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

Role of Vα14+ NKT cells in host defense and Th1 response to cryptococcal infection

The role of Vα14+ NKT cells in the host defense and Th1 response to cryptococcal infection was elucidated using mice genetically lacking Vα14+ NKT cells. Both the development of Th1 cells specific for cryptococcal antigens (as shown by IFN-γ production by re-stimulated spleen cells from the infected mice), and the delayed-type hypersensitivity (DTH) response (as indicated by footpad swelling in response to challenge immunization), were hampered in Vα14+ NKT cell-deficient mice, compared with the control. Furthermore, the clearance of *C. neoformans* was significantly delayed in these mice. Based on these data, NKT cells were identified as a key cell population, which regulated the Th1-mediated host resistance by bridging the innate and acquired immune responses.

Therapeutic effect of α-GalCer in cryptococcal infection

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

treatment were completely abrogated in Vα14+ NKT cell-deficient mice. IL-12 was essential for the IFN-γ production and host protection induced by α-GalCer administration, although a critical role for IL-18 was not proven [57].

Concluding remarks

Many scientists have been focusing attention on NKT cells, because many recent investigations have revealed that these cells play crucial roles in various immune responses. α-GalCer, a ligand of Vα14+ and Vα24+ NKT cells, appears to be useful in protecting hosts against tumor metastasis, microbial infection and autoimmune disease. Although the number of Vα24+ NKT cells in the peripheral blood is very small, such a problem might be solved by a novel approach of expanding the population of Vα24+ NKT cells by using α-GalCer-loaded DCs with IL-7 and IL-15 [111]. Some modification, such as combination therapy of G-CSF and α-GalCer [34], or use of OCH, an analogue of α-GalCer [22], may augment the effect of α-GalCer in treating patients with cancer or autoimmune disease. α-GalCer might therefore be an attractive tool as a new immunotherapeutic agent to treat patients with intractable cancer, infection and autoimmune disease.

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7

Innate Immunity in the Lungs to Cryptococcal Infection

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1. Introduction

Humans breathe about 10,000 liters of air per day. Therefore, the airway is always exposed to infectious agents, such as bacteria, fungi, and viruses, and environmental hazards, including cigarette smoke, pollutants, and allergens. To protect against these harmful stresses, highly sophisticated immune systems are developed in the lung. These systems are largely divided into two distinct categories: innate and acquired immunity. The former consists of humoral antimicrobial molecules, complements, phagocytic cells, such as neutrophils and alveolar macrophages, and other innate immune cells, including dendritic cells (DCs), natural killer (NK) cells, NKT cells, antigen receptor γδ-bearing T (γδ T) cells, and B1-B cells. On the other hand, acquired immunity is characterized by antigen-specific cell-mediated and antibody-mediated immune responses. Recent development in research of the innate immune system includes the identification of Toll-like receptors (TLRs) that specifically recognize various microbial components called pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan, lipoarabinomannan (LAM), and lipopeptides. In addition, other cell surface molecules recognizing PAMPs have been identified, which include receptors for complement components, mannose, and β-glucan. These discoveries accelerate the understanding of innate immune mechanisms operating against infectious pathogens.

Cryptococcus neoformans is a yeast-like fungal pathogen with a thick polysaccharide capsule. Infection 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 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 microbe to the brain has attracted attention as a serious problem, particularly with the increased number of patients with acquired immunodeficiency syndrome (AIDS).

The host defense against *C. neoformans* is critically regulated by cell-mediated immunity (Lim and Murphy, 1980), and CD4+ T cells play a central role in eradicating this infection (Mody et al., 1990; Hill and Harmsen, 1991; Huffnagle et al., 1991). 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 (Kawakami et al., 1997; Koguchi and Kawakami, 2002). Mice depleted of Th1-type cytokines (e.g., IFN-γ and TNF-α) are highly susceptible to cryptococcal infection (Huffnagle et al., 1996; Kawakami et al., 1996), while the infection is less severe in mice lacking Th2 cytokines (e.g., IL-4 and IL-10) than control mice (Decken et al., 1998; Blackstock et al., 1999). Differentiation of

naïve helper T cells into Th1 cells absolutely requires the presence of IL-12 (Trinchieri, 1995), and this response is strongly potentiated by IL-18 (Robinson et al., 1997). In recent investigations (Decken et al., 1998: Kawakami et al., 2000a), targeted disruption of the gene for IL-12 or IL-18 resulted in attenuated host resistance and Th1 response to *C. neoformans*, indicating the role of these cytokines in the development of host protective response.

