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A syngeneic mouse B-cell lymphoma model for pre-clinical evaluation of CD19 CAR T-cells

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Cover letter

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Dear Indrani,

Please find enclosed our manuscript entitled:

"**A syngeneic mouse B-cell lymphoma model for pre-clinical evaluation of CD19 CAR T-cells**"

Thank you for inviting me to submit this manuscript.

As discussed, I have included a protocol for the pre-clinical evaluation of CD19 CAR T-cells in both lymphodepleted and lymphoreplete mice.

Lymphodepleted mice are representative of the vast majority of patients receiving CAR T-cell therapy. This model allows testing of CAR T-cells without the xenegenis GvHD observed when using human T-cells in NSG mice.

Lymphoreplete mice allow the testing of consequences of interactions of therapies with endogenous immune cells. Something we have shown can be important in eradication of lymphoma and the induction of long term immunity.

Despite phenomenal success, CD19 CAR T cell therapy still has challenges such as with cost, toxicity and overcoming antigen escape and relapse. Perhaps just as pertinent, is that CD19 CAR T-cells can be used as a model system for making improvements to this mode of therapy that can then be applied to the solid tumour setting. These protocols will help those looking to address these challenges. In a wider sense, this syngeneic lymphoma model will be useful for testing a range of immunotherapy agents that interact with the host immune system to achieve tumour clearance.

Some figures from this manuscript have been previously published by me, or are adapted from my previously published works. The authors all approve submission of this manuscript and declare no potential conflicts of interest apart from Dr David Gilham who works for Celyad, who are developing NK-CAR T-cells receptors.

We look forward to your response
Kind regards

Gray Kueberuwa
Postdoctoral Research Associate

TITLE:

A Syngeneic Mouse B-Cell Lymphoma Model for Pre-Clinical Evaluation of CD19 CAR T Cells

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KEYWORDS:

CAR T cells, TRUCKs, adoptive T-cell therapy, syngeneic mouse model, A20, immunotherapy, IL-12, pre-conditioning, CD19, lymphoma

SHORT ABSTRACT:

Here, we present a protocol for the production and pre-clinical testing of murine CD19 CAR T cells by retroviral transduction and utilization as a therapy against established syngeneic A20 B-cell lymphoma in BALB/c mice with or without lymphodepleting pre-conditioning.

LONG ABSTRACT:

The astonishing clinical success of CD19 chimeric antigen receptor (CAR) T-cell therapy has led to the approval of two second generation chimeric antigen receptors (CARs) for acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL). The focus of the field is now on emulating these successes in other hematological malignancies where less impressive complete response rates are observed. Further engineering of CAR T cells or co-administration of other treatment modalities may successfully overcome obstacles to successful therapy in other cancer settings.

We therefore present a model in which others can conduct pre-clinical testing of CD19 CAR T cells. Results in this well tested B-cell lymphoma model are likely to be informative CAR T-cell therapy in general.

This protocol allows the reproducible production of mouse CAR T cells through calcium phosphate transfection of Plat-E producer cells with MP71 retroviral constructs and pCL-Eco

packaging plasmid followed by collection of secreted retroviral particles and transduction using recombinant human fibronectin fragment and centrifugation. Validation of retroviral transduction, and confirmation of the ability of CAR T cells to kill target lymphoma cells *ex vivo*, through the use of flow cytometry, luminometry and enzyme-linked immunosorbent assay (ELISA), is also described.

Protocols for testing CAR T cells *in vivo* in lymphoreplete and lymphodepleted syngeneic mice, bearing established, systemic lymphoma are described. Anti-cancer activity is monitored by *in vivo* bioluminescence and disease progression. We show typical results of eradication of established B-cell lymphoma when utilizing 1st or 2nd generation CARs in combination with lymphodepleting pre-conditioning and a minority of mice achieving long term remissions when utilizing CAR T cells expressing IL-12 in lymphoreplete mice.

These protocols can be used to evaluate CD19 CAR T cells with different additional modification, combinations of CAR T cells and other therapeutic agents or adapted for the use of CAR T cells against different target antigens.

INTRODUCTION:

Chimeric antigen receptor (CAR) T-cell therapy has shown astonishing clinical success in the treatment of CD19^{+} malignancies leading to the approval of tisagenlecleucel for relapsed acute lymphoblastic leukaemia[1] and axicabtagene ciloleucel for progressive large B-cell non-Hodgkin lymphoma[2] in 2017.

The importance of Interactions between cancer and the immune system in both disease progression and therapeutic mechanisms is becoming increasingly recognized[3-5]. For example, it is well documented that the tumor microenvironment (TME) is awash with factors that can suppress the effector functions of immune cells[6-8]. Alternatively priming of endogenous immune cells and epitope spreading can be key in tumor eradication and long term resistance to tumor challenge[9,10]. Both of these phenomena cannot be evaluated in xenogeneic models that lack an immune system. Likewise, systems utilizing transgenic proteins do not accurately reflect the challenge of breaking immune tolerance which is required for epitope spreading[11,12]. A syngeneic model with a fully functional immune system is, therefore, paramount for modeling these important aspects of cancer disease progression and immune therapeutics.

An important caveat of CAR T-cell therapy is that lymphodepleting pre-conditioning is required for therapeutic success[13,14]. This is typically achieved in patients by administering chemotherapy prior to infusion of CAR T cells[15,16]. As a standard method, in order to mimic lymphodepletion used in the patient setting, we administer 5 Gy total body irradiation (TBI) to achieve lymphodepletion prior to administration of therapeutic CAR T cells to mice bearing systemic A20 B-cell lymphoma.

While lymphodepleting pre-conditioning is not an issue for the majority of patients, toxicity that comes with chemotherapeutic agents means that patients of low performance status are not eligible for CAR T-cell therapy. To create a test system that represents the patients ineligible for

lymphodepletion, we established a lymphoreplete syngeneic mouse model in which we model CAR T-cell therapy of lymphoma. In this model, we showed that the secretion of IL-12 from within CAR T cells could lead to eradication of established lymphoma with a success rate of ~ 25%[17]. Moreover, we showed that endogenous immune cells were involved in cancer eradication.

Here we describe in detail the protocol for the production of mouse CAR T cells, establishing lymphoma in syngeneic mice, and treatment of lymphoma with CAR T cells with or without the use of lymphodepleting pre-conditioning. This can be used for combination studies of CAR T cells with other agents, testing CAR T cells with other transgenes or for the use of other adoptive cell therapy or immunotherapy strategies against lymphoma.

PROTOCOL:

All animal experiments were conducted under the auspices of the Animals (Scientific Procedures) Act 1986 and under UK Coordinating Committee for Cancer Research guidelines. All animal studies were conducted at the CRUK-Manchester institute and approved by the local animal welfare and ethics review body (CRUK-MI AWERB).

1. Preparations

1.1. Maxiprep pMP71 retroviral construct plasmid and pCL-Eco retrovirus packaging plasmids[18].

Note: pMP71 encodes mCherry and the CAR separated by an FMDV2A sequence. This is interchangeable with other retroviral constructs. pCL-Eco encodes gag, pol and the ecotropic envelope proteins.

1.2. Prepare complete T cell medium (TCM) for culturing mouse T cells using RPMI 1640 medium, 10% FCS, 1% 100x penicillin-streptomycin-glutamine (PSG).

Note: The solution contains 100 IU/mL penicillin, 100 µg/mL of streptomycin and 2 mM of L-glutamine), 50 µM β-mercaptoethanol and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

1.3. Culture A20 cells in RPMI 1640, 10% FCS and 0.05 mM β-mercaptoethanol at 37 °C, 5% CO_2.

1.4. Culture the Platinum-E (Plat-E) cells in complete Dulbecco's modified eagle medium (DMEM) (DMEM with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 µg/mL puromycin and 10 µg/mL blasticidin) at 37 °C, 5% CO_2.

Note: Plat-E cells are derived from 293T cells and express gag, pol and ecotropic envelope retroviral proteins.

1.5. Prepare transfection media solutions 1 and 2 immediately prior to transfection. Prepare solution 1 (pH 7.9) to contain DMEM + 10% FCS + 25 mM HEPES, solution 2 (pH 7.1) to contain DMEM + 25 mM HEPES.

1.6. Prepare 10 µg/mL recombinant human fibronectin fragment solution by diluting with sterile phosphate-buffered saline (PBS) and store at -20 °C until use.

1.7. Sterile filter all media through 0.2 µm filters prior to use (excluding recombinant human fibronectin fragment).

2. Retroviral Transduction of T cells

2.1. Day 1: Preparation for transfection

2.1.1. Seed 7.5 x 10^6 Platinum-E (Plat-E) cells in 15 cm^2 tissue culture dishes in 18 mL of complete DMEM and incubate overnight at 37 °C, 5% CO_2.

2.2. Day 2: Transfection of Plat-E retroviral packaging cell line

2.2.1. Remove DMEM media from the 15 cm^2 dishes and replace with 12 mL of transfection solution 1.

CAUTION: When changing the media, the 15 cm^2 dishes can dry at the center. This can cause substantial death of transfected Plat-E cells. Work swiftly and remove media from just 1-2 plates at a time.

2.2.2. Prepare 20.4 µg of pcl-Eco packaging vector DNA, 39.6 µg of plasmid DNA encoding retroviral CAR construct and 150 µL of 1 M $CaCl_2$ to final volume of 3 mL transfection solution 2 per 15 cm^2 dish.

2.2.3. Vortex for 10 s and rest for 5 min. Add 3 mL transfection solution 2 containing DNA and $CaCl_2$ to each 15 cm^2 dish drop-wise, evenly across each plate. Gently rock plates with a side to side motion for 10 s. Incubate at 37 °C, 5% CO_2 overnight.

2.3. Day 3: Preparation of virus-containing supernatant for transduction

2.3.1. Replace the media of transfected Plate-E cells with 18 mL complete TCM.

CAUTION: When changing the media 15 cm^2 dishes can dry at the center. This can cause substantial death of transfected Plat-E cells. Work swiftly and remove media from just 1-2 plates at a time.

2.4. Day 3: Isolation and *in vitro* activation of mouse splenic T cells

2.4.1. Remove spleens from 6-8-week-old BALB/c mice as previously described by Parkinson *et al.*[19] and immerse them in sterile, ice-cold, PBS in a 50 mL conical tube.

2.4.2. Use tweezers to transfer a spleen to a 1.5 mL microcentrifuge tube and homogenize using a pestle with minimal force.

2.4.3. Use a 1000 µL pipette and ~ 800 µL PBS to transfer homogenate to a 100 µm pore cell strainer affixed to a 50 mL tube containing 5 mL PBS to attain a single cell suspension. Repeat step 2.4.2 for the additional spleens. Do not exceed 3 spleens per tube.

CAUTION: Splenocytes passed through filter can form clumps if left standing. Manually swirl tubes intermittently if processing several spleens to avoid cell clumping. Remaining fragments on the cell strainer can be further mashed using a plunger from a 5 mL syringe using minimal force.

