

Stimulating natural killer cells to protect against cancer: recent developments

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Current cancer immunotherapies have begun to target cell types involved in innate immunity, such as natural killer (NK) cells that recognize and kill tumor cells. Recent advances in the study of NK cell biology have generated interest in manipulating these cells to generate anti-tumor responses. A rise in the number of activated NK cells has been shown to prevent and treat cancer in many preclinical models and is a positive clinical factor in human tumors. This article will focus on recent research on the ability of IL-15 and Toll-like receptor ligands to stimulate NK cell activity against cancer. The potential of these therapies, both alone and in conjunction with traditional and other vaccine platforms, will be reviewed. The current status of these therapies in clinical trials will also be discussed. Targeting these cell types in the context of human cancers may be an essential factor in future cancer treatments.

KEYWORDS: cancer • IL-15 • immunotherapy • NK cells • TLR agonists

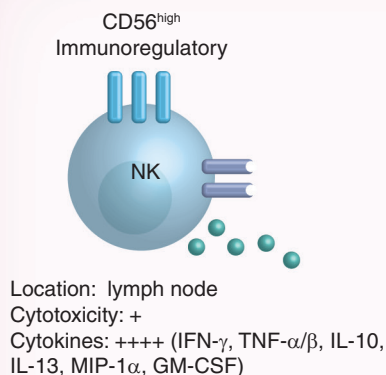
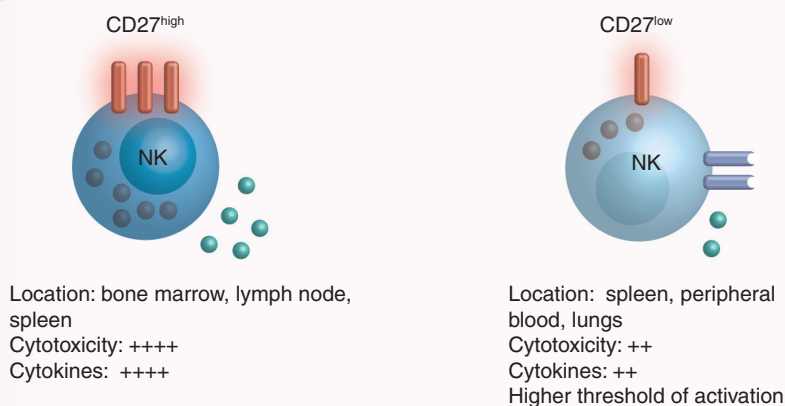
Damaged precancerous cells are a common occurrence in the body. The innate immune system is a key player in performing 'immunosurveillance' to remove these cells before they form tumors [1–3]. One of the major cell types that perform this surveillance are called natural killer (NK) cells. NK cells are large granular lymphocytes that were first discovered because of their ability to kill tumor cells with no prior sensitization [4]. They are found throughout the body in lymphoid and nonlymphoid tissues and make up 10–15% of human peripheral blood mononuclear cells [5]. NK cells are known to exert many biological functions. These include acting as regulatory cells, influencing the adaptive immune response, controlling viral infection and eliminating tumor cells [6]. The activation state of a NK cell determines its ability to act against tumor cells. While it is known that certain subsets of NK cells are able to kill cancer cells, it is obvious that they do not always perform this function effectively. Tumor cells have developed ways to inhibit NK cells. First of all, tumor growth decreases NK cell numbers and maturation in the periphery [7,8]. In addition, tumor cells can increase expression of surface molecules that inhibit NK cells while decreasing expression of those that activate NK cells [9]. Immunosuppression by tumor-secreted molecules such as TGF- β and indoleamine 2,3-dioxygenase also decreases NK cell

activity [10,11]. Research aimed at counteracting these subversive measures is currently underway. The ability to shift NK cells towards a more activated phenotype is essential for NK cells to overcome the influence of the tumor. Multiple methods have been employed in an attempt to harness the power of NK cells in the fight against cancer. After a review of NK cell biology, we will focus on two major treatment strategies to stimulate NK cell activity against cancer that show promise as future therapies: IL-15 and Toll-like receptor (TLR) agonists. These strategies activate NK cells to a high degree, both directly or indirectly, and have shown less side effects than other therapies aimed at stimulating NK cells [12–16].

NK cells

Human NK cells

NK cells can be divided into functional subsets based on the expression of surface markers. These subsets differ in their anatomical distribution as well as their function. This distinction differs between humans and mice (FIGURE 1). In humans, there is a CD56^{high} and a CD56^{low} subset [17]. The CD56^{low} subset is commonly termed cytotoxic NK cells. These cells make up 90% of NK cells (peripheral blood and splenic NK cells) [6,17]. Cytotoxic NK cells express perforin and produce the cytokine IFN- γ when they interact with tumor cells, which results in tumor cell death

A Human NK cells**B Mouse NK cells****C Tissue subsets**

- NK22: produce IL-22 at mucosal sites (human and mouse)
- Thymic NK cells: mature in the thymus, GATA-3⁺, home to the lymph node (mouse)
- Uterine NK cells: secrete VEGF, similar to human CD56^{high} and mouse CD27^{low} NK cells (human and mouse)

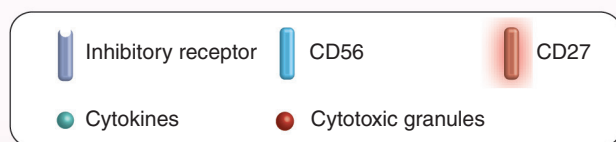


Figure 1. Summary of natural killer cells subsets. (A) Human NK cell subsets are separated on the basis of CD56 expression. CD56^{high} NK cells are immunoregulatory and produce high levels of cytokines, whereas CD56^{low} NK cells are highly cytotoxic. (B) In mice, CD27 expression defines NK cell subsets. CD27^{high} NK cells are highly cytotoxic and produce high levels of cytokines, whereas CD27^{low} NK cells have a higher threshold of activation owing to increased inhibitory receptors. In humans and mice, the subsets also differ in their anatomical distribution. (C) New subsets of NK cells are being discovered. In mucosal tissues, NK22 cells (NK cells that express IL-22) act on epithelial cells and in the thymus there is production of thymic NK cells. MIP-1 α : Macrophage inflammatory protein-1 α ; NK: Natural killer.

cytokines such as IFN- γ , TNF- α , TNF- β , IL-10, IL-13, macrophage inflammatory protein-1 (MIP-1 α) and GM-CSF [17,18].

