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Review

# Natural Killer Cells: Diverse Functions in Tumor Immunity and Defects in Pre-neoplastic and Neoplastic Stages of Tumorigenesis

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**Natural killer (NK) cells are the key immune effectors with the ability to mediate selection and differentiation of a number of different cancer stem cells/undifferentiated tumors via lysis, and secreted or membrane-bound interferon (IFN)-γ and tumor necrosis factor (TNF)-α, respectively, leading to curtailment of tumor growth and metastasis. In this review, we present an overview of our recent findings on the biology and significance of NK cells in selection and differentiation of stem-like tumors using *in vitro* and *in vivo* studies conducted in humanized-BLT mice and in cancer patients. In addition, we present current advances in NK cell expansion and therapeutic delivery, and discuss the utility of allogeneic supercharged NK cells in the treatment of cancer patients. Moreover, we discuss the potential loss of NK cell numbers and function at the neoplastic and pre-neoplastic stages of tumorigenesis in induction and progression of pancreatic cancer. Therefore, because of their indispensable role in targeting cancer stem-like/undifferentiated tumors, NK cells should be placed high in the armamentarium of tumor immunotherapy. A combination of allogeneic supercharged NK cells with other immunotherapeutic strategies such as oncolytic viruses, antibody-dependent cellular cytotoxicity (ADCC)-inducing antibodies, checkpoint inhibitors, chimeric antigen receptor (CAR) T cells, CAR NK cells, and chemotherapeutic and radiotherapeutic strategies can be used for the ultimate goal of tumor eradication.**

## Natural Killer Cells

Natural killer (NK) cells develop in bone marrow and are known to regulate innate and adaptive immunity.[1,2] They constitute 5%–10% of the peripheral blood and are cytotoxic effectors with the ability to recognize and lyse a number of different cancer stem cells (CSCs) and undifferentiated or poorly differentiated tumors, exhibiting lower levels of major histocompatibility complex (MHC) class I, CD54, and PD-L1 and higher expression of CD44.[3–6] NK cells recognize and target tumor cells and virally-infected cells[7] by releasing perforin and granzyme B, which are regulated by cystatins and cathepsins, and are known to induce necrotic as well as apoptotic cell death in susceptible targets.[8,9] NK cells also mediate direct and antibody-dependent cellular cytotoxicity (ADCC) against tumors, and they are known to regulate the functions of other cells through the secretion of cytokines and chemokines.[10] Activation of NK cells is governed by a series of activating and inhibitory receptors.[11–13]

Two distinct subpopulations of NK cells were previously identified.[14] The $CD16^{bright}CD56^{dim}$ subset of NK cells with predominance in circulation and the $CD16^{dim}CD56^{bright}$ subset with predominance in mucosa were shown to have cytotoxic and regulatory functions, respectively.[15] We have identified four stages of NK cell maturation in humans using CD16, CD56, and CD69 surface receptors.[16,17] $CD16^{bright}CD56^{dim}CD69^{-}$ NK cells, which constitute ~90% of peripheral blood NK cells, are found to select and kill CSCs/undifferentiated tumors, whereas the low or non-cytotoxic/split-anergized $CD16^{low}CD56^{bright}CD69^{bright}$ NK cells which are induced by tumors (please see Split Anergy in NK Cells for a description of split anergy) may regulate the function of other cells and are known to induce differentiation of tumor cells.[14] NK cells may lose the ability to mediate cytotoxicity or secrete interferon (IFN)-γ, thereby giving rise to the third stage of NK cell maturation. Finally, NK cells may undergo apoptosis, giving rise to stage 4.[18] In many cancer patients, the numbers and frequencies of stage 3 and 4 NK cells increase substantially, leading to ineffective NK cell-mediated functions.[19,20] Decreased NK cell cytotoxicity in the tumor microenvironment and peripheral blood, as well as downmodulation of CD16 receptors on the surface of NK cells of cancer patients, have been reported previously.[21–24] Decreased function of NK cells is associated with increased

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cancer risk, whereas higher NK activity and increased infiltration of tumor cells by the NK cells are associated with better prognosis.[25,26]

### Split Anergy in NK Cells

To fully understand how NK cells function within the tumor microenvironment we need to understand how they are regulated and what mechanisms they use in order to either kill or differentiate tumors. We have reported previously that NK cells down-modulate CD16 receptors and exhibit decreased ability to mediate cytotoxicity upon interaction with CSCs/undifferentiated tumors while maintaining secretion of IFN-γ and TNF-α, which is a functional state we previously coined as "split anergy."[27–29] We think that this stage of NK cell maturation is important for the differentiation of neighboring undifferentiated cells. In contrast, interaction of differentiated tumor cells with the NK cells does not induce either activation or split anergy in NK cells, indicating that differentiated tumors are not targeted by the NK cells.[12,30] We have previously demonstrated that split anergy in NK cells can also be induced by the treatment of NK cells with recombinant human interleukin (IL)-2 and activating CD16 monoclonal antibodies (anti-CD16 mAbs), mimicking the activation process that NK cells undergo upon interaction with tumor cells.[17,28,29]

Split anergy in NK cells is different from that of T cell anergy in which all of the functions of T cells are inhibited. Thus, split anergy in NK cells is defined as selective loss or decrease in cytotoxicity in the presence of increased cytokine and chemokine secretion.[27,31,32] As indicated above, this functional stage of NK cells is important for mediating differentiation of tumors as described in Key Roles of Split-Anergized NK Cells in Differentation of Stem-like/Poorly Differentiated Cells, potentially resulting in the termination of NK cell activation, since well differentiated tumor cells are not targeted or are targeted less by the primary activated NK cells.[12,27,33]

