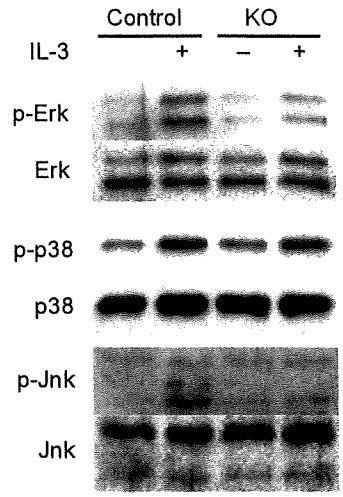
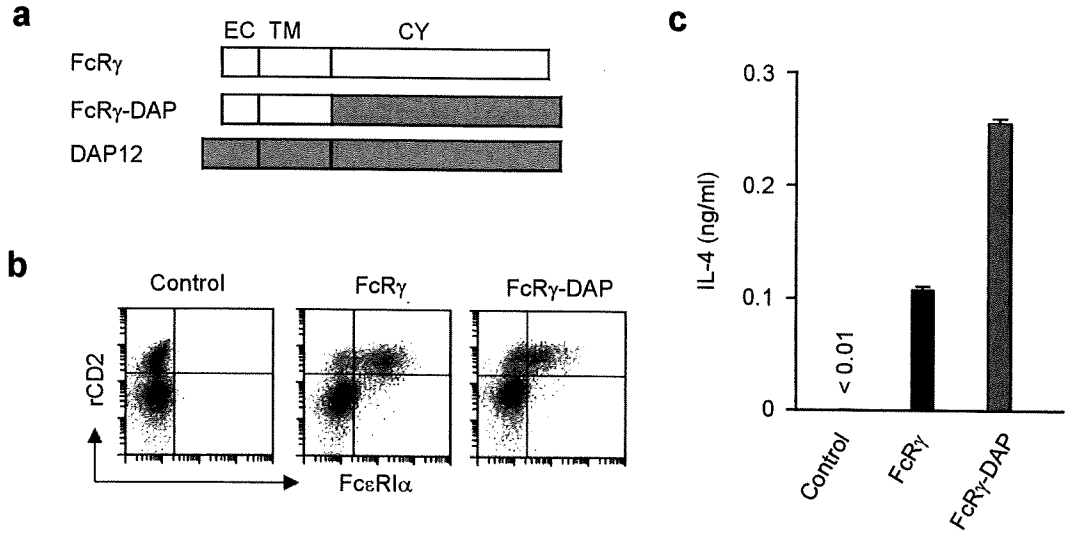
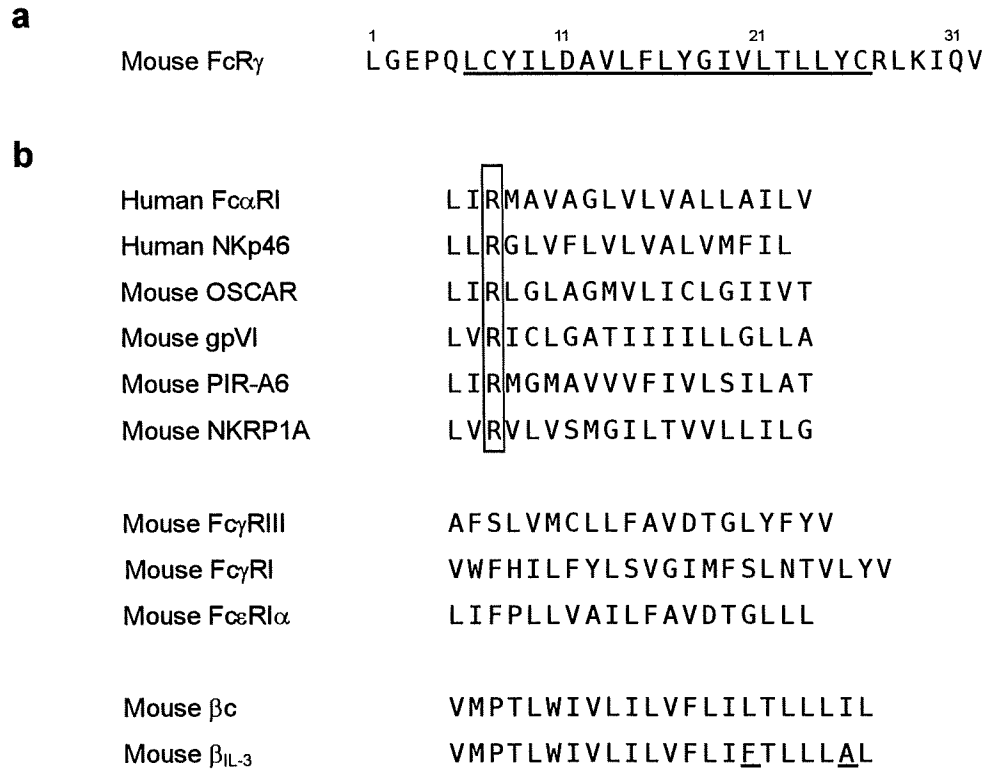


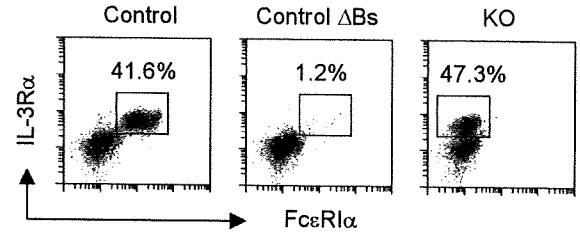
Supplementary figure 8. MAPK activation induced by IL-3 in the absence of FcRγ. Whole cell lysates were prepared from wild-type and FcRγ-deficient (KO) basophil-enriched BM cells (38.1% and 32.0% pure, respectively) stimulated for 10 min as in **Fig.3b,** and subjected to immunoblot analysis with antibodies against the native and phosphorylated forms of the MAPK p38, Erk and Jnk. Representative of two independent experiments.



