The Journal of Immunology

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J Immunol 2001; 167:6525-6532; ;
doi: 10.4049/jimmunol.167.11.6525
http://www.jimmunol.org/content/167/11/6525

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Monocyte Chemoattractant Protein-1-Dependent Increase of Vα14 NKT Cells in Lungs and Their Roles in Th1 Response and Host Defense in Cryptococcal Infection[1]

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To elucidate the role of NKT cells in the host defense to cryptococcal infection, we examined the proportion of these cells, identified by the expression of CD3 and NK1.1, in lungs after intratracheal infection with *Cryptococcus neoformans*. This population increased on day 3 after infection, reached a peak level on days 6–7, and decreased thereafter. In Vα14 NKT cell-deficient mice, such increase was significantly attenuated. The proportion of Vα14 NKT cells, detected by binding to α-galactosylceramide-loaded CD1d tetramer, and the expression of Vα14 mRNA increased after infection with a similar kinetics. The delayed-type hypersensitivity response and differentiation of the fungus-specific Th1 cells was reduced in Vα14 NKT cell-deficient mice, compared with control mice. Additionally, elimination of this fungal pathogen from lungs was significantly delayed in Vα14 NKT cell-deficient mice. Production of monocyte chemoattractant protein (MCP)-1 in lungs, detected at both mRNA and protein levels, increased on day 1, reached a peak level on day 3, and decreased thereafter, which preceded the increase in NKT cells. Finally, the increase of total and Vα14$^+$ subset of NKT cells after infection was significantly reduced in MCP-1-deficient mice. Our results demonstrated that NKT cells, especially Vα14$^+$ subset, accumulated in a MCP-1-dependent manner in the lungs after infection with *C. neoformans* and played an important role in the development of Th1 response and host resistance to this fungal pathogen. *The Journal of Immunology,* 2001, 167: 6525–6532.

Natural killer T cells express not only TCR but also NK markers including NK1.1 (1, 2). Specific features of this cell type include an extremely limited repertoire with an invariant Vα chain consisting of Vα14-Jα281 gene segment and highly skewed Vβ chains, Vβ8.2, Vβ7, and Vβ2 in mice (1, 2). These cells are found in large numbers in the liver, thymus, and bone marrow and in small numbers in the spleen and lung (2). Although the natural ligand for NKT cells remains to be defined, GPIs and a synthetic glycolipid, α-galactosylceramide (α-GalCer),[3] have been demonstrated to be presented in the context of CD1d (1–4). NKT cells secrete large amounts of IFN-γ and IL-4 in a prompt manner after engagement of the Ag receptor (1, 2, 4, 5) and contribute to the differentiation of both Th1 and Th2 cells (1, 2, 6–10). It has been reported that NKT cells play an important role in various aspects of the regulation and effector arms of the immune response, including the regulation of allergic and autoimmune diseases (11–14) and prevention of tumor metastasis (15–18).

The role of NKT cells in host defense to infectious pathogens has been elucidated by several investigators, and three different results were reported. First, the clinical course of *Mycobacterium tuberculosis* infection is not much different in CD1d-deficient and control mice, the former of which lack NKT cells (19), and is minimally affected by treatment with anti-CD1d mAb (20). Similarly, genetic depletion of Vα14 NKT cells did not result in worsening of *Salmonella choleraesuis* infection (21). Second, infection with *Listeria monocytogenes* or *Toxoplasma gondii* improved by manipulations designed to suppress the activity of NKT cells (22, 23). Finally, mice lacking Vα14 NKT cells were more susceptible to *Leishmania major* infection than were control mice (24). A ligand-specific activation of Vα14 NKT cells by α-GalCer has been shown to protect mice against protozoan and fungal infections (25, 26). Thus, the role of NKT cells seems different among infectious pathogens.

Cryptococcus neoformans causes granulomatous lesions in the lungs, a primary infected organ, and hematogenously disseminates to the central nervous system, frequently leading to lethal meningoencephalitis, particularly in AIDS patients. The host defense against this pathogen is critically regulated by cell-mediated immunity (27), and CD4$^+$ T cells play a central role in limiting infection (28, 29). The balance between Th1 and Th2 cytokines markedly influences the outcome of infection; the predominant synthesis of Th1 cytokines over Th2 protects mice against infection (30, 31), whereas infection is exacerbated under Th2-dominant conditions (32, 33). In recent studies (31), targeted disruption of the gene for IL-12 or IL-18, both of which play important roles in the differentiation of Th1 cells and IFN-γ synthesis by T and NK cells, resulted in reduced host resistance and Th1 response to *C. neoformans*. However, the role of NKT cells in the development of

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Received for publication July 19, 2001. Accepted for publication October 5, 2001.

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[1] This work was supported in part by a grant-in-aid for science research (C12670261) from the Ministry of Education, Science and Culture, Japan, and by grants from the Ministry of Health and Welfare, Japan.

