Clinical-scale NK Cell Engineering Using mRNA Electroporation for the Development of Novel Cell-based Mono- and Combination Immunotherapies

Abstract

The ability to engineer sensitive cells, such as primary and stems cells, with high efficiency and cell viability is key to harnessing late-breaking scientific discoveries and exploring novel therapeutic approaches. This white paper highlights the use of scalable, mRNA electroporation to engineer NK cells with the efficiency and viability needed for therapeutic efficacy. The case studies presented are based on published research and customer partnerships using mRNA electroporation of NK cells that demonstrate: 1). High-efficiency, high-viability genetic modification of primary & *ex vivo*-expanded NK cells; 2). Translation to the clinic using a scalable, 1-day engineering method; and 3). The ability to support multiple therapeutic approaches including enhanced NK cell activation and tumor targeting via chimeric antigen receptor (CAR) expression and improved antibody dependent cellular cytotoxicity (ADCC) via high-affinity CD16 expression, as well as novel approaches such as improving NK cell migration via CCR7 expression. At the conclusion of the paper are general guidelines for NK cell mRNA electroporation.

Introduction

NK cells represent a promising avenue for adoptive cell transfer as they recognize and rapidly kill cancer cells in an antigen-independent fashion, and they lack many of the surface receptors responsible for graft-vs-host disease (GvHD) while maintaining graft-vs-tumor (GvT) activity. This opens the door to developing off-the-shelf allogeneic products that significantly reduce manufacturing complexity, compress production timelines and widen the treatable patient population.

Enhancements in clinical-grade NK cell isolation, *ex vivo* expansion and stimulation regimens, as well as improvements in NK cell genetic engineering have significantly advanced the field of NK cell-based therapies.[1-4] Extensive preclinical work is now underway to genetically engineer NK cells to further enhance their efficacy – taking a variety of scientific approaches including improving persistence, tumor migration, tumor targeting and cytotoxic effector functions – with the end goal of translation to the clinic. [5-7]

One critical aspect of clinical translation is the ability to establish a streamlined, clinical-scale and regulatory-compliant manufacturing process for NK cell engineering, while maintaining key performance parameters such as engineering efficiency, cell viability and reproducibility. Therapeutic developers that integrate a high-performance, scalable engineering platform early within their pipelines are thus able to greatly accelerate time to clinic and reduce overall risk.

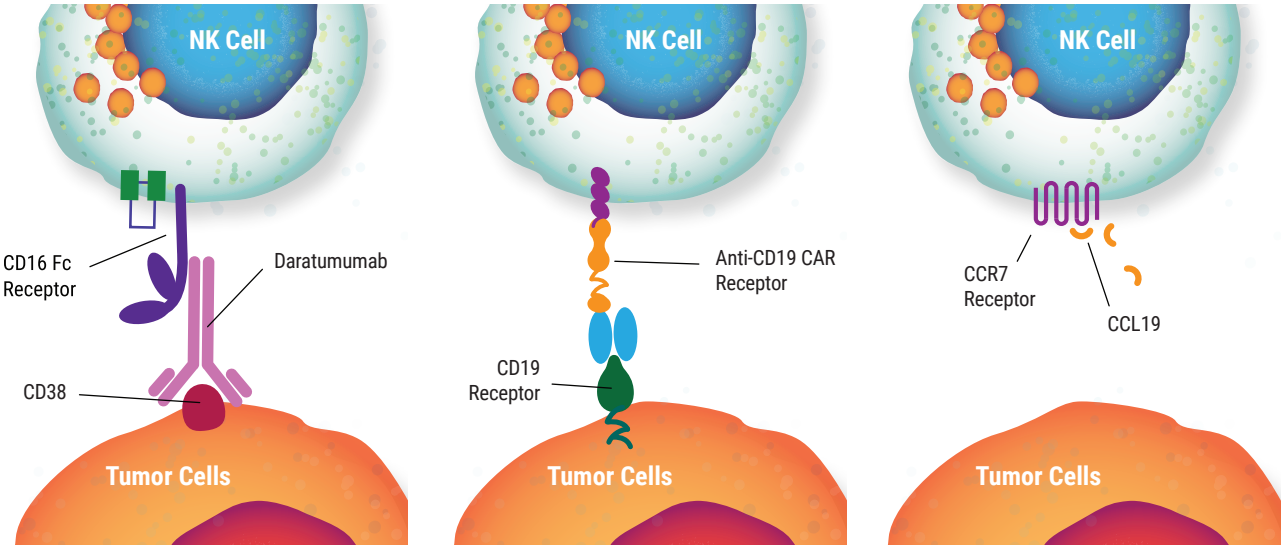
This paper presents 3 case studies – expression of an anti-CD19 CAR, a high-affinity CD16 Fc receptor and the CCR7 chemokine receptor – that demonstrate the high performance of mRNA electroporation using MaxCyte's ExPERT Platform and its proven ability to compress development timelines for diverse therapeutic approaches.

