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MINIREVIEW

Role of CD1d-Restricted NKT Cells in Microbial Immunity

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The discovery that T cells recognize lipid and glycolipid molecules presented by CD1 proteins has greatly expanded the number of potential microbial antigens targeted by the immune system following infection. The ability of CD1d-restricted NKT cells to activate innate and adaptive immune responses has led to the idea that these cells can modulate immunity to infectious agents. In addition, CD1d-restricted NKT cells may directly contribute to host resistance as they express a variety of effector molecules that could mediate an antimicrobial effect. Although much has been learned about CD1d-restricted NKT cells through the use of the synthetic antigen α-galactosylceramide (αGalCer), the field has been hampered by the paucity of information about the physiological self and microbial lipid antigens that can be presented by CD1d. Here we review the literature stating that CD1d-restricted NKT cells contribute to host defense against microbial pathogens.

The biology of CD1d and CD1d-restricted T cells. The CD1 proteins are antigen-presenting molecules that present lipid antigens to T cells. Similar in structure to major histocompatibility complex (MHC) class I, the CD1 heavy chain associates with β2 microglobulin to form a heterodimer that is expressed on the cell surface of the antigen-presenting cell (APC) (76). However, in contrast to MHC molecules, CD1 proteins have a deep hydrophobic antigen binding pocket that is well suited to binding lipid antigens (35, 96). The human CD1 locus is located on chromosome 1 and contains five distinct genes: CD1A, -B, -C, -D, and -E. Based on sequence homology, the CD1 family is divided into group 1 (CD1a, -b, and -c) and group 2 (CD1d) proteins (18). The group 1 CD1 proteins are found in a variety of mammalian species, including humans, but not in mice or rats (78). In contrast to group 1 CD1, CD1d is found in humans, rodents, and most mammalian species that have been studied. The discovery that CD1d is the antigen-presenting molecule that restricts NKT cells provided an important insight into the function of group 2 CD1 (12). Murine NKT cells were originally defined as a population of T cells that express an invariant T-cell receptor (TCR) α chain (Vα14/Jα281) in association with Vβ2, -7, or -8 and express the NK1.1 antigen (NKR-P1C), a cell surface C-type lectin that is also expressed by NK cells and activated T cells (13, 60). Phenotypically, $NK1^+$ T cells are either $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^-$ and this T-cell population represents a major fraction of the mature T cells in thymus, nearly 50% of α/β TCR^+ T cells in liver and up to 5% of splenic T cells, but are rare in lymph nodes (LN). These cells are notable for their rapid production of interleukin 4 (IL-4) and gamma interferon (IFN-γ) after activation with anti-CD3 monoclonal antibody (MAb). Human invariant Vα24-JαQ/Vβ11T cells are phenotypically and functionally homologous to murine $NK1^+$ T cells and, like their murine counterparts, are CD1d restricted and express NKR-P1. The degree of conservation is remarkable, as mouse CD1d-restricted T cells can recognize human CD1d and vice versa, establishing mice as an excellent model for the study of human CD1d and NKT cells (15).

Not surprisingly, defining NKT cells has become more complicated. Conventional human and murine α/β TCR^+ and γ/δ TCR^+ T cells can also express NK cell markers, especially following infection. For example, NKT cells have been detected in CD1d knockout (−/−) and $J\alpha281^{-/-}$ mice, showing that coexpression of the α/β TCR-CD3 complex with the NK1.1 antigen is not sufficiently specific to identify CD1d-restricted NKT cells. To complicate matters further, two subsets of CD1d-restricted T cells have been identified: one that expresses the invariant TCR (i.e., invariant NKT cells or iNKT) and one that uses a diverse TCR repertoire (diverse NKT cells) (9). The synthetic ligand, αGalCer (see below), activates iNKT cells but not diverse NKT cells. Although exceptions may emerge, this has been a useful distinction, as iNKT cells can be specifically identified by flow cytometry with αGalCer-loaded CD1d-multimers that bind to the invariant TCR (34, 41). The in vivo function of the two NKT cell subsets can sometimes be distinguished, since $CD1d^{-/-}$ mice lack both subsets of NKT cells, while $J\alpha281^{-/-}$ mice lack only iNKT cells. In this review, the more inclusive term "CD1d-restricted NKT cell" will be used to include both invariant and diverse CD1d-restricted NKT cells. When appropriate, the term "iNKT" cell will be used to refer to NKT cells that stain with αGalCer-loaded CD1d tetramers, respond to αGalCer, or are absent from $J\alpha281^{-/-}$ mice.