### Key Roles of Split-Anergized NK Cells in Differentiation of Stem-like/Poorly Differentiated Cells

Tumors contain a stem-like population expressing CD133, ALDH, and CD44 phenotype, with a specific genetic signature known as cancer stem-like cells, which sustain tumor growth because of their self-renewal capacity and their high rates of proliferation.[14,34–36] In addition, CSCs/undifferentiated or poorly differentiated tumors are known to have aggressive phenotypes with a potential to give rise to metastatic growth after implantation of a few tumor cells in mice.[35,37–39] We as well as others have previously demonstrated that NK cells target both normal Mesenchymal Stem Cells (MSCs) as well as a number of different CSCs.[10,40] Indeed, it has been speculated for a long time that NK cells are important in shaping the size of many of the body organs and are involved in targeting and regulating the adipose tissue stem cells, thereby countering obesity.[41,42] In addition, our recent work using several different CSCs/undifferentiated tumors, such as pancreatic, oral, melanoma, glioblastoma, and lung tumors, has established NK cells as the major immune effectors with the capability to target and differentiate these tumors.[4,43,44] In contrast to our findings, breast CSCs were found to be resistant to NK cell-mediated functions.[45] It is presently unknown why susceptibility of breast CSCs to NK cell-mediated cytotoxicity is different from many other tumor CSCs examined by different laboratories.[45,46] Indeed, in all different types of CSCs that we have examined and reported previously, a generalized profile of surface receptor expression was identified, demonstrating a lack or a decrease in the levels of MHC class I, CD54, and PD-L1 and in an increase in CD44 expression.[43,44] We have used these surface markers to successfully differentiate between a number of distinct stem-like/poorly differentiated and well differentiated tumor types.[4,16,47–49] In addition, CSCs/undifferentiated tumors have the ability to proliferate significantly and give rise to much larger numbers of tumor cells, whereas their differentiated counterparts proliferate less and give rise to much lower numbers of tumor cells.[44]. Consequently, CSCs/undifferentiated tumors form larger tumors and are able to invade and metastasize to other organs, whereas their differentiated counterparts form smaller tumors and are not able to metastasize.[44,50,51] Interestingly, although CSCs/undifferentiated tumors are highly susceptible to NK cell-mediated cytotoxicity, they are greatly resistant to chemotherapeutic drugs.[4,50]

We have reported previously that split-anergized NK cells, either paraformaldehyde fixed or their supernatants, trigger differentiation of CSCs/undifferentiated tumors primarily via secreted and membrane-bound forms of IFN-γ and TNF-α, with IFN-γ having a more dominant role. NK cell-induced differentiation of stem-like/poorly differentiated tumors was also shown to decrease the rate of tumor growth and induce resistance of tumor cells to NK cell-mediated cytotoxicity.[27,44,47,48,50]

We have also shown that the addition of each of the anti-IFN-γ and anti-TNF-α antibodies alone to IL-2 and anti-CD16 mAb activated NK cells, and the subsequent use of their supernatants for differentiation of the above-mentioned tumors had a slight effect on reversal of resistance of tumors to NK cell-mediated cytotoxicity, whereas the addition of the combination of anti-IFN-γ and anti-TNF-α antibodies to activated NK cells before their use in differentiation of tumors abrogated the NK cell-induced resistance of tumors to NK cell-mediated cytotoxicity, indicating prevention of differentiation of tumors by both antibodies. More importantly, NK cell-induced tumor differentiation and induction of resistance of tumors to NK cell-mediated cytotoxicity were correlated with the increased expression of CD54, PD-L1, and MHC class I and downmodulation of CD44 on tumors, criteria that were shared by most NK cell-differentiated or patient-derived naturally occurring well differentiated tumors. The addition of a combination of anti-TNF-α and anti-IFN-γ antibodies to activated NK cells before their use in differentiation of tumors prevented the upregulation of the above-mentioned receptors on tumors.[27,47,48] In contrast, blocking NK cell-mediated INF-γ induction with anti-IFN-γ antibody but not anti-TNF-α antibody during NK cell activation, as well as the use of the supernatants to treat tumors before their co-culture with freshly isolated NK cells, exhibited increased secretion of IFN-γ by the NK cells by keeping the tumor cells in an undifferentiated state, thereby maintaining secretion

of IFN-γ by the NK cells.[27] Thus, it appears that the NK cell-induced IFN-γ but not TNF-α effect on tumors primarily regulates tumor differentiation by the NK cells, whereas the effect of both TNF-α and IFN-γ induced by the NK cells on tumors is important for regulating the cytotoxicity of NK cells.[27] No effects of IL-6 or IL-8 in tumor differentiation were seen.[43] Therefore, differentiation of the above-mentioned tumor cells by split-anergized NK cells increases key differentiation receptors on tumor cells, induces tumor cell resistance to NK cell-mediated cytotoxicity, and inhibits inflammation due to a decrease or shutdown of cytokines and chemokines induced by the NK cells.[27,47] Differentiation by the NK cells is also responsible for decreased tumor growth and metastasis.[27,47] Thus, both cytotoxic and split-anergized NK cells are important in shaping the interacting cells since cytotoxic NK cells may initially select the cells by lysis of a subset of the cells, whereas split-anergized NK cells may promote the differentiation of selected undifferentiated cells.[27,52] This will not only limit tumor cell growth, which should keep the size of the tumors under control, but also will promote a less inflammatory microenvironment. While it is important to understand how NK cells shape the interacting cells, it is equally important to understand how the interacting cells within the microenvironment shape the function of NK cells. The next section describes the mechanisms by which NK cells may become activated.

### Increase in the Function of NK Cells by Tumors and in Mice with Knockout or Knockdown of Cellular Genes

We have previously reported the list of cellular genes that augment NK cell function when deleted or decreased in tumors.[53] We have shown that knockdowns of nuclear factor κB (NF-κB) in HEp2 tumors, as well as CD44 in breast and melanoma tumors, were able to increase expansion and functional activation of NK cells significantly.[4,54,55] In addition, targeted knockdown of COX2 in non-transformed healthy myeloid cells and mouse embryonic fibroblasts was also found to increase expansion and functional activation of NK cells significantly.[53] Indeed, more than 19 gene knockout studies by us and those in other laboratories were found to demonstrate increased activation of NK cells in the gene knockout cells or in mice,[53] and this is likely a limited number of studies listed and a gross underestimation of the gene knockout studies that have been shown to increase NK function. One of the common underlying mechanisms for activation of NK cells in our gene knockout studies was found to be related to the downmodulation of MHC class I expression on both transformed and non-transformed healthy cells.[4,53–55] Surprisingly, hyperresponsiveness of the NK cells was also seen in mice with knockouts of genes that mediate inflammation, in particular even those that are involved in NK cell signaling and activation, such as DAP10/DAP12, indicating that NK cell activation is much more complex than we have previously envisioned, involving many genes and pathways, and it is likely dependent on modulation of the stage of differentiation of the cells by these genes and pathways.[56] In addition, when CD44, which is upregulated in many stem-like tumors and is widely used as a marker of stemness, was knocked down in breast and melanoma tumors, a decrease in differentiation antigens, mainly CD54, MHC class I, and PD-L1, was observed on the tumors with a corresponding increase in NK cell activation when these tumors were cultured with the NK cells.[4] Therefore, such increases in responsiveness of NK cells when key cellular genes were knocked out or knocked down in interacting cells and tumors may point to the fundamental function of NK cells in targeting cells that lose the ability to differentiate optimally, and that the degree of differentiation of the cells is likely the key in regulating the NK cell expansion and function.