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[3] Abbreviations used in this paper: α-GalCer, α-galactosylceramide; DTH, delayed-type hypersensitivity; WT, wild type; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein.

0022-1767/01/$02.00

fungus-specific Th1 cells and host defense remains to be elucidated, although α-GalCer-induced activation of NKT cells resulted in the protection of mice against this infection (26).

In the present study, the role of Vα14 NKT cells in Th1 response and host resistance to *C. neoformans* infection was elucidated. For this purpose, we examined the kinetics of accumulation of this subset in the lungs after intratracheal inoculation and the effects of Vα14 NKT cell deficiency on the development of Th1 cells and delayed-type hypersensitivity (DTH) response and elimination of microorganisms from infected organ. We further addressed the mechanism of NKT cell increase in lungs after intratracheal infection with *C. neoformans* by testing the role of monocyte chemoattractant protein (MCP)-1, a β-chemokine.

Materials and Methods

Animals

Vα14 NKT cell-deficient mice were established by targeted deletion of the Jα281 gene segment (34) and back-crossed eight times with C57BL/6 mice. MCP-1-deficient mice with a genetic background of C57BL/6 mice (35) 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 Breeding Laboratories (Osaka, Japan) and used as a control wild-type (WT) animal. All mice were used at 8–15 wk of age. All experimental protocols described in the present study were approved by the Ethics Review Committee for Animal Experimentation of University of the Ryukyus.

Microorganisms

A serotype A-encapsulated strain of *C. neoformans*, designated as YC-13, was established from a patient with pulmonary cryptococcosis (36). Infection with this pathogen was self-limited in the lungs of WT mice and did 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 per 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 (37). Briefly, the chest of the mouse was opened and the lung vascular bed was flushed by injecting 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 (Life Technologies, Grand Island, NY) 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 the 50-μm nylon mesh. After centrifugation, the cell pellet was resuspended in 4 ml of 40% (v/v) Percoll (Pharmacia Biotech, 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 contain lymphocytes, macrophages, and neutrophils.

Flow cytometric analysis

The following Abs were used for flow cytometry: FITC-conjugated anti-CD3 and PE-conjugated anti-NK1.1 mAbs (clones 145-2C11 and 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 as a control staining. In some experiments, cells were stained with PE-conjugated α-GalCer-loaded or unloaded CD1d tetramer (kind gifts of M. Kronenberg, La Jolla Institute for Allergy and Immunology, San Diego, CA) (38). The stained cells were analyzed using a EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA). Data were collected from 15,000–20,000 individual cells using parameters of forward scatter and side scatter to set a gate on lymphocyte population.

Extraction of RNA and RT-PCR

Total cellular RNA was extracted from the lungs after various time periods of infection using Isogen (Wako Pure Chemical, Osaka, Japan) followed by reverse transcription (30). The obtained cDNA was then amplified in an automatic DNA thermal cycler (PerkinElmer/Cetus, Norwalk, CT) using specific primers 5′-TCCATGCAGGTCCCTGTCATGCTT-3′ (sense) and 5′-CTAGTTCACTGTCACACTGGTC-3′ (antisense) for MCP-1, 5′-CTAAGCACAGCACGCTGCACA-3′ (sense) and 5′-AGGTATGACAATCAGCTGAGTCCC-3′ (antisense) for TCR Vα14, and 5′-GTTGGATACAGGCCAAGACTTTGTTG-3′ (sense) and 5′-GATTCAACTTGCGCTCATCTTAGGC-3′ (antisense) for hypoxanthine phosphoribosyl transferase (HPRT). We added 1.0 μl of the sample cDNA solution to 49 μl of the reaction mixture, which contained the following concentrations: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 10 μg/ml gelatin, dNTP (each at a concentration of 200 μM), 1.0 μM sense and antisense primer, and 1.25 U of AmpliTaq DNA polymerase (PerkinElmer/Cetus). The mixture was incubated for 1 min at 94°C, 1 min at 62°C, and 1 min 45 s at 72°C for MCP-1 and incubated for 1 min at 94°C, 1 min at 54°C, and 1 min 30 s at 72°C for HPRT. The number of cycles was determined for samples not reaching the amplification plateau (28 cycles for HPRT and 32 cycles for MCP-1). For TCRVα14, the samples were heated at 94°C for 5 min and then subjected to 35 cycles of incubation for 45 s at 94°C, 45 s at 55°C, and 90 s at 72°C, followed by a final extension for 7 min at 72°C. The PCR products were electrophoresed on 2% agarose gels, stained with 0.5 μg/ml ethidium bromide, and observed with a UV transilluminator. The obtained bands of amplified DNA were quantitated using NIH Image software (version 1.61; National Institutes of Health, Bethesda, MD), and the production of Vα14 mRNA was expressed as a relative value to that of HPRT mRNA.

In vitro stimulation of lung lymphocytes

Pulmonary leukocytes were prepared from three mice on day 7 after infection with *C. neoformans* and cultured at 4×10^5/well in flat-bottom microculture plates (Falcon no. 3072; BD Biosciences, Franklin Lakes, NJ) with various doses of viable organisms for 48 h. The culture supernatants were collected and measured for the concentration of IFN-γ and IL-4 using ELISA kits.