In this chapter, recent research developments in innate immune defense against cryptococcal infection in the respiratory system are highlighted with particular emphasis on the role of innate immune lymphocytes, such as NKT cells and γδ T cells, based on our data using a murine model of pulmonary cryptococcal infection.

2. General Characteristics of Host Defense in the Lung

Host defense mechanisms against infectious microbial pathogens develop in the upper and lower respiratory tracts. Initially, anatomical and mechanical host defense systems trap large-sized particles in the inhaled air. These systems include nasal hairs, nasopharyngeal channels, glottis, and highly divided branches of bronchi. The particles are caught by the mucous blanket lining the bronchial surface that contains viscid glycoproteins called mucins and cleared by ciliary movement and coughing up to the oropharynx. In contrast, small-sized particles less than 5 μm in diameter, including most infectious pathogens, reach the alveolar spaces where they can cause pulmonary infection. *C. neoformans* in the inhaled air from environment is usually in an acapsular form, the size of which is 1 to 5 μm in diameter (Powell et al., 1972), suggesting the ability of this fungal pathogen to penetrate into the terminal air spaces. In order to keep lung sterility, additional host defense mechanisms are found in these areas, which are largely divided into 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 antibody-mediated and cell-mediated immune responses. Furthermore, in the airway, specific mucosal immune systems composed of nasal-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) play important roles as the mucosal barriers in the acquired phase of host defense (Pabst, 1992; Wu and Russell, 1997). AQ1

3. Humoral Components Operate in Innate Host Defense

In the respiratory tract, many antimicrobial molecules are secreted by various immune and non-immune cells, which include lysozyme, fibronectin, lactoferin, transferin, defensins, cathelicidin, collectins, and complements (Zhang et al., 2000). I will discuss here the role of defensins, collectins, and complements in conjunction with host defense against cryptococcal infection.

3.1. Defensins

Defensins are antimicrobial cationic peptides of a small molecular weight (2 to 6 kDa) that contain six cysteines and three intramolecular disulfide bonds. Based on their size and pattern of disulfide bonds, these molecules are largely classified into two categories, α- and β-defensins (Lehrer et al., 1993). In human, so far eight defensins have been identified, including six α-defensins [human defensins (HD)-1 to 6] and two β-defensins [human β-defensins (HBD)-1 and 2]. HD-1, -2, -3, and -4 constitute 30 to 50% of the total protein in azurophilic granules of neutrophils and are also released from these cells into the airway lining fluids, while

HBD-1 and -2 are synthesized by the airway epithelial cells (Zhang et al., 2000). Defensins exert a broad-spectrum antimicrobial activity by permeabilizing the membrane of microorganisms, including bacteria, fungi, and viruses (Lehrer et al., 1993; Zhang et al., 2000). *C. neoformans* is directly killed by α-defensin from rabbit and rhesus macaque in a dose-dependent manner (Alcouloumre et al., 1993; Tang et al., 1999).

In addition, recent studies have identified a variety of immunomodulatory roles for defensins (Yang et al., 2002; Ganz, 2003). These include the activation of complement system, induction of IL-8 production by epithelial cells, augmentation of expression of adhesion molecules on neutrophils and their adhesion responses, induction of chemotaxis of leukocytes, and enhancement of proliferation and cytokine production of CD4+ T cells. Thus, defensins may be involved in the innate and acquired phase host defense against cryptococcal infection through their immunoregulatory as well as antimicrobial activities.

