# NK Cell-based Immunotherapies in Pediatric Oncology

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## Abstract

The past decade has seen several anti-cancer immunotherapeutic strategies transition from "promising preclinical models" to treatments with proven clinical activity or benefit. In 2013, the journal Science selected the field of Cancer Immunotherapy as the overall number-1 breakthrough for the year in all of scientific research. In the setting of cancer immunotherapy for adult malignancies, many of these immunotherapy strategies have relied on the cancer patient's endogenous anti-tumor T cell response. While much promising research in pediatric oncology is similarly focused on T cell reactivity, several pediatric malignancies themselves, or the chemo-radiotherapy used to achieve initial responses, can be associated with profound immune suppression, particularly of the T cell system. A separate component of the immune system, also able to mediate anti-tumor effects and less suppressed by conventional cancer treatment, is the NK cell system. In recent years, several distinct immunotherapeutic approaches that rely on the activity of NK cells have moved from preclinical development into clinical testing, and some have shown clear antitumor benefit. This review provides an overview of NK cell-based immunotherapy efforts that are directed towards childhood malignancies, with an emphasis on protocols that are already in clinical testing.

### Keywords

immunotherapy; natural killer cells; child; neoplasms

## Background

Natural Killer (NK) cells comprise approximately 10% of lymphocytes in normal humans[1,2] and their main function is to destroy virally infected, damaged, or transformed cells. NK cells also influence function of the adaptive immune system through the secretion of cytokines and chemokines that affect T cell function and dendritic cell (DC) maturation. Additionally, NK cell-mediated lysis of immature DCs selects for a more immunogenic subset of DCs during adaptive immune responses[3,4].

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In general, NK cells can be divided into two subsets, distinguished by density of CD56 surface expression (CD56 is an adhesion molecule known as Neural Cell Adhesion Molecule or NCAM). The CD56^{bright} subset is localized primarily in lymph nodes and secondary lymphoid tissue and constitutes only 10% of circulating NK cells. This subset is proliferative and secretes abundant amounts of cytokines in response to cytokine stimulation[5,6]. The CD56^{dim} subset is found predominantly in blood, bone marrow and spleen and comprises 90% of circulating NK cells. This subset: 1) expresses FcγRIIIa receptors (CD16) and mediates antibody-dependent cellular cytotoxicity (ADCC), 2) has limited proliferative capacity, and 3) secretes cytokines in response to target cell recognition[5,6]. Resting NK cells in the CD56^{dim} subset are more cytotoxic against NK-sensitive targets compared to CD56^{bright} NK cells. However, CD56^{bright} NK cells have similar cytotoxic capability after stimulation with Interleukin-2[7].

## NK Cell Functions

### Cytokine production

NK cells secrete the cytokines Interferon- γ (IFN-γ), Tumor Necrosis Factor-α (TNF-α), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Interleukin-10 (IL-10), Interleukin-13 (IL-13) and the chemokines Macrophage Inflammatory Protein-1α (MIP-1α), Macrophage Inflammatory Protein-1β (MIP-1β), and Regulated on Activation Normal T Cell Express and Secreted (RANTES) in response to stimulation[5,8]. NK cells in the CD56^{bright} subset require co-stimulation for cytokine secretion (usually Interleukin-12 in addition to another cytokine or the ligation of an activation receptor [e.g., natural killer group 2, member D, or NKG2D]) while those in the CD56^{dim} subset secrete cytokines in response to target cell recognition. Through the secretion of TNFα, GM-CSF, and IFN-γ, NK cells regulate DC maturation. The secretion of IFN-γ has a number of effects, including activation of macrophages, up-regulation of class I expression by antigen presenting cells (APC), Type 1 T-helper cell (Th1) polarization, and a direct antiproliferative effect on tumor cells. Thus, NK cells can directly inhibit tumor cell proliferation and augment the adaptive immune response to tumors via cytokine secretion.

### Cytotoxicity

NK cells are able to kill virally infected, damaged and transformed cells by two mechanisms (Figure 1). The first involves the secretion of lytic granule contents and destroys the target within hours. The second involves the interaction of death receptor ligands expressed on NK cells with surface death receptors on transformed or damaged cells, inducing apoptosis of the latter within a day. In the first instance, NK cell secretory lytic granules containing perforin and granzymes are released into the intercellular space between the NK cell and its target at a zone of cell-to-cell contact, called the immune synapse. The immune synapse (IS) is a highly organized supra-molecular structure comprised of adhesion molecules, receptors, signaling molecules, co-stimulatory ligands, and cytoskeletal elements. The initial step in formation of a functional IS involves close association between NK cell and target cell that results in initial signaling events, and in the absence of overriding inhibitory signaling, firm adhesion occurs. Active cytoskeletal processes result in reorganization of cellular elements at the point of contact. A synaptic "cleft" is formed at the IS between the two cells into

which cytolytic molecules are secreted. NK cell cytoskeletal reorganization results in the recruitment of lytic granules, that are distributed throughout the cell, toward the IS. Actin reorganization produces "conduits" within the NK cell cortex through which the lytic granules pass to accumulate at the synaptic cleft region of the IS. There, the lytic granules dock at the membrane with subsequent priming, fusion and secretion. Each intracellular event in the process, from initiation of IS formation to the directed release of lytic molecules, is highly regulated to enable NK cells to carefully direct their potent cytotoxic capability. The most well studied components of lytic granules are perforin and granzyme molecules. Perforin is a protein that forms pores in the target cell membrane allowing the entry of ions and small molecules. Cell death may ensue from the resultant disruption of osmotic equilibrium. Alternatively, target cell death can result from the action of granzymes that enter the cell through the perforin-induced pores or by endocytosis after granzyme-binding of mannose 6-phosphate receptors on the surface of the target cell. Interestingly, endocytosis in the target cell is increased due to cellular wound healing in the region of membrane disruption caused by perforin-induced pores. Granzymes entering the cell via endocytotic vesicles then gain entry to the cytoplasm via perforin-induced pores in the endocytotic vesicle membrane. Granzyme A induces cell death by indirectly generating single-stranded DNA nicks and Granzyme B produces rapid induction of caspase-dependent apoptosis[9].

The second mechanism by which NK cells eliminate target cells involves the interaction of tumor cell surface death receptors with death receptor ligands expressed on NK cells (e.g., TNF-related apoptosis-inducing ligand or TRAIL, Fas-ligand) to induce apoptosis. When Fas-ligand (FasL) or TRAIL on NK cells bind their respective receptors on target cells, apoptosis of the target cell ensues through the activation of caspases 8 and 9 of the extrinsic pathway[10,11]. It has been suggested[12,13] that NK cell-mediated lysis of tumor cells through ligation of death receptors may function without inhibitory KIR (killer immunoglobulin-like receptor) input. This may represent an important mechanism by which allogeneic NK cells may effectively kill tumor cells of a KIR/KIR ligand matched individual (see below).

### Effect of inhibitory and activating receptors

Whether an NK cell is activated to elicit effector functions (cytokine secretion or secretion of contents of lytic granules) depends on the balance of inhibitory and activating signals received through germ-line encoded, cell surface receptors (Figure 2). An important feature of the immune synapse is its role in integrating these signals through clustering of receptors and signal molecules at the point of cell-to-cell contact. Activating receptors include natural cytotoxicity receptors (e.g., NKp30, NKp44, NKp46), some C-type lectin-like receptors (e.g., NKG2D, NKG2C, NKG2E), CD2 family molecules (2B4, CRACC), receptors for nectin or nectin-like molecules (e.g., DNAM-1), several members of the KIR family, and Toll-like receptors 3 and 9 (figure 2)[14,15]. Another activating receptor on NK cells that is particularly important in the context of immunotherapy is FcγRIIIa (CD16). The CD56$^{dim}$ subset of NK cells possesses a high density of CD16, while the less mature CD56$^{bright}$ subset expresses limited CD16. The ligand for FcγRIIIa is the Fc domain of IgG antibody. Antibody bound to target cell surface antigens, via $F_v$ domains, results in a lattice of bound antibody molecules with exposed Fc domains that essentially "tags" the tumor cell for

destruction by NK cells by a process called antibody-dependent cellular cytotoxicity (ADCC).