Mouse NK cells

In mice, the delineation between mature NK cell subsets involves the surface marker CD27 [19]. The CD27^{high} NK cells are found predominately in the bone marrow and lymph nodes (LNs), whereas the CD27^{low} NK cells are excluded from the LNs and are dominant in the peripheral blood and lungs [19,20]. CD27^{high} NK cells are able to produce higher levels of cytokines in response to stimulus and possess a greater cytotoxic capability [19,20]. CD27^{low} NK cells are able to respond to stimuli, but have a much higher threshold of activation and are more tightly regulated [19,20]. There is no direct comparison between the NK cell subsets in the mouse and human. For example, CD56^{high} human NK cells and CD27^{high} mouse NK cells both primarily reside in the LNs, but the CD27^{high} NK cells are highly cytotoxic whereas the CD56^{high} cells are immunoregulatory.

Additional NK cell subsets

Recently, it has come to light that in addition to the aforementioned NK cell subsets, there are tissue-specific NK cell subsets in mice and humans. For example, a new subset of NK cells that express IL-22 (NK22) was recently discovered at mucosal sites [21]. These NK22 cells act differently to traditional NK cell subsets as they stimulate epithelial cells exclusively to secrete antimicrobial molecules as well as anti-inflammatory cytokines such as IL-10 [21]. There is also a subset of thymic NK cells that develop in the thymus instead of the bone marrow and have unique surface molecules as well as distinct functions [22,23]. While NK cells have previously been regarded as innate immune cells, there have recently been reports of 'memory' NK cells [24,25]. In the context of a cytomegalovirus infection in mice, these long-lived NK cells possess immunologic memory for particular viral antigens [24,25]. Although studies are just beginning on these 'memory' NK cells, they may be particularly effective at preventing tumor recurrence. The increasing knowledge regarding NK cell subsets reveals that not all NK cells are equal in the fight against cancer. Some NK cells are desirable for treating tumors,

as well as the promotion of an adaptive immune response [6]. By contrast, the CD56^{high} population is termed immunoregulatory NK cells [6,17]. These cells lack perforin, but are able to make

venting tumor recurrence. The increasing knowledge regarding NK cell subsets reveals that not all NK cells are equal in the fight against cancer. Some NK cells are desirable for treating tumors,

but others may actually assist tumors if they are of the immunoregulatory phenotype. This makes the appropriate activation of NK cells an important consideration for cancer therapeutics.

NK cell activation

Owing to their potential to cause damage, NK cell activation is tightly regulated. In order to regulate the activation of NK cells, there are both inhibitory and activating receptors on the NK cell surface. It is the balance between these two types of receptors that determines whether or not a NK cell is able to kill. There are two major superfamilies of NK receptors (NKR) that allow NK cells to determine if a cell is dangerous: the immunoglobulin superfamily (i.e., KIRs and NCR) and the C-type lectin superfamily [9]. In both superfamilies, there are a multitude of both inhibitory and activating receptors. Some of the most important inhibitory NKRs in humans are the KIRs, Ly49 in mice and CD94-NK group 2 (NKG2) in both species [26]. These receptors bind to MHC class I molecules on target cells and produce an inhibitory signal in the NK cell [27]. This function is very important in preventing NK cells from killing normal host cells that possess MHC class I. In both tumor cells and viral/bacterially infected cells, MHC class I expression is frequently downregulated, and as a result, the NK cell will no longer receive an inhibitory signal [26]. This alone is not enough to activate the NK cell. It is a balance between lack of inhibition and activation. Cells under stress express certain ligands that can activate NK cells via NKRs. For example, an extremely interesting NKR in both humans and mice is NKG2 member D (NKG2D) [28]. This receptor has several different ligands including retinoic acid early inducible-1 (RAE-1) proteins in mice and RAET1 proteins and MHC class I chain-related proteins A and B in humans [28–30]. The ligands for NKG2D are expressed on cells that are undergoing stress such as DNA damage [31,32]. Studies have indicated that this activating receptor is important for NK cells to perform immunosurveillance of tumors [33–35]. Another group of important activating receptors is the NCRs: NKp46, NKp44 and NKp30. Although the ligands for this family of receptors are not well known, NKp46-deficient mice have increased tumor formation [36]. Also, blocking these receptors prevents NK cell killing of tumor cells *in vitro* [26]. Therefore, it is believed that these receptors also play a role in NK cell mediated tumor surveillance. In addition, NK cells also express TLRs that bind infectious non-self molecules such as dsRNA, leading to increased cytokine production as well as cytotoxicity of the NK cells [15]. The balance between activating and inhibiting receptors on NK cells can also be controlled by the cytokine environment. Increasing knowledge regarding how NK cells are able to kill tumor cells has made them an attractive target for cancer therapy.

NK cells & cancer

Preclinical evidence (mouse models)

In a multitude of mouse tumor types, including methylcholanthrene-induced carcinoma, spontaneous mammary carcinoma, colon carcinoma and B16 melanoma, it has been confirmed that NK cells play an important role in tumor destruction [37–40]. The majority of these studies involved mice that were either depleted

of NK cells or lacked NK cell function [37–40]. For example, mice that lack the NKG2D-activating receptor are more susceptible to spontaneous epithelial and lymphoid malignancies [41]. Various studies have shown that NK cells are able to kill tumor cells directly via granzyme/perforin apoptosis [42,43], antibody-dependent cellular cytotoxicity or death receptor ligands on their surface such as Fas ligand and TNF-related apoptosis-inducing ligand [44,45]. It is also known that NK cells can impact tumors indirectly owing to cytokine release (IFN- γ), which promotes effective adaptive immune responses via dendritic cell (DC) activation and downstream activation of CD8⁺ T cells [46–48].

As described, mice and humans have differences in their NK cell subsets, thus the information derived from mouse studies may not directly extend into the clinic. In an effort to address this, a humanized mouse model that supports human NK cell development was created [49,50]. Newborn BALB/C Rag2^{-/-}/ γ c^{-/-} mice were irradiated and injected intrahepatically with human cord blood CD34⁺ stem cells [49,50]. After engraftment, mice had low levels of functional CD56^{bright} and CD56^{low} NK cells that expressed various inhibitory and activating receptors [50]. These NK cells were functional as they were able to protect from K562 tumor cell challenge [50]. Recently, it has also been reported that IL-15/IL-15 receptor (IL-15R) α treatment of these mice is able to increase the amount of NK cells, as well as promote a more mature activated NK cell phenotype [51,52]. While the functional relevance of the humanized mouse model is clear, research has yet to be conducted on potential therapies in this model.

