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SPOTLIGHT

INNOVATOR INSIGHT

Manufacturing NK cells for the clinic: the Spanish experience

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This article will discuss a standardized method for manufacturing a high number of clinical-grade NK cells ideal for infusion into patients, expand on the optimization of protocols, and provide a glimpse into the clinical results of infused NK cells at a hospital in Madrid, Spain.

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WHY NATURAL KILLER CELLS?

Natural killer (NK) cells are the main cells in the innate immune system and recognize their targets in a human leukocyte antigen (HLA)-unrestricted manner. This mechanism differs from that of T cells, which bind to specific receptors. NK cell effector function is controlled by a complex array of activating and inhibitory receptors that can differentiate between healthy and stressed cells. It is hypothesized that NK cells recognize their targets by two mechanisms: missing-self recognition, where they attack tumor cells that downregulate the expression of major histocompatibility complex (MHC) class I to evade T cell response, and induced self-recognition, where target cells are recognized due to overexpression of activating ligands that are induced by stress, such as DNA damage or malignant transformation.

These functions are performed in the context of a learning process ('licensing') regulated mainly by inhibitory killer cell immunoglobulin-like receptors (KIR) and their ligands (HLA class I molecules, in humans).

NK CELL ISOLATION

NK cells can be isolated from an initial leukapheresis product by a series of

enrichment steps using CliniMACS Prodigy® devices to achieve 99% NK cells and very few residual T cells (Table 1).

NK CELL THERAPY IN HSCT

In hematopoietic stem cell transplantation (HSCT), – NK cells need to be either alloreactive or activated in order to induce killing of the viral or tumor cells. It has been observed that utilizing alloreactivity in mismatched HSCT for adult and pediatric acute myeloid leukemia (AML patients has resulted in very low incidence of transplant related mortality/graft-versus-host disease and event-free survival of nearly 70%

NK CELLS IN THE CLINIC

Based on previously published data and our own pre-clinical and clinical experience, the use of NK cells to treat cancer patients is safe and feasible. However, the anti-cancer efficacy is limited in extent and duration; around 50% of patients experience remission but this can take 3 months to take effect, and most have begun to relapse within 15 months.

To overcome these limitations, we propose in the future to carry out NK cell engineering to:

1. Improve cytotoxic capacity by creating CAR-NK cells;

TABLE 1

Isolation of NK cells from a leukapheresis product.

n=45	Median	q3	q1	IQR
Patient's age	9.50	12.25	5.00	7.25
Patient's weight	29.00	45.25	21.25	24.00
Donor's age	39.00	44.00	36.00	8.00
Leukapheresis product				
WBC	1.43×10^{10}	1.87×10^{10}	1.24×10^{10}	6.26×10^{9}
NK (%)	7.80	13.00	5.32	7.69
NK cells	1.19×10^{9}	2.18×10^{9}	8.05×10^{8}	1.38×10^{9}
T cells (%)	55.14	67.64	42.86	24.78
T cells	8.02×10^{9}	1.03×10^{10}	6.11×10^{9}	4.14×10^{9}
Viability (%)	99.00	100.00	98.00	2.00
After CD3 depletion				
WBC	6.04×10^{9}	8.44×10^{9}	4.52×10^{9}	3.92×10^{9}
NK (%)	19.57	39.13	10.17	28.97
NK cells	1.44×10^{9}	2.71×10^{9}	5.99×10^{8}	2.11×10^{9}
T cells (%)	0.06	0.13	0.02	0.11
T cells	3.62×10^{6}	8.06×10^{6}	1.13×10^{6}	6.93×10^{6}
Viability (%)	98.00	99.50	98.00	1.50
After CD56 enrichment				
WBC	5.76×10^{8}	1.00×10^{9}	4.35×10^{8}	5.65×10^{8}
NK (%)	99.00	99.33	98.02	1.32
NK cells	5.70×10^{8}	9.92×10^{8}	4.12×10^{8}	5.81×10^{8}
T cells (%)	0.02	0.07	0.01	0.06
T cells	1.40×10^{5}	3.13×10^{5}	3.55×10^{4}	2.77×10^{5}
Viability (%)	100.00	100.00	98.00	2.00
NK cells ($\times10^{6}$)/kg	1.98×10^{7}	3.82×10^{7}	9.53×10^{6}	2.86×10^{7}
T cells ($\times10^{6}$)/kg	2.54×10^{-3}	8.24×10^{3}	0	8.24×10^{3}
Efficiency	50.21	63.90	38.52	25.38