Normal somatic cells do not generally express ligands for NK activating receptors but cells undergoing genotoxic or cellular stress, as occurs during transformation, do. For instance, many human cancer cells up-regulate the expression of a B7 gene family member designated B7-H6, a ligand for the NK cell activating receptor NKp30, while B7-H6 is absent from the surface of normal cells[16]. In addition, the ligands PVR (Poliovirus Receptor) and Nectin-2 that are present on some tumor cells, including freshly isolated neuroblastoma cells[17] and neuroblastoma cell lines[18], bind and activate DNAM-1 (DNAX Accessory Molecule 1) receptors on NK cells[19]. Moreover, many tumor cells express MICA and/or MICB (major histocompatibility complex class I-related chain glycoprotein A/B), ULBP-1 and/or ULBP-3 (UL-16 binding proteins 1/3), which are all ligands for the activating receptor, NKG2D[20,21]. In fact, tumors secrete NKG2D ligands as a form of NK cell immune evasion[22]. Interestingly, distinct signaling cascades are induced by different activating receptors in contrast to a common signaling pathway employed by most inhibitory receptors[14].

The most well studied NK cell inhibitory receptors are members of the KIR family and CD94/NKG2A. The ligands for these receptors are major histocompatibility class (MHC) I molecules (classical and non-classical, respectively) which are expressed on all nucleated cells and therefore, serve as an excellent means by which NK cells can distinguish cells that are "self" from "non-self". In fact, all NK cells that have been "licensed to kill" through the maturation process[23], express at least one inhibitory receptor that recognizes an MHC class I molecule[24,25]. The binding affinity of ligands for inhibitory receptors is generally greater than that for ligands of activating receptors[14]. In this way, NK cell-mediated destruction of "self" is usually avoided unless self cells have reduced expression of MHC class I molecules or substantially increased expression of ligands for NK cell activating receptors, both of which frequently occur in the process of transformation. Many tumor cells have diminished MHC class I expression, likely through selective pressure to avoid recognition by T cells. Many neuroblastomas, for instance, have been shown to express very low levels of MHC class I molecules[26–29].

When an NK cell is stimulated to kill a target cell, the target cell death that results is the sum of cytotoxicity induced by secretion of lytic granule contents and cytotoxicity induced through ligation of death receptors by NK cell surface TRAIL and FasL. In the absence of antibody, the NK cell-induced target cell lysis is called, "natural cytotoxicity" or antibody-independent cytotoxicity. For natural cytotoxicity, the cumulative integrated signal includes inputs from all NK cell receptors that are bound to target cell ligands at the IS except for FcγRIIIa receptors, since they are not bound to antibody. When antibody is bound to the target cell surface and NK cell FcγRIIIa receptors bind to the Fc domain, the resulting cytotoxicity is called ADCC. For ADCC, the cumulative integrated signal includes inputs from all NK cell receptors that are bound to target cell ligands at the IS including FcγRIIIa receptors. So in effect, the cytotoxicity in the presence of antibody is the sum of natural cytotoxicity (all receptors except FcγRIIIa receptors) plus the additional cytotoxicity resulting from stimulation of FcγRIIIa receptors. Interestingly, FcγRIIIa receptor ligation is

the only activating input that does not require additional co-stimulation to result in an activation signal in NK cells[30].

## Enhancing NK Cell Function with Cytokines, Drugs and Toll-like receptor Ligands

### Cytokines

Cytokines are secreted or membrane-bound molecular messengers that are produced by cells of the immune system to allow intercellular communication. Recombinant DNA manufacturing technology allows the production of sufficient quantities of these molecules for systemic administration for cancer immunotherapy. This section will focus on cytokines that influence NK cell proliferation, phenotype or function and thus, may produce anti-tumor responses, at least in part, through effects on NK cells.

**Interleukin-2**—Interleukin-2 (IL-2) is a well-studied gamma (c) cytokine that is FDA approved to treat renal cell carcinoma and melanoma. IL-2 stimulates the proliferation of NK cells, particularly the $CD56^{bright}$ subset, and promotes their functional maturation by inducing the expression of FcγRIIIa, NCRs (natural cytotoxicity receptors), NKG2D and production of perforin[31–33]. Thus, IL-2 renders this NK cell subset cytotoxic to NK-sensitive targets. Moreover, IL-2 activates $CD56^{dim}$ NK cells, augmenting the production of perforin[15] and granzymes[15,34], enhancing ADCC and antibody-independent (natural) cytotoxicity as well as IFN-γ production[15,35–43]. Activating receptors on this subset are also up-regulated by IL-2, including NCRs[44–46], NKG2D[15,33,44], and DNAM-1[44–46].

Although systemic IL-2 therapy has not demonstrated antitumor efficacy in pediatric trials as monotherapy[47–49], it augments ADCC when administered with therapeutic antibodies[50]. In fact, IL-2 administered with alternating cycles of GM-CSF plus the mAb, ch14.18, along with standard therapy (isotretinoin) increased event-free survival by 20% after 2 years compared to standard therapy alone in children with high-risk neuroblastoma[51]. An ongoing multi-center clinical trial in Europe and Israel that is nearing completion (NCT01701479) is evaluating the long-term continuous infusion of an anti-GD2 mAb, ch14.18/CHO, along with subcutaneous IL-2 in children with high-risk neuroblastoma. In addition, a single center phase I trial is currently evaluating the combination of the anti-GD2 mAb, hu-3F8, along with subcutaneously administered IL-2 in children with $GD2^+$ tumors (NCT01662804).

Monotherapy with inhaled IL-2 to treat pulmonary metastases of renal cell carcinoma was shown to be tolerable and controlled progressive disease for considerable periods of time[52]. There is an ongoing single institution, phase I/II trial that employs aerosolized IL-2 for the treatment of pulmonary metastases in individuals who are age 12 and older (NCT01590069).

IL-2 effectively activates NK cells *ex-vivo* for use in adoptive therapy regimens[53] and several ongoing clinical trials employ *ex-vivo* activated allogeneic NK cells to treat cancer, mostly in adults. Chemotherapy is typically administered prior to allogeneic cell infusion in order to induce a state of lymphodepletion, which is thought to promote expansion of the adoptively transferred cells. In the setting of lymphodepletion, low-dose IL-2 therapy following administration of allogeneic NK cells facilitates the *in vivo* expansion of donor

NK cells[39,53,54]. Successful, albeit transient, engraftment of donor NK cells using this approach has been demonstrated in adults[53] and children whose acute myeloid leukemia responded to allogeneic NK cell therapy[55]. A number of current clinical trials involve the administration of low dose IL-2 in conjunction with NK cell adoptive therapy.

**Interleukin-15**—Interleukin-15 (IL-15) is also a member of the gamma (c) cytokine family and shares the use of two receptor subunits, IL2Rβ and γc (an intermediate affinity heterodimer), with IL-2 although they each bind to a unique alpha subunit (creating unique high affinity heterotrimers). Since the cytokines share an intermediate affinity receptor, IL-2 and IL-15 have similar functions, as might be expected. Like interleukin-2, IL-15 enhances ADCC[56–64] and upregulates perforin[65,66] and granzyme B[67]. In addition, IL-15 induces maturation[67] and proliferation[56] of the CD56^{bright} NK cell subset and promotes NK cell survival through induction of Bcl-2[68].

IL-2 and IL-15 possess distinct roles as well, likely resulting from the differential distribution of their α-subunits, the distinct temporal and spatial patterns of expression of the two cytokines and the predominant mode of presentation of each cytokine. IL-15 is generally presented *in trans* by IL15Rα to neighboring cells bearing the IL2Rβ/γc receptor while IL-2 is usually presented by IL2Rα *in cis* to IL2Rβ/γc on the same cell via movements within microdomains of the extracellular membrane. IL-2 is involved in the elimination of self-reactive T cells by eliciting activation-induced cell death (AICD) and it promotes the activity and survival of regulatory T cells (Tregs), that function to regulate (or inhibit) immune responses to prevent autoimmunity. By contrast, IL-15 inhibits IL-2 induced AICD of CD8^{+} memory-phenotype T cells and does not activate Treg cells. Since IL-2 down-modulates the immune response by promoting AICD of T cells and by increasing Treg activity while IL-15 supports the maintenance of CD8^{+} memory T cells and does not have a significant effect on Treg cells, IL-15 may produce superior overall anti-tumor effects. IL-15 is currently being evaluated in phase I clinical trials in adults with solid tumors (NCT01727076) or with AML (NCT01385423) to determine safety and tolerability. An NCI sponsored phase I clinical trial (NCT01875601) is evaluating IL-15 in children and young adults with advanced solid tumors. Patients receive infusions of autologous NK cells that have been activated and expanded *ex vivo* followed by at least 12 doses of daily IL-15. A single institution clinical trial in Spain using IL-15 activated allogeneic NK cells in the transplant setting for pediatric refractory solid tumors was terminated to evaluate potential toxicity (NCT01337544).