2.4.4. Top up to 20 mL with PBS. Layer the 20 mL cell suspension gently onto 20 mL of density gradient media (**Table of Materials**) in a 50 mL tube. Centrifuge the resultant overlaid suspension at 800 x g for 20 min with no brake applied.

2.4.5. Harvest cells at interface layer using a sterile Pasteur pipette and transfer to a 50 mL tube. Top up to 50 mL with PBS and centrifuge at 800 x g for 10 min to wash. Discard the supernatant and re-suspend cells in complete TCM.

2.4.6. Count the number of cells using a hemocytometer.

2.4.7. Culture cells at a density of 5 x 10^6 cells/mL in complete TCM with 30 ng/mL anti-CD3ε antibody (Clone 145-2C11), 30 ng/mL anti-CD28 antibody (Clone 37.51), 100 U/mL recombinant human IL-2 and 2 ng/mL recombinant murine IL-7. Use an appropriately sized tissue culture flask for the volume of cells harvested.

Note: Antigen-presenting cells are required for T-cell activation by CD3 and CD28 antibodies, if working with purified T cells it is necessary to coat plates with antibodies, or use magnetic beads (**Table of Materials**)

2.4.8. Incubate mouse splenocytes at 37 °C, 5% CO_2 overnight.

2.5. **Day 3: Preparation of plates for transduction**

2.5.1. Coat non-tissue-culture 6-well plates with 2 mL of 10 µg/mL recombinant human fibronectin fragment and incubate overnight at 4 °C.

2.6. **Day 4: Transduction of mouse T cells**

2.6.1. Transfer recombinant human fibronectin fragment from coated plates to fresh non-tissue-culture 6-well plates. Incubate these plates overnight at 4 °C for round 2 of transduction.

2.6.2. Add 2 mL of TCM to each well of recombinant human fibronectin fragment-coated plates and leave for 30 min at room temperature to block non-specific binding.

2.6.3. Harvest retrovirus-containing supernatant from transfected Plat-E cells in 15 cm tissue culture dishes and replace with 18 mL of complete TCM.

CAUTION: Work swiftly to avoid drying of Plat-E cells.

Note: Success of transfection can be checked at this stage by fluorescence microscopy if utilizing a fluorescent marker gene such as mCherry (**Figure 1**).

2.6.4. Filter the retrovirus-containing supernatant through 0.45 µm filter to remove cell debris. Remove TCM from recombinant human fibronectin fragment-coated 6-well plates and add 2.5 mL of filtered retrovirus-containing supernatant or to each well (use complete TCM for mock transfection). Label each well as to the addition of retrovirus or mock media.

2.6.5. Centrifuge the plates at 1200 x g for 30 min at room temperature.

2.6.6. Whilst plates are spinning, collect activated T cells and count using a hemocytometer.

2.6.6.1. Transduction is carried out with 5 x 10^6 activated splenocytes in a total of 5 mL/well. Pellet the required number of splenocytes for mock/transduction in separate tubes by centrifugation at 500 x g for 5 min.

2.6.6.2. Re-suspend splenocytes at a density of 5 x 10^6 cells per 2.5 mL of filtered retrovirus-containing supernatant or complete TCM for control. Add recombinant human IL-2 (hIL-2) and recombinant mouse IL-7 (mIL-7) to a final concentration of 200 IU/mL and 4 ng/mL respectively.

2.6.7. Collect 6-well plates from the centrifuge at the completion of step 2.6.5 and add 2.5 mL/well re-suspended splenocytes into appropriate wells to make a final volume of 5 mL/well and a final concentration of 100 U/mL hIL-2 and 2 ng/mL mIL-7.

2.6.8. Centrifuge the plates at 1200 x g for 90 min at room temperature. After centrifugation, incubate the plates at 37 °C, 5% CO_2 overnight.

2.7. **Day 5: Round 2 of transduction**

2.7.1. Collect the recombinant human fibronectin fragment from the plates as this can be re-used. Repeat steps 2.6.2 - 2.6.5.

2.7.2. Whilst plates are spinning, collect cells from the 1st round of transduction using a Pasteur pipette. Rinse each well with 2 mL PBS, swirl and collect any remaining cells in each well.

Note: Pipette up and down to re-suspend sedimented cells. Collect each control/transduction group in separate tubes.

2.7.3. Centrifuge tubes at 500 x g for 5 min. Re-suspend cells in 2.5 mL per well of transduction with 200 IU/mL IL-2 and 4 ng/mL IL-7. Repeat steps 2.6.7 - 2.6.8.

2.7.4. Remove cells from the centrifuge and incubate at 37 °C, 5% CO_2 for 4 h. Collect transduced cells as in steps 2.7.2-2.7.3.

2.7.5. Count cells, centrifuge at 500 x g for 5 min and re-suspend in complete TCM at a density of 1 x 10^6 cells/mL with 100 U/mL hIL-2 and 2ng/mL mIL-7. Transfer to a suitably sized culture flask and incubate at 37 °C, 5% CO_2.

2.7.6. Add fresh TCM media containing 100U/mL hIL-2 and 2ng/mL mIL-7 every 2 days, maintaining a cell density of 1 x 10^6 cells/mL.

Note: Harvested splenocytes contain a variety of cell types. Under these culture conditions, non T cells die off over the course of 2-3 days. After ~ 4 days in cell culture, the number of T cell is typically equivalent to the total number of harvested splenocytes on day 0.

3. Measurement of Transduction efficiency

3.1. On day 4 post transduction, collect a sample of transduced or non-transduced T cells (approximately 3 x 10^5 cells). Centrifuge the cell suspension at 500 x g for 5 min, discard the supernatant, wash the pelleted cells once with PBS and centrifuge again.

3.2. Discard the supernatant and add 100 µL of PBS containing a suitable amine reactive dye (*e.g.*, live/dead stain, 1 in 100 dilution) per well. Incubate for 15 min at room temperature in the dark.

3.3. Wash twice with PBS and centrifuge at 500 x g for 5 min. Discard the supernatant and incubate with 50 µL of FACS buffer containing anti-mouse CD16/CD32 antibodies for Fc receptor blocking (1 in 100 dilution). Incubate for 10 min at 4 °C.

3.4. Directly add 50 µL of antibody staining master mix containing anti-mouse CD4-BV786 and CD8-BV711 antibodies (final concentration of 1 µL/well in FACS buffer). Incubate for 30 min at 4 °C in dark. Repeat the wash step 3.3. Re-suspend the cells in 1% PFA buffer and keep the in the dark at 4 °C until analysis by flow cytometry.

3.5. Analyze cells with equivalent suitable cytometer using BV711, BV785 and mCherry fluorescence as markers of CD4 and CD8 subset and CAR expression respectively gating as in (**Figure 2**).

4. *In vitro* Validation of CAR T cell Activity

4.1. Seed syngeneic target $CD19^+$ tumor cells with or without luciferase expression at a density of 1 x 10^4 cells in 100 µL TCM/well in a 96-well U-bottom tissue culture plate.

4.2. Add 1 x 10^4 CD19 CAR T cells/well in a volume of 100 µL/well to achieve an effector to target (E:T) ratio of 1:1.

Note: E:T ratios should be established for each CAR construct and target cell line.

4.3. Use T cells alone and tumor cells alone as negative controls and T cells stimulated by phorbol-myristate-acetate (PMA) (50 ng/mL) and ionomycin (1 µg/mL) as positive control for Interferon gamma (IFNγ) release. Co-culture cells at 37 °C, 5% CO_2 for 16-24 h.

4.4. Following co-culture, centrifuge the plates at 500 x g for 5 min and collect the supernatant for further IFNγ and IL-12p70 ELISA analysis.

Note: This can be stored at -80 °C.

4.5. Re-suspend cell pellets in 100 µL of PBS containing luciferin (final concentration of 1.5 mg/mL). Incubate the plates for 10 min at 37 °C. Then measure the luminescence from each well with a suitable luminometer.

Note: Exposure times must be optimized for cell lines and density. Representative results are shown in **Figure 3a**. *Ex-vivo* cytotoxicity of CAR T cells can be modified to express luciferin by co-culture with cell lines expressing target antigen. As CAR T cells kill target cells, luciferin is released, therefore a reduction in luminometry signal is correlated with cell kill. Non-transduced cells can often have an effect on target cell viability, particularly over long incubation periods. Measure the concentration of murine IFNγ and IL-12p70 in the supernatant according to the manufacturer's ELISA protocols. Representative results are shown in (**Figure 3b** and **3c**). *Ex-vivo* activation of CAR T cells by co-culture with cell lines expressing target antigen can be assayed by analyzing supernatant contents using ELISA. The ratio of CAR T cell to target cells and length of co-culture period must be optimized for each CAR construct, target cell line and analyte. PMA and ionomycin treatment can be used as a positive control to confirm quality of T cells and their ability to respond.

5. Assess Anti-cancer Activity in Mice

5.1. **Protocol 1**

5.1.1. Perform 100 mg/kg intravenous (IV) delivery of cyclophosphamide into 6 to 8-week BALB/c mice. This allows tumor engraftment without significant lymphodepletion[17] (**Figure 4**).

Note: Establishing A20 lymphoma can take over 2 months with a suboptimal take rate. This can be improved by the use of cyclophosphamide 1 day prior to the delivery of lymphoma cells. In order to study lymphoreplete mice, we identified a dose of cyclophosphamide that could increase efficiency of lymphoma without causing lymphodepletion.

5.1.2. The next day, inject 100 µL of 5 x 10^5 syngeneic A20 B-cell lymphoma cells modified to express luciferase and green fluorescent protein (GFP) into mice by intravenous (IV) injection.

5.1.3. Allow the mice to develop systemic lymphoma for ~ 17 days.

5.1.4. Confirm the presence of systemic lymphoma by intraperitoneal (IP) injection of 100 µL of 30 mg/mL luciferin and imaging using an *in vivo* bioluminescence imaging system.

5.1.4.1. Use separators to avoid signal spillover into adjacent mice. Expose mice for 1 min on the ventral side with a constant sized region of interest.

5.1.4.2. Display relative light units (RLU) as photons per second (p/s). Settings must be optimized for each tumor model; use an exposure that can pick up early detection of tumors but does not lead to saturation as tumors reach endpoints.

5.1.4.3. Record total RLU for each mouse with a constant sized region of interest. (**Figure 5a** and **b**).

5.1.5. Inject a single dose of 1 x 10^6 CAR T cells by IV injection into lymphoreplete mice bearing established lymphoma.

Note: (Important) Dosing levels must be established for each CAR construct using a dose escalation schedule to ensure that any possible toxicities arising from CAR T cells are characterized and can be addressed. Though anti-mouse CD19 CAR T cells do not display toxicities, CAR T cells can give rise to unexpected toxicities. Where multiple CAR constructs and transduction efficiencies are not identical, the total number of T cells administered should be kept equal by the addition of non-transduced T cells into cell preparations.

