Standardizing ex vivo CAR-T cell cytotoxicity evaluation via multimodal 2-D and 3-D imaging of CAR-T target dual reporter cell lines

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Abstract 98

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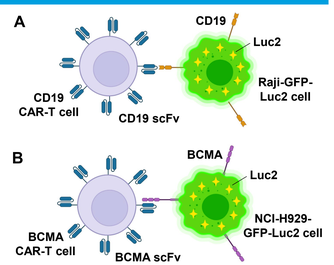
Abstract

Chimeric Antigen Receptor T (CAR-T) cell therapy has emerged as an advanced and highly effective approach for treating hematologic malignancies with its scope rapidly expanding to encompass diverse applications including solid tumors. This fast-paced development calls for highly sensitive and quantitative ex vivo assays for assessing CAR-T cell cytotoxicity, particularly in models that recapitulate 3-D tumor environments. To address this need, we generated GFP-luciferase dual reporter cancer cell lines that endogenously express high levels of the key CAR-T target antigens CD19 and BCMA. Using both antigen-specific and mock-engineered CAR-T cells, we assessed cytotoxicity via a multimodal imaging strategy, integrating a luciferase-based bioluminescence assay with phase contrast and fluorescence live-cell imaging. To further demonstrate dynamic visualization of CAR-T activity in an environment that mimics in vivo conditions, we embedded dual reporter spheroids in 3-D matrices, which allowed for time-lapse imaging of CAR-T cell infiltration. Our multimodal imaging approach revealed significantly enhanced cancer cell killing by targeted CAR-T cells compared to controls. Employing 2-D and 3-D co-culture assays of dual reporter cancer cells with targeted and mock-engineered CAR-T cells, we found that targeted CAR-T cells exhibited higher killing of cancer cells than mock CAR-T cells in both live imaging and luciferase assays. In 3-D fluorescence imaging experiments, we identified a reduction in both the size of cancer cell spheroids and in the GFP signal in response to targeted CAR-T cells as compared to mock CAR-T cells. Additionally, in both 2-D and 3-D co-culture assays, we noticed a significant decrease in relative luciferase signal from reporter cancer cells in conditions with targeted CAR-T cells, implying targeted cancer cell killing by CAR-T cells. Moreover, time-lapse confocal imaging of spheroids in 3-D matrices captured active infiltration of CAR-T cells into tumor spheroids over time. These results showcase the benefit of multimodal methodology combining bioluminescent and live fluorescence imaging to quantitatively examine CAR-T cytotoxicity as well as visualize the spatial and temporal interplay of CAR-T cells and cancer cells in both 2-D and 3-D co-culture systems. The scalability and sensitivity of this assay platform makes it a versatile tool for standardizing CAR-T cytotoxicity evaluation, advancing CAR-T therapeutic development in industrial and translational research settings.

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 encodes 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 GFP and luciferase transgenes. CD19 CAR-T cells target endogenously expressed CD19 on the surface of Raji-GFP-Luc2 cells (A), resulting in Raji-GFP-Luc2 cell death and loss of expression of both reporters. Similarly, the plasmacytoma line NCI-H929-GFP-Luc2 (ATCC® CRL-3580-GFP-LUC2™) is a target for BCMA CAR-T cells (B). Figure created with Biorender.com.



Results

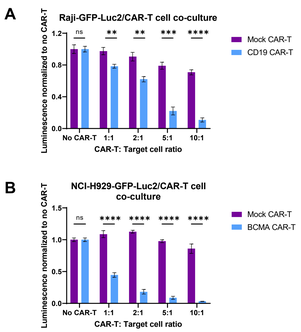


Figure 1: Targeting CAR-T cells kill dual reporter cancer cells in 2-D co-culture in a dose-dependent manner as measured by luminescence assay. (A) Raji-GFP-Luc2 (ATCC® CCL-86-GFP-LUC2™) cells were co-cultured with donor-matched mock (purple) or CD19 (blue) CAR-T cells (ProMab) for 24 hours using varying ratios of CAR-T cells to target cells. Luciferase expression was detected using the Bright-Glo system (Promega). Luminescence values were normalized to no CAR-T wells. ns = not significant, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, unpaired t-test. Error bars represent the standard deviation of three biological replicates. (B) NCI-H929-GFP-Luc2 (ATCC® CRL-3580-GFP-LUC2™) cells were co-cultured with donor-matched mock (purple) or BCMA (blue) CAR-T cells (ProMab) for 24 hours using varying ratios of CAR-T cells to target cells. Luciferase expression was detected using the Bright-Glo system (Promega). Luminescence values were normalized to no CAR-T wells. ns = not significant, **** = $p < 0.0001$, unpaired t-test. Error bars represent the standard deviation of three biological replicates.