Case Study 1: Shifting the Balance of Activation Signaling and Tumor Targeting via Anti-CD19 CAR

Genetic modification of NK cells to shift the balance of receptor signaling towards NK cell activation to overcome immunosuppression and bolster anti-tumor cytotoxicity is of great interest and being attacked via a variety of receptors, including engineered expression of CARs to improve activation and tumor targeting.

First generation NK-CAR studies demonstrated improved cytotoxic activity of anti-CD19 CAR NK cells against B-cell malignancies, however, engineering using viral transduction posed challenges for more sophisticated engineering, commercial manufacturing, and ultimately sacrificed clinical translation.

The goal of the work described in this case study and reported in *Cytotherapy*, 2012, 14(7): 830-840 was to develop and validate mRNA electroporation using the ExPERT cell engineering platform as a safe, clinically-feasible means of anti-CD19 CAR expression in primary and *ex vivo*-expanded NK cells. Specifically, these studies demonstrate high-efficiency, high-viability, large-scale manufacturing of anti-CD19 CAR NK cells as well as establish *in vitro* functionality, *in vivo* safety, and anti-tumor activity of NK-CAR cells that supported the initiation of a clinical trial.[8]



NK Cell Type	Electroporation	Cell Viability (Median)	Cell Viability (Range)	Transfection Efficiency: CAR Expression (Median)	Transfection Efficiency: CAR Expression (Range)
Primary NK Cells (n=9 donors)	Small-scale, unoptimized conditions	91%	79-96%	38%	21-60%
Ex vivo-expanded NK Cells (n=9 donors)	Small-scale, unoptimized conditions	90%	81-95%	69%	58-82%
Ex vivo-expanded NK Cells (n=12 donors)	Large-scale, optimized conditions	87%	85-93%	82%	32-88%

Table 1: Reproducible, High-efficiency, High-viability Engineering of Primary & Ex vivo-expanded NK Cells. *NK cells were isolated from 9 healthy donors and used immediately (primary NK cells) or expanded* ex vivo *for 7 days. Primary and expanded NK cells were transfected via small-scale electroporation with mRNA encoding an anti-CD19 CAR using unoptimized conditions on the MaxCyte's GTx. Expanded NK cells from 12 healthy donors underwent large-scale electroporation using optimized conditions. CAR expression and NK cell viability were assessed 24 hours post electroporation.*

>80% Efficiency & >85% Viability Using Large-scale Electroporation of Expanded NK Cells

NK cells were isolated from 9 healthy donors and electroporated with mRNA encoding the anti-CD19-BB-ζ receptor either immediately following isolation (primary NK cells) or following *ex vivo* expansion to evaluate baseline transfection efficiency, cell viability, and consistency using unoptimized conditions (i.e. mRNA concentration and NK cell density).

