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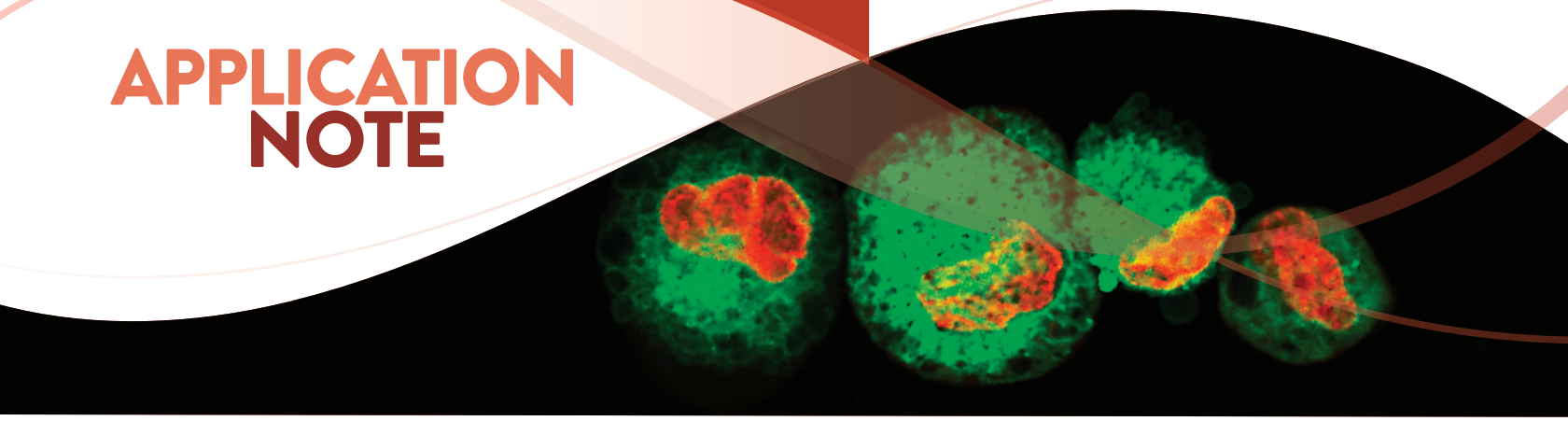
APPLICATION NOTE



LUCIFERASE REPORTER CANCER CELL LINES FACILITATE CAR-T DEVELOPMENT

John Foulke, MS; Luping Chen, BS; Brian Della Fera, BS; Hyeyoun Chang, PhD; Kevin Tyo, PhD; Zhizhan Gu, PhD; and Fang Tian, PhD
Immuno-oncology Group, Cell Biology R&D, ATCC, Gaithersburg MD 20877

ABSTRACT

Chimeric antigen receptor (CAR)-T cells have displayed remarkable efficacy in treating malignant cancers, particularly liquid tumors. CAR-T cells have proven to be a new type of "living" therapeutic that harnesses the patient's immune system to recognize specific tumor-associated antigens and redirects the engineered T cells to more specifically target tumor cells.[1] Considerable research efforts have been invested into developing new CAR structures to increase the scope of targeted cancer types and raise their antitumor efficacy.[2] Evaluating the biofunction of CAR-T cells in vitro typically involves a series of labor-intensive co-culture experiments and immunoassays, where reproducibility remains a challenge during the validation of new CAR-T cells due to donor-to-donor variation and other possible factors.[3] In this study, we present CAR-T Target Luciferase Reporter Cells that have high endogenous expression of CD19, CD20, and HER2, which makes them more physiologically relevant as in vitro tools to develop adoptive CAR-T cell therapies. These liquid and solid tumor cell lines exhibit sensitive and stable luciferase reporter expression that can be used to measure the potency and efficacy of a wide range of CAR structures engineered into T cells for autologous therapy.

INTRODUCTION

CAR-T cell-based therapeutics have emerged as a promising immunotherapy for treating specific leukemias, lymphomas, and myelomas. In this exciting approach to treating refractory cancers, T cells are isolated from a patient's blood via apheresis. The cells are then incubated with the cytokine interleukin 2 and anti-CD3 antibodies to stimulate proliferation. Ex vivo silencing of genes involved in graft rejection is conducted to aid in improving T-cell stability after infusion. Once expanded, the appropriate CAR is introduced into the cells by retroviral transduction. These effector CAR-T cells are then infused back into the patient where they can exert their cytotoxic effects on tumor cells.[1]

Considerable research efforts have been invested into developing new CAR structures to increase the scope of targeted cancer types and raise their antitumor efficacy. One of the bottlenecks in the process of CAR-T development is evaluating the biofunction of CAR-T cells. This in vitro process typically involves a series of labor-intensive co-culture experiments and immunoassays, where reproducibility remains a challenge during the validation of new CAR-T cells due to donor-to-donor variation and other possible factors. In addition to reproducibility being an issue, the validation assays themselves can be problematic.

