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Daniel Tylawsky April 15, 2015

Drug Resistance Immunotherapy for Cancer Treatment Using a Chimeric Antigen Receptor Approach

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Abstract

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The toxicity of nucleoside analogue chemotherapeutics on rapidly proliferating cells is a well documented and understood side effect of cancer treatment. One of the most consequential trade-offs of this off-tumor cytotoxicity is that it acts on immune system cells that have a natural capacity to fight tumors. As such, making immunocompetent cells resistant to a chemotherapeutic, so that they can stay alive during it's administration, is a desirable approach to enhance cancer treatment with chemotherapy. This is the basis for drug resistant immunotherapy (DRI), which we implement in our studies. We think that by combining novel immunotherapy strategies with currently used chemotherapy protocols, a potent and enduring cancer treatment can be achieved. In this study, we investigated the ability of overexpressed cytidine deaminase to confer resistance to the chemotherapeutic Ara-C and showed that this could be achieved in MV4-11 cells. For the immunotherapy arm of our studies, we experimented with engineering a chimeric antigen receptor (CAR) to enhance the specificity and activation of immunocompetent cells. We took a novel approach in constructing a CAR by utilizing a variable lymphocyte receptor (VLR) derived from lampreys, to function as the specific antigen-binding portion of the construct. This was done because the independently evolved adaptive immune system of jawless fish, which utilizes VLRs, can potentially recognize epitopes distinct from those recognized by mammalian antibodies. In this study, we experimented with an anti-CD5 VLR-CAR construct. We observed that Jurkat cells transduced with anti-CD5 VLR-CAR encoded lentivirus, were in fact successfully activated in the presence of CD5+ Jurkat cells. This proved that not only can VLRs be effectively used in a CAR construct, but also, for the first time, can activate T cells against a therapeutically relevant antigen.

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Tables and Figures

Introduction

Chemotherapy is well established and widely used in cancer treatment. While it has been largely successful for some diseases, there are several drawbacks. Chemotherapy comes at the cost of toxicity and morbidity in patients receiving treatment. One of the reasons for this is lack of specificity. Although many chemotherapeutics are effective killers of tumor cells, they are also effective killers of the body's own cells. This occurs because a common mechanism of action for chemotherapeutics is to attack rapidly proliferating cells, which would include tumor cells. However, other rapidly dividing cell populations of the body, such as white blood cells are targeted and killed as well, which can lead to leukopenia. The consequential immunosuppression makes patients vulnerable to opportunistic pathogens. This scenario is further complicated when multiple chemotherapeutic agents are used simultaneously. Many agents share the same toxicities, which can be additive or even synergistic. Additionally, the condition a patient is in when they begin treatment must be taken into account in determining a proper dose of chemotherapy. Sometimes the extent or distribution of tumors may be outside of curative potential of the chemotherapeutic. In such cases, treatment is more likely to be palliative in an effort to sustain a higher quality of life.[1] As such, the delicate compromise between efficacy and toxicity shows the need for the development of alternative, non-chemotherapy based treatments.

Another avenue currently being explored for cancer treatment is immunotherapy. This approach takes advantage of the body's own immune system to fight cancer. Cells that function in the immune system can be harvested from a patient and subsequently modified *in vitro* to specifically target a tumor cell. When these modified cells are returned to the patient, they effectively arm the patient with the proper means to mount an immune response against a tumor.[2] Some immunotherapy-based methods have been very effective in clinical trials. For example, in

patients with metastatic melanoma, adoptive transfer was performed with highly selected tumor-reactive T cells directed against overexpressed self-derived differentiation antigens. This resulted in a persistent clonal repopulation of T cells in patients, which displayed functional activity, and trafficked to tumor sites. This led to regression of the patients' metastatic melanoma.[3] While widely researched and promising, immunotherapeutic treatments are in their infancy with regards to clinical trials and approved applications. Today, there are only 4 active immunotherapies that have been approved for cancer treatment.[4]

