Electroporation characteristics of human primary T cells

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Abstract

Genetic manipulation of primary human T cells is a valuable technique for basic research in immunology to explore gene function and clinical applications that involve T cells. Among other techniques such as viral-based methods, electroporation is the most feasible material delivery system for manipulating human T cells. In this study, we used electroporation to either induce exogenous gene expression in human primary T cells by plasmids and in vitro transcribed (IVT) mRNA or target endogenous genes by Cas9 ribonucleoproteins (RNPs). We characterized the electroporation conditions both for activated and unstimulated T cells. Although, naive cells are non-dividing and their metabolism is slower compared to activated T cells; we were able to manipulate both naive and memory cells within the unstimulated T cell population by IVT mRNA- and Cas9 RNP-electroporation with more than 95% and 80% efficiency, respectively, and by plasmids with more than 50% efficiency. Here, we outline the best practices for achieving highly-efficient and non-viral genetic manipulation in primary T cells without causing significant cytotoxicity to the cells. Because there is increasing evidence for younger cells to have better anti-tumor activity for immunotherapy, manipulating naïve T cells with high efficiency is of high importance to clinical applications.

Introduction

Adoptive cell transfer (ACT) is an immunotherapy method in which cancer patients' own immune cells (*e.g.* T cells) are infused back to the patient. These cells can be genetically edited to improve their anti-tumor activity. Genetic manipulation of T cells is achieved after activating the cells with CD3/CD28 antibody-coated beads (Dynabeads™). However, activation of the cells also push them towards their differentiation program and the longer the cells are cultured *ex vivo* to achieve a certain number the more exhausted they become. Several studies have shown superior anti-tumor effect of "younger" cells in ACT–*i.e.* naive cells do better than memory cells and central memory (CM) cells do better than effector memory cells (EMs) (Gattinoni et al. 2005; Hinrichs et al. 2011; Hinrichs et al. 2009). Manipulation of T cells in general and naive T cells specifically has been challenging; therefore, activation has been a prerequisite for T cell engineering in clinics. Transfection of T cells through commonly-used transfection reagents has not been possible due to high toxicities associated with the reagents, such as lipofectamine

(Ebert et al. 1997). Another way of delivering materials into cells is by electroporation– *i.e.* opening pores on the cell membrane. Electroporation has been widely used since its first introduction in 1982 (Neumann et al. 1982). In recent years, relatively more efficient electroporation devices have been made commercially available (e.g. Lonza's nucleofector or Thermo Fisher's Neon electroporation devices).

To study gene function or manipulate cells with the expression of exogenous proteins, hard-to-transfect cells can be electroporated with plasmids or mRNAs. mRNA electroporation has been shown to be more gentle on the cells (Zhao et al. 2006; Gerer et al. 2017). Moreover, for protein expression, cytoplasmic delivery of the material is sufficient for mRNA, whereas nuclear delivery is necessary for plasmids. As a result, exogenous protein expression starts faster for mRNA electroporation compared to plasmid delivery–*i.e.* in 30 minutes (Zhao et al. 2006). On the other hand, mRNA electroporation results in transient protein expression whereas plasmids persist relatively longer compared to mRNA (Hardee et al. 2017). Moreover, plasmid electroporation has the potential to lead to higher protein expression, since more mRNA copies can be generated from a single plasmid. However, mRNA electroporation is safer because there is no possibility of genomic integration and the expression dosage can be adjusted through controlling the amount of mRNA delivered (Yin et al. 2014).

CRISPR/Cas9 technology has recently emerged as a powerful tool for gene targeting (Cong et al. 2013; Mali et al. 2013). Cas9-mediated gene targeting can be achieved by delivering the Cas9 enzyme together with the guide RNAs (gRNAs). These molecules can be delivered into the cells in different formats, such as plasmids, RNA, or protein (only Cas9). For T cells, it has been shown that the most efficient gene targeting is possible when the molecules are delivered as Cas9 ribonucleoproteins (Cas9 RNPs) (Schumann et al. 2015). Cas9 RNP method is efficient because the complex is prepared *in vitro* and it is delivered to the cells as a pre-assembled complex. This way of gene targeting is less toxic and it starts more rapidly compared to delivery of both molecules as plasmids or RNA.

Here, we electroporated both activated and unstimulated T cells, which were isolated from healthy human donors, with either plasmids, mRNA, or Cas9 RNPs. We showed that unstimulated cells could efficiently be electroporated and either protein expression (via plasmid or mRNA) or gene knockout (via Cas9 RNPs) could be achieved in these cells at a level comparable to that of activated cells. More importantly, characterization within the subpopulations of unstimulated cells using these methods showed that we achieved efficient genetic manipulation not only in memory cells (CM and EM) but also in naive T cells. Our results suggest that, in theory, genetic manipulation of T cells in clinics could be achieved even without activating the cells and therefore these "younger" cells could potentially do better since they are not differentiated and exhausted before infusing them to the patients.

Results

Electroporation of plasmids into activated and unstimulated T cells

Plasmid electroporation into activated cells

We wanted to explore the genetic manipulation options for T cells using the Neon electroporation device. We first started with plasmids and CD3/CD28 Dynabead-activated cells. On the second day of activation, we debeaded the cells and electroporated them with a GFP plasmid containing the PEST domain and a nuclear localization signal (NLS). The cells were electroporated at a concentration of 7.5 ug DNA per million cells. The next day, frequency of GFP+ cells were detected by flow cytometer. The electro-transfection efficiency was, on average, 50% based on 3 independent experiments with 3 donors (Figure 1a). The viability of the plasmid-electroporated cells were always worse than mock-electroporated counterparts.Normalized against mock-electroporated samples, the average frequency of live cells that were electroporated with plasmids was 65% as determined by the live-cell gate on forward versus side scatter (FSC vs SSC) plot by flow cytometer (Figure 1b).

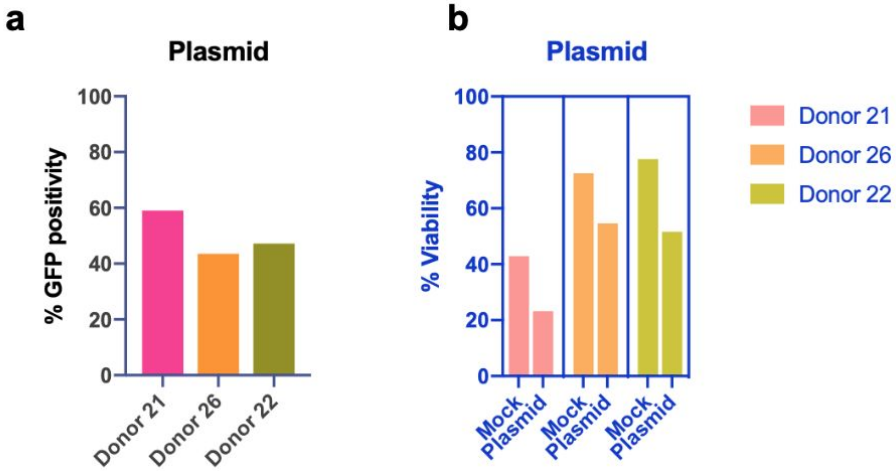

Figure 1: Plasmid electroporation of activated T cells. a) CD3/CD28 Dynabead-activated T cells were electroporated with a GFP plasmid at a concentration of 7.5 ug DNA per million cells. The frequency of GFP-expressing cells were analyzed by flow cytometer 24h after electroporation. The average transfection efficiency was 49.9% **b)** The viability of plasmid-electroporated cells were consistently lower (on average, 39.5%) than the mock electroporated counterparts (on average, 72%).

