Non-Viral Gene Delivery to T Cells with Lipofectamine LTX

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Abstract

Retroviral gene delivery is widely used in T cell therapies for hematological cancers. However, viral vectors are expensive to manufacture, they integrate genes in semi-random patterns, and their transduction efficiency is highly variable. In this study, several non-viral gene delivery vehicles, promoters, and additional variables were compared to optimize non-viral transgene delivery and expression in both Jurkat and primary T cells. Overall, transfecting Jurkat cells in X-VIVOTM 15 media with Lipofectamine LTX provided a high transfection efficiency (63.0±10.9% EGFP+). However, the same method yielded a much lower transfection efficiency in primary T cells (8.1±0.8% EGFP+). Subsequent confocal microscopy revealed that a majority of the lipoplexes did not enter the primary T cells, which might be due to relatively low expression levels of heparan sulfate proteoglycans (HSPGs) detected via mRNA-sequencing. PYHIN DNA sensors (e.g., AIM2, IFI16) were also expressed at high levels in Primary T cells, which can induce apoptosis when bound to cytoplasmic DNA. Therefore, transfection of primary T cells appears to be limited at the level of cellular uptake and/or DNA sensing in the cytoplasm, so both of these factors should be considered in the development of future viral and non-viral T cell gene delivery methods.

Introduction

Chimeric antigen receptor (CAR) T cell therapy is a type of immunotherapy in which a patient's T cells are harvested, genetically modified to express a chimeric antigen receptor, and then infused back into the patient to seek out and eliminate any cells that express a target antigen that is bound by the CAR. For example, anti-CD19 CAR-T cell therapy is approved by the FDA for the treatment of B cell leukemia (e.g., non-Hodgkin's lymphoma,[1] chronic lymphocytic leukemia,[2] and acute lymphoblastic leukemia[3–5]). In these treatments, the CAR is delivered to the T cells using a gammaretrovirus or lentivirus.[6,7] These viruses are generally regarded for their high transduction efficiency, but it is worth noting that their genomic integration patterns are semi-random.[11]For example, gammaretroviral vectors have been shown to have a preference for integrating near transcriptional start sites. This type of semi-random integration could potentially lead to mutagenesis, but years of clinical scrutiny have demonstrated these vectors to be safe thus far.[8–10,12,13] Nonetheless, these vectors are still considered by the FDA to be potentially oncogenic and thus must be tested for replication competence during manufacturing and patients must be monitored for up to 15 years after receiving treatment.[13–15] It is also important to note that the transduction efficiency achieved with retroviral delivery systems can vary significantly between patients (2.3-80%),[1–6]although this variation may be due to differences between their genotypes and treatment regimens.

These challenges have motivated researchers to investigate non-viral transfection methods for delivery of the CAR gene, which has been a formidable task. Indeed, T cells have proven to be notoriously hard to transfect, perhaps because they are uniquely adapted to clear the body of viral infections and restrict viral replication.[20]For example, lymphocytes have been shown to expel mitochondrial DNA in inflammatory webs after recognizing CpG oligodeoxynucleotides (a pathogenic signature unique to bacteria).[7]Nonetheless, several groups have shown that transfection efficiencies as high as 60-70% can be achieved in primary T

cells with electroporation.[21,22] This technique applies an electric field to a sample of cells that exceeds the capacitance of the cell membrane to create pores in the cell membrane that allow delivery of DNA.[15,23] This physical method of introducing DNA to T cells is generally quick and inexpensive, but it can be difficult to scale up and is relatively harsh, leading to significant decreases in T cell viability.[24–27] Alternatively, a few groups have investigated the use of cationic polymers (e.g., PEI and pDMAEMA) for gene delivery to T cells.[82-85] These studies have demonstrated highly efficient delivery of siRNA and mRNA to T cells, but the maximum transfection efficiencies for pDNA with these vehicles (1.5-25%) tend to be relatively low compared to electroporation and lentiviral transduction.[86]

The goal of this work was to investigate the use of Lipofectamine LTX as a lipid-based alternative to cationic polymers, electroporation, and lentiviral transduction for T cell gene delivery. Transfection with the cationic lipid Lipofectamine (i.e., Lipofection) involves the formation of a lipoplex consisting of negatively charged plasmid DNA and positively charged liposomes. The lipoplex can then enter the cell via endocytosis and escape into the cytoplasm by lysing the endosome through the proton-sponge effect.[28,29] The effects of additional transfection variables (e.g., media type, promoter, et al.) on Lipofectamine transfection efficiency were also investigated to determine the most efficient means of non-viral gene delivery to both Jurkat (a T cell leukemia line) and primary T cells. Finally, the transcriptome of the T cells was also analyzed to detect potential mechanisms of resistance to transfection or transduction.

