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Accumulation of γδ T Cells in the Lungs and Their Regulatory Roles in Th1 Response and Host Defense against Pulmonary Infection with *Cryptococcus neoformans*[1]

Kaori Uezu,* Kazuyoshi Kawakami,[2]* Kazuya Miyagi,* Yuki Kinjo,[3]* Takeshi Kinjo,* Hiromichi Ishikawa,† and Atsushi Saito*

The present study was designed to elucidate the role of γδ T cells in the host defense against pulmonary infection with *Cryptococcus neoformans*. The γδ T cells in lungs commenced to increase on day 1, reached a peak level on day 3 or 6, and then decreased on day 10 after intratracheal infection. The increase of these cells was similar in monocyte chemoattractant protein (MCP)-1-deficient mice, although that of NK and NKT cells was significantly reduced. The number of live microorganisms in lungs on days 14 and 21 was significantly reduced in mice depleted of γδ T cells by a specific mAb compared with mice treated with control IgG. Similarly, elimination of this fungal pathogen was promoted in γδ T cell-deficient (TCR-$\delta^{-/-}$) mice compared with control littermate mice. Finally, lung and serum levels of IFN-γ on days 7 and 14 and on day 7 postinfection, respectively, were significantly higher in TCR-$\delta^{-/-}$ mice than in littermate mice, whereas levels of TGF-β showed the opposite results. IL-4 and IL-10 were not different between these mice. IFN-γ production by draining lymph node cells upon restimulation with cryptococcal Ags was significantly higher in the infected TCR-$\delta^{-/-}$ mice than in control mice. Our results demonstrated that γδ T cells accumulated in the lungs in a manner different from NK and NKT cells after cryptococcal infection and played a down-modulatory role in the development of Th1 response and host resistance against this fungal pathogen. *The Journal of Immunology,* **2004, 172: 7629–7634.**

Cryptococcus neoformans, a yeast-like fungal pathogen, frequently causes fatal meningitis in hosts with a compromised immune system, such as AIDS (1). This fungus resists the killing mechanism of macrophages and grows within these cells (2). The host defense against cryptococcal infection is mediated largely by cellular immune response (3) and $CD4^+$ T cells play an important role (4–6). In previous investigations using gene-disrupted mice, it was demonstrated that Th1-related cytokines, including IFN-γ, IL-12, IL-18, and TNF-α, are essential for the host protection (7–10), whereas Th2 cytokines, such as IL-4 and IL-10, play suppressive roles in these responses (8, 11).

During infection, microbial pathogens are recognized by host cells via pattern-recognition receptors, including Toll-like receptors, mannose receptors, and complement receptors. This process leads to the phagocytosis of microorganisms and the activation of macrophages and dendritic cells, followed by the development of innate-phase immune responses (12, 13). Innate immune lymphocytes, consisting of NK, NKT (4) cells, and γδ T cells, are activated by IL-12 secreted by macrophages and dendritic cells and play regulatory roles in the establishment of adaptive immune responses (14–16). Earlier studies indicated the critical roles for NK cells in eliminating *C. neoformans* from the infected tissues. NK cells directly kill this fungal microorganism and up-regulate macrophage fungicidal activity through the production of IFN-γ (17–20). In contrast, we recently demonstrated that NKT cells recruited into the infected lungs and played an important role in the host defense against this fungal pathogen by inducing Th1-type immune responses (21). These observations indicate the contribution of these cells not only to the innate-phase host protection but also to the development of adaptive immune responses.

The third type of innate immune lymphocytes, γδ T cells, is also known to modulate the development of inflammatory lesions (22). In experimental animal models of infectious diseases, γδ T cells exert different patterns of influences on the host protection. Manipulations that result in ablation of γδ T cells, e.g., genetic disruption and treatment with a specific Ab, rendered mice susceptible to infection with a variety of microorganisms (23–30). Interestingly, similar manipulations improved the infection caused by *Listeria monocytogenes*, *Salmonella choleraesuis*, *Candida albicans*, and *Eimeria vermiformis* (31–34). In chlamydial infection, γδ T cells showed contrast roles at early and late stages (35). Thus, γδ T cells seem to act in a complex manner from one microbe to another and in the stage of infection.

