL-17 levels in BALF were significantly higher in GKO nude than in WT nude mice. IFN-γ could be detected only in BALF of WT nude mice (Fig. 3A). IL-4 was not detected in both mice (data not shown).

Only a trace of IL-17 and a significant amount of IFN-γ was produced by lung cells in WT nude mice stimulated with anti-CD3 mAb. In contrast, a significant amount of IL-17 was produced by anti-CD3 mAb-stimulated lung cells of GKO nude mice (Fig. 3B and C). To determine which subset of T cells produce IL-17, intracellular staining of BALF cells was carried out. Greater number of BALF $CD4^+$ T cells from infected GKO nude mice produced IL-17 than those from WT nude mice (Fig. 4). $CD8^+$ T cells from both mice did not show IL-17 production (data not shown).

4. Discussion

In this paper we demonstrated that the number of cysts was greater and histopathology in the lung was severer in GKO nude than in WT nude mice. This suggests that IFN-γ seems to have advantage to *Pneumocystis* infection. Our finding is similar to the results reported previously [16–18]. It has been shown that recombinant IFN-γ enhanced the efficacy of trimethoprim-sulfamethoxazole to resolve PCP in cortisone-treated rats [16]. In addition, exposure to aerosolized IFN-γ significantly lowered *Pneumocystis* burden in $CD4^+$ T cell-depleted mice [17]. After primed with IFN-γ in vitro, alveolar macrophages (AM) enhanced their production of nitrogen oxides which are toxic to *Pneumocystis* [18]. Our result was also consistent with reported one that prolonged and exacerbated inflammatory response in lungs was demonstrated in

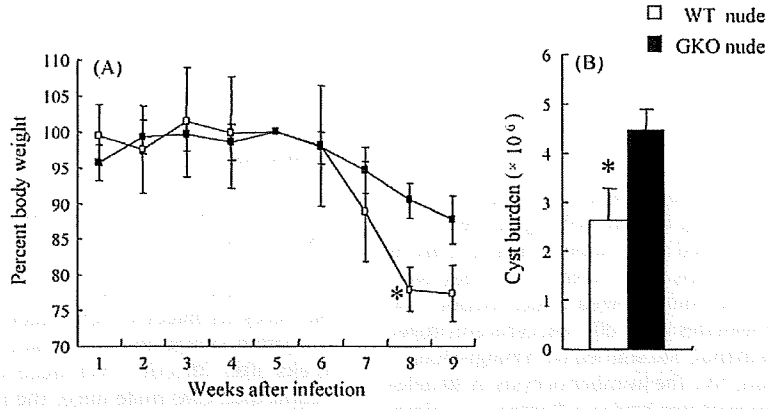

Fig. 1. The changes in the body weight and the number of *Pneumocystis*. GKO nude (■) and WT nude (□) mice were co-housed with *Pneumocystis*-infected scid mice. (A) Mice were weighed every week. Data represented the means ± SD of percent body weights to peak body weight at week 5 after infection ($n = 5$). (B) Lungs of GKO and WT nude mice were homogenized 9 weeks after infection. Aliquots of the homogenates were diluted, cytocentrifuged onto glass slides, fixed in methanol, and stained with Fungi-Fluor kit. Cysts were counted under the fluorescent microscope. Data represent means ± SD ($n = 5$). *Significantly smaller in WT nude mice ($P < 0.05$).

SCID mice inoculated with *Pneumocystis* and reconstituted with splenocytes from GKO mice compared to those reconstituted with splenocytes from WT mice [19]. These results suggest that IFN-γ plays an important role in regulating the inflammatory response to the *Pneumocystis*.

Granulomatous lesions were seen more frequently in GKO nude mice (Fig. 2C), with a large number of multinucleated giant cells. This resembles to the observation reported using a murine model of chlamydial infection, where higher IL-10 production is correlated with lower IFN-γ production, weaker delayed hypersensitivity (DTH), and slower organism clearance followed by granuloma formation at the later stages of infection [20]. Inability of fungicidal activity of alveolar macrophages in GKO nude mice seems to lead the granuloma formation in our experiments. Cases of granulomatous PCP were reported, although not frequently, in HIV and non-HIV patients [21,22]. It is suggested that the pathogenesis of the granulomatous response to *P. jirovecii* may more likely be related to host factors [22], although the precise mechanism is still uncertain. Our experimental model would be a good tool for further investigating the immunological mechanism of granulama formation in PCP.