Plasmid electroporation into unstimulated T cells

We also tried manipulating unstimulated cells by plasmids. When we kept all of the electroporation settings the same as for activated cells (1600 V 10 ms 3 pulses), there was almost no GFP expressing cells the day after electroporation. These results made us question the electroporation efficiency of unstimulated cells. To better understand electroporation efficiency of unstimulated cells, we labeled an empty plasmid with Cyanine-5 (Cy5) and electroporated it into both activated and unstimulated cells obtained from the same donors. The frequency of Cy5+ (plasmid positive) cells was higher than 60% for unstimulated cells (Figure 2a) and 90% for activated cells (Figure 2b) 15 minutes after electroporation. The frequencies of positive cells declined for both groups the next day, but it was still higher than 40% for unstimulated cells (Figure 2a) and almost 80% for activated cells (Figure 2b).

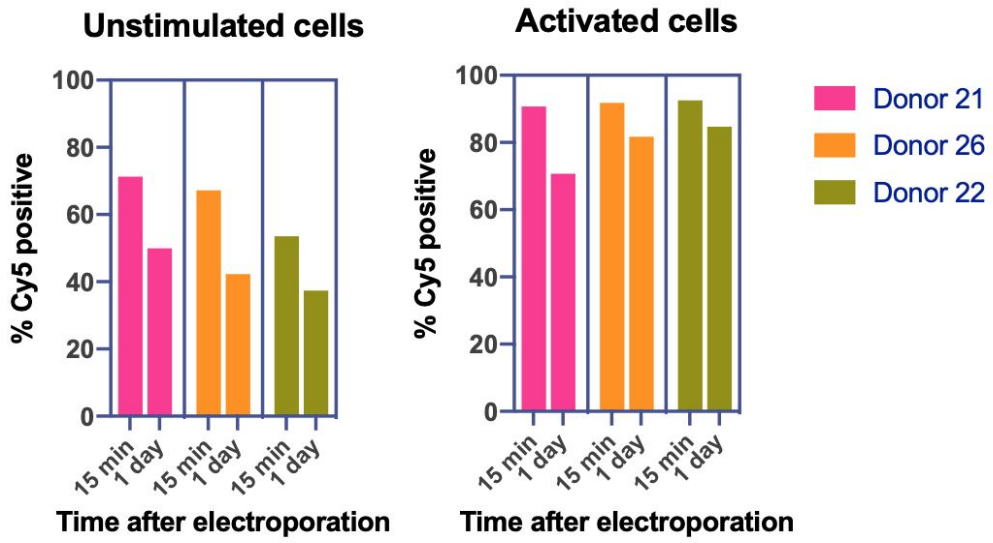

Figure 2: Electroporation of a Cy5-labeled plasmid into activated and unstimulated T cells. Unstimulated (a) or activated cells (b) were electroporated with a Cy5-labeled empty plasmid. Frequency of Cy5+ cells were determined by flow cytometer either 15 minutes or 24 hours after electroporation. Both unstimulated (on average, 64%) and activated (on average, 91.6%) cells had higher frequency of Cy5+ cells on the same day of electroporation, compared to 24 hours after electroporation (43.2% for unstimulated cells and 79% for activated cells).

These results suggested that unstimulated cells were able to take up materials by electroporation but they were not as efficient as activated counterparts for gene expression. We then imaged the cells by fluorescence microscopy and found that 60% of activated cells were positive for nuclear plasmids whereas unstimulated cells were only 20% positive (Figure S1). Unstimulated cells are smaller compared to activated T cells (Iritani et al. 2002). Therefore, their optimal electroporation settings might be different given that smaller cells require higher voltage (Shirley et al. 2014; Gehl 2003). Jay Levy's group electroporated unstimulated CD8+ T cells

with plasmids and achieved 59.6% electro-transfection efficiencies with a viability of 34.6% at 2200 V 20 ms 1 pulse setting using the same electroporation device (Neon, Thermo Fisher) that we used in this study (Liu et al. 2011). When we tried the same settings for electroporating unstimulated cells with our GFP plasmid, we achieved an average of %54.3 electro-transfection efficiency across 3 donors (Figure 3a, orange bars). We also stained the cells for CD45RO and CCR7 surface proteins to estimate the frequency of naive (CCR7+CD45RO-), central memory (CM, CCR7+CD45RO+); effector memory (EM, CCR7-CD45RO+), and effector memory RA (EMRA, CCR7-CD45RO-) subpopulations that were also GFP+ (Sallusto et al. 1999; Mahnke et al. 2013). Our analyses showed that naive cells were mostly GFP positive at this electroporation setting (Figure 3b). The viability of plasmid-electroporated cells were around 55%, compared to mock-electroporated counterparts (Figure 3d). The viabilities of plasmid-electroporated cells at the 1600V setting were better compared to the ones that were electroporated at the 2200V setting (Figure 3c); however, their electro-transfection efficiency was close to zero (Figure 3a, pink bars).

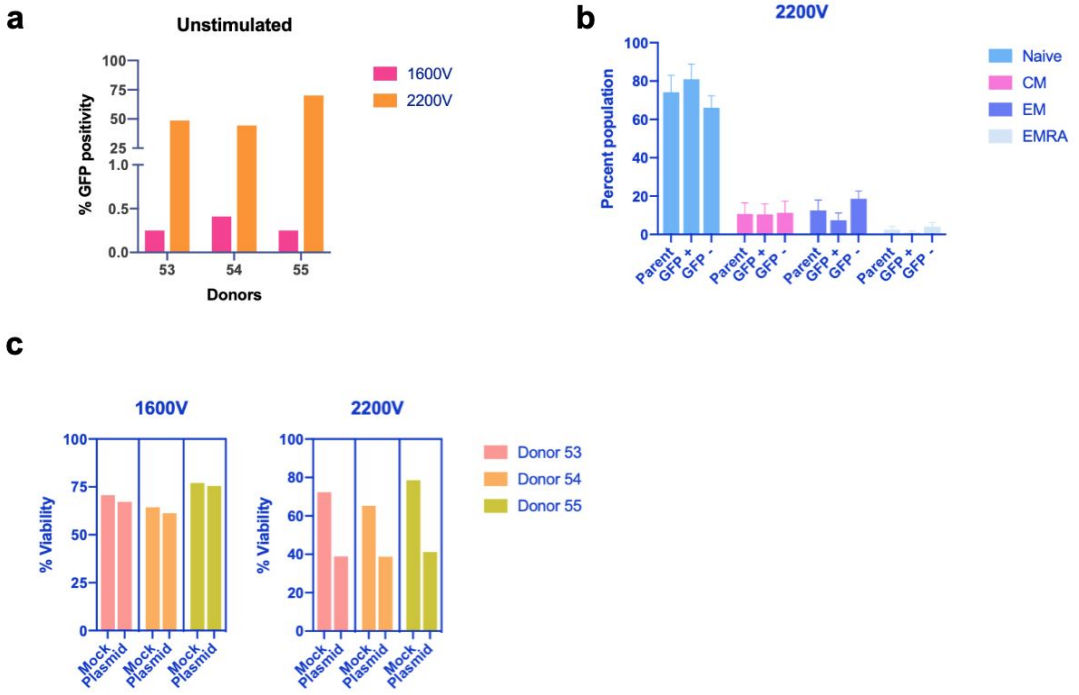

Figure 3: Plasmid electroporation of unstimulated cells at 1600V and 2200V settings. Unstimulated cells from 3 donors were electroporated with a GFP plasmid. Frequency of GFP+ cells were analyzed by flow cytometer 24h after electroporation. **a)** Electroporation at 2200V settings is more efficient than the 1600V settings (on average, 54.3% for 2200V and 0.3% for 1600V). **b)** Subpopulations within the unstimulated cell population were analyzed by staining the cells for CCR7 and CD45RO antibodies. The frequency of GFP+ naive cells were higher than the naive cell frequency in the parent population (80.96% and 74.2%, respectively; n=3) **c)** The viability of the plasmid-electroporated cells was better at the 1600V settings compared to 2200V settings (1600V, mock: 70.6%, plasmid: 68.1%; 2200V mock: 72%, plasmid 39.5%; n=3)

