Characterisation of human NK cells for therapeutic use

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Abstract:

As a part of the innate immune system, Natural killer cells are cytotoxic lymphocytes that can exert cytotoxic activity against infected or transformed cells. Furthermore, due to their expression of a functional Fc receptor, have also been eluded as a major effector fraction in antibody dependent cellular cytotoxicity. These characteristics have led to multiple efforts to use them for adoptive immunotherapy against various malignancies. There are now at least 70 clinical trials testing the safety and efficacy of NK cell products around the world in early phase clinical trials. NK cells are also being tested in the context of tumor retargeting via chimeric antigen receptors, other genetic modification strategies as well as tumor specific activation strategies such as bispecific engagers with or without cytokine stimulations. One advantage of the use of NK cells for adoptive immunotherapy is their potential to overcome HLA barriers. This has led to a plethora of sources such as cord blood hematopoietic stem cells and induced pluripotent stem cells that can generate comparatively high cytotoxic NK cells to peripheral blood counterparts. However, the variety of the sources have led to a heterogeneity in the characterization of the final infusion product.

Therefore, in this review, we will discuss a comparative assessment strategy, from characterization of NK cells at collection to final product release by various phenotypic and functional assays, in an effort to predict potency of the cellular product.

Introduction

The ability of human NK cells to kill a variety of cell lines isolated from leukemias, lymphomas and solid tumours was recognised in the 1970's [1-4] and these cells became some of the first cellular immunotherapies to enter clinical trials [5]. Despite early enthusiasm and a few notable successes [6] most trials failed. The demonstration that the immune-mediated anti-leukemia

effect of allogeneic bone marrow transplantation was mediated by T cells [7] and the subsequent proof-of-concept of donor lymphocyte infusions in the resolution of disease relapse [8] focused attention to T cell-based immunotherapies for cancer. However, better understanding of NK cell biology and the recognition that human NK cells differ from those of inbred mice has led to a resurgence of interest in autologous and allogeneic NK cell-based immunotherapies; and this has triggered multiple academic and commercial clinical trials as discussed below. Since 2006-2007, the production of NK cell preparations as cellular medicinal products officially requires optimised and reproducible assays for quality control of identity, purity and potency to meet current regulatory standards.

NK cells are initially defined by their ability to lyse tumour cells and virally infected cells in the absence of prior exposure and without MHC class I restriction or prior sensitization[1-4,9,10]. This historic definition is not as useful for therapeutic products where the focus is on immunophenotypic criteria. Surface expression of CD56 in the absence of CD3 are typically used and this strategy would identify more than 98% of NK cells in the peripheral blood of healthy donors.

Phenotypic identification of NK cell subsets can be based upon expression of the activatory and inhibitory receptors discussed below, or of maturation/exhaustion markers and effector molecules. Unlike T and B cells, NK cells express an array of germline-encoded surface receptors that enable them to scan cells for markers of intracellular infection, transformation and general signs of cellular stress. Once activated, they can rapidly release preformed cytotoxic granules containing perforin and granzymes [11-13]. NK cells also kill by transmitting death signals vie Fas ligand and TRAIL to the target cell [12,14]. Furthermore, the may recognize and kill antibody-covered cells with their Fc-receptor CD16, a process termed antibody-dependent cellular cytotoxicity (ADCC) [15,16]. They secrete a broad range of cytokines and chemokines and thus have a key role in setting the stage for subsequent immune reactions to infected or cancerous cells, by regulating cells of the adaptive immune system, e.g. DCs or T cells [17,18].

Activating NK cell receptors are expressed by the majority of NK cells, and each individual NK cell expresses a whole array of activating and inhibitory receptors [19]. The integration of the activating and inhibitory signals determines the reactivity of individual NK cells [20]. In addition to expression of activating receptors, processes such as education, differentiation, location

and memory formation can influence the activity of NK cells and lately, several different subsets have been identified which are thought to represent distinct cell types, maturational stages or have distinct terminal differentiation due to their location in different organs [21-23]. The activating receptors recognize pathogen encoded or stress induced ligands and molecules, and thus allow recognition of cells with a stressed or altered self-phenotype[24]. Furthermore, NK cells can express pattern recognition receptors (PRRs) and intracellular innate recognition sensors such as RIG-I, MDA-5 and STING [25].

