

## Development of 'enhanced' potency immunotherapy products using nonviral approaches

In the next 5–10 years we could see cellular-based pharmaceuticals, or cell therapy, meeting the unmet medical needs of thousands of people. How this therapy will meet these needs depends on the ability of researchers and manufacturers to successfully and cost effectively manufacture and deliver engineered cell-based therapeutic products that are safe and exhibit enhanced potency with resulting durable, meaningful clinical efficacy. The ability to engineer such enhanced potency using nonviral, cGMP-compliant, automated and closed system manufacturing processes will represent a significant advantage. To outline how such a process might work, we have summarized the application of a scalable, cGMP-compliant, electroporation platform for engineering dendritic cells (DCs), NK cells and T cells for development of cellular immunotherapies targeting hematological malignancies and solid tumors. Autologous cellular immunotherapy refers to a class of therapies that are designed to stimulate a specific immune response against cancer cells or other disease targets. Many autologous cellular immunotherapy protocols involve *ex vivo* modification of a patient's immune cells to enhance biological potency. Cellular modification strategies include pulsing DCs with tumor antigens/lysate and expression of chimeric antigen receptors or modified T-cell receptors (TCRs) on T cells and NK cells. DC pulsing is a process that stimulates uptake of tumor antigens or tumor cell lysate by DCs for the purpose of presenting tumor antigens on the DC surface in complex with major histocompatibility class molecules. DC pulsing is used in autologous cellular therapies for treating cancer. A chimeric antigen receptor (CAR) is a recombinant protein that typically consists of an extracellular antigen recognition domain (usually derived from an immunoglobulin variable region or TCR), a transmembrane domain, and one or more intracellular signaling domains (typically derived from TCRs and other proteins that are involved in T-cell signal transduction). Expression of CARs on the surfaces of NK cells or T cells following viral gene delivery or transfection with mRNA allows specific targeting of autologous immune cells to cancer cells. Scalable electroporation is a method for loading molecules into large numbers of cells based on the application of an electric field that produces temporary permeabilization of cell membranes. In contrast to conventional electroporation, which typically takes place in small cuvettes, scalable electroporation enables the processing of larger numbers of cells by flowing cells and loading agents between a pair of electrodes. mRNA transfection involves introducing mRNA molecules into cells for the purpose of expressing exogenous proteins. Compared to transfection of cells with plasmid DNA, mRNA transfection typically results in higher levels of cell viability and higher percentages of cells that express the encoded proteins. Transfection with mRNA also eliminates the potential for undesirable genomic insertion events that are associated with viral gene delivery methods.

**Keywords:** cancer immunotherapy • cell therapy • chimeric antigen receptors • dendritic cells • electroporation • GMP-compliant • mRNA • NK cells • T cells

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### Dendritic cell cancer vaccines

Cell-based vaccines are showing tremendous promise for treating a range of cancers. Solid tumors, which are often intractable to conventional cancer treatments, can now be targeted effectively by cellular therapies that trigger an immune response specifically against cells expressing tumor antigens. Modified dendritic cells (DCs) provide an especially potent platform for cellular immunotherapy because, as professional antigen-presenting cells, they are capable of stimulating naive and memory T cells, natural killer (NK) cells and B cells while producing important immunomodulatory cytokines that are essential for cell-mediated killing [1]. One such cellular immunotherapy is Provenge<sup>®</sup>, which was approved in April 2010 by the US FDA for advanced prostate cancer [2].

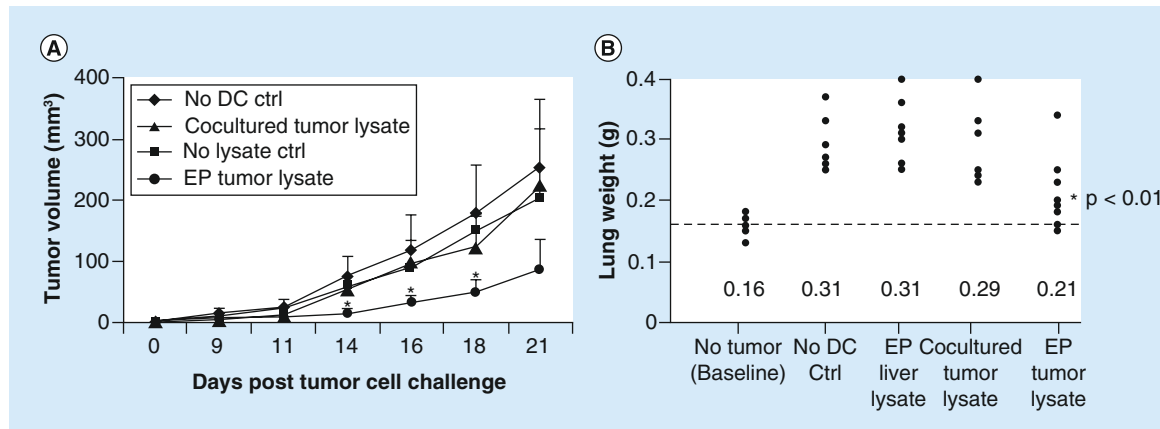
A number of approaches have been used to harness DCs for cancer therapy, all based on modifying autologous DCs to present tumor antigens on the cell surface in conjunction with major histocompatibility complex (MHC) molecules. Most DC modification protocols involve co-incubation of immature DCs with peptides, antigens, or whole cell tumor lysates. However, these passive delivery methods, which rely on endogenous endocytic functions of the DC, are hampered by low efficiency [3–6]. Active delivery approaches based on transfection of DCs with tumor-encoding mRNAs, peptides, or cell lysates are much more efficient, but they are often impractical from a clinical perspective due to lack of GMP compliance [7–9]. Below we present several examples of active DC loading that use a scalable, GMP-compliant electroporation-based technology that can load either immature or mature DCs. In addition to high loading efficiency and regulatory acceptance, clinical electroporation offers enhanced biological efficacy based on *in vitro* tumor-killing assays and patient responses.