Measurement of cytokines

Murine IFN-γ, IL-4, and MCP-1 were measured by respective ELISA kits (Endogen, Cambridge, MA, for IFN-γ and IL-4; and Toyobo, Osaka, Japan, for MCP-1). The detection limits of assays for IFN-γ, IL-4, and MCP-1 were 15, 5, and 9 pg/ml, respectively.

DTH response

Seven days after pulmonary infection, 1×10^6 yeast cells of *C. neoformans* (50 μl) were injected into the left hind foot pads of the mice and the same volume of PBS was injected into the right hind foot pads. The foot pad swelling was blindly measured 24 h later and expressed as the difference of thickness between left and right foot pads.

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 potato dextrose agar plates and cultured for 2–3 days, followed by counting the number of colonies.

Statistical analysis

Analysis of data was conducted using StatView, Japanese version (Hulinks, Tokyo, Japan), on a Macintosh computer (Apple Computer, Cupertino, CA). Data are expressed as mean $\pm$ SD. Differences between groups were examined for statistical significance using one-way ANOVA with a posthoc analysis (Fisher PLSD test). A p value < 0.05 was considered significant.

Results

Accumulation of Vα14 NKT cells in the lungs after infection with C. neoformans

Initially, we elucidated whether NKT cells accumulated in the lungs after infection with *C. neoformans* by determining the proportion of these cells, identified as a lymphocyte subset double positive for CD3 and NK1.1, among lung parenchymal leukocytes

obtained from mice infected intratracheally with this pathogen. As shown in Fig. 1*A*, NKT cells formed only 0.5–0.7% of the lung lymphocytes before infection, but their proportion commenced to increase on day 1, reached a peak level on day 7, and then decreased on day 14 postinfection. During the observation period, the proportions of NK and T cells, identified as lymphocyte subsets positive for either NK1.1 or CD3, respectively, increased with similar kinetics as in NKT cells, although such increase was less pronounced than in the latter cells (Fig. 1*B*). The absolute number of NKT cells also markedly increased with a peak level on day 6 postinfection (Fig. 1*C*).

To define the contribution of Vα14$^+$ subset to the increase of NKT cells, we compared the kinetics in the expansion of

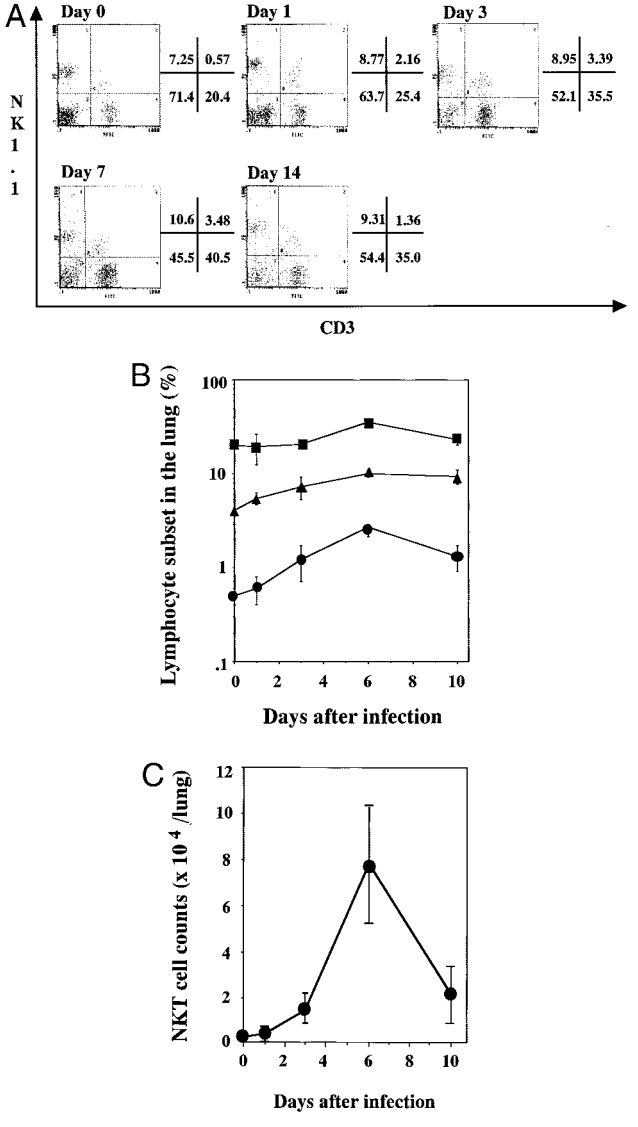

FIGURE 1. Increase of NKT cells in lungs after *C. neoformans* infection. *A*, WT mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The lung leukocytes were prepared and stained with FITC-anti-CD3 and PE-anti-NK1.1 mAbs before (0 day) and 1, 3, 7, and 14 days after infection. The lymphocyte population was analyzed by flow cytometry. The number in each quadrant represents the percentage of each subset. *B* and *C*, Similar experiments were conducted and the lung leukocytes were prepared before (0 day) and 1, 3, 6, and 10 days after infection. The percentages of NKT (●), NK (▲), and T cells (■) in lymphocyte population were analyzed (*B*), and the absolute number of NKT cells was calculated (*C*). Each symbol represents the mean ± SD of four mice.