Electroporation of mRNA into activated and unstimulated T cells

mRNA electroporation into activated cells

Due to decreased viabilities upon plasmid electroporation in both activated and unstimulated T cells, we tried to manipulate the cells with *in vitro* transcribed (IVT) GFP mRNA. We used the same plasmid that we used for plasmid electroporation experiments for in vitro transcribing the mRNA. Activated T cells from 3 donors were electroporated with IVT mRNA (6 ug RNA/million cells) on the second day of activation following debeading. Analysis by flow of GFP-positive cells was performed the next day. Using IVT GFP mRNA, we achieved more than 80% GFP+ cells with comparable viabilities to the mock-electroporated counterparts (Figure 2 a and b). These results suggested that mRNA electroporation, compared to plasmids, yields better electro-transfection efficiencies and viabilities for activated T cells.

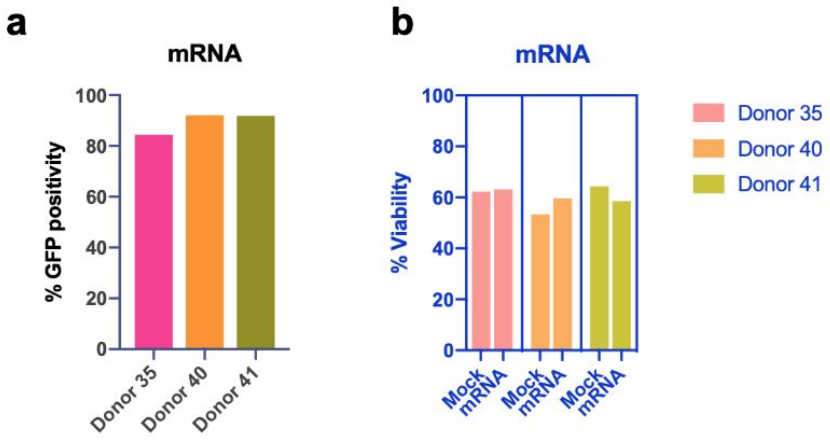

Figure 4 mRNA electroporation of activated T cells. a) Activated T cells were electroporated with IVT GFP mRNA (6 ug RNA/million cells) after debeading the cells on the second day of activation. The frequency of GFP+ cells were analyzed by flow cytometer 24h after electroporation and it was higher than 80%. **b)** Viability of the mRNA electroporated cells were similar to the mock-electroporated ones.

mRNA electroporation into unstimulated cells

Similar to activated cells, manipulating unstimulated cells with plasmids also resulted in decreased viability (Figure 3c, 2200V). mRNA electroporation of activated cells, however, was not as harsh as plasmid electroporation and the frequency of GFP+ cells was also higher. Therefore, we used the same IVT mRNA (8 ug RNA/million cells) to electroporate unstimulated cells. We electroporated the cells at both 1600V and 2200V settings to compare the efficiency at two different settings.

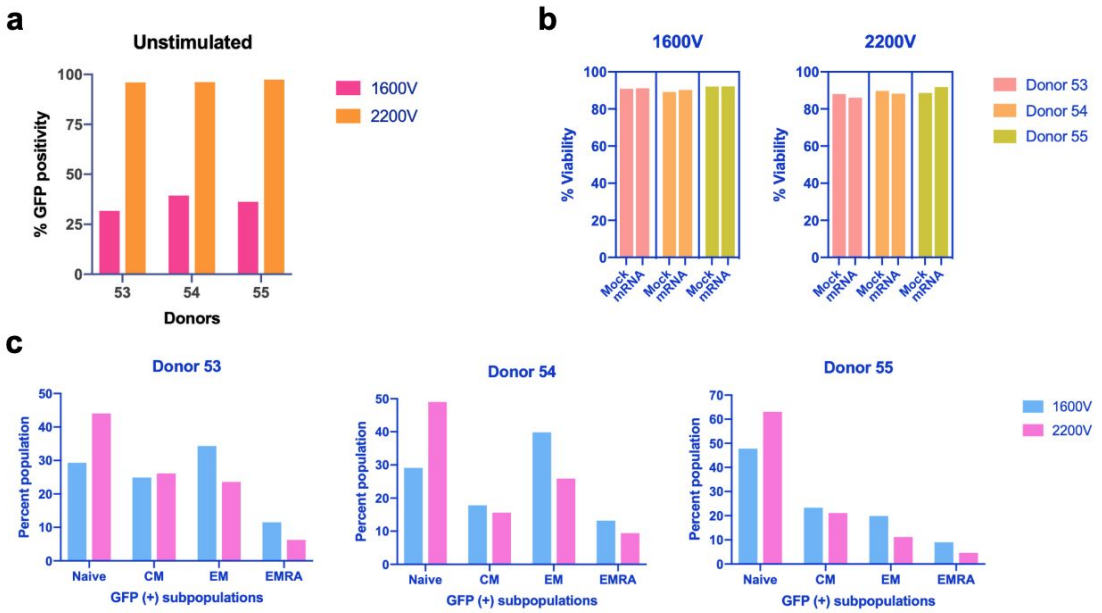

Figure 5: mRNA electroporation of unstimulated cells. Unstimulated T cells were electroporated with IVT GFP mRNA (8 ug RNA/million cells) either at 1600V or 2200V settings. **a)** Electro-transfection efficiency of unstimulated cells was on average 95% with the 2200V settings and 35% with the 1600V settings. **b)** Viabilities of the mRNA-electroporated cells were similar to the mock-electroporated controls at both electroporation settings. **c)** Frequencies of naive, CM, EM, and EMRA subpopulations were analyzed based on their GFP positivity after electroporation at 1600V and 2200V. Naive cells were highly GFP+ at 2200V setting. The bar graphs show the frequencies of GFP+ subpopulations at both settings.

Electroporating unstimulated cells with mRNA at 1600V settings resulted in 35% GFP+ cells and no apparent cell death (Figure 5a pink bars and 5b). However, electroporating the cells at 2200V settings resulted in over 95% GFP+ cells and the cell viability was still comparable to the mock-electroporated controls (Figure 5a orange bars and 5b). When the subpopulations within the unstimulated cells were analyzed for GFP expression, we found that the main difference was the GFP-positivity of the naive population between the two settings (Figure 5c). These results suggested that 2200V settings was more successful for introducing the mRNA into naive cells.

CRISPR in activated and unstimulated T cells

Targeting CD4 and CD25 in activated T cells

To accomplish gene editing via CRISPR/Cas9 system in T cells, similar to other cell types, two components should be delivered into the cell: Cas9 and gRNA. These components can be delivered into target cells with viral-vectors or via electroporation. Cas9 and gRNAs can be electroporated as plasmids, as RNA or as Cas9 RNP complex. Among all of these methods, the

number of studies using Cas9 RNP in T cells has been increasing for the last couple of years given its efficiency and low toxicity compared to plasmids and also given its transient nature (Schumann et al. 2015; Roth et al. 2018; Seki and Rutz 2018).

