Published in final edited form as:

Clin Immunol. 2011 August ; 140(2): 160–166. doi:10.1016/j.clim.2010.12.010.

Harnessing Natural Killer T (NKT) cells in Human Myeloma: Progress and Challenges

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Abstract

Multiple myeloma is a hematologic malignancy characterized by growth of malignant plasma cells in the bone marrow. Tumor cells in myeloma express CD1d and are sensitive to lysis by CD1d restricted natural killer T (NKT) cells. Here we discuss recent studies to harness the properties of these cells in the context of human myeloma. In spite of large body of preclinical data, attempts to fully harness the properties of these cells in the clinic are in early stages. Early phase clinical studies document the capacity of human monocyte-derived dendritic cells to expand NKT cells *in vivo* in myeloma patients. These results have set the stage for ongoing studies combining NKT activation with immune-modulatory drugs. Lessons learnt from these studies may inform the optimal application of human NKT based therapies in other settings as well.

Natural Killer T cells: subsets and functions

Natural killer T cells (NKT) cells are innate lymphocytes that recognize lipid antigens in the context of CD1d family of antigen presenting molecules [1,2]. NKT cells provide a distinct niche for the immune system, as a cellular arm for the recognition of lipid antigens. A striking property of at least a major subset of these cells is to rapidly secrete cytokines in response to ligand dependent stimulation. This leads to rapid downstream activation of several other immune cells, including NK cells, dendritic cells and T cells [3]. Thus NKT cells may serve as an important bridge between innate and adaptive immunity in the context of signals derived from self or pathogen derived lipid antigens. In view of their broad effects in the immune system, NKT cells have been implicated in immune regulation of diverse disease states, including regulation of responses to pathogens and cancer, as well as allergy, inflammation and autoimmunity[4,5]. In this review, we will primarily focus on the current state of studies harnessing NKT cells in the context of myeloma. However it is likely that many of the principles of NKT biology gleaned from the study of these patients or models may be applicable more broadly in medicine, particularly in the context of inflammation.

Although NKT cells were originally discovered as a T-cell subset expressing both T and NK markers, there is now consensus in the field, that these cells are part of a broader repertoire of lipid-specific T cells[6]. At least 3 distinct subsets of NKT cells are recognized. The best studied of the subsets are those expressing the invariant T cell receptor (V α 14J α 18 TCR in the mouse or V α 24J α 18 in the human), called type I NKT or invariant NKT (iNKT) cells. The discovery of α -galactosylceramide (α -GalCer; KRN-7000), derived from a marine

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sponge (or microorganisms symbiotic with the sponge) as a potent agonist for these cells spurred considerable research on the properties of both human and murine type I NKT cells. In humans, Type I NKT cells can be further divided into CD4⁺ and CD4[−]CD8[−] DN subsets, and a small subset of human NKT cells may even express CD8αα[7,8]. NKT cells have also been distinguished by their tissue localization. In the mouse, these cells have their highest prevalence in the liver, where they may represent up to 30% of CD3⁺ T cells [9]. The liver-resident type I NKT cells are not only more prevalent, but also show different functional characteristics, in that they have been shown to be more protective against tumors than NKT cells from the spleen or thymus[10]. Type I NKT cells have a phenotype of activated T cells, and can be rapidly activated by a synthetic glycolipid ligand, α-GalCer. This property also allows facile detection of these cells using α-GalCer loaded CD1d multimers. NKT activation by α-GalCer leads to rapid downstream activation of NK cells and induction of adaptive immunity via activation of dendritic cells and T cells[11]. This property of NKT cells is being targeted to help improve the efficacy of adjuvants. Invariant NKT cells are evolutionarily conserved, thus suggesting an important role in the immune system.

Another subset of NKT cells that are also CD1d-restricted but thought to express more diverse (not invariant) TCRs were termed type II NKT cells[12]. Compared to type I NKT cells, the current understanding of the biology of type II NKT cells in humans is very limited. Finally, there is another heterogeneous subset of T cells with diverse TCRs that express NK markers, but is not CD1d-restricted or glycolipid-reactive, and termed type III NKT cells. As discussed below, there is a growing body of evidence that the distinction between different subtypes of NKT cells is relevant with regard to their potential role in tumor immunity.