NK1.1$^+$CD3$^+$ cells in the lungs after infection with *C. neoformans* between Vα14 NKT cell-deficient and WT mice. As shown in Fig. 2*A*, the proportion of NKT cells increased even in Vα14 NKT cell-deficient mice, with a peak level on day 6, although such accumulation was significantly lower on day 6 compared with the control mice. These results suggested increased proportion of Vα14 NKT cells in infected lungs. To confirm this conclusion, two different experiments were conducted. First, using the RT-PCR method, we examined the expression of Vα14 mRNA in the lungs during the course of infection. As shown in Fig. 2, *B* and *C*, such expression was weak in uninfected lungs, while a significant increase of Vα14 mRNA was observed on days 3 and 6 after infection with *C. neoformans*. Second, α-GalCer-reactive cells were examined by detecting the cells bound to fluorescence-labeled α-GalCer-loaded CD1d tetramer, which has been reported to bind to Vα14 NKT cells in a specific manner (38). Significant increase of positive cells was observed in the lungs with a similar kinetics as in NKT cells postinfection, while there was no change in the proportion of cells bound to α-GalCer-unloaded CD1d tetramer (Fig. 2, *D* and *E*).

Reduced Th1 response and host defense to C. neoformans *in Vα14 NKT cell-deficient mice*

Previous studies demonstrated the involvement of Vα14 NKT cells in both Th1 and Th2 responses by producing large amounts of IFN-γ and IL-4 upon stimulation through Ag receptors (1, 2, 4, 5), but the role of these cells in host defense to infection remains to be fully understood (19–26). Therefore, we elucidated the role of Vα14 NKT cells in Th1 response and host defense in cryptococcal infection. In the first step, we compared the concentration of IFN-γ in the culture supernatants of lung lymphocytes derived from infected WT and Vα14 NKT cell-deficient mice upon stimulation with various doses of live microorganisms. The proportion of T cells was not different in the two strains of mice (38.3 ± 0.4% vs 38.9 ± 4.3% in WT and Vα14 NKT cell-deficient mice, respectively). As shown in Fig. 3, production of IFN-γ by lung lymphocytes was significantly lower in Vα14 NKT cell-deficient mice compared with WT mice. In contrast, there was no significant difference in the production of IL-4 by restimulated lung lymphocytes between these mice (data not shown). Second, we examined the DTH response to *C. neoformans* by measuring the foot pad swelling of mice rechallenged with Ags 7 days after infection. As shown in Fig. 4, such response was strongly reduced in Vα14 NKT cell-deficient mice compared with WT mice. Finally, to test the role of Vα14 NKT cells in host defense against infection with *C. neoformans*, the clinical course of this infection was compared between Vα14 NKT cell-deficient and WT mice by measuring the number of live microorganisms in lungs. As shown in Fig. 5, elimination of *C. neoformans* was significantly delayed in the former type of mice compared with that in the latter.

Contribution of MCP-1 to NKT cell accumulation in the lungs after infection with C. neoformans

Several investigators previously demonstrated the involvement of chemokines in the trafficking of NK cells to the sites of inflammation. These chemokines included α-chemokines such as IP-10 (39, 40), β-chemokines including MCP-1, -2, -3, macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (40–43), and γ-chemokines such as lymphotactin (39, 44). In the present study, we focused on MCP-1 to elucidate the regulatory mechanism for NKT cell accumulation in infected organs. In the initial experiment, we examined the kinetics in the production of this chemokine at both mRNA and protein levels in the lungs after infection with *C. neoformans*. As shown in Fig. 6*A*, only a weak

