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The role of natural killer cells in cancer therapy

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1. ABSTRACT

Natural killer (NK) cells are innate immune cells that have long been known to be involved in the recognition and lysis of tumor cells. Despite significant gains in our understanding of the mechanisms that regulate NK cell function, the development of successful NK cell-based therapies has not yet been achieved. However, recent advances in our ability to modulate NK receptor signals and the sensitivity of tumor cells to NK cell-mediated lysis have led to a number of clinical trials testing novel methods to enhance NK cytotoxicity against cancer. Here, we present an overview of current therapies.

2. NATURAL KILLER CELLS

Natural killer (NK) cells are essential lymphocytes of the innate immune system, which are located in peripheral blood, lymphatics and tissues (1-3). They provide a first line of defense against tumors, viruses, certain bacterial and parasitic infections (4). The recognition of infected and tumor cells is governed by inhibitory and activating receptor-mediated signals (5, 6). Recent genomic analysis identified NKp46 as a unique marker expressed on NK cells of different species including humans, mice and rats (7). Traditionally, however, mouse NK cells have been defined as $CD3^-$ lymphocytes that

ither express NK1.1 or DX5 depending on the mouse strain (8). In humans NK cells comprise a $CD56^{+}CD3^{-}$ subpopulation of lymphocytes. Approximately 90% of peripheral human NK cells express low levels of CD56 ($CD56^{dim}$). The $CD56^{dim}$ population is characterized by highly effective cytotoxicity upon stimulation and their ability to mediate antibody-dependent cellular cytotoxicity due to their expression of the FcγR III (CD16) receptor. The remaining 10% of peripheral human NK cells express high levels of CD56 ($CD56^{bright}$). In response to activation signals, $CD56^{bright}$ NK cells secrete high levels of cytokines such as interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF), but do not efficiently lyse target cells. Here, we review current strategies that are based on our improved understanding of tumor recognition by NK cells and discuss novel approaches for future NK cell based immunotherapies of cancer.

2.1. Regulation of NK cell functions

The qualitative and quantitative response of NK cells depends on cytokines and interactions with other immune cells, such as T cells, dendritic cells (DCs) and macrophages (9). Human NK cell effector function is regulated by cytokines such as interleukin-2 (IL-2), IL-12, IL-15, IL-18, IL-21, IFN-α, IFN-β, TGF-β and Toll-like receptor (TLR) ligands (10, 11). IL-2 has long been used *in vitro* and *in vivo* to promote proliferation, cytotoxicity, and partially the cytokine secretion of NK cells (lymphokine activated killer cells - LAK cells), which are capable of lysing tumor cell lines (see table 1 for a summary of clinical trials) (4, 12-16). Infusion of LAK cells with concomitant injection of recombinant IL-2, however, only showed a response in 10-20% of the renal cancer patients in a clinical trial (17). Further increase in number of LAK cells or IL-2 proved to be ineffective in part because IL-2 promotes the expansion of regulatory T cells, which appear to suppress NK cell effector functions (18).

Injection of recombinant human IL-12 intratumorally resulted in measurable immunological response levels and tumor regression of human and murine melanoma (19-23). However, IL-12 showed dose-limiting toxicities at 500 ng/kg (24-26). Repeated administrations of IL-12 were also associated with persistently elevated plasma levels of IL-10, presumably due to IL-12-induced IL-10 production by T cells (19, 20, 27). Finally, administration of IL-12 transiently diminished NK cells numbers in the blood of patients, which may be caused by migration of NK cells from blood to lymph nodes, lungs, liver, and spleen (28).

The effectiveness of several other cytokines that regulate NK cell function has been assessed in clinical trials. IL-15 plays an important role in the development, survival and probably activation of NK cells (29, 30). Treatment of NK cells with IL-15 increases the expression of cell survival genes such as BcI-2, BcI-xL, survivin and NK-related effector molecules like perforin and granzyme B in splenic NK cells (31, 32). Administration of IL-15 in combination with chemotherapeutic agents such as cyclophosphamide enhances anti-tumor response in tumor-bearing mice (33, 34).

In a phase I clinical study, IL-18 infusion increased IFN-γ serum levels and FasL expression on NK cells (35). It remains to be determined if the unconfirmed partial antitumor responses observed in two patients correlated with NK cell activation.