Both lysis and differentiation of tumor cells by the NK cells are important for the containment of the tumor cells. The next section describes the potential underlying mechanisms for targeting and killing undifferentiated tumor cells by the NK cells, which is important for the selection of more differentiated tumors that are not targeted by the NK cells.

### Regulation of NK Cell Cytotoxicity by Cystatins and Cathepsins

The granule-based cytotoxicity of NK cells represents the main mechanisms for target cell killing. It is dependent on the activity of granzymes B and A and perforin.[57] They are all synthetized as inactive precursors requiring the proteolytic removal of N-terminal peptide for their activation.[57] The proteolytic activation of granzymes and perforin is performed predominantly by cysteine cathepsins. Cathepsin C,[58] cathepsin H,[58] cathepsin L, and legumain were all suggested to play a role in the proteolytic processing of granzymes and perforin.[59,60]

The activity of cysteine cathepsins is regulated by endogenous inhibitors known as cystatins.[61] Cystatins act mainly as reversible, tight-binding inhibitors of cysteine peptidases. Cystatin F is localized in endosomal and lysosomal vesicles and is thus capable of direct regulation of cathepsins' activity. In NK cells, cystatin F is present within the same secretory granules as cathepsins and granzymes.[62] We have shown that increased levels of the monomeric active form of cystatin F are associated with decreased activities of cathepsins C and H and consequently decreased activation of pro-granzymes in split-anergized NK cells.

As indicated above, both lysis and differentiation of tumors are important for the control of tumor growth and metastasis in cancer patients; unfortunately, however, these functions of NK cells are severely suppressed in cancer patients, which is the subject of the next section in this review.

### Loss of NK Cell Cytotoxicity and Decreased Secretion and Dysfunctional IFN-γ Produced by NK Cells from Cancer Patients

It is well known that both cytotoxic function and secretion of IFN-γ by NK cells are compromised in cancer patients,[23] and these are important mechanisms for the survival and maintenance of tumors in cancer patients since NK cells are the primary effectors to target aggressive tumors. Many mechanisms have been attributed to the loss of NK cell function, including downmodulation and a decrease in important receptors, such as CD16,[63] NKG2D,[64] and ζ chain,[65] as well as decreased survival and expansion of NK cells[18] and

decreased secretion of important cytokines such as IFN-γ.[18] Loss of NK cells not only is the key factor in decreased lysis of CSCs/undifferentiated tumors, but it is also important in decreased secretion of IFN-γ, which contributes to the increase in the frequency and load of undifferentiated tumors.[44] In addition, when using the same amounts of IFN-γ secreted from healthy and cancer patients' NK cells, those from cancer patients had much lower ability to differentiate tumors when compared to IFN-γ secreted from healthy individuals.[18] This finding is of particular interest since it has translational significance. Not only is restoration of IFN-γ secretion by patient NK cells important, but also the functional capability of secreted IFN-γ in differentiation of tumors is of significance for the effective control of tumors in cancer patients.

### Similar to Cancer Patients' NK Cells, Those from Tumor-Bearing Humanized-BLT Mice Are Also Functionally Suppressed

NK cells from tumor-bearing humanized-BLT (hu-BLT) mice were found to have defective NK cell functions similar to those of cancer patients (K.K., data not shown).[18,50,66] Because of similarities between cancer patients and hu-BLT mice with regard to decreased numbers and function of NK cells, humanized mice could serve as an appropriate model to study NK cell-tumor interactions.[50,67] In addition, we have recently shown that NK cells from oral and pancreatic tumor-bearing hu-BLT mice lose significant function and decrease in expansion when compared to non-tumor-bearing mice (K.K., data not shown).[18] Differentiated pancreatic and oral tumors, both patient derived and NK cell induced, were unable to grow or metastasize to vital organs in hu-BLT mice and were highly susceptible to chemotherapeutic drugs, whereas their stem-like tumors grew rapidly and metastasized and were resistant to chemotherapeutic drugs (K.K., data not shown).[50] Poorly differentiated oral and pancreatic tumors grew and metastasized in NSG and hu-BLT mice, and the intravenous injection of highly potent supercharged NK cells (see Novel Strategy to Expand Supercharged NK Cells for Immunotherapy Using Osteoclasts as Feeder Cells) prevented tumor formation in hu-BLT mice (K.K., data not shown).[50] Interestingly, identical percentages of $CD4^+$ and $CD8^+$ T cell subsets and B cells were seen in the peripheral blood of humans and hu-BLT mice, whereas the frequencies of NK cells in hu-BLT mice were approximately half of those seen in peripheral blood of humans, suggesting a plausible reason for the successful establishment of stem-like/poorly differentiated tumors in these mice.

Tumors resected from NK cell-injected hu-BLT mice exhibited a differentiated phenotype, grew very slowly, and did not expand, whereas tumors obtained from non-NK cell-injected tumor-bearing mice grew faster, expanded at a higher rate, and remained highly susceptible to NK cell-mediated cytotoxicity, indicating their poor differentiation phenotype. 18- to 22-fold more human $CD45^+$ immune cells were recruited to tumors dissociated from tumor-bearing mice injected with NK cells in the presence and absence of feeding with AJ2 probiotic bacteria when compared to tumor-alone implanted mice (K.K., data not shown).[50] Loss of NK cell cytotoxicity and decreased IFN-γ secretion in tumor-bearing mice within PBMCs, splenocytes, bone marrow-derived immune cells, enriched NK cells, and purified T cells were restored by injection of NK cells in the presence and absence of feeding with AJ2 probiotic bacteria in hu-BLT mice (K.K., data not shown).[50]

Although suppression of NK cell function in cancer patients is a fairly well-established phenomenon, it is not entirely clear whether suppression of NK cell function is due to the induction and progression of cancer or whether inhibition of NK cell function preceded establishment of cancer and could be an underlying mechanism for the generation of tumors. The next section is focused on the studies conducted on the suppression of NK cell function at the pre-neoplastic stage of cancer.