What antigens are presented by CD1d? A significant advance in understanding the biology of the group 1 CD1 proteins (CD1a, -b, and -c) was the finding that these proteins can present foreign microbial lipid antigens, including several mycobacterial antigens (6, 7, 71, 80). In contrast, the antigens presented by CD1d remain poorly characterized. CD1d-restricted NKT cells were first described as self-reactive, as both human and murine CD1d-restricted NKT cells can recognize

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CD1d in the absence of exogenously added antigen (11, 12, 20). The direct recognition of CD1d may arise in part from the use of T-cell clones and hybridomas with a low activation threshold and the use of tumor cell transfectants as APC that express supraphysiological levels of CD1d, both of which enhance the detection of low-affinity interactions between CD1d, self-lipid antigens, and the TCR. Directly reactive CD1d-restricted NKT cells are antigen dependent, and some recognize endogenous cellular lipid antigens (42). Although the self-lipid antigens remain largely unidentified, we have shown that certain iNKT cells recognize phospholipids, including phosphatidylinositol and phosphatidylethanolamine, and the endogenous antigen for at least one CD1d-restricted T cell has been successfully purified (41; S. Behar, J. E. Gumpez, D. Young, D. B. Moody, M. B. Brenner, C. E. Costello, and J. Rauch, abstract from the 2nd International Workshop on CD1 Antigen Presentation and NKT Cells 2002, abstr. 1, 2002). A more complicated question is whether CD1d can present microbial lipids to NKT cells. Microbial pathogens produce many lipid and glycolipid molecules that are sufficiently different from mammalian molecules so that they could be recognized as foreign antigens by the mammalian immune system (24). One class of candidate lipid antigens are the glycosylphosphatidylinositols (GPI), which are found in the cell membrane of mammalian and protozoan cells and function as a membrane anchor for some cell surface proteins. Mammalian GPI has been reported to be one of the major ligands bound to CD1d, although it is not thought to be recognized by CD1d-restricted NKT cells (70). Protozoan GPI, which is structurally different from mammalian GPI, could be presented by CD1d and recognized as a foreign antigen (49).

αGalCer specifically activates CD1d-restricted iNKT cells. The compound αGalCer is a synthetic glycolipid based on the structure of related lipids purified from marine sponges, which were shown to induce tumor regression in experimental animal models (73). Taniguchi et al. showed that the antitumor effect of αGalCer was dependent upon iNKT cells and that the α-glycosylceramides were antigens presented by CD1d (25, 56). The recognition of αGalCer is a general feature of both human and murine iNKT cells (15, 57, 85). αGalCer binds to purified CD1d protein in cell-free systems, and the resulting αGalCer/CD1d complex can activate iNKT cell hybridomas, proving that αGalCer is a CD1d-presented antigen (42, 74). Although their structure resembles those of other CD1-presented antigens, α-glycosylceramides are not known to be produced by mammalian cells or pathogenic microbes and their physiological relevance is unknown (56, 71, 85). Despite this, the ability to specifically activate iNKT cells has made αGalCer a critical reagent for the study of iNKT.

In vivo administration of αGalCer has profound immunological consequences that are mediated by CD1d-restricted iNKT cells, and αGalCer-dependent modulation of the immune response does not occur in mice that lack CD1d or iNKT cells. These effects include activation of NK cells, B cells, and memory $CD8^+$ and $CD4^+$ T cells within 3 to 24 h, as determined by the induction of early cell activation markers such as CD69 (B, T, and NK cells) and CD80 and CD86 (B cells) (17, 21, 81). For example, following αGalCer treatment, iNKT cells activate NK cells to produce IFN-γ, which may contribute to the transient increase in serum IFN-γ induced by αGalCer (21, 63). Although $CD1d^{-/-}$ and $J\alpha281^{-/-}$ mice have intact T-helper (Th2) responses, administration of αGalCer can skew the immune response of both iNKT and conventional antigen-specific T cells toward a Th2 phenotype (17, 23, 68, 81, 83). Under other conditions, αGalCer-activated iNKT cells inhibit Th2 cell differentiation and, during certain infections, αGalCer induces IFN-γ production but not Th2-type cytokines (reference 26 and see below). These seemingly contradictory data may reflect our incomplete understanding of the role of APC in the activation of iNKT cells (58, 92). For example, iNKT cell recognition of αGalCer presented by dendritic cells (DC) leads to CD40/CD40 ligand-dependent IL-12 production by the DC. Thus, under the influence of iNKT cells, DC mature into Th1-promoting APC. In contrast, the production of IL-4 by iNKT cells is independent of IL-12 (92). Thus, complex interactions and feedback regulatory networks between APC and iNKT cells may determine whether activated iNKT cells promote a Th1 or Th2 immune response.

In addition to these effects on the immune response, αGalCer has important consequences on iNKT cells themselves. In contrast to conventional T cells, expansions of NKT cells have only been infrequently detected following activation (2, 93). In fact, following stimulation with anti-CD3 MAb or αGalCer, it can be difficult to detect iNKT cells because of their tendency to undergo apoptosis (32, 62). Despite being a minor T-cell population, the restricted TCR repertoire of CD1d-restricted NKT cells may result in a higher precursor frequency for a particular antigen than what is typically observed for MHC-restricted T cells. If the number of NKT cells recognizing a particular antigen is high to begin with, clonal expansion may not be required. Furthermore, expansion may not be required for iNKT cell function because their modulation of other cells can occur locally through the production of cytokines.

ROLE OF CD1d-RESTRICTED NKT CELLS IN BACTERIAL INFECTIONS

***Mycobacterium*.** The group 1 CD1 proteins present lipids found in the *Mycobacterium tuberculosis* cell wall, such as mycolic acid, glucose monomycolate, and isoprenoids, to human T cells (8, 71, 72). These antigens are presented by CD1 when the purified lipids are provided to APC and are presented by the CD1 pathway after intracellular processing in macrophages infected with *M. tuberculosis*. Antigen-specific CD1-restricted human T-cell lines can kill macrophages infected with *M. tuberculosis*, and since some of these CD1-restricted T cells express granulysin, they can kill the bacteria as well (87, 88). That increased CD1-restricted T-cell responses to mycobacterial lipid antigens can be detected in tuberculosis patients suggests that these T cells are generated as part of the adaptive immune response following infection of people with *M. tuberculosis* (72).

