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

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1 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 adds 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.

2 Keywords
Natural killer cells, NK cells, CQA, CPP, expansion process, culture platform, static, dynamic

3 Background
3.1 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 number 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.

3.2 Challenges of natural killer 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 pre-processed 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 pre-processing step is increasing the likelihood of a pure NK cell expansion, as also other cell types, such as T lymphocytes, can proliferate under the conditions used for NK cells. The fraction of cells resulting from the pre-processing steps are 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 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 process parameters (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 Quality Attributes (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 that further increases the challenge of cellular therapies.

3.3 Process parameters of natural killer 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 factors are 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 wellbeing 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 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, anti-apoptotic 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 interleukins, 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 interleukins 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 (EBV-TM-LCL), 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 a cellular therapy very different to other biopharmaceuticals where such methods can be applied at the end of the process (34).

![Diagram of process parameters and quality attributes](image)

Figure 1 Overview of the different process parameters and quality attributes of NK cell expansion processes.

### 3.4 Quality attributes of natural killer 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 are 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 the maturity. About 5-15% of the circulating NK cell population is characterized as CD56bright (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 CD56dim 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 (KIRs), which are involved in recognizing and targeting specific cellular
stress signals (4). This population can be further separated into a \( \text{CD16}^{\text{dim}} \) \( \text{NKG2C}^{\text{high}} \) population which provides memory-like properties with enhanced functional properties (35). The second subgroup of the \( \text{CD56}^{\text{dim}} \) population is characterized by \( \text{CD16}^{-} \) 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 is 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 receptors, called natural killer 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.

### 3.5 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 process parameters (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 (47).

The identification of CPPs and CQAs was challenging because the scope of the experiments in the literature are 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 medium 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.

4 Data extraction and analysis methods

4.1 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 natural killer 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 were 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 (Figure 2). Similarly, all reported cellular parameters or behavior were considered as a QA (Figure 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).
Figure 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.
### Table 1: Categorization of process parameters and quality attributes including the ranges and differences in source or quality

<table>
<thead>
<tr>
<th>PP category</th>
<th>Content or range</th>
<th>QA category</th>
<th>Content or range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Human or animal source (e.g. FBS)</td>
<td>Fold change low</td>
<td>1-100-fold</td>
</tr>
<tr>
<td>IL-2 low</td>
<td>10-100IU/mL</td>
<td>Fold change medium</td>
<td>100-1,000-fold</td>
</tr>
<tr>
<td>IL-2 medium</td>
<td>100-500IU/mL</td>
<td>Fold change high</td>
<td>&gt;1,000-fold</td>
</tr>
<tr>
<td>IL-2 high</td>
<td>500-1,000IU/mL</td>
<td>Viability high (dynamic/static format)</td>
<td>≥85%/≥95%</td>
</tr>
<tr>
<td>Depletion of other cell types</td>
<td>All other cell types than CD3+ cells (e.g. CD4+, CD19+, CD20+, CD33+)</td>
<td>Purity low</td>
<td>&lt;50%</td>
</tr>
<tr>
<td>Seeding density low</td>
<td>&lt;1x10⁶ cells/mL</td>
<td>Purity medium</td>
<td>50-90%</td>
</tr>
<tr>
<td>Seeding density high</td>
<td>≥ 1x10⁶ cells/mL</td>
<td>Purity high</td>
<td>≥90%</td>
</tr>
<tr>
<td>Short culture duration</td>
<td>1-5 days</td>
<td>Cytotoxicity high</td>
<td>&gt;70%</td>
</tr>
<tr>
<td>Long culture duration</td>
<td>&gt; 5 days</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 4.2 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 that 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) (48). 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 (46) 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 (49). 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% (50). 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 QAs with statistical significance, however, this does not implicate that the results calculated later on in the meta-analysis are statistically significant.

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

\[
n = \frac{z^2 \times p(1-p)}{e^2} \left(1 + \frac{z^2 \times p(1-p)}{e^2 \times N}\right)
\]

- 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.

