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Application of tissue-specific NK and NKT cell activity for tumor immunotherapy

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Abstract

Natural killer (NK) and NKT cells are a first line of defense against pathogens and transformed cells. However, dysregulation of their function can lead to autoimmune disease. A better understanding of the mechanisms controlling NK and NKT effector function should lead to the development of improved strategies for the treatment of many diseases. The site in which NK and NKT cells reside should be taken into account, because accumulating evidence suggests that the tissue microenvironment strongly influences their function. In this regard, the liver represents a unique immunologic organ in which the balance between the need for tolerance and the ability to respond rapidly to pathogens and tissue injury is tightly regulated. NK cells in the liver have augmented cytolytic activity as compared to other organs, which is consistent with a role for liver-associated NK cells in being critical effector cells for inhibiting tumor metastasis in the liver. Several studies also suggest that hepatic NKT cells have different functions than those in other organs. Whereas splenic and thymic NKT cells have been shown to suppress diabetes development, facilitate the induction of systemic tolerance and are regulated by IL-4 and other Th2 cytokines, certain subsets of NKT cells in the liver are important sources of Th1 cytokines such as Interferon gamma, and are the primary mediators of anti-tumor responses. The unique properties and roles as critical effector cells make NK and NKT cells within the liver microenvironment attractive targets of immunotherapeutic approaches that have the goal of controlling tumor metastasis in the liver.

Keywords

NK cells; NKT cells; liver microenvironment; immunotherapy; cancer

I. Introduction

Natural Killer (NK) and NKT cells are innate immune cells critical for the first line of defense against infections and tumor genesis. NK cells were first identified and named by their capacity to spontaneously lyse some tumor targets. NKT cells are a subset of T-cells that express some NK receptors (NKR). NK and NKT-cells derive from a common lymphoid progenitor in the bone marrow [1,2] but while NKT cells develop further in the thymus [1], the NK cell's principal development site is the bone marrow [2] and to a lesser extent in the thymus and

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lymph node[3]. Following development, NK and NKT cells migrate to peripheral lymphoid and non-lymphoid tissues, where their function is uniquely shaped by the organ-specific milieu. NK and NKT cells are found in lymphoid and nonlymphoid organs with a high concentration of these cells found in the liver. In the milieu NK and NKT cells survey the microenvironment and help direct the type and magnitude of the ensuing response by releasing a burst of inflammatory cytokines. Improper regulation of these cells can lead to autoimmunity [4,5], while an inability of these cells to activate during infection or tumor formation can give rise to uncontrolled disease. Their swift response and involvement in a broad range of diseases from autoimmunity to cancer make them excellent targets for immunotherapy.

In this review we discuss how NK and NKT cells are activated, their effector functions and anti-metastatic immunotherapeutic approaches focused on the liver that take advantage of these properties.

II. The unique immunological properties of the liver

The liver has unique immune requirements. As the only organ that receives arterial blood and venous blood from the gut, a tightly regulated balance between tolerance to innocuous gut antigens and the ability to quickly respond to foreign pathogens is required. A unique cellular composition of professional and non-professional antigen presenting cells (APC) such as Kupffer cells, plasmacytoid dendritic cells, liver sinusoidal endothelial cells (LSEC) and hepatocytes with a high proportion of immature dendritic cells, all of which have been shown to induce tolerance in the liver [6] The importance of the induction of liver tolerance is highlighted in numerous transplantation models. Transplantation of mismatched or allogeneic tissue usually leads to immunological rejection by the host. However, the unique immunological properties of the liver milieu allow allogeneic tissues to be tolerated in many animal models. Liver tolerance is conferred systemically as shown by the acceptance of transplanted organs at other sites when accompanied by successful tissue grafts within the liver. This demonstrates that immunological events that occur in the liver can have wide-ranging, systemic effects.

