



Role of natural killer T cells in host defence against cryptococcal infection

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Summary

Cryptococcosis is an opportunistic fungal infectious disease that often occurs in severely immunocompromised patients. Host defence against the causative microorganism is largely mediated by cellular immunity, and Th1 cytokines, such as IFN- γ , play central roles in the host protective responses. IL-12 and IL-18 activate the synthesis of IFN- γ by innate immune cells, including NK, NKT and $\gamma\delta$ T cells and promote the differentiation of Th1-type acquired immune responses. Recently, NKT cells, which are involved in the recognition of glycolipid antigens, have attracted much attention based on their potent immunomodulating activities. Several studies have reported the role of this particular component of innate immune responses in tumor immunity and pathogenesis of autoimmune diseases. In this review, I outline the recent findings on the role of NKT cells in host defence against infectious microorganisms, with a special focus on our data emphasizing the importance of this subset of immunocytes in the development of acquired as well as early host protection against cryptococcal infection.

Key words

NKT cells, Host defence, Cryptococcosis

Papel de las células T agresoras naturales (NKT) en la defensa del huésped frente a la infección criptocócica

Resumen

La criptococosis es una micosis oportunista que se observa con frecuencia en pacientes con severa inmunodeficiencia. Las defensas del huésped contra los agentes etiológicos está mediada principalmente por la inmunidad celular, y las citocinas de los Th1, como el IFN- γ , juegan un papel central en las respuestas protectoras del huésped. IL12 e IL18 activan la síntesis de IFN- γ por parte de las células inmunes innatas, que incluyen a las células NK, NKT y $\gamma\delta$ T y promueven la diferenciación de respuestas inmunológicas adquiridas de tipo Th1. Recientemente, las células NKT implicadas en el reconocimiento de los antígenos glicolípidos han llamado la atención de los investigadores debido a sus potentes actividades inmunomoduladoras. Se han descrito varios estudios sobre el papel de este componente concreto de la s respuesta inmunes innatas en la inmunidad antitumoral y en la patogenia de enfermedades autoinmunes. En esta revisión, resalto los hallazgos recientes sobre el papel de las células NKT en la defensa del huésped frente a los agentes infecciosos, con especial atención sobre nuestros datos que enfatizan la importancia de esta subpoblación de inmunocitos en el desarrollo de la protección tanto temprana como adquirida del huésped frente a la infección criptocócica.

Palabras clave

Células NKT, Defensa del huésped, Criptococosis

After intracellular infectious pathogens invade the host, innate immune mechanisms mediated by the complement system, phagocyte cells and primitive immune lymphocytes, such as natural killer (NK), natural killer T (NKT), $\gamma\delta$ antigen receptor-bearing T ($\gamma\delta$ T) cells and B1 cells, are activated to limit the infection. However, the innate immune response fails to get rid of the infection, because the intracellular pathogens resist the killing mechanisms by phagocyte cells. Although innate immune lymphocytes potentiate the macrophage killing activity via production of IFN- γ , the overall host defence 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 these properties, the innate immune mechanism has been recognized merely as a "temporary protection" until the acquired immune response is established. However,

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recent investigations disagree with this concept. In this respect, Tateda and co-workers [1] argued for the involvement of early recruited neutrophils in the polarization process of Th1-mediated acquired immune responses, rather than the original role as "phagocyte cells", during *Legionella pneumophila* pneumonia. Similar observations were reported by Pedrosa *et al.* [2] in mice infected with *Mycobacterium tuberculosis*. Furthermore, accumulating evidence suggests that innate immune lymphocytes, including NK and NKT cells, are the cells that bridge between innate and acquired immune responses [3-5]. Thus, the early host protective responses mediated by innate immune mechanism is more than a "temporary protection" before development of acquired immunity. This review deals with the role of NKT cells in the host defence against infectious pathogens, with an emphasis on our recent data in a murine model of cryptococcal infection.