In contrast, NK cell inhibitory receptors mainly recognize HLA-I and other surface molecules expressed by healthy "self-cells" [26-29]. There are several different inhibitory receptors but the main inhibitory receptor family expressed on human NK cells is the killer-cell-immunoglobulin-like receptor (KIR) family. This group of transmembrane signalling molecules was first identified as mediators of NK-inhibition through recognition of "self" MHC. However, paired family members with different intracellular signalling domains were subsequently identified as activating receptors [30,31] . Another widely expressed inhibitory receptor is NKG2A, which recognizes correctly assembled HLA-E and thus allows NK cells to probe the entire pathway involved in HLA class I synthesis [32-35]. It was thought that the absence of "self" MHC molecules (e.g. on certain tumour cells which evolve loss of MHC moieties) was sufficient to induce NK cell activation through "missing self" but it is now realised that absence of inhibition is not sufficient to lead to activation. Indeed, even the prototypical HLA class I negative NK target cell line, K562, is resistant to NK lysis if the CD15 activating signal on the K562 is blocked [36]. Thus it is the integration of inhibitory and activating signals which leads to NK cells activation and target cell lysis and/or cytokine secretion.

In addition to the molecules described above, NK cells express a range of adhesion molecules, such as LFA-1, DNAM-1, CD11b, CD11c, integrins and selectins, which enable both migration to sites of inflammation and facilitate interactions with other cells by initiating and maintaining immune synapse formation and these may be developed as surrogate potency markers [37-42]. Appropriate formation of the immune synapse is essential for NK cell function and the absence of sufficient adhesion cell molecule ligands on the target cell is probably the most common mechanism of immune evasion by cancer cells.

A wide range of cytokines and chemokines can influence NK cell activation, proliferation, differentiation and migration, which results in different NK cell subsets with distinct effector functions [40]. It is therefore of interest to assess expression levels of the receptors for these

mediators expressed by NK cells, which include IL-2R, IL-12R, IL-15R, IL-18R, IL-21R, and chemokine receptors CXCR1-4, CCR5 and CX3CR1 (Susek et al, Frontiers in Immunology; manuscript in press).

Recently, adaptive features of NK cells have been recognized and a whole body of studies show that NK cells can respond better to secondary stimulation[43-46]. Furthermore, NKG2C+ NK cells from CMV+ donors show increased functional responses towards malignant cells[47-52]

Therapeutic use of NK cells

Since the 1980's the potential of NK cells as a therapeutic agent against cancer has been recognized when administration of autologous lymphokine-activated killer (LAK) cells led to complete tumor regression in some patients with metastatic cancers[5]. Shortly after this initial trial it was demonstrated that LAK cells turned out to be NK cells[53]. At about the same time, the antileukemic effect of NK cells after bone marrow transplantation (BMT) was recognized [54], where NK cells are among the first lymphocytes to reconstitute from the transplanted BM. After these initial trials, much of the attention focused on T cell therapies, but since the early 2000's, autologous and allogeneic NK cells have been used for adoptive therapy of haematological malignancies [55-59]. While the initial studies focused on the use of autologous NK cells, focus has shifted later on to the use of haplo-identical NK cell sources. From these studies we learned that the relationship between the donor's inhibitory and activating KIR expression and the patients' HLA genotype can have an impact on the clinical outcome in allogeneic settings. A longer progression-free survival post-transplantation was observed in leukaemia patients that lack the KIR-ligand for the donors inhibitory KIRs[58-64]. These studies also showed that NK cells reduce the risk of GVHD [59] by limiting activation of allogeneic T cells of the graft. The impact of activating KIRs is demonstrated by studies showing that patients receiving NK cell allografts from donors expressing activating KIRs (e.g. KIR2DS1) but lacking the ligand (eg HLA-C2) had less relapse that patients which received grafts where both KIR and KIR-ligand were expressed [64-66]. Haploidentical (partly HLA-I mismatched) NK cells have been used to treat patients with AML, CML, ALL, MDS with variable success rates[58,60,62-64,67,68]. Differences in conditioning regimen, T cell depletion or immunosuppressive treatment have been suggested to provide an explanation for the differences observed in clinical outcome. Furthermore, different sources of NK cells have been used [69-72] but, to date, there is no consensus on what parameters are critical on the final NK cell product for adoptive transfer.