Role of CD1d restricted T cells in tumor immunity

Type I NKTs cells have been implicated to play a protective role against tumors in several studies [13]. The importance of the CD1d-NKT cell axis in immune regulation has been clearly demonstrated in many murine models of tumor immunity[12]. Several studies have confirmed the anti-tumor activity of type I NKT cells stimulated by α-GalCer in vivo [14,15]. An important role for NKT cells in immune-surveillance of tumors was also suggested based on the use of mice deficient in type I NKT cells. These mice have increased propensity for cancer in both spontaneous as well as carcinogen and oncogene-induced models, and this may be particularly relevant in the setting of p53 deficiency[14-17]. The antitumor effects of NKT cells are due to several mechanisms, including enhancement of immune effectors, particularly IFN-γ mediated activation of NK cells and antiangiogenesis[16,17]. Syngeneic DCs pulsed with α-GalCer were able to treat established liver metastases of the B16 melanoma[18]. More recent studies show that a C-glycoside analogue of α-GalCer, skewed NKT cells more toward an interferon-γ response, and was even more effective against melanoma metastases[19]. In addition to the role of IFN-γ in recruiting NK cells, NKT cells were shown to activate DCs to make IL-12, and also lead to activation of conventional T cells and B cells. This capacity of NKT cells to link innate and adaptive immunity may be important for their role in tumor immunity [20,21]. Another major target of type I NKT mediated anti-tumor effects may involve inhibition of myeloid derived suppressor cells[22,23].

As with mice, a role for human type I NKT cells in tumor immunity has also been postulated[24]. Human Vα24⁺ NKT cells can mediate antitumor effects in vitro; a deficiency of NKT cells, or defects in their function, have been described in cancer patients[25-27]. These studies have served as the basis for approaches to enhance anti-tumor immunity by specifically targeting type I NKT cells[24,28]. Studies in some mouse models

suggested that NKT cells could also suppress tumor immunosurveillance based on their production of the Th2 cytokine IL-13[12,29,30]. However it is now clear that these cells are type II NKT cells which may inhibit protective immunity. Together these studies support a model wherein the balance of activation of type I versus type II NKT cells may regulate the anti-tumor effects of NKT cells.

NKT cells in human multiple myeloma

Multiple myeloma (MM) is a hematologic malignancy characterized by the accumulation of transformed plasma cells in the bone marrow. Myeloma is an interesting target for NKT mediated anti-tumor immunity due to several considerations. In prior studies, we have shown that progression to clinical myeloma is associated with loss of interferon- γ secreting function of NKT cells[26]. We studied the function of NKT cells from the blood and tumor bed in patients with premalignant gammopathy, indolent or progressive multiple myeloma. NKT cells from patients with progressive disease, but not indolent or premalignant disease, had a marked deficiency of ligand-dependent interferon- γ production[26]. This functional defect could be overcome in vitro using dendritic cells pulsed with the NKT ligand, α -GalCer. These data suggested that clinical progression in patients with monoclonal gammopathies is associated with an acquired but potentially reversible defect in NKT cell function and support the possibility that these innate lymphocytes play a role in controlling the malignant growth of this incurable tumor. Thus measurement of NKT cell function may be a useful predictor of clinical outcome in these patients. These data also provided impetus for further clinical studies to boost or restore NKT cell function in malignancy.

It is worth noting that in myeloma, tumor cells grow predominantly in the bone marrow and that a significant proportion of T cells in the human bone marrow may be CD1d restricted, albeit non-invariant T cells [31]. Moreover, we have previously shown that myeloma cells express CD1d, are sensitive to lysis by NKT cells, and therefore are a particularly interesting target for NKT directed therapies[26]. Our group and others have observed that CD1d is highly expressed in tumor cells in pre-malignant and early myeloma [32][26]. Expression of CD1d is reduced with disease progression and eventually lost altogether in most myeloma cell lines, suggesting that CD1d impacts negatively on myeloma cell survival. Consistent with this, engagement of CD1d by anti-CD1d mAbs induces cell death of myeloma cell lines with restored CD1d expression and primary myeloma cells [32]. As discussed above, NKT activation may also mediate anti-myeloma effects by anti-angiogenesis, as well by activation of NK cells, and tumor specific T cells.