5.1.6. Monitor disease progression weekly through IP injection of 100 µL of 30 mg/mL luciferin and imaging using an *in vivo* bioluminescence imaging system (**Figure 5c**).

5.1.7. Closely monitor mice for signs of toxicity and euthanize any mice that show early signs of hind limb paralysis (HLP) or pathological tumor burden before any suffering can arise.

Note: Toxicities from A20 lymphoma can include hind limb paralysis through tumor invasion of the meninges. Check regularly for early signs of altered gait. Likewise, large IP tumors can arise which can lead to discomfort shown by altered behavior.

5.1.8. Monitor survival of mice for 60 - 100 days (**Figure 5d**). Perform euthanasia by a schedule-1 method upon conclusion of the experiment.

5.2. **Protocol 2**

5.2.1. Deliver 200 mg/kg cyclophosphamide to 6 to 8-week old BALB/c mice by tail vein injection in 100 µL of PBS per mouse.

5.2.2. On the following day, inject of 5 x 10^5 syngeneic A20 B-cell lymphoma cells expressing luciferase and GFP in 100 µL PBS *via* tail vein injection.

5.2.3. Allow the mice to develop systemic lymphomas for ~ 7-14 days

5.2.4. Confirm systemic lymphoma by IP injection of 100 µL of 30 mg/mL luciferin and imaging using an *in vivo* bioluminescence imaging system.

5.2.5. Perform 5 Gy total body irradiation (TBI) at 0.02 Gy/min for lymphodepletion.

Note: Patients undergoing CAR T-cell treatments undergo a range of regimens to achieve lymphodepletion before the administration of CAR T cells which significantly increases the engraftment of adoptively transferred CAR T cells. This can be replicated in mice with total body irradiation (TBI) (**Figure 6**).

5.2.6. On the next day, inject 1 x 10^6 CAR T cells in 100 µL of PBS *via* tail vein injection into mice bearing established tumors.

5.2.7. Collect blood samples *via* tail vein bleeds after 7 days.

5.2.8. Add red cell lysis buffer to each blood sample, then prepare for flow cytometry as described in section 3. Analyze CAR T cell persistence in the circulation by flow cytometry (**Figure 2**).

Note: Addition of counting beads immediately prior to cytometry allows determination of the number of CAR T cells per milliliter of blood.

5.2.9. Monitor disease progress as described in steps 5.1.5 - 5.1.8 (**Figure 7**).

REPRESENTATIVE RESULTS:

For high efficiency transduction of T cells, it is necessary to obtain fresh retroviral particles. Transfection of the Plat-E cell line with pCL-Eco producer plasmid and pMP71 retrovirus plasmid gives rise to the secretion of retroviral particles into the cell supernatant. When a fluorescent marker gene, such as mCherry, is encoded in the retrovirus, successful transfection can be confirmed by fluorescence microscopy (**Figure 1**). Virus-containing supernatant from transfected Plat-E cells is used to transduce T cells *via* 2 rounds of spin-fection on fibronectin fragment-coated plates. The efficiency of transduction can be determined 4 days post transduction *via* flow cytometry. Successfully transduced cells express the marker gene encoded in the retrovirus (**Figure 2**). Transduction efficiencies range from ~ 50 - 90% efficiency with first generation receptors to ~ 10 - 40% with CAR constructs close to the retroviral packaging capacity. While marker gene expression shows successful retroviral transduction, it is paramount to show

functionality of CAR T cells upon engaging with cells that express target antigen on their surface. Target cell lines modified to express luciferase can be used in luciferase assays to test the degree of cell-kill by CAR T cells directly (**Figure 3A**). The release of effector cytokines from CAR T cells upon co-culture with target cells, determined by ELISA, can also be used as an indirect measure of CAR T cell cytotoxicity (**Figures 3B** and **3C**).

CAR T cells produced in this protocol can be evaluated in lymphoreplete mice by establishing systemic A20 lymphoma with a 100 mg/kg dose of cyclophosphamide (injected intravenously), 1 day prior to IV injection of 5 x 10^5 A20 cells (**Figure 4**). IP injection with luciferin and image capture using an *in vivo* bioluminescence imager can be used to monitor tumor burden using a constant ROI and exposure time throughout (**Figure 5A**-**C**). CAR T cells modified to express IL-12 are capable of eradicating systemic lymphoma with lymphodepleting pre-conditioning giving disease-free survival in about 25% of mice (**Figure 5D**). Lymphodepleting preconditioning, achieved by 5 Gy TBI 1 day prior to the IV administration of CAR T cells, significantly improves engraftment (**Figure 6**). In this model, first generation CAR T cells are capable of eradicating systemic A20 lymphoma, typically inducing disease-free survival in 100% of mice (**Figure 7**).

FIGURE AND TABLE LEGENDS:

Figure 1. Confirmation of successful transfection of Plat E cells. Plat-E cells transfected with retroviral CAR construct and pMP71 and pcl-Eco packaging vector plasmid DNA. Successful transfection is shown by expression of the mCherry fluorescent marker gene. **A)** Bright field microscopy, **B)** fluorescence microscopy and **C)** merged images are shown. Magnification = 50X.

Figure 2. Determining transduction efficiency by flow cytometry. Flow cytometry is used to determine the transduction efficiency of the mouse T cells on day 4 post transduction, using Zombie UV live/dead, mCherry, BV711 and BV785 for the detection of the live, CAR construct, CD4 and CD8 cells, respectively. Representative results of **A)** Non-transduced, **B)** mCherry.αmCD19.mCD3z and **C)** mCherry.αmCD19.mCD3z.mIL12 are shown with gating of 1) Singlets → 2) Live cells → 3) CD4 and CD8 → 4) and 5) Assessment of mCherry positive cells expressing CAR.

Figure 3. Validation of CAR T-cell activity. αmCD19 CAR T cells were co-cultured with A20 lymphoma cells modified to express luciferase (1 x 10^4:1 x 10^4) for 16 h in a U-bottom 96-well plate. After co-culture, cells were pelleted, and supernatant was collected. **A)** Cells were re-suspended in PBS and luminometry was used to assess the viability of the target cells. Supernatant from co-culture was assessed for the presence of IFNγ (**B**) and IL-12 (**C**). The ratio of CAR T cell to target cells and length of co-culture period must be optimized for each CAR construct and target cell line. PMA and ionomycin treatment can be used as a positive control to confirm quality of T cells and their ability cells to respond. Error bars show SD. Statistical analysis was performed using one-way ANOVA. *** $p < 0.001$). This figure has been modified from[17].

Figure 4. Establishing A20 lymphoma without lymphodepletion. Cyclophosphamide can increase efficiency of lymphoma induction without causing lymphodepletion. **A)** Blood counts of

6-8-week-old BALB/c mice after IV delivery of 100 mg/kg of cyclophosphamide. Error bars show SD **B)** Lymphoma burden of 6-8-week-old BALB/c mice after IV delivery of 100 mg/kg of cyclophosphamide or saline on day -1 and IV delivery of 5 x 10^5 A20 cells on day 0 measured using a luminometer. **C**) Survival of mice in **B)**. Error bars show SD. Statistical analysis was performed using 2-way ANOVA. ** $p < 0.01$, *** $p < 0.001$). This figure has been modified from Kueberuwa *et al.*[17].

Figure 5. Monitoring lymphoma burden and survival. Mice bearing A20 lymphoma expressing luciferase receive 100 µL intraperitoneal (IP) injections of 30 mg/mL luciferin and were imaged using an *in vivo* bioluminescence imaging system. **A)** Mice were exposed for 1 min on the ventral side and immediately flipped over to image dorsal to pick up tumor masses on both sides of the bodies **(B)**. **C)** Representative results of the lymphoma burden of BALB/c mice receiving varying αmCD19 CAR T cells without lymphodepletion. Error bars show SEM. **D)** Survival rate of the same mice. This figure has been modified from Kueberuwa *et al.*[17].

Figure 6. Effects of lymphodepletion. A) Blood counts of 6-8-week-old BALB/c mice after receiving 5 Gy TBI at a dose rate of 0.02 Gy/min; error bars show SD. Statistical analysis by two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **B)** Monitoring of $CD4^+$ and $CD8^+$ CAR T cells in the peripheral blood of mice by flow cytometry for the mCherry marker gene 7 days post administration. Error bars show SD. This figure has been modified from Kueberuwa *et al.*[17].

Figure 7. CAR T cell activity with lymphodepleting pre-conditioning. Typical results showing the effect of 5 Gy TBI the day prior to CAR T-cell administration. **A)** Imaging and **(B)** graphical displays of imaging of mice after 100 µL intraperitoneal (IP) injections of 30 mg/mL luciferin using an *in vivo* bioluminescence imaging system. Error bars show SEM. **C)** Survival of the same mice. This figure has been modified from Kueberuwa *et al.*[17].

DISCUSSION:

Syngeneic mouse models allow the testing of disease progression and therapy while maintaining an intact immune system. This is paramount when it comes to therapies that interact with the immune system and in particular for immunotherapeutic agents.

The protocol described here has two critical work streams, the first one is genetically modifying mouse T cell to express CARs. This requires 7 days from initiation to the validation of the transduction. Concomitant with production of CAR T cells is establishment of systemic lymphoma in mice. Should CAR T cell production fail, or quality be insufficient, there is typically not enough time to produce replacement cells before mice succumb to lymphoma. It is therefore critical that researchers using these models accurately perform tumor dosing and disease progression studies in order to successfully time the production of CAR T cells for therapeutic administration.

Typical reasons for low T-cell transduction efficiency includes poor transfection efficiency of producer cells, typically caused by poor plasmid purity or inaccurate determination of the pH of transfection media. It is recommended to check the efficiency of producer cell transfection before proceeding with the full protocol as poor transfection will limit the efficiency of T-cell

transduction. Recombinant human fibronectin fragments can be collected and stored at -20 °C for re-use, however, multiple freeze thaws result in reduced transduction efficiency. Swift processing of mouse spleens after collection is also important for obtaining high yields of viable T cells.

It should be noted that the protocol described here utilizes A20 cells expressing luciferase. This is preferred as it provides the ability to measure systemic tumor burden by bioluminescence imaging. However, in the presence of a functional immune system, responses to luciferase could skew the results. We have previously tested immune reactions of surviving mice to marker transgenes[17]. It is key to replicate key experiments using A20 cells free of transgenes to validate that these do not play a significant role in tumor eradication by immune cells.