Twenty four hours post electroporation 69% of expanded NK cells and 38% of primary NK cells expressed the anti-CD19 CAR with cell viabilities >90% (Table 1, Figure 1). Subsequent large-scale electroporation of *ex vivo*-expanded NK cells from 12 individuals using optimized conditions resulted in 82% CAR expression and 87% cell viability, levels well above those obtained using viral transduction.

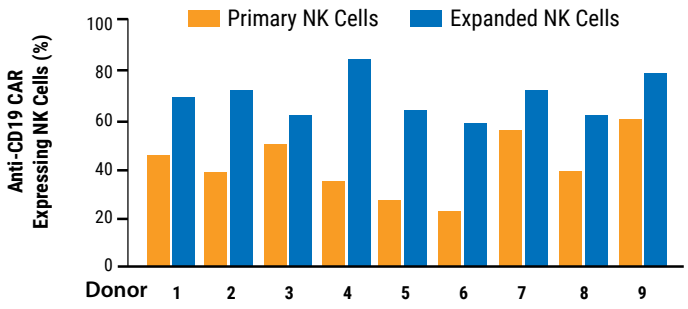


Figure 1: Robust CAR Expression Following mRNA Electroporation of NK Cells. *NK cells were isolated from healthy donors and used immediately (primary NK Cells) or expanded* ex-vivo *for 7 days prior to use. Cells were transfected using small-scale electroporation and unoptimized conditions with anti-CD19 CAR mRNA and CAR expression assessed via flow cytometry 24 hours post electroporation.*

10 Day Reduction in Manufacturing

Side-by-side experiments were conducted using expanded NK cells from three donors in which cells were either transduced with a retrovirus containing a CD19-BB-ζ construct using previously established protocols[9] or electroporated with CD19-BB-ζ mRNA.

Retroviral transduction required two consecutive days of infection followed by 8-10 days of culturing to reach peak CAR expression resulting in an average of 60% (range = 39, 59 & 80%) of cells expressing the anti-CD19 CAR. In contrast, mRNA electroporation was performed in <1 hour with peak CAR expression averaging 87% (range = 75, 91 & 94%) as early as 24 hours post electroporation, saving as much as 10 days in the manufacturing process while improving consistency (Figure 2).

No differences in CD19-specific cytotoxic activity were detected between the two engineering methods on a per cell basis, however, the absolute number of NK cells expressing the CAR was substantially higher following electroporation.

In addition to improved manufacturing consistency and substantially reduced engineering timelines, MaxCyte mRNA electroporation further reduces time and costs as a result of the relative simplicity of mRNA preparation versus the necessary manufacturing and testing of viral stocks.

Successful Translation to the Clinic

NK cell expression of anti-CD19 CAR following mRNA electroporation induced strong, tumor-specific *in vitro* effector functions including augmented IFN-γ production and considerably higher cytotoxicity against CD19+ B cell lymphoma cell lines, including 380, OP-1, RS4; 11, Raji and Ramos (data reported in Cytotherapy, 14(7): 830-840). Importantly, CAR-expressing cells did not exhibit increased *in vitro* cytotoxicity against CD19-negative tumor cell lines or against non-transformed allogeneic mesenchymal cells.

In subsequent studies using xenograft models of B-cell leukemia, administration of anti-CD19 CAR-expressing NK cells resulted in 10-fold lower tumor burdens demonstrating the ability of these cells to safely mediate *in vivo* anti-tumor activity.