### Loss of Numbers and Function of NK Cells at the Pre-neoplastic Stage of Pancreatic Cancer by Genetic and Environmental Factors

Pancreatic ductal adenocarcinoma (PDAC) induced by KRAS mutation is the most severe form of pancreatic malignancy.[68] We have recently demonstrated that feeding a high-fat, high-calorie diet (HFCD) to mice with pancreatic KRAS mutation severely inhibited NK cell function at the pre-neoplastic stage of pancreatic cancer.[66,69] NK cell cytotoxicity was decreased in the peripheral blood, spleen, pancreas, and peri-pancreatic tissue in mice with KRAS mutation that were fed with HFCD, whereas in bone marrow, NK cell cytotoxicity was increased when compared to wild-type (WT) mice fed with a lean control diet (CD).[69] Mice with KRAS mutation fed with HFCD demonstrated the highest reduction in the expansion and function of NK cells, followed by mice with KRAS mutation that were fed with a CD and WT mice fed with HFCD as compared to WT mice fed with CD.[69] Interestingly, NK cells cultured with autologous monocytes from mice with KRAS mutation and WT mice fed with HFCD exhibited decreased expansion, cytotoxicity, and IFN-γ secretion. However, when NK cells from WT mice fed with CD were cultured with osteoclasts from KC mice fed with HFCD, NK cells exhibited no or lower cytotoxicity in the presence of increased cytokine secretion.[69] Thus, although NK cell cytotoxicity was always suppressed, depending on the extent of defect either in NK cells or in their interacting cells or both within the microenvironment, distinct profiles of IFN-γ secretion could be obtained.[69] These results suggested that NK cell functional loss could be selective depending on the extent and levels of suppression that NK cells receive from the microenvironment. Loss of expansion and decreased cytotoxicity could precede loss of IFN-γ secretion, and under the most significant inactivation of NK cells all of the key functions of NK cells can be compromised.

Osteoclasts from mice with KRAS mutation fed with HFCD expressed much lower levels of MHC class I inhibitory ligands and RAE1-delta-activating ligands, suggesting that both inhibitory and activating ligands for signaling of NK cells were decreased.[69] The loss of expression was much more severe on osteoclasts from mice with KRAS mutation fed with HFCD as compared to those with KRAS mutation that were fed with CD, and the highest expressions

were seen on the surface of WT mice fed with CD.[69] The decreased levels of MHC class I and RAE1-delta detected on osteoclasts correlated with the generation of pre-neoplastic lesions (pancreatic intraepithelial neoplasias [PanINs]) in mice with KRAS mutation, indicating that the loss of surface receptors on osteoclasts in combination with decreased expansion and function of NK cells may be a better indicator of PanIN induction.[66,69] Thus, our results suggested that NK cell defects induced by both genetic and environmental factors at the pre-malignant stage of pancreatic cancer may drive establishment and progression of pancreatic cancers.[66,69]

PanINs express less CD44 expression and are resistant to NK cell-mediated cytotoxicity, demonstrating more of a differentiated phenotype, whereas KC tumors express higher levels of CD44 expression and are susceptible to NK cell-mediated cytotoxicity, exhibiting more of a poorly differentiated phenotype.[69] Therefore, based on these profiles, one may expect to observe a lower NK cell inactivation in mice at the pre-neoplastic stage than when overt cancer is established.[69] Indeed, severe inhibition of NK cell functions is seen in pancreatic cancer patients and in hu-BLT mice implanted with pancreatic and oral tumors, whereas at the pre-neoplastic stage of tumorigenesis the severity of NK cell suppression may be lower, with a gradual increase in intensity when overt cancer is generated.[18] In agreement, loss of NK cell-mediated cytotoxicity and severe decreases in IFN-γ secretion are likely contributing factors for oral and pancreatic tumor progression in hu-BLT mice (K.K., data not shown).[18]

Interestingly, in contrast to other tissues, bone marrow from mice with KRAS mutation in the presence and absence of feeding with HFCD in comparison to WT mice fed with CD or HFCD demonstrated higher NK cell-mediated cytotoxicity in the presence of lower IFN-γ secretion.[50,69] At the moment, it is unclear why NK cells from bone marrow behave differently as compared to the other tissues examined; however, because NK cells develop and mature within bone marrow, it is possible that the microenvironment provides the primary activating signals that drive their initial activation, and when such activated NK cells exit to the periphery, additional signals from the microenvironment drive these cells to second, third, and fourth stages of NK cell maturation depending on the levels and intensities of signals they receive.

A synergistic increase in IL-6 secretion in the presence of decreased IFN-γ secretion during interaction of peri-pancreatic adipose-derived cells with NK cells could be one mechanism by which the adipose tissue can contribute to the increased proliferation of pancreatic tumors.[69] Indeed, IL-6 is one of the major drivers of PDAC proliferation[70–74] and suppression of NK cell function.[75] Moreover, addition of IL-6 to tumor/NK cell cultures inhibited NK cell-mediated IFN-γ secretion in our previous studies.[4,9,16,43,54] Therefore, since tumors have a predilection to grow in the adipose tissue, these tissues are likely to convert tumor-suppressive NK cells to tumor-promoting cells. Blocking IL-6 may not only inhibit tumor growth, but it may also rescue the NK cells from suppression induced by the peri-pancreatic adipose tissue or those infiltrating the tumor tissues and offer an attractive and effective therapeutic strategy to target pancreatic tumors.

The synergistic contributions of both KRAS mutation and obesity in the generation of pre-neoplastic lesions and their association with the increased loss of NK cell function at the pre-neoplastic stage of pancreatic cancer should be important in the design of the future immunotherapeutic strategies to prevent establishment, growth, invasion, and metastasis of pancreatic tumors.[66,69] In addition, demonstrating the loss of NK cell function associated with a lifestyle factor, such as diet, should also provide the incentive for the establishment of programs in public education and the promotion of lifestyle changes to combat cancer. Our studies support the long-standing notion that both genetic and environmental factors are important in tumorigenesis, and they place NK cells within the list of potential factors for the establishment and progression of pancreatic cancers.