**Alpha-interferon (IFNα)**—Alpha-interferon (IFNα) is a cytokine that likely exerts antitumor effects through a number of mechanisms involving cells of the innate (NK cells, DCs, macrophages) and adaptive (T cells) immune system. Although the antitumor mechanisms of action are not fully understood, IFNα has been shown to activate NK cells[69,70]. IFNα-2a is FDA approved for use in adults to treat melanoma, CML, hairy cell leukemia, and AIDS-related Kaposi's sarcoma. IFNα-2a is also approved as combination therapy with bevacizumab for metastatic renal cell carcinoma. In children, a limited number of studies have evaluated the use of IFN-alphas to treat cancer. Tolerability and feasibility of high dose IFN-α2a administered for 4 weeks followed by a lower maintenance dose for 48

weeks was demonstrated in children with stage III melanoma[71]. A phase II study of pegylated IFN-α2a in 32 children with diffuse intrinsic pontine glioma was recently reported to delay the time to progression without significantly improving 2-year survival[72].

A number of ongoing clinical trials are evaluating immunotherapy with IFN-α. A single institution phase I trial (NCT00855452) in Israel involves administration of low-dose IFN-α after allogeneic lymphocyte infusion in patients (12 years of age and older) with metastatic solid tumors. A phase II trial that incorporates pegylated IFN-α2b (NCT00539591) to treat children with high-risk melanoma is ongoing and pegylated IFN-α2b is being evaluated in the treatment of plexiform neurofibromas (NCT00678951). A combination of chemotherapy, pegylated IFN-α2b and surgery is being tested in a phase III COG trial to treat patients with osteosarcoma (NCT00134030) and a combination of IFN-α2b plus GM-CSF is being investigated to treat ALL, AML, blast phase CML and myelodysplastic syndrome in a phase I clinical trial (NCT00548847).

### Drugs

**Lenalidomide**—Lenalidomide, a structural analog of thalidomide, was shown to increase the percentage and absolute number of NK cells in a dose-dependent manner in children with solid tumors or myelodysplastic syndrome[73]. After 3 weeks of lenalidomide therapy in a dose finding phase I trial, NK cells from these subjects demonstrated elevated granzyme content and enhanced antibody independent cytotoxicity. Interestingly, the authors reported a decrease in the percentage of Treg in these patients as well. Since Treg diminish antitumor immune responses elicited by tumor specific CD8^{+} cells[74], tumor specific CD4^{+} cells[75], and NK cells[76], the authors suggest that lenalidomide-induced Treg inhibition may contribute to lenalidomide's potential antitumor activity. These results were in agreement with preclinical findings of several studies that evaluated the effect of lenalidomide or thalidomide on NK cell number and function[77–79].

**Bortezomib**—Bortezomib is an ubiquitin-proteasome pathway inhibitor that induces bcl-2 phosphorylation and is associated with G2-M phase cell cycle arrest and induction of apoptosis[80]. However, the mechanism(s) by which it elicits antitumor effects are not entirely clear. Bortezomib has been shown to upregulate surface expression of the death receptor TRAIL-R2 (DR5) on tumor cells sensitizing them to NK cell killing through the engagement of NK cell TRAIL[81,82]. Additionally, bortezomib decreases expression of MHC class I on tumor targets[83] rendering them more sensitive to NK cell lysis.

**b-AP15**—A novel proteasome deubiquitinating inhibitor, currently called b-AP15, was also shown to sensitize numerous tumor cell lines to killing by NK cells through the upregulation of TRAIL-R2 on tumor cells in vitro[84]. Interestingly, T cell recognition of targets is reduced for tumor cells treated with bortezomib due to altered proteasomal processing and presentation of antigens[81]. b-AP15 inhibits the deubiquitinating activity of a different proteasomal regulatory particle than does bortezomib leaving immunoproteasome processing and presentation of antigenic peptides to T cells intact. Thus, b-AP15 may enhance NK cell killing without attenuating antigen processing and T cell-mediated killing[84]. It is not yet being tested in clinical trials.

## Toll-Like Receptors

Toll-Like Receptors (TLRs) bind to molecules that possess highly conserved structural and molecular patterns associated with pathogens (e.g., double stranded DNA or lipopolysaccharide) or self-peptides released during cellular damage or death (alarmins). Identification of these self-peptides, known as damage associated molecular patterns or DAMPs, is an active area of investigation. TLR ligands can cause NK cell activation indirectly via binding of TLRs on other immune cells (e.g., DCs, macrophages) that become activated and secrete NK cell activating cytokines (e.g., IL-12, IL-18). Alternately, TLR ligands may produce direct NK cell activation through binding of intracellular and cell surface TLRs in and on NK cells. Sivori and colleagues demonstrated the presence of TLR3 and TLR9 in NK cells[85]. In the presence of cytokines, CpG and double-stranded RNA induce NK cell secretion of IFN-γ and TNF-α and enhance NK cell cytotoxicity of tumor cells. Several groups have reported the expression of TLR2 and TLR4 on NK cells[86,87] and TLR4 receptor binding on NK cells was associated with release of IFN-γ and TNF-α, upregulation of perforin and enhanced cytotoxicity[87]. The cell-wall skeleton of Bacillus Calmette-Guerin (BCG) is a TLR2 and TLR4 agonist that is FDA approved for the treatment of bladder cancer in adults. Preclinical studies showed that BCG enhanced NK cell antibody independent cytotoxicity and ADCC[88] and increased NK cell lysis of NK-resistant tumor targets[89]. A phase I clinical trial in children with GD2+ tumors that combined BCG treatment with the anti-idiotypic mAb A1G4 (NCT00003023) is now complete although results have not yet been reported.

## Enhancing NK Cell Function with Monoclonal Antibodies

Normal cells are resistant to NK cell lysis due to the presence of MHC class I molecule expression and the lack of activating ligands (Figure 2). In this situation, inhibitory signals predominate, NK cell natural cytotoxicity does not occur and the cell is considered "NK cell-resistant". As mentioned above, tumor cells frequently express activating ligands and reduced levels of MHC class I, which results in the tumor cell being sensitive to NK cell natural cytotoxicity. Unfortunately, in some instances, tumor cells are resistant to NK cell natural cytotoxicity due to inhibitory signals predominating over activating signals and NK cell natural cytotoxicity does not occur. However, if antibodies are bound to the target cell, as occurs when tumor specific antibodies are employed as cancer immunotherapy, then activating signals through NK FcγRIIIa receptor ligation may predominate and result in ADCC.

Selectively targeting tumor cells for destruction via ADCC elicited by immune effector cells (e.g., NK cells, monocytes, neutrophils) became possible through hybridoma technology and the development of monoclonal antibodies (mAb) for which Milstein and Kohler received the Nobel Prize in 1984[90]. Transformed cells frequently express abnormal molecules, or abnormal amounts of normal molecules on their cell surface, and monoclonal antibodies (mAb) can be generated to specifically bind antigenic determinants on these molecules. The ideal tumor antigen against which a therapeutic antibody is generated is one that is not expressed, or expressed in very limited amounts, by normal cells. An example of such an antibody is RAb[DMvIII91] that binds to the most common variant of the epidermal growth

factor receptor known as EGFRvIII that is exclusively expressed on certain malignant cells including some pediatric gliomas[92]. Alternatively, tumor antigens can serve as excellent targets if tumor cells are exposed to circulating mAb while normal cells (bearing the same antigen) are sequestered from circulating mAb. For example, anti-GD2 mAbs (e.g., 3F8, ch14.18, hu14.18) are specific for the disialoganglioside, GD2, which is expressed on cells of neuroectodermal origin. Expression of GD2 on normal tissues is generally restricted to cells that are protected from circulating mAb by the blood-brain-barrier or blood-nerve-barrier.