In this study, we also explored the success of gene editing via Cas9 RNP in both activated and unstimulated T cells. On the second day of CD3/CD28 Dynabead activation, the activated cells from 2 donors were debeaded and electroporated with Cas9 RNPs (7.5 pmol sgRNA and 1250ng Cas9 per 200,000 cells, as recommended by the manufacturer, either against CD25 or CD4. We used one chemically modified synthetic target gene–specific CRISPR RNAs (crRNA) per target. Because both target proteins were cell surface proteins, we were able to check the knockout efficiencies by flow cytometer 3 days after electroporation. For each target, we had 3 replicates from both donors (Figure 6a). We achieved a knockout efficiency of 86% for CD4 and of 84.4% for CD25 (Figure 6b). The cell viabilities were similar to the mock-electroporated samples (Figure 6c).

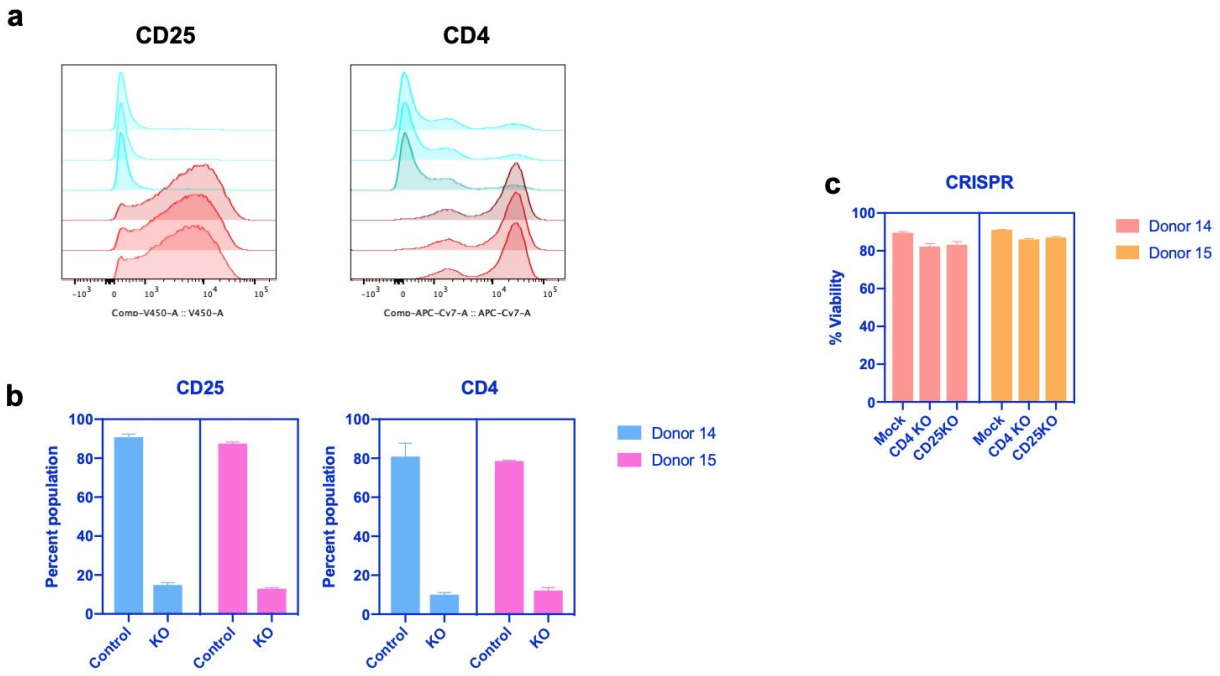

Figure 6: CRISPR in activated T cells Activated T cells were electroporated with Cas9 RNPs against CD25 or CD4 and the protein levels were measured 72h after electroporation. **a)** The histograms show the expression levels of mock-electroporated samples (red) and CD25-Cas9 RNP- or CD4-Cas9 RNP-electroporated samples (cyan). **b)** CD25 frequencies decreased from 89.1% to 13.9% in CD25-Cas9 RNP electroporated samples and CD4 frequencies decreased from 79.75% to 11.08% in CD4-Cas9 RNP electroporated samples. The bar graphs were plotted using the frequencies of CD25 or CD4 positive cells from each treatment group with 3 experimental replicates and the mean is shown with standard deviation (SD). **c)** Viabilities of the Cas9 RNP-electroporated cells were similar to the mock-electroporated controls. KO: samples that were electroporated with the corresponding Cas9 RNPs.

Targeting CXCR4 and CD127 in unstimulated CD4 (+) cells

To our knowledge, there is only one CRISPR study that showed efficient knockout in unstimulated human T cells (Seki and Rutz 2018). In the study, the group used Lonza's Nucleofector to knockout CXCR4, CD127, and CCR7 in human CD4+ T cells by delivering 3 crRNAs per target. They achieved around 90% knockout efficiency with 60% viability, 3 days after electroporation.

To replicate these findings, we used the same crRNA sequences against CXCR4 and CD127. Instead of isolating CD4+ cells directly from fresh PBMCs, we thawed the T cells that we isolated from healthy human blood and then enriched CD4+ cells by depleting CD8+ cells. We then electroporated the unstimulated CD4+ T cells with Cas9 RNPs (3 crRNAs against one gene) using Neon transfection system either at the 1600V or the 2200V setting. The knockout efficiencies were checked by flow cytometer on day 3 and day 6 to account for potentially slow protein turn-over due to the nature of the unstimulated cells. The cells were also stained with CD45RO and CCR7 antibodies to estimate the subpopulation frequencies and the knockout efficiency within each subpopulation.

Using the 1600V settings and CD127 Cas9 RNPs, we did not detect successful knockout events in any of the subpopulations for any of the 4 donors (Figure 7a). However, there was a small decrease in the CD127 protein levels within the CM and EM subpopulations as measured by the mean fluorescence intensity (MFI) using flow cytometer (Figure 7b). When the same Cas9 RNPs were electroporated into the cells at the 2200V setting, all of the subpopulations –including the naive cells– predominantly lost the CD127 protein at the cell surface (Figures 7c and d).

Similar to the CD127 CRISPR experiments, electroporation at the 1600V setting did not result in efficient knockout of the CXCR4 protein (Figure 8a). For CXCR4, the decrease in MFIs within all subpopulations were more prominent than the decrease in CD127 levels at the 1600V setting (Figure 8b). However, electroporating the CXCR4 Cas9 RNPs at the 2200V setting resulted in efficient knockout of the protein within all subpopulations (Figure 8c and d).