Nevertheless, the host must be able to mount a rapid immune response to harmful pathogens that may be present in the liver, thereby switching from a tolerant state to a responsive state. The liver is also a frequent target for the metastatic spread of many solid tumors. These observations suggest that immunosurveillance is suppressed in the liver even against antigenic tumors such as renal cell carcinoma and melanoma. Additionally, viruses such as Hepatitis B and C may persist even when an immune response is mounted. To overcome these challenges, the liver contains an atypical proportion of NK and NKT lymphocytes that target transformed and/or infected cells, as compared to other lymphoid tissues (Table 1) . Previous work from our laboratory and others has characterized the contributions of chemokines and the adhesion molecule vascular cell adhesion molecule (VCAM)-1 in the recruitment of NK cells to the liver [7,8]. Although NKT cells represent 1~2% of the lymphocytes in the spleen, they account for ≈30% of the lymphocytes in the liver (Table 1). Hepatic NKT cells also express higher levels of the adhesion molecule lymphocyte function-associated antigen (LFA)-1 and the chemokine receptor CXCR6 [9,10] as well as NK1.1 which controls their activation (Table 1). Interactions between these molecules and their respective ligands may therefore contribute to the accumulation and/or activation of NK and NKT cells in the liver. Hepatic NK and NKT cells reside in the liver sinusoids, adhering to endothelial cells and are therefore in a strategic location to kill metastasizing tumor cells [11]. Not only are NK and NKT cells activated rapidly without prior sensitization, those residing in the liver exhibit particularly augmented activity, as compared to NK and NKT cells present in other tissues (Table 2). It is likely that the unique cellular and molecular composition of the liver contributes to the distinct phenotype of NK and NKT cells as compared to those from other organs. Taken together, NK and NKT cells are

critical components in overcoming the tolerogenic environment to mount necessary immune responses in the liver. Immunotherapeutic approaches targeting hepatic NK and/or NKT cells should break tolerance and prove useful in the induction of more effective immune responses for the improved treatment of liver diseases.

III. NK and NKT cell activation provide host protection against transformation and infection

To promptly defend against infected or transformed cells while preventing unchecked activation, NK cells express a pattern of inhibitory and activating receptors that recognize MHC class I on target cells, termed LY49s in mice and human killer cell Ig-like receptors (KIRs) in humans [12]. In an elegant study utilizing human NK cells, it was found that simultaneous crosslinking of several different activating receptors was capable of overcoming the inhibition imposed by an inhibitory KIR. However the ligation of a single activating receptor failed to overcome KIR-mediated inhibition or only partially induced activation [13]. Thus, inhibitory receptor functions appear to dominate over effector responses. This complex regulation enables NK cells to potentially eliminate tumors or infected cells that are missing MHC class-I but only after a series of prerequisite and redundant signals are present, in a process termed 'missing-self'[14]. Distinct from NK cells that have activating and inhibitory LY49/KIRs, NKT cells only have inhibitory LY49/KIRs [15].

Another activating receptor that is found on NK as well as NKT cells is the NKG2D receptor. Separate from LY49/KIRs receptors that sense cells with missing ligands, NKG2D receptors detect stress-induced ligands that are expressed on transformed, infected or damaged cells [14,16]. A key additional receptor expressed by NKT cells is the T-cell receptor. However, in contrast to T-cells that respond to foreign peptides presented by MHC class I or II molecules, NKT cells recognize glycolipids presented by CD1d molecules, a MHC class I like molecule [17]. Thus, antigen stimulation via this receptor represents an important way NKT cells can be activated that is distinct from NK cells. There are two types of NKT cells based upon their defined TCR rearrangement diversity. Type I NKT cells have an invariant TCR (iNKT), while type II NKT cells have a variant TCR [18]. Type I (invariant) NKT cells can mediate both protective and regulatory immune functions, including antitumor responses, protection against pathogens the maintenance of transplant tolerance and inhibition of autoimmunity [19] whereas type II NKT cells have been shown to suppress tumor surveillance in certain model systems [20,21]. The suppressive function of Type II NKT cells have also been in a liver inflammatory model. Triggering type II NKT cells with a self-ligand, sulfatide derived from myelin, prevented concanavalin A-induced hepatitis [22]. However, good markers are lacking that identify type II NKT cells and more work needs to be done to determine if these cells have a dual role like type I NKT cells or whether they are more restricted in nature as suppressive cells.