4.3 Visualization of PP:QA frequencies

To visualize the generated results, heatmaps were created using the relative frequencies of dynamic and static cultures, separately (Figure 3 and Figure 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 (Error! Reference source not found. and Error! Reference source not found.).
5 Influence of process parameters on quality attributes

5.1 Identified process parameters and quality attributes

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 (Figure 3 and Figure 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 (Error! Reference source not found.). 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 (Error! Reference source not found.). 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 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.

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

<table>
<thead>
<tr>
<th>No.</th>
<th>QAs studied in dynamic cultures</th>
<th>Count of QA</th>
<th>QAs studied in static cultures</th>
<th>Count of QA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NKG2D upregulation</td>
<td>13</td>
<td>NKG2D upregulation</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>NKp30 upregulation</td>
<td>12</td>
<td>NKp30 upregulation</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>NKp44 upregulation</td>
<td>12</td>
<td>NKp44 upregulation</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>Proliferation fold change low</td>
<td>10</td>
<td>Proliferation fold change medium</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>E:T 10:1 cytotoxicity high</td>
<td>10</td>
<td>Proliferation fold change low</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>NKG2C upregulation</td>
<td>8</td>
<td>E:T 5:1 cytotoxicity high</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>TRAIL upregulation</td>
<td>8</td>
<td>DNAM1 upregulation</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>FasL upregulation</td>
<td>8</td>
<td>E:T 10:1 cytotoxicity high</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>Proliferation fold change high</td>
<td>7</td>
<td>E:T 2.5:1 cytotoxicity high</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>CD25 upregulation</td>
<td>7</td>
<td>CD69 upregulation</td>
<td>8</td>
</tr>
<tr>
<td>11</td>
<td>Proliferation fold change medium</td>
<td>5</td>
<td>CD107a upregulation</td>
<td>7</td>
</tr>
<tr>
<td>12</td>
<td>Purity high</td>
<td>5</td>
<td>NKP46 upregulation</td>
<td>6</td>
</tr>
<tr>
<td>13</td>
<td>DNAM1 upregulation</td>
<td>5</td>
<td>CD56 upregulation</td>
<td>6</td>
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<tr>
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5.2 Influence of process parameters on quality attributes 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, 51, 52), however, plasma was only used in 14% of all instances (Figure 3 cyan QAs) (53). Similarly, in static cultures serum was used in 80% of all experiments that showed high proliferation (Figure 4 cyan QAs) (25, 54-56). 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 1,000-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, 52, 57).

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 was achieved in 80% when CD3+ cells were depleted or in 60% when other, non-NK cell types were depleted (29, 52, 53). Additionally, a prolonged culture period and the use of feeder cells resulted for 60% of all analyzed experiments in high purity (29, 52, 53).

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 IL-2 QAs) (29, 51, 53, 58, 59). Nevertheless, high proliferation fold changes were observed in 71% when low level IL-2 concentrations were used (60, 61). 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, 54, 56, 62). 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 (63). 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, 51, 53, 54, 56, 58, 59, 62, 64-72). 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 (63). 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 time, 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, 54, 56, 62). Feeding fresh medium during culture, or a long culture duration of more than five days was also done in 80% or 100% of experiments, respectively when high proliferation was achieved in static cultures (25, 54-56, 62). 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, 54, 57, 67, 68, 71-74). 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 a viability greater than 95% (Figure 4 cyan QAs) (67, 68). 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) (67, 68). In contrast, the enrichment of CD56+ cells was never done in dynamic cultures when high viability was achieved.
Figure 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 section 4.2. 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.

5.3 Influence of process parameters 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, 51-53, 58, 61, 64). 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, 54, 56, 62, 65-69, 72). 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 (Figure 3 and Figure 4 blue QAs) (25, 28-30, 53, 58, 61, 62, 64, 65, 67-69, 72). 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, 51, 52, 54-58, 61, 64, 68, 69, 72). 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) (58, 61). 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 (54, 56), but only in 16% and 18% when NKp30 and NKp44 were upregulated, respectively (25, 54, 56, 65, 72). Contrary, in dynamic cultures OKT3 was never used when an upregulation of NKp46 was reported.