For example, the use of the radioactive ^{51}Cr release assay has a major drawback; data can only be acquired at a single time point. Moreover, reagent half-life, protective measures, and waste disposal are critical factors because of the assay's intrinsic radioactivity. Nonradioactive assays for CAR-T functional evaluation are available, although these require a time-consuming labeling step and suffer from intra- and inter-assay variability stemming from inconsistent dye uptake and spontaneous dye leakage over the course of the assay.

A method of studying CAR-T effector function that eliminates the concerns of ^{51}CR release and dye loading assays is the bioluminescence (BLI) reporter assay. In BLI reporter assays, target cells that constitutively express luciferase are co-cultured with the candidate effector cells and cytotoxicity is monitored via loss of BLI signal.[4] In addition to their ease of use, the luciferase-expressing target cells in BLI assays can improve interexperimental reproducibility.

To provide target cells for immuno-oncology researchers adopting BLI assays, we generated CAR-T Target Luciferase Reporter Cells that can be used to examine the function of CAR-T cells. These reporter cells naturally express high levels of clinically relevant CAR-T target antigens on the cell surface.

ATCC's CAR-T Target Luciferase Reporter Cells were derived from a variety of highly malignant liquid and solid cancer types, namely B cell lymphoma, Burkitt's lymphoma, Non-Hodgkin's B cell lymphoma, and ductal breast carcinoma. These novel target cells were generated from parental tumor cells that have high endogenous expression of target antigens such as CD19, CD20, and HER2. Stable luciferase-expressing clones were engineered to display high signal-to-noise ratios, aiding in data interpretation.

To generate the CAR-T Target Luciferase Reporter Cells, antibiotic selection and single cell sorting were performed to isolate stable clones with high luciferase expression via the introduction of a Lenti-LUC2 luciferase reporter into the parental cell lines. The target antigen and luciferase were then verified to have expression stability by comparing the low-passage and the high-passage reporter cells. Once stable clones were selected and the expression of antigen and luciferase was verified, the reporter cell lines were characterized and authenticated using tried-and-true methods such as short tandem repeat (STR) profiling, mycoplasma detection, and cell growth rate and morphology assays.

The performance of the CAR-T Target Luciferase Reporter Cells was verified in T cell co-culture experiments. Commercially available CAR-T cells targeting CD19, CD20, and HER2 were employed in this study, with which empty vector-transduced T cells from the same donor were paired as controls. The cytotoxicity of the CAR-T cells against target tumor cells was measured using a luciferase assay, a commercially available potency assay, and a bright field and fluorescence live cell imaging assay. Our results demonstrate that the luciferase reporter system is a simple, robust, and highly sensitive means to measure biological processes in cancer and T cell ex vivo co-cultures. In summary, CAR-T Target Luciferase Reporter Cells from ATCC are well-characterized tools with high reproducibility for studying CAR-T biofunction and validating new CAR-T agents for cancer immunotherapy.