Materials and Methods

Plasmid/Promoter Cloning

Initial screening experiments for the comparison of different vehicles in Jurkat cells used either the luciferase (luc2 gene)[30] expression plasmid pGL4.50 (Promega, #E1310) or the EGFP expression plasmid pEF-GFP (Addgene, #11154). The pEF-Luc plasmid used in Figures 1 & 2 was prepared by replacing the EGFP gene in pEF-GFP with the luc2 gene. For the promoter comparison experiments in Figure 2, the EF1α promoter in pEF-Luc was replaced with the CAG promoter (cloned from Addgene plasmid #48138) and the CMV promoter (cloned from pGL4.50).

Jurkat T Cell Growth and Transfection

Human T lymphocyte leukemia cells (Jurkat, clone E6-1, ATCC® TIB-152™) were seeded in 24 well plates at a density of 2x10^5 cells/well in fetal bovine serum-containing RPMI-1640 media immediately prior to transfection. Polymer-mediated transfections were performed by mixing either branched PEI (MW = 25 kDa), linear PEI (MW = 10 kDa), jetPEI®, or TurboFect™ with plasmid DNA (1,000 ng DNA/well) and then incubating the mixture for 20 minutes at room temperature to form polyplexes. Transfections with Lipofectamine® LTX were performed according to the manufacturer's protocol. Branched PEI (BP), linear PEI (LP), and jetPEI (JP) were all tested with 5:1 and 10:1 nitrogen:phosphate (N:P) ratios. N:P ratios could not be calculated for TurboFect and Lipofectamine LTX because their formulations are proprietary, so the following manufacturer-recommended volumes of each vehicle were tested for the delivery of 1 μg pDNA/well in a 24-well plate: "LipoHi" = 2.75 μL/well of Lipofectamine® LTX, "LipoLo" = 1.25 μL/well of Lipofectamine® LTX, and Turbofect = 6 μL/well of TurboFect™. Polyplexes and lipoplexes were then added to the cells, which were subsequently incubated for 48 hours at 37°C. Cells were then either analyzed for EGFP expression via flow cytometry or lysed with 150 μL of cell culture lysis reagent/well (125 mM Tris-HCl pH 7.8, 10 mM DTT, 10 mM EDTA, 50% glycerol, and 5% Trition X-100) to quantify luciferase expression with a Promega Luciferase Assay Kit (#E1501).

Primary T Cell Growth and Transfection

Primary T cells from three healthy donors were purchased from Astarte Biologics and grown in X-VIVO 15 media with Human T Activator CD3/CD28 Dynabeads (Thermo Fisher Scientific #11131D) at a 1:1 ratio of beads to cells. The culture media was also supplemented with IL-2 at 0.5 ng/mL to stimulate T cell growth. Beads were replaced every 7-10 days during passaging, while IL-2 was added every 2-3 days. Transfections of primary T cells followed the protocol that was optimized for the Jurkat T cells (2.75 μL Lipofectamine

and 1 μg of pDNA per well in a 24-well plate).

MTT Assay

Following transfection and a 48 hour incubation period, 10 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well of cells and incubated for 4 hours at 37°C to allow proliferating cells to metabolize MTT into purple formazan. After MTT incubation, 100 μL of detergent (10% SDS in 0.01% HCl/water) was then added to the wells and incubated for another 2 hours at 37°C to release and solubilize the formazan. A sample of 150 μL from each well was then transferred to a clear 96-well plate and the absorbance at 570 nm was recorded. Raw absorbance values at 570 nm for each sample were then normalized to a non-transfected drug-free control sample to obtain a relative measure of cell metabolic activity.

Annexin V/Propidium Iodide Cell Viability Assay

Samples of approximately 100,000 cells were spun down at 400 g for 4 minutes, then aspirated and resuspended in BioLegend Cell Staining Buffer two times before finally pelleting and resuspending the cells in 20 μL of BioLegend Annexin V Binding Buffer. 1 μL of propidium iodide (Ex/Em = 493/636 nm) and 0.5 μL of annexin V R-PE (Ex/Em = 565/578 nm) (Biotium #29045) were added to each cell sample and incubated in the dark for 20 minutes. Binding buffer (80 μL) was then added to each sample and then samples were transferred to a 96-well plate for flow cytometry.

Flow Cytometry Analysis

Cells transfected with pEF-EGFP were analyzed 24 or 48 hours following transfection to determine the percentage of transfected cells (%$EGFP^+$) and EGFP expression levels (mean EGFP) using either a FACSCalibur Flow Cytometer (Figure 1) or a Millipore Sigma Guava easyCyte HT sampling Flow Cytometer (Figures 2-6). Cells were analyzed via side scatter analysis to differentiate live cell populations from cell debris, while gates for fluorescent ($EGFP^+$) populations were defined using non-transfected ($EGFP^-$) control samples.

mRNA Sequencing

To detect potential changes in host cell gene expression patterns after transfection, parallel cultures of PC-3, Jurkat, and primary T cells were passaged into fresh plates or flasks. Control (untransfected) cultures were left to grow for 48 hours, while the remaining cultures were transfected with pEF-GFP one day after passaging. On the following day, a small fraction (10%) of each culture was then analyzed using flow cytometry and fluorescent microscopy to confirm EGFP expression, then total RNA samples were extracted from the remaining cells with a Qiagen RNEasy kit. The RNA samples were then submitted to the Beijing Genomics Institute (BGI, Hong Kong, China) for library preparation, next-generation sequencing, and data analysis to obtain the FPKM values shown in Tables 1-3.