Characterization of NKT cells

NKT cells not only express T cell receptor but also NK markers including NK1.1 [6,7]. Specific features of this cell type include extremely limited repertoire with an invariant V α chain consisting of V α 14-J α 281 gene segment and highly skewed V β chains, V β 8.2, 7 and 2 in mice [6,7]. These cells are found in large numbers in the liver, thymus and bone marrow and in small numbers in the spleen and lungs [7]. Although the natural ligand for NKT cells remains to be defined, glycosylphosphatidyl inositols (GPIs) and a synthetic glycolipid, α -galactosylceramide (α -GalCer), which was originally identified as a novel anti-cancer agent from marine sponge, have been demonstrated to be presented in context of CD1d, a MHC class I-like surface molecule, which is composed of monomorphic α chain and β 2 microglobulin [6-9]. NKT cells secrete large amounts of IFN- γ and IL-4 in a prompt manner after engagement of the antigen receptor [6,7,9,10] and contribute to the differentiation of both Th1 and Th2 cells [6,7,11-15]. NKT cells exhibit perforin-dependent cytotoxicity against tumour cells upon receptor-mediated stimulation with α -GalCer. Administration of α -GalCer suppresses the growth and metastasis of tumour cells through activation of NKT cells [16-19]. Thus, NKT cells are considered to play an important role in the regulation of tumour immunity. In other investigations, it has been demonstrated that NKT cells are also involved in other pathogenic conditions, including allergic and autoimmune diseases [20-23]. Manipulations that increase the activity of NKT cells lead to the attenuation of experimental autoimmune diseases, such as diabetes mellitus in NOD mice [21], while the opposite treatment worsens the disease conditions [20,22]. Consistent with these findings, the proportion of peripheral blood NKT cells is reduced in patients with active collagen diseases, and their number correlate with the severity of disease [23].

Regulation of Th1-Th2 cytokine balance by NKT cells

In earlier studies, Yoshimoto and co-workers [24] demonstrated that activation of NKT cells by *in vivo* administration of anti-CD3 mAb resulted in a rapid production of IL-4 and proposed that this cell population may be the major source of early IL-4 production that contributes to the differentiation of Th2 cells. However, several subsequent studies indicated that Th2 response was not hampered in β 2-microglobulin- or CD1d-deficient mice, which have markedly reduced numbers of NKT cells [25-

28]. These findings made the role of these cells in Th2 cell development questionable. Recent investigations, however, revealed that stimulation of NKT cells via their antigen receptors expedited and increased the production of IFN- γ and IL-4 [6,7], suggesting the dual roles of this particular lymphocyte subset in the differentiation of both Th1 and Th2 cells. In this respect, V α 14+ TCR transgenic mice showed elevated serum level of IgE and IL-4 [29], and activation of V α 14+ NKT cells by α -GalCer induced T cell response to ovalbumin (OVA) polarized toward Th2-dominant condition [14]. In contrast, accumulating evidence emphasizes a positive role for NKT cells in the development of Th1 cells. Activation of V α 14+ NKT cells by α -GalCer led to the rapid production of IFN- γ by themselves and other bystander cells, such as NK cells, *in vitro* [30] and suppressed *in vivo* Th2 differentiation and subsequent IgE synthesis induced by OVA immunization or infection with *Nippostrongylus brasiliensis* through the induction of IFN- γ production [12]. In other studies, NKT cells were found to contribute to Th1-mediated responses, including granuloma formation caused by mycobacterial lipid antigen [31] and the IFN- γ -mediated protection of mice against infection with malaria parasites through the ligand-specific activation of NKT cells by α -GalCer [32].

NKT cells and infection

Although the significance of NKT cells in infectious diseases remains to be fully elucidated, to date there are several published studies on this issue. These studies were conducted in anti-CD1d mAb-treated and CD1d gene-disrupted (CD1d $^{-/-}$) mice, which manipulations abolished most of NKT cells, and J α 281-deficient (J α 281 $^{-/-}$) mice, which lacked particular NKT cell subset bearing V α 14J α 281+ antigen receptors. Other studies examined the effect of α -GalCer, a specific activator of V α 14+NKT cells, on the clinical course of infectious diseases. In these investigations, three roles were identified for NKT cells in host defence against infectious pathogens (Table 1). First, the clinical course of *M. tuberculosis* infection in CD1d-deficient mice was not much different from that in control mice [33,34] and minimally affected by treatment with anti-CD1d monoclonal antibody (mAb) [35]. Similarly, genetic depletion of V α 14+ NKT cells did not result in exacerbation of infection with *M. tuberculosis*, *Mycobacterium bovis* BCG and *Salmonella choleraesuis* [34,36,37]. Second, infection with *Listeria monocytogenes* or *Toxoplasma gondii* was rather improved by manipulations designed to suppress the activity of NKT cells [38,39]. Administration of anti-CD1d mAb resulted in prolongation of *Listeria* infection in mice, which was associated with increased secretion of Th1-type cytokines and decreased TGF- β production [38]. Similarly, depletion of NKT cells enhanced host protection of mice from *T. gondii* infection by increasing Th1-polarized cytokine production [39]. Finally, mice lacking V α 14+NKT cells were more susceptible to *Leishmania major* and *Trypanosoma cruzi* infection than control mice [40,41]. Similar results were reported in CD1d $^{-/-}$ mice infected with *Borrelia burgdorferi*, *Plasmodium yoelii* and *T. cruzi* [41-43]. Thus, the role of NKT cells seems different among infectious pathogens.