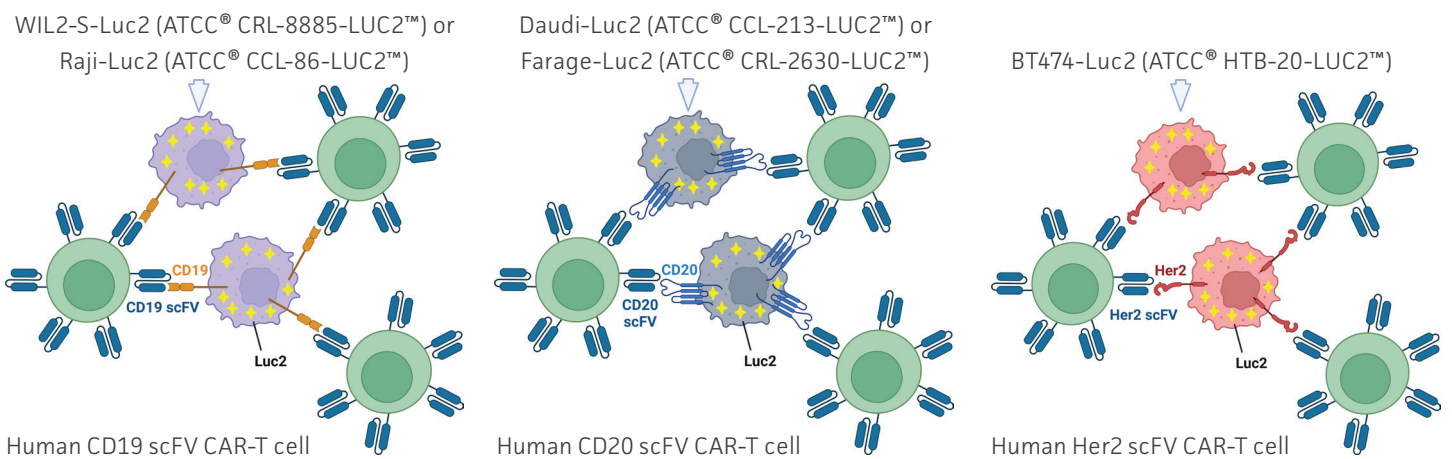


Figure 1: CAR-T Target Luciferase Reporter Cells. Schematic showing CAR-T target cells with expression of CD19-positive WIL2-S-Luc2 and Raji-Luc2, CD20-positive Daudi-Luc2 and Farage-Luc2, and HER2-positive BT-474-Luc2 being surrounded and attacked by CD19-, CD20-, and HER2-targeting CAR-T cells, respectively. Created with BioRender.com.

Table 1: CAR-T Target Luciferase Reporter Cells

Designation	ATCC® No.	Disease	Target
WIL2-S-Luc2	CRL-8885-LUC2™	B Cell Lymphoma	CD19
Raji-Luc2	CCL-86-LUC2™	Burkitt's Lymphoma	CD19
Daudi-Luc2	CCL-213-LUC2™	Burkitt's Lymphoma	CD20
Farage-Luc2	CRL-2630-LUC2™	Non-Hodgkin's B Cell Lymphoma	CD20
BT-474-Luc2	HTB-20-LUC2™	Breast Ductal Carcinoma	HER2

RESULTS WITH MATERIAL AND METHODS

To generate the CAR-T Target Luciferase Reporter Cells, we selected human tumor cell lines exhibiting high endogenous expression of clinically relevant CAR-T target antigens (CD19, CD20, HER2) on the cell surface. WIL2-S (ATCC® CRL-8885™), Raji (ATCC® CCL-86™), Daudi (ATCC® CCL-213™), Farage (ATCC® CRL-2630™), and BT-474 (ATCC® HTB-20™) were transduced with a lentiviral plasmid expressing luciferase under the control of an EF1A promoter. The genetically modified cells were grown under antibiotic selection, and single cell sorting was performed to isolate individual clones that were verified to stably express luciferase (Table 1).

To demonstrate the performance of the CAR-T Target Luciferase Reporter Cells, we used cancer and T cell co-culture experiments. Commercially available CAR-T cells targeting CD19, CD20, and HER2 were employed in this study; empty vector-transduced T cells from the same donor were paired as controls. The cytotoxicity of the CAR-T cells against target tumor cells was measured using a luciferase assay, a commercially available potency assay, and a bright field and fluorescence live cell imaging assay. A luminescence assay was used to evaluate the functionality of the luciferase reporter cell lines in which the cells were co-cultured with antigen-specific CAR-T cells (ProMab) or mock CAR-T (ProMab) cells derived from the same donor as a control at various target to effector ratios (1:1, 2:1, 5:1, and 10:1). After co-culturing for 24 hours, the Bright-Glo™ Luciferase Assay System (Promega) was used and luminescence was measured on a plate reader.

Analysis showed a clear dose-dependent decrease in luminescence, indicating the antigen-specific CAR-T killing potential was greater than the non-specific killing observed when co-culturing with mock CAR-T cells (Figures 2A, 2B, 3A, 4A, and 5A). Live cell imaging studies were conducted using the same method and were observed microscopically every 30 minutes to 1 hour using the Cytation 1 system (Agilent). The luciferase-expressing cells were either pre-stained with Vybrant™ (Thermo Fisher) DiO (Figure 2C and 2D) or co-cultured in the presence of Incucyte® Cytotox red dye (Sartorius)[5] (Figures 4B and 5B). Incucyte Cytotox red, which stains dead cells, shows a dose-dependent increase in fluorescence intensity when cells are co-cultured with antigen-specific CAR-T cells (Figures 4C, 4D, 5C, and 5D). BT-474-Luc2 when co-cultured with HER2 CAR-T cells or mock CAR-T cells from the same donor were measured in a real-time cell analysis assay using the xCelligence (Agilent)[6] (Figure 3B) system and impedance was measured every 15 minutes. BT-474-Luc2 cells co-cultured with HER2 CAR-T cells show a greater decrease in cell impedance as the cells lift off the plate as compared to co-culturing with mock CAR-T cells or BT-474-Luc2 cells alone.

