

The invariant NKT cell subset in anti-viral defenses: a dark horse in anti-influenza immunity?

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ABSTRACT

iNKT cells, a small subset of $\alpha\beta$ TCR⁺ T cells, are capable of producing large amounts of cytokines upon activation through their TCR. Unlike conventional T cells that express highly diverse TCRs, iNKT cells express a glycolipid-reactive invariant TCR- α chain paired with a limited number of β chain(s). These cells recognize glycolipid antigens when presented on CD1d molecules found on APC or other cells. Although the immunoregulatory roles of iNKT cells in the context of autoimmune disease are fairly well characterized, several lines of evidence highlight the importance of this cell type in immune responses against microbial insults caused by bacterial, viral, and parasitic pathogens. Recent studies that have investigated the role of iNKT cells in immune responses against influenza virus have suggested an important role for these cells in innate defense mechanisms as well as antibody- and cell-mediated responses. This review highlights the important contributions of iNKT cells to immune responses against viral pathogens with particular emphasis on immunity to influenza infections. *J. Leukoc. Biol.* **88**: 635–643; 2010.

Introduction

iNKT cells constitute a unique subset of T lymphocytes with a hybrid phenotype of NK cells and conventional T cells. A thymic and an extra-thymic origin of NKT cells have been suggested [1, 2], however, there is little evidence that the extra-thymic pathway plays a significant role for NKT cell develop-

ment. iNKT cells express the c-type lectin CD161 (also known as NK1.1 found in certain but not all mouse strains) and an invariant TCR. Although the majority of NKT cells belongs to the CD4⁺CD8[−] (double-negative) or CD4⁺CD8⁺ populations, a subset of these cells that CD8⁺CD4[−] exists in humans [3, 4].

NKT cells are generally divided into two subtypes: type I and type II [5]. The majority of NKT cells belongs to type I and express an invariant TCR unlike conventional T cells, hence their classification as iNKT cells. A significant body of evidence gathered over the last decade signifies the importance of these cells in immune responses against several infectious agents. In contrast, type II NKT cells (vNKT, nonclassical, or non-invariant NKT cells) express a more diversified TCR repertoire, whose precise role in immune activation or regulation is understood only vaguely [6–8].

iNKT cells are found in small numbers within thymus, spleen, bone marrow, blood, and lymph nodes, and unlike many other tissues, mouse iNKT cells are found in abundance (40% of intrahepatic lymphocytes) in liver [9]. This number is reduced to ~12% in human liver [5]. In mice, iNKT cells express a V α 14-J α 18 rearrangement with an invariant CDR3 region that is typically coexpressed with either of the more diverse V β 8.2, V β 2, or V β 7 chains. In humans, NKT cells express an invariant V α 24-J α 18 rearrangement with V β 11, which are the human orthologs of mouse V α 14 and V β 8, respectively [10]. The TCRs of iNKT cells typically recognize glycolipid antigens presented through CD1d molecules on the APC, such as DC, macrophages, and B cells. Upon the engagement of their TCR, iNKT cells become activated and produce large amounts of Th1-type (e.g., IFN- γ) and/or Th2-type (e.g., IL-4) cytokines. Their importance in the regulation of immune response in preventing autoimmune diseases has been well documented previously [11–13].

In addition to their immunoregulatory roles, iNKT cells play a role in recognition and response to microbial infections

Abbreviations: α -C-GC=synthetic analog of α -galactosylceramide, α -GalCer= α -galactosylceramide, ASC=antibody-secreting cell, bcl2=prosurvival gene, CD40L=CD40 ligand, DC=dendritic cell(s), EMCV=encephalomyocarditis virus, HA=hemagglutinin, HBV=hepatitis B virus, IAV=influenza A virus, IDO=indoleamine 2, 3-dioxygenase, iGB3=iso-globotrihexosylceramide, iNKT cell=invariant NK T cell, IPR8=inactivated Puerto Rico/8/34 (H1N1), KSHV=Kaposi sarcoma-associated herpes virus, LCMV=lymphocytic choriomeningitis virus, AMP=associated molecular pattern, MDSC=myeloid-derived suppressor cell, ODN=oligodeoxynucleotide, pDC=plasmacytoid dendritic cell(s), PR8=Puerto Rico/8/34 (H1N1), PRR=pattern recognition receptor, RSV=respiratory syncytial virus, vNKT cell=variant NK T cell

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[14]. This review will focus on the important roles of iNKT cells in the context of viral infections with particular emphasis on the influenza infection.

INKT CELL RECOGNITION OF GLYCOLIPID ANTIGENS

iNKT cells recognize glycolipid antigens presented by MHC-I-like but less polymorphic CD1d molecules on APC. The mode and binding of the antigen in the deep hydrophobic pockets of the CD1d molecule and its interaction with iNKT-TCR have been described elsewhere in detail [15]. These structure-function analyses of CD1d-TCR interactions have been greatly facilitated by the advent of a potent iNKT-TCR activator, α -GalCer, a synthetic glycolipid antigen. α -GalCer was isolated initially from marine sponges, but some researchers have speculated recently that this glycolipid might have originated from some microbes present in the sponge [14]. CD1d molecules bind endogenous as well as microbe-derived foreign lipids, mainly glycolipids [16, 17]. iGB3 was the first endogenous ligand identified for binding to CD1d, which has facilitated the evaluation of iNKT cell-mediated activation in the absence of a foreign lipid antigen using mice deficient in iGB3 ($\text{hexB}^{-/-}$) [18]. However, much less is known about the endogenous ligands for iNKT cells, and moreover, the importance of iGB3 as a physiological ligand for iNKT cells is still debatable.