Host defence against cryptococcal infection

Cryptococcosis is an opportunistic fungal infectious disease often seen in patients with impaired cellular

Table 1. NKT cells and infections.

Role of NKT cells in host defence against infection	Microbes	α -GalCer	Anti-CD1d Ab, CD1dKO or J α 281KO mice	Reference
Protective	<i>L. major</i>	- ^a	Exacerbated	40
	<i>B. brugdorferi</i>	-	Exacerbated	42
	<i>P. yoelii</i> & <i>P. berghei</i>	Protected	-	32
	<i>T. cruzi</i>	-	Exacerbated	41
	<i>P. aeruginosa</i>	Protected	Exacerbated	71
	<i>C. neoformans</i>	Protected	-	69,70
	<i>C. neoformans</i>	-	Exacerbated	54
No effect	<i>M. tuberculosis</i>	-	Not exacerbated	33
	<i>M. tuberculosis</i>	-	Not exacerbated	34
	<i>M. bovis</i> BCG	-	Not exacerbated	36
	<i>S. choleraesuis</i>	-	Not exacerbated	37
Suppressive	<i>L. monocytogenes</i>	-	Protected	38
	<i>T. gondii</i>	-	Protected	39

a - : not examined.

immunity, such as haematological malignancies and acquired immunodeficiency syndrome (AIDS). The causative microbial agent, *Cryptococcus neoformans*, infects lungs through an airborne route and causes granulomatous lesions, which prevent the microorganism from spreading out from the primary infected sites. However, the infection can disseminate haematogenously to the central nervous system, frequently leading to lethal meningoencephalitis particularly in AIDS patients. The host defence against this fungal pathogen is critically regulated by cell-mediated immunity [44] and CD4⁺ T cells play a central role in limiting infection [45,46]. The balance between 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 Th2 dominant conditions [47,48]. Mice depleted of Th1-type cytokines (e.g., IFN- γ and TNF- α) are highly susceptible to cryptococcal infection [49,50], while the infection is less severe in mice lacking Th2 cytokines (e.g., IL-4 and IL-10) than control mice [51,52]. Differentiation of naive helper T cells into Th1 cells absolutely requires the presence of IL-12, and this response is strongly potentiated by IL-18 [53]. In recent studies from our laboratory [49], targeted disruption of the gene for IL-12 or IL-18 resulted in reduced host resistance and Th1 response to *C. neoformans*, indicating the prerequisite role for these cytokines in the development of host protective response.

Role of NKT cells in host defence against cryptococcal infection

To my knowledge, there are no published studies that define the role of NKT cells in host defence against infection caused by fungal microorganisms. Here, I review this issue with emphasis on our recent data from a murine model of cryptococcal infection [54].

