Surface-Engineered Lentiviral for Targeted Chimeric Antigen Receptor Transduction on T-memory stem cells

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Introduction

Cancer immunotherapy is a fast growing field in biotechnology with CAR T-Cells being one of the major associated therapies. However, there are several large barriers to CAR T-Cell development. These include price (as shown by Novartis' $475,000 USD Kymriah drug) due to the ex-vivo editing process, adverse effects due to off target CAR transduction, and overall lack of patient response. To combat this, a 2016 study was able to show specific targeting towards CD8 receptors using LVs with Nipah-virus (NiV) glycoproteins and single-chain variable fragments (scFv). (Bender et al., 2016) Following that, another study used the aforementioned findings to induce *in vivo* CAR T-cell therapy in mice. (Pfeiffer et al., 2018) However, the results still showed the need for improvement as signs of cytokine-release syndrome were present and the treatment efficacy did not outperform the control group.

Recent studies have shown that a subtype of T-cell known as the T-memory stem cell (T_{SCM}) has unique capabilities making it an ideal candidate for CAR T-cell therapy. (Flynn & Gorry 2015)The presence of T-memory stem cells has been correlated with greater in-vivo expansion during CAR T-cell therapy. (Xu et al., 2014) Simultaneously, T_{SCM}'s also secrete lower levels of IFN-gamma compared to effector T-cells which can eliminare adverse effects cytokine release syndrome. By modifying the previously mentioned NiV-CD8-LV *in vivo* CAR T-cell therapy to target T-memory stem cells, CAR T-cell therapy could come more affordable, less

resource intensive, feature an increased response, and decrease off-target transduction.

Experimental Design

The study aims to measure the transduction efficacy of the CD19-CAR system into lentiviral vectors targeted towards T-memory stem cells. In the T_{SCM}-model, LVs are pseudotyped with receptor-targeted Nipah virus (NiV) glycoproteins and necessary packaging plasmids. The LV's are transfected with single-chain variable fragments (scFvs) targeted towards the CD8 receptor on CD8+ T-cells along with T_{SCM} specific receptors (CD45RA+, CD62L+, CCR7+, CD95+). Finally, the LV will be transfected with a CD19-CAR.

Our first stage is directed towards identifying the optimal dilution of the models by analyzing titration. Required plasmids are transferred to Lenti-X 293T cells to produce two populations of LVs one targeting all CD8+ T-cells and the second targeting specifically T_{SCM}. A CD8+ cell line is used to analyze titration of the CD8-LV model. For the T_{SCM}-LV model, the CD8+ cell line is used to create induced T_{SCM} through co-culture with Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines as well as DLL1-expressing OP9 cells.

The second stage will test the CAR expression and CD19+ B-cell depletion *in vitro* and involves LVs from Lenti-X 293T cells being transferred to a population of peripheral blood mononuclear cells (PBMCs) to express the CD19-CAR. Three groups will be subjected to test the efficacy of B-cell depletion and LV transduction efficacy (where applicable). The first group is the control group with no B-cell targeting methods introduced. The second is the group with the CD19-CAR on T_{SCM}'s and the third group has CD19-CARs on all CD8+ T-cells.

Results and Interpretation

Lentiviral Titration: Lentiviral titration is measured to analyze the efficiency of LV production. Titration is only measured in the first stage where several gradient dilutions of the lentiviral particles are produced by adding the associated amount of DMEM complete media along with 10µg/mL polybrene.1.5mL of each viral dilution are added to wells and then cells are counted and incubated for up to 48-72 h. After aspiration and replacement with phosphate-buffered saline, the percentage of CAR+ cells are determined by flow-cytometry. Titration is subsequently calculated.