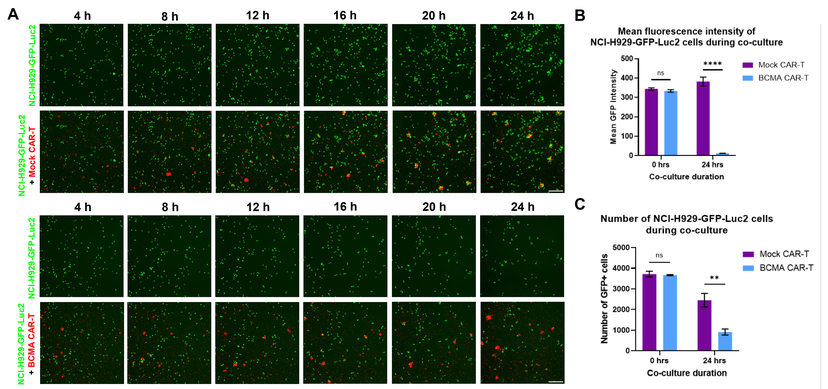


Figure 2: CAR-T cell in vitro killing assay of NCI-H929-GFP-Luc2 dual reporter cells in 2-D co-culture using fluorescence live imaging. (A) Mock (top) or BCMA (bottom) CAR-T cells were stained with Vybrant DiD dye (Invitrogen) and co-cultured with NCI-H929-GFP-Luc2 (ATCC® CRL-3580-GFP-LUC2™) cells at a 5:1 ratio of CAR-T cells to target cells. Images were acquired every 2 hours for 24 hours using the Leica Mica microscope. NCI-H929-GFP-Luc2 cells are in green, dyed CAR-T cells are in red. Scale bars, 200 µm. (B) Mean GFP fluorescence intensity or (C) number of GFP+ cells of entire NCI-H929-GFP-Luc2/CAR-T co-culture wells quantified at 0 hours and after 24 hours of co-culture with either mock (purple) or BCMA (blue) CAR-T cells at a 10:1 CAR-T cell to target cell ratio. Error bars indicate the standard deviation of three biological replicates. ns = not significant, ** = $p < 0.005$, **** = $p < 0.0001$, unpaired t-test.

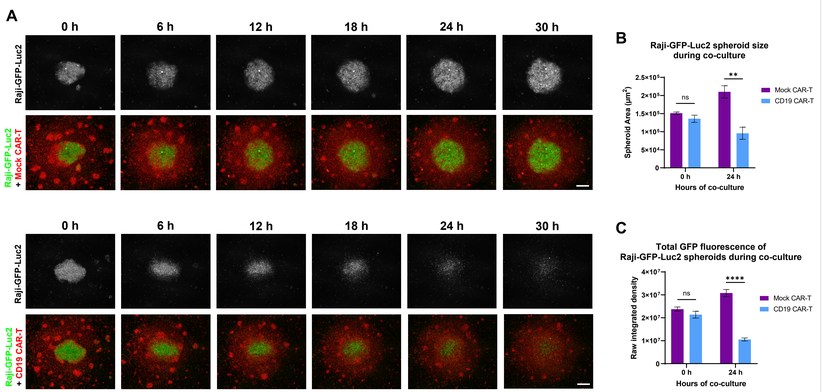


Figure 3: CAR-T cell in vitro killing assay of Raji-GFP-Luc2 dual reporter cells in 3-D co-culture using fluorescence live imaging. (A) Raji-GFP-Luc2 (ATCC® CCL-86-GFP-LUC2™) cells (green) were formed into spheroids and DiD-dyed mock (top) or CD19 (bottom) CAR-T cells (red) were added the next day. 5 µm Z stacks were acquired every 3 hours for 36 hours on a Leica Mica confocal microscope. Maximum projections are shown. Scale bars, 200 µm. (B) Raji-GFP-Luc2 spheroid area during co-culture with mock (purple) or CD19 (blue) CAR-T cells. Spheroid area was measured using the GFP channel in ImageJ at 0 hours and after 24 hours of co-culture. n.s. = not significant, ** = $p < 0.005$. Error bars represent the standard deviation of three biological replicates. (C) Raw integrated density (sum of pixel values) of Raji-GFP-Luc2 spheroids during co-culture with mock (purple) or CD19 (blue) CAR-T cells. Raw integrated densities were measured using the GFP channel in ImageJ (NIH) at 0 hours and after 24 hours of co-culture. n.s. = not significant, **** = $p < 0.0001$. Error bars represent the standard deviation of three biological replicates.