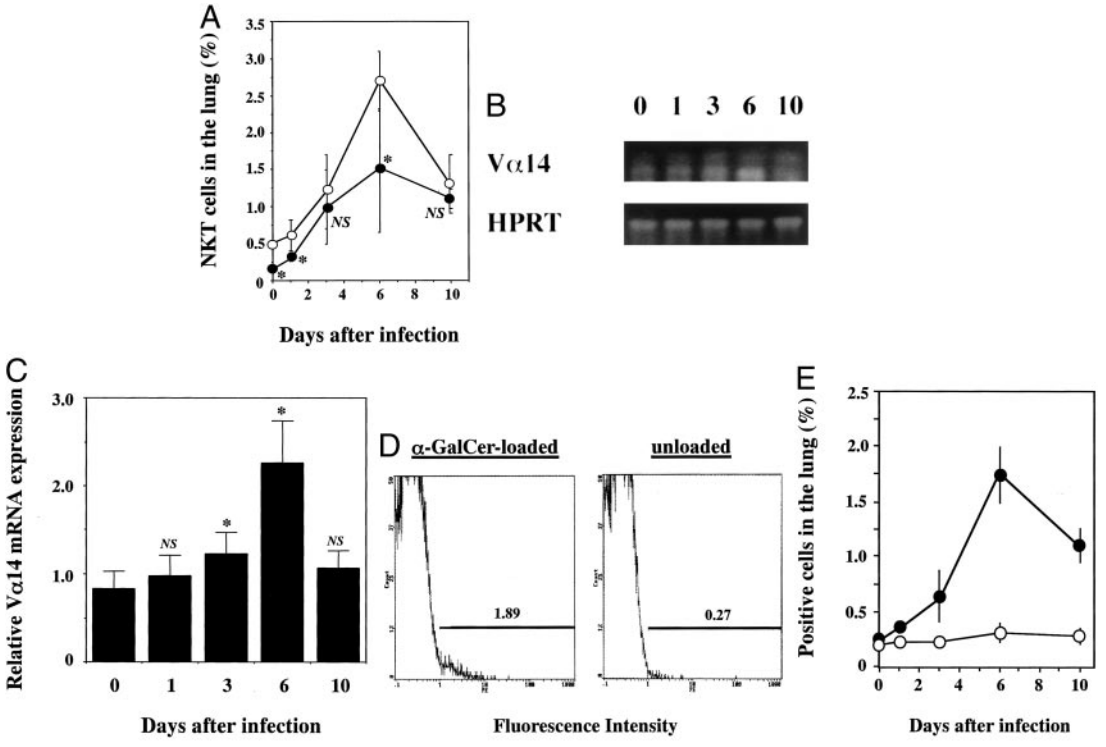

FIGURE 2. Increase of Vα14 NKT cells in lungs after *C. neoformans* infection. *A*, WT and Vα14 NKT cell-deficient mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The lung leukocytes were prepared and stained with FITC-anti-CD3 and PE-anti-NK1.1 mAbs before (0 days) and 1, 3, 6, and 10 days after infection. The lymphocyte population was analyzed by flow cytometry. Each symbol represents the mean ± SD of NKT cell proportion in four mice. Open circles, WT mice; closed circles, Vα14 NKT cell-deficient mice. *NS*, not significant; ∗, $p < 0.05$, compared with WT mice. *B* and *C*, Total RNA was extracted from lung homogenates of WT mice before (0 day) and 1, 3, 6, and 10 days after infection. RT-PCR was conducted to examine the expression of Vα14 and HPRT mRNA. The obtained bands were quantitated and the production of Vα14 mRNA was expressed as a relative value to that of HPRT mRNA *C*, Each bar represents the mean ± SD of four mice. *NS*, not significant; ∗, $p < 0.05$, compared with uninfected (day 0) mice. *D* and *E*) Lung leukocytes were prepared from WT mice before (0 day) and 1, 3, 6, and 10 days after infection and stained with α-GalCer-loaded (●) or unloaded CD1d (○) tetramer. The lymphocyte population was analyzed by flow cytometry. Each histogram shows the representative staining profile of four mice on day 6 postinfection. The number described in the histogram represents the percentage of positive cells (*D*). Each symbol represents the mean ± SD of four mice (*E*).

band of MCP-1 mRNA was identified in the uninfected lungs and the expression commenced to increase on day 1, reached a peak level on day 3, and then decreased on days 6–10. A similar kinetics was observed in MCP-1 protein synthesis in the lungs during the course of infection (Fig. 6*B*). These results indicated the production of MCP-1 preceded the increase of NKT cells in the lung and suggested that this chemokine may be involved in the accumulation of NKT cells in the lungs after infection with *C. neoformans*.

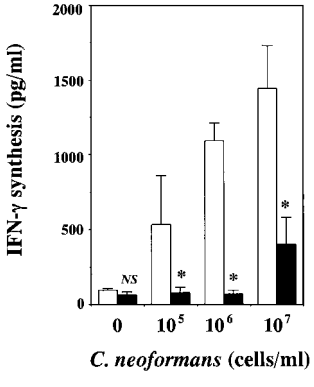

FIGURE 3. Reduced Th1 cell development in the lungs of Vα14 NKT cell-deficient mice. WT (open bar) and Vα14 NKT cell-deficient mice (filled bar) were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The lung leukocytes were prepared from three mice and cultured at 4×10^5/well in flat-bottom microculture plates with indicated doses of live microorganisms for 48 h, and the concentration of IFN-γ was measured. Each bar represents the mean ± SD of triplicate cultures. Experiments were repeated three times with similar results. *NS*, not significant; ∗, $p < 0.05$, compared with WT mice.