Many studies have shown that mAbs are capable of inducing ADCC of tumor cells by immune effector cells *in vitro*[93]. The importance of the antibody-FcR interaction for mAb antitumor efficacy *in vivo* has been demonstrated in pre-clinical models. Using murine xenograft models of HER2$^+$ human breast carcinoma and CD20$^+$ human B-cell lymphoma, Clynes et al.[94] showed that the antitumor effect of Herceptin and Rituximab®, respectively, were attenuated in FcγR knockout mice compared to wild-type. Similar results were obtained using an immunocompetent, syngeneic melanoma murine model[95]. Although mouse models lend strong support for the role of ADCC in the antitumor effect of mAbs, the most convincing evidence that activating FcRs play a role in the clinical response to therapeutic antibodies (likely through ADCC, but potentially also via augmented antigen presentation) comes from studies evaluating the relationship between certain activating FcγR polymorphisms and clinical benefit of mAb. A polymorphism of the activating FcγR, FcγRIIIa, exists at position 158 where the V/V (homozygous for valine at position 158) genotype results in an FcγRIIIa that binds human IgG1 antibody with high affinity. The F/F (homozygous for phenylalanine at position 158) and V/F genotypes result in a low and intermediate affinity FcγRIIIa, respectively. In non-Hodgkin's Lymphoma, Rituximab® was most effective in those patients with the 158 V/V genotype and thus, those patients with the high affinity FcRγIIIa[96]. Likewise, HER2$^+$ metastatic breast cancer patients treated with Trastazumab[97] and metastatic colorectal cancer patients treated with Cetuximab[98] showed greater response to therapy if they had the 158 V/V genotype. If ADCC plays no role in the therapeutic efficacy of these antibodies, then polymorphisms of FcγRIIIa would not be expected to be associated with clinical outcome. Moreover, antitumor efficacy of antibodies induced by immunization to idiotypic antigens on B cell tumors[99] and antigens on colon cancer[100] are associated with possession of the genotypes for high affinity FcγRIIa and FcγRIIIa. However, some have argued that higher affinity FcRs might provide clinical benefit via enhanced antigen processing which may augment an adaptive immune response, rather than facilitating ADCC and this issue has not been resolved.

Therapeutic mAbs can be engineered in a number of ways to increase their utility for clinically relevant NK-mediated ADCC. First, they can be engineered to be less foreign to the human immune system, thus attenuating the development of anti-antibodies that may neutralize the therapeutic mAb. The constant region of a human immunoglobulin gene can be grafted to the variable region of mouse immunoglobulin genes to create a chimeric mAb (e.g., ch14.18; ~80% human). Alternately, a humanized antibody can be created by grafting the nucleotide sequence for the complementarity-determining region (CDR) of a murine antibody to the appropriate CDR location of a human immunoglobulin gene (e.g., hu14.18;

~98% human). Additional modifications of therapeutic mAbs include modified glycosylation of Fc-linked oligosaccharides to enhance ADCC (e.g., hu14.18K322A) and amino acid substitution in the heavy chain constant region to alter the binding affinity of mAb to complement (e.g., hu14.18K322A).

Therapeutic mAbs can also be conjugated to cytokines (e.g., ch14.18-IL2, hu14.18-IL2) by fusing the human gene for the cytokine to the mAb gene. By linking cytokine directly to the tumor specific mAb, the immunomodulatory effect of the cytokine should be more localized to the tumor microenvironment. For example, ch14.18-IL2 has two functional molecules of interleukin-2 attached to the Fc terminal end of the ch14.18 mAb. Preclinical studies showed that this fusion protein (or immunocytokine) had far superior antitumor activity when compared to comparable doses of ch14.18 mAb and IL-2 given simultaneously as individual agents[101]. A study evaluating immunocytokine-facilitated conjugate formation between NK cells and tumor cells demonstrated that tumor cells coated with immunocytokine were bound to NK cells not only through the Fc region of the immunocytokine but also through the IL-2 moiety[102,103]. NK cells bound to tumor cells formed activated immune synapses, defined by the localization of LFA-1 and CD2, along with clustering of NK cell CD25 into the synapse. In fact, in the presence of immunocytokine, NK cells with minimal or no FcγRIIIa were able to form conjugates with tumor cells demonstrating an important role for IL2Rα. Thus, immunocytokine therapy may: 1) target NK cells to tumor via tumor antigen-mAb moiety-FcγIIIa interactions, 2) deliver immune-modulatory cytokine directly to the tumor microenvironment, and 3) participate in immune synapse formation via the IL2 component of the immunocytokine bound by the IL2 receptors on the NK cells (Fig. 3).

A recent approach to augment ADCC employing the use of monoclonal antibodies involves a separate means to activate NK cells *in vivo*. When NK cells become activated following engagement of their FcRs by tumor bound mAb, they up-regulate expression of cell surface CD137 molecules, which are activating receptors. Provision of an agonistic anti-CD137 mAb at this critical time, *in vivo*, can augment ADCC capabilities and function of the NK cells with improved *in vivo* anti-tumor effects in tumor bearing mice[104]. This approach is now being pursued clinically in studies of Rituximab® for lymphoma[104,105].

Table 1 shows open or recently completed clinical trials employing mAb therapy for pediatric cancers that may involve NK-mediated mechanisms. Excellent recent reviews that include all mAb therapy for pediatric cancers are available[106–108]

## Antitumor effects of NK cells in Allogeneic HSCT

The rationale for using allogeneic hematopoietic stem cell transplant (HSCT) in an attempt to improve survival for individuals with recurrent or refractory hematologic malignancies is two-fold. First, stem cell rescue provides the opportunity to use high doses of chemotherapy and radiation that may potentially overcome drug resistance but are likely to produce severe myelosuppression. Second, donor immune cells can react to and destroy recipient tumor cells producing a "graft versus leukemia" (GVL) effect. Unfortunately, donor immune cells may also attack normal recipient tissues resulting in graft versus host disease (GVHD). Initial pediatric allogeneic HSCTs were generally performed in patients who had a human

leukocyte antigen (HLA)-matched sibling donor. The frequent lack of a suitable HLA-matched donor led to the use of parents as a donor. Most parents share only one HLA haplotype with their child (haploidentical) and this degree of HLA mismatch would be expected to cause severe GVHD. However, since T lymphocytes are predominantly responsible for eliciting GVHD, the depletion of T cells from haploidentical grafts reduces the incidence and severity of this untoward effect. Although T lymphocytes were known to play a significant role in mediating the GVL effect of allogeneic HSCT, T-depleted grafts were still associated with an anti-leukemic effect[109]. This antitumor activity was mediated, in large part, by NK cell effector mechanisms. Interestingly, although NK cells are able to elicit a GVL (or graft versus tumor) effect, they do not appear to cause GVHD.

Because the genes for HLA and KIR are on different chromosomes and they sort independently, matching for HLA genes between donor and recipient does not result in matched KIR genes. In fact, ~50–75% of transplants from HLA identical sibling donors will be KIR/KIR ligand mismatched. The probability of KIR/KIR ligand mismatch in the haploidentical transplant setting is similarly 50–75%. A pivotal study by Ruggeri and colleagues[110] clearly demonstrated the effectiveness of KIR-mismatched donor NK cells, predicted to be alloreactive, to mediate a graft-versus-leukemia effect in adults with acute myelogenous leukemia (AML). Using haploidentical HSCT, they showed that susceptibility of tumor cells to NK cell cytotoxicity correlated with the predicted existence of a mismatch between KIR receptors expressed by donor NK cells and MHC class I alleles expressed by the recipient. Another study in the setting of haploidentical stem cell transplant showed that NK cell alloreactivity appeared to be a major factor influencing the risk of relapse for pediatric patients with both acute lymphoblastic leukemia (ALL) and AML suggesting that a GVL effect, mediated by allogeneic NK cells, was not limited to patients with AML[111]. A recent study in children with high-risk leukemias who received haploidentical HSCT showed that donor-derived alloreactive NK cells are generated and persist for many years in the transplanted recipients[112].

