Cytotherapy 26 (2024) 1285–1298

Contents lists available at ScienceDirect

CYTOTHERAPY

journal homepage: www.isct-cytotherapy.org

Review Article

A review and statistical analysis to identify and describe relationships between CQAs and CPPs of natural killer cell expansion processes

Valentin von Werz, Oliver Spadiut, Bence Kozma*

Research Area Biochemical Engineering, Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, Vienna, Austria

ARTICLE INFO

Article History:
Received 22 December 2023
Accepted 29 May 2024

Key Words:
CPP
CQA
dynamic
expansion process
natural killer cells
NK cells

ABSTRACT

Natural killer (NK) cells make only a small fraction of immune cells in the human body, however, play a pivotal role in the fight against cancer by the immune system. They are capable of eliminating abnormal cells via several direct or indirect cytotoxicity pathways in a self-regulating manner, which makes them a favorable choice as a cellular therapy against cancer. Additionally, allogeneic NK cells, unlike other lymphocytes, do not or only minimally cause graft-versus-host diseases opening the door for an off-the-shelf therapy. However, to date, the production of NK cells faces several difficulties, especially because the critical process parameters (CPPs) influencing the critical quality attributes (CQAs) are difficult to identify or correlate. There are numerous different cultivation platforms available, all with own characteristics, benefits and disadvantages that add further difficulty to define CPPs and relate them to CQAs. Our goal in this contribution was to summarize the current knowledge about NK cell expansion CPPs and CQAs, therefore we analyzed the available literature of both dynamic and static culture format experiments in a systematic manner. We present a list of the identified CQAs and CPPs and discuss the role of each CPP in the regulation of the CQAs. Furthermore, we could identify potential relationships between certain CPPs and CQAs. The findings based on this systematic literature research can be the foundation for meaningful experiments leading to better process understanding and eventually control.

Background

Importance of natural killer cells

Natural killer (NK) cells are an important component of the immune system as they play a crucial role in the defense against viral infections and malignant tumors [1]. As therapy, they have some advantages over other lymphocytes, such as T cells, as they do not or only minimally cause any graft-versus-host disease and they are equipped with multiple different mechanisms that allow the cells to eliminate other cells [2,3]. Utilizing the abilities of NK cells as immunotherapy, targeting cancer could remedy patients for whom conventional cancer therapies were unsuccessful. NK cells naturally occur in the bloodstream only in limited numbers and they proliferate relatively slowly, therefore to enable the treatment of tumors the number of NK cells in the patients' system must be increased [4]. The obvious solution to increase the amount of NKs in the blood is the *in vitro* expansion and subsequent administration into the patient, described first in 1985 [5]. However, one of the key challenges in the production of cellular therapies, including NKs, is exactly the efficient *in vitro* expansion. Their sensitivity to culture conditions, complex activation and slow proliferation characteristics make it difficult to develop a robust process that can safely, reliably and reproducibly generate large numbers of these cells for therapeutic use even from different donors.

Challenges of NK cell expansion

An expansion process of NK cells usually starts with freshly isolated cells, typically from peripheral blood mononuclear cells or umbilical cord blood [6]. As blood is consisted of only a small fraction of NK cells [7], it is usually preprocessed before starting a culture. Two commonly used methods are available, performing either a positive selection by extracting only NK cells and leaving all other cell types behind [8]. Alternatively, a negative selection can be performed where all non-NK cells are depleted from the starting material, resulting in a pure NK cell population [8,9]. This preprocessing step is increasing the likelihood of a pure NK cell expansion, as also other cell types, such as T lymphocytes, can proliferate under the

List of abbreviations: CQA, critical quality attribute; CPP, critical process parameter; QA, quality attribute; PP, process parameter**; NK, natural killer; IL-, interleukin-; E:T, effector to target

* Correspondence: Bence Kozma, PhD, Research Area Biochemical Engineering, Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, Gumpendorferstraße 1a, 1060 Vienna, Austria.
E-mail address: bence.kozma@tuwien.ac.at (B. Kozma).

https://doi.org/10.1016/j.jcyt.2024.05.025

conditions used for NK cells. The fraction of cells resulting from the preprocessing steps is highly pure NK cells with only minimal contamination by other cell types. These NK cells are then used to initiate the expansion process.

Several different culturing platforms are available for NK cell expansion, offering different advantages or limitations (Figure 1) [10]. These platforms are hard to compare and there are process parameters (PPs) that are characteristics of each platform. The biggest difference in culturing formats is the differentiation between static and dynamic cultures. Static cultures are usually plates, flasks or bags located inside an incubator that require repetitive refilling or replacing of culture medium and additives to fulfill the demand of the growing cells [10]. This system usually requires manual operation inside a laminar flow workbench to protect the vessels and medium supply as these must be opened to access and are not connected in a closed sterile system. Dynamic cultures on the other hand are characterized by some sort of agitation of the culture, for example by an orbital shaker or a stirred vessel. This culture format is not necessarily dependent on an incubator. Refilling or replacing of the culture medium can be performed in a closed system, depending on the setup. Dynamic systems often provide automated controllability, such as the aeration that can be controlled by adjusting the desired gas composition and flow. In static cultures, however, gas supply can only be controlled indirectly by the atmosphere of the incubator [10].

The development of an optimized expansion process requires a good understanding of the PPs as well as the behavior of the cells during the process. Furthermore, it is very important to characterize the quality of the product with quality attributes (QAs), so the development of the process can be tailored specifically to these QAs. However, so far one of the major concerns related to NK cell expansion process development is exactly how to characterize the product itself and which quality characteristics to aim for. The critical QAs (CQAs) of these cells and especially their ranges are not as well defined and consolidated yet as it is the case for other biopharmaceuticals such as monoclonal antibodies. Clearly, it is easier to characterize a molecule than a living entity which further increases the challenge of cellular therapies.

PPs of NK cell expansion

In general, all mammalian cells require several different factors to proliferate *in vitro*, as these conditions attempt to mimic the *in vivo* conditions [11]. The most important factor is a controlled atmosphere of 37°C in a water-based culture medium with different supplements [11]. The medium is primarily consisted of essential nutrients, salts, vitamins, amino acids and growth factors (Figure 1). The pH is usually buffered to 7.4 and serum frequently added to support cell growth [11]. Depending on the cultured cell type and origin the composition can vary significantly, and even more supplements can be needed. Some cells, such as NK cells, also need an active component in the medium to stimulate growth and cytotoxic activation. These stimulants can be for example interleukins (IL-) or cytokines. All of these PPs contribute to the well-being of the cells *in vitro*.

The serum plays a role in cell culture medium by acting as a transportation vehicle for nutrients as well as by reducing the shear forces by its matrix characteristics (Figure 1) [12,13]. This effect can help to protect the sensitive, human cells, especially under dynamic conditions [12,13]. Plasma, has similar properties as serum, as it is from the same raw material, however, plasma does not contain anticoagulants [14]. Both serum and plasma are commonly used as cell culture supplements providing optimized environments for cells *in vitro* [14]. Nevertheless, the application of any human or animal-derived product in the production of a medicinal product is not favorable due the uncertainty of content, high batch-to-batch variability, potential limitations in the supply chain as well as ethical concerns [15].

An important PP in NK cell cultures is IL-2. IL-2 is involved in the development, homeostasis and function of the NK cells (Figure 1) [16]. Upon binding of IL-2 to the IL-2R on the surface of the NK cells the Janus kinases Jak1 and Jak3 are activated [17]. The activated Janus

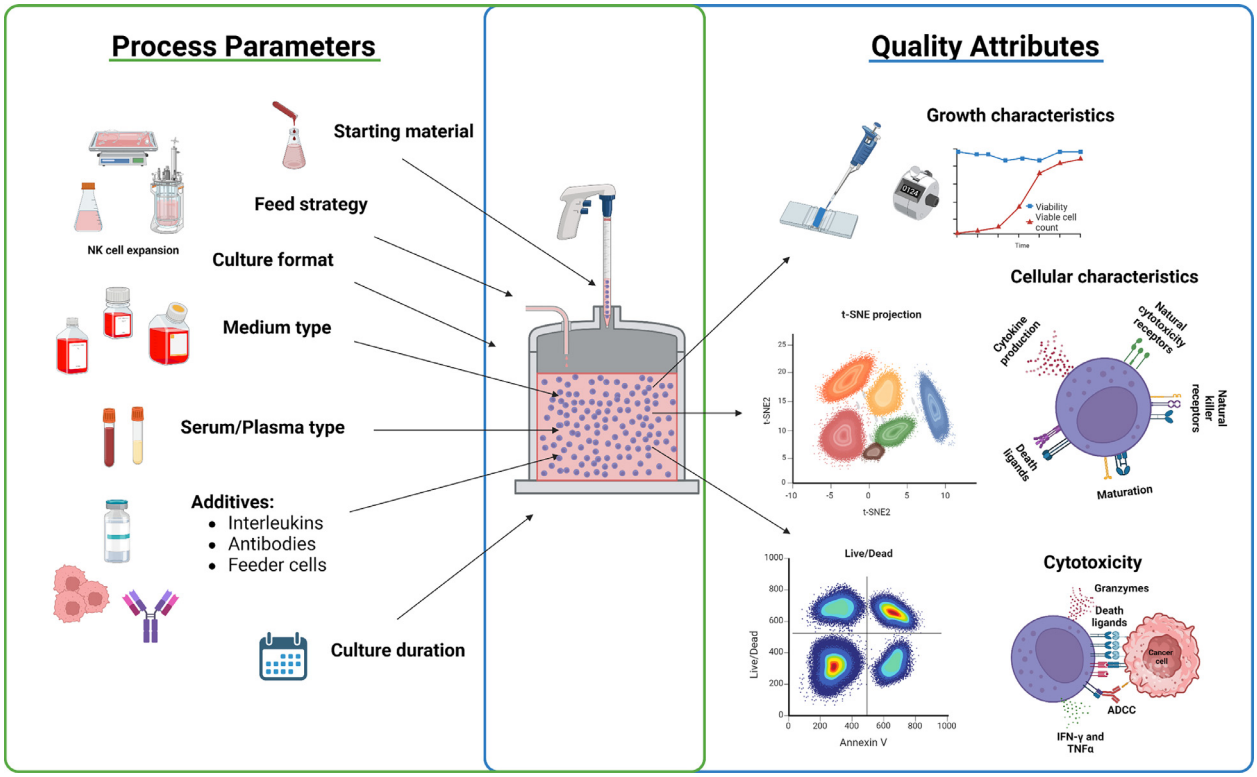

Fig. 1. Overview of the different process parameters and quality attributes of NK cell expansion processes. (Color version of figure is available online.)

kinases phosphorylate tyrosine residues on the IL-2 receptor which leads to the dimerization of STAT proteins [17]. Upon translocation to the nucleus, the STAT dimers act as transcription factors regulating gene expression that is controlling cell cycle progression, antiapoptotic signals and effector molecule production [17]. It is commonly accepted, that IL-2 plays a pivotal role in NK cell expansion, even if the ideal concentration or frequency of addition to the culture is not standardized, yet. Other cytokines and IL, such as IL-6, IL-7, IL-12, IL-15 or IL-21 are also said to have effects on the growth or cytokine production of NK cells, however, none of these IL were as frequently studied as IL-2 [18–20].