The present study was designed to define the role of γδ T cells in the development of Th1 response and the host defense against *C. neoformans*. For this purpose, we analyzed the kinetics of γδ T cells accumulation in the infected tissues after intratracheal inoculation and the effect of deficiency of these cells on the clearance of microorganisms and development of Th1 responses. We also

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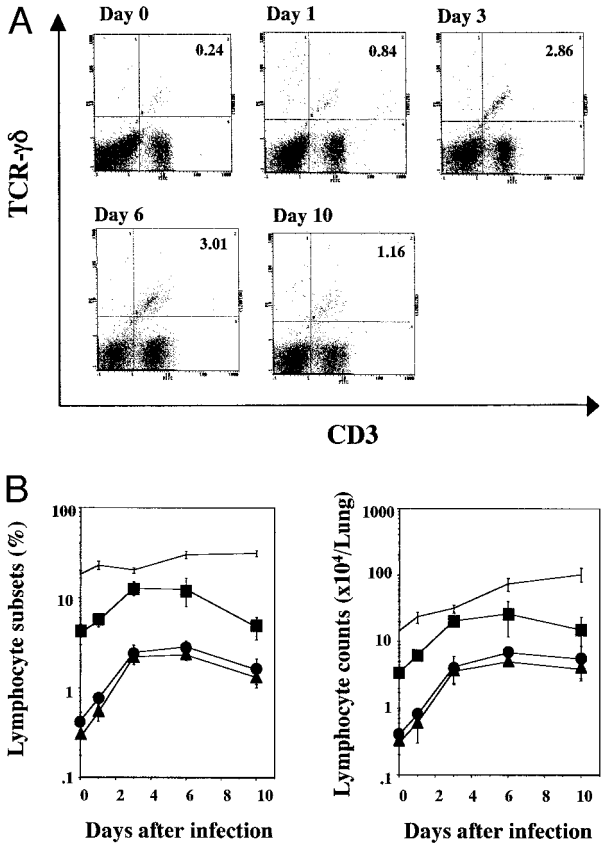


FIGURE 1. Increase of γδ T cells in lungs after *C. neoformans* infection. *A,* Mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The lung leukocytes were prepared and stained with FITC-anti-CD3 and PE-anti-TCR-γδ mAbs before (0) and 1, 3, 6, and 10 days after infection. The lymphocyte population was analyzed by flow cytometry. The number in each quadrant represents the percentage of each lymphocyte subset. *B,* Similar experiments were conducted and the lung leukocytes were prepared before (0) and 1, 3, 6, and 10 days after infection. The percentages and actual numbers of γδ T (●), NKT (▲), NK (■), and T cells (dots) in lymphocyte population were analyzed. Each symbol represents the mean of four mice.

determined the mechanism of γδ T cell accumulation in the infected lungs by testing the role of monocyte chemoattractant protein (MCP)[4]-1, which is involved in the recruitment of NK and NKT cells after cryptococcal infection (21).

Materials and Methods

Animals

TCR-δ mutant (TCR-$\delta^{-/-}$) mice were established as described previously (36). These mice were backcrossed six times to C57BL/6 mice in the Department of Microbiology, Keio University School of Medicine (Tokyo, Japan). We obtained TCR-$\delta^{-/-}$ and littermate (LM) mice by crossing TCR-$\delta^{+/-}$ and TCR-$\delta^{+/-}$ or TCR-$\delta^{-/-}$ mice. Mice were typed by using PCR analysis of tail DNA with a set of primers for the neomycin resistance gene (5′-CTT GGG TGG AGA GGC TAT TC-3′ and 5′-AGG TGA GAT GAC AGG AGA TC-3′, 280-bp PCR fragment), and for the wild-type (WT) TCR-δ gene (5′-AAA AGC CAG CCT CCG GCC AAA-3′ and 5′-AAC TGA ACA TGT CAC TGA ATT-3′, 222-bp PCR fragment). MCP-$1^{-/-}$ mice with a genetic background of C57BL/6 mice (37) were kindly provided by B. J. Rollins (Harvard Medical School, Boston, MA). These mice were bred in a pathogen-free environment in the Laboratory Animal Center for Biomedical Science, University of the Ryukyus (Okinawa, Japan). C57BL/6 mice were purchased from Charles River Japan (Osaka, Japan) and used as WT control. All mice were used at 8–15 wk of age. All experimental protocols described in the present study were approved by the Ethics Review Committees for Animal Experimentation of our universities.