IL-21 was shown to enhance tumor rejection through NKG2D-dependent mechanisms in mice (36). Administration of IL-21 in melanoma patients in phase I trials induced a dose dependent transient decrease in circulating NK cells and T cells, but enhanced the ability of NK cells to kill sensitive targets *ex vivo* and increased the perforin and granzyme B mRNA levels (37, 38).

Infusion of IFN-α resulted in 60 to 80% response in CML patients, which correlated with NK activity (39, 40). IFN-α and IFN-β (Type I interferons) have also been shown to be critical for tumor surveillance by NK cells *in vivo* (41). 3-methycholanthrene (MCA) treated type I IFN receptor deficient mice ($IFNAR1^{-/-}$ and $IFNAR2^{-/-}$) show increased susceptibility to fibrosarcoma formation (42). *In vivo*, type I IFN expression is induced by pathogen associated molecular patterns (PAMPs), uniquely expressed by certain microbes (43). Many PAMPs bind to TLRs, the intracellular RIG-I like helicases or NOD molecules expressed by most cells types (44). Upon binding of their respective ligands these receptors induce a signal cascade that activates transcription factors such as NF-κB and interferon-regulated factor 3 (IRF3), leading to the expression of type I IFNs and IL-18 (45). Interestingly, TLR2, TLR3 and TLR9 agonists can directly activate human NK cells in the presence of cytokines (46-48). Hence, human NK cells may contribute to the adjuvant effects of TLR agonists, which appear to be safe in triggering NK and T cell function in phase I clinical trials of lymphoma patients (49-52). TLR3 agonists have also been used to treat patients with the chronic fatigue syndrome, a disease associated with defects in NK cell functions (53, 54). Bacillus Calmette-Guerin immunotherapy (containing TLR2, 4, and 9 agonists) for superficial bladder cancer also depends on NK cell functions in mice (55). In summary, TLR agonists may have great potential in NK cell based therapies by inducing type I IFN expression and activating NK cells directly. Combination with other cytokines may further improve their efficacy.

2.2. Recognition of target cells by NK cells

NK cell activation is regulated by a balance between a variety of activating receptors such as NKp46, NKp44, NKp30 (collectively called NCRs), DNAM-1 and NKG2D, as well as inhibitory NK cell receptors (5, 6). Inhibitory receptors include members of at least three families of proteins: the lectin-like Ly49 family present in mice and rats, but not in humans; the killer cell immunoglobulin-like receptor (KIR) found in humans and other primates, but not in mice; and the CD94/NKG2A receptor shared by all species so far examined. Many of these inhibitory receptors expressed by NK cells are specific for major histocompatibility complex (MHC) class

