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Review

Supercharged NK Cells Are a Unique Population of Cells Based on Transcriptomic, Single-Cell RNAseq, Proteomic, and Functional Characteristics; Comparison with Other NK Cell Subpopulations

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Abstract: Natural killer (NK) cells are large granular lymphocytes with potent anti-tumor activity. However, their use in clinical trials has resulted in less than favorable outcomes due to the lack of reliable methods to augment their expansion and anti-tumor potential. Based on the previous publications and our ongoing studies, we present the characteristics of sNK cells that make these cells optimal for clinical trial applications. sNK cells have significantly high anti-tumor activity as well as increased survival and augmented expansion when they are cultured with osteoclasts and probiotic bacteria. In this review, we compare the expansion and function of sNK cells with other NK cell expansion methodologies to indicate the similarities and differences between sNK cells and other NK cell expansion methodologies. We also describe the uniqueness of sNK cells in terms of increased survival, expansion, and heightened function as determined by proteomic, transcriptomic, single-cell RNAseq, and functional characteristics. These cells not only target and kill poorly differentiated/stem-like tumors, but also can target differentiated tumors. In addition, they can restore the functional loss of autologous NK cells, allowing the patient's NK cells to become functional. These attributes of sNK cells make these cells not only unique population of NK cells but also cells that can effectively be used in clinical trials of cancer patients.

Keywords: NK cells; supercharged NK cells; osteoclasts; probiotic bacteria; cytotoxicity; IFN-γ

1. Why Do We Need NK Cell-Based Cancer Immunotherapies?

Current treatments for the most aggressive tumors include surgery, radiation therapy, chemotherapy (examples include but are not limited to Paclitaxel, Gemcitabine, Fluorouracil, Irinotecan liposome injection, etc.), targeted therapy, T-cell therapy, and other forms of immunotherapy. In advanced cases, these treatments are not likely to offer a benefit; thus, the physician will focus on palliative care for as long as possible [1,2]. Most explored cancer immunotherapies include: monoclonal antibodies, checkpoint inhibitors, bispecific antibodies, T Cell redirecting antibodies, vaccination strategies, gene-modified T cells, donor lymphocyte infusions (DLI) and antigen-specific DLI, CAR T-cells, and TCR gene-modified T cells. Interest in adoptive cell therapy for treating cancer is exploding, given the early clinical successes of autologous chimeric antigen receptor (CAR) T lymphocyte therapy [3–5]. However, limitations using T cells and autologous cell products are apparent as they (A) take weeks to generate, (B) utilize a 1:1 donor-to-patient model, (C) are expensive, and (D) are prone to heterogeneity and manufacturing failures [6]. CAR T cells are also associated with significant toxicities, including cytokine release syndrome, immune effector cell–associated neurotoxicity syndrome, and prolonged cytopenia [7,8]. To overcome these issues, natural killer cells (NK) are being explored as an alternative cell source for

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allogeneic cell therapies [9–11], and are currently being investigated in ongoing clinical trials as single therapy or combination therapy [12–15], and have thus far yielded many encouraging clinical results [16].

NK cells may represent a valid alternative to T cells, due to their inherent nature as part of the innate immune response to aggressively attack major histocompatibility complex (MHC) class I-deficient or mutated cells [17]. Although tumors may develop multiple resistance mechanisms to endogenous NK cell attack, in vitro activation, expansion, and genetic modification of NK cells can greatly enhance their anti-tumor activity and give them the ability to overcome drug resistance [18,19]. Unlike T cells, NK cells activation is managed by the interaction of NK cells receptors with their ligands on the target cell; this process is not dependent on antigen processing or presentation [20]. NK cells can be obtained from blood or cord blood, or be derived from hematopoietic stem and progenitor cells or induced pluripotent stem cells, and can be expanded and cryopreserved for off-the-shelf availability [20–22]. NK cell progenitors or mature NK cells can be infused with other cells as part of the Hematopoietic stem-cell transplantation (HSCT) or alone following the pre-enrichment process. Inhibitory receptors on donor allogeneic NK cells (e.g., KIR) do not recognize human leukocyte antigen (HLA) class I on recipient cells in case of a class mismatch. Therefore, the donor NK cells are relieved of their repressive receptor-triggered inhibition. In this case, cancer cells lack the suitable class I MHC ligands to engage the repressive KIR, and thus, they are removed by allo-reactive NK cells [16,23,24]. Numerous reports have revealed that allogeneic NK cells potentially trigger remission or suppress relapse of the tumor in cancer patients [25,26]. In a clinical trial of haploidentical NK cells for AML, the authors reported the induction of complete remission in dismal prognosis or elderly individuals and a 100% event-free survival rate at 18 months in a pediatric cohort [1,2]. Through these advances, NK cell–based therapies provide a complementary clinical strategy to, and overcome limitations of, US Food and Drug Administration–approved chimeric antigen receptor T-cell therapies [27–29].