ROLE OF INKT CELLS IN MICROBIAL INFECTIONS

The discovery that iNKT cells are activated directly by recognition of certain glycolipid antigens and subsequently, produce large amounts of cytokines on a per-cell basis led to intense studies geared toward identification of various forms of lipid antigens of a variety of microbial and parasite species. iNKT cells have been demonstrated to have a protective role in many infections, such as those caused by *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Leishmania major*, and *Plasmodium berghei* [19, 20]. However, in some cases, iNKT cells have also been implicated in exertion of detrimental effects in infections caused by *Salmonella choleraesuis* and *Escherichia coli* infections [21–24]. For example, in the case of *E. coli* infection, liver injuries characterized by formation of necrotic, inflammatory foci, cell death in hepatocytes, and lymphocyte infiltration were observed in wild-type mice, and these lesions were reduced significantly in $\text{J}\alpha 18^{-/-}$ mice [21]. Furthermore, it was discovered that purified intra-hepatic NKT cells, which expressed TLR2 mRNA, were activated in vitro in response to a synthetic lipoprotein, a ligand for TLR2 [21]. Shimizu and colleagues [22] found similar effects of increased lesions in the livers of wild-type mice following *S. choleraesuis* infection, compared with $\text{J}\alpha 18^{-/-}$ mice. As these lesions were reduced significantly in $\text{TLR2}^{-/-}$ mice, it was suggested that NKT-mediated liver injury was, in part, dependent on interactions between TLR2 and its ligand [22].

Similar to conventional T cells, iNKT cells may require signals from APC for their activation. In case of microbial organ-

isms, recognition of structural components of these organisms (collectively named pathogen-associated molecular patterns) by APC through their PRR leads to expression of cytokines and other mediators that can activate iNKT cells. Several types of PRRs have been identified, among which TLRs are of note. PAMPs, such as nucleic acids, flagellin, peptidoglycan, and LPS, can bind to TLRs and trigger downstream intracellular pathways, leading to cell activation and cytokine production [25, 26]. A recent study by Nagarajan and Kronenberg [27] showed an increased IFN- γ and TNF- α production by iNKT cells in response to *E. coli* LPS, which was TCR-independent but required IL-12 and IL-18 release from DC. This group subsequently showed that DC primed with a TLR9 agonist (CpG-ODN) could activate iNKT cells to produce IFN- γ , a phenomenon that was IL-12-dependent [28]. iNKT cells are also shown to have a functional TLR4/myeloid differentiation protein-2 complex as determined by intracellular staining [29]. It is interesting to note that the expression of TLR2, -4, and -5 but not TLR9 has been shown in NKT cells at the transcript level [22]. Overall, these studies suggest that iNKT cells may be able to receive signals, directly or indirectly, generated from the interactions between TLRs and their PAMP ligands. However, despite the expression of certain TLRs in iNKTs and in the absence of evidence for a direct stimulation of iNKT cells with TLR agonists, it seems that APC are essential for transmitting TLR-derived signals to iNKT cells and their subsequent activation and production of cytokines.

The studies aimed at elucidating the role of iNKT cells in microbial infections have been greatly facilitated by the availability of experimental tools to enrich selectively iNKT cells and the knockout mouse models that allowed further dissection of the role of these cells in a disease context. Two genetic knockout models have been used primarily for this purpose: $\text{J}\alpha 18^{-/-}$ and $\text{CD1d}^{-/-}$ mice, and the former model is particularly more definitive. As $\text{J}\alpha 18^{-/-}$ mice are devoid of iNKT cells only, whereas $\text{CD1d}^{-/-}$ mice do not express CD1d, which is required for positive selection of iNKT and vNKT cell populations during thymic development, therefore, these mice lack both types of NKT cells. In addition, these mice are devoid of some CD1d-reactive, conventional T cells. In some cases, blocking antibodies to CD1d are also used to determine the role of iNKT cells in disease models.

In recent years, several reviews have attempted to describe the role of iNKT cells in microbial infections, particularly those involving bacterial and parasitic pathogens [5, 14, 19, 30]. Therefore, the present review will summarize our current knowledge and understanding of the involvement of iNKT cells in viral infections, particularly in influenza infections.

INKT CELL INTERACTIONS WITH VIRUSES

Interactions between TLRs and virus-associated molecular patterns may be required for activation and exertion of effector function of iNKT cells. A recent study revealed that human iNKT cells expand and increase their IFN- γ production when PBMCs are treated with TLR3, -7, and -9 agonists [31]. Further, similar effects were observed when PBMCs were treated

with UV-inactivated HSV-1. Depletion of pDC from PBMCs reduced NKT cell activation significantly, suggesting the importance of pDC and likely pDC-derived cytokines, such as IFN- α , for NKT cell activation. Interestingly, exposing PBMC to LPS results in a significant reduction of IFN- γ and increase in IL-10 production, suggesting that DC-iNKT cell interactions are finely tuned to the type of TLR-derived signal received by DC [31]. A similar role of pDC and IFN- α produced by these cells in iNKT activation was demonstrated in PBMCs treated with a TLR9 agonist (CpG-ODN) [32]. Recently, Tyznik et al. [28] showed that coculture of DC exposed to TLR7 and -9 agonists with purified mouse splenic iNKTs resulted in high levels of IFN- γ production. Furthermore, iNKT cell activation and IFN- γ production were observed in mice in response to mouse CMV infection, which was TLR9- and IL-12-dependent.