Microorganisms

A serotype A-encapsulated strain of *C. neoformans*, designated as YC-13, was established from a patient with pulmonary cryptococcosis (38). In the WT mice, infection with this pathogen is self-limited to the lungs and does not disseminate to the brain. The yeast cells were cultured on potato dextrose agar plates for 2–3 days before use. To induce pulmonary infection, mice were anesthetized by i.p. injection of 70 mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL) and restrained on a small board. Live *C. neoformans* (1×10^6 cells) were inoculated at 50 μl/mouse by insertion of a 25-gauge blunt needle into and parallel to the trachea.

Preparation of pulmonary intraparenchymal leukocytes

Pulmonary intraparenchymal leukocytes were prepared as described previously (39). Briefly, the chest of the mouse was opened and the lung vascular bed was flushed by injection of 3 ml of chilled physiological saline into the right ventricle. The lungs were then excised and washed in physiological saline. The lungs, teased with the stainless mesh, were incubated in RPMI 1640 (Nippro, Osaka, Japan) with 5% FCS (Cansera; Rexdale, Ontario, Canada), 100 U/ml penicillin G, 100 μg/ml streptomycin, 10 mM HEPES, 50 μM 2-ME, and 2 mM L-glutamine containing 20 U/ml collagenase (Sigma-Aldrich, St. Louis, MO) and 1 μg/ml DNase I (Sigma-Aldrich). After incubation for 60 min at 37°C with vigorous shaking, the tissue fragments and the majority of dead cells were removed by passing through a 50-μm nylon mesh. After centrifugation, the cell pellet was resuspended in 4 ml of 40% (v/v) Percoll (Pharmacia, Uppsala, Sweden) and layered onto 4 ml of 80% (v/v) Percoll. After centrifugation at $600 \times g$ for 20 min at 15°C, the cells at the interface were collected, washed three times, and counted with a hemocytometer. The obtained cells were a mixture of lymphocytes, macrophages, and neutrophils.

Flow cytometric analysis

The following Abs were used for flow cytometry: FITC-conjugated anti-CD3, anti-TCR-αβ, and PE-conjugated anti-TCR-γδ, anti-NK1.1 mAbs (clones 145-2C11, H57-59, and GL3, PK136, respectively; BD PharMingen, San Diego, CA). Cells were preincubated with anti-FcγRIII mAb (clone 2.4G2; BD PharMingen) on ice for 15 min in PBS containing 1% FCS and 0.1% sodium azide, stained with these Abs for 25 min, and then washed three times in the same buffer. Isotype-matched irrelevant Abs were used for control staining. The stained cells were analyzed using an EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA). Data were