Table 1. Summary of clinical trials that modulate NK cell function

Treatment	Phase	Patient #	Exp. Design	Cancer type	Response	Effect on NK cells	Ref.
Cytokines							
IL-2[1]	1, 2	23	i.v.[2] + BMT[3]	Breast cancer, NHL[4]	0%	ND[5]	12
IL-2	2, 3	270	i.v.	Mel[6]	16%	ND	14
IL-2	1, 2, 3	1712	i.v. or s.c.[7]	RCC[8]	15%	ND	15
IL-2	1	19	s.c.	Advanced cancer	ND	Expansion	16
IL-12	1	26	s.c.	RCC	ND	↓[9] in blood	19
IL-12	2	10	i.t.[10]	HNSCC[11]	ND	↓ in blood/↑[12] in LN[13] and tumors	20
IL-12	1	40	i.v.	Advanced cancer	ND	Cytotoxicity	22
IL-12	1	28	i.v. + IL-2	RCC, Mel	1 partial	ND	23
IL-12	1	14	i.v.	RCC, Mel	1 partial	ND	24
IL-12	1	40	i.v.	RCC, Mel, CC[14], PC[15], CerC[16], ACC[17]	2 partial	ND	26
IL-18	1	28	i.v.	RCC, Mel, HL[18]	2 partial	↓ in blood	35
IL-21	1	29	i.v.	Mel	1 CR[19]	Cytotoxicity	36
IL-21	1	72	i.v.	RCC, Mel	ND	↓ in blood, Cytotoxicity	38
IFN-alpha[20]		26	i.v.	CML[21]	60–80%	Cytotoxicity	39
IFN-alpha		26	i.v.	CML	58% remission	Cytotoxicity	40
TLR Agonists[22]							
CpG	1	23	i.v.	NHL	2	Cytotoxicity	50
Cell Therapy							
LAK cells[23]	1, 2	34	i.v. + IL-2 + BMT	Lymphoma, Breast cancer	0%	Cytotoxicity	12
HSC[24] (KIR mismatched)		120	IR[25], Thiotepa, Flu[26] or Cy[27], ATG[28]	AML	34% (6% Ctrl)	Alloreactivity, Cytotoxicity	77
HCT[29] (KIR mismatched)		130		ALL[30], AML[31], CML, MDS[32], NHL, HD[33], MM[34]	Survival 87% (48% Ctrl[35])	ND	78
HCT (KIR mismatched)		2026		AML, CML, MDS	0.54 relative risk	ND	79
HCT (KIR mismatched)		175		ALL, AML, CML, SAA[36], Leukemia, MD[37]	No response	ND	80
HCT (KIR mismatched)		1571		AML, CML, MDS	No response	ND	81
Haploidentical NK cells		43	Cy + Flu	AML, Mel, RCC, HD	5 CR (AML)	Expansion	82
NK-92	1	12	i.v.	RCC	1 minor	ND	83
Antibody Therapeutics							
Rituximab	1	43	+ IL-2 s.c.	B-cell NHL	53%	ADCC[38]	93
Rituximab	2	57	+ IL-2 s.c.	Indolent NHL	8.80%	ND	95
Rituximab	1	10	+ IL-2 i.v.+ LAK	B-cell NHL	1 partial, 4 SD[39]	ADCC	94
CD16/CD30 specific Ab[40]	1, 2	15	i.v.	HD	25%	Cytotoxicity	96
CD16/CD30 specific Ab	2	16	IL-2 + GM-CSF[41]	HD	29%	ADCC	97
Chemotherapy							
Ara-C[42]	3	320	s.c. + Histamine + IL-2	AML	40% (Ctrl 26%)	ND	13
Daunorubicin + Ara-C	1	32	i.v. + IL-2	AML	55% relapse free	Cytotoxicity	119
Flu	2	40	i.v. + Rituximab	Low-Grade or Follicular Lymphoma	80% complete	Transient, modest ↓	121

Abbreviations: IL, Interleukin[1]; i.v., Intravenous[2]; BMT, Bone Marrow Transplantation[3]; NHL, Non-Hodgkin's Lymphoma[4]; ND, Not Determined[5]; Mel., Melanoma[6]; s.c., Subcutaneous[7]; RCC, Renal Cell Carcinoma[8]; ↓, Decrease[9]; i.t., Intratumoral[10]; HNSCC, Head and Neck Squamous Cell Carcinoma[11]; ↑, Increase[12]; LN, Lymph node[13]; CC, Colon Cancer[14]; PC, Parotid Cancer[15]; CerC, Cervical Cancer[16]; ACC, Adenoid Cystic Cancer[17]; HL, Hodgkin's Lymphoma[18]; CR, Complete Response[19]; IFN, Interferon[20]; CML, Chronic Myeloid Leukemia[21]; TLR, Toll-like receptor[22]; LAK, Lymphokine Activated Killer[23]; HSC, Hematopoietic Stem Cell[24]; IR, Irradation[25]; Flu, Fludarabine[26]; Cy, Cyclophosphamide[27]; ATG, Antithymocyte Globulin[28]; HCT, Hematopoietic Cell Transplantation[29]; ALL, Acute Lymphoblastic Leukemia[30]; AML, Acute Myeloid Leukemia[31]; MDS, Myelodysplastic Syndrome[32]; HD, Hodgkin's Disease[33]; MM, Multiple Myeloma[34]; Ctrl, Control[35]; SAA, Severe aplastic anemias[36]; MD, Metabolic Disorder[37]; ADCC, Antibody-dependent Cell-mediated Cytotoxicity[38]; SD, Stable Disease[39]; Ab, Antibody[40]; GM-CSF, Granulocyte Macrophage-Colony Stimulating Factor[41]; Ara-C, Cytosine Arabinoside[42].

