Process Engineering of Natural Killer Cell-based Immunotherapy

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ABSTRACT

Cell therapy offers the potential for curative treatment of cancers. While T cells have been the predominantly used cell type, natural killer (NK) cells have attracted much attention because of their capability to kill cancer cells and the advantage of being naturally suitable for allogeneic applications. Upon stimulation by cytokines or activation by a target cell, NK cells proliferate and expand their population. These cytotoxic NK cells can be cryopreserved and used as an off-the-shelf medicine. The production process of NK cells will thus differ from autologous cell therapies. This article will briefly outline key biological features of NK cells, review the manufacturing technologies for protein biologics and their adaptation for developing robust NK cell biomanufacturing processes.

NK CELL-BASED CANCER IMMUNOTHERAPY

Cell therapy has emerged as a promising treatment for cancers, especially hematologic cancers [1]. The potential of curative treatment of cancers using genetically modified chimeric antigen receptor (CAR)-T cells to recognize and kill tumors expressing specific antigens has raised the hope that cell therapy may spur another rapid expansion of cell-based biomanufacturing as seen in the growth of protein biologics in the past quarter century. While initial breakthroughs in cell-based immunotherapies were accomplished using **autologous (see Glossary)** CAR-T cell therapies, in recent years, significant research efforts and several clinical trials have been directed towards developing **allogeneic** CAR-T cell therapies, which offer the potential for broader accessibility and cost-effectiveness [2]. Natural killer (NK) cells have also emerged as a promising type of allogeneic immunotherapy due to their intrinsic ability to be employed in such applications [3]. Unlike cytotoxic T cells, which are part of the adaptive immune response and recognize specific antigens on diseased cells, NK cells are involved in innate immunity, and they recognize and kill tumor cells and virus infected cells without relying on specific peptide presentation by human leukocyte antigens (HLAs) [4]. NK cells also have an added advantage of not causing **graft-versus-host disease (GvHD),** making them a favorable candidate for allogeneic cell therapies [1]. The success of allogeneic cell therapies will likely change the face of cell therapy biomanufacturing, moving it from boutique operations to banked cell-based, well-controlled processes. However, challenges remain in the forthcoming transformation akin to the transition of protein biologics production from exploratory research endeavors to multi-thousands kilogram therapeutic protein manufacturing. The path taken in cell culture engineering for protein biologic production in the past quarter century could be a guide for advancing cell therapy

biomanufacturing. This article will highlight the technological advances that will help move cell therapy processing forward and the opportunities for innovations ahead.

NK CELLS AND RECEPTORS

NK cells are cytotoxic lymphocytes constituting approximately 5-15% of human adult **peripheral blood mononuclear cells (PBMCs)**. They characteristically express the adhesion molecule CD56 while lacking surface expression of the TCR-CD3 complex [5]. In our bodies, NK cells serve as a first line of defense within the innate immune system to respond rapidly to malignant and infected cells without the need for extensive priming. This contrasts with cytotoxic T cells within the adaptive immune system that recognize peptide antigens presented by HLA molecules on the surface of malignant cells and require several priming steps to achieve full effector function. NK cells survey tissues throughout the body using an array of activating and inhibitory surface receptors with specificity for various ligands (Figure 1). Cancer cells and virus-infected cells often express stress proteins that serve as activating ligands and mark them as potentially dangerous cells that ought to be destroyed. The major inhibitory ligands on target cells are HLAs, which NK cells recognize through inhibitory killer immunoglobulin-like receptors (KIRs) to distinguish “self” versus “non-self” [6]. Cancer cells frequently downregulate class I HLAs to escape recognition by cytotoxic T cells. However, the reduced expression and binding to the inhibitory receptors makes them susceptible to killing by NK cells. The balance of activating and inhibitory signaling dictates whether NK cells initiate natural cytotoxicity.

In addition to killing cancer and infected cells, NK cells also mediate antibody-dependent cellular cytotoxicity (ADCC). In ADCC, CD16, which is the low-affinity Fc receptor found on the surface of most peripheral blood NK cells, interacts with the Fc region of antibodies bound to their corresponding surface antigen on a target cell. This triggers a strong cytotoxic response and the

secretion of inflammatory cytokines. The expression level of activating ligands and inhibitory molecules varies widely among different cancer cells [6]. Through the binding of different combinations of activating and inhibitory receptor-ligand pairs, NK cells employ a robust pattern recognition system for the discrimination and destruction of abnormal cells (Figure 1).

NK CELL PROLIFERATION AND PERSISTENCE

A consequence of NK activation upon killing a target cell (see Box 1 for more details) is vigorous proliferation, though stimulation with cytokines alone can also trigger NK cell effector responses and drive limited proliferation [7]. Cytokine stimulation activates JAK/STAT signaling pathways that interact with the pathways downstream of NK activating receptor signaling [8]. The term "activation" is often used loosely to refer to the triggering of cytokine release and cytotoxicity, promotion of limited proliferation, or induction of vigorous proliferation.

The doubling time of NK cells in healthy human blood was estimated to be 1-2 weeks [9], certainly rather slow as most NK cells are not activated. *In vitro*, NK cells have a doubling time of about 1.25 days when cocultured with engineered K562 **feeder cells** [10]. NK cells are also subject to turn-over, and their half-life in circulation is around two weeks [9]. Upon adoptive transfer of allogeneic NK cells into a recipient, the representation of transplanted NK cells in the blood is determined by the balance of their expansion, turn-over, and migration to other organs such as secondary lymphoid organs. Unless the transferred cells continue to proliferate, they will eventually diminish and disappear. Previous studies indicate that the persistence of adoptively transferred NK cells in patients' blood, which is determined by the detectability of donor-specific HLA using flow cytometry or PCR, correlates with treatment outcome [11, 12]. Unlike autologous CAR-T cells, which have been shown to persist in the patient's body for months to years, allogeneic NK cells persist for only a few weeks after adoptive transfer [11-14]. Importantly,

allogeneic NK cell therapies require lymphodepletion, in which the patient is treated with chemotherapy to kill patient's T cells, before NK cells administration to mitigate against rapid host rejection and to generate niches for homeostatic expansion. However, the recipient's adaptive immune system often recovers after 2-3 weeks and eliminates the allogeneic donor NK cells, limiting the persistence of allogeneic NK cells [15]. Therefore, multi-dose treatment may be required to increase the efficacy of allogeneic NK cell therapies [12, 16]. Cytomegalovirus (CMV) seropositive people frequently harbor an **adaptive NK cell** population defined as $NKG2C^{+}CD57^{+}$ that can persist for up to 35 months [17, 18]. Several ongoing clinical trials are examining the anti-tumor efficacy of adaptive NK cells (NCT03383055, NCT03081780, and NCT03319459, registered with ClinicalTrials.gov). Ex vivo treatment of peripheral blood NK cells with cytokines such as IL-15, IL-12, and IL-18 also creates memory-like NK cells which were shown to persist in acute myeloid leukemia (AML) patients for more than two months in immune-compatible settings. Feeder-expanded NK cells also have shown improved persistence *in vivo* compared with IL-2 treated and untreated NK cells [19]. Recently, it was reported that anti-CD19 CAR NK cells can be detected at low levels in patients for at least 12 months after infusion despite HLA mismatching, suggesting addition of CARs to NK cells could improve their *in vivo* persistence [20]. However, depending on the source of NK cells, the efficiency of CAR engineering is quite different [21].

