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WhitePaper

Quality by Design Driven Process Development of NK Scalable Manufacturing

15 Sep 2025

About Author

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Chia-Yu has over nine years of experience in cell therapy, specializing in process development and scale-up of immune cell therapeutics. She has expertise in dendritic cells, T cells, NK cells, and mesenchymal stem cells, with a focus on building robust, scalable, and GMP-compliant manufacturing platforms to support clinical translation.

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About Mycenax

Mycenax is a dedicated CDMO specializing in process development and cGMP manufacturing of biologics and biosimilars. We provide integrated DNA-to-Drug Product services for mAbs, ADCs, plasmid DNA, cell therapies, vaccines, and more through advanced platforms and PIC/S GMP-compliant facilities. Our GMP site is approved by EMA, PMDA, and Health Canada, and has successfully passed over 70 global client audits, ensuring the highest standards of quality and compliance.

Emerging and Challenges in Allogeneic NK Cell Therapy

Following the clinical successes of autologous CAR-T therapy in $CD19^+$ liquid malignancies, NK cells have considered as attractive alternative "CAR-drivers" effectors[1]. Allogeneic NK therapies are grown exponentially as a key player in the landscape of immune-oncology, offering advantages including universal "off-the-shelf" accessibility, eliminate the need for procuring autologous cell sources from ill patients, no eliciting GvHD side effects (without severe side effects such as cytokine release syndrome and neurotoxicity) which are common in CAR-T therapy. Various NK-based therapies are emerged including NK cells, CAR-NK cells, and antibody combination with NK therapy that triggers antibody-dependent cellular cytotoxicity pathway[2].

Despite the immense promise of NK-based therapies, scaling manufacturing of allogeneic NK cells with consistent quality to meet clinical and commercial demands still faces crucial challenges **(Figure 1)**. NK cells expand poorly and vary by donor ex vivo, and tend to be exhausted during expansion and have limited lifespans in vivo. Another bottleneck is that NK cells are not resistant to cryopreservation and are labile after thawing. Balance between cost-effectiveness, scalability and clinical efficacy remains the key concern of NK manufacturing strategy. In order to develop robust and scalable process for clinical production of off-the-shelf NK products, we implemented quality-by-design (QbD) strategy to kick-off these hurdles in process development stage and representative results were showcased in this article.

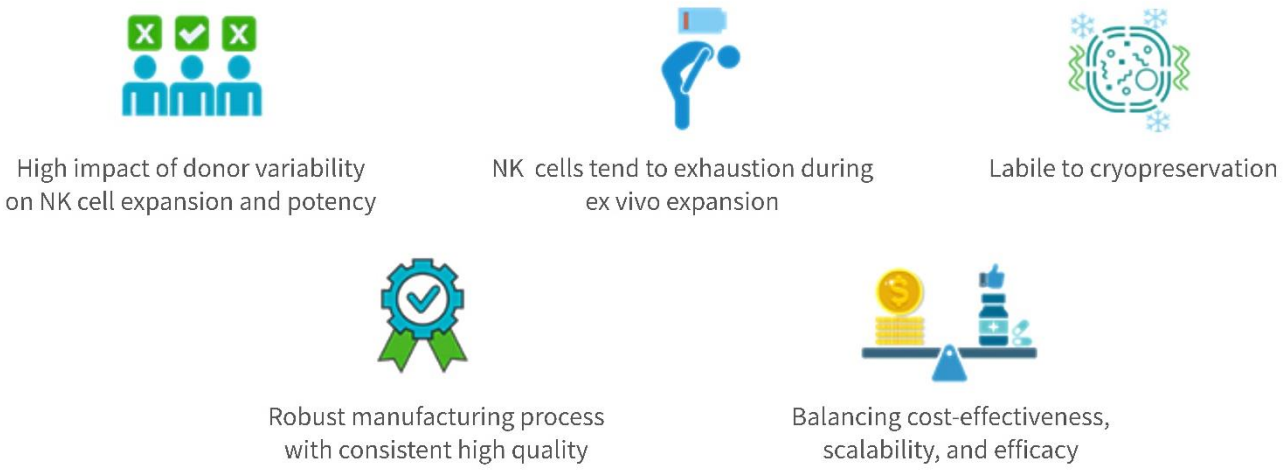


Figure 1. Current Challenges Hinder Allogeneic NK Cell Manufacturing.

Developing NK Scalable Process with QbD Strategy

Implementing QbD strategy enables systematic identification and characterization of Critical Process Parameters (CPPs) and Critical Quality Attributes (CQAs) of NK product development **(Figure 2)**. This approach ensures high-quality of NK products via robust process control and continuous improvement through the whole product lifecycle management, seamlessly the scaling-up production with consistent Quality Target Product Profile (QTPP) for guaranteeing the success of clinical outcomes[3].