3.2. Collectins

Collectins, collagenous C-type lectins, are oligomeric proteins composed of carbohydrate-recognition domains (CRDs) attached to collagenous regions and recognize PAMPs through calcium-dependent binding to mannose-specific carbohydrates (Clark et al., 2000). In addition to mannose-binding lectin (MBL) and conglutinin in plasma, the surfactant protein A and D (SP-A and SP-D), present in the surface layer of alveolar spaces, belong to the collectin superfamily. SP-A and SP-D bind a variety of infectious pathogens including Gram-positive and -negative bacteria, mycobacteria, fungi, and viruses, mainly via their CRD domains (Clark et al., 2000; McCormack and Whitsett, 2002). Such binding results in enhanced phagocytosis of microorganisms by alveolar macrophages and neutrophils (McCormack and Whitsett, 2002). SP-A binds to both acapsular and encapsulated forms of *C. neoformans* in a concentration-dependent manner, although three-fold better binding is observed with the acapsular form. Despite this feature, SP-A fails to function as an opsonin in the phagocytosis of this fungal pathogen by macrophages (Walenkamp et al., 1999). On the other hand, SP-D binds to the acapsular form, but either does not bind to the encapsulated form of *C. neoformans*, or does bind to a lesser extent. In contrast to SP-A, SP-D causes aggregation of acapsular yeast cells, which may stimulate their removal by enhancing the mucociliary clearance (Schelenz et al., 1995; van de Wetering et al., 2004).

3.3. Complement

The complement system is activated via three distinct pathways: classical, alternative, and lectin pathways. Whereas the activation of the classical pathway occurs from C1q subcomponent only in the presence of antigen–antibody complex, the alternative pathway is directly activated by interaction of C3 component with microbial surfaces and does not require the existence of antibody for its activation. The activation of recently identified lectin pathway is initiated by the binding of MBL with a structure similar to that of C1q, to carbohydrate (Fujita, 2002). The activated components of the complement pathways play various roles in the host defense. The deposition of C3b and iC3b fragments on the surface of microorganisms facilitates their phagocytosis by neutrophils and macrophages through interaction with complement receptor type 1 (CR1) and type 3 (CR3), respectively. In addition, C3a and C5a fragments initiate the inflammatory responses by attracting the recruitment of neutrophils into the sites of inflammation.

C. neoformans is a potent activator of the complement system. Previous studies showed that encapsulated *C. neoformans* directly activates the alternative pathway, which results in the deposition of C3 fragments on the capsular surface of this fungal microbe (Kozel et al., 1989, et al 1991). Similar deposition was

detected on the surface of encapsulated cryptococci derived from the infected tissues in mice (Truelsen et al., 1992). In non-immune hosts, this pathway is considered dominant at the innate phase of infection, although the classical pathway may be triggered through binding to naturally occurring antibodies to the cell wall polysaccharide components. In contrast, further studies will be required for understanding the contribution of lectin pathway.

Early studies showed that fresh serum enhanced the engulfment of *C. neoformans* by phagocytes, and that such activity was lost by heating at 56°C (Mitchell and Friedman, 1972; Diamond et al., 1974; Davies et al., 1982;). The opsonic potential of serum was attributed to the activation of complement system in in vitro experiments showing the blocking by treatment with antibodies specific for complement receptors (Levitz and Tabuni, 1991; Collins and Bancroft, 1992). The importance of the complement system in the host defense to cryptococcal infection was also documented in vivo in animals depleted of C3 by treatment with cobra venom factor or neutralizing antibody. These animals died of this infection earlier than did untreated animals (Graybill and Ahrens, 1981; Cross et al., 1997). Similar results are reported using congenitally C5-deficient mice, which were associated with increased susceptibility to the infection and with attenuated recruitment of neutrophils (Rhodes, 1985; Lovchik and Lipscomb, 1993). Thus, the complement system appears to play an important role in the innate phase resistance against cryptococcal infection by operating as opsonins and chemotactic factors in the lung.