Another often-used supplement that is added to the culture medium to achieve enhanced NK cell growth and performance is the anti-CD3 antibody OKT3, which was originally used for T cell activation (Figure 1) [21]. Interestingly, NK cells do not express CD3 on their surface but T cells indirectly activate NK cells with cytokines that are produced upon OKT3 activation [22]. Besides the anti-CD3 antibody, researcher also used antibodies directed against CD2 or CD335 coated on beads for co-stimulation of two receptors [23].

Besides the direct, cytokine-based activation, another commonly used activation and proliferation method is the use of feeder cells. Here, target cells (usually cancer cell lines) are used in co-culture with the NK cells to constantly activate and drive the proliferation of the NK cells (Figure 1) [6]. Frequently used feeder cells include irradiated peripheral blood mononuclear cells (PBMCs) [24], Epstein-Barr virus-transformed lymphoblastoid B cell lines, engineered K562 cells expressing a membrane-bound form of IL-15 linked to the T-cell receptor CD8α and the 41BB ligand [25–30], or K562 cells expressing IL-21 [31]. The activation usually happens by several parallel effects. Examples of these are the secretion of cytokines, produced by the feeder cells or the direct cell-to-cell contact [6]. It has been demonstrated that the membrane-bound presentation of cytokines, for example on the surface of feeder cells can be beneficial compared to soluble stimulation with the same cytokine [6]. Furthermore, the combination of various stimuli, detected by cell-cell contact can further enhance the stimulatory effect on NK cells, as shown for 41BBL and membrane-bound IL-21 [32].

The release of soluble factors such as growth factors can also play a stimulatory role [33]. These mechanisms independently trigger the activation of the NK cells as described above. All PPs contributing to activation and proliferation are commonly used for efficient cell expansion, however, the effect of each PP can have its own limitations. For example, it can be problematic to generate a final product purely consisting of NK cells when the process was performed with feeder cells or if certain antibodies were used. As the final product is the living NK cells, no filtration or chemical elimination of impurities can be performed, as this could harm the product. This makes the production of cellular therapy very different to other biopharmaceuticals where such methods can be applied at the end of the process [34].

QAs of NK cell expansion

The most important QAs give information about the growth characteristics (Figure 1). The growth of NK cells is of high priority, as the cells themselves are the product. Hence, a rapid expansion process is important for two reasons. First, the product should be available within short time for the patient in need, but more importantly, the NK cells should not spend too much time in the expansion process to avoid exhaustion and thereby reduced therapeutic efficacy. To characterize the growth characteristics of a culture, usually the viable cell count per milliliter, as well as the viability as the proportion of viable cells from the total cell count in the culture is used. The speed of proliferation is usually given as the growth rate or as a fold change value for a certain time. Nevertheless, besides the growth characteristics, more factors are important to characterize the product and the expansion process, such as the phenotypic expression pattern or the cytotoxicity. NK cells can have three major phenotypes, also referred to as maturation (Figure 1) [4,35]. Besides the proliferation, the understanding of the phenotypes and their differences in functionality are important factors in the expansion processes. For NK cells the marker CD56 is commonly used for assessing maturity. About 5–15% of the circulating NK cell population is characterized as $CD56^{bright}$ [4]. These cells are known to be immature and tend to proliferate faster as well as producing higher levels of cytokines and growth factors [35]. The $CD56^{dim}$ population, representing about 85–95% of the circulating NK cell population, is known to be more involved in direct cytotoxicity, showing higher levels of killer cell immunoglobulin-like receptors, which are involved in recognizing and targeting specific cellular stress signals [4]. This population can be further separated into a $CD16^{dim}$ $NKG2C^{high}$ population which provides memory-like properties with enhanced functional properties [35]. The second subgroup of the $CD56^{dim}$ population is characterized by $CD16^{+}$ expression and the production of the cytotoxicity effector molecules granzyme A and B as well as perforin [35]. The proportion of each phenotype group can be flexible, as the expression of each marker and the functionality of the NK cells are influenced by stimulating agents such as chemokines or surface markers presented by other immunomodulatory cells [36,37].

NK cells are equipped with several different surface receptors, which are used to scan the surface of other surrounding cells (Figure 1). With the help of these receptors, the NK cells can decide if a surrounding cell is classified as healthy or normal which would not require any action. If the surrounding cell is not normal, it would trigger a response from the NK cell. The response is usually triggered by the sum of all activating and inhibiting signals produced upon binding to the surrounding cell. One of these receptors is a group called natural cytotoxicity receptors [38,39]. The natural cytotoxicity receptors belong to the group of activating receptors, generating potent activating signals upon binding to the target cell in order to eliminate the target cells [38,39]. Examples of the receptors from this group are NKp30, NKp44, NKp46, NKp80 [38,39]. Upon binding of these receptors to the target cell, the NK cell can perform a lysis, even without prior exposure to the antigen.

NK cells also express another type of regulatory receptor, called NK receptors (Figure 1). These include the NKG2C, NKG2D, receptors that deliver activating signals upon binding as well as NKG2A and NKG2B that deliver inhibitory signals upon binding [39–41]. These receptors alongside other important receptors such as DNAM1 and 2B4 are a key regulating system on NK cells when evaluating the fate of the contacted cell [42,43]. Upon activation, the response can be the use of the cytotoxic potential of the NK cells which leads to the death of the target cell.

Cytotoxicity mechanisms can be divided into two main groups based on how they affect the target cells (Figure 1). One general way is direct when the release of perforins or granzymes directly harms the target cell. Another also common, way is indirect, which is driven by the attraction of other immune cells by NK-cell-produced cytokines [44]. Alternatively, NK cells can eliminate the target cells by binding to the so-called death ligands (FasL or TRAIL) on the target cell. Upon binding, a cascade involving caspase enzymes leads to the apoptosis of the target cells [44,45].

There are several different potentially important QAs that could be classified as CQAs, however, besides obvious CQAs, such as the viable cell count and the viability, uncertainty dominates the nomination of the remaining CQAs (Figure 1). The careful selection of the CQAs is of highest importance for the quality, safety and efficacy of the product. Additionally, to control the process, it is not enough to know only the desired CQAs of the product. Moreover, it is important to understand, which PPs can drive the process in the direction of the desired outcome.

Problem statement

The aim of this review was to contribute to the understanding of NK cell expansion processes by analyzing in a systematic manner the reported results of NK cell expansion processes. Our goal was to extract the PPs and QAs from all papers dealing with NK cell expansion processes and search for a general trend in PPs and their influence on QAs, leading to the identification of CQAs and critical PPs (CPPs) that influence CQAs. To our knowledge, this type of analysis has not been reported before for NK cells. Only a more general approach was presented in previous works [6]. The identification of CPPs and CQAs was challenging because the scope of the experiments in the literature is usually diverse, not necessarily targeting process development to understand the influence of PPs on QAs but analytical development or to better understand the biology of NK cells. Experimental work published often varied largely in terms of the use of PPs and QAs. Different mediums used with different additives and stimulants and different starting conditions all could cause process uncertainty and these issues are rarely covered in their complexity in the papers. Also, it can be difficult to compare different experiments using the same culture type, as one experiment might be planned as a static, batch process while the other is not truly static as regular manipulation (e.g., medium exchange) happens. The same issue applied for the measured QAs as, understandably, each QA was not monitored in all experiments. Even though the available results were diverse and there was only limited experimental data available for *in vitro* NK cell expansion, we hypothesized that the main PPs and QAs could still be identified from the research data by the incidence of a certain PP when it was reported together with a certain QA that was affected by the PP. Moreover, this method could be used to discover potential causalities between PPs and QAs.

Data Extraction and Analysis Methods

Literature research and data extraction

Our work was conducted as a meta-analysis when the assumption was that in case there are multiple reports of a certain PP mentioned in connection with positive influence on a certain QA that is not coincidence but there could be causality. Following this strategy, we can identify relationships between PPs and QAs. However, to do this, data on PPs and QAs had to be extracted from the papers and processed.

Literature research was done by screening for research articles that described results of NK cell expansion processes for cellular therapy applications. The keywords included amongst others “Natural Killer,” “Expansion,” “Process Parameter,” “Quality Attribute.” Since this review focused on enhancing process understanding for the optimization of NK cell expansion processes and the relationship between PPs and QAs, literature, describing only the biological importance of PPs was not collected. To keep the focus on NK cell manufacturing and to ensure an unbiased review process, it was decided to not include more basic science work about the effects of certain, subjectively selected PPs. Only published and peer-reviewed articles from indexed journals were selected. From all available literature in the PubMed database, a differentiation into two categories was made based on the chosen culture format. Any mentioned process condition, starting or raw material was considered as a PP and was extracted from the papers (Supplementary Tables 1 and 2). The data included the name of the PP, as well as the value or the range used in the experiment (e.g., 5% heat-inactivated serum or 500 IU/mL IL-2) (Figure 2). Similarly, all reported cellular parameters or behavior were considered as a QA (Supplementary Table 2). However, the extraction was only done, when a statistically significant change in the QA was reported by the authors as a consequence of the process. The data included the name of the QA, as well as the value or the range of the reported change (e.g., 100-fold change in cell proliferation since inoculation or 90% viability) (Figure 2).

Normalization of extracted data

The truncated raw matrix contained information about how often a certain PP was used when a certain QA was significantly impacted during the expansion and multiple PPs were related to multiple QAs. Some PPs and some QAs were overrepresented in the dataset because their impact was studied more often in more experiments which led to a biased importance. To avoid overrepresentation, the incidences of each PP-QA pair were normalized by the total number of experiments in which the certain QA was reported. This resulted in relative frequencies that explained how often one particular PP was used in all analyzed experiments when a certain QA was impacted treating all QAs separately (e.g., in 50% of the experiments in dynamic cultures when the NK cells were co-cultured with feeder cells, NKG2C was upregulated). This information is now unbiased from the number of experiments therefore it is more useful to understand which PPs appear to play a role in the process of NK cell expansion.

Although after the previous step the relative frequencies eliminated the bias of the number of experiments, the resulting information might be misleading in cases when a significantly impacted QA was only reported in a few experiments. By a high relative frequency, a PP is considered of high importance for a certain QA, however, if this QA was only reported with a low count, the importance of the PPs would be falsely judged because of the QAs low true incidence. In other words, if the QA count was low, even a low count of PPs can lead to high relative frequencies of this PP-QA pair. Hence, each QA can only be accurately judged when its count is a representative sample of the overall QA count for each culturing format. To avoid interpretation of underrepresented QAs a threshold was calculated that describes the lower limit of the representative sample size in static and dynamic cultures separately using the Cochran formula (Equation 1) [46]. After this step, only those QAs were included in the analysis that were studied often enough for an unbiased interpretation of the PPs potentially influencing this QA. In case QAs were included in the data analysis that were below this threshold, the interpretation of data would be from underrepresented amount of data compared to other QAs. The Cochran formula was chosen, as it allows the calculation of the representative sample size from small datasets [47] and the available publications, from which QAs and PPs could be extracted for this meta-analysis, were limited. For the calculation of the QA threshold, the confidence interval was set to 95%, a commonly used value for high certainty of the calculation. In the Cochran formula, the confidence interval must be transformed to the *z*-score, which represents the number of standard deviations a data point is away from the mean of a standard normal distribution. The *z*-score can be found in the normal tables for each confidence interval and was 1.96 in this case [48]. The p-value is the estimated or expected proportion of the population. In cases where prior knowledge about the population distribution is available, the value can be set accordingly, however, as no prior knowledge was available the value was set to 0.5 to generate the largest sample size estimation which ensures a higher level of confidence. The margin of error represents the acceptable range of variation around the estimated population proportion and was set to 10% [49]. Including only the QAs above the threshold, the relative frequencies were not biased by the number of experiments, while in the meantime, the number of observations of each QA was high enough to assume a high level of certainty for the influence that the PP had on the QA. Researcher should be advised though to avoid misinterpretation of the calculated results, as the Cochran formula determines a sample size, in this case, a QA count that represents the total number of QA with statistical significance, however, this does not implicate that the results calculated later on in the meta-analysis are statistically significant.