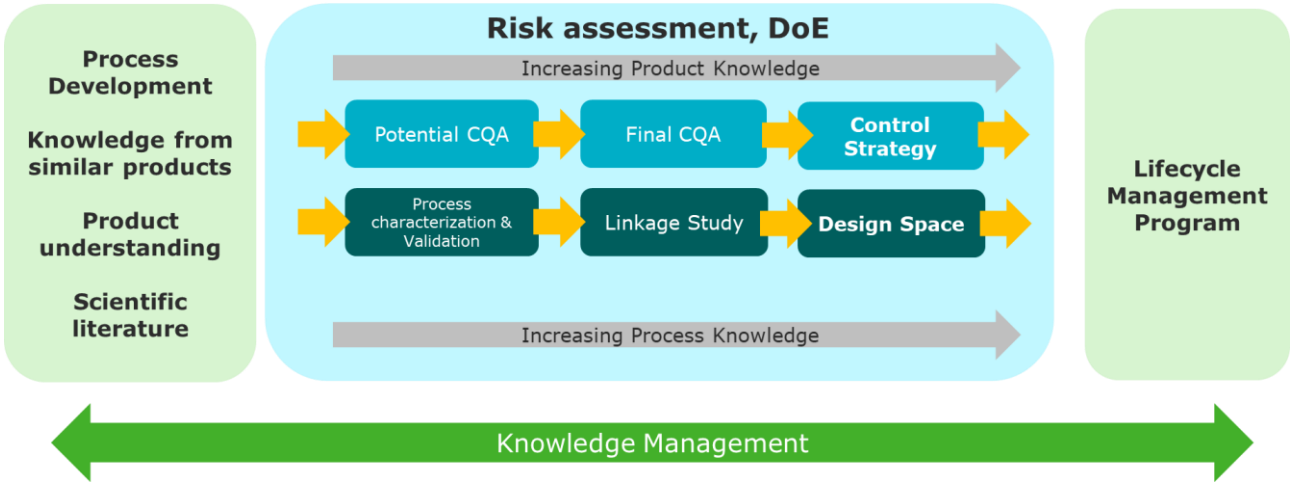


Figure 2. Roadmap of Quality-by-Design Product Development Based on Product and Process Knowledge Management.

Cell maturation stages, cytotoxicity, and cell expansion capacity of NK cells are potential CQAs to define the expected QTPP of NK products. NK cell development and maturation can be broadly categorized into 5 stages based on multiple marker expression levels and functional characteristics including cytokine production, proliferation and cytotoxicity[4] **(Figure 3)**. NK's cytotoxicity and cell vitality (persistence or exhaustion) is closely correlated to the maturation stage and sub-population composition after ex vivo expansion. NK cytotoxic capabilities gradually strengthened accompanied with cell exhaustion during NK maturation. Therefore, we have developed a proprietary quantitative tool to simulate sub-population composition of expanded NK cells based on representative immunophenotyping expression levels (in-process and release testing), to accumulate process knowledge through product characterization and establish the process control strategy.