Both immunotherapy and chemotherapy have their contributions and limitations in cancer treatment, however it is our lab's focus to combine the two approaches and take advantage of the benefits of both. As mentioned, chemotherapy results in toxicity and cell death of the body's good cells as well as tumor cells. Our immune system has a natural capacity to recognize and mount an immune response against tumor cells.[5] However, in the presence of a chemotherapeutic agent, immune system cells die and lose their tumor fighting potential. This complicates combining immunotherapy and chemotherapy because if modified immunocompetent cells are administered concurrently with a chemotherapeutic, they too will die, thus negating their therapeutic advantage. A possible solution to this problem is to make the modified immunocompetent cells resistant to the chemotherapeutic agent. Doing so could achieve a drug resistant immunotherapy (DRI), in which immunotherapeutic cells can effectively be combined with chemotherapy for potentially improved efficacy. If modified immunocompetent cells can be kept alive to fight in the presence of chemotherapy, then it can be hypothesized that the combined treatment would be more efficacious. Additionally, if such an approach is successful, it could incorporate sub-recommended doses for chemotherapy, which would result in less

toxicity throughout treatment. Furthermore, higher efficacy with less chemotherapeutic toxicity can allow for previously incurable prognoses to be successfully treated.

One possible target for DRI is acute myeloid leukemia. AML is a cancer of the blood and bone marrow in which myeloid stem cells have limited differentiation capacity preventing proper maturation of blood cells. The immature blood cells or myeloblasts cannot carry out normal blood cell functions, and their accumulation in the blood and bone marrow leaves less room for other blood cells to function normally. This can subsequently lead to problems such as infection, anemia, or excessive bleeding.[6] Current methods of treating leukemia in general include combinations of cytotoxic drugs, which have been more successful against ALL than AML. Using such methods, the 5-year survival rate for pediatric ALL is above 80% while that for pediatric AML is roughly 60%. As such, there is room for improvement in treatment of AML that extends beyond current chemotherapy regimens. Also, relapses and subsequent malignancies are especially difficult to treat with chemotherapy.[7] Dose intensification and introduction of new chemotherapeutic agents is limited since they come at the expense of increased toxicity.[8] Another important consideration is that in the context of pediatric oncology, stabilization of a disease for several months does not carry the same weight in children as it does in adults. This prompts a need to develop both a more effective and more permanent treatment for pediatric AML. The goal for future treatments must be to effectively cure the child; to have not only complete but also durable remission as to maintain a high quality of life after cancer.

One conventional approach to treating AML is through the use of Ara-C (cytosine arabinoside). Ara-C is a chemotherapeutic agent that functions as a highly cytotoxic cytidine analog. Cell death is achieved through Ara-C's ability to incorporate into DNA and interfere with DNA synthesis.[9] For over 40 years, this drug has been at the forefront of AML treatment

and it has been shown to lead to remission in about 85% of children with AML. Following induction therapy, higher doses of Ara-C are typically used for consolidation and 30-40% of patients will be cured from chemotherapy alone.[10] While it is a good drug, chemotherapeutic toxicity is a major obstacle for treatment with Ara-C, especially at high doses, which becomes increasingly problematic in consolidation and post relapse treatment.[11]

To lessen the toxicity of Ara-C against immunocompetent cells, we think these cells can be modified to express a gene that will allow them to be resistant to the drug. One method to achieve this is through the action of cytidine deaminase (CDA). CDA normally functions in the body as part of the pyrimidine salvage pathway where it catalyzes the irreversible hydrolytic deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively.[12] With regards to the drug Ara-C, CDA deaminates Ara-C to a much less active compound, arabinosyluracil (Ara-U) which is subsequently excreted.[13] Bearing this in mind, it is expected that increased expression of cytidine deaminase in a cell would render it resistant to the toxic effects of Ara-C.

