Preclinical Evaluation of Anti-ROR1 CAR T-cells Employing a ROR1 Binding scFv Derived From the Clinical Stage mAb Cirmtuzumab

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Introduction We generated chimeric antigen receptor T (CAR T) cells that target the extracellular domain of ROR1, which is expressed on many human cancers but not on post-partum tissues. The anti-ROR1 CAR utilizes the humanized binding domain of UC-961 (cirmtuzumab), which currently is being evaluated in Phase 1/2 studies for patients with hematologic and solid-tumor malignancies (NCT 02222688, 02776917). Cirmtuzumab has demonstrated high affinity for human ROR1 with no off-target binding[1], a prolonged half-life, and excellent safety profile in Phase 1 clinical studies[2]. The humanized scFv of cirmtuzumab represents an excellent ROR1 targeting moiety for use in CAR-based cell therapies.

We developed multiple candidate 2nd and 3rd generation CAR constructs with different signaling and spacer regions. To evaluate these constructs, we generated candidate CAR T-cells in a serum-free growth media supplemented with IL-2. Using this protocol, we produced CAR T-cells from over 20 healthy donors and examined their activity against lymphoid cancers in vitro using chromium release and impedance assays. The 2nd generation CARs using the 4-1BB co-stimulation domain demonstrated activity even at low effector to target (E:T) ratios, eliminating both primary B-cell leukemias and lymphomas, as well as tumor cell lines that express the ROR1 target.

Methods To evaluate ROR1 CAR T-cells, we created a luciferase/RFP lentiviral expression vector to generate labeled human MEC1-ROR1 leukemia cells[3]. When injected into immune-deficient NSG mice, MEC1-ROR1 cells infiltrate the marrow (femur), kidney, spleen, and liver, resulting in progressive and fatal leukemia/lymphoma. From multiple 2nd generation CAR T-cell products, we selected the CAR with 4-1BB and CD3z signaling domains that demonstrated the best activity in *in vitro* assays.

To introduce the CAR into cells, we created lentiviral vectors encoding the ROR1 CAR (Figure 1).

Figure 1. Structure of the anti-human anti-ROR1 CAR. The 2nd generation CAR employing the humanized anti-ROR1 cirmtuzumab variable regions of heavy chain (VH) and light chain (VL).

The *in vitro* killing activity of this highly active and specific CAR product is shown below (Figure 2).

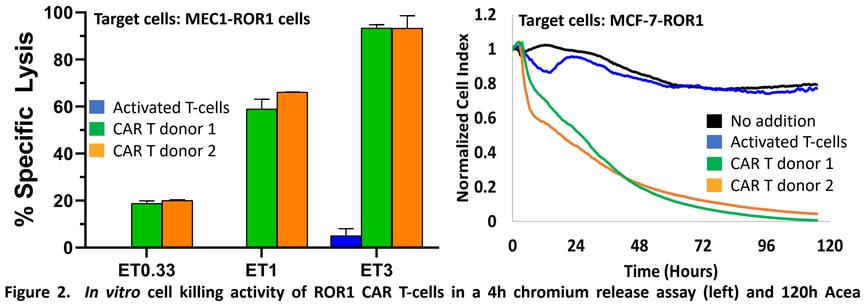

Figure 2. ***In vitro*** **cell killing activity of ROR1 CAR T-cells in a 4h chromium release assay (left) and 120h Acea impedance assay (right).** ROR1 CAR T-cell products were generated from 2 healthy donors as described above. The generated CAR T-cells were tested at the indicated E:T ratios. The anti-ROR1 CAR T-cells demonstrated high and specific cytotoxicity without significant killing of ROR1-negative target cells [less than 10% of specific killing (data not shown)]. ATC = activated T-cells

References: 1) Choi, Clin Lymphoma Myeloma Leuk, 2015; 2) Choi, Cell Stem Cell, 2018; 3) Yu et al., J Clin Invest, 2015