The differences in PP stimulation between culture formats continues 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, 54, 55, 57, 62, 65-69, 72) or in 56%, 77% and 83%, respectively when seeded initially at a low seeding density (25, 28, 54, 55, 65, 66, 68). 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, 53, 64) 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 (51, 53). 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, 51-53, 55, 57, 58, 65-67, 69, 72) or culturing them for a longer period (>5 days) (25, 30, 54-56, 62, 65-69, 72) 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 (Figure 3 and Figure 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.
5.4 Influence of process parameters on natural killer receptors and other activating markers

The natural killer 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 natural killer 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 (75). 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, 51, 52, 58, 61) and 75%, 71% and 70% of static cultures, respectively (Figure 3 and Figure 4 green QAs) (25, 28, 30, 54-57, 62, 68, 69, 72). CD69 was enhanced 88% of all static cultures when serum was used in the process (57, 68, 69). 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 (63). 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, 54-57, 62, 65-69, 72) as well as CD69 in static format (65, 68, 69).

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, 51-53, 58). 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) (54, 55, 69). 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, 54, 55, 66, 67, 69, 72). Also, in dynamic cultures NKG2C, NKG2D were upregulated in 50% and 62% when medium was fed during culture, respectively (29, 51-53, 58). 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, 54, 55, 62, 65-68, 72) and more than 60% in dynamic cultures (29, 51-53, 58)). Another similarity in the upregulation of activating natural killer 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, 53, 58, 61). Similarly, in static cultures NKG2D and DNAM1 were upregulated in 64% and 50% when CD3+ cells were depleted (25, 28, 30, 62, 67-69, 72). 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 natural killer 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, 54, 55, 57, 62, 65-69, 72) as well as 2B4 in 67% of dynamic cultures (Figure 3 green QAs) (51). 2B4 upregulation appeared to be positively influenced when the anti CD3 antibody OKT3 was used (60% of all experiments), however only in static cultures (54, 56). 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).

5.5 Cytotoxicity influencing process parameters

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 evidences 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, an enhanced cytotoxicity was observed with a relative frequency of 60% in all experiments that were performed with serum (29, 51), and in 90% with a medium level IL-2 concentration (29, 51, 53) (Figure 3 orange QAs). In static cultures, a similar picture could be drawn, as serum was used in 50%-75% (25, 28, 54, 55, 57, 69, 70), and a medium IL-2 concentration 42-62% of all instances with enhanced cytotoxicity, depending on the E:T ratio (25, 54, 62, 65, 67, 70) (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, 51, 53), while 40-88% of static cultures used feeder cells depending on the E:T ratio (Figure 4 orange QAs) (28, 54, 57, 62, 65, 67, 69). 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 (76). 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 (51, 53) and more than 67% of static experiments, depending on the E:T ratio (Figure 3 and Figure 4 orange QAs) (25, 28, 54, 55, 65, 67, 70). 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, 51, 77) and static cultures, respectively (25, 28, 54, 55, 62, 65, 67, 69, 70). 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, 51, 53) and in all static cultures at an E:T of 5:1 and 2.5:1 (25, 28, 54, 55, 57, 62, 65, 67). 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 (78).
5.6 Process parameters 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 (79, 80). 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 were 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, 58, 61, 64) 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, 51, 58, 61, 64) (Figure 3 and Figure 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, 58, 61, 64), 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, 58, 64). 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, 64). 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, 58). Most probable reason was that the upregulation of the death ligands had happened only upon adaptation to the in vitro conditions that 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 (Figure 3 and Figure 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 an upregulation of both death ligands were used (55, 57). Also, feeding the culture with fresh medium upregulated the expression of the two death ligands in all experiments (55, 57). The only similarity between static and dynamic culture was the use of serum (63% in dynamic and 100% in static format) (55, 57) as well as a long culture period (75% in dynamic and 50% in static format) (55).