Interestingly, when NK cell reconstitution is monitored following haploidentical HSCT using $CD34^+$-selected cells, it appears that immature, poorly functioning $KIR^-NKG2A^+$ NK cells predominate in the early transplant period[112,113]. Thus, allogeneic HSCT using $CD34^+$ selected grafts may have limited GVL effect in the immediate post-transplant period due to the lack of mature donor NK cells in the graft. This finding provides a rationale for graft processing methods that selectively remove unwanted GVHD-inducing cells (α/β T cells) and thus, leave desirable GVL effector cells in the graft (e.g., NK cells, γ/δ T cells)[114]. Alternatively, mature donor NK cells can be adoptively transferred during the post-transplant period. A number of ongoing clinical trials are using haploidentical HSCT, with and without donor NK cell adoptive therapy, for the treatment of both hematologic and solid tumor malignancies (Table 2).

The influence of NK cell alloreactivity on post-transplant survival is not limited to the setting of haploidentical HSCT, but has also been demonstrated in patients receiving HLA matched sibling or unrelated donor grafts. Hsu et al. documented a significant improvement in disease-free survival among AML and MDS patients who were KIR-KIR ligand mismatched with their HLA matched sibling donors[115]. This survival benefit was not

observed for patients with ALL or CML, suggesting that the leukemias that benefit from donor NK cell alloreactivity depends on multiple variables. First, it should be noted that HSCT therapy in various studies is dissimilar with respect to: 1) conditioning regimen 2) graft composition and dose 3) donor type, 4) cell source (peripheral blood, bone marrow, umbilical cord blood), and 5) use of post-transplant immunosuppression. These disparate HSCT processes will affect NK cell recovery, proliferation, maturation, and activation in the post-transplant period. Second, other families of inhibitory receptors on NK cells besides inhibitory KIRs may play a role in the anti-leukemia effect of NK cells. Miller and colleagues[116] have shown that families of inhibitory receptors (e.g., NKG2A, LIR-1) present on both KIR+ and KIR– NK cells are involved in leukemia cell killing. They assert that blockade of multiple MHC class I-recognizing NK cell receptors may overcome ALL blast resistance to NK alloreactivity. Moreover, while most investigators have focused on the influence exerted by donor inhibitory KIRs, Cooley et al. found that donor activating KIRs influenced survival in AML patients following unrelated donor transplantation[117]. Patients transplanted from donors possessing the B KIR haplotype were found to have a 30% improvement in the relative risk of relapse-free survival compared to donors lacking this genotype. The B KIR haplotype is associated with a greater number of activating KIR genes.

## Adoptive therapy with NK cells

In addition to utilizing the antitumor potential of adoptive NK cell therapy during the post-allogeneic hematopoietic transplant period, allogeneic NK cells have been successfully administered in the non-transplant setting. Patients are initially treated with lymphodepletive chemotherapy to avoid immediate rejection of allogeneic NK cells and to decrease competition for endogenous cytokines. The efficacy of this approach was first demonstrated by Miller and colleagues, who administered *ex-vivo* IL-2 stimulated, allogeneic NK cells to lymphodepleted patients along with IL-2 therapy[53]. In this study, 75% of patients who were KIR/KIR ligand mismatched (in the graft-versus-host direction) achieved complete remission while only 13% of patients achieved complete remission in the group without KIR/KIR ligand mismatch status. Another trial to treat AML in adults involved the infusion of unstimulated, KIR/KIR ligand mismatched haploidentical donor NK cells along with IL-2 administration. The complete response rate in this elderly adult cohort was encouraging[118]. Patients with solid tumors, for example ovarian or breast cancer, have also been treated with allogeneic NK cells in the non-transplant setting[119]. A study in adults with advanced renal cell carcinoma or melanoma that involved the infusion of IL-2 stimulated, haploidentical donor NK cells along with IL-2 administration showed disease stabilization when measured six weeks following the infusion[53]. Iliopoulou et al., treated adult patients with advanced non-small cell lung cancer with repetitive infusions of donor NK cells in combination with chemotherapy and showed disease stabilization or a partial response in half of the patients. In this study, only one donor was reported to be KIR/KIR ligand mismatched to the recipient[120]. A recent report described this approach in children. Rubnitz and colleagues[55] administered non-activated, haploidentical donor NK cells, followed by IL-2 administration, to children with AML (most with favorable risk disease) and reported that all patients remained in remission for at least 2 years following adoptive NK cell therapy. The donor NK cells were KIR/KIR ligand mismatched in 9/10 of these patients. There are several open

pediatric clinical trials employing adoptive NK cell therapy in the non-transplant setting (Table 2).

## Role of Autologous KIR/KIR ligand Mismatch

The genes for KIRs and MHC class I are located on different chromosomes and they segregate independently in human pedigrees[121]. Thus, approximately 60% of individuals have KIRs for which they have no corresponding MHC class I; these individuals are considered to be autologous, or "self-" KIR/KIR ligand mismatched. During the maturation process, an NK cell must express at least one inhibitory KIR that recognizes an MHC class I molecule to be "licensed to kill"[23]. NK cells that do not encounter an MHC class I ligand for one of its KIRs during development are unlicensed and functionally less potent than "licensed" NK cells, yet they are still somewhat reactive. Recent studies suggest that licensing may actually be a continual process by which NK cells respond to their environment[122].

A study evaluating the relationship between KIR/KIR ligand mismatch and clinical outcome following autologous HSCT in children with solid tumors and lymphoma showed that relapse was significantly reduced in individuals with self-KIR/KIR ligand mismatch[123]. A similar study in high-risk neuroblastoma patients who had undergone autologous HSCT found that self-KIR/KIR ligand mismatch was associated with improved disease-free survival[124]. Another study in children with neuroblastoma who were treated with hu14.18-IL2 reported a better response to therapy in those that were self-KIR/KIR ligand mismatched[125]. These studies support the notion that unlicensed, hyporesponsive NK cells (self-KIR/KIR ligand mismatched) may gain potent effector function in the proper milieu that is provided in the setting of HSCT and subsequent immune reconstitution. The selection of appropriately KIR/KIR-ligand mismatched donors for allogeneic cell therapy, and selection of autologous KIR/KIR-ligand mismatched patients for NK based forms of immunotherapy (such as hu14.18-IL2 therapy) seems to be supported by the associations of improved outcome or responses for KIR/KIR-ligand mismatched individuals. One way to potentially circumvent the apparent disadvantage of a KIR/KIR-ligand matched setting may be the additional administration of a mAb that binds to and blocks inhibitory signals transmitted by inhibitory KIRs[126]. Preclinical models indicate that blocking inhibitory receptors can cause a potent NK cell mediated anti-tumor effect[127].

## Adoptive NK cell therapy using expanded, activated NK Cells

A limitation of NK cell adoptive therapy is the relatively small number of NK cells that can be obtained from apheresis of a healthy adult. In order to increase the number of NK cells available for adoptive therapy, several new protocols have been developed to expand NK cells *ex-vivo*. Co-culturing donor NK cells with irradiated "feeder" cells (that present activating antigens to NK cells) substantially increases NK cell numbers and their activity compared to simple IL-2 cytokine stimulation. Several of these feeder cell lines have been developed to expand donor NK cells and are currently in use. The first feeder cell line, EBV-TM-LCL (Epstein-Barr virus-transformed B-cell line), was reported to expand NK cells nearly 500-fold in 3 weeks[128]. Several different feeder cell lines are genetic

modifications of the myelogenous leukemia cell line, K562, that have been induced to express different molecules on the cell surface. The K562-MICA-41BBL-mbIL15 feeder cell line was shown to expand NK cells 550-fold in about 3 weeks[129] and the K562-mb15–41BBL feeder cell line expanded NK cells more than 275-fold in the same period of time[130]. An additional feeder cell line that has reported dramatic NK cell expansion in 3 weeks (median of > 21,000-fold) is K562-Clone9-mbIL21[131].