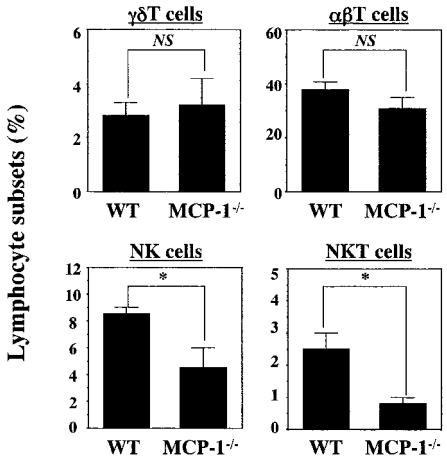


FIGURE 2. Role of MCP-1 in the increase of γδ T cells in lungs after infection with *C. neoformans.* WT and MCP-$1^{-/-}$ mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The lung leukocytes were prepared and stained with FITC-anti-TCR-αβ and PE-anti-NK1.1 mAbs or FITC-anti-CD3 and PE-anti-TCR-γδ mAbs on day 6 after infection. The percentages of γδ T, NKT, NK, and T cells in the lymphocyte population were analyzed by flow cytometry. Each bar represents the mean ± SD of four mice. *, $p < 0.05$.

[4] Abbreviations used in this paper: MCP, monocyte chemoattractant protein; WT, wild type; LM, littermate; LN, lymph node.

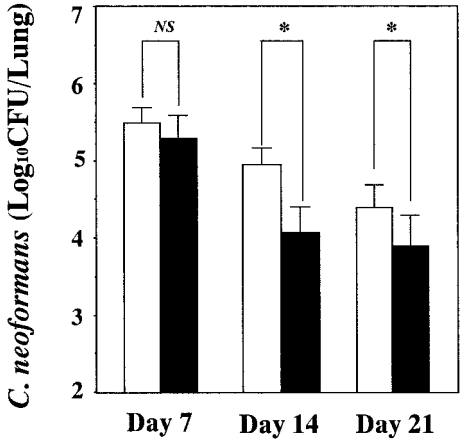


FIGURE 3. Effect of γδ T cell-depletion on the host defense against *C. neoformans*. Mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). These mice received i.p. injections of anti-TCR-γδ mAb or control IgG (400 μg) on days −3, 0, +3, +7, and +14 after infection. The number of live colonies in lung was examined on days 7, 14, and 21. Each bar represents the mean ± SD of six mice. □, Control IgG; ■, anti-TCR-γδ mAb. *, $p < 0.05$.

collected from 15,000 to 20,000 individual cells using parameters of forward scatter and side scatter to set a gate on lymphocyte population.

Antibodies

Monoclonal anti-TCR-γδ (hamster IgG) was purified by using a protein G column kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD) from the culture supernatants of hybridomas (clone UC7-13D5). To delete γδ T cells, mice were injected i.p. with anti-TCR-γδ mAb at 400 μg on days −3, 0, +3, +7, and + 14 after infection. Hamster IgG (Organon Teknika, Durham, NC) was used as the control Ab.

Enumeration of viable C. neoformans

Mice were sacrificed 1, 2, and 3 wk after infection and lungs were dissected carefully and excised, then separately homogenized in 10 ml of distilled water by teasing with a stainless mesh at room temperature. The homogenates, appropriately diluted with distilled water, were inoculated at 100 μl on PDA plates, cultured for 2–3 days followed by counting the number of colonies.

Preparation of lung homogenates

Mice were sacrificed on days 7 and 14 after infection and lungs were separately homogenized in 2 ml of PBS by teasing with a stainless mesh. The homogenates were centrifuged, filtered through 0.22-μm filter (Millipore, Bedford, MA) and kept at −70°C before use.

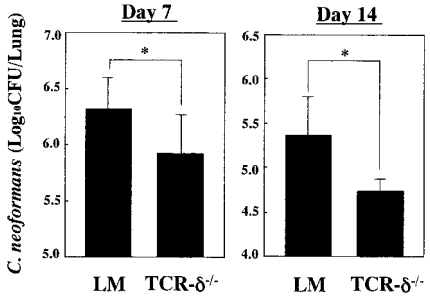


FIGURE 4. Enhanced clearance of *C. neoformans* in TCR-$\delta^{-/-}$ mice. TCR-$\delta^{-/-}$ or LM mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The number of live colonies in lung was examined on days 7 and 14 after infection. Each bar represents the mean ± SD of 10 and 6 mice for days 7 and 14, respectively. *, $p < 0.05$.