Natural killer (NK) cells are the large granular Lymphocytes with potent anti-tumor and anti-viral functions. However, clinical use of these cells is hampered due to a lack of reliable methods to augment their expansion and anti-tumor potential. Based on the previous publications, in this review, we summarize the characteristics of supercharged NK (sNK) cells that make these cells optimal to be used in cell therapy. We provide a comprehensive review on sNK, the NK cell subsets with significantly increased anti-cancer activity (cytotoxic function and the secretion levels of cytokines). The technology to develop supercharged NK cells was demonstrated in several publications generated from our laboratory [30–33]. We also discuss the preclinical efficacy and safety of sNK cells in humanized mice [31,32,34–38] and clinical efficacy and safety in human patients infused with sNK cells [39].

2. Supercharged NK Cells

To generate highly potent supercharged NK cells (sNK), PBMC-derived NK cells are co-cultured with osteoclasts (OCs) and sonicated probiotic bacteria (sAJ2). Osteoclasts were selected among several feeder cells because they express ligands for NK cell receptors such as MICA/B, KLRG1, and ULBPs on their surface and secrete cytokines and chemokines, including IL-12, IL-15, IFN-γ, and IL-18, which are known to activate NK cells [40,41]. Probiotic bacteria sAJ2 is a combination of seven gram-positive probiotic bacteria *Streptococcus thermophiles*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Lactobacillus paracasei*. These strains were selected based on their activation in NK cells. The combination of these probiotic treatments in NK cells was found to increase cytokine secretion by NK cells, including IFN-γ, which could facilitate signals required for NK cell expansion [42–46]. Therefore, a combination treatment of both probiotics and OCs in NK cells results in the induction of signals participating in cell expansion and functional activation of NK cells, generating sNK cells. sNK cells have demonstrated increased life-span, cell expansion, cytotoxicity, and secretion of cytokines; these sNK

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cell characteristics ultimately result in increased differentiation and killing of oral cancer both *in vivo* and *in vitro* [32,35,44].

