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APPLICATION NOTE

DUAL REPORTER CANCER CELL LINES ACCELERATE CAR-T CELL THERAPY DEVELOPMENT

Catherine McManus, PhD; John Foulke, MS; Meghan Sikes, MS; Luping Chen, BS; Hyeyoun Chang, PhD; and Fang Tian, PhD

ABSTRACT

In the ongoing battle against cancer, chimeric antigen receptor (CAR)-T cell therapy has emerged as a promising avenue of treatment. A total of seven therapies are currently FDA-approved for several refractory liquid tumors. Despite this progress, researchers are still actively focused on identifying optimal antigen targets, refining CAR constructs, and expanding the therapeutic scope of this modality. To improve the efficacy and the number of applications of this revolutionary therapy, researchers require reliable, sensitive assays to quickly test CAR-T cell function ex vivo. Here, we describe two dual reporter cancer cell lines—Raji-GFP-Luc2 (ATCC® CCL-86-GFP-LUC2™) and NCI-H929-GFP-Luc2 (ATCC® CRL-3580-GFP-LUC2™)—that stably express luciferase and GFP transgenes along with high endogenous levels of either CD19 or BCMA, antigens targeted by FDA-approved CAR-T therapies. We demonstrate that the expression levels of these transgenes can be used as a readout for CAR-T cytotoxicity in two distinct assays: bioluminescence and live fluorescence imaging. Upon co-culture with antigen-specific CAR-T cells, we observed marked reductions in luciferase and GFP signals, demonstrating effective cancer cell lysis. Collectively, these dual reporter lines are useful tools for quickly and dependably assessing CAR-T cell cytotoxicity ex vivo and will aid researchers in improving the effectiveness of this promising therapy.

INTRODUCTION

Chimeric antigen receptor (CAR)-T cell therapy is a revolutionary therapy in the treatment of cancer. A patient's T cells are isolated and transduced with a CAR construct encoding a receptor that targets the patient's own T cells to a specific marker enriched on the surface of the cancer cells.[1,2] These targeting CAR-T cells are then transfused back into the patient and are highly efficient in the treatment of several lymphomas, leukemias, and myelomas.[3] Despite these advancements, CAR constructs are still being optimized to enhance cytotoxicity. In addition, researchers are actively exploring if CAR-T cell therapy can be applied to new target antigens and cancer types and if other immune cells can be utilized.

To improve the efficacy of CAR-T cell therapy and to increase the number of treatable cancers, researchers require efficient and reliable assays to test CAR-T cells ex vivo. However, the gold-standard cytotoxicity assay in the field—the chromium-51 (^{51}Cr) release assay—quantifies levels of cell death during co-culture through the use of radioactive materials, which require special handling and disposal.[4] Furthermore, the ^{51}Cr release assay can only be used as an end-point assay.[5] To circumvent these limitations, other assays such as bioluminescence and live imaging can be used. In the bioluminescence assay, a plate reader is used to detect light emitted when the luciferase enzyme reacts with substrates provided in commercially available reagents. As luciferase expression is correlated with cell number, a decrease in detected light corresponds to lower cell numbers, and thus increased cell death.[6] Similarly, cells expressing a fluorescent transgene can be monitored in real time for loss of transgene expression using fluorescence microscopy. This method yields critical insight into the dynamics of CAR-T cell targeting and cancer cell killing. The main drawback of both the bioluminescence and live-imaging assays is the requirement for cells that stably express the required transgene(s).

To that end, we developed the dual reporter cancer cell lines Raji-GFP-Luc2 (ATCC® CCL-86-GFP-LUC2™) and NCI-H929-GFP-Luc2 (ATCC® CRL-3580-GFP-LUC2™) for use as targets in CAR-T cytotoxicity assays. The cell lines endogenously express high levels of the FDA-approved CAR-T target antigens[3] CD19[7] and BCMA[8], respectively, and exhibit strong and stable expression of both GFP and luciferase (Figure 1). The bioluminescence and live-imaging assays can be used in conjunction to determine cytotoxicity levels of CAR-T cells in co-culture with the dual reporter cancer cells. Decrease in signal of either transgene indicates high levels of cancer cell killing by the CAR-T cells. Bioluminescence functions as a sensitive and efficient readout for cytotoxicity, while live imaging provides spatial and temporal information in real time. Here, we demonstrate the use of these cell lines in both assays and observe significant cell killing of the reporter cells by antigen-specific CAR-T cells. We further enrich the live-imaging assay by quantifying the loss of GFP fluorescence during co-culture. Overall, we show that these reporter cell lines are robust, sensitive tools for precisely determining the levels of CAR-T cell cytotoxicity ex vivo.