4. Recognition of Cryptococcus

4.1. Nonopsonic Phagocytosis

Upon entry into alveolar spaces, *C. neoformans* are first recognized and then phagocytosed by macrophages. In this process, complement acts as opsonins via interaction with particular receptors, such as CR1, CR3, and CR4 (Levitz and Tabuni, 1991; Collins and Bancroft, 1992). Recently, however, nonopsonic phagocytosis by macrophages has been reported by several investigators. In earlier studies, the ingestion of acapsular *C. neoformans* was thought to be independent of complement, as indicated by the failure of anti-CR3 mAb to inhibit this response and efficient phagocytosis without prior opsonization (Cross and Bancroft, 1995). For nonopsonic engulfment of cryptococci, several cell surface receptors on phagocytic cells appear to be involved. CR3 mediates nonopsonic binding of *Mycobacterium tuberculosis* via a binding site distinct from the complement-binding site, which leads to the entrance of this bacterium into macrophages (Cywes et al., 1996). In the case of *C. neoformans*, a direct interaction between glucuronoxylomannan (GXM) and CR3 may facilitate phagocytosis by macrophages (Zaragoza et al., 2003). In previous investigations, receptors for mannose and β-glucan were suggested to mediate nonopsonic ingestion of the acapsular strain of *C. neoformans* and synthesis of proinflammatory cytokines by macrophages (Cross and Bancroft, 1995). Mannose receptor is also likely to participate in the phagocytosis of this fungal microbe for antigen presentation to T cells by dendritic cells (Syme et al., 2002). Recently, dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) and Dectin-1 have been identified as the receptors for mannose and β-glucan, respectively (Feinberg et al., 2001; Brown et al., 2002), which are found to act in the nonopsonic recognition of *Candida albicans* and zymosan by macrophages (Cambi et al., 2003; Taylor et al., 2004). The role of these receptors in phagocytosis of cryptococci remains to be elucidated.

4.2. Toll-like Receptors (TLRs)

The TLRs are evolutionally conserved from *Drosophila* to mammalian. *Drosophila* Toll are originally discovered as molecules that deliver

signals for the expression of antifungal peptides (Lemaitre et al., 1996). TLRs are expressed on macrophages and dendritic cells and involved in the recognition of PAMPs from various infectious pathogens, followed by signaling to nuclei via NFκB and MAP-kinases for the expression of cytokines and cell surface molecules (Akira et al., 2001; Takeda et al., 2003). TLR2 is activated by peptidoglycan, bacterial lipoproteins, and mycobacterial lipoarabinomannan, TLR3 by double-stranded RNA, TLR4 by LPS, TLR5 by flagellin, and TLR9 by bacterial CpG-DNA (Takeda et al., 2003). Upon ingestion by macrophages, *C. neoformans* secrete polysaccharides, including GXM, galactoxylomannan and mannoproteins, into phagosomes, in which various sets of TLRs are expressed. Recently, GXM was shown to interact with TLR4 and to activate NFκB in phagocytic cells, although such interaction did not result in the activation of MAP-kinases and production of TNF-α (Shohaml et al., 2001). Thus, further effort is still required to uncover the contribution of these receptors in the innate immune response against this fungal microbe.

5. Innate Cellular Host Defense

In addition to activation of the cellular response, the invading pathogens also activate the humoral components of the innate immune mechanisms. In the airways, alveolar-resident macrophages, which permanently reside in the alveolar spaces, first encounter these pathogens, followed by recruitment of other cellular components including phagocytic cells and innate immune lymphocytes. Here, I discuss the role of alveolar macrophages, neutrophils, and dendritic cells (DCs) in the local host defense against pulmonary cryptococcal infection.

5.1. Alveolar Macrophages

In the lung, alveolar macrophages (AMϕ) play a key role in the local host defense mechanism by exerting both phagocytic and immunoregulatory functions. After invading the terminal respiratory tract, AMϕ phagocytize infectious pathogens opsonized by complement fragments through complement receptors (CR1, CR3, and CR4). The microorganisms are also recognized via interaction with other PAMP receptors present on the surface of AMϕ. AMϕ express TLR2, TLR4, DC-SIGN, and Dectin-1 (Soilleux et al., 2002; Oshikawa and Sugiyama, 2003; Steele et al., 2004), all of which are considered important in the recognition of fungal PAMPs (Shohaml et al., 2001; Cambi et al., 2003; Steele et al., 2004; Roeder et al., 2004; Taylor et al., 2004). AQ2
Following such recognition, AMϕ release proinflammatory cytokines and chemokines and express co-stimulatory molecules, which results in the recruitment of neutrophils, macrophages, DCs, and innate immune lymphocytes, from the peripheral circulation into alveolar spaces and in the activation of these inflammatory cells.