Fluorescent Plasmid Transfections

Transfections of Mirus Bio Label IT fluorescein-labeled plasmid (MIR 7906) were conducted by mixing 2.75 μL Lipofectamine with 1 μg pDNA for each well of $2x10^5$ Jurkats and $CD3^+$primary T cells, while wells with $1x10^5$ PC-3 cells were transfected with 1 μL Lipo and 500 ng pDNA. Jurkat and primary T cells were both cultured and transfected in serum-free X-VIVO 15 media, while PC-3 cells were cultured and transfected in RPMI 1640 media supplemented with 10% FBS. Following transfection, all cells were incubated for 24 hours and then spun down at 300 g for 4 minutes to remove excess pDNA in the media. The cells were then resuspended in trypsin-EDTA solution and incubated for 10, 20, or 30 minutes to disrupt interactions between cell surface proteins and lipoplexes. Once the incubation was complete, the trypsin was quenched with serum, the cells were spun down again, resuspended in phosphate buffered saline (PBS) that was supplemented with 10% FBS, and then analyzed for fluorescein labeling via flow cytometry. The same

protocol was followed for the PC-3 cells, but with the addition of an initial trypsinization to detach the cells from the culture plate.

Confocal Microscopy Slide Preparation

Slides were prepared for confocal microscopy with a standard fixing, washing, staining, and mounting protocol.[9] PC-3 cells were grown for 48 hours on #1.5 glass coverslips after transfection and then excess media was removed before fixing for 10 minutes in 4% formaldehyde solution, followed by three washes with 1X PBS. Biotium Red CellBrite Cytoplasmic Membrane dye was diluted according to the manufacturer's protocol (5 μL of cell staining solution in 1 mL of PBS) and then added to the coverslips, which were incubated for 10 minutes in the dark and then washed three more times. Two drops of Thermo Fisher NucBlue Live ReadyProbes Reagent (Hoescht 33342) were then added to the slips in 1 mL of PBS and incubated in the dark for 20 minutes. Finally, 10 μL of 1X PBS was used as mounting medium to mount the coverslips onto AmScope microscope slides (BS-72P) and sealed with clear nail polish. The same protocol was followed for primary T cells with 300 g for 4 minute centrifugation spins in between each step.

All images were obtained using a Leica TCS SP8 Four Channel Confocal Microscope with a 63X oil immersion lens. Lasers were set for 407 nm (DAPI), 488 nm (EGFP), and 633 nm (AlexaFluor 647). A three-sequence run was used to maximize reading while minimizing background and crosstalk. DAPI was set to PMT while EGFP and Alexa Fluor 647 were set to HyD. Images were taken using z-stacking from the bottom to the top of each cell.

Statistical Analysis

Results and Discussion

Non-Viral Gene Delivery Vehicles

An initial screen of several cationic polymers (branched PEI, linear PEI, jetPEI, & Turbofect) and lipids (Lipofectamine LTX) in Jurkat T cells revealed that the vehicles could be separated into three significantly different cohorts (Figure 1A). For example, branched PEI and some of the other polymeric vehicle formulations provided no detectable luciferase expression, while Turbofect and a specific nitrogen:phosphate ratio (N:P = 10:1) of linear PEI yielded modest luciferase expression. The highest luciferase expression levels were obtained with Lipofectamine LTX (Lipo-Lo = 1.25 μL and 1μg DNA; Lipo-Hi = 2.75 μL and 1 μg DNA) and jetPEI (N:P = 5:1).

Subsequent transfections of pEF-GFP (Figure 1B) using the vehicles that provided the highest luciferase expression levels (Lipofectamine, Turbofect, and jetPEI) showed that the higher dose of Lipofectamine LTX (Lipo-Hi) provided the highest transfection efficiency for Jurkat T cells (25% $EGFP^+$), while the transfection efficiencies obtained with Turbofect and jetPEI were significantly lower (4.5-4.8% $EGFP^+$ cells). As observed with the luciferase assays, the higher amount of Lipofectamine (Lipo-Hi) appeared to provide a higher number of $EGFP^+$ cells than the lower amount (Lipo-Lo), but the difference was not statistically significant. The histograms in Figure 1D-F also illustrate a distinct increase in the number of fluorescent cells obtained with Lipo-Hi compared to Lipo-Lo and Turbofect.