NK cells that are activated *ex vivo* by these feeder cell methodologies show increased expression of activation receptors, including the activating receptors, DNAM-1, NKp46, NKp44, NKp30[132–135] and NKG2D[130,132,133,135]. By up-regulating the density of activating surface receptors on the NK cell, the balance of signals at the NK cell interface with a ligand-expressing tumor cell may be "tipped" toward activation. Therefore, some authors have proposed that the rule of KIR mismatch do not apply to *ex vivo* expanded NK cells due to the high degree of activation[135]. Up-regulation of chemokine receptors, CXCR3, CXCR4 and CXCR6, has also been reported[132,135], which may enhance NK localization to the tumor microenvironment. In addition, activated, expanded NK cells show increased expression of adhesion molecules CD54 and CD56[132,135] which may enhance adherence to target cells, the first stage of immune synapse formation. Activated, expanded NK cells have also shown potent *in vitro* cytotoxicity against numerous tumor cell types[130,132–136]. Despite the high cytotoxicity against tumor cells demonstrated by these NK cells, they have been shown to spare healthy cells[130,132,134].

In light of the finding that approximately 60% of individuals are self-KIR/KIR ligand mismatched, the notion of adoptive therapy using expanded, highly activated autologous NK cells is particularly attractive. In fact, Liu and colleagues[131] have demonstrated that NK cells from children with neuroblastoma can be effectively expanded and activated using the irradiated K562-derived Clone9.mbIL21 feeder cell line. These cells were shown to possess potent anti-neuroblastoma activity, even following cryopreservation. The authors propose that clinical testing using autologous expanded and activated NK cells combined with ch14.18 in the minimal disease setting is warranted.

Four ongoing adult clinical trials use one of the aforementioned methodologies to expand and activate either autologous or allogeneic NK cells prior to NK cell infusion and two additional adult trials are registered but not yet open to accrual. A pediatric trial has recently been completed that involved the expansion and activation of haploidentical allogeneic NK cells *ex vivo* prior to adoptive therapy (NCT00640796).

## NK cell CARs (Chimeric Antigen Receptors)

A first generation CAR is a genetically engineered "hybrid" receptor comprised of an extracellular single-chain antibody fragment, or scFv, (that recognizes the tumor associated antigen) linked by a transmembrane domain to an intracellular signaling moiety, usually CD3ζ. Second and third generation CARs also incorporate one or two costimulatory activating motifs (e.g., 2B4, CD28, 4-1BB, OX-40), respectively, into the intracellular segment of the CAR. The most common approach to introduce CARs into effector cells uses γ retroviruses that stably integrate the genetic sequence for the receptor into the target cell

genome[137,138]. Alternate approaches use lentiviral transduction, direct RNA transfection and transposon-based systems.

The incorporation of tumor specific CARs into NK cells provides the opportunity for NK cells to directly bind a specific antigen on tumor cells. Receptor binding is agonistic in nature and an activation signaling cascade ensues. The nature of the immune synapse formed has not been explored but it appears that the activation signal resulting from CAR activation may be sufficient to override inhibitory receptor signals[139–141]. Several *in vitro* studies demonstrate markedly enhanced cytotoxicity of tumor cells by NK cells bearing CARs specific for tumor antigen[140,142,143]. A single institution, phase I trial (NCT00995137) in children with B-cell ALL is investigating the safety of NK cells bearing CD19-specific CARs. Donor NK cells are first expanded by co-culture with feeder cells (K562-41BBL-mbIL15) and then transduced to express the signaling CAR, anti-CD19-BB-zeta. Preclinical studies are exploring the antitumor activity of expanded cord blood NK cells bearing CD20-specific CARs[143].

## Summary

A number of immunotherapy strategies employ the effector functions of NK cells to kill malignant cells. NK cells can be activated by cytokines or specific TLR ligands, enhancing their proliferative capacity, survival and effector functions (cytotoxicity and secretion of cytokines and chemokines). In addition, select drugs can enhance the expression of proteins on the surface of tumor cells that serve as ligands for activating receptors on NK cells. Moreover, tumor cells can be targeted for destruction by NK cells using tumor specific mAbs. NK cells are also involved in the success of both autologous and allogeneic HSCT in treating malignancies and evidence supports a role for KIR/KIR ligand mismatch in the associated antitumor effect. Adoptive therapy with allogeneic NK cells, in the setting of transplant or lymphodepletion, has demonstrated some success, particularly in hematologic malignancies. An exciting new means by which to exploit the potent tumor killing potential of natural killer cells is through the introduction of tumor specific CARs into NK cells using genetic engineering. Finally, new methodologies that allow the production of large numbers of highly active NK cells may provide an avenue to utilize autologous, allogeneic, and tumor specific CAR possessing NK cells for cancer immunotherapy. Numerous clinical trials are ongoing to explore several possible therapeutic uses of NK cells to treat pediatric cancer. Some of these utilize technology that requires highly specialized clinical support/ production laboratories (i.e., *ex vivo* expansion of NK cells for infusion, or *ex vivo* transfection with CARs), while others involve approaches that are "off the shelf" and readily administered in standard pediatric oncology clinics or inpatient units (i.e., infusions of NK-activating cytokines, or use of mAbs that facilitate NK induced ADCC). Many of these approaches are already showing clear evidence of antitumor activity or clinical benefit. This next decade will involve efforts of the pediatric oncology community to integrate these forms of NK based immunotherapy (along with other forms of immunotherapy) into the conventional treatment for childhood malignancies. The goal, as always, will be to improve disease free survival while minimizing acute and long-term toxicities from treatment.

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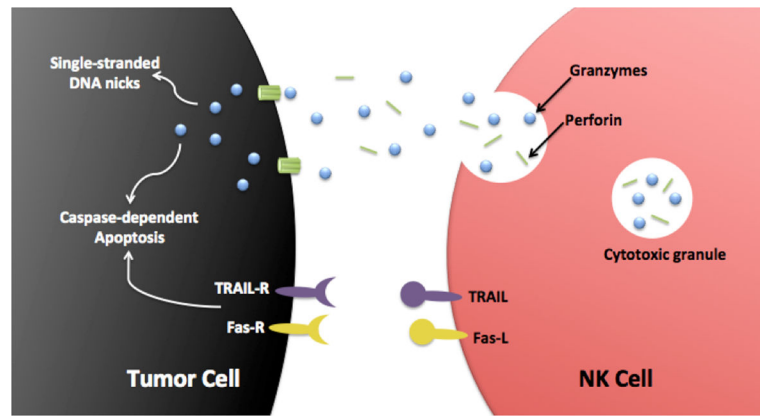
**Figure 1.**
Two major mechanisms by which NK cells kill tumor cells: NK cells can use the perforin/
granzyme containing granule exocytosis pathway or the TRAIL/Fas-L death-receptor
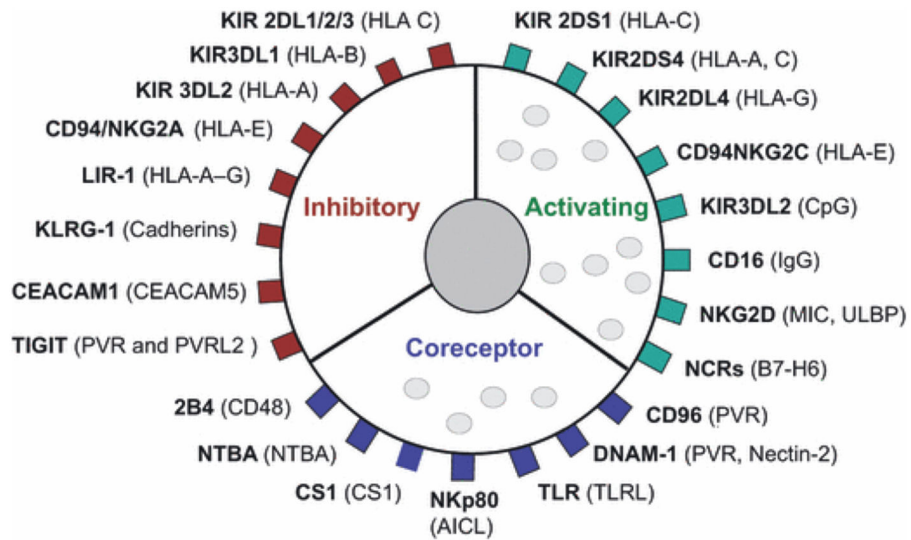pathway.