Human evidence

Evidence for the role of NK cells in tumor formation is less clear in humans than in mice. There is a correlation between some hereditary cancers and low NK cell activity [53,54]. In several human cancers, including colorectal and gastric cancer, the presence of intratumoral NK cells is a positive clinical factor [9,55,56]. By contrast, in certain renal, head, neck and lung cancers the presence of IL-15, an NK cell-activating cytokine, is a negative prognostic indicator [57,58]. This is due to the presence of membrane-bound IL-15 on certain types of cancer cells that when bound to soluble IL-15R α (also produced by these tumor cells) can actually promote tumor progression [59,60]. Therefore, the type of cancer must be considered when NK cell-stimulating treatments are to be used. Many tumor cell lines derived from human tumors are susceptible to NK cell killing, and a recent study suggests that NK cells are even capable of killing melanoma cells that have cancer stem cell characteristics [61]. If this is true for other tumor types, it indicates that NK cells may be a successful treatment after chemotherapy as these cancer stem cells are not easily killed by traditional methods. While there is much less information regarding NK cells and human tumors, it is obvious that, as in the mouse, NK cells are involved in tumor cell killing.

IL-15

IL-15 is the key cytokine involved in the development, survival and activation of NK cells [17,62–64]. IL-15 was discovered in 1994 by two groups that found IL-15 was similar to IL-2 in its ability

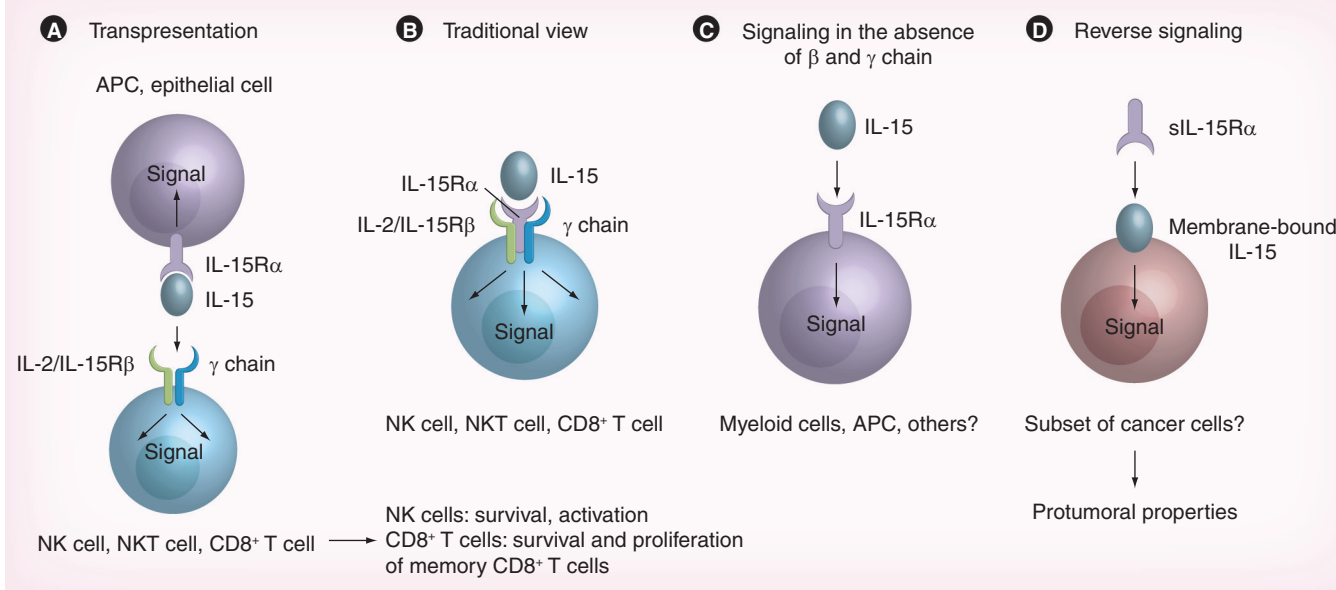


Figure 2. Summary of IL-15 and IL-15 receptor interactions. (A) It was recently discovered that the main mode of IL-15 action *in vivo* is via transpresentation. IL-15 and IL-15R α expressed by the same cell (i.e., APCs and epithelial cells) meet in the Golgi apparatus and proceed to the cell surface. Here they are presented to NK and CD8⁺ T cells that have IL-2/IL-15R β and the γ chain. In the NK cell, IL-15 binding induces IL-2/IL-15R β to bind JAK1/STAT3, whereas the γ chain binds JAK3/STAT5 as well as the Src family of tyrosine kinases. Ultimately, PI3K and MAPK pathways are activated leading to proliferation, differentiation and survival of the cells. IL-15R α also has signaling abilities; it can bind Syk kinase and TRAF2. (B) Traditionally, IL-15 was thought to bind to IL-15R α , IL-2/IL-15R β and the common γ chain. (C) IL-15 is also capable of binding to IL-15R α in the absence of the IL-2/15 γ chain. (D) In reverse signaling, membrane-bound IL-15 binds to soluble IL-15R α and membrane-bound IL-15 induces signals such as MAPK and focal adhesion kinase. In a subset of cancer cells this type of signaling can promote tumor formation.

APC: Antigen-presenting cell; IL-15R: IL-15 receptor; NK: Natural killer; NKT: Natural killer T cell.

to cause T-cell proliferation [65,66]. In fact, IL-2 and IL-15 share many aspects, but also have very distinct features. For example, both bind a trimeric receptor complex with a common β and γ chain, but each has its own specific α chain [67]. Also, IL-2 promotes activation-induced cell death, whereas IL-15 prevents it [68]. IL-15 mRNA is expressed by a broad range of cell types, but its protein product is mostly found in monocytes/macrophages, DCs, fibroblasts and epithelial cells [67]. This is caused by the tight regulation of IL-15 expression during transcriptional, translational and intracellular trafficking [69]. Recently, it was determined that the major mode of IL-15 signaling *in vivo* is via transpresentation (FIGURE 2) [70]. IL-15R α subunit on antigen-presenting cells (APCs; especially DCs) binds to IL-15 and acts as a chaperone through the Golgi apparatus before being presented on the cell surface to other cells expressing IL-2/IL-15R β and the common γ chain [70–72]. This research revealed that expression of both IL-15 and IL-15R α on the same cell type is important for IL-15 signaling [73,74]. It has also been found that IL-15 can exist in a membrane-bound form and perform reverse signaling in certain cell types [75,76]. IL-15 binding signals via the β and γ chain to the JAK1/JAK3, STAT3/STAT5 as well as Src family tyrosine kinases (lck, fyn, lyn), PI3K and MAPK pathway [67]. IL-15R α is also able to induce signals within the cell upon IL-15 binding [77]. As mentioned, in an NK cell, IL-15 stimulation leads to increased survival, development and activation. In fact, in IL-15-knockout (KO) mice there are no NK cells [78]. By contrast, in mice that

overexpress a modified IL-15 transcript that lacks post-transcriptional control, we see a large increase in NK cells (IL-15 transgenic) [79]. In addition, NK cells exposed to IL-15 have increased activating receptors such as NKG2D [80]. IL-15 also has effects on many other cell types that are important in cancer defense such as T cells, DCs and macrophages [6].