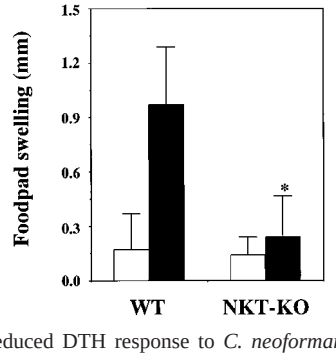

FIGURE 4. Reduced DTH response to *C. neoformans* in Vα14 NKT cell-deficient mice. WT and Vα14 NKT cell-deficient mice were instilled intratracheally with *C. neoformans* (1×10^6/mouse) or normal saline. The DTH response was examined on day 7 after instillation. Each bar represents the mean ± SD of six mice. Open bar, uninfected; filled bar, infected. NKT-KO, Vα14 NKT cell-deficient mice. ∗, $p < 0.05$, compared with WT mice.

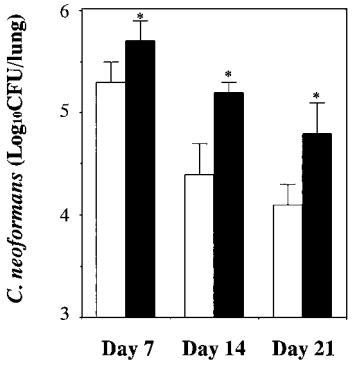

FIGURE 5. Impaired host defense to *C. neoformans* in Vα14 NKT cell-deficient mice. WT (open bar) and Vα14 NKT cell-deficient mice (filled bar) were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The number of live colonies in lung was examined on days 7, 14, and 21 after infection. Each bar represents the mean ± SD of six mice. *, $p <$ 0.05, compared with WT mice.

To confirm this conclusion, we compared the proportion of NKT cells in the lungs after infection with *C. neoformans* in MCP-1-deficient and WT mice. As shown in Fig. 7*A*, the increase of NKT and NK cells in lungs caused by infection was significantly less marked in MCP-1-deficient mice than in WT mice on days 6 and 10 and days 1, 3, 6, and 10, respectively, while such difference was not detected when the numbers of conventional T cells were examined (data not shown). The absolute number of NKT and NK cells in lungs was also significantly lower in MCP-1-deficient mice than in control on days 3, 6, and 10 postinfection (Fig. 7*B*). In addition, we examined the effect of MCP-1 expression on the increase of Vα14 NKT cells by measuring the number of α-GalCer-loaded CD1d tetramer-binding cells on day 6 postinfection. As shown in Fig. 7*C*, the increase of Vα14 NKT cells caused by infection was significantly less pronounced in MCP-1-deficient mice compared with WT mice.

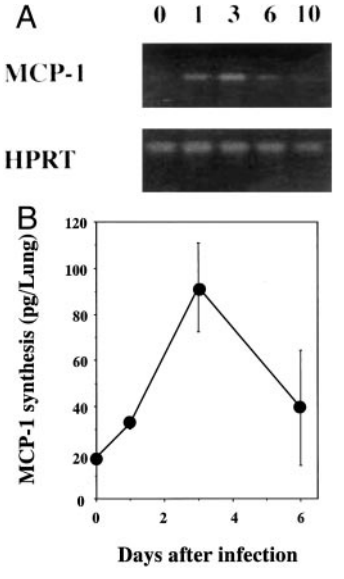

FIGURE 6. Production of MCP-1 in lungs after infection with *C. neoformans*. *A*, WT mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The total RNA was extracted from the lung homogenates before (0 day) and 1, 3, 6, and 10 days after infection. RT-PCR was conducted to examine the expression of MCP-1 and HPRT mRNA. *B*, In similar experiments, the concentration of MCP-1 in lung homogenates obtained before (0 day) and 1, 3, and 6 days after infection was measured. The results are expressed as the total amount per lung. Each symbol represents the mean ± SD of six mice.

