Review Article

Clinical Cancer Therapy by NK Cells via Antibody-Dependent Cell-Mediated Cytotoxicity

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Natural killer (NK) cells are powerful effector cells that can be directed to eliminate tumor cells through tumor-targeted monoclonal antibodies (mAbs). Some tumor-targeted mAbs have been successfully applied in the clinic and are included in the standard of care for certain malignancies. Strategies to augment the antitumor response by NK cells have led to an increased understanding of how to improve their effector responses. Next-generation reagents, such as molecularly modified mAbs and mAb-cytokine fusion proteins (immunocytokines, ICs) designed to augment NK-mediated killing, are showing promise in preclinical and some clinical settings. Continued research into the antitumor effects induced by NK cells and tumor-targeted mAbs suggests that additional intrinsic and extrinsic factors may influence the antitumor response. Therefore more research is needed that focuses on evaluating which NK cell and tumor criteria are best predictive of a clinical response and which combination immunotherapy regimens to pursue for distinct clinical settings.

1. Introduction

Natural killer (NK) cells are innate immune effector cells capable of recognizing and destroying virally infected and neoplastic cells. The importance of NK cell-mediated immunosurveillance in the control of tumor growth has been evaluated in NK cell-deficient mouse models with limited information in humans. Humans with NK cell deficiencies are plagued with persistent acute viral infections, especially herpes simplex virus [1]. However, mouse models with defects in NK cell effector function clearly demonstrate an increased susceptibility to neoplastic disease as they age [2].

NK cell effector functions can be exploited for the treatment of some tumors through their ability to mediate antibody-dependent cellular cytotoxicity (ADCC). The NK cell Fc receptor, CD16 (FcγRIIIa), contains an immunotyrosine-activating motif (ITAM) in the cytoplasmic domain. NK cell recognition of an antibody-coated target cell results in rapid NK cell activation and degranulation [3]. mAbs that specifically target tumor cells take advantage of the ADCC effector pathway to tip the balance of an interrogating NK cell in the favor of the activating receptors resulting in tumor cell destruction and an anti-tumor immune response [4].

Tumor-targeted mAbs that initiate NK cell ADCC have been used clinically. Antibodies targeting CD20, Her2/neu, epidermal growth factor receptor (EGFR), and disaloganglioside (GD2) are examples of clinically successful antibodies whose mechanisms include NK cell-mediated ADCC [5–9]. GD2 is overexpressed on tumors of neuroectodermal origin, such as neuroblastoma and melanoma, and minimally expressed in normal tissues making it a good target for tumor-specific mAb. Anti-GD2 mAbs work through NK cell-mediated ADCC and have demonstrated clinical benefit for children with neuroblastoma [10]. In this paper, we will use examples from mAbs targeting GD2 and other tumor antigens to discuss antibody-facilitated NK cell-mediated cancer immunotherapy strategies.

2. NK Cell Responses to Tumor-Specific mAbs

There are numerous Fc receptors for IgG (FcγR) that are widely expressed on immune cells. The FcγR family consists of four classes of receptors, FcγRI, FcγRII, FcγRIII, and FcγRIV, that have been identified in both mice and humans. There are significant similarities in the functions of the FcγR receptors between mice and humans, but there is limited homology in receptors themselves [11]. To date, only one inhibitory FcγR, FcγRIIb, has been identified and is the only receptor to have complete homology between mice and humans [11]. FcγRs can be found on virtually all hematopoietic cells except T cells; in most cases, cells coexpress activating and inhibitory FcγR, allowing for the balance between activating and inhibitory receptors to dictate their response [11]. NK cells are an exception to this rule and express only the activating FcγRIIIa. NK cells do not express the inhibitory FcγRIIb.

