Durable Multiplex Epigenetic Editing for Generation of Allogeneic CAR T Without Chromosomal Rearrangements

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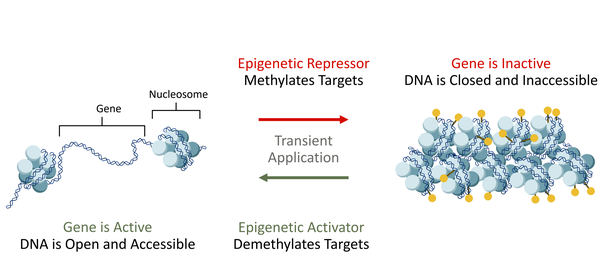
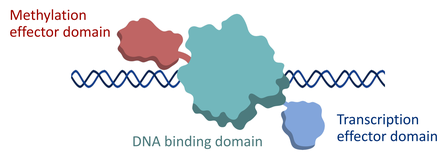
Epigenetic editing leverages the cell's endogenous system to precisely control gene expression

Transient application of our epigenetic editors causes a durable change in phenotype without a change in genotype



Chroma's epigenetic editor



DNA binding domain precisely localizes effector domains to target sequence

Transcription effector domain transiently represses target gene

Methylation effector domain durably silences target gene

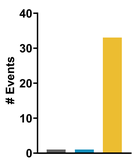
Multiplex epigenetic editing without translocations

Epigenetic gene regulation has the potential to be efficient, specific, and durable without any cuts, nicks, or changes to the underlying DNA

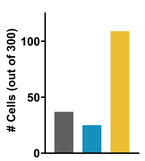
1. **Has the potential to enable multiplexing without genotoxic risk**
   - Simultaneous silencing of several targets without introducing DNA damage
2. **Streamlines manufacturing**
   - Accomplish a high number of multiplex edits in a single step
   - Reduce requirement for in-depth characterization of edited T cells for translocations and chromosomal rearrangements

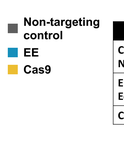
Multiplexing with epigenetic editors preserves genomic integrity

Translocation Events Detected in Targeted FISH Assay



Number of Cells With Genomic Rearrangement Events





Translocation frequency in sequencing and FISH assays

	Sequencing	FISH
Cas9 Nuclease	7%	9%
Epigenetic Editor	<0.1%	<0.3%
Controls	<0.1%	<0.3%

Gross chromosomal abnormalities evident in Cas9 nuclease-treated cells

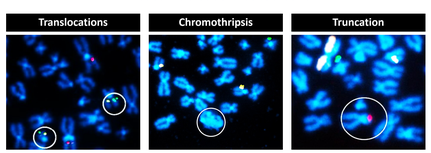


Figure 1. Multiplexing with epigenetic editors does not induce translocations or genomic rearrangement events[1]. Primary T cells were edited at three target genes in multiplex with gRNA and epigenetic editor or Cas9 nuclease, or with a non-targeting control gRNA that does not correspond to any target site in the human genome. Edited cells were sorted to enrich for lack of target expression and were analyzed at day 3 post-editing using both a single cell fluorescent in situ hybridization assay (KromaTiD InSite), in which images from 300 metaphase cells were analyzed, and a sequencing assay (UDiTaS). Genomic rearrangement events quantified include translocations, centromere abnormalities, chromothripsis, loss, gain, sister chromatid exchanges/inversions and truncations. EE = epigenetic editor; FISH = fluorescent in situ hybridization.

Durable epigenetic editing for allogeneic CAR T

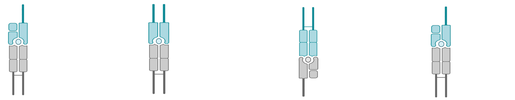
Our technology enables multiplexed allogeneic edits to eliminate GvHD and resist CD8+ T, CD4+ T, and NK cell responses

B2M — CD8+ T resistance
- Reduce MHC class I surface expression
- Reduce CD8+ T cell alloresponse

RFXAP — CD4+ T resistance
- Reduce MHC class II expression
- Reduce CD4+ T cell alloresponse

CD3D, CD3G — Eliminate GvHD
- Reduce expression of TCR
- Reduce risk of ex vivo T cell GvHD

HLA-E — NK resistance
- NK inhibitory ligand
- Reduce NK cell response to MHC class I-negative cells



Durable silencing of MHC class I, MHC class II, and TCR as single targets and in multiplex