The proliferation and survival of a subset of memory CD8⁺ T cells that are very important to tumor cell killing are controlled by IL-15 [81,82]. In IL-15 transgenic mice, we see increased memory CD8⁺ T cells [68,79]. By contrast, IL-15 KO mice have impaired CD8⁺ T-cell responses and slightly lower absolute numbers of CD8⁺ T cells [78,83]. It has been reported that a particular subset of memory CD8⁺ T cells called central memory cells are particularly upregulated by IL-15 [84]. IL-15 treatment of cell lines or mice has the ability to increase both memory and effector CD8⁺ T cells [85,86]. Part of this expansion is thought to be due to the ability of IL-15 to prevent apoptosis in CD8⁺ cells by increasing anti-apoptotic molecules (Bcl-2) and decreasing proapoptotic molecules (Bim, Bax) [87,88]. While this expansion can be antigen nonspecific, adoptive immunotherapy studies have shown that CD8⁺ T cells stimulated with antigen in the presence of IL-15 lead to central memory phenotype T cells that protect efficiently from tumor challenge [89]. It has also been reported that IL-15 treatment is able to rescue inactive tolerized T cells that escaped peripheral deletion so that they can regain effector function against leukemia [90]. The effect of IL-15 on CD4⁺ T cells is less well characterized.

IL-15 can expand memory CD4⁺ T cells in a similar manner to memory CD8⁺ T cells [91]. It has also been reported that IL-15 can drive the expansion of CD4⁺ Tregs [92,93]. Further studies revealed that while IL-15 can increase CD4⁺ Treg numbers, it can also make other CD4⁺ and CD8⁺ effector cells more resistant to the suppressive effects of these Tregs [92,94]. The presence of increased CD4⁺ Tregs is usually a negative prognostic factor in cancer, but it is possible that this effect may be counteracted by the effect of IL-15 on other CD4⁺ and CD8⁺ T cells. It is likely that the effect of IL-15 on CD4⁺ T cells will depend on the system under investigation.

Antigen-presenting cells are very important cells in the response to cancer as they link the innate immune response to the adaptive immune response. IL-15 can directly and indirectly affect APC activation. IL-15-activated NK cells release IFN- γ , which can lead to DC and macrophage activation [46,47]. For DCs in particular, IL-15 can directly promote the maturation of DCs and their expression of costimulatory molecules [95,96]. IL-15 and IL-15Rs are important in APC function as demonstrated in IL-15 KO mice that have impaired functioning of their DCs [96]. Several groups have reported that transferred IL-15-treated tumor antigen-primed DCs were able to activate cytotoxic T lymphocyte responses that ultimately eliminated resistant tumors [95,97]. Although few studies have examined the direct effect of IL-15 on macrophages, it is known that they can respond to and produce IL-15. Cytokines such as IFN- γ derived from activated NK cells can also affect macrophage activity and phenotype [98–100]. High levels of IFN- γ promote the formation of classically activated macrophages (M1) which prevent tumor formation instead of alternatively activated macrophages (M2) which promote tumor formation [101]. Tumor-associated macrophages belong to the M2 group of macrophages [102]. Several studies in both human samples and transgenic mouse models have revealed that tumor-associated macrophages greatly aid in tumor development [103–105]. M2 macrophages have also been found to aid in metastasis, both at the primary tumor site and the site of metastasis [104,106]. Therefore, IL-15 is an important cytokine both for innate immune cells like NK cells and APCs, but it also promotes an effective adaptive immune response involving CD8⁺ T cells.

IL-15 & immunotherapy

Preclinical research (mouse models)

Owing to the ability of IL-15 to promote activation of NK cells, DC maturation and increase the amount of highly effective CD8⁺ memory T cells, it is unsurprising that it has been a successful immunotherapeutic. The majority of *in vivo* studies using IL-15 have focused on subcutaneous injection or lung metastasis models. In IL-15-transgenic mice, protection from subcutaneous MHC class I-negative melanoma (B16F10) was due to an NK cell-dependent mechanism, whereas protection from an MHC class I-positive B16F10 was also dependent on CD8⁺ T cells [107]. Other groups have treated subcutaneous tumors with systemic or intratumoral (plasmid based) IL-15 and achieved successful tumor regression [108,109]. IL-15 has also been administered

with success in combination with other treatments such as chemotherapy, radiofrequency thermal ablation and other cytokines [110–113]. Recent work has focused on using IL-15 already bound to IL-15R α as an immunotherapeutic as this is known to increase its half-life and biological activity [73,114]. Studies using this method for melanoma, spontaneous tumors of the pancreas, colorectal cancer and hepatocellular carcinoma have proved efficacious [115–119]. In some of these studies, the protection observed was NK cell dependent, but in others tumor-specific CD8⁺ T cells were key. It has also been shown that NK cell-dependent rejection of a tumor is able to promote a CD8⁺-based resistance to secondary tumor challenge [48]. Therefore, while IL-15 can act directly on CD8⁺ T cells, the action of IL-15 on NK cells may contribute indirectly to CD8⁺ T-cell generation. To date, mouse studies on the effects of IL-15 on tumor formation have largely centered on injectable tumor models and more research needs to examine its potential in solid spontaneous cancers. While many treatment strategies have been successful, it is unknown how this will translate into human cancers.