I molecules, which are expressed by most vertebrate cells and may protect normal cells from NK cell attacks. In addition, several inhibitory receptors exist that are specific for non-MHC class I ligands such as the NKR-P1B and NKR-P1D receptors, which bind to Clr-b/Ocil, a member of a distinct family of lectin-like cell surface glycoproteins (56, 57). Inhibitory receptors play a central role in 'missing self-recognition' by NK cells; the capacity of NK cells to attack cells that lose or downregulate expression of MHC class I molecules (58, 59). Lowered or absent MHC class I expression often occurs in tumor cells and infected cells, presumably as a means for these cells to evade an adaptive immune response. However, the ability of NK cells to lyse cells does not always correlate with MHC class I expression. These findings suggested the existence of activating receptors on NK cells whose engagement by tumor cell ligands is necessary to trigger NK-mediated cytotoxicity.

One of the best-characterized NK cell-activating receptor in the context of cancer is NKG2D (60-62). All NK cells constitutively express NKG2D. In humans its surface expression requires association with the adaptor protein DAP10. Engagement of NKG2D leads to cytokine secretion and cytotoxicity that is mediated via phosphatidylinositol-3-kinase and phospholipase C (63). NKG2D recognizes MHC class I chain-related (MIC) A and B proteins and UL16 binding proteins (ULBP) in humans and Rae1, H60 and Mult1 molecules in mice (60). NKG2D ligand expression has been observed on tumors of many origins, in particular in solid tumors, lymphomas and myeloid leukemia (64, 65). Although cellular ligands of NCRs on target cells have not yet been identified, blocking experiments suggest an important role for NCRs in the NK cell recognition of tumor cells (66). Recently DNAM-1 was shown to be critical for the ability of NK cells to recognize and lyse MCA and 7,12-dimethylbenzanthracene (DMBA) induced tumors (67). *In vitro* studies further suggest that DNAM-1 is also required for NK cell-mediated killing of tumor cells, such as neuroblastoma and myelomas, expressing the DNAM-1 ligands CD155 and CD112 (68, 69).

2.3. Blocking of inhibitory signals in NK cell therapy

A novel concept to enhance NK cell function is to block inhibitory NK receptors. Antibodies that block inhibitory receptors have been shown to enhance tumor rejection in preclinical models of leukemia and melanoma (70). Phase I clinical trials are currently investigating the effects of a humanized monoclonal antibody that blocks the interactions between KIR2DL1, 2, 3 inhibitory receptors and HLA-C ligands in patients with AML and multiple myeloma in remission after chemotherapy (71). Furthermore, blocking of KIRs enhances the NK cell-mediated lysis of B cell tumors by rituximab, an anti-CD20 antibody (72). Other attempts at reducing inhibitory signaling rely on proteosome inhibitors (f. e. Bortezomib), which reduce MHC expression on target cells (73). Proteosome inhibitors also sensitize tumor cells to NK cell-mediated lysis via upregulation of the TRAIL receptor and NKG2D ligands (73-75).

Another promising approach is to exploit NK alloreactivity in allogeneic transplantations. NK cell alloreactivity derives from a mismatch between inhibitory receptors for self-MHC class I molecules on donor NK cell subsets and the MHC class I ligands on recipient cells resulting in lack of KIR-mediated inhibition. Results from murine models and patients showed that NK cell alloreactivity could eliminate leukemic cells and reduce graft-versus-host disease (GVHD) thereby improving survival (76-79). Alloreactive NK cells also eradicated recipient DCs and T cells improving hematopoietic engraftment (76). However, some subsequent studies were not able to confirm the beneficial effects of allogeneic transplantation (80, 81). The discrepancies between these studies may partially be explained by differences in the degree of KIR mismatches, number of stem cell in the graft and the degree of T cell removal from the grafts.