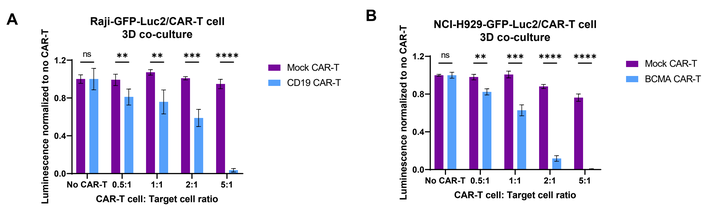


Figure 4: Targeting CAR-T cells kill dual reporter spheroids in a dose-dependent manner as measured by luminescence assay. (A) Raji-GFP-Luc2 (ATCC® CCL-86-GFP-LUC2™) or (B) NCI-H929-GFP-Luc2 (ATCC® CRL-3580-GFP-LUC2™) cells were formed into spheroids and the next day mock (purple) or targeting (blue) CAR-T cells were added at varying ratios of CAR-T cells to target cells. After 24 hours (Raji-GFP-Luc2) or 16 hours (NCI-H929-GFP-Luc2) of co-culture, luciferase expression was detected for each well using the Bright-Glo system. Luminescence values were normalized to no CAR-T wells. ns = not significant, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, unpaired t-test. Error bars represent the standard deviation of three biological replicates.

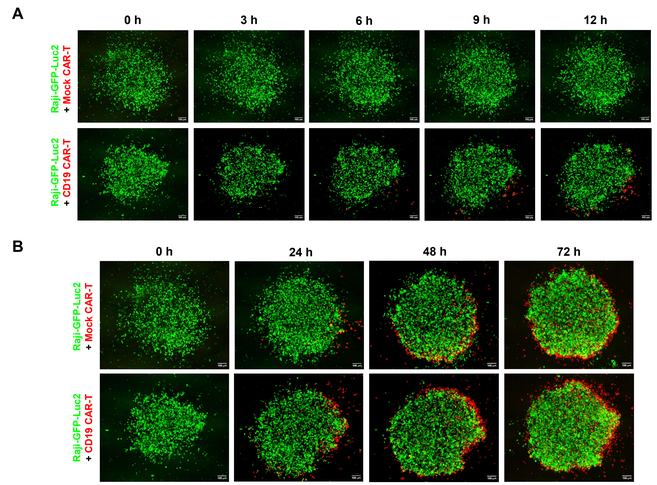


Figure 5: CAR-T cells infiltrate gel-embedded Raji-GFP-Luc2 spheroids within 12 hours. Raji-GFP-Luc2 transduced pool cells were formed into spheroids and embedded in a BME/collagen gel the next day. DiD-dyed CAR-T cells were added the day after embedment at a 5:1 CAR-T cell: target cell ratio. 10 µm Z stacks were acquired (A) every 3 hours for 12 hours or (B) every 24 hours for 72 hours using a 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- and BCMA-targeting CAR-T cells kill Raji-GFP-Luc2 (ATCC® CCL-86-GFP-LUC2™) and NCI-H929-GFP-Luc2 (ATCC® CRL-3580-GFP-LUC2™) cells, respectively, in a dose-dependent manner in both 2- and 3-D co-culture.
- NCI-H929-GFP-Luc2 (ATCC® CRL-3580-GFP-LUC2™) cells show a decrease in cell number and GFP fluorescence after 2-D co-culture with BCMA CAR-T cells as assayed by widefield live imaging.
- Raji-GFP-Luc2 (ATCC® CCL-86-GFP-LUC2™) spheroids show a decrease in GFP fluorescence and spheroid size after 3-D co-culture with CD19 CAR-T cells as assayed by confocal live imaging.
- Raji-GFP-Luc2 (ATCC® CCL-86-GFP-LUC2™) spheroids can be embedded to assay T cell infiltration in a solid tumor model.

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