In contrast to group I CD1, there are no definitive examples of CD1d presentation of mycobacterial antigens to NKT cells. Mycobacterial lipoarabinomannan (LAM) can bind to purified CD1d protein; however, purified iNKT cells do not recognize LAM, nor is the anti-LAM antibody response CD1d dependent (14, 16). On the other hand, preliminary studies do indicate that at least some iNKT cells may recognize certain mycobacterial phosphatidylinositolmannosides (e.g., PIM4) (E.

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TABLE 1. Mice rendered genetically deficient in CD1d-restricted NKT cells exhibit a spectrum of susceptibility to infectious disease

Finding	αGalCer enhances host resistance in +/+ mice	αGalCer not tested
CD1d$^{-/-}$ or Jα281$^{-/-}$ mice have more-severe disease than do +/+ mice	*P. aeruginosa*, *C. neoformans*, HSV, ECMV-D[b]	*B. burgdorferi*, Plasmodium-infected erythrocytes,[a] *L. major*, RSV[a]
CD1d$^{-/-}$ or Jα281$^{-/-}$ mice have disease similar to +/+ mice	*M. tuberculosis*, *Plasmodium* sporozoite, *T. cruzi*[c]	
CD1d$^{-/-}$ or Jα281$^{-/-}$ mice have less-severe disease than do +/+ mice	Coxsackievirus B3,[b] RSV[a]	*S. choleraesuis*, *L. monocytogenes*,[d] *Plasmodium*-infected erythrocytes[a]

[a] The phenotype depends on the mouse strain.
[b] A role for diverse CD1d-restricted T cells is suggested by the data.
[c] The phenotype depends upon virulence of pathogen.
[d] Only anti-CD1d antibody blockade was tested.

Scotet, S. Maillet, K. Fischer, U. E. Schaible, and M. Bonneville, abstract from the 2nd International Workshop on CD1 Antigen Presentation and NKT cells 2002, abstr. 2, 2002). *M. tuberculosis* cell walls treated to remove most protein induce granuloma formation when injected subcutaneously into mice, and under these conditions, the majority of infiltrating T cells are iNKT cells (3). The critical *M. tuberculosis* cell wall constituent appears to be PIM, which can also induce granulomas containing infiltrating iNKT cells (37). Interestingly, the recruitment of iNKT cells into the granulomatous lesions is independent of CD1d (67). Perhaps this is not surprising, since the migration of iNKT cells is thought to be dependent upon chemotactic signals induced by local inflammation rather than upon antigen recognition. Although the recruitment of iNKT cells to inflammatory sites is independent of CD1d, the presentation of microbial lipids could lead to their activation and retention. Why would this be beneficial to the host? Recent studies have highlighted the ability of both CD1-restricted T cells to induce DC maturation (64, 95). Presentation of either self or foreign antigens by tissue resident immature DC to CD1d-restricted NKT cells may induce DC maturation and migration to regional LN. Thus, in addition to acting as early effector cells, rapid recruitment of iNKT cells may contribute to the initiation of adaptive immune response through their interactions with DC.

The initial impetus to examine the role of CD1d in the host response to *M. tuberculosis* was based on the finding that group 1 CD1 proteins presented mycobacterial lipid antigens to human T cells. As discussed above, there is little evidence to date that CD1d presents microbial lipid antigens to NKT cells; instead, it is thought that CD1d-restricted NKT cells play an immunoregulatory role during the immune response. Following intravenous (i.v.) inoculation with *M. tuberculosis*, CD1d$^{-/-}$ mice are not more susceptible than controls, indicating that CD1d-restricted NKT cells are not absolutely required for protective immunity (10). Our results have been confirmed by other labs following respiratory and i.v. infection by *M. tuberculosis* and *Mycobacterium bovis* BCG (30, 53, 84). In contrast, Sugawara et al. showed Jα281$^{-/-}$ mice that lack CD1d-restricted iNKT cells were marginally more susceptible to *M. tuberculosis*, and Szalay et al. found that anti-CD1d MAb administered in vivo impaired early immunity to *M. tuberculosis* (89, 91). Although interesting, the latter study is difficult to interpret since CD1d is expressed by a variety of murine cell types and one cannot be certain that this effect was mediated by the blockade of CD1d antigen presentation, instead of by a different mechanism such as antibody-dependent lysis of CD1d-expressing APC. However, these studies suggest that, under certain conditions, CD1d-restricted NKT cells could participate in the host response to *M. tuberculosis*. To further examine this possibility, αGalCer, a potent activator of CD1d-restricted NKT cells, was given to mice infected with *M. tuberculosis* (Table 1). It was found that administration of αGalCer increases lymphocyte recruitment into the lung, reduces the lung mycobacterial CFU count, and prolongs the survival of infected mice (22). Thus, although CD1d-restricted T cells are not absolutely required for optimum immunity, their specific activation enhances host resistance to disease.