Unfortunately, most of the studies have characterized the NK cells either in different aspects or in different time points before administration. Clearly, a proposal for homogenization is needed.

Characterisation of NK cells as advanced therapy medicinal product (ATMP)

Cellular therapies differ greatly from conventional drug design and drug delivery. Determining the efficacy and toxicity, pharmacokinetics and pharmacodynamics, as well as purity and potency, is not trivial, and different groups/medical centres apply different criteria for assessing quality of the final cellular product. Both the European Medicines Agency (EMA) and The Food and Drug Administration (FDA) have published guidelines and regulations for (current) Good Manufacturing Practice ($_{(C)}$GMP).

When it comes to potency testing, guidelines are provided that allow the researcher/clinician to use assays specific for the function of the specific cellular product. The regulations and guidelines by EMA and FDA demand documentation concerning identification, quality, purity and strength of all processes, equipment and reagents to be used for generating the final cell product (chemistry, manufacturing and control, CMC), the use of standard operating procedures (SOPs) and quality control to assure product safety. How these guidelines can be applied for manufacturing of NK cell products has been discussed with focus on manufacturing procedures and quality and safety assessment, and general considerations concerning release criteria and potency[73]. Which markers to use for identification and characterisation of the final product though, is still a matter of debate and the list of potential markers of NK cell subsets and potential predictors of *in vivo* function is discussed below.

Previous reviews, including ours, have focused on the source of the NK cells, such as expansion and activation on undifferentiated cells from peripheral blood, cord blood or bone marrow; differentiated cells from cord blood hematopoietic stem cells as well as pluripotent sources such as induced pluripotent stem cells and embryonic stem cells. Recent studies show that NK cells form different sources can behave similarly, therefore we now propose that NK cells should be characterized based on their phenotypic and functional profile. For this purpose, we have characterized the NK cells based on their final product instead of source type. Furthermore, we suggest that the characterization of the product should be done at least as a two-step procedure, once as an initial characterization before the freezing of the

cells as the final characterization after the thawing immediately before administration of the cellular product to the patient. We recommend that the characteristics of the thawed product are determined during produce development and process validation rather than as a test on every product at time of administration. In the EU it is common to regard the NK cell product prior to cryopreservation as the "drug substance" and the thawed cells post cryopreservation as the "drug product". Typically we determine the effect of cryopreservation and thawing on the absolute number of viable CD3-/CD56+ NK cells in the drug substance during process development and validation and use those data to calculate the median cell loss and adjust the drug substance dosing to accommodate. This allows the drug to be dosed prior to cryopreservation and remove any need for testing of each drug product at the time of infusion.

Characterization of NK cells as therapeutic product

Phenotypic characterization can be done by flow cytometry, with a panel of antibodies directed against surface markers that allow the distinction of different subsets, maturation and activation status. A detailed list of markers is given below, however, there is still no consensus about which markers should be included in such a general phenotypical assessment. We recommend that the minimal product definition to determine identity and purity is based on the proportion of CD3-/CD56+ cells in the drug product with other markers selected on the basis of the perceived or proven mode of action of the product. In one of our recent clinical trials of allogeneic primed NK cells we included CD69 expression as part of the drug substance definition since it identified the subset of NK cells which we had shown medicated anti-leukemic effect in vitro [68]. In the cases of allogeneic NK cell immunotherapy, where contaminating T cells represent a significant risk of acute graft-versus-host disease, it is important to determine the absolute number of contaminating T cells and the dose of NK cells may be limited by the T cell contaminant. Most trials of primary allogeneic NK cells have used MHC haploidentical donors and the limit of T cell contamination has been restricted to an absolute dose of $1x10^4$ T cells per Kg patient body weight [57,68].

