Preclinical Development of LYL119, a ROR1-Targeted CAR T-Cell Product Candidate Incorporating Four Novel T-Cell Reprogramming Technologies to Overcome Barriers to Effective Cell Therapy for Solid Tumors

Abstract 1118

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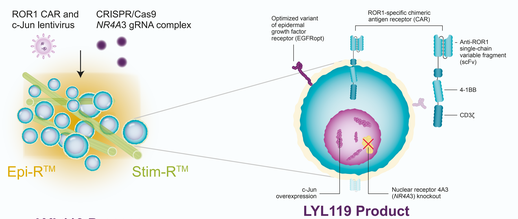
Background

- T-cell exhaustion and lack of durable stemness, defined as the ability of cells to proliferate, persist, and self-renew, are key barriers to effective T-cell therapy in solid tumors[1–2]
- Ongoing efforts to overcome these barriers include genetic and epigenetic T-cell reprogramming:
  - Genetically reprogramming T cells through c-Jun overexpression delays exhaustion and results in increased proliferation, sustained cytokine production, and durable antitumor activity[1,3–4]
  - NR4A transcription factors may contribute to exhaustion and reduced T-cell function by limiting the activity of c-Jun,[5–6] *NR4A3* gene KO synergizes with c-Jun overexpression to further enhance resistance to exhaustion, resulting in improved antitumor activity[7]
  - Epigenetic reprogramming with Lyell's Epi-R™ manufacturing protocols preserves stem-like qualities by controlling T-cell proliferation and differentiation with optimized proprietary cell culture media and other manufacturing steps[1,8–10]
  - Epigenetic reprogramming with Lyell's Stim-R™ technology, a synthetic biomimetic designed to present precise and more physiological T-cell activation signals during manufacturing, further improves T-cell polyfunctionality, persistence, and antitumor activity[11]
- These four T-cell reprogramming technologies are combined to create LYL119, an investigational ROR1-targeted CAR T-cell product enhanced with c-Jun overexpression, *NR4A3* KO, and Epi-R and Stim-R technologies to overcome barriers to successful T-cell therapy in solid tumors (**Figure 1**)

Figure 1: LYL119, an investigational ROR1-targeted CAR T-cell product enhanced with Lyell's T-cell reprogramming technologies



Methods

- Healthy donor T cells were transduced with a ROR1 CAR tri-cistronic lentiviral vector with (c-Jun–2A–ROR1 scFv-BBz–2A–EGFRt) or without (CD19t–2A–ROR1 scFv-BBz–2A–EGFRt) c-Jun overexpression
- *NR4A3* was knocked out using CRISPR/Cas9 ribonucleoprotein delivery via electroporation. Control ROR1 CAR T-cells were edited at a control locus (human CD19) that is not expressed in T cells
- T cells were stimulated with an optimized Stim-R formulation or a standard benchmark activation reagent ("Reagent")
- T-cell products for all conditions were produced using the Epi-R manufacturing protocol
- CAR T-cell cytotoxicity and cytokine production were evaluated in vitro after primary and serial antigen-stimulation assays designed to promote exhaustion
- CAR T cells were evaluated in vivo using ROR1-expressing A549 or H1975 human lung cancer xenograft models in NSG HLA dKO mice

Abbreviations

4-1BB, tumor necrosis factor ligand superfamily member 9; AUC, area under the curve; CAR, chimeric antigen receptor; Cas9, CRISPR associated protein 9; CCL5, chemokine ligand 5; CD, cluster of differentiation; CRISPR, clustered regularly interspaced short palindromic repeats; dKO, double knockout; EGFR, epidermal growth factor receptor; EGFRopt, optimized variant of epidermal growth factor receptor; GNLY, granulysin; gRNA, guide RNA; HLA, human leukocyte antigen; HSD, honest significant difference; IFNγ, interferon gamma; IL-2, interleukin 2; KO, knockout; NR4A, nuclear receptor subfamily 4A; ns, not significant; RFU, relative fluorescent units; RNA, ribonucleic acid; ROR1, receptor tyrosine kinase-like orphan receptor; scFv, single-chain variable fragment; SEM, standard error of the mean; TCF7, transcription factor 7; TIGIT, T-cell immune receptor with immunoglobin and ITIM domains; UMAP, uniform manifold approximation and projection.