Dendritic Cell mediated activation of human NKT cells in cancer

Deficiency in NKT numbers and/or function in the setting of cancer is not unique to MM and has been demonstrated in several settings including in patients with solid tumors[25]. This loss of NKT function appears to correlate with clinical stage of tumors, and may be reversible. Therefore, the ability to manipulate NKT cells in humans has attractive therapeutic potential. The discovery and availability of α -GalCer as a clinical grade reagent has allowed studies employing this ligand to enhance NKT cells in vivo. Despite encouraging preclinical data using α -GalCer, injection of the ligand alone in humans with cancer led to only modest and transient effects without substantial NKT expansion[33]. In contrast, injection of glycolipid loaded DCs led to prolonged NKT activation in mice[18]. These results have led the investigators to test alternative approaches utilizing targeted antigen presenting cells to enhance NKT cell activation.

To understand the impact of different APCs on the activation of human NKT cells we conducted study on freshly isolated human NKT cells and quantified them by an enzyme-linked immunospot (ELISPOT) assay[34]. We observed that human NKT cells have a Th1

profile after stimulation with α -GalCer loaded DCs. We evaluated APC requirements for human NKT cell activation in fresh blood and showed that monocyte derived DCs are more effective than monocytes/macrophages for detecting and activating NKT cells in fresh blood, with mature α -GalCer pulsed DCs being optimal. In this study, DCs were also efficient APCs for expanding NKT cells in culture and generating NKT cell lines. NKT cells expanded with DCs were functional, secreting both IFN- γ and IL-4, and killing NKT-sensitive targets. Optimal activation of these lines was seen using mature DCs loaded with α -GalCer. DCs matured with several different stimuli were effective. These data helped to establish the conditions for loading DCs with α -GalCer for immune therapeutic targeting of NKT cells.

Based on these data, we carried out a phase I clinical trial to test the safety and tolerability of α -GalCer loaded mature DCs in patients with advanced cancer[35]. Monocyte-derived mature DCs that were loaded with α -GalCer and matured using a cocktail of inflammatory cytokines were intravenously injected in patients with advanced cancer. Injection of α -GalCer-pulsed DCs led to greater than 100-fold expansion of several subsets of NKT cells in all patients. Interestingly, these expansions could be detected for up to six months after vaccination. NKT activation was associated with an increase in serum levels of IL-12 and IFN- γ -inducible protein-10. In addition, we observed enhancement of antigen specific T cell responses. There was also an increase in cytomegalovirus specific memory CD8⁺ T cells after injection of GalCer loaded DCs. These data demonstrated the feasibility of sustained expansion of NKT cells in vivo in patients with advanced cancer, and suggested that NKT activation may lead to activation of adaptive immunity via NKT mediated activation of DCs in vivo. There is already considerable body of preclinical evidence for the ability of NKT cells to activate DCs and resultant activation of adaptive immunity[11,19,36,37].

In spite of the ability to expand iNKT cells in vivo, we observed defects in IFN γ secreting function of NKT cells expanded in vivo. Since the capacity of iNKT cells to mediate anti-tumor effects is strongly linked to their ability to secrete IFN γ , this has led us and others to explore novel approaches to overcome this defect. Recent preclinical studies by the Exley lab have shown that NKT dysfunction may be related in part to direct activation by CD1d+ tumor cells and is corrected by coadministration of IL12 with α -GalCer [38]. Another approach being considered is to test different glycolipids, with potentially enhanced immune-stimulatory function. We have reasoned that yet another approach may be to combine NKT targeted therapy with pharmacologic approaches that might lead to enhanced iNKT activation, as discussed below.

Immuno-modulatory drugs as adjuvants for NKT activation

Given the complex interaction between malignant cells, immune effectors and microenvironment of the tumor bed, simply activation of NKT cells in vivo may not be sufficient for significant anti-tumor effects. A more effective strategy might be to use combined modalities to attain maximal anti-tumor effects of immunotherapy. Thalidomide and its derivative lenalidomide (LEN) can enhance costimulation of human T cells[39]. These agents have anti-inflammatory, antiangiogenic, and immunomodulatory properties, and may target tumor cells by direct cytotoxicity and indirectly by interfering with several components of the bone marrow microenvironment. LEN retains antitumor activity equal to or greater than the parent compound, but carries less toxicity. Importantly, LEN demonstrates greater potency at T cell costimulation. LEN has been shown to have significant clinical activity in multiple myeloma and LEN based regimens have become a common frontline approach in myeloma[40].