Our results indicated that greater number of Th17 cells infiltrated and produced higher levels of IL-17 in the lungs of *Pneumocystis*-infected GKO nude than in WT nude mice. Th1 immune response took the place of Th17 response in WT nude mice. IL-17 was known as a proinflammatory cytokine that mediates multiple chronic inflammatory responses including angiogenesis, recruitment of inflammatory cells, and induction of proinflammatory mediators by endothelial and epithelial tissues [23]. IFN-γ was considered to suppress Th17 differentiation in our experimental model, as was also reported previously [24]. Upregulated production of IL-17 must be the factors of the severer PCP in GKO nude

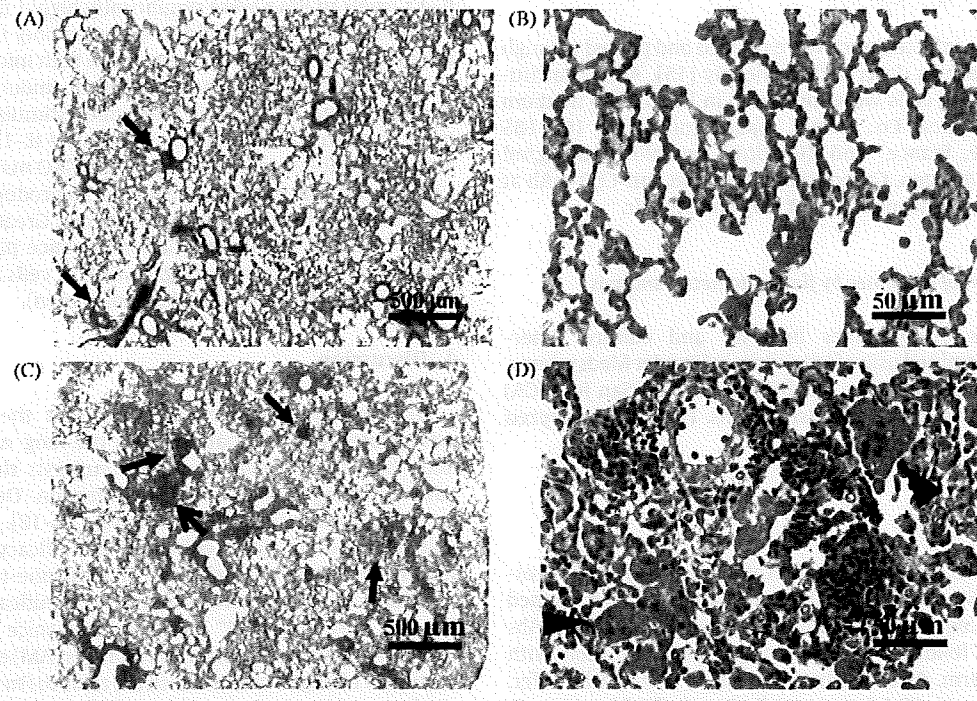

Fig. 2. Sections of lungs from WT nude (A and B) and GKO nude (C and D) mice were stained with hematoxylin and eosin 9 weeks after infection. Arrows indicate granulomatous lesion (A and C). Arrowheads indicate multinucleated giant cells (D).