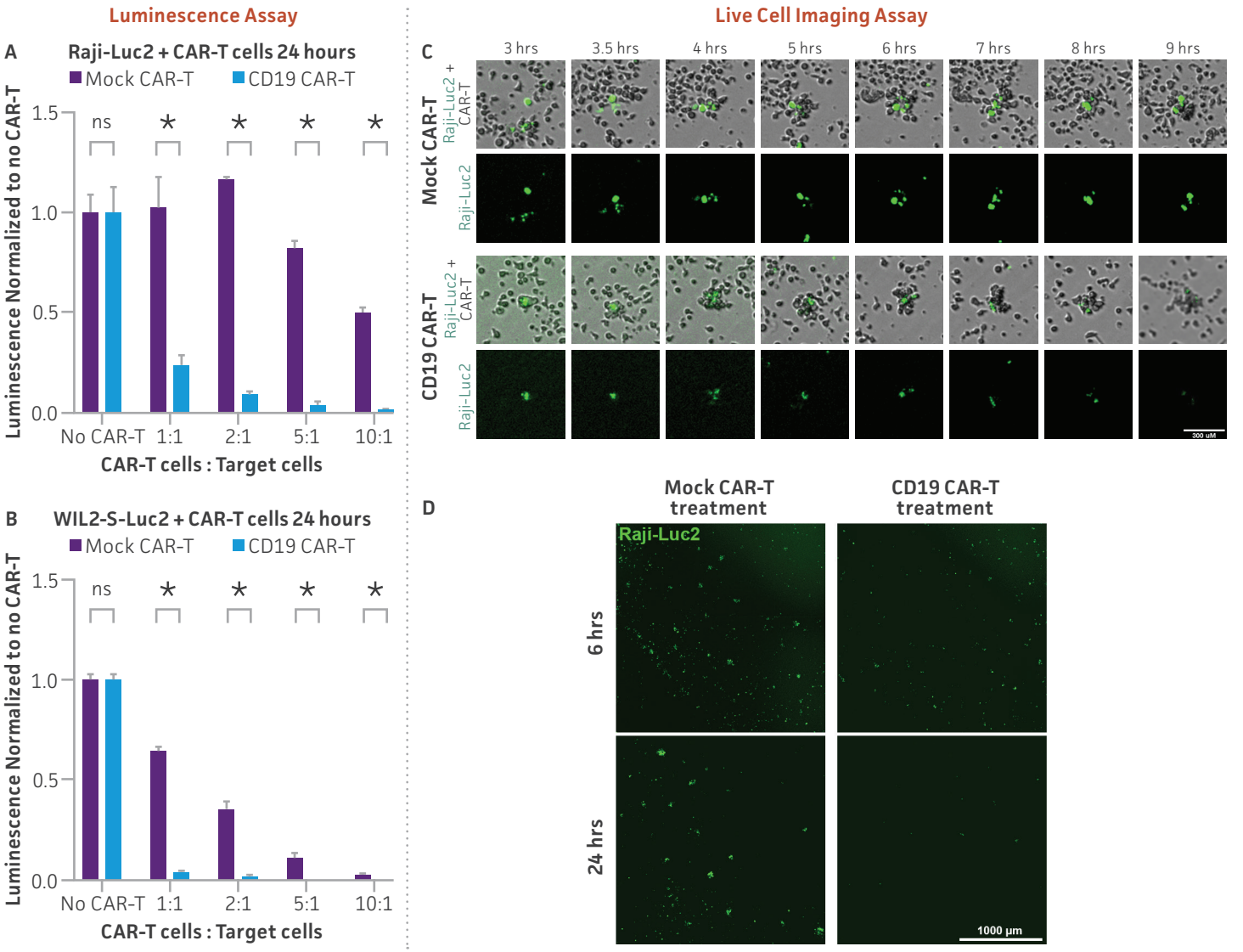


Figure 2: CD19 CAR-T in vitro killing assay of Raji-Luc2 and WIL2-S-Luc2 measured using luminescence and live cell imaging. (A) CD19-positive Raji-Luc2 cells (5 x 10^3) or (B) WIL2-S-Luc2 cells (5 x 10^3) were seeded into a 96-well plate and were used as target cells for either CD19 CAR-T or Mock CAR-T (control) from the same donor, which were seeded at various ratios of CAR-T cells to target Raji-Luc2 or WIL2-S-Luc2 cells (1:1, 2:1, 5:1, and 10:1). After 24 hours of co-culture, Bright-Glo was added to the indicated wells. The luminescence of the plate was read within 10 minutes using a luminescence plate reader and determined to have a dose-dependent-specific killing with CD19 CAR-T cells that was greater than the non-specific killing observed with mock CAR-T cells (* = significant difference and ns = not significant using unpaired t test, with a single pooled variance). (C) Raji-Luc2 cells were stained with Vybrant DiO dye and real-time fluorescent imaging was measured every 30 minutes for 24 hours during the co-culture of Raji-LUC2 cells with CAR-T cells. (D) Two stained Raji-Luc2 cells (Green) from the co-culture experiment were tracked for 6 hours and became surrounded by CAR-T cells, resulting in a decrease of fluorescence when treated with CD19 CAR-T