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REVIEW ARTICLE

Kazuyoshi Kawakami

Promising immunotherapies with Th1-related cytokines against infectious diseases

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Abstract In recent years, there has been an increase in the number of individuals with compromised immune systems. This is due to the rise in the numbers of aging people, patients receiving immunosuppressive treatment after organ transplantation, patients with hematological malignancies, and patients with AIDS. These individuals frequently fall into helper T cell (Th)1–Th2 cytokine imbalance due to a shift towards a Th2-dominant condition. Such a pathological condition puts them at a high risk for developing infectious diseases caused by a variety of microbial pathogens which are often refractory to conventional chemotherapy. Therefore, the administration of Th1-related cytokines is expected to be promising immunotherapy against these intractable infectious diseases. In a series of investigations, we have demonstrated the effectiveness of treatment with Th1-related cytokines, such as interferon (IFN)-γ, interleukin (IL)-12, and IL-18, in protecting animals from experimental infectious diseases caused by *Mycobacterium tuberculosis* and *Cryptococcus neoformans*. Recently, several investigators reported successful clinical treatment with IFN-γ or IL-12 in patients with intractable tuberculous and nontuberculous mycobacteriosis. Thus, now is an appropriate time for scientific evaluation to clinically confirm the effectiveness of these novel immunotherapies.

Key words Immunotherapy · Th1–Th2 cytokine balance · IFN-γ · IL-12 · IL-18, infectious disease

Introduction

In recent years, circumstances in infectious diseases have shown considerable changes. In the twentieth century, a variety of antimicrobial agents were exploited after the discovery of penicillin by Fleming in 1928. Because of these successes, many clinicians temporarily felt that infectious diseases had already been overcome. However, many problems, including emerging and re-emerging infectious diseases, antibiotic-resistant infectious diseases, and hospital-acquired infectious diseases, have been manifested since the 1980s, most of which have been carried over to the new century. In particular, since the 1990s, various drug-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, β-lactamase nonproducing ampicillin-resistant *Hemophillus influenzae*, extended-spectrum β-lactamase-producing bacteria, metallo-β-lactamase-producing bacteria, multidrug-resistant *Pseudomonas aeruginosa*, vancomycin-resistant *S. aureus*, and multidrug-resistant *Mycobacterium tuberculosis*, have emerged, and some of them have posed a clinical threat. In addition, recently, the numbers of immunocompromised hosts have increased with the aging of populations, progress in organ transplantation, and with the spread of the acquired immune deficiency syndrome (AIDS), which has made opportunistic infections, as intractable diseases, an important clinical problem.

On the other hand, studies in infection and immunity have developed with rapid progress in molecular biology since the mid-1980s, and these have enabled the host defense mechanism against infectious pathogens to be understood at a molecular level, with the understanding of cytokines, chemokines, adhesion molecules, and intracellular signal transducing molecules. In addition to acquired immune responses, a central thesis of immunology, innate immune responses have been well investigated since the discovery of Toll-like receptors, γδ T cells, natural killer T (NKT) cells, and B1-B cells. Furthermore, the Th1–Th2 theory proposed by Mosmann and co-workers[1] was proved true in human as well as mouse immune systems, and extensive studies of its significance in the development of diseases have been conducted in the fields of infection, tumor surveillance, allergy, and so on.

Against this background, new developments have been progressing in vaccine and immunotherapy against intrac-

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table infectious diseases, and such progress has led to these areas becoming more practical. In this review, the significance of the Th1–Th2 cytokine balance in the host defense mechanism against infection is described, and the possibility of immunotherapy with Th1-related cytokines in intractable infectious diseases is discussed.

Infection and Th1–Th2 balance

Th1 and Th2 cells

Mosmann and co-workers[1] found that longterm cultured helper T (Th) cells in mice were of two different types, Th1 and Th2 cells, characterized on the basis of cytokine production. Th1 cells secrete interleukin (IL)-2, interferon (IFN)-γ, and lymphotoxin (LT), but not IL-4, IL-5, IL-10, and IL-13, while Th2 cells are characterized by the opposite pattern of cytokine synthesis. Naïve Th cells differentiate into each subset under particular microenvironments after activation through their antigen-specific receptors: Th1 cells differentiate in the presence of IL-12 secreted by dendritic cells (DC), while Th2 cells differentiate under the influence of IL-4 secreted by NKT cells, basophils, eosinophils, and mast cells. IL-18, IFN-γ-inducing factor, potentiates the differentiation of Th1 cells caused by IL-12,[2] although the same cytokine is reported to contribute to the development of Th2 cells.[3,4] More importantly, these polarized subsets of Th cells are elegantly related to their functional properties in the immune responses: Th1 cells play a central role in the development of cell-mediated immunity and host defense against intracellular bacteria, protozoa, and some fungal pathogens,[1,5-7] while Th2 cells are a prerequisite for IgE synthesis and activation of eosinophils, and therefore play important roles in the host defense against helminthic infection and the development of allergic diseases.[1,8]

Role of Th1-related cytokines in the host defense against infection

In an early study by Heinzel and co-workers,[9] it was demonstrated that IFN-γ protected mice from infection with *Leishmania major*, while IL-4 exacerbated the same infection. Since this important observation, a novel idea has been proposed, that a balance between Th1 and Th2 cytokine production determines the fate of infection.

