

infection with *S. choleraesuis*, although these cells may be a major effector population for liver injury in this infection.

Role of NKT cells in host defense against infection with *Cryptococcus neoformans*

Host defense mechanism against cryptococcal infection

C. neoformans, a yeast-like fungal pathogen with a thick polysaccharide capsule, causes granulomatous lesions in the lungs and hematogenously disseminates to the central nervous system, leading to fatal meningoencephalitis. Host resistance to this fungal pathogen is critically regulated by cellular immunity [85] and CD4⁺ T cells play a central role in limiting infection [86-88], as predicted by the high morbidity rate observed in AIDS patients. The balance between Th1 and Th2 cytokines influences the outcome of infection [89]. Th1 cytokines, such as IFN- γ , TNF- α and IL-12, are essential for protection against *C. neoformans* infection [90-93], and administration of these cytokines increases host resistance [90,94-96]. In contrast, Th2 cytokines, such as IL-4 and IL-10, inhibit Th1-mediated host defense against this infection [93,97]. IL-12 is absolutely required for Th1 development and IL-18 potentiates this response [98]. We have demonstrated that both IL-12 and IL-18 play important roles in host defense and Th1 response to *C. neoformans* [99-102].

MCP-1-dependent accumulation of NKT cells in pulmonary cryptococcosis

In order to elucidate the role of NKT cells in host defense against cryptococcal infection, we first examined whether NKT cells (CD3⁺NK1.1⁺) accumulated in the lungs after infection with *C. neoformans* [55]. NKT cells constituted only 0.5-0.7% of lung lymphocytes before infection, but commenced increasing on day 1 (2.16%), reached a peak level on day 6-7 (3.48%) and thereafter decreased. NKT cells increased most profoundly at the infected sites, compared to other lymphocyte populations, T and NK cells, although these cells increased with similar kinetics as for NKT cells. Next, we examined the expression of V α 14 mRNA in the lungs and the proportion of α -GalCer-reactive cells, as detected using a α -GalCer-loaded CD1d tetramer, which has been reported to bind to V α 14⁺ NKT cells in a specific manner [103]. The expression of V α 14 mRNA and the proportion of α -GalCer-reactive cells increased in lungs. Thus, V α 14⁺ NKT cells were found to increase at the primary infected sites after pulmonary infection with *C. neoformans*.

How do NKT cells increase in the lungs after infection? Migration of inflammatory leukocytes is critically regulated by a variety of chemoattracting cytokines. NK cells are attracted by many chemokines, including MCP-1, -2, -3,

macrophage inflammatory protein (MIP)-1 α , RANTES, IP-10 and lymphotactin [104-109]. In contrast, MIP-2 was the only cytokine to traffick NKT cells [110]. Based on this background, we focused on the role of monocyte chemoattractant protein (MCP)-1 to elucidate the regulatory mechanism of NKT cell accumulation. First, we examined the kinetics of MCP-1 production at both the mRNA and protein levels at the infected site. Both parameters of MCP-1 production increased on day 1, peaked on day 3, and decreased thereafter. Interestingly, MCP-1 production preceded the increase of NKT cells in the lung, which suggested involvement of this chemokine in the increase of NKT cells during infection. In our next experiments, this supposition was addressed using mice with a genetic depletion of MCP-1. The increase of NKT cells observed after induction of cryptococcal infection was significantly less pronounced in MCP-1KO mice compared to control mice. Furthermore, significant pulmonary accumulation of NKT cells was detected by intratracheal instillation of recombinant MCP-1. Thus, MCP-1 was identified as a regulatory chemokine in the inflammatory response mediated by NKT cells during infection with *C. neoformans*.

Role of V α 14+ NKT cells in host defense and Th1 response to cryptococcal infection

The role of V α 14+ NKT cells in the host defense and Th1 response to cryptococcal infection was elucidated using mice genetically lacking V α 14+ NKT cells. Both the development of Th1 cells specific for cryptococcal antigens (as shown by IFN- γ production by re-stimulated spleen cells from the infected mice), and the delayed-type hypersensitivity (DTH) response (as indicated by footpad swelling in response to challenge immunization), were hampered in V α 14+ NKT cell-deficient mice, compared with the control. Furthermore, the clearance of *C. neoformans* was significantly delayed in these mice. Based on these data, NKT cells were identified as a key cell population, which regulated the Th1-mediated host resistance by bridging the innate and acquired immune responses.

Therapeutic effect of α -GalCer in cryptococcal infection

The therapeutic effect of α -GalCer on the host protective responses to cryptococcal infection was investigated using a murine model of systemic infection [56]. Administration of α -GalCer resulted in a vigorous increase in serum IFN- γ concentration and enhanced Th1 cell development, as shown by increased IFN- γ production by re-stimulated spleen cells from infected mice. Consistent with this, the same treatment promoted clearance of *C. neoformans* from spleen and lung, which was mediated by IFN- γ . These effects of α -GalCer

treatment were completely abrogated in V α 14+ NKT cell-deficient mice. IL-12 was essential for the IFN- γ production and host protection induced by α -GalCer administration, although a critical role for IL-18 was not proven [57].