IQR: Interquartile range; NK: Natural killer cells; WBC: White blood cells.

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TABLE 2

Activated and expanded natural killer cells (day +14–19 culture), release criteria.

Test	Specification
Total cell counts	$0.5–20\times10^6$ cells/ml
$CD45^+$ cells viability	≥70%
Phenotype:$CD45^+CD3^-CD56^+$ and $CD45^+CD3^+CD56^+$	≥80%
$CD45^+CD3^+CD56^+$	$\leq10\times10^{10}$/kg
Potency	≥50% versus K562 cell line
Mycoplasma	Negative
Sterility	Sterile blood culture and negative gram
Endotoxins	<0.25 UE/mL
Contaminating cells: K562-mb-IL 15-4BBI	Absence of BCR/ABL (%)

2. Improve delivery into the tumor with chemokine receptor-expressing NK cells;
3. Minimize exhaustion of cells with 'memory phenotype' NK cells.

NK CELL MANUFACTURING

Despite the benefits, NK cells have some important limitations, notably that NK cells represent only a minor fraction of human lymphocytes and large numbers are needed to achieve clinical benefits. These limitations can be overcome by developing good manufacturing practice (GMP) methods for NK expansion, for example using cytokines, different sources of NK cells, or co-culture with irradiated feeder or artificial antigen-presenting cells (aAPCs).

A more than 85-fold NK-cell expansion was reported by Klöss and colleagues in 2017 [1] and we have used a similar process in our laboratory to manufacture two products: IL-15-stimulated NK cells for use in the HSCT setting and activated and expanded NK (NKAE) cells for allogeneic transplantation for sarcoma. Spanish regulators have approved these manufactured cell products for use in patients, with release criteria as shown in Tables 2 & 3.

OPTIMIZING POTENCY & QUALITY OF NK CELL PRODUCT

Culture media

To optimize the potency and quality of NK cell products, different media were compared: RPMI, stem cell growth medium (SCGM), TexMACS, and NKMACS [2]. No significant differences were seen in the numbers of total expanded NK cells, but NKMACS yielded the highest fold increase in NK cells (Figure 1), followed by TexMACS. At the time of this project, NKMACS was approved for research use only, so TexMACS was chosen as the initial culture medium for this work. Subsequently, NKMACS has been approved for GMP use and will be used going forward.

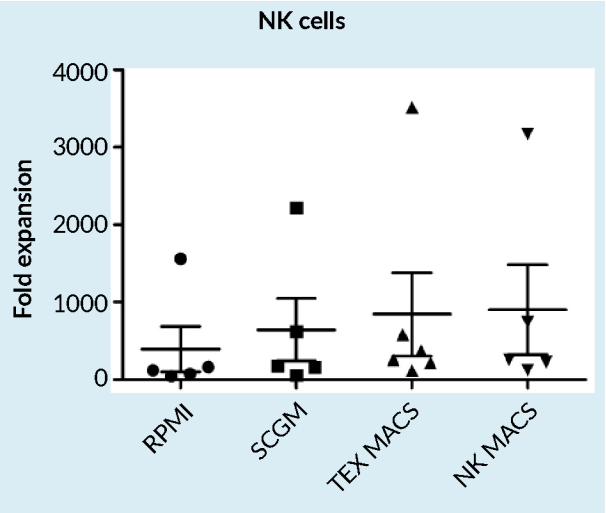
TABLE 3

Overnight IL-15 stimulated NK cells, release criteria.