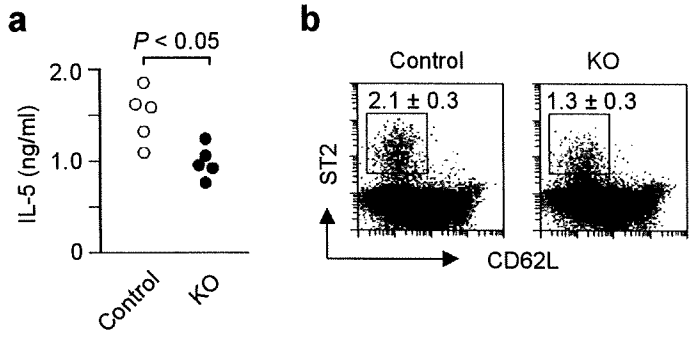
Supplementary figure 9. The cytoplasmic portion of DAP12 could replace that of FcRγ. (**a**) Schematic representation of the structure of a chimeric molecule comprising of the extracellular (EC) and transmembrane (TM) portions of FcRγ fused with the cytoplasmic (CY) portion of DAP12 (FcRγ-DAP). (**b**) Surface FcεRIα expression on FcRγ-deficient BM-derived basophils transduced with the vectors for wild-type FcRγ or FcRγ-DAP. Shown are the data for c-kit$^-$ cells. (**c**) IL-3-induced IL-4 production by 'starved' FcRγ-deficient BM-derived basophils expressing none (Control), wild-type FcRγ or FcRγ-DAP. In the absence of IL-3 stimulation, IL-4 production was undetectable (< 0.01 ng/ml). Data represent the means and s.d. of duplicate cultures. Note that FcRγ-DAP was more potent in transducing IL-3 signals for IL-4 production (**c**), despite its apparent inefficiency in transporting FcεRI to the surface (**b**), compared with wild-type FcRγ, potentially suggesting that FcRγ and DAP12 cytoplasmic portions might not be functionally identical. Repeated twice with similar results.



Supplementary figure 10. Primary structure of FcRγ and various FcRγ-associated receptors. **(a)** Amino acid sequence of the extracellular and potential TM portions of FcRγ is shown in the single letter format. TM sequence is underlined, and amino acid positions in the mature protein are given. **(b)** Amino acid sequences of the potential TM portions of various FcRγ-associated receptors. The canonical arginine residues are boxed, and the two amino acids in β_{IL-3} different from those in βc underlined. These sequences were taken from the UniPlot database (http://www.pir.uniprot.org/).



Supplementary figure 11. BM cell preparations used for supplementing the cultures of FcRγ-deficient OT-II TCR transgenic spleen cells. Basophil-enriched BM cells were prepared from control or *Fcer1g*$^{-/-}$ (KO) mice as in **Fig.2a**. Basophils (DX5^{+}) were additionally depleted from the control cell preparation (Control ΔBs). In 'Control' and 'Control ΔBs' basophil preparations, CD11c^{high} cells (DCs) were less than 0.5% and 1%, respectively (not shown). Shown are the surface staining profiles for IL-3Rα and FcεRIα. The numbers are for the percentages of cells within the gates that represent basophils. Repeated at least three times with similar results.



Supplementary figure 12. Observations suggesting impaired T_H2 development *in vivo* in *Fcer1g*$^{-/-}$ mice. Serum IL-5 levels (**a**) and the expression of ST2 and CD62L on CD4$^+$ T cells in mesenteric lymph nodes (**b**) in control or *Fcer1g*$^{-/-}$ (KO) mice at 10 day after infection with *T. spiralis*. In **a**, each symbol represents an individual animal (n = 5 for each genotype). Cumulative data of two independent infections. $P < 0.05$. Dot plots in **b** are representative of three animals of each genotype. Numbers shown are the means and s.d. of values in these animals that differ significantly between control and *Fcer1g*$^{-/-}$ mice ($P < 0.05$).

Supplementary Methods.

PCR primers for quantitative RT-PCR. PCR primers used for quantitative RT-PCR were as follows:
β-actin (Applied Biosystem), sense: 5 -GCTTCTTTGCAGCTCCTTCGT-3 , antisense: 5 -AGCGCAGCGATATCGTCAT-3 ,
IL-4 (Takara Bio), sense: 5 -TCTCGAATGTACCAGGAGCCATATC-3 , antisense: 5 -AGCACCTTGGAAGCCCTACAGA-3

Plasmid construction for FcRγ-DAP12 fusion protein. The extracellular and transmembrane portions of FcRγ, corresponding to amino acid position 1-26, and the cytoplasmic portion of DAP12, corresponding to amino acid position 47-93, were separately cloned by RT-PCR. These cDNAs were sequentially cloned into pMX-IRES-rCD2.