Human/primate research

Research on the effects of IL-15 on human NK cells and cancers is less well studied. As an initial indication of the effect of IL-15 on human NK cells, in a humanized mouse model, IL-15/IL-15R α treatment greatly increased mature NK cell numbers [51,52]. These NK cells were able to protect from tumor challenge [50]. Recently, it was shown that with a variety of dosing schedules, low and high doses of recombinant human IL-15 treatment had low toxicity in rhesus macaques and were able to greatly increase NK cell and CD8⁺ memory T-cell numbers [12,120,121]. This paves the way for future clinical trials. As yet, no reports have been published on the use of IL-15/IL-15R α combination therapy in macaques. It would be of interest to determine if this combination treatment that has efficacy in mice, works more effectively in a system closer to that of humans. It has been reported that the NIH is currently starting the first clinical trials for IL-15. If the results are at all similar to what has been observed in the preclinical research setting, IL-15 holds great promise as an immunotherapeutic with the potential to safely treat many forms of cancer. It is likely that as in mouse studies, IL-15/IL-15 R α in conjunction with traditional treatments or vaccines will be particularly useful. In addition, it is believed that NK cell-mediated strategies will be most effective in cases of minimal residual disease and may be important in preventing metastasis.

NK cell adoptive immunotherapy in humans

Treatment of NK cells with IL-15 is also being investigated in the context of human adoptive immunotherapy. Initial studies in this field involved the administration of IL-2 to stimulate the proliferation and activation of NK cells in an *ex vivo* setting. While this showed promise, the clinical results have been mixed [122]. It has now been demonstrated that IL-15, alone or in combination with IL-2, is capable of stimulating the proliferation of human NK cells, which are more cytotoxic to tumor cells than IL-2 treatment alone [123–125]. In patients with acute myeloid leukemia,

it has been demonstrated that if patients' NK cells are cultured with IL-15, there is an increase in NK cell-activating receptor expression and cytotoxicity against autologous leukemic cells [126]. This study greatly supports the use of IL-15 in *ex vivo* NK cell adoptive immunotherapy. The most recent evidence indicates that IL-15 presented in a manner mimicking transpresentation is more effective at generating human NK cells *in vitro*, which are more capable of killing tumor cells than those treated with IL-15 alone [127]. Another interesting area that holds promise is the adoptive transfer of allogeneic NK cells. This work is based on pioneering studies by Ruggeri *et al.* [128]. In these studies, NK cells were transplanted with mismatched inhibitory MHC class I receptors (KIRs) into the host acute myeloid leukemia patients and survival was greatly increased [128]. The lack of inhibitory ligands on host cells allowed the NK cells to attack the cancerous cells of the host more effectively. This approach was shown to be successful in patients with many different tumor types [129]. A recent Phase I trial of non-small-cell lung cancer (NSCLC) patients revealed that IL-15 and hydrocortisone-treated allogeneic NK cell injection, in conjunction with chemotherapy, was both safe and possibly effective [130]. Therefore, the evidence thus far indicates that IL-15/IL-15R α -treated NK cells may be more effective than previously employed methods in adoptive therapy of autologous and allogeneic NK cells.

TLRs, NK cells & cancer

Toll-like receptors are pattern recognition receptors (PRRs) that have evolved in the innate immune system to recognize infection. TLRs detect highly conserved pathogen-associated molecular patterns (PAMPs) that are found on pathogens. Humans have ten TLRs, while mice have 12, but TLRs 1–9 are common [131]. TLRs are found in various locations within the cell; TLR-1, -2, -4, -5, -6 and -11 are distributed on the cell surface and recognize mostly bacterial components, whereas TLR-3, -7, -8 and -9 are found on intracellular vesicles and recognize pathogen nucleic acids [131]. There are also cytosolic PRRs including RIG-I-like receptors and NOD-like receptors [131]. TLRs can be expressed on many cell types including immune cells such as macrophages, DCs, B cells, monocytes, Tregs and NK cells [132,133]. Each TLR recognizes a specific repertoire of PAMPs and is differentially expressed on various cell types. For example, TLR-9 recognizes unmethylated CpG repeats found in pathogen DNA, and is highly expressed on plasmacytoid DCs [131]. In addition, the binding of a ligand to a TLR initiates a unique set of signaling molecules that ultimately leads to the production of inflammatory cytokines and/or type I interferons (IFNs). Signaling from the various TLRs has been thoroughly reviewed recently [131]. In short, all TLRs except TLR-3 have signaling cascades that involve the adaptor molecule MyD88 and lead to the activation of NF- κ B and MAPK and ultimately to the production of inflammatory cytokines [131]. TLR-3 and TLR-4 can also signal via the signaling adaptor TRIF, which leads to NF- κ B and IRF3 activation, which can activate inflammatory cytokines and type I IFNs [131]. TLR-7 and -9 signaling in plasmacytoid DC is unique as they signal through MyD88, but can also produce type I IFNs

owing to the presence of IRF7 [131]. We will now focus on several TLRs and their ligands that have been investigated for their use as immunotherapeutics in cancer.

The potential for TLRs in cancer immunotherapy has been of interest for over a decade. Immune cell recognition of PAMPs by a DC leads to activation/maturation of that cell and therefore better antigen presentation, secretion of proinflammatory cytokines (i.e., IFNs and IL-12), which activate other cell types that act against cancer (e.g., NK cells, CD8⁺ T cells), as well as the promotion of a Th1-based secondary adaptive immune response [16,133–136]. In addition, certain TLR ligands are able to cause direct apoptosis of tumor cells that express that TLR [137]. Recently, it has also been shown that some TLR agonists can directly stimulate NK cell activity against cancer cells [13]. As a result of these effects, TLR agonists could be applied to multiple cancer therapy strategies: monotherapy, adjuvants to cancer vaccines and in conjunction with other traditional therapies such as chemotherapy. We will focus on the potential of these TLR agonists to affect NK cell activity.

TLR-9 agonist CpG

CpG is the most studied TLR agonist in terms of application to cancer therapy. In studies involving TLR-9^{-/-} mice, TLR-9 was found to be the key molecule in immune cell recognition of unmethylated CpG repeats that are frequently found in bacterial DNA [138]. TLR-9 is strongly expressed by human plasmacytoid DC and B cells and shows low expression in NK cells (also expressed on macrophages on mice) [13,136,139]. CpG stimulates the activation and maturation of DCs and induces NK cell activity in human and murine lymphocytes. The majority of studies have found that this effect is not direct, but rather that CpG activates APCs to produce IFN- α /IL-12, which then activates NK cells [136,140]. Some studies report that under certain circumstances, NK cell cytotoxicity may be increased directly by CpG [13,15]. There are different forms of CpG that are distinguished by length (known as A, B and C) and these forms activate different cell types and therefore induce different cytokine responses [132].