sNK cells are a unique population of NK cells with completely distinct profiles from those of primary peripheral blood-derived NK cells at the RNA seq analysis at the single cell level based on UMAP (an algorithm that takes a high-dimensional dataset) and regulon profiles, and in terms of cell cycle analysis, granule content, and functional capabilities [31]. sNK cells have very high anti-tumor cytotoxicity and are polyfunctional [31]. sNK cells demonstrated higher expression of activating receptors and down-modulation of inhibitory receptors [44]. In addition, sNK cells demonstrated increased secretion of IFN-γ and TNF-α, elevated levels of Trail expression at the single-cell transcriptomic analysis, higher levels of BCL2, and were able to resist the induction of cell death and loss of cytotoxicity within the tumor microenvironment [47]. Moreover, in contrast to primary activated NK cells, sNK cells were able to lyse both stem-like and differentiated tumors [48]. sNK cells were found to be superior to many other NK cell treatments, including IL-2, IL-2 and anti-CD16 mAbs, IL-2 and anti-CD16 mAbs, and sAJ2 [47,49], treatment with other NK-specific cytokines such as IL-12, IL-15, and IL-18 and OSCSCs or K562 expanded NK cells [44,47,50,51]. In addition, when compared to cord blood-derived NK cells, iPSC-derived NK cells, NK92, and several other NK cells, sNK cells exhibited much higher levels of cytotoxicity and cytokine secretion [36,48]. sNK cells induce tumor killing irrespective of MHC-class I expression on tumor cells, making them a unique NK cell-based treatment strategy in heterogeneous tumors [36]. We recently explored the proteomic, transcriptomic, and functional characterization of sNK cells and found that sNK cells were less susceptible to split anergy and tumor-induced exhaustion. Split anergy is the stage of NK cells previously defined in our studies [52–54]. At this stage, NK cells reduced their cytotoxic function in the presence of significant secretion of cytokines [52–54]. Proteomic analyses revealed that sNK cells significantly increase their cell motility and proliferation. Single-cell transcriptomes indicated that sNK cells undertake a unique differentiation trajectory and turn on several important regulons essential for augmenting anti-tumor effector functions and proliferation. Both proteomic and single-cell transcriptomes revealed that an increase in Cathepsin C augmented the quantity and function of Granzyme B. The results obtained support the use of sNK cells for clinical utilization and delineate the molecular mechanisms associated with their maturation [31,36,55] (manuscript in press).

3. History: A Historical View of NK Expansion Methodologies and Their Differences with Supercharged NK Cells

Since its discovery, the NK biologists have attempted to discover strategies that could be safely used to effectively treat cancer patients. Even though these cells were shown to be extremely safe in cell therapy, the level of efficacy has always been challenged due to several issues. Unlike T cells, which can comprise 60 to 40% of lymphocytes, NK cells are only 5-10% on average in the peripheral blood [56]. Also, the functions of NK cells are impacted during preneoplasia and cancer [57,58]. NK cells are quick to become inactivated in the tumor microenvironment, even when they are activated by cytokines or other methods. There is a lack of persistence of NK cells when injected into the patients. These qualities of NK cells have made these cells less desirable for cell therapy. However, recently, several methodologies have been found to overcome the lack of adequate expansion of NK cells ex vivo [30,59–67]. Unfortunately, many of such methodologies give rise to larger numbers of NK cells with poor quality of for a methodology to not only expand well but also retain NK cell functional capabilities [56]. Even though we are getting closer to having effective cells, we are still not entirely there. Although many studies claim that they have a significant expansion of NK cells with the use of cytokines, the majority of them have been less desirable due to the variability of the donor-derived NK cells' expansion and functional capabilities. Thus, the search for super donors makes NK cell-based therapy very limited. Feeder layer-dependent expansion, such as K562 or OSCSCs, has been less than desirable for expansion [30,31]. Also, genetic manipulation expressing 4IBBL and IL-15 or IL-21 has gained popularity for the expansion of the NK cells [30,65,68–75]. It is not clear whether such expansion will increase the functional activation of NK cells maximally and

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for how long. Knockdown of genes in NK cells was shown to increase the functional activation of NK cells [76]. This characteristic is very common for the function of NK cells since either the knockdown is in the target cells or the NK cells will result in the activation of the NK cells [76,77]. Again, no experiments have been performed to compare this platform to the other existing expansion methodologies; as such, it is difficult to compare with other platforms.

Although we did not have access to all the different kinds of platforms that have been engineered for expansion and functional activation of NK cells, we had the opportunity to compare the sNK cells with a few established strategies. When comparing sNK cells to either IL-2-activated cord blood-derived NK cells or iPSC-derived NK cells, no NK cell-mediated cytotoxicity and slight IFN-γ secretion could be seen, whereas there were substantial increases in cytotoxicity and secretion of IFN-γ by the sNK cells. We have characterized NK92 cells for their function previously and found them to have no or very slight cytotoxicity and secretion of IFN-γ [48]. In addition, using K562 or OSCSCs as feeder cells, we could not see appreciable levels of expansion and function [30,31]. Likewise, although we could see an increase in NK cell function with the combination of cytokine treatment, we could not see increased expansion of the cells [30,31]. When we used K562 or OSCSCs or MP2 or PBMCs as feeder cells to expand NK cells, and compared to NK expanded using OCs as feeder cells, an increased rate of NK cell proliferation, increased NK cell survival in culture, much higher cytotoxicity, and higher levels of cytokine secretions were seen in NK cells expanded using OCs as feeder cells [30,31].