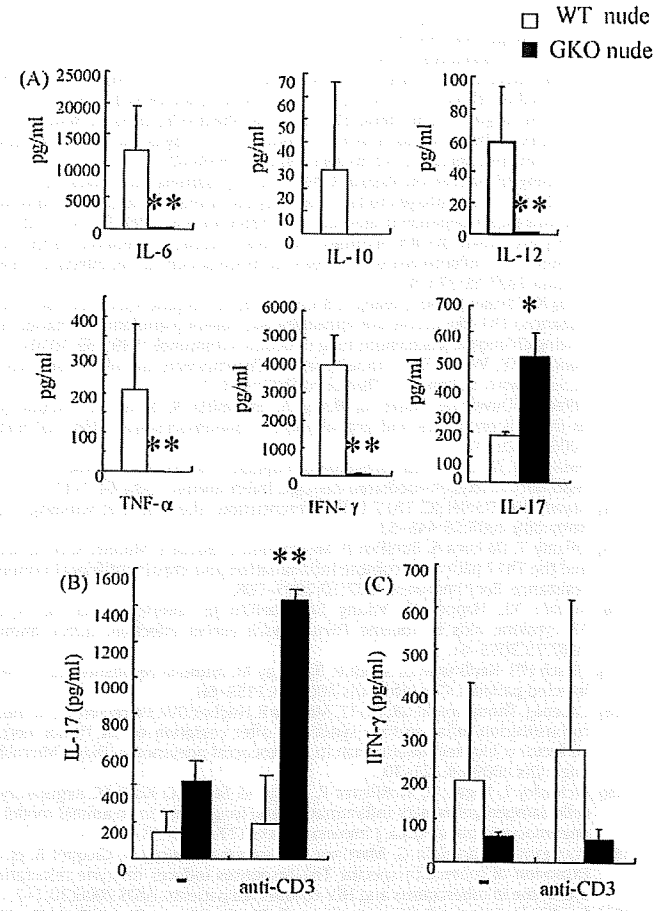

Fig. 3. Levels of cytokines in BALF of GKO nude and WT nude mice 9 weeks after infection. (A) Lung airways were lavaged with 0.5 ml PBS through an intratracheal cannula and concentrations of cytokines in the first lavage were determined. Data represent means ± SD ($n = 5$). IL-17 (B) and IFN-γ (C) production by lung T cells from GKO and WT nude mice. Lung cells from infected GKO and WT nude mice were incubated with anti-CD3 mAb for 48 h. Concentrations of IL-17 and IFN-γ in the supernatant were determined. Data represent means ± SD ($n = 5$). *Significantly different from WT nude mice ($P < 0.05$). **Significantly different from WT nude mice ($P < 0.01$).

mice, although the loss of body weight was greater in WT nude than in GKO nude mice. This may be because TNF-α production was also reduced in GKO nude mice. This should be addressed in the future study.

The roles of IL-17 in fungal infection remain controversial. It has recently been reported that IL-23/IL-17 developmental pathway negatively regulates Th1 responses to *Aspergillus fumigatus* and *Candida albicans* and permits more extensive growth of fungi in vivo. Moreover, IL-17 inhibits antifungal activity in vitro [25]. In contrast, lower levels of IL-17 in the lung caused by IL-23 deficiency or neutralization with anti-IL-17 mAb resulted in transient decrease (3 weeks after infection) in clearance of *Pneumocystis* organisms [26]. $CD4^+$ T cells were not depleted and *Pneumocystis* were cleared afterwards in their experiments, suggesting that IL-17 might have fungicidal activity but is not necessary for clearance of *Pneumocystis* organisms in immunocompetent hosts. In our study, the number of Th17 cells and production of IL-17 increased in the lungs of GKO nude mice, although *Pneumocystis* burden in the lung was not decreased compared with WT nude ones. This suggested that Th17 cells seem not to be effective to protect *Pneumocystis* infection in immunocompromised hosts.

IRIS has been well described in AIDS patients receiving antiretroviral therapy. This paradoxical worsening of clinical symptoms after immunological recovery has been associated with an excessive inflammatory response to either intercurrent or previously unrecognised (subclinical) opportunistic infections [27]. There are reports of similar paradoxical symptoms occurring in patients without HIV infection following chemotherapy for *Mycobacterium* species infections [9] or cessation of a TNF-α blocker [28]. Because few longitudinal sample sets are available to characterize the immune response, the immunopathology of IRIS is poorly understood.

$CD25^+CD4^+$ Treg cells has been reported to control pulmonary inflammation and lung injury in IRIS with *Pneumocystis* infection [6,29]. Transfer of $CD25^-CD4^+$ T cells without $CD25^+CD4^+$ Treg cells to *Pneumocystis*-infected SCID resulted in hyperinflammatory response that lead to lung injury and death with strong IFN-γ in the lung [29]. Increased levels of IFN-γ and chemokines induced by IFN-γ such as IP-10 in IRIS patients were also reported [30,31]. These data suggested that there might be a deficit in the ability to control or turn off antigen-specific immune responses following antigen clearance. Absence of Treg cells results in severe deregulation of the immune system, leading to lymphoproliferation and autoimmune disease [32]. Therefore, it is considered that Treg cells might be deficient in numbers or in function in IRIS patients. Instead Treg cells increased at the peak of IRIS symptoms [33]. In addition, a significant expansion of $CD127^{lo}Foxp3^+CD25^+$ Treg cells was observed in IRIS patients compared with healthy controls and also compared with late-stage HIV-infected patients who commenced combination antiretroviral therapy without developing an IRIS [31]. It is important to clarify the role of Treg cells in IRIS patients.