Because of their rapid and unopposed responses to mAb, NK cells play a major role in the anti-tumor response elicited by tumor-specific mAbs. Early studies demonstrated that NK cells are the primary mediators of the immune response elicited by tumor-targeted mAbs. However, recent data show that granulocytes may also play a significant role in anti-tumor responses generated by tumor-targeted antibodies [11, 12]. NK cells are important effectors in the mAb-driven immune response to tumors, and data continue to accumulate on their importance [13]. Multiple clinically successful mAbs utilize NK-mediated ADCC as a mechanism of action. Rituximab (anti-CD20), Herceptin (anti-Her-2/neu), Cetuximab (anti-EGFR), and the anti-GD2-mAbs 3F8 and ch14.18 are examples of tumor-specific mAbs whose clinical activity can be attributed, at least in part, to NK cells.

3. Augmentation of mAb Responses through the Activation of NK Cells

Various strategies of NK cell activation alongside antibody administration have been evaluated. NK cell activation can occur through a variety of stimuli including cytokine administration, toll-like receptor (TLR) agonists, or agonist antibodies directed toward activating receptors on NK cells. The coadministration of an NK-activating cytokine, interleukin-2 (IL-2), enhances the anti-tumor activity of NK cells [14, 15]. The TLR9 agonist CpG can activate numerous innate immune effectors, including NK cells [16]. Combination of CpG with Rituximab increases NK-mediated ADCC in vitro and anti-tumor responses in a mouse model of CD20-expressing tumors [17].

Further activation of NK cells through numerous receptors including 4-1BB or the Fc receptor FcγRIIIa increases ADCC activity [18]. Antibodies with Fc regions that have higher affinity for FcγRIIIa are better at activating NK cells while simultaneously initiating additional NK effector pathways [19]. High-affinity Fc-antibodies can be used at lower concentrations than traditional antibodies and maintain anti-tumor activity [19]. Antibodies with higher affinity for FcγRIIIa may be beneficial in a clinical setting by reducing the amount of antibody necessary to produce an antitumor response and therefore reduce mAb-related toxicities [20]. 4-1BB (CD137) is an activating receptor on the surface of NK cells [21]. The activation of NK cells with an agonistic antibody to 4-1BB has recently been described in a mouse model of B-cell lymphoma [18]. 4-1BB is increased on CD56^{dim} effector NK cells after CD16-mediated activation. Activation of NK cells with a 4-1BB agonist antibody between Rituximab courses in vivo led to complete regression of subcutaneous murine lymphoma tumors by NK cells [18].

The combination of mAb therapy with cytokines is another strategy used to increase their activity. Combination of Herceptin with interleukin-12 (IL-12), an important cytokine to NK cell responsiveness and IFNγ production, increases the response of NK cells to Her2-expressing breast tumor cells in a mouse model of breast cancer [22]. Clinical development of this concept is underway [23]. Augmentation of NK cell responses by the addition of exogenous IL-2 has been extensively demonstrated to increase the anti-tumor response of antibody therapy. IL-2-activated lymphokine-activated killer (LAK) cells have increased ADCC activity against mAb-coated tumor cells [14, 15, 24]. IL-2 lowers the required amount of antibody necessary for NK cells to effectively lyse antibody-coated tumor targets [25]. Increased NK cell effector function after IL-2 activation is true of NK cells isolated from humans, mice, and dogs [26, 27].

4. Altered mAbs That Increase NK Cell Effector Functions

Following the production of the initial 14.18 murine anti-GD2 mAb, several molecular modifications have resulted in 2nd and 3rd generation reagents, designed to have improved function. First, there was the class switch to murine IgG2a to augment ADCC (creating the 14.G2a mAb). This was followed the by creation of a chimeric antibody (ch14.18), a humanized antibody (hu14.18), and multiple altered hu14.18 antibodies to enhance the anti-tumor response [10]. Humanized anti-GD2 mAb hu14.18K322A (K322A) is a new-generation anti-GD2 mAb that has been designed to stimulate NK cell effector mechanisms and simultaneously reduce some of the toxicities associated with anti-GD2 therapy [5]. hu14.18K322A has two key differences from its hu14.18 parent. First, hu14.18K322A was produced in a rat hybridoma line, YB2/0. YB2/0 cells have low fucosyltrasferase activity and therefore produce antibodies with fewer fucose side chains on the Fc portion. IgG antibodies that have low or absent fucose side chains are more effective at eliciting ADCC [28, 29]. Second, hu14.18K322A has a point mutation at the 322 position resulting in the replacement of lysine 322 with an alanine. This specific mutation reduces the ability of hu14.18K322A to activate complement compared to its anti-GD2 relatives [30]. Allodynia, the major clinical toxicity associated with anti-GD2 therapy, is likely the result of complement fixation. Therefore, hu14.18K322A is designed to retain or potentially enhance NK-mediated anti-tumor responses while reducing the antibody's toxicity [30].