In vitro studies indicate that AMϕ can phagocytize, kill, and present antigens in *C. neoformans* infection. AMϕ efficiently phagocytize the fungal microorganisms opsonized by serum (Bulmer and Tacker, 1975). The fungicidal activity against encapsulated *C. neoformans* in unstimulated human AMϕ is limited compared with other natural effector cells, peripheral blood monocytes, and neutrophils, although the acapsular strain is killed to a higher degree (Vecchiarelli et al., 1994a). Cryptococcus-laden AMϕ show a potent antigen-presenting activity to CD4+ T cells in context of HLA class II DR molecules, as indicated by their proliferative response and IFN-γ production (Vecchiarelli et al., 1994b). In addition, culture of AMϕ with this fungal microbe causes the production of proinflammatory cytokines, including IL-1β, TNF-α, IL-6, IL-12, and IL-18, and chemokines, such as monocyte chemoattractant protein-1 (MCP-1) (Vecchiarelli et al., 1994b; Li and Mitchell, 1997; He et al., 2003; our unpublished data), which may contribute to the regulation of host immune responses that takes place in the bron-

choalveolar spaces. Interestingly, these activities in human AMϕ are down-regulated by encapsulation of *C. neoformans* (Vecchiarelli et al., 1994b). In contrast, the in vivo role of AMϕ in the local protection against pulmonary cryptococcosis remains to be fully understood, although earlier investigations demonstrated that depletion of macrophages by systemic administration of silica markedly decreased the host resistance to cryptococcal infection (Monga, 1981).

Thus, AMϕ play a key role in the initiation of host protective response against *C. neoformans* at the gateway of the airway tract. In addition to the phagocytic and fungicidal functions, AMϕ exert immunoregulatory activities through the production of various proinflammatory cytokines and chemokines, which could provide a great influence on the quality of subsequent immune responses, recruitment of innate immune cells, and the activation and development of acquired immune responses.

5.2. Neutrophils

Although the development of cryptococcosis is not clinically associated with neutropenia, neutrophils are highly active in engulfing and killing *C. neoformans* in vitro (Miller and Mitchell, 1991). Neutrophils are thought to contribute to the innate defenses against cryptococcosis, particularly early in the course of infection before the development of acquired immune responses. In animal models, an influx of neutrophils into tissues is observed soon after cryptococcal challenge and is associated with rapid, but partial, clearance of the organisms (Gadebusch and Johnson, 1966; Perfect et al., 1980). Neutropenia caused by administration of cyclophosphamide is associated with exacerbation of this infection, although the same treatment also affects lymphocyte function (Graybill and Mitchell, 1978). In mouse models of cryptococcosis, boosting neutrophil-mediated host defenses by administration of granulocyte colony-stimulating factor (G-CSF) resulted in reduced brain tissue burden and prolonged survival of mice treated with fluconazole (Graybill et al., 1977). In human, neutrophils are frequently found in pathology specimens taken from patients with cryptococcosis (Baker and Haugen, 1955; Lee et al., 1996).

Recently, accumulating evidence highlights the immunoregulatory role of neutrophils in the host defense against infectious pathogens. In pulmonary infection with *Legionella pneumophila*, neutrophils play an important role in protection through polarizing the immune response toward a Th1-dominant condition (Tateda et al., 2001). Similarly, neutropenic mice are more susceptible to *M. tuberculosis* infection than mice bearing normal counts of neutrophils, which is associated with reduced expression of IFN-γ and iNOS (Pedrosa et al., 2000). Interestingly, Mednick and co-workers (2003) reported opposite results, indicating the enhanced survival of mice infected with a lethal dose of *C. neoformans* probably by coordinating inflammatory responses via modulation of cytokine synthesis in the lung.