The effects of Lipofectamine, Turbofect, and jetPEI (5:1) on cell metabolic activity were also measured with an MTT assay (Figure 1C). Each vehicle significantly decreased cell metabolic activity approximately 30-40% relative to control samples of untransfected cells, but no significant differences were observed between the vehicles themselves.

Overall, Lipofectamine LTX appeared to be more efficient than Turbofect and branched or linear PEI. This observation is in agreement with previous studies that have compared the transfection efficiencies of cationic lipids and polymers in other cell lines.[31,32] These studies attribute the higher transfection efficiency of the lipid vehicles to their higher affinity for cell membranes, which allows them to be endocytosed more quickly than cationic polymers like PEI.[31]Consequently, Lipofectamine (Lipo-Hi) was selected as the vehicle for our subsequent studies.

Promoters and Plasmids

While cationic polymers and lipids play an important role in delivering genes to cells, promoters play an equally important role in ensuring the expression of the gene that must not be overlooked. Indeed, transgene expression has been shown to vary significantly for different promoters between cell types.[33,34] For example, the mammalian EF1α promoter provides high levels of transgene expression in mouse embryonic cell lines, while expression from the viral cytomegalovirus (CMV) promoter is significantly lower in those cells.[35]

Figure 2 shows a comparison of three commonly used promoters (EF1α, CMV, and the CMV early enhancer/chicken beta-actin/rabbit beta globin hybrid promoter known as "CAG") that were used to drive expression of luciferase (Figure 2A) and EGFP (Figure S1) in Jurkat T cells. Similar trends in luciferase expression were observed with both Lipofectamine and jetPEI, with the EF1α promoter showing the highest luciferase levels followed by a slightly lower (but not statistically significant) decrease in the level of luciferase expression provided by the CAG promoter. A significantly lower level of luciferase expression was observed with the viral CMV promoter, which was the only promoter in these experiments that lacked an intron. This may explain the decrease in luciferase expression levels, since introns have been previously shown to increase transgene expression in other cell types.[36] However, it is also interesting to note that no significant difference in GFP expression was observed between the promoters (Figure S1).

Effects of Cell Culture Media

In addition to vehicles and promoters, the effects of several other variables were also tested to optimize transfection efficiency in Jurkat T cells. For example, cells were treated with small molecule inhibitors that have previously been shown to enhance transgene expression in other cell types (e.g., BX795, MS-275, AG-490, HMN-214 et al).[37,38] One of the inhibitors (iCRT14) enhanced luciferase expression approximately 3-fold, but did not significantly enhance transfection efficiency (i.e., %EGFP$^+$ cells, shown in Figure S2). We also compared the conventional pEF-GFP plasmid to EGFP-expressing nanoplasmids (Nature Technologies, #NTC9385R), which are much smaller (1,594 bp) than pEF-EGFP (5,051 bp) and have been shown to increase transfection efficiency and the duration of EGFP expression in other cell lines and *in vivo* .[39,40] However, at both 24 and 48 hour time points following transfection, no significant differences in EGFP expression were observed between pEF-GFP and the nanoplasmids in Jurkats. Finally, varying amounts of Lipofectamine did not have a significant effect on transfection efficiency was observed (Figure 3A), but the lowest dose of Lipofectamine was significantly less toxic to the Jurkat T cells (Figure 3B).

One variable that did significantly influence transfection efficiency was the type of media used during the transfection. For example, the initial experiments shown in Figures 1 and 2 were conducted in serum-containing RPMI-1640 media, since that is the media recommended by ATCC for Jurkat cells (clone E6-1, TIB-152). In contrast, chemically-defined serum-free media formulations like X-VIVO 15 are typically used in primary T cell culture to avoid the potentially problematic effects of serum components *in vivo* . As shown in Figure 3C, culturing Jurkat cells in X-VIVO media significantly increased the transfection efficiency of Lipofectamine in Jurkat cells (63.0±10.9% EGFP$^+$), relative to the modest transfection efficiency observed in RPMI media (23.1±5.5% EGFP$^+$). Similar results were also observed with transfections of nanoplasmids (Figure S3).

Since albumin and other components present in serum-containing (SCM) RPMI are known to inhibit transgene delivery,[41,42]additional transfections were performed with serum-free (SFM) RPMI and serum-containing X-VIVO media. The exclusion of serum from RPMI had no significant effect on transfection efficiency, but the addition of serum to the X-VIVO media did significantly decrease transfection efficiency. However, the transfection efficiency obtained with serum-containing X-VIVO media was still higher than both SCM- and SFM-RPMI at 24 and 48 hours post-transfection, which suggests that some component of the X-VIVO media may enhance transfection. For example, one component that is present in X-VIVO but absent in RPMI is recombinant transferrin, which has previously been shown to increase Lipofection efficiency by enhancing endocytosis and nuclear targeting.[43,44]