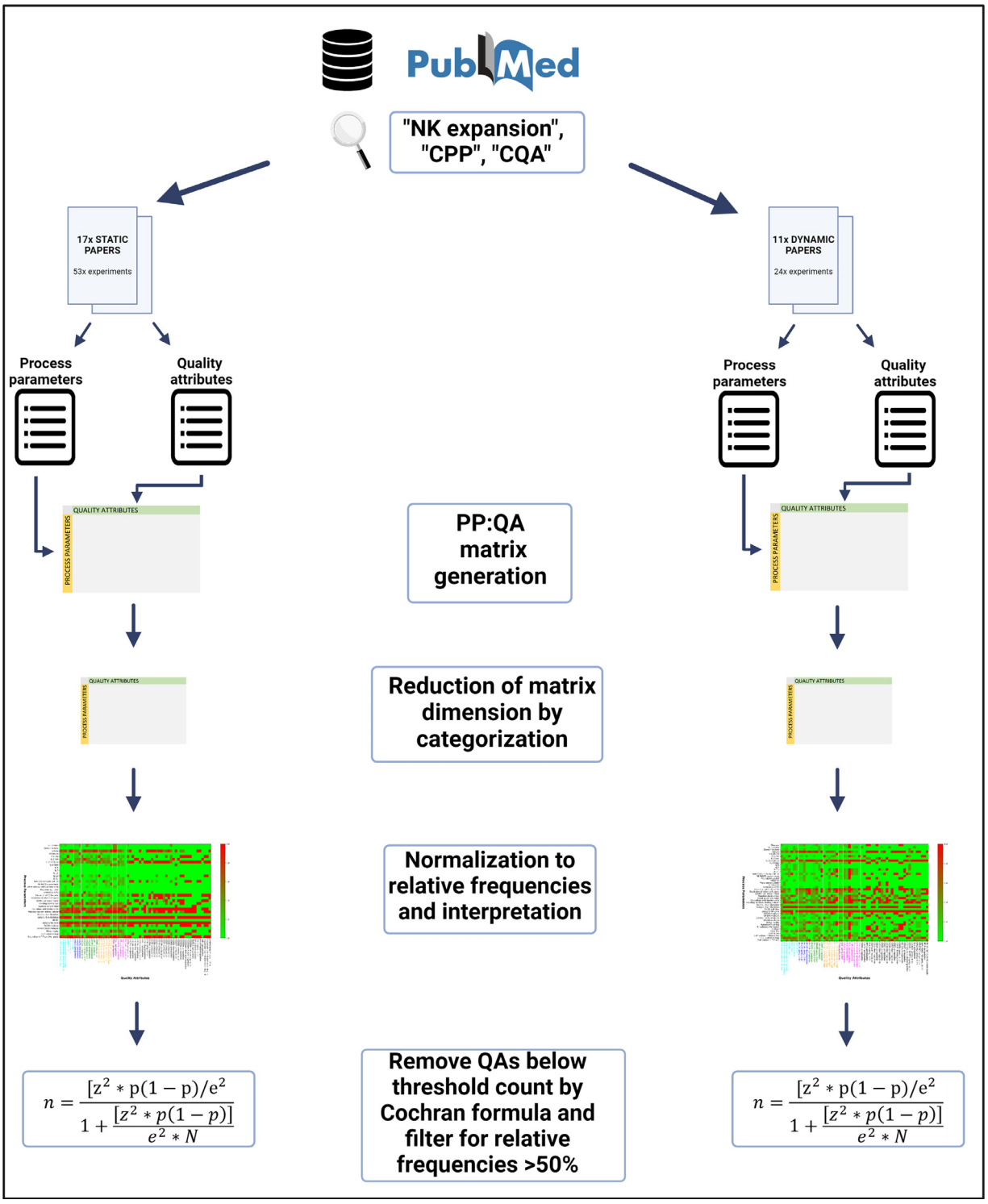

Fig. 2. Graphical overview of the literature research and data processing workflow. Literature was divided into experiments describing dynamic or static culture formats of NK cell expansion. The QAs and PPs were extracted and formed a PP:QA matrix for dynamic and for static cultures. If the same PP was used or if the same QA was changed but in a different experiment the names and values of the PPs or QAs were not added again to the matrix but only the incidence of the PPs and QAs was increased at their intersection. QA and PP categories were defined where applicable to reduce the dimensions of the matrices (Table 1). Relative frequencies were calculated for each PP:QA observation and plotted in heatmaps. Filters were applied for relative PP:QA frequencies of >50% as well as a filter for QA counts above a calculated threshold. (Color version of figure is available online.)

Equation (1) Cochran formula for the calculation of the minimal sample size

$$n = \frac{z^2 \times p(1-p)/e^2}{1 + \frac{z^2 \times p(1-p)}{e^2 \times N}}$$

- n is the required sample size
- z is the z-score corresponding to the desired level of confidence
- p is the estimated proportion or the expected proportion of the population
- e is the desired margin of error

The results and conclusions are based only on those QAs that passed the calculated threshold for a minimal QA count, if not stated otherwise. When the QA count threshold was fulfilled for one culture format but not for the other culture format, the QA below the threshold was still useful to include in the analysis to add some context to the discussion. For instance, when comparing high and low proliferation fold change between static and dynamic cultures, high proliferation fold change in dynamic cultures did not pass the threshold, however as it was very close to the threshold and the overall picture would benefit if it was discussed, it was included. Nevertheless, in any case, the inclusion from below the threshold was always clearly mentioned in the text because causality could not be assumed for those QAs.

Visualization of PP:QA frequencies

To visualize the generated results, heatmaps were created using the relative frequencies of dynamic and static cultures, separately (Figures 3, 4). From these heatmaps, it could be read, which PP was used at which frequency for each affected QA, allowing the identification of potential causalities between the incidence of a PP and the effect on the QA. The QAs were collected in seven groups on the heatmaps and in the sections below to collect similar characteristics for comparison. As not every single experiment was targeting the same QAs, the QA count could be interpreted as a weight factor describing a higher certainty of correctness for the calculated relative frequencies for QAs with higher counts (Table 2). Additional heatmaps were generated with further modifications to enhance the visibility of more pronounced effects (Supplementary Figures 1 and 2).

Influence of PPs on QAs

Identified PPs and QAs

The papers were analyzed firstly for the identification of all QAs and PPs, leading to a matrix of 145 PPs and 104 QAs for dynamic cultures and 129 PPs and 146 QAs for static cultures. To increase the interpretability of these matrices, some PPs and some QAs were grouped together, in cases the PP or QA ranges were widely spread (Table 1). After the generation of these groups, the PP:QA matrix contained 44 PPs and 59 QAs for dynamic cultures and 34 PPs and 63 QAs for static. These second matrices, consisting of absolute frequencies, were then normalized with the individual QA count to obtain relative frequencies and are shown on heatmaps for dynamic and static culture (Figures 3, 4, respectively). All QAs that were underrepresented by the count of studies were identified and removed from the matrices in order to reduce the bias caused by these QAs (Supplementary Figure 1). The threshold, as determined by the Cochran formula, was a QA count of 8 incidences for dynamic cultures and 5 incidences for static cultures (Table 2). The thresholds were used as filters by which the QA counts were reduced from 59 without application of the threshold filter to 8 after the threshold filter in dynamic cultures and from 63 before to 16 QAs after the threshold filter in static cultures (Supplementary Figure 3). The QAs remaining after the threshold filter application had the highest chance of being studied so often in different experiments that a high relative frequency could be with higher chance interpreted as causality and not only coincidence. Several, seemingly interesting QAs were excluded by the application of the QA count threshold such as the information about

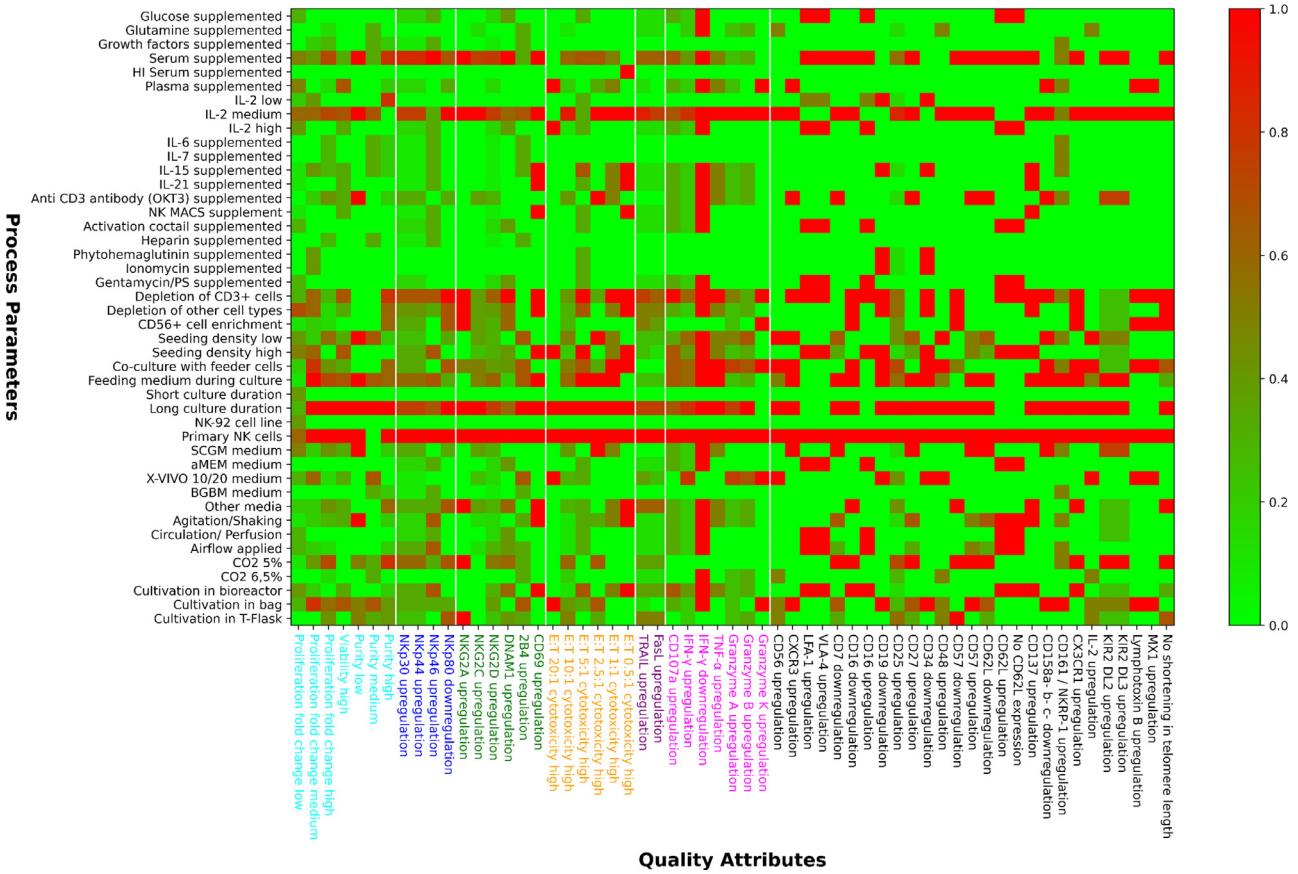

Fig. 3. Heatmap of all relative QA-PP frequencies in dynamic cultures. The range of the colors in the heatmap is from bright green (0% or 0.0 on the axis label) to bright red (100% or 1.0 on the axis label) according to the relative frequency generated as described in the section 'Normalization of extracted data'. No filters were applied for a minimal QA count or for a minimal relative frequency. The QAs are grouped in NK cells growth characteristics (cyan), natural cytotoxicity receptors (blue), natural killer receptors (green), death ligands (purple), cytotoxicity (orange), cytokines and related QAs (magenta) or other QAs (black) and color-coded accordingly. (Color version of figure is available online.)