In our previous studies, accumulation of neutrophils was not associated with protective host responses against lethal pulmonary infection with *C. neoformans* (Kawakami, 1999). The infected mice showed little or no expression of CC- and ELR^- CXC-chemokines that attract lymphocytes and macrophages, but ELR^+ CXC-chemokines that recruit neutrophils. These observations were consistent with poor infiltration of mononuclear leukocytes, but accumulation of neutrophils at the site of infection. Administration of IL-12 that rescued mice from lethal infection polarized the chemokine and cellular inflammatory responses toward the preferential accumulation of lymphocytes and macrophages, but not of neutrophils. These findings may provide evidence arguing against the protective role of neutrophils in infection with *C. neoformans*.

Thus, the contribution of neutrophils to the host defense against cryptococcal infection remains controversial. However, any such role is not likely pronounced according to the previous observations reported.

5.3. Dendritic Cells

DC is an efficient antigen-presenting cell for naïve T lymphocytes. Immature DCs that develop from $CD34^+$ progenitors in the bone marrow are characterized by their ability to capture antigens by endocytosis and phagocytosis. After capturing antigens, DCs undergo maturation that is associated with the expression of processed antigens in context of MHC class II and upregulation of co-stimulatory molecules, such as CD40, CD80, and CD86, expressed on their surface (Banchereau et al., 2000). During these changes, DCs migrate from the infected tissues into the T cell area of draining lymph nodes, where they encounter naïve antigen-specific CD4+ T cells (Guermonprez et al., 2002). DCs have an important role in determining the profile of cytokine production by T cells, i.e., Th1 or Th2 cells. In human, monocyte-derived DCs seem to promote the differentiation of Th1 cells by producing IL-12, while Th2 cells are likely induced by plasmacytoid-derived DCs (Moser and Murphy, 2000). DCs express many receptors for PAMPs, including TLRs, mannose receptors, and β-glucan receptors. Interaction of these receptors with the PAMPs leads to the production of proinflammatory cytokines and increased expression of co-stimulatory molecules by DCs. IL-12 and IL-18 released from these cells strongly promote the production of IFN-γ by innate immune lymphocytes, such as NK, NKT, and γδ T cells, as well as the differentiation of Th1 cells (Trinchieri, 1995; Robinson et al., 1997; Okamura et al., 1998; Qureshi et al., 1999; Baxevanis et al., 2003).

The cell-mediated immune responses are essential for the host defense against cryptococcal infection. The involvement of DCs in the development of cell-mediated immunity against this fungal microbe is not well understood. Bauman and co-workers (2000) examined the kinetics of DC accumulation in the draining lymph nodes after subcutaneous immunization with cryptococcal antigens. The protective anticryptococcal immune responses are associated with preferential accumulation of myeloid DCs in the draining lymph nodes, while lymphoid DC is the major subset in the unprotected mice. Accumulation of DCs in the lymph nodes is regulated by TNF-α (Bauman et al., 2003). In our unpublished data, DCs, identified as cells expressing both CD11c and class II MHC molecules, migrate into paratracheal lymph nodes after pulmonary infection with *C. neoformans*, although further investigation is required for understanding the role of these cells in the local host defense in the lung.

6. Innate Immune Lymphocytes

C. neoformans show the features of intracellular parasitism within phagocyte cells, as is well known in *M. tuberculosis*, *Listeria monocytogenes*, and *Salmonella typhimurium* (Feldmesser et al., 2001). Because such pathogens resist the killing mechanisms, phagocytes fail to eradicate them without any activation. The innate immune lymphocytes, such as NK, NKT, and γδ T cells, can enhance their killing activity through the production of IFN-γ, although the overall potential is not sufficient for complete eradication of the infection, which needs more potent protective mechanisms by developing 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 disagree with this concept. In this respect, accumulating evidences suggest that innate immune lymphocytes are the cells that determine the quality of acquired immune responses (Horwitz et al., 1999; Nishimura et al., 2000; Schaible and Kaufmann, 2000). Thus, the early host protective responses mediated by these cells is more than a "temporary protector" before development of acquired immunity.