4. Current Findings:

4.1. Infusion of sNK Cells in Humanized Mice or Humans Leads to a Significant Increase in the Percentages of NK Cells and Restores or Increases Function in Autologous NK Cells

When assessing the expansion and function of autologous NK cells after sNK infusion, either in mice (31, 32, 34, 35) or humans (manuscript in press), we could see a substantial improvement in the NK function in pancreatic, oral, and melanoma-implanted humanized mice [31,32,34,35]. There was a 47% to 94% improvement in NK cytotoxicity depending on the tissue examined when sNK cells were infused in pancreatic tumor-bearing mice (Table 1A). Likewise, there was 69% to 97% improvement in secretion of IFN-γ, depending on the tissue examined, when sNK cells were infused in pancreatic tumor-bearing mice (Table 1A). Similarly, there was 78% to 98% improvement in NK cytotoxicity depending on the tissue examined when sNK cells were infused in oral cancer-bearing mice (Table 1B). There was 76% to 98% improvement in secretion of IFN-γ, depending on the tissue examined, when sNK cells were infused in oral cancer-bearing mice (Table 1B). There was 69% to 85% improvement in NK cytotoxicity, depending on the tissue examined, when sNK cells were infused in melanoma-bearing mice (Table 1C). There was a 56% to 93% improvement in the secretion of IFN-γ, depending on the tissue examined, when sNK cells were infused in melanoma-bearing mice (Table 1C). In humans, we observed increased percentages of autologous NK cells and increased function of NK cells in cancer patients (manuscript in press).

Table 1. Efficacy of sNK cells in restoring immune function in tumor-bearing humanized mice.

A. Pancreatic tumor-bearing humanized mice

Tissues or isolated cells	**P values (tumor untreated mice vs. tumor mice treated with sNK cells)**		**(LU or IFN-γ of sNK cell-treated tumor mice/LU or IFN-γ of healthy mice)*100**		**# of mice**
	Cytotoxicity	**IFN-γ**	**Cytotoxicity**	**IFN-γ**	
Peripheral blood	**	*	94%	69%	9
Bone marrow	*	*	75%	67%	9

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Spleen	*	*	66%	72%	9
Pancreas/pancreatic tumor	**	**	**88%**	**97%**	9
Oral mucosa	**	*	92%	84%	6
NK cells isolated from spleen	*	*	64%	59%	9
NK cells isolated from PBMCs	*	*	47%	69%	9

B. Oral tumor-bearing humanized mice

Tissues or isolated cells	**P values (tumor untreated mice vs. tumor mice treated with sNK cells)**		**(LU or IFN-γ of sNK cell treated tumor mice/LU or IFN-γ of healthy mice)*100**		**# of mice**
	Cytotoxicity	**IFN-γ**	**Cytotoxicity**	**IFN-γ**	
Peripheral blood	**	**	98.00%	97%	8
Bone marrow	**	**	96.70%	98%	8
Spleen	**	**	92.80%	96%	8
Oral mucosa	**	*	94%	87%	5
NK cells isolated from spleen	*	*	77.80%	76%	8
NK cells isolated from PBMCs	**	***	88%	98%	8

B. Melanoma-bearing humanized mice

Tissues or isolated cells	**P values (tumor untreated mice vs. tumor mice treated with sNK cells)**		**(LU or IFN-γ of sNK cell-treated tumor mice/LU or IFN-γ of healthy mice)*100**		**# of mice**
	Cytotoxicity	**IFN-γ**	**Cytotoxicity**	**IFN-γ**	
Peripheral blood	*	*	73%	76%	6
Bone marrow	*	*	69%	77%	6
Spleen	NA	*	NA	56%	6
NK cells isolated from the spleen	**	**	85%	88%	6
T cells isolated from PBMCs	**	**	83%	93%	6