Derivation and 'starvation' of BM-derived basophils. Whole BM cells (2.5×10^7) were cultured in 10 ml of 10% fetal calf serum-containing RPMI1640 medium (Nissui, Japan) supplemented with recombinant murine IL-3 (5 ng/ml) for 13 days with medium changed every 3 days. Recombinant murine IL-3 was produced by a cell line transfected with murine IL-3 expression vector and partially purified. BM-derived basophils were enriched by depleting mast (c-kit$^+$) cells using IMag beads, washed extensively, seeded at 2×10^6/ml and 'starved' for 12 to 18 hours. 'Starved' BM-derived basophils were used for stimulation with IL-3.

Introduction of shRNA expression vectors. Wild-type BM cells cultured for only 4 days in IL-3 were infected with the retrovirus for pSINsi-mu6 vector for targeting Syk (Syk-shRNA) or control vector (see **METHODS**) for 2 days. Infected cells were then selected for successfully transduced cells by culturing with G418 (Roche Applied Science, 0.5 mg/ml) in the presence of IL-3 for 6 days, starved and stimulated with IL-3 or ionomycin for 24 hours for IL-4 production.

Examination of the effects of FcRγ deficiency in CD4$^+$ T cells, APCs and basophils on T_H2 generation *in vitro*. In the experiments depicted in **Fig. 7b**, 1×10^6 CD4$^+$ T cells purified from spleen by IMag beads (regularly >90% pure) prepared from OT-II TCR tg wild-type or *Fcer1g*$^{-/-}$ mice were stimulated with 0.5 μM chicken ovalbumin peptide in the presence of 2.5×10^5 splenic APCs purified by removing TCRβ$^+$, B220$^+$,

NKG2D$^+$ and TER119$^+$ cells from spleen cells from C57BL/6 mice. In other experiments (**Fig. 7c**), CD4$^+$ T cells purified similarly from wild-type OT-II TCR-tg mice were stimulated as above in the presence of 2.5 x 10^5 splenic APCs prepared from control or *Fcer1g*$^{-/-}$ mice. For supplementing exogenous basophils (**Fig. 7d**), BM basophils enriched from wild-type or *Fcer1g*$^{-/-}$ mice (see legends for **Fig. 7** and **Supplementary Fig. 11** for the purities) were added to the cultures of FcRγ-deficient OT-II TCR-tg spleen cells (1 x 10^6) prepared by removing B, NK and erythroid cells. Recombinant mouse IL-4 (25 ng/ml; R&D systems) was also included as a positive control.

Research

Exposure to Bisphenol A Prenatally or in Adulthood Promotes T_H2 Cytokine Production Associated with Reduction of $CD4^+CD25^+$ Regulatory T Cells

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BACKGROUND: Bisphenol A (BPA) is a widespread endocrine-disrupting chemical that can affect humans and animals.

OBJECTIVES: We investigated the effects of adult or prenatal exposure to BPA on T-helper (T_H)1/T_H2 immune responses and the mechanisms underlying these effects.

METHODS: To evaluate the effects of exposure to BPA in adulthood, male *Leishmania major*–susceptible BALB/c and –resistant C57BL/6 mice were subcutaneously injected with 0.625, 1.25, 2.5, and 5 μmol BPA 1 week before being infected with *L. major*. To evaluate prenatal exposure, female mice were given BPA-containing drinking water at concentrations of 1, 10, and 100 nM for 2 weeks, then mated, and given BPA for another week. Male 10-week-old offspring were infected with *L. major*. Footpad swelling was assessed as a measure of the course of infection.

RESULTS: Mice exposed to BPA prenatally or in adulthood showed a dose-dependent increase in footpad swelling after being infected with *L. major*. Exposure to BPA in adulthood significantly promoted antigen-stimulated production of interleukin (IL)-4, IL-10, and IL-13 but not interferon-γ (IFN-γ). However, mice prenatally exposed to BPA showed increased production of not only IL-4 but also IFN-γ. The percentages of $CD4^+CD25^+$ cells were decreased in mice exposed to BPA either prenatally or in adulthood. Effects of prenatal BPA exposure were far more pronounced than effects of exposure in adulthood.

CONCLUSION: BPA promotes the development of T_H2 cells in adulthood and both T_H1 and T_H2 cells in prenatal stages by reducing the number of regulatory T cells.

KEY WORDS: bisphenol A, cytokine, endocrine-disrupting chemicals, prenatal exposure, regulatory T-cells. *Environ Health Perspect* 116:514–519 (2008). doi:10.1289/ehp.10829 available via *http://dx.doi.org/* [Online 29 January 2008]

Bisphenol A (BPA), an estrogenic endocrine-disrupting chemical (EDC), has drawn attention because of its potential for human exposure. BPA is widely used, including in dentistry, food packaging, and lacquers to coat food cans and water pipes. It can enter the environment, resulting in chronic exposure of humans and wildlife. In fact, BPA has been detected not only in food and water (Heemken et al. 2001; Shin et al. 2001; Thomson and Grounds 2005) but also in human urine and blood samples as well as in the placenta and amniotic fluid of pregnant women (Ikezuki et al. 2002; Matsumoto et al. 2003; Schonfelder et al. 2002; vom Saal and Hughes 2005). BPA is one of the most widespread EDCs.