Since previous efforts in reversing suppression of NK cell function have yielded disappointing results in solid tumors, new strategies are desperately needed to combat tumors and restore NK function. The next sections are focused on studies conducted in enhancing the NK cell functions in combination with other therapeutic strategies.

### Novel Strategy to Expand Supercharged NK Cells for Immunotherapy Using Osteoclasts as Feeder Cells

In order to compensate for the lower frequencies of NK cells observed in hu-BLT mice, and to generate large numbers of NK cells with potent function, we generated efficient *ex vivo* human NK cells for adoptive NK cell transfer therapy of human CSCs, using osteoclasts as feeder cells. We have previously shown that this myeloid-derived subset is a potent activator of NK cells, and their effect in the induction of cytotoxicity and secretion of cytokines and chemokines by NK cells is much stronger than that of monocytes or dendritic cells.[76] Human osteoclasts produce IL-15, IL-12, IL-18, and IFN-α, but not IFN-γ, and express lower levels of MHC class I and II, CD14, CD11b, and CD54, and they minimally upregulate MHC class I surface expression when treated with either the combination of TNF-α and IFN-γ or when treated with activated NK cell supernatants known to increase MHC class I expression.[76] Low expression of MHC class I together with increased release of IL-15, IL-12, IL-18, and IFN-α may represent some of the mechanisms by which osteoclasts are able to expand functionally potent NK cells. More importantly, osteoclasts also exhibit higher expression of NKG2D ligands.[76]

Several *in vitro* NK expansion techniques have been developed to allow for a higher therapeutic cell dose.[77,78] Using our strategy, we expanded highly functional NK cells at the levels that were significantly more superior to those established by other methodologies.[18] In addition, expansion of purified cancer patients' NK cells, unlike purified NK cells from healthy individuals, was significantly limited due to the faster expansion of a very small fraction of contaminating T cells (0.2%–1%) that eventually crowded out the NK cells by their

faster proliferating capability. The mechanism for the faster expansion of patient T cells was found to correlate with decreased NK cell cytotoxic function.[18] As mentioned earlier, it is possible that functionally competent NK cells are required for the maintenance of decreased expansion of T cells, especially T regulatory cells (Tregs) and MDSCs, both of which are known to suppress NK cell function.[79] Indeed, $CD4^+$ but not $CD8^+$ T cells are targeted and lysed by the NK cells (K.K. and M.W.K., data not shown). Faster expansion of contaminating T cells within purified NK cells was also seen in tumor-bearing hu-BLT mice.[18]

Not only is good expansion of NK cells under different experimental conditions important for the eventual efficacy of NK cells in cancer therapy, but also their functional competency is important for targeting tumors. Our ongoing studies indicated that cord blood-derived and induced pluripotent stem cell (iPSC)-derived NK cells are able to expand large numbers of cells with the NK cell phenotype, but they are not capable of targeting and lysing CSCs/poorly differentiated tumors or producing sufficient amounts of IFN-γ (K.K. and M.W.K. data not shown) when either compared to primary NK cells derived from peripheral blood or to supercharged NK cells. Standardization among all different NK cell platforms for immunotherapeutics and their functional comparisons should provide the basis for the selection of the best products to be used in immunotherapy. In addition, it may also provide the basis for why the use of such products was not successful in controlling the disease in the past clinical trials.

### Different Efficacy of NK Cell Expansion and Function Using Allogeneic versus Autologous NK Cells from Healthy or Cancer Patients

Not only tumor cells but also non-transformed stromal cells within the tumor microenvironment, in particular other immune effectors, may affect the expansion and function of NK cells. We have previously shown that monocytes, dendritic cells, and osteoclasts can each increase NK expansion and function to varying degrees, with osteoclasts being the best.[18] The best NK cell expansion and function were seen when NK cells from healthy donors were used in cultures with their autologous osteoclasts. In contrast, patient NK cells with autologous osteoclasts had the most severe defect in NK cell expansion and function (K.K., data not shown). Similar results to those of cancer patients were also seen in tumor-bearing hu-BLT mice (K.K., data not shown). Thus, when designing immunotherapeutic strategies using autologous or allogeneic NK cells, such differences in the levels of NK cell expansion and function should be considered and may be crucial for the success of the therapy. In addition, to increase the effect of NK cell therapy, the treatment may be combined with other therapeutic strategies detailed in the following sections.

### Immunotherapy Is Essential in Combination with Chemotherapy: Chemotherapy Only Targets Differentiated Tumors and Not Cancer Stem-like Cells

CSCs/undifferentiated tumors were shown to be chemoresistant due to their increased expression of multi-drug resistance and DNA mismatch repair genes.[80] Increased survival and selection of CSCs/undifferentiated tumors after chemotherapy result in relapse, metastasis, and invasion of tumors.[81,82] CSCs/undifferentiated tumors, although they are highly susceptible to NK cell-mediated cytotoxicity, they are quite resistant to either *cis*-diamminedichloroplatinum (CDDP), a chemotherapeutic drug also known as cisplatin, or radiation-induced cell death, whereas their differentiated counterparts, even though are resistant to NK cell-mediated cytotoxicity, they are susceptible to both CDDP- and radiation-induced cell death.[4]

CDDP induced significant cell death in OSCCs (oral squamous cell carcinomas) when compared to OSCSCs (oral squamous CSCs). Similarly, OSCCs were highly susceptible to radiation treatment, whereas OSCSCs did not undergo cell death after radiation.[4] Differentiation of OSCSCs or MP2 (MiaPaCa-2, pancreatic poorly differentiated/CSCs) tumors with supernatants from split-anergized NK cells resulted in a significant increase in their susceptibility to CDDP, whereas undifferentiated OSCSCs or MP2 cells remained relatively resistant to CDDP. Melanoma tumors stably expressing short hairpin RNA (shRNA) against firefly luciferase (A375shLUC) demonstrated higher differentiation antigens and were significantly more susceptible to CDDP-mediated cell death when compared to those that had knockdown of CD44 (A375shCD44). Differentiation with split-anergized NK cell supernatants increased CDDP-mediated cell death of A375shCD44 cells substantially.[4] Therefore, although stem-like oral and pancreatic tumor cells are highly susceptible to NK cell-mediated cytotoxicity, they remain quite resistant to either CDDP-mediated or radiation-induced cell death, whereas their differentiated counterparts are killed efficiently by these treatments.[4] These data suggest that knockdowns of cellular genes and their reversion to a less differentiated phenotype may activate NK cell-mediated cytotoxicity, but this may also lead to the resistance of these cells to chemotherapeutic agents. Thus, stage of differentiation is a determinant of tumor susceptibility to NK cell-mediated cytotoxicity as well as their response to chemotherapeutic drugs. Therefore, a tailored therapeutic scheme using a combination of NK cell immunotherapy and chemotherapy is important for efficient elimination of both CSCs/undifferentiated and differentiated tumors present in the tumor nest.

