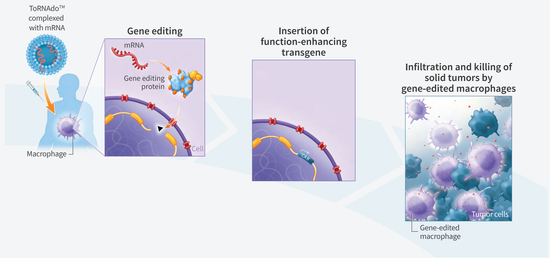
mRNA Cell Engineering Enables Rapid Prototyping of Macrophage Gene-Editing Strategies for Cancer Immunotherapy Applications

Ian Hay[1], Abigail Blatchford[1], Christopher B. Rohde[1], Matthew Angel[1]
[1]Factor Bioscience Inc., Cambridge MA

Thank you to Taeyun Kim, Claire Abel and Mackenzie Parmenter for their support.
This work is protected by one or more pending patent applications.

Summary

Macrophages are attractive candidates for cell therapies in oncology and infectious disease due to their innate functionality in many tissues and infiltrative capacity. However, much of the focus for macrophage cellular engineering has thus far used chimeric antigen receptors (CARs) designed for T-cells, which are fundamentally different immune cells. We aimed to create a rapid prototyping procedure to assess novel CAR designs directly in macrophages. We developed a protocol to differentiate iPSCs to macrophages to generate a large, homogenous cell population. These cells are then transfected with mRNA encoding an ROR1-targeting CAR, displaying high expression and quantifiable functionality.



Conclusions

mRNA transfected into iPSC- and PBMC-derived macrophages demonstrates fast and efficient expression, and 5moU mRNA shows more consistent and brighter expression in macrophages than mRNA synthesized with canonical nucleotides. mRNA encoding CARs enables rapid evaluation of CAR design, including antigen binding domains, for functional optimization. ROR1-CAR macrophages demonstrated increased functionality following activation with ROR1, displaying quantifiable CD3ζ phosphorylation, Zymosan bead phagocytosis, and ROR1-expressing cancer cell cytotoxicity. mRNA gene editing enables efficient bi-allelic insertion of optimized target transgene.

1 iPSC-Macrophage Differentiation

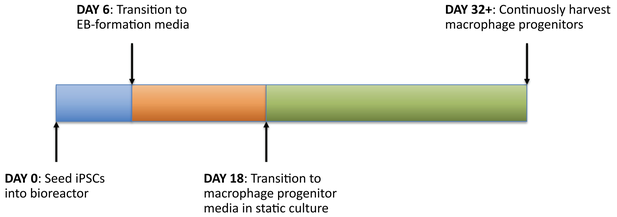


Figure 1. **Efficient differentiation of iPSCs into macrophages.** iPSCs were differentiated into macrophages using a 3-stage process. iPSCs are initially recovered from thaw in a PBS-Mini 0.1L bioreactor with mTeSR media, then form embryoid bodies (EBs) using EB formation media from StemCell Technologies. The EBs are transferred to a static culture plate for macrophage progenitor formation in X-VIVO 15 with 25 ng/mL IL-3 and 100 ng/mL M-CSF.

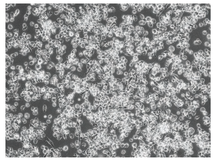


Figure 2. **iPSC-Macrophages display macrophage-like morphology following differentiation.** Following the 3-stage differentiation over the course of 32 days, the harvested macrophages were plated into IMDM media with 50 ng/mL M-CSF to terminally differentiate into mature macrophages. The macrophage progenitors efficiently adhered within 4 hours and were imaged on an EVOS M5000 microscope.

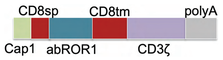


Figure 3. Structure of mRNA encoding ROR1-CAR containing CD3zeta activation domain (CD3ζ), CD8 signal peptide and transmembrane domains (CD8sp and CD8tm), ROR1 antibody domain (abROR1), a $^{m7}GpppN_{2'Ome}N$ cap (Cap1) and poly adenosine tail (polyA).

2 Macrophage mRNA Transfection Nucleotide Optimization

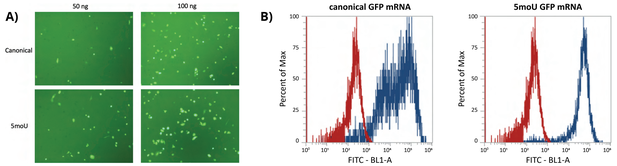


Figure 4. **GFP-encoding mRNA transfection into macrophages.** While macrophages are known to efficiently uptake nucleotides including mRNA, they can elicit an immune response and potentially impair translation. We assessed the expression of mRNA encoding GFP using either canonical RNA nucleotides or 5-Methoxy-UTP (5moU). **A)** Microscope images taken 4 hours after transfection demonstrating efficient expression of mRNA GFP in macrophages. 5moU nucleotides shows brighter expression relative to canonical (AUGC). Images taken with EVOS M5000 microscope. **B)** Flow cytometry analysis after 4 hours following mRNA transfection. 5moU transfected cells showing more consistent and efficient GFP expression. Data collected using an Attune NxT flow cytometer.