***Pseudomonas*.** In a murine model of *Pseudomonas aeruginosa*-induced pneumonia, Nieuwenhuis et al. showed that activated CD1d-restricted iNKT cells were critical in clearance of the acute infection (75). Twenty-four hours after infection by the respiratory route, the bacterial burden was 100-fold higher in the lungs of either CD1d$^{-/-}$ mice or anti-CD1d MAb-treated animals than in untreated control mice. Although *P. aeruginosa* infection has a high mortality rate in recombination-activating gene 2 (RAG2)$^{-/-}$ mice, it was not reported whether the impaired clearance of bacteria observed in the CD1d$^{-/-}$ mice affected their survival. Treatment of mice with αGalCer prior to infection facilitated the rapid clearance of bacteria from the lungs and resolution of the inflammatory response. In contrast, the untreated mice suffered from lung hemorrhage, swelling, and loss of normal alveolar architecture. The lungs of infected CD1d$^{-/-}$ mice had decreased neutrophils and less of the neutrophil chemotactic factor macrophage inflammatory protein 2, suggesting that CD1d-restricted NKT cells may play an immunomodulatory role in this model.

***Listeria* and *Salmonella*.** In contrast to the beneficial effect of activated iNKT cells on *M. tuberculosis* and *P. aeruginosa* infection, the presence of NKT cells had a detrimental effect on infection with *Salmonella enterica* subsp. *enterica* serotype Choleraesuis and *Listeria monocytogenes* (46, 90). Hepatocyte destruction, as measured by an increase in serum alanine transaminase, was observed in C57BL/6 (B6) mice during the 1st week of infection with serotype Choleraesuis. This effect was abolished in B6 Jα281$^{-/-}$ mice, indicating that iNKT cells may mediate the liver damage. Since β_2 microglobulin$^{-/-}$ mice, which lack iNKT cells, also have elevated serum alanine

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transaminase levels following infection, other mechanisms must also be involved in determining liver pathology. In another model of intracellular bacterial infection, mice treated with anti-CD1d MAb at the time of infection with *L. monocytogenes* survived longer than did mice coinjected with a control MAb. Furthermore, splenocytes from infected mice treated with anti-CD1d MAb produced more of the proinflammatory cytokines tumor necrosis factor alpha, IL-12, and IFN-γ but less transforming growth factor β2 after in vitro restimulation with heat-killed listeria. These data suggest that, under certain conditions, activation of CD1d-restricted NKT cells may adversely affect the outcome of infection, although confirmatory studies are still required.

***Borrelia*.** CD1d-restricted NKT cells were also proposed to have an early impact on immunity to the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease (61). B6 and B6 × 129 mice do not develop arthritis following infection with *B. burgdorferi*. In contrast, CD1d$^{-/-}$ mice developed joint inflammation typical of murine Lyme arthritis within a week of infection. At the same time, an altered humoral immune response in CD1d$^{-/-}$ mice was observed. Antibody titers against *B. burgdorferi* antigens were markedly elevated, particularly the immunoglobulin G2a subclass, which is normally produced in mice that are susceptible to *B. burgdorferi* and is typically associated with the Th1 immune response. These data show that CD1d-restricted NKT cells can influence antibody production. Still, how NKT cells exert their effector function in this model and why susceptible mouse strains expressing CD1d are not protected are questions that remain to be answered.

ROLE OF CD1d-RESTRICTED NKT CELLS IN PARASITIC INFECTIONS

Malaria. Schofield et al. proposed that protozoan GPI anchors were presented by CD1d to murine NKT cells. Splenocytes from mice immunized with the purified lipids or infected with plasmodium sporozoites produced IL-4 and proliferated after in vitro stimulation with purified GPI, and this appeared to be dependent upon CD1d (79). Although suggestive, CD1d was not definitively shown to be the antigen-presenting molecule, nor were iNKT cells shown to be the responding cell. Since protozoan GPI is a ligand for Toll-like receptor 2, an alternate interpretation of the data is that GPI may induce IL-12 production by APC, which subsequently activates NKT cells (1, 19).

CD1d-restricted NKT cells play a role in host defense following infection with parasitized erythrocytes. After infection with erythrocytes parasitized by *Plasmodium yoelii*, B6 CD1d$^{-/-}$ mice had a more prolonged parasitemia than did B6 mice. Both knockout and control mice had an increased percentage of hepatic NKT cells, which serves to emphasize that not all NKT cells are CD1d-restricted NKT cells. Despite the persistent parasitemia, Mannoor et al. found that B6 CD1d$^{-/-}$ mice had a more Th2-polarized immune response and less hepatic injury than did B6 controls (66). Hansen et al. also used parasitized erythrocytes to infect CD1d$^{-/-}$ mice, both the resistant BALB/c and susceptible B6 background mice (43). The absence of CD1 increased the susceptibility of BALB/c mice but made B6 mice more resistant. The results with the B6 and B6 CD1d$^{-/-}$ mice were similar to the findings of Mannoor et al. (66). Following infection, BALB/c CD1d$^{-/-}$ produced more serum IFN-γ and tumor necrosis factor alpha than did BALB/c control mice; B6 CD1d$^{-/-}$ mice produced less IFN-γ than B6 control mice. Antigen-stimulated T cells from infected BALB/c CD1d$^{-/-}$ mice produced more IFN-γ than did BALB/c control mice and failed to develop a protective Th2 response compared to BALB/c control mice. This change in the Th1/Th2 balance of the immune response correlated with the change in susceptibility of the knockout mice.