SOURCES OF NK CELLS

Primary NK cells for cell therapy applications can be isolated from adult donor peripheral blood mononuclear cells (PBMCs) or **umbilical cord blood (UCB)** (Figure 2A-B). NK cells constitute around 10% of PBMCs [22] and 30% of lymphocytes in UCB [23]. NK cells are typically enriched from PBMCs by magnetic depletion to remove T cells and B cells. From a 450-ml of donor

peripheral blood, multiples of 10^7 to 10^8 NK cells can be isolated. NK cells isolated from PBMCs are subdivided into a less mature and less abundant (~5-10%) subtype of $CD56^{bright}CD16^-$ ($CD56^{bright}$) NK cells and a more mature $CD56^{dim}CD16^+$ ($CD56^{dim}$) NK cell subtype [22]. While both subtypes of NK cells can produce cytokines and mediate cellular cytotoxicity toward infected or malignant cells, $CD56^{bright}$ cells show higher cytokine secretion and lower cytotoxicity. The majority of NK cells in UCB are less mature $CD56^{bright}$ cells, which have higher proliferation capacity. However, they exhibit lower levels of multiple activating and inhibitory receptors and are less potent in killing target cells compared to NK cells from adult PBMCs [23].

With a typical treatment requiring ~10^9 NK cells per dose [24], a common strategy is to expand the enriched primary NK population *ex vivo* prior to adoptive transfer (Figure 2E). Expanded NK cell products exhibit cytotoxicity and robust cytokine production in response to a broad array of cancer cell lines [25, 26]. Early results from NK cell clinical trials are encouraging, but with variable outcomes based on the tumor type and method of NK expansion [27]. In one study, for instance, a 72% overall response rate was reported in patients with relapse or refractory acute leukemia with feeder cell expanded PBMC-derived NK cells [28]. While cytokine stimulation of purified NK cells elicits only limited cell expansion, co-culture of NK cells with feeder cells results in much faster growth rate and higher fold-expansions [25], enabling large-scale production of primary NK cells for immunotherapy.

NK cells can also be derived from **human induced pluripotent stem cells (hiPSCs)** or **human embryonic stem cells (hESCs)** by directed differentiation first to hematopoietic lineage then to the NK cell lineage (Figure 2C) [29]. The directed differentiation yields a relatively homogeneous NK cell population with a reduced risk of contamination from residual T cells, which can be an issue with primary NK cell products [29, 30]. Unlike NK cells isolated from PBMCs, which have

very low transduction efficiency and are hard to genetically modify, hiPCSs and hESCs can be genetically modified at the stem cell state to establish clonal lines that give rise to NK cells with enhanced functionalities or differentiation characteristics [31]. By using hiPSCs to derive NK cells, one can distribute different degrees of cell expansion to hiPSCs, $CD34^+$ hematopoietic progenitors, and NK cell stages instead of relying on all of the cell expansion to take place at the differentiated NK cell stage, as in the case of primary NK cells. Even early passage NK cells can be acquired in large quantities and banked for further NK expansion for final product, allowing for more product consistency and potentially unlimited sources for biomanufacturing.

Another source of NK cells for therapeutic applications is the NK-92 cell line (Figure 2D). These cells can be grown to very large quantities and can be genetically modified to express CARs for cancer cell targeting. However, they lack certain receptors which are commonly found on primary NK cells, such as CD16 and most of the KIR family receptors [32]. Furthermore, because of their tumorigenic nature, they need to be irradiated to deprive them of proliferative capacity. This significantly reduces their persistence *in vivo* [33].

NK CELLS IN CULTURE: IMPLICATION OF BIOLOGY ON PROCESS DESIGN

NK cells for adoptive transfer may originate from patient PBMCs, donor PBMCs, UCB, or hiPSCs. Regardless of the source, these NK cells all undergo *ex vivo* activation using short- to long-term expansion protocols [34]. Activation of NK cells using the cytokines IL-2 or IL-15 results in increased cytotoxicity and elicits moderate cell expansion before adoptive transfer. This is sufficient for cryopreservation and multiple transfers to the patient [35]. However, the degree of cell expansion with protocols that rely on cytokine stimulation alone is insufficient for wide-spread allogeneic applications regardless of the NK cell source. In an optimized biomanufacturing setting, one would seek to generate a very large number of doses in order to benefit from economy of scale

and to increase the consistency of product quality. As a reference point, in the production of protein biologics, tens of thousands of doses may be produced in a single batch. A biomanufacturing process of NK cells for allogeneic therapy may aim to produce hundreds or even thousands of doses, or up to a trillion (10^{12}) cells per run. While such a scale may be years away, it is prudent to bear that in mind in making process choices.

To expand NK cells beyond the level that cytokine stimulation can achieve, most have relied on co-culture of NK cells with feeder cells, or in some cases feeder cell-free systems based on receptor-ligand pairs [36-38]. Commonly used feeder cell lines include RPMI8866, EBV-LCL, and K562, or their genetically engineered variants [39]. These feeder cells likely induce expansion by presenting activating ligands to NK cells. They are irradiated to deprive them of the capacity to multiply and are co-cultured at a high effector-to-target (E:T) ratios (1:1 or 2:1) with NK cells. Over time they are killed and lysed by NK cells. However, it is not clear whether other biological processes such as synapse formation or granule release also play a role in NK cell expansion after interaction with feeder cells.

The feeder cells that elicit the greatest levels of expansion are engineered K562 cells that express membrane bound 4-1BB ligand (4-1BBL) and membrane bound IL-21 or IL-15 (herein denoted as K562/mbIL-15-4-1BBL or K562/mbIL-21-4-1BBL) [40]. A similar engineered K562 line expressing OX40 has also been reported[41]. Upon feeder cell activation, particularly with the use of the engineered K562/mbIL-21-4-1BBL line, NK cells grow vigorously. Periodic re-stimulation by feeder cells extends the growth period to increase population expansion [25]. A 10^4- to > 10^6-fold NK cell expansion in 3-7 weeks was observed with activation by co-culture with K562/mbIL-21-4-1BBL feeder cells, outperforming the 10^4 increase over 7 weeks observed after NK cell co-culture with K562/mbIL-15-4-1BBL feeder cells [25].

Upon persistent exposure to antigens, T cells exhibit reduced cytotoxicity and other effector functions both *in vivo* and during in *ex vivo* culture. Such T cell exhaustion is marked by the upregulation of markers like PD-1, LAG-3, TIGIT, and TIM-3 [42]. Similarly, chronic stimulation of NK cells through activating receptors *ex vivo* has been reported to result in decreased effector functions [43, 44]. But the nature of NK exhaustion is not as well defined as it is for T cells [45]. Hence, whether serial periodic feeder cell stimulation could result in the exhaustion of *ex vivo* cultivated NK cells is still an open question.

NK cell proliferation may also be constrained by senescence, an irreversible cell cycle arrest resulting from multiple cell divisions and mediated by telomere shortening [45]. While senescence is a universal byproduct of active cell proliferation, it was shown that the process can be delayed by using K562 feeder cells engineered with mbIL-21 instead of mbIL-15 [25]. However, with the inevitability of replicative senescence, the number of NK cell fold expansions is an important consideration during the *ex vivo* expansion process.