Immunocytokines (ICs) are antibodies with linked cytokines at the Fc terminal end. The anti-GD2 IC hu14.18-IL-2 is a humanized mAb with two functional interleukin-2 proteins at the Fc terminal end [31, 32]. ICs may have certain advantages over traditional mAbs [33]. In several preclinical models, using 3 different ICs, the IC provided far greater antitumor effects than the same amount of the naked mAb infused with the same amount of IL2 (but infused simultaneously as separate molecules rather than as the IC fusion protein). This may be because ICs transport cytokine to the site of tumor and can support an ongoing local anti-tumor immune response [34, 35]. Direct delivery of IC into the tumor itself elicits a more potent local effect. Intratumoral injection of IC in tumor-bearing mice induces better antitumor responses than systemic administration. This effect can be attributed to its activating effects on intratumoral NK cells [34].

One advantage of using IC is its effect on the formation of an immune synapse between the Ab-coated tumor cell and the NK cell. Recent data from our laboratory suggest that NK recognition of an IC involves not only the Fc receptor, but also IL2 receptors [36, 37]. The involvement of the IL2R increases IC-facilitated conjugate formation between NK cells and tumor cells (Figure 1) [36, 37]. Furthermore, IC may facilitate NK: tumor cell conjugation in the absence of Fc receptors. Using an NK cell line with minimal, if any, expression of FcγRIIIa, we recently demonstrated that the IL2Rα chain plays an important role in NK: tumor cell conjugation [36]. The association of NK cells with IC-coated tumor cell results in the formation of an activated immune synapse (AIS), defined by the localization of LFA-1and CD2 [37]. AIS formation facilitated by an IC is hallmarked by clustering of NK cell CD25 into the synapse and can also be abrogated by CD25 blockade [37]. The potential benefit of IL-2 containing ICs in activating and assisting NK cells in tumor cell destruction is a relatively new research area for clinical NK-mediated tumor immunotherapy.

5. Recent Clinical Results with Anti-GD2 mAb-Induced ADCC

High-risk childhood neuroblastoma remains a disease that has not shown major improvements in cure rates over the past 2 decades [38]. Preclinical and early clinical work suggested that anti-GD2-based mAb therapy would be most effective if given in the setting of minimal residual disease, and in combination with cytokines that augment ADCC. A large randomized study was conducted by the Children's Oncology Group (COG) to test these concepts. Following initial response to combined agent chemotherapy, surgery, ablative chemotherapy, and autologous hematopoietic stem cell transplant, children received isotretinoin and were randomized to receive immunotherapy (the ch14.18 chimeric mAb + IL2 + GM-CSF) or no immunotherapy. Two-hundred twenty-six children were randomized, and the group receiving immunotherapy showed a 2-year event-free survival of 66% versus 46% for the no-immunotherapy group ($P = .012$) [39]. In the USA, through the COG, this

immunotherapy regimen has thus become the "standard of care" maintenance treatment for high-risk patients that have responded to their initial therapy.

The next-generation immunotherapy approach has involved the hu14.18-IL2 IC. Our hypothesis, based on our preclinical data [27], was that the IC approach would work best for children with smaller amounts of refractory/relapsed neuroblastoma. A recent phase II COG study in children with relapsed or refractory neuroblastoma showed that 7 of 24 patients with "nonbulky" disease showed evidence of antitumor activity, while 0 of 13 with bulky disease had evidence of antitumor activity [40]. The results of this study are consistent with the hypothesis of better activity for nonbulky disease ($P = .03$). We are continuing to develop this agent and are hoping that a future large COG trial will enable us to test this genetically engineered molecule for children in remission in order to prevent relapse, as was done for the separate ch14.18 mAb with GM-CSF and IL2 regimen [39].