Recently, we have elucidated the role of Th1-related cytokines, such as IFN-γ, IL-12, and IL-18, in the host defense against infection with *Mycobacterium tuberculosis* and *Cryptococcus neoformans*, using mice genetically lacking the genes for these cytokines. Cell-mediated immunity is essential for eradicating *M. tuberculosis*, a prototypic intracellular pathogen. Although *C. neoformans* was originally recognized as an extracellular infectious pathogen, evidence has been accumulated showing its intracellular parasitism within macrophages.[10] For both microorganisms, the infection was exacerbated in mice with a genetic disruption of IFN-γ, IL-12, or IL-18, and such exacerbation was greater with IFN-γ and IL-12 than with IL-18.[11,12] On the other hand, treatment with neutralizing anti-IL-4 antibody rendered mice more resistant to cryptococcal infection than treatment with control IgG, indicating the negative regulation of the host defense mechanism by Th2 cytokines.[13]

However, it has been unclear whether the Th1–Th2 theory established in mice could be applied in humans. Several investigations indicated that IFN-γ did not potentiate, but rather, suppressed the bactericidal activity against *M. tuberculosis* of human macrophages obtained from peripheral blood monocytes, although this cytokine was known to activate mouse macrophages.[14,15] The discovery of patients with abnormalities in the genes for IFN-γ receptor, IL-12, and IL-12 receptor resolved this issue: these patients were highly susceptible to infection with intracellular infectious pathogens, such as acid-fast bacilli, *Salmonella*, and *Legionella*.[16] Thus, the importance of Th1-related cytokines in the host defense was substantiated in humans as well as in mice.

Increasing numbers of clinical studies have indicated the relationship between the Th1–Th2 cytokine balance and susceptibility to infection. In Table 1, factors promoting the onset and aggravation of tuberculosis are indicated, most of which are suggested to be associated with an imbalance in the production of Th1 and Th2 cytokines. Previous studies pointed out that many tuberculosis patients had diabetes mellitus and that these patients were prone to the exacerbation and recurrence of tuberculosis. In an earlier study, Saiki and co-workers[17] reported using a mouse model of diabetes mellitus in which this condition resulted in impaired cellular immune responses to *M. tuberculosis*. Mencacci et al.[18] demonstrated, using a similar model, that a condition with high blood glucose caused a Th2-dominant condition of the Th1–Th2 cytokine balance, which resulted in increased susceptibility to *Candida albicans* infection. Long term treatment with glucocorticoid causes various opportunistic infections by reducing the host resistance to infection. Glucocorticoid is well known to impair the migration, phagocytosis, and bactericidal activity of neutrophils and to show a strong suppression of cell-mediated immune responses. Recent studies have reported that this immunosuppressive agent shifted the Th1–Th2 cytokine balance

Table 1. Factors aggravating tuberculosis

1. General
   (1) Sex, (2) age, (3) ethnic group, (4) malnutrition, (5) alcoholism
2. Pathological conditions
   (1) Diabetes mellitus
   (2) Chronic renal failure (hemodyalysis)
   (3) Hematological malignancies: leukemia, malignant lymphoma
   (4) Malignant diseases (solid tumors)
   (5) Congenital immune deficiencies
   (6) Acquired immune deficiency syndrome (AIDS)
3. Iatrogenic
   (1) Longterm treatment with glucocorticoid
   (2) Immunosuppressive treatment after organ transplantation
   (3) Anticancer chemotherapy
   (4) Irradiation

toward a Th2-dominant condition through the specific inhibition of Th1 cytokine production.[19,20] In AIDS patients, cell-mediated immunity is severely impaired because of the reduction of CD4 + T cell counts in peripheral blood, which makes these patients highly susceptible to *M. tuberculosis* infection. Clerici and co-workers[21] reported polarized production of Th2 cytokines over Th1 in parallel to the progression of HIV infection, which suggested not only quantitative but also qualitative alterations of Th cells leading to the immunosuppression in these patients.

Immunotherapy with Th1-related cytokines against infectious diseases

Cytokines exert profound modulating effects on the cellular immune response. Another beneficial aspect of cytokines is their convenience of administration to patients, similar to that of chemotherapeutic agents. Thus, cytokines are potentially promising therapeutic agents to improve the depressed host defense mechanism and increase the effectiveness of treatment with antimicrobial agents in such patients. In this section, I will focus on the potential clinical application of IFN-γ, IL-12, and IL-18 in infectious diseases and discuss the putative problems that may interfere with the development of this novel therapy.

IFN-γ

Among Th1 cytokines, IFN-γ was the first cytokine tested clinically for the treatment of infectious diseases. Badaro et al.[22] reported the use of this cytokine in combination with pentavalent antimony in patients with drug-resistant visceral leishmaniasis, and demonstrated that such treatment was effective in 14 of 17 patients. In an earlier study by Nathan and co-workers,[23] patients with lepromatous leprosy, in which condition Th2 cytokine production is predominant over that of Th1 cytokines, were treated with intradermal injections of IFN-γ. This treatment resulted in the induction of delayed-type hypersensitivity (DTH) reactions, similar to tuberculoid leprosy, in which lesions Th1 cytokine production is greater than that of counterpart ones,[24] and a reduction in acid-fast bacilli in some cases. Holland et al.[25] used conventional chemotherapy combined with IFN-γ for the treatment of refractory disseminated nontuberculous mycobacterial infection in patients with various underlying immunodeficient states, including idiopathic CD4+ T-lymphocytopenia. All 7 treated patients showed significant clinical improvement, with resolution of fever, reduction in the number of bacteria in the sputum and blood, resolution of skin lesions, and radiographic improvement. In a subsequent study by the same group, they reported that peripheral blood monocytes from 3 of these patients showed abnormal IL-12 production in vitro upon stimulation with *S. aureus* Cowan I strain. Furthermore, IFN-γ is currently being used for patients with chronic granulomatous diseases, a group of disorders of the oxidative microbicidal capacity of phagocyte cells,[26] and for Omen's syndrome, a genetic disorder characterized by the presence of an imbalance in the production of Th2 cytokines relative to Th1 cytokines and associated with hypereosinophilia and hyper IgE in the serum.[27] This treatment is reported to reduce the frequency of opportunistic infections in the former group of patients and to improve the Th1–Th2 imbalance in the latter group.