Concluding remarks

Many scientists have been focusing attention on NKT cells, because many recent investigations have revealed that these cells play crucial roles in various immune responses. α -GalCer, a ligand of V α 14+ and V α 24+ NKT cells, appears to be useful in protecting hosts against tumor metastasis, microbial infection and autoimmune disease. Although the number of V α 24+ NKT cells in the peripheral blood is very small, such a problem might be solved by a novel approach of expanding the population of V α 24+ NKT cells by using α -GalCer-loaded DCs with IL-7 and IL-15 [111]. Some modification, such as combination therapy of G-CSF and α -GalCer [34], or use of OCH, an analogue of α -GalCer [22], may augment the effect of α -GalCer in treating patients with cancer or autoimmune disease. α -GalCer might therefore be an attractive tool as a new immunotherapeutic agent to treat patients with intractable cancer, infection and autoimmune disease.

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Accumulation of $\gamma\delta$ T Cells in the Lungs and Their Regulatory Roles in Th1 Response and Host Defense against Pulmonary Infection with *Cryptococcus neoformans*¹

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The present study was designed to elucidate the role of $\gamma\delta$ T cells in the host defense against pulmonary infection with *Cryptococcus neoformans*. The $\gamma\delta$ 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 $\gamma\delta$ T cells by a specific mAb compared with mice treated with control IgG. Similarly, elimination of this fungal pathogen was promoted in $\gamma\delta$ 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 $\gamma\delta$ 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.

C*ryptococcus 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 $\gamma\delta$ 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, $\gamma\delta$ T cells, is also known to modulate the development of inflammatory lesions (22). In experimental animal models of infectious diseases, $\gamma\delta$ T cells exert different patterns of influences on the host protection. Manipulations that result in ablation of $\gamma\delta$ 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, $\gamma\delta$ T cells showed contrast roles at early and late stages (35). Thus, $\gamma\delta$ 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 $\gamma\delta$ T cells in the development of Th1 response and the host defense against *C. neoformans*. For this purpose, we analyzed the kinetics of $\gamma\delta$ 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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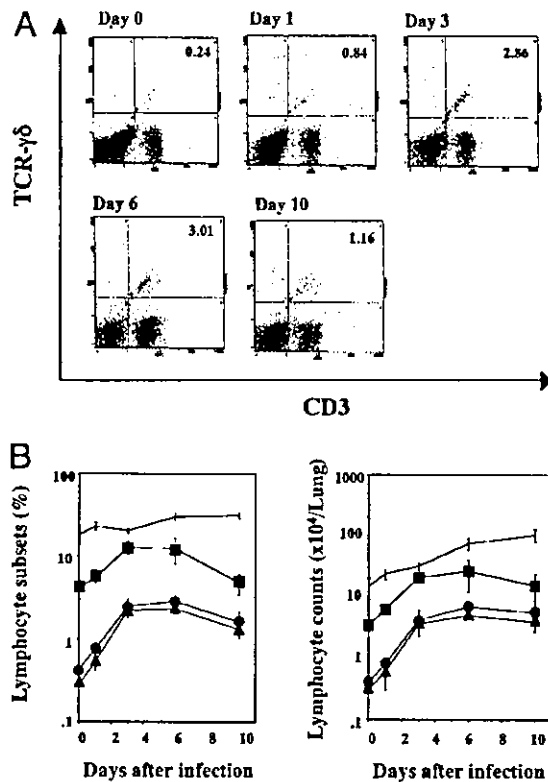


FIGURE 1. Increase of $\gamma\delta$ T cells in lungs after *C. neoformans* infection. **A**, Mice were inoculated intratracheally with *C. neoformans* (1×10^6 /mouse). The lung leukocytes were prepared and stained with FITC-anti-CD3 and PE-anti-TCR- $\gamma\delta$ mAbs before (0) and 1, 3, 6, and 10 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 $\gamma\delta$ T (\bullet), NKT (\blacktriangle), NK (\blacksquare), and T cells (dots) in lymphocyte population were analyzed. Each symbol represents the mean of four mice.

determined the mechanism of $\gamma\delta$ T cell accumulation in the infected lungs by testing the role of monocyte chemoattractant protein (MCP)-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

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

(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 μ l/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 μ g/ml streptomycin, 10 mM HEPES, 50 μ M 2-ME, and 2 mM L-glutamine containing 20 U/ml collagenase (Sigma-Aldrich, St. Louis, MO) and 1 μ g/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- μ m 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- $\alpha\beta$, and PE-conjugated anti-TCR- $\gamma\delta$, anti-NK1.1 mAbs (clones 145-2C11, H57-59, and GL3, PK136, respectively; BD Pharmingen, San Diego, CA). Cells were preincubated with anti-Fc γ RIII 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

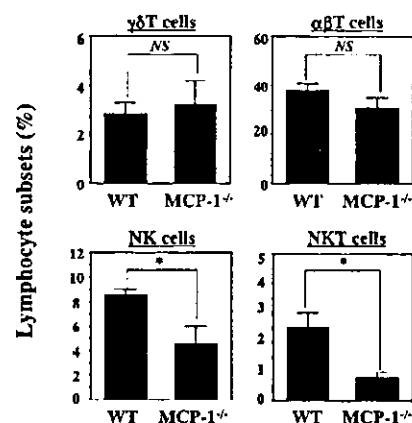


FIGURE 2. Role of MCP-1 in the increase of $\gamma\delta$ T cells in lungs after infection with *C. neoformans*. WT and MCP-1 $^{-/-}$ mice were inoculated intratracheally with *C. neoformans* (1×10^6 /mouse). The lung leukocytes were prepared and stained with FITC-anti-TCR- $\alpha\beta$ and PE-anti-NK1.1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