To influence of LEN on NKT cell function, we evaluated the capacity of LEN to modulate the function of iNKT cells in culture[41]. We showed that LEN enhances ligand dependent expansion of NKT cells in response to α -GalCer in both healthy donors and in patients with myeloma. NKT cells activated with α -GalCer loaded DCs in the presence of LEN had greater ability to secrete interferon- γ . Antigen-dependent activation of NKT cells was greater in the presence of dexamethasone (DEX) plus LEN than with DEX alone. Together these data demonstrate that LEN and its analogues enhance CD1d-mediated presentation of glycolipid antigens and support combining these agents with other NKT targeted approaches. Preclinical studies suggest that CD4-subset of NKT cells may be more proficient at anti-tumor effects. We were unable to show that the enhancement of interferon- γ secretion specifically targets a particular subset of iNKT cells. However it is ligand dependent and seems to target all NKT cells responsive to the ligand. Another group recently explored the effects of lenalidomide on iNKT cells from newly diagnosed and advanced multiple myeloma patients, and confirmed the capacity of LEN to enhance Th1 polarization of iNKT cells[42]. This latter study provides particular support for combining adoptive NKT cell transfer with LEN in the context of MM. Together, these results provide preclinical evidence for the iNKT cell-mediated immunotherapy and a rationale for combining NKT targeted therapy with lenalidomide in multiple myeloma. Based on the results discussed above, we are currently carrying out a phase II trial testing the combination of α -GalCer-loaded DCs and LEN in the setting of asymptomatic / early stage myeloma in order to exploit their adjuvant properties to expand NKT cells, and boost both innate and adaptive immunity to tumors. We chose to target patients with early stage disease as tumor cells in more advanced cancer often lose the expression of CD1d. In this study patients receive 3 monthly cycles of LEN along with injection of α -GalCer loaded DCs on the 8th day of each cycle. To date, the study has progressed well, but it is too early to draw any conclusions regarding clinical or immunologic effects of the combination. It should be noted that the effects of LEN on NKT cells appear to be ligand dependent. A recent study in patients with myelodysplastic syndrome found no consistent changes in frequency of NKT cells in myelodysplastic syndrome patients responding to LEN and only modest increase in NKT cell function in culture [43]. Evaluation of NKT function in this study was however tested using PMA based stimulation and not with a NKT ligand.

Ligands for CD1d restricted T cells in Myeloma

As discussed earlier, the cause of loss of ligand dependent IFN γ secreting function in NKT cells from MM patients is not known and is an area of active investigation. However the finding that the effector function of conventional T cells was preserved in at least some of the patients suggested that the effects on NKT function were not due to a global alteration of T cell function[26]. Therefore we posited that understanding the nature of ligands recognized by NKT cells may provide novel insights into the mechanism of observed NKT dysfunction. Over the past few years, there has been considerable interest in trying to understand the nature of endogenous ligands recognized by NKT cells. Studies have shown that type I NKT cells can recognize some microbial glycolipids (such as those from sphingomonas and Borrelia) and self-antigens [44]. However the nature of antigens specifically recognized by type II NKT cells is even less clear. Some studies have shown sulfatide and nonlipidic small molecules as capable of binding these T cells [45,46]. In some settings, GD3 and tumor-derived glycosphingolipids have also been implicated as ligands for iNKT cells[47]. However, the nature of the specific endogenous ligands recognized by either type I or II NKT cells in inflammation or cancer in humans remains obscure. In order to identify the nature of CD1d binding ligands in the context of human myeloma, we isolated bulk lipids from plasma of myeloma patients, and used it to load CD1d multimers and stain human T cells. Interestingly, these CD1d multimers stained a subpopulation of both V α 24+ and V α 24- T cells. Elution of CD1d bound lipid led to the discovery of lyso-