Lipofection of Primary T Cells

After determining the optimum vehicle (Lipofectamine LTX), promoter (EF1α), and media formulation (serum-free X-VIVO) in Jurkat cells, we used the same conditions to transfect $CD3^+$ primary T cells. Surprisingly, this protocol yielded much lower transfection efficiencies in the primary T cells (8.1±0.8% $EGFP^+$) than the Jurkat T cells (Figure 4A). This dramatic decrease in transgene expression is also illustrated by the histograms in Figures 4C/D and was visually apparent when observing the cells with fluorescent microscopy, since the Jurkat cells fluoresced brightly while the primary T cells were relatively dim. It is worth noting that primary T cells are cultured with additional components (recombinant IL-2 and anti-CD3/CD28 Dynabeads) that could potentially interfere with transfection, but culturing Jurkat T cells with Dynabeads and IL-2 did not significantly decrease transfection efficiency (data not shown).

A previous study using cationic pHEMA-g-pDMAEMA polymers observed a similar disparity, in which the polymer provided 50% transfection efficiency in Jurkat T cells but only 18-25% transfection efficiency in $CD4^+$ and $CD8^+$ primary T cells.[45] This same group later showed that endosomes in primary T cells acidify at a slower rate than endosomes in HeLa cells, which could slow the rate of endosomal escape for non-viral gene delivery vehicles that rely upon the proton sponge effect to destabilize the endosome and escape into the cytoplasm.[73]

Another variable that significantly influence transfection efficiency in primary T cells was the timing (0-3 days) between activation and transfection (Figure 4B). Indeed, other groups have previously shown that the time between these two steps significantly impacts gene delivery with viruses, electroporation, and cationic polymers.[25,45,12–14] In our experiments, cells were either transfected 30 minutes after the addition of anti-CD3/28 Dynabeads to the cells (i.e., Day 0) or 24, 48, and 72 hours later (i.e., Days 1-3). Interestingly, transfection efficiency was highest when lipoplexes were added to the cells on Day 0 or Day 1, but decreased significantly on Days 2 and 3. This downward trend in transfection may be due to the fact that cell size and substrate uptake are both maximized immediately after activation.[74,75]Additionally, cell division spikes after activation, which would allow for more effective entry into the nucleus compared to cells that are in a resting state.[76]

Tracking Plasmid Delivery Using a Fluorescent Plasmid in T cells

The relatively low transfection efficiency that was obtained with Lipofectamine in the primary T cells could be due to multiple different steps in the transfection process, including cellular uptake, nuclear translocation, transcription, and translation of the transgene. However, since other groups have achieved high transfection efficiencies (up to 70%) with electroporation of similar plasmids and synthetic mRNAs in primary T cells,[24,46–48] we hypothesized that cellular uptake may be the key limiting step for Lipofectamine (instead of nuclear uptake or transcription/translation). To test this hypothesis, we used a fluorescein-labeled plasmid (Mirus Bio, MIR 7906) to track the delivery of fluorescent plasmid DNA to the cells with flow cytometry and confocal microscopy.

First of all, as shown in Figure 5A, transfection of adherent PC-3 cells (a cell line that is relatively permissive to transfection) with Lipofectamine and pEF-GFP provided a very high percentage of $EGFP^+$ cells (91+-1.1% $EGFP^+$). Likewise, a similarly high percentage of the PC-3 cells fluoresced when transfected with the fluorescein-labeled plasmid (96+-0.94% $fluorescein^+$), demonstrating that the plasmid was successfully delivered to almost all the PC-3 cells. Interestingly, the transfection efficiency observed with pEF-GFP for the Jurkat cells (51+-9.7% $EGFP^+$) was more modest, but the percentage of fluorescein+ cells (74+-4.5%) was significantly higher. The mean fluorescence (Figure 5B) of the PC-3 cells was also significantly higher than the mean fluorescence of the Jurkat cells in both types of transfections.

Similar trends were also observed with the primary T cells (Figure 5A/B). The percentage of $fluorescein^+$ primary T cells and the mean fluorescence of the $fluorescein^+$ primary T cells was significantly lower than both Jurkat T cells and the PC-3 cells. There was also a more dramatic disparity between the fraction of $fluorescein^+$ primary T cells (51+-11.2% $EGFP^+$) and the percentage of primary T cells expressing EGFP (8.1+-0.8% $EGFP^+$). This stark difference suggests that the lipoplexes bind to a large fraction of the primary T cells, but many of those lipoplexes may fail to either enter the cell via endocytosis, escape the endosome

into the cytosol, or enter the nucleus.