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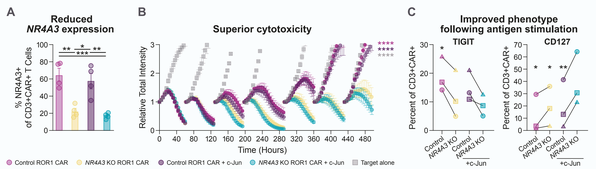
Results

NR4A3 KO and c-Jun overexpression synergize to reduce exhaustion and maintain ROR1 CAR T-cell function in vitro and in vivo

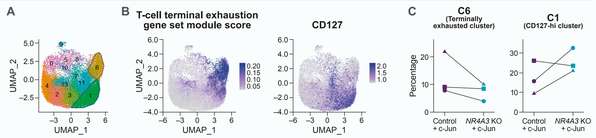
- *NR4A3* KO + c-Jun ROR1 CAR T cells demonstrated improved tumor cell killing and produced high levels of cytokines (data not shown), and reduced surface expression of inhibitory receptors after repetitive antigen stimulation compared with ROR1 CAR T cells with c-Jun overexpression alone (**Figure 2**)
- *NR4A3* KO + c-Jun ROR1 CAR T cells displayed transcriptional profiles consistent with reduced terminal exhaustion and increased retention of memory T cells following serial stimulation (**Figure 3**)
- *NR4A3* KO + c-Jun ROR1 CAR T cells had robust antitumor efficacy in vivo with activity observed at a 7-fold reduction in CAR T-cell dose and a 10-fold greater expansion in blood compared with control CAR T cells enhanced with c-Jun overexpression alone (**Figure 4**)

Figure 2: *NR4A3* KO and c-Jun overexpression synergize to enhance ROR1 CAR T-cell function in vitro



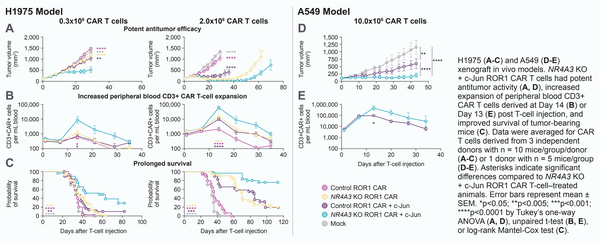
(**A**) Significant NR4A3 protein reduction in edited ROR1 CAR T cells does not affect the expression of ROR1 CAR T cells or c-Jun (data not shown). (**B**) *NR4A3* KO + c-Jun ROR1 CAR T cells had superior cytotoxicity with significant differences at the end of the seventh sequential stimulation with H1975 target cells. (**C**) *NR4A3* KO + c-Jun ROR1 CAR T cells have reduced expression of TIGIT and increased CD127 expression after four stimulations. Shapes correspond to CAR T cells derived from each donor. Asterisks indicate significant differences compared to *NR4A3* KO + c-Jun ROR1 CAR T cells. Error bars represent mean ± SEM. *p<0.05; **p<0.005; ***p<0.001; ****p<0.0001 by unpaired t-test (**A**, **B**) or paired t-test (**C**).

Figure 3: Transcriptomic analysis revealed that *NR4A3* KO reduces terminal exhaustion and enhances memory-like cell proportions following serial restimulation with antigen



(**A**) Single-cell RNA-seq clustering analysis identified a terminally exhausted cluster (C6, brown) and a CD127-*hi* cluster (C1, green) of CD8+ *NR4A3* KO and control-edited ROR1 CAR T cells with c-Jun overexpression following 7 days of in vitro antigen restimulation using A549 target cells. (**B**) Cluster C6 was enriched for expression of genes identified in a previously published T-cell terminal exhaustion gene set,[4] whereas cluster C1 was enriched for expression of CD127. (**C**) The proportion of cells in the terminally exhausted cluster C6 was reduced, and the proportion in the CD127-hi cluster C1 was increased in *NR4A3* KO + c-Jun ROR1 CAR T cells compared to control-edited + c-Jun ROR1 CAR T cells. Closed shapes represent CAR T cells derived from one of three independent donors.

