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The Contrasting Roles of NKT cells in Tumor Immunity

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Abstract

NKT cells are true T cells that serve as a bridge between the innate and adaptive immune system, acting as first responders. They recognize lipid antigens rather than peptides, and respond to these when presented by a non-classical class I MHC molecule, CD1d. NKT cells can play a pathogenic role in asthma or a protective role against several autoimmune diseases, in part based on their cytokine profile. In cancer, they can play opposite roles, contributing to anti-tumor immunity or suppressing it. The protective NKT cells were found to be primarily type I NKT cells defined by use of a semi-invariant T cell receptor involving Vα14Jα18 in mice and Vα24Jα18 in humans and responding to α-galactosylceramide, and the most protective were among the minority that are $CD4^-$. The suppressive NKT cells were found to be $CD4^+$ and to be primarily type II NKT cells, that have diverse T-cell receptors and respond to other lipids. Further, the type I and type II NKT cells were found to counter-regulate each other, forming a new immunoregulatory axis. This axis may have broad implications beyond cancer, as NKT cells play a role in steering other adaptive immune responses. The balance along this axis could affect immunity to tumors and infectious diseases and responses to vaccines.

Keywords

NKT cells; tumor immunity; immunosurveillance; immunoregulation; α-galactosylceramide; cancer; IL-13; TGF-β

There are many mechanisms by which the immune system polices itself to prevent an irrationally exuberant response. Tumors have learned to subvert the immune system in part by exploiting these natural mechanisms. Conversely, the immune system has many armaments to target tumors, including parts of both the innate and the adaptive immune systems. We will focus on one class of cell, the NKT cell, that bridges the innate and adaptive immune systems by having properties of both. These cells, although not prevalent in large numbers, can have a disproportionate impact on either a protective or suppressive anti-tumor immune response.

NKT cells are true T cells with αβ T cell receptors (TCRs) and CD3, but they also may have some receptors characteristic of NK cells, such as NK1.1, and are able to kill in an NK-like fashion [1–7]. They were originally defined as T cells in the thymus with a relatively low level of CD3 expression and expressing NK1.1 [8–13]. However, they were soon found to be unusual in their MHC restriction, in that they were dependent on a nonclassical MHC molecule, CD1d [1–7,14]. As the usage has evolved, the CD1d restriction has become the primary defining characteristic of NKT cells, so that even ones that are NK1.1 negative are called NKT cells if they are CD1d restricted [5]. Another major difference between NKT cells and conventional T cells is that the NKT cells primarily recognize glycolipid antigens rather than peptide fragments of proteins, and the CD1d molecule preferentially presents lipids. Thus, NKT cells,

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as part of the adaptive immune system, provide a means by which the immune system can distinguish and respond to lipid or glycolipid antigens. These lipids include not only self antigens from mammalian cells [15–18], but also microbial lipid antigens [19–22]. Indeed, a breakthrough in characterizing these cells first came when it was discovered that a large segment of them recognized a glycolipid from a marine sponge or microorganisms symbiotic with the sponge, α-galactosylceramide (αGalCer) [23]. This allowed these cells to be specifically stimulated so that their function could be examined. It turned out that the major subset of NKT cells that respond to αGalCer have a relatively invariant TCR, expressing a unique TCR Vα14Jα18 chain in the mouse and a Vα24Jα18 chain in the human, along with a small number of possible TCR Vβ chains, accounting for their ability to all recognize the same antigen. These have been called classical or type I NKT cells, also known as iNKT for their invariant TCRα. Functionally, they are defined by their response to αGalCer and by their ability to bind fluorescent tetramers of CD1d loaded with particular lipids like αGalCer.

In addition to specific antigen recognition like part of the adaptive immune system, NKT cells (at least type I) also behave partly like members of the innate immune system, being among the first responders on the scene [24,25]. They make cytokine responses within a few hours, and their early response has been shown to be one potential source of early IL-4 to initiate a Th2 response [26]. The early response seems to be due to the fact that these cells have pre-formed mRNA for various cytokines (possibly due to self reactivity) [24,25], so that they can be activated even by IL-12 made by macrophages or DCs stimulated by LPS from bacteria [27].