To determine if the fluorescent lipoplexes were bound to the surface of the cells or taken up into the cytoplasm or nucleus, cells transfected with the fluorescein-labeled plasmid were treated with trypsin-EDTA for up to 30 minutes to disrupt any potential interactions between cell surface proteins on the cell surface and the lipoplex (Figure 5C/D). Hypothetically, if the fluorescent lipoplexes were simply bound to the cell membrane, then this trypsinization would detach them, leading to a significantly lower mean fluorescence and percentage of fluorescein$^+$ cells. Indeed, trypsinization had no significant effect on the percentage of fluorescein$^+$PC-3 cells or their mean fluorescence, suggesting that most of the fluorescent lipoplexes were in the cytosol or nucleus of the PC-3 cells. In contrast, trypsinization significantly decreased the mean fluorescence and the percentage of fluorescein$^+$ Jurkat cells to a level that was comparable to the percentage of EGFP$^+$ cells shown in Figure 5A. A similar, although not statistically significant, decrease in the percentage of fluorescein$^+$ cells was also observed in the primary T cells. However, the mean fluorescence of the primary T cells significantly decreased and was virtually eliminated after 30 minutes of trypsinization (Figure 5D). Altogether, these findings suggest that cellular uptake may be a limiting step for gene delivery in both Jurkat and primary T cells.

To further investigate the localization of the fluorescein-labeled plasmid in the PC-3 and primary T cells, fluorescent microscopy was used to visualize the fluorescent lipoplexes after transfection (Figure 6). In these experiments, cells were also stained with Hoescht 33342 nuclear stain and the Biotium CellBrite red cytoplasmic membrane dye to visualize the nucleus and cytoplasm, respectively. Overall, similar trends in fluorescence were observed across the cell lines. A widespread distribution of bright EGFP fluorescence within the cytoplasm was observed in PC-3 cells transfected with pEF-EGFP (indicating successful transgene expression), while cells transfected with the fluorescein-labeled plasmid exhibited small regions of concentrated fluorescence inside the cytoplasm and nucleus that indicated successful plasmid uptake and nuclear delivery (Figure 6A). In addition, z-stacking with 0.5 μm slices from the top edge to the bottom edge of the PC-3 cells also confirmed that the fluorescent lipoplexes were inside the cell (individual images areshown in Figure 6C, while compiled z-stacks are shown in a video in Figure S5).

In contrast, transfection of CD3$^+$ primary T cells with pEF-EGFP only yielded a small fraction (3.9±0.24%) of EGFP$^+$ cells and the primary T cells that did fluoresce were much dimmer than the PC-3 cells (in agreement with the flow cytometry data shown in Figure 5B). Confocal microscopy z-stacking images also showed that the fluorescent lipoplexes appeared to be localized to the outside of the cell membrane instead of being internalized by the cell (Figures 6C and S6). These observations further support the notion that lipoplexes may successfully bind to primary T cells, but endocytosis of the lipoplexes appears to be limited.

Transcriptome Analysis of Primary T Cells

The experiments with the fluorescein-labeled plasmid (Figures 5-6) seemed to suggest that impaired cellular uptake of the plasmid may be partially responsible for the T cells' resistance to transfection, but the observation that 30-40% of the primary T cells remained fluorescein$^+$ after trypsinization suggested that other intracellular mechanisms may also impair endosomal escape, nuclear delivery, and/or expression of the transgene. In an effort to detect any additional mechanisms that could inhibit transgene delivery or expression and provide a rationale for the relatively low transfection efficiency of primary T cells, mRNA sequencing was used to investigate the transcriptomes of PC-3, Jurkat, and primary T cells in the absence or presence of lipoplexes. These experiments were motivated by previous studies that showed both viral vectors and Lipofectamine can activate innate immune response pathways which trigger the expression of anti-viral genes (TLRs, MyD88, IRFs) that can hinder transduction in some cell types.[54]

The complete mRNA-seq data (fastq and bam files, along with a spreadsheet of all FPKM values) from these experiments are available at the NCBI GEO repository (GEO Accession# GSE151759). Overall, one of the most significant differences that was observed in the gene expression profiles of the PC-3 cells and both types of T cells (Table 1) was the absence of several heparan sulfate proteoglycans (HSPGs), which are also known as syndecans (SDCs). As shown in Table 1, HSPG2 and all the sydecans were expressed in

PC-3 cells, but mostly absent in both Jurkat and primary T cells (with and without lipoplexes). SDC3 was the only syndecan expressed in Jurkat cells, but its role in endocytosis and gene delivery has not yet been established. SDC4 was expressed in primary T cells (as expected for activated T cells), but SDC2 was not detected. Similar results were reported by one study that showed 100-fold lower levels of HSPG expression in Jurkat T cells compared to HeLa cells.[77] Low levels of HSPG expression have also been observed in primary T cells, but T cell activation can upregulate SDC2 and SDC4 expression.[58,78]