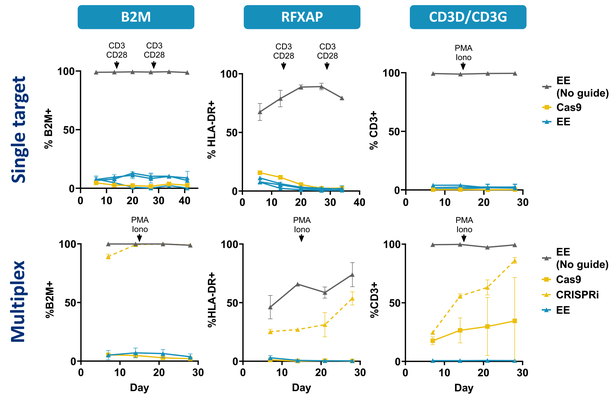


Figure 2. Durable single target[3] and multiplex silencing achieved for three allogeneic targets in primary T cells. T cells were nucleofected with mRNA (Cas9, CRISPRi, or EE) and gRNA targeting one or all allogeneic targets. Cells were then cultured for several weeks in cytokine-supplemented media and stained for cell surface expression of B2M, HLA-DR, and CD3 at the indicated timepoints post-nucleofection. T cells were restimulated at day 14 and/or day 28 post-nucleofection with either PMA/ionomycin or a CD3/CD28 antibody cocktail to increase T cell proliferation and pressure-test durability of silencing. Data presented as mean +/-SD, N=2-3 biological replicates from 1 donor. EE = epigenetic editor.

Epigenetically edited silenced T cells do not exhibit GvHD response and are resistant to T cell alloresponses

T cells silenced at CD3D/CD3G do not exhibit a GvHD response

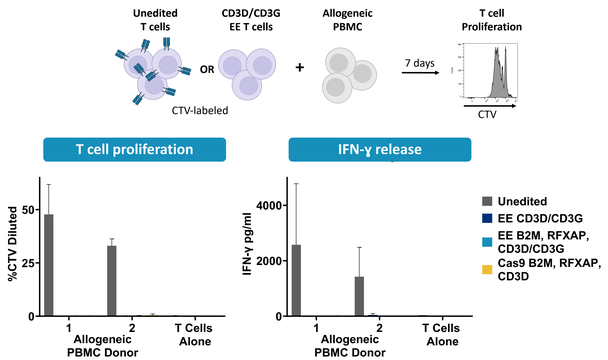


Figure 3. CD3D/CD3G-silenced T cells do not exhibit an in vitro GvHD response. Edited T cells were cocultured with allogeneic PBMC at a 1:1 ratio. Cell supernatant was harvested at 5 days for IFN-γ quantification by MSD and flow cytometry analysis for proliferation by CTV dye dilution was conducted after 7 days of coculture. Data presented as mean +SD, N= 2 biological replicates from 2 donors. EE = epigenetic editor.; GvHD = graft-versus-host disease. Schematic created with BioRender.com.

T cells silenced at B2M and RFXAP evade T cell alloresponses

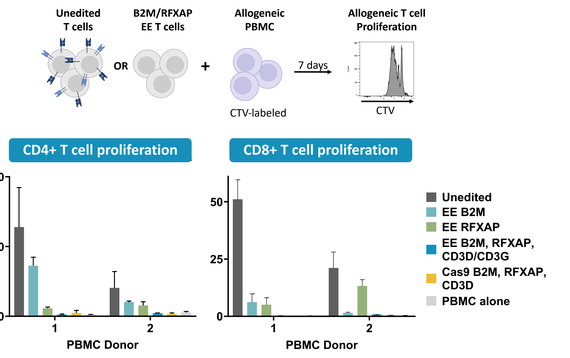
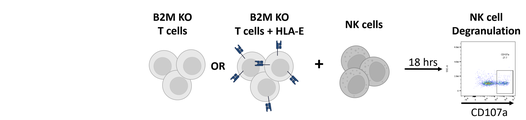


Figure 4. Reduced allogeneic response in vitro to epigenetically-edited T cells. Mitomycin C-treated T cells were cocultured with allogeneic PBMC at a 1:1 ratio. Flow cytometry analysis for proliferation by CTV dye dilution was conducted after 7 days of coculture. Data presented as mean +SD, N= 2 technical replicates from 1 donor, representative of results obtained with cells from 2 donors. EE = epigenetic editor; GvHD = graft-versus-host disease. Schematic created with BioRender.com.