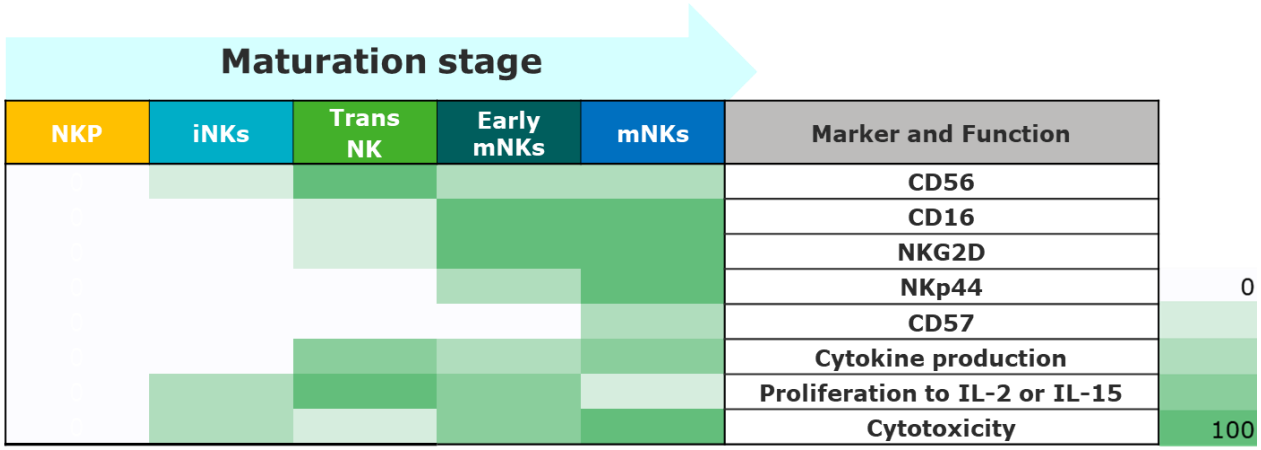


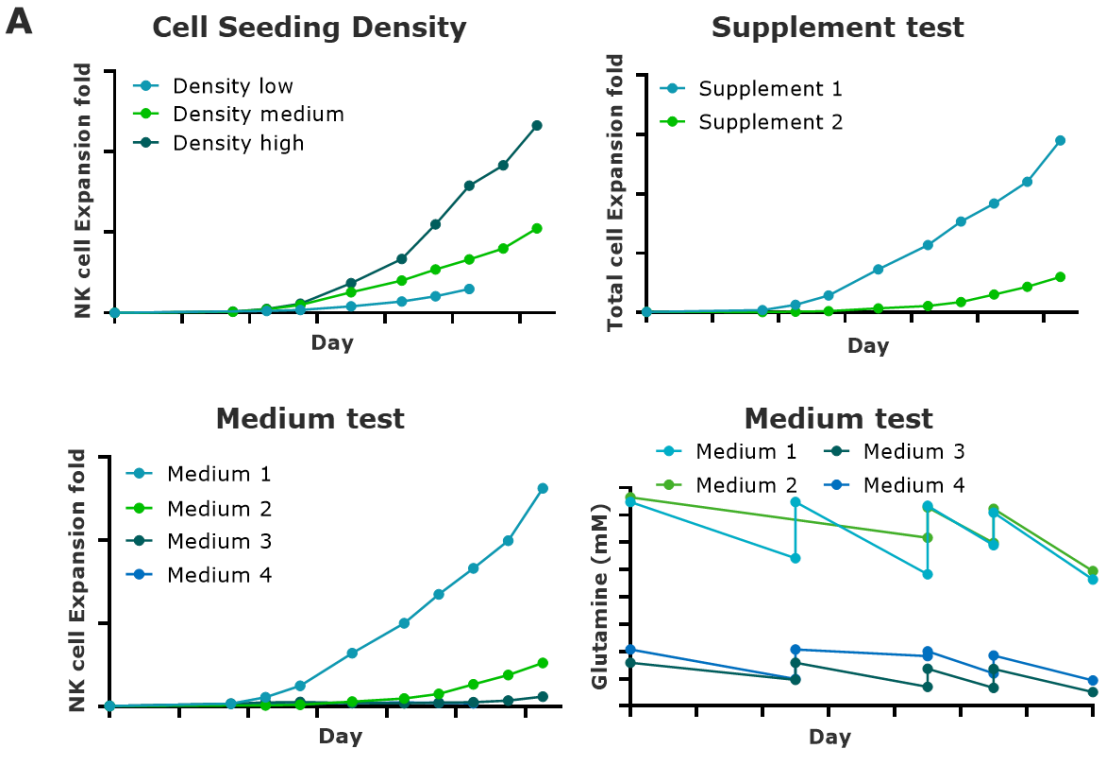
Figure 3. Schematic Representation of Human NK Cell Development and Terminal Differentiation.

NK cell development progresses through a series of well-defined stages, each marked by distinct phenotypic (grouped by expression levels of these common surface markers CD56, CD16, NKG2D, NKp44 and CD57), proliferation capacity and functional changes. This progression can be divided into 5 stages, including NK-cell precursors (NKPs), immature NK (iNK), TransNK (transition from immature to mature NK), early mature NK (early mNK) and mature NK (mNK). The intensity of NK markers and functions (cytokine production, proliferation and cytotoxicity) is visually represented by a gradient green color from 0% to 100%.

Identify CPPs and CQAs by Process Characterization for Defining Control Strategy of NK Manufacturing

To address the challenges of robust and scalable ex vivo NK expansion, co-culture NK cells with irradiated K562 cells engineered to express stimulatory molecules like 4-1BBL and membrane-bound IL-21, are widely used to enhance NK cell proliferation and activation to realize large-scale production for clinical demand[1,5]. In this study, we demonstrated how to establish process control strategy for scalable process of co-cultured NK cell with irradiated engineered feeder cells according to the principle of QbD.

Design of Experiments (DoE) is systematically employed to screen the impact of potential process parameters and material attributes on NK cell expansion capacity. At the beginning, a screening design is conducted to evaluate the effects of multiple potential factors. Our results indicated that cell seeding density is the CPP, and medium and supplement are Critical Material Attributes (CMAs) which significantly correlate to NK expansion **(Figure 4A)**. After identifying CPP and CMA of NK process in 1st run study, an in-depth DoE optimization study is conducted to establish a design space model focused on seeding density and Supplement 1. Using Response Surface Methodology (RSM) to analyze the interactions and nonlinear effects of cell seeding density and supplement 1, the design space and control space (normal operating ranges) can be well-defined **(Figure 4B)**.