While clinical agents can only be used *in vivo* in immune deficient mice, the use of mouse CAR T cells against mouse cancer cells allows us to evaluate the contributions of the immune system to therapeutic efficacy or disease progression. This protocol could be utilized for the pre-clinical evaluation of CARs targeting B-cell lymphoma or other CARs with additional modifications such as secretion of IL-12 as described here. It must be noted that although interplay between immune cells can be evaluated in syngeneic mouse models, they may not accurately recapitulate interaction in humans *in vivo*. Of particular note, human and mouse CARs will vary in structure which may have downstream consequences; optimal activation and cell culture conditions for growth of T cells are different[20], tissue distribution of target antigen expression may vary between humans and mice and experienced toxicities may be radically different. It is therefore essential to utilize *ex vivo* and xenogeneic models to corroborate the results.

In summary, the syngeneic lymphodepletion and lymphoreplete model of lymphoma recapitulate the patients with and without prior chemo/radiotherapy. This provides a model system in which to mimic the clinical settings to allow the testing a range of therapeutic strategies that will be important with the coming wave of new immune therapy agents.

With the use of pre-conditioning, it will be noted that all the mice typically clear the lymphoma. With up to 90% complete response rates in humans, this is representative. However, the challenges for CD19 CAR T-cell therapy will hinge on preventing the high frequency of relapses observed that are often $CD19^-$. Relapses have not been observed in this model up to, and often beyond 100 days. Modifications to mimic the relapses seen in the clinic could help with the future challenges of CD19 CAR T-cell therapy.

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DISCLOSURES:
David Gilham works for Celyad which is involved in the production of CAR T cells. The rest of the authors have nothing to disclose.

REFERENCES:

1 Brian, W. Food and Drugs Administration Biologics Licence Application Approval letter. *https://www.fda.gov/downloads/BiologicsBloodVaccines/CellularGeneTherapyProducts/ApprovedProducts/UCM574106.pdf.* (2017).

2 Malarkey, M., Brian, W. Food and Drugs Administration Biologics Licence Application Approval letter. *https://www.fda.gov/downloads/biologicsbloodvaccines/cellulargenetherapyproducts/approvedproducts/ucm581259.pdf.* (2017).

3 Liu, Y. & Zeng, G. Cancer and Innate Immune System Interactions: Translational Potentials for Cancer Immunotherapy. *Journal of Immunotherapy (Hagerstown, Md. : 1997).* **35** (4), 299-308, doi:10.1097/CJI.0b013e3182518e83, (2012).

4 Janssen, L. M. E., Ramsay, E. E., Logsdon, C. D. & Overwijk, W. W. The immune system in cancer metastasis: friend or foe? *Journal for ImmunoTherapy of Cancer.* **5** (1), 79, doi:10.1186/s40425-017-0283-9, (2017).

5 Pandya, P. H., Murray, M. E., Pollok, K. E. & Renbarger, J. L. The Immune System in Cancer Pathogenesis: Potential Therapeutic Approaches. *Journal of Immunology Research.* **2016**, 13, doi:10.1155/2016/4273943, (2016).

6 Vinay, D. S. *et al.* Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Seminars in Cancer Biology.* **35,** S185-S198, doi:https://doi.org/10.1016/j.semcancer.2015.03.004, (2015).

7 Gajewski, T. F., Meng, Y. & Harlin, H. Immune Suppression in the Tumor Microenvironment. *Journal of Immunotherapy.* **29** (3), 233-240, doi:10.1097/01.cji.0000199193.29048.56, (2006).

8 Munn, D. H. & Bronte, V. Immune suppressive mechanisms in the tumor microenvironment. *Current opinion in immunology.* **39,** 1-6, doi:10.1016/j.coi.2015.10.009, (2016).

9 Vanderlugt, C. L. & Miller, S. D. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nature Reviews Immunology.* **2**, 85, doi:10.1038/nri724, (2002).

10 Hardwick, N. & Chain, B. Epitope spreading contributes to effective immunotherapy in metastatic melanoma patients. *Immunotherapy.* **3** (6), 731-733, doi:10.2217/imt.11.62, (2011).

11 Makkouk, A. & Weiner, G. Cancer Immunotherapy and Breaking Immune Tolerance-New Approaches to an Old Challenge. *Cancer research.* **75** (1), 5-10, doi:10.1158/0008-5472.CAN-14-2538, (2015).

12 Jackson, S. R., Yuan, J. & Teague, R. M. Targeting CD8(+) T-cell tolerance for cancer immunotherapy. *Immunotherapy.* **6** (7), 833-852, doi:10.2217/imt.14.51, (2014).

13 Brentjens, R. J. *et al.* Lymphodepletion and tumor burden govern clinical responses in patients with B-cell malignancies treated with autologous, CD19-targeted T cells. *Journal of Clinical Oncology.* **29** (15_suppl), 2534-2534, doi:10.1200/jco.2011.29.15_suppl.2534, (2011).

14 Brentjens, R. J. *et al.* Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood.* **118** (18), 4817 (2011).

15 Hay, K. A. *et al.* Kinetics and Biomarkers of Severe Cytokine Release Syndrome after CD19 Chimeric Antigen Receptor-modified T Cell Therapy. *Blood.* **130**, 2295-2306, (2017).
16 Zhang, T. *et al.* Efficiency of CD19 chimeric antigen receptor-modified T cells for treatment of B cell malignancies in phase I clinical trials: a meta-analysis. *Oncotarget.* **6** (32), 33961-33971 (2015).
17 Kueberuwa, G., Kalaitsidou, M., Cheadle, E., Hawkins, R. E. & Gilham, D. E. CD19 CAR T Cells Expressing IL-12 Eradicate Lymphoma in Fully Lymphoreplete Mice through Induction of Host Immunity. *Molecular Therapy - Oncolytics.* **8**, 41-51, doi:10.1016/j.omto.2017.12.003, (2018).
18 Engels, B. *et al.* Retroviral vectors for high-level transgene expression in T lymphocytes. *Human Gene Therapy.* **14** (12), 1155-1168, doi:10.1089/104303403322167993, (2003).
19 Parkinson, C. M. *et al.* Diagnostic Necropsy and Selected Tissue and Sample Collection in Rats and Mice. *Journal of Visualized Experiments : JoVE.* (54), 2966, doi:10.3791/2966, (2011).
20 Kueberuwa, G. *et al.* CCR7(+) selected gene-modified T cells maintain a central memory phenotype and display enhanced persistence in peripheral blood *in vivo*. *Journal for Immunotherapy of Cancer.* **5**, 14, doi:10.1186/s40425-017-0216-7, (2017).

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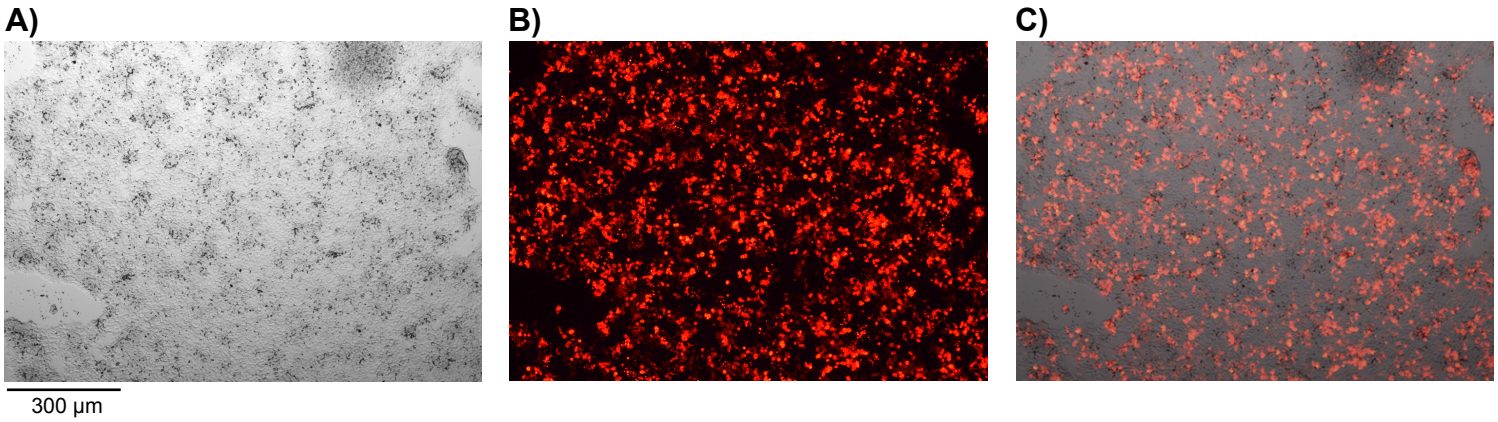

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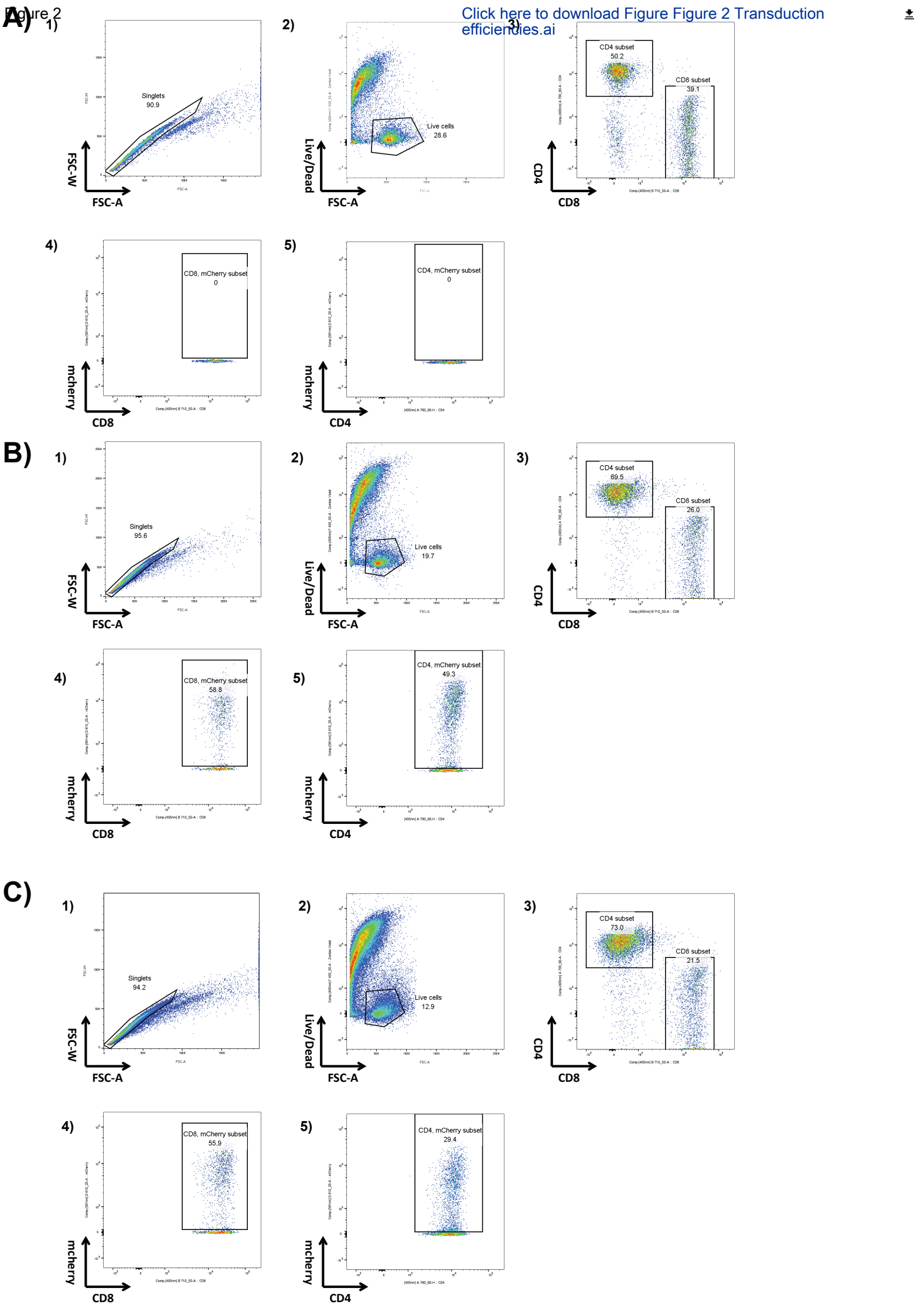