Fig. 4. Heatmap of all relative QA-PP frequencies in static cultures. The range of the colors in the heatmap is from bright green (0% or 0.0 on the axis label) to bright red (100% or 1.0 on the axis label) according to the relative frequency generated as described in the section 'Normalization of extracted data'. No filters were applied for a minimal QA count or for a minimal relative frequency. The QAs are grouped in NK cells growth characteristics (cyan), natural cytotoxicity receptors (blue), natural killer receptors (green), death ligands (purple), cytotoxicity (orange), cytokines and related QAs (magenta) or other QAs (black) and color-coded accordingly. (Color version of figure is available online.)

high proliferation fold change, high viability or high NK cell purity in dynamic cultures and similar QAs in static cultures (Table 2). A possible reason for this might be that only a few experiments were able to produce NK cells that meet the criteria of certain "high" QAs so far.

Influence of PPs on QAs characterizing cell growth

The growth of the NK cells is a key factor of cell production for therapy. The quality of NK cell growth is not solely characterized by the cells' doubling time or expansion fold change, but also the viability and the purity of the cells play an important role, as it is the goal to have as many live and active cells as possible with a minimal impurity from other cell types in the final product.

In dynamic cultures, the relative frequencies for high proliferation fold change, high viability and high purity could be interpreted only with caution because the filters for minimal QA count would have excluded them being just below the QA count threshold (Table 2). The same applied to high viability and purity in static cultures. Nevertheless, in case of dynamic cultures, a high proliferation fold change was observed in more than 70% of the time when serum was used [29,50,51], however, plasma was only used in 14% of all instances (Figure 3 cyan QAs) [52]. Similarly, in static cultures, serum was used in 80% of all experiments that showed high proliferation (Figure 4 cyan QAs) [25,53–55]. This led to the assumption that for beneficial growth behavior of NK cells, the use of serum played a more important role than plasma, as in both static and dynamic culture formats serum was used more often than plasma when the proliferation resulted in fold changes above 1000-fold. Also, serum was used in 80% and 100% of all observations of high purity in dynamic or static cultures, respectively, highlighting the importance of serum, regardless of the culture platform [29,51,56]. Serum is commonly known as a critical factor in cell culture acting as a transportation vehicle for nutrients as well as reducing the shear forces by its matrix characteristics, hence it was not entirely surprising to be used in almost all experiments [12,13]. Furthermore, high purity in dynamic conditions

Table 1
Categorization of process parameters and quality attributes including the ranges and differences in source or quality.

PP category	Content or range	QA category	Content or range
Serum	Human or animal source (e.g., FBS)	Fold change low	1–100-fold
IL-2 low	10–100 IU/mL	Fold change medium	100–1000-fold
IL-2 medium	100–500 IU/mL	Fold change high	>1000-fold
IL-2 high	500–1000 IU/mL	Viability high (dynamic/static format)	≥85%/≥95%
Depletion of other cell types	All other cell types than $CD3^+$ cells (e.g., $CD4^+$, $CD19^+$, $CD20^+$, $CD33^+$)	Purity low	<50%
Seeding density low	$<1 \times 10^6$ cells/mL	Purity medium	50–90%
Seeding density high	$\geq 1 \times 10^6$ cells/mL	Purity high	≥90%
Short culture duration	1–5 days	Cytotoxicity high	>70%
Long culture duration	>5 days		

PP: process parameter; QA: quality attribute.

Table 2
List of all QAs in dynamic and static cultures.

No.	QAs studied in dynamic cultures	Count of QA	QAs studied in static cultures	Count of QA
1	**NKG2D upregulation**	**13**	**NKG2D upregulation**	**28**
2	**NKp30 upregulation**	**12**	**NKp30 upregulation**	**25**
3	**NKp44 upregulation**	**12**	**NKp44 upregulation**	**22**
4	**Proliferation fold change low**	**10**	**Proliferation fold change medium**	**22**
5	**E:T 10:1 cytotoxicity high**	**10**	**Proliferation fold change low**	**20**
6	**NKG2C upregulation**	**8**	**E:T 5:1 cytotoxicity high**	**12**
7	**TRAIL upregulation**	**8**	**DNAM1 upregulation**	**10**
8	**FasL upregulation**	**8**	**E:T 10:1 cytotoxicity high**	**8**
9	Proliferation fold change high	7	**E:T 2.5:1 cytotoxicity high**	**8**
10	CD25 upregulation	7	**CD69 upregulation**	**8**
11	Proliferation fold change medium	5	**CD107a upregulation**	**7**
12	Purity high	5	**NKp46 upregulation**	**6**
13	DNAM1 upregulation	5	**CD56 upregulation**	**6**
14	IFN-γ upregulation	5	**IFN-γ upregulation**	**6**
15	Granzyme A upregulation	4	**Proliferation fold change high**	**5**
16	KIR2 DL2 upregulation	4	**2B4 upregulation**	**5**
17	KIR2 DL3 upregulation	4	FasL upregulation	4
18	Viability high	3	CD25 upregulation	4
19	Purity medium	3	Viability high	4
20	NKp46 upregulation	3	NKG2A upregulation	4
21	NKp80 downregulation	3	E:T 50:1 cytotoxicity high	4
22	2B4 upregulation	3	NKG2C upregulation	4
23	E:T 5:1 cytotoxicity high	3	E:T 1:1 cytotoxicity high	4
24	E:T 2.5:1 cytotoxicity high	3	CD158e upregulation	4
25	CD107a upregulation	3	TRAIL upregulation	4
26	Granzyme B upregulation	3	CD158a upregulation	4
27	CD7 downregulation	3	TNF-α upregulation	3
28	CD27 upregulation	3	Purity medium	3
29	No shortening in telomere length	3	CD57 upregulation	3
30	Purity low	2	E:T 20:1 cytotoxicity high	3
31	NKG2A upregulation	2	CD16 upregulation	3
32	E:T 1:1 cytotoxicity high	2	NKG2A downregulation	2
33	TNF-α upregulation	2	CD54 upregulation	2
34	CD56 upregulation	2	CD62L upregulation	2
35	LFA-1 upregulation	2	CD137 upregulation	2
36	VLA-4 upregulation	2	CD158b+e+ downregulation	1
37	CD16 downregulation	2	KLRD-1 upregulation	1
38	CD48 upregulation	2	Purity high	1
39	CD57 downregulation	2	CXCR3 upregulation	1
40	CD57 upregulation	2	CD8 upregulation	1
41	CD161/NKRP-1 upregulation	2	CD48 upregulation	1
42	IL-2 upregulation	2	KIR2Dl3downregulation	1
43	E:T 20:1 cytotoxicity high	1	CXCR6 upregulation	1
44	E:T 0.5:1 cytotoxicity high	1	LFA-1 upregulation	1
45	IFN-γ downregulation	1	CD3 downregulation	1
46	Granzyme K upregulation	1	CD11a upregulation	1
47	CD69 upregulation	1	CD58 upregulation	1
48	CXCR3 upregulation	1	CD158a+e+ downregulation	1
49	CD16 upregulation	1	Perforin upregulation	1
50	CD19 downregulation	1	CXCR4 upregulation	1
51	CD34 downregulation	1	CXCR4 downregulation	1
52	CD62L downregulation	1	CCR7 downregulation	1
53	CD62L upregulation	1	CD19 upregulation	1
54	No CD62L expression	1	CD49b upregulation	1
55	CD137 upregulation	1	CD62L downregulation	1
56	CD158a- b- c- downregulation	1	CD158 downregulation	1
57	CX3CR1 upregulation	1	CD158a+b+e+ downregulation	1
58	Lymphotoxin B upregulation	1	LIR-1 downregulation	1
59	MX1 upregulation	1	LIR-1 upregulation	1
60			KIRs upregulation	1
61			Expansion reached plateau on day 14	1
62			Expansion reached plateau on day 15	1
63			Proliferation of CD3+ T cells	1

The QAs that passed the calculated QA count threshold are placed on top of the table in descending order and highlighted by bold font. The threshold was a minimal QA count of 8 for dynamic cultures and a minimal QA count of 5 for static cultures.
QA, quality attribute; E:T, effector to target.

was achieved in 80% when CD3+ cells were depleted or in 60% when other, non-NK cell types were depleted [29,51,52]. Additionally, a prolonged culture period and the use of feeder cells resulted for 60% of all analyzed experiments in high purity [29,51,52].

When studying the results of medium level IL-2 concentrations in dynamic cultures no clear association with a specific proliferation level could be made, because medium level IL-2 was used in processes with low, medium and high proliferation fold changes as well

(Figure 3 cyan QAs) [29,50,52,57,58]. Nevertheless, high proliferation fold changes were observed in 71% when low-level IL-2 concentrations were used [59,60]. Hence, we concluded that lower level of IL-2 was potentially beneficial for a high proliferation rate in dynamic cultures. In static 80% of cultures, high proliferation fold changes could be observed when medium IL-2 concentrations were used (Figure 4 cyan QAs) [25,53,55,61]. IL-2 is a known critical simulation factor for NK cells, however, it does not only promote the growth of NK cells but also stimulates for example the cytotoxic activity or upregulation of CD69 [62]. We could confirm the general importance of IL-2 as in 74% of all impacted QAs in dynamic cultures and in 65% of all impacted QAs in static cultures IL-2 was added to the media and at a medium level concentration [25,29,50,52,53,55,57,58,61,63–71]. Since IL-2 was found to be involved in different stimulations impacting numerous QAs, it was important to observe that the concentration in the culture medium needed to be carefully selected and that the concentration was probably also determined by the targeted QA. The stimulation effect of IL-2 on the cells' growth was found to be ideal at medium level concentration in static cultures but low-level of IL-2 in dynamic cultures was sufficient. Similarly, CD69, which is also known to be influenced by IL-2, was upregulated most often at medium level IL-2 concentrations (50%) in static cultures [62]. A big difference between static and dynamic culture formats is the presence of a nutrient gradient in static cultures, hence it would be expected, that the concentration of available IL-2 reduces over time along the gradient from the surface of the medium in the culture vessel to the cells at the bottom of the vessel as the cells constantly consume it. Consequently, static cultures should theoretically need a higher IL-2 concentration than homogeneously mixed dynamic cultures to provide enough IL-2 for the cells at all times, which was indicated by the studied experiments.