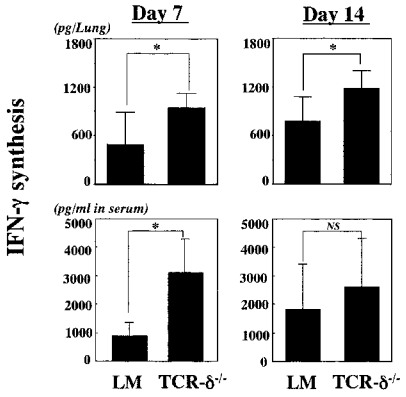


FIGURE 5. Increased production of IFN-γ in TCR-$\delta^{-/-}$ mice after *C. neoformans* infection. TCR-$\delta^{-/-}$ or LM mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The levels of IFN-γ in lung homogenates and serum were measured on days 7 and 14 after infection. Each bar represents the mean ± SD of 10 and 6 mice for days 7 and 14, respectively. *, $p < 0.05$.

In vitro stimulation of lymph node (LN) cells

Paratracheal LN cells were prepared from four mice on day 7 after infection with *C. neoformans* and cultured at 2×10^6/ml in flat-bottom culture plates (Falcon 3047; BD Labware, Franklin Lakes, NJ) with various doses of viable organisms or purified protein derivatives (Nihon BCG, Tokyo, Japan) for 48 h. The culture supernatants were collected and kept at −70°C before use.

Measurement of cytokines

Murine IFN-γ, IL-4, IL-10, and TGF-β were measured by ELISA kits (Endogen, Cambridge, MA for IFN-γ, IL-4, and IL-10; R&D Systems, Minneapolis, MN for TGF-β). The detection limits of assays for IFN-γ, IL-4, IL-10, and TGF-β were 10, 5, 12, and 2.89 pg/ml, respectively.

Statistical analysis

Analysis of data was conducted using StatView II software (Abacus Concept, Berkeley, CA) on a Macintosh computer. Data are expressed as mean ± SD. Differences between groups were examined for statistical significance using one-way ANOVA with a post hoc analysis (Fisher's PLSD test). A $p < 0.05$ was considered to be significant.

Results

Accumulation of γδ T cells in the lungs after cryptococcal infection

Initially, we elucidated the kinetics of γδ T cells in the lungs after infection with *C. neoformans* by determining the proportion of

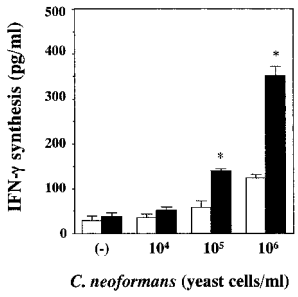
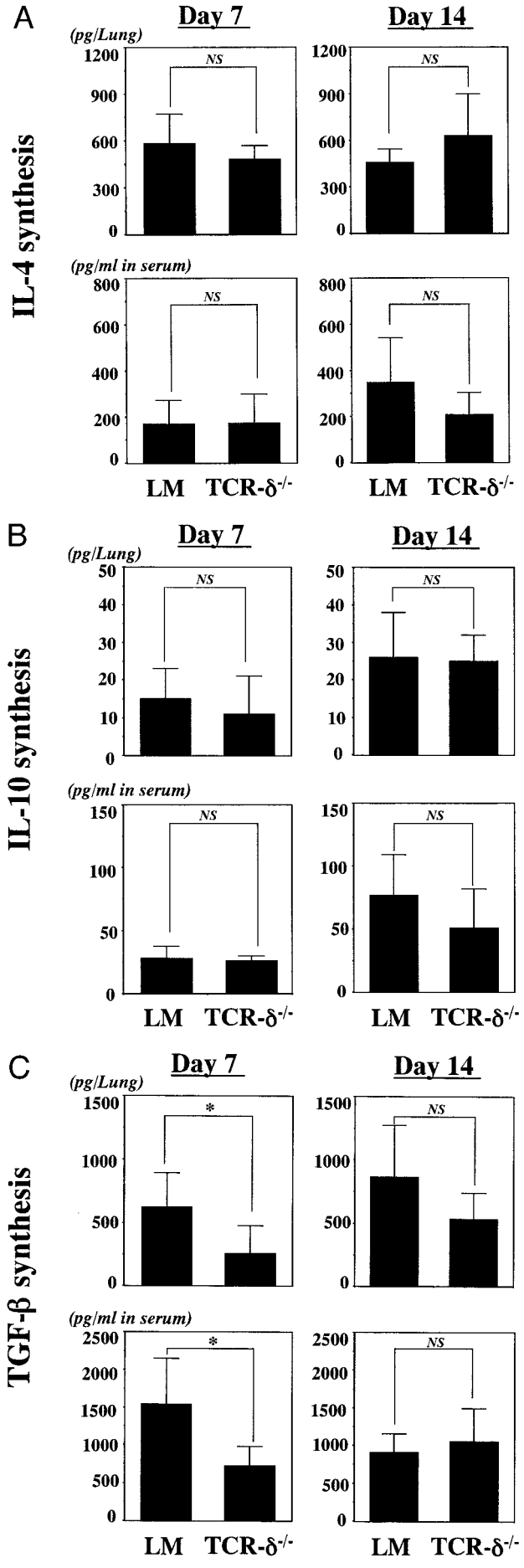


