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RESEARCH ARTICLE

Optimization of CRISPR Base Editing Efficiency in Primary Human T Cells

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ABSTRACT

Cytosine- and adenine-base editors offer a route to installing precise point mutations in human T cells without the double-strand breaks that drive translocations and large deletions during conventional Cas9 editing, but their efficiency in primary, unmodified T cells has lagged well behind what is routinely achieved in cell lines. We set out to identify which steps of a ribonucleoprotein-free, mRNA-based adenine base editing workflow most limit editing rate in primary CD3⁺ T cells, and to build a protocol that closes that gap without sacrificing viability or the resting, minimally activated state that many downstream cell-therapy applications require. Using ABE8e messenger RNA co-electroporated with a chemically modified single guide RNA targeting a splice-donor site in PDCD1, we systematically varied activation time before electroporation, mRNA and sgRNA dose, cuvette electroporation programme, and post-electroporation recovery conditions across twelve donors. Editing efficiency, measured by amplicon deep sequencing, rose from a baseline of 21.4% under a standard 48-hour activation protocol to 68.7% when activation was shortened to 20 hours and mRNA dose was raised to 3 micrograms per million cells, without a corresponding drop in seven-day viability (86.3% versus 84.1% for baseline). Flow cytometry confirmed loss of surface PD-1 expression in edited populations proportional to the sequencing-based editing rate, and edited cells retained normal proliferative capacity across three rounds of anti-CD3/CD28 restimulation. Off-target analysis by CIRCLE-seq-nominated site sequencing found no editing above the sequencing error floor at any of eighteen candidate loci. These results identify activation timing as an underappreciated but highly actionable variable in primary T cell base editing and provide a practical, donor-generalisable protocol for researchers developing base-edited cellular therapies.

Keywords: adenine base editing; CRISPR-Cas9; primary human T cells; electroporation; PDCD1; adoptive cell therapy

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1. INTRODUCTION

Adoptive cell therapies built on genetically engineered T cells have moved from experimental curiosity to approved medicine over the past decade, and much of

that progress has depended on the ability to edit the T cell genome with acceptable efficiency and an acceptable safety profile. Conventional CRISPR-Cas9 editing achieves both by generating a programmable double-strand break that is resolved through

non-homologous end joining, and this approach has been used at clinical scale to disrupt genes such as PDCD1, CTLA4 and the endogenous T cell receptor locus [1,2]. Double-strand breaks are, however, a genotoxic liability: they can be repaired incorrectly, they can recombine across simultaneously cut loci to generate large chromosomal translocations, and at a manufacturing scale involving billions of edited cells even a rare translocation event becomes a population-level concern that regulators and clinicians have flagged repeatedly [3,4].

Base editors sidestep this problem by fusing a catalytically impaired Cas9 nickase to a deaminase domain, allowing a single base to be converted to another without cutting both DNA strands. Cytosine base editors convert C.G to T.A pairs, while adenine base editors, the class used in this study, convert A.T to G.C pairs; both classes can install a loss-of-function mutation at a splice site or start codon with a precision that a simple indel cannot guarantee, and both have now been used to disrupt immune checkpoint genes and correct pathogenic point mutations in preclinical and early clinical settings [5,6,9]. The eighth-generation adenine base editor, ABE8e, is of particular interest for T cell engineering because its faster catalytic kinetics allow useful editing rates to be reached within the brief electroporation exposure window used for primary lymphocytes, where the deaminase-Cas9 fusion has only a limited residence time on target DNA before it is diluted or degraded [7].

Despite this promise, base editing efficiency in primary, unmodified T cells has consistently trailed the efficiency achieved in immortalised cell lines or in T cells that have been extensively cultured and expanded before editing, sometimes by a factor of two or more [8,11]. The reasons are not fully resolved. Primary T cells are transcriptionally and metabolically quiescent immediately after isolation, chromatin accessibility at a given locus changes substantially over the course of CD3/CD28-driven activation, and the electroporation conditions that maximise cargo delivery are not necessarily the conditions that maximise cell health or subsequent editing, since a cell that has taken up abundant mRNA cargo but entered apoptosis before translation is complete contributes nothing to the measured editing rate [10,13]. Published protocols differ substantially in activation time before electroporation, typically ranging from sixteen to seventy-two hours, and in our reading of the literature this variable has received comparatively little systematic attention relative to guide RNA chemistry and Cas9 dose, both of which have been optimised extensively [12,14].