as compared to co-cultures with Mock-CAR-T cells. After 24 hours of co-culture, CD19 CAR-T cells showed a decrease in fluorescent cells as compared to 6 hours; in a co-culture with Mock CAR-T cells numerous Raji-LUC2 cells were present.

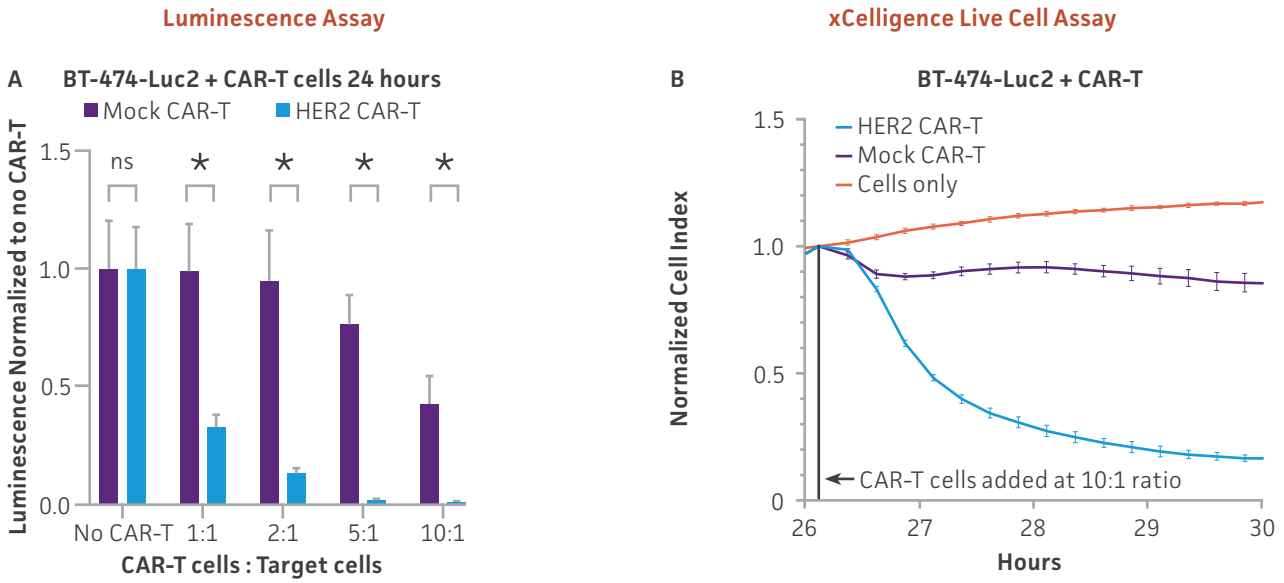


Figure 3: HER2 CAR-T in vitro killing assay of BT-474-Luc2 measured using luminescence assay and xCelligence. (A) HER2-positive BT-474-Luc2 cells (5 x 10^3) were seeded into a 96-well plate and were used as target cells for either HER2 CAR-T or Mock CAR-T (control) from the same donor, which were seeded at various ratios of CAR-T cells to target BT-474-Luc2 cells (1:1, 2:1, 5:1, and 10:1). After 24 hours of co-culture, Bright-Glo was added to the indicated wells. The luminescence of the plate was read within 10 minutes using a luminescence plate reader and determined to have a dose-dependent-specific killing with HER2 CAR-T cells, which was greater than the non-specific killing observed with mock CAR-T cells (* = significant difference and ns = not significant using unpaired t test, with a single pooled variance). (B) HER2 CAR-T cells were used to target 2 x 10^4 HER2-positive BT-474-Luc2 at a 10:1 ratio and cell killing was measured using the xCELLigence system. Mock CAR-T cells from the same donor were used as a control.