In addition to interactions between TLRs and virus-associated molecular patterns, it is possible that viral antigens can activate iNKT cells directly through their TCR. However, given the fact that unlike bacteria and parasites, viruses do not contain any lipid antigens, it is somewhat puzzling how iNKT cells respond to antigens of these infectious agents. The most likely explanation is that viral infections induce endogenous lipid synthesis, and iNKT cells are activated in response to endogenous ligands, coupled with cytokine help from APC. In this case, one can also expect that the infected cells, presenting endogenous lipids in the context of CD1d molecules, may be targeted for cytotoxic effects exerted by iNKT cells. Interestingly, a recent study showed that a synthetic acylpeptide, N-acyl glycine dodecamer peptide (lipo-12), which mimics lipoproteins and is produced by cells and viruses, was presented by CD1c molecules to human T cells [33]. This response was specific for the acyl linkage as well as the peptide length and sequence. Therefore, the possibility that viruses may acquire endogenous lipids from the host cells while budding out, thereby initiating CD1d-mediated glycolipid presentation to iNKT cells, does not seem too far-fetched.

Viruses have developed strategies to evade the host immune system, including the CD1d-dependent antigen presentation pathway (reviewed by Van Kaer and Joyce [34]). A key piece of evidence that viral infections down-regulate CD1d expression in APC has interested many research groups to investigate the role of CD1d-restricted cells in antiviral responses. Many viruses, including HIV, RSV, HSV, and KSHV, block CD1d expression by interfering at one or more steps involved in its intracellular trafficking pathway, leading to CD1d cell surface expression [35]. A well-studied example of viral interference with CD1d function is the Nef protein of HIV and mannosyltransferase, inositol trisphosphate receptor, and ryanodine receptor proteins of KSHV, which are thought to interfere with the transport of the CD1d molecule from the trans-Golgi network to the cell surface and the endocytosis of surface CD1d molecules [36].

ROLE OF iNKT CELLS IN IMMUNITY TO VIRAL INFECTIONS

In the context of HBV infection, a few studies have used an experimental mouse hepatitis B transgenic model. Kakimi et

al. [37] showed a rise in IFN- γ and IFN- α/β transcripts in livers of mice within 24 h of injection with α -GalCer, which was also associated with increased viral clearance from the liver. This effect was temporally associated with a rapid decline of iNKT cells in the liver and accompanied by recruitment of NK cells into this organ. The disappearance of iNKT cells from the liver was thought to reflect activation-induced cell death or the down-regulation of their TCR and NK1.1 upon activation, hence making them undetectable by flow cytometry. However, the possibility of recruitment of iNKT cells to other tissues was not addressed, which could have been done by confirming the presence or absence of iNKT cells by real-time PCR to quantify the mRNA transcripts for rearranged *V α 14-J α 281* or by in situ hybridization methods as described previously [38]. Subsequently, these authors also showed that iNKT cell-mediated HBV clearance from the liver was not dependent on inflammatory cell recruitment [39]. Interestingly, another study using the aforementioned transgenic hepatitis mouse model found an increase in the number of activated, IFN- γ -producing, non-classical NKT cells in the liver and demonstrated that these cells were activated in response to HBV surface (envelope) antigens presented by liver parenchymal cells in the context of CD1d [40]. These NKT cells were "nonclassical" in the sense that although they were CD1d-restricted, they were not reactive to α -GalCer. However, this finding is somewhat surprising, as viral antigens are generally nonlipid in nature and thus, CD1d-independent. Therefore, it is also possible that these were conventional T cells that coexpressed NK receptors or that the HBV surface envelope may have contained host lipids. It should also be noted that the role of iNKT cells in immunity against HBV is not known. In a recent study, α -GalCer treatment of chronically infected patients with HBV did not result in HBV clearance, which raises the possibility that NKT cells are not involved in HBV immunity [41].

An important role of iNKT cells in HCV infection was suggested based on the observation that patients suffering from HCV-induced chronic hepatitis had elevated numbers of NKT cells in liver with an up-regulated expression of CD1d molecules in liver cells [42]. Furthermore, Yamagiwa and colleagues [43] found a significant increase in the proportion and absolute numbers of NK and NKT cells in livers of HCV-infected patients who were receiving an antiviral therapy, which included IFN- α . This increase in NKT cells suggested a beneficial role for these cells in mediating HCV clearance following therapy. In a recent study, NKT cells were also shown to be important in mounting an anti-HCV response in HCV-infected liver transplant recipients [44]. Interestingly, an earlier study by Exley et al. [45] investigated the role of NKT cells in HCV infection in humans and reported high frequencies of CD1d-reactive hepatic NKT cells, which were Th1-biased but were noninvariant (type II NKT). These authors found that unlike in mice, human liver and bone marrow, but not blood, contain a large proportion of CD1d-reactive non-invariant NKT cells and suspected a novel lineage for these Th1-biased NKT cells in liver. Further, it was also suggested that IFN- γ , produced by these liver NKT cells, may cause tissue damage in response to HCV infection. In particular, hepatic NKT cells with variant TCR need to be studied in further detail to deter-