Type I NKT cells can make both Th1 cytokines like interferon-γ (IFN-γ) and Th2 cytokines like IL-4 and IL-13. Through the latter they appear to play an important role in allergic asthma in mice [28,29] as well as in humans [30], although their role in human asthma is less well established [31]. In autoimmune diseases, they can be protective against experimental allergic encephalitis by mechanisms involving type II cytokine production when they are stimulated by a weaker αGalCer analog, OCH, that skews the response toward Th2 cytokines [32], but also by non-Th2 cytokine mechanisms in other situations [33,34]. They can also protect against collagen-induced arthritis in mice through a Th2 cytokine-dependent mechanism [35]. In diabetes-prone NOD mice, type I NKT cells appeared to protect against development of diabetes through several mechanisms, including production of Th2 cytokines [36–39], when activated by αGalCer [36], or when adoptively transferred into NOD mice [40,41]. A newer analog of αGalCer that skews toward Th2 cytokines, C20:2, was found to be even more potent in suppressing diabetes in NOD mice [42]. Further, type I NKT cells appeared to be less frequent in NOD mice [37,38] as well as human patients with autoimmune diabetes (or had a reduced production of Th2 cytokines) [43,44], although the lowered level in humans was questioned when these cells were identified in patients and controls using a combination of CD1d tetramers and antibodies to Vα24 [45]. Thus, type I NKT cells can play both pro-inflammatory and anti-inflammatory roles.

In addition to type I NKT cells, a second category of NKT cell was discovered that had diverse TCRs, not using the invariant Vα14Jα18 combination, and not responding to αGalCer. These type II NKT cells do respond to other lipids presented by CD1d, and so they fit within the definition of CD1d restricted T cells as NKT cells. They were first found by Cardell and colleagues [46] among $CD4^+$ T cells remaining in class II MHC deficient mice, and their physiologic function is much less well understood. They were found again among T cells responding to a tumor that was negative for conventional class I and II MHC molecules but expressed CD1d [47]. They seemed to respond to a different set of lipids from type I NKT cells in that they did not require endosomal loading of CD1d as did type I NKT cells [48]. One lipid that was found to specifically target type II and not type I NKT cells, was sulfatide from myelin sheaths [49]. CD1d tetramers loaded with this lipid identified a population of NKT cells that

did not overlap with the population stained with CD1d tetramers loaded with αGalCer, providing a potential marker for at least a portion of type II NKT cells, as not all type II cells respond to sulfatide [49]. A crystallographic structure of sulfatide bound to CD1d has been obtained [50]. These cells suppressed a response to experimental autoimmune encephalitis, a mouse model of multiple sclerosis [49]. Only a few other physiologic functions for type II NKT cells have been reported. Other immunosuppressive roles have been found in human mixed lymphocyte reactions [51] and autoimmune diabetes mellitus in NOD mice [52]. Some other functions found are pro-inflammatory. Type II NKT cells were increased in the livers of human hepatitis C patients and found to make Th1 cytokines there [53], and were also implicated in a mouse model of hepatitis B infection [54]. In hepatitis, their activity was reported to be dependent on the NK cell activating receptor NKG2D [55]. They have also been shown to play a pro-inflammatory role in ulcerative colitis in humans, dependent on their production of IL-13 [56].

NKT cells in tumor immunity

A role for NKT cells in tumor immunity was first detected when it was found that αGalCer injection into mice could protect against some tumors [57–59] and then that this molecule specifically stimulated invariant NKT cells [23]. Subsequent studies confirmed in multiple murine tumor models that NKT cells had protective activity when stimulated by αGalCer in vivo [60,61] or by IL-12 [62]. Moreover, type I NKT cells were found to have a protective role in immunosurveillance against tumors even without any specific stimulation by αGalCer, in a methylcholanthrene-induced tumor model, as demonstrated using CD1d KO mice that lack all NKT cells and Jα18 KO mice that lack type I NKT cells, as well as using adoptive transfer of NKT cells [61,63–65]. The protective activity was dependent on production of IFN-γ and on activation of NK cells, but not on perforin in the NKT cells themselves, suggesting that the protective mechanism was indirect, involving IFN-γ-mediated activation of NK cells and possibly $CD8^+$ T cells that lysed the tumor cells, rather than direct lysis by NKT cells [63,66, 67]. The protective activity was especially mediated by a subset of type I NKT cells found in the liver, rather than the spleen or thymus, and that were negative for CD4 and CD8 [68]. This finding is consistent with the finding in humans that the double negative NKT cells are more skewed toward IFN-γ production whereas the $CD4^+$ NKT cells produce a more balanced mix of both Th1 and Th2 cytokines [69,70]. Thus, among the three dichotomies mentioned, TCR type, CD4/8 expression and tissue location, it appears the most protective NKT cells are those with the invariant TCR (i.e. type I), that are CD4-CD8-double negative, and that are found in the liver.

Dendritic cells pulsed with αGalCer appeared to be more potent at inducing protective NKT cell activity than did free αGalCer in vivo, whereas free αGalCer tended to anergize NKT cells after activating them [71], or to skew them more towards Th2 cytokine production [72]. Further, dendritic cells matured by interaction with NKT cells made IL-12 and IL-15 and were more effective at eliciting T cell responses [73]. Thus, αGalCer pulsed DCs could be used rather than free αGalCer to treat tumors [60]. Similarly, B cells pulsed with αGalCer and tumor peptides, or tumor cells themselves pulsed with αGalCer, were effective at inducing NKT cell activity and protection [74,75]. Human DCs pulsed with αGalCer also activated human NKT cells and skewed their cytokine profile toward Th1 cytokines [76].