6. Fc Receptor Polymorphisms and NK Responses to mAbs and ICs

Two allelic polymorphisms have been identified in human FcγRIIIa at position 158. The aa at this location in the receptor interacts with the hinge region of IgG antibodies and affects the magnitude of response at subsaturating concentrations of IgG [41]. The one aa difference of either a phenylalanine (f) or a valine (v) in FcγRIIIa may have implications for mAb therapy [42]. NK cells containing a valine at position 158 have a higher affinity for IgG. NK cells isolated from individuals bearing this receptor contain more cytophilic IgG when examined directly ex vivo [42]. FcγRIIIa[158v] is associated with a less favorable prognosis for autoimmune sufferers [43]. FcγRIIIa[158v] NK cells are more sensitive to activation and have a higher calcium influx and more rapid induction of activation-induced cell death (AICD) when stimulated [44]. A more sensitive activating receptor that produces a stronger intracellular response, such as Ca^{2+} influx, may support a more productive immune synapse by localizing granules faster to the centrosome, as has been shown with T-cell responses to TCR stimulation [45]. Faster granule localization results in more granules loaded into the synapse for target cell destruction [45]. Therefore, NK cells with FcγRIIIa[158v] receptors may have a twofold advantage in the setting of mAb-mediated cancer immunotherapy: (1) enhanced ability to recognize and bind to tumor cells coated with mAb molecules and (2) the release of more granules for each tumor cell they encounter.

Patients with an FcγRIIIa[158v] genotype respond better to therapy that utilizes an ADCC-mediating mAb as a mechanism of action. Studies using patient samples during treatment with Herceptin, Rituximab, and Cetuximab have correlated an FcγRIIIa[158v] receptor genotype with better response to therapy [46–48]. The response of FcγRIIIa[158v] NK cells in an in vitro assay of ADCC against antibody-coated tumor cells can be used as a predictor for patient response to therapy [49]. The role of Fc receptor polymorphisms on the response to immunocytokine in comparison
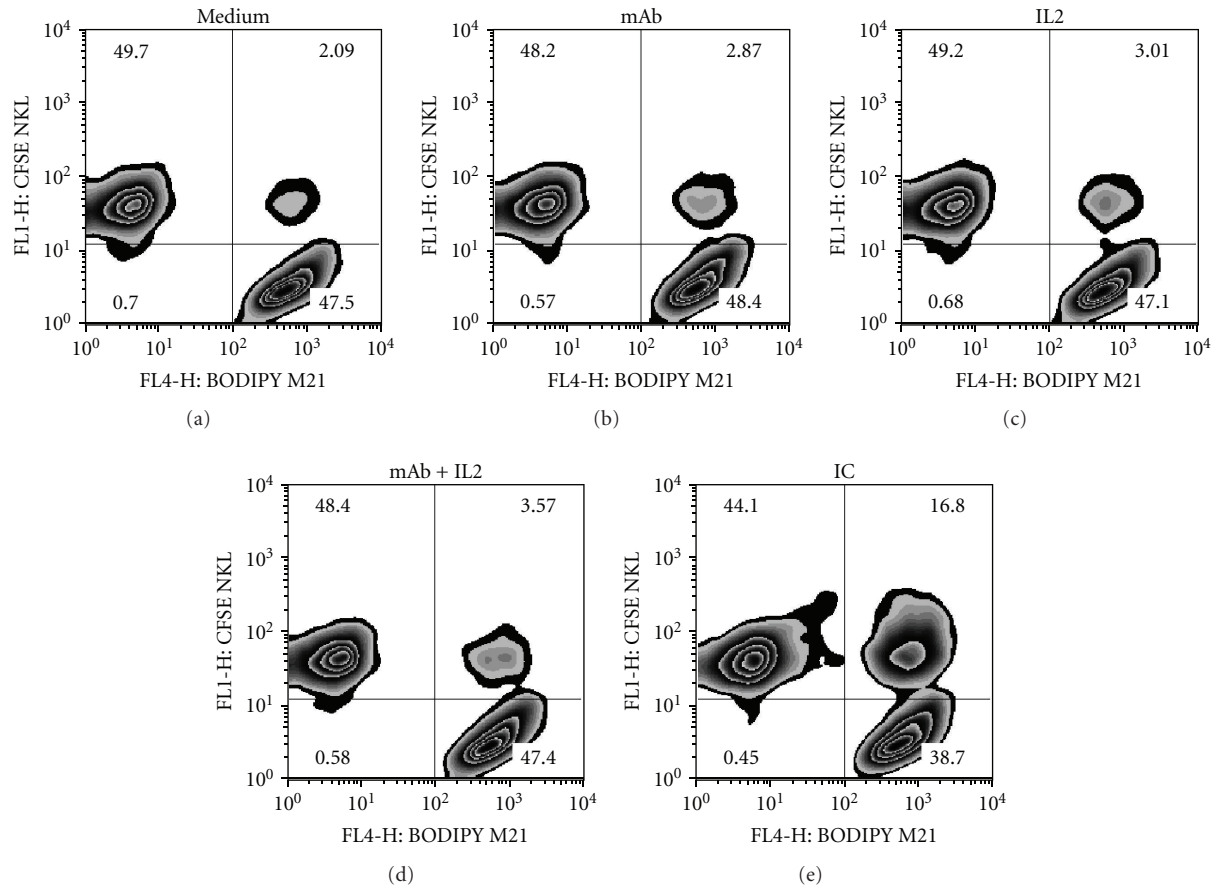
FIGURE 1: Immunocytokine facilitates adhesion of NK cells to tumor cells. This figure, adapted from Buhtoiarov et al. [36], shows the adhesion of M21 human melanoma tumor cells to NKL human NK cells after coincubation for 30 minutes. Tumor cells were prelabeled with CFSE, and NK cells were prelabeled with BODIPY and coincubated in medium alone, with hu14.18 (mAb) alone, soluble IL2, hu14.18 + soluble IL2, or hu14.18-IL2 immunocytokine (IC). The percentage of cells that had formed conjugates was defined as double positive cells as determined by flow cytometry.