Figure 4: *NR4A3* KO + c-Jun ROR1 CAR T cells demonstrate potent antitumor activity in vivo

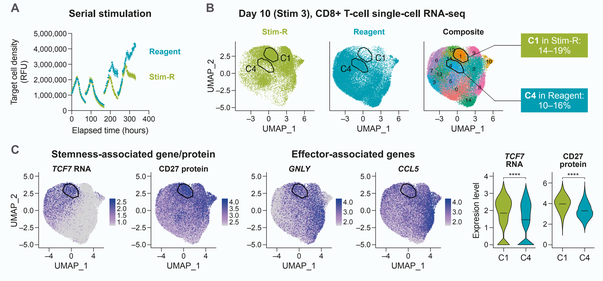


H1975 (**A**-**C**) and A549 (**D**-**E**) xenograft in vivo models. *NR4A3* KO + c-Jun ROR1 CAR T cells had potent antitumor activity (**A**, **D**), increased expansion of peripheral blood CD3+ CAR T cells derived at Day 14 (**B**) or Day 13 (**E**) post T-cell injection, and improved survival of tumor-bearing mice (**C**). Data were averaged for CAR T cells derived from 3 independent donors with n = 10 mice/group/donor (**A**-**C**) or 1 donor with n = 5 mice/group (**D**-**E**). Asterisks indicate significant differences compared to *NR4A3* KO + c-Jun ROR1 CAR T-cell–treated animals. Error bars represent mean ± SEM. *p<0.05; **p<0.005; ***p<0.001; ****p<0.0001 by Tukey's one-way ANOVA (**A**, **D**), unpaired t-test (**B**, **E**), or log-rank Mantel-Cox test (**C**).

Stim-R technology generates ROR1-targeted CAR T-cell products with enhanced and prolonged functional activity in vitro and in vivo

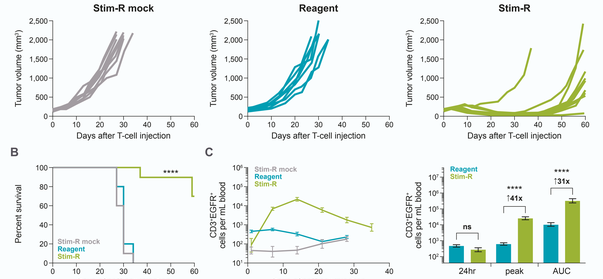
- ROR1 CAR T cells produced with Stim-R technology show:
  - Enhanced cytotoxicity in vitro following serial antigen stimulation (**Figure 5**)
  - Retained persistence of a unique cell population enriched in both stemness and effector-associated gene signatures following repeat tumor cell stimulation in vitro (**Figure 5**)
  - Improved antitumor efficacy and CAR T-cell expansion in vivo (**Figure 6**)

Figure 5: Following serial antigen stimulation, ROR1 CAR T cells generated with Stim-R technology have enhanced cytotoxicity, are less exhausted, and retain a unique subset of stem-like cells with effector-associated gene signatures from transcriptomic analyses



(**A**) ROR1 CAR T cells manufactured with Stim-R technology showed superior target clearance compared to ROR1 CAR T cells manufactured with Reagent. (**B**) Single-cell and bulk RNA-seq of CD8+ ROR1 CAR T cells following 10 days of in vitro antigen restimulation using H1975 target cells. CD8+ T cells generated with Stim-R technology and Reagent are separated on UMAP plot generated in single-cell RNA-seq analysis. Putative stem-like clusters identified in Stim-R-derived cells (C1) and Reagent-derived cells (C4), respectively, are highlighted, with proportions shown in text box annotations. (**C**) C1 in Stim-R–generated cells (highlighted) exhibited high expression of stemness-associated gene marker *TCF7* and protein marker CD27, as well as positivity for known effector-associated genes *GNLY* and *CCL5* (left panels). C1 in Stim-R-generated cells showed significantly higher *TCF7* RNA and CD27 protein expression compared to C4 in Reagent-generated cells (right panel). ****p<0.0001 by Wilcoxon test.