Although humans naturally express CDA, it is in such quantities that there is little to no effect on the action of Ara-C. To increase the expression of CDA in immunocompetent cells to potentially Ara-C resistant levels, CDA encoded lentiviral vectors can be utilized. Lentiviruses are a subclass of rertroviruses that are able to infect both dividing and non-dividing cells. The use of lentiviruses is an attractive method for gene delivery because lentiviruses integrate into the host genome. This allows for a permanent copy of the gene of interest to remain in the genome. Therefore, when the infected cells divide, they carry the transgene with them.[14]

Along with chemotherapeutic resistance, a further consideration for DRI is the specificity of immunocompetent cells. To further augment their tumor suppressive quality, immunocompetent cells can be engineered to express monoclonal anti-tumor receptors. One way that this can be achieved is by developing a chimeric antigen receptor (CAR) for effector cells. CARs are constructed by combining a monoclonal antigen recognition site with the signal activating machinery of T cells. When such a receptor is engineered to be expressed on the surface of a cytotoxic lymphocyte, it can effectively target a specific tumor antigen and result in activation of immunocompetent cells that can thereafter mount an anti-tumor cytotoxic response. This is particularly effective because it frees antigen recognition from MHC restriction.[15] As such, the antigens that can be targeted are not limited to protein peptides, but also carbohydrate markers.

The conventional structure for a CAR combines a single chain variable fragment (scFv) from a monoclonal antibody with a CD3-zeta transmembrane signaling motif from a T cell receptor (TCR). To ensure that the heavy and light chain of an scFv fold over one another properly, a short linker fragment (usually rich in glycine for flexibility) is incorporated. Finally, a hinge region is added to bridge the antigen-binding and signaling portions of the CAR. This portion is generally comprised of the Fc domain of an IgG1 antibody. CARs that only incorporate a CD3-zeta signaling domain are considered first generation. Such CARs still require antigen presenting cells to provide co-stimulation for the CAR modified cell to be fully activated. However, 2nd and 3rd generation CARs are engineered to incorporate additional stimulatory domains that are typically derived from intracytoplasmic portions of costimulatory molecules. Accordingly, when such CARs bind antigens, multiple costimulatory signals are

delivered to the cell. This effectively bypasses the need for costimulation from other cells and lessens the likelihood of anergy or apoptosis from lack of costimulation.[16]

It should be noted that while the potential of CAR diversity is immense, there are some limitations to the use of monoclonal antibodies for construction. For one, immunoglobulin structure is restricted by mammalian genetic diversity. To develop monoclonal antigen-binding regions for the CARs in our study, we are exploring lamprey variable lymphocyte receptors (VLRs). Lampreys independently evolved an adaptive immune system using leucine-rich repeat motifs that form antigen-recognizing VLRs.[17] These VLRs have extensive diversity similar to antibodies and it is anticipated that the independently evolved VLRs can recognize epitopes that are unique from those that would be recognized by monoclonal antibodies. An additional advantage to VLRs is that they are naturally single chain molecules, which makes them easier to work with than single chain variable fragments that have to be constructed from an antibody. To generate an scFv, the sequences of a heavy chain and a light chain have to be isolated and cloned out from the gene of an antibody of interest. A proper linker sequence then has to be incorporated between the two chains to facilitate linkage between them. As such, achieving proper protein folding for an scFv can be a painstaking process. Since VLRs are already single chained structures, this additional modification to construct the antigen-recognizing region for a CAR is circumvented.

In our study we are exploring an Anti-CD5 VLR-CAR. The sequence for the anti-CD5 VLR was published by a collaborator and could easily be integrated into a CAR construct that had already been verified in our lab.[18] CD5+ malignancies to which such an immunotherapeutic agent can be applied include a variety of lymphomas such as chronic lymphocytic leukemia, small lymphocytic lymphoma, and mantle cell lymphoma.[19]