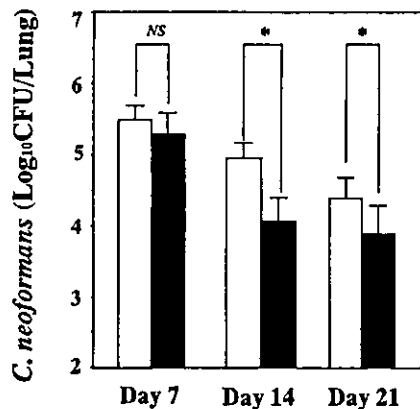


FIGURE 3. Effect of $\gamma\delta$ T cell-depletion on the host defense against *C. neoformans*. Mice were inoculated intratracheally with *C. neoformans* (1×10^6 /mouse). These mice received i.p. injections of anti-TCR- $\gamma\delta$ mAb or control IgG (400 μ g) on days -3, 0, +3, +7, and +14 after infection. The number of live colonies in lung was examined on days 7, 14, and 21. Each bar represents the mean \pm SD of six mice. \square , Control IgG; \blacksquare , anti-TCR- $\gamma\delta$ 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- $\gamma\delta$ (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 $\gamma\delta$ T cells, mice were injected i.p. with anti-TCR- $\gamma\delta$ mAb at 400 μ g 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 μ l 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- μ m filter (Millipore, Bedford, MA) and kept at -70°C before use.

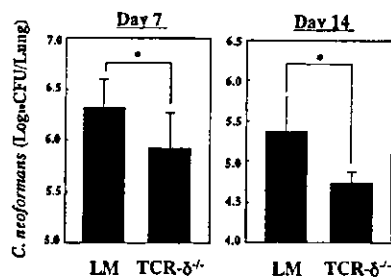


FIGURE 4. Enhanced clearance of *C. neoformans* in TCR- $\delta^{-/-}$ mice. TCR- $\delta^{-/-}$ or LM mice were inoculated intratracheally with *C. neoformans* (1×10^6 /mouse). The number of live colonies in lung was examined on days 7 and 14 after infection. Each bar represents the mean \pm SD of 10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

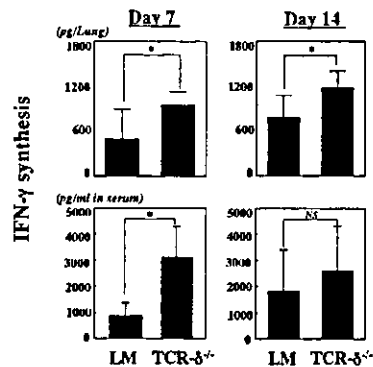


FIGURE 5. Increased production of IFN- γ in TCR- $\delta^{-/-}$ mice after *C. neoformans* infection. TCR- $\delta^{-/-}$ or LM mice were inoculated intratracheally with *C. neoformans* (1×10^6 /mouse). The levels of IFN- γ in lung homogenates and serum were measured on days 7 and 14 after infection. Each bar represents the mean \pm 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 \pm 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 $\gamma\delta$ T cells in the lungs after cryptococcal infection

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

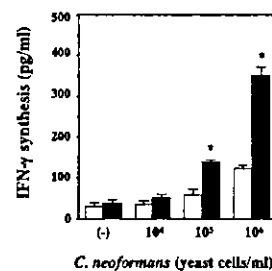
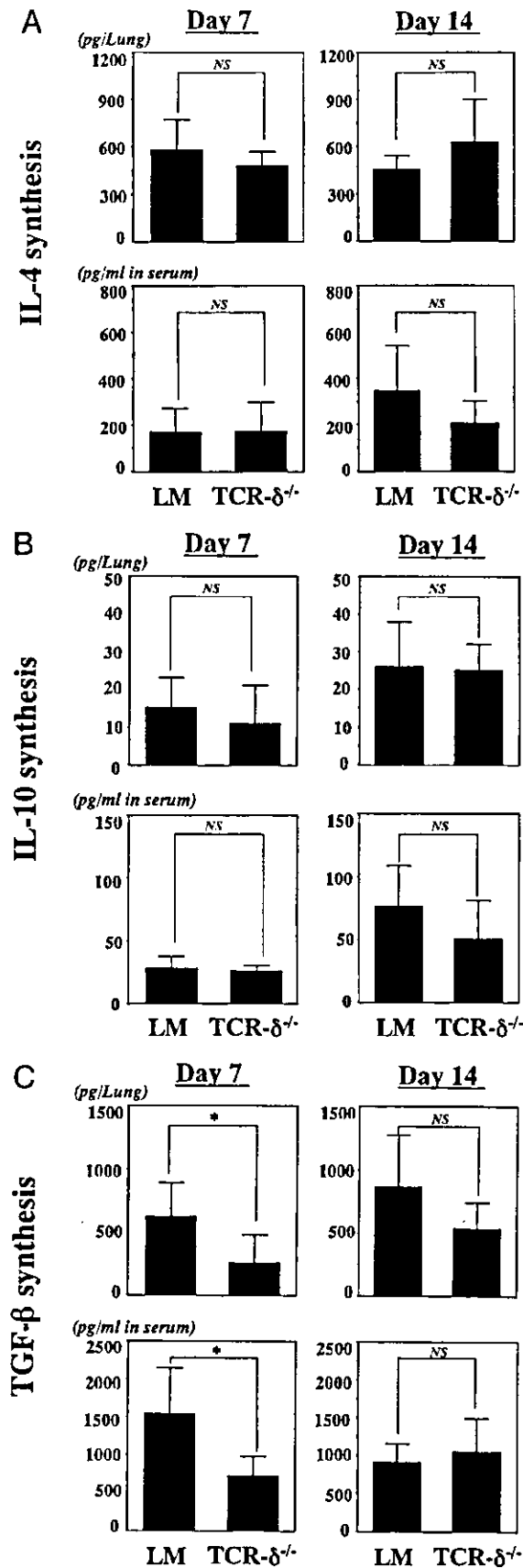