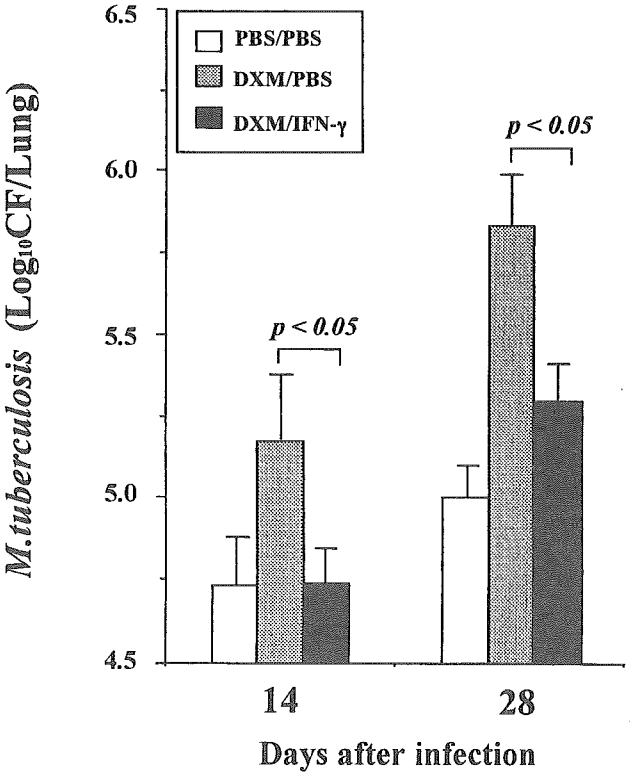

Fig. 1. Effectiveness of interferon γ (*IFN-γ*) treatment in experimental tuberculosis in immunosuppressed mice. Mice immunosuppressed by dexamesathone (*DXM*) were infected intravenously with *Mycobacterium tuberculosis*. These mice were treated with IFN-γ or phosphate-buffered saline (*PBS*), and the number of live colonies of *M. tuberculosis* in lung was measured 14 and 28 days after infection

Previously, we have demonstrated that IFN-γ treatment improved the survival rate in mice infected with a highly virulent strain of *C. neoformans*, and reduced the lung and brain burdens of this pathogen.[28] Similar effects were observed in *M. tuberculosis* infection in mice immunocompromised by the administration of dexamethasone. This infection was aggravated in these mice, and IFN-γ treatment significantly lowered the number of live acid-fast bacilli, as shown in Fig. 1.[29] In another study, Joly and co-workers[30] demonstrated that combined treatment with amphotericin B and IFN-γ resulted in more beneficial effects; prolongation of the mean survival time of mice infected with the same pathogen and reduction in *C. neoformans* counts in the lungs, spleens, and brains, relative to the effects observed when either the cytokine or the antifungal agent was used alone.

IL-12

In animal studies, many investigators have demonstrated the effectiveness of IL-12 in enhancing the host defense activity against a variety of infectious pathogens.[31] A single treatment regimen of cytokine was used in all such studies. However, at present, it seems that combined treatment with conventional chemotherapeutic agents is essential for clinical application. Nabors et al.[32] demonstrated that IL-12 promoted the clearance of *L. major* infection in mice only when combined with antimicrobial chemotherapy. Based on these findings, it seems that the effect of these agents is mediated by converting the augmented Th2 responses, which may be associated with a high antigen load, into the curative Th1 responses.

Recently, Doherty and Sher[33] showed that combined treatment with IL-12 and an antimycobacterial agent, clarithromycin or rifabutin, reduced the number of live *M. avium* in mice to levels that were significantly lower than those obtained by either the cytokine or the agent alone. However, in the same study, IFN-γ failed to enhance the antimycobacterial effects of these agents. In cryptococcal infections, two groups have reported the effects of combined treatment with IL-12 and antifungal agents using murine models. Clemons and co-workers[34] demonstrated that IL-12 promoted the therapeutic efficacy of the antifungal agent fluconazole in a central nervous system infection with *C. neoformans*. However, the same treatment did not show such an effect in the lungs, livers, and spleens. In contrast, our group reported that IL-12 treatment clearly prolonged the survival of mice infected with a highly virulent strain of *C. neoformans* by reducing the pathogen counts in the lung and brains.[28] Furthermore, using the same infection model, we demonstrated that combined treatment with IL-12 and fluconazole was more effective in protecting the animals than either treatment alone.[35]

In addition, persistently high levels of IL-12 were present in the blood of treated animals, with a half-life of 2.5h in rats and 17h in rhesus monkeys;[36] these half-lives are much longer than those of other cytokines. Thus, IL-12 treatment is useful, particularly when combined with antimicrobial agents, in the treatment of infectious diseases in animal models. However, clinical application of this cytokine has not yet been conducted in such diseases in humans, except in AIDS patients,[37] because of its adverse effects, as will be discussed in a later section.

IL-18

Many investigations have demonstrated the important role of IL-18 in the host defense against infection, using mice with a targeted disruption of the gene for this cytokine or its specific neutralizing antibody (Ab).[38] A few studies have described the usefulness of this cytokine in the treatment of infectious diseases. Exogenous administration of IL-18 promoted the clearance of microorganisms such as *C. neoformans*, *Candida albicans*, *Toxoplasma gondii*, and herpes simplex virus from infected tissues.[39-42] However, such an effect seemed to be less potent than that of IL-12.