Test	Specification
Total cell counts	$20–50\times10^6$ /kg
$CD56^+$ cell viability	≥70%
Phenotype: $CD45^+CD3^-CD56^+$	≥75%
T cells $CD45^+CD3^+CD56^-$	$\leq1\times10^4$ cells/kg
Potency	CD107a ≥ 10%
Mycoplasma	Negative
Sterility	Sterile blood culture and negative gram

FIGURE 1

Fold expansion of NK cells with different culture media. Reproduced from [2].



NKMACS and TexMACS media also resulted in the highest purities at day 21 (91 versus 92%, respectively) and the lowest residual T cell rates (3.5 versus 4%). NKMACS and TexMACS also gave the highest percentage of NK dim versus NK bright cells. This is important because bright NK cells lack the KIR CD16$^+$ receptor and are consequently unable to induce antibody-dependent cellular cytotoxicity, leading to a dependence on T cell toxicity.

Starting materials

An important factor in NK cell expansion is the starting material used. Mobilized apheresis yielded fewer NK cells than non-mobilized apheresis and peripheral blood mononuclear cells (PBMCs). After expansion, PBMCs yielded better expansion with IL-21-stimulated compared with IL-15-stimulated cell lines (Figure 2). There was no difference between starting materials in terms of NK receptors or NK cytotoxicity. The chosen starting material was therefore CD56$^+$ PBMCs obtained with non-mobilized apheresis.

Transcriptomic analysis

Transcriptomic analysis of the final products revealed differences between basal NK cells and NKAE cells. There were 2185 differentially expressed genes (1178 upregulated, 1007 downregulated) in NKAE cells. These genes were concentrated in 30 pathways, largely related to cell growth, proliferation, cell death, and metabolism.

Comparing IL-21-stimulated and IL-15-stimulated APCs, more than 600 differentially expressed genes were observed, of which 29 showed upregulation in NKAE cells. Transcriptomic analysis showed that enriched pathways were related to inflammatory and immune system responses.

Comparing PBMC with CD45RA$^+$ cells as starting material, 37 genes were upregulated, and 11 genes were downregulated in PBMC-derived NKAEs versus CD45RA$^+$-derived NKAEs. Two pathways were enriched, associated with hematopoietic cell lineage and metabolism of arachidonic acid.

GMP manufacturing

NK cells used by University Hospital La Paz were manufactured in a GMP facility, using an automated activation expansion process performed with the CliniMACS Prodigy instrument. The CliniMACS T520 tubing set and T cell transduction protocol were used.

At day 0, the co-culture was initiated by using 2×10^6–2.5×10^6 NK cells and 4×10^7 K562mbIL15 or K562mbIL21 cells previously irradiated with 100 Gy. Cells were cultured in 70 mL of GMP-grade TexMACs medium supplemented with 5% human AB serum (Sigma) and 100 IU/mL of IL-2 (Miltenyi Biotec). NK cells were incubated in the culture chamber (37°C and 5% CO2) in a static culture for the first week. At day +7, agitation was started, and 70 mL of fresh complete medium was added to the culture. Cells were expanded for 14 days before being harvested.

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Sampling was performed at day +7 for process controls, including cell counts, viability, $CD56^+/CD3^-$ cell content, mycoplasma, and sterility. When the expansion was complete, cells were automatically collected in 0.9% sodium chloride solution supplemented with 0.5% human serum albumin (Albutein 20%, Grifols, Barcelona, Spain), in a sterile bag. Release quality controls included total cell counts, viability, $CD56^+/CD3^-$, $CD3^+/CD56^-$and $CD56^+/CD3^+$ cell content, cytotoxicity against K562 cells, Gram staining, endotoxins, cell impurities (K562mbIL15 or K562 mbIL21), mycoplasma, and sterility.