FIGURE 6. Enhanced Th1 cell development in TCR- $\delta^{-/-}$ mice after *C. neoformans* infection. TCR- $\delta^{-/-}$ (\blacksquare) or LM mice (\square) were inoculated intratracheally with *C. neoformans* (1×10^6 /mouse). The paratracheal LN cells were prepared from four mice and cultured at 2×10^6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



these cells, identified as a lymphocyte subset double positive for CD3 and TCR $\gamma\delta$, among lung parenchymal leukocytes obtained from mice infected intratracheally with this pathogen. As shown in Fig. 1A, $\gamma\delta$ 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 $\gamma\delta$ T cells (Fig. 1B). 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. 1B).

Accumulation of $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cells in the infected tissues from that of NK and NKT cells.

Enhanced host protection against cryptococcal infection in $\gamma\delta$ T cell-deficient mice

To elucidate the role of $\gamma\delta$ 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, $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cells played a regulatory role in the host defense against cryptococcal infection.

Increased IFN- γ levels in the lung and serum of $\gamma\delta$ 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 $\gamma\delta$ 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 \pm 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 $\gamma\delta$ T cells were deleted by administration of the specific mAb (data not shown). These results suggested that $\gamma\delta$ T cells down-regulate the development of Th1 cells specific for cryptococcal Ags.

Enhanced Th1 cell development in $\gamma\delta$ 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 $\gamma\delta$ T cells were deleted by administration of the specific mAb (data not shown).

Effect of lack of $\gamma\delta$ T cells on the production of Th2 cytokines and TGF- β

Finally, we examined the effect of lack of $\gamma\delta$ 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. 7C). Similar data were obtained when $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cells at the site of infection using mice with a gene disruption of this chemokine. The major findings of this study were 1) $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cells, because mice deficient of IL-15R α or IL-2/IL-15R β 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 $\gamma\delta$ T cells. The kinetics of $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cell accumulation.

$\gamma\delta$ 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, $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T cells play regulatory roles in the host defense to cryptococcal infection via the TGF- β -mediated mechanism.

The role of $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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.

Acknowledgments

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Invited Review

Regulation by Innate Immune T Lymphocytes in the Host Defense against Pulmonary Infection with *Cryptococcus neoformans*

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SUMMARY: Recently, innate immune lymphocytes, such as natural killer (NK) T cells and $\gamma\delta$ antigen receptor-bearing T ($\gamma\delta$ T) cells, have garnered much attention, and their biological significance in the tumor immunity, allergic diseases and infectious diseases is extensively exploited. We have addressed the role of these cells in the host defense using a mouse model of pulmonary infection with *Cryptococcus neoformans*, which frequently causes fatal meningoencephalitis in AIDS patients. Host defense to this fungal pathogen is largely mediated by cellular immunity, and type-1 helper T (Th1) cells play a central role in this process. This infection causes a prompt accumulation of both NKT and $\gamma\delta$ T cells in the lung tissues in a monocyte chemoattractant protein (MCP)-1-dependent or -independent manner, respectively. Genetic deletion of V α 14+ NKT cells ameliorates the Th1 response and clearance of microorganisms in the lungs, whereas these host protective responses are rather enhanced in mice lacking $\gamma\delta$ T cells. Thus, in some aspect, these innate immune lymphocytes may co-regulate the Th1-mediated response for induction of the moderate host defense. $\gamma\delta$ T cells may act to keep the balance of Th1-Th2 responses in a proper manner by suppressing the exaggerated Th1 response caused by NKT cells. In this review, I describe the recent research development in the innate immune host defense against cryptococcal infection in respiratory organs with emphasis on our data in the regulatory role of NKT cells and $\gamma\delta$ T cells.