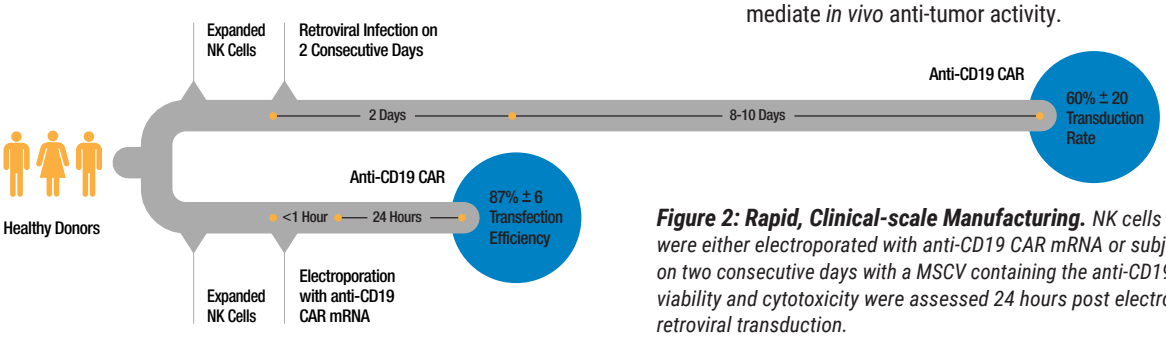


Figure 2: Rapid, Clinical-scale Manufacturing. *NK cells expanded from three donors were either electroporated with anti-CD19 CAR mRNA or subjected to retroviral transduction on two consecutive days with a MSCV containing the anti-CD19-BB-ζ construct. CAR expression, viability and cytotoxicity were assessed 24 hours post electroporation or 8-10 days post retroviral transduction.*

These preclinical studies opened the path to an ongoing clinical trial (NCT01914479) using large-scale MaxCyte engineering of NK cells, and clearly highlight the performance and clinical feasibility of mRNA electroporation.

Case Study 2: Augmenting Duratumumab Efficacy through Expression of the High-Affinity Fc Receptor

Following the trend of other immuno-oncology therapies, NK cells are likely not to stand alone, but instead extend to combination therapies. For example, engineering of NK cells to express a high-affinity CD16 Fc receptor holds promise to augment the efficacy of biotherapeutic antibodies, BiTEs and TriKEs by increasing NK-cells' natural ADCC antibody dependent cellular cytotoxicity (ADCC).[10, 11]

Several clinical studies have observed that patients homozygous for the high-affinity CD16 receptor polymorphism (CD16-158V) have improved clinical outcomes upon monoclonal antibody treatment highlighting the importance of NK cell Fc receptor engagement of antibodies.[12-15] *In vitro* studies using mRNA electroporation to express CD16-158V in *ex vivo*-expanded NK cells demonstrated increased rituximab-mediated cytotoxicity against CD20+ B cell lymphomas cells supporting the therapeutic potential of engineering high-affinity Fc receptor expression.[16]

Highly Efficient CD16-158V Engineering of KHYG1 NK Cells

Darzalex® (daratumumab) is an FDA-approved monoclonal antibody directed against CD38 used to treat patients with multiple myeloma. While CD38 is commonly found on the surface of myeloma cells, many NK cells naturally express CD38 presenting a therapeutic challenge -- the population of CD38+ NK cells that is important in mediating ADCC is depleted by daratumumab treatment.[17]

The KHYG1 NK cell line displays >10-fold lower levels of CD38 when compared to either *ex vivo*-expanded primary NK cells or the NK-92 cell line (Figure 3), thereby representing a potential NK cell source for engineering.

In this case study, researchers sought to overexpress high-affinity CD16 on CD38low NK cells as a combination immunotherapy to potentiate daratumumab efficacy against multiple myeloma. Specifically, they demonstrate efficient expression of CD16-158V on KHYG1 CD38low NK cells using MaxCyte mRNA electroporation with minimal effects on cell viability and improved duratumumab-mediated cytotoxic activity against primary multiple myeloma cells.

KHYG1 cells electroporated with mRNA encoding CD16-158V demonstrated >95% cell viability with a significant increase in CD16 surface expression. CD16 expression peaked 24 hours post electroporation and remained higher than mock-electroporated NK cells during the 5-day post electroporation culture period (Figure 4). Due to the short persistence of adoptively transferred NK cells, the transient nature of CD158-V expression is not thought to negatively impact the therapeutic potential.