The acceptance criteria included viability higher than 70%, cytotoxicity against K562 higher than 50% at a ratio of 1:8 for effector and target cells, Mycoplasma spp negativity, sterility (zero-colony forming units), endotoxins less than 0.25 EU/mL, and undetectable BCR-ABL.

CONCLUSION

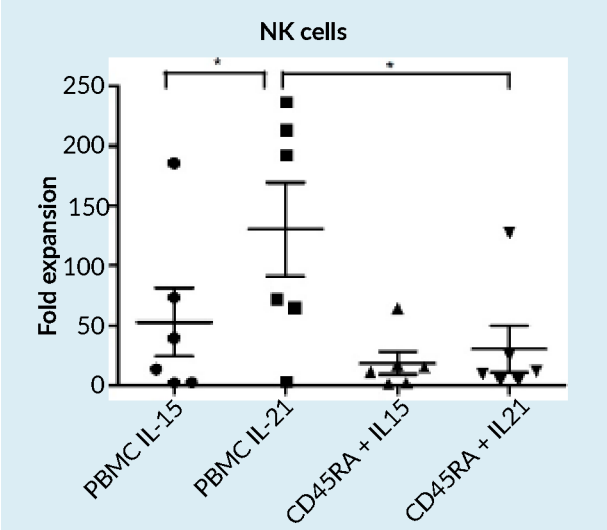
An optimized protocol is described to obtain NKAE cells by using four different culture growth media (RPMI, SCGM, TexMACs, and NKMACs) and two different NK cell sources [PBMC or $CD45RA^+$ cells and two distinct irradiated aAPC (K562mbIL15 or K562mbIL21)]. TexMACs was determined to be the most suitable cell culture medium to expand NK cells (although this has now been replaced with NKMACS).

NK cells could be activated and expanded from $CD45RA^+$ cells obtained from non-mobilized apheresis, although the use of PBMC as the NK cell source yielded the highest numbers of purified NKAE cells. When K562mbIL21 was chosen as the APC, the highest numbers of NKAE cells and lowest T cell contamination were achieved regardless of the NK cell source used. All NKAE cells obtained from either PBMC or $CD45RA^+$ expanded with K562mbIL15 or K562mbIL21 showed comparable antitumor ability against sarcoma, T-ALL, CML, neuroblastoma, and rhabdomyosarcoma cells.

Finally, clinical manufacturing of NKAE cells was fulfilled in an automated closed system CliniMACS Prodigy by using $CD56^+$ cells and either irradiated K562mbIL15 or K562mbIL21. In both processes, sufficient numbers of NKAE cells with high purity and low T cell contamination were manufactured after 14 days in culture. The release tests showed that manufactured NKAE cells met the requirements and specifications from the regulatory agency and thus, were suitable for clinical use. The NKAE cells manufactured are suitable for direct infusion to the patient or cryopreservation – and could also serve as a platform for more advanced NK cell therapies such as a combination with bi-specific killer engagers (BiKEs) or genetic modification to express chimeric antigen receptors (CARs).

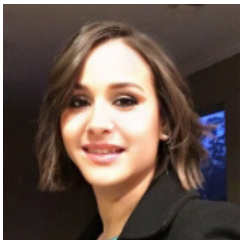
FIGURE 2

Fold expansion of NK cells with different starting materials. Reproduced from [2].



ASK THE EXPERTS





Charlotte Barker, Editor, BioInsights speaks to (pictured left to right) **Marty Giedlin, Senti Bio**, **Antonio Perez-Martinez, La Paz University Hospital, Madrid** and **Mariam Ammari, MD Anderson Cancer Center** answer your questions on NK cell manufacturing.

Q How big an impact does the starting material have on the final product?

MG: Based on Antonio's experiences and the experience at Senti in developing an alloreactive NK CAR, the starting material from healthy donors is critical. Our screening paradigm first and foremost includes manufacturability – whether we can make the dose that we plan to put into phase 1. We also consider whether the cells that we make are functional and able to provide a therapeutic effect for the patient post-infusion. We spend a lot of time characterizing our starting material and trying to come up with algorithms that will show us what fits our process, and then we recall suitable donors for GMP apheresis to start making cell banks for manufacturing.