1. Introduction

Airway is directly connected to the outer environment and always exposed to infectious agents, such as bacteria, fungi and viruses. To protect from these harmful agents, respiratory organs of hosts develop a highly sophisticated defense system. Initially, anatomical and mechanical systems trap large size particles in the inhaled air, which include nasal hairs, nasopharyngeal channels, glottis and highly divided branches of bronchi. These particles are caught by the mucous blanket

lining the bronchial surface and cleared by ciliary movement into the upper airway. In contrast, small size particles less than 5 μ m in diameter including most infectious pathogens reach the alveolar spaces. In order to keep the sterility in lung, additional mechanisms are equipped in these areas, which are largely divided into the two categories: innate and acquired host defense systems. The former consists of humoral components including antimicrobial proteins and complements, phagocytic cells like neutrophils and macrophages, dendritic cells and innate immune lymphocytes, while the latter is associated with antigen-specific responses mediated by antibody and cellular immunity.

Fungal infection is believed to relate with dysfunction of innate immunity. Infection of *Cryptococcus neoformans*, a yeast-like fungal pathogen with a thick polysaccharide capsule takes place by inhaling the desiccated yeast cells into the lungs. The organisms reach the subpleural area to establish the primary lesions. In normal hosts, the infection is usually self-limiting, since host defense mechanisms can eliminate the infection. In contrast, in immunocompromised

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patients with impaired cell-mediated immunity, the infection is not limited to the primary site of infection; it frequently disseminates to the central nervous system, which is often associated with a high mortality in these patients. Disseminated infection of this fungal pathogen to the brain has attracted clinical attention as a serious problem, particularly with the increased number of patients with AIDS (1,5).

In addition to conventional T cells that recognize peptide antigens in context of MHC class I or class II molecules, a number of unconventional T cell subsets have been identified (1,2). These subsets include natural killer (NK) T cells and $\gamma\delta$ antigen receptor-bearing T ($\gamma\delta$ T) cells, which are recognized as the innate immune lymphocytes. When infectious pathogens invade the tissues, these cells promptly respond by producing a variety of cytokines, which results in the promoted host protective responses. However, their overall potential is not sufficient for complete eradication of the infection, which needs more potent protective mechanisms by developing the subsequent acquired immune responses. Based on this property, the innate immune lymphocytes have been recognized merely as a "temporary protector" until the acquired immune response is established. However, recent investigations have accumulated evidences indicating that innate immune lymphocytes determine the quality of acquired immune responses (2-5), which may allow the early host protective responses mediated by these cells to be identified as more than a "temporary protector" before development of acquired immunity.

In this review, I describe the recent research development in the innate immune host defense against cryptococcal infection in respiratory organs with emphasis on our data in the regulatory role of NKT cells and $\gamma\delta$ T cells in a murine model of pulmonary infection with *C. neoformans*.

2. Basics in the host defense to cryptococcal infection

C. neoformans shows the features of intracellular parasitism within phagocyte cells, as well known in *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Salmonella typhimurium* (6). Because such pathogens resist the killing mechanisms, phagocytic cells fail to eradicate them without activation by interferon (IFN)- γ . Compatibly, the host defense against *C. neoformans* is critically regulated by cell-mediated immunity (7), and CD4⁺ T cells play a central role in eradicating this infection (8-10). The balance between type-1 helper T (Th1) and Th2 cytokines markedly influences the outcome of infection; the predominant synthesis of Th1 cytokines over Th2 protects mice from infection, whereas infection is exacerbated under a Th2 dominant condition (11,12). Mice depleted of Th1-type cytokines (e.g., IFN- γ and TNF- α) are highly susceptible to cryptococcal infection (13,14), while the infection is less severe in mice lacking Th2 cytokines (e.g., IL-4 and IL-10) than control mice (15,16). Differentiation of naive helper T cells into Th1 cells absolutely requires the presence of IL-12 (17), and this response is strongly potentiated by IL-18 (18). In recent investigations (15,19), targeted disruption of the gene for IL-12 or IL-18 resulted in attenuated host resistance and Th1 response to *C. neoformans*, indicating the prerequisite role for these cytokines in the development of host protective response.

3. Role of NKT cells

3-1. General features of NKT cells

NKT cell is a unique T cell subset that shares the features of NK cells. Originally, this population was discovered as a lymphocyte subset expressing both T cell receptor (TCR) $\alpha\beta$ and NK1.1 or NKR-P1 (CD161) in mice (20-22). Specific characteristics of this cell type include highly limited repertoire with an invariant V α chain consisting of V α 14-J α 18 gene segment and highly skewed V β chains, V β 8.2, 7 and 2 in mice and with V α 24-J α 18 and V β 11 in human. By this meaning, these cells are called invariant (*i*)NKT cells. The mouse *i*NKT cells constitute CD4⁺ and double negative (DN) subsets. They usually do not express CD8, although CD8⁺ subset can be found in human. The development of *i*NKT cells is dependent on the non-classical MHC class I molecule CD1d, which is composed of non-polymorphic heavy chain and β 2 microglobulin, as shown by the findings that they disappear in CD1d gene-disrupted (CD1d-KO) mice. The glycosphingolipid, α -galactosylceramide (α -GalCer) that was originally discovered from marine sponge as a novel anti-cancer agent is recognized by *i*NKT cells in context of CD1d, which results in their strong activation. These cells are found in large numbers in the liver, thymus and bone marrow and in small numbers in the spleen and lungs.