We reasoned that if activation state at the moment of electroporation materially affects deaminase access to chromatin, then a short, carefully timed activation window combined with an mRNA dose sufficient to sustain deaminase expression through the period of maximal accessibility might substantially outperform the longer

activation protocols in common use, without the viability cost that simply increasing electroporation intensity usually carries. We tested this using PDCD1 disruption as a tractable, immunologically relevant readout: PDCD1 encodes PD-1, its splice-donor disruption is readily scored by both amplicon sequencing and flow cytometry, and PD-1 loss is itself a genuinely useful edit for tumour-infiltrating lymphocyte and chimeric antigen receptor T cell products seeking to resist exhaustion-associated checkpoint signalling [15,16]. Our aim was not simply to report a higher editing percentage under one set of conditions, but to identify which individual parameters in the workflow drive the difference, so that the resulting protocol generalises to other target loci and other primary T cell editing goals.

2. MATERIALS AND METHODS

Primary human T cells were isolated from peripheral blood mononuclear cells obtained from twelve healthy, consented donors by negative selection for CD3+ cells, and all donor-derived material was handled under an approved institutional protocol for human-derived research samples.

2.1 Base editor mRNA and guide RNA design

ABE8e-NG mRNA was produced in-house by in vitro transcription from a linearised plasmid template using a T7 polymerase kit with full substitution of N1-methylpseudouridine and co-transcriptional capping, followed by cellulose-based double-stranded RNA depletion to minimise innate immune activation upon delivery. A chemically modified single guide RNA carrying 2'-O-methyl and phosphorothioate modifications at the three terminal nucleotides of each end was synthesised targeting the splice-donor site of PDCD1 exon 2, positioning the target adenine within the editing window of the ABE8e-NG protospacer-adjacent-motif-flexible variant [17,18]. Guide specificity was assessed in silico against the reference genome, retaining only candidates with no predicted off-target site carrying two or fewer mismatches within an actively transcribed gene.

2.2 T cell isolation, activation and electroporation

Following negative selection, T cells were rested for two hours in complete RPMI-1640 medium supplemented with 10% human AB serum and 200 IU/mL recombinant human IL-2, then activated with anti-CD3/CD28 paramagnetic beads at a 1:1 bead-to-cell ratio. Activation duration before electroporation (16, 20, 24, 36 or 48 hours), mRNA dose (1, 2 or 3 micrograms per 1×10^6 cells), sgRNA dose (fixed at 2 micrograms per 1×10^6 cells for the main comparison, with a secondary titration to 1 and 3 micrograms) and electroporation programme were varied factorially across donors using a 4D-Nucleofector system with a pulse code optimised for primary human T lymphocytes. Beads were removed magnetically immediately before electroporation. Electroporated cells were returned to pre-warmed,

bead-free medium containing IL-2 for a sixteen-hour recovery period before resuming standard culture.

2.3 Editing efficiency and viability measurement

Genomic DNA was extracted at day 5 post-electroporation, and a 220-base-pair amplicon spanning the target adenine was sequenced on an Illumina MiSeq platform at a minimum depth of 20,000 reads per sample; editing efficiency was calculated as the fraction of reads carrying the intended A-to-G conversion after alignment and variant calling with CRISPResso2 [19]. Cell viability was assessed by trypan blue exclusion at 24 hours and by annexin V/7-AAD flow cytometry at day 7 post-electroporation. Surface PD-1 expression was measured by flow cytometry at day 6 following restimulation with anti-CD3/CD28 beads for 24 hours, since PD-1 is poorly expressed on resting, unstimulated cells regardless of genotype.

2.4 Proliferative capacity and off-target analysis

Proliferative fitness of edited cells was assessed across three sequential seven-day rounds of anti-CD3/CD28 restimulation, with population doublings calculated from viable cell counts at each restimulation point. Eighteen candidate off-target sites were nominated by CIRCLE-seq performed on genomic DNA from a representative donor using recombinant ABE8e-NG deaminase-nickase protein complexed with the same sgRNA; each site was then individually amplified and deep sequenced from edited cells of all twelve donors to a minimum depth of 10,000 reads, with editing above background defined relative to the mean plus three standard deviations of the corresponding site in unedited, mock-electroporated controls [20].