AM-P: I agree with Marty. The starting material is important. The most important learning point from our experience was not to use mobilized apheresis because it yields fewer NK cells and they do not expand as well as those obtained with non-mobilized apheresis.

MA: I agree with both Marty and Antonio that the starting material makes a big difference in the final product. In our case, we usually begin with a frozen starting material like umbilical cord blood. We found out that how the product is cryopreserved before activation and expansion makes a big difference to the final product. NK cells survive for only 2 weeks, so we need to understand the starting material and the quality of our NK in the starting material, and how that might affect the final product, the expansion, and also the exhaustion of NK cells after they are infused into patients.

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Q What are the most important criteria to consider when selecting donors and what screening methods and approaches should be used?

MA: For manufacturing products, the criteria for the starting material should be the viability, the age of the donor (if starting from PBMCs), the type of disease, and whether the product is autologous. We take a medical history and perform serology for infectious diseases to see what the impact of the starting material will be on the manufacturing process and the final product.

AP-M: Initially, we were worried about how to select the best donor and tried to look for donors with the best KIR haplotypes or alloreactive NK cells. However, helpful reviewers of our work pointed out that it is unnecessary to use alloreactive NK cells to expand because the cells are going to be modified and the phenotype is going to change. So, the most important thing when selecting a donor of NK cells for expansion is to choose a young, healthy donor, as Mariam said. Of course, if you are not going to expand the cells and plan to infuse fresh NK cells without any modifications in a haploidentical setting, you should try to select alloreactive NK cells from a KIR-matched donor.

MG: I agree with both Antonio and Mariam. Expansion is extremely important in manufacturing, but it is also important to measure that against what is 'left in the tank' post-infusion: is there still some replicative capacity of the NK cells that can seek and kill tumors? Therefore, we measure both aspects and try to set up screening so that we have the appropriate time of expansion – post-activation transduction in our case – and still some life left in the cells afterward. Part of our small-scale screening operation aims to identify donors who are most applicable to the process.

Q What are the biggest challenges when considering the manufacturing of NK cells in a hospital setting, specifically?

MA: Based on my experience working in GMP manufacturing in a hospital setting, I think the most important challenge for hospitals is insufficient funds and not being up to date with the technology and equipment used in commercial manufacturing. Also, there are limited pharmaceutical investments in long-term projects with hospitals. If big pharmaceutical companies invested in hospital settings, it would help to make cell therapy accessible for more patients in more hospitals.

AP-M: Our institution in Spain is a public hospital, so the main challenge is human resources. When trying to incorporate a new strategy in cell transplantation or new cell therapy, first you have to convince your colleagues and your group. Second, you need human resources and facilities to perform the processes. In Spain, this would normally be performed in a research hospital but there are very few. It would be helpful to incorporate researchers in university hospital departments.

Q Could the panel comment on novel bioprocessing technologies with the potential to reduce manufacturing timeframes and requirements for technical expertise?

AP-M: Many of the processes remain very work-intensive. More closed and systematic ways of working will reduce the demand for human resources and make results more repeatable. Of the novel bioprocessing technologies, CliniMACS Prodigy has helped us to make more homogenous products across different locations using fewer resources.

MA: I agree with Antonio. Having a closed system that decreases human resource demands, improves reproducibility, and makes the manufacturing process scalable and functional will be great for the future of cell therapy.

MG: I think the other area to focus on is analytics. Particularly what is being termed 'integrated analytics.' As well as closed systems, systems that continuously monitor viability, metabolic state, residuals, etc. in cell culture will be very helpful. Ideally, those systems would be built-in and reactive; for example, sending notifications to your cell phone that a certain parameter has fallen out of range but has been automatically corrected. That would help to make better cultures but also to take the pain out of constant sampling and eliminate a lot of 'touches' to your cultures going forward.