mine the mechanisms of their adverse effects in HCV infection. Moreover, there are reports that livers from chronically infected HBV and HCV patients had increased NKT activity with an altered effector phenotype characterized by Th2 cytokines [24]. Therefore, it has been suggested that hepatic NKT cells in patients with chronic viral hepatitis may actually be detrimental [5]. The protective role of vNKT or iNKT cells in HBV and HCV infections is somewhat questionable, as there seem to be certain subtle differences in liver NKT populations within species and also between mice and humans. Recent data indicate that iNKT cells and vNKT cells may have opposing roles in immune regulation, for instance, in the context of anti-tumor immunity [6–8]. For example, selective stimulation of vNKT cells was shown to suppress the anti-tumor immunosurveillance activity of iNKT cells, and stimulation of iNKT cells resulted in protection against tumor growth even when responses were somewhat skewed toward Th2 cytokines [7]. Interestingly, when both of these cell subsets were stimulated simultaneously, vNKT cells appeared to suppress the activation and protective effect of iNKT cells in vivo [7]. Therefore, it would be interesting to examine the role of both subsets and their interplay in antiviral immune responses.

A protective role for iNKT cells is suggested in HSV-1 infection in the mouse model [46]. HSV-1 (SC16 strain) infection in C57BL/6 wild-type mice was compared with infection in CD1d^{-/-} and J α 18^{-/-} mice. It was shown that the knockout mice had an impaired clearance of the virus from skin and dorsal root ganglion tissues, suggesting an important role for these cells against HSV-1 infection. Along similar lines, Ashkar and Rosenthal [47] suggested a protective role for these cells in an intravaginal mouse model of infection with HSV-2. In contrast with the findings of Grubor-Bauk and colleagues [46], a subsequent study using CD1d^{-/-} mice showed that iNKT cells were not necessary for resolving or controlling the severity of HSV-1 (KOS strain) infection [48]. It is important to note that such discrepancies might have resulted from the differences in the virulence of virus strains used in these studies, as the KOS strain of HSV-1 is less virulent than the SC16 strain.

A few studies have investigated the role of CD1d-reactive cells in infection of mice with EMCV-D, a picorna virus that causes acute diabetes, paralysis, and myocarditis within the first few days of infection [49, 50]. These studies revealed that CD1d^{-/-} mice had a greater susceptibility to infection compared with wild-type mice, and resistance to EMCV-D infection was mediated by IL-12 and IFN- γ [49]. Depletion of NK and NKT cells in wild-type mice by treatment with anti-asialo GM1 antibodies led to enhanced susceptibility of mice to EMCV-D infection, and the increased susceptibility of CD1d^{-/-} mice to infection prevailed, implying that this response was iNKT-mediated but not NK-mediated. However, the possibility of involvement of other CD1d-reactive T cells cannot be ruled out, as J α 18^{-/-} mice that selectively lack iNKT cells were not used in this study. It is also important to note here a controversy that anti-asialo GM1 antibodies only deplete NK cells but not NKT cells, and anti-NK1.1 antibody depletes both [51]. Recently, Ilyinskii et al. [50] evaluated the CD1d-dependent T cell response to a secondary infection with EMCV in vitro and

in vivo. The in vitro results demonstrated that EMCV persisted in high levels in splenocytes from CD1d^{-/-} mice, and infected cells produced less IFN- γ and IFN- α . Consistent with these in vitro findings, CD1d^{-/-} mice were more susceptible to EMCV infection and had reduced lymphocyte activation and IFN- α production, implying a protective role for NKT cells in EMCV infection in mice.

Similar to HCV infection, the role of iNKT cells in the mouse model of RSV infection is somewhat controversial, as the disease outcome depends on the genetic background of mice [52]. For example, in CD1d^{-/-} mice on a C57BL/6 background, severity of disease is increased compared with wild-type mice. However, CD1d knockout mice on a BALB/c background exhibit resistance to infection. Furthermore, these resistant mice, when injected with α -GalCer showed increased viral clearance with Th1 and Th2 cytokine profiles. However, the finding common to both of these backgrounds was an effective expansion of CD8⁺ T cells, implying an important role of NKT cells in RSV infections. Similar results were also noted in a murine model of cerebral malaria by *P. berghei* [53]. Therefore, it seems that the role of iNKT cells in an infection model depends on the genetic background as well as the pathogen.