This general lack of syndecan expression may explain the significantly lower transfection efficiencies shown in Figure 5A for the Jurkat and primary T cells relative to the PC-3 cells (which express HSPG2 and all the syndecans). Indeed, while HSPGs are best known for their roles in the attachment of adherent cells to the extracellular matrix or tissue culture plates, they are also directly involved in gene delivery, since they regulate endocytosis and their negatively charged sulfate groups are involved in the initial binding of several viruses and positively charged polyplexes or lipoplexes.[79] Indeed, overexpression of SDC1, SDC2, and SDC4 enhances the transfection efficiency of liposomes in K562 cells, although overexpression of SDC2 has also been shown to inhibit PEI-mediated gene delivery.[67,80] Alternatively, blocking the sulfation of SDCs has also been shown to inhibit endocytosis and gene delivery.[57,67]

As previously mentioned, mRNA-sequencing was performed in the different cell lines both in the absence and presence of lipoplexes. The rationale for comparing transfected and untransfected cells was to determine if there were any host cell genes that were upregulated in response to transfection of double-stranded plasmid DNA. Indeed, there are many examples of genes that are induced or upregulated when dsDNA is detected in the cytoplasm and many of these upregulated genes have potent anti-viral functions that are designed to inhibit the replication of viruses.[68,69] Unfortunately, many of these genes can also inhibit non-viral transgene delivery or expression.[70]

Transfection of PC-3 cells led to the induction or upregulation of hundreds of cytokines and cytokine-stimulated genes (CSGs, data not shown), some of which can inhibit transgene delivery (e.g., IFITM 1, 2, and 3) or translation (e.g., IFIT 1 and 2). Nonetheless, the transfection efficiency and mean GFP levels were still high in PC-3 cells, suggesting that many of these upregulated genes may be inconsequential for non-viral pDNA or transgene delivery and expression in PC-3 cells.

In contrast to PC-3 cells, only a few differentially expressed genes were significantly upregulated at least 3-fold in the Jurkat and primary T cells (Table 2). This result is somewhat unanticipated, since the requisite cytosolic DNA sensors (e.g., cGAS and IFI16) and the downstream effectors (e.g., STING, TBK1, and IRFs) that are necessary to detect foreign DNA and induce the expression of cytokines and CSGs were detected in the Jurkat and primary T cells (data not shown). This lack of an innate immune response to dsDNA has been previously reported by other groups, suggesting that T cells may lack an unknown component of the DNA sensing pathways or they somehow repress CSGs.[8] Nonetheless, some metallothioneins were significantly upregulated in the Jurkat (MT1F, MT2A) and primary T cell lines (MT1H) after transfection. Metallothioneins are involved mainly in metal binding, often to zinc, but they have also been implicated in immune regulation and the response to bacterial and viral infections.[60,61] However, the role of metallothioneins in transgene delivery and expression has not yet been determined.

Although T cells did not significantly upregulate cytokines or CSGs in response to transfection, one interesting trend that emerged when comparing the transcriptomes of the T cells to the PC-3 cells was that multiple CSGs that were upregulated in PC-3 cells after transfection were constitutively expressed at relatively high levels in the T cell lines (Table 2). For example, each member of the pyrin and HIN (PYHIN) domain DNA sensor family (IFI16, AIM2, and PYHIN1/IFIX) was detected in both untransfected and transfected primary T cells. In contrast, AIM2 was only expressed in PC-3 cells after transfection and IFI16 was highly upregulated in transfected PC-3 cells, suggesting that these DNA sensors play an important role in the innate immune response to dsDNA in PC-3 cells. This is a particularly intriguing observation, because after AIM2 and IFI16 bind dsDNA in the cytoplasm, they form an inflammasome complex with PYCARD, Caspase 1/8, and Gasdermin D (all of which were expressed at detectable levels in primary T cells, but not Jurkat cells) that can induce inflammation, apoptosis, and pyroptosis.[64]Pyroptosis has also been observed in primary T

cells during abortive HIV infection, in which double-stranded cDNA is generated in the cytoplasm by the virus.[63] Therefore, while the AIM2 and IFI16 inflammasome pathways must be induced (AIM2 & CASP1) or upregulated in PC-3 cells, it appears that primary T cells constitutively express the genes in these pathways, which may lead to higher levels of apoptosis upon transfection with plasmid DNA and the decrease in proliferation shown in Figure 1. Indeed, several other studies have reported that dsDNA is highly toxic in T cells.[71,72]

Conclusions

Overall, this study shows that Lipofectamine can be used to transfect a moderately high percentage of Jurkat cells (51±9.7%) but a much lower fraction (8.1±0.8%) of primary T cells. Nonetheless, this modest transfection efficiency may still be suitable for the generation of CAR-T cells, since some studies using lentiviral vectors have obtained complete responses with transduction efficiencies as low as 2.3% CAR^+ T cells.[81] In either case, our results emphasize that cellular uptake (due to a lack of HSPGs) and an increased risk of apoptosis induced by the PYHIN DNA sensors AIM2 and IFI16 are two particularly important issues that should be considered in the development of both viral and non-viral gene delivery methods for T cells.

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Statement of contribution of all authors: DZ and EH performed the research, JE designed the research study, AB assisted in conducting and analyzing the results of flow cytometry experiments, DZ and EH analyzed the data, JE and EH wrote the paper.