3-2. Regulatory role of NKT cells in Th1-Th2 cytokine balance

In earlier investigations (23), Yoshimoto and co-workers demonstrated that *in vivo* activation of NKT cells resulted in a rapid production of IL-4. Based on this observation, they speculated that this cell population may be the major source of early IL-4 production in the differentiation of Th2 cells, although it was not confirmed by subsequent studies (24,25). Recent investigations revealed that *i*NKT cells promptly secrete large amounts of both IFN- γ and IL-4 after engagement of the antigen receptor (20-22,26,27), suggesting the dual roles of this subset in the differentiation of both Th1 and Th2 cells. V α 14 TCR transgenic mice showed elevated serum levels of IgE and IL-4 (28), and activation of *i*NKT cells by α -GalCer induced T cell response to ovalbumin (OVA) polarized toward Th2-dominant condition (29). In contrast, other studies emphasized a positive role for NKT cells in the development of Th1 cells. Administration of α -GalCer led to the rapid production of IFN- γ by *i*NKT cells and other bystander cells, such as NK cells, *in vitro* (30) and suppressed *in vivo* Th2 differentiation and subsequent IgE synthesis caused by OVA immunization or infection with *Nippostrongylus brasiliensis* through the induction of IFN- γ production (31). Further evidences supporting this notion were accumulated: granuloma formation caused by mycobacterial lipid antigen (32) and IFN- γ -mediated protection of mice against infection with malaria parasites through ligand-specific activation of *i*NKT cells (33).

3-3. Significance of NKT cells in infectious diseases

There are several published studies addressing the role of NKT cells in the host defense against infectious pathogens. These studies are conducted using anti-CD1d mAb-treated and CD1d-KO mice, which manipulations abolish most of NKT cells, and J α 18 gene-disrupted (KO) mice, which lack particular NKT cell subset bearing V α 14-J α 18 antigen receptors. Based on these investigations, three roles are identified for NKT cells in the host defense to infection. First, the clinical course of *M. tuberculosis* infection in CD1d-KO mice is not much different from that in control mice (34,35) and

minimally affected by treatment with anti-CD1d mAb (36). Similarly, genetic depletion of *i*NKT cells does not result in exacerbation of infection with *M. tuberculosis*, *M. bovis* BCG and *S. choleraesuis* (34,37). Second, infection with *L. monocytogenes* or *Toxoplasma gondii* is rather improved by manipulations designed to suppress the activity of NKT cells (38,39). Administration of anti-CD1d mAb results in prolongation of *Listeria* infection in mice, which is associated with increased secretion of Th1-type cytokines and decreased TGF- β production (38). Similarly, depletion of NKT cells by anti-IL-2R β mAb enhances the host protection of mice from *T. gondii* infection by increasing Th1-polarized cytokine production (39). Finally, mice lacking V α 14⁺ NKT cells are more susceptible to *Streptococcus pneumoniae*, *Leishmania major* and *Trypanosoma cruzi* infection than control mice (40-42). Similar results are reported in CD1d-KO mice infected with *Pseudomonas aeruginosa*, *Borrelia burgdorferi* and *Plasmodium yoelii* (43-45). Thus, the significance of NKT cells in infectious diseases seems different from pathogen to pathogen.

3-4. Recruitment of NKT cells in the lung after cryptococcal infection

To understand the role of NKT cells in the host defense against pulmonary infection with *C. neoformans*, we elucidated whether these cells increased in the infected tissues (46). Inflammatory leukocytes obtained from the homogenates of infected lungs were stained with anti-TCR $\alpha\beta$ and -NK1.1 mAbs to discriminate conventional T, NK and NKT cells. The proportions of conventional T, NK and NKT cells, as indicated by TCR $\alpha\beta$ ⁺NK1.1⁻, TCR $\alpha\beta$ ⁻NK1.1⁺ and TCR $\alpha\beta$ ⁺NK1.1⁺ cells, respectively, started to increase on day 1, reached a peak level on day 6 and then decreased on day 10 post-infection. Interestingly, NKT cells most profoundly increased at the infected sites among these cells. We further defined the dynamics of *i*NKT cells bearing V α 14 TCR in the infected lungs by detecting cells bound to either anti-V α 14 mAb or α -GalCer-loaded CD1d tetramer. Similar kinetics was observed in this particular subset of NKT cells using both strategies for detection. Thus, *i*NKT cells as well as conventional T and NK cells were found to accumulate in the lungs after intratracheal infection with *C. neoformans*.

Migration of inflammatory leukocytes is critically regulated by a variety of chemokines, which are classified into two major subgroups, CXC- and CC-chemokines, based on the arrangement of two N-terminal cysteine residues (47). ELR⁺ CXC-chemokines, including IL-8, are neutrophil-mediated inflammatory responses, while ELR⁻ CXC-chemokines (e.g., IP-10 and Mig) and CC-chemokines (e.g., MCP-1, MIP-1 α , -1 β and RANTES) predominantly attract lymphocytes and macrophages. Many investigators have reported that resting or activated NK cells are attracted by many chemokines, including MCP-2, -3, MIP-1 α , RANTES, IP-10 and lymphotactin, under various conditions (48-53). In contrast, MIP-2 had been the only chemokine that functions in trafficking NKT cells before we identified MCP-1 as a chemoattractant for this lymphocyte subset (54). In MCP-1KO mice, accumulation of NKT cells in lungs was profoundly attenuated after infection with *C. neoformans* (46). Consistent with these data, MCP-1 production preceded the kinetics of NKT cell-mediated inflammatory responses. Thus, NKT cell trafficking into the fungus-infected tissues involves at least in part the production of MCP-1, although other chemokines may contribute, as observed in NK cells.