Rationale: CAR T Targeting ROR1 Designed to Avoid Common CAR T Challenges

Unmet Need: Emerging CAR T Issues	Advantages to Targeting ROR1
Treatment failures • Increasing number of patient relapses following CAR T therapy, frequently due to mutations or loss of target tumor antigen (e.g. CD19), evading CAR T-cells	**Potential for fewer antigen negative relapses** • ROR1 expression associated with aggressive tumor phenotype • ROR1 mutation or antigen loss might render cancer cells less aggressive and susceptible to chemotherapy
Safety concerns • Persistent CAR T safety issues including deaths potentially related to activation by normal cells expressing the target antigen ("on-target/off-tumor")	**Potential safety advantages** • Cirmtuzumab did not bind to normal human tissues in GLP tissue cross-reactivity studies • No serious adverse events related to cirmtuzumab-only reported in clinical studies

Conclusions:

- Single dose of ROR1 CAR T-cells expanded in MEC1-ROR1 xenografts and homed to disease sites.
- By week four, rapidly-expanding leukemia cells were cleared from major tissue reservoirs, including bone marrow, kidneys and spleen.
- CAR T-cell-treated animals survived longer than 90 days vs 21 days for animals in control groups.
- CAR -T-cells were highly active and detected in mouse tissues more than 2 months after injection.

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Results

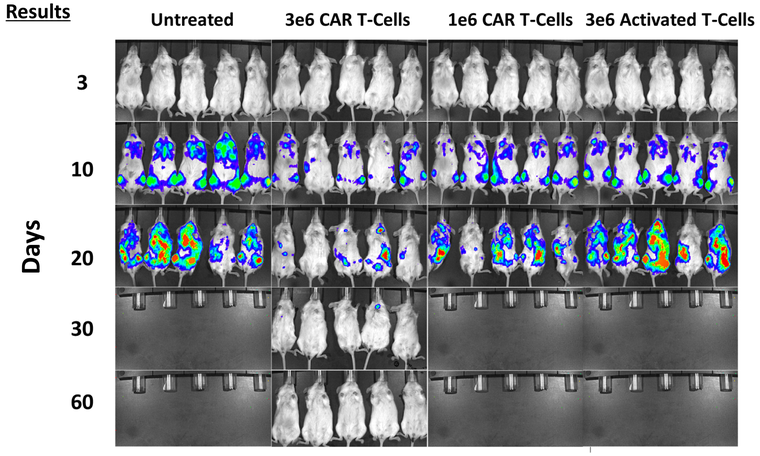

Figure 3. Bioluminescence imaging of mice inoculated with MEC1-ROR1 cells and with ROR1 CAR T-cells. Animals treated with CAR T-cells had reduced disease burden compared to controls. The highest dose (3e6 CAR Ts) cohort had only minimal amounts of disease at the end of the study.

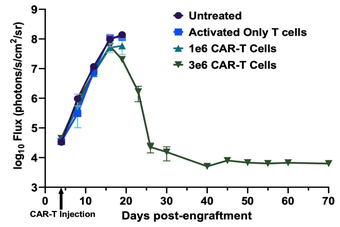

Figure 4. Bioluminescence imaging of MEC1-ROR1 cells following treatment with ROR1 CAR T-cells. Mice treated with 3e6 CAR T reduced the leukemic burden to background levels by day 30 and controlled disease for remainder of study. Animals in the control groups (untreated, ATC or lower 1e6 dose) had to be sacrificed on day 20.

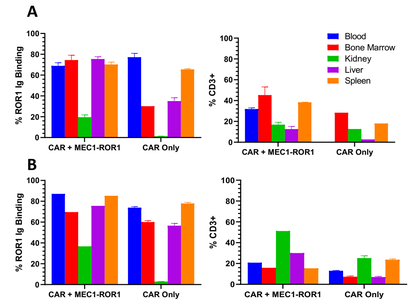

Figure 5. Detection of CAR T-cells in mouse tissues. Following CAR T administration, animals were sacrificed on days 11 (Panel A), and 25 (Panel B), blood and organs collected and subjected to flow analysis for CAR expression and confirmatory ROR1 binding activity. The CAR T-cell number was substantially greater in mice bearing MEC-1 ROR1 cells (CAR + MEC1-ROR1) vs control (CAR only) demonstrating elevated expansion of CAR T-cells in animals with tumor burden.
Bars represent the mean values from the five mice in each group and error bars represent the S.E of the mean.

Future Directions for Research:

Results of these studies strongly encourage clinical studies evaluating safety and efficacy of our ROR1-directed CAR T cell therapy for patients with hematological malignancies.