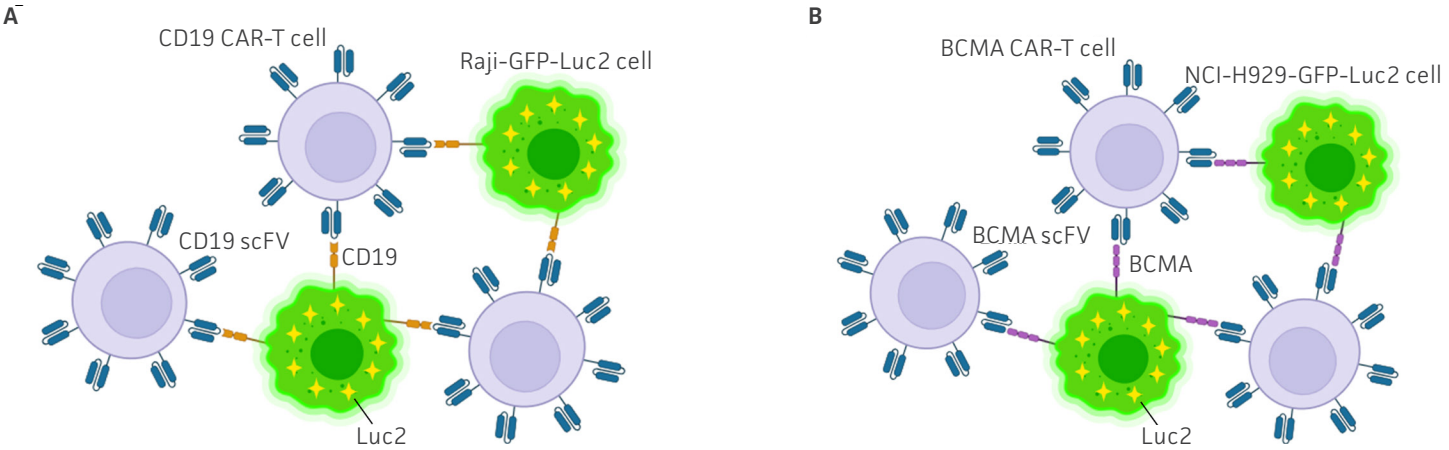

Figure 1: CAR-T target dual reporter cells. Schematic showing (A) Raji-GFP-Luc2 (ATCC® CCL-86-GFP-LUC2™) and (B) NCI-H929-GFP-Luc2 (ATCC® CRL-3580-GFP-LUC2™) cells (green) expressing CD19 or BCMA on their cell surfaces. Antigen-specific CAR-T cells (purple) target CD19 or BCMA on the cell surface of the reporter cells. Figure created with BioRender.com.

RESULTS WITH MATERIALS AND METHODS

To generate dual reporter cell lines for use in ex vivo CAR-T cell cytotoxicity assays, we selected human cancer cell lines with high endogenous expression of FDA-approved CAR-T target antigens. Raji (ATCC® CCL-86™) and NCI-H929 (ATCC® CRL-3580™) cell lines were selected for their high endogenous expression of CD19 and BCMA, respectively.[7,8] The selected cell lines were transduced with a lentiviral vector expressing luciferase and GFP under the control of the EF1A and CMV promoters, respectively. The transduced cells were grown under puromycin selection and single cell sorting was performed to isolate individual clones, which were verified to stably express both luciferase and GFP. For each line, the clone with the strongest expression of both transgenes as well as similar growth and morphology to the parental line was selected for expansion. High expression of the CAR-T target antigen was verified in the selected clone by flow cytometry. Finally, the single clonal lines were authenticated using short tandem repeat (STR) profiling and were verified for sterility.