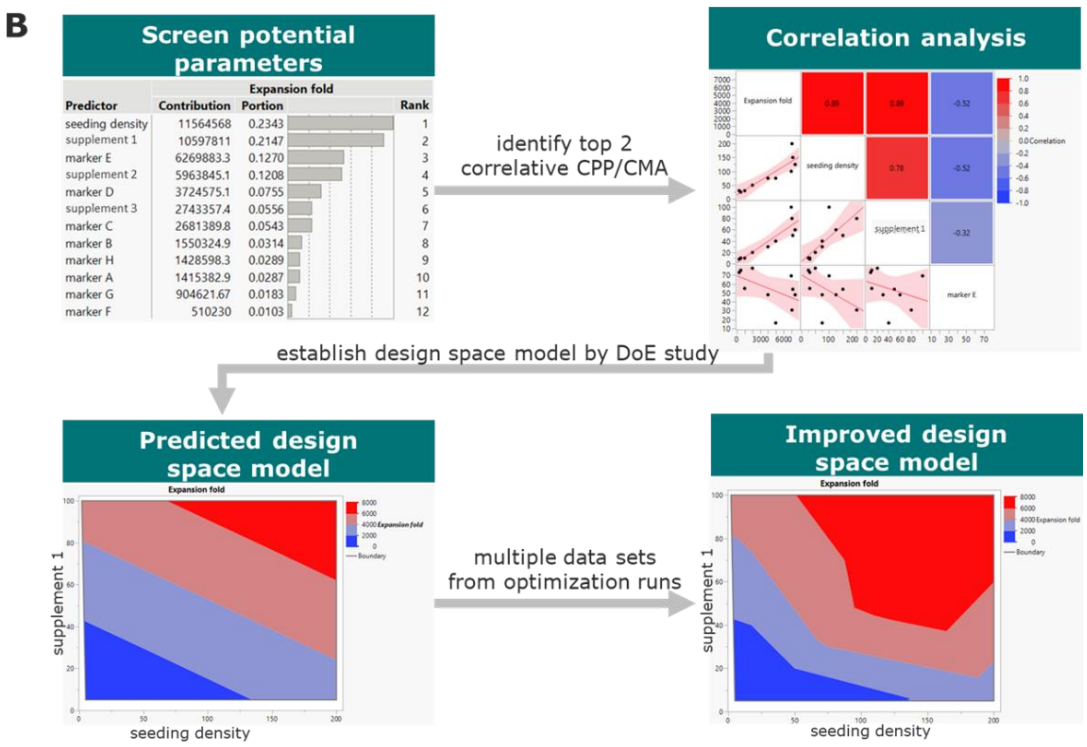


Figure 4. Identification of CPPs/CMAs and Establishing Design Space Model of NK Expansion Process by DoE Studies. (A) A DoE study identified cell seeding density (CPP) and culture medium with supplement composition (CMA) as key drivers of NK cell expansion. (B) Seeding density and Supplement 1 were further optimized using Response Surface Methodology (RSM) to refine the design space and predict optimal expansion conditions.

Secondly, we compared NK characteristics expanded from feeder-free and feeder-dependent cytokine-induced NK processes side-by-side **(Figure 5A)**. Feeder-free NK process shows limited cell growth potential (~8 CPDs, 1370-fold) and is mainly varied by donor variability. On the other side, feeder-dependent NK process demonstrates dramatically intensive and robust cell growth capacity (~17.8 CPDs, 230,000-fold), with a 168-fold increase compared to feeder-free NK process after 3-week culture **(Figure 5B)**. Meanwhile, extensive expansion capacity of feeder-dependent NK process is reproducible among different donors with negligible variations, indicating the possibility of by-pass labored donor screening and maintaining donor pool for re-called donation. NK purity (CD3-CD56+) is consistently over 95% whereas CD3+ T cell impurities are under 1% between these two groups. Interestingly, we found that the immunophenotyping profile is quite different between feeder-free and feeder-dependent process (NKp44, NKG2D, PD1 and CD57 is higher in feeder-free group). Cytotoxicity is comparable among NK cells expanded from feeder-free day14, feeder-free day21 and feeder-dependent day21 process; however, NK cytotoxicity from feeder-dependent day14 process is significantly lower than the others. In order to investigate this phenomenon, we evaluated NK maturation stage by

simulating NK subset composition using our proprietary computational tool (calculation model based on representative immunotyping marker expression levels) **(Figure 5B)**. The result indicated that NK cells from feeder-dependent day14 process was predominantly immature NK subsets composed of TransNKs followed by iNKs, with minor mature subsets of early mNKs and mNKs, thereof resulting in lower cytotoxic capabilities. On day21 of feeder-dependent NK process, the subset composition shift toward maturation which was positively correlated to enhanced cytotoxicity. Due to the high impact of NK maturation stage on cell proliferation capacity and cytotoxicity, it's critical to establish process control strategy for in-process testing and control the maturation progression of expanded NKs during process development stage.