IV. Immunotherapeutic activation of NK and NKT cells can mediate tumor rejection

As early responders to infection and tissue injury, NK and NKT cells are important targets for immunotherapeutic regimens which have the goal of directing and amplifying the ensuing inflammatory response. Table 3 summarizes a series of elegant studies that not only convincingly demonstrate the critical role for NK and NKT cells in controlling liver inflammation but moreover highlights the clinical relevance of manipulating hepatic NK and/or NKT cells using targeted immunotherapies for the improved treatment of liver disease and tumor metastasis within the liver microenvironment. It is well established that a Th1 response is critical for a successful anti-tumor immune response. IL-12 is a potent Th1 polarizer of T-

cells, inducer of IFNγ production and was recently listed by the immunotherapy community among the top immune reagents needed for more preclinical and clinical testing [23]. IL-12 has also shown great potential in pre-clinical tumor models particularly in combination with other cytokines [24]. Immediately following IL-12 activation, NK and NKT cells produce theTh1 polarizing cytokine IFNγ. Furthermore, our lab has found IFNγ is critical, but not sufficient, for the therapeutic effects of our preclinical immunotherapeutic approaches tried to date[20,25]. NK and NKT cells, but not naïve T-cells, constitutively express the IL-12 receptor, and these cells are the first to respond to IL-12 and can contribute to the protective effects IL-12 has against tumors in some experimental models[26]. NK-derived IFNγ production serves as an important indicator of the generation of durable anti-tumor responses and a predictor of long-term survival in cancer patients receiving immunotherapy[27].

Much of the observed effector function of activated NKT cells is through the production of inflammatory cytokines that promote cytotoxic effects of other T-cells and NK cells. In contrast, activated NK cells have been well characterized in their ability to directly mediate anti-tumor responses through the delivery of cytotoxic effector molecules such as granzymes and perforin [28]. NK-mediated perforin production was critical for the rejection of tumor cells expressing NKG2D ligands, defining NKG2D as a cytotoxicity-inducing receptor [16]. Another mechanism whereby NK and NKT-cells directly eradicate target cells is through the expression of cell death inducing ligands FasL [29] and TRAIL [30]. Many successful preclinical immunotherapeutic approaches against tumors augment these cytolytic pathways. For example, IL-2, IL-12, IL-15 and IL-21 each increased expression of NKG2D, TRAIL and perforin on NK cells that was concomitant with increased tumor cytolysis[31,32]. Furthermore, IL-12 and IL-15 treatment of NK cells increased TRAIL and perforin expression, resulting in enhanced NK effector function. Consistently, Fehniger, et al showed that IL-2, IL-15 or IL-18-stimulated NK cells exhibited higher cytolytic activity due to significant increases in granzyme B and perforin expression [28]. Our laboratory also found that combinations of cytokines exert biological effects that could not be achieved by single agent treatment. For example, IL-2 synergizes with IL-18, resulting in increased NK cell numbers, IFNγ production and cytolysis of NK target tumor cells [33]. Combinations of IL-2/IL-12, IL-2/IL-18 or IL-12/IL-18 similarly led to synergistic augmentation of IFNγ production by NK and NKT cells that was critical for the generation of anti-tumor responses in mice[20,25].

NK and NKT cells also produce chemokines that are important for the recruitment of effector T cells, B cells, neutrophils and other NK and NKT cells to the disease site. IL-2- or IL-12-activated NK cells secrete several T cell-recruiting chemokines, including MIP-1α, MIP-1ß, IL-8, macrophage-derived chemokine (MDC), and regulated on activation, normal T-cell expressed and secreted (RANTES), particularly when these cytokines are used in combination with TLR agonists [34]. NK or NKT-derived IFNγ also upregulates expression of the chemokine receptor CXCR3 that mediates the subsequent recruitment of $CXCR3^+$ T and NK cells to infected tissues [35]. The early production of chemokines by NK and NKT cells is likely to have a profound role in shaping the ensuing inflammatory response.