5.7 Process parameters 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 interferon gamma (IFN-γ) were enhanced when serum was added to static cultures in 57% and 67% of experiments, respectively (54, 57) (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 (54, 57, 65, 67). Furthermore, feeding the cells with medium during culture was also beneficial for CD107a upregulation in 67% of all experiments (54, 65, 67). 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 (54, 57, 65, 67). Low level IL-2 concentrations however, were never used when an upregulation of CD107a was observed, leading to the conclusion that a 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 (54, 65, 67, 69, 81). The production of interleukin 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 (82).

5.8 Process parameters influencing NK cell maturation

NK cells can be grouped in two populations based on the level of CD56 expression. Firstly, 5-15% of the circulating NK cell population is characterized as CD56\textsuperscript{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. Secondly, the CD56\textsuperscript{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 (KIRs), 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\textsuperscript{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) (67, 68). Also, an upregulation of CD56 was observed more often in combination with plasma as medium supplement, compared to serum (67% and 50%, respectively) (55, 67, 68). 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) (67, 68). 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\textsuperscript{bright} cells, characterized by faster proliferation than the CD56\textsuperscript{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 five days culture duration appeared to be beneficial for CD56 upregulation, as it was used in 83% of all experiments (Figure 4 black QAs) (55, 67, 68). Additionally, the depletion of CD3\textsuperscript{+} cells (67, 68), and the co-culture with feeder cells was reported in 67% when CD56 was upregulated (55, 67). As described earlier CD56\textsuperscript{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 \((55, 67, 68)\).

![Figure 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 section 2.4. 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.](image-url)
5.9 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 (83). Similarly, a decreased oxygen concentration could have been studied, as the oxygen level in the blood, tissue or a solid tumor can vary drastically (84). 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 (85, 86). 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 (85, 86). 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 affects several different CQAs highlighting its inevitable and probably one of the most important roles in NK expansion and activation processes (Table 3).
Table 3 Summary list of NK cell CQAs in static and dynamic cultures and CPPs that influence these CQAs. 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.

<table>
<thead>
<tr>
<th>Affected CQA group</th>
<th>Culture format</th>
<th>Influential CPP [range where applicable]</th>
</tr>
</thead>
</table>
| Proliferation/cell growth        | Dynamic cultures | • Serum  
• IL-2 low level [10-100IU/mL]  
• Feeding fresh medium during culture  
• Initial seeding density low [<1x10⁶ cells/mL]                                                                 |
|                                  | Static cultures | • Serum  
• IL-2 medium level [100-500IU/mL]  
• Anti CD3 antibody OKT3  
• Initial seeding density low [<1x10⁶ 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 [100-500IU/mL] |
|                                  | Static cultures | • IL-15  
• CD56⁺ cell enrichment |
| Cytotoxicity                     | Dynamic cultures | • Serum  
• IL-2 medium level [100-500IU/mL]  
• Feeding fresh medium during culture  
• Initial seeding density low [<1x10⁶ cells/mL]  
• Co-culture with feeder cells  
• Long culture (> 5 days) |
|                                  | Static cultures | • Serum  
• IL-2 medium level [100-500IU/mL]  
• Initial seeding density low [<1x10⁶ cells/mL]  
• Co-culture with feeder cells  
• Long culture (> 5 days) |
| Degranulation and cytokine production | Dynamic cultures | • IL-2 medium level [100-500IU/mL]  
• CD3⁻ cell depletion |
|                                  | Static cultures | • Serum  
• IL-2 medium level [100-500IU/mL]  
• Feeding fresh medium during culture  
• Co-culture with feeder cells  
• Long culture (> 5 days) |

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 the 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.

6 Conclusion
In this contribution, process parameters and quality attributes 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.

7 List of abbreviations
- Critical Quality Attribute ........................................CQA
- Critical Process Parameter ......................................CPP
- Quality Attribute ..................................................QA
- Process Parameter ................................................PP
- Natural Killer .......................................................NK
- Interleukin .............................................................IL-
- Effector to target ....................................................E:T
- Stem Cell Growth Medium .....................................SCGM
- Minimum Essential Medium α .................................αMEM
- Effector to target ....................................................E:T

8 Declarations
8.1 Ethics approval and consent to participate
Not Applicable.

8.2 Declaration of interest
'Declarations of interest: none'

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

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Not Applicable.

9 References

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