Besides IL-2 several other PPs were used when high proliferation fold changes were achieved, such as the supplementation of the anti-CD3 antibody OKT3 or the co-culture with feeder cells because these PPs were used in 60% of all observations in static cultures (Figure 4 cyan QAs) [25,53,55,61]. Feeding fresh medium during culture, or a long culture duration of more than 5 days was also done in 80% or 100% of experiments, respectively, when high proliferation was achieved in static cultures [25,53–55,61]. The use of the above-discussed PPs appeared intuitive for the generation of high proliferation fold changes, however, some of the PPs involved in high proliferation were surprisingly used also when only a low proliferation fold change was reported such as feeding of fresh medium during culture (85%), long culture duration (85%) or seeding at a low cell density (50%) [28,53,56,66,67,70–73]. These contradictory results highlight the difficulty to choose from available PPs for enhanced growth behavior in static cultures, as no clear pattern of ideal PPs arose.

The viability is of highest importance for the expansion process, hence even if the QA count for high viability (more than 95%) in static cultures was just below the QA count threshold it is still discussed here but without the intention to assume causality (Table 2). The addition of IL-15 was beneficial to achieve high viability as it was used in 75% of all static experiments that ended with viability greater than 95% (Figure 4 cyan QAs) [66,67]. Contrary, IL-15 was used in only 30% of all experiments when high viability was reached in dynamic cultures (Figure 3 cyan QAs) (30). Another potential option to generate high viability in static cultures was the enrichment of $CD56^+$ cells at the beginning of the culture or if the cells were fed with fresh medium during culture (75% of all experiments) [66,67]. In contrast, the enrichment of $CD56^+$ cells was never done in dynamic cultures when high viability was achieved.

Influence of PPs on natural cytotoxicity receptor expression

The viable cell count alone does not determine the quality of expanded NK cells because the cells do not only need to be administered into the patient in large quantities, but they also need to have the potential to kill their target cells. The level of natural cytotoxicity receptors can give an insight to the cells' potency therefore they are often measured. In the studies about dynamic cultures, an upregulation of the receptors NKp30 and NKp44 could be observed when one or a combination of the following PPs were used (Figure 3 blue QAs): serum in the medium at a relative frequency of 83% of all observations and medium level IL-2 concentration (75%) [29,50–52,57,60,63]. Interestingly, the upregulation of natural cytotoxicity receptors was differently influenced by IL-2 in static cultures. In 56% of experiments, medium level IL-2 was used for the upregulation of NKp30, however, NKp44 and NKp46 were more often (45% and 50%, respectively) upregulated when low-levels of IL-2 were used compared to medium levels IL-2 (36% and 33%, respectively) (Figure 4 blue QAs) [28,53,55,61,64–68,71]. This is contradictory to the observations made earlier regarding the impact of IL-2 concentration on the growth of NK cells comparing static and dynamic cultures because there a concentration dependency could be observed. Potential explanation could be, that the stimulatory effect of IL-2 on the natural cytotoxicity receptors is more effective, therefore the final concentration is only of low importance.

In both formats, the upregulation of NKp30 and NKp44 happened after the depletion of $CD3^+$ cells at the initiation of culture in 67% of all experiments in dynamic, and in 68% and 59%, respectively, in static cultures (Figures 3, 4 blue QAs) [25,28–30,52,57,60,61,63,64,66–68,71]. Same upregulation was observed with the use of serum in 83% of all incidences in dynamic and 64% and 59%, respectively, of all static cultures [25,28–30,50,51,53–57,60,63,67,68,71]. The upregulation of NKp46 in static cultures was not observed when the $CD3^+$ cells were depleted, it was reported however in 67% of dynamic experiments (QA count of NKp46 was below the threshold in dynamic cultures (Table 2) [57,60]. Same dichotomy continued when analyzing the use of the OKT3 anti-CD3 antibody between culture formats. OKT3 was used in 50% of all static experiments that resulted in an upregulation of NKp46 [53,55], but only in 16% and 18% when NKp30 and NKp44 were upregulated, respectively [25,53,55,64,71]. Contrary, in dynamic cultures OKT3 was never used when an upregulation of NKp46 was reported.

The differences in PP stimulation between culture formats continue further as in static cultures, NKp30, NKp40 and NKp46 were upregulated in 68%, 82% and 50%, respectively, of all observations when NK cells were co-cultured with feeder cells (Figure 4 blue QAs) [30,53,54,56,61,64–68,71] or in 56%, 77% and 83%, respectively, when seeded initially at a low seeding density [25,28,53,54,64,65,67]. These observations could not be confirmed in dynamic cultures as only 33% of all experiments that used feeder cells reported an upregulation of NKp30 and NKp44 [29,52,63] and no experiment was reported where NKp46 was upregulated in combination with the use of feeder cells (Figure 3 blue QAs). Also, seeding at low density was only reported in 25% when NKp30 and NKp44 were upregulated in dynamic cultures [50,52]. Perhaps, the seeding density plays a more important role in static cultures, as the space per cell is more limited than in dynamic cultures where cells are suspended in a three-dimensional space rather than sinking only to the bottom of the dish.

Culture format dichotomy was not present when feeding the cells during culture with fresh medium [25,29,50–52,54,56,57,64–66,68,71] or culturing them for a longer period (>5 days) [25,30,53–55,61,64–68,71] because these PPs seemed to be beneficial in both dynamic (more than 58% and in more than 67% incidence, respectively) and static cultures (more than 86% and in more than 73% of all incidence, respectively) for the upregulation of all three cytotoxicity receptors (Figures 3, 4 blue QAs). The influence of these PPs appeared to be similar on the upregulation of all three receptors, yet still with some differences as the observation frequencies differed.

Influence of PPs on NK receptors and other activating markers

The NK receptors are a key feature of NK cells when balancing their response to other cells, as the NKG2C, NKG2D, DNAM1 and 2B4 receptors deliver activating signals, while the NKG2A&B receptors induce inhibitory signals. The expression level of the activating NK receptors therefore can be useful when assessing the activation status of the expanded cells. Hence, also the marker CD69 was added to this group as it is commonly known to be upregulated in activated immune cells [74]. With certainty, we can make conclusions for NKG2D, DNAM1, 2B4 and CD69 in static culture formats as well as NKG2C and NKG2D in dynamic culture formats, as the QA count describing an upregulation of these markers was above the calculated threshold (Table 2). The analysis and interpretation of the results reporting upregulation of DNAM1 and 2B4 in dynamic cultures as well as NKG2C in static cultures have to be made with caution as the QA count was just below the threshold.

In both, dynamic and static cultures, NKG2C, NKG2D and DNAM1 upregulation appeared to be positively influenced by serum in the medium at a relative frequency of 75%, 77% and 100% of all observations of dynamic cultures [29,50,51,57,60] and 75%, 71% and 70% of static cultures, respectively (Figures 3, 4 green QAs) [25,28,30,53–56,61,67,68,71]. CD69 was enhanced 88% of all static cultures when serum was used in the process [56,67,68]. As discussed above, IL-2 is known for its stimulatory effects on various QAs in NK cells, one of which is the upregulation of CD69 [62]. Furthermore, a medium level IL-2 concentration was used in more than 50% of all cultures when NKG2C, NKG2D, DNAM1, 2B4, upregulated in both culture formats [25,30,53–56,61,64–68,71] as well as CD69 in static format [64,67,68].

Additionally, feeding of fresh medium during culture was also beneficial for NKG2C, NKG2D and 2B4 in 50%, 62% and 67% of all dynamic experiments, respectively (Figure 3 green QAs) [29,50–52,57]. Similarly to dynamic cultures, where 2B4 appeared to be the most affected marker by the addition of fresh medium, 2B4 was also heavily influenced in static cultures, as all experiments that were fed during culture reported an upregulation (Figure 4 green QAs) [53,54,68]. Similarly, NKG2C, NKG2D and DNAM1 were upregulated in 75%, 82% and 80% of all experiments when fresh medium was fed in static cultures, respectively [25,28,53,54,65,66,68,71]. Also, in dynamic cultures NKG2C, NKG2D were upregulated in 50% and 62% when medium was fed during culture, respectively [29,50–52,57]. The similarities in upregulation between the two culture formats continued as a long culture duration was beneficial for all activating markers (more than 75% in static [30,53,54,61,64–67,71] and more than 60% in dynamic cultures [29,50–52,57]). Another similarity in the upregulation of activating NK cell receptors of both platforms was the depletion of $CD3^+$ cells. The depletion of $CD3^+$ cells was performed in 62% and 100% of all dynamic experiments when NKG2D and DNAM1 were upregulated, respectively [29,52,57,60]. Similarly, in static cultures, NKG2D and DNAM1 were upregulated in 64% and 50% when $CD3^+$ cells were depleted [25,28,30,61,66–68,71]. Interestingly, the upregulation of NKG2D and DNAM1 was not reported when non-$CD3^+$ cells were depleted in static cultures. Potentially, the NK cell source was so pure, that the remaining cells after isolation were mostly $CD3^+$ cells. This would explain why only the $CD3^+$ cell depletion was beneficial and the depletion of other cells showed no effect. The similarity in upregulation of activating NK cell receptors is not given, however, for the use of feeder cells. Feeder cells were only beneficial in static culture formats in 50–80% of all observations for NKG2C, NKG2D, DNAM1 and CD69 (Figure 4 green QAs) [28,30,53,54,56,61,64–68,71] as well as 2B4 in 67% of dynamic cultures (Figure 3 green QAs) [50]. 2B4 upregulation appeared to be positively influenced when the anti-CD3 antibody OKT3 was used (60% of all experiments), however only in static cultures [53,55]. It must be noted, that NK cells do not express CD3 on their surface, but there is evidence, that NK cells can be activated by T cells indirectly after they produced cytokines upon OKT3 has activated them [22].

Cytotoxicity influencing PPs

From all potential CQAs cytotoxicity must be a CQA that is most looked at regardless of the culture format because NK cells that do not have the potential to eliminate target cells are meaningless in the fight against tumor cells. In the underlying studies, a cytotoxicity higher than 70% was discussed with evidence for an effector to target (E:T) ratio of 10:1 in dynamic cultures as well as E:T ratios of 10:1, 5:1 and 2.5:1 in static cultures. Only these ratios were studied frequently enough to be above the calculated significance threshold (Table 2).

In dynamic cultures, enhanced cytotoxicity was observed with a relative frequency of 60% in all experiments that were performed with serum [29,50], and in 90% with a medium level IL-2 concentration [29,50,52] (Figure 3 orange QAs). In static cultures, a similar picture could be drawn, as serum was used in 50–75% [25,28,53,54,56,68,69], and a medium IL-2 concentration 42–62% of all instances with enhanced cytotoxicity, depending on the E:T ratio [25,53,61,64,66,69] (Figure 4 orange QAs). The different effects of IL-2 on NK cells were discussed earlier, however, these findings added to them, confirming the important role of IL-2 for the stimulation of NK cells to become highly cytotoxic. This effect could not be a coincidence and it was present regardless of the culture format.