**Figure 2.**
NK cell surface receptors and their ligands. Receptors are broadly classified based on their primary function (inhibitory receptors, activating receptors, and activating co-receptors). Known ligands are denoted in parentheses. Despite the multitude of receptors shown, other families of receptors are not illustrated, including cytokine receptors (e.g., IL-1, IL-2, IL-12, IL-15, IL-18, IL-21, IFNα), chemotactic receptors (CCR2, CCR5, CXCR1, CXCR3, CXCR4, CXCR6, CX3CR1 and Chem23R), adhesion receptors (CD2 and β1 and β2 integrins), and inhibitory co-receptors (CD300A, LAIR-1 and Siglec7). Adapted with permission from: Wing Leung, *Use of NK cell activity in cure by transplant,* British Journal of Haematology 155, 14–29, 2011, Blackwell Publishing Ltd.
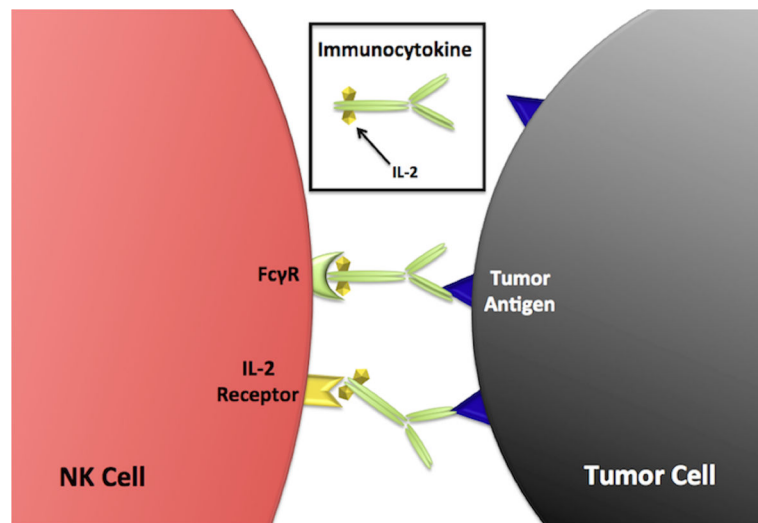
**Figure 3.**
Schematic representation of immunocytokine-facilitated tumor cell-NK cell conjugate formation. Tumor cells coated with immunocytokine bind NK cells via interaction between 1) Fc region of the immunocytokine and the NK cell FcγR, and 2) IL-2 moiety of immunocytokine and the NK cell IL-2 receptor. Bound FcγRs and IL-2Rs transmit activation signals leading to enhanced NK cell function.
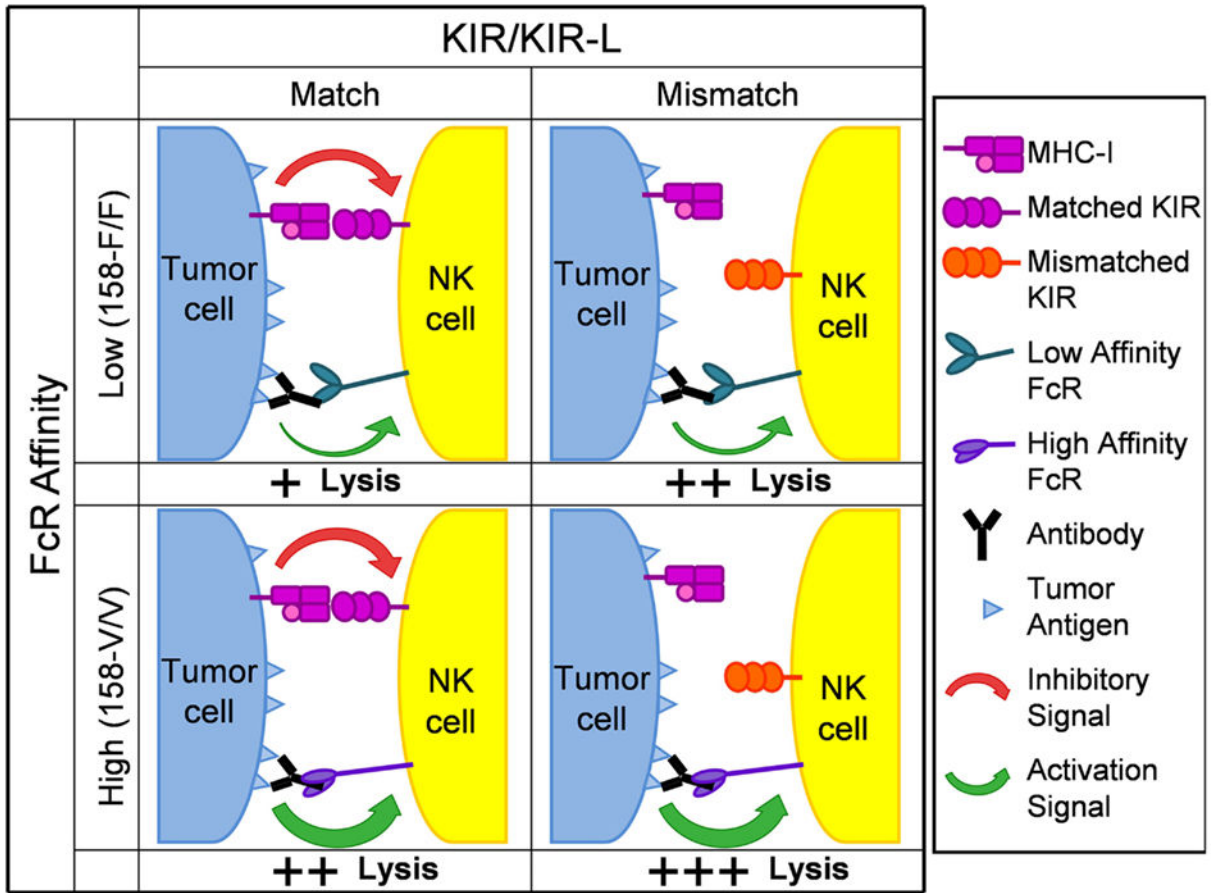
**Figure 4. Impact of KIR/KIR-Ligand relationship and FcγR genotype on tumor cell lysis following mAb treatment**

KIR molecules are depicted as matched (left boxes) or mismatched (right boxes) for the corresponding MHC class I molecule on the tumor cell. The NK cell FcγIIIa receptors (FcR) are depicted as lowest affinity (homozygous for phenylalanine at position 158, upper two boxes) or highest affinity (homozygous for valine at position 158, lower two boxes). This simplified example demonstrates the interplay of inhibitory and activating signals that are integrated within the NK cell to produce a lytic response. Adapted with permission from: Koehn TA *et al.*, *Increasing the clinical efficacy of NK and antibody-mediated cancer immunotherapy: potential predictors of successful clinical outcome based on observations in high-risk neuroblastoma*, Frontiers in Pharmacology 3, Article 91, 2013, Nature Publishing Group.

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**Table 1**

Open and Recently Completed Clinical Trials for Pediatric Cancers Employing Monoclonal Antibodies and Immunocytokines that may involve NK-mediated mechanisms