The potential contribution of allogeneic NK cells to mediate graft versus leukemia effects has prompted clinical studies of adoptive allogeneic NK cell immunotherapy. Previous studies using autologous NK cells found no consistent efficacy in cancer patients when compared with cohorts of matched controls (12). Adoptive transfer of haploidentical NK cells in AML patients induced complete remission in 5 out of 19 patients (82). Remission depended on high dose chemotherapy prior to NK cell infusion. High-dose immunosuppressive regimen allowed for transient NK engraftment and *in vivo* expansion, which correlated with increased systemic levels of IL-15. Infusion of a human NK cell line NK-92, which lacks KIR receptors, in patients with advanced refractory renal cell cancer and melanoma has been shown to be safe in a phase I trial (83). The possibility of large-scale expansion and relative safety of administering allogeneic NK cells or NK cells that lack KIR receptors may enable NK cell-based therapy although their antitumor activity against different cancers remains to be determined.

2.4. Modification of positive signals in NK cell activation

A number of different approaches are used to enhance activating receptor signals. The low affinity Fcγ receptor IIIa, CD16, is unique among the activating receptors as engagement of CD16 is sufficient for lysis of target cells by both resting and IL-2-activated NK cells (84, 85). Thus CD16 allows NK cells to recognize and lyse antibody-coated tumor cells (86). This antibody-dependent cellular cytotoxicity (ADCC) has been suggested as a mechanism that may contribute at least in part to the efficacy of monoclonal antibody therapies against tumors, such as rituximab (anti-CD20) and trastuzumab (anti-Her2/Neu) (87-91). Responsiveness to rituximab in patients with non-Hodgkin's lymphoma correlates with polymorphisms in CD16 and CD32 (88, 92). Furthermore, it was shown that blocking the inhibitory receptor KIR3DL1 with an antibody enhanced the ADCC response of NK cells against a lymphoma cell line (72). Phase I clinical trials combining monoclonal antibody therapy with IL-2 or infusion of activated NK cell appeared to increase their efficiency (93, 94). However, some phase 2 trials have shown no beneficial effects (95-97). Different approaches are being tested to render ADCC by NK cells more efficient. The affinity of the Fc region for CD16 can be enhanced using protein-engineering approaches (89). A related therapeutic approach is the use of bispecific antibodies specific for CD16 on NK cells and tumor antigens such as CD20, CD19 or ERB2 (97-99). Hodgkin's lymphoma patients that were treated with bispecific antibodies against CD16 and CD30 showed some antitumor response (97). Finally, soluble proteins in which the constant region of human IgG1 was fused to the extracellular portion of an activating receptor, NKp30 have been shown to inhibit the growth of two different human prostate cancer cell lines *in vivo* (100).

Recent advances to genetically modify NK cells have been used to introduce transgenes for activating chimeric receptors into human NK cells. Human NK-92 cells which ectopically express a chimeric receptor consisting of a CD20-specific single-chain variable antibody fragment connected to the intracellular CD3ζ chain display markedly enhanced cytotoxicity against CD20 positive target cells, when compared to CD20

negative cells (101). Similarly, the transduction of *ex vivo* expanded NK cells with a chimeric anti-CD19-CD3ζ-4-1BB receptor dramatically enhanced their ability to kill CD19-expressing malignant B cells (102). This study also highlights the importance of the choice of the intracellular domain, as DAP10, an adaptor associated with several activating receptors, was less efficient in stimulating NK cells (102, 103). Careful testing of different intracellular domains may allow to further increase the effector functions of NK cells.