NK Cell Biomanufacturing:

Current manufacturing process

Since NK cell therapy is still at the clinical trial stage, cell production is at a smaller scale, and the selection of cell cultivation methods is less constrained. An automated instrument (called the CliniMACS Prodigy®) that integrates centrifugation, antibody-conjugated magnetic bead cell separation and cell cultivation in a closed system has been reported to expand an initial population of 2×10^6 $CD56^+$ NK cells to 1.4×10^9 NK cells by co-culturing with K562/mbIL-21-4-1BBL feeder cells and an initial population of 2.5×10^6 $CD56^+$ NK cells to about 0.6×10^9 NK cells by co-culturing with K562/mbIL-15-4-1BBL feeder cells [46]. A multi-parallel channel, perfusion

bioreactor with an antibody/matrix cocktail coated surface has also been used to expand NK cells [47]. Commonly used culture systems for cell production for clinical trials are similar for NK and T cells, and include bag systems and stationary G-Rex® flasks with gas-permeable membranes for enhanced oxygen transfer [48]. The scale of future biomanufacturing of NK cells for allogeneic applications will be substantially larger than those used for T cell culture. It is conceivable that mixing bioreactors such as stirred tanks of 10-100 L size at 10^{10} cells/L will be necessary to produce a batch quantity of 10^{11}-to-10^{12} cells. That reactor size range is well within the comfortable operation zone to produce biologics. The cell expansion process will thus be distinct from that of CAR-T cells for autologous application. It will likely be more similar to recombinant protein production, starting from the thawing of cryopreserved vials of banked NK cells, through multiple stages of cell expansion in **seed bioreactors**, until reaching the production bioreactor in which high concentrations of highly active cells are generated and harvested. However, the similarity in their process flowchart belies a major distinction. Periodically, irradiated feeder cells are added to activate NK cells for continued expansion. Since the feeder cells need to be added at 1:1 ratio weekly in serial transfer to the next larger reactor, a parallel production process of a large quantity of irradiated and well-characterized feeder cells will be needed. Due to these issues, feeder cell-free expansion methods using magnetic/agarose bead-bound antibodies [37, 49] or plasma membrane particles [50] were explored. However, it is not clear whether these systems can sustain weeks of active growth for robust cell expansion. Hence, understanding the mechanisms by which feeder cells activate NK cells and developing feeder cell-free activation methods that can expand NK cells to large numbers will greatly impact the economy of NK cell biomanufacturing.

Learning from success of protein biologics manufacturing

In past three decades, the production of therapeutic proteins has grown from burgeoning explorations to highly efficient and robust biomanufacturing processes. This has reduced the cost of these proteins by two orders of magnitude [51]. The general path taken for the development of protein biomanufacturing can serve as a guide for NK cell therapy. Both better cell lines and intensified processes have contributed to the success of biologics production. Through more efficient screening of cell clones in conjunction with cell line engineering, the producing cells nowadays have higher specific productivity, enhanced growth kinetics, and desired product glycosylation profiles (For review see [52, 53]). In a similar vein, there have been efforts toward making NK cells more potent effectors. In order to increase cytotoxicity and specificity of NK cells, many groups have incorporated CARs that target specific antigens on tumor cells (for review see [54]). To improve the persistence and cytotoxicity of NK cells, expression of soluble and membrane-bound IL-15 constructs have been reported [55, 56]. Several other strategies, including deletion of *CISH*, *ADAM17*, and *PDCD1*, have also been shown to improve NK cell potency *in vivo*[57, 58].

In the early years of cell culture industrialization, many innovative efforts were devoted to developing bioreactors or cell culture systems, including many membrane devices and cell entrapment systems. In a few years' time, the traditional mixing vessels, especially stirred tanks, became the norm once the required reactor size went beyond pilot plant scale. NK cell culture for allogeneic applications is likely to employ mixing vessels as the production platform.

Key to the transformation of cell culture operations to industrial processes was the adoption of fed-batch culture or continuous operation with cell retention (commonly called perfusion culture) to increase the cell concentration and hence the productivity (Figure 3) beyond what can be

achieved in a batch operation. In a fed-batch culture, nutrients are added intermittently or continuously to avoid depletion and to prolong the cell growth phase for a sustained production period. Eventually, the accumulation of metabolites exerts inhibitory effects on growth, and the process is terminated. A continuous perfusion culture has a feed stream to supply nutrients continuously and an effluent stream of culture fluid taking cells and metabolites out to alleviate metabolite accumulation. A cell separation device is typically used to return a portion of cells from the effluent stream into the bioreactor. A perfusion culture is operated in two stages: initially cells are completely retained in the reactor to allow cell concentration to increase rapidly. As the cell concentration approaches the target level, some cells are allowed to be discharged so that cell growth and discharge are balanced to sustain the reactor at a steady state.

NK cell biomanufacturing will face the same need of increasing cell concentration and productivity, with the additional constraints of maintaining high levels of viability and functionality since the cells themselves are the end product. Mammalian cells in culture, including NK cells, produce lactate due to the Warburg effect, generate ammonium through anaplerosis of glutamine, and excrete degradation catabolites of aromatic and branched-chain amino acids, which may accumulate to inhibitory levels at high cell concentrations especially in fed-batch cultures [59, 60]. Keeping them in check, by continuous removal of metabolites, might be important in maintaining functionally active NK cells. With periodic stimulation to sustain active growth, NK cells can expand for several weeks and increase the population size up to multiple-thousand-fold. An NK cell expansion process will thus entail a series of bioreactors with increasing volume. A perfusion type of approach, albeit with total cell retention since cells are the product (Figure 3C), or a hybrid form of fed-batch and perfusion is an attractive alternative to batch or fed-batch culture. A number of cell retention membrane devices used in industrial operations for recombinant protein

production, such as tangential flow filtration (TFF) or alternative tangential filtration (ATF), are adoptable for NK cell culture [61].

A major advance in cell culture-based manufacturing in the past few decades is the shift away from serum-containing media toward chemically-defined media. The use of feeder cells and human serum in NK cell cultivation protocols increases media complexity, poses challenges in quality control, and risks fouling of cell retention membrane devices. Recent studies have examined the use of different serum-free conditions for NK cell expansion and have demonstrated that serum replacement supplements can potentially be used in manufacturing settings[37, 62].

Prior to their use as a cellular product, expanded NK cells need to be washed to remove medium components, feeder cell debris, and other additives including elements for feeder cell replacement. Some automated devices for clinical scale cell washing and harvesting could be integrated to median scale cell manufacturing processes (See [63] For review). For allogeneic applications, the off-the-shelf NK cell product will likely be cryopreserved. Studies have shown that NK cells are sensitive to the freeze/thaw cycle and exhibit poor cytotoxicity post-thaw [64, 65]. NK cell cryopreservation methods typically employ DMSO- and serum-containing freezing media with controlled-rate freezing [65]. It was shown that cryopreservation conditions could affect the recovery of NK cells and their cytotoxicity after thawing [66]. A systematic assessment of the impact of cryopreservation on NK cell cytotoxicity will be necessary in process development.

In-line monitoring of NK expansion

In the early 2000s, the US Food and Drug Administration (FDA) launched **process analytical technology (PAT)** and **quality by design (QbD)** initiatives to promote innovations in process monitoring technology and to ingrain product quality control in pharmaceutical manufacturing [67]. The PAT initiative has advanced bioprocess monitoring in recent years. Several commonly

used **in-line** and real-time sensors for monitoring and control of a cell culture bioreactor are shown in Figure 4. Classical in-line oxygen sensing is now widely used to measure oxygen uptake rates for real-time metabolic monitoring [68]. pH sensing as an indirect measurement of lactate production has been used to control nutrient feeding rates to modulate metabolic fluxes [69]. **Raman spectroscopy** in the middle infrared and near infrared ranges has been widely used in line to measure concentrations of cells and nutrients including glucose and glutamine in biomanufacturing settings [70-72] and in T cell expansion protocols [73]. These in-line measurements are typically complemented by off-line or on-line assays, which are measured less frequently and subject to time delays. Like in-line Raman spectroscopy, commercial instrumentation has made capacitance measurement of viable cell concentration readily accessible. Since viable cells have intact cell membranes and measure at low conductance, whereas dead cells are permeable and conductive, capacitance measurement can provide a good estimate of total viable cell volume. This contrasts with classical in-line turbidity particle measurements which give total cell concentration. NK cells derived from PBMCs often display large donor-to-donor variability in their growth behavior [74]. The combination of on-line oxygen consumption rate determination, in-line capacitance, and Raman spectroscopy measurements may allow for real time sensing and control of growth and metabolic activities.