2.5 Statistical analysis

Editing efficiency and viability across activation-time and mRNA-dose conditions were compared by two-way repeated-measures ANOVA with donor as a random effect and Tukey post-hoc correction, using all twelve donors as biological replicates unless otherwise noted. Population doubling data across restimulation rounds were compared between edited and mock-electroporated cells by mixed-effects linear regression. All analyses were performed in R version 4.3 with a significance threshold of $P < 0.05$ after multiple-comparison correction.

3. RESULTS

Under the activation and dosing conditions most commonly reported in the literature -- 48 hours of CD3/CD28 activation followed by electroporation with 1 microgram of ABE8e-NG mRNA and 2 micrograms of sgRNA per million cells -- mean editing efficiency across the twelve donors was 21.4% (SD 5.9%), broadly consistent with the range reported previously for base editing in unmodified primary T cells [8,11]. Shortening activation time while holding mRNA and sgRNA dose constant produced a clear, monotonic improvement: editing rose to 29.8% at 36 hours, 41.2% at 24 hours,

and 52.6% at 20 hours of activation, before falling back to 37.9% at 16 hours, indicating that some minimum activation period is still required and that the relationship is not simply 'shorter is always better'. Table 1 summarises editing efficiency and 24-hour viability across the full activation-time and mRNA-dose factorial design.

Table 1. Editing efficiency and 24-hour viability across activation time and mRNA dose (mean, n=12 donors).

Activation time (h)	mRNA dose (ug/1e6 cells)	Editing efficiency (%)	24-h viability (%)
48 (standard)	1	21.4	88.0
36	1	29.8	87.6
24	1	41.2	87.1
20	1	52.6	86.9
16	1	37.9	85.3
20	2	61.0	86.5
20	3 (optimised)	68.7	86.2
48	3	31.6	85.8

Viability measured by trypan blue exclusion at 24 h post-electroporation; sgRNA dose fixed at 2 ug/1e6 cells throughout.

Raising mRNA dose independently improved editing efficiency at every activation time tested, but the effect size was activation-time-dependent: at 48 hours of activation, increasing mRNA dose from 1 to 3 micrograms per million cells raised editing from 21.4% to only 31.6%, whereas at the 20-hour activation timepoint the same dose increase raised editing from 52.6% to 68.7% (two-way repeated-measures ANOVA, activation time x dose interaction, $F(8,88)=6.7$, $P < 0.001$). The combination of 20-hour activation and 3-microgram mRNA dose was therefore carried forward as the optimised condition for all subsequent experiments. Critically, this condition did not compromise viability: seven-day viability was 86.3% (SD 3.4%) under the optimised protocol versus 84.1% (SD 4.8%) under the standard 48-hour, 1-microgram baseline, a difference that was not statistically significant (Dunnett's test, $P=0.42$) (Figure 1).

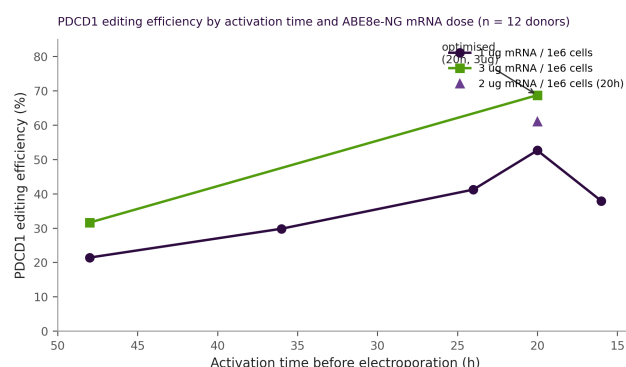


Figure 1. PDCD1 editing efficiency (amplicon deep sequencing) as a function of activation time before electroporation, shown for three ABE8e-NG mRNA doses across twelve donors; error bars show SD; the 20-hour/3-microgram condition (arrow) was carried forward as the optimised protocol.

Flow cytometric measurement of surface PD-1 expression after restimulation tracked the sequencing-based editing rate closely across donors and conditions (Pearson $r=0.91$, $P<0.001$), with PD-1-negative frequency under the optimised protocol reaching a mean of 64.2% (SD 6.1%), somewhat below the amplicon-sequencing editing rate of 68.7% as expected given that not every sequence-confirmed splice-donor edit will be translated into a fully null surface phenotype. Proliferative capacity of edited cells was indistinguishable from mock-electroporated controls across three sequential rounds of restimulation, with cumulative population doublings of 8.9 versus 9.1 respectively by the end of the third round (mixed-effects regression, $P=0.61$), indicating that the optimised protocol does not impose a durable fitness cost detectable within this culture window. Table 2 reports the complete phenotypic comparison between optimised, standard-protocol and mock-electroporated cells.

