Optimizing ex vivo CAR-T cell infiltration and cytotoxicity assays using 2- and 3-D multimodality imaging

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Abstract 118

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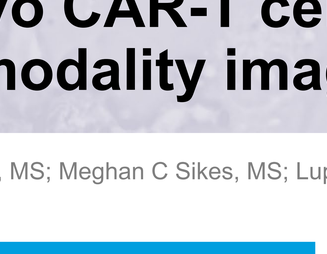
Abstract

CAR-T cell therapy is a revolutionary cancer treatment that is highly efficient in treating liquid tumors and continues to expand in its applications, including which immune cells are engineered, what constructs are utilized, and which cancers are targeted. In such a rapidly evolving field, sensitive, quantitative assays to test the cytotoxicity of CAR cells ex vivo are required to continue improving the efficacy of this treatment. Furthermore, testing infiltration of engineered CAR-T cells into solid tumor models is critically important to gain more specific insight into the mechanisms of how cold tumors become hot with CAR cells infiltrating, targeting and killing cancer cells. We developed a single clone reporter cancer cell line, Raji-Luc2 (ATCC® CCL-86-Luc2™), that endogenously expresses high levels of the key CAR-T target antigen CD19. Using CD19 and mock-engineered CAR-T cells, we targeted Raji-Luc2 cells and assessed the killing efficacy of the CAR-T cells by a multimodality imaging approach that included a bioluminescence luciferase assay as well as a phase contrast and fluorescence live-cell imaging assay. Next, we engineered a dual reporter line, Raji-GFP-Luc2 (ATCC® CCL-86-GFP-Luc2™), and demonstrated CAR-T cell infiltration into Raji-GFP-Luc2 spheroids by confocal live imaging in 3-D. We combined the luciferase assay with live imaging to create a multimodal cytotoxicity assay that provides superior sensitivity as well as spatial and temporal resolution as compared to existing assays. Using the luciferase assay, we discovered that the CD19 CAR-T cells killed cancer cells at more significant levels relative to mock CAR-T control cells. Furthermore, we developed a CAR-T cell infiltration assay by co-culturing dyed CAR-T cells with Raji-GFP-Luc2 spheroids. By imaging in 3-D and generating time-lapse videos, we show how CAR-T cells infiltrated non-gel-embedded as well as gel-embedded spheroids in real time. Overall, we show that these engineered cell lines are highly useful for assaying CAR-T cell cytotoxicity as well as infiltration into 3-D solid tumor models such as spheroids and organoids. The quantitative readout of the luciferase assay combined with the dynamic, real-time information provided by multiple live-imaging techniques in both 2-D and 3-D results in a sensitive, multimodal assay that can be used in a variety of applications. Importantly, visualization of T cell infiltration in 3-D is critical to understanding how CAR cells infiltrate into solid tumors and then target and kill cancer cells; these more physiologically relevant conditions enable the efficacy of this treatment to be further improved.

Background

In CAR-T cell therapy, a cancer patient's T cells are isolated from their blood and transduced with a CAR (chimeric antigen receptor) construct that codes for a receptor that targets the patient's T cells to the cancer cells. The lymphoblast line Raji (ATCC® CCL-86™) expresses endogenously high levels of CD19, an FDA-approved CAR-T target antigen. To develop ex vivo CAR-T cell cytotoxicity assays, we engineered Raji cells to express a single luciferase reporter or a dual GFP and luciferase reporter. CD19 CAR-T cells should target endogenously expressed CD19 on the surface of Raji-Luc2 (ATCC® CCL-86-Luc2™) or Raji-GFP-Luc2 (ATCC® CCL-86-GFP-Luc2™) cells, resulting in Raji-Luc2/Raji-GFP-Luc2 cell death and loss of reporter expression. Figure created with Biorender.com.