QbD in NK manufacturing

QbD principles are now well integrated in the development of biologics and have contributed to the enhanced robustness of cell culture biologic manufacturing [75]. Even though NK cell therapy and manufacturing is still in its infancy, given the complexity of the product, it is prudent to keep QbD principles in mind during process development. For such efforts, a document on a

potential QbD framework for cell therapy products recently released by a working group can serve as a guide[76].

The general framework of QbD starts with best knowledge of mechanism of action, safety, and efficacy of the product to identify a set of product quality characteristics in qualitative or quantitative terms, known as the quality target product profile (QTPP) (Figure 5). The QTPP is critical for accomplishing clinical goals and should contemplate attributes that relate specifically to product safety, identity, strength, purity, potency and quality (SISPQ), as well as attributes that physically describe the product. For example, QTPP for an NK cell product may include sterility, the identity of the NK cell product, the quantity for each dose, non-cellular impurities, potency, as well as delivery form. A comprehensive list of quality attributes which impact the QTPP and hence the product's quality is then developed. From this list, critical quality attributes (CQAs) are identified via risk assessment. CQAs are defined to be "physical, chemical, biological, or microbiological properties or characteristics that should be within an appropriate limit, range, or distribution to ensure the desired product quality" [77].These CQAs are to be measured and quantified, how each CQA varies by process conditions in each manufacturing unit operation is to be evaluated and the range each CQA must be controlled must be established in process development. For NK cell therapy, the QTPP and CQAs are still being refined since we are still learning from clinical outcomes and process development. As a cell therapy product, NK cell identity, viability, quantity, and functionality in terms of cytotoxicity will certainly be considered as CQAs, along with safety attributes such as sterility [78]. Additionally, cellular impurities may be considered as an important quality attribute. To prevent possible GvHD, especially for NK cells products derived from PBMCs and with limited cell expansion, T cell numbers in the final product must be below a defined level. In instances where feeder cells are used for NK activation, their

absence in the final product needs to be confirmed. For iPSC-derived NK cells, the number of undifferentiated cells needs to be below a maximal level tolerable. In each case, monitoring and controlling levels of cellular impurities within the final product will be an essential element of a process control strategy.

During process development, those process parameters and material components that have a significant impact on CQAs, known as critical process parameters (CPPs) and critical material attributes (CMAs) respectively, need to be defined. The operating range of the CPPs and CMAs within which the CQAs of the product can be controlled within the acceptable bounds must also be defined. Since some CPPs are likely to interactively impact the CQAs, a **design of experiment (DOE)** approach can be taken to explore a wide range of operating parameter space (called **Design Space**). From the outcome of DOE in Design Space, a smaller **control space** of CPPs or CMAs is defined, the manufacturing will be conducted within the control space. Since such studies are carried out for each unit operation, they require large resources and effective scale-down models using multiplex miniature equipment. Possible CPPs include cytokine concentration during cell expansion, cell freezing and thawing conditions in cell banking, and in cell-thawing for initiating seed culture. Prominent among possible CMAs are materials involved in NK activation, like feeder cells and cytokines. The post-thaw viability of feeder cells may influence their potency in NK cell activation. The amount and frequency of feeder cell delivery, the scheme of setting the serial reactor transfer time and the medium perfusion rate, might significantly affect the cytotoxicity, a likely CQA, of the product.

CONCLUDING REMARKS

The prospect of allogeneic cancer therapy afforded by NK cells has generated much excitement. NK cells can be activated and expanded in culture and have the potential of providing off-the-shelf

medicine for treating cancers. By scaling up the process, expanding the cell population, and increasing the production efficiency, the NK cell product can also be produced at an affordable cost. The development of manufacturing processes for NK cells can benefit from adapting cell culture technologies established for producing protein biologics. However, large-scale expansion of NK cells is critically distinct from the production of recombinant proteins in that NK cells are the final product. This is very much unlike the production of biologics for which cells are akin to catalysts and are discarded at the end of the production. The quality of the NK cell product is critically coupled to the biological state of the cell. The development of NK cell manufacturing processes will need to place the focal point on the product quality, i.e., the cell's biological features that drive therapeutic potency (see Outstanding questions). Adapting the QbD and PAT frameworks, even in the early stage of process development, will help establish quality-centered NK cell manufacturing. By continued integration of developing biological insights and process advances, NK cell expansion can become a robust manufacturing technology.

BOX 1. Activation, cytotoxicity, and effector function of NK cells upon interaction with target cells

Engagement of activating receptors with their ligands results in phosphorylation of immunoreceptor tyrosine-based activating motifs (ITAMs) in cytoplasmic regions of activating receptors by Src family tyrosine kinases, leading to recruitment of Syk and Zap70, which eventually activate extracellular signal-regulated kinases (ERKs) and mitogen-activated protein kinases (MAPKs) [77]. When inhibitory receptors engage with their ligands, immunoreceptor tyrosine-based inhibition motifs (ITIMs) are phosphorylated and recruit phosphatases that dephosphorylate key molecules downstream of activating receptors such as Fyn, Syk, Zap70, and Lck to disrup activation [77]. If the engagement of activation/inhibitory receptors culminates in a

net activating signal, the NK cell undergoes cytoskeleton reorganization, leading to a widened contact area between the NK cell and its target. This eventually results in the formation of an immunological synapse and localization of granzyme- and perforin-containing lytic granules to the synapse [78]. The engaged NK cell then releases lytic granules into the target cell to trigger apoptosis. Upon target cell engagement, NK cells also release cytokines such as IFN-γ and TNF-α that recruit other immune populations and help orchestrate the adaptive immune response [79].

In addition to releasing granzymes and perforin, NK cells can also kill target cells by inducing death receptor-mediated apoptosis through Fas ligand binding to Fas death receptors on the surface of target cells [80]. The lytic granule-mediated killing occurs in a shorter time scale of minutes, while the death-receptor mediated process takes a few hours [81]. After killing, immunological synapses dissociate and the NK cell disengages.

Since killing involves the release of around 10% of stored lytic granules from each NK cell, after a few serial killing events the granules need to be replenished. Using imaging tracking of NK killing, it was shown that only a small subpopulation (~5.6%) of NK cells are responsible for serially killing target cells, which accounts for 26% of total kills [82]. It was observed that NK cells employ lytic granule-mediated killing and switch to death-receptor-mediated killing after granule exhaustion [81]. Target cell engagement and killing is an important mechanism of NK cell activation and can drive NK cells into a proliferative state. However, among the series of cellular events, the mechanistic links between NK cell activation and proliferation are not clearly defined. Further interrogation into the kinetics of target cell killing and understanding the nature of serial killing by NK cells will be helpful in improving the potency of NK cells generated as immunotherapy products.

Glossary

Adaptive NK cells: memory-like NK cells that can persist for several months in CMV seropositive individuals

Allogeneic: cells that are obtained from different individuals of the same species

Adoptive transfer: the process of transferring cells from one individual to another

Autologous: cells that are obtained from an individual's own body

Control space: the defined range of critical process parameters or critical material attributes within which the critical quality attributes of a product can be controlled and maintained with a high probability

Design space: the range of operating parameters and material attributes within which a product's CQAs can be controlled within acceptable bounds

Design of Experiment (DOE): a statistical approach used to explore the relationships between test variables and outcome variable by varying the range of test variables and considering their interactions

Feeder cells: cells that are co-cultured with the principal cells in order to support their growth

Graft-versus-Host-Disease (GvHD): a complication that can occur after hematopoietic cell transplantation, in which the donor's T cells attack the recipient's cells

Human induced Pluripotent Stem Cells (hiPSCs): human cells that have been genetically reprogrammed from adult cells into an embryonic-like pluripotent state

Human Embryonic Stem Cells (hESCs): cells found in the early-stage human embryo that can develop into all lineages of cells in the body

In-line sensing: the use of sensors or monitoring devices that are integrated into a unit operation in order to continuously gather data about the process or the products being produced