HLA-E variant does not activate NKG2C+ NK cells and protects MHC class I-deficient cells from NK lysis



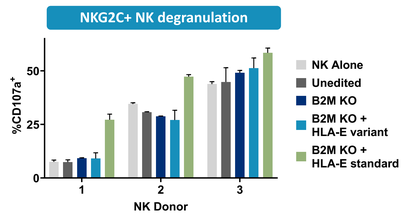


Figure 5. HLA-E variant does not elicit degranulation response by NKG2C+ NKG2A- NK cells. T cells (B2M KO with and without overexpression of an engineered HLA-E) and NK cells were cocultured overnight followed by flow cytometry analysis for NK cell CD107a expression. HLA-E standard.[2] Data presented as mean +/- SD, N= 2 technical replicates from 1 donor, representative of results obtained with T cells from 2 donors. KO = knock out; NK = natural killer. Schematic created with BioRender.com.

Multiplex epigenetically-edited CAR T have comparable cytotoxic function to unedited CAR T

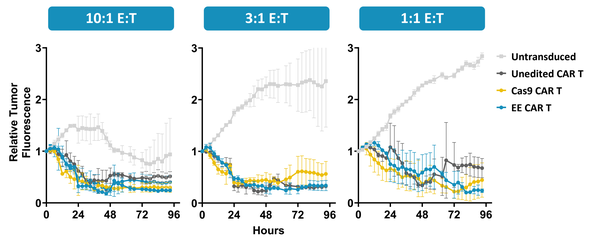
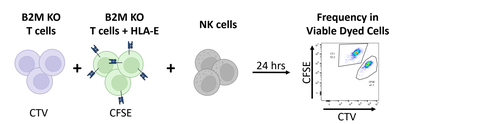


Figure 7. Multiplex epigenetically-edited CAR T kill tumor cells as effectively as unedited CAR T and triple Cas9 knock out CAR T for the same three targets. BCMA CAR T cells were multiplex edited by Cas9 or EE at B2M, RFXAP, and CD3D/CD3G loci. CAR T and MM.1S tumor cells were cocultured at a range of E:T ratios and tumor killing monitored in an Incucyte assay. Data presented as mean +/- SD, N= 2 technical replicates from 1 donor, representative of results obtained with cells from 3 donors. EE = epigenetic editor; E:T = effector:target.



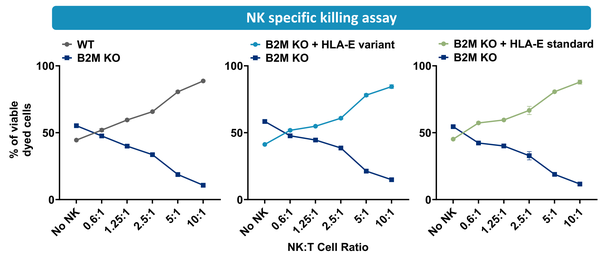


Figure 6. HLA-E variant protects MHC class I-deficient T cells from NK cell lysis. A 50:50 mix of two different T cell populations as indicated, one CTV-labeled, the other CFSE-labeled, were cocultured with NK cells at varying NK:T cell ratios. After 24 hours, flow cytometry analysis was conducted to assess the frequency of CTV+ and CFSE+ cells among total dyed viable cells to measure relative survival of the two T cell populations. Data presented as mean +/- SD, N= 2 technical replicates from 1 donor, representative of results obtained with two donors. KO = knock out; NK = natural killer. Schematic created with BioRender.com.

Summary

These data support multiplex epigenetic editing to produce allogeneic CAR T that:

- ✓ **Do not exhibit a GvHD response**
- ✓ **Resist CD8+ and CD4+ T cell alloresponses**
- ✓ **Resist the NK cell missing-self response to reduced MHC class I expression**

Acknowledgements

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References

1. Abubucker S, Collins M, Hildebrand E, et al. Multiplexed editing without chromosomal rearrangements using epigenetic editors, ASGCT 2023.
2. Gornalusse GG, Hirata RK, Funk SE, et al. HLA-E-expressing pluripotent stem cells escape allogeneic responses and lysis by NK cells. *Nat Biotechnol*. 2017;35(8):765-772. doi:10.1038/nbt.3860.
3. Schafer J, Durable epigenetic editing for generation of multiplex-edited T cells without chromosomal rearrangements, ASGCT IO 2023.