To demonstrate the use of the CAR-T target dual reporter cell lines in CAR-T cell cytotoxicity assays, we co-cultured Raji-GFP-Luc2 (ATCC® CCL-86-GFP-LUC2™) or NCI-H929-GFP-Luc2 (ATCC® CRL-3580-GFP-LUC2™) cells with commercially available CAR-T cells (ProMab) targeting CD19 or BCMA, respectively. T cells from the same donor were transduced with a mock construct and used as negative controls. Co-cultures were performed at varying ratios of CAR-T cells to target cells (1:1, 2:1, 5:1, or 10:1). After 24 hours of co-culture, luciferase assays were performed using the Bright-Glo Luciferase Assay System (Promega). We observed a decrease in luminescence when Raji-GFP-Luc2 or NCI-H929-GFP-Luc2 cells were co-cultured with targeting CAR-T cells as compared to mock CAR-T cells (Figure 2, Figure 3). This decrease in luminescence was dose-dependent, with more dramatic decreases in luminescence occurring at higher CAR-T cell to target cell ratios. This result indicates that levels of antigen-specific CAR-T cell killing of the reporter cell lines were greater than the non-specific killing observed in mock CAR-T cell co-cultures.

We also monitored co-cultures in real time by live fluorescence imaging. CAR-T cells were pre-stained with Vybrant DiD dye (Invitrogen) one day prior to co-culture. Green and red fluorescence images of the co-cultures were acquired every two hours using an environmentally controlled microscope (Leica Mica) at a 5:1 ratio of CAR-T cells to target cells (Figure 4, Figure 5). Over the course of a 24-hour co-culture, we observed dramatic loss of GFP expression in co-cultures with targeting CAR-T cells, suggesting cell death of Raji-GFP-Luc2 or NCI-H929-GFP-Luc2 cells. This decrease in fluorescence was not observed in co-cultures with mock CAR-T cells. We performed additional co-culture experiments using unstained CAR-T cells at a 10:1 ratio of CAR-T cells to target cells and quantified the mean GFP fluorescence intensity after 24 hours using ImageJ (NIH). In addition, we quantified either the number of GFP+ cells or, in the case of Raji-GFP-Luc2, which grows in clusters at higher cell densities, the average size of GFP+ particles (Figure 6, Figure 7). These analyses verified a loss in mean fluorescence intensity after 24 hours of co-culture with targeting CAR-T cells for both dual reporter lines. Furthermore,

the average size of Raji-GFP-Luc2 clusters was significantly decreased after 24 hours of co-culture with CD19-targeting CAR-T cells. Similarly, the total number of GFP+ cells for NCI-H929-GFP-Luc2 decreased after 24 hours of co-culture with BCMA CAR-T cells.

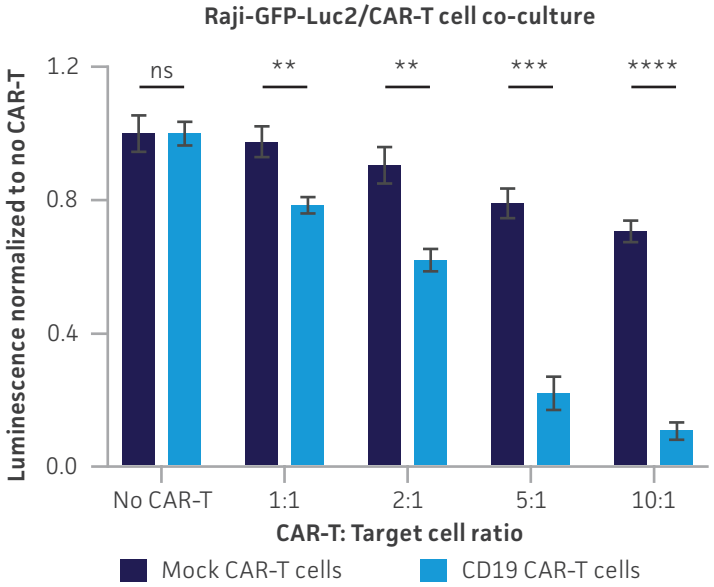

Figure 2: CD19 CAR-T cell in vitro killing assay of Raji-GFP-Luc2 cells measured using luminescence. 5 x 10^3 Raji-GFP-Luc2 cells were co-cultured with either mock or CD19 CAR-T cells from the same donor. Co-cultures were performed at varying ratios of CAR-T cells to target cells (1:1, 2:1, 5:1 or 10:1). After 24 hours of co-culture, Bright-Glo reagent was added and luminescence was determined using a plate reader. Luminescence values were normalized to no CAR-T wells. Error bars indicate the standard deviation of three biological replicates. ns = not significant, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, unpaired t-test.