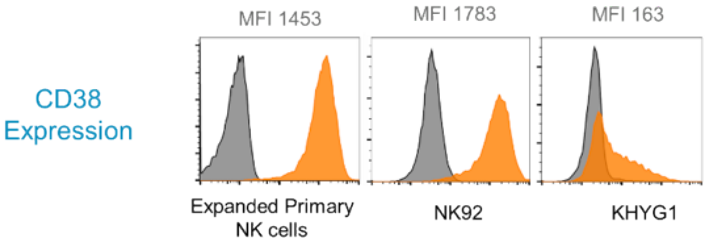


Figure 3: Low CD38 Expression by NK Cell Line KHYG1. Ex vivo-*expanded NK cells, NK-92® cells or KHYG1 cells were evaluated via flow cytometry for the expression of CD38. Mean fluorescence intensity values are reported.*

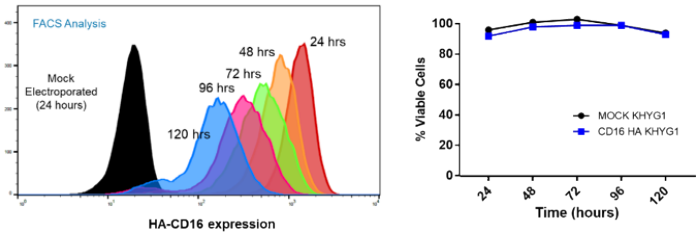


Figure 4: High Viability and CD16-158V Expression for 5 days Post mRNA Electroporation. *KHYG1 cells were electroporated with mRNA encoding the high-affinity CD16 receptor (HA-CD16). Cell viability and HA-CD16 expression were evaluated over the course of a 5-day,* in vitro *culture period. Mean fluorescence intensity and % viable cells are reported. Mock electroporated KHYG1 cells were used as a control.*

CD16-158V Expression Translated to Enhanced Duratumumab-mediated Anti-Tumor Cytotoxicity

In vitro duratumumab-mediated cytotoxic activity of CD16-158V engineered or non-engineered KHYG1 cells was assessed against primary CD38+ multiple myeloma cells. Significantly increased cytotoxicity was observed in the presence of CD16-158V-electroporated KHYG1 cells compared with mock-electroporated cells, even at low (0.5:1) effector to target cell ratios (Figure 5).

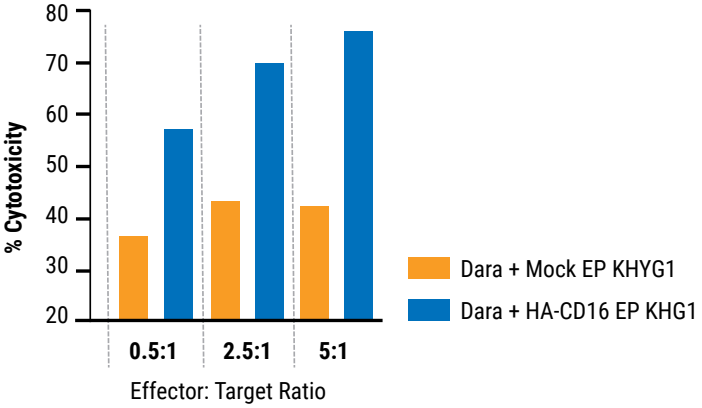


Figure 5: Improved Daratumumab-mediated Lysis of Primary Myeloma Cells. *HA-CD16 electroporated or mock electroporated KHYG1 cells were co-cultured with CD38+ primary multiple myeloma cells at various target to effector cell ratios in the presence of daratumumab and cytotoxicity examined.*

These ongoing studies illustrate the accelerated development of an NK cell engineering process for the expression of the high-affinity CD16 Fc receptor based on mRNA electroporation. The use of the ExPERT platform provided high levels of efficiency and cell viability which can now be rapidly translated to the clinic, compressing the commercialization timeline of combination NK cell immunotherapies that augment the anti-tumor activity of currently approved biotherapeutic antibodies.