Combined treatment with IL-12 and IL-18

Many investigations have demonstrated a synergistic effect for IL-12 and IL-18 in inducing the production of IFN-γ by NK cells, B cells, T cells, macrophages, and dendritic cells.[43] In a series of studies, we have also demonstrated that IL-12 and IL-18 synergistically induced the production of IFN-γ by cultured NK cells and augmented the fungicidal activity of peritoneal exudate cells against *C. neoformans*.[44] Furthermore, in our in vivo study,[45] such combined treatment was therapeutically effective against a fatal form of murine infection caused by *C. neoformans*, as indicated by reduced fungal burdens in the lungs and brains and prolonged survival of infected mice, while the use of either IL-12 or IL-18 alone did not result in such an effect. The in vivo effect was mediated by the local production of IFN-γ, not only by NK but also by γδ T cells in the lungs. Similar findings were reported in animal models of other infectious diseases by Okamura and co-workers.[46] Combined treatment using these two IFN-γ-inducing cytokines diminished the mortality of mice infected with *L. major* or *P. berghei*.[46,47]

Clinical application of Th1-related cytokines in infectious diseases

At present, cytokines clinically available in Japan are granulocyte-colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF), IL-2, and IFN-γ. The clinical use of IL-2 and IFN-γ is limited, and these cytokines are not used for the treatment of infectious diseases. However, IFN-γ has been tried in the treatment of intractable mycobacterial infectious diseases because of its strong activity in inducing macrophage killing of microbial pathogens. In an earlier report by Holland and co-workers,[25] this cytokine was administered in a systemic manner and showed a therapeutic effect against nontuberculous mycobacteriosis in patients with various underlying diseases. In contrast, Condos et al.[48] used an inhalation of IFN-γ in combination with chemotherapy in five patients with multidrug-resistant tuberculosis, as indicated in Table 2. This IFN-γ therapy, performed 3 days per week for 4 weeks, resulted in negative sputum smears in four of the five patients and in significant improvement in one, no change or slight improvement in one, and reduced cavitary lesions in three patients on chest computed tomography (CT) findings. Some of these patients complained of myalgia and cough, but no other severe adverse effects were observed.

Previously, we tried IFN-γ treatment in a patient with intractable multidrug-resistant tuberculosis. The patient was a 34-year-old man who suffered from pulmonary tuberculosis 2 years after he had been diagnosed with diabetes mellitus. The causative agent obtained from his sputum was multidrug-resistant *M. tuberculosis*, which showed

Table 2. Inhalation therapy with interferon (IFN)-γ in intractable tuberculosis

Chemotherapy	Cases				
	No. 1	No. 2	No. 3	No. 4	No. 5
	CPEX, CPM, CLFZ, RBT	INH, OFLX, TH, CS	CPM, CPFX, PZA, CS, TH	EB, OFLX, CPM, TH, phenylaminosalicylate	AMK, CS, TH, CLFZ phenylaminosalicylate
Period before IFN-γ therapy (months)	24	12	13	10	5
Sputum smear					
Before IFN-γ	++	++	++++	+	+++
After IFN-γ	−	−	−	−	+
Chest CT findings	Improved	Improved	Improved	Improved	No change or slightly improved

Modified from reference 48
CLFZ, clofazimine; RBT, rifabutin; OFLX, ofloxacin; AMK, amikacin; CPFX, ciprofloxacin; CPM, capreomycin; INH, isoniazid; TH, ethionamide; CS, cycloserine; PZA, pyrazinamide; EB, ethambutol; CT, computed tomography

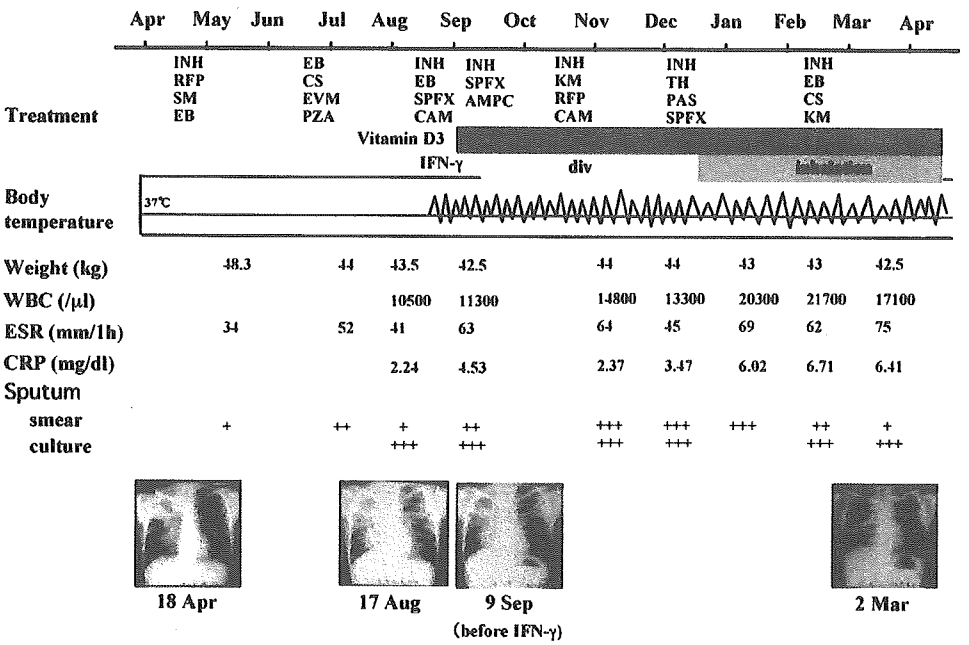

Fig. 2. Clinical course of a patient with intractable tuberculosis who received immunotherapy with IFN-γ. A 34-year-old man with multidrug-resistant, intractable pulmonary tuberculosis and insulin-dependent diabetes mellitus received immunotherapy with IFN-γ in combination with vitamin D3. The clinical course in this patient is indicated. *INH*, isoniazid; *RFP*, rifampicin; *SM*, streptomycin; *EB*, ethambutol; *CS*, cycloserine; *EVM*, enviomycin; *PZA*, pyrazinamide; *SPFX*, sparfloxacin; *CAM*, clarithromycin; *AMPC*, amoxicillin; *KM*, kanamycin; *TH*, ethionamide; *PAS*, paraamino salicylic acid; *ESR*, erythrocyte sedimentation rate; *CRP*, C-reactive protein; *div*, drip infusion