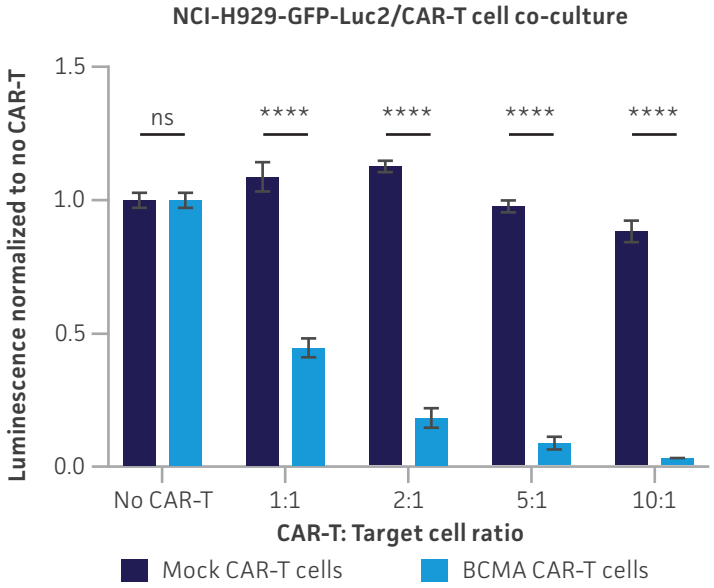

Figure 3: BCMA CAR-T cell in vitro killing assay of NCI-H929-GFP-Luc2 cells measured using luminescence. 5 x 10^3 NCI-H929-GFP-Luc2 cells were co-cultured with either mock or BCMA CAR-T cells from the same donor. Co-cultures were performed at varying ratios of CAR-T cells to target cells (1:1, 2:1, 5:1 or 10:1). After 24 hours of co-culture, Bright-Glo reagent was added and luminescence was determined using a plate reader. Luminescence values were normalized to no CAR-T wells. Error bars indicate the standard deviation of three biological replicates. ns = not significant, **** = $p < 0.0001$, unpaired t-test.