V. NK and NKT cells mediate tumor surveillance in the liver microenvironment

To better elucidate NK and NKT cell function in the liver, our lab first developed a method for isolating leukocytes from the liver [11]. These methodologies enabled us to provide clear evidence that NK activity in the liver were several-fold higher than those observed in spleen and blood (Table 2) [36]. A clear role for NK cells in guarding against developing tumors has been established[14]. NK cells control the growth of liver tumors and mice treated with the NK-depleting agent anti-asialo GM1 had increased formation of experimental metastases in the liver and lung after i.v. challenge with B16 melanoma or Lewis lung carcinoma [36]. Additionally, hepatic NK cells induced cytolysis in tumor cells that were otherwise resistant

to splenic or blood NK cells [37]. This increased cytolytic activity was shown to be mediated by the perforin/granzyme pathway [37] as well as IFNγ [38] and through the IFNγ-dependent upregulation of TRAIL. Hepatic NK cells express TRAIL and lack expression of Ly-49 inhibitory receptors, that allows for the potential to self-target hepatocytes upon activation [39] and spontaneously reject tumor metastasis [40]. TRAIL induction on NK cells plays a critical role in the antimetastatic effects of IL-12 and alpha-GalCer, particularly in the liver which was the only organ in which alpha-GalCer augmented TRAIL-mediated function [41]. Furthermore, the susceptibility of TRAIL-sensitive tumors to apoptosis was abrogated in NK cell-depleted animals [42], further defining the important role that NK cells play in immune surveillance against tumor development within the liver microenvironment. Because the liver has a higher frequency of $NK1.1^{(+)}$ NKT cells (Table 1) and NK1.1 regulates the recognition of "missing-self", the activation of iNKT cells regulates ensuing host immune responsesand suggests NKT cells from the liver are more active in resisting tumor growth. Indeed, Crowe et al. found B16 melanoma or MCA-1 sarcoma were rejected following transfer of liver $CD4^{(-)}$ iNKT, but not spleen and thymus $CD4^{(-)}$ iNKT cells [43]. These studies suggest that the liver microenvironment is an important determining factor in shaping NK and NKT effector function.

While the role for NK cells in immunosurveillance is fairly clear, the role for NKT cells in this process remains ambiguous. Terabe et al found NKT cells suppressed immunosurveillance of 15-12RM tumors implanted subcutaneously through the production of IL-13 [44]. Our lab also found NKT suppressed immunosurveillance when it was revealed that the Renca murine renal cancer formed fewer tumor nodules in mice lacking NKT cells or in mice depleted of NKT cells (Figure 1) [20]. In contrast, another group found NKT cells had effector function when they reported that mice lacking NKT cells had an increase in tumors induced by methylcholanthrene (MCA) [45]. There are several possibilities for the observed differences in NKT cell function between these studies. First, the studies that found a suppressive role for NKT cells used transplantable tumors, whereas the study that found an effector function for NKT cells used a chemical carcinogen to induce tumor formation. If resting NKT cells are immunosuppressive and activated NKT cells have effector function, then it may be that MCA activates NKT at the site of the injection, while the transplantable tumors do not. Another possibility is that NKT cells are suppressing inflammatory irritation needed for MCA to induce tumors, whereas mice lacking NKT cells are unable to effectively suppress this inflammation. Another possibility for the differences observed in NKT cell function might be due to tumor location. As discussed above, NKT cell function is shaped by the microenvironment in which they reside and NKT cell function could therefore be influenced by tumor location in different and distinct microenvironments. Specifically, MCA induced tumors were studied in the intradermis, 15-12RM tumors were examined subcutaneously, and Renca tumors were examined in the liver microenvironment. Finally, a key study recently showed functional differences between type I NKT cells and type II NKT cells. In this study, type II NKT cells were sufficient for suppression of immune surveillance [46]. These authors further found type II NKT cells activated with sulfatide antigen could inhibit the effector function of type I NKT cells. This important study highlights the potential for a novel immunoregulatory axis mediated by NKT cell subsets and which may play an important role in many inflammatory conditions [18].

VI. Immunotherapeutic regimens that target hepatic NK and NKT cells are promising cancer treatments

The targeting of NK and NKT cells within the liver microenvironment represents an important goal for immunotherapy of liver disease (Table 3). Many combination immunotherapies have been described that augment NK cell numbers and activity within the liver microenvironment. One tactic has been the use of pathogens, such as attenuated *Listeria monocytogenes*, that