There is much evidence that exposure to BPA through contamination of the environment or the treatment of experimental animals disrupts developmental programs to alter sexual phenotypes and reproductive functions (Farabollini et al. 2002; Herath et al. 2004). BPA antagonizes the actions of thyroid hormone (Moriyama et al. 2002). Exposure of pregnant rats to BPA resulted in the chemical's transplacental transfer to the fetuses (Takahashi and Oishi 2000; Welshons et al. 2006), suggesting that developing embryos or fetuses might be affected by BPA. Prenatal exposure to BPA has been shown to alter a variety of reproductive endocrine parameters, such as testosterone and luteinizing hormone levels in rats (Ramos et al. 2003; Rubin et al. 2001) and the early onset of sexual maturation of female mice (Honma et al. 2002). In addition, behavioral changes have been reported in offspring of mice exposed to BPA during pregnancy and lactation (Dessi-Fulgheri et al. 2002). BPA may also be a potentially important modulator of immune responses. It inhibits adhesion capacity and promotes cytokine production in macrophages *in vitro* (Segura et al. 1999; Yamashita et al. 2005). Exposure to BPA also enhances the production of autoantibodies by B1 cells (Yurino et al. 2004). Furthermore, imbalanced T-helper (T_H)1/T_H2 immune responses have been demonstrated on exposure to BPA. BPA inhibits the secretion of interferon-γ (IFN-γ) in C57BL/6 and female NZB/NZW mice (Sawai et al. 2003). In contrast, BALB/c mice treated with BPA exhibit augmented T_H1 immune responses alone (Alizadeh et al. 2006), or both T_H1 and T_H2 responses (Yoshino et al. 2003). Our previous study indicated that BPA promotes T_H2 cytokine production *in vitro* and *in vivo* (Tian et al. 2003). However, the effects of prenatal exposure to BPA on immune responses have not been clarified.

In this study, we used mice infected cutaneously with *Leishmania major* to investigate the effect of BPA on T_H1/T_H2 immune responses in adulthood and prenatal stages. The model provides an excellent system with which to study the factors controlling the generation and regulation of T_H1 and T_H2 cells *in vivo*. Experimental infections of different strains of mice with *L. major* result in the development of either a predominant T_H1 response and resistance or a predominant T_H2 response and susceptibility. The early production of interleukin-12 (IL-12) and IFN-γ promotes a T_H1 response and healing, whereas IL-4 production is necessary for the development of a T_H2 response and of progressive disease. We also focused on $CD4^+CD25^+$ regulatory T cells (Treg cells), one of the $CD4^+$ T cell populations constitutively expressing the IL-2 receptor α-chain (CD25) playing a central and prominent role in the maintenance of the immunologic balance (Maloy and Powrie 2001; Shevach 2002) by inhibiting the proliferation of and the production of cytokines by $CD4^+$ and $CD8^+$ T cells (Dieckmann et al. 2005; Stassen et al. 2004). We evaluated whether $CD4^+CD25^+$ Treg cells were affected by exposure to BPA, resulting in the alteration of cytokine production by $CD4^+$ T cells.

Materials and Methods

Mice. Six- to 8-week-old *L. major*–susceptible BALB/c and *L. major*–resistant C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). Mice were housed in polymethylpentene (TPX) cages and fed sterile standard chow (FR-2; Funabashi Farm, Chiba, Japan). Drinking water was provided *ad libitum* in glass bottles. All animals were handled according to the guidelines of the Ethics Committee for Animal Experiments of Shinshu University. Animals were treated humanely and with regard for alleviation of suffering.

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The authors declare they have no competing financial interests.

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Monoclonal antibodies and reagents. BPA was purchased from Nacalai Tesque (Kyoto, Japan). Phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)–conjugated anti-CD25 monoclonal antibodies (mAbs) were obtained from BD, Biosciences (San Diego, CA, USA). The cytometric bead array (CBA) kits were also from BD Biosciences.

Leishmania major. L. major (MHOM/SU/73/5ASKH) was kept in a virulent state by continuous passage in BALB/c mice. A cell suspension of popliteal lymph node from an infected BALB/c mouse was cultured in Schneider's medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 20% heat-inactivated fetal calf serum (FCS; Biocell Laboratories, Carson, CA, USA). Stationary phase promastigotes were collected by centrifugation and washed with saline. Mice were infected in the right hind footpad with 5×10^6 promastigotes. The course of infection was monitored by making weekly measurements of footpad thickness with a metric caliper. The results were expressed as the difference between the thickness of the infected right footpad and that of the noninfected left one.

To prepare soluble *L. major* antigen, 1×10^9 promastigotes were homogenized by three cycles of freezing and thawing in phosphate-buffered saline. Aliquots were stored at –30°C before use.

BPA treatment. **Exposure of adult male mice to BPA.** BPA was dissolved in corn oil and injected subcutaneously into the right hind leg at doses of 0.625, 1.25, 2.5 and 5 µmol, which is equivalent to 5.7, 11.4, 22.8, and 45.6 mg/kg body weight (bw). These doses were based on our previous study in which 1 µmol BPA was shown to increase IL-4 and IL-10 production in *Trichinella spiralis*–infected mice (Tian et al. 2003). The control mice received corn oil vehicle alone. One week later, the mice were injected with *L. major* promastigotes in the footpad of the same leg.