Immunoreceptor tyrosine-based activation motifs (ITAMs): a sequence of amino acids in the cytoplasmic domain of receptor proteins that can be phosphorylated and activate the immune response

Immunoreceptor tyrosine-based inhibition motifs (ITIMs): a sequence of amino acids in receptor proteins that can be phosphorylated and inhibit the immune response

Peripheral Blood Mononuclear Cells (PBMCs): blood cells that have a round nucleus including monocytes, T cells, B cells, NK cells, and dendritic cells

Process Analytical Technology (PAT): a system for designing, analyzing, and controlling manufacturing process by taking timely measurements of process parameters during production in order to ensure the quality of the final product

Quality by Design (QbD): A systematic approach to development that links the quality of a product to the way it is manufactured, and uses this connection as an approach to guide the development process

Raman spectroscopy: a technique that uses the scattering of light to measure the vibrational energy of molecules, which can be used to measure the concentrations of cells, nutrients, and other substances

Seed bioreactor: a series of bioreactors with increasing volume used to grow cells with increasing quantity that will later be used to initiate a larger production bioreactor

Umbilical cord blood (UCB): the blood that remains in the placenta and umbilical cord after birth

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REFERENCES

1. Lamb, M.G. et al. (2021) Natural killer cell therapy for hematologic malignancies: successes, challenges, and the future. Stem Cell Res Ther 12 (1), 211.

2. Depil, S. et al. (2020) 'Off-the-shelf' allogeneic CAR T cells: development and challenges. Nat Rev Drug Discov 19 (3), 185-199.

3. Du, N. et al. (2021) NK Cell Therapy: A Rising Star in Cancer Treatment. Cancers (Basel) 13 (16).

4. Liu, S. et al. (2021) NK cell-based cancer immunotherapy: from basic biology to clinical development. J Hematol Oncol 14 (1), 7.

5. Paul, S. and Lal, G. (2017) The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. Front Immunol 8, 1124.

6. Barrow, A.D. et al. (2019) The Natural Cytotoxicity Receptors in Health and Disease. Frontiers in Immunology 10.

7. Uppendahl, L.D. et al. (2019) Cytokine-induced memory-like natural killer cells have enhanced function, proliferation, and in vivo expansion against ovarian cancer cells. Gynecol Oncol 153 (1), 149-157.

8. Miyazato, K. and Hayakawa, Y. (2020) Pharmacological targeting of natural killer cells for cancer immunotherapy. Cancer Sci 111 (6), 1869-1875.

9. Zhang, Y. et al. (2007) In vivo kinetics of human natural killer cells: the effects of ageing and acute and chronic viral infection. Immunology 121 (2), 258-265.

10. Liu, Y. et al. (2013) Growth and activation of natural killer cells ex vivo from children with neuroblastoma for adoptive cell therapy. Clin Cancer Res 19 (8), 2132-43.

11. Miller, J.S. et al. (2005) Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood 105 (8), 3051-7.

12. Zhu, H. and Kaufman, D.S. (2019) Engineered human pluripotent stem cell-derived natural killer cells: the next frontier for cancer immunotherapy. Blood Sci 1 (1), 4-11.

13. Kennedy, P.R. et al. (2022) Challenges to the broad application of allogeneic natural killer cell immunotherapy of cancer. Stem Cell Res Ther 13 (1), 165.

14. Geller, M.A. et al. (2011) A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. Cytotherapy 13 (1), 98-107.

15. Gang, M. et al. (2020) Memory-like natural killer cells for cancer immunotherapy. Semin Hematol 57 (4), 185-193.

16. Handgretinger, R. et al. (2016) Exploitation of natural killer cells for the treatment of acute leukemia. Blood 127 (26), 3341-9.

17. Sheppard, S. and Sun, J.C. (2021) Virus-specific NK cell memory. J Exp Med 218 (4).

18. Schlums, H. et al. (2017) Adaptive NK cells can persist in patients with GATA2 mutation depleted of stem and progenitor cells. Blood 129 (14), 1927-1939.

19. Granzin, M. et al. (2016) Highly efficient IL-21 and feeder cell-driven ex vivo expansion of human NK cells with therapeutic activity in a xenograft mouse model of melanoma. Oncoimmunology 5 (9), e1219007.

20. Liu, E. et al. (2020) Use of CAR-Transduced Natural Killer Cells in CD19-Positive Lymphoid Tumors. New England Journal of Medicine 382 (6), 545-553.

21. Schmidt, D. et al. (2022) Engineering CAR-NK cells: how to tune innate killer cells for cancer immunotherapy. Immunother Adv 2 (1), ltac003.

22. Angelo, L.S. et al. (2015) Practical NK cell phenotyping and variability in healthy adults. Immunol Res 62 (3), 341-56.

23. Sarvaria, A. et al. (2017) Umbilical Cord Blood Natural Killer Cells, Their Characteristics, and Potential Clinical Applications. Front Immunol 8, 329.

24. Lupo, K.B. and Matosevic, S. (2019) Natural Killer Cells as Allogeneic Effectors in Adoptive Cancer Immunotherapy. Cancers (Basel) 11 (6).

25. Denman, C.J. et al. (2012) Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. PLoS One 7 (1), e30264.

26. Rizwan Romee, M.R., et al. (2016) Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. Science Translational Medicine 8 (357).

27. Suen, W.C. et al. (2018) Natural Killer Cell-Based Cancer Immunotherapy: A Review on 10 Years Completed Clinical Trials. Cancer Invest 36 (8), 431-457.

28. Vela, M. et al. (2018) Haploidentical IL-15/41BBL activated and expanded natural killer cell infusion therapy after salvage chemotherapy in children with relapsed and refractory leukemia. Cancer Lett 422, 107-117.

29. Zhu, H. and Kaufman, D.S. (2019) An Improved Method to Produce Clinical-Scale Natural Killer Cells from Human Pluripotent Stem Cells. Methods Mol Biol 2048, 107-119.

30. Euchner, J. et al. (2021) Natural Killer Cells Generated From Human Induced Pluripotent Stem Cells Mature to CD56(bright)CD16(+)NKp80(+/-)In-Vitro and Express KIR2DL2/DL3 and KIR3DL1. Front Immunol 12, 640672.

31. Saetersmoen, M.L. et al. (2019) Off-the-shelf cell therapy with induced pluripotent stem cell-derived natural killer cells. Semin Immunopathol 41 (1), 59-68.

32. Fabian, K.P. and Hodge, J.W. (2021) The emerging role of off-the-shelf engineered natural killer cells in targeted cancer immunotherapy. Mol Ther Oncolytics 23, 266-276.

33. Heipertz, E.L. et al. (2021) Current Perspectives on "Off-The-Shelf" Allogeneic NK and CAR-NK Cell Therapies. Front Immunol 12, 732135.

34. Granzin, M. et al. (2017) Shaping of Natural Killer Cell Antitumor Activity by Ex Vivo Cultivation. Front Immunol 8, 458.

35. Williams, S.M. et al. (2018) Clinical-scale production of cGMP compliant CD3/CD19 cell-depleted NK cells in the evolution of NK cell immunotherapy at a single institution. Transfusion 58 (6), 1458-1467.

36. Oyer, J.L. et al. (2016) Natural killer cells stimulated with PM21 particles expand and biodistribute in vivo: Clinical implications for cancer treatment. Cytotherapy 18 (5), 653-63.

37. Johnson, C.D.L. et al. (2022) Feeder-Cell-Free and Serum-Free Expansion of Natural Killer Cells Using Cloudz Microspheres, G-Rex6M, and Human Platelet Lysate. Front Immunol 13, 803380.

38. Gurney, M. et al. (2022) Feeder Cells at the Interface of Natural Killer Cell Activation, Expansion and Gene Editing. Front Immunol 13, 802906.