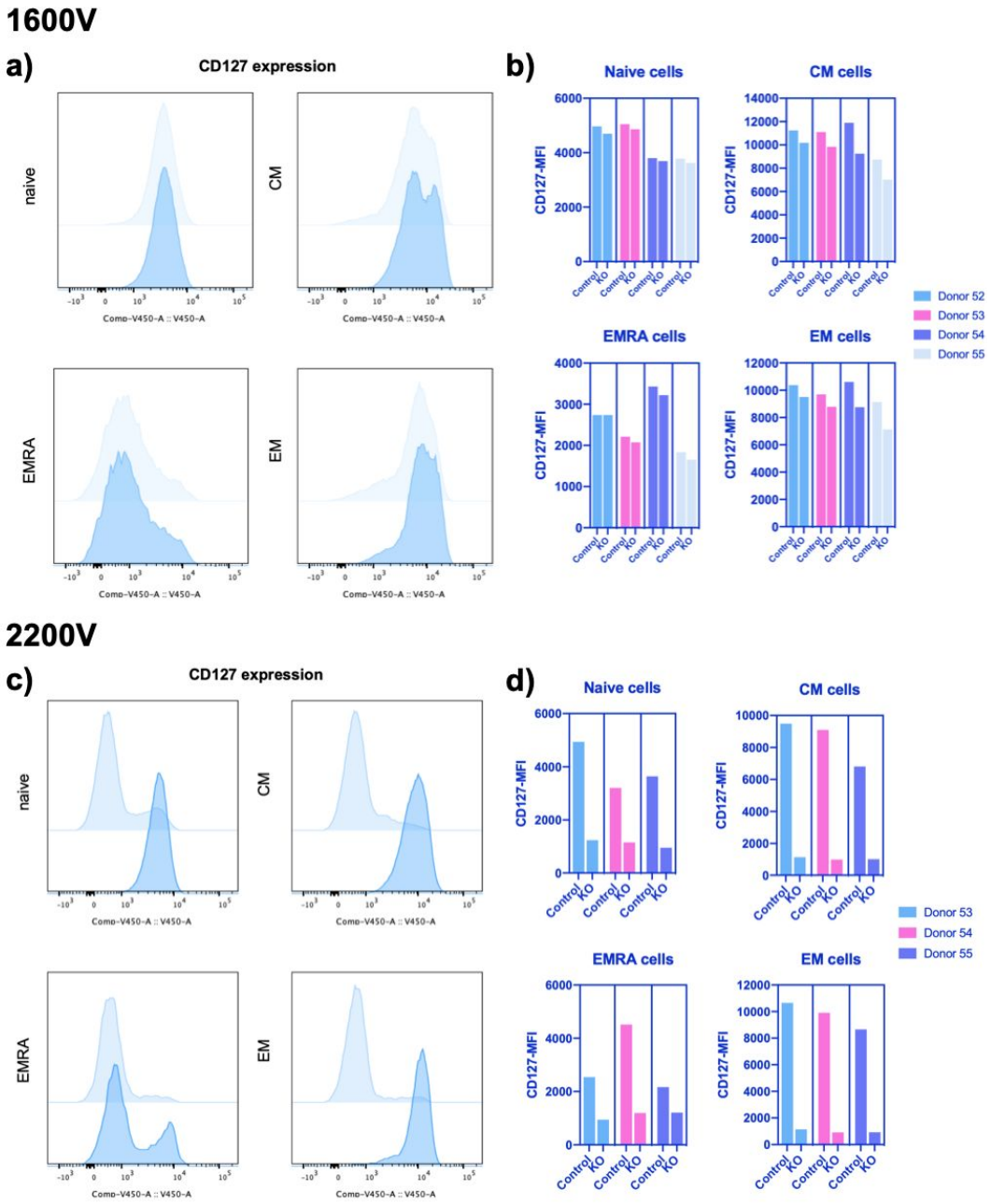

Figure 7: CRISPR in unstimulated T cells: CD127 Unstimulated CD4+ T cells were electroporated with CD127-Cas9 RNPs either at 1600V **(a, b)** or 2200V **(c, d)** settings by Neon electroporation machine. CD127 expression was checked 72h after electroporation. The histograms **(a, c)** show the CD127 expression levels as detected by flow cytometer within each subpopulation from a single donor. Dark blue histograms are for the mock-electroporated sample and the light blue histograms are for the CD127 Cas9 RNP-electroporated sample either at 1600V **(a)** or 2200V **(c)**. **(b and d)** The bar graphs were plotted using the MFI values of CD127 stains within each subpopulation. Control bars show the MFI values of the mock-electroporated samples.

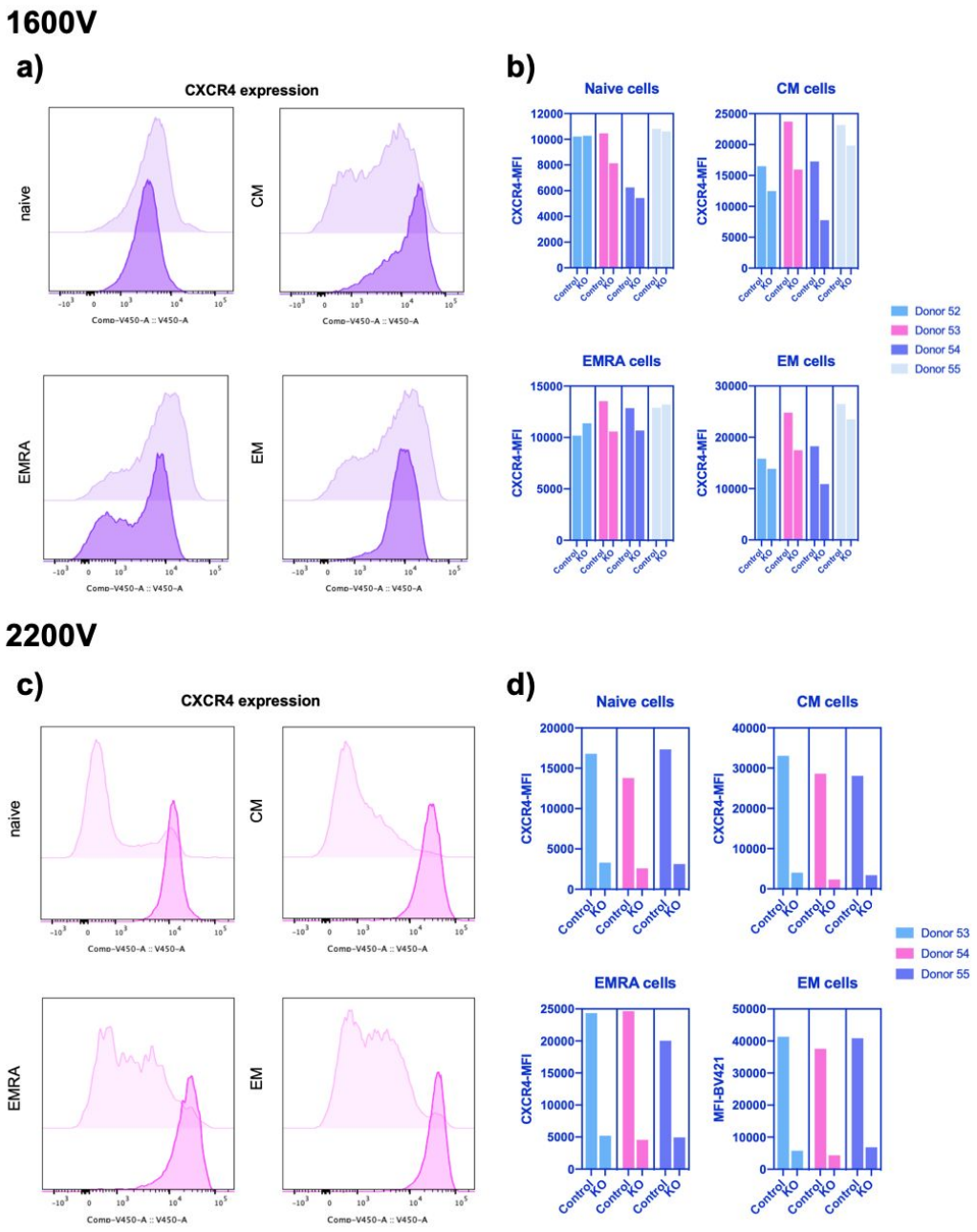

Figure 8 CRISPR in unstimulated T cells: CXCR4 Unstimulated CD4+ T cells were electroporated with CXCR4-Cas9 RNPs either at 1600V **(a, b)** or 2200V **(c, d)** settings by Neon electroporation machine. CXCR4 expression was checked 6 days after electroporation. The histograms **(a, c)** show the CXCR4 expression levels as detected by flow cytometer within each subpopulation from a single donor. Dark-colored histograms are for the mock-electroporated sample and the light-colored histograms are for the CXCR4 Cas9 RNP-electroporated sample either at 1600V **(a)** or 2200V **(c)**. **(b and d)** The bar graphs were plotted using the MFI values of CXCR4 stains within each subpopulation. Control bars show the MFI values of the mock-electroporated samples.