Weiss *et al.* demonstrated the practicality and efficacy of electroporation-based DC pulsing by loading murine DCs with whole cell lysates generated from tumor cell lines [10]. The authors noted several advantages to loading whole-tumor lysate versus single antigens. First, there is a limited supply of well-characterized tumor antigens for many types of cancer. Second, lysates provide a more comprehensive repertoire of tumor-associated antigens (TAAs), which should reduce the likelihood for tumor escape [11–12]. Third, single antigens will be presented in complex with a particular human leukocyte antigen type, leading to induction of only cytotoxic T-cell responses. In contrast, by using a whole-tumor lysate approach, different antigens should be presented in class I and class II MHC pathways. This dual pathway presentation could also be expected to induce simultaneous CD4

and CD8 T-cell stimulation, leading to more efficient tumor killing [13].

Immature DCs derived from murine bone marrow were first subjected to electroporation in the absence of loading agent and assayed by flow cytometry to confirm that the electroporation process did not alter the normal repertoire of MHC class II and costimulatory molecules, such as CD80, CD83 and CD86. Following treatment with a cytokine maturation cocktail, the electroporated DCs also exhibited expected expression of maturation markers and secreted cytokines. Thus, electroporation appeared to have no discernable impact on normal DC cell biology. Next, whole cell lysates from multiple tumor cell lines or from control cells were loaded either by electroporation or by co-incubation into immature DCs. Following overnight maturation, DCs were cocultured with naïve syngeneic splenocytes. After several rounds of priming, stimulated splenocytes were cocultured with tumor cell lines matching the lysate source or with control cells, and tumor cell killing was quantified by a chromium release assay. DCs electroloaded with tumor lysate exhibited significantly higher levels of tumor cell-specific killing compared with DCs loaded by co-incubation. Enhanced CD8<sup>+</sup> T cell stimulation was also evidenced by robust IFN- $\gamma$  production following co-incubation with electroloaded DCs. *In vivo* efficacy of DC electroloading was demonstrated by injecting loaded DCs into syngeneic mice, which were also injected with tumor cells either post DC injection (tumor challenge model) or prior to DC injection (metastatic reduction model). Significantly lower rates of tumor growth and metastasis were observed in mice treated with DCs that were electroloaded with tumor lysate versus DCs loaded by co-incubation or control DCs (Figure 1).

Building on the above studies with murine immature DCs (imDCs), Wolfrim *et al.* demonstrated efficacy and scalability of an electroporation-based loading approach using human DCs. Monocytes enriched from human peripheral blood mononuclear cells were first differentiated into imDCs. Based on other studies that reported enhanced *in vitro* potency with DCs that were matured prior to loading, the authors then converted the imDCs to mDCs using standard protocols [13]. As an initial proof of concept, mDCs were electroloaded with lysate from cultured tumor cells that were engineered via transient transfection to express the influenza virus matrix protein (M1). A head-to-head comparison was performed with co-incubation loading, and efficacy was measured based on *in vitro* expansion of antigen specific CD8<sup>+</sup> T cells. Data generated with mDCs from nine different donors showed a significant advantage to electroloading versus co-incubation (Figure 2). Upon restimulation, the majority of the antigen-spe-