Preclinical research (mouse models)

There are many animal studies that support the use of CpG as an adjuvant, particularly with tumor antigen vaccines [141,142]. In these studies, CpG was found to increase both antibody and cytotoxic T lymphocyte responses to tumors. It has also been used successfully as a monotherapy administered subcutaneously or intratumorally in both prophylactic and treatment models [143–145]. In cases where the immune response was characterized, CD8⁺ T cells appeared to be the main cell type that had increased tumor killing ability, although some indicated that NK cells may play a role [144,145]. CpG is not a successful treatment in all cases; in a recent rat glioma model, tumors were actually found to increase in size following CpG treatment [146]. Lastly, CpG used in conjunction with chemotherapy has been shown to be of benefit in multiple animal models and leads to reversal of the immunosuppression created by chemotherapy [147,148]. The positive results from these animal studies has led to a plethora of Phase I and II clinical trials using CpG as a monotherapy, adjuvant and in conjunction with chemotherapy.

Table 1. Summary of some published clinical trials using CpG or polyI:C for cancer treatment.

Author (year)	Treatment type	Disease	Phase	Outcome	Ref.
Traditional therapy + TLR agonist					
Manegold <i>et al.</i> (2008)	Taxin/platin chemotherapy + CpG (PF-3512676)	NSCLC	II	1-year survival: 50 vs 33% for chemotherapy alone	[151]
Weber <i>et al.</i> (2009)	Dacarbazine + CpG (PF-3512676)	Melanoma	II/III	Did not continue to Phase III	[152]
Rosenfeld <i>et al.</i> (2010)	Radiation + temozolomide + polyI:C (poly-ICLC)	Glioblastoma	II	Median overall survival: 18.3 vs 14.6 months in EORTC 26981/22981 trial without polyI:C*	[177]
Butowski <i>et al.</i> (2009)	Radiation + polyI:C (poly-ICLC)	Glioblastoma	II	1-year survival: 69.4 vs 35% for radiation alone	[178]
Vaccines + TLR agonist					
Speiser <i>et al.</i> (2005)	CpG 7909 + MART1 peptide	Melanoma	I	No OR, but increased anti-tumor CD8 ⁺ T cells found	[153]
Fourcade <i>et al.</i> (2008)	CpG 7909 + montanide ISA + analog peptide (NY-ESO-1)	Melanoma	I	Generated anti-tumor CD8 ⁺ T cells	[154]
Monotherapy					
Hofmann <i>et al.</i> (2008)	CpG (PF-3512676)-intralesion	Melanoma	I	One CR (out of five)	[157]
		BCC	I	One CR, two PR (out of five)	
Pashenkov <i>et al.</i> (2006)	CpG (PF-3512676)-subcutaneous	Melanoma	II	Two PR, three SD, increased NK cytotoxicity (out of 20)	[156]
Carpentier <i>et al.</i> (2010)	CpG (PF-3512676)-intralesion	Glioblastoma	II	1-year survival: 24 vs 15% in several other Phase II studies on glioblastoma	[155]
Butowski <i>et al.</i> (2009)	Poly-ICLC	Anaplastic glioma	II	No improvement over control	[179]

*The authors compare their result with a similar study that did not involve polyI:C but did include radiation with temozolomide.

BCC: Basal cell carcinoma; CR: Complete response; NK: Natural killer; NSCLC: Non-small-cell lung cancer; OR: Objective response; Poly-ICLC: Poly-I-lysine and carboxymethylcellulose; PR: Partial response; SD: Stable disease; TLR: Toll-like receptor.

Human research

Phase I and II trials using a variety of CpG treatment schedules have been conducted mostly with melanoma, non-Hodgkin's lymphoma, renal cell carcinoma, NSCLC and a few in breast carcinoma, glioblastoma and basal cell carcinoma (TABLE 1). These trials have been thoroughly reviewed in several recent publications [139,149,150]. The results from these trials are very hard to interpret at this point. However, it can be concluded that CpG treatment is well tolerated and at least in some cases has led to tumor regression or lack of progression. In one particularly promising Phase II study with taxane/platin chemotherapy and CpG (PF-3512676) against NSCLC, survival was 50% in the dual treatment group versus 33% in the chemotherapy alone group [151]. On the basis of this study, a similar Phase III study was initiated, but the trial was stopped when it failed to produce any evidence that CpG had additional benefits over chemotherapy alone [152]. Some suggest that NSCLC is not a good tumor type to treat with this method since it is generally TLR-9 negative and refractory to chemotherapy [149,150]. The majority of the Phase I and II trials that have used CpG (7909/Agatolimod) as an adjuvant have involved tumor antigens for melanoma [153,154]. These studies showed benefits to the patients who received CpG, including increased tumor-specific CD8⁺ T cells, and therefore demonstrated the utility of CpG as a good adjuvant for these purposes as well as

its safety [153,154]. Since these promising results, several other clinical trials have been launched to determine the most appropriate treatment combinations to best improve patient survival. Lastly, CpG has also been used as a monotherapy to treat multiple tumor types, including melanoma and glioblastoma [155–157]. The results have been mixed. For melanoma, different CpG regimens have been used including subcutaneous injection and intratumoral injections in Phase I and II trials, respectively [156,157]. Both studies found that a few patients had partial and complete regression and that this regression was associated with increased NK cell cytotoxicity [156,157]. The results are not as positive for patients with recurrent glioblastoma. In a recent Phase II study, intratumoral infusions with CpG had a disappointing effect on tumor progression, however, there was still an increase in 1-year survival from less than 15 to 24% [155]. What may be concluded from these studies is that each tumor type is unique and will require experimentation with dosing, administration schedules and combination therapies to determine the best application of CpG immunotherapy.

TLR-3 agonist polyI:C

Double-stranded RNA from pathogens is a natural ligand for TLR-3 and RIG-I/MDA5. PolyI:C is a synthetic dsRNA that has been studied in cancer immunotherapy. PolyI:C can activate TLR-3