Discussion

Achieving successful genetic manipulation of primary human T cells is of importance to both basic immunology research and clinical applications involving genetically-altered human T cells. The first published study to show plasmid electroporation in unstimulated human T cells achieved 37% efficiency and 32% viability (Bell et al. 2001). In the same study, Bell et al also showed that in 24 hours, frequency of both GFP-expressing cells and viable cells declined compared to 7 hours post-electroporation. An earlier study using phytohemagglutinin (PHA)-activated human T lymphocytes resulted in very low transgene expression (15%) (Van Tendeloo et al. 2000). In 2011, a broad optimization study using Neon electroporation machine showed 59.6% efficiency and 34.6% viability in unstimulated CD8+ T cells (Liu et al. 2011). Later, in 2013, another group showed that CD3- and CD28-activated T cells were vulnerable to plasmid electroporation by nucleofection and because of this, plasmid electroporation in activated cells was not achieved (Chicaybam et al. 2013). The same study showed ~45% electro-transfection efficiency and 25% viability in unstimulated PBMCs. They also showed that when the PBMCs were activated 24 hours after plasmid electroporation, GFP expression frequencies remained higher than 30% for 7 days (Chicaybam et al. 2013). A more recent paper from 2018 showed that plasmid electroporation could yield 40% efficiency in CD3/CD28 Dynabead-activated human T cells, however it also concluded that unstimulated cells could not be efficiently electroporated with plasmids (<5% efficiency) (Zhang et al. 2018).

Studies from the 2000s investigated mRNA electroporation of PBMCs with contradicting results. One paper claimed that both unstimulated and CD3-stimulated T cells could be efficiently electroporated with GFP mRNA (Zhao et al. 2006). An earlier paper concluded that PHA-stimulated T cells could efficiently be electroporated with GFP mRNA, however unstimulated PBMCs could not (Smits et al. 2004). The most recent paper on RNA electroporation of unstimulated CD8+ T cells described a double sequential electroporation method to knock down endogenous TCRs and then insert a tumor-specific TCR mRNA (Campillo-Davo et al. 2018). Another set of papers showed successful gene knockouts by Cas9 RNPs in both unstimulated and activated cells. In 2015, Marson Lab reported successful utilization of Cas9 RNPs for gene editing in activated human T cells (Schumann et al. 2015). However, editing unstimulated cells has remained a challenge for the last couple of years. A paper from 2018 was the first to show efficient knockout in both human and mice unstimulated T cells using Ca9 RNPs (Seki and Rutz 2018). In this 2018 paper, the group optimized the buffers and electroporation settings using Lonza's nucleofector and most importantly showed that combination of 3 sgRNAs increased target gene knockout efficiency compared to a single-gRNA-mediated-targeting.

In this study, we characterized the most efficient and less cytotoxic ways of electroporating unstimulated and CD3/CD28 bead-activated T cells. By using Neon electroporation device at two different electroporation settings, 1600 V 10 ms 3 pulses (1600V) for activated cells and 2200 V 20 ms 1 pulse (2200V) for unstimulated T cells, we achieved high electro-transfection

efficiencies through delivering in vitro transcribed (IVT) mRNA or synthetic Cas9, to both activated and unstimulated cells. Plasmid electroporation yield (50-55%) was relatively low compared to these two methods for both types of T cells.

Our first attempt of plasmid electroporation in unstimulated cells failed when the electroporation was performed at the 1600V settings. Observing almost 0% efficiency upon plasmid electroporation made us question the abilities of unstimulated cells to take up material by electroporation. We then electroporated activated and unstimulated cells with a fluorescently-labeled empty plasmid at the 1600V settings. The flow cytometry results showed that unstimulated cells were, indeed, able to take up the labeled plasmid at a level similar to activated cells (Figure 2). Then, we repeated the experiment and imaged the cells 24 hours after electroporation. Imaging results showed that 60% of the activated cells had plasmids in their nucleus whereas the frequency was only 20% for the unstimulated cells (Figure S1). Since unstimulated cells are, on average, smaller than the activated cells, we wanted to test whether a higher voltage setting would improve the efficiency as others have noted (Shirley et al. 2014; Gehl 2003). Then, we switched to a higher voltage setting (2200V) as was suggested by Jay Levy's group for plasmid electroporation of unstimulated CD8+ T cell using Neon electroporation machine (Liu et al. 2011). By electroporating unstimulated cells at the 2200V setting, we achieved a relatively higher efficiency even within the naive subpopulation. Our plasmid and IVT-mRNA electroporation results suggest that although the naive cells are not proliferating and do not have high gene expression activity, they can efficiently be electro-transfected to a level that is comparable to the activated cells. Similarly, we were able to get relatively high Cas9 RNP-mediated KO in naive cells with the 2200V setting. Using CD4+ unstimulated cells, we were able to knockout CXCR4 and CD127 genes in both naive cells and memory cells with similar efficiencies (Figures 7 and 8).

In summary, electroporation-based transfection of primary cells has been around for decades but its utility as a non-viral alternative to genetic manipulation of human primary T cells has recently been re-evaluated. This is mostly due to the emergence of highly efficient CRISPR/Cas9-mediated gene knockout techniques and their potential for studying basic T cell biology and translational application for T-cell-mediated immunotherapies. Although many other groups have attempted to show the utility of electro-transfection in (mostly activated) human primary T cells, the use of this technique has not been extensively characterized in unstimulated T cells side-by-side with the activated ones. In this study, we systematically profiled the genetic manipulation efficiency of unstimulated and activated T cells through electro-transfection to better evaluate their utility for basic T cell biology and its feasible translation to clinic. . We show that both electroporation of IVT mRNA for transient gene expression and Cas9 RNP for gene knockout are highly efficient not only in the activated but also in unstimulated cells, including naive T cells. We expect to see wide adoption of these techniques in the near future.

Materials and Methods

Human primary T cell culture

PBMCs were isolated from healthy human donors by Ficoll centrifugation (Lymphocyte separation medium; Corning, Corning, NY). T cells were isolated using Dynabeads Untouched Human T Cells Kit using manufacturer's protocols (Thermo Fisher, Waltham, MA). Isolated T cells were kept in T cell media: RPMI with L-glutamine (Corning, Corning, NY), 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 50 uM 2-mercaptoethanol (EMD Millipore), 25 mM HEPES (HyClone, GE Healthcare, Chicago, IL), 1% Penicillin-Streptomycin (Thermo Fisher, Waltham, MA), 1X sodium pyruvate (HyClone, GE Healthcare, Chicago, IL), and 1X non-essential amino acids (HyClone, GE Healthcare, Chicago, IL). T cells were activated for 2 days with anti-CD3/CD28 magnetic dynabeads (Thermo Fisher, Waltham, MA) at a beads to cells concentration of 1:1, with supplement of 200 IU/ml of IL-2 (National Cancer Institute).