iNKT cells appear to play a role in HIV infection [54]. It has been demonstrated that the CD4⁺ iNKT cell subset is targeted selectively by the HIV-1 virus because of its tropism to cells that coexpress CD4 with CCR5 and/or CXCR4 receptors [55]. Subsequently, it was found that the CD4⁺ iNKT cells from human PBMCs express high levels of CCR5 in contrast with conventional CD4⁺ T cells that expressed higher levels of CXCR4 compared with CD4⁺ iNKT cells. The high permissiveness of CD4⁺ iNKT cells to HIV-1 strains with CCR5 tropism was demonstrated further by showing that GFP-tagged HIV-1 viruses could infect up to 50% of α -GalCer-pulsed, DC-stimulated CD4⁺ iNKT cells in contrast to 5% of conventional CD4⁺ T cells [55]. Although, resting iNKT cells were also susceptible to HIV-1, the infection of these cells by the virus was much lower compared with TCR-stimulated iNKT cells. The findings of this study also revealed that iNKT cells in PBMCs of HIV-1-infected individuals were reduced dramatically in numbers as compared with healthy donors. However, the CD4⁺ iNKT cell subset also seemed to be targeted in some cases, as the number of these cells was found to be reduced in HIV-infected patients, but the reduction occurred at a slower rate compared with the CD4⁺ NKT cell population [55]. Although the reason for this finding was not clear, authors speculated about the possibility of an activation-induced cell death as a result of direct (TCR) or indirect (IL-12) activation of these CD4⁺ iNKT cells during HIV-1 infection. However, it is also possible that the CD4⁺ NKT cells may have reached systemic tissues such as lymph nodes and spleen, thus disappearing from circulation. Overall, HIV can cause a rapid decline in the total number of iNKT cells in the circulation at a rate faster than that observed for conventional CD4⁺ T cells. Nevertheless, further studies are required to understand the iNKT kinetics in acute versus chronic stages of HIV-1 infection.

Similar findings of a selective but transient loss of iNKT cells were reported in livers of mice infected with the nonhepato-

tropic Armstrong strain of LCMV within 2–3 days of infection with an apparent recovery by 8–14 days [56]. There was no evidence of these cells migrating to other tissues, as splenic and peritoneal iNKT cell numbers were unaltered, and this loss was found to be independent of IFN- γ and IL-12 production but was observed in mice treated with polyinosinic:polycytidylic acid, a potent inducer of IFN- α/β and a TLR3 agonist. However, the Armstrong strain of LCMV induces acute infection, and such effects of iNKT cells in the chronic infection model, using the C13 strain of LCMV, remain to be investigated further. Moreover, it was also found that liver iNKT cells from LCMV-infected mice were infected with the virus. These findings are suggestive of an immune-evasive property of viruses, such as HIV and LCMV in infecting NKT cells that are apparently crucial in the first line of defense against these infectious agents. Further studies are needed to elucidate the effects of iNKT enrichment as a means of therapeutic intervention.

Collectively, all of the above lines of evidence indeed indicate an important role for iNKT cells in antiviral defense with some exceptions, such as HCV infections, where a detrimental role is suggested. The role of nonclassical NKT cells (vNKT) in these infections also needs to be investigated further.

INKT CELLS IN IMMUNITY TO INFLUENZA INFECTIONS

Influenza virus infections are considered a major threat to human health as a result of the possibility of emergence of pandemics. At the same time, seasonal flu viruses are also a major burden on the healthcare system because of their ability to undergo antigenic drift, leading to emergence of viruses against which humans may not have sufficient immunity, hence requiring generating new vaccines and using antiviral drugs.

In general, influenza viruses are negative sense ssRNA viruses belonging to the Orthomyxoviridae family and are categorized into three types (A, B, and C). As IAV can infect many hosts and possess the capacity to cause pandemic infections, they have been studied extensively. Most experiments have used mouse models for this purpose, as although the mouse is not a natural host for IAV, it can be infected experimentally using mouse-adapted IAV strains, such as PR8. Immunity to IAV is dependent on antibody- and T cell-mediated immune response components [57, 58], and a collective and corroborative cross-talk between different cell types of the innate and adaptive immune components seems to be important in immunity against IAV. In this context, the iNKT cell subset has gained attention in the last 5 years, and many studies have already begun to explore the importance of iNKT cells in anti-influenza immunity.

ROLE OF iNKT CELLS IN INNATE IMMUNE RESPONSES TO INFLUENZA VIRUS

A recent study investigated the role of iNKT cells in innate defense against IAV and showed that mice injected with

α -GalCer had reduced viral titers in lungs, as well as reduced weight loss compared with controls, indicating an improvement in disease outcome [59]. A relatively milder, less virulent strain of IAV (E61-13-H17; H3N2 reassortant) was used to study changes in iNKT responses to IAV over time. Mice that received α -GalCer i.p. but not intranasally at the point of viral challenge had a 100-fold reduction in early viral titers in lungs. The underlying mechanism for this protection was thought to be mediated by an early response (by Day 2 postinfection) through cytokines such as IL-12 and IFN- γ , as measured in the serum, since iNKT cell activation did not affect CD8⁺ T cell responses at the early time-point or at the peak (Days 6–8 postinfection) of the response. Assessment of CD8⁺ T cell responses in this study included enumerating influenza nucleoprotein-MHC-I tetramer-positive CD8⁺ T cells and their ability to produce IFN- γ , as measured by intracellular cytokine staining. However, this study did not address a possible secondary activation of NK cells by iNKT cells leading to IFN- γ production or cytotoxicity [60]. Nevertheless, iNKT cells seem to play an important direct and/or indirect role during early IAV infection. In contrast to these findings, Benton et al. [61] used various gene knockout mice in an attempt to identify critical immune elements involved in providing heterosubtypic immunity to IAV infections and showed that iNKT cells were dispensable in this context. This was because priming of CD1d^{-/-} mice with sublethal doses of IAV led to an increased rather than a decreased survival rate after challenge with lethal doses of IAV. These two contrasting studies used a mild, less virulent strain of IAV or sublethal doses of IAV for infection of mice, which preclude any definitive conclusion regarding a possible protective role for iNKT cells during influenza infection. Therefore, experimental mouse models, a dose of the virus challenge, and the route of α -GalCer administration may have differently contributed to these apparently conflicting outcomes.