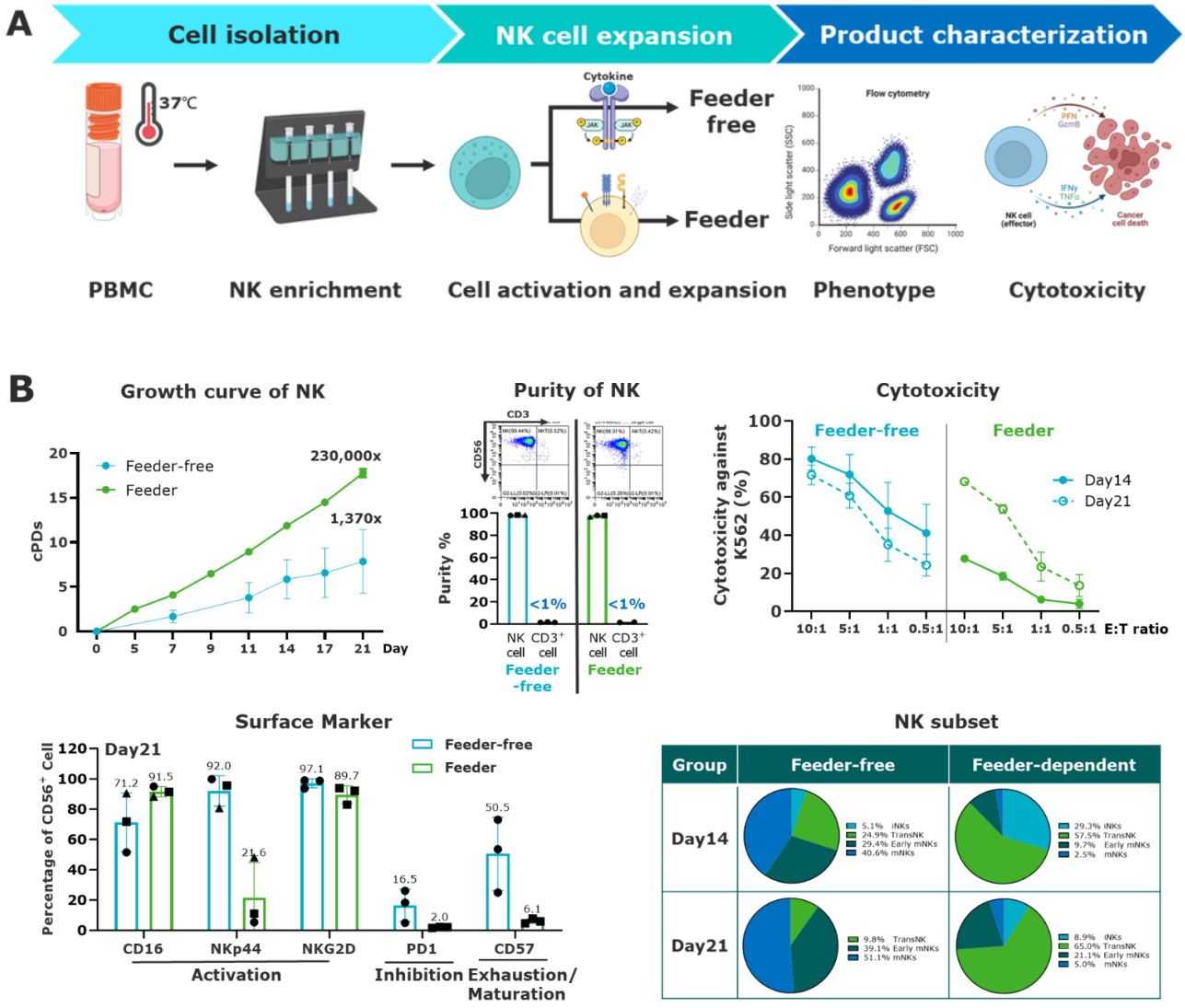
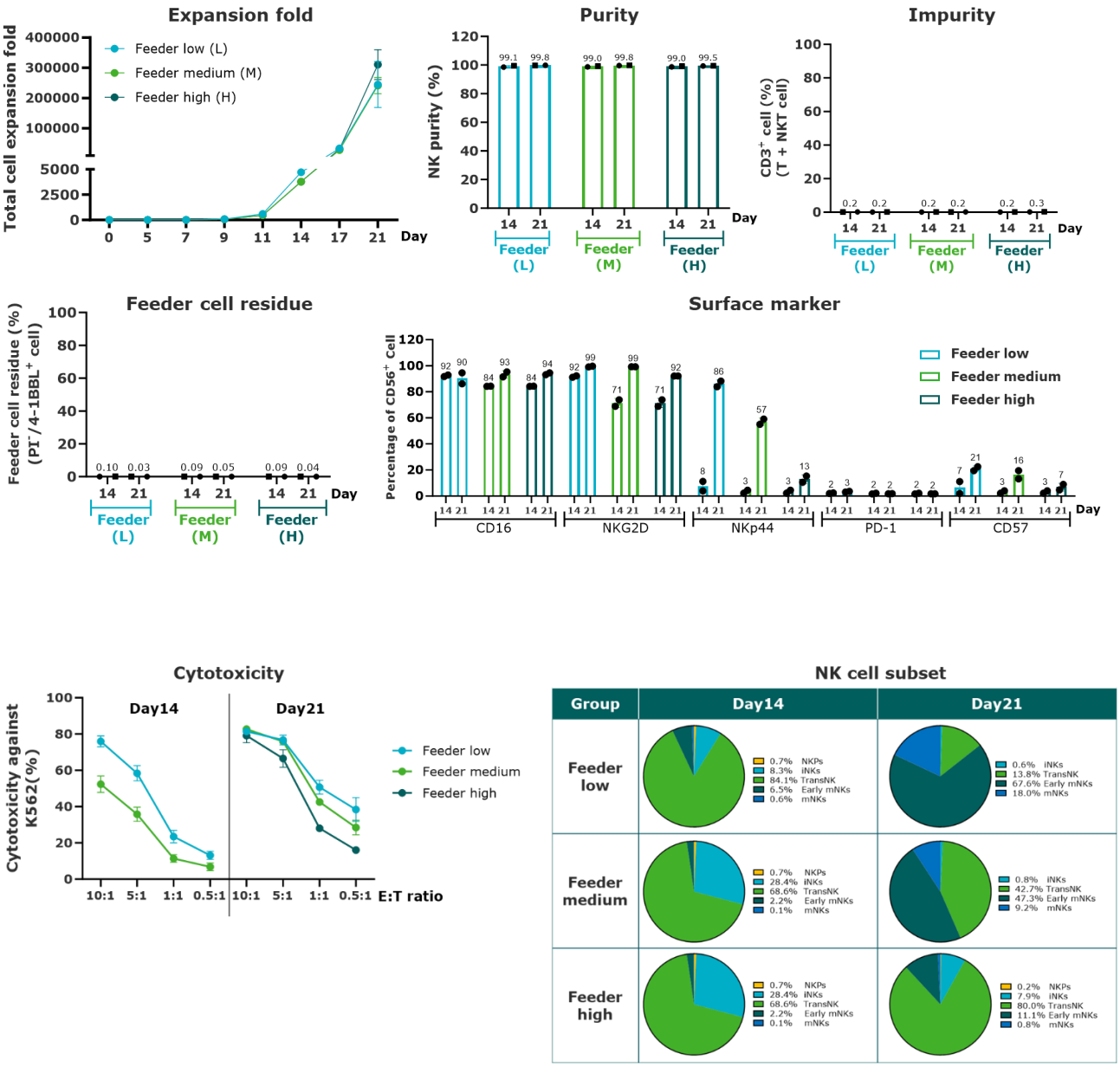