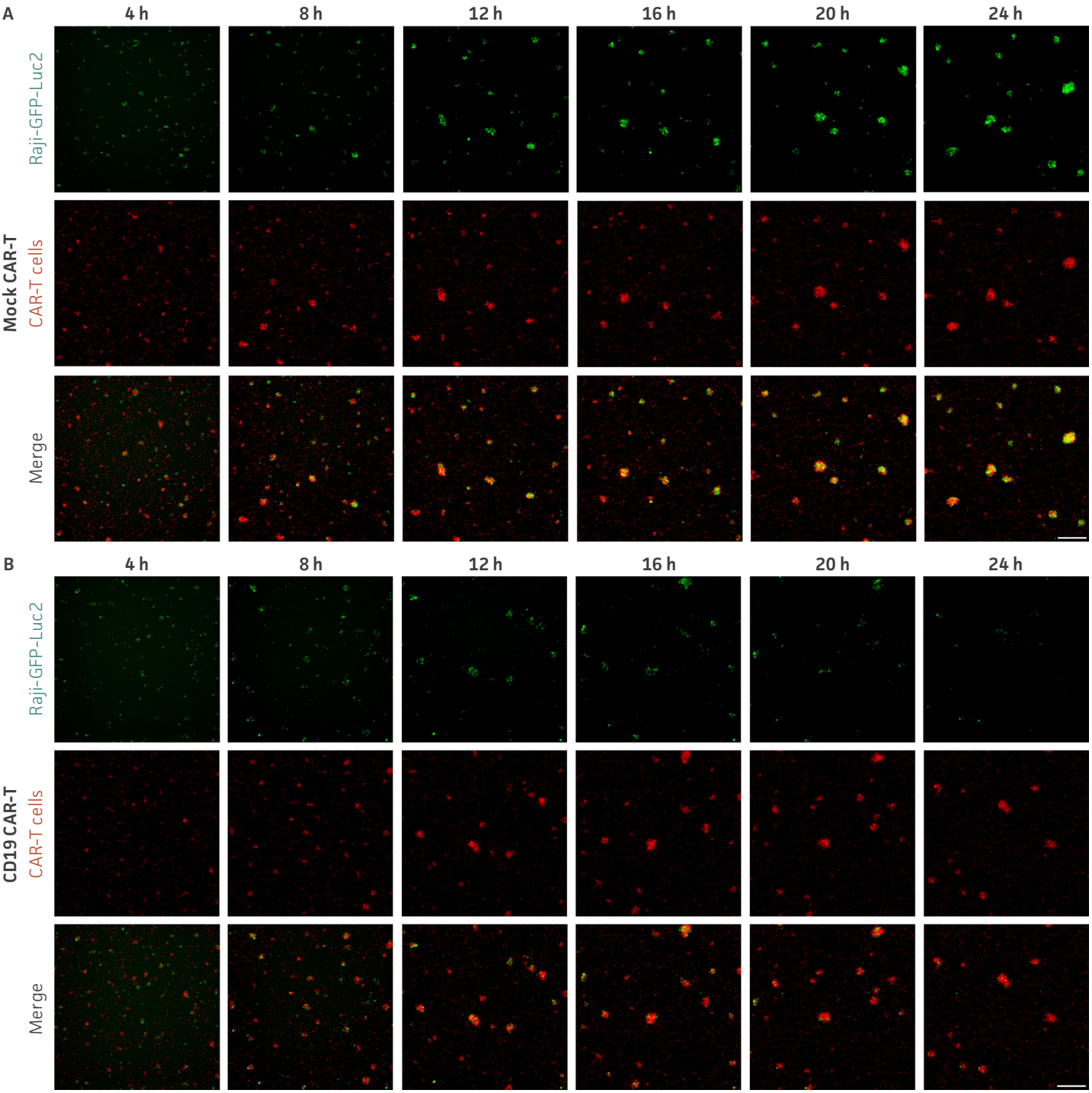

Figure 4: CD19 CAR-T cell in vitro killing assay of Raji-GFP-Luc2 cells monitored by live fluorescence imaging. (A) Mock or (B) CD19 CAR-T cells were pre-stained with Vybrant DiD dye and co-cultured with Raji-GFP-Luc2 cells at a 5:1 ratio of CAR-T cells to cancer cells. Green and red fluorescence images were captured every two hours for 24 hours using an environmentally controlled widefield microscope. Raji-GFP-Luc2 cells are labeled in green, dyed CAR-T cells are labeled in red. Scale bars, 200 µm.