Thus, human clinical trials of αGalCer either alone [77,78], or pulsed onto autologous DCs [79–82] have been tried as a therapy for cancers. Also, NKT cells from glioma patients, expanded ex vivo with αGalCer-pulsed DCs, were able to lyse glioma cells [83]. Adoptive transfer of NKT cells stimulated in vitro with αGalCer and IL-2 was also studied in a phase I human trial in cancer patients [84]. Although some immune responses have been obtained, no clinical benefit has yet been achieved with any of these approaches to date. Whether this lack

of success in humans so far reflects the lower frequency of type I NKT cells in humans [6], or a difference between the adoptive tumor experimental models in mice and the advanced human cancer patients, is not yet understood.

In view of all this evidence for a protective role of NKT cells in tumor immunity, at least in mice, it was surprising when it was first discovered that NKT cells could also suppress tumor immunity [85–88]. The suppressive cell was $CD4^+$ and its suppressive activity against tumor immunosurveillance depended on its production of IL-13 (not IL-4) in two different tumor models, the 15-12RM fibrosarcoma that grows, regresses, and then recurs, and the CT26 colon carcinoma lung metastasis model [85,89,90]. In these models, CD1d KO mice that lacked all NKT cells were protected from tumor recurrence in the first model and had reduced metastases in the second model, and blockade of IL-13 showed a similar effect, whereas blockade or deficiency in IL-4 had no effect. Since the cytotoxic T lymphocyes mediating tumor immunosurveillance did not have IL-13 receptors, it was clear that the effect of IL-13 depended on other steps downstream that suppressed the cytotoxic T cells. These were identified when it was found that the IL-13 was necessary to induce $Gr\text{-}1^+CD11b^+$ myeloid cells to make TGF-β, and that it was the TGF-β that suppressed the protective response [87]. Further, the presence of NKT cells and of IL-13 in vivo was necessary for these non-lymphoid cells to be induced to make TGF-β. Thus, the TGF-β that was suppressing tumor immunity was coming not from the tumor itself, but from the immune system via this newly identified immunoregulatory pathway. NKT cells were also found to suppress tumor immunity in a renal carcinoma liver metastasis model [91]. Although the primary immunoregulatory pathway involving NKT cells appears to be the one described above involving IL-13 and TGF-β, there is at least one example in which NKT cells can suppress tumor immunity against an orthotopic osteosarcoma independent of IL-13 or STAT 6 (the downstream signal transducer of IL-13) and independent of TGF-β, so NKT cells may work by additional mechanisms not yet identified [92]. Further, in the case of the 4T1 orthotopic mammary carcinoma, although CD1d KO mice lacking NKT cells are protected, as are mice lacking STAT6, blockade or deficiency of IL-13 or IL-4 does not protect, suggesting that other mechanisms can play a role in that tumor as well [93]. In contrast, $CD4^+CD25^+$ T reg cells did not seem to play a role in these tumor models [94].

These findings led to a paradox, in that NKT cells had been found to contribute to protective anti-tumor immunity and immunosurveillance as well as suppress it [94–96]. It was possible that type I NKT cells were doing both, depending on their cytokine production. Indeed, type I NKT cells were shown to suppress immunity against a lymphoid malignancy [97]. However, the alternative was that two different NKT cell subsets were mediating these opposite effects. This possibility was explored by comparing CD1d KO mice that lacked type I and type II NKT cells with Jα18 KO mice that lacked only type I, in four different tumor models studied in different labs around the world [94]. In all these tumor models, Jα18 KO mice that lacked only type I NKT cells did not show the protection that had been observed in CD1d KO mice that lacked both. Those results led to the conclusion that the type II NKT cells still present in Jα18 KO mice were at least sufficient, in the absence of type I NKT cells, to suppress tumor immunity [94]. Indeed, in the absence of type I NKT cells in Jα18 KO mice, tumor growth was accelerated at an early stage, suggesting that suppression by type II NKT cells was more active in the absence of type I NKT cells [98]. This finding also provided a likely explanation for an earlier finding that CpG oligodeoxynucleotide-mediated tumor immunity was enhanced in CD1d KO mice independent of type I NKT cells [99]. The idea that type I and type II NKT cells played opposite roles in tumor immunity was also consistent with observations in two parasitic disease models, *Trypanasoma cruzi* [100] and *Schistosoma mansoni* [101]. Both of these models found differences between CD1d KO mice and Jα18 KO mice, and in the latter case, CD1d mice lacking both type I and type II NKT cells had reduced Th2 cytokine production, whereas Jα18 KO mice lacking only type I had reduced interferon-γ production. [101]. Thus, one resolution

of the paradox was that type I NKT cells enhanced tumor immunity whereas type II NKT cells suppressed it.