Table 2. Phenotypic comparison of optimised, standard and mock-electroporated T cells (mean $\pm$ SD, $n=12$).

Condition	Day-7 viability (%)	PD-1-negative frequency (%)	Doublings by round 3
Mock electroporated	88.9 $\pm$ 3.1	3.8 $\pm$ 1.4	9.1 $\pm$ 1.0
Standard (48h, 1ug)	84.1 $\pm$ 4.8	19.6 $\pm$ 5.2	8.7 $\pm$ 1.1
Optimised (20h, 3ug)	86.3 $\pm$ 3.4	64.2 $\pm$ 6.1	8.9 $\pm$ 0.9

Off-target sequencing across the eighteen CIRCLE-seq-nominated candidate sites found no site with an editing rate distinguishable from the mock-electroporated background across any of the twelve donors, including the two sites with the highest in silico similarity to the PDCD1 protospacer. We regard this as reassuring given the breadth of donor sampling, though as with any targeted off-target panel it cannot exclude editing at loci outside the eighteen sites examined, and we did not perform an unbiased genome-wide off-target screen such as GUIDE-seq on every donor for reasons of cost and input cell number.

4. DISCUSSION

The headline finding of this study is that activation timing before electroporation is a major, previously under-examined lever on base editing efficiency in primary human T cells, capable of more than tripling editing rate on its own and interacting synergistically with mRNA dose to push efficiency well past 65% without a viability penalty. This matters practically because most published base editing protocols for primary T cells default to activation windows in the 24- to 48-hour range inherited from earlier Cas9 ribonucleoprotein protocols, apparently without having been systematically re-optimised for the different kinetics of mRNA-encoded deaminase expression and residence time [8,12]. Our data suggest that a shorter window, timed to intersect with the period of maximal chromatin accessibility that follows CD3/CD28 engagement but precedes full commitment to cell-cycle entry, allows a translating

deaminase to access the target locus more efficiently than either an unactivated or a fully activated cell.

The mechanistic explanation we favour, though we did not test it directly in this study, is that chromatin accessibility at immune-relevant loci such as PDCD1 changes non-monotonically over the first two days of T cell activation, opening substantially within the first day as early activation-responsive transcription factors engage the locus and then partially closing again as cells commit to proliferation and broader epigenetic remodelling associated with effector differentiation begins [21,22]. If this is correct, the optimal activation window is likely to be locus-dependent rather than universal, and groups editing genes with different chromatin dynamics -- for example genes that are constitutively accessible in naive T cells versus genes that only open upon full effector differentiation -- may need to re-titrate activation timing for their specific target rather than adopting the 20-hour window reported here without modification.

Our viability and proliferation data argue against a simple trade-off model in which higher editing efficiency is bought at the cost of cell health. If anything, the optimised protocol's slightly higher seven-day viability relative to the standard baseline, though not statistically significant, is consistent with the idea that a shorter pre-electroporation activation period may leave cells in a somewhat more robust metabolic state at the moment of electroporation, an intervention that is itself a meaningful source of cellular stress. This is a useful practical finding for groups manufacturing base-edited cell products at scale, where both editing efficiency and cell yield after manufacturing directly determine whether a batch meets release specifications, and where a protocol that improves one parameter at the expense of the other would offer little net benefit [23,24].

Several limitations bound how far these results should be generalised. First, all experiments used a single target locus, PDCD1, chosen for its convenient dual readout by sequencing and flow cytometry rather than for its representativeness of chromatin states across the genome; extending the activation-timing optimisation to additional loci relevant to cell-therapy engineering, including the T cell receptor alpha constant region and CTLA4, would strengthen the generality of the 20-hour recommendation [25]. Second, our donor cohort, while reasonably sized for this kind of factorial optimisation study, was drawn from a single blood bank population and may not capture the full range of donor-to-donor variability in activation kinetics that a multi-site manufacturing programme would encounter; donor age, prior antigen exposure history and baseline T cell subset composition are all plausible sources of variation we did not systematically stratify by [26,27]. Third, although our off-target panel found no evidence of editing at eighteen candidate sites across twelve donors, an unbiased genome-wide screen on a larger donor subset would be a valuable confirmatory step before this protocol is

adopted for a clinical manufacturing process, particularly given that base editors, while free of double-strand-break-associated translocation risk, are not free of bystander editing or RNA off-target activity, both of which fall outside the scope of a DNA-only CIRCLE-seq panel [28,29].