### Combination of NK Cell and Tumor-Specific Antibody Therapy: NK Cells Can Target CSCs and Their Differentiated Counterparts through Direct Lysis and/or ADCC, Respectively

NK cells can eliminate both undifferentiated and differentiated tumors through direct killing and through ADCC, respectively.[13,83,84] Indeed, if antibodies against specific receptors expressed on differentiated tumors are available, even though NK cells will not be able to lyse these cells directly, they can eliminate such tumors through ADCC. Such observations were made with regard to the increased expression of MICA/MICB on differentiated tumors, and much less on CSCs/undifferentiated tumors, and the differentiated tumors were found to be targeted greatly through NK cell-mediated ADCC but minimally through direct lysis.[4] Similarly, antibodies targeting specific receptors on CSCs/undifferentiated tumors should be able to lyse these tumors through NK cell-mediated ADCC as well as direct lysis. In addition, sensitizing antibodies such as IL-4 mAbs

have been used in combination with standard chemotherapeutic strategies to successfully target CD133$^{bright}$ CSCs of colon cancer.[34] A large number of antibodies targeting tumors such as anti-EGFR, anti-HER2, and anti-PD-L1 antibodies, to name a few, have been generated and are currently being used to treat patients. These antibodies could potentially not only kill tumors directly but also indirectly through NK cell-mediated ADCC.[85]

### NK Cells Are Activated by Oncolytic Virus-Infected Pancreatic Cancers

Genetically modified oncolytic viruses (OVs) have become an exciting therapeutic modality for cancer patients with approved agents for cancer therapy.[86] Genetically engineered viruses kill cancer both directly by infection and lysis of tumors, and indirectly by inducing host cytotoxic immunoresponses to cancer by modulating the immunosuppressive nature of the tumor microenvironment. Immune cell infiltration in tumors is predictive of response to immunotherapies, with the type I and type II IFN pathways being the key players in activation of adaptive immunity.[87]

OVs enhance immune activation against tumors through multiple mechanisms. OVs enhance infiltration of tumors by immune cells, upregulate costimulatory molecules on cancer cells, enhance immunogenic cell death and antigen release,[88] and activate the type I IFN pathway. Pre-clinical[89] and clinical evidence[90] clearly indicates that OVs turn immunologically "cold" tumors into "hot" tumors. Treatment of refractory disseminated melanoma with agents targeting immune checkpoint inhibition is greatly enhanced by oncolytic herpes viral therapy.[91]

OV therapies not only activate T cells, but also NK cells. In a recent preclinical study of Newcastle disease virus (NDV), intra-tumoral therapy of B16 melanoma with oncolytic NDV was shown to induce inflammatory responses, leading to lymphocytic infiltrates and an antitumor effect locally and in distant tumors without virus spread. Immune cell depletion studies showed that both NK and CD8$^+$ T cells were essential for the therapeutic effect of OVs.[89] NDV was also shown to activate NK cells.[91] It appears that NK cells are key immune cells for early inflammatory responses and IFN-γ production after OV therapy, while CD8$^+$ T cells are responsible for long-term antigen-specific tumor control.[89]

Although NK cells may kill infected cancer cells and limit the amplification of OVs, studies have found that NK cells often have positive effects on therapeutic outcomes of OVs.[92–95] Different types of OVs have been engineered to target and lyse pancreatic tumors. They have been engineered to not only be effective in lysing the tumors but also to allow effective activation of immune effectors, in particular NK cells.[96,97] Virus-infected cancer cells tend to downregulate their MHC class I, making them good targets for NK cells.[92,93]

This ability of the virus to down-modulate MHC class I on tumors occurs both in poorly differentiated as well as in well differentiated tumors. We have found that different preparations of OVs have different potencies in lysing the tumor and activating the function of NK cells. Infection of differentiated PL-12 pancreatic tumor cells with different preparations of OVs demonstrated different effects on the decrease in MHC class I, as well as their ability to increase functional activation of NK cells (Figures 1A and 1B). Whereas significant inhibition of MHC class I could be observed in the presence of 33-GFP OV, no or lower levels of MHC class I decrease can be seen by 189 OVs when compared to mock-infected PL-12 pancreatic tumors. As indicated before, differentiated tumors express higher levels of MHC class I, PD-L1, and CD54 and lower levels of CD44. Treatment of PL-12 with 33-GFP OVs also decreased the expression of CD54 and PD-L1. Interestingly, the expression of CD44 was slightly elevated with 33-GFP OV-treated PL-12 tumors when compared to either 189 OV- or mock-treated tumors (Figure 1A). Accordingly, higher induction of IFN-γ secretion by the purified NK cells was observed when these cells were cultured with 33-GFP-infected PL-12 cells as compared to either mock- or 189 OV-infected PL-12 cells (Figure 1B). These data indicated that OVs may be able to decrease key surface antigen expression on differentiated tumors, making them more susceptible to NK cells. Future investigations should be directed to demonstrate whether there are differences in the capability of viruses to infect CSCs/undifferentiated tumors versus differentiated tumors, and the levels of their infection, or whether such virus-infected tumors will allow higher NK cell-mediated ADCC.