Another similarity between both dynamic and static culture formats was the enhancement of cytotoxicity when feeder cells or a low seeding density were used. In dynamic cultures, 50% of the experiments reporting high cytotoxicity had feeder cells (Figure 3 orange QAs) [29,50,52], while 40–88% of static cultures used feeder cells depending on the E:T ratio (Figure 4 orange QAs) [28,53,56,61,64,66,68]. It seemed obvious that NK cells showed high cytotoxicity after co-cultivation, as these cells have been activated for prolonged time by the presence of target cells, enabling the full cytotoxic potential. However, it was still not clear if this was a favorable culturing strategy, because the cells might become exhausted if they were cultured in this highly activated state for too long and they might not be as effective in the body as they could be. Therefore, the level of exhaustion could be also monitored by markers like TIGIT or PD-1, when NKs are co-cultured with feeder cells [75]. Unfortunately, these exhaustion markers were not analyzed in the present studies.

High cytotoxicity was also reported when cells were seeded at low density in 50% of dynamic [50,52] and more than 67% of static experiments, depending on the E:T ratio (Figures 3, 4 orange QAs) [25,28,53,54,64,66,69]. The effect of a culture duration longer than 5 days was also similar for both culture formats, as high cytotoxicity was observed in 100% and more than 88% in dynamic [29,50,76] and static cultures, respectively [25,28,53,54,61,64,66,68,69]. This suggested, that NK cells needed time to develop under the new *in vitro* culture conditions before showing their full cytotoxic potential, otherwise, high cytotoxicity should be reported in cultures of shorter duration. This development time could be for example caused by a change in availability of nutrients or stimulants compared to the *in vivo* conditions which could cause a change in metabolism and the development of the highest cytotoxic potential. Feeding with fresh medium was done in both culture formats when high cytotoxicity was reported during culture, which happened for 60% of dynamic cultures [29,50,52] and in all static cultures at an E:T of 5:1 and 2.5:1 [25,28,53,54,56,61,64,66]. Perhaps, also other medium components that were not analyzed in detail so far helped to enhance the cytotoxicity. These could be for example certain amino acids that contributed to the production of granzymes [77].

PPs influencing death ligand expressions

Death ligands on NK cells are crucial for the fight against abnormal cells because, upon binding with their FasL or TRAIL on the target cells' surface, caspases trigger the activation of apoptosis [78,79].

Even though understanding the influence of PPs on death ligand expression would have been important to analyze in detail for both culture formats, the QA count was only above the threshold in dynamic cultures. Still, the differences between the different culture formats were highlighted, because the QA count for static cultures was just below the threshold (Table 2).

Upregulation of FasL and TRAIL was observed when one or a combination of the following PPs was used: serum at a relative frequency of 63% of all experiments [29,57,60,63] and medium level IL-2 concentration in 88%, and 75% of all experiments that both resulted in upregulation of FasL or TRAIL expression, respectively [29,50,57,60,63] (Figures 3, 4 purple QAs). As mentioned before, IL-2 stimulates several characteristic activating receptors, so it is not surprising, that FasL and TRAIL were upregulated in experiments that used IL-2.

$CD3^+$ cells were depleted, in 63% and 75% of dynamic cultures, when FasL and TRAIL were upregulated, respectively [29,57,60,63], similar to the depletion of all other cell types except $CD3^+$ which was done in 50% when both markers were upregulated (Figure 3 purple QAs) [29,57,63]. This might be explained in a way that other immune cells that were present in the original starting donor sample, primarily induced inhibitory signals on the NK cells' receptors. Hence, the NK cells were stimulated by fewer binding events to the inhibitory receptors and consequently, they started to upregulate the activating receptors to adapt to the new situation. It was reassuring to see that TRAIL was upregulated in 50% of all experiments when $CD56^+$ cells were enriched at the beginning of culture [29,63]. However, it was surprising that none of the markers were upregulated in the presence of feeder cells because it would have been expected to see an upregulation of the death ligands in the presence of target cells as the feeder (cancer) cells should trigger the killing mechanisms of the NK cells, thereby promoting the ligand expression.

When the culture period was long under dynamic conditions, an upregulation of both death markers was reported in 75% of the experiments (Figure 3 purple QAs) [29,57]. Most probable reason was that the upregulation of the death ligands had happened only upon adaptation to the *in vitro* conditions which likely took time. Even if the QA count of static cultures was just below the threshold (Table 2) it was still interesting to see that the PPs that were used when an upregulation of the death ligands was reported were different to the PPs that were used in dynamic cultures that reported the same upregulation (Figures 3, 4 purple QAs). In static cultures, feeder cells in co-culture with the NK cells at a relative frequency of 75% of the experiments that reported upregulation of both death ligands were used [54,56]. Also, feeding the culture with fresh medium upregulated the expression of the two death ligands in all experiments [54,56]. The only similarity between static and dynamic culture was the use of serum (63% in dynamic and 100% in static format) [54,56] as well as a long culture period (75% in dynamic and 50% in static format) [54].

PPs influencing cytokine production and degranulation

NK cells are capable of killing the target cells by a series of different mechanisms. These include the above-mentioned mechanisms but the release of granzymes, that cause pore formation in the target cells' membrane, is another one. The monitoring of granzyme release can be done either by quantification of released granzymes or by analyzing the degranulation marker CD107a. In static cultures, the QA count of experiments describing the enhanced production of interferon-gamma (IFN-γ) and the upregulation of CD107a was above the calculated threshold (Table 2).

The degranulation marker CD107a and the production of IFN-γ were enhanced when serum was added to static cultures in 57% and 67% of experiments, respectively [53,56] (Figure 4 magenta QAs). Additionally, when NK cells were co-cultured with feeder cells, CD107a was upregulated in 86% and IFN-γ in each of the experiments [53,56,64,66]. Furthermore, feeding the cells with medium during culture was also beneficial for CD107a upregulation in 67% of all experiments [53,64,66]. The optimal concentration of IL-2 for CD107a upregulation was not clearly addressable, as both medium and high IL-2 levels were used in 57% and 43%, respectively, when enhanced CD107a expression was observed [53,56,64,66]. Low-level IL-2 concentrations, however, were never used when an upregulation of CD107a was observed, leading to the conclusion that at least a medium level of IL-2 is needed.

CD107a and IFN-γ markers were upregulated in 57% and 83% of cultures of long duration, respectively [53,64,66,68,80]. The production of IL or granzyme appeared to be positively influenced by a long culture duration. Perhaps, it is not the production and the release of the final product itself, but also the production of other factors such as enzymes that are involved earlier in the production pathway. It would have been expected, that even though cells need time to adapt to a new culture situation, the production of cytokines would have been independent of the culture situation, as the release of cytokines occurs primarily upon contact to target cells [81].

PPs influencing NK cell maturation

NK cells can be grouped in two populations based on the level of CD56 expression. First, 5–15% of the circulating NK cell population is characterized as $CD56^{bright}$ [4]. These cells are known to be immature and tend to proliferate faster as well as producing higher levels of IFN-γ or TNF-α. Second, the $CD56^{dim}$ population, representing about 85–95% of the circulating NK cells, is known to be more involved in direct cytotoxicity, showing higher levels of killer cell immunoglobulin-like receptors, which are involved in recognizing and targeting specific cellular stress signals [4]. The QA count of static cultures, describing CD56 expression levels allowed the discussion of the PPs that were used when upregulation was observed (Table 2).

Upregulation of the CD56 NK cell marker to the $CD56^{bright}$ population could be observed in 67% of all experiments in static cultures when a medium level IL-2 concentration was used (Figure 4 black QAs) [66,67]. Also, an upregulation of CD56 was observed more often in combination with plasma as medium supplement, compared to serum (67% and 50%, respectively) [54,66,67]. This is unusual, as the majority of the above-discussed markers were more influenced by serum than by plasma.

Surprisingly, IL-15 was used in 67% of all experiments that reported upregulation of CD56 (Figure 4 black QAs) [66,67]. Most other markers reviewed here were not changed when IL-15 was used. This effect of IL-15 was very similar to the effect it had on achieving high viabilities in static cultures (75% of the experiments resulted in high viability and 67% resulted in enhanced CD56 expression). This is interesting, as with the application of IL-15 NK cells of high viability could be produced that belonged to the group of $CD56^{bright}$ cells, characterized by faster proliferation than the $CD56^{dim}$ population. IL-15 could therefore be a CPP candidate for enhancing the growth of NK cells *in vitro* before activation for enhanced cytotoxicity.

It was also found, that a low seeding density and a longer than 5 days culture duration appeared to be beneficial for CD56 upregulation, as it was used in 83% of all experiments (Figure 4 black QAs) [54,66,67]. Additionally, the depletion of $CD3^+$ cells [66,67], and the co-culture with feeder cells was reported in 67% when CD56 was upregulated [54,66]. As described earlier $CD56^{bright}$ cells are known to produce enhanced levels of IFN-γ [4]. Here, we found that a low IL-2 concentration level, a long culture duration or the co-culture with feeder cells were all used in experiments that reported enhanced IFN-γ production, which confirmed that an overlap between these PPs and QAs existed) [54,66,67].

Discovered relationships between CPPs and CQAs

Based on all the details above, a list of CQAs and CPPs for NK cell expansion processes with the focus on which CPP could be potentially used to control the level of which CQA could be generated (Table 3). According to our knowledge, this is the first list of CQAs and CPPs derived from the reported NK cell expansion processes available in the literature. Obviously, the content of the list has to be experimentally validated to verify the trueness and the level of the relationship between the listed CPPs and CQAs because the different research papers often aimed to answer different questions than CPP-CQA relationships, which made them difficult to compare. Nevertheless, information about PPs and QAs could be extracted and their relationship could still be studied by later processing the collected information. The presented method of meta-analysis allowed to observe indirect relationships of PPs on QAs that were not targeted to be discovered by the experiments but as overarching trends were hidden in the data.

Inevitably, several aspects of NK cell production processes could not be covered in this review, because only limited experimental research was available to date for some PPs or QAs. Hence, these were underrepresented in the analysis and could have biased the analysis. However, even if several PPs and QAs had to be excluded from the analysis, some might still be of high importance but were so far simply understudied. The temperature was usually set to the physiological 37°C, however, there could be an effect that increased temperatures would be in favor for NK cell proliferation, mimicking a site of inflammation [82]. Similarly, a decreased oxygen concentration could have been studied, as the oxygen level in the blood, tissue or a solid tumor can vary drastically [83]. The effect of pH change in the cultures, as well as the varying dissolved oxygen or carbon dioxide, were also not sufficiently studied, yet. Only little attention was given to the optimization of culture medium composition, even though, this is the basis to supply the need of NK cells for ideal *in vitro* cultivation. Additionally, so far, only little attention was given to the characterization of the starting material [84,85]. It was therefore not entirely surprising that different researchers could achieve different results, as the donor-to-donor variability is known to be usually high [84,85]. These important questions should also be taken into consideration for future experimental planning when aiming for cultivation process optimization. On the other hand, the versatile effect of other stimulants such as IL-2 became obvious. As described above, IL-2

Table 3
Summary list of NK cell CQAs in static and dynamic cultures and CPPs that influence these CQAs.