Accumulation of NKT cells at the site of infection. Initially, we examined the kinetics of NKT cells in lungs after intratracheal infection with *C. neoformans*. Inflammatory leukocytes were obtained from the homogenates of infected lungs by density-gradient centrifugation after treatment with collagenase and DNase. The obtained cells were stained with anti- $\alpha\beta$ TCR and -NK1.1 mAbs to discriminate conventional T, NK and NKT cells. The proportions of conventional T, NK and NKT cells, as indicated by $\alpha\beta$ TCR+NK1.1-, $\alpha\beta$ TCR-NK1.1+ and $\alpha\beta$ TCR+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 (5-6 and 1.5-2 fold, compared with uninfected condition in NKT cells and other cells, respectively). We further defined the dynamics of V α 14J α 281 antigen receptor-bearing cells, a dominant subset of NKT cells in the infected lungs by detecting cells bound to either anti-V α 14 mAb or α -GalCer-CD1d tetramer. Similar kinetics were observed in particular subsets of NKT cells using both strategies for detection. Thus, V α 14⁺ 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 is critically regulated by a variety of chemokines, which are classified into two major subgroups, CXC or α and CC or β , based on the arrangement of two N-terminal cysteine residues [55]. ELR⁺ CXC-chemokines, including IL-8, are neutrophil-mediated inflammatory responses, while ELR⁻ CXC-chemokines (e.g., IP-10 and Mig) and CC-chemokines (e.g., MCP-1, MIP-1 α , -1 β and RANTES) predominantly attract lymphocytes and macrophages [55]. Many investigators have reported that resting or activated NK cells are attracted by a variety of chemokines, including MCP-2, -3, MIP-1 α , RANTES, IP-10 and lymphotactin, under various conditions [56-61]. In contrast, MIP-2 was the only chemokine that functions in trafficking NKT cells until we identified MCP-1 as a chemoattractants for these cells [62]. In mice genetically lacking MCP-1 synthesis, accumulation of NKT cells in lungs was not observed after infection with *C. neoformans* [54]. 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. Other alternate mechanisms for the increase of NKT cells include the possible their local proliferation at the site of infection, rather than migration from the circulation. IL-15 is known to act as a major growth factor for NKT cells, because mice deficient of IL-15R α or IL-2/IL-15R β lacked such cells [63]. However, in our study, this possibility remains open for further investigation.

Role of NKT cells in bridging innate immunity to Th1-mediated acquired protective immune responses. A remarkable feature of NKT cells is the expeditious and abundant production of IFN- γ and IL-4 upon stimulation via their antigen receptors [6,7]. Accumulating evidence suggests that NKT cells are involved in the regulation of Th1 and Th2 cell development. On the other hand, host

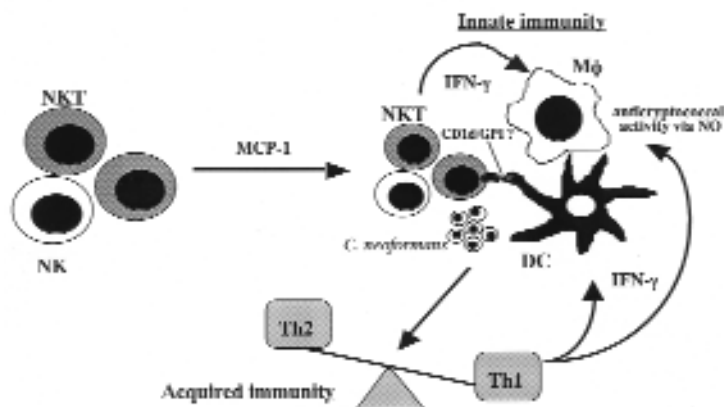


Figure 1. Bridging role of NKT cells between innate and acquired immunity. Dendritic cells (DCs) may express yet-unknown glycolipid antigen derived from *C. neoformans* coupled with CD1d molecule on their surface. This antigen-CD1d complex may be presented to the antigen receptor of NKT cells, which accumulate at the infected site by particular chemokines, such as MCP-1. The activated NKT cells may promptly secrete a large amount of IFN- γ , which potentiates macrophage (M ϕ) anti-cryptococcal activity as well as facilitates the differentiation of Th1 cells. Thus, NKT cells are likely to bridge the early protective response mediated by innate immunity to the more potent acquired immune responses for eradication of fungal infection. NO: nitric oxide.

defence against cryptococcal infection is critically regulated by the balance between Th1- and Th2-mediated immune responses [47,48]. These findings suggest that NKT cells may affect the host immune responses and protection against infection with this fungal microorganism. In our recent study [54], 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 J α 281^{-/-} mice genetically lacking V α 14⁺ NKT cells, compared with control wild-type mice. In contrast, Th2 cytokine synthesis was not influenced by the genetic disruption of J α 281 gene. Furthermore, the clearance of fungal microorganisms from the infected sites was significantly delayed in J α 281^{-/-} mice, compared with control mice. These findings demonstrate that NKT cells function not only in the innate immune phase but also in bridging to the establishment of Th1-mediated acquired immune responses, which leads to host protection against cryptococcal infection. These relations are summarized in Figure 1.