### Combination Therapy with NK Cells and Immune Checkpoint Inhibitors

Treatment with checkpoint inhibitors has revolutionized the field of immunotherapy and shown impressive results in a selected group of patients. Many recent reports have indicated the ability of NK cells to become activated through checkpoint inhibitions.[98–100] In particular, the role of the anti-PD-1/PD-L1 axis in NK cell inhibition and the ability of antibody to anti-PD-1 to activate NK cells have been studied in many laboratories, including ours.[98,100] We have shown previously that when tumors are implanted in the oral cavity[50] or in the pancreas of hu-BLT mice (Figure 2), and injected either with supercharged NK cells or with anti-PD-1 antibody, both were able to activate immune cells to secrete higher amounts of IFN-γ when compared to tumor-implanted mice in the absence of these agents (Figure 2). Remarkably, infusion of NK cells and treatment with anti-PD-1 antibody had a synergistic effect and increased IFN-γ secretion substantially when compared to the infusion of each agent alone (Figure 2). The increased secretion of IFN-γ can be both from the NK cells and T cells since activated NK cells can also activate T cells, thereby providing increased expression of PD-1 on the T and NK cells, making them more susceptible to activation through anti-PD-1 antibody. Anti-PD-1 antibody treatment had a differential effect on cytotoxicity versus IFN-γ secretion in hu-BLT mice implanted with oral tumors and infused with supercharged NK cells and fed with AJ2 probiotic bacteria. Whereas NK cell-mediated cytotoxicity was increased by the treatment of anti-PD-1 antibody, secretion of IFN-γ was variable due likely to the plateauing effect of IFN-γ secretion by the treatment of supercharged NK cells in the presence of AJ2 feeding.[50] Nonetheless, there is a clear effect of anti-PD-1antibody

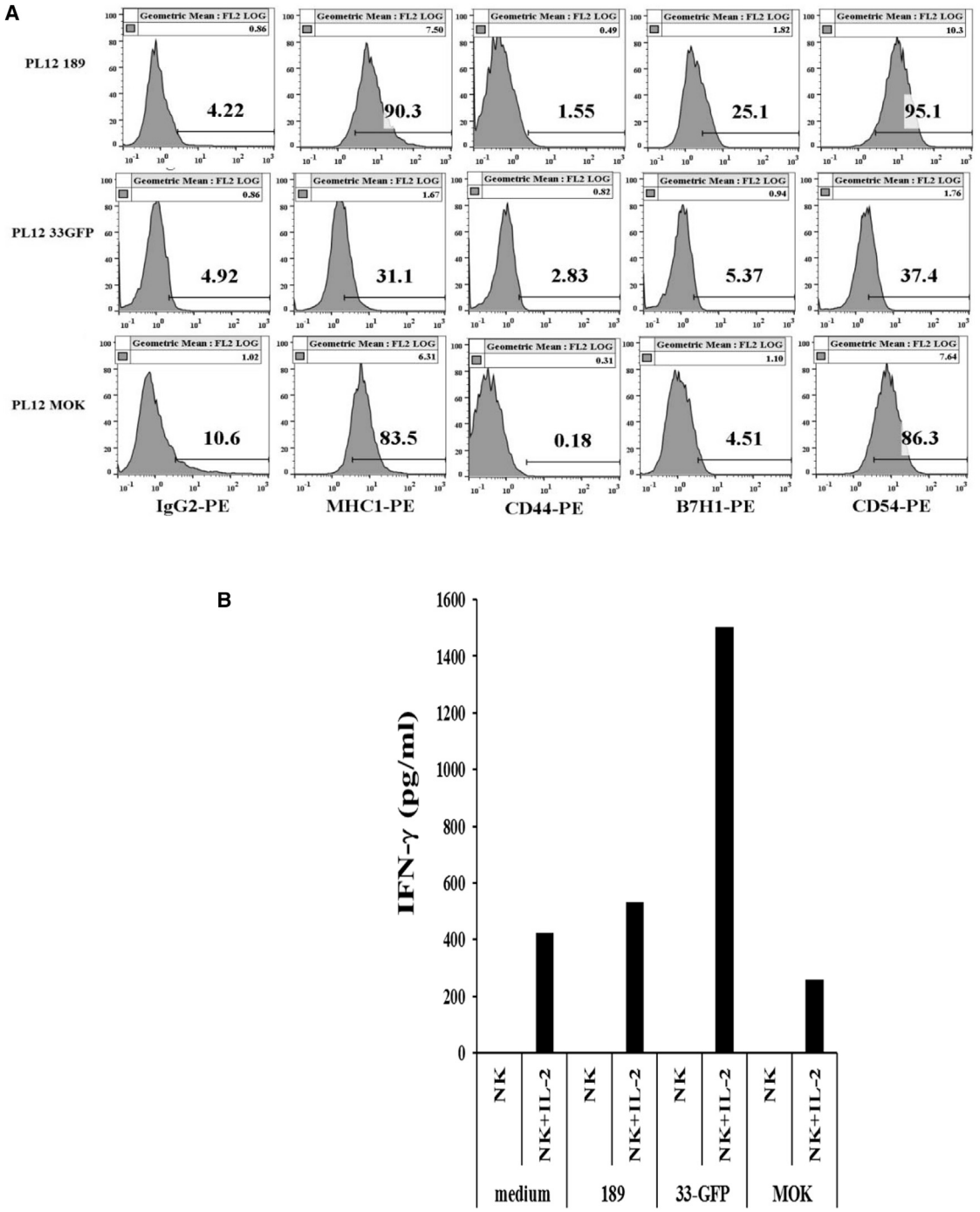


**Figure 1. 33-GFP OV-Infected PL-12 Tumors Down-modulate MHC Class I, CD54, and PD-L1 Receptors and Trigger IFN-γ Secretion from Purified NK Cells**
PL-12-differentiated pancreatic tumors were infected with 189 OV, 33-GFP OV, or mock OV and the levels of MHC class I, CD54, B7H1, and CD44 receptors were determined. These tumors express higher levels of MHC class I, CD54, and B7H1 and much lower levels of CD44. (A) After 33-GFP OV infection, the levels of MHC class I, CD54, and B7H1 decreased substantially on the surface of the tumors when compared to either mock-infected or 189 OV-infected PL-12 tumors. (B) 33-GFP-infected PL-12 tumors cultured with the NK cells induced higher IFN-γ secretion from the NK cells when compared to either mock-infected or 189 OV-infected PL-12 tumors.