39. Phan, M.T. et al. (2016) Expansion of NK Cells Using Genetically Engineered K562 Feeder Cells. Methods Mol Biol 1441, 167-74.

40. Chang, M. et al. (2022) Differential effects on natural killer cell production by membrane-bound cytokine stimulations. Biotechnol Bioeng 119 (7), 1820-1838.

41. Kweon, S. et al. (2019) Expansion of Human NK Cells Using K562 Cells Expressing OX40 Ligand and Short Exposure to IL-21. Front Immunol 10, 879.

42. van der Heide, V. et al. (2022) Advancing beyond the twists and turns of T cell exhaustion in cancer. Science Translational Medicine 14 (670), eabo4997.

43. Merino, A. et al. (2019) Chronic stimulation drives human NK cell dysfunction and epigenetic reprograming. J Clin Invest 129 (9), 3770-3785.

44. Merino, A.M. et al. (2020) Unraveling exhaustion in adaptive and conventional NK cells. J Leukoc Biol 108 (4), 1361-1368.

45. Judge, S.J. et al. (2020) Characterizing the Dysfunctional NK Cell: Assessing the Clinical Relevance of Exhaustion, Anergy, and Senescence. Front Cell Infect Microbiol 10, 49.

46. Fernandez, A. et al. (2021) Optimizing the Procedure to Manufacture Clinical-Grade NK Cells for Adoptive Immunotherapy. Cancers (Basel) 13 (3).

47. Broker, K. et al. (2019) Mass Production of Highly Active NK Cells for Cancer Immunotherapy in a GMP Conform Perfusion Bioreactor. Front Bioeng Biotechnol 7, 194.

48. Ralf Pörtner, C.S., Shreemanta K. Parida, and Hans Hoffmeister (2019) Single-Use Bioreactors for Manufacturing of Immune Cell Therapeutics. In Single-Use Technology in Biopharmaceutical Manufacture, Second Edition (Regine Eibl, D.E. ed), pp. 327-334, John Wiley & Sons, Inc.

49. Gratch, Y.S. et al. (2018) A semi-automated, high-purity process for natural killer (NK) cell manufacturing in a rocking bioreactor. Cytotherapy 20 (5), e8-e9.

50. Oyer, J.L. et al. (2015) Generation of highly cytotoxic natural killer cells for treatment of acute myelogenous leukemia using a feeder-free, particle-based approach. Biol Blood Marrow Transplant 21 (4), 632-9.

51. Farid, S.S. et al. (2020) Benchmarking biopharmaceutical process development and manufacturing cost contributions to R&D. MAbs 12 (1), 1754999.

52. Le, H. et al. (2015) Cell line development for biomanufacturing processes: recent advances and an outlook. Biotechnol Lett 37 (8), 1553-64.

53. Hong, J.K. et al. (2018) Towards next generation CHO cell line development and engineering by systems approaches. Current Opinion in Chemical Engineering 22, 1-10.

54. Zhang, L. et al. (2022) CAR-NK cells for cancer immunotherapy: from bench to bedside. Biomark Res 10 (1), 12.

55. Woan, K.V. et al. (2021) Harnessing features of adaptive NK cells to generate iPSC-derived NK cells for enhanced immunotherapy. Cell Stem Cell 28 (12), 2062-2075.e5.

56. Liu, E. et al. (2018) Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity. Leukemia 32 (2), 520-531.

57. Zhu, H. et al. (2020) Metabolic Reprograming via Deletion of CISH in Human iPSC-Derived NK Cells Promotes In Vivo Persistence and Enhances Anti-tumor Activity. Cell Stem Cell 27 (2), 224-237 e6.

58. Pomeroy, E.J. et al. (2020) A Genetically Engineered Primary Human Natural Killer Cell Platform for Cancer Immunotherapy. Mol Ther 28 (1), 52-63.

59. Dean, J. and Reddy, P. (2013) Metabolic analysis of antibody producing CHO cells in fed-batch production. Biotechnol Bioeng 110 (6), 1735-47.

60. Buchsteiner, M. et al. (2018) Improving culture performance and antibody production in CHO cell culture processes by reducing the Warburg effect. Biotechnol Bioeng 115 (9), 2315-2327.

61. Su, Y. et al. (2021) Optimized process operations reduce product retention and column clogging in ATF-based perfusion cell cultures. Appl Microbiol Biotechnol 105 (24), 9125-9136.

62. Moseman, J.E. et al. (2020) Evaluation of serum-free media formulations in feeder cell-stimulated expansion of natural killer cells. Cytotherapy 22 (6), 322-328.

63. Li, A. et al. (2021) Advances in automated cell washing and concentration. Cytotherapy 23 (9), 774-786.

64. Mark, C. et al. (2020) Cryopreservation impairs 3-D migration and cytotoxicity of natural killer cells. Nat Commun 11 (1), 5224.

65. Li, R. et al. (2019) Preservation of cell-based immunotherapies for clinical trials. Cytotherapy 21 (9), 943-957.

66. Min, B. et al. (2018) Optimization of Large-Scale Expansion and Cryopreservation of Human Natural Killer Cells for Anti-Tumor Therapy. Immune Netw 18 (4), e31.

67. Rathore, A.S. et al. (2010) Process analytical technology (PAT) for biopharmaceutical products. Anal Bioanal Chem 398 (1), 137-54.

68. Zhao, L. et al. (2015) Advances in process monitoring tools for cell culture bioprocesses. Engineering in Life Sciences 15 (5), 459-468.

69. Konakovsky, V. et al. (2016) Metabolic Control in Mammalian Fed-Batch Cell Cultures for Reduced Lactic Acid Accumulation and Improved Process Robustness. Bioengineering (Basel) 3 (1).

70. Rangan, S. et al. (2020) Applications of Raman spectroscopy in the development of cell therapies: state of the art and future perspectives. Analyst 145 (6), 2070-2105.

71. Kozma, B. et al. (2019) On-line glucose monitoring by near infrared spectroscopy during the scale up steps of mammalian cell cultivation process development. Bioprocess Biosyst Eng 42 (6), 921-932.

72. Berry, B.N. et al. (2016) Quick generation of Raman spectroscopy based in-process glucose control to influence biopharmaceutical protein product quality during mammalian cell culture. Biotechnol Prog 32 (1), 224-34.

73. Baradez, M.O. et al. (2018) Application of Raman Spectroscopy and Univariate Modelling As a Process Analytical Technology for Cell Therapy Bioprocessing. Front Med (Lausanne) 5, 47.

74. Granzin, M. et al. (2015) Fully automated expansion and activation of clinical-grade natural killer cells for adoptive immunotherapy. Cytotherapy 17 (5), 621-32.

75. Abu-Absi, S.F. et al. (2010) Defining process design space for monoclonal antibody cell culture. Biotechnol Bioeng 106 (6), 894-905.

76. Amr Eissa, L.G., Josephine Lembong, Michael Lehmicke, Marie Daghlian, Carol Collier, Michaela Flatley, A-CELL, A case study-based approach to integrating QbD principles in Cell-based Therapy CMC programs, 2022.

77. (2009) ICH Harmonised Tripartite Guideline: Pharmaceutical Development Q8 (R2). ICH.

78. Lipsitz, Y.Y. et al. (2016) Quality cell therapy manufacturing by design. Nat Biotechnol 34 (4), 393-400.

79. Phung, S.K. et al. (2021) Bi-specific and Tri-specific NK Cell Engagers: The New Avenue of Targeted NK Cell Immunotherapy. Mol Diagn Ther 25 (5), 577-592.

80. Mace, E.M. et al. (2014) Cell biological steps and checkpoints in accessing NK cell cytotoxicity. Immunol Cell Biol 92 (3), 245-55.

81. Bald, T. et al. (2020) The NK cell-cancer cycle: advances and new challenges in NK cell-based immunotherapies. Nat Immunol 21 (8), 835-847.