Prenatal exposure to BPA. Female mice were given BPA in drinking water at doses of 1, 10, and 100 nM for 2 weeks. Each group of mice was then mated with a male and treated with BPA-containing drinking water for another week. Offspring born within 16–19 days after BPA treatment was complete were used in this experiment. The 100 nM (about 3 µg/kg bw/day) dose of BPA was based on recent studies showing that administration of low doses of BPA at 2 and 20 µg/kg bw/day to pregnant animals caused permanent changes in reproductive organs of offspring (Honma et al. 2002; Nagel et al. 1997). The mice in all groups drank approximately 3–4 mL water per day. The total dose received by each female mouse during the period of experiment was about 0.07, 0.7, or 7 nmol. Offspring of dams who received drinking water without BPA were used as controls. Male 10-week-old offspring were infected with *L. major.*

In vitro ***culture of splenocytes.*** A single-cell suspension containing 2×10^6 splenocytes from each mouse was incubated in 24-well tissue-culture plates (Greiner, Nurtingen, Germany) in 1 mL RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FCS (Biocell Laboratories), penicillin (100 IU/mL), and streptomycin (100 µg/mL) (Gibco BRL) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were stimulated with *L. major* antigen (3 µg/mL) during the cultivation. Culture supernatants were collected 48 hr later and stored frozen until used.

Cytokine analysis. Concentrations of IL-4, IL-10, IL-13, and IFN-γ in culture supernatants were determined using CBA kits according to the manufacturer's instructions.

Flow cytometric analysis. Single-cell suspensions containing 1×10^6 splenocytes were stained with PE-conjugated anti-CD4 mAb and FITC-conjugated anti-CD25 mAb. The cells were washed, then analyzed using fluorescence-activated cell sorting (FACS) with a FACSCalibur flow cytometer (BD Biosciences) with CellQuest software (BD Biosciences).

Statistical analysis. Results are presented as the mean ± SE. The statistical significance of the values was evaluated using Student's *t*-test. The significance was assessed at the $p < 0.05$ level of confidence.

Results

Effects of BPA on footpad swelling and cytokine production in **L. major–*infected adult male mice.*** Adult male mice injected with different doses of BPA were infected with promastigotes of *L. major* 1 week later. *L. major*–susceptible BALB/c mice developed a continuous increase in footpad thickness whether or not they were injected with BPA. The degree of swelling increased dose-dependently in mice treated with BPA. Mice exposed to 2.5 and 5 µmol of BPA developed significantly larger swelling than nonexposed control mice at weeks 6 and 8 after infection. Eight weeks after infection, footpad swelling was 1.49-fold greater in mice treated with 5 µmol of BPA than in controls (Figure 1A). However, infection with *L. major* among resistant C57BL/6 mice resulted in minimal swelling that began to resolve by 4 weeks after infection. There was no significant difference in footpad swelling among the groups (Figure 1B).

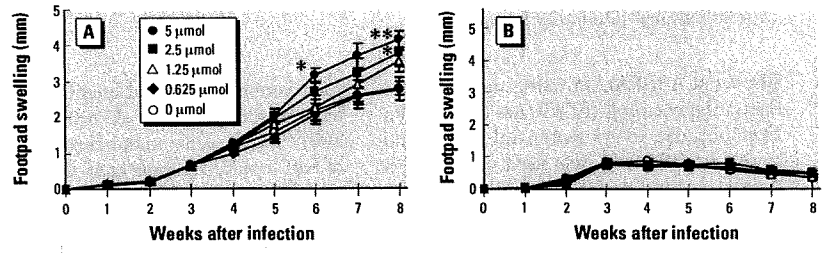

Figure 1. Effects of exposure to BPA in adult male BALB/c (*A*) and C57BL/6 (*B*) mice on footpad swelling after infection with *L. major.* Values represent mean ± SE ($n = 3$–4).
*$p < 0.05$ and **$p < 0.01$ compared with the nonexposed control group.

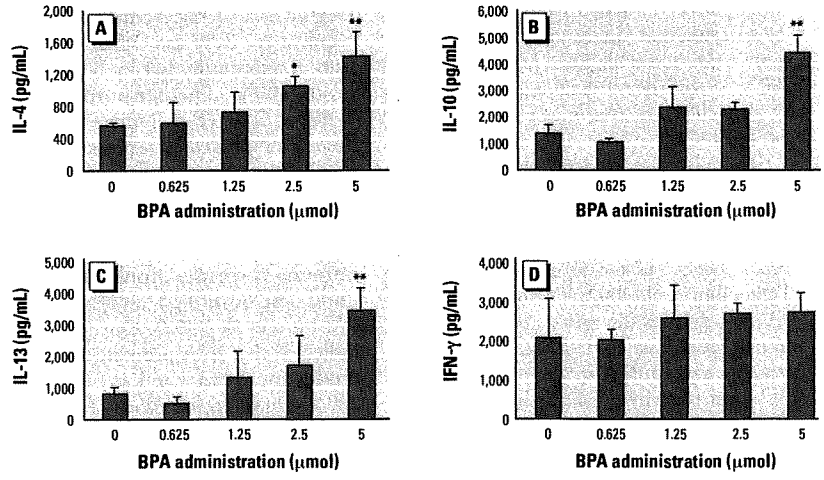

Figure 2. Effects of exposure to BPA in adult male BALB/c mice on IL-4 (*A*), IL-10 (*B*), IL-13 (*C*), and IFN-γ (*D*) cytokine production after infection with *L. major.* Values represent mean ± SE ($n = 3$–4).
*$p < 0.05$ and **$p < 0.01$ compared with the nonexposed control group.