complete resistance to major antituberculous agents, such as isoniazid, rifampicin, pyrazidomide, streptomycin, and ethambutol. His condition became progressively aggravated, with extended shadows on chest X-ray, in spite of chemotherapy with the first- and second-line antituberculous agents and other antibiotics, including fluoroquinolone and clarithromycin. Therefore, we decided to start cytokine therapy with IFN-γ, with the chemotherapy being continued, after written informed consent was obtained from the patient and his family. Three million units of IFN-γ per day was administered every day, through an intravenous route, for the initial 3 months, followed by switching to intratracheal administration by inhalation, because worsening of diabetes mellitus was observed, as shown by an increase in the dose of insulin needed to control his blood sugar level. This finding was suspected to be due to the adverse effect of IFN-γ treatment. Administration of vitamin D3 was combined throughout this treatment because of its potentiating effect for macrophage mycobactericidal activity.[14] Such treatment showed clear immunological effects: e.g., potentiated NK cell activity, indicated by the cytolytic effect of peripheral blood mononuclear cells (PBMC) against K562 cells, and increased production of proinflammatory cytokines by PBMC. However, unfortunately, no clinical improvement was obtained in this patient even after this treatment. His clinical course is shown in Fig. 2.

Recently, a randomized, double-blind pilot clinical trial of IFN-γ treatment was conducted in AIDS patients with acute cryptococcal meningitis.[49] These patients received standard therapy with amphotericin B ± flucytosine daily for 2 weeks, followed by fluconazole daily for 8 weeks, with adjunctive IFN-γ (100 or 200μg) or placebo administered three times weekly during the whole period of chemotherapy. Seventy-eight patients were enrolled in this trial, of whom 70 patients satisfied all entry criteria, and 63 patients completed at least 2 weeks' therapy. As shown in Table 3,

Table 3. A randomized double-blind pilot study of IFN-γ therapy in AIDS patients with cryptococcal meningitis

Sandard chemotherapy[a] plus	Culture-negative rate in CSF		Reduction rate of CrAg titer in CSF[b]		Survival	Adverse events[c]
	2 Weeks	10 Weeks	2 Weeks	10 Weeks		
Placebo	18%	6%	8-Fold	24-Fold	90%	48%
IFN-γ (100μg)	38%	6%	20-Fold	27-Fold	93%	63%
IFN-γ (200μg)			11-Fold	52-Fold		

Modified from reference 49
[a] Amphotericin B ± flucytosine for 2 weeks, followed by fluconazole for 8 weeks
[b] Cryptococcal antigen (CrAg) titers were measured by latex agglutination test in cerebrospinal fluid (CSF)
[c] Fever, rigors, and malaise

there was little difference among the placebo and lower and higher dose IFN-γ groups in clinical parameters, including symptoms, negative cerebrospinal fluid (CSF) culture rates, rates of reduction of cryptococcal antigens in CSF, and survival. Adverse effects were also similar among the groups, although fever, rigors, and malaise were slightly more common in the IFN-γ-treated group than in the placebo group. This is an important report because it is the first randomized, blinded clinical trial to use IFN-γ in infectious diseases.

Greinert et al.[50] for the first time, tried immunotherapy using another cytokine, IL-12, in a patient with intractable tuberculosis. The patient was a 24-year-old, HIV-uninfected man who suffered from miliary tuberculosis. His condition became exacerbated despite directly observed therapy (DOT) with standard antituberculous agents for 8 months and then combined therapy with IFN-γ for 4 months. Therefore, they decided to use IL-12, which had been clinically tried for the treatment of cancer. Surprisingly, IL-12 treatment for 3 months led to dramatic improvement in this patient, without any severe adverse events.

Potential complications in treatment with Th1-related cytokines

Cytokines are known to act on a variety of cells, including lymphocytes, macrophages, neutrophils, epithelial cells, fibroblasts, and endothelial cells, and they exhibit multiple biological functions. These properties suggest that cytokines not only produce beneficial effects but that they can also produce harmful effects if used clinically for disease control.

IFN-γ has already been used for the therapy of various diseases, including chronic granulomatous disease, malignant diseases, and infectious diseases. In these clinical trials, it has been reported that IFN-γ improved the condition in patients with these diseases without any life-threatening adverse effects. However, a flu-like syndrome was described in many patients, and bone marrow suppression and hepatocyte toxicity were noted in a few cases.[51] In addition, some patients with leprosis developed erythema nodosum leprosum (ENL) at the site of intradermal injections of IFN-γ,[52] which was probably due to the overproduction of tumor necrosis factor α (TNF-α).[53] Thus, overcoming these side effects is also essential for the safe use of this cytokine in infectious diseases.

IL-12 is reported to induce bone marrow suppression, hepatotoxicity, skeletal muscle necrosis, and fluid leak with pleural effusion and ascites in mice.[36] The use of IL-12 at daily doses of up to 10μg/kg, which exhibit antitumor effects in mice, is well tolerated and produces almost no side effects. In contrast, at higher doses, e.g., 50μg/kg, serious side effects occur, including bone marrow suppression, hepatotoxicity, and pulmonary edema.[36] In an earlier clinical study, this cytokine was used for the treatment of patients with renal cell carcinoma. However, this treatment resulted in serious side effects in most of the patients.[54]