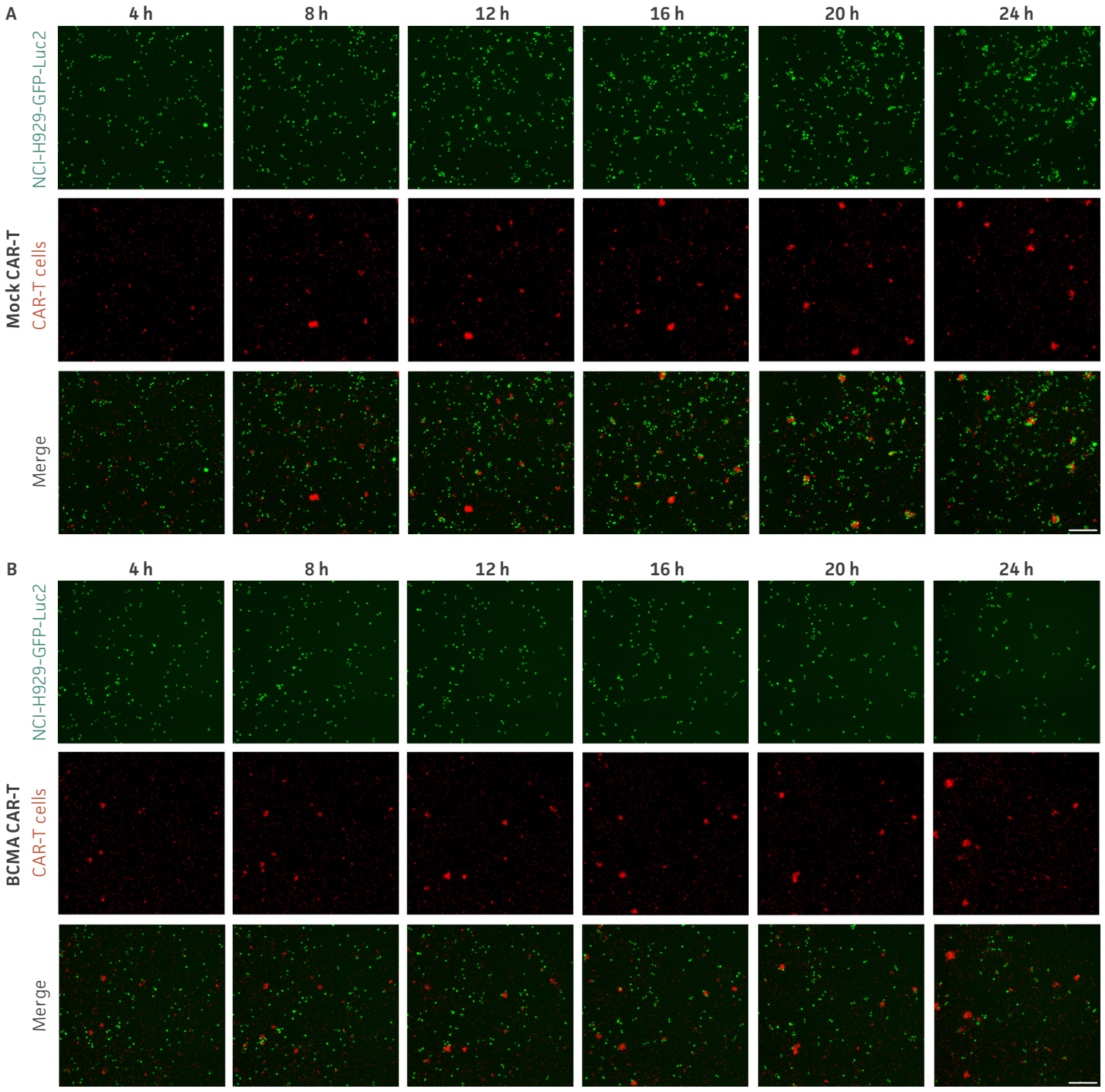

Figure 5: BCMA CAR-T cell in vitro killing assay of NCI-H929-GFP-Luc2 cells monitored by live fluorescence imaging. (A) Mock or (B) BCMA CAR-T cells were pre-stained with Vybrant DiD dye and co-cultured with NCI-H929-GFP-Luc2 cells at a 5:1 ratio of CAR-T cells to cancer cells. Green and red fluorescence images were captured every two hours for 24 hours using an environmentally controlled microscope. NCI-H929-GFP-Luc2 cells are labeled in green, dyed CAR-T cells are labeled in red. Scale bars, 200 µm.