6.1. NK Cells

NK cells play a role in the innate cellular host defense mechanisms to eliminate virus-infected cells and tumor cells (Trinchieri, 1989). NK cells express their killing activity through a non-phagocytic mechanism, which is mediated by several killing molecules including perforin and granzyme B (Kagi et al., 1996). In host defense against infectious pathogens, NK cells regulate the innate defense mechanisms through the production of cytokines such as IFN-γ (Dunn and North, 1991; Laskay et al., 1993; Scharton and Scott, 1993). This process operates especially in the early phase of infection before the establishment of a specific immune response mediated by T cells, through the enhancement of the antimicrobial activity of phagocytic cells. IL-12 and IL-18 potentiate the tumoricidal activity as well as production of IFN-γ by NK cells and act synergistically when these cytokines are administered in combination (Okamura et al., 1995, 1998; Trinchieri, 1995; Zhang et al., 1997; Hyodo et al., 1999).

Earlier studies also indicated the role of NK cells in eliminating *C. neoformans* from the host. In a series of studies, Murphy and coworkers demonstrated that NK cells inhibited the growth of fungal microorganisms by directly binding to them (Murphy and MacDaniel, 1982; Nabavi and Murphy, 1985; Hidore and Murphy, 1989; Hidrore et al., 1990, et al 1991a, et al b; Murphy et al., 1991, et al 1993). Other studies by various investigators indicated that mice defective in NK cell activity were more susceptible to intravenous challenge with *C. neoformans* than control animals (Hidore and Murphy, 1986; Lipscomb et al., 1991; Scharton and Scott, 1993). These early observations emphasized the role of NK cells in eliminating *C. neoformans* from infected organs through a direct fungicidal activity. In contrast, our studies showed that SCID mouse-derived splenic NK cells, which were stimulated with a combination of IL-12 and IL-18, did not show any direct cryptococcocidal effect, although the production of IFN-γ and cytolytic activity to NK-sensitive tumor cells were markedly induced by the same treatment. NK cells rather upregulated the nitric oxide (NO)-mediated antifungal activity against *C. neoformans* through the production of IFN-γ (Kawakami et al., 2000b). Thus, NK cells contribute to the host defense against cryptococcal infection by regulating the immune response as well as by directly killing this fungal microbe.

The in vivo role of NK cells, which form approximately 5 to 6% of the lymphocyte population in lung (Kawakami et al., 2001a), in the local host defense against cryptococcal infection in airway tissues remains to be fully understood. In earlier studies, lung infection was not aggravated in mice depleted of NK cells by administration of anti-NK1.1 mAb (Lipscomb et al., 1987), although the same treatment deleted not only NK cells but also NKT cells. However, NK cells appear to be the source of IFN-γ to control *C. neoformans* infection in mice receiving a combined treatment with IL-12 and IL-18 (Qureshi et al., 1999) or in mice with a genetic disruption of IL-12p40 gene (Kawakami et al., 2000a). In contrast, IFN-γ production and host protection from cryptococcal infection caused by administration of unmethylated synthetic DNA-containing CpG-motif do not involve NK cells (our unpublished data). Thus, the in vivo role of NK cells in anticryptococcal host response appears to vary in different settings; further studies will be necessary to better understand this role.

6.2. NKT Cells

6.2.1. Characteristics

NKT cell is a unique T cell subset sharing some features with NK cells. Originally, this population was identified as a lymphocyte subset that expresses both T cell receptor (TCR) αβ and NK1.1 or NKR-P1 (CD161) in mice (Godfrey et al., 2000; Taniguchi and Nakayama, 2000). Specific characteristics of

this cell type include highly limited repertoire with an invariant Vα chain consisting of Vα14-Jα18 (formerly Jα281) gene segment and highly skewed Vβ chains, Vβ8.2, 7, and 2 in mice and with Vα24Jα18 and Vβ11 in human. Accordingly, these cells are called invariant (*i*)NKT cells. The mouse *i*NKT cells are either CD4+ or double negative (DN) and usually do not express CD8, while 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 because this population disappears in CD1d gene-disrupted (CD1d-KO) mice. The glycosphingolipid, α-galactosylceramide (α-GalCer) that was originally discovered in marine sponge as a novel anticancer agent, is recognized by *i*NKT cells in context with 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.