3-5. Role of NKT cells in Th1 response and host defense to cryptococcal infection

NKT cells promptly produce a large amount of IFN- γ and IL-4 upon stimulation via their antigen receptors (20-22). Accumulating evidences indicate that NKT cells are involved in the regulation of Th1 and Th2 cell development. On the other hand, host defense to cryptococcal infection is critically regulated by the balance between Th1- and Th2-mediated immune responses (11,12). These findings suggest that NKT cells may affect the host immune responses and protection against infection with this fungal pathogen. In our study (46), Th1-mediated immune responses, as indicated by antigen-specific IFN- γ production by T cells and delayed-type hypersensitivity reaction, were significantly ameliorated in J α 18-KO mice lacking *i*NKT cells, compared with control wild-type mice. In contrast, Th2 cytokine synthesis was not influenced in these mice. Furthermore, the clearance of fungal pathogen from the infected tissues was significantly delayed in J α 18-KO mice, compared with control mice. These findings demonstrate that *i*NKT cells function not only in the innate immune phase but also in bridging to the Th1-mediated acquired immune responses, which leads to host protection against cryptococcal infection.

3-6. Natural ligands

*i*NKT cells express antigen receptors with an invariant V α chain and highly skewed V β chains. Based on this property, many investigators had predicted a particular molecule to be the ligand for this lymphocyte subset. Kawano and co-workers (26) are the first group which reported that α -GalCer is a specific ligand for antigen receptors of these cells. However, the endogenous natural ligands of *i*NKT cells have not been defined, because mammals do not generate α -GalCer, originally discovered from marine sponge. Using crystal structure and mass spectrometry analyses, Joyce and co-workers (55) found glycosylphosphatidylinositol (GPI) to be a candidate molecule that could bind to CD1d and present to NKT cell antigen receptors. In addition, it was demonstrated by Schofield et al. (56) that NKT cells regulated IgG production against GPI-anchored surface antigens of protozoans, *Plasmodium* and *Trypanosoma*. Similar results were reported by Duthie et al. (42) in mice infected with *T. cruzi*. They indicated the attenuation in chronic phase of antibody response to GPI-anchored surface antigens in NKT cell-deficient mice. From these observations, GPI is speculated as a molecule recognized by NKT cells as the endogenous and exogenous natural ligand. However, conflicting data are recently provided by other investigators (57,58). The IgG response to malarial GPI-anchored proteins is dependent on MHC class II, but not on CD1d (57). In other study, GPI-anchored mucin-like glycoproteins from *T. cruzi* bind to CD1d but do not elicit dominant innate or adaptive immune responses via the CD1d/NKT cell pathway (58). Thus, no defined ligand has so far been discovered. In order to understand the precise mechanism in which NKT cells contribute to the host defense against infection, identification of pathogen-derived ligands is desired. In this regard, Brigl and co-workers have recently reported a putative contribution of endogenous, but not pathogen-derived ligands for CD1d-dependent activation of NKT cells after infection with *S. typhimurium* and *Staphylococcus aureus* in mice (59). Further approaches will be required before understanding the mechanism of pathogen-related activation of NKT cells.

3-7. Induction of Th1 response and host defense to cryptococcal infection by ligand-specific activation

iNKT cells recognize α -GalCer by their antigen receptors in context of CD1d molecules expressed on DCs (20-22,27). Such engagement causes prompt secretion of both IFN- γ and IL-4 by these cells and emergence of their cytolytic activity against tumour cells. Toura et al. (60) indicated that administration of DCs pulsed with α -GalCer induced potent antitumor activity through specific activation of iNKT cells, and resulted in the complete suppression of melanoma metastasis in the liver.

In infectious diseases, Gonzalez-Aseguinolaza and co-workers (33) for the first time demonstrated the effectiveness of α -GalCer treatment in improving the clinical course of murine malaria. The development of liver stage, but not blood stage, malaria was strongly inhibited via induction of IFN- γ synthesis by α -GalCer. The same group recently revealed that co-administration of α -GalCer potentiated the protective effect against this infection caused by immunization with irradiated malaria parasite (61). Our group observed similar effects of this treatment in a murine model of cryptococcal infection (62). Administration of α -GalCer strongly enhanced the production of IFN- γ by NK and Th1 cells and significantly reduced the number of live colonies of *C. neoformans* in the infected organs, compared with vehicle treatment. These effects were not detected in J α 18-KO mice, indicating the involvement of iNKT cells. IFN- γ production induced by α -GalCer was totally mediated by IL-12, but not IL-18 (63). Similar findings are recently reported in *P. aeruginosa* and *M. tuberculosis* by other investigators (43, 64), although the contribution of iNKT cells to the host defense against the latter infection remains not clearly defined (34). These observations suggest that α -GalCer can be a promising immunotherapeutic agent for the treatment of certain intractable infectious diseases including cryptococcal meningitis complicated in immunodeficient patients.