Treatment of BALB/c and B6 mice with αGalCer 1 to 2 days before i.v. infection with *Plasmodium berghei* and *P. yoelii* sporozoites reduced the level of parasitemia (38). Treatment with αGalCer was protective only against sporozoites and not against the blood form of the parasite. The protective effect of αGalCer was dependent upon CD1d, Jα281, IFN-γ, and IFN-γR and was independent of IL-12 p40, NK, B, and conventional T cells. An increase in the number of IFN-γ-secreting hepatic lymphocytes was detected following treatment with αGalCer. Thus, activated iNKT cells may directly reduce the level of parasitemia by increasing the production of IFN-γ in the liver. Administration of αGalCer during immunization with irradiated *P. yoelii* sporozoites or recombinant viruses containing CSZ protein epitopes enhances vaccine-induced protection as measured by a greater reduction in parasitemia than that caused by administration of vaccine alone (39). Immunologically, an increase in anti-CSZ IFN-γ secreting cells was observed in αGalCer-treated vaccinated mice. Thus, activation of iNKT cells can modulate the adaptive immune response and enhance host resistance to microbial pathogens.

Therefore, it appears that CD1d-restricted NKT cells are not required for immunity to the malaria sporozoite, which is primarily IFN-γ mediated; however, αGalCer enhances host resistance, most likely by inducing hepatic iNKT cells to secrete IFN-γ. In contrast, immunity following infection with parasitized erythrocytes is more complex, and iNKT cells modulate host resistance, possibly by altering the Th1/Th2 balance. In the absence of CD1d-restricted T cells, naturally resistant Th2-dominant BALB/c mice become more susceptible as their immune response becomes Th1 polarized. In B6 mice, the absence of CD1d-restricted T cells leads to a Th2-like cytokine profile and consequently the mice are more resistant.

Trypanosomiasis. The cell membrane of *Trypanosoma cruzi* contains abundant GPI-anchored mucin-like glycoproteins (GPI mucins) and glycolipids, some of which are targets of the host immune response. Fragments of these antigens could potentially be presented by CD1d, and consequently there is great interest in whether CD1d-restricted T cells play a role in host defense against *T. cruzi*. Although trypanosomal GPI mucins and glycoinositolphospholipids are not recognized by iNKT cells, both the intact molecule and the purified lipid portion bind to CD1d and can compete with the presentation of αGalCer (77). Procopio et al. showed that the T-cell and antibody responses to the GPI mucins are MHC class II restricted, and no role for CD1d was detected (77). It has not been resolved whether CD1d-restricted NKT cells participate in immunity to *T. cruzi*. Duthie and Kahn (31) observed a modest prolongation in the duration of parasitemia in mice lacking CD1d or iNKT cells, but the *T. cruzi* strain was not very virulent, and ultimately the infection was cleared in all mice. Pretreating mice with αGalCer enhanced the ability of mice to

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clear the infection, which was dependent on iNKT cells and IFN-γ but was independent of IL-12 p40 (31). The results were quite different when a virulent strain of *T. cruzi* was used: no difference was observed in the degree of parasitemia or survival of $CD1d^{-/-}$ mice compared to that in healthy control mice (69, 77). Nor did the absence of CD1d-restricted NKT cells impair the immunological response to infection as measured by serum cytokines or production of cytokines by splenocytes. Furthermore, no added benefit was observed when αGalCer was combined with traditional chemotherapy. Finally, in contrast to the adjuvant-like effect observed for αGalCer when administered with malarial vaccines, simultaneous administration of αGalCer and trypanosomal DNA vaccines abolished the protective effect of immunization (69).

Other parasitic infections. The role of CD1d-restricted NKT cells has also been studied following infection with *Leishmania major* (47). Depletion of $NK1^+$ cells (NK and NKT cells) but not asialo-$GM1^+$ cells (NK cells) led to an increase in the parasite burden, suggesting that CD1d-restricted NKT cells contribute to host resistance. This hypothesis was confirmed by showing that $J\alpha281^{-/-}$ mice were more susceptible to infection. Denkers et al. observed that vaccination of class II $MHC^{-/-}$ mice with an attenuated strain of *Toxoplasma gondii* provided some protection against challenge with a virulent strain of *T. gondii* (29). Just as in normal mice, the vaccine-induced protection was mediated by $CD8^+$ T cells that produced IFN-γ and could kill infected cells. Interestingly, the generation of $CD8^+$ effector cells required the presence of $CD4^+$ $NK1.1^+$ T cells at the time of immunization, suggesting that NKT cells may have the capacity to provide T cell help.

ROLE OF CD1d-RESTRICTED NKT CELLS IN FUNGAL DISEASE

Cryptococcus neoformans is a fungal pathogen that causes pulmonary disease and disseminates hematogenously to the central nervous system, especially in patients with AIDS but also in other individuals with impaired cell-mediated immunity. The Th1/Th2 balance is important in determining susceptibility to infection: Th1-polarized responses are protective, with IL-12, IL-18, and $CD4^+$ T cells playing a critical role (28, 55, 59, 65). Studies by Kawakami et al. have examined the role of NKT cells and CD1d in immunity to *C. neoformans* by using the strain YC-13, which causes a self-limited infection in B6 mice without any evidence of central nervous system invasion (52, 54).