with mAb remains to be answered. A recent study from our laboratory [12] evaluated patient samples from our COG phase II anti-GD2 IC trial for FcγRIIIa genotypes [40]. This study only had two patients with an FcγRIIIa$^{158v/v}$ genotype and was therefore inconclusive. The identification of FcγR polymorphisms that affect clinical responses to mAb therapy may suggest a new criterion for more tailored patient selection for mAb therapy. The importance of FcγRIIIa polymorphisms in IC-induced anti-tumor responses will be of considerable interest because of the involvement of the IL2R.

7. KIRs and the Response to mAbs and ICs

An important factor in NK-mediated therapy is the intrinsic ability for NK cells to respond to stimuli. The licensing hypothesis of NK cell function was developed after the observation that NK cells from mice lacking MHC-I, an important NK inhibitory ligand, respond poorly [50]. The intricate system of killer cell immunoglobulin-like receptors (KIRs) in humans and Ly-49 receptors in mice recognizes

normally expressed MHC-I antigens on neighboring cells and inhibit NK cell effector functions [51]. According to the licensing hypothesis, an NK cell that does not encounter a ligand for one of its KIR or Ly49 inhibitory receptors during development is functionally deficient [50]. However, KIR and Ly49 genes are inherited on different chromosomes from MHC, and therefore many individuals and mouse strains have at least one KIR or Ly49 that lacks a corresponding ligand [51]. These individuals and mouse strains have at least one population of functionally deficient NK cells [50], reflecting the NK population that contains the KIR receptor corresponding to the KIR/KIR-L mismatch. Clinical studies evaluating KIR/KIR-L matching have estimated that ∼60% of people have at least one KIR for which they lack a corresponding receptor and are therefore KIR/KIR-L mismatched for at least one locus [51].