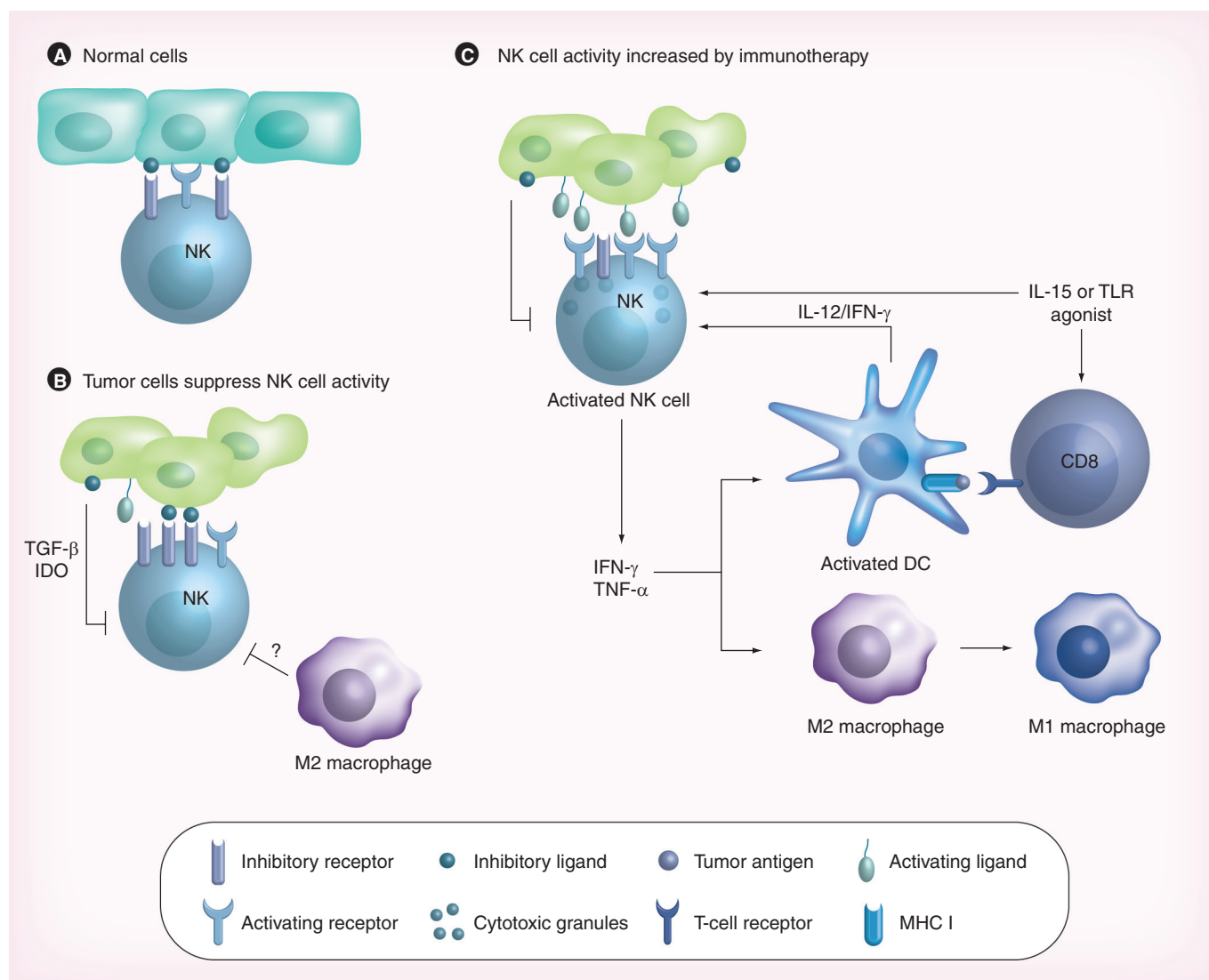


Figure 3. The activity of natural killer cells in different situations. (A) When an NK cell encounters a normal cell, it receives inhibitory signals and moves on. (B) Upon transformation, tumor cells express signs of damage such as activating ligands for NK cells. The tumor can upregulate inhibitory ligands to prevent NK cell killing, and secrete immunosuppressive molecules such as TGF- β to prevent NK cell activation. In this case, NK cells do not become activated. Also, M2 tumor macrophages play protumoral and immunosuppressive roles. (C) When IL-15 or TLR ligands are added, NK cells are stimulated, both directly and indirectly, to increase activating receptors/cytotoxicity and subsequently eliminate tumor cells. DCs are activated to present antigens and promote adaptive CD8 $^{+}$ T-cell responses and macrophages are converted from M2 protumoral to M1 anti-tumoral phenotypes. Cytokine cross-talk between the APCs and NK cells reinforces these actions. APC: Antigen-presenting cell; DC: Dendritic cell; IDO: Indoleamine 2,3-dioxygenase; NK: Natural killer; TLR: Toll-like receptor.

and acts via a TRIF-dependent pathway, rather than a MyD88-dependent pathway, to induce type I IFNs and proinflammatory cytokines [158,159]. It can also activate MDA5 and act via an IPS-1 pathway [158,159]. The cellular expression pattern of TLR-3 and MDA5 are different and this allows multiple cell types to respond to dsRNA with a different signaling cascade [158,159]. In fact, polyI:C signaling in mice and humans is different as a result of this. In mice, polyI:C induces maturation of CD8 α^{+} DCs via TLR-3 and MDA5 and via IL-12/IFN- β secretion, and this can indirectly lead to the activation of NK cells [160,161]. In addition, MDA5 activation in stromal cells leads to IFN- α/β production and NK cell activation [160].

NK cells are not thought to be activated directly by polyI:C in mice, but this is not what has been observed in studies of human NK cells. Human NK cells express TLR-3, and TLR-3–polyI:C binding can lead to activated NK cells with very high levels of cytokine secretion and cytotoxicity [13,15,136,162]. In preclinical models of cancer, polyI:C has been investigated as an adjuvant, monotherapy and in combination treatments such as radiation/chemotherapy.

Preclinical research (mouse models)

As a monotherapy, polyI:C is administered intraperitoneally or intratumorally and has been used both prophylactically and to

treat established tumors such as B16 melanoma, mesothelioma and prostate cancer [163–166]. In all cases, treatment correlated with a decrease in tumor formation and this decrease was associated with a polyI:C-mediated increase in NK cell activity [163–166]. In terms of polyI:C adjuvant studies, multiple studies have been conducted that combine polyI:C with a tumor antigen and this results in regression of established tumors or prophylactic prevention of tumor formation [167–170]. The correlates of protection include one or more of the following: activation of NK cells, cytotoxic CD8⁺ T cells or a skew toward Th1 antibody responses [167–170]. PolyI:C has also been tested in the context of more traditional cancer therapies. In mouse models of melanoma or lung cancer, irradiation followed by polyI:C administration increases survival [171]. In another interesting study in rats, surgery to remove MADB106 mammary tumors was followed by polyI:C treatment and tumors returned less frequently [172].