Results

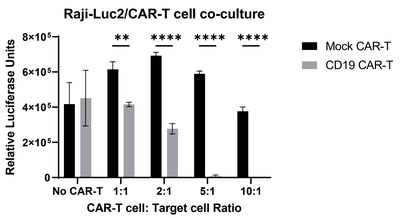


Figure 1: CD19 CAR-T in vitro killing assay of Raji-Luc2 in 2-D co-culture using bioluminescence. Raji-Luc2 (ATCC® CCL-86-Luc2™) cells were co-cultured with donor-matched mock (black) or CD19 (grey) CD8+ CAR-T cells for 72 hours using varying ratios of CAR-T cells to target cells. Luciferase expression was detected using the Bright-Glo™ system (Promega®). ** = p < 0.005, **** = p < 0.0001. Error bars represent the standard deviation of three biological replicates.

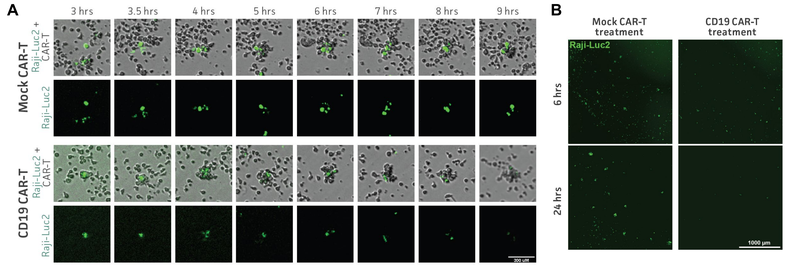


Figure 2: CD19 CAR-T in vitro killing assay of Raji-Luc2 in 2-D co-culture using live cell imaging. (A) Raji-Luc2 (ATCC® CCL-86-Luc2™) cells were stained with Vybrant™ (Invitrogen®) DiO dye and co-cultured with mock (top) or CD19 (bottom) CAR-T cells at a 5:1 ratio of CAR-T cells to target cells. Images were acquired every 30 minutes for 24 hours using the Cytation® 1 plate reader (Agilent®). Raji-Luc2 cells are labeled in green. Scale bar, 300 µm. (B) GFP channels only for co-cultures of Raji-Luc2/mock CAR-T cells (left) or Raji-Luc2/CD19 CAR-T cells (right). 6-hour (top) and 24-hour (bottom) time points are shown for a large field of cells. Scale bar, 1000 µm.

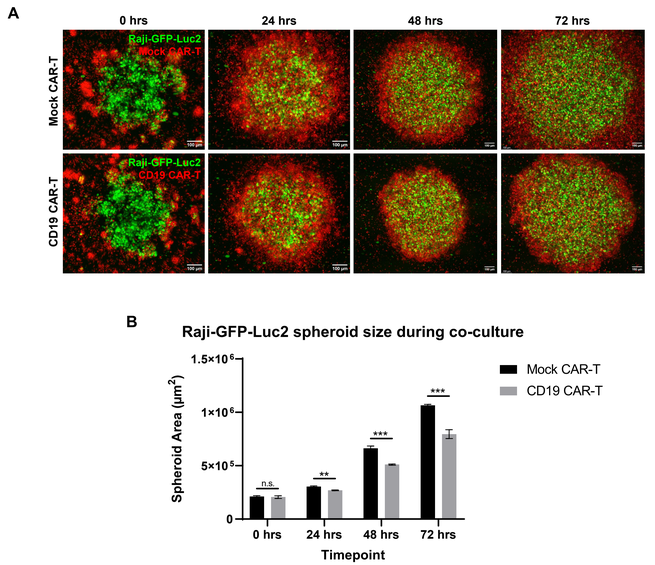


Figure 3: CD19 CAR-T cells infiltrate and kill Raji-GFP-Luc2 spheroids in 3-D co-culture. (A) Raji-GFP-Luc2 (ATCC® CCL-86-GFP-Luc2™) transduced pool cells (green) were formed into spheroids and Vybrant™ (Invitrogen®) DiD-dyed mock or CD19 CAR-T cells (red) were added the next day. 5 µm Z stacks were acquired every 24 hours for 72 hours on the Leica® Mica™ confocal microscope. Maximum projections are shown. Scale bars, 100 µm. (B) Raji-GFP-Luc2 spheroid area during co-culture with mock (black) or CD19 (grey) CAR-T cells. Spheroid area was measured using the GFP channel only in ImageJ. n.s. = not significant, ** = p < 0.01, *** = p < 0.001. Error bars represent the standard deviation of three biological replicates.