specifically target NK cells. Bahjat, et al showed that this approach resulted in the migration and activation of NK cells within the liver and the concomitant NK-dependent destruction of hepatic tumors [47]. This study demonstrates that microbial stimuli are capable of potent immune activation resulting in the establishment of tumor-specific immune responses. Immune modulating cytokines comprise another, major approach for the manipulation of NK cells. Smyth, et al showed that IL-2 and IL-12 each resulted in the suppression of tumor metastases through an NKG2D-dependent pathway that involved perforin-mediated cytolysis [48]. These two cytokines were more effective against tumors expressing NKG2D ligands. In contrast, IL-18 was found to mediate the NKG2D-independent, Fas ligand-mediated rejection of tumors [48]. The implications of this important study are that the Fas ligand-sensitivity and expression of NKG2D ligands on tumors needs to be monitored as this may reflect the tumor responsiveness to a particular immunotherapy. In our own studies, we utilized plasmid DNA encoding cytokine genes with the rationale that these may serve as useful adjuvants for cancer vaccines and might also be potentially efficacious in combination with other immunomodulatory agents. We reported that the intradermal injection of plasmid DNA encoding murine IL-12 elicited the systemic expression of IL-12 as well as IFNγ and IFNγ-inducible chemokines within 24 hours [49]. The expressed cytokine was functional in that NK cell activity was augmented even in mice deficient in endogenous IL-12 p40 expression. In another study, we showed similarly that hydrodynamically delivered IL-2 cDNA caused a sustained increase in NK cell numbers and NK-mediated cytolytic activity in liver and spleen leukocytes [50]. Furthermore, the treatment of mice bearing established lung and liver metastases showed that IL-2 plasmid DNA was an effective treatment against liver metastasis and had moderate effectiveness against lung metastasis as well.

Early and ongoing studies from our laboratory have characterized the mechanisms that regulate the recruitment of NK and NKT cells to the liver in response to proinflammatory cytokines. We showed that a variety of exogenously added cytokines resulted in the recruitment and activation of hepatic NK cells. For example, systemic IL-2 administration resulted in the rapid and sustained recruitment of NK cells in the liver[50]. IL-12 also induced NK recruitment to the livers of treated mice through an IFN-γ dependent pathway [7,20]. Less is known about the recruitment of NKT cells to the liver following activation, however the chemokine receptor CXCR6 plays a crucial role in NKT cell homeostasis and for patrolling the liver sinusoid [10]. Human NKT cells were examined for chemokine receptor profiles and were found to express receptors associated with inflammatory chemokines [51]. In contrast to conventional T cells, only a low percentage of NKT express CCR7, a chemokine receptor found on naïve or memory T-cells. This chemokine receptor profile suggests NKT cells intrinsically have an activated/primed phenotype permitting quick mobilization to sites of inflammation. Certain viral infections similarly augment NK cell number and/or activity within the liver, and this is frequently associated with the production of proinflammatory cytokines. The mechanisms whereby this occurs include the induction of chemokines, such as macrophage inflammatory protein (MIP) 1-α that mediates the CCR5-dependent recruitment of NK cells into the liver [8]. Our lab and others have shown an important role for cytokines such as TNFα in the recruitment of NK cells into the liver[52]. Based on the well established ability of TNFαto upregulate adhesion molecule expression on endothelial cells, we also demonstrated the critical role for vascular cell adhesion molecule-1 / very late activation antigen-4 interactions in mediating NK recruitment and subsequent activation within the livers of poly-ICLC and IL-2 treated mice[7]. Although the complex milieu of cytokines produced *in vivo* by resident hepatocytes, Kupffer cells, endothelial cells as well as infiltrating leukocytes makes it challenging to assign the relative importance of a single cytokine for the recruitment of NK cells into the liver, it is evident that a more complete understanding of the molecular mechanisms regulating NK recruitment and activity within the liver microenvironment is essential for the design of improved immunotherapy to treat liver diseases.