Q What are the best methods for assessing the purity and potency of NK cells?

AP-M: Not necessarily the best method but probably the fastest, is a panel flow cytometry to check the purity of cells. To check the potency, you can use decannulation by flow cytometry or any functional test against different cell lines. In my opinion, if you are going to use your expanded cells against a solid tumor, these functional tests should be against cell lines similar to the target cells. However, this is not easy.

MG: One thing that is becoming more acceptable, or maybe more characteristic, is the use of single-cell assays. In the NK field, you're starting with only a few cells, and your expansions are still somewhat limited, so you need as many cells as possible for the patient. For potency in particular, there are systems available that look at single-cell RNA expression. There are also ways to assess potency by looking at pathways or what your cell can do after activation. I am looking forward to getting beyond the two-dimensional mast cell assays that have been used for the past 25–30 years, and into something more indicative of true potency (e.g., whether NK cells can find and kill the tumor). Maybe some of the three-dimensional models that are emerging for assessing trafficking and killing will provide another way of looking at the potency of NKs.

MA: Similar to Antonio and Marty, we use flow cytometry to assess the purity of our CAR NK cells, but we also have some cytotoxicity assays that we developed

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in-house. I also agree that we need more single-cell sequencing technologies to be integrated into our process.

Q When it comes to ensuring the quality and compliance of your final drug product, what are the key considerations and approaches?

MA: When it comes to ensuring final product quality, we measure safety and we have some release assays for sterility and Mycoplasma spp, but, since we use virus transduction, we do replication-competent retrovirus testing. We also measure vector copy number, and we assess the transduction by flow cytometry.

MG: We are inserting CARs and other genes into our NKs as a way of turning them into 'mini-computers', so we have to show activity for up to four different genes. We must ensure that copy numbers are within the U.S. Food and Drug Administration (FDA) ranges and assess the transduction efficiency and potency of the end product. We also have irradiated feeders that have been genetically manipulated and we use a polymerase chain reaction and a flow cytometry assay to show that they are not present in the final product. Last, but not least, because we are making bags of frozen NKs from healthy donors, these must be extensively tested for viruses according to FDA regulations. There is a lot of testing going on for these allogeneic products that may have not been necessary for an autologous approach, but certainly, the FDA is very interested in making sure that we're not transmitting any viruses or introducing any oncologic events via our transduction techniques.

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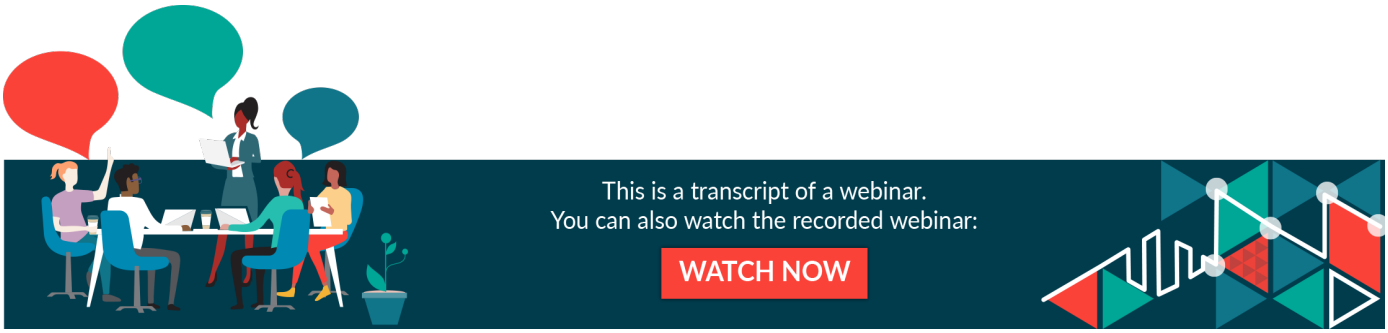

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