3 ROR1-CAR mRNA Transfection

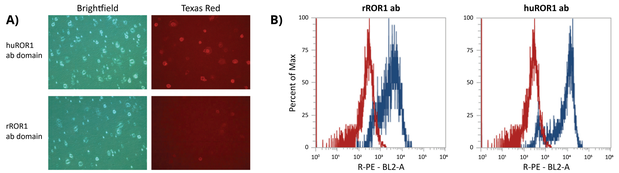


Figure 5. **Macrophage transfection with mRNA encoding an ROR1-CAR described in Figure 3.** Two different ROR1 antibody domains, the rabbit R12 (rROR1) and human (huROR1) domains, were assessed for their ability to bind to PE-labelled recombinant ROR1 protein. **A)** Microscope images demonstrating ROR1 binding. 24 hours post transfection the wells were washed twice with DPBS and incubated with PE-ROR1 for 30 minutes. Cells were washed in DPBS and imaged in flow cytometry staining buffer on a Nikon Ti Eclipse inverted microscope. **B)** Flow cytometry analysis of ROR1 binding. Following ROR1-PE staining of mRNA transfected macrophages with the ROR1-CAR, cells were lifted, fixed with 4% paraformaldehyde, and assessed on the Attune NxT flow cytometer. Cells transfected with the huROR1 antibody domain showed greater binding affinity to the recombinant ROR1-PE protein compared to the rROR1 domain.

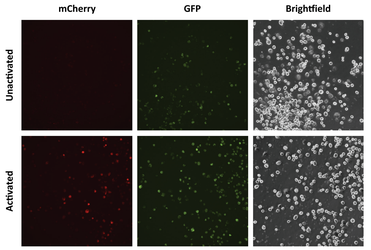


Figure 6. **Zymosan bead phagocytosis assay.** huROR1-CAR transfected macrophages were seeded into multiple wells. In one well macrophages had ROR1-PE added to their culture to activate CAR signaling, while the other had no ROR1-PE protein added. Both wells were subsequently incubated with GFP-labelled pHrodo Zymosan particles for 4 hours. ROR1 activation increased the intensity of Zymosan bead fluorescence caused by acidity in the phagosomes and lysosomes of macrophages.

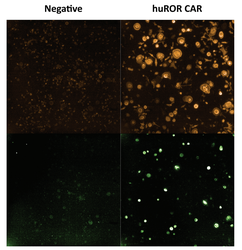


Figure 7. **CD3ζ phosphorylation.** huROR1-CAR transfected macrophages were activated with ROR1-PE protein (orange). Following activation, they were permeabilized with Triton X-100 and stained from intracellular CD3ζ phosphorylation (green). Cells were imaged with the PerkinElmer Operetta CLS High Content Analysis System.

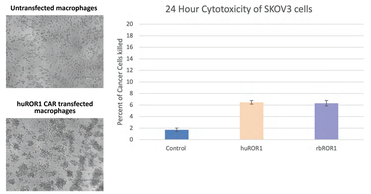


Figure 8. **SKOV3 cytotoxicity assay.** Macrophages were co-cultured with ROR1-expressing SKOV-3 cancer cells at 5:1 E:T ratio for 24 hours. The wells were imaged on the EVOS M5000 microscope, then enzymatically dissociated, stained for CD45 and Scarlet Live/Dead, and assessed on the Attune NxT flow cytometer. The proportion of dead cancer cells was determined as the dead population of CD45- cells. Experiments done in technical triplicate wells.

4 ROR1-CAR mRNA Gene Editing-mediated Knock In

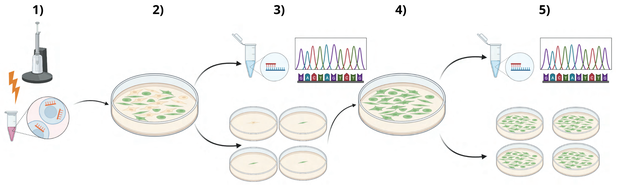


Figure 9. **5-step transgene insertion protocol for iPSC engineering. 1)** iPSC electroporation with gene-editing mRNA and repair template ssDNA. **2)** Plating and recovery of the heterogonous edited population. **3)** Single-cell sorting and simultaneous verification of insertion in the bulk population. **4)** Recovery and expansion of single-cell wells. **5)** Verification of insertion in the isolated clonal cell lines.

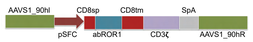


Figure 10. **Structure of ROR1-CAR transgene.** AAVS1_90hL and AAVS1_90hR: 90 nucleotide AAVS1 left and right homology arms. pSFC: SFC promoter. CD8sp: CD8 signal peptide. abROR1: ROR1 antibody domain. CD3ζ: CD3zeta activation domain. CD8tm: CD8 transmembrane domain. SpA: synthetic polyadenylation sequence.

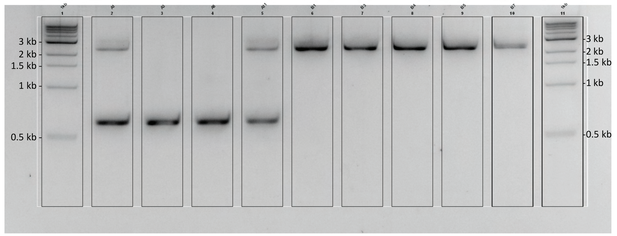


Figure 11. **Insertion of ROR1-CAR transgene into iPSC lines.** Following the protocol outlined in Figure 9 and using the transgene described in Figure 10, we successfully inserted the transgene into 7 of 9 picked iPSC colonies, with 5 displaying biallelic insertion. Colonies were passaged and split, with a portion of cells seeded for expansion and the remainder isolated for gDNA extraction. gDNA was amplified around the AAVS1 locus to verify insertion via PCR. Expected amplicon size is 600 bases long uninserted, 2.4kb with transgene insertion. Columns 1 and 11 show a 1 kilobase ladder, while the remaining show isolated iPSC colonies after electroporation and single-cell sorting. kb: kilobase pair.