**Figure 1. Delay of tumor burden and reduction of tumor metastases with electropoloaded dendritic cells.**

**(A)** Delay of tumor burden by electropoloaded DCs versus DCs loaded via co-incubation. DCs derived from BalbC mouse bone marrow were co-incubated or electropoloaded with RENCA tumor lysate. Following DC maturation, approximately 1E6 mock or treated DCs were injected subcutaneously in syngeneic mice. After 10 days, the mice were challenged with 5E5 RENCA tumor cells. Significantly smaller tumor volumes were detected in mice that received tumor lysate–electropoloaded DCs compared with no lysate DCs and no DC control mice (\* $p = 0.05$ ). **(B)** Reduction of lung metastases by electropoloaded DCs. C57BL6 mice were injected intravenously with 5E5 Lewis lung carcinoma (LLC) cells. DCs derived from C57BL6 bone marrow were co-incubated or electropoloaded with LLC whole-tumor cell lysate, matured and cryopreserved. On days 3 and 6 post LLC injection, mice were dosed with 1E6 DCs via tail vein injection. Mice were killed and lungs were weighed 15 days post LLC injection. There was a significant reduction in LLC lung metastases following administration of DCs electropoloaded with LLC lysate compared with lungs from mice given no DCs, DCs electropoloaded with control liver lysate, or DCs co-incubated with LLC lysate. ( $n = 2$ ;  $p = 0.01$ ; each dot on the graph represents one mouse from a group of at least eight mice).

DC: Dendritic cell.  
Adapted from [10].

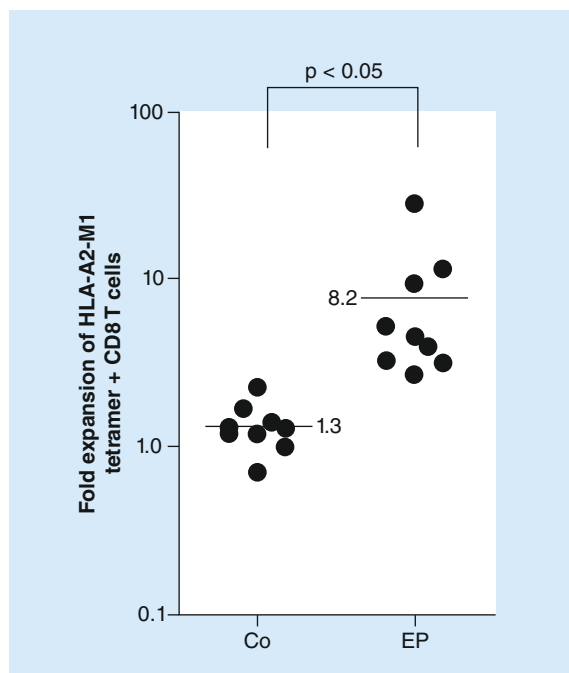
cific CD8<sup>+</sup> T cells secreted high levels of IFN- $\gamma$ , indicating that the electropoloaded cells were functional and not anergic. Superiority of electroporation-based DC pulsing was validated with lysate from a melanoma cell line. mDCs loaded with melanoma lysate elicited expansion of CD8<sup>+</sup> T cells specific to the TAA MART-1/MelanA. To demonstrate robustness and scalability of the loading process, large volume flow electroporation was used to pulse  $>1 \times 10^8$  mDCs with tumor lysate. In experiments with cells from three different donors, mDCs loaded by large-scale flow electroporation and small-scale static electroporation elicited statistically identical levels of T cell expansion. Finally, the authors noted that electroporation required two- to five-fold less lysate than co-incubation. Additionally, loading mDCs rather than imDCs significantly reduced antigen loss during the maturation step, resulting in a more potent cell product.

Safety and efficacy of electroporation-based DC loading were demonstrated in a clinical setting by Kamigaki *et al.* [14]. During a Phase I clinical trial, autologous tumor lysates, prepared from resected tumors, were loaded by electroporation into imDCs from ten cancer patients suffering from various types of solid tumor malignancies. Only mild adverse events were noted, and there were no signs of autoimmune responses. Subsequently, 52 patients with 41 types of

solid tumors were treated using autologous electropoloaded DC vaccines. In some cases, the DC vaccine was used as an adjuvant therapy post surgery or it was used in combination with other forms of therapy. The overall response rate (complete remission + partial remission) was 4.9%, and the clinical benefit rate (complete remission + partial remission + stable disease) was 37.1%. The authors also noted that a delayed type hypersensitivity response was present in 91.7% of patients exhibiting clinical benefit.

### Expression of chimeric antigen receptors in NK cells & T cells

In addition to DCs, natural killer (NK) cells and T cells are two other components of the immune system that have been at the center of efforts to develop autologous cellular cancer therapies. The challenge of targeting NK and T cells to cancerous cells while avoiding deleterious off-target immune responses has been addressed in recent years by applying *ex vivo* cellular modification and gene delivery strategies to cells isolated from the peripheral blood of cancer patients. Two of the most promising approaches to immune cell modification involve expression of modified T-cell receptors (TCRs) and chimeric antigen receptors (CARs) that specifically interact with TAAs [15,16]. TCRs recognize antigens presented in complex with



**Figure 2. Electroloading tumor lysate into dendritic cells produces a more potent vaccine than co-incubation.** Dendritic cell (DCs) were isolated from nine healthy human leukocyte antigen-A2+ donors and pulsed with lysate from JCOB cells expressing the influenza matrix protein M1, either by co-incubation or by electroloading. Following a 48-h maturation period, DCs were co-incubated with primary blood lymphocytes, and antigen-specific expansion of CD8<sup>+</sup> T cells was quantified by M1 peptide tetramer staining. Statistically greater expansion of T cells was observed with the electroloaded DCs versus the co-incubation DCs. Adapted from [13].

major histocompatibility antigen (MHC) molecules. CARs have potential advantages over modified TCRs in that they typically have higher affinities for tumor antigens, and they recognize antigens on the cell surface without the requirement for a particular context of MHC presentation [15–20]. Below, we discuss strategies for engineering NK and T cells to express CARs, and we describe several published studies that highlight the advantages of a nonviral, electroporation-based mRNA delivery method for CAR expression for treatment of solid cancers [21].