82. Prager, I. and Watzl, C. (2019) Mechanisms of natural killer cell-mediated cellular cytotoxicity. J Leukoc Biol 105 (6), 1319-1329.

83. Prager, I. et al. (2019) NK cells switch from granzyme B to death receptor-mediated cytotoxicity during serial killing. J Exp Med 216 (9), 2113-2127.

84. Vanherberghen, B. et al. (2013) Classification of human natural killer cells based on migration behavior and cytotoxic response. Blood 121 (8), 1326-34.

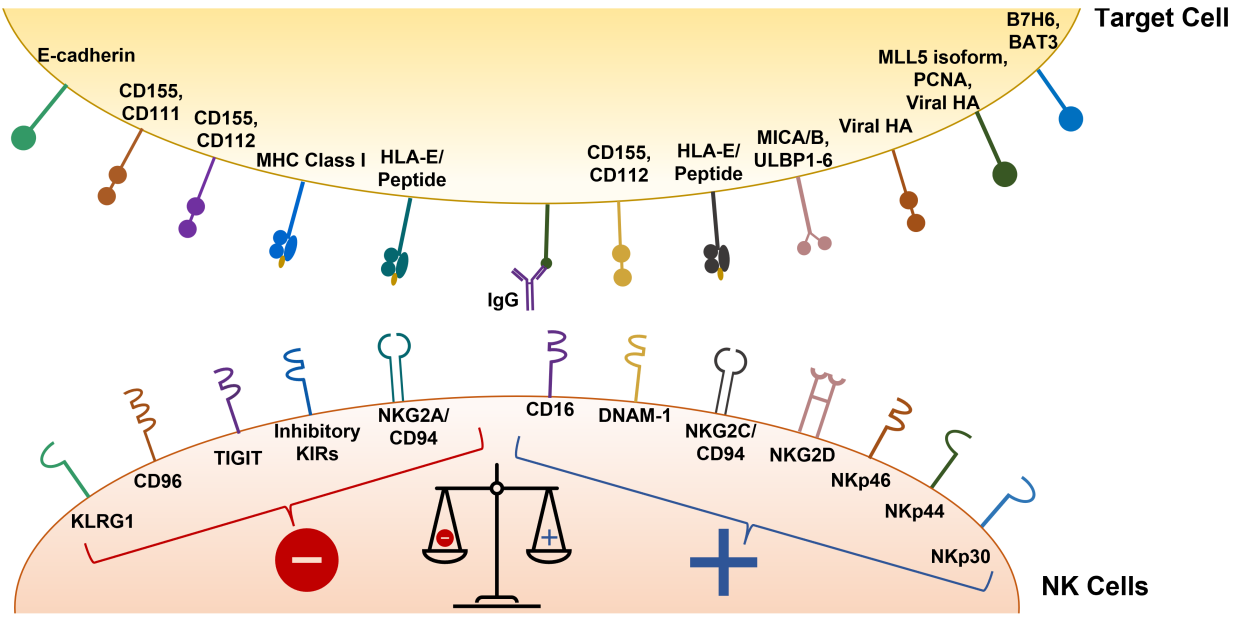

Figure 1. **Main NK cell activating and inhibitory receptors and their ligands**. Inhibitory receptors are shown on the left side of the NK cell (in red bracket), activating receptors are on the right side (blue bracket). Each pair of NK cell receptor and the cognate ligand on the target cell are shown with the same colors. Activating or inhibitory signals are triggered upon engagement of their ligands. The net balance of activating (positive) and inhibitory (negative) signals determines the response of NK cells toward the target cell. Abbreviations: HA, hemagglutinin; MHC, major histocompatibility complex; IgG, Immunoglobulin G.

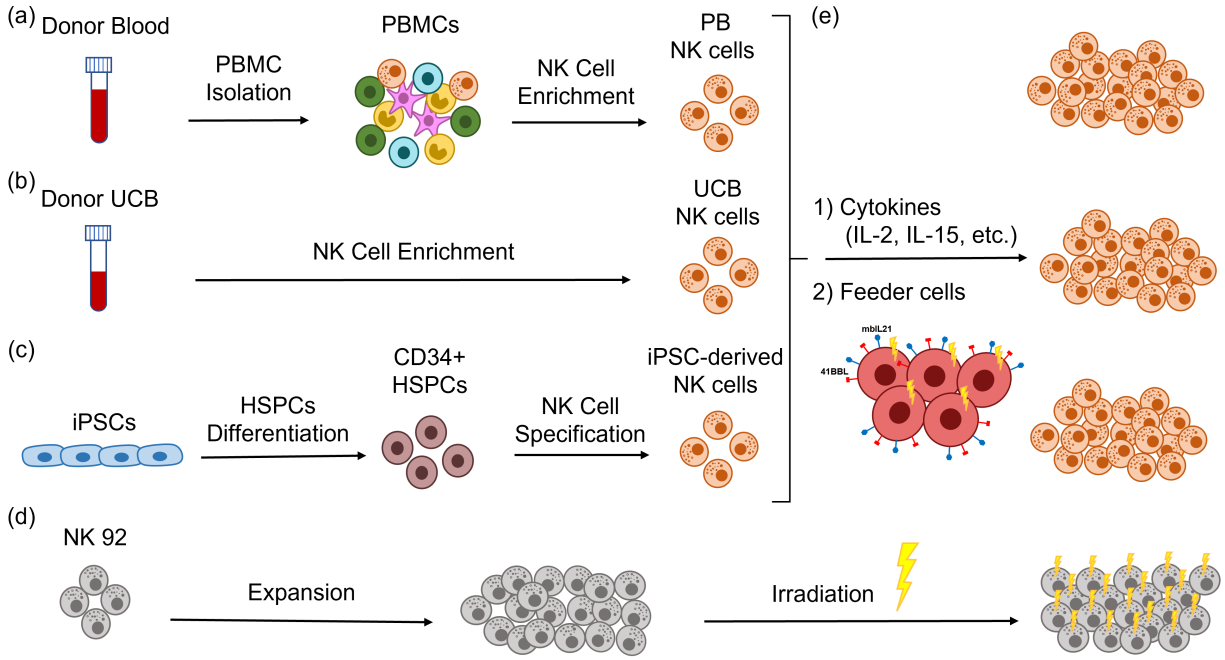

Figure 2. Different NK cell sources for cell therapy. (a) Human PBMCs can be isolated from donor blood using density gradient centrifugation. NK cells can be enriched from PBMCs using negative or positive antibody-based magnetic beads selection. (b) NK cells can be enriched from donor UCB using magnetic beads. (c) Human iPSCs can be differentiated into CD34+ hematopoietic stem and progenitor cells (HSPCs) and subsequently into NK cells by adding different soluble molecules. (d) NK 92 cell line can be expanded to large numbers and then irradiated for cell therapy applications. (e) PB NK cells, UCB NK cells, and iPSC-derived NK cells often need to be expanded to large numbers. This can be achieved by different methods such as cytokine stimulation or coculture with feeder cells such as mb41BBL/mbIL21 K562 cells. The expanded cells then can be used as allogeneic NK cell therapies. Abbreviations: PB, peripheral blood; mbIL21, membrane-bound IL-21.