Recently, De Santo et al. [62] evaluated the survival rate of CD1d^{-/-} and J α 18^{-/-} mice injected with the PR8 strain of IAV to examine the role of iNKT more directly in IAV infection models. The findings of this study showed that although most of the wild-type mice survived the IAV infection, none of the J α 18^{-/-} and CD1d^{-/-} mice could survive the infection. An analysis of cross-talk between iNKT cells and MDSCs, capable of suppressing T cell proliferation, in protection against influenza infection showed a reduction of the suppressive activity of MDSCs by iNKT cells in a CD1d- and CD40-dependent manner. The susceptible J α 18^{-/-} and CD1d^{-/-} mice were found to have high numbers of MDSCs with suppressive activity, which was markedly reduced when iNKT cells were adoptively transferred into these knockout mice. Further, it was shown in vitro that MDSCs activated by TLR3 and TLR7 agonists were able to activate iNKT cells to exert their MDSC-modulatory role. This activation was apparently CD1d-dependent and likely involving endogenous lipid antigen presentation to the TCR of iNKT cells, as MDSCs from Hexb^{-/-} mice lacked the ability to activate iNKT cells. Hexb^{-/-} mice lack hexosaminidase B enzyme required for the presentation of an endogenous antigen (iGB3) by APC to the TCR of iNKT cells. These findings implied that the MDSCs stimulated by viral nu-

cleic acids in vivo were modulated toward their maturation by iNKT cells through a direct recognition, thus contributing to anti-IAV innate immune responses (Fig. 1A).

ROLE OF iNKT CELLS IN ADAPTIVE IMMUNITY AGAINST INFLUENZA VIRUS

Accumulating evidence in the last few years suggests a versatile role for iNKT cells, not only as innate lymphocytes but also as major contributors to adaptive immune responses (Fig. 1B). Similar to conventional CD4⁺ and CD8⁺ T cells, iNKT cells act in a somewhat antigen-specific manner, although the nature of all of the lipid antigens they recognize is not clearly known. In addition to cytotoxicity against target cells [3], iNKT cells secrete various cytokines characteristic of Th1, Th2, and Th17 cells [30]. Therefore, Kronenberg and colleagues [30] have described recently a role for iNKT cells in microbial infections in association with Th0 cells and proposed a new paradigm: Th1 + Th2 = Th0, suggesting that the iNKT cells capable of rapidly producing a mixture of Th1 (IFN- γ) and Th2 (IL-4) cytokines are Th0-like cells.

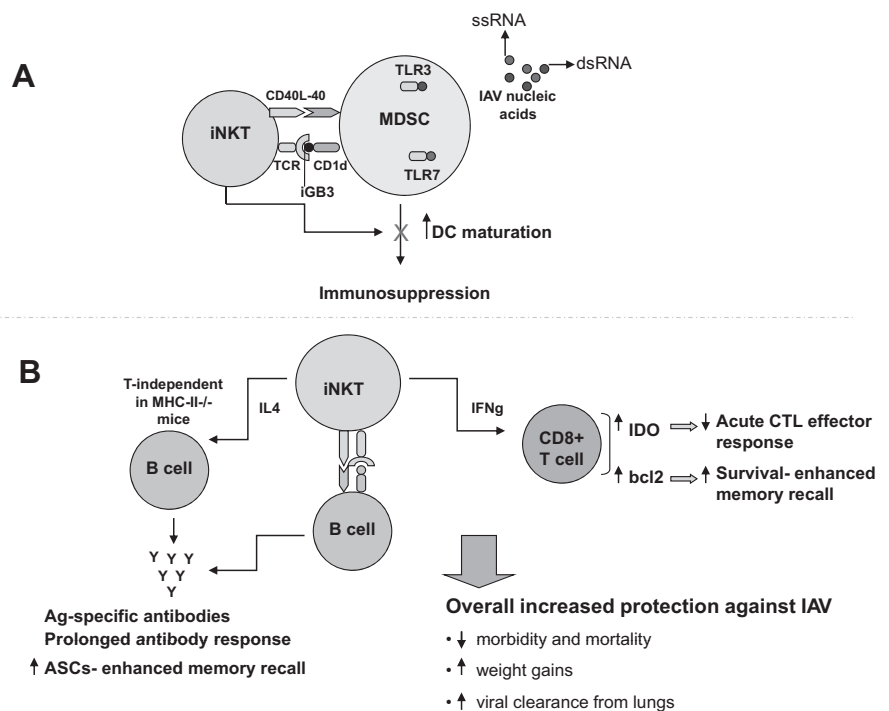
The importance of iNKT cells in adaptive immunity against influenza has been signified in a recent study that evaluated the use of α -GalCer as a nasal adjuvant in protection against a lethal challenge with influenza virus in mice [63]. This study used intranasal immunization (three times at 1-week intervals) of BALB/c mice with PR8-HA (split-product virus vaccine), with or without α -GalCer, followed by an intranasal challenge with a 20-LD₅₀ dose of PR8 influenza virus 2 weeks after the third immunization. Mice that received α -GalCer had high levels of HA-specific IgA antibodies in the nasal and lung washings and HA-specific serum IgG lev-