Protocol details:

- Culture media: DOI:10.17504/protocols.io.qu5dwy6
- PBMC isolation from buffy coat: DOI:10.17504/protocols.io.qu2dwye

Plasmids

- pcDNA3.3_NDG was a gift from Derrick Rossi (Addgene plasmid # 26820).
- pCMV6-Entry Tagged Cloning Vector was purchased from OriGene (#PS100001).

Plasmid labeling with Label-IT kit

100 ug of pCMV6 plasmid was labeled with 55 ul of Cy5 Label-IT kit for 1 hour at 37°C (Mirus Bio, Madison, WI). The labeled plasmid was purified by ethanol precipitation. In brief, 0.1 volume of 5M sodium chloride and 2.5 volumes of ice cold 100% ethanol was added to the reaction. The solution was mixed and the tube was kept at -20°C for at least 30 minutes. Following the centrifugation and ethanol wash, the DNA pellet was resuspended in 10 mM Tris-Cl buffer (pH 8.5) and the DNA absorbance was read at A260 by NanoDrop One (Thermo Fisher, Waltham, MA) to quantify the eluted DNA.

Staining and imaging of T cells

The cells were collected and centrifuged at 300 x g for 5 minutes. The supernatant was discarded and the cells were washed once with PBS. Then, the cells were resuspended in PBS and 16% formaldehyde (Thermo Fisher #28908, Waltham, MA) was added at a final concentration of 4%. The cells were fixed for 30 minutes at 4°C. After incubation, the cells were pelleted and washed twice with 1X BD Perm/Wash buffer (BD Biosciences #554714, Franklin Lakes, NJ). After the wash, the cells were stained with Alexa Fluor 488 Phalloidin (Thermo Fisher #A12379, Waltham, MA) for 30 minutes at room temperature in dark. After the

incubation, the cells were pelleted and washed with PBS. In the end, the cells were resuspended in PBS and cytospinned on microscope slides by centrifugation for 5 minutes at 500 x g. After the spin, 1 drop of ProLong Glass Antifade Mountant with NucBlue Stain (Thermo Fisher #P36983, Waltham, MA) was added on the slide and the cells were covered with a coverslip. The cells were visualized by Keyence BZ-X710 fluorescence microscope at 60X.

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Image analysis with Cytokit

Image analysis was conducted using Cytokit pipelines configured to segment nuclei over U-Net probability maps (McQuin et al. 2018) followed by secondary (cell boundary) and tertiary (plasmid body) object detection using threshold images resulting from Phalloidin and labeled plasmid channels. All image objects were subjected to morphological and minimum intensity filters before establish nucleus localization frequencies for plasmid objects, and parameters for this filtering were varied in a sensitivity analysis to ensure that findings are robust to processing configuration. Single cell image visualizations were generated using Cytokit Explorer. Raw imaging data sets are publicly available at the following Google Storage URL: `gs://cytokit/datasets/dna-stain`.

In vitro transcription

IVT was performed using the T7 promoter of the pcDNA3.3_NDG plasmid and HiScribe T7 ARCA mRNA kit with tailing (NEB #E2060S, Ipswich, MA). Whole kit was used with 20 ug DNA following manufacturer's protocol. Final RNA product was eluted in 330 ul nuclease-free water.

Electroporation of T cells

After 2 days of activation, the cells were collected and put in a centrifuge tube. The tube was placed on DynaMag (Thermo Fisher, Waltham, MA) and the magnetic beads were removed. Activated and unstimulated cells were centrifuged for 7 minutes at 300 x g, the supernatant was aspirated and the cell pellet was washed once with PBS and then resuspended in electroporation buffer (R for activated cells, T for unstimulated cells) (Thermo Fisher, Waltham, MA). When working with Neon 10 ul tip, 200,000 cells were resuspended in 9 ul of T buffer and 1.5 ug DNA was added. Electroporation was performed at 1600 V 10 ms 3 pulses settings for activated cells and at both 2200 V 20 ms 1 pulse and at the same settings as activated cells for unstimulated cells using Neon electroporation device (Thermo Fisher, Waltham, MA). For DNA electroporation experiments in activated cells, 5 reactions were seeded on a 24-well-plate (a total of 1 million cells) with 0.5 ml T cell media. For DNA electroporations in unstimulated T cells, Neon 100 ul tip was used and 2 million cells were electroporated per reaction and then plated on a 24-well-plate with 1 ml media. For mRNA electroporations, cell pellet needs to be washed thoroughly with PBS. For mRNA electroporation of activated cells, Neon 100 ul tip was used and 1 million cells were electroporated per reaction and then plated on a 24-well-plate with 1 ml media and 200IU/ml IL-2. For mRNA electroporation of unstimulated cells, Neon 100 ul tip was

used and 1-1.5 million cells were electroporated per reaction and then plated on a 24-well-plate with 1 ml media. For the microscope imaging experiment, 3 Neon 100 ul reactions (6 million cells and 45 ug labeled DNA in total) were electroporated and plated on a 12-well-plate with 3 ml T cell media.

Cas9 RNP preparations and electroporation

Cas9 RNPs were prepared immediately before the experiment. For activated cells, only one single crRNA (Thermo Fisher, Waltham, MA) was mixed with tracRNA (Thermo Fisher, Waltham, MA) and incubated at a thermocycler for 5 mins at 95C and 25 mins at 37C. After incubation, the newly formed sgRNA (7.5 pmol sgRNA for 200,000 cells) was mixed with TrueCut v2 Cas9 protein (0.25 ul of Cas9 for 200,000 cells; #A36499,Thermo Fisher) and incubated in the cell culture incubator (at 37C) for 15-20 mins. Then, the cells were added on top of the prepared Cas9 RNPs and immediately were electroporated. For the unstimulated cells, 3 crRNAs were used per target. Individual crRNAs were incubated with equal volumes of tracRNA. After thermocycler incubation of the individual sgRNAs were completed, 3 sgRNAs were mixed together and then Cas9 protein was added. The protocol for Cas9 RNP preparation for unstimulated cells and the crRNA sequences for CXCR4 and CD127 were adapted from Seki and Rutz (Seki and Rutz 2018).

crRNA sequences

Name	Sequences (5’ to 3’)
CD4 (Roth et al. 2018)	GGCAAGGCCACAATGAACCG
CD25 (Broad Institute's GPP Web Portal)	GGATACAGGGCTCTACACAG
CXCR4 (Seki and Rutz 2018)	#1: GAAGCGTGATGACAAAGAGG #2: AGGGAAGCGTGATGACAAAG #3: ACGGCATCAACTGCCCAGAA
CD127 (Seki and Rutz 2018)	#1: TCAGGCACTTTACCTCCACG #2: CAGGCACTTTACCTCCACGA #3: CAAGTCGTTTCTGGAGAAAG

Antibodies

Name	Vendor	Catalog #
CD45RO	Biolegend	304210
CCR7	Biolegend	353212
CXCR4	Biolegend	306518
CD127	Biolegend	351310
CD4	Biolegend	317418
CD25	Biolegend	302627
CD8 (depletion)	Biolegend	344702

Flow Cytometry

Flow cytometric analysis was performed on BD FACSVerse Flow Cytometer. Cells were collected and centrifuged at 300 x g for 5 minutes. The supernatant was aspirated. The cells were resuspended in flow buffer (PBS with %20 FBS) and the labeled-antibodies were added at the recommended concentration. The cells were stained at room temperature for 20-30 minutes at dark. After incubation, the cells were pelleted and resuspended in PBS. Flow cytometry results were analyzed by FlowJo v10 (TreeStar, Ashland, OR, USA). The graphs were generated using GraphPad Prism8 software (GraphPad Software, San Diego, CA, USA).