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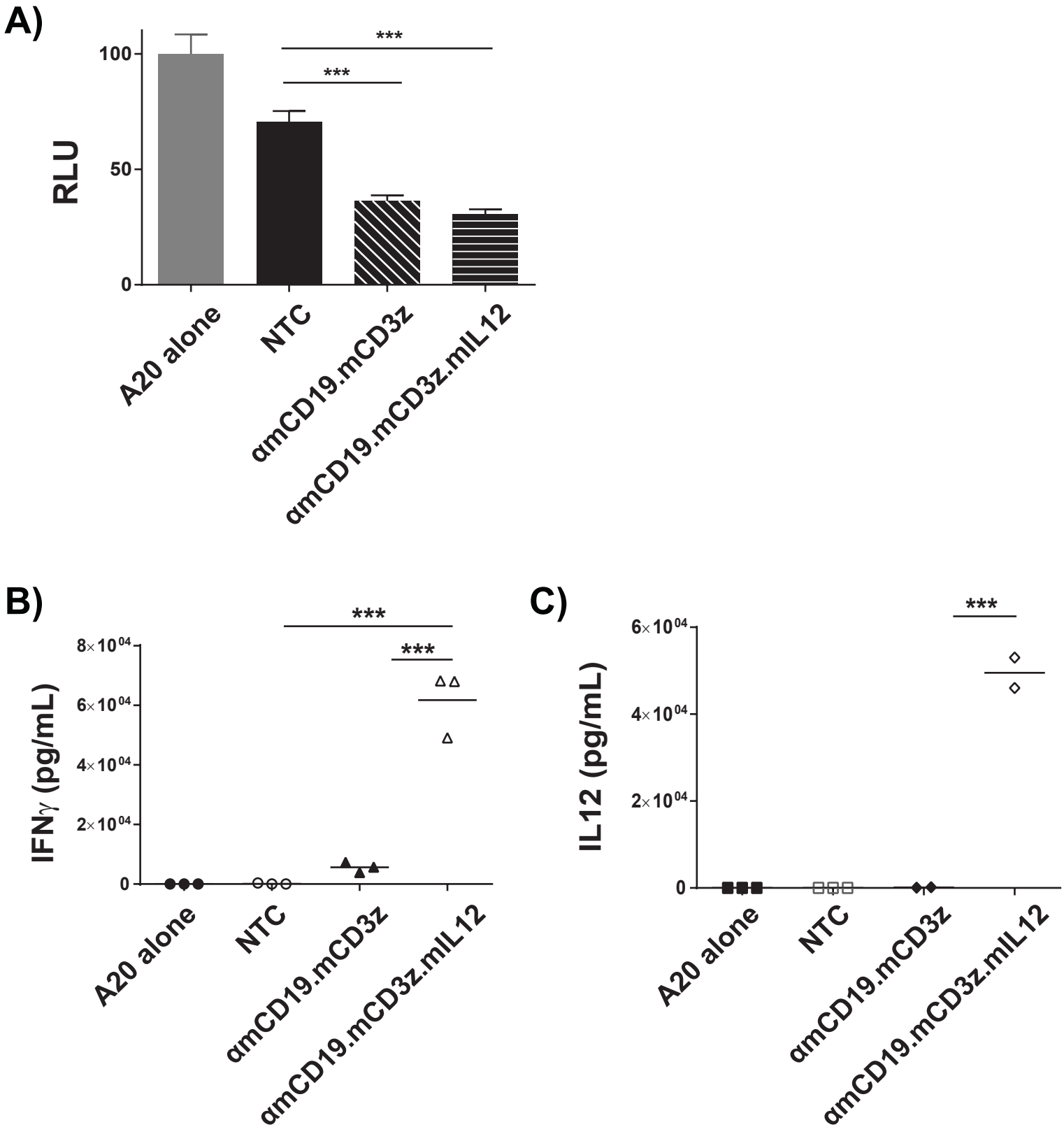

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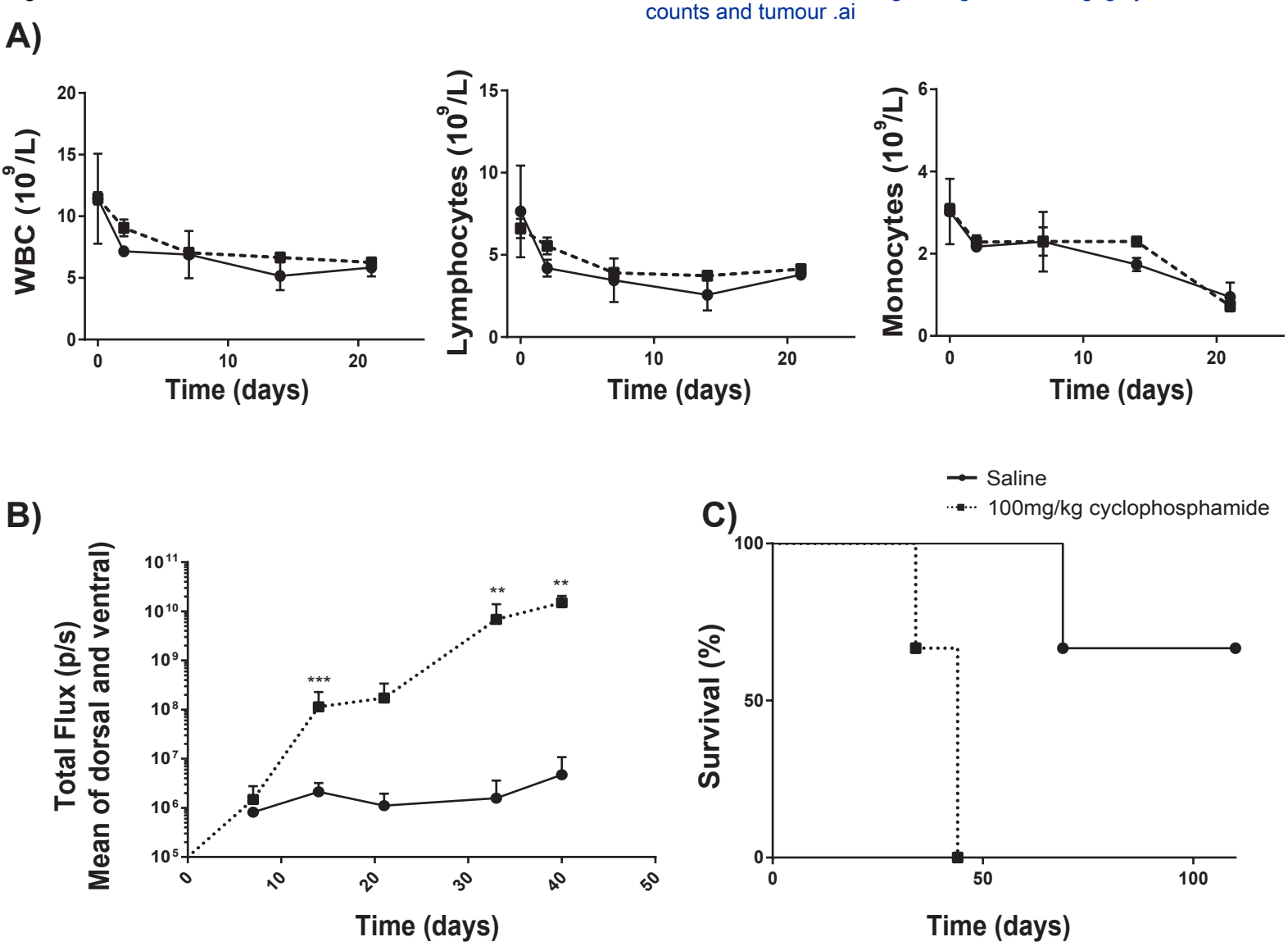

