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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

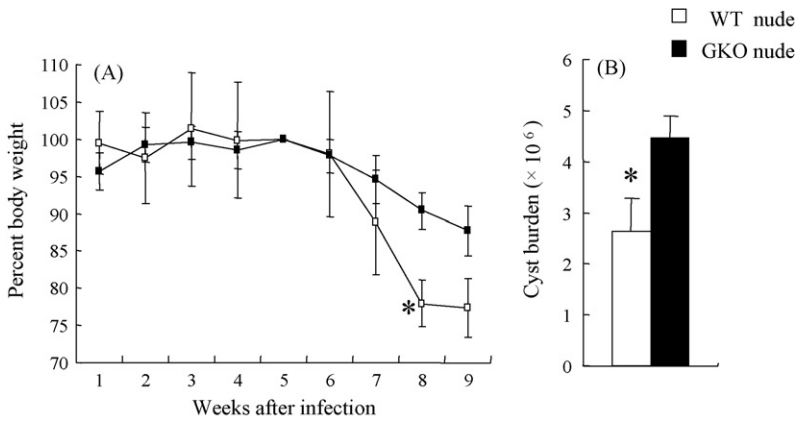

Fig. 1. The changes in the body weight and the number of *Pneumocystis*. GKO nude (■) and WT nude (□) mice were co-housed with *Pneumocystis*-infected scid mice. (A) Mice were weighed every week. Data represented the means ± SD of percent body weights to peak body weight at week 5 after infection ($n = 5$). (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

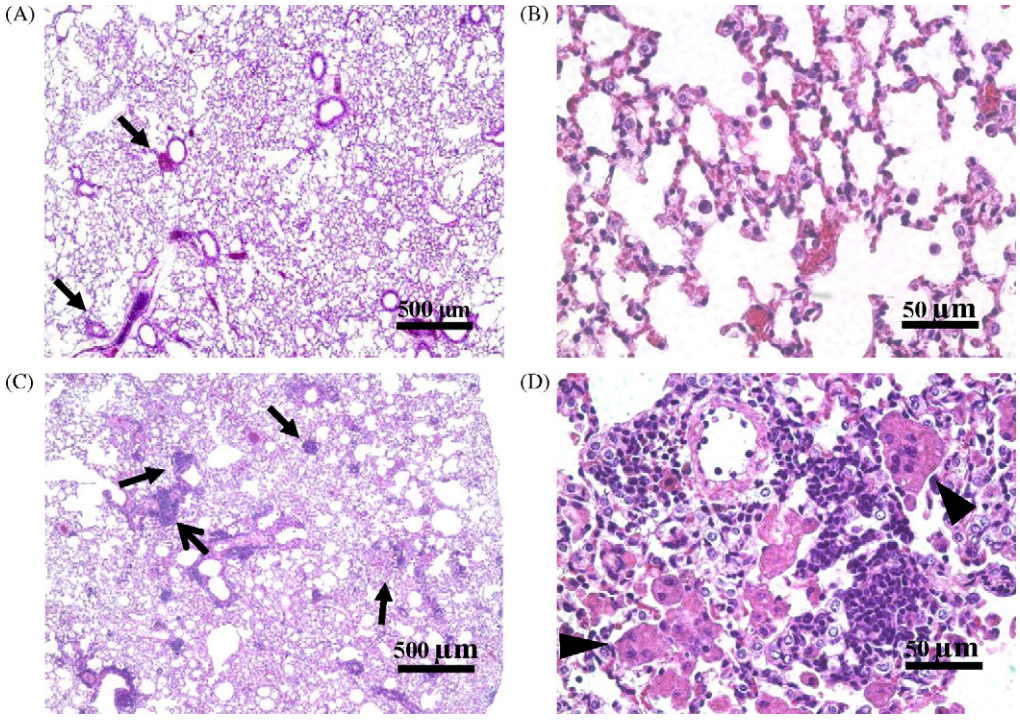

Fig. 2. Sections of lungs from WT nude (A and B) and GKO nude (C and D) mice were stained with hematoxylin and eosin 9 weeks after infection. Arrows indicate granulomatous lesion (A and C). Arrowheads indicate multinucleated giant cells (D).