| Clinical Trial Number | Title | Sponsor |
|---|---|---|
| NCT01055314 | Temozolomide, Cixutumumab, and Combination Chemotherapy in Treating Patients With Metastatic Rhabdomyosarcoma | National Cancer Institute |
| NCT01598454 | Use of Racotumomab in Patients With Pediatric Tumors Expressing N- glycolylated Gangliosides | Laboratorio Elea S.A.C.I.F. y A. |
| NCT01595048 | Combination Chemotherapy With or Without Rituximab in Treating Younger Patients With Stage III-IV Non-Hodgkin Lymphoma or B-Cell Acute Leukemia | Children's Oncology Group |
| NCT01279707 | Monoclonal Antibodies in Recurrent or Refractory B Cell Acute Lymphoblastic Leukaemia (ALL) (MARALL) | Queen Mary University of London |
| NCT01900496 | Study of Rituximab and Brentuximab Vedotin for Relapsed Classical Hodgkin Lymphoma | Sidney Kimmel Comprehensive Cancer Center |
| NCT01552434 | Bevacizumab, Temsirolimus, Valproic Acid, Cetuximab | M.D. Anderson Cancer Center |
| NCT01767194 | Irinotecan Hydrochloride and Temozolomide With Temsirolimus or Monoclonal Antibody Ch14.18 in Treating Younger Patients With Refractory or Relapsed Neuroblastoma | National Cancer Institute |
| NCT00026312 | Isotretinoin With or Without Dinutuximab, Aldesleukin, and Sargramostim Following Stem Cell Transplantation in Treating Patients With Neuroblastoma | National Cancer Institute |
| NCT01711554 | Lenalidomide and Dinutuximab With or Without Isotretinoin in Treating Younger Patients With Refractory or Recurrent Neuroblastoma | National Cancer Institute |
| NCT01526603 | High Dose Chemotherapy and Autologous Transplant for Neuroblastoma | Masonic Cancer Center, University of Minnesota |
| NCT01419834 | Humanized 3F8 Monoclonal Antibody (Hu3F8) in Patients With High-Risk Neuroblastoma and GD2-Positive Tumors | Memorial Sloan-Kettering Cancer Center |
| NCT01662804 | Humanized 3F8 Monoclonal Antibody (Hu3F8) When Combined With Interleukin-2 in Patients With High-Risk Neuroblastoma and GD2-positive Solid Tumors | Memorial Sloan-Kettering Cancer Center |
| NCT00877110 | Anti-GD2 3F8 Antibody and Allogeneic Natural Killer Cells for High-Risk Neuroblastoma | Memorial Sloan-Kettering Cancer Center |
| NCT01183429 | 3F8/GM-CSF Immunotherapy Plus 13-Cis-Retinoic Acid for Consolidation of First Remission After Non-Myeloablative Therapy in Patients With High-Risk Neuroblastoma | Memorial Sloan-Kettering Cancer Center |
| NCT01757626 | Combination Therapy of Antibody Hu3F8 With Granulocyte- Macrophage Colony Stimulating Factor (GM-CSF) in Patients With Relapsed/Refractory High-Risk Neuroblastoma | Memorial Sloan-Kettering Cancer Center |
| NCT01183897 | 3F8/GM-CSF Immunotherapy Plus 13-Cis-Retinoic Acid for Primary Refractory Neuroblastoma in Bone Marrow | Memorial Sloan-Kettering Cancer Center |
| NCT01183884 | 3F8/GM-CSF Immunotherapy Plus 13-Cis-Retinoic Acid for Consolidation of Second or Greater Remission of High-Risk Neuroblastoma | Memorial Sloan-Kettering Cancer Center |
| NCT00445965 | Iodine I 131 Monoclonal Antibody 3F8 in Treating Patients With Central Nervous System Cancer or Leptomeningeal Cancer | Memorial Sloan-Kettering Cancer Center |
| NCT01704716 | High Risk Neuroblastoma Study 1 (1.5) of SIOP-Europe (SIOPEN) | St. Anna Kinderkrebsforschung, Austria |
| NCT01701479 | Long Term Continuous Infusion ch14.18/CHO Plus s.c. Aldesleukin (IL-2) (LTI) | St. Anna Kinderkrebsforschung, Austria |

| Clinical Trial Number | Title | Sponsor |
|---|---|---|
| NCT01576692 | A Safety/Feasibility Trial of the Addition of the Humanized Anti-GD2 Antibody (hu14.18K322A) With and Without Natural Killer Cells to Chemotherapy in Children and Adolescents With Recurrent/Refractory Neuroblastoma (GD2NK) | St. Jude Children's Research Hospital |
| NCT00743496 | A Phase I Trial Of The Humanized Anti-GD2 Antibody In Children And Adolescents With Neuroblastoma, Osteosarcoma, Ewing Sarcoma and Melanoma | St. Jude Children's Research Hospital |
| NCT01857934 | Therapy for Children With Advanced Stage Neuroblastoma | St. Jude Children's Research Hospital |
| NCT01592045 | Ch14.18 Pharmacokinetic Study in High-risk Neuroblastoma | United Therapeutics |
| NCT01748721 | MORAb-004 in Treating Young Patients With Recurrent or Refractory Solid Tumors or Lymphoma | Morphotek |
| NCT01334515 | Biological Therapy, Sargramostim, and Isotretinoin in Treating Patients With Relapsed or Refractory Neuroblastoma | COG |
| NCT00003750 | Biological Therapy in Treating Children With Refractory or Recurrent Neuroblastoma or Other Tumors | aaaaaaaaaCOG |

**Table 2**

Open and Recently Completed Clinical Trials for Pediatric Cancers Employing Adoptive NK cell therapy

| Clinical Trial Number | Title | Sponsor |
|---|---|---|
| NCT00582816 | Haploidentical Transplant With NK Cell Infusion for Pediatric Acute Leukemia and Solid Tumors | University of Wisconsin, Madison |
| NCT00640796 | Pilot Study of Expanded, Donor Natural Killer Cell Infusions for Refractory Non-B Lineage Hematologic Malignancies and Solid Tumors | St. Jude Children's Research Hospital |
| NCT01287104 | A Phase 1 Study of NK Cell Infusion Following Allogeneic Peripheral Blood Stem Cell Transplantation From Related or Matched Unrelated Donors in Pediatric Patients With Solid Tumors and Leukemias | National Cancer Institute |
| NCT01386619 | NK DLI in Patients After HLA-haploidentical HSCT | University Hospital, Basel, Switzerland |
| NCT01700946 | Therapy for Pediatric Relapsed or Refractory Precursor B-Cell Acute Lymphoblastic Leukemia and Lymphoma | St. Jude Children's Research Hospital |
| NCT01875601 | NK White Blood Cells and Interleukin in Children and Young Adults with Advanced Solid Tumors | National Cancer Institute |
| NCT01795378 | Safety and Efficacy Study of Donor Natural Killer Cells Given After Haploidentical Hematopoietic Cell Transplantation (DNKI-II) | Asan Medical Center |
| NCT00896701 | Relationship Between Natural Killer Cells' Ability to Kill Leukemia Cells and the Outcome of Patients With Acute Myeloid Leukemia Previously Treated With Interleukin-2 | Cancer and Leukemia Group B |
| NCT00789776 | Fludarabine Phosphate, Cyclophosphamide, Total-Body Irradiation, and Donor Bone Marrow Transplant Followed by Donor Natural Killer Cell Therapy, Mycophenolate Mofetil, and Tacrolimus in Treating Patients With Hematologic Cancer | Fred Hutchinson Cancer Research Center/ University of Washington Cancer Consortium |
| NCT01823198 | Natural Killer (NK) Cells With HLA Compatible Hematopoietic Transplantation for High Risk Myeloid Malignancies | M.D. Anderson Cancer Center |
| NCT00871110 | Anti-GD2 3F8 Antibody and Allogeneic Natural Killer Cells for High-Risk Neuroblastoma | Memorial Sloan-Kettering Cancer Center |
| NCT00526292 | Chemotherapy and a Donor Natural Killer Cell Infusion in Treating Patients With Relapsed or Persistent Leukemia or Myelodysplastic Syndrome After a Donor Stem Cell Transplant | Memorial Sloan-Kettering Cancer Center |
| NCT01621477 | T-Cell Replete Haploidentical Donor Hematopoietic Stem Cell Plus Natural Killer (NK) Cell Transplantation in Patients With Hematologic Malignancies Relapsed or Refractory Despite Previous Allogeneic Transplant | St. Jude Children's Research Hospital |
| NCT01576692 | A Safety/Feasibility Trial of the Addition of the Humanized Anti-GD2 Antibody (hu14.18K322A) With and Without Natural Killer Cells to Chemotherapy in Children and Adolescents With Recurrent/Refractory Neuroblastoma (GD2NK) | St. Jude Children's Research Hospital |
| NCT00145626 | HLA-Nonidentical Stem Cell and Natural Killer Cell Transplantation for Children Less the Two Years of Age With Hematologic Malignancies | St. Jude Children's Research Hospital |
| NCT01857934 | Therapy for Children With Advanced Stage Neuroblastoma | St. Jude Children's Research Hospital |
| NCT00995137 | Genetically Modified Haploidentical Natural Killer Cell Infusions for B- Lineage Acute Lymphoblastic Leukemia | St. Jude Children's Research Hospital |
| NCT00703820 | Clofarabine Plus Cytarabine Versus Conventional Induction Therapy And A Study Of NK Cell Transplantation In Newly Diagnosed Acute Myeloid Leukemia | St. Jude Children's Research Hospital |
| NCT01807611 | KIR Mismatched Haploidentical Donor Hematopoietic Progenitor Cell and NK Cell Transplantation for Hematologic Malignancy | St. Jude Children's Research Hospital |