Human research

TLR-3 is unique as it appears to be expressed on many human tumor cell lines such as breast, head and neck and prostate cancers [173–176]. In studies that have examined the effect of polyI:C binding to tumor cell TLR-3, it was found that there is inhibition of cell proliferation, expression of inflammatory mediators that recruit immune cells and the induction of apoptosis. Therefore, polyI:C may be a particularly useful strategy for attacking TLR-3-expressing tumors. In terms of clinical trials, few have been performed with polyI:C (TABLE 1). There have been a few recent Phase II studies using a polyI:C that has been stabilized with poly-L-lysine and carboxymethylcellulose (poly-ICLC) to treat recurrent and primary glioblastoma [177–179]. Poly-ICLC has been used as a monotherapy and with chemotherapy/radiation and has proven to be safe and slightly improved overall survival [177–179]. Another clinical grade polyI:C analog (Ampligen®, Bioclones Ltd, Sandton, South Africa) has recently been developed and is proposed as an optimal adjuvant for cancer immunotherapy as it stimulates maturation of human myeloid DCs and promotes Th1-type cytokines such as IL-12 in controls and cancer patients [180,181]. It is of great interest to determine if stimulation of TLR-3 by polyI:C or other analogues will have additional benefits to TLR-9/CpG activation in human clinical trials.

TLR-4 agonist FimH

Our center recently discovered a new PAMP that binds to TLR-4 called FimH [182]. FimH is the adhesion portion on type I fimbriae of Enterobacteriaceae [183]. FimH acts on different cells than lipopolysaccharide (LPS; another TLR-4 ligand), as it does not require the coreceptor CD14 and its signaling requires the MyD88 pathway [182]. FimH has been shown to induce anti-pathogen responses by stimulating IFN- β production from macrophages [183]. Recently, it was found that FimH can also directly activate both human and murine NK cells via TLR-4 [14]. We have shown that, unlike LPS, FimH can activate NK cells which are positive only for TLR-4, not CD14. Activation of

NK cells by LPS requires both TLR-4/MD-2 complexes as well as CD14. In addition, treatment of animals with FimH 2 days prior to B16 melanoma tumor injection led to decreased tumors in the lung [14]. Protection provided by FimH was found to be NK cell mediated. While a long way from clinical applications, discoveries like this reveal that there are many possible routes to stimulate an effective innate immune response that remain to be explored. There is also a possibility that certain TLR ligands will be efficacious for some tumors and harmful for others. A recent study in human head and neck cancer shows that TLR-4 stimulation via LPS actually promotes tumor development [184]. It is very likely that different tumor types will require a particular combination of TLR ligands to achieve successful protection. TLR ligand therapy may be most effective as a personalized therapy in the future.

Expert commentary & five-year view

While NK cells have the ability to directly and/or indirectly kill tumor cells, tumor cells and tumor-infiltrated leukocytes produce potent immunosuppressive cytokines and factors, including TGF- β and IDO, to suppress NK cell activities (FIGURE 3). Knowledge regarding advances in NK cell activating and inhibitory receptors and the factors involved in their regulation provide optimism for the manipulation of NK cells through immunotherapy. To overcome the ability of tumors to escape from NK cells, upregulation of activating receptors, downregulation of inhibitory receptors and an increase in the killing capacity of NK cells is crucial. Recent advances in IL-15/IL-15R α biology and information about the effectiveness of transpresentation on NK cell activation, as well as the effect of NK cells on the promotion of CD8⁺ T-cell responses may open new avenues for immunotherapy. In particular, thus far, IL-15 treatment seems to be better tolerated than IL-2 and has several characteristics that make it more effective. One area that must be addressed by future studies is the type of NK cell, either memory, cytotoxic or regulatory, that is generated by various immunotherapies. This will help delineate the effectiveness of NK-activating techniques and determine which type of NK cell is best for cancer prevention or treatment. This knowledge would also be useful in developing NK cell adoptive immunotherapy. Another area of future focus should be the production of NK cells from embryonic stem cells in order to engineer highly active and compatible NK cells. The ability to produce large quantities of NK cells *in vitro* with the desired uniform phenotype bodes well for the future of adoptive immunotherapy strategies.

Use of TLR ligands may be particularly effective in cancer immunotherapy as they not only activate NK cells, but also promote activities of M1 macrophages while suppressing the activity of tumor-associated macrophages. The success of this therapy in mouse models, however, has not fully translated into human studies, at least for the TLR-9 agonist CpG. We are discovering new TLR ligands that directly activate NK cells, such as FimH, that may hold even more promise as therapeutics. More studies on the effects of IL-15/IL-15R α complexes and TLR ligands on

various immune cells, including NK cells, and tumor types will be important in the future. We know that different subsets of immune cells express different PRRs (in both mice and humans). Understanding the effects of each ligand in a human context will allow better delineation of what type of stimulation produces the desired immune reactions for a given cancer. The humanized mouse model may be an effective tool to evaluate the efficacy of promising therapies on human NK cells against human tumors. The ability of certain TLR ligands to directly kill tumor cells has attracted the attention of many researchers. If this finding translates into a human *in vivo* setting, the administration of TLR ligands could be a very effective treatment. It is likely that therapies involving NK cell stimulation will be most effective in cases where there is minimal residual disease or to prevent metastasis. Since established tumors have many mechanisms to prevent NK cell activity, it is unlikely that NK cell therapy with IL-15 or TLR ligands alone will be enough. To increase the efficacy of NK cell-based immunotherapy, it must be combined with treatments that

block immunosuppressive factors, particularly TGF- β , which is produced in the tumor microenvironment. Therefore, successful therapies will involve combination treatments that employ NK cell activation with blockade of immunosuppressive factors, more traditional therapies and/or vaccine platforms. This strategy will lead to effective therapy that will eliminate tumors and provide long-lasting immunity to prevent recurrences.

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Key issues

- Natural killer (NK) cells are effective killers of tumor cells and can be activated by IL-15 or certain Toll-like receptor (TLR) ligands.
- In preclinical models, IL-15 has been very effective in stimulating NK cells as well as CD8⁺ T cells, resulting in increased survival in several tumor models. IL-15/IL-15 receptor α combinations show even more promise.
- Several recent studies indicate that IL-15 appears to be safe and effectively increases the quantity of NK cells and memory CD8⁺ T cells in macaques, and hence clinical trials with IL-15 and cancer will soon be underway.
- TLR ligands such as CpG have been used in immunotherapy models as a monotherapy, adjuvant and in addition to chemotherapy. Clinical trials have indicated efficacy, but Phase III trials have not yet been completed.
- Other TLR ligands, such as polyI:C and FimH, have shown promise in preclinical models, but their effectiveness in human cancers is unknown.
- It is likely that therapeutic strategies that activate NK cells will be most effective when used in conjunction with other therapies that decrease immunosuppression or remove the majority of the tumor mass.

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