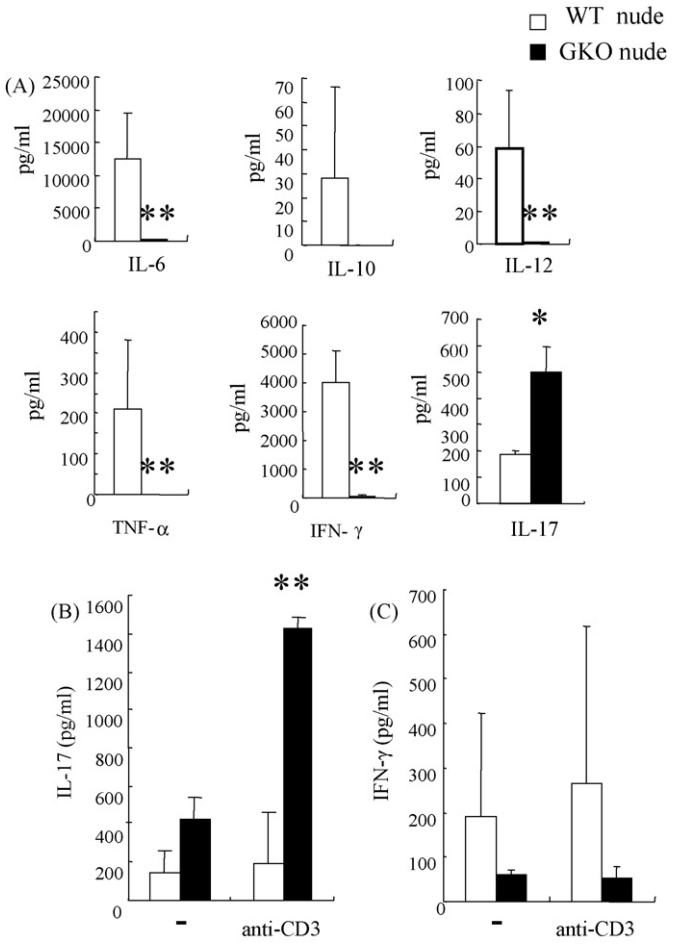

Fig. 3. Levels of cytokines in BALF of GKO nude and WT nude mice 9 weeks after infection. (A) Lung airways were lavaged with 0.5 ml PBS through an intratracheal cannula and concentrations of cytokines in the first lavage were determined. Data represent means ± SD ($n = 5$). IL-17 (B) and IFN-γ (C) production 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.

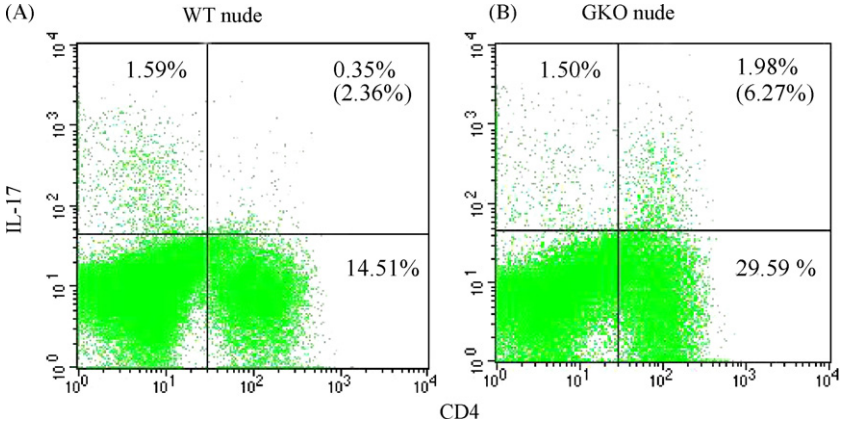

Fig. 4. Intracellular staining of $CD4^+$ T cells from BALF of GKO and WT nude mice. Percentages in the parentheses indicate the percentage of IL-17 producing $CD4^+$ cells in $CD4^+$ T cells.

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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