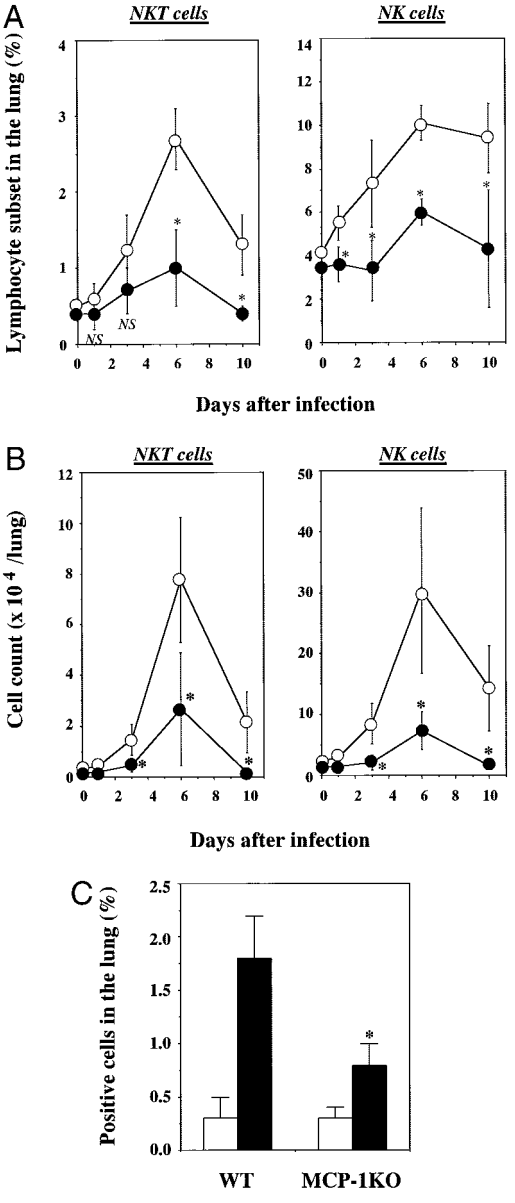

FIGURE 7. MCP-1-dependent increase of NKT cells in lungs after infection with *C. neoformans*. *A* and *B*, WT (open circles) and MCP-1-deficient (closed circles) mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The lung leukocytes were prepared and stained with FITC-anti-CD3 and PE-anti-NK1.1 mAbs before (0 day) and 1, 3, 6, and 10 days after infection. The percentages of NKT and NK cells in the lymphocyte population were analyzed by flow cytometry (*A*), and the absolute number of each subset was calculated (*B*). Each symbol represents the mean ± SD of four mice. *C*, In similar experiments, the lung leukocytes obtained on day 6 after infection were stained with α-GalCer-loaded (filled bar) or unloaded CD1d (open bar) tetramer, and the lymphocyte population was analyzed by flow cytometry. Each bar represents the mean ± SD of four mice. MCP-1KO, MCP-1-deficient mice. *NS*, not significant; *, $p <$ 0.05, compared with WT mice.

Discussion

To understand the role of NKT cells in host defense to *C. neoformans* infection, we examined the kinetics of Vα14 NKT cells in lungs after infection with this pathogen and the role that NKT cells

play in the induction of immunity to cryptococcal infection. Furthermore, we elucidated the effect of MCP-1 on Vα14 NKT cells at the site of infection using mice with a gene disruption for this chemokine. The major findings of this study were 1) Vα14 NKT cells increased in the lungs after intratracheal inoculation of *C. neoformans*, 2) Vα14 NKT cells played an important role in the induction of Th1 response and host resistance to this pathogen, and 3) MCP-1 was involved in the recruitment of Vα14 NKT cells to the lungs after *C. neoformans* infection.

Several studies have recently demonstrated the expansion of NKT cells during infection. In leishmanial infection, the proportion of NKT cells, as identified by double staining with anti-CD3 and anti-IL-2Rβ mAbs, increased in regional lymph nodes after s.c. infection in resistant, but not susceptible, mice (24). In another study, Pied et al. (45) revealed the expansion of NKT cells, especially $CD4^-CD8^-$ double negative subset, in the liver of mice after infection with *Plasmodium yoelii*. Interestingly, $CD4^+$ NKT cells were rather decreased in these mice (45). Similar results were reported by Weerasinghe and coworkers (46). In the present study, the major subset of expanding NKT cells during cryptococcal infection was negative for the expression of both CD4 and CD8 (data not shown). We also showed the expansion of Vα14 NKT cells, as identified by specific binding of α-GalCer-loaded CD1d tetramer and expression of Vα14 mRNA, along with an increase in the total number of NKT cells. In malaria infection, one study indicated increased expression of Vβ7 and Vβ2 gene segments, which have been known to form semi-invariant $\alpha\beta$ TCRs with Vα14 (45), while another study showed a reduction in the expression of Vα14 mRNA (46). These observations suggested a regulatory role for $V\alpha14^+$ in addition to other subsets of NKT cells in the early host defense to infection, although their contribution might be distinct among infectious pathogens.

In earlier studies, Yoshimoto and coworkers (47) demonstrated that activation of NKT cells by in vivo administration of anti-CD3 mAb resulted in a rapid production of IL-4 and proposed that this population may be the major source of early IL-4 production which contributes to the differentiation of Th2 cells. However, several studies subsequently indicated that Th2 response was not hampered in β_2-microglobulin- or CD1d-deficient mice, which have markedly reduced numbers of NKT cells (48–51). Although these findings made the role of these cells in Th2 cell development obscure, such possibility was not completely excluded. For example, Vα14 TCR-transgenic mice showed elevated serum levels of IgE and IL-4 (52), and activation of Vα14 NKT cells by α-GalCer induced T cell response to OVA polarized toward Th2-dominant condition (9). In contrast, accumulating evidence emphasizes the positive role of NKT cells in the development of Th1 cells. Activation of Vα14 NKT cells by α-GalCer led to the rapid production of IFN-γ by themselves and other bystander cells, such as NK cells, in vitro (53), and suppressed in vivo the Th2 differentiation and subsequent IgE synthesis induced by OVA immunization or infection with *Nippostrongylus brasiliensis* through the induction of IFN-γ production (7). In other studies, NKT cells were found to contribute to Th1-mediated responses, including granuloma formation caused by mycobacterial lipid Ag (54) and the IFN-γ-mediated protection of mice against infection with malaria parasites (25) and *C. neoformans* (26) through the ligand-specific activation of NKT cells by α-GalCer. In the present study, we demonstrated that Vα14 NKT cells played a critical role in the induction of Th1 response, such as IFN-γ production and DTH response, during cryptococcal infection. Thus, NKT cells are likely to contribute to the development of both Th1 and Th2 responses under distinct situations. Further studies are necessary to clarify the precise mechanism of this regulation.