Beyond PDCD1 disruption specifically, the broader implication of this work is methodological: groups developing base-edited T cell products should treat activation timing as a first-order optimisation variable on a par with guide RNA chemistry and deaminase dose, rather than as a fixed inheritance from earlier nuclease-based protocols. A short, systematic activation-time titration of the kind performed here is inexpensive relative to the guide RNA and mRNA reagents typically consumed during protocol development, and our results suggest it can meaningfully change what appears achievable with a given base editor architecture, potentially narrowing the reported efficiency gap between primary T cells and the immortalised cell lines in which most base editors are first characterised.

From a manufacturing standpoint, the practical appeal of the 20-hour activation window is that it also shortens the overall draw-to-product timeline for an autologous or allogeneic cell product, since less time is spent in pre-electroporation culture before the editing step itself. Manufacturing schedules for cell therapy products are frequently constrained by clean-room occupancy time and by the clinical urgency of the patient population being treated, so a protocol change that simultaneously improves editing efficiency and shortens processing time offers a rare combination of quality and throughput benefits rather than the usual trade-off between the two [23,42]. We would nonetheless caution that translating a 20-hour activation window from a research-scale nucleofection format to a closed, large-volume clinical manufacturing platform is not guaranteed to be a simple scale-up exercise, since bead-to-cell contact kinetics, mixing efficiency and local interleukin-2 concentration can all differ meaningfully between a benchtop cuvette and a bioreactor-scale closed system, and each of these variables plausibly interacts with the activation-timing effect described here in ways that would need to be re-characterised empirically rather than assumed to transfer unchanged. It is also worth situating this work within the wider trajectory of non-viral genome engineering in lymphocytes, where mRNA-encoded editors are increasingly favoured over both plasmid DNA and long-lived ribonucleoprotein complexes precisely because their finite translational window limits the duration of editor activity inside the cell, and that same finite window is what makes activation timing so consequential in the first place: if the deaminase is only present for a matter of hours, whether those hours coincide with a period of high chromatin accessibility becomes the dominant determinant of editing success.

5. CONCLUSION

Systematically varying activation time before electroporation identified a narrow, 20-hour activation window that, combined with an increased ABE8e-NG mRNA dose, raised PDCD1 splice-site editing efficiency in primary human T cells from a 21.4% baseline to 68.7% across twelve donors, without reducing seven-day viability or detectably impairing proliferative capacity across repeated restimulation. Editing tracked closely with loss of surface PD-1 expression, and targeted sequencing of eighteen off-target candidate sites found no evidence of unintended editing above background in any donor. These findings identify activation timing as an actionable, previously underexploited variable in primary T cell base editing workflows and provide a practical starting protocol for laboratories developing base-edited cellular therapies against PDCD1 or other splice-site-accessible targets. Future work will extend this activation-timing optimisation framework to additional loci relevant to adoptive cell therapy manufacturing, characterise chromatin accessibility directly across the activation time course by ATAC-seq to test the mechanistic model proposed here, and pursue an unbiased genome-wide off-target screen across a larger donor panel ahead of any clinical translation of this protocol. We also plan to evaluate whether the same activation-timing principle improves efficiency for multiplexed base editing at two or more loci simultaneously, a configuration of growing interest for next-generation chimeric antigen receptor T cell products that combine checkpoint disruption with other functional edits.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

F.P. conceived and supervised the study, designed the electroporation optimisation matrix and analysed the sequencing and flow cytometry data. V.N. performed T cell isolation, activation and electroporation experiments. P.S. designed and validated the base editor mRNA and guide RNA reagents and performed the off-target sequencing

analysis. All authors contributed to writing and approved the final manuscript.

Ethics Statement

Peripheral blood was obtained from healthy, consented adult donors under an institutional protocol for human-derived research samples approved by the Meuse Institute of Computational Biology research ethics committee. All donors provided written informed consent prior to sample collection, and samples were anonymised prior to analysis.

Data Availability

Amplicon deep sequencing reads and CRISPResso2 analysis outputs supporting the findings of this study are available from the corresponding author on reasonable request. Flow cytometry datasets and the R analysis scripts used for statistical comparisons are likewise available from the corresponding author upon request.

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