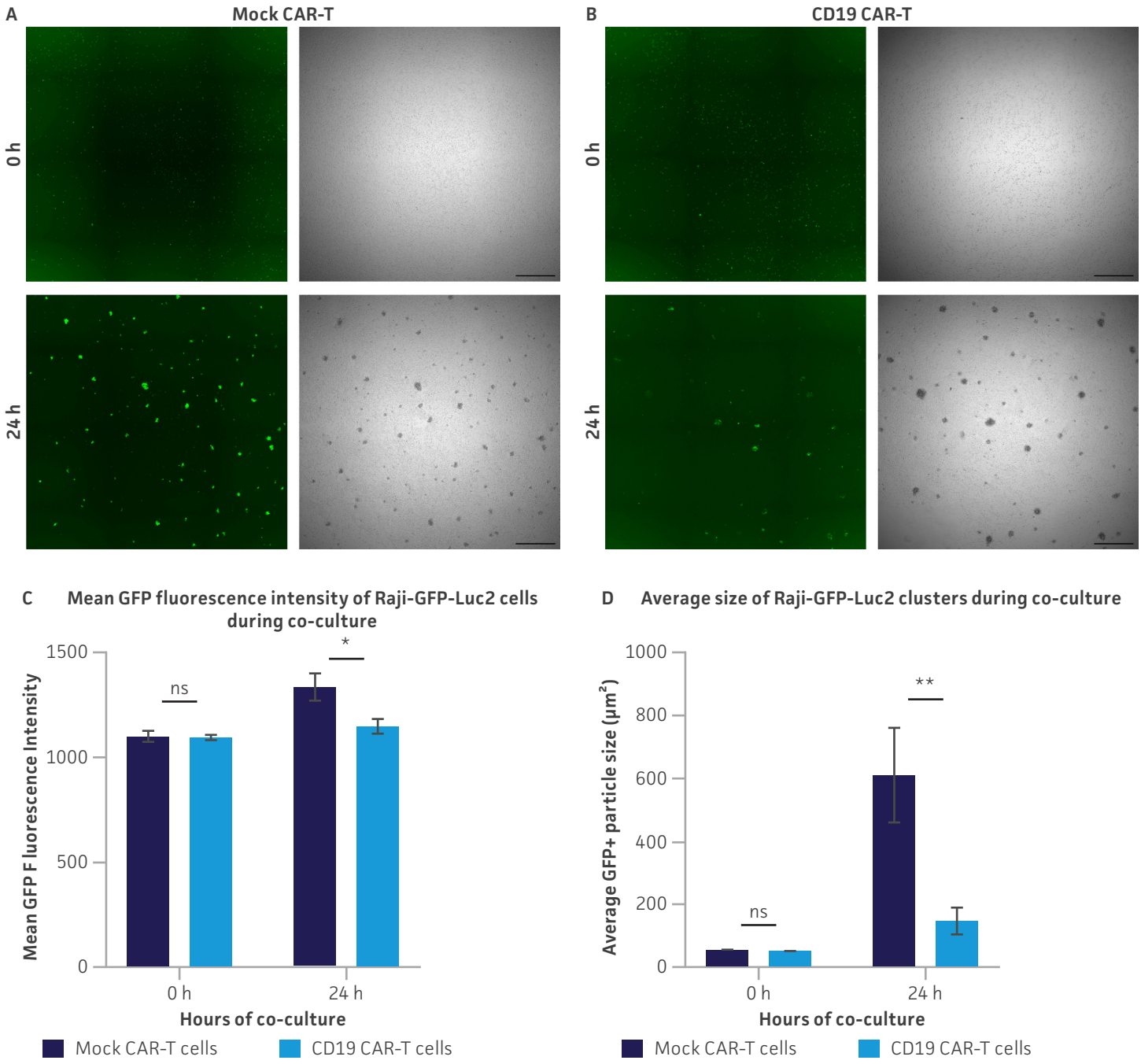

Figure 6: CD19 CAR-T cell in vitro killing assay of Raji-GFP-Luc2 cells quantified using live fluorescence imaging. Raji-GFP-Luc2 cells were co-cultured with either (A) mock or (B) CD19 CAR-T cells at a 10:1 ratio of CAR-T cells to cancer cells. GFP and brightfield images were captured at 0 h and after 24 h of co-culture using widefield settings on a Leica Mica microscope. Raji-GFP-Luc2 cells are shown in green. Scale bars, 500 µm. (C) Mean GFP fluorescence intensity of entire Raji-GFP-Luc2/CAR-T co-culture wells quantified at 0 hours and after 24 hours of co-culture. Error bars indicate the standard deviation of three biological replicates. ns = not significant, * = $p < 0.05$, unpaired t-test. (D) Average size of GFP+ clusters quantified at 0 hours and after 24 hours of Raji-GFP-Luc2 co-culture with either mock or CD19 CAR-T cells. Error bars indicate the standard deviation of three biological replicates. ns = not significant, ** = $p < 0.01$, unpaired t-test.