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

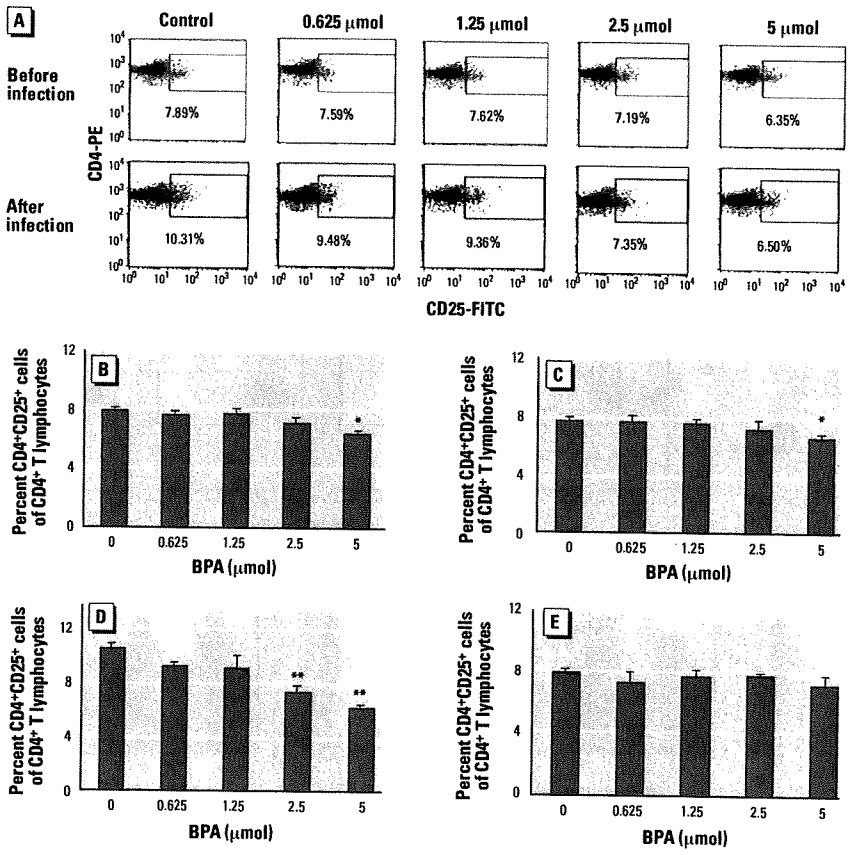

Figure 3. Effects of exposure to BPA on the percentage of $CD4^+CD25^+$ cells among $CD4^+$ T cells in adult male mice. (*A*) The representative FACS data of BALB/c mice before and after infection with *L. major*. (*B–E*) Splenocytes from BALB/c (*B, D*) and C57BL/6 (*C, E*) mice before (*B, C*) and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

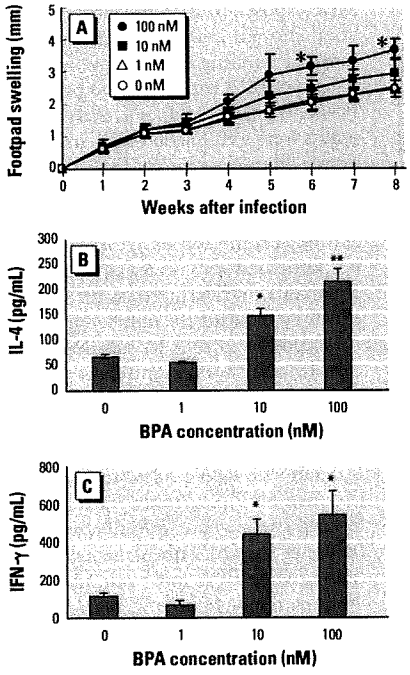

Figure 4. Effects of prenatal exposure to BPA on the course of *L. major* infection and T_H1- and T_H2-related cytokine production. (*A*) Footpad swelling after infection with *L. major*. (*B, C*) Splenocytes were obtained at 8 weeks after infection and cultured for 48 hr with *L. major* antigen. Concentrations 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.

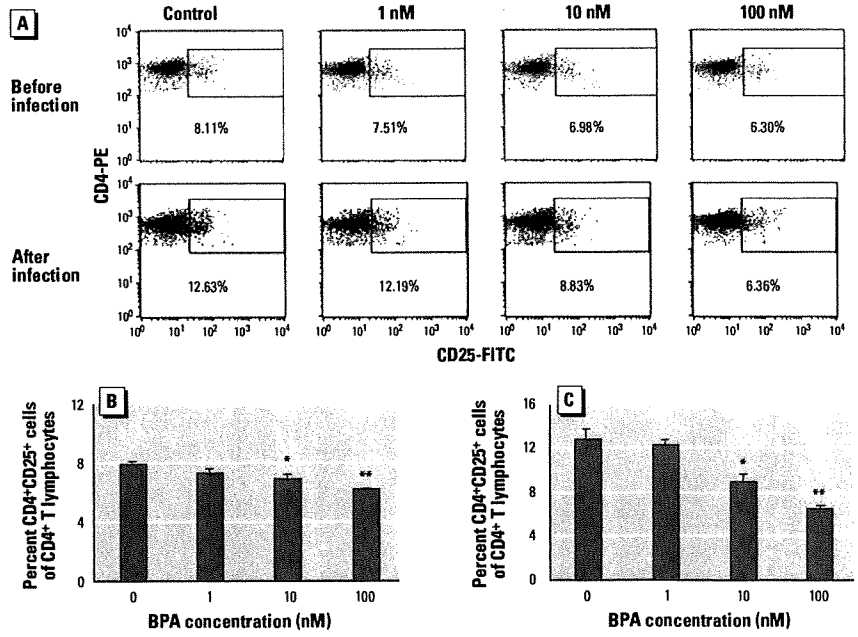

Figure 5. Effects of prenatal exposure to BPA on the percentage of $CD4^+CD25^+$ cells among $CD4^+$ T cells. (*A*) The representative FACS data of offspring on day 22 of birth (before infection) and after *L. major* infection. (*B, C*) Splenocytes from offspring of dams exposed to the indicated dose 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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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 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