Only a few studies have reported the therapeutic efficacy of another IFN-γ-inducing cytokine, IL-18, in animal models of infectious diseases. Further studies should be conducted to evaluate the use of this cytokine as a putative immunotherapeutic agent for such diseases. As discussed above, the combined use of IL-12 and IL-18 is likely to produce a potent effect on host defense against infection, because these cytokines synergistically induce the production of IFN-γ when used in combination.[43] Furthermore, the studies of the combined use of IL-12 and IL-18 suggest that the doses of both cytokines can be reduced when they are used together, compared with the doses used in treatment with either agent alone. This may help to reduce the development of serious side effects. In preliminary studies in our laboratory, we have found, however, that high doses of these cytokines in animals were associated with the development of serious side effects, including wasting disease, and even the death of some mice (unpublished data). These effects were likely to have been mediated, at least in part, by the overproduction of TNF-α. Chikano and co-workers[55] injected high doses of IL-12 and IL-18 in mice and reported the development of serious pathological changes or manifestations, such as severe diarrhea and rapid weight loss. Histopathological examination of the affected tissues showed hemorrhagic erosions in the intestine and colon, fatty degeneration of the liver, necrotic change in the pancreas, and marked atrophy of the thymus. These changes are thought to be due to Fas-independent apoptosis. Thus, these problems definitely need to be resolved before any combination cytokine therapy can be used clinically.

Perspective of therapy with Th1-related cytokines in infectious diseases

The potential use of cytokine therapy for infectious diseases is now being considered by many investigators. The important issue that interferes with the development of such novel therapy is that related to the side effects described above.

Several efforts have been made to resolve this problem. Kaplan and co-workers (Sampaio et al.[52]) demonstrated that prolonged treatment with recombinant IFN-γ induced ENL in lepromatous leprosy patients. ENL is an acute inflammatory condition characterized by the appearance of a painful vasculitic rash and systemic symptoms of fever, malaise, lymphadenopathy, and weight loss, occurring in approximately 30% of patients.[56] TNF-α is thought to play an important role in the pathogenesis of ENL.[53] The same group also demonstrated that therapy with thalidomide, a known sedative drug, promptly reduced the painful subcutaneous nodules and systemic symptoms.[57,58] The mechanism of action of thalidomide is thought to be the suppression of TNF-α production, because this agent selectively inhibits TNF-α production by stimulated peripheral blood monocytes.[58–60] Because thalidomide also suppresses other TNF-α-mediated reactions,[58] its combined use may reduce the side effects associated with exogenously administered IFN-γ, IL-12, and IL-18, some of which side effects may be mediated by the overproduction of TNF-α. In regard to other possible agents, IL-10 might be useful to reduce the detrimental effects of Th1-related cytokines caused by excessive production of TNF-α, as recently indicated by Inoue.[61]

Other approaches, such as the local administration of cytokines (e.g., intratracheal or intrathecal injection) may be effective in reducing the systemic side effects. The use of these modes of delivery may also increase the concentration of cytokines at the local site, compared with systemic administration. However, it is essential to use frequent doses of a recombinant cytokine, because of its short half-life, which may make local treatment difficult. To resolve this problem, the application of gene transfer techniques might be useful.[62–64] These techniques aim at the prolongation of cytokine expression in vivo to increase therapeutic efficacy at local sites. On the other hand, many efforts have been made to obtain better carriers for gene transfer, such as the replication-defective adenovirus vector. Thus, this technique is a promising candidate for the immunotherapy of refractory infectious diseases.

Concluding remarks

Major efforts to overcome infectious diseases have been focused on the development of stronger antibiotics to defeat resistant pathogenic microorganisms. However, history indicates that antimicrobial agents do not always overcome the offending microbes. Moreover, the recent increase in the number of immunocompromised individuals has largely changed aspects of infectious diseases. On the other hand, recent progress in immunology and genetic engineering has led to the discovery of a number of new cytokines. Several studies using animal models have confirmed the effectiveness of these cytokines against various infectious diseases. Thus, it is now an appropriate time to consider the development of a novel immunotherapy, to be combined with chemotherapeutic agents, to improve depressed host defense mechanisms. Such combined therapy may provide human beings with a powerful weapon against microbial pathogens in this new century.

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

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

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

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

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

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

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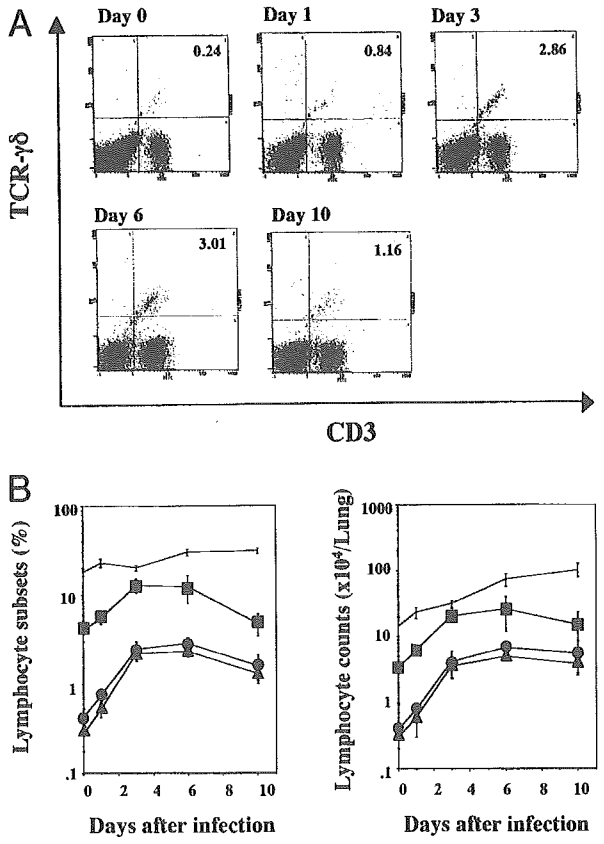


FIGURE 1. Increase of γδ T cells in lungs after *C. neoformans* infection. *A*, Mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The lung leukocytes were prepared and stained with FITC-anti-CD3 and PE-anti-TCR-γδ mAbs before (0) and 1, 3, 6, and 10 days after infection. The lymphocyte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