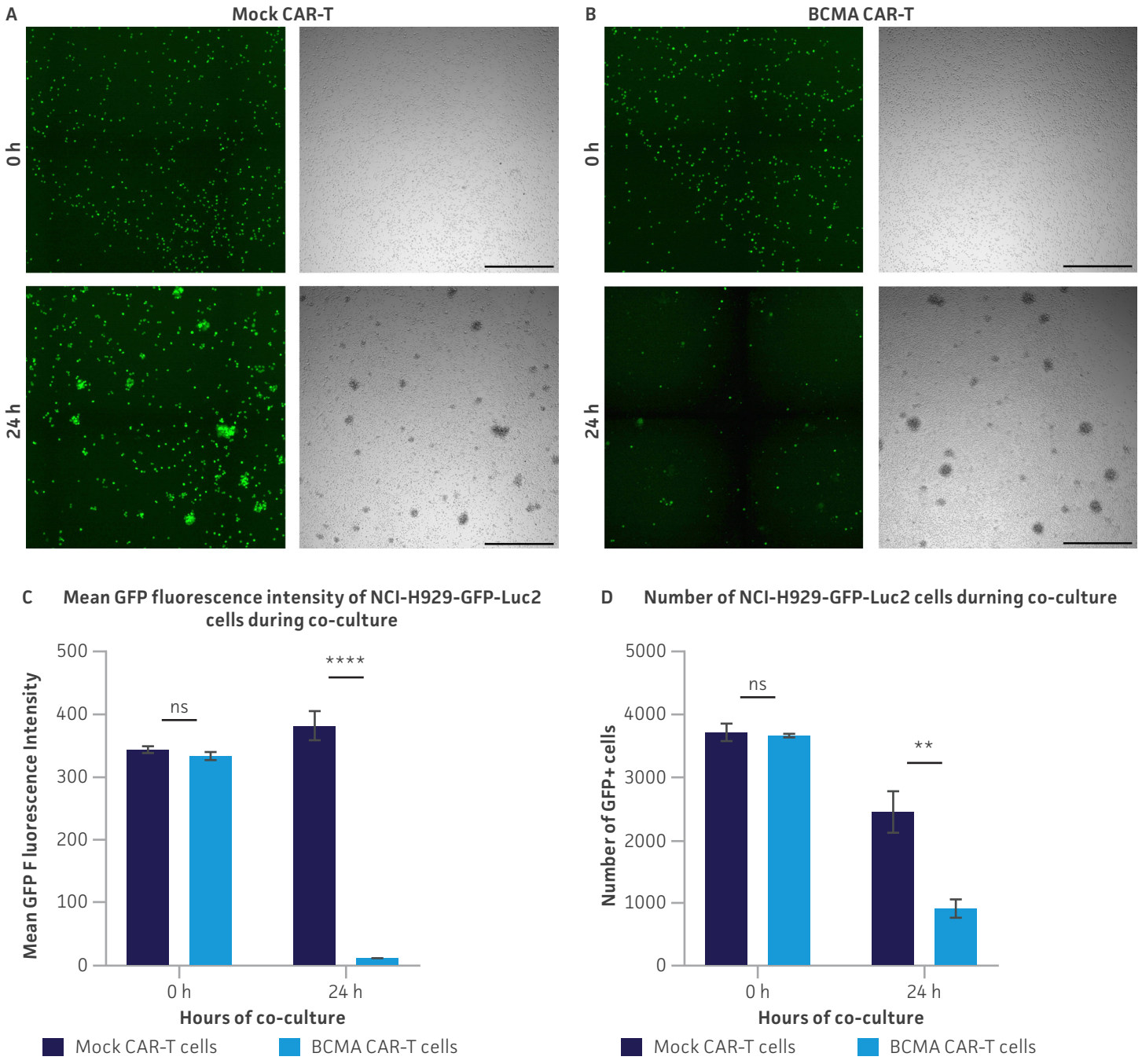

Figure 7: BCMA CAR-T cell in vitro killing assay of NCI-H929-GFP-Luc2 cells quantified using live fluorescence imaging. (A) NCI-H929-GFP-Luc2 cells were co-cultured with either (A) mock or (B) BCMA CAR-T cells at a 10:1 ratio of CAR-T cells to cancer cells. GFP and brightfield images were captured at 0 h and after 24 h of co-culture using widefield settings on a Leica Mica microscope. NCI-H929-GFP-Luc2 cells are shown in green. Scale bars, 500 µm. (C) Mean GFP fluorescence intensity of entire NCI-H929-GFP-Luc2/CAR-T co-culture wells quantified at 0 hours and after 24 hours of co-culture. Error bars indicate the standard deviation of three biological replicates. ns = not significant, **** = $p < 0.0001$, unpaired t-test. (D) Number of GFP+ cells quantified at 0 hours and after 24 hours of NCI-H929-GFP-Luc2 co-culture with either mock or BCMA CAR-T cells. Error bars indicate the standard deviation of three biological replicates. ns = not significant, ** = $p < 0.005$, unpaired t-test.

CONCLUSIONS

Here, we developed the dual reporter cell lines Raji-GFP-Luc2 (ATCC® CCL-86-GFP-LUC2™) and NCI-H929-GFP-Luc2 (ATCC® CRL-3580-GFP-LUC2™) and demonstrated their use in evaluating CAR-T cell cytotoxicity via bioluminescence and live fluorescence imaging. The endogenous expression of CD19 in Raji-GFP-Luc2 and BCMA in NCI-H929-GFP-Luc2 facilitates efficient targeting by the appropriate CAR-T cells and represents a more physiologically relevant system for CAR-T cell research as compared to cell lines engineered to overexpress antigen targets on their cell surface. To further delve into the dynamics of how CAR-T cells target cancer cells, we performed quantification of our live fluorescence imaging data and verified loss of fluorescence from the reporter cancer cells after co-culture with targeting CAR-T cells. Our results highlight how the bioluminescence and live fluorescence imaging assays are simple, robust, and highly sensitive for determining cytotoxicity levels of CAR-T cells. Overall, the reporter cell lines can be effectively used in these assays and will assist researchers in determining the best conditions for improved CAR-T cell targeting and cancer cell killing.

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10801 University Boulevard
Manassas, Virginia 20110-2209

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