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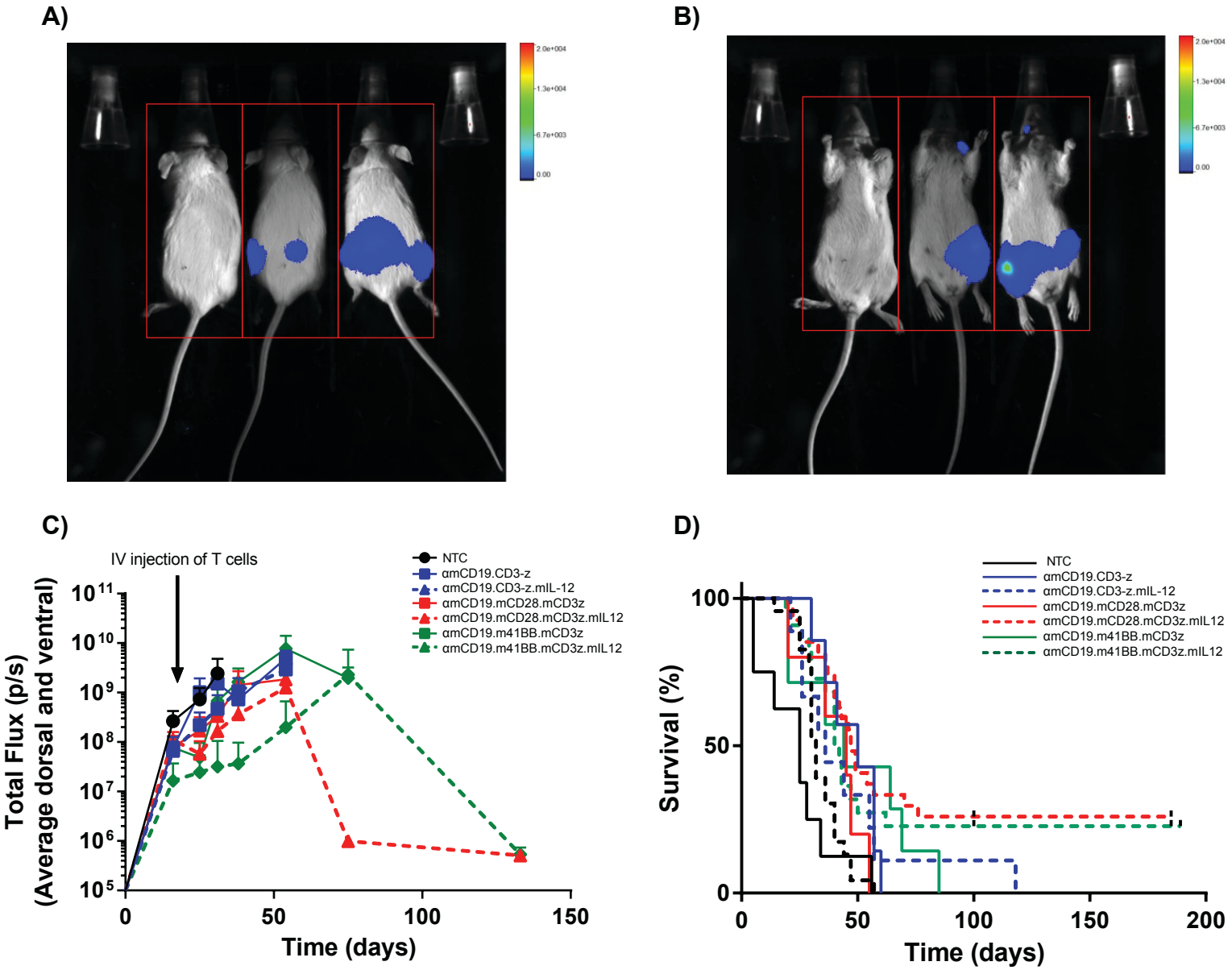

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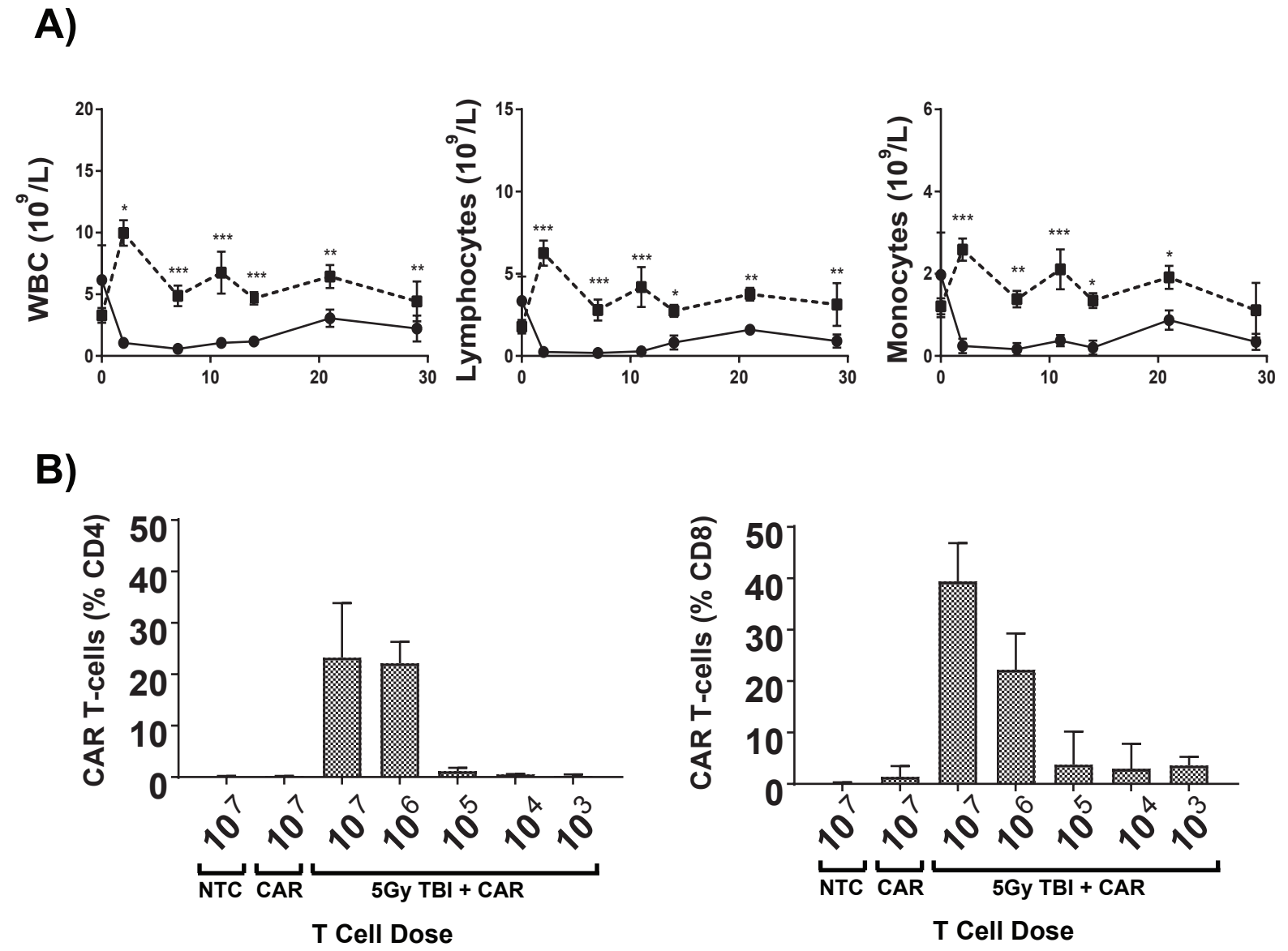

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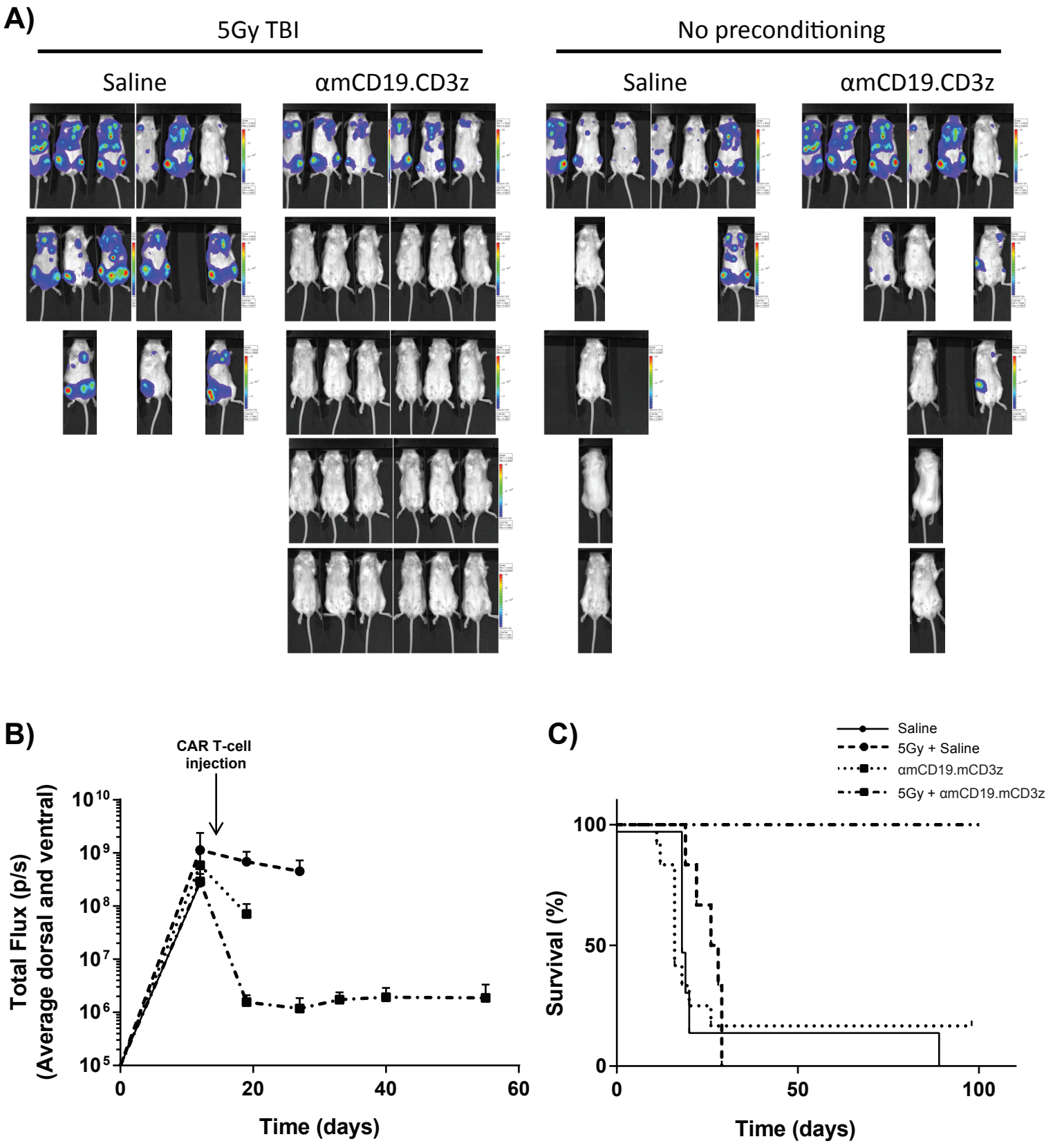

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Name	Company	Catalog Number
0.2 µm syringe filter	Appleton Woods	FC121
0.45 µm syringe filter	Appleton Woods	FC122
1.5ml pestle and microtube	VWR	431-0098
100X penicillin-streptomycin-glutamine (PSG)	Gibco	10378016
2-Mercaptoethanol (50 mM)	Gibco	*31350 -010*
Blasticidine S hydrochloride	Sigma- Aldrich	15205
Bottle Top Filter (0.2 µm)	Scientific Laboratory Supplies	FIL8192
Brilliant Violet 711 anti-mouse CD8a Antibody	BioLegend	100759
Brilliant Violet 785 anti-mouse CD4 Antibody	BioLegend	100552
Calcium chloride dihydrate	Sigma- Aldrich	C7902
Cell counting beads – CountBright absolute counting beads	Molecular Probes	C36950
Cell Strainer 100µm	VWR	734-0004
Cyclophosphamide Monohydrate	Merck	239785-1GM
Dulbecco's Modified Eagle medium (DMEM) - High Glucose	Sigma Aldrich	D6546
Dynabeads	Gibco	11131D
Ficoll Paque Plus	GE Healthcare	GE17-1440-03
Flow cytometer - LSR Fortessa x20	BD Biosciences	658222R1
Foetal Bovine Serum	Gibco	10270
Haemacytometer	Appleton Woods	HC001
HEPES solution	Sigma- Aldrich	H0887
IL-12 p70 Mouse Uncoated ELISA Kit	Invitrogen	88-7121-76
IL2, Proleukin	Novartis	PL 00101/0936
in vivo bioluminescence imaging system – in vivo xtreme II imaging system	Bruker	T149094