A typical CAR protein has an extracellular domain comprised of a single chain variable fragment (scFv) encompassing variable regions from heavy and light chain immunoglobulins that recognize a particular TAA. The TAA-specific scFv is adjoined by a membrane spanning domain, which in turn, is linked to an intracellular signaling domain. Various signaling domains have been tested in CAR cell therapies, either alone or in tandem with other signaling domains.

Commonly used CAR signaling domains include those derived from the CD3 zeta chain and from costimulatory molecules such as CD28, OX40 and 41BB [22,23].

Viral vectors are often used for *ex vivo* delivery of CAR expression constructs into primary hematopoietic cells [22,23]. Although the process is relatively efficient, viral gene delivery presents significant safety concerns stemming from the random nature of viral insertion. In addition, a commercial cellular therapy product based on viral transduction presents challenges for both manufacturing and clinical administration. Transient transfection of NK and T cells with plasmid DNAs encoding CARs obviates many of the safety and logistical issues related to viral transduction. However, plasmid DNA induces apoptosis soon after it is introduced into most hematopoietic cells [24–26].

To overcome the safety concerns of viral transduction and the toxicity of DNA delivery, Li *et al.* developed a strategy for introducing CAR-encoding mRNAs into NK cells using a scalable, GMP-compliant electroporation-based transfection process [27–29]. Transfection efficiency greater than 90% with minimal impact on cell viability was achieved in human NK cells following electroporation with mRNA encoding GFP. Unstimulated NK cells were then transfected with mRNA encoding a CAR targeting CD19, a surface antigen expressed on B cells. Flow cytometry analysis showed robust cell-surface expression of CAR proteins in over 50% of the transfected cells. Similar results were achieved by transfecting expanded NK cells that were stimulated by coculture with irradiated K562 cells expressing a membrane-bound form of interleukin 15 and 4-1BB ligand (K562-mb15–41BBL). Scaling up the electroporation process, achieved by flowing cells through an electroporation chamber, yielded the same CAR expression levels as smaller scale transfections performed via static electroporation. Finally, biological efficacy was demonstrated by quantifying the ability of transfected NK cells to induce lysis of CD 19 expressing tumor cells. Figure 3 illustrates that >80% cell killing could be achieved at effector:target cell ratios of 1:2 or above when transfected NK cells were cocultured with OP-1 cells [27–29].

*In vivo* antitumor activity of NK cells transfected with anti-CD19 CAR mRNA was demonstrated by Shimasaki *et al.* [30–34] and with T cells by collaborators at the University of Pennsylvania [35,36]. NK cells from healthy human donors were expanded by coculturing peripheral blood mononuclear cells with irradiated K562-mb15–41BBL cells for 7 days. A 94% pure population of CD56<sup>+</sup>, CD3<sup>+</sup> cells was generated following depletion of T cells. After demonstrating efficient *in vitro* cell killing with several CD19<sup>+</sup> tumor cell lines, NK cells loaded with anti-CD19 CAR mRNA via flow electroporation were tested for antitumor activity in a

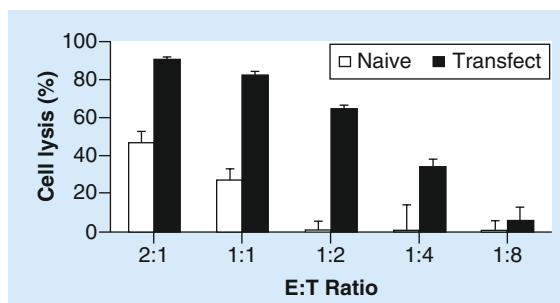
murine model of acute lymphocytic leukemia (ALL). NOD scid gamma (NSG) mice, which lack mature B cells, T cells and NK cells, were injected with cells from an ALL cell line. 2 days later, the mice were injected with CAR-expressing NK cells, either one time or daily for 3 days. After 30 days, leukemic cell burden was significantly lower in the CAR-NK cell-treated mice versus mice receiving unmodified NK cells or no NK cells (Figure 4).