Putative natural ligands for recognition by V α 14⁺ NKT cells in cryptococcal infection. NKT cells express antigen receptors with a limited repertoire, consisting of invariant α chain with V α 14 gene segment and highly skewed β chains with V β 8.2, 7 and 2 in mice [6,7]. Based on these properties, many investigators had predicted a particular molecule to be the ligand for NKT cells. Kawano and co-workers [9] were the first group to report that α -GalCer, a marine sponge-derived glycolipid, is a specific ligand for antigen receptors of these cells. α -GalCer activates NKT cells to produce both IFN- γ and IL-4 and to acquire cytotoxic potential in a specific manner [6,7]. However, the endogenous natural ligands of NKT cells have not been defined, because mammals do not generate α -GalCer. Using crystal structure and mass spectrometry analyses, Joyce and co-workers [8] recently found GPIs to be candidate molecules that could bind to CD1d and present to NKT cell antigen receptors. In addition, it was demonstrated by Schofield *et al.* [64] that NKT cells regulated IgG production against GPI-anchored surface antigens of protozoans, *Plasmodium* and *Trypanosoma*. Similar results were reported by Duthie *et al.* [41] in mice infected with *T. cruzi*. They showed shortening of the chronic phase of antibody response to GPI anchored surface antigens in NKT cell-deficient mice. Thus, GPIs seem likely molecules that are recognized by NKT cells as the endogenous and exogenous natural ligands, although conflicting data have been also reported by other investigators [65]. In this regard, certain GPI-anchored antigens might be involved in activation of NKT

cells caused by cryptococcal infection. Compatible with this hypothesis, it was recently reported that *C. neoformans* could potentially secrete GPI-anchored surface antigens [66] and that this fungal pathogen produced major T cell-stimulating antigens, which were composed of mannoproteins with GPI-binding sites and incorporated by antigen presenting cells via mannose receptor [67]. In order to understand the precise mechanism of involvement of NKT cells in the host defence against this infection, identification of pathogen-derived ligands of these cells is desired.

Induction of Th1 response and protection against cryptococcal infection by ligand-specific activation of NKT cells. V α 14⁺ NKT cells recognise α -GalCer, a synthetic glycolipid, by their antigen receptors in the context of CD1d molecules expressed on dendritic cells (DCs) [6-9]. Such engagement causes prompt secretion of both IFN- γ and IL-4 by these cells and emergence of their cytolytic activity against tumour cells [6,7]. Taura *et al.* [19] indicated that administration of DCs pulsed with α -GalCer induced potent antitumor activity through specific activation of V α 14⁺ NKT cells, and resulted in the complete suppression of melanoma metastasis in the liver.

In infectious diseases, Gonzalez-Aseguinolaza *et al.* [32] 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 revealed that co-administration of α -GalCer potentiated the protective effect against this infection caused by immunization with irradiated malaria parasite [68]. Our group observed similar effects for this treatment in a murine model of cryptococcal infection [69]. 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 mice genetically lacking V α 14J α 281 gene, indicating the involvement of V α 14⁺ NKT cells. IFN- γ production induced by α -GalCer was totally mediated by IL-12, but not IL-18 [70]. The protective effects by the ligand-specific activation of NKT cells against *Pseudomonas aeruginosa* were recently reported by other investigators [71].

The aforementioned effects suggest that α -GalCer is a potentially promising immunotherapeutic agent for the treatment of certain intractable infectious diseases as well as malignant and autoimmune diseases.

Concluding remarks

Acquired immunity, an antigen-specific host defence mechanism, had been the central dogma of previous studies on immunological response to infection. Recently, however, the roles of innate immune responses, mediated by NK, NKT and $\gamma\delta$ T cells, have garnered much attention by many investigators and the biological significance of these cells is being extensively explored. In the host immune response to infectious pathogens, as demonstrated in our series of investigations on cryptococcal infection, the important roles of innate immunity, mediated especially by NKT cells, have been clarified, and many evidences have been provided that support the involvement of this particular lymphocyte subset in determining the balance of Th1-Th2 immune responses.

Thus, NKT cells seem to participate in bridging early host protection, by innate immune cells, to antigen-specific acquired immune responses. Furthermore, NKT cells could be potentially useful clinically for immunotherapy of intractable infectious diseases by ligand specific activation with α -GalCer. However, there are still many issues to be resolved before such immunotherapy could be applied: e.g., the reasons why NKT cells play distinct roles in host defence against different pathogenic microorganisms, the relationship with other innate immune cells like NK and $\gamma\delta$ T cells and the precise mechanism for the anti-infective effect of α -GalCer.

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