els. Further, none of the mice that received PR8-HA plus α -GalCer died after lethal virus challenge and had reduced weight loss, indicating a complete protection against influenza. These effects were absent in CD1d^{-/-} mice, indicating iNKT help in an antibody-mediated response against influenza antigens. A subsequent study used α -GalCer in conjunction with the iPR8 virus to immunize BALB/c mice against a lethal challenge with the PR8 virus in a single intranasal administration regimen [64] compared with three immunizations used in the previous study [63]. Intranasal administration of iPR8 in conjunction with α -GalCer resulted in high levels of IgG and IgA antibodies in serum and respiratory mucosa, respectively, but the overall response was suggested to be Th2-dominated compared with mice that received iPR8 alone. The Th2-dominant response was characterized by increased IL-4 and IL-5 and reduced IFN- γ production by splenocytes, which were restimulated with iPR8 in vitro. However, distinct CTL responses against PR8-infected target cells were also observed in mice that received iPR8 plus α -GalCer compared with mice that received iPR8 alone. Authors speculated that the induction of CTL responses in the presence of little IFN- γ production can be attributed to the α -GalCer-induced DC maturation and increased T cell proliferation as described previously [65]. Further, the immunized mice were protected, although partially, against a highly lethal challenge (100 LD₅₀ live PR8 virus; 1.9×10^6 pfu), and the antibody response lasted for 3 months, suggesting a role for iNKT cells in conferring prolonged immunity against influenza.

The role of iNKT cells in sustaining specific B cell responses and memory has also been investigated, as illustrated in Figure 1. Mice immunized with α -GalCer plus a

Figure 1. Role of iNKT cells in innate and adaptive anti-influenza immune responses.

(A) iNKT cells regulate the suppressive effects of MDSCs in IAV infections. MDSCs stimulated by TLR3 and -7 ligands and viral dsRNA and ssRNA, respectively, present iGB3-like endogenous lipids on CD1d molecules to iNKT-TCR, along with CD40-CD40L costimulation. This interaction results in activation of iNKT cells, which helps the maturation process of these DC, thus blocking MDSC-mediated suppression. (B) iNKT cells enhance B cell responses through IL-4 secretion or coupled with direct cell-cell interaction, resulting in higher and prolonged antibody production with maintenance of memory response. iNKT cells can induce antigen (Ag)-specific antibodies, to some extent, in a T-independent manner in mice lacking MHC-II molecules. iNKT cells can also regulate CD8⁺ T cell functions through IFN- γ (IFN γ) by inhibiting the expansion of CTL effectors at the peak of response of IAV infection while enhancing CD8⁺ T cell memory.



subunit vaccine containing influenza HA and neuraminidase from an H3N2 subtype (A/Panama/2007/99) had increased antibody titers following primary and secondary immunizations compared with untreated controls [66]. Interestingly, $J\alpha 18^{-/-}$ mice also had similar antibody titers, but the decay in their titers was rapid compared with wild-type mice, raising the possibility of the involvement of iNKT cells in the maintenance of B cell memory to influenza antigens. Furthermore, it was found that in a recall antibody response experiment, after a boost with H3N2 alone given at Week 30, mice that received H3N2 plus α -GalCer in the first two immunizations (Weeks 0 and 2) developed higher antibody titers compared with mice that received H3N2 alone. In addition, in support of this, higher frequencies of H3N2-specific ASC precursors were found in the spleens of α -GalCer-treated mice, indicating a role for iNKT cells in homeostatic maintenance of memory. Further investigations into mechanisms of protection revealed that iNKT cells are able, at least in part, to help B cells through direct CD40L-CD40 interaction to produce antigen-specific IgG responses in a CD4⁺ T cell-independent manner in mice lacking MHC-II expression.

iNKT cells may also influence T cell responses (Fig. 1B). It has been demonstrated recently that iNKT cells can inhibit the expansion of CTL effectors at the peak of response to IAV infection and enhance CD8⁺ T cell memory [67]. In this study, an inactivated IAV preparation was combined with α -GalCer to immunize mice to evaluate iNKT influence on CTL generation and maintenance. This study revealed a reduction in percentage and number of CTLs specific for nucleoprotein- and acid polymerase-derived immunodominant epitopes of IAV, namely, NP366 and PA224, respectively, during the acute phase of response (Day 7) in spleen and bronchial lymph nodes with an increase in NKT cell numbers compared with untreated controls. In contrast to an early time-point (Day 7), an increase in the proportion and number of NP366-specific memory CTLs was found 6 weeks later (Day 42) in the α -GalCer-treated mice compared with untreated controls, suggesting the importance of iNKT cells in CTL memory generation and maintenance. Mechanistic experiments suggested that iNKT cells mediated these responses through IFN- γ -induced IDO expression in lymph nodes. IDO in turn may inhibit T cell proliferation and also up-regulate the prosurvival gene, *bcl2*, in memory CD8⁺ cells, thus prolonging their life for a subsequent viral challenge. This study also showed a protective role for the α -GalCer-enhanced CTL response against other IAV subtypes. Taken together, these findings demonstrate an important role of iNKT cells in B and T cell effector and memory responses.