In order to examine the effects of these NKT cell subsets by direct stimulation rather than by their absence in different knockout mice, Ambrosino et al [98] took advantage of the fact that αGalCer selectively activated only type I NKT cells [23] whereas sulfatide selectively activated type II NKT cells, albeit not all of them [49]. In both the 15-12RM fibrosarcoma model and the CT26 lung metastasis model, αGalCer protected mice in vivo [98], consistent with earlier studies cited above. To test whether a weaker Type I NKT cell agonist, OCH, that skewed the cytokine profile more toward Th2 [32] would suppress, OCH was tested but this was found to protect nearly as well as αGalCer [98]. Thus, any stimulation of type I NKT cells led to protection, regardless of the cytokine balance within the range testable, but it remained possible that a complete skewing to exclusive production of Th2 cytokines might make type I NKT cells suppress tumor immunity. Conversely, sulfatide treatment of mice actually increased tumor growth, consistent with the hypothesis that type II NKT cells were suppressive. This suppressive activity of sulfatide was indeed dependent on $CD4^+$ type II NKT cells because it was abrogated by depletion of $CD4^+$ cells and was effective in Jα18 KO mice but not CD1d KO mice. Thus, selective activation of type I NKT cells protected against tumor growth whereas selective activation of type II NKT cells suppressed tumor growth [96,98].

The finding of opposing roles of the two subsets of NKT cells raised the question whether there was cross-talk, or more particularly, counter-regulation between them, as had been observed in the now classical dichotomy between Th1 and Th2 cells [102]. This was tested in vitro and in vivo [98]. First, in vitro, when spleen cells were stimulated with both αGalCer and sulfatide, the proliferation induced by αGalCer was partially inhibited by concurrent stimulation with sulfatide [98]. This was true whether the proliferation was measured by thymidine incorporation, in which case it might represent proliferation of a combination of type I NKT cells and bystander cells stimulated by those NKT cells, or by CFSE dilution among type I NKT cells gated by binding of a CD1d-αGalCer tetramer, in which case it was proliferation only of type I NKT cells that was being measured. Further, it was not simply due to competition by sulfatide inhibiting binding of αGalCer to CD1d, because the inhibition could be seen when separate populations of antigen presenting cells were pulsed with sulfatide and αGalCer and then mixed, to separately stimulate both types of NKT cells [98]. Similar inhibition of interferon-γ production was also observed.

In vivo, simultaneous administration of αGalCer and sulfatide tended to skew the cytokine response ratio more toward IL-13 production, consistent with the role of IL-13 in NKT-mediated immunoregulation. Most importantly, sulfatide inhibited the protective effect of αGalCer in vivo in two different tumor models. In the 15-12RM fibrosarcoma model, αGalCer completely prevented tumor recurrence, whereas sulfatide had no effect or somewhat accelerated tumor growth. However, when sulfatide was given shortly after αGalCer administration, it completely abrogated the protection induced by αGalCer [98]. Likewise, in the CT26 colon carcinoma lung metastasis model, αGalCer completely protected the mice, whereas sulfatide increased the number of tumor nodules. When sulfatide was given shortly after αGalCer, it reduced but did not completely eliminate the protective effect [98]. Thus, in both tumor models in vivo, stimulation of type II NKT cells with sulfatide can reduce or eliminate the protective effect of stimulation of type I NKT cells with αGalCer. This counter-regulation may be direct or indirect, and its mechanism needs to be further explored. It does not appear to be cytokine mediated, based on experiments with culture supernatants and inhibitory antibodies. Further, Halder et al recently found a suppression of type I NKT cells by type II NKT cells in a hepatitis model mediated through an effect on plasmacytoid dendritic cells [103]. Conversely, suppression by type II NKT cells was increased in the absence of type I NKT cells in Jα18 KO mice [98]. Overall, whether direct or indirect, we conclude that type

I and type II NKT cells counter-regulate each other in tumor immunity. In doing so, they form a new immunoregulatory axis, analogous to that of Th1 and Th2 cells that counter-regulate each other [96]. Studies are underway to determine the relationship between this immunoregulatory axis and $CD4^+CD25^+$ T reg cells, as well as what determines which regulatory mechanism predominates in any particular tumor situation. This axis may have profound implications for other immune responses in addition to anti-tumor immunity, because of the pivotal role played by NKT cells in initiating the adaptive immune response. Therefore, altering the balance along this axis could have a significant impact on other natural immune responses to cancer or infection, and could influence the outcome of vaccination.

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