2.5. Induction of ligands for activating receptors

A similar approach is to specifically induce or increase the expression of ligands for activating receptors and thereby rendering tumor cells more susceptible to NK cell-mediated lysis. An attractive target for enhancing the therapeutic activity of NK cells against cancer is the NKG2D ligand system. A number of reports suggest that ectopic expression of NKG2D ligands in rare tumor cell lines that lack endogenous NKG2D ligands renders the cells sensitive to NK cell lysis *in vitro* and increases their immunogenicity *in vivo*. In some cases long-lasting T cell-mediated immunity against the tumor cells was observed (61, 104). In addition, administration of a DNA-vaccine encoding NKG2D ligands and tumor antigens, but not tumor antigens by themselves, induced immune responses that were able to eradicate established tumors (105). NKG2D has also been implicated in recent studies to be important in controlling the incidence and progression of cutaneous carcinogenesis and in surveillance of carcinogen-induced tumors (62, 106). In summary, a large body of evidence suggests a role for NKG2D-mediated immune activation in tumor rejection, but more experimental evidence is needed. Recent studies have provided a number of candidate approaches for optimizing NKG2D-dependent killing *in vivo*. Chemotherapeutic agents, radiation and histone deacetylase inhibitors have been reported to induce NKG2D ligand up-regulation on tumors, sensitizing them to NKG2D-dependent NK cell cytolysis *in vitro* (107-111). In addition, chemotherapy also upregulated the expression of PVR, a ligand for the activating receptor DNAM-1 on multiple myeloma cells (112). Our data indicate that *in vitro* NKG2D ligand and PVR induction is independent of p53, which is required for self-intrinsic apoptosis in response to chemotherapeutic agents (107, 113 and our unpublished observations). As p53 function is often disrupted in cancer, it is possible that increased sensitivity of treated cells to NK cell-mediated lysis accounts for some of the efficacy of chemotherapeutic drugs. A number of studies using mouse models have shown that low doses of some chemotherapeutic agents enhance anti-tumor immunity (114, 115). In a few cases it correlated with an increased NK cell activity (16, 116-118). In AML patients chemotherapy in combination with injection of low dose IL-2 increased NK cells and T cells numbers and enhanced cytolytic activity against leukemia cells (119). Studies using low dose chemotherapy suggest that it may be equal or even superior to high-dose chemotherapy, which is frequently immunosuppressive (120). Furthermore, chemotherapy may help antibody-based therapies although the role of NK cells has not been explored (121-123). It remains to be shown if the efficacy of chemotherapeutic agents partially relies on NK cells *in vivo*.

3. CONCLUSION AND PROSPECTS

The anti-tumor activity of NK cells has long been observed *in vitro* and *in vivo*. The molecular characterization of the inhibitory and activating receptor-ligand systems has allowed novel NK-cell based immunotherapeutic strategies against human cancer. A promising idea is to reduce inhibitory signals in NK cells by using haploidentical NK cells in adoptive transfer therapies, which suggest clinical anti-tumor effects without adverse side effects. Current efforts are focused on understanding which factors allow the survival, expansion and activation of adoptively transferred NK cells *in vivo*. Such strategies may also be used in combination with other treatments such anti-tumor antibodies, potentially leading to synergistic anti-tumor activities.

In tumors that express insufficient amounts of activating ligands combination therapies using antibodies targeting tumor antigens that bind with high affinity to FcγRIII, together with NK cell adjuvants are worthwhile options to be validated in clinical trials. Direct manipulation of activating ligand levels on tumor cells by chemotherapeutic agents may offer a new exciting possibility to render tumor cells more susceptible to NK cell-mediated killing. It will be important to carefully evaluate the different chemotherapy agents and the optimal dose to achieve ligand upregulation without negatively affecting NK cell activation. Combination of chemotherapy with subsequent adoptive transfer of NK cells may in part circumvent toxic effects of the chemotherapy on the patient's NK cells. It will be important to minimize the use of immunosuppressants, such as chemotherapeutic agents and steroids. The development of biomarkers to check the different NK cell functions and tumor susceptibility to NK cell throughout clinical trials will be an important issue that needs to be investigated to a greater extent in the future.

Finally, the development of immune evasion mechanisms during treatment needs to be taken into account and monitored. Recent reports suggest that tumors can evade immune attacks by controlling the NKG2D ligand expression. The activation of metalloproteinases leads to proteolytic shedding of the human NKG2D ligands MICA, MICB and ULBP2 by tumor cells, which correlates with a markedly reduced susceptibility to NKG2D-mediated cytotoxicity (124-128). Similarly, shedding of Fas by matrix metalloproteinases correlates with tumor progression in cancer patients (129-131). Tumor cells may also evade cytotoxic effector function of NK cells by upregulating the expression of proteinase inhibitor 9 (PI-9, SerpinB9), an intracellular granzyme B inhibitor (132, 133). In summary NK cells have great potential to play an important role in future therapies against certain human cancers, both alone and in combination with other therapies, however more efforts to translate our basic understanding of NK cell biology to the clinic are required.

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