Flow Cytometry: Flow cytometry is performed to identify cell specificity of the different cell-targeting models. A 5 polychromatic system is used to identify CAR expression on T_{SCM}'s. Anti-CD3 antibody to identify T-cell count, anti-CD8 antibody to identify CD8+ T-cells, and then anti-CD45RA, anti-CD95 and anti-CD62L (or anti-CCR7) to identify T_{SCM}'s. Simultaneously, anti-CD19 CAR antibody is used to identify which PBMCs have CAR expression. (O'Donnell et al., 2013)

Furthermore, a 3 polychromatic system is used to identify CAR expressions on all CD8+ T-cells. Anti-CD3, anti-CD8, and anti-CD19 CAR antibodies are used. (Mousset et al., 2019)

In-vitro Killing Assay: An *in vitro* immune-mediated tumor killing assay is used to predict the *in vivo* cytotoxicity and B-cell depletion of CD19-CARs. CD19+ cells are labelled with CFSE as well as pre-labelled CAR+ cells are separated and isolated from PBMCs by fluorescence-activated cell sorting (FACS). $5x10^4$ CD19+ cells are placed in 96-well plates.

CAR+ cells are added to wells in effector to target (E:T) ratios starting at 1:2 up to 3:1. (Zhang et al., 2018) Wells are incubated at 37 °C for 4 h. Resulting dead cells are labelled and used to calculate the percentage of total CD19+ cells that were killed.

Materials and Methods

Lentiviral Production: 2.5×10^7 Lenti-X 293T cells are placed into a T175 flask 24 hours before transfection. After media aspiration, 10mL of DMEM is added containing an autophagy inhibitor (chloroquine diphosphate). The solution is then incubated for 5 hours.

Optimized plasmid concentrations are diluted to a total volume of 500 μl using a serum-free median (SFM). Add polyethyleneimine (PEI) to the solution to have a 1:3 ratio of DNA:μg of PEI. Transfer plasmid solution to the Lenti-X 293T cells. Centrifuge viral supernatant at 500g for 5 minutes and then filter through 0.45μm polyethersulfone (PES) filter to pellet and remove packaging cells. Resulting vectors are frozen at -80 °C until further use. (Addgene, 2016)

T-memory stem cell Induction: CD8 cell line is labelled with cell-tracing dye and co-cultured with Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines (LCLs) and cells are transferred onto new medium after day 2 and day 4 of co-culture. After 7 days, activated CD8+ T-cells at a cell density of 1×10^5 cells/mL are co-cultured with DLL1-expressing OP9 stromal cells for the start of the induction phase. Cells are transferred to a new layer of DLL1-expressing O9 stromal cells on day 3, 7, and 11. After day 11, cells are counted and sorted to isolate T memory stem cells. (Kondo et al., 2018)

Sample PBMC Preparation: Cryopreserved B-cell lymphoma PBMC samples are placed into a water bath at 37°C until the last ice crystal remains to efficiently thaw. 50µl of RPMI-1640 growth medium along with 10% fetal bovine serum (FBS), L-glutamine, and 1% penicillin/streptomycin is added to the PBMC sample. Sample is then transferred into 15-30 mL falcon tubes where it is further supplemented with 10mL RPMI-1640 medium.

Solution is centrifuged to wash off toxic DMSO and other chemicals at 500g for 5 minutes, supernatant is removed, solution is re-supplemented with medium, then centrifugation and washing is repeated once over. To increase viable yield, PBMCs are rested overnight to remove apoptotic cells. Thawed PBMCs are activated on plates with anti-human CD3 and CD28 mAbs.

Cell Transduction: 1.5×10^5 PBMC are coated per well of a 48-well plate. LV vectors are added to cells and centrifuged at 850g for 90min at 32°C for spinfection.

Timeline of Project

Week 1 & 2: LVs production for CD8+ and T_{SCM} targets

Week 3 & 4: Transduce CD8+ T-cells and T_{SCM}'s and analyze titration

Week 5 & 6: Prepare and transduce PBMCs

Week 7: Flow cytometry and killing assay analysis

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