Clearly, much more work needs to be done in order to define the contributions of NKT cells as cytolytic cells. NKT cells were first shown as anti-tumor effector cells when it was found that the potent anti-tumor ceramide αGalCer, activated NKT cells in a CD40/CD40L and B7 dependent manner [53]. Subsequent studies examining the inflammatory response of αGalCer-activated NKT cells found a coordinated interaction between NKT cells and APCs expressing αGalCer that leads to activation of NK cells. Kitamura found αGalCer activated NKT cells express CD40L that engages CD40 on APCs triggering IL-12 production that stimulated NKT cells to produce IFNγ [54]. In turn, NKT-derived IFNγ activates NK cells causing them to become more cytotoxic and induce the activation of $CD8^+$ cytotoxic T lymphocytes [55]. Smyth, et al expanded these findings by demonstrating that αGalCer activated NKT cells stimulate the proliferation and cytolytic activity of NK cells and that this newly-expanded NK cell population could be made to be even more cytolytic by the subsequent administration of systemic IL-21 [56]. Crosstalk cascades have been found with dendritic cells for both NK and NKT cells. Adam, et al showed that primary rejection of lymphoma and the establishment of long-term T cell memory was dependent upon reciprocal cellular receptor- and cytokine-mediated interactions between NK cells and DC [57]. αGalCer-pulsed DC also result in NK-dependent tumor rejection in a number of hepatic metastasis models [29,58]. These crosstalk studies further confirmed a critical role for IL-12 production by the APC and the ensuing IFNγ production by NK and NKT cells. Because IL-12 is a central player in the αGalCer-induced immune cascade, other therapies using IL-12 have demonstrated remarkably similar anti-tumor activities and a dependence on NKT and NK cells [26]. However, a study examining B16.F10 melanoma in the liver found that IL-12 had therapeutic effects in the absence of NKT cells and found NK cells were the essential cells for tumor rejection [59]. Thus, contrasting roles for hepatic NK and NKT cells may exist and may possibly be dependent upon the cellular microenvironment. The unique cellular interactions between immune cells are likely to play an important role in the priming and regulation of both innate and adaptive immune responses against liver tumors. It is critical that we now define the molecules involved in these cell-cell interactions, so that improved therapies can be developed.

Intriguingly, the use of cytokine combinations to treat tumor-bearing mice has suggested that the liver microenvironment, but not that of other organs may be particularly sensitive to immune surveillance by NK cells. Although synergistic anti-tumor responses against orthotopic kidney tumors have been achieved using IL-2/IL-12 or IL-2/IL-18 combinations, these anti-tumor effects were maintained in mice depleted of NK cells [25]. Although, for a variety of transplantable and chemically-induced murine tumors, the depletion of NK cells (using anti NK1.1 antibody) resulted in the growth and metastatic spread of several transplantable and chemically-induced murine tumors [60], a caveat of these studies however, is that anti-NK1.1 depletes not only NK cells, but also NKT cells. The development of mice deficient in NK cells, but not NKT or T cells, would therefore be optimally suited for the refinement of NK cell contributions towards anti-tumor responses. For tumors arising in the liver, however, we believe that the augmented NK cytolytic activity within this particular microenvironment endows them with a critical role for the control of hepatic tumors. We showed recently that NK cells were an important component for tumor regression in a murine liver tumor model (Figure 1) [20]. In this study, the combined administration of IL-18 and IL-12 augmented the numbers of and production of IFNγ by hepatic NK cells concomitantly with the removal of immunosuppressive NKT cells, which resulted in significantly reduced tumor nodules in the livers of treated mice (Figure 1). Ongoing experiments in our laboratory are examining whether the IL-12/IL-18 regimen uniquely primes NK cells for enhanced anti-tumor responses. Taken together, these data suggest that although NK cells may not be principal mediators of the anti-tumor response in certain tumor models, they are critical components of effective anti-tumor responses in the liver microenvironment and possibly other sites.

Although this work focuses on tumor immunotherapy, it is equally clear that there are significant roles for NK, NKT cells and of course innate immunity in the regulation of the normal immune response as well as in autoimmunity. We will not review this literature but will cite recent work [61-71]. We believe it likely that the most effective immunotherapeutic approaches will be those that take advantage of the unique cellular microenvironment of the liver for robust NK cell activation and the coordinated engagement of NKT cells.