Ionomycin Calcium Salt	Sigma- Aldrich	I0634
Live/dead stain - Zombie Violet Fixable Viability Kit	BioLegend	423114
Luminometer - Lumistart Omega	BMG Labtech	415-301
Murine IFN-γ ELISA kit	Diaclone	861.050.010
Paraformaldehyde	Sigma- Aldrich	16005
pCL-Eco	Novus Biologicals	NBP229540
Phorbol 12-myristate 13-acetate (PMA)	Sigma- Aldrich	**P8139**
Platinum E cell line	Cell Biolabs	RV-101
Purified NA/LE Hamster Anti-Mouse CD28	BD Biosciences	553294
Purified NA/LE Hamster Anti-Mouse CD3ε	BD Biosciences	553057
Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block)	BD Biosciences	553142
Puromycin Dihydrochloride	Sigma- Aldrich	P8833
Recombinant human fibronectin fragment - RetroNectin Reagent	TaKaRa	T100B
Recombinant Mouse IL-7 (carrier-free)	BioLegend	577806
Red cell lysis buffer	eBioscience	004-4333-57
RPMI 1640 Medium	Lonza	BE12-167F
Trypsin - EDTA solution	Sigma- Aldrich	T3924
XenoLight D-Luciferin	Perkin Elmer	122799

Comments
1 in 100 staining dilution Clone 53-6.7
1 in 100 staining dilution Clone RM4-5
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Clone 37.51

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Clone 2.4G2

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C: weiming.zheng@postgrad.manchester.ac.uk, milena.kalaitsidou@manchester.ac.uk, dgilham@celyad.com, robert.e.hawkins@manchester.ac.uk

Dear Dr. Kueberuwa,

Your manuscript, JoVE58492 A syngeneic mouse B-cell lymphoma model for pre-clinical evaluation of CD19 CAR T-cells, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.

Your revision is due by **Jul 10, 2018**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Alisha DSouza, Ph.D.
Senior Review Editor
JoVE
617.674.1888
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Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

GK: Proofread

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

GK: A word document with a link and snapsjots of licence page, as well as a pdf showing communication with the EAGCT, in which they give explicit permission for use, is attached.

3. Figure 2: Please increase the size of the text in figures to make it easier to read.

GK: Size of scale bar increased, as well as size of text. Time and date stamps removed.

4. Figure 3: Please change "ml" to "mL".

GK: changed

5. Figures 3-7: Please define error bars and asterisk symbols in the figure legend.

GK: error bars and asterisks and statistical tests defined

6. Please shorten the figure legends. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

GK: Figure legends shortened. Additional descriptive text pertaining to figures has been moved to protocol text

7. Please provide an email address for each author.

GK: Provided on title page under "contact details"

8. Please define all abbreviations before use.

GK: checked

9. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

GK: edited

10. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

GK: edited

11. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

GK: edited

12. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

GK: checked

13. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: RetroNectin, Falcon, Zombie Violet, LSR Fortessa, Lumistart Omega, Xtreme II Bruker, etc.

GK: Commercial names removed and restricted to materials table

14. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

GK: ethics statement added

15. 1.1: Please write the text in the imperative tense in complete sentences.

GK: Explanatory elements are now under a "note"

16. 1.3, 1.4: Please mention the conditions for culturing cells.

GK: at 37 °C, 5 % CO_2 added

17. 2.9: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.

GK: reference added

18. 2.10: What container is used in this step? What volume of PBS is used?

GK: additional description has been added

19. 2.14: What is centrifuged? Please add the missing information.

GK: added – resultant cell suspension overlay

20. 5.7: Please write the text in the imperative tense.

GK: altered

21. 5.16: Please add more details to this step or add references to published material specifying how to perform the protocol action.

GK: added to description and referred to section 3

22. What happens to mice after the experiment?

GK: added instructions for schedule 1 method of euthenasia

23. Please include single-line spaces between all paragraphs, headings, steps, etc. After that, please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

GK: implemented single spacing. In order to reduce to 2.75 pages removed some in vivo injections that will be easily found elsewhere. However, in order to maintain cohesion, about 3 pages are highlighted.

24. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

GK: N/A

25. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included.

GK: updated with paragraph to explain representative results.

26. Discussion: Please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

GK: paragraph two outlines the importance of T-cell transduction relative to tumour growth. Causes for poor transduction efficiency have been added, with recommended checks to reagents. Limitations are discussed in paragraph 4.

27. Please include volume and issue numbers for all references.

Two references are web links to FDA approval docs therefore no volume included

28. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.

GK: Multiple lot numbers used. RRID not available for antibodies, only available for Plat-E cell line which is now added.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Preclinical studies of CAR T-cells usually rely on the use of human CAR T-cells in immunodeficient mice that cannot recapitulate the immunosuppressive tumor microenvironment of human tumors. This makes the preclinical evaluation of strategies designed to overcome the tumor microenvironment almost impossible. Here is presented a strategy for the generation of murine CD19.CAR T-cells for use in syngeneic mice bearing murine lymphoma. This system can be modified for the evaluation of different CARs and modifying transgenes in different tumor systems. This system will be useful for evaluating concepts, but not for evaluating clinical CAR safety or CAR structure.

Major Concerns:

I would like to have seen the viability, recovery and subsequent proliferation of the murine T-cells after transduction using this method. It would also help to describe the results quantitatively in terms of transduction efficiency, phenotype, cytokine secretion and killing rather than simply referring to representative

figures. The disadvantages of syngeneic mouse models as well as their advantages should be mentioned (clinical CARs cannot be tested, antigen distribution is often different, murine immune systems and tumors are different)

GK: Viability and recovery is variable depending on which CAR T-cell construct. Those expressing IL-12 lead to much lower viability, possible dues to upregulation of Fas ligand signaling. Figure 2 includes representative results showing the proportion of live cells 4 days post transduction. These numbers vary greatly depending on donor cells.

The advantage/disadvantages of syngeneic models are discussed in several parts of this manuscript. Added description to ensure the reader appreciates the limitations has been added.

*Line 64, a major point of CARTs is that they do not have to break tolerance

GK: this was referring to epitope spreading and general immune based approaches. Clarification added.

*5 GY of irradiation cannot be said to mimic the patient situation since Cytoxan and fludarabine are the standard means to produce lymphodepletion with a very different mechanism from irradiation that induces substantial inflammation.

GK: The standard for human lymphodepletion in humans is now 5FU and fludarabine, however, several methods, including radiation have been used in the past. We have tested several methods for lymphodepletion in mice. We prefer total body irradiation as it is less invasive than an IV injection, more consistent and at the low dose rate used here, induces minimal discomfort to mice. There are several publications with differing opinions on the mechanisms of lymphodepletion. Our own work shows that TBI causes efficient lymphodepletion and a drastic enhancement of CAR T-cell engraftment – (shown in Figure 6 of this manuscript).

*Two paragraphs later, you state that you established a lymphoreplete model. This is confusingly presented, although later it becomes clear.

GK: clarification added by describing standard method, and referring to patients ineligible for lymphodepletion.

*Please provide source of packaging plasmids and cell lines and more description of Plat-E cells

GK: Plat-E and pCL-Eco added to materials list. Description of PlatE cell line added to section 1 of protocol.

*For how long do you rock the plates of Plat E cells with the CaCl2 DNA solution

GK: added

*Not clear why parts of the text are highlighted?

GK: highlighted for filming

*Step 2.19. What culture vessels are used to culture the murine T-cells?

GK: this depends on the number of cells obtained – cells are kept at 5e6/ml. added text to say appropriate sized flask.

*Step 2.40. "Resuspend cells in 2.5 ml per well of transduction with 200 IU/ml and 4 ng/ml" (IL2 and IL7 is missing)

GK: added

*2.45. What type of culture vessel and cell densities do you recommend? Wells or flasks?

GK: cell density is stated, flasks now added to text for clarity.

*Centrifugation of splenocytes for 90 minutes at 1200 seems quite harsh. What is the viability and recovery of the cells after the second round of transduction?

GK: This speed is required to have an effect on viral particles. A note on viability has not been added as in the first 2-3 days, all non T-cells die off under these tissue culture.

*What fold expansion do you achieve over the 4 days after transduction?

GK: A statement about overall viability over first 4 days has been added to a note on point 2.46

*3.5. Wash twice with PBS and (missing centrifuge) 500 x g for 5 min

GK: added

*4.0 What is total "n" in the experiments shown in Figure 3b and 3c

GK: representative results. N added to figure legend.

*5.5. What unexpected toxicities have you observed and using what transgenes?

GK: We have not observe toxicities from these CAR T-cells, however if adapting this protocol it is of vital importance to keep this caution in mind. Clarified in text

*It would be helpful to describe the results: What transduction efficiencies did you get (median and range, including n)

GK: this varies based on length of CAR construct size. Ranges for first generation and large vectors now added.

*Is it surprising to see so little IFNg production from CART cell cultured with tumor cells. Very high background levels would be seen with human CART cells on day 5 or 6 after stimulation with CD3 and CD28.

GK: Can't comment without seeing protocol for human cells. Our lab stimulates human cells for much longer periods than mouse. This protocol is overnight before CD3 and CD28 antibodies are removed. It is noted in the manuscript that non transduced cells can often display non specific activity.

*Should the tumors fail to :"take", can the CART cells be cryopreserved for future use?

GK: We have not validated this yet. The freeze thaw process causes substantial cell death and can have effects on the T-cell phenotype so it is avoided.

*Line 403 sense?

GK: Clarified- concomitant production

*How do you measure epitope spreading?

GK: there is not a standard measure, but presence is confirmed by lack of response to initial epitope. Not in the scope of this protocol.

Reviewer #2:

Manuscript Summary:

The manuscript describes the production and testing of mCD19-specific CAR T-cells. Testing is done in vitro and in vivo in a syngeneic tumor model using lymphodeplete and lymphoreplete recipients. The protocols are generally well described but there are concerns regarding the presentation of some data, lack of some details, and questions regarding the range of transduction efficiencies obtained.

Major Concerns:

1. CAR T-cell patients are typically given a lymphodepleting dose of cyclophosphamide (often with fludarabine) prior to CAR T-cell transfer. It therefore is not clear why the authors use TBI to lymphodeplete, especially since they wish "to mimic the patient setting" (lines 68-69). Lymphodepletion by chemotherapy administration prior to CAR T-cell transfer is more clinically relevant.