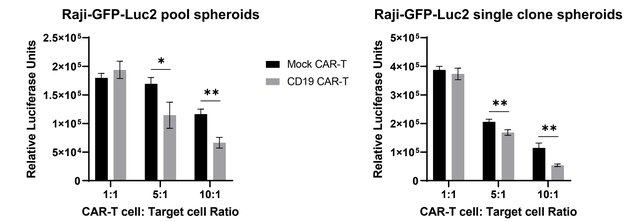


Figure 4: CD19 CAR-T cells kill Raji-GFP-Luc2 spheroids in a dose-dependent manner. Raji-GFP-Luc2 (ATCC® CCL-86-GFP-Luc2™) transduced pool (left) or single clone (right) cells were formed into spheroids and mock (black) or CD19 (grey) CAR-T cells were added the next day. After 72 hours of co-culture, luciferase expression was detected for each well using the Bright-Glo™ system (Promega®). * = p < 0.05, ** = p < 0.01. Error bars represent the standard deviation of three biological replicates.

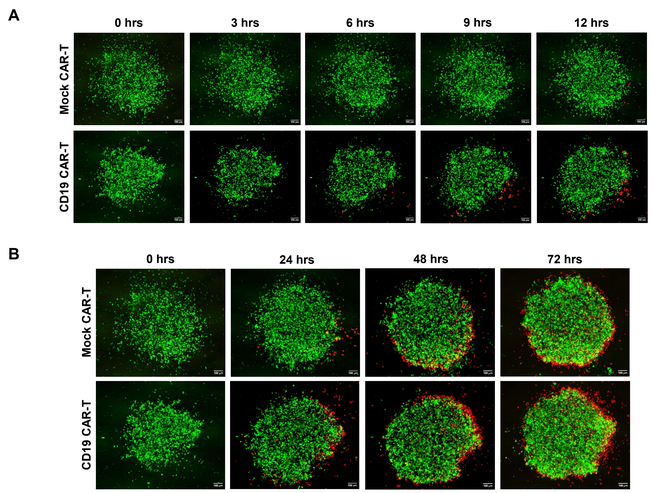


Figure 5: CAR-T cells infiltrate gel-embedded Raji-GFP-Luc2 spheroids within 12 hours. Raji-GFP-Luc2 (ATCC® CCL-86-GFP-Luc2™) transduced pool cells were formed into spheroids and embedded in a BME/collagen gel the next day. Vybrant™ (Invitrogen®) DiD-dyed CAR-T cells were added the day after embedment at a 5:1 CAR-T cell: target cell ratio. 10 µm Z stacks were acquired (A) every 3 hours for 12 hours or (B) every 24 hours for 72 hours using the Leica® Mica™ confocal microscope. Maximum projections are shown. Raji-GFP-Luc2 cells are labeled in green and CAR-T cells are labeled in red. Scale bars, 100 µm.

Conclusions

- CD19 CAR-T cells target and kill Raji-Luc2 (ATCC® CCL-86-Luc2™) cells in 2-D co-culture and Raji-GFP-Luc2 (ATCC® CCL-86-GFP-Luc2™) cells in 3-D co-culture at higher levels compared to mock CAR-T cells
- Raji-GFP-Luc2 (ATCC® CCL-86-GFP-Luc2™) spheroids can be embedded to assay T cell infiltration in a solid tumor model
- Raji-Luc2 (ATCC® CCL-86-Luc2™) and Raji-GFP-Luc2 (ATCC® CCL-86-GFP-Luc2™) reporter cell lines can be used in a variety of cytotoxicity assays, including bioluminescence and live imaging

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