Some preclinical studies have challenged the importance of licensing in certain models. Orr et al. evaluated the response of licensed versus unlicensed NK cells in response to murine cytomegalovirus (MCMV). C57Bl/6 mice have a "licensed" subset of NK cells expressing Ly49C/I, the receptor for H2^b and "unlicensed" NK cells expressing an

activating Ly49H. After infection of B6 mice with MCMV, a virus for which NK cell function is necessary for control, LY49C/I^{neg} Ly49H$^+$ ("unlicensed") NK cells were able to respond to infection [52]. This paper elegantly demonstrated that unlicensed NK cells still maintain some functionality to activating signals, although their effector responses are still less powerful than that of licensed NK cells.

New data suggest that "licensing" may not be a process restricted to NK cell development but is actually a continuous process that even mature NK cells use to respond to their environment [53, 54]. Recently, two separate reports compared adoptive transfer studies between wild type (WT) and β2m knockout (MHC-I deficient) mice to show that mature NK cells become hyporesponsive when they are put into an environment lacking MHC and vice versa [53, 54]. In these studies, only NK cells expressing an inhibitory Ly49 for which there was a cognate MHC-I ligand present could respond in the new surroundings. These studies suggest that "licensing" is a continuous (rather than absolute) mechanism that NK cells use to judge and appropriately respond to a changing environment.

While studies using murine models have been valuable in dissecting the potential factors affecting NK cell responsiveness, some observations of better anti-tumor responses by patients that are self-KIR/KIR-L mismatched have been made at multiple institutions [12, 55, 56]. Two studies evaluated anti-tumor responses in pediatric cancer patients following autologous stem cell transplant [55, 56]. These two independent studies both observed an association between disease-free survival and autologous KIR/KIR-L mismatch. Our laboratory recently evaluated the KIR/KIR-L status [12] of neuroblastoma patient samples from our COG phase II study of the anti-GD2-IC, hu14.18-IL2 [40]. In this study, we observed a better response to IC in patients that were KIR/KIR-L mismatched. These data suggest that the "unlicensed" NK cells in these patients were still involved in mediating the observed antitumor effect after IC treatment, consistent with some retained NK function by "unlicenced" NK cells. Previous reports have suggested that KIR-deficient NK cells have impaired responses to CD16-mediated stimulation [57]. In this study using NK cells from healthy donors, fewer KIR-deficient CD56^{dim} NK cells produced IFNγ or upregulated CD107a in response to either antibody-coated tumor cells or plate bound anti-CD16. However, our study using patient samples [12] and evaluating response to IC rather than naked mAb warrants a closer examination of NK cell "licensing" in the context of IC-mediated ADCC.

8. Conclusions

In the relatively short time since the first description of NK cells, just over 35 years ago [58], we have learned a great deal about their function. This knowledge has allowed us to design therapeutic strategies that utilize the powerful effector mechanisms of NK cells for multiple malignancies [6–8, 40]. Our understanding of NK cells, NK cell effector responses, and the signals that drive them, continues to expand. NK cells are capable of eliminating tumor cells coated with an IgG antibody in vitro and in some patients, and activation of NK cells with cytokines such as IL2 increases the anti-tumor effect [14, 15, 24, 46]. This approach has led to successful therapeutic mAbs for certain tumors but still do not elicit a response in all patients [38].

Our continued understanding of the factors that affect the response to Ab-coated tumor cells in the tumor environment is important for the creation of the next-generation mAbs and therapeutic strategies. Important intrinsic factors that affect NK cell responsiveness to Ab-coated tumor cells include the expressed variants of FcγRIIIa and the intrinsic ability of individual NK cell subsets to respond to stimuli due to their "licensing" status [12, 50]. Separate factors include the mass of tumor present when immunotherapy is given, the activation state of the effector cells mediating ADCC, and potential other receptor-ligand interactions that influence the synapse formed between NK and tumor cells. Next-generation immunocytokines may have a functional advantage over traditional mAbs in activation of certain NK cell subsets because of the contribution of cytokine receptors [33, 36, 37].

Acknowledgments

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