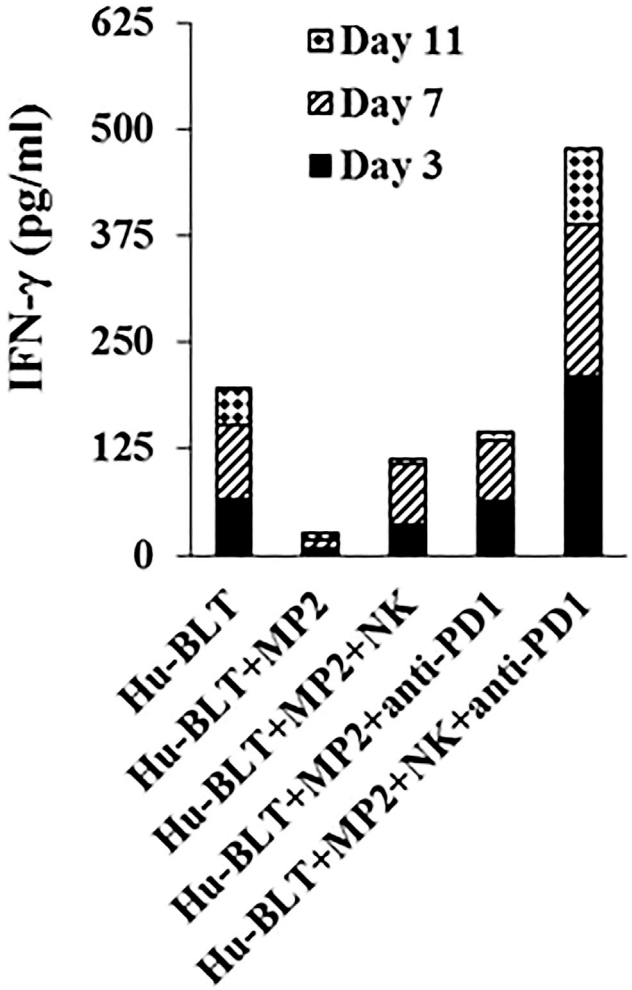


**Figure 2. Combination of Supercharged NK Cells with Anti-PD1 Antibody Injection Increased IFN-γ Secretion by PBMCs from hu-BLT Mice**

Reconstituted hu-BLT mice were orthotopically implanted with $1 \times 10^6$ of human MP2 tumors in the pancreas. 10–14 days after tumor implantation, hu-BLT mice received $1.5 \times 10^6$ supercharged NK cells via tail vein injection. Seven days later, anti-PD1 (50 μg/mouse) was injected via tail vain injection. At the end of experiment, mice were sacrificed and PBMCs were obtained and cultured with IL-2 (1,000 U/mL), and the supernatants were harvested at the indicated days in the figure, and the levels of IFN-γ were determined using ELISA.

treatment when combined with supercharged NK cells in the presence of probiotic bacteria AJ2.[50]

More recent work has implicated other checkpoint inhibitors such as TIGIT in NK cell function, and their blockade was shown to activate NK cells.[101,102] Future immunotherapeutic strategies may make use of the blockade of several different checkpoint inhibitors for effective targeting of tumor cells by NK cells.

### Conclusion

Significant advances have been made in the studies of NK cells in cancer patients in recent years. Studies from our laboratory and those of others have shown significant suppression in the functions of NK cells and T cells in cancer patients, indicating that successful cancer therapy will require restoration of both NK and T cell functions in cancer patients since each is likely designed to target different subsets of tumor cells with opposing degrees of cellular differentiation. NK cells are likely targeting CSCs/undifferentiated tumors with no or lower MHC class I expression, and T cells are targeting differentiated tumors with higher expression of MHC class I. NK cells mediate successful control of the tumor cells by their direct cytolytic effect and/or through antibody-mediated ADCC or indirectly through differentiation of tumor cells by IFN-γ, which increases the efficacy of chemotherapeutic and radiotherapeutic targeting strategies. Because of significant changes in the immune environment of cancer patients, selection of allogeneic or autologous NK cell immunotherapy should be considered carefully since most cancer patients have defective NK cell function. In addition, strategies should be designed to allow maintenance of good NK expansion and function in cancer patients since NK cells are likely to limit the expansion of tumor-associated macrophages (TAMs), Tregs, and MDSCs, all of which are the hallmarks of aggressive tumors. Large numbers of allogeneic supercharged NK cells can be combined with other immunotherapeutic strategies such as OVs, ADCC-inducing antibodies, checkpoint inhibitors, chimeric antigen receptor (CAR) T cells, CAR NK cells, and chemotherapeutic and radiotherapeutic strategies for the ultimate goal of tumor eradication (Figure 3).

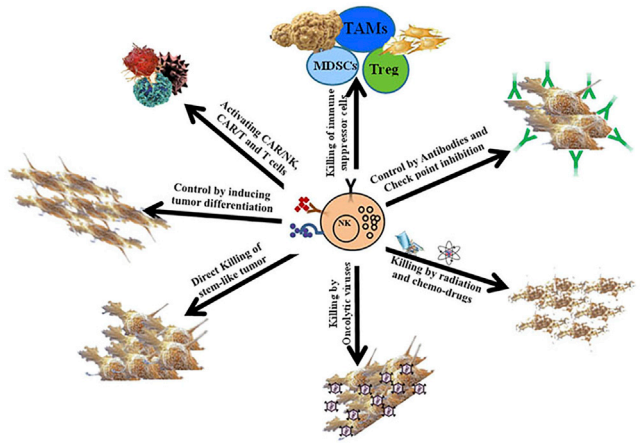


**Figure 3. Schematic Representation of Diverse NK Cell Functions against Cancer**

NK cells limit the expansion of tumor cells by their direct targeting of CSCs and by differentiation of tumor cells, which halts their expansion and growth. In addition, they are known to target and kill tumor-associated macrophages (TAMs), Tregs, and MDSCs, which are known to inhibit the function of NK and T cells. Large numbers of allogeneic supercharged NK cells can be combined with other immunotherapeutic strategies such as oncolytic viruses, ADCC antibodies, checkpoint inhibitors, CAR T cells, CAR NK cells, and chemotherapeutic and radiotherapeutic strategies for the ultimate goal of tumor eradication.

## AUTHOR CONTRIBUTIONS

A.J. oversaw the design of the experiments and data analysis, prepared grant proposals, and wrote the manuscript. J.K. and Y.F. reviewed and

edited the manuscript. K.K. performed the majority of the experiments, performed data analysis and literature searches, and assisted in the preparation of the manuscript. T.S., C.S., and M.W.K. performed experiments and were responsible for review and editing of the manuscript. W.C., P.W., A.K.N., and C.F. assisted in performing the experiments and reviewed and edited the manuscript.

## CONFLICTS OF INTEREST

The authors declare no competing interests.

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