Following intratracheal infection with a clinical isolate of *C. neoformans*, an accumulation of NKT cells was observed in the lungs of infected mice (52). The number and percentage of NKT cells peaked 3 days after infection. A similar increase was observed in $J\alpha281^{-/-}$ mice, which lack iNKT cells, and again illustrates the problem with using the NK1.1 antigen as a marker of iNKT cells. Nevertheless, some iNKT cells were present, since Vα14-Jα281 RNA was detected in the lungs of infected mice. The appearance of iNKT cells in the lungs following infection does not necessarily imply that microbial antigens are being presented to iNKT cells by CD1d, since other investigators have described how NKT cells can be recruited to inflammatory foci independently of CD1d (67). On the other hand, Kawakami et al. observed that $J\alpha281^{-/-}$ mice have an impaired in vitro recall response and impaired delayed-type hypersensitivity reaction to cryptococcal antigen, suggesting that iNKT cells are playing a physiological role in the host response to cryptococcal infection. These findings were accompanied by impairment in the ability of $J\alpha281^{-/-}$ mice to control the infection.

Consistent with the impairment of host defense observed in $J\alpha281^{-/-}$ mice is the finding that treatment with αGalCer enhances host resistance following cryptococcal infection. Administration of αGalCer starting on the day of infection increases the serum IFN-γ level in infected mice from that found in αGalCer-treated, uninfected mice or vehicle-treated, infected mice (54). The IFN-γ peaks 7 days after infection and is dependent upon iNKT and CD4 cells but is independent of NK cells. Furthermore, activation of iNKT cells enhanced the production of IFN-γ in response to cryptococcal antigen in vitro, which could be detected within 3 days after infection in mice treated with αGalCer. In contrast, the recall response was not detected until day 7 in vehicle-treated mice. Lastly, αGalCer treatment reduced the pathogen burden in the lungs and spleens, and although the effect was modest (<0.5 log), the kinetics of the clearance of the organisms did appear to be altered. These results signify a physiological role for iNKT cells in the modulation of host immunity following cryptococcal infection.

ROLE OF CD1d-RESTRICTED NKT CELLS IN VIRAL INFECTIONS

CMV. Several studies show that activation of iNKT cells with αGalCer ameliorates disease caused by viruses. One likely mechanism is the downstream activation of NK cells, given their importance in viral immunity. Using murine cytomegalovirus (CMV) as a model to evaluate the role of CD1d-restricted NKT cells in immunity to viral infections, van Dommelen et al. ruled out a critical role for iNKT cells in early clearance of CMV infection when they used $J\alpha281^{-/-}$ mice (94). Nevertheless, in vivo activation of iNKT cells by using αGalCer transiently reduced the viral load in infected organs. The ability of GalCer treatment to reduce the viral load was diminished if mice were pretreated with anti-asialo-GM1. This experiment indicates that the antiviral effect of αGalCer was mediated in part by NK cells. Still, αGalCer treatment reduced the viral load in NK cell-depleted mice, suggesting that, in addition to activating NK cells, iNKT cells were either directly killing virus-infected cells or were exerting their effector function by a different mechanism.

HSV-1. A role for CD1d-restricted NKT cells and iNKT cells in another herpesvirus, herpes simplex virus type 1 (HSV-1), was reported by Grubor-Bauk et al. (40). Resistant B6 control mice were compared to B6 $CD1d^{-/-}$ and B6 $J\alpha281^{-/-}$ mice. $CD1d^{-/-}$ mice were more susceptible to HSV-1 by a number of criteria. They developed larger skin lesions containing more viral particles, and HSV-1 spread more rapidly to the peripheral nervous system in $CD1d^{-/-}$ mice than in controls. Both $CD1d^{-/-}$ and $J\alpha281^{-/-}$ mice were strikingly defective in their ability to clear the virus from the skin or nervous system compared to controls. The authors suggest that, in the absence of a CD1d-restricted NKT cell response, initiation of the virus-specific adaptive immune response may be impaired. While

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this hypothesis is well reasoned, given the importance of $CD8^+$ and $CD4^+$ T cells in HSV-1 viral clearance, it remains a possibility that iNKT cells are having a direct effect in this model.

HBV. In a transgenic hepatitis B virus (HBV) model, liver iNKT cells play a role in a transient, IFN-α/β- and IFN-γ-dependent inhibition of HBV transcription after i.v. injection of αGalCer (50). Within 24 h of αGalCer treatment, a burst of hepatic cytokine transcription occurs, which coincides with reduced viral replication. Although there is a rapid influx of macrophages, T cells and activated NK cells entering the liver or resident hepatic NK or iNKT cells are likely to mediate the downstream antiviral effects of αGalCer in this model, since reduced viral replication precedes the recruitment of leukocytes to the liver (50, 51). Although these mice are tolerant to the transgenically expressed viral proteins, transfer of naïve wild-type splenocytes into HBV-transgenic $RAG2^{-/-}$ recipients leads to the development of acute hepatitis. Baron et al. proposed that induction of acute hepatitis in this transfer model was mediated by CD1d-restricted NKT cells (5). It remains to be shown why CD1d-restricted NKT cells become activated in the recipient mice and why, when activated, they induce acute hepatitis.