The immunocompetent cells that we are studying include NK92 cells and gamma delta (γδ) T cells. NK92 cells are a cell line of natural killer cells. NK92 cells make for a good model for ordinary NK cells for several reasons. First, they express the characteristic CD56 surface marker and are similarly CD3-. They also have an analogous mechanism of action in that NK92 cells recognize foreign cells only if those cells do not express the proper self-MHC molecules. Additionally, NK92 cells can induce apoptosis via several mechanisms including secretion of perforin and subsequent granzyme release or through the Fas-Fas Ligand process. Additionally, they are capable of producing cytokines like TNF-alpha that can kill cancer cells, as well as
cytokines that will stimulate and expand other immune cells such as interferon.[19]

We are also studying γδ T cells as potential immunocompetent effectors. While the large majority T cells use an αβ T cell receptor as an antigen recognition structure, this second population of T cells expresses an alternative TCR comprised of one γ and one δ glycoprotein chain. As such, γδ T cells recognize qualitatively distinct antigens from αβ T cells. It is also notable that γδ T cells are not restricted to recognizing peptides that are bound to MHC molecules displayed by antigen presenting cells. In addition, they can function to signal and regulate other immune cells by their capacity to produce diverse cytokines and chemokines. Furthermore, γδ T cells can directly lyse and eliminate infected or stressed cells through the production of granzymes. Another reason they are of particular interest is their ability to recognize stress induced ligands and subsequently have antitumor cytotoxicity.[20] In fact, it has recently been observed that γδ T cells can specifically recognize and kill AML myeloblasts which makes them of particular relevance.[21]

In this study there are two main branches of focus: drug resistance and immunotherapy. With regards to the former, using AML as a target disease, we sought to confer resistance in cells to the prevalently used chemotherapeutic Ara-C. To accomplish this, we constructed a cytidine deaminase encoded lentivirus, transduced a target human AML cell line (MV4-11), and determined the extent of resistance from survival curves. We began experimentation using one variant of human cytidine deaminase (K27) and moved into a comparative study with an alternative variant (Q27). The Q27 variant differs from K27 by one non-conservative amino acid substitution at position 27 of the amino acid sequence, yielding a glutamine (Q) residue instead of a lysine (K) residue at that position. Notably, the Q27 variant has been characterized as a more efficient enzyme for Ara-C as a substrate, showing a lower K_m and a higher V_{max}.[12] For immunotherapy experiments, we investigated the effectiveness of an anti-CD5 VLR CAR that we constructed. We tested to see if a T cell line (Jurkat) transduced with our construct demonstrated increased activation in the presence of CD5+ cells.

Methods

Ara-C Resistance Studies:

Cytidine Deaminase Gene

The correct cDNA sequences for the K27 and Q27 variants of human cytidine deaminase (CDA) were identified by searching publicly available DNA sequences on the NIH genetic database GenBank ®. Once verified, the CDA sequences were flanked with EcoRI and BamHI restriction enzyme recognition sequences to create distinctive cohesive ends and thereby facilitate directional cloning of the gene fragment. The flanked CDA sequences were then submitted to Integrated DNA Technologies (IDT) for synthesis as codon optimized double stranded gene fragments (gBlocks®). Once synthesized, the lyophilized (dried) DNA product was re-suspended in 20µL of water to give a final concentration of 10 ng/µL.

Restriction Enzyme Digest

For each variant, the re-suspended gene fragment was digested with EcoRI-HF (high fidelity) and Bam HI-HF in a solution of CutSmart® buffer and water. Similarly, a FUGW lentiviral expression plasmid was digested with the aforementioned enzymes to excise GFP so that the CDA gene could be inserted in its place (Figure 1). After a 2-hour incubation period, gel electrophoresis was performed on the FUGW sample using a 0.8% agarose gel, which yielded two bands. The band corresponding to the FUGW backbone (larger band) was cut out from the gel and afterwards a gel purification assay was performed using a Qiagen gel extraction kit. The concentration of the extracted plasmid sample was determined using a NanoDrop™

spectrophotometer (as were all subsequent measures of concentration of plasmid sample). Next, a ligation assay was performed. The digested CDA gBlock® fragments and digested FUGW vector were incubated in solutions of QuickLigase, QuickLigase buffer, and water for 5 minutes at room temperature.