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

Materials and Methods

Animals

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

Microorganisms

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

Preparation of pulmonary intraparenchymal leukocytes

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

Flow cytometric analysis

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

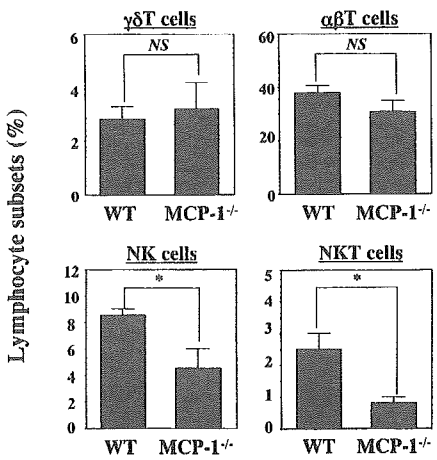


FIGURE 2. Role of MCP-1 in the increase of γδ T cells in lungs after infection with *C. neoformans*. WT and MCP-$1^{-/-}$ mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The lung leukocytes were prepared and stained with FITC-anti-TCR-αβ and PE-anti-NK1.1 mAbs or FITC-anti-CD3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

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

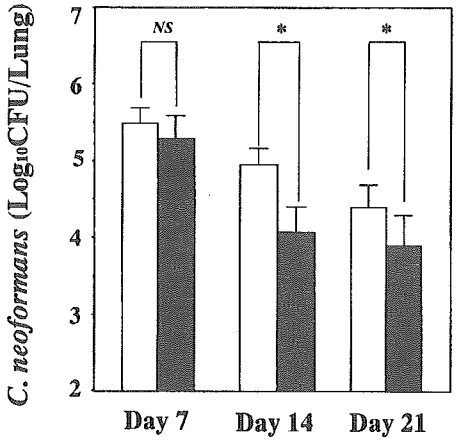


FIGURE 3. Effect of γδ T cell-depletion on the host defense against *C. neoformans*. Mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). These mice received i.p. injections of anti-TCR-γδ mAb or control IgG (400 μg) on days −3, 0, +3, +7, and +14 after infection. The number of live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

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

Antibodies

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

Enumeration of viable C. neoformans

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

Preparation of lung homogenates

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

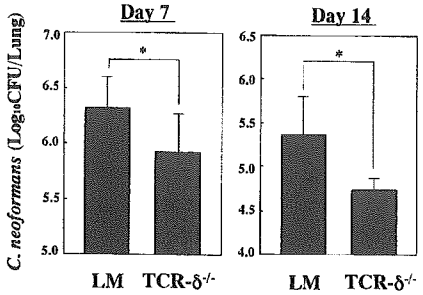


FIGURE 4. Enhanced clearance of *C. neoformans* in TCR-$\delta^{-/-}$ mice. TCR-$\delta^{-/-}$ or LM mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The number of live colonies in lung was examined on days 7 and 14 after infection. Each bar represents the mean ± SD of 10 and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

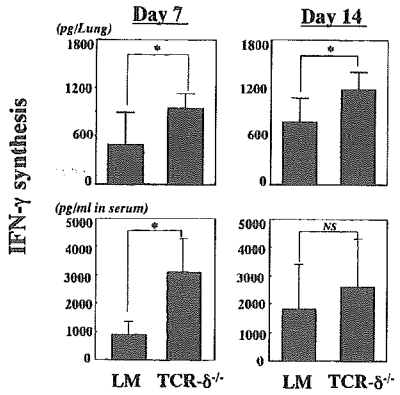


FIGURE 5. Increased production of IFN-γ in TCR-$\delta^{-/-}$ mice after *C. neoformans* infection. TCR-$\delta^{-/-}$ or LM mice were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The levels of IFN-γ in lung homogenates and serum were measured on days 7 and 14 after infection. Each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

In vitro stimulation of lymph node (LN) cells

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

Measurement of cytokines

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

Statistical analysis

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

Results

Accumulation of γδ T cells in the lungs after cryptococcal infection

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

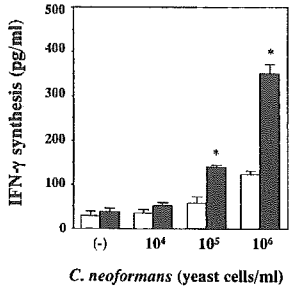
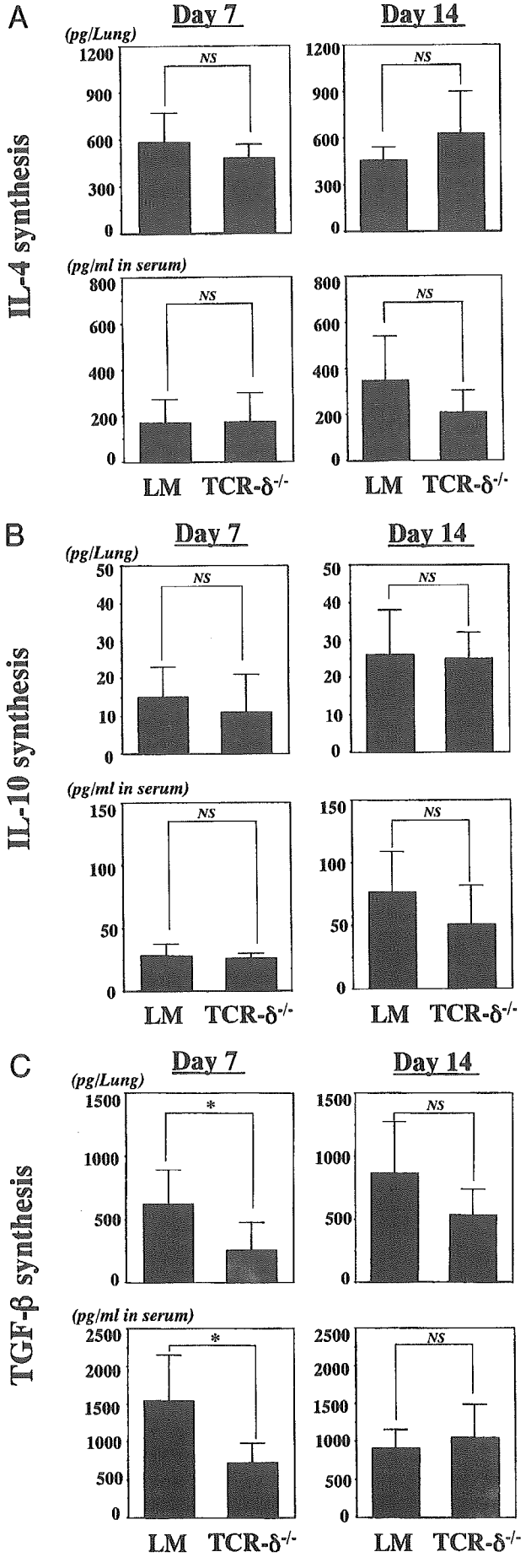