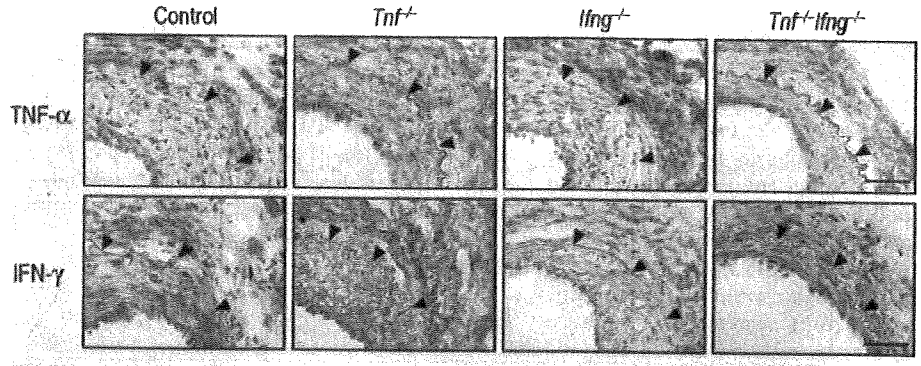

Figure 1 Expression of TNF-α and IFN-γ in neointimal lesion. Wire-mediated vascular injury was produced in the control, $Tnf^{-/-}$, $Ifng^{-/-}$, and $Tnf^{-/-}Ifng^{-/-}$ mice. The femoral arteries were excised at 28 days after injury. Immunohistochemical staining for TNF-α and IFN-γ was performed.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

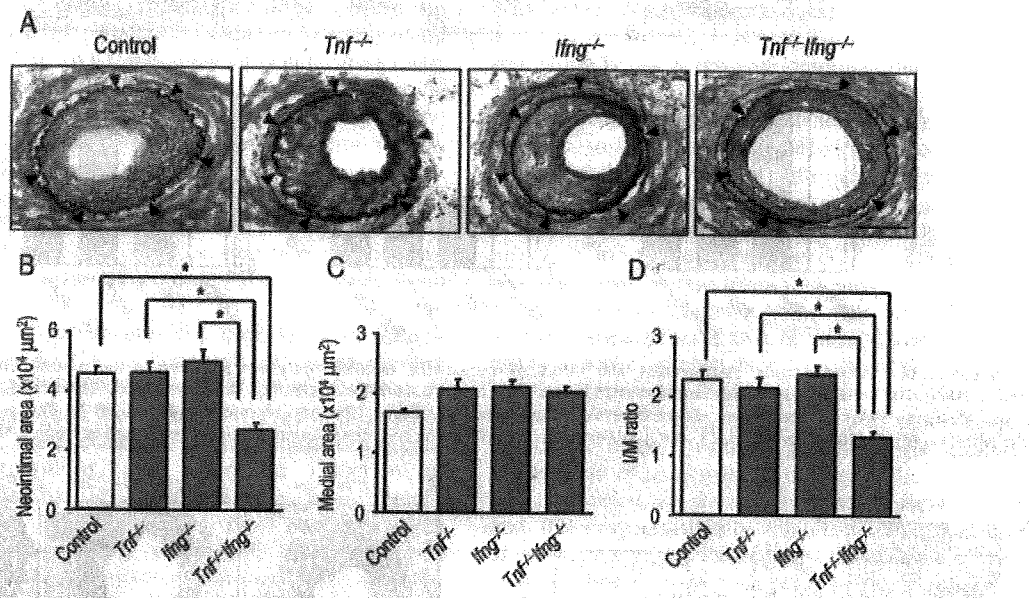

Figure 2 Effect of TNF-α and/or IFN-γ deficiency on neointimal formation. Wire-mediated vascular injury was produced in the control ($n = 10$), $Tnf^{-/-}$ ($n = 18$), $Ifng^{-/-}$ ($n = 18$), and $Tnf^{-/-}Ifng^{-/-}$ ($n = 14$) mice. The femoral arteries were excised at 28 days after injury. The sample 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*).