GK: Similar comment to reviewer 1. Addressed above:

The standard for human lymphodepletion in humans is now 5FU and fludarabine, however, several methods, including radiation have been used in the past. We

have tested several methods for lymphodepletion in mice. We prefer total body irradiation as it is less invasive than an IV injection, more consistent and at the low dose rate used here, induces minimal discomfort to mice. There are several publications with differing opinions on the mechanisms of lymphodepletion. Our own work shows that TBI causes efficient lymphodepletion and a drastic enhancement of CAR T-cell engraftment – (shown in Figure 6 of this manuscript).

2. There are no measures of reproducibility or range. For example, what is the range of transduction efficiencies over a number of attempts? Does transduction efficiency vary between different CAR constructs?

GK: Also similar comment to reviewer 1. Addressed above: range of efficiencies added, as well as an explanation that the larger inserts have reduced efficiency.

3. The quality of the dot plots shown in Figure 1 is poor and the embedded text is illegible.

GK: Text in figure 1 has been altered as per suggestion by editor.

4. The number of animals in some figures (e.g. Figure 3C) is too low to inspire confidence in the results, despite the statistical significance.

GK: These are representative results. For simplicity, killing assay, IFN gamma ELISA and IL12 ELISA were taken from the same experiment. This can be edited if needs be.

5. No indications of statistical significance are provided in some figures (e.g. Figures 5 and 7).

GK: figures 5 and 7 contain tumour burden and survival curves.

Statistical analysis of tumour burden at the time of apparent therapeutic efficacy cannot be performed by classical two-way ANOVA because mice in control groups have been euthenised. Performing analysis without these mice skews results as clearly they attained high tumour burden before being euthenised.

have classical survival curve tails associated with immunotherapies. Analysis through classical comparison of median overall survival is therefore not suitable for this data. Analysis on an arbitrary level such as 80% rather than 50% could be performed, however it does not add value to statement of long term survival proportion.

6. In addition to CD4 and CD8 expression, the transduced cells should be phenotyped for activation/exhaustion markers as different culture conditions yield different differentiated T-cell products.

GK: This is beyond the scope of this protocol. Whilst T-cell phenotype is impacted upon by activation method, cell culture conditions, length of cell culture and transgenes added to CAR T-cell constructs. Previous research from our lab analysing phenotypic chnge with culture mmethods has been added to the text

Minor Concerns:
1. One line 149, a word is missing after, "Centrifuge the..."

GK: changed

2. The hybridoma clone designations for each monoclonal antibody should be added to the material/equipment table.

GK: added to materials list

3. The in vivo BLI data are presented incorrectly as counts (photons/sec); they should be presented as radiance units (photons/sec/cm²/sr). "Counts are a relative measure of the photons incident on the CCD camera and radiance is in absolute physical units that measure the photon emission from the subject." (https://mbi-ctac.sites.medinfo.ufl.edu/files/2017/02/Concept-Tech-Note-2-Image-Display-and-Measurement.pdf)

GK: As described in the protocol, ROI and exposure time are constant for each mice and each time point. Distance from camera and steradian is equivalent

throughout, therefore results of total flux are displayed, which is measured in photons/sec

4. On line 285, the frequency of BLI measurements should be noted (i.e. q.a.d? weekly?)

GK: added the description of weekly imaging.

Reviewer #3:

Manuscript Summary:

The authors present protocols for setting up a preclinical system for testing chimeric antigen receptor T cells in a syngeneic background. This includes the retroviral transduction of murine T cells and also the engraftment of B-cell lymphoma cells into BALB/c mice with and without lymphodepleting pre-conditioning.

The protocol itself and experimental variables are well described. While these models have been published in the literature--and thus are not novel--the description here is concise and brings attention to the importance of studying CAR T cells in immunocompetent backgrounds, which can have significant effects on antitumor activity and the overall immunological milieu of the host.

Major Concerns:

The authors should comment on using target tumor lines that express luciferase, as it is a foreign protein and indeed is known to impact the tumorgenicity of several tumor lines. Certainly, an immune response against luciferase may play a role in the durability of antitumor responses. This should be addressed, for example by using experiments/cell lines without luciferase that monitor overall survival. In the very least, the appropriate caveats and the question of endogenous immunity to luciferase should be discussed so that readers are aware of this limitation when attempting to recapitulate this model.

GK: added in a description, reference to the molecular therapy oncolytics which contains examination of immune response to luciferase is added.

Reviewer #4:

Manuscript Summary:

Kueberuwa et. al provide a detailed protocol on CD19 CAR T cell production and CAR T cell validation assays. They also show how to test their CAR T cells in lymphoreplete and lymphodepleted mouse models.

Minor Concerns:

2.23 - Takara (Clonetech) suggests the use of sterile 2% BSA to block non-specific binding instead of TCM.

GK: we have used both BSA and media containing FCS. As T-cell media is used in the next step in the protocol it is more convenient.

2.35 - Here you state that you reuse the Retronectin collected from the first and second transduction and save it for future use. Do you re-freeze the Retronectin at -20C after collection? Have you seen loss of transduction efficiency due to recycling Retronectin?

GK: This point is addressed in the critical steps and modifications section of the discussion. Re-freezing leads to reduced transduction efficiency over time

2.40 - Please clarify that the 200 IU/mL and 4 ng/mL are respective to IL-2 and IL-7

GK: added

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"CD19 CAR T Cells Expressing IL-12 Eradicate Lymphoma in Fully Lymphoreplete Mice through Induction of Host Immunity" - https://doi.org/10.1016/j.omto.2017.12.003 was published under the following licence.

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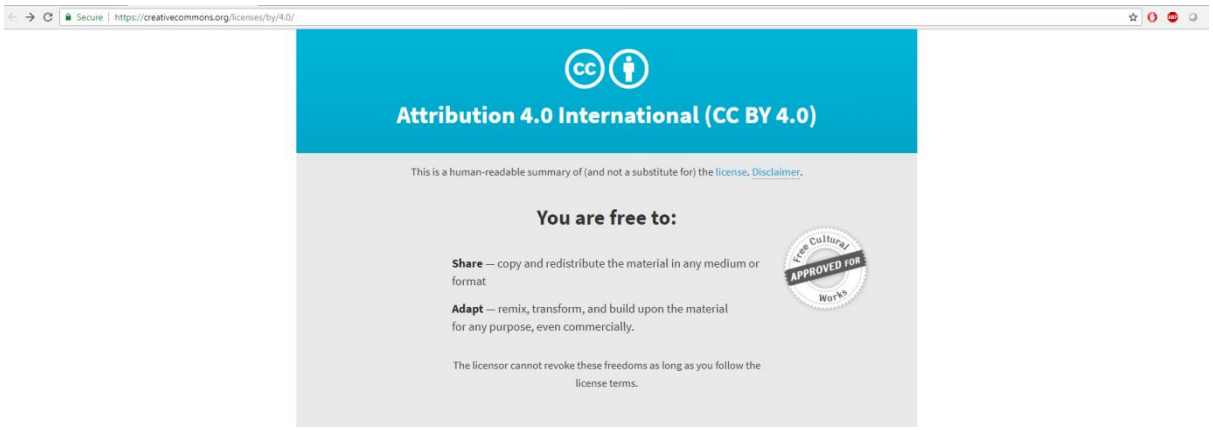

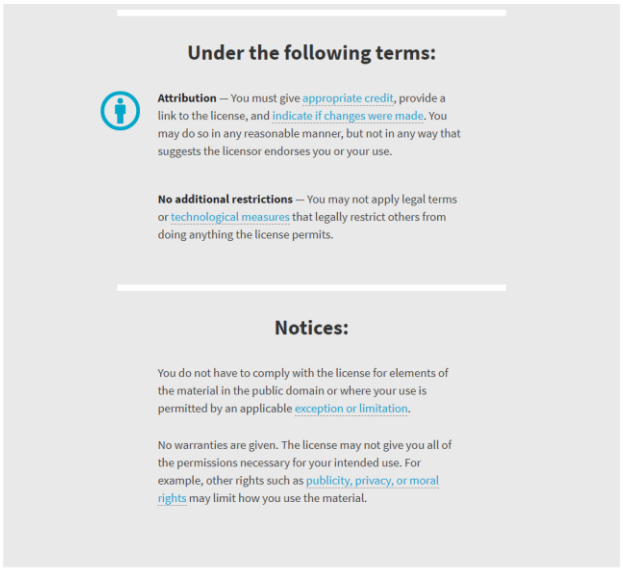

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RE: licence query

Gray Kueberuwa
Sent: 20 June 2018 15:00
To: Susan Brettingen (ASGCT) [SBrettingen@asgct.org]

Dear Sue,

Thanks very much for your reply
Gray

Dr Gray Kueberuwa
Postdoctoral Research Associate
Clinical and Experimental Immunotherapy Group
Division of Cancer Sciences
Manchester Cancer Research Centre
Manchester
M204GJ

From: Susan Brettingen (ASGCT) [SBrettingen@asgct.org]
Sent: 20 June 2018 14:16
To: Gray Kueberuwa
Subject: RE: licence query

Hi Gray,

Sorry it has taken so long to respond to your message. We had some glitches in our software here that routed some emails to junk folders and other places they should not have gone. As a result, we encountered delays in receiving genuine emails, such as yours.

Having said that, I thank you for the inquiry. You have permission to use the figures in line with your request.

Again, thank you for your patience.

Best,

Sue Brettingen
Journal Coordinator
American Society of Gene & Cell Therapy
414.918.3070

From: info (ASGCT)
Sent: Wednesday, June 20, 2018 8:07 AM
To: Susan Brettingen (ASGCT) <SBrettingen@asgct.org>
Subject: FW: licence query

From: Gray Kueberuwa [mailto:gray.kueberuwa@manchester.ac.uk]
Sent: Wednesday, June 20, 2018 5:44 AM
To: info (ASGCT)
Subject: FW: licence query

Hello,

I have not received a reply to my inquiry below.

Can you advise please?

Many thanks
Gray

Dr Gray Kueberuwa
Postdoctoral Research Associate
Clinical and Experimental Immunotherapy Group
Division of Cancer Sciences
Manchester Cancer Research Centre
Manchester
M204GJ

From: Gray Kueberuwa
Sent: 03 May 2018 16:48
To: info@asgct.org
Subject: licence query

Hello,

I have been approached by the Journal of online video entries (JoVE) to publish a detailed protocol about the methods used in :

"CD19 CAR T Cells Expressing IL-12 Eradicate Lymphoma in Fully Lymphoreplete Mice through Induction of Host Immunity" - https://doi.org/10.1016/j.omto.2017.12.003

It may be good to use one or more of the figures from the paper as representative data. This paper was published open access and I just want to check if this is possible? If not we will use other representative data showing similar results.

Could you advise?

Many thanks
Gray

Dr Gray Kueberuwa
Postdoctoral Research Associate
Clinical and Experimental Immunotherapy Group
Division of Cancer Sciences
Manchester Cancer Research Centre
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