Whilst immunophenotype is useful in defining NK cell dose and purity it is rarely a reliable marker of NK cell potency. Regulatory agencies recommend and often require some measurement of potency as part of the release criteria for the drug substance or drug product. Our two groups have used functional assessment as a criterion for release of the

medicinal cellular product[68,69] although we have not shown correlation of *in vitro* potency with clinical outcome and a recent review of 16 NK cell trials in the US and EU showed that potency was not a quantitative release assay in any of them [69]. Historically, the most common assay for determining cellular cytotoxicity has been a 4h chromium release assay, where target tumour cells are labelled with radioactive chromium, co-incubated with effector NK cells, and the radioactive chromium released from dead cells is measured after 4 hours. This assay, however, is beset with problems; it requires the use of a radioactive isotope, it requires appropriate selection of a relevant tumour target cell and it is restricted to a maximum of a 4 hour assay since the background cell death from the isotope labelling precludes extended assays. Over the past 20 years laboratories have moved towards flow cytometric methods to measure cell cytotoxicity in which the target cells are labelled with a fluorescent-membrane dye and the absolute number of "live" target cells calculated by internal bead standard or volumetric measurement on the basis of dye exclusion (e.g. To-pro-3 iodide) [74].

In parallel with the development of dye-exclusion based assays, the field of has adopted measurement of NK cell degranulation as a surrogate of target cell lysis[75]. NK cells are co-incubated with tumour target cells in the presence of brefeldin and stained for expression of the degranulation marker CD107a. This has the benefit of being able to be combined with multiple NK cell surface and intracellular markers such as IFN-gamma, TNF and others to give a comprehensive analysis of the NK cell product.

Challenges remain in the selection of the appropriate tumour target cells for analysis. It is common to use the NK-sensitive cell line, K562. Another unresolved question is the timing of the characterization of the cellular product. At what time-point should the NK cells be characterized, directly after apheresis (or collection for the other cellular sources), after separation of NK cells (CD3/CD19 depletion vs. $CD3^{-}CD56^{+}$ selection), at the end of the expansion phase, i.e. before freezing, or after thawing of the cells? We think that the final characterization should be performed directly before infusion, as this is the final cellular medicinal product. However, we suggest to perform an initial characterization before the expansion phase, and a more thorough assessment of phenotype and function before freezing of the cells. We propose that the ultimate characterization, should be done on the frozen product, i.e. on a batch/aliquot of the frozen product. Freezing and thawing may have

a positive or negative impact on NK phenotype and function, and, ultimately, the characterization of importance is of the infusion product and not of the process.

Functional molecules on the surface of NK cells

Resting human NK cells are divided into two subsets on the basis of CD56 expression; so-called $CD56_{bright}$ and $CD56^{dim}$. $CD56^{bright}$ NK cells are a rare subset and primarily reside in primary and secondary lymphoid organs, and the majority of lack CD16 expression. When studied at rest, these cells are less potent mediators of *in vitro* tumour cell lysis, probably due to the lower levels of perforin, granzymes A, B & M. However, after cytokine priming with IL-2 they show no significant functional difference to $CD56^{dim}$ NK cells [76]. The expression levels of CD56 and CD16 allow researchers to classify NK cells into two major subtypes in humans, which differ in localization and function [77]. $CD56^{dim}CD16^{+}$ show strong cytolytic activity upon activation, and rapidly release cytokines when their activating receptors are triggered and may express KIRs. $CD56^{bright}CD16^{-}$ NK cells are poorly cytotoxic, express no a little KIRs but secrete large amounts of cytokines upon cytokine-induced stimulation. $CD56^{dim}$ and $CD56^{bright}$ cells differ in their expression of chemokine receptors, which may account for the difference in tissue distribution. While the majority of NK cells are $CD56^{dim}$, $CD56^{bright}$ NK cells represent only 10% of blood NK cells but are enriched in secondary lymphoid organs and are recruited to inflamed tissues[77,78]. The $CD56^{bright}$ subset secretes cytokine such as IL-10, Il-13, TNF-□ and GM-CSF and they are the predominant source of IFN-□□after IL-2 or tumour-mediated priming. Until recently, it was debated whether $CD56^{bright}$ represent a developmental stage of NK cells which can mature into $CD56^{dim}$ but current consensus is that this pathway does exist but does not exclude the possibility that a subset of $CD56^{bright}$ NK cells persist as terminally mature cells[79]. The functional differences of the two subsets with respect to direct cytotoxicity, secretion of inflammatory cytokines and interactions with the acquired immune response through dendritic cell activation demonstrate that, irrespective of their ontological relationships, it is important to distinguish them when characterising an NK immunotherapeutic product. It has now been shown that CD56 is involved in the recognition of fungal pathogens[80] and that it may be a trigger for fungal-mediated NK activation. In addition, CD56 is required for NK cell maturation by enabling synapse formation between developing NK cells and BM stromal cells [81].