FIGURE 6. Enhanced Th1 cell development in TCR-$\delta^{-/-}$ mice after *C. neoformans* infection. TCR-$\delta^{-/-}$ (■) or LM mice (□) were inoculated intratracheally with *C. neoformans* (1×10^6/mouse). The paratracheal LN cells were prepared from four mice and cultured at 2×10^6/ml with indicated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



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

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

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

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

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

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

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

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

LM mice. As shown in Fig. 5, lung and serum levels of this cytokine on days 7 and 14 and on day 7, respectively, were significantly higher in TCR-$\delta^{-/-}$ mice than those in control mice. Similar data were obtained when $\gamma\delta$ 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. 7*C*). 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-15Rα or IL-2/IL-15Rβ lacked such cells (40, 41). In the present study, we did not test the expression of this cytokine at the site of infection and its contribution to the increase of $\gamma\delta$ 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.

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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 Vα14+ 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 μm 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 Vα chain consisting of Vα14-Jα18 gene segment and highly skewed Vβ chains, Vβ8.2, 7 and 2 in mice and with Vα24-Jα18 and Vβ11 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 β2 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. Vα14 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 Jα18 gene-disrupted (KO) mice, which lack particular NKT cell subset bearing Vα14-Jα18 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-2Rβ mAb enhances the host protection of mice from *T. gondii* infection by increasing Th1-polarized cytokine production (39). Finally, mice lacking Vα14+ 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 Vα14 TCR in the infected lungs by detecting cells bound to either anti-Vα14 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-1α, -1β 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-1α, 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 Jα18-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 Jα18-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 Vα chain and highly skewed Vβ 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 glycosylphosphatidyl inositol (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

*i*NKT 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 *i*NKT 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 Jα18-KO mice, indicating the involvement of *i*NKT 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 *i*NKT 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 γδ T cells

4-1. General features of γδ T cells

Besides conventional T cells bearing TCRαβ, a distinct subset of T cells expressing novel antigen receptors consisting of γ and δ chains, designated as γδ T cells, was discovered approximately 20 years ago (65). In a sharp contrast to αβ T cells, which are the major population in lymphoid tissues, such as lymph node and spleen, γδ T cells are preferentially localized in non-lymphoid tissues, including epidermis, where γδ 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, TCRγ gene segments are located on chromosome 7, while TCRδ gene segments are interspersed with TCRα gene on chromosome 14. Before birth, Vγ8 and Vγ9 subsets associate with Vδ2 subset, which distribute to peripheral blood and tonsil. After birth, Vγ2, Vγ3, Vγ4, Vγ5 and Vγ8 gene segments are rearranged and associated with Vδ1 subset, which are found located preferentially in mucosal tissues, such as intestine (65,66). On the other hand, mouse TCRγ gene segments are found on chromosome 13 and TCRδ gene segments are dispersed with TCRα gene on chromosome 14 (65). Similar to human, mouse γδ T cell subsets show particular localization patterns. Vγ5, Vγ6 and Vγ4 subsets are formed during gestation and Vγ1 and Vγ7 subsets at birth and shortly thereafter. These subsets are localized in defined anatomical sites: Vγ5 in skin, Vγ6 in uterus and lung, Vγ4 in spleen, lung and tongue, Vγ1 in spleen, and Vγ7 in intestine. In an analysis with quantitative PCR technique, Vγ6 subset is the only γδ T cells found in lung when mice are born. After birth, however, Vγ4, Vγ5 and Vγ7 subsets were detected and by 2-3 months of age, Vγ4 subset becomes predominant among pulmonary γδ T cells (66-69).

4-2. Natural ligands

The number of V gene segments of γδ T cells that determine their diversity is very limited when compared with that in αβ 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 γδ T cells is assumed to be limited in contrast to αβ 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 Vγ9/Vδ2+ γδ 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 γδ T cells. Human Vγ9/Vδ2+ γδ T cells recognize tetanus toxoid in context of MHC class II molecules (72). Mycobacterial heat-shock proteins stimulate both human and mouse γδ 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 γδ T cells in infectious diseases

From previous observations showing that γδ T cells accumulate at the sites of infection and chronic inflammation (76), their involvement in regulating the immune response has been suggested. γδ 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. brasilliensis*. Furthermore, some γδ 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, γδ T cells exert different patterns of influences on the host protection. Manipulations that result in ablation of γδ T cells, e.g., genetic disruption and treatment with a specific Ab, rendered mice susceptible to infection with *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, γδ T cells showed contrast roles at early and late stages (95). Thus, γδ 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 γδ 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). γδ T cells quickly increased in a similar kinetics as observed in NK and