LCMV. As discussed elsewhere in this review, T-cell expression of cell surface markers normally associated with NK cells is not synonymous with CD1d-restricted NKT cells. The lymphocytic choriomeningitis virus (LCMV) infection model was used to examine whether MHC class I- and class II-restricted T cells can express NK cell markers (82). LCMV-specific $CD4^+$ and $CD8^+$ MHC-restricted T cells rapidly and persistently expressed several different NK cell markers, including NK1.1, DX5, and asialo-GM1. A less careful interpretation would have suggested that CD1d-restricted NKT cells undergo expansion following LCMV infection. In fact, Hobbs et al. showed the opposite. The loss of NKT cells occurred within 3 days of LCMV infection, as indicated by a reduction of Vα14-Jα281 TCR transcripts in the liver (44). Hepatic $TCR\beta^+$ $NK1.1^+$ cells upregulated the active form of caspase 3, showing that the reduction of NKT cells was a consequence of apoptosis within this T-cell subset. In any case, the loss of CD1d-restricted NKT cells does not hamper NK cell activation following LCMV infection, nor does the absence of CD1d-restricted NKT cells impair the generation of LCMV-specific MHC-restricted cytotoxic T cells (86).

RSV. The rapid production of cytokines by activated iNKT cells has led to the hypothesis that these cells may help elicit an appropriate adaptive immune response, although only a few studies have presented data in favor of this hypothesis. One example is respiratory syncytial virus (RSV) infection (48). The infiltration of $CD8^+$ T cells into the lungs was reduced in BALB/c $CD1d^{-/-}$ mice compared to BALB/c controls after infection with RSV. By using MHC class I tetramers loaded with viral peptides, it was shown that the number of virus-specific $CD8^+$ T cells in the lungs of BALB/c $CD1d^{-/-}$ mice was reduced 7 and 10 days after infection compared to that found in the lungs of BALB/c control mice. These data suggest that CD1d-restricted NKT cells influence the adaptive immune response. Illness, as measured by body weight, was affected by the presence or absence of CD1d-restricted NKT cells but was also dependent on the genetic background. Following infection, 129 × B6 and BALB/c mice were less ill in the absence of CD1d, while B6 mice lost more weight in the absence of CD1d and CD1d-restricted NKT cells. This more or less correlated with IFN-γ being produced to a larger extent in mice with reduced body weight during primary RSV infection. In contrast, viral titers did not typically reflect the health status of the animals, suggesting that the cytokine profile of the host's immune response following RSV infection, rather than the virus itself, determines the severity of the disease. NKT cell activity during the natural course of the infection likely influences this cytokine milieu and eventually the adaptive immune responses. As opposed to the role of NKT cells during the natural course of the infection, αGalCer injection into RSV-infected BALB/c wild-type mice had a beneficial effect on the host, as measured by body weight and delayed viral clearance in this situation despite increased IFN-γ production and a larger number of virus-specific $CD8^+$ T cells in the lungs than in the lungs of the vehicle-treated control animals.

ECMV-D. Two reports have suggested that diverse CD1d-restricted NKT cells play a role in viral infections (33, 45). Exley et al. (33) reported that BALB/c $CD1d^{-/-}$ mice were more susceptible to diabetogenic encephalomyocarditis virus (ECMV-D) infection. Intraperitoneal infection with ECMV-D leads to paralysis within a week in highly sensitive BALB/c mice; however, the incidence and severity of paralysis were greater in BALB/c $CD1d^{-/-}$ mice. Interestingly, BALB/c $J\alpha281^{-/-}$ mice were no more susceptible than BALB/c mice, suggesting a role for the diverse subset of CD1d-restricted NKT cells and not iNKT cells. Although iNKT cells did not appear to be required for host resistance to ECMV-D infection, activation of iNKT cells by using αGalCer prior to ECMV-D infection had a dramatic effect on the development of disease in infected mice. αGalCer-treated BALB/c wild-type mice had less severe paralysis and were protected from virally induced diabetes. These effects of αGalCer may arise from its ability to induce IFN-γ, which is known to be protective in this model.

Coxsackievirus. The coxsackievirus B3 virus causes myocarditis in BALB/c and BALB/c $J\alpha281^{-/-}$ mice but not in BALB/c mice lacking CD1d (45). Although the viral titers did not differ between mouse strains 7 days after infection, the $CD1d^{-/-}$ mice had dramatically less inflammation of the myocardium, suggesting that the CD1d-restricted immune response was mediating the disease. A role for γ/δ TCR^+ T cells in the pathology of coxsackievirus B3-induced myocarditis has been described, and $V\gamma4^+$ T effector cells isolated from coxsackievirus B3-infected recipients were able to kill infected target cells in a CD1d-dependent manner.

HOW IS THE ANTIMICROBIAL EFFECT OF CD1d-RESTRICTED NKT MEDIATED?