CD16 is a low-affinity receptor on $CD56^{dim}$ NK cells which binds the Fc portion of antibodies (IgG1 and IgG3 in humans)[12] and induces ADCC[81]. Human CD16 signals via FcRgamma and CD3zeta[30]. However, there is significant proportion of NK cells which expresses CD56 but lacks CD16 constitutively and furthermore, CD16 may be shed upon activation [82,83], a process mediated by matrix metalloproteinases [83]. While this may make CD16 a poor marker for NK identification, it is nonetheless a very important molecule since CD16 mediates ADCC it is an essential subset marker for characterisation of NK products in settings where ADCC is a proposed mode of action. In addition, CD56 expression is quite common on aberrant cells in some cancer setting, e.g. in 70-80% of all cases of multiple myeloma[84], and therefore may not be a good marker for selection of NK cells for therapy in an autologous setting. Therefore, a combination of CD3- $CD56^{dim/bright}$ and $CD16^{+/-}$ as baseline characterization has been commonly applied by many researchers.

There are conflicting opinions with regards to preference of CD16 expression, the affinity of CD16 and the shedding of CD16 on NK cells. Regarding the affinity, there's growing evidence that a variant of CD16 (V158) that occurs in approximately 10% of the Caucasian population has a better overall response rate to antibody therapies that utilizes ADCC[85-88]. This discovery consecutively led to generation of genetically modified NK cell therapy candidates expressing the high affinity CD16 variant[89]. To our knowledge, there are no clinical trials at this moment to test this hypothesis. On the other hand, shedding of CD16 and blockade thereof has generated a lot of interest during the recent years. Upon identification of ADAM 17, a metalloproteinase, as the critical enzyme for shedding of CD16[90], studies have focused on generating a noncleavable CD16 variant for adoptive gene modified NK cell immunotherapy[91]. These studies have led to an iPS derived NK cell clinical product candidate that has cleavage resistant CD16 expression (http://fatetherapeutics.com/wp-content/uploads/2017/06/2017-AACR-FATE-iNK-Poster.pdf).

CD16 shedding might be extremely important for secondary synapse formation, and thus, may have a significant impact on the serial killing capacity of the modified NK cells[92]. Further studies in understanding the optimal expression level and the persistence of CD16 expression is therefore warranted.

In addition, studies show an induced $CD56^{bright}$ population after ex vivo expansion which doesn't really fall into the classical $CD56^{dim/bright}$ separation but may exert very strong ADCC function[93-95].

In addition to CD56 and CD16, many activating receptors have been described to have beneficial effects in tumor cell killing. NKG2D is a C-type lectin family which detects structures that are expressed at low levels by healthy cells but induced upon cellular stress such as DNA damage, heat shock response or excessive proliferation[96-99]. Thus NKG2D seems to be an important receptor in NK cell-mediated immunesurveillance, but given the fact that it is expressed by almost all NK cells, it probably may be omitted when characterizing NK cells as medicinal product. The natural cytotoxicity receptors (NCRs) NKp46, NKp30 and NKp44 also trigger cytotoxicity against tumor targets [100]. The NCRs bind to primarily intracellular proteins, that may be expressed on the surface of stressed, proliferating and otherwise activated and stressed cells [100-102]. Furthermore, recognition of viral hemagglutinins has been reported[103-105]. NKp46 is also expressed on almost all NK cells, and has be used as pan NK cell marker. NKp30 and NKp44 have not been widely used when screening NK cells as cellular products.
The activating receptor NKG2C, which recognizes HLA-E coupled to leader peptides of classical HLA class I molecules, appears to be involved in the development of adaptive NK cell population in CMV^+ individuals. The increased functionality of the $NKG2C^+$ NK cell population of these CMV^+ NK cells has triggered the search for donors with a large $NKG2C^+$ NK cell population. Assessment of the expression and the levels of this receptor will probably be soon incorporated in most clinical characterizations of NK cells as medicinal product.