FIGURE 6. Enhanced Th1 cell development in TCR-$\delta^{-/-}$ mice after *C. neoformans* infection. TCR-$\delta^{-/-}$ (■) or LM mice (□) were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The paratracheal LN cells were prepared from four mice and cultured at 2×10^6/ml with indicated doses of live microorganisms for 48 h and the concentrations of IFN-γ were measured. Each bar represents the mean ± SD of triplicate cultures. *, $p < 0.05$ compared with LM mice.



these cells, identified as a lymphocyte subset double positive for CD3 and TCRγδ, among lung parenchymal leukocytes obtained from mice infected intratracheally with this pathogen. As shown in Fig. 1*A*, γδ T cells formed only 0.2% of the lung lymphocytes before infection, but their proportion commenced to increase on day 1, reached a peak level on day 3 or 6, and then decreased on day 10 postinfection. During the same observation period, the proportions of NK and NKT cells, identified as NK1.1$^+$TCR$\alpha\beta^-$ and NK1.1$^+$TCR$\alpha\beta^+$ lymphocyte subsets, respectively, and the actual number of each subset increased with similar kinetics as in γδ T cells (Fig. 1*B*). The proportion and number of $\alpha\beta$ T cells, identified as NK1.1$^-$ TCR$\alpha\beta^+$ lymphocytes, showed a continuing increase at the later stage of infection (Fig. 1*B*).

Accumulation of γδ T cells in the lung is independent of MCP-1

Recently, we demonstrated that MCP-1 was involved in the increase of NK and NKT cells in the lungs after infection with *C. neoformans* (22). Therefore, we asked whether a similar mechanism regulated the increase of γδ T cells in the infected tissue by comparing the proportion of these cells between WT and MCP-1$^{-/-}$ mice. As shown in Fig. 2, the proportion of γδ T cells in the lungs on day 6 postinfection was not reduced in MCP-1$^{-/-}$ mice compared with that in WT mice. In contrast, the proportion of both NK and NKT cells was significantly lower in MCP-1$^{-/-}$ mice than in WT mice, while there was no significant difference detected in the proportion of $\alpha\beta$ T cells. These results indicated that a different mechanism regulated the increase of γδ T cells in the infected tissues from that of NK and NKT cells.