Case Study 3: Reprogramming NK Cell Migration via CCR7 Expression

The ability of adoptively transferred NK cells to exert anti-tumor activities against solid tumors relies on their capacity to migrate to tumor sites. Studies have shown that cytokine-activated and *ex vivo*-expanded NK cells -- the most likely source of adoptively transferred NK cells -- lack, or express at extremely low levels, homing receptors required to achieve tissue-specific tumor targeting, and display altered migration patterns when compared to adoptively transferred non-expanded NK cells.[18, 19]

The feasibility of altering NK cell migration via chemokine receptors was demonstrated in a study in which CCR7, a homing receptor normally present on only a small subset of NK cells, was transferred to NK cells via trogocytosis using feeder cells which resulted in redirected migration.

The goal of the work described in this case study and reported in *Front. Immunol.*, 2016, 7:105 was to use clinical-grade genetic modification of NK cells to enhance homing to secondary lymphoid tissues including lymph nodes, key sites where hematological malignancies reside upon adoptive transfer. Specifically, they establish high-efficiency expression of the CCR7 lymph node-associated homing receptor in *ex vivo*-expanded human NK using MaxCyte mRNA electroporation, with minimal effects on viability, NK cell phenotype, and native cytotoxicity functions, while inducing dose-dependent migration towards the CCR7 ligand, CCL19.

High-efficiency, Low-toxicity Engineering of NK Cells

NK cells isolated from healthy donor peripheral mononuclear cells (PBMCs) were expanded *ex vivo* for 11 – 15 days prior to electroporation. The expanded cells were transfected using small-scale electroporation with increasing concentrations (0.5 – 8 µg/10^6 cells) of mRNA encoding CCR7. Strong CCR7 expression was detected as early as 8 hours post electroporation and correlated with the CCR7 mRNA concentration (Figure 6).

Examination of 15 NK cell activating and inhibitory receptors demonstrated no impact of mRNA electroporation on cell phenotype except for mild increases in TRAIL expression (see detailed data in *Front. Immunol.*, 2016, 7:105). Additionally, electroporated cells maintained potent anti-tumor cell cytotoxicity functions, again pointing to the low impact of mRNA electroporation on cell health and normal functions.

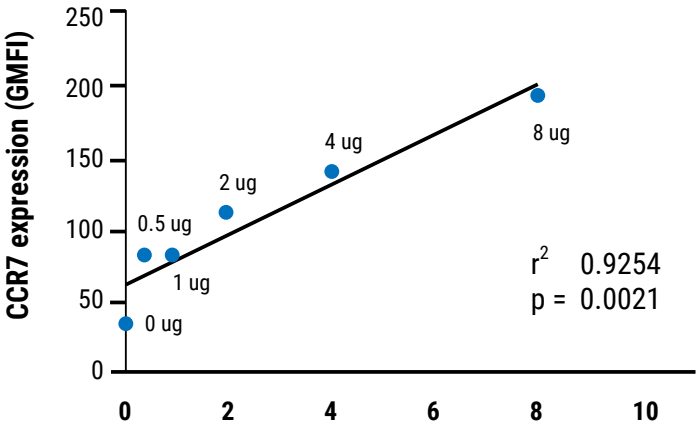


Figure 6: CCR7 Expression Levels Correlate with mRNA Concentration. *Expanded NK cells were electroporated with various concentrations of mRNA encoding the CCR7 homing receptor. NK cell CCR7 expression was assessed 8 hours post transfection.*

Improved Migration Towards CCL19, a CCR7 Ligand

CCR7 mRNA-electroporated and non-electroporated NK cells were examined via transwell assays for migration towards the CCL19 chemokine, a ligand for CCR7. Electroporated NK cells showed augmented and dose-dependent *in vitro* migration towards CCL19 compared to non-electroporated cells (Figure 7). Exposure to CCL19 also lead to a dose-dependent reduction in CCR7 (see detailed data in Front. Immunol., 2016, 7:105) similar to that seen for other CCR7-expressing lymphocytes indicating wild-type behavior of the expressed homing receptor.