Affected CQA group	Culture format	Influential CPP (range where applicable)
Proliferation/cell growth	Dynamic cultures	• Serum • IL-2 low-level (10–100 IU/mL) • Feeding fresh medium during culture • Initial seeding density low ($<1 \times 10^6$ cells/mL)
	Static cultures	• **Serum** • **IL-2 medium level (100–500 IU/mL)** • **Anti-CD3 antibody OKT3** • **Initial seeding density low ($<1 \times 10^6$ cells/mL)** • **Co-culture with feeder cells** • **Feeding fresh medium during culture** • **Long culture (>5 days)**
Viability	Dynamic cultures	• Plasma • Long culture (>5 days) • IL-2 medium level (100–500 IU/mL)
	Static cultures	• IL-15 • $CD56^+$ cell enrichment
Cytotoxicity	**Dynamic cultures**	• **Serum** • **IL-2 medium level (100–500 IU/mL)** • **Feeding fresh medium during culture** • Initial seeding density low ($<1 \times 10^6$ cells/mL) • Co-culture with feeder cells • **Long culture (>5 days)**
	Static cultures	• **Serum** • **IL-2 medium level (100–500 IU/mL)** • **Initial seeding density low ($<1 \times 10^6$ cells/mL)** • **Co-culture with feeder cells** • **Long culture (>5 days)**
Degranulation and cytokine production	Dynamic cultures	• IL-2 medium level (100–500 IU/mL) • $CD3^+$ cell depletion
	Static cultures	• **Serum** • **IL-2 medium level (100–500 IU/mL)** • **Feeding fresh medium during culture** • **Co-culture with feeder cells** • **Long culture (>5 days)**

The CPPs are followed by their ranges if applicable or known. The bold CQAs and CPPs were above the calculated CQA threshold, therefore these effects were considered more certain than a coincidence. The rest of the CPPs showed some effect but less prominent.
CQA, critical quality attribute; CPP, critical process parameter.

affects several different CQAs highlighting its inevitable and probably one of the most important roles in NK expansion and activation processes (Table 3).

Another open question in the expansion process is the selection of the cultivation method and platform. There are already numerous options available on the market, all promising to be an ideal solution, however, the selection of a certain platform already determines several CPPs and thereby the influenceability of CQAs. For example, agitation can have a direct influence on a desired QA such as proliferation. To enhance this QA a lower IL-2 concentration was sufficient in dynamic cultures, whereas a higher concentration was ideal in static cultures (Table 3). Information about the expansion characteristics or cytotoxicity from such a study would be very valuable to refine the knowledge about CPP-CQA relationships.

Conclusion

In this contribution, PPs and QAs of NK cell cultures were systematically reviewed. Our method of literature review and data processing was the first of its kind for NK cells. We have applied true objectivity as we have only included data extracted from literature and did not include any PPs or QAs that are commonly known in the scientific community but contrary to this were not published. By preprocessing the data, we ensured comparability between the studies, even if the experimental scope was diverse. With the overview generated here, we provided an unbiased view on all PPs that are commonly used and all QAs that are frequently measured. Furthermore, we offered a feasible solution of how to get closer to understand the expansion process of NK cells. Moreover, we were able to identify the potential relationship between CPPs and CQAs as well as the ideal ranges of CPPs. Nonetheless, further experimental work is needed to validate the suggested roles of CPPs on CQAs from this review. Furthermore, we offered a feasible solution of how to get closer to understand the expansion process of NK cells. Moreover, we were able to identify the potential relationship between CPPs and CQAs as well as the ideal ranges of CPPs. Nonetheless, further experimental work is needed to validate the suggested roles of CPPs on CQAs from this review.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Ethics Approval and Consent to Participate

Not applicable.

Submission Declaration and Verification

The work has not been published previously and it is not under consideration or approved for publication elsewhere.

Funding

This research was funded in whole or in part by the Austrian Science Fund (FWF) [grant DOI: 10.55776/I5910]. For open access purposes, the author has applied a CC BY public copyright license to any author accepted manuscript version arising from this submission.

Author Contributions

VW screened the literature about expansion processes of NK cells and prepared the draft of the manuscript. OS provided supervision and scope-related guidance. BK was a major contributor in formulating and structuring the document. All authors read and approved the final manuscript.

Acknowledgments

Not applicable.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2024.05.025.

References

[1] Mishra R, Welsh R, Szomolanyi-Tsuda E. NK cells and virus-related cancers. Crit Rev Oncog 2014;19:107–19.
[2] Zhang P, Yang S, Zou Y, Yan X, Wu H, Zhou M, et al. NK cell predicts the severity of acute graft-versus-host disease in patients after allogeneic stem cell transplantation using antithymocyte globulin (ATG) in pretreatment scheme. BMC Immunol 2019;20:46.
[3] Lodoen MB, Lanier LL. Natural killer cells as an initial defense against pathogens. Curr Opin Immunol 2006;18:391–8.
[4] Koepsell SA, Miller JS, McKenna Jr. DH. Natural killer cells: a review of manufacturing and clinical utility. Transfusion 2013;53:404–10.
[5] Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, et al. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N Engl J Med 1985;313:1485–92.
[6] Fang F, Xie S, Chen M, Li Y, Yue J, Ma J, et al. Advances in NK cell production. Cell Mol Immunol 2022;19:460–81.
[7] Angelo LS, Banerjee PP, Monaco-Shawver L, Rosen JB, Makedonas G, Forbes LR, et al. Practical NK cell phenotyping and variability in healthy adults. Immunol Res 2015;62:341–56.
[8] Becker PS, Suck G, Nowakowska P, Ullrich E, Seifried E, Bader P, et al. Selection and expansion of natural killer cells for NK cell-based immunotherapy. Cancer Immunol Immunother 2016;65:477–84.
[9] Farace F, Le Ridant A-M, Escudier B, Hercend T, Triebel F. Studies on NK cell purification by negative selection in human peripheral blood. Biotherapy 1992;5:11–20.
[10] Garcia-Aponte OF, Herwig C, Kozma B. Lymphocyte expansion in bioreactors: upgrading adoptive cell therapy. J Biol Eng 2021;15:13.
[11] Phelan K, May KM. Basic techniques in mammalian cell tissue culture. Curr Protoc Toxicol 2016;70:A.3b.1–22.
[12] Legazpi L, Laca A, Diaz M. Kinetic analysis of hybridoma cells viability under mechanical shear stress with and without serum protection. Bioprocess Biosyst Eng 2009;32:717–22.
[13] Shah G. Why do we still use serum in the production of biopharmaceuticals? Dev Biol Stand 1999;99:17–22.
[14] Yu Z, Kastenmüller G, He Y, Belcredi P, Möller G, Prehn C, et al. Differences between human plasma and serum metabolite profiles. PLoS One 2011;6:e21230.
[15] Davie DABNL, Culme EJ, Chris S, W MD, Rowley SJA. Peak serum: implications of serum supply for cell therapy manufacturing. Future Medicine 2012;7:7–13.
[16] Becknell B, Caligiuri MA. Interleukin-2, interleukin-15, and their roles in human natural killer cells. Adv Immunol 2005;86:209–39.
[17] Damoiseaux J. The IL-2 – IL-2 receptor pathway in health and disease: the role of the soluble IL-2 receptor. Clin Immunol 2020;218:108515.
[18] Wang G, Tschoi M, Spolski R, Lou Y, Ozaki K, Feng C, et al. In vivo antitumor activity of interleukin 21 mediated by natural killer cells. Cancer Res 2003;63:9016–22.
[19] Habib T, Nelson A, Kaushansky K. IL-21: a novel IL-2-family lymphokine that modulates B, T, and natural killer cell responses. J Allergy Clin Immunol 2003;112:1033–45.
[20] Strengell M, Matikainen S, Sirén J, Lehtonen A, Foster D, Julkunen I, et al. IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells. J Immunol 2003;170:5464–9.
[21] Landegren U, Andersson J, Wigzell H. Mechanism of T lymphocyte activation by OKT3 antibodies. A general model for T cell induction. Eur J Immunol 1984;14:325–8.
[22] Yu H, Chen W, Li C, Lin D, Liu J, Yang Z, et al. Large scale ex vivo expansion of clinical-grade effector cells for adoptive immunotherapy. Exp Ther Med 2017;14:5678–86.
[23] Johnson CDL, Zale NE, Frary ED, Lomakin JA. Feeder-cell-free and serum-free expansion of natural killer cells using Cloudz microspheres, G-Rex6M, and human platelet lysate. Front Immunol 2022;13:803380.
[24] Miller JS, Oelkers S, Verfaillie C, McGlave P. Role of monocytes in the expansion of human activated natural killer cells. Blood 1992;80:2221–9.
[25] Siegler U, Meyer-Monard S, Jorger S, Stern M, Tichelli A, Gratwohl A, et al. Good manufacturing practice-compliant cell sorting and large-scale expansion of single KIR-positive alloreactive human natural killer cells for multiple infusions to leukemia patients. Cytotherapy 2010;12:750–63.
[26] Berg M, Lundqvist A, McCoy Jr. P, Samsel L, Fan Y, Tawab A, et al. Clinical-grade ex vivo-expanded human natural killer cells up-regulate activating receptors and death receptor ligands and have enhanced cytolytic activity against tumor cells. Cytotherapy 2009;11:341–55.