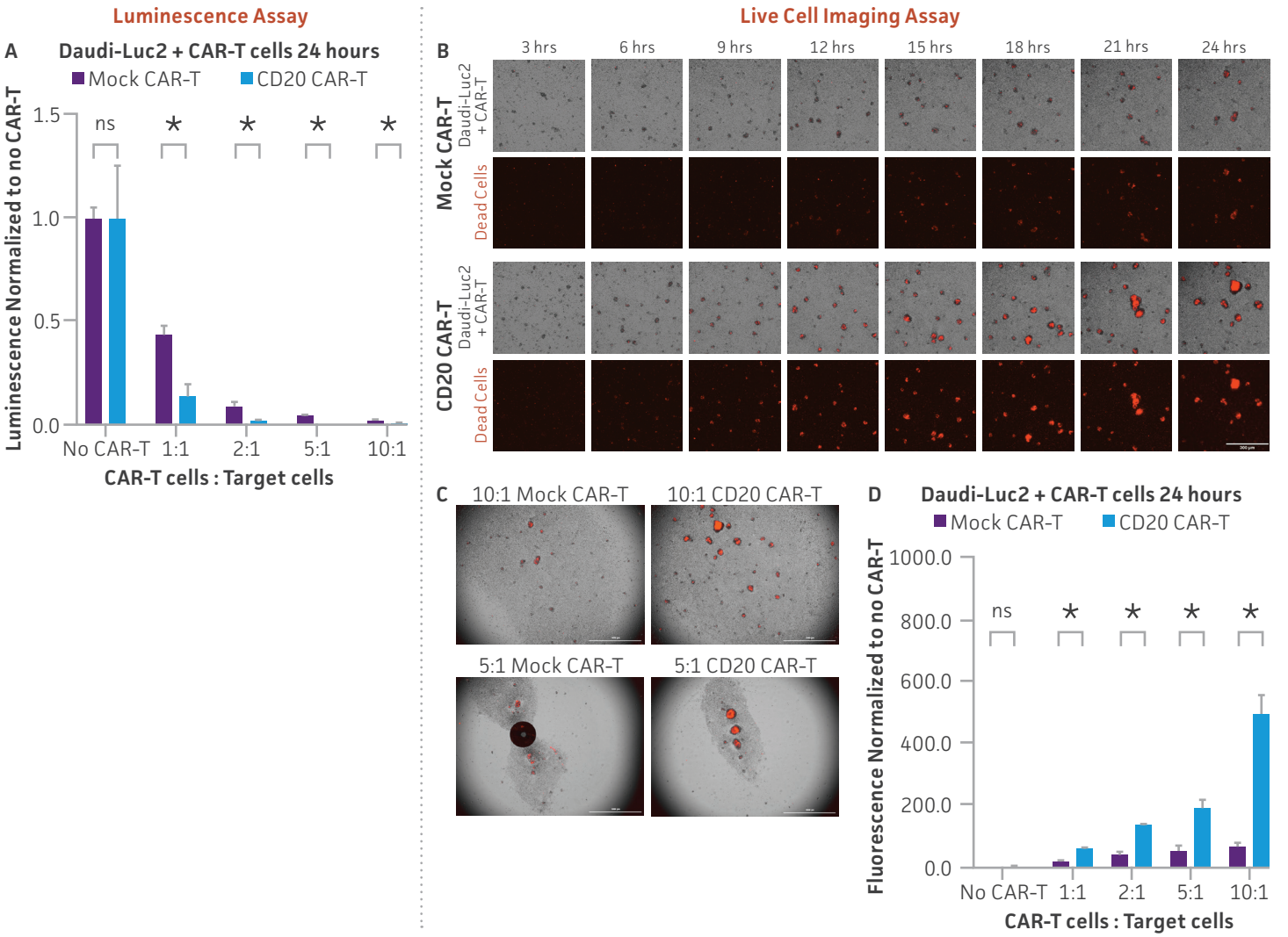


Figure 4: CD20 CAR-T in vitro killing assay of Daudi-Luc2 measured using luminescence and live cell imaging. (A) CD20-positive Daudi-Luc2 cells (5 x 10^3) were seeded into a 96-well plate and were used as target cells for either CD20 CAR-T or Mock CAR-T (control) from the same donor, which were seeded at various ratios of CAR-T cells to target Daudi-Luc2 cells (1:1, 2:1, 5:1, and 10:1). After 24 hours of co-culture, Bright-Glo was added to the indicated wells. The luminescence of the plate was read within 10 minutes using a luminescence plate reader and determined to have a dose-dependent-specific killing with CD20 CAR-T cells, which was greater than the non-specific killing observed with mock CAR-T cells (* = significant difference and ns = not significant using unpaired t test, with a single pooled variance). (B) Daudi-Luc2 cells (5 x 10^3) were co-cultured with CD20 CAR-T cells or Mock CAR-T cells in the presence of Incucyte Cytotox red dye in the medium and real-time fluorescent imaging was measured every hour for 24 hours, resulting in an increase of fluorescence intensity when co-cultured with CD20 CAR-T as compared to co-cultures with Mock-CAR-T cells. (C) After 24 hours of co-culture with CD20 CAR-T cells, Daudi-Luc2 showed an increase in the number dead (red) fluorescent cells as compared co-culture with Mock CAR-T cells. (D) The clustered red fluorescence was quantified and compared (* = significant difference and ns = not significant using unpaired t test, with a single pooled variance).