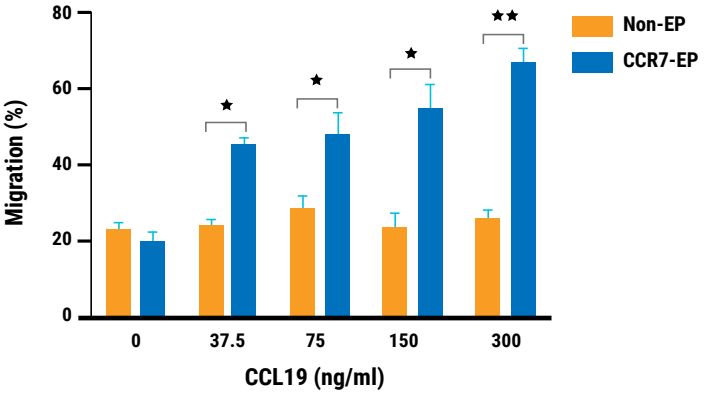


Figure 7: Improved Migration of CCR7-expressing NK Cells. *Transwell migration assay of CCR7 electroporated or non-electroporated NK cells against a gradient of CCL19. Error bars, SEM. Paired t-test used in analysis. *p<0.05, **p<0.01.*

These studies demonstrate high efficiency mRNA electroporation of primary NK cells as well as its low impact on cell health and native cell phenotype. This foundation of high-performance engineering of historically difficult-to-engineer primary cells is key to harnessing late-breaking scientific discoveries and bringing to market novel cell therapies.

Conclusion

The ever-expanding understanding of NK cell biology and the improved ability to genetically engineer NK cells using clinically-feasible, streamlined manufacturing are allowing scientists to develop mono- and combination immunotherapies using this powerful, yet untapped cell type. The three case studies presented here demonstrate the ability of MaxCyte's regulatory-compliant ExPERT™ Platform to engineer primary or *ex vivo*-expanded NK cells at clinical-scale with high viability, efficiency, and reproducibility, with low impact on cell health and native phenotype. This foundation of performance, payload flexibility and scalability enable enhanced persistence, cytolytic activity, tumor targeting, and cell migration of engineered NK cells -- that ultimately provides for improved therapeutic efficacy, accelerated translation to the clinic, and rapid commercialization of novel therapies.

General NK Cell Electroporation Methods

Below are general guidelines for NK cell transfection using MaxCyte electroporation.

- Resuspend primary, *ex vivo*-expanded, or NK cell lines in MaxCyte electroporation buffer at 1 – 3x10^8 cells/mL. While 2x10^8/mL is the recommended cell concentration at the time of transfection, higher or lower cell concentrations can be used. Contact MaxCyte for recommendations.
- Add mRNA to the resuspended cells at 0.5 – 4 µg/10^6 NK cells. Various mRNA levels should be tested to optimize expression of the desired protein.
- Transfer cells to the appropriate MaxCyte processing assembly and transfect on MaxCyte's ATx or GTx using the MaxCyte-identified NK cell electroporation protocol.
- Incubate electroporated NK cells at 37°C for 20 minutes and then dilute in media of choice and culture as desired or cryopreserve.
- Results for electroporation of freshly isolated and *ex vivo*-expanded NK cells using mRNA encoding GFP are detailed in: *Front. Immunol.* (2016), 7: 105, & *Cancer Gene Ther.*, (2009), 17(3):147-154. Transfection efficiency, cell viability, and characterization of NK cell phenotype, growth and native cytotoxic activity are detailed.

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info@maxcyte.com | maxcyte.com