243 NR4A3 GENE EDITING AND C-JUN OVEREXPRESSION SYNERGIZE TO LIMIT EXHAUSTION AND ENHANCE FUNCTIONAL ACTIVITY OF ROR1 CAR T CELLS *IN VITRO* AND *IN VIVO*

Viola Lam*, Jessica Barragan, Christina Cheung, Jia Lu, David Chian, Rowena Martinez, Candace Sims, Purnima Sundar, Hajime Hiraragi, Shobha Potluri, Rachel Lynn. *Lyell Immunopharma, Inc., South San Francisco, CA, USA*

Background Next-generation strategies to improve T-cell functional activity, persistence, and durability are needed for effective cellular immunotherapy against solid tumors. Overexpression of the activator protein 1 (AP-1) family transcription factor c-Jun reduces chimeric antigen receptor (CAR) T-cell exhaustion thereby improving functional activity in multiple preclinical models.[1] Nuclear receptor subfamily 4A (NR4A) transcription factors may contribute to exhaustion and limit T-cell function by restraining expression of AP-1 regulated genes.[2,3] Thus, we hypothesize that NR4A knockout (KO) and c-Jun overexpression may synergize to further limit exhaustion and enhance CAR T-cell function.

Methods Healthy donor T cells were transduced with a ROR1 CAR lentiviral vector with (+) or without (-) c-Jun overexpression. NR4A family genes (NR4A1, NR4A2, or NR4A3) were disrupted using CRISPR/Cas9 ribonucleoprotein delivery via electroporation (EP). CAR T-cell cytotoxicity and cytokine production were evaluated *in vitro* after primary and repeated antigen-stimulation assays designed to promote exhaustion. Cell phenotypes (flow cytometry) and transcriptional profiling (bulk and single-cell RNA-Seq) were also assessed. Finally, CAR T cells were evaluated *in vivo* using a ROR1-expressing H1975 lung cancer xenograft model in mice.

Results NR4A3 KO ROR1 CAR T cells consistently demonstrated superior cytotoxic activity and prolonged cytokine production upon repeated antigen stimulation compared to NR4A1 KO, NR4A2 KO, and EP control ROR1 CAR T cells. NR4A3 KO showed significant synergy in ROR1 CAR T cells overexpressing c-Jun (figure 1).

NR4A3 KO + c-Jun ROR1 CAR T cells were phenotypically and functionally indistinguishable at primary antigen stimulation. However, this combination resulted in the highest levels of cytokine production (IFN-γ, IL-2, and TNF-α), increased CAR T-cell persistence, and reduced surface expression of inhibitory receptors after repetitive antigen stimulation, suggesting a mechanism of resistance to exhaustion-induced dysfunction. Transcriptomic analysis indicated that NR4A3 KO + c-Jun increased effector and interferon response-associated T-cell subsets, yet reduced terminal exhaustion compared to control + c-Jun ROR1 CAR T cells following antigen restimulation.

NR4A3 KO + c-Jun ROR1 CAR T cells showed the most robust anti-tumor efficacy *in vivo* with activity at a 7-fold reduced CAR T-cell dose and demonstrated more than 20-fold greater CAR T-cell expansion in blood compared to control + c-Jun ROR1 CAR T cells (figure 2).

Conclusions These data suggest that reducing NR4A3 expression in combination with c-Jun overexpression has the potential to further limit exhaustion and provide durable ROR1 CAR T-cell functional activity compared to either strategy alone, which may improve cellular immunotherapy against ROR1-expressing solid tumors.

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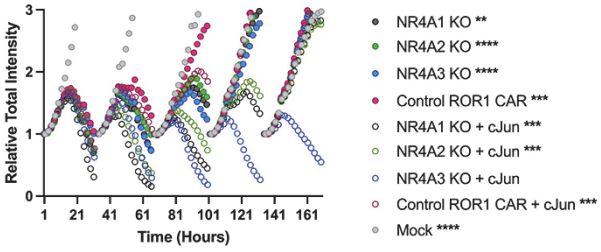
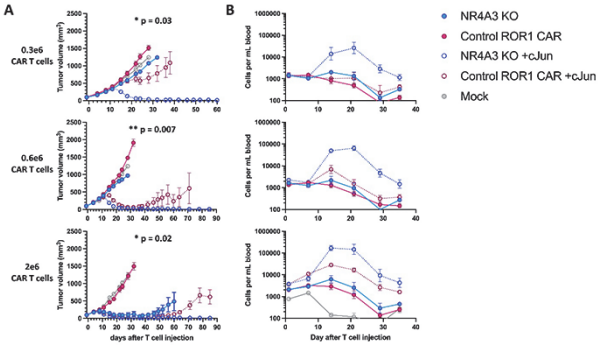
Ethics Approval Experiments presented in this abstract relied on human donor material that was obtained from commercial vendors. These vendors use their own IRB-approved protocol and consent process. In vivo experiments presented in this abstract were approved by Lyell Immunopharma's IACUC (EB17-010-117).



Abstract 243 Figure 1 Successive lysis of ROR1-expressing H1975-NucLight Red (NLR) target cells by NR4A1 KO, NR4A2 KO, NR4A3 KO, or control unedited ROR1 CAR T-cells with (+) or without (-) c-Jun overexpression, and mock untransduced T cells in one representative donor of four donors tested. Lysis of H1975-NLR target cells was quantified by measuring total NLR intensity. NLR intensity was normalized relative to the starting intensity after replating for each round of stimulation. NR4A3 KO + c-Jun showed significant synergy at the last timepoint of the fifth stimulation compared to other T-cells tested. Asterisks represent p-value significance of each condition compared to NR4A3 KO + c-Jun (unpaired t-test, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$).



Abstract 243 Figure 2 Anti-tumor efficacy at three CAR T-cell doses (A) and CAR T-cell expansion (B) of NR4A3 KO or control edited ROR1 CAR T cells with (+) or without (-) c-Jun overexpression, and mock untransduced T cells in one representative experiment (n=3 healthy donors) tested in an in vivo H1975 xenograft model. NR4A3 KO + c-Jun CAR T cells significantly impaired tumor growth at all three CAR T cell doses tested compared to control + c-Jun (* $p < 0.05$, ** $p < 0.005$, Tukey one-way ANOVA). At the 0.6x106 CAR T-cell dose, NR4A3 KO + c-Jun CAR T cells demonstrated a significantly higher day 21-fold expansion compared to control + c-Jun (mean of 40.1 vs 1.7, ****$p <$ 0.0001, unpaired t-test, n=10 mice per group). Day 21-fold expansion was normalized relative to day 1.

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