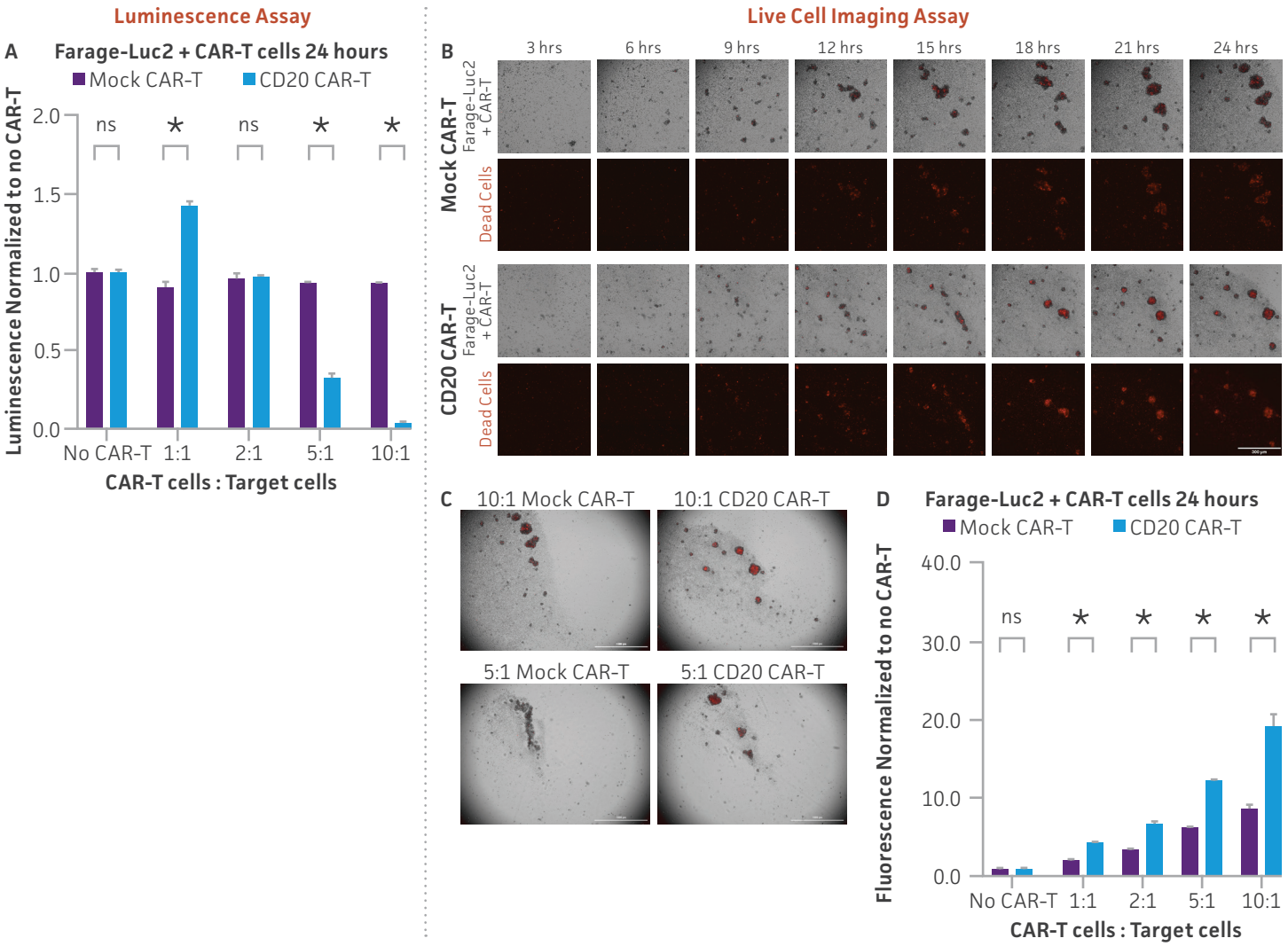


Figure 5: CD20 CAR-T in vitro killing assay of Farage-Luc2 measured using luminescence and live cell imaging. (A) CD20-positive Farage-Luc2 cells (5 x 10^3) were seeded into a 96-well plate and were used as target cells for either CD20 CAR-T or Mock CAR-T (control) from the same donor, which were seeded at various ratios of CAR-T cells to target Farage-Luc2 cells (1:1, 2:1, 5:1, and 10:1). After 24 hours of co-culture, Bright-Glo was added to the indicated wells. The luminescence of the plate was read within 10 minutes using a luminescence plate reader and determined to have a dose-dependent-specific killing with CD20 CAR-T cells, which was greater than the non-specific killing observed with mock CAR-T cells (* = significant difference and ns = not significant using unpaired t test, with a single pooled variance). (B) Farage-Luc2 cells (5 x 10^3) were co-cultured with CD20 CAR-T cells or Mock CAR-T cells in the presence of Incucyte Cytotox red dye in the medium and real-time fluorescent imaging was measured every hour for 24 hours, resulting in an increase of fluorescence intensity when co-cultured with CD20 CAR-T as compared to co-cultures with Mock-CAR-T cells. (C) After 24 hours of co-culture with CD20 CAR-T cells, Farage-Luc2 showed an increase in the number dead (red) fluorescent cells as compared co-culture with Mock CAR-T cells. (D) The clustered red fluorescence was quantified and compared (* = significant difference and ns = not significant using unpaired t test, with a single pooled variance).

CONCLUSIONS

The CAR-T Target Luciferase Reporter Cells described in this study provide an advantage in measuring target cell killing without the use of a radioactive ^{51}Cr release assay or pre-labeling the cells for CAR-T functional evaluation.[4] In addition, these reporter cell lines were characterized and authenticated using cell morphology, growth kinetics, and STR analysis.