4. Role of $\gamma\delta$ T cells

4-1. General features of $\gamma\delta$ T cells

Besides conventional T cells bearing TCR $\alpha\beta$, a distinct subset of T cells expressing novel antigen receptors consisting of γ and δ chains, designated as $\gamma\delta$ T cells, was discovered approximately 20 years ago (65). In a sharp contrast to $\alpha\beta$ T cells, which are the major population in lymphoid tissues, such as lymph node and spleen, $\gamma\delta$ T cells are preferentially localized in non-lymphoid tissues, including epidermis, where $\gamma\delta$ T cells are very common as dendritic epidermal T cells, and mucosal/epithelial tissues, such as intestine, lung, tongue, mammary, uterine and vaginal epithelia. Such characteristic localization suggests the role of these cells in a first line host defense against infectious agents and other antigens.

In human, TCR γ gene segments are located on chromosome 7, while TCR δ gene segments are interspersed with TCR α gene on chromosome 14. Before birth, V γ 8 and V γ 9 subsets associate with V δ 2 subset, which distribute to peripheral blood and tonsil. After birth, V γ 2, V γ 3, V γ 4, V γ 5 and V γ 8 gene segments are rearranged and associated with V δ 1 subset, which are found located preferentially in mucosal tissues, such as intestine (65,66). On the other hand, mouse TCR γ gene segments are found on chromosome 13 and TCR δ gene segments are dispersed with TCR α gene on chromosome 14 (65). Similar to human, mouse $\gamma\delta$ T cell

subsets show particular localization patterns. V γ 5, V γ 6 and V γ 4 subsets are formed during gestation and V γ 1 and V γ 7 subsets at birth and shortly thereafter. These subsets are localized in defined anatomical sites: V γ 5 in skin, V γ 6 in uterus and lung, V γ 4 in spleen, lung and tongue, V γ 1 in spleen, and V γ 7 in intestine. In an analysis with quantitative PCR technique, V γ 6 subset is the only $\gamma\delta$ T cells found in lung when mice are born. After birth, however, V γ 4, V γ 5 and V γ 7 subsets were detected and by 2-3 months of age, V γ 4 subset becomes predominant among pulmonary $\gamma\delta$ T cells (66-69).

4-2. Natural ligands

The number of V gene segments of $\gamma\delta$ T cells that determine their diversity is very limited when compared with that in $\alpha\beta$ T cells. In addition, particular subsets are localized in the defined anatomical areas and at the different developmental stages. From these features, the diversity of antigen recognition by $\gamma\delta$ T cells is assumed to be limited in contrast to $\alpha\beta$ T cells that recognize broad spectrum of antigens (65,66). Previous investigations have identified a variety of antigens recognized by these cells from microbial products. Human V γ 9/V δ 2+ $\gamma\delta$ T cells react with low molecular weight nonproteinaceous antigens, such as prenyl pyrophosphate and nucleotide triphosphate from *M. tuberculosis* and alkylamine from *Proteus morganii*, in non MHC-restricted manner (70,71). In addition, protein antigens can be a ligand for the activation of $\gamma\delta$ T cells. Human V γ 9/V δ 2+ $\gamma\delta$ T cells recognize tetanus toxoid in context of MHC class II molecules (72). Mycobacterial heat-shock proteins stimulate both human and mouse $\gamma\delta$ T cells (73-75). However, no ligand of these cells has so far been identified from fungal microorganisms, including *C. neoformans*.

4-3. Significance of $\gamma\delta$ T cells in infectious diseases

From previous observations showing that $\gamma\delta$ T cells accumulate at the sites of infection and chronic inflammation (76), their involvement in regulating the immune response has been suggested. $\gamma\delta$ T cells secrete a variety of cytokines, including TNF- α , GM-CSF, IFN- α , IFN- β , IFN- γ , IL-2, IL-4, IL-5 and IL-10, under particular conditions, such as infection by microorganisms (77-81). Ferrick and co-workers (82) indicated that these cells produce Th1-type cytokines in mice infected with *L. monocytogenes*, while Th2 cytokines in infection with *N. brasiliensis*. Furthermore, some $\gamma\delta$ T cells can express cytolytic activity against infected cells and tumor cells in a perforin and Fas-L-dependent manner (83).

In experimental animal models of infectious diseases, $\gamma\delta$ T cells exert different patterns of influences on the host protection. Manipulations that result in ablation of $\gamma\delta$ T cells, e.g., genetic disruption and treatment with a specific Ab, rendered mice susceptible to infection with *Klebsiella pneumoniae*, *Escherichia coli*, *L. monocytogenes*, *M. tuberculosis*, *L. major* and *T. gondii* (84-91). Interestingly, similar manipulations rather improved the infection caused by some microorganisms (92-94). In chlamydial infection, $\gamma\delta$ T cells showed contrast roles at early and late stages (95). Thus, $\gamma\delta$ T cells seem to act in a complex manner from one microbe to another and in the stage of infection.

4-4. Regulatory role in host defense to cryptococcal infection

In our recent study, the role of $\gamma\delta$ T cells in the development of Th1 response and the host defense against pulmonary infection with *C. neoformans* has been investigated using a mouse model of pulmonary cryptococcosis (96). $\gamma\delta$ T cells quickly increased in a similar kinetics as observed in NK and