Solid tumors are also amenable to treatment via adoptive immune cell transfer. Noting that retroviral transduction of T cells can produce insertional mutagenesis, Zhao *et al.* [37,38] leveraged the advantages of an electroporation-based mRNA transfection strategy for introducing CARs into T cells. Human T cells purified by elutriation were electroporated with mRNA encoding a CAR targeting mesothelin, a glycosylphosphatidylinositol-linked molecule that is overexpressed on ovarian and pancreatic cancer and mesothelioma cells. Different combinations of CD3 $\zeta$ , CD28 and 4-1BB intracellular activation domains were tested in conjunction with an extracellular antimesothelin scFv region.

An optimized CAR construct was identified, based on an *in vitro* tumor cell lysis assay and tested for *in vivo* efficacy in a mouse tumor model. Mesothelin-positive tumors expressing firefly luciferase were established in NSG mice. Then beginning 8 weeks later, the mice were injected periodically with stimulated, autologous T cells that were isolated from the tumor cell donor. The mice were divided into three treatment groups: injection with T cells electroporated with antimesothelin CAR mRNA, injection with T cells electroporated with anti-CD19 CAR mRNA, and saline-injected controls. Tumor burden, measured by bioluminescence imaging, was significantly reduced in the mice treated with antimesothelin CAR T cells, whereas the tumor continued to grow in the other two treatment groups. The 50% median survival was also significantly higher and the mean change in total body weight was lower in the antimesothelin CAR T group, compared with the other two groups.

## Conclusion

Cellular therapies based on modifying autologous cells from cancer patients represent a promising new avenue for targeting diseases that respond poorly to conventional treatment methods. Loading dendritic cells with tumor lysates and expressing modified/chimeric receptors on the surfaces of T cells and NK cells are two emerging therapeutic approaches that have led to the regression of both solid and hematologic malignancies. A number of autologous cellular therapies currently in the clinic rely on viral-based methods for introducing genes into primary T cells and other immune cells. While viral gene therapy is effective for many cell



**Figure 3. Specific killing of tumor cells by natural killer cells electroloaded with chimeric antigen receptor mRNA.** Unstimulated natural killer (NK) cells were transfected with anti-CD19-BB-z mRNA, and 3 h post transfection, transfected cells were mixed with OP-1 tumor cells at five different relative ratios. Target cells were loaded with calcein-AM (the non-fluorescent acetomethoxy derivative of the dye calcein) prior to addition of NK cells, and cell killing was measured via flow cytometry.

Adapted from [28].

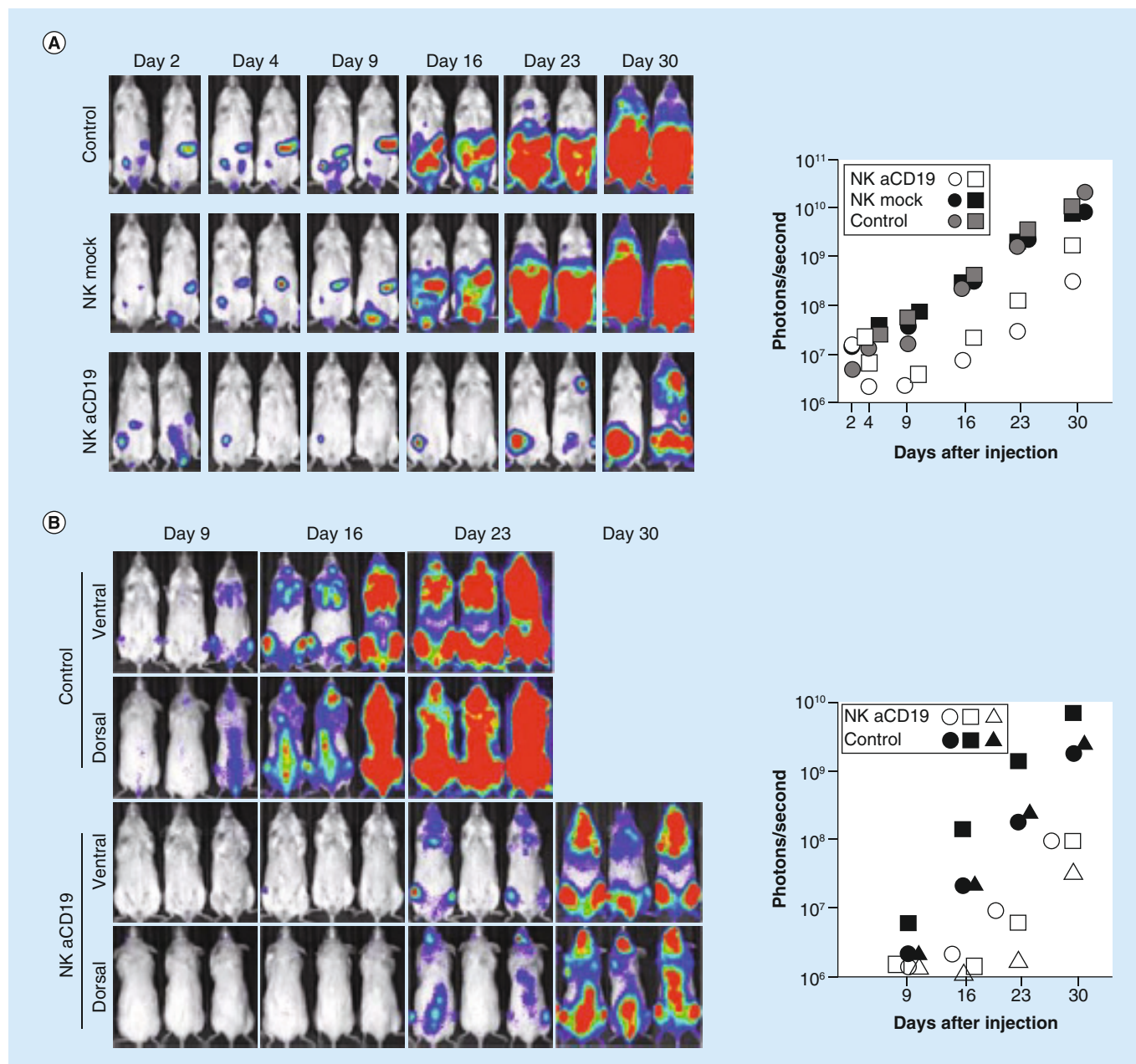
types, it presents a number of safety concerns, as well as manufacturing challenges. In addition, viral gene transfer is of limited use for dendritic cell pulsing.