[7] The expression stability of both the target antigen and luciferase was verified by comparing the low-passage and the high-passage reporter cells. Importantly, cytotoxicity in these luciferase-expressing cells could be measured by the loss of BLI signal in real time, and this loss was confirmed by live cell imaging and cytotoxic dye uptake assays. In summary, the well-characterized luciferase reporter cell lines enable convenient and consistent signal quantification, and they are easy-to-use tools for studying CAR-T biofunction and validating new CAR-T agents for cancer immunotherapy. These robust cell models are representative of the most predominant patient-derived carcinoma and lymphoma cancer lines used in oncology research. The CAR-T Target Luciferase Reporter Cells were selected from ATCC's extensive catalog of established cancer cell lines that contain high endogenous expression of some of the most prevalent cancer antigens, which makes them more physiologically relevant as in vitro tools to develop adoptive CAR-T cell therapies.

REFERENCES

1. Kiesgen S, et al. Comparative analysis of assays to measure CAR T-cell-mediated cytotoxicity. Nat Protoc 16: 1331-1342, 2021. PubMed: 33589826
2. Jackson HJ, et al. Driving CAR T-cells forward. Nat Rev Clin Oncol 13: 370-383, 2016. PubMed: 27000958
3. Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. Blood Cancer J 11: 69, 2021. PubMed: 33824268
4. Riss T, et al. Cytotoxicity Assays: In Vitro Methods to Measure Dead Cells, in Assay Guidance Manual. (eds. S. Markossian et al.) (Bethesda (MD); 2004). PubMed: 31070879
5. Meijer TWH, et al. Targeting glucose and glutamine metabolism combined with radiation therapy in non-small cell lung cancer. Lung Cancer 126: 32-40, 2018. PubMed: 30527190
6. Lisby AN, et al. Evaluation of CAR-T cell cytotoxicity: Real-time impedance-based analysis. Methods Cell Biol 167: 81-98, 2022. PubMed: 35153000
7. Capes-Davis A, et al. Check your cultures! A list of cross-contaminated or misidentified cell lines. Int J Cancer 127: 1-8 2010. PubMed: 20143388

10801 University Boulevard
Manassas, Virginia 20110-2209

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