Plasmid Transformation and Screening

The ligation mixtures were then added to a vial of competent One Shot® Stbl3™ (Life Technologies) and incubated on ice. After 30 minutes, the cells were heat-shocked for 45 seconds in a 42 °C water bath. The cells were removed from the bath and placed on ice for an additional 2 minutes, after which 250uL of S.O.C. Medium were added to each vial. Vials were then placed in a horizontal shaker at 37°C for 1 hour at 225 rpm. Finally, 50µL of the transformation mixtures were spread onto an ampicillin selective agar plates for overnight incubation. The following day, individual colonies were picked from the plates and placed in cultures containing 3mL of LB broth and 3µL of 1000x ampicillin solution. The bacteria cultures were grown overnight. Plasmids were extracted from 1.5mL of the 3mL cultures using a Qiagen mini-prep kit and then screened via multiple restriction enzyme digests and gel electrophoresis (Table 1).

Virus Production

To generate more plasmids, the remaining 1.5mL of the initial bacterial cultures were expanded to a culture of 100mL LB broth, and 100µL of 1000x ampicillin solution. The following day plasmids were extracted using a Qiagen midi-prep kit. The plasmids isolated from this assay were once again screened using the same screening strategy as before. The correctly screened

plasmids were then used for virus production performed by Andrew Fedanov (lab member) and we appreciate his help in providing us with CDA encoded lentivirus. The viral titer for the Q27 encoded virus was $8.29x10^8$ viral particles/mL and that of the K27 encoded virus was $1.44x10^8$ VP/mL.

Transduction of MV4-11 Cells and Jurkat Cells

The resulting lentivirus was subsequently used as the vector to transfer the CDA genes into naïve cells. To set up MV4-11 transductions, $1x10^6$ cells were plated in 1mL of IMDM Media (10%FBS, 1%Pen/Strep) in a 6-well plate. Next, Q27 and K27 encoded viruses were added to cells at volumes of either 100µL or 300µL. One of the plated wells was left untransduced as a naïve control. Additionally, 1µL of 1000x polybrene was added to each well to improve transduction. Jurkat cells were similarly plated with the exception of using RPMI Media (10%FBS, 1%Pen/Strep). To transduce the cells, Q27 and K27 encoded viruses were added to cells at volumes of either 1µL or 5µL. Smaller volumes were used for Jurkat cells because they are more easily transduced. Once again, one well of cells was left untransduced as a control and 1µL of 1000x polybrene was added to each well. In both experiments, media was changed the following day to remove viral particles.

Real-time PCR (qPCR)

Once the cells were assessed to be growing stably, genomic DNA was extracted from an aliquot of cells using a Qiagen DNeasy Blood and Tissue kit. This genomic DNA was tested in a qPCR assay using RRE primers to determine copy number.

Survival Curves

After performing copy number analysis, we continued experimentation using the groups of K27 and Q27 transduced cells that showed the most comparable average number of copies. We proceeded with examining the ability of these transduced cells to show resistance to Ara-C. Survival curves were generated for MV411 cells and Jurkat cells using an identical procedure. Ara-C was added to cells at concentrations of 0μM, 0.5μM 1μM, 2.5μM, 5μM, and 10μM. On day 3 post-addition of drug, cell counts were performed to determine the amount of cells per well Following a cell count, 200μL aliquots of cells were taken from each well and plated in 2 mL of fresh media. We then allowed the cells to grow for an additional 7 days and added an additional 2mL of media on day-6 post-addition of drug. Finally, on day 10-post addition of drug, we performed a second cell count for each well.

Cytotoxicity Assays

In a cytotoxicity assay of NK cells versus MV4-11 AML cells we utilized flow cytometry. For this experiment, we stained target cells (MV4-11) with the cell membrane dye PKH-26 and we left effector cells (NK92) unstained. We then incubated target and effector cells together for 4 hours at several ratios of effector to target: 0:1, 1:1