DNAM-1 is a molecule that functions both as activating receptor and adhesion molecule, and has received much attention lately due to the fact that DNAM-1^{high} NK cells seem to be more responsive towards tumor cells [42,106-110]. In addition, DNAM-1 expression is higher on adaptive NK cells and even seems to be play a role in the emergence of NK cell memory[111].
Other adhesion molecules may also be of interest, as initiation of NK function is the result of multiple cell surface interactions leading to diapedesis from the vasculature into the extravascular space though ligation of critical adhesion molecules including LFA-1, VLA-1, VLA-4 and PECAM-1. NK cell products which lack expression of LFA-1 and VLA-1 will not adhere to the vascular endothelium and will not extravasate to the tumour site. If they are unable to upregulate VLA-4 and PECAM they will be unable to diapedese between the endothelial junctions. NK cells not only express LFA-1 but also express the cognate ligand, ICAM-1. During diapedesis, LFA-1 locates on the leading edge of NK cells and ICAM-1 to the trailing edge

providing a bridge to other NK cells. This ICAM-/LFA-1 interaction may be an early initiator signal for NK cytotoxicity [41].

The signals involved in the activation and triggering of function by NK cells are extremely complex and still subject to debate. Undoubtedly NK cells receive a balance of activating and inhibitory signals upon target cell binding and the outcome is dependent upon the relative contributions of these opposing signals. It is therefore also of interest to assess the expression of inhibitory receptors on the NK cell therapy product. As mentioned above, expression of certain KIRs may be favourable in patients with a known HLA class I setup. In addition, expression of inhibitory receptors may increase the overall functionality of NK cells in a process termed education [113-116]. However, the assessment of KIR expression and whether there is a missing KIR-ligand in the patient, is not trivial. The KIR locus, similar to the HLA class I locus, is one of the most polymorphic regions in the human genome. However, detection of mRNA by PCR does not allow assessment of expression levels, which differ between individuals and which has an impact on NK cell function. Due to the high sequence homology among different KIRs, the antibodies used for KIR detection have cross-reactivity to several KIRs, which makes Flow cytometric assessment of KIR expression difficult and cannot distinguish between activatory and inhibitory KIRs in most cases. Whether or not KIR assessment will be performed on a regular basis in future clinical trials using NK cells, is therefore questionable.

The inhibitory receptor KLRG1 is associated with NK cell maturation, recognizes cadherins on Given the complexity of NK cell activation both *in vitro* and *in vivo* it is paramount that developers of NK cells therapies make every effort to understand the biology of the specific disease they are targeting and the critical aspects required of the NK cells they are delivering.

Identity and purity by flow cytometry –

Minimum standards:

CD	Name	Expression	Rationale
CD3	T cell co-receptor	Negative	Excludes T cells and NKT cells. CD3 is strongly expressed on T and NKT cells and thus this exclusion marker can be analysed with a weak fluorochrome such as FITC or PerCp
CD56	NCAM	Positive	The relative contribution of CD56bright versus CD56dim cells to the function you are claiming for your immunotherapy should be determined during pre-clinical development and process validation so that these subsets can be enumerated individually. CD56dim NK cells require the use of a fluorochrome with a high signal:noise ratio such as PE or PECy5.
CD14		Negative	To exclude monocytes
CD16	Fcγ receptor	Variable	NK immunotherapies which are planned to mediate ADCC must express one or more isoforms of CD16 whereas NK cells primed ex-vivo to mediated direct tumour cell cytotoxicity may be defined by their lack of CD16.
CD45	Leucocyte common antigen	Positive	All NK cells express CD45 strongly and purity can be presented as the percentage of CD56+/CD3-ve cells within the "live" CD45+ve mononuclear cells
Intercalating dye exclusion	"viability"	>20%	Cell viability remains a controversial topic but dye exclusion assays remain the minimum standard for cell therapy QC and release tests. DAPI and Hoescht dyes are not recommended as they are not truly membrane impermeable and will be taken up by live cells if incubated at 37°C. 7-AAD and propidium iodide remain the common viability dyes for 488nm laser excitation and to-pro 3 iodide is suitable for excitation by the red laser lines. Trypan blue exclusion by microscopy is less sensitive than analysis of fluorofores by flow cytometry. The added value of analysis of apoptosis by forward angle light scatter versus propidium iodide dye exclusion is another reason for using a flow cytometric viability assay. This approach also negates the need for Annexin V staining to determine early apoptosis since "FSC ^{low} / PI ^{low}" account for early apoptotic cells.

Additional markers:

CD	Name	Expression	Rationale
CD2	T11	Positive	All NK cells express CD2 and an essential ligand for CD58 co-stimulation. CD2 may also be ligated by CD15 on tumour cells. K562 lysis is dependent upon CD2:CD15 interactions [36] and transfection of NK-resistant tumours to express CD15 renders them NK sensitive [117].
CD8	T8	Variable	A subset of NK cells expresses the CD8α/α homodimer and these have been shown to mediate higher level of tumor cell killing [118] and to recover from cytolysis-induced apoptosis to mediated repeated target cell killing [119].
CD11a	LFA-1	Variable	Essential for extravasation if the therapy is to be delivered i.v.
CD25	TAC	Variable	resting NK cells express the intermediate affinity IL-2R$\beta\gamma$ and exposure to members of the common gamma chain interleukins leads to upregulation of CD25 and the formation of the IL-2R□□□ high affinity receptor. CD25 expression is not the optimal marker for NK cell activation as it may be an indicator of proliferative capacity rather than lytic function [120].
CD44	HCAM	Positive	Constitutively expressed on resting NK cells but upregulated and transformed to the activated form by exposure to IL-2 or IL-15. Natural ligand is hyaluronan. Acts as a costimultory signal in human NK cells mediating ADCC.
CD54	ICAM-1	Variable	Acts as an adhesion and costimulatory molecule on resting NK cells and is upregulated following activation through CD16
CD57	HNK-1	Variable	NK maturation antigen. Defines a subset with high cytotoxicity and low responsiveness to cytokines
CD58	LFA-3	Variable	The common ligand for CD2 and, like CD2, is constitutively expressed on resting NK cells forming an essential component in the NK:target cell:NK synapses.
CD62L	L-selectin	Variable	Marker of polyfunctionality. CD62L+ human NK secrete cytokines and are cytolytic. Possibly represent an intermediate stage in NK cell differentiation [121].
CD69	Early activation antigen	Variable	This is the earliest activation markers expressed by NK cells after tumor cell or cytokine stimulation. It is detectable on the cell surface within 1 hour of activation and maintained for over 4 hours. Its role is unknown as is its ligand but blocking of NK:target synapse formation with rCD69 in vitro may inhibit NK function [82].

NK triggering ligands:

CD96 – expressed on all resting human NK cells and is the ligand for CD155 on human tumour cells

DNAM1 – CD226 expressed by resting NK cells and is a ligand for CD112 which is expressed on tumour cells. The interaction of NK cells with dendritic cells is also dependent on CD266-CD155 and the potentiation of the adaptive immune response

NKp30 – NK ligand for B7-H6 expressed on tumour cells

NKp46 – activating receptor which is required for immune synapse formation with tumour targets and appears to drive polarisation of lytic granules.

NKp80 – constitutively expressed on resting NK cells and induces NK activation after ligation by AICL expressed on cytokine-primed NK cells. It may therefore be an essential molecule involved in the expansion of the NK cell response to tumour *in vivo*.