Figure 5. Comparison of NK Cell Expansion from Feeder-free and Feeder-dependent Processes. (A) Schematic representation of the experimental workflow for NK cell expansion using feeder-free and feeder-dependent processes. (B) Comparison of NK cell characteristics expanded from feeder-free and feeder-dependent processes, including

cell expansion capacity, purity ($CD3^{-}CD56^{+}$), surface marker expression, cytotoxicity against K562, and NK subset composition.

Several potential CPPs of feeder-dependent NK process on NK characteristics (cell growth capacity, maturation stage and cytotoxicity) were further evaluated including the co-culture ratio of NK-to-feeder cells, feeder stimulation frequency and culture duration. The expected goal is the develop a process control strategy to ensure expanded NK cell products balancing between quantity and quality. In **Figure 6**, effect of feeder stimulation frequency and culture duration on NK characteristics was demonstrated. There is no significant difference on NK expansion fold, NK purity, between feeder stimulation frequency low, medium and high. $CD3^{+}$T impurity and feeder cell residue are extremely low (<1%) which meets the current clinical criteria previously described[6]. While feeder stimulation frequency is increased, TransNKs and iNKs subsets (immature subsets) tend to be dominant, corresponding to lower cytotoxicity compared to low and medium group (day21). Along with culture progression from day14 to day21 of these 3 groups, NK composition shifted toward maturation subsets (early mNK and mNK) and cytotoxicity was correspondingly elevated to different extents in each group. In conclusion, our results revealed that feeder-dependent NK expansion retains higher proportion of TransNKs and early mNKs subsets which preserve steady proliferation capacity, compared to the counterpart from feeder-free NK process. Furthermore, the maturation stage and cytotoxicity of NK products can be fine-tuned via process control of feeder cell stimulation frequency and culture duration by our feeder-dependent NK expansion process.