VII. Conclusions

Recent advances in our understanding of NK and NKT cell biology make it possible to design combinational immunotherapeutic strategies that result in enhanced anti-tumor responses. We believe that optimal immunotherapeutic approaches against cancer are likely to be those that target the critical effector pathways in ways that take advantage of NK and NKT cell's distinct immunomodulatory attributes in relation to the unique microenvironment that these cells are exposed to. In this regard, combinations of cytokines hold great promise for the augmentation of cell-type specific regulation of host anti-tumor responses. However, these therapies may need to be tailored specifically to the target organ. One unresolved question is how the tissue microenvironment shapes NK and NKT cell functions. Also, it is important to understand why NK cells appear to be more important in certain tumor studies than in others and whether this reflects important differences in the tumor model, cytokine therapy and possibly tumor location. Finally, as others have already noted in this special issue of the Journal of Autoimmunity, we are pleased to contribute this work in recognition of Noel Rose's lifetime contributions in autoimmunity and the improvement of patient care. This issue is part of the Journal of Autoimmunity's series that recognizes great figures in autoimmunity [72-75].

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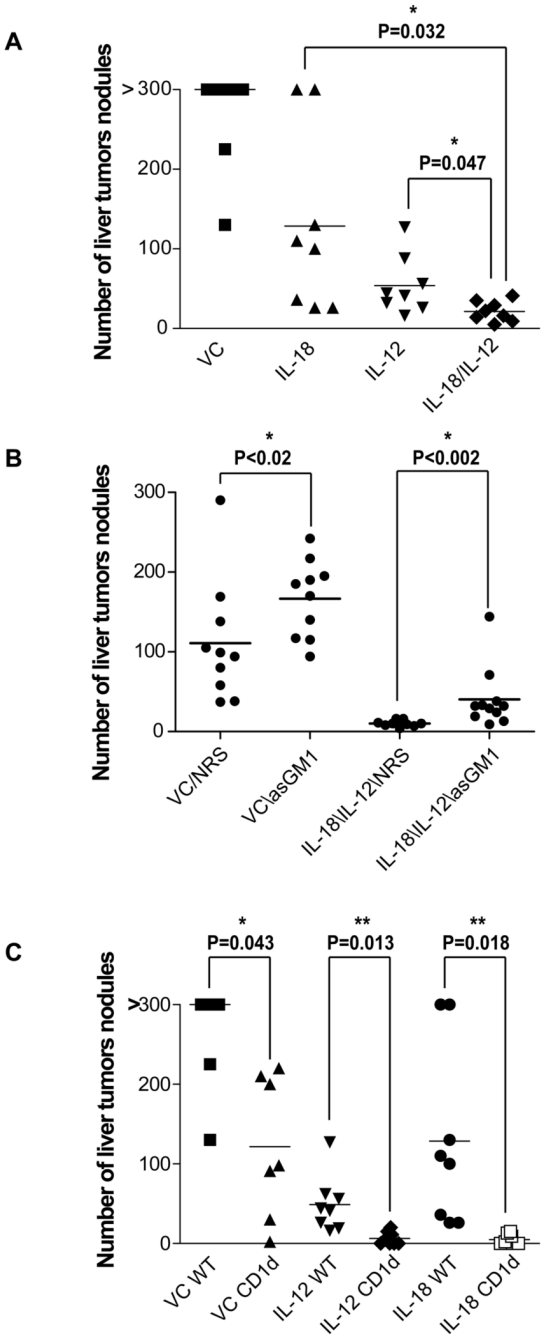

Figure 1. IL-18/IL-12 therapy reveals an effector role for NK cells and an endogenous immunosuppressive effect for NKT cells in the liver

(A) Renca tumor was implanted in the livers of Balb/c mice intrasplenically. Three days after tumor implantation, mice were treated with vehicle control (VC), IL-18, IL-12 or IL-18/IL-12 for three weekly cycles. On day 14, livers were harvested and tumor nodules were counted. (B) Wildtype Balb/c Mice were treated with normal rabbit serum (NRS) or anti-asGM1 to deplete NK cells concurrent with intrasplenic implantation of Renca tumors. (C) CD1d −/− mice deficient in NKT cells were inoculated intrasplenically with Renca. Three days after tumor implantation, mice received daily i.p. administration of vehicle control (VC), IL-12, IL-18 or IL-18/IL-12. On day 17 after tumor implantation, livers were harvested and the number

of tumor nodules was counted. Each treatment group consisted of 8-10 mice and results are representative of three experiments.