Many of the technical and safety hurdles associated with viral gene therapy can be overcome with the application of clinical electroporation. Electroporation of DCs with tumor cell lysates promotes a more robust T-cell response compared to conventional methods of DC pulsing, and the method is amenable to both mature and immature DCs. T cells and NK cells can be electroporated with mRNAs encoding chimeric antigen receptors or modified T-cell receptors. In addition to high levels of transgene expression, mRNA electroporation produces higher cell viability and eliminates the potential for integration of transgenes into deleterious loci. Non-viral methods of cellular modification have already been demonstrated in multiple clinical trials, and with more data emerging daily, we appear to be entering an exciting new era that will bring new hope to cancer patients.

## Future perspective

In the next 5–10 years, traditional cancer therapies based on nontargeted delivery of chemical toxins or harsh radiation treatments will be replaced by precise cellular modification strategies that direct the patients' own immune systems to specifically eradicate cancers while greatly minimizing off target side effects. These more effective strategies for combating cancer have been made possible by advances in our understanding of the molecular mechanisms of immune system regulation and by innovative approaches for genetic modification of immune cells. Although, the field of gene therapy has advanced significantly from its early days, the inherent risks of viral-based gene delivery are still present and





**Figure 4. Antileukemic activity of natural killer cells electroperated with anti-CD19 chimeric antigen receptor. (A)** Ability of NK cells electroloaded with anti-CD19 CAR mRNA to kill tumor cells *in vivo*. NOD scid gamma mice were injected ip. with cells from the acute lymphocytic leukemia cell line 380 stably expressing luciferase, followed 2 days later by intraperitoneal injection of expanded NK cells electroloaded with or without anti-CD19 chimeric antigen receptor (CAR) mRNA. Leukemia progression was monitored by *in vivo* imaging. **(B)** Reduction of systemic leukemia cell engraftment by NK cells expressing anti-CD19 CAR. NSG mice were injected with 380 cells intravenously followed 2 days later by 3 daily intravenous administrations of expanded NK cells expressing the anti-CD19 CAR or RPMI-1640 (control mice). NK: Natural killer.

Adapted from [30].

offer significant safety and manufacturing challenges to widespread implementation of autologous cellular cancer therapies. Thus, large-scale, transient delivery of mRNA using electroporation will enable reproducible cellular modification on a commercially viable scale while eliminating the safety concerns stemming from

random integration events that are associated with viral vectors and other DNA-based gene delivery methods. Messenger RNA electroporation has proven effective in the laboratory as well as in the clinic for modifying DCs, NK cells, T cells and B cells to serve as autologous cancer therapies. In addition to advancing cellular

immunotherapy treatments for cancer, the advantages of mRNA electroporation over viral transduction have been proven in other applications, such as the derivation of induced pluripotent stem cells [39]. Therefore, in the coming years, we can expect future advances in cellular therapy and regenerative medicine to depend more and more on the application of electroporation-based cell loading technology.

#### Financial & competing interests disclosure

All authors are employed by MaxCyte. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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#### Executive summary

- A cGMP-compliant, scalable electroporation technology offers significant safety and efficacy advantages over cellular modification strategies that require viral vectors.
- Cancer immunotherapies based on electroloading autologous dendritic cells (DCs) with tumor lysate are more efficient than traditional approaches to pulsing DCs based on co-incubation, and a whole cell lysate approach offers a more comprehensive repertoire of tumor antigens, thus reducing the potential for tumor escape.
- Unlike conventional DC pulsing strategies that typically work best with immature DCs, electroporation enables DCs to be loaded with tumor lysate post maturation, reducing the potential for antigen loss.
- Electroporation of natural killer (NK) cells and T cells with mRNA yields high levels of transfection efficiency while overcoming the toxicity associated with DNA transfection and eliminating the random integration issues associated with viral vectors.
- NK cells and T cells transfected with mRNA encoding chimeric antigen receptors showed specific, efficient killing of tumor cells *in vitro*.
- NK cells and T cells electroloaded with chimeric antigen receptor mRNA reduced the expansion of hematologic and solid cancers in mice, demonstrating *in vivo* efficacy of mRNA-based approaches to cellular cancer immunotherapy.