Several studies addressed the role of NKT cells in the host defense to infectious pathogens. Behar and coworkers (19) compared the clinical course of *M. tuberculosis* infection between CD1d-deficient and control mice. They indicated that the lack of CD1d expression, leading to deficiency of NKT cells, did not result in exacerbation of mycobacterial infection. In another study, a significant but small inhibition of host resistance to the same pathogen was observed in mice treated with anti-CD1d mAb (20). However, these authors concluded that NKT cells had a minimal contribution, if any, to the host defense against mycobacterial infection (55). Similar results were also reported in *S. choleraesuis* infection by Ishigami et al. (21); that is, the clinical course of this infection was not different between Vα14 NKT cell-deficient and control mice, although the hepatic injury was reduced in the former mice (21). Szalay et al. (22) reported that treatment with anti-CD1d mAb led to a transient amelioration of infection with *L. monocytogenes* through enhanced production of Th1-type cytokines. Similarly, depletion of NKT cells by anti-IL-2Rβ mAb treatment, but not of NK cells by anti-asialo GM1 Ab, resulted in the protection of mice against *T. gondii* infection, suggesting the negative regulatory role of NKT cells in host defense to this pathogen (23). In contrast, the recent studies of Ishikawa and colleagues (24) showed opposite results; Vα14 NKT cells played a crucial role in the early stage of protective immunity against *L. major* infection. Thus, the role of NKT cells varies depending on the infectious pathogen. Our study demonstrated the protective role of these cells in the host defense against infection with *C. neoformans*. At present, the precise mechanism of their different roles among infectious pathogens remains to be elucidated, and further studies will be required to determine the ligand of pathogens responsible for activation of NKT cells before understanding such a mechanism.

There are two possible mechanisms for the increase of Vα14 NKT cells in lungs after infection with *C. neoformans*: 1) local growth at the infected sites and 2) recruitment from the peripheral circulation. IL-15 is known to act as a major growth factor for NKT cells, because mice deficient of IL-15Rα or IL-2/IL-15Rβ lacked such cells (56). In the present study, we did not examine the expression of this cytokine at the site of infection and its contribution to the increase of Vα14 NKT cells. Thus, the first mechanism remains open for further study. In contrast, migration of NKT cells into the lungs is a plausible mechanism, because we found that NKT cells did not migrate to lungs of MCP-1-deficient mice in response to cryptococcal infection. Compatibly, the expression of MCP-1 mRNA preceded the increase of NKT cells after infection. In addition, we also identified accumulation of NKT cells in the lungs after intratracheal instillation of rMCP-1 (our unpublished data). To our best knowledge, this is the first report showing the involvement of MCP-1 in the in vivo accumulation of NKT cells, although MIP-2 has been recently demonstrated as a chemoattractant for these cells (57). At the same time, we also demonstrated the contribution of MCP-1 to the recruitment of NK cells to the lungs infected with *C. neoformans*. Similarly, other investigators have reported the ability of this chemokine to attract resting or activated NK cells under various conditions (41–43). Other chemokines, including MCP-2 and -3, MIP-1α, RANTES, IP-10, and lymphotactin, were also identified as trafficking chemokines of NK cells (39–44), suggesting that these chemokines may act on NKT cells in addition to MCP-1.

In conclusion, we demonstrated in the present study the MCP-1-dependent increase of Vα14 NKT cells in the lungs after infection with *C. neoformans* and their important roles in the development of Th1 response and host resistance to this infection. The natural ligand of NKT cells remains to be elucidated, although

some glycolipids, such as GPIs, represent a potential candidate (3). At present, it is not clear whether this fungal pathogen produces glycolipids recognized by NKT cells. In this regard, Doering and coworkers (58) recently provided evidence indicating the secretion of GPIs from *C. neoformans*, as previously reported in malaria parasite and *Trypanosoma brucei* (59). To understand the precise mechanism of how NKT cells are involved in the host defense to this infection, identification of pathogen-derived ligands of these cells is desired.

Acknowledgments

We thank Dr. B. J. Rollins (Harvard Medical School) for the kind gift of MCP-1-deficient mice and Dr. M. Kronenberg (La Jolla Institute for Allergy and Immunology) for the kind gift of α-GalCer-loaded and unloaded CD1d tetramers.

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