Enhanced host protection against cryptococcal infection in γδ T cell-deficient mice

To elucidate the role of γδ T cells in the host defense against *C. neoformans*, we examined the effect of lack of these cells on the clinical course of this infection, as indicated by the fungal loads in lung. For this purpose, γδ T cells were depleted from C57BL/6 mice by injection of anti-TCR-δ mAb. As shown in Fig. 3, the numbers of live fungal microorganisms were significantly reduced on days 14 and 21 in mice depleted of γδ T cells when compared with those of mice treated with control IgG, although there was no significant reduction detected on day 7 postinfection. In additional experiments, we compared the fungal loads in lung between TCR-δ$^{-/-}$ and control LM mice on days 7 and 14 after *C. neoformans* infection. As shown in Fig. 4, the numbers of live microorganisms were significantly lower in TCR-δ$^{-/-}$ mice than those in control mice at both time points. These results clearly indicated that γδ T cells played a regulatory role in the host defense against cryptococcal infection.

Increased IFN-γ levels in the lung and serum of γδ T cell-deficient mice

The host defense against cryptococcal infection has been well documented to absolutely require IFN-γ-mediated responses (7, 9). Therefore, to address the mechanism of enhanced host resistance against *C. neoformans* in mice lacking γδ T cells, we initially compared the concentrations of IFN-γ in lung homogenates and serum on days 7 and 14 after this infection between TCR-δ$^{-/-}$ and

FIGURE 7. Production of Th2 cytokines and TGF-β in TCR-δ$^{-/-}$ mice after *C. neoformans* infection. TCR-δ$^{-/-}$ or LM mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The levels of IL-4 (*A*), IL-10 (*B*), and TGF-β (*C*) in lung homogenates and serum were measured on days 7 and 14 after infection. Each bar represents the mean ± SD of 10 and 6 mice for days 7 and 14, respectively. *, $p < 0.05$.

LM mice. As shown in Fig. 5, lung and serum levels of this cytokine on days 7 and 14 and on day 7, respectively, were significantly higher in TCR-$\delta^{-/-}$ mice than those in control mice. Similar data were obtained when γδ T cells were deleted by administration of the specific mAb (data not shown). These results suggested that γδ T cells down-regulate the development of Th1 cells specific for cryptococcal Ags.

Enhanced Th1 cell development in γδ T cell-deficient mice

To address this possibility, we compared the in vitro synthesis of IFN-γ by draining LN cells obtained from TCR-$\delta^{-/-}$ and LM mice on day 7 after cryptococcal infection upon restimulation with live microorganisms. As shown in Fig. 6, LN cells from control mice produced IFN-γ at concentrations dependent on the amount of the added Ags, and such production was significantly elevated at 10^5 and 10^6 yeast cells/ml in TCR-$\delta^{-/-}$ mice compared with that in control mice. IFN-γ production was not detected when purified protein derivatives was added to the cultures (data not shown), indicating that this response was specific for cryptococcal Ags. Similar data were obtained when γδ T cells were deleted by administration of the specific mAb (data not shown).

Effect of lack of γδ T cells on the production of Th2 cytokines and TGF-β

Finally, we examined the effect of lack of γδ T cells on the synthesis of Th2 cytokines, such as IL-4 and IL-10, and TGF-β after infection with *C. neoformans*. As shown in Fig. 7, *A* and *B*, lung and serum levels of IL-4 and IL-10 did not significantly differ on both days 7 and 14 postinfection between TCR-$\delta^{-/-}$ and control LM mice. By contrast, levels of TGF-β on day 7 were significantly lower in TCR-$\delta^{-/-}$ mice than those in LM mice, although no significant difference was detected on day 14 (Fig. 7*C*). Similar data were obtained when γδ T cells were deleted by administration of the specific mAb (data not shown). In addition, there was no significant difference in the synthesis of IL-4 by LN cells from TCR-$\delta^{-/-}$ and control LM mice upon restimulation with live microorganisms (data not shown).

Discussion

To elucidate the role of γδ T cells in the host defense against *C. neoformans*, we examined their kinetics in lungs after intratracheal infection with this pathogen and the role that these cells play in the development of host protective immune responses. Furthermore, we elucidated the contribution of MCP-1 on the accumulation of γδ T cells at the site of infection using mice with a gene disruption of this chemokine. The major findings of this study were 1) γδ T cell counts increased in the lungs after infection of mice with *C. neoformans*; 2) the accumulation of these cells was independent of MCP-1, which contributed to the recruitment of NK and NKT cells at the infected tissues; and 3) depletion of γδ T cells resulted in the enhancement of IFN-γ synthesis and Th1 cell development and promoted eradication of *C. neoformans* infection.