#### References

Papers of special note have been highlighted as: • of interest

- Pizzurro GA, Barrio MM. Dendritic cell-based vaccine efficacy: aiming for hot spots. *Front. Immunol.* 6, 91 (2015).
- US Food and Drug Administration. April 29, 2010 Approval Letter – Provenge. [www.fda.gov/biologicsbloodvaccines/cellulargenetherapyproducts/approvedproducts/ucm210215.htm](http://www.fda.gov/biologicsbloodvaccines/cellulargenetherapyproducts/approvedproducts/ucm210215.htm)
- Xi H-B, Wang G-X, Fu B, Liu W-P, Li Y. Surviving and PSMA loaded dendritic cell vaccine for the treatment of prostate cancer. *Biol. Pharm. Bull.* 38(6), 827–835 (2015).
- Schuetz V, Burgdorf S. The ins-and-outs of endosomal antigens for cross-presentation. *Curr. Opin. Immunol.* 26, 63–68 (2014).
- Apostolopoulos V, Thalhammer T, Tzakos AG, Stojanovska L. Targeting antigens to dendritic cell receptors for vaccine development. *J. Drug Deliv.* 2013 869718 (2013).
- Cohn L, Delamarre L. Dendritic cell-targeted vaccines. *Front. Immunol.* 5, 1–11 (2014).
- Pollard C, De Koker S, Saelens X, Vanham G, Grooten J. Challenges and advances towards the rational design of mRNA vaccines. *Trends Mol. Med.* 19(12), 705–713 (2013).
- Chiang CL, Balint K, Coukos G, Kandalafi LE. Potential approaches for more successful dendritic cell-based immunotherapy. *Expert Opin. Biol. Ther.* 15(4), 569–582 (2015).
- Dattaa J, Terhunea JH, Lowenfelda L, Cintoloa JA, Xua S, Rosa RE, Czernieck BJ. Optimizing dendritic cell-based approaches for cancer immunotherapy. *Yale J. Bio. Med.* 87, 491–518 (2014).
- Weiss JM, Allen C, Shivakumar R, Feller S, Li LH, Liu LN. Efficient responses in a murine renal tumor model by electroloading dendritic cells with whole-tumor lysate. *J. Immunother.* 28(6), 542–550 (2005).
- **Demonstrates the efficiency and potential benefits of electroporation-based dendritic cell (DC) loading compared with conventional co-incubation strategies.**
- Graner MW, Lillehei KO, Katsanis EO. Endoplasmic reticulum chaperones and their roles in the immunogenicity of cancer vaccines. *Front. Immunol.* 4, 1–12 (2015).
- González FE, Gleisner A, Falcón-Beas F, Osorio F, López MN, Salazar-Onfray F. Tumor cell lysates as immunogenic sources for cancer vaccine design. *Human Vac. Immunother.* 10(11), 3261–3269 (2014).
- Wolfrim LA, Takahara M, Viley AM *et al.* Clinical scale electroloading of mature dendritic cells with melanoma whole tumor cell lysate is superior to conventional lysate co-incubation in triggering robust *in vitro* expansion of functional antigen-specific CTL. *Int. Immunopharmacol.* 15(3), 488–497 (2013).
- Kamigaki T, Kaneko T, Naitoh K *et al.* Immunotherapy of autologous tumor lysate-loaded dendritic cell vaccines by a closed-flow electroporation system for solid tumors. *Anticancer Res.* 33(7), 2971–2976 (2013).
- **Demonstrates clinical efficacy of electroporation-based DC loading is demonstrated.**