CD1d-restricted NKT cells can clearly play a role in immunity to bacteria, parasites, yeasts, and viruses. In some cases, mice that lack CD1d or iNKT cells are more susceptible to certain pathogens; in others, iNKT cell activation by αGalCer ameliorates disease. One clear principle is that αGalCer modulation of host resistance to microbial pathogens requires that it be administered early during the course of infection. Although several protocols have been used and found to be beneficial, all require pretreatment or treatment shortly after

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infection. For example, in the *P. aeruginosa* model, repeated i.v. injection of αGalCer starting a week prior to infection dramatically reduces the bacterial burden (75). At the other end of the spectrum, the ability of αGalCer treatment to prolong the survival of mice infected with *M. tuberculosis* was compromised if its administration was delayed more than 5 days following infection (22). From these observations, we may infer that the greatest impact of iNKT cells on infection is during the initiation of the immune response. iNKT cells are likely to be functioning either as direct effector cells that transiently lower the microbial burden in the host, leading to long-term benefits, or as regulatory cells that modulate the immune response. These interpretations are consistent with the data from the *M. tuberculosis* model showing that αGalCer treatment has no effect during established infection and that repeated administration of αGalCer provides no additional benefit compared to a single dose (23). The therapeutic use of αGalCer during infection provides us a glimpse of the potential of iNKT cells to ameliorate disease and may provide us with some insight into how they mediate their effect.

A more fundamental question is whether CD1d-restricted NKT cells have a physiological role in host defense against infection. We have seen how mice that lack CD1d or iNKT cells are more susceptible to certain infections and in a limited number of examples are more resistant. These findings imply that CD1d-restricted NKT cells become activated as a consequence of microbial infection. How CD1d-restricted NKT cells become activated during infection remains a central question. NKT cell recognition of microbial lipid and glycolipid antigens presented by CD1d could lead to activation, as occurs for group 1 CD1-restricted T cells. Alternately, the upregulation of CD1d and presentation of endogenous self-antigens to autoreactive CD1d-restricted NKT cells may transmit a danger signal to the host. A third possibility is that non-TCR-mediated signals activate NKT cells, such as IL-12 or cross-linking of NK1.1 (4, 58, 92). A better understanding of how CD1d-restricted NKT cells are activated in vivo will be critical to understanding the beneficial effect of CD1d-restricted NKT cells on host resistance to infection.

Three distinct paradigms are emerging that may explain how CD1d-restricted NKT cells exert their antimicrobial effect. CD1d-restricted NKT cells may act (i) as direct effector cells, (ii) by modulating adaptive immunity, or (iii) by modulating innate immunity. Like $CD8^+$ T cells, CD1d-restricted NKT cells can be cytolytic and both human and murine NKT cells have the capacity to kill $CD1d^+$ target cells (12, 86). Human iNKT cells can express both perforin and granulysin, and granulysin-expressing iNKT cell clones have been shown to kill *M. tuberculosis* (36, 41). Furthermore, CD1d-restricted NKT cells can produce IFN-γ, which enhances the ability of infected cells to kill intracellular microbes. This is likely to be the mechanism by which αGalCer-activated iNKT cells reduce parasitemia following infection with malaria sporozoites (38) and inhibits HBV replication (50). On the other hand, it is also clear that, in certain models, the influence of CD1d-restricted NKT cells on the innate immune system plays an important role in host defense. For example, NKT cells appear to enhance the recruitment of granulocytes to the lung following infection with *P. aeruginosa* (75). Following infection with viruses, NKT cell-mediated activation of NK cells contributes to host resistance in CMV infection (94). Finally, there is evidence from several models that CD1d-restricted NKT cells can modulate the adaptive immune response. This was observed as an alteration of the Th1/Th2 polarization in malaria (39, 43, 66) or *B. burgdorferi*-caused disease (61) or a change in T-cell responsiveness after infection with *C. neoformans* (52) and RSV (48).

THERAPEUTIC POTENTIAL OF CD1d-RESTRICTED NKT CELLS

Our ability to modulate the activity of CD1d-restricted NKT cells may provide new therapeutic options for the treatment and prevention of infectious diseases. First, vaccines that use antigens presented by CD1 have the advantage that CD1 is not polymorphic and so a greater number of individuals will potentially respond if the antigen is presented by the CD1 antigen-processing pathway. Vaccines targeting mycobacterial antigens presented by group 1 CD1 have been tested in animal models (27). Second, the activation of CD1d-restricted NKT cells could represent a therapeutic strategy, either in combination with traditional antimicrobial chemotherapy or as a distinct strategy. Compounds such as αGalCer offer some ability to pharmacologically activate iNKT cells. Presently, the use of αGalCer appears to be limited to postexposure therapy, which may have some applications for biodefense or the exposure to pathogens for which antimicrobial therapy does not exist. As we gain an understanding of how NKT cells become activated during infection and how they mediate their antimicrobial effect, it is possible that other ways will be developed to activate this lymphocyte population. Finally, the adjuvant-like properties of αGalCer raise the possibility that it could enhance the efficacy of certain vaccines. Clearly, a better understanding of the ligands that CD1d-restricted NKT cells recognize, their activation requirements, and the way in which they mediate their antimicrobial effect will provide us with greater insight into the role of CD1d-restricted NKT cells in host defense against infection. Such an understanding may ultimately provide us with an immunological pathway amenable to modulation that can be used for new therapeutic challenges in infectious diseases.

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