Figure 6: ROR1 CAR T cells manufactured with Stim-R technology exhibited higher peak CAR T-cell numbers in the blood, prolonged persistence, and improved antitumor activity in vivo

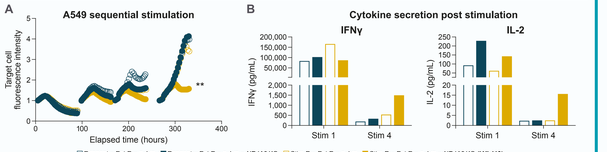


ROR1 CAR T cells produced with Stim-R technology showed improved antitumor activity (**A**) and overall survival (**B**) compared to mock untransduced Stim-R T cells (Stim-R mock) and Reagent CAR T cells. (**C**) ROR1 CAR T cells produced with Stim-R technology showed higher peak CAR T-cell numbers, prolonged persistence, and higher total CAR T cells detected over the course of the study as assessed by calculating the AUC compared to ROR1 CAR T cells produced with Reagent. Data in (**A**) represent individual tumor growth curves of n = 10 mice per condition. Data in (**C**) represent mean ± SEM of n = 10 mice per condition. ****p<0.0001 by Mantel-Cox test (**B**) or one-way ANOVA, followed by Tukey's HSD post hoc test for each metric (**C**); only Reagent and Stim-R-produced CAR T-cell conditions shown.

LYL119 ROR1 CAR T cells, incorporating *NR4A3* KO + c-Jun + Epi-R + Stim-R technologies, demonstrate superior in vitro and in vivo activity

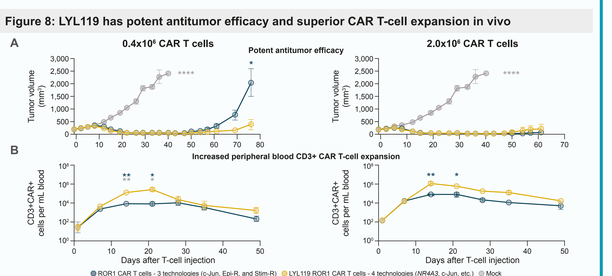
- LYL119 demonstrates prolonged cytotoxicity and enhanced cytokine production in vitro compared with control cells (**Figure 7**)
- LYL119 has potent antitumor efficacy and superior CAR T-cell expansion in vivo compared with control-edited ROR1 CAR T cells (with only c-Jun and Epi-R and Stim-R technologies; **Figure 8**)

Figure 7: LYL119 demonstrates prolonged cytotoxicity and enhanced cytokine production in vitro



(**A**) LYL119 CAR T cells showed superior target clearance compared to controls. (**B**) LYL119 CAR T cells showed a trend of higher IFNγ and IL-2 production after repeated stimulation. Representative data from 1 in 3 donors is shown (**A**, **B**). **p=0.0017 compared to "Reagent + Epi-R + c-Jun + *NR4A3* KO". Statistical comparisons were performed using the AUC of the final round of restimulation in three donors using a one-way ANOVA analysis.

Figure 8: LYL119 has potent antitumor efficacy and superior CAR T-cell expansion in vivo



LYL119 has potent antitumor activity (**A**) and enhanced expansion of peripheral blood CD3+ CAR T cells (**B**) in the H1975 xenograft model. Data are CAR T cells derived from 1 independent donor with n = 5 mice/group. Asterisks indicate significant differences compared to LYL119-treated animals. Error bars represent mean ± SEM. *p<0.05; **p<0.005; ***p<0.001; ****p<0.0001 by Tukey's one-way ANOVA (**A**) or unpaired t-test (**B**).

Conclusion

- *NR4A3* KO and c-Jun overexpression synergize to reduce CAR T-cell exhaustion and enhance ROR1 CAR T-cell function
- Stim-R technology generates ROR1 CAR T cells with enhanced proliferative capacity and sustained antitumor activity
- Preliminary data suggest that ROR1 CAR T cells genetically modified to knock out *NR4A3* expression and overexpress c-Jun and epigenetically reprogrammed using Epi-R and Stim-R technologies demonstrate additive functional enhancements to further improve CAR T-cell antitumor efficacy in vitro and in vivo
- To test whether this strategy can enhance the efficacy of T-cell therapy in patients with ROR1-expressing solid tumors, Lyell is developing LYL119, an investigational ROR1-targeted CAR T-cell product candidate that incorporates these four reprogramming technologies

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