Figre 6. Impact of Feeder Stimulation Frequency on NK Cell Characteristics.

The effects of feeder stimulation frequency on NK expansion, purity, immunophenotyping and cytotoxicity were evaluated. While NK expansion and purity remained consistent across conditions, higher stimulation frequency favored immature subsets (TransNKs and iNKs), leading to reduced cytotoxicity. Enhanced cytotoxicity correlated with increased proportions of early mNKs and mNKs.

Process Optimization of NK Cryopreservation Procedure

Process optimization for NK cryopreservation is essential to minimize ice crystal formation and osmotic stress, preserving NK cell integrity so as to maintain their viable cell numbers, stability and functionality post-thawing. Key CPPs and CMAs were systematically screened including freezing buffers, optimal cell density for freezing, and GMP-compliant controlled rate freezing program. As shown in **Figure 7A**, NK cell recovery rate, viability and cytotoxicity can be well-maintained utilizing freezing buffer 1 and high freezing cell density condition. Meanwhile, NK cell recovery rate and viability post-thawing was comparable between conventional CoolCell device and controlled-rate freezer whereas freezing temperature profile was recorded. Furthermore, we combined these optimal conditions to evaluate the long-term stability of cryopreserved NK products. Within 12-month cryopreservation under liquid nitrogen vapor phase environment (< -190 °C), NK cell recovery rate (>85%), viability (>96%) and potency (50~70% cytotoxicity against K562 target cells at 5:1 E/T ratio) were well-maintained after thawing (**Figure 7B**). Our results demonstrated the efficient measures overcoming NK's nature of labile to cryopreservation through process optimization. The possibility and feasibility of cryopreserved off-the-shelf NK products for clinical demands was highlighted.