Table 1: Humanized mice were orthotopically injected with 1 x 10^6 human MIA PaCa-2 (MP2) (A), oral tumor CSCs (B), or melanoma tumor cells (C) into the pancreas, oral, and subcutaneously, respectively. For pancreatis and oral tumors, one week post-tumor implantation, mice were administered 1 x 10^6 sNK cells via tail-vein injection. For melanoma, one week post-tumor

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implantation, mice were administered 2 x 10^6 sNK cells every 7 days via tail-vein injection. At week 5, the mice were sacrificed, and tissues were collected. Single-cell isolation from tissues, NK cells, or T cells were cultured with IL-2 (1000 U/mL) for 7 days. After incubation, cells were used as effectors against OSCSCs in a standard 4-hour ^{51}Cr release assay (cytotoxicity), and the supernatants were collected to measure IFN-γ secretion levels using ELISA. The restoration of cytotoxic function and IFN-γ secretion was quantified using the formula:(LU or IFN-γ of sNK-treated tumor mice / LU or IFN-γ of healthy mice) × 100.

4.2. Safety of sNK Cells for Human Use

In terms of safety, there was no evidence of treatment-related toxicities or graft vs. host disease [31,32,34,35]. Indeed, when infused into humans, patients indicated that they felt more energy and all their aches and pains had disappeared. The safety of these cells was determined both in adult populations and in pediatric populations. Unlike sNK cell infusion, treatment-related toxicities were very frequently seen in other well-established cancer therapeutics, such as CAR-T therapies, which are found to be associated with cytokine-release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) [78–84]. In addition, in CAR-T or CAR-NK cells, if the tumor downmodulates or loses the particular CAR, it has to be manufactured again, whereas sNK cells target all different types of tumors. Unlike CART cells, sNK cells do not lead to cytokine-release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome**.** sNK cells do not undergo cell death while interacting with tumor cells, therefore, they have longer circulation times and more effective function. sNK cells are easy to scale up, and we can obtain greater than a billion sNK cells for therapy. Finally, sNK cells can be used with other therapeutics to further enhance the eradication of tumors. Since sNK cells are known to expand CD8+ T cells, the combination of sNK with CD8+ T cells will result in better tumor clearance. Finally, sNK cells can target both poorly differentiated/stem-like tumors and the differentiated tumors [36]. These findings make sNK cells an effective cell therapeutic strategy to eliminate tumors and hopefully achieve a cure for the patients.

These data indicate that sNK cells not only can target and eliminate tumors, but they can also increase the percentages and function of autologous NK cells. This increase was observed greater than 9 months in cancer patients.

4. Conclusions

The methodology to expand sNK cells does not involve any genetic manipulation of cells. sNK cells work differently than CART or CARNK cells since they can target tumors with/without a wide range of antigens, on a wide variety of cancers. sNK cells can target both CSCs as well as differentiated tumors, facilitating the targeting of the heterogeneous population of tumors, ultimately leading to complete eradication of cancer. sNK cells secrete significantly higher levels of cytokines, especially those known to induce differentiation of tumors. sNK-differentiated tumors can be further cleared by CD8+ T cells or chemotherapy. Thus, sNK cells could enhance the efficacy of other therapies. sNK cells induce beneficial effects with no signs of adverse events, particularly they don't induce cytokine-release syndrome (CRS) or immune effector cell-associated neurotoxicity syndrome. Restored or increased immune function post-sNK cell therapy in patients results in an immune system boost so that autologous NK cells can further fight against the disease. Last, but not least, it is easy to scale up the sNK cells' production for cancer therapeutics as the starting material is the immune cells from the peripheral blood of a healthy donor, which are easily available.

Author Contributions: KK performed the data generation, literature search, and wrote the manuscript. AJ was the principal investigator on research articles cited in this review, and she also participated in writing and editing the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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