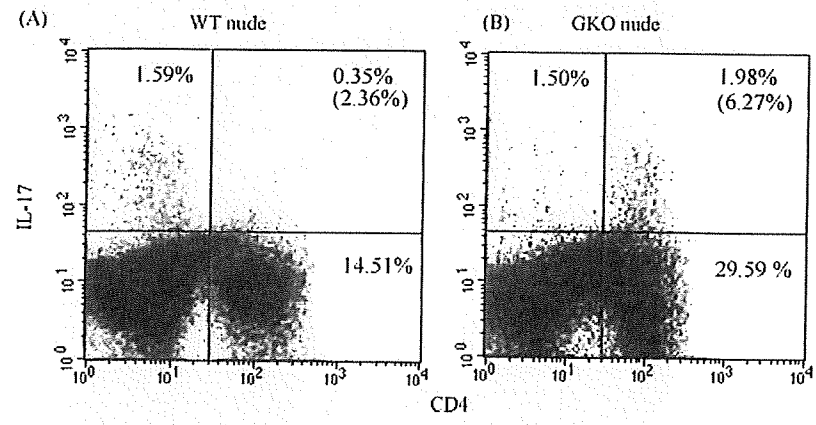

Fig. 4. Intracellular staining of $CD4^+$ T cells from BALF of GKO and WT nude mice. Percentages in the parentheses indicate the percentage of IL-17 producing $CD4^+$ cells in $CD4^+$ T cells.

Our results clearly demonstrated using a nude mouse model that deficiency in IFN-γ induces the differentiation of Th17 and that IL-17 is responsible for inflammatory response in PCP. Introducing modified gene into nude animals would be a good tool for evaluating inflammatory reactions against *Pneumocystis* and other opportunistic infections.

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特集 子どもと動物―上手にふれあうためは―

Ⅳ．動物由来感染症各論

1．小児のイヌ・ネコ回虫症

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KEY WORDS　動物由来回虫症，好酸球増多症，イヌ・ネコ回虫症，幼虫移行症

はじめに

動物由来の寄生虫がヒトに侵入して起きる感染症のなかで，イヌやネコの腸管内に寄生する回虫の幼虫を原因とするものをイヌ・ネコ回虫症と呼んでいる（どちらもトキソカラ属の回虫なのでトキソカラ症と呼ばれることもある）。ヒトの体内で分裂増殖することはなく，１個の虫卵（幼虫包蔵卵）が経口摂取されると１隻の幼虫が体内で孵化して消化管粘膜から侵入し，肝臓や網膜，あるいは中枢神経系へと移行し，様々な症状を引き起こす。

臨床的にトキソカラ症は内臓型，眼型，中枢神経型，潜在型の４型に分けられる（表１）。以下に典型的な内臓型小児トキソカラ症を紹介する[1]。

Ⅰ．症例

患児：１歳５カ月　女児

主訴：発熱，肝腫大，好酸球増多

現病歴：１カ月前から発熱と咳嗽を認め，近医にて上気道炎と診断され，鎮咳剤と抗生剤を処方されていた。通院中に肝腫大と好酸球増多を指摘され総合病院小児科に紹介入院となった。

入院時現症：体温39.2℃，意識清明で黄疸，全身のリンパ節腫脹や皮膚発疹は認めず，呼吸音，心音正常。肝臓は右季肋下に6 cm触知し，弾性軟。脾臓は触知しなかった。胸部X線，心電図所見ともに正常で，神経学的異常も認めなかった。

入院時検査所見：白血球数61,200/mm³のうち好酸球が80％を占めていた。貧血なく血小板数は正常。血液生化学検査では軽度の肝機能障害を認め，CRP は6.0mg/dℓ，赤沈65 mm/hr と亢進していた。γグロブリン値は正常。血液細菌培養は陰性であった。腹部超音波検査では低エコーレベルの多発性占拠病変を認め，腹部 CT では肝内に低吸収域が多数存在していた。糞便検査では虫卵陰性。患児の家庭ではペットを飼育していないが，公園での砂遊びの最中にしばしば砂を口に入れる行動があったという。

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