There may be two possible mechanisms for the accumulation of γδ T cells in the lungs after *C. neoformans* infection: 1) local growth at the infected sites and 2) recruitment from the peripheral circulation. In relation to the first mechanism, IL-15 is known to act as a major growth factor for γδ T cells, because mice deficient of IL-15Rα or IL-2/IL-15Rβ lacked such cells (40, 41). In the present study, we did not test the expression of this cytokine at the site of infection and its contribution to the increase of γδ T cells. The kinetics of γδ T cell accumulation in the lungs after *C. neoformans* infection paralleled that of NK and NKT cells. Recently, we demonstrated that MCP-1 played a key role in the accumulation of NK and NKT cells in the infected lungs (21), raising a possibility that a similar mechanism operates in the increase of γδ T cells. Thus, we addressed this possibility by comparing the number of these cells in the lungs of WT and MCP-$1^{-/-}$ mice after infection. In contrast to NK and NKT cells, accumulation of γδ T cells was not reduced, but rather slightly enhanced, in the absence of MCP-1 synthesis. These data suggested that MCP-1 is not involved in the lung accumulation of γδ T cells, although these cells are reported to express CCR2, the receptor for this chemokine, in previous studies (42). Thus, further investigations on the roles of IL-15 and other chemokines will be necessary to define the precise mechanism of γδ T cell accumulation.

γδ T cells play complex roles in the host protective response to infection in experimental animal models (23–34). In the present study, depletion of these cells resulted in increased synthesis of IFN-γ and enhanced development of Th1 cells after *C. neoformans* infection, and, compatibly, such manipulation rendered mice more resistant to this infection than the control group. Based on our data, γδ T cells may be identified as a lymphocyte subset that down-regulates the host protection against *C. neoformans* by interfering with the development of Th1 responses. Earlier investigations reported anti-inflammatory γδ T cells that produced Th2 cytokines and TGF-β (43, 44). These previous observations suggest that these cytokines mediated the down-regulatory effect observed in our study. The reduced production of TGF-β in TCR-$\delta^{-/-}$ mice in the earlier (day 7), but not later (day 14), phase of cryptococcal infection was compatible with this hypothesis, although no significant difference in the synthesis of IL-4 and IL-10 was detected. TGF-β has been known to suppress the host defense to infectious microorganisms (45–48). Furthermore, other investigations revealed that γδ T cells down-regulate the host defense against infection caused by *L. monocytogenes*, *S. choleraesuis*, *C. albicans*, and *E. vermiformis* (31–34). Thus, our present results suggested that γδ T cells play regulatory roles in the host defense to cryptococcal infection via the TGF-β-mediated mechanism.

The role of γδ T cells in the host defense against *C. neoformans* was likely quite different from that of NKT cells shown in our recent study (21). Interestingly, Nakano and coworkers (49, 50) reported the inverse relationship in the roles of these cells in host defense against *Toxoplasma gondii* infection. Depletion of γδ T cells led to reduced production of IFN-γ and aggravation of this infection, whereas the opposite results were observed in mice lacking NKT cells. At present, it remains elusive what mechanisms determine the different roles of these innate immune lymphocytes in the host protective responses against *C. neoformans* and *T. gondii*. However, these observations suggested that NKT and γδ T cells counterregulate the development of Th1-mediated host defense against some infectious pathogens to avoid exaggerated inflammatory responses that may be detrimental to host tissues.

In conclusion, we demonstrated in the present study the down-modulatory role of γδ T cells in the induction of Th1-mediated immune responses and host defense against cryptococcal infection. Our findings enhance our understanding of the innate-phase host defense against *C. neoformans* and might be useful for the development of effective vaccines against this fungal microorganism.

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