NKGD2 – Ligand for MICA, MICB and ULBPs

TIGIT – Expressed on most NK cells at rest and after cytokine activation. Like DNAM1, it is a ligand for CD112 on tumour cells but may bind with higher affinity and act as an inhibitor of DNAM1-medited activation [122].

Potency assays:

Measurement of NK cell potency should be tailored to the clinical effect envisaged but can, broadly, be covered by assays for cytotoxicity (granule exocytosis or apoptosis), cytokine production and proliferation. There is insufficient scope in this review to discuss the pro's and con's of different assay platforms but some basic tenets can be applied.

First, a pool of NK cells from healthy donors should be established as "positive control" effectors. This can be purified NK cells from multiple donors which is pooled and cryopreserved in dosed aliquots. In some cases an NK cell line such as NK-92 can act as a positive control but one should bear in mind that these cells require culture in IL-2 and are thus not a direct control for resting human NK cells.

Second, cytotoxicity assays should be evaluated to optimise the chosen tumour target cells, co-culture time and the effector:target cell ratios (E:T).

A tumour target cell line which reflects the chosen target in the planned clinical trial may be appropriate but it should be remembered that a successful trial of an NK product in one disease may lead to trials in other cancer settings and it would be preferable to avoid having to validate a

different functional assay for every trial. Choice of a tumour cell line which is relatively resistant to resting NK lysis (e.g. Daudi cells) may be a good option since that will test the product for its degree of activation.

Historically, NK killing has been measured by 4 hour ^{51}Cr release assays but these are becoming increasingly difficult to establish due to tighter controls on radioisotopes. Better alternatives are available which allow measurement of target cell lysis of protracted incubation which will be closer to the physiological setting and have greater sensitivity. As described above, many of these assays are flow cytometric which has the benefit of rapid data acquisition and analysis but can lead to under-estimation of the degree of killing. Flow-based lysis assays should determine the "absolute number of viable target cells" at the end of the incubation period and compare that with the number of target cells at the outset. The percentage reduction is the "percent specific lysis". Measurement of the "percent dead cells" by flow cytometry is unable to detect those target cells which have broken down into fragments and thus fall outside of the analysis region.

A popular alternative to target cell lysis is measurement of LAMP-1 (CD107a) expression on NK cells after co-incubation with a target cell. This can be a robust assay but it is important perform it as it was originally described [75] by maintaining anti-CD107a in the co-culture throughout the assay and adding monensin to the co-culture after 1 hour to prevent internalisation and degradation of the CD107a.

Most cytotoxicity tests use a variety of E:T ratios but this makes analysis of the data as a release assay very difficult. An optimal E:T ratio should be established during assay development which provides a reproducible result whilst using a small number of effector cells.

Measurement of NK function by cytokine release may be easier than a lysis assay but it must be proven that the cytokines are released from the NK cells and not from the target cells in the co-culture. An easy alternative is to use PMA as an NK cell mitogen to induce cytokine secretion in the absence of target cells. This says nothing about the target cell recognition ability but does give a measure of function. If you chose to use a target cell line then intracellular staining of the NK cells for cytokine synthesis may be the best option to avoid measurement of cytokines derived from the target cells.

Finally, measurement of NK cell proliferation is rarely used currently but this may become more relevant in the field of CAR-NK where the delivered dose may not be sufficient to achieve clinical benefit. The CFSE-dilution assay can be sufficiently robust if reported as "percentage of cells which have divided" rather than attempting to calculate the median number of divisions or a proliferation index. Thus using the assay as a qualitative measure rather than attempting a quantitative criterion.

Conclusions –

Adoptive NK cell therapies are, once again, in the ascendant and the number of clinical trials increases year-on-year. An important determinant of success will be the accurate definition of the functional NK cell product and sophisticated analysis of the complex interactions of adhesion molecules, inhibitory and activatory receptors and their cognate ligands will be essential. Here we have reviewed the current understanding of these complex interactions and proposed some phenotypic and functional characterisations to be used to harmonise product definition and allow comparison of trial outcomes from different groups.

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