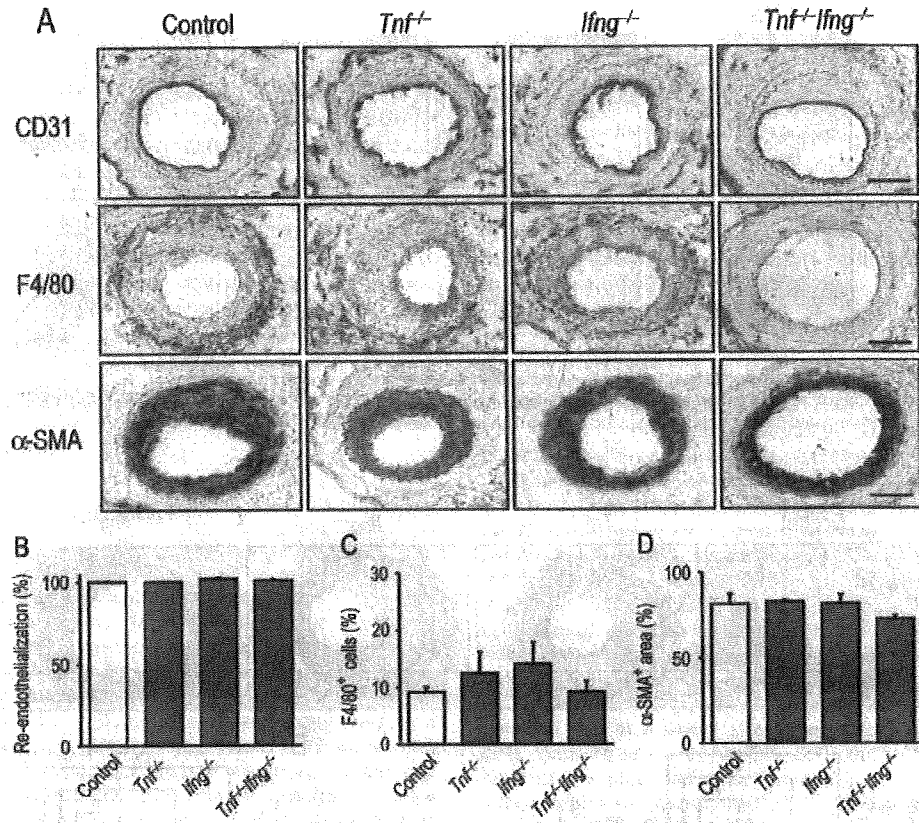

Figure 3 Detection of endothelial cells, macrophages, and VSMCs. Wire-mediated vascular injury was produced in the control, $Tnf^{-/-}$, $Ifng^{-/-}$, and $Tnf^{-/-}Ifng^{-/-}$ mice. The femoral arteries were excised at 28 days after injury. (*A*) Immunohistochemical staining for endothelial cells (CD31), 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).

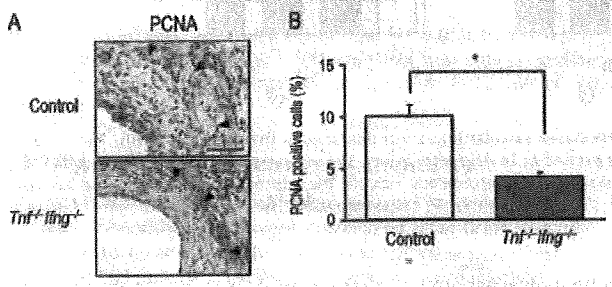

Figure 4 Proliferation activity in neointimal lesion. Wire-mediated vascular injury was produced in the wild-type and $Tnf^{-/-}$ $Ifng^{-/-}$ mice. The femoral arteries were excised at 28 days after injury. Immunohistochemical staining for PCNA was performed. (*A*) Representative photographs of PCNA 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

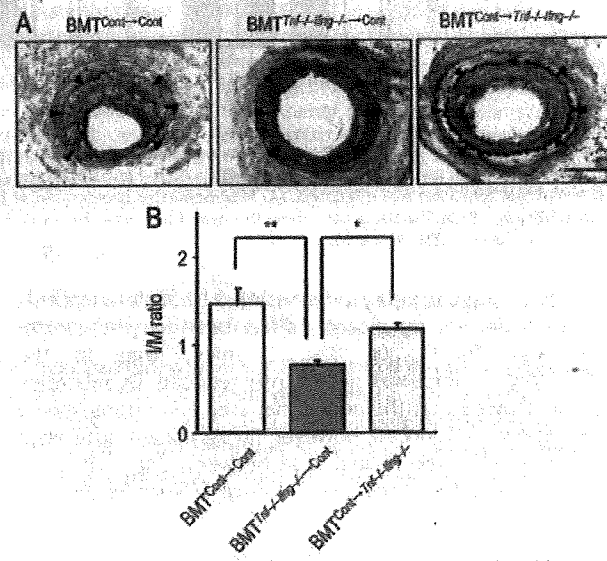

Figure 5 Contribution of bone marrow cells to neointimal formation. Bone marrow-transplanted mice [$BMT^{Cont \rightarrow Cont}$ ($n = 7$), $BMT^{Tnf-/-Ifng-/- \rightarrow Cont}$ ($n = 6$), and $BMT^{Cont \rightarrow Tnf-/-Ifng-/-}$ mice ($n = 6$)] were developed, and wire-mediated vascular injury was 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 $BMT^{Cont \rightarrow Cont}$ mice tended to be reduced when compared with that in the wild-type mice (no irradiation) (*Figure 5A* and *B*). Moreover, neointimal formation in $BMT^{Tnf-/-Ifng-/- \rightarrow Cont}$ was significantly decreased when compared with that in $BMT^{Cont \rightarrow Cont}$ (I/M ratio, $P < 0.01$) and $BMT^{Cont \rightarrow Tnf-/-Ifng-/-}$ ($P < 0.05$). 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 $BMT^{Cont \rightarrow Cont}$ 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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