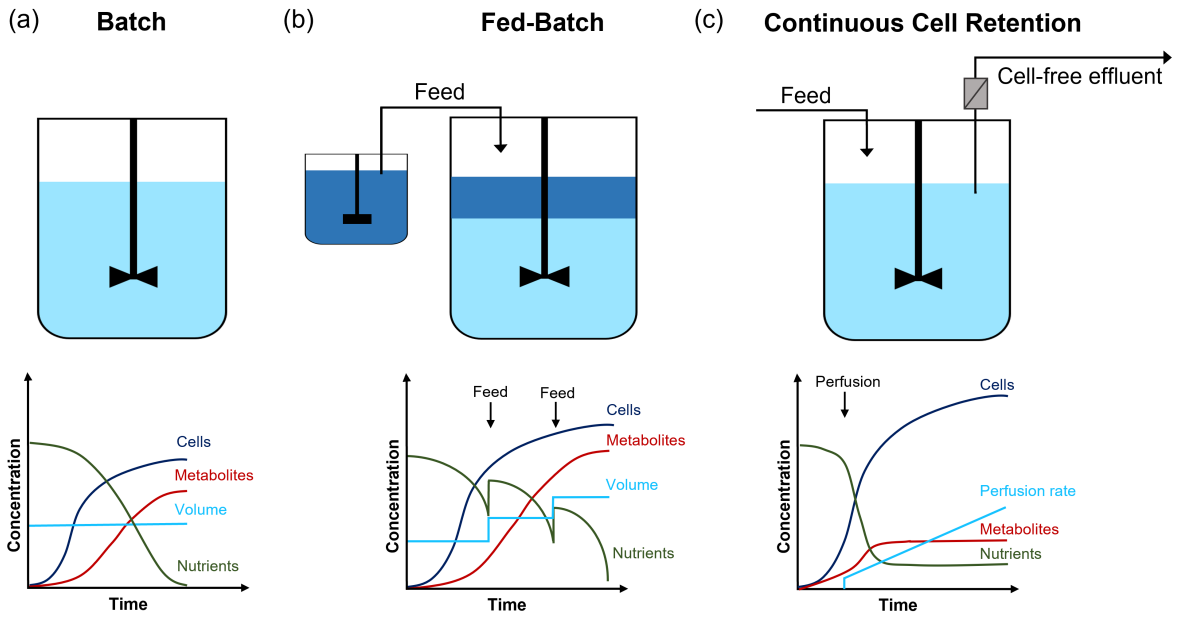

Figure 3. Different types of bioreactors commonly used for cell expansion manufacturing. (a) A batch bioreactor in which cells and nutrients are added in the beginning of the process and there is no input or output of medium until the end of the process. (b) In a fed-batch culture nutrients can be added intermittently or continuously over-time as the cells grow. The metabolite may accumulate to growth inhibitory level to end the process. (c) A continuous cell retention bioreactor is initiated like a batch culture, after reaching a certain level, medium is continuously fed and simultaneously removed through a cell separation device to return the cell into the reactor. The effluent stream removes inhibitory metabolites to prolong the growth period and increase cell concentration.

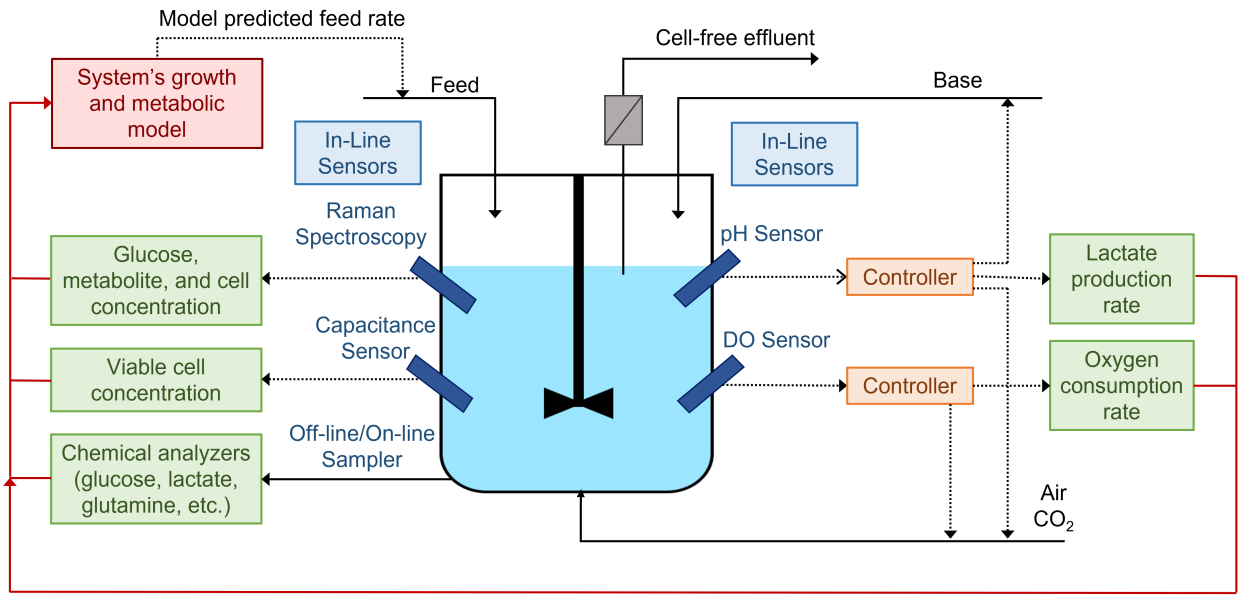

Figure 4. Different types of in-line sensors used in bioreactors. Dissolved oxygen (DO) and pH electrodes are commonly used to measure and control DO and pH at set points. By monitoring the dynamics of DO and the amount of base and CO2 added to maintain pH, the oxygen uptake rate and lactate production rate can be determined in real time. Raman spectroscopy can be used to measure the levels of various nutrients and metabolites, as well as cell concentration in the system, while a capacitance sensor can be used to measure viable cell concentration. By utilizing the lactate consumption rate, oxygen uptake rate, nutrient concentration, and viable cell concentration, the cell's metabolic and growth state can be determined. A system's growth and metabolic model is then used to determine the level of feed required to direct the culture towards the target state along an optimal trajectory.

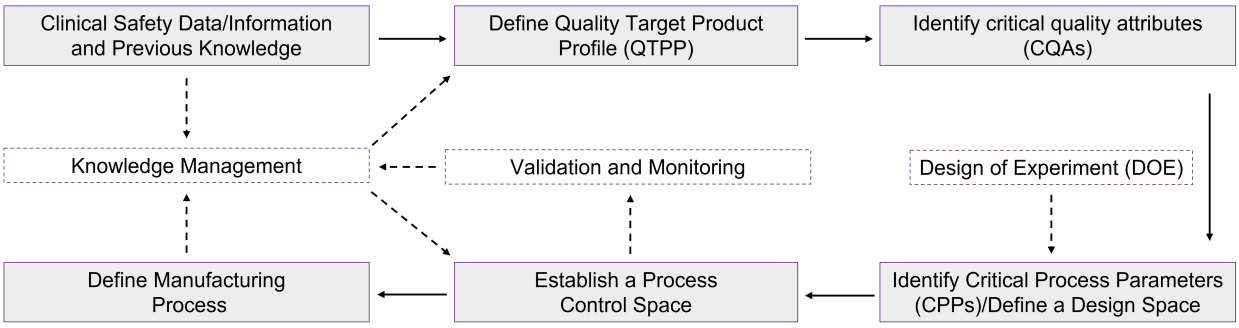

Figure 5. A workflow for Incorporation of QbD in process development and manufacturing.

QbD starts with the collection of information from available data and previous knowledge on the mechanism of action of the product. From the collected information, QTPP can be defined. Next, CQAs are identified from the QTPP. The process is then carried out and product quality is characterized to identify CPPs and to define a design space of CPPs within which the product quality will be within the desired bound. This may be achieved by taking a DOE approach. Subsequently, a control space, which is smaller than the design space, could be established to attain a high probability that the product quality is acceptable. Based on the data generated from control space, QTPP can be refined and redefined. The final step includes defining the manufacturing process for the production of the cell therapy product. As more clinical and manufacturing data are gathered, and potentially new QTPP elements and CQAs are identified, the control space and manufacturing process could be redefined to include the new information.