Table 1

The murine liver contains an increased frequency of NK and NKT cells and a majority of hepatic NKT cells express NK1.1

Leukocytes from three individual 8-10 week old C57/BL6 mice were isolated from liver, spleen, lung, bone marrow, thymus and lymph node as previously described [29] and stained with anti-CD45, anti NK1.1 and CD1d-tetramer loaded with PBS57 (NIH tetramer facility). The percentage of NK and NKT cells were examined using flow cytometric analysis and gating on the lymphocyte population determined by forward and side scatter. Student's unpaired t test was used to compare the frequency of liver NK cells and NKT cells to other organs.

	Frequency of NK cells	Frequency of NKT cells	Percentage of NKT cells expressing NK1.1
Liver	5.06 ± 0.33% **	41.64 ± 2.96% ***	81.82 ± 2.26% ***
Spleen	2.33 ± 0.13%	2.17 ± 0.11%	31.47 ± 1.91%
Lung	6.47 ± 0.17% *	2.27 ± 0.19%	28.88 ± 1.14%
Bone Marrow	2.30 ± 0.05%	2.63 ± 0.24%	44.90 ± 0.77%
Thymus	0.07 ± 0.02%	1.13 ± 0.23%	25.60 ± 4.35%
Lymph Node	0.28 ± 0.03%	0.44 ± 0.03%	7.71 ± 2.87%

The percentage of NKT cells that expressed NK1.1 were also examined using flow cytometric analysis. Student's unpaired t test was used to compare the percentage of liver $NK1.1^+$ cells to $NK1.1^+$ NKT cells in different organs (*** $p<0.0003$ as compared to all other organs).

** ($p<0.002$ as compared to spleen, bone marrow, thymus or lymph nodes, but not lungs)

*** ($p<0.0003$ as compared to all other organs).

* The frequency of lung NK cells was significantly greater than other organs, including the liver ($p<0.02$).

Table 2

Hepatic NK and NKT cells have distinct effector properties as compared to splenic and/or thymic NK or NKT cells

	NK cells	Refs	NKT cells	Refs
Effector Molecule Expression	• Higher TRAIL, perforin, and granzyme expression. • Augmented TRAIL function. • Lack of Ly-49 inhibitory receptors.	[37, 40,41]	• Higher frequency of $NK1.1^+$ cells	Table 1, [9]
Anti-Tumor Activity	• Augmented as compared to spleen. • Kill tumor cells otherwise resistant to splenic NK cells.	[11,36, 37,39, 40,42,55]	• Augmented as compared to spleen or thymus. • Greater resistance to IL-4 mediated regulation, as compared to thymus.	[43]

Table 3

Critical analysis of NK and NKT cell function in tumor immunology and potential clinical implications.

Study	Conclusion	Immunotherapeutic Implications	Ref
Wiltrout, et al (1985)	NK activity in the lungs and liver were several-fold higher than those in spleen and blood.	Immunotherapies can augment NK activity in target organs to an even greater extent than in the blood and spleen.	[36]
Terabe et al (2000)	NKT cells and IL-13 are necessary for the downregulation of tumor immunosurveillance.	IL-13 inhibitors may prove to be a useful tool in cancer immunotherapy.	[44]
Smyth, et al (2005)	AlphaGalCer in combination with cytokine therapy potently increased antitumor responses that were dependent upon perforin-activity of NK cells.	This study demonstrates the feasibility of augmenting NK-mediated anti-tumor activity indirectly by specifically activating NKT cells.	[56]
Crowe et al (2005)	NKT cells from liver rejected sarcoma development *in vivo* better than those from spleen or thymus.	First study to demonstrate the existence of functionally distinct NKT cell subsets in vivo and provides insight into the paradox that NKT cells function as immunosuppressive cells in some disease models, whereas they promote cell-mediated immunity in others.	[43]
Geissmann et al (2005)	NKT cells patrol liver sinusoids to provide intravascular immune surveillance and CXCR6 regulates their abundance.	The chemokine receptor CXCR6 is a potential target for strategies that aim to control NKT-associated immune responses in the liver.	[10]
Subleski, et al (2006)	Mice lacking NK or NKT cells had increased or decreased liver tumor formation, respectively.	Immunotherapeutic approaches that enhance NK cell function while eliminating or altering NKT cells could be effective in the treatment of cancer in the liver.	[20]