[27] Lundqvist A, Berg M, Smith A, Childs RW. Bortezomib treatment to potentiate the anti-tumor immunity of ex-vivo expanded adoptively infused autologous natural killer cells. J Cancer 2011;2:383–5.
[28] Fujisaki H, Kakuda H, Shimasaki N, Imai C, Ma J, Lockey T, et al. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. Cancer Res 2009;69:4010–7.
[29] Granzin M, Soltenborn S, Muller S, Kollet J, Berg M, Cerwenka A, et al. Fully automated expansion and activation of clinical-grade natural killer cells for adoptive immunotherapy. Cytotherapy 2015;17:621–32.
[30] Lapteva N, Durett AG, Sun J, Rollins LA, Huye LL, Fang J, et al. Large-scale ex vivo expansion and characterization of natural killer cells for clinical applications. Cytotherapy 2012;14:1131–43.
[31] Denman CJ, Senyukov VV, Somanchi SS, Phatarpekar PV, Kopp LM, Johnson JL, et al. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. PLoS One 2012;7:e30264.
[32] Chang M, Tang X, Nelson L, Nyberg G, Du Z. Differential effects on natural killer cell production by membrane-bound cytokine stimulations. Biotechnol Bioeng 2022;119:1820–38.
[33] Gazitt Y, Shaughnessy P, Devore P. Mobilization of dendritic cells and NK cells in non-Hodgkin's lymphoma patients mobilized with different growth factors. J Hematother Stem Cell Res 2001;10:177–86.
[34] Wigginton KR, Kohn T. Virus disinfection mechanisms: the role of virus composition, structure, and function. Curr Opin Virol 2012;2:84–9.
[35] Vivier E, Rebuffet L, Narni-Mancinelli E, Cornen S, Igarashi RY, Fantin VR. Natural killer cell therapies. Nature 2024;626:727–36.
[36] Walzer T, Vivier E. G-protein-coupled receptors in control of natural killer cell migration. Trends Immunol 2011;32:486–92.
[37] Walzer T, Jaeger S, Chaix J, Vivier E. Natural killer cells: from CD3(-)NKp46(+) to post-genomics meta-analyses. Curr Opin Immunol 2007;19:365–72.
[38] Hudspeth K, Silva-Santos B, Mavilio D. Natural cytotoxicity receptors: broader expression patterns and functions in innate and adaptive immune cells. Front Immunol 2013;4:69.
[39] Paul S, Lal G. The molecular mechanism of natural killer cells function and its importance in cancer immunotherapy. Front Immunol 2017;8:1124.
[40] Siemaszko J, Marzec-Przyszlak A, Bogunia-Kubik K. NKG2D natural killer cell receptor—a short description and potential clinical applications. Cells 2021;10:1420.
[41] Siemaszko J, Marzec-Przyszlak A, Bogunia-Kubik K. Activating NKG2C receptor: functional characteristics and current strategies in clinical applications. Arch Immunol Ther Exp (Warsz) 2023;71:9.
[42] de Andrade LF, Smyth MJ, Martinet L. DNAM-1 control of natural killer cells functions through nectin and nectin-like proteins. Immunol Cell Biol 2014;92:237–44.
[43] Buller CW, Mathew PA, Mathew SO. Roles of NK cell receptors 2B4 (CD244), CS1 (CD319), and LLT1 (CLEC2D) in cancer. Cancers (Basel) 2020;12:1755.
[44] Prager I, Watzl C. Mechanisms of natural killer cell-mediated cellular cytotoxicity. J Leukoc Biol 2019;105:1319–29.
[45] Zamai L, Ahmad M, Bennett IM, Azzoni L, Alnemri ES, Perussia B. Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. J Exp Med 1998;188:2375–80.
[46] Heinisch O. Cochran WG. Sampling Techniques, 2. Aufl. John Wiley and Sons, New York, London 1963. Preis sBiometrische Zeitschrift19657203
[47] Grafe AVollmar J.Small numbers in mutagenicity testsArch Toxicol1977382734
[48] Owen DB. A table of normal integrals. Commun Stat Simul Comput 1980;9:389–419.
[49] Alageel RA, Bukhari AE, Alotaibi AS, Alsalman RS, Aldakhilallah MM, Siddiqui JJ, et al. Perception of stretch marks risk factors among adults in Riyadh, Saudi Arabia. Cureus 2021;13:e19561.
[50] Sutlu T, Stellan B, Gilljam M, Quezada HC, Nahi H, Gahrton G, et al. Clinical-grade, large-scale, feeder-free expansion of highly active human natural killer cells for adoptive immunotherapy using an automated bioreactor. Cytotherapy 2010;12:1044–55.
[51] Spanholtz J, Preijers F, Tordoir M, Trilsbeek C, Paardekooper J, de Witte T, et al. Clinical-grade generation of active NK cells from cord blood hematopoietic progenitor cells for immunotherapy using a closed-system culture process. PLoS One 2011;6:e20740.
[52] Min B, Choi H, Her JH, Jung MY, Kim HJ, Jung MY, et al. Optimization of large-scale expansion and cryopreservation of human natural killer cells for anti-tumor therapy. Immune Netw 2018;18:e31.
[53] Lee HR, Son CH, Koh EK, Bae JH, Kang CD, Yang K, et al. Expansion of cytotoxic natural killer cells using irradiated autologous peripheral blood mononuclear cells and anti-CD16 antibody. Sci Rep 2017;7:11075.
[54] Oyer JL, Igarashi RY, Kulikowski AR, Colosimo DA, Solh MM, Zakari A, et al. Generation of highly cytotoxic natural killer cells for treatment of acute myelogenous leukemia using a feeder-free, particle-based approach. Biol Blood Marrow Transplant 2015;21:632–9.
[55] Alici E, Sutlu T, Bjorkstrand B, Gilljam M, Stellan B, Nahi H, et al. Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components. Blood 2008;111:3155–62.
[56] Kloss S, Oberschmidt O, Morgan M, Dahlke J, Arseniev L, Huppert V, et al. Optimization of human NK cell manufacturing: fully automated separation, improved ex vivo expansion using IL-21 with autologous feeder cells, and generation of anti-CD123-CAR-expressing effector cells. Hum Gene Ther 2017;28:897–913.
[57] Oberschmidt O, Morgan M, Huppert V, Kessler J, Gardlowski T, Matthies N, et al. Development of automated separation, expansion, and quality control protocols for clinical-scale manufacturing of primary human NK cells and alpharetroviral chimeric antigen receptor engineering. Hum Gene Ther Methods 2019;30:102–20.
[58] Nowakowska P, Romanski A, Miller N, Odendahl M, Bonig H, Zhang C, et al. Clinical grade manufacturing of genetically modified, CAR-expressing NK-92 cells for the treatment of ErbB2-positive malignancies. Cancer Immunol Immunother 2018;67:25–38.
[59] Luhm JBJ, Koritke P, Höppner M, Kirchner H, Frohn C. Large-scale generation of natural killer lymphocytes for clinical application. J Hematother Stem Cell Res 2002;11:651–7.
[60] Broker K, Sinelnikov E, Gustavus D, Schumacher U, Portner R, Hoffmeister H, et al. Mass production of highly active NK cells for cancer immunotherapy in a GMP conform perfusion bioreactor. Front Bioeng Biotechnol 2019;7:194.
[61] Ojo EO, Sharma AA, Liu R, Moreton S, Checkley-Luttge MA, Gupta K, et al. Membrane bound IL-21 based NK cell feeder cells drive robust expansion and metabolic activation of NK cells. Sci Rep 2019;9:14916.
[62] Ma D, Cao W, Kapur A, Felder M, Scarlett CO, Patankar MS, et al. Differential expression of proteins in naive and IL-2 stimulated primary human NK cells identified by global proteomic analysis. J Proteomics 2013;91:151–63.
[63] Park KU, Jin P, Sabatino M, Feng J, Civini S, Khuu H, et al. Gene expression analysis of ex vivo expanded and freshly isolated NK cells from cancer patients. J Immunother 2010;33:945–55.
[64] Lim O, Lee Y, Chung H, Her JH, Kang SM, Jung MY, et al. GMP-compliant, large-scale expanded allogeneic natural killer cells have potent cytolytic activity against cancer cells in vitro and in vivo. PLoS One 2013;8:e53611.
[65] Voskens CJ, Watanabe R, Rollins S, Campana S, Hasumi L, Mann DL. Ex-vivo expanded human NK cells express activating receptors that mediate cytotoxicity of allogeneic and autologous cancer cell lines by direct recognition and antibody directed cellular cytotoxicity. J Exp Clin Cancer Res 2010;29:134.
[66] Torelli GF, Rozera C, Santodonato L, Peragine N, D'Agostino G, Montefiore E, et al. A good manufacturing practice method to ex vivo expand natural killer cells for clinical use. Blood Transfus 2015;13:464–71.
[67] Peragine N, Torelli GF, Mariglia P, Pauselli S, Vitale A, Guarini A, et al. Immunophenotypic and functional characterization of ex vivo expanded natural killer cells for clinical use in acute lymphoblastic leukemia patients. Cancer Immunol Immunother 2015;64:201–11.
[68] Lim SA, Kim TJ, Lee JE, Sonn CH, Kim K, Kim J, et al. Ex vivo expansion of highly cytotoxic human NK cells by cocultivation with irradiated tumor cells for adoptive immunotherapy. Cancer Res 2013;73:2598–607.
[69] Carlens S, Gilljam M, Chambers BJ, Aschan J, Guven H, Ljunggren HG, et al. A new method for in vitro expansion of cytotoxic human CD3-CD56+ natural killer cells. Hum Immunol 2001;62:1092–8.
[70] Klingemann HG, Martinson J. Ex vivo expansion of natural killer cells for clinical applications. Cytotherapy 2004;6:15–22.
[71] Besser MJ, Shoham T, Harari-Steinberg O, Zabari N, Ortenberg R, Yakirevitch A, et al. Development of allogeneic NK cell adoptive transfer therapy in metastatic melanoma patients: in vitro preclinical optimization studies. PLoS One 2013;8:e57922.
[72] Carlens S, Gilljam M, Chambers BJ, Aschan J, Guven H, Ljunggren H-G, et al. A new method for in vitro expansion of cytotoxic human CD3−CD56+ natural killer cells. Human Immunol 2001;62:1092–8.
[73] Freedman RS, Tomasovic B, Templin S, Atkinson EN, Kudelka A, Edwards CL, et al. Large-scale expansion in interleukin-2 of tumor-infiltrating lymphocytes from patients with ovarian carcinoma for adoptive immunotherapy. J Immunol Methods 1994;167:145–60.
[74] Ziegler SF, Ramsdell F, Alderson MR. The activation antigen CD69. Stem Cells 1996;12:456–65.
[75] Whang M, Xie M-H, Jamboretz K, Lemar H, Guo C, Rahman N, et al. Potentiating the large-scale expansion and engineering of peripheral blood-derived CAR NK cells for off-the-shelf application. J. Immunotherap. Cancer. 2021;9. https://doi.org/10.1136/jitc-2021-SITC2021.151.
[76] Koehl U, Esser R, Zimmermann S, Tonn T, Kotchetkov R, Bartling T, et al. Ex vivo expansion of highly purified NK cells for immunotherapy after haploidentical stem cell transplantation in children. Klin Padiatr 2005;217:345–50.
[77] Trapani JA. Granzymes: a family of lymphocyte granule serine proteases. Genome Biol 2001;2. reviews3014.1−7.
[78] Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 2000;1:489–95.
[79] Seol DW, Li J, Seol MH, Park SY, Talanian RV, Billiar TR. Signaling events triggered by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL): caspase-8 is required for TRAIL-induced apoptosis. Cancer Res 2001;61:1138–43.
[80] Gritzapis AD, Dimitroulopoulos D, Paraskevas E, Baxevanis CN, Papamichail M. Large-scale expansion of CD3(+)CD56(+) lymphocytes capable of lysing autologous tumor cells with cytokine-rich supernatants. Cancer Immunol Immunother 2002;51:440–8.
[81] Aktas E, Kucuksezer UC, Bilgic S, Erten G, Deniz G. Relationship between CD107a expression and cytotoxic activity. Cell Immunol 2009;254:149–54.
[82] Idorn M, Hojman P. Exercise-dependent regulation of NK cells in cancer protection. Trends Mol Med 2016;22:565–77.
[83] Liu S, Shah SJ, Wilmes LJ, Feiner J, Kodibagkar VD, Wendland MF, et al. Quantitative tissue oxygen measurement in multiple organs using 19F MRI in a rat model. Magn Reson Med 2011;66:1722–30.
[84] Foley B, Ta C, Barnes S, de Jong E, Nguyen M, Cheung LC, et al. Identifying the optimal donor for natural killer cell adoptive therapy to treat paediatric B- and T-cell acute lymphoblastic leukaemia. Clin Transl Immunol 2020;9:e1151.
[85] Barnes SA, Trew I, de Jong E, Foley B. Making a killer: selecting the optimal natural killer cells for improved immunotherapies. Front Immunol 2021;12:765705.