- 15 Gill S, June C. Going viral: chimeric antigen receptor T-cell therapy for hematological malignancies. *Immunol. Rev.* 263(1), 68–89 (2014).
- 16 Wang X, Rivière I. Manufacture of tumor- and virus-specific T lymphocytes for adoptive cell therapies. *Cancer Gene Ther.* 22, 85–94 (2015).
- 17 Duong CP, Yong CS, Kershaw MH, Slaney CY, Darcy PK. Cancer immunotherapy utilizing gene-modified T cells: from the bench to the clinic. *Mol. Immunol.* 67(2 Pt A), 46–57 (2015).
- 18 Fujiwara H. Adoptive immunotherapy for hematological malignancies using T cells gene-modified to express tumor antigen-specific receptors. *Pharmaceuticals* 7, 1049–1068 (2014).
- 19 Lamers CH, van Steenbergen-Langeveld S, van Brakel M *et al.* T cell receptor-engineered T cells to treat solid tumors: T cell processing toward optimal T cell fitness. *Human Gene Ther. Meth.* 25(6), 345–357 (2014).
- 20 Lee DW, Kochenderfer JN, Stetler-Stevenson M *et al.* T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a Phase 1 dose-escalation trial. *Lancet* 7(385), 517–528 (2015).
- 21 Beatty GL, Haas AR, Maus MV *et al.* Mesothelin-specific chimeric antigen receptor mRNA-engineered t cells induce antitumor activity in solid malignancies. *Cancer Immunol. Res.* 2(2), 112–120 (2014).
- 22 Guedan S, Chen X, Madar A *et al.* ICOS-based chimeric antigen receptors program bipolar TH17/TH1 cells. *Blood* 124(7), 1070–1080 (2014).
- 23 Jonnalagadda M, Mardiros A, Urak R *et al.* Chimeric antigen receptors with mutated IgG4 Fc spacer avoid Fc receptor binding and improve T cell persistence and antitumor efficacy. *Mol. Therap.* 1–12 (2014).
- 24 Li LH, Sen A, Murphy SP, Jahreis GP, Fuji H, Hui SW. Apoptosis induced by DNA uptake limits transfection efficiency. *Exper. Cell Res.* 253(2), 541–550 (1990).
- 25 Miyahara T, Umeda Y, Yoshikawa S, Matsuno Y, Iwata H, Takemura H. Selective blockade of apoptosis by *in vivo* electroporation-mediated gene transfer combined with portal infusion of plasmid DNA attenuates liver cirrhosis. *Minerva Chir.* 67(3), 249–255 (2012).
- 26 Chicaybam L, Sodre AL, Curzio BA, Bonamino MH. An efficient low cost method for gene transfer to T lymphocytes. *PLoS ONE* 8(3), e60298 (2013).
- 27 Li L, Allen C, Shivakumar R, Peshwa MV. Large volume flow electroporation of mRNA: clinical scale process. *Methods Mol. Biol.* 969, 127–138 (2013).
- 28 Li L, Liu LN, Feller S *et al.* Expression of chimeric antigen receptors in natural killer cells with a regulatory-compliant non-viral method. *Cancer Gene Ther.* 17(3), 147–167 (2010).
- **The authors showed that natural killer cells expressing chimeric antigen receptors (CARs) following mRNA transfection exhibited high viability, robust transgene expression and potent functional activity in tumor cell killing assays.**
- 29 Singh N, Liu X, Hulitt J *et al.* Nature of tumor control by permanently and transiently modified GD2 chimeric antigen receptor T cells in xenograft models of neuroblastoma. *Cancer Immunol. Res.* 2(11), 1059–1070 (2014).
- 30 Shimasaki N, Fujisaki H, Cho D *et al.* A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies. *Cytotherapy* 7, 830–840 (2012).
- 31 Shimasaki N, Campana D. Natural killer cell reprogramming with chimeric immune receptors. *Methods Mol. Biol.* 3(969), 203–220 (2013).
- 32 Koh S, Shimasaki N, Suwanarusk R *et al.* A practical approach to immunotherapy of hepatocellular carcinoma using T cells redirected against hepatitis B virus. *Mol. Ther. Nucleic Acids* 2, 1–9 (2013).
- 33 Chang Y-H, Connolly J, Shimasaki N *et al.* A chimeric receptor with NKG2D specificity enhances natural killer cell activation and killing of tumor cells. *Cancer Res.* 73(6), 1777–1786. 2012.
- 34 Kudo K, Imai C, Lorenzini P *et al.* Lymphocytes expressing a CD16 signaling receptor exert antibody-dependent cancer cell killing. *Cancer Res.* 74(1), 93–103 (2013).
- 35 Singh N, Liu X, Hulitt J *et al.* Nature of tumor control by permanently and transiently modified GD2 chimeric antigen receptor T cells in xenograft models of neuroblastoma. *Cancer Immunol. Res.* 2(11), 1059–1070 (2014).
- 36 Barrett DM, Liu X, Jiang S *et al.* Regimen-specific effects of RNA-modified chimeric antigen receptor T cells in mice with advanced leukemia. *Hum. Gene Ther.* 24, 717–727 (2013).
- 37 Barrett DM, Zhao Y, Liu X *et al.* Treatment of advanced leukemia in mice with mRNA engineered T cells. *Human Gene Ther.* 22, 1575–1586 (2011).
- 38 Zhao Y, Moon E, Carpenito C *et al.* Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. *Cancer Res.* 70(22), 9053–9061 (2010).
- **Demonstrated *in vivo* antitumor activity of T cells expressing CARs following mRNA electroporation.**
- 39 Warren L, Manos PD, Ahfeldt T *et al.* Highly efficient reprogramming to pluripotency and directed differentiation of human cells using synthetic modified mRNA. *Cell Stem Cell* 7(5), 618–630 (2010).