6.2.2. Role in Host Defense to Infection

NKT cells contribute to development of both Th1 and Th2 responses under different experimental conditions (Lehuen et al., 1998; Carnaud et al., 1999; Cui et al., 1999; Singh et al., 1999). Although the significance of NKT cells in infectious diseases remains to be fully elucidated, to date there are several published studies on this issue. Three roles are identified for these cells in host defense against infectious pathogens. First, the clinical course of *M. tuberculosis* and *Salmonella choleraesuis* infection is not much affected by manipulations designed to suppress the activity of NKT cells (Behar et al., 1999; Ishigami et al., 1999). Second, infection with *Listeria monocytogenes* or *Toxoplasma gondii* is rather improved by the similar manipulations (Szalay et al., 1999; Nakano et al., 2001). Finally, mice lacking NKT cells are more susceptible to infection caused by *Leishmania major*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Borrelia burgdorferi*, and *Plasmodium yoelii* than control mice (Ishikawa et al., 2000; Kumar et al., 2000; Mannoor et al., 2001; Nieuwnhuis et al., 2002; Kawakami et al., 2003). Thus, the role of NKT cells seems different among infectious pathogens.

6.2.3. Accumulation of iNKT Cells in Lung after Cryptococcal Infection

Recently, we reported the increase of NKT cells in lungs after intratracheal infection with *C. neoformans* (Kawakami et al., 2001a). Inflammatory leukocytes obtained from the homogenates of infected lungs were stained with anti-TCRαβ 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 peak values 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, $V\alpha14^+$ NKT cells as well as conventional T and NK cells were found to increase in the lungs after intratracheal infection with *C. neoformans*.

Migration of inflammatory leukocytes from the peripheral circulation to the site of infection 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 (Rossi and Zlotnik, 2000). 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 to the site of infection by many chemokines, including MCP-2, -3, MIP-1α, RANTES, IP-10, and lymphotactin, under various conditions (Allavena et al., 1994; Maghazachi et al., 1994, 1997; Taub et al., 1995; Giancarlo et al., 1996; Loetscher et al., 1996 et al). In contrast, MIP-2 was the only chemokine known to function in trafficking NKT cells until MCP-1 was identified as a chemoattractant for these cells (Faunce et al., 2001). In MCP-1KO mice, accumulation of NKT cells in lungs was not observed after infection with *C. neoformans* (Kawakami et al., 2001a). Consistent with these data, MCP-1 production preceded the kinetics of NKT cell-mediated inflammatory responses. Thus, NKT cell trafficking into the fungus-infected sites involves at least in part the production of MCP-1, although other chemokines may contribute, as observed in NK cells.

6.2.4. Role of iNKT cells in Th1 Response and Host Defense to Cryptococcal Infection

A remarkable feature of NKT cells is the expeditious and abundant production of IFN-γ and IL-4 upon stimulation via their antigen receptors (Godfrey et al., 2000; Taniguchi and Nakayama, 2000). Accumulating evidence suggests that NKT cells are involved in the regulation of Th1 and Th2 cell development. On the other hand, host defense against cryptococcal infection is critically regulated by the balance between Th1- and Th2-mediated immune responses (Kawakami et al., 1997; Koguchi and Kawakami, 2002). These findings suggest that NKT cells may affect the host immune responses and protection against infection with this fungal microorganism. In our study (Kawakami et al., 2001), the Th1-mediated immune responses, as indicated by antigen-specific IFN-γ production by T cells and delayed-type hypersensitivity reaction, were significantly ameliorated in Jα281-KO mice lacking *i*NKT cells, compared with control wild-type mice. In contrast, Th2 cytokine synthesis was not altered in these mice. Furthermore, the clearance of fungal microorganisms from the infected sites was significantly delayed in Jα281-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 the establishment of Th1-mediated acquired immune responses, which leads to host protection against cryptococcal infection.

6.2.5. Induction of Th1 Response and Protection against Cryptococcal Infection by Ligand-Specific Activation of iNKT Cells

*i*NKT cells recognize α-GalCer by their antigen receptors in the context of CD1d molecules expressed on DCs (Kawano et al., 1997; Godfrey et al., 2000; Taniguchi and Nakayama, 2000). Such engagement causes prompt secretion of both IFN-γ and IL-4 by these cells and emergence of their cytolytic activity against tumor cells. Toura et al. (1999) 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 et al. (2000) were the first group to demonstrate 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