α -GALCER ANALOGUES AS INKT CELL AGONISTS AND THEIR USE FOR ENHANCING IMMUNITY TO VIRUSES

With the discovery of α -GalCer, there has been a "storm" of experiments dissecting the usefulness of iNKT cells in mouse models of infectious diseases and other disorders. As described above, these cells are gaining a momentum in many microbial infections including but not limited to influenza. α -GalCer-

mediated activation of iNKT cells results in a robust release of cytokines, such as IFN- γ and IL-4, from these cells, as well as activating other neighboring cells in the microenvironment. Transactivation of bystander cells such as NK cells by iNKTs forms an important event in the bulk production of IFN- γ , especially during the later phases of a Th1 response in α -GalCer-treated mice [60]. Similarly, IL-4 production is also partly contributed during this process by DC and Th2 cells [68].

Attempts have been made to achieve a desired Th1 or Th2 response by studying the structural binding between CD1d-loaded glycolipid antigen and iNKT-TCR. These attempts have focused predominantly on modifying the lipid chain or the sugar head of α -GalCer [4]. Obtaining a Th2-biased response by iNKT cells has been achieved by substantially shortening the length of the sphingosine chains (lipid tails) of α -GalCer, and one such successful example was described first by Miyamoto et al. [69]. This iNKT agonist, known as OCH, has a truncated phytosphingosine chain and when used therapeutically in the experimental autoimmune encephalomyelitis model, resulted in an early IL-4 production, skewing the response away from Th1, thus exerting beneficial effects. Similarly, we showed prolonged cardiac allograft survival in mice treated with OCH and early production of IL-4, which was suggestive of the effective priming of CD4⁺ Th0 cells at an early stage to differentiate into the Th2 phenotype [70]. In addition to OCH, the Th2-promoting activity of the glycolipid C20:2 has been described [71]. C20:2, a diunsaturated N-acyl-substituted analog of the prototypical α -GalCer (KRN7000), was shown to induce higher levels of IL-4 and IL-2 compared with OCH but comparable levels with that of α -GalCer [71]. On the other end, a synthetic analog of α -GalCer, referred to as α -C-GC (or C-glycoside), which was designed by modifying the polar glycoside head of α -GalCer, was shown to be a potent Th1 agonist in vitro and in vivo [72, 73].

More recently, α -C-GC has been used as an adjuvant with a live, attenuated IAV with a truncated NS1 gene (A/PR8 mutant) to immunize mice and showed an increased immunogenicity of the vaccine, resulting in greater protection against a challenge compared with the vaccine alone [72]. The choice of NS1 mutation in this study was based on the findings that the truncated NS1 mutants can immunize animals effectively against IAV infections. BALB/c mice vaccinated with the adjuvant had greater virus-specific, total IgG, IgG1, and IgG2a antibodies as well as IFN- γ -secreting CD8⁺ T cells. Collectively, although these α -GalCer analogs are able to drive a favorable Th1 or Th2 response, possessing therapeutic potentials, the mechanisms underlying these cell-cell interactions have yet to be fully elucidated. In the context of immunity against influenza, it is important to note that α -GalCer, in the mouse model, has provided a superior/complete protective response against a lethal challenge with H1N1 and H3N2 strains of IAV [59, 62–64, 66, 67]. As α -GalCer stimulation of iNKT cells results in a mixed Th1 and Th2 response and as immunity to influenza is cell- and antibody-mediated, it seems reasonable to assume that α -GalCer can be an ideal adjuvant to boost protective responses conferred by influenza vaccines.

CONCLUDING REMARKS

Recent findings support a protective role for iNKT cells in viral infections and in particular, immunity to influenza. α -GalCer-mediated activation and expansion of iNKT cells in tissues, such as spleen, lungs, and liver, result in resistance to influenza infection through enhanced innate and adaptive defenses. The underlying mechanisms of these innate defense mechanisms suggest iNKT help in DC maturation, regulation of MDSCs, bystander NK cell activation, and recruitment of B and T cells to the site of infection through secretion of various cytokines. In addition, iNKT cells help to enhance IAV-specific B cells, CTL, and memory recall responses by increasing survival of memory B cells and CTLs. Although certain mechanisms by which iNKT cells interact with other immune system cell types are known, the question of how these cells respond differently to different stimuli and more importantly, their precise role in memory response remains largely unknown.

A few α -GalCer analogs identified recently seem promising therapeutically to induce desired Th1/Th2 immune responses. However, the fine-tuning in their binding to iNKT cell-TCR seems subtle and requires further understanding of the complete events taking place in an interactive microenvironment in vivo. Nevertheless, α -GalCer or its analogs are potential candidates to be used as vaccine adjuvants to boost immune responses to influenza vaccines. Moreover, iNKT cell-mediated orchestration of help to B and T cells suggests that the iNKT cell can be considered a “dark horse” that may occupy a center place one day in anti-influenza immunity.

AUTHORSHIP

R.R.K. wrote the paper, and S.M.H. and S.S. wrote and edited the paper.

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KEY WORDS:

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