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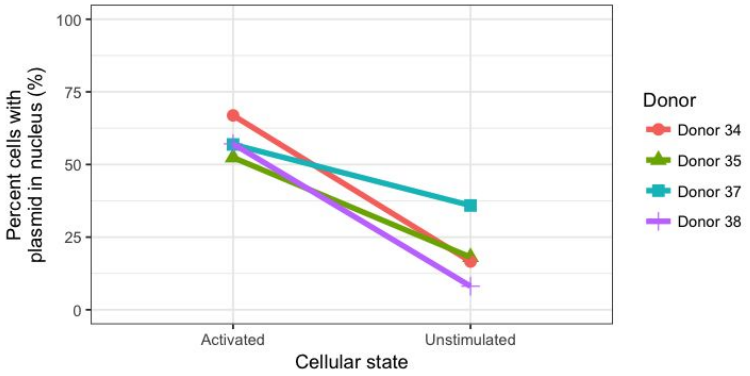
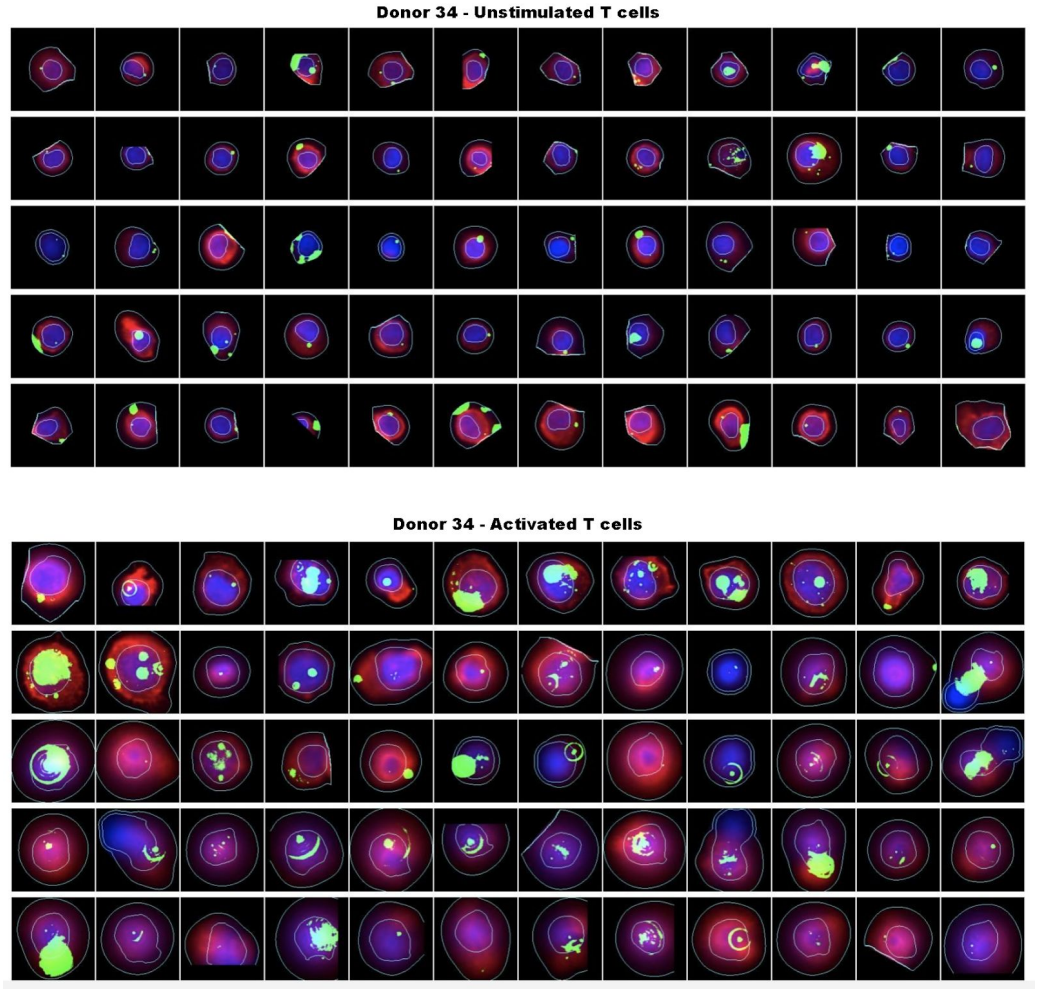
Supplemental Data



(a)



(b)

Figure S1. Imaging of the labeled plasmid electroporated cells 24 hours after electroporation. Cy5-labeled-pCMV6 was electroporated into unstimulated and activated cells on the second day of activation. The cells were incubated for 24h and then they were fixed on slides. (a) The frequency of cells with nuclear plasmid was higher in activated T cells compared to unstimulated T cells. See this notebook for detailed data and analysis. (b) Cytokit was used to analyze the microscope images (Czech et al. 2019). Each sub-panel shows 60 representative individual cells that were plasmid positive. Cell, nucleus, and plasmid signal borders as well as the signal intensities are shown as inferred via Cytokit's detection algorithm (Red: phalloidin, green: Cy5-labeled-plasmid, blue: DAPI). See Table S1 for detailed inferred cellular characteristics.

Table S1. Details of cellular characteristics inferred from fluorescence microscopy images of activated and unstimulated cells via Cytokit.

Experiment	20180911-D35-activated-labeled-60X-11by11	20180911-D35-unstimulated-labeled-60X-11by11	20180921-D34-act-lab-60X-15by15	20180921-D34-us-lab-60X-15by15	20181005-d37-act-lab-60x-19x19-take2	20181005-d37-unstim-lab-60x-19x19-take2	20181016-d38-act-lab-19by19-60x	20181016-d38-unstim-lab-19by19-60x
cells_per_sqmm_overall	1210.11	8709.36	627.975	4269.4	971.647	1233.35	171.665	1456.49
cells_per_sqmm_target	100.971	128.719	17.6165	135.129	17.826	9.68805	0.954452	4.36321
mean_cell_diameter	13.6997	8.25876	15.5385	8.59266	12.2213	8.37917	15.122	10.1386
mean_nucleus_diameter	8.02582	4.85701	9.01983	4.85302	7.20999	5.43356	9.25999	5.35117
mean_nucleus_to_cell_ratio	0.352117	0.367877	0.35295	0.335657	0.363202	0.439678	0.390034	0.299249
median_nucleus_to_cell_ratio	0.349692	0.357499	0.336009	0.321522	0.360909	0.441738	0.412458	0.292977
n_cells	262	334	85	652	138	75	7	32
pct_plasmid_in_nucleus	0.524109	0.180952	0.668675	0.166028	0.569231	0.358696	0.571429	0.081081
plasmid_count_dist	1 - 146 2 - 67 3 - 20 4 - 14 5 - 12 6 - 2 9 - 1	1 - 277 2 - 42 3 - 6 4 - 6 5 - 1 6 - 2	1 - 42 2 - 21 3 - 13 4 - 5 5 - 2 6 - 1 7 - 1	1 - 547 2 - 88 3 - 13 4 - 3 9 - 1	1 - 98 2 - 30 3 - 5 4 - 3 5 - 2	1 - 63 2 - 8 3 - 3 4 - 1	1 - 7	1 - 28 2 - 3 3 - 1