Data Availability Statement

The complete mRNA-seq data (fastq and bam files, along with a spreadsheet of all FPKM values) from these experiments are available at the NCBI GEO repository (GEO Accession# GSE151759).

Conflict of Interest Declaration

The authors have no conflicts of interest to disclose.

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Tables

Table 1 – Expression levels (FPKM) of HSPGs in PC-3, Jurkat, and primary T cells.

	Primary T cells	*Primary T cells*	*Jurkat T cells*	*Jurkat T cells*	*PC-3 cells*	*PC-3 cells*
Gene	Control	Trans.	Control	Trans.	Control	Trans.
HSPG2	0.1	0.1	0.1	0.1	17.7	14.6
SDC1	0.1	0.1	0.1	0.1	74.0	31.0
SDC2	0.0	0.0	0.1	0.0	12.8	6.5
SDC3	0.0	0.0	10.0	11.6	5.4	3.9
SDC4	95.3	103.9	0.0	0.0	30.0	44.7

Table 2 – Expression levels (FPKM) of upregulated host cell genes in PC-3, Jurkat, and primary T cells.

Gene
MT1H
MT1E
MT1F

n.d. = not detected, asterisks (*) indicate significant upregulation vs. untransfected controls, values shown are the average o

Table 3 – Expression levels of PHYIN DNA sensor pathway genes in PC-3, Jurkat, and primary T cells

Gene
IFI16
AIM2
PYHIN1
PYCARD
CASP1
CASP8
GSDMD

n.d. = not detected, asterisks (*) indicate significant upregulation vs. untransfected controls, values shown are the average o

Figure Legends

Figure 1: Comparison of non-viral gene delivery vehicles in Jurkat T cells, with the lead vehicle (Lipo-Hi) represented by black bars. Each vehicle was used to deliver either the luciferase expression plasmid pGL4.50 (A) or the EGFP expression plasmid pEF-GFP (B-F). Panels D-F show representative histograms (cell count vs. cell fluorescence, FL1-H) for Turbofect and 2 concentrations of Lipofectamine. Gates used to calculate %EGFP$^+$ cells are shown as horizontal bars near the top of each panel. Panel C shows the effects of lead vehicles from (A) on cell metabolic activity as measured with MTT as an indication of overall cell viability. Statistically similar cohorts are grouped with letters in (A) and (B), while an asterisk (*) indicates significantly lower values than the control ($p<0.05$) in (C).

Figure 2: Comparison of common promoters (EF1α, CAG, and CMV) in Jurkat T cells. (A) Expression of luciferase (LUC) from each promoter following delivery with either Lipofectamine (Lipo-Hi) or jetPEI (N:P = 5:1). (B) Transfection efficiencies (%EGFP$^+$

Figure 3: Lipofectamine volume (μL) impact on transfection efficiency and viability in Jurkat cells. (A) Transfection efficiency at 24 and 48 hours post-transfection and (B) Annexin V/PI viability staining at 24 hours post-transfection. (C) Comparison of the effects of media type (RPMI-1640 and X-VIVO^{TM}

Figure 4: Lipofection of Primary CD3$^+$ T cells. (A) Transfection efficiency of Jurkats and primary CD3$^+$ T cells at optimized conditions (2.75 μL Lipo, 1 μg pEF-EGFP, X-VIVO media, transfected 24 hours post-passage and 30 min after activation). (B) Transfection efficiency of CD3$^+$T cells at different days post-activation with Dynabeads. (C) Representative FACS histogram denoting untransfected and transfected Jurkat T cells.

Figure 5: Flow cytometry analysis of PC-3, Jurkat, and CD3$^+$ primary T cells transfected with either pEF-GFP or a fluorescein-labeled plasmid. (A) Percentage of fluorescent cells 24 hours transfection with either pEF-GFP or the fluorescein-labeled plasmid (B) Mean fluorescence of transfected cells 24 hours post-transfection. (C) Effects of trypsinization on the percentage of fluorescein$^+$ cells and their (D) mean fluorescence levels. Error bars denote one standard deviation. For (A) and (B), CRD ANOVA and Kruskal-Wallis test used to calculate significance within cell lines. For (C) and (D), significance calculated within cell lines using CRD ANOVA and Tukey post-hoc or Kruskal-Wallis test, across time points.

Figure 6: Confocal microscopy images of PC-3 and CD3$^+$ primary T cells transfected with pEF-EGFP and Fluorescein fluorescent plasmid. (A) Confocal microscopy images of PC-3 cells transfected with pEF-EGFP and Fluorescein at 48 hours post-transfection (B) Confocal microscopy images of CD3$^+$ primary T cells at 24 hours post-transfection. (C) Confocal microscopy images/Z-stacks of PC-3 and Primary T cells transfected with fluorescein-labeled pDNA and then stained with Hoescht 33342 and CellBrite Red.