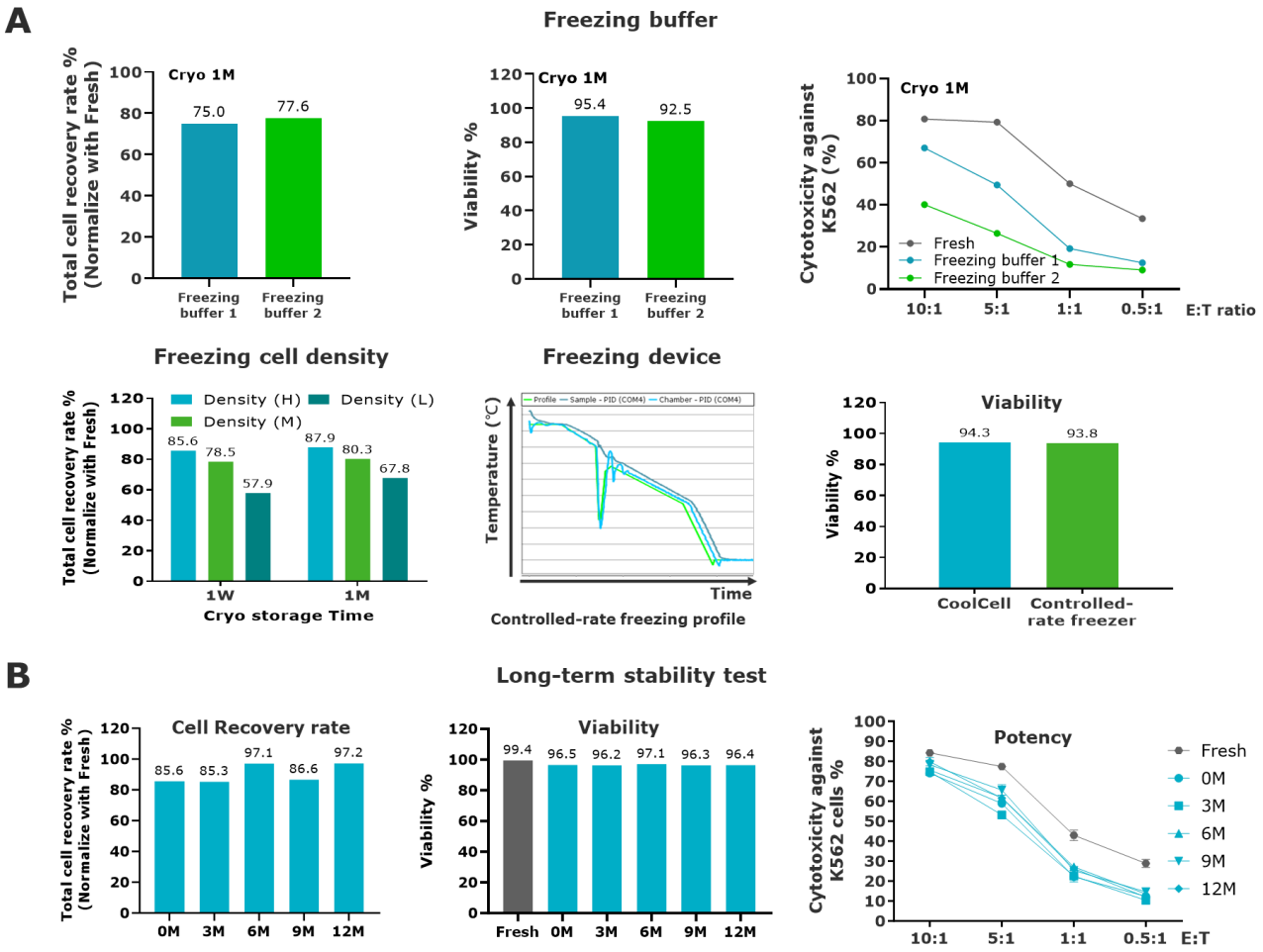


Figure 7. Process Optimization and Long-term Stability Study of NK Cryopreservation.
(A) Ex vivo expanded NK cells were cryopreserved with different conditions such as freezing buffer (1 and 2), freezing cell density (low, medium and high), and freezing devices (CoolCell and controlled-rate freezer) for 1 week or 1 month. NK cell recovery, viability, and cytotoxicity were analyzed after thawing.
(B) NK cells were frozen with freezing buffer 1 at high freezing cell density by controlled-rate freezer and then cryopreserved at the vapor phase of LN2 tank for long-term stability study. At the time points of Month 0 (as the cryopreserved control),3, 6, 9 and 12, the cell recovery, viability and cytotoxicity (potency) were evaluated after NK thawing.

Summary

We have solved the key challenges of allogeneic NK manufacturing, and our proprietary NKMX platform, a feeder-dependent NK scalable process, has been established to provide CRDMO services for CGT developers to accelerate translating allogeneic off-the-shelf NK product development into clinical application **(Figure 8)**. Workflow of NKMX platform ensures GMP-compliant and GMP-friendly with high-level quality control. By 2-stage process design, NK intermediates after stage I pre-expansion can be aliquoted and cryopreserved for subsequent multiple batches of scale-up mass production, theoretically achieving more than $2x10^{13}$ NK cells expanded from single leukopak (equal to 20,000 clinical doses estimated by $1x10^{9}$ cell per dose).

With extensive experiences of process development, we have developed precise control strategies on CPPs, CMAs, procedures and testing for NK scalable manufacturing with consistent CQAs, maximizing their therapeutic potentials. In conclusion, NKMX platform with feeder-dependent process offers unique advantages on allogeneic NK manufacturing, including eliminating donor variation effect, high cell yields & purity, supporting scalable mass production up to 50L bioreactor per batch, ensuing batch-to-batch consistent quality and affordability by cost effectiveness. As NK-based cell therapies advance toward widespread clinical and commercial application, continuing improvement and innovations will foster delivering safer, affordable, and more effective allogeneic NK therapeutics for unmet clinical needs.

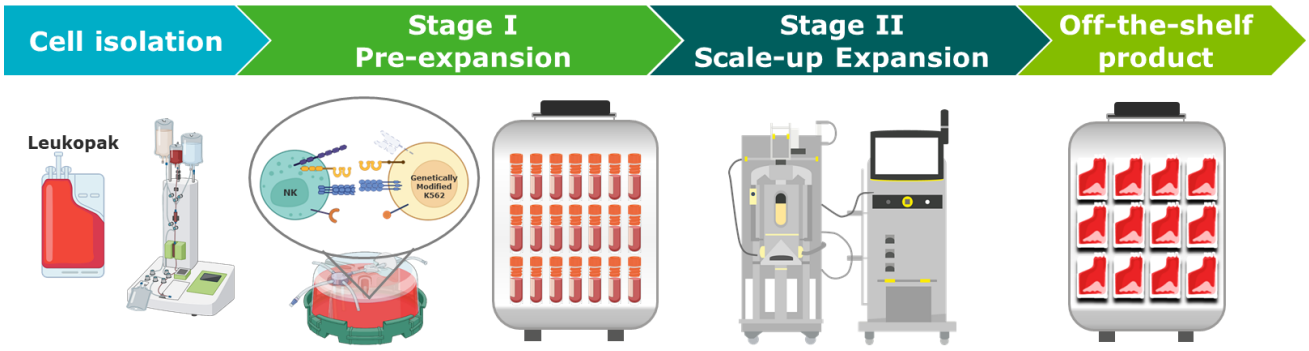


Figure 8. Workflow of Mycenax's NKMX Platform for Allogeneic NK Scalable Manufacturing.

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Acknowledgment

Gratitude to Chia-Yu Chen, Hsiang-Yu Chang, Michelle Lin and Wei-I Chen from the CGT Development Department of Mycenax for their contributions to the figures in this white paper.

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