phosphatidylcholine (LPC) as a CD1d binding ligand recognized by human T cells, particularly type II NKT cells[48]. Several species of LPC were identified in these experiments. The presence of LPC-specific T cells could also be demonstrated in MM patients. These T cells were skewed towards a Th2 phenotype, particularly expressing IL13 upon TCR based stimulation. The concept that PC and LPC can bind CD1d has now been confirmed by several groups. Using a mass spectrometry based approach, the Cresswell lab recently demonstrated the binding of phospholipids to CD1d[49]. The Th2 bias of T cells in response to such ligands may relate to the nature of binding to CD1d[50]. Gumperz et al. had initially identified phospholipids as CD1d binding ligands in mice[51]. More recently, this group also analyzed human NKT cell recognition of CD1d associated cellular ligands[52-54]. In these studies, the most antigenic species was LPC. In addition, lyso-sphingomyelin, which shares the phosphocholine head group of LPC, also activated NKT cells. Antigen-presenting cells pulsed with LPC were capable of stimulating increased cytokine responses by NKT cell clones and by freshly isolated peripheral blood lymphocytes. These results demonstrate that human NKT cells recognize cholinated lyso-phospholipids as antigens presented by CD1d. At present the status of information about human type II NKT cells in particular is limited. We are currently trying to better understand the TCR repertoire and signaling pathways in LysoPC reactive NKT cells in human MM. Since these lyso-phospholipids serve as lipid messengers in normal physiological processes and are present at elevated levels during most inflammatory responses including cancer, these emerging insights point to a novel link between NKT cells and cellular signaling pathways that are associated with human cancer and inflammation[55]. It is therefore of interest to examine whether LysoPC specific T cells are enriched in other human tissues characterized by inflammation, and whether tissue inflammation modifies the properties of these cells. It is notable that while the detection of these cells adds another dimension to inflammation associated signaling in MM, the nature of tumor-derived CD1d binding lipid ligands still remains obscure. Whether or how inflammation associated ligands might alter the properties of iNKT cells or other immune cells in the tumor bed is an area of active research, and may provide novel insights into how inflammation may alter or disable this innate arm of the immune system.

Challenges to harnessing NKT cells in the clinic

In spite of the large body of preclinical data suggesting a regulatory role for NKT cells in diverse medical conditions including cancer, approaches to specifically harness these cells towards clinical benefit remain in their infancy[4]. One reason behind this may be the species specific differences in the relative frequencies of NKT cells in mice versus humans. In particular, NKT cells are much more frequent in mice than in humans[1]. Therefore the effects of NKT activation may be more amplified in mice versus humans. As a result, approaches targeting NKT cells in humans are now considering a combination approach to both increase their frequency either by vaccination or adoptive transfer and target their functional properties ex vivo or in vivo. However most of the current studies to date have been based on small numbers of patients and are in early phase of testing. Therefore while the current data provide evidence of feasibility, there are serious limitations for drawing conclusions about any clinical impact. There are also several key gaps in knowledge that need to be addressed to improve NKT targeted therapies. In many settings including in MM, the reason behind defects in NKT function in patients with cancer remain poorly understood. Improved understanding of naturally occurring ligands for these cells, and understanding the balance between type I and II NKT cells in humans may facilitate therapeutic targeting of these cells in the clinic. A particular property of these cells that has not yet been fully exploited in the clinic relates to their ability to provide adjuvant function and enhance the capacity of vaccines [3]. Several groups are trying to develop approaches to either enhance NKT activation or optimize targeting of the NKT ligands, Chemical modification of α -

GalCer backbone has been attempted towards the development of second generation NKT agonists, with greater capacity for polarization of responses towards IFN γ secretion[56]. NKT targeted therapies may also benefit from combination approaches targeting checkpoint blockade[57]. Preclinical studies suggest that BCR mediated targeting of CD1d antigens may be a potentially useful approach to elicit antibody responses against pathogens or tumors in vivo [58]. This approach may be useful in MM wherein humoral deficiency is an early feature of disease. Stirnemann et al. described a novel approach for activation and tumor targeting of NKT cells using a fusion protein containing α -GalCer-CD1d with scFv anti-HER2 antibody[59]. Yet another approach involves directly loading α -GalCer onto tumor cells, which provides the opportunity to then link NKT cells to adaptive anti-tumor immunity[60]. All of these approaches can in principle be applied to hematologic malignancies including MM. Continued careful investigation into the biology of human NKT cells may permit harnessing the properties of these cells towards clinical benefit in human cancer including in MM.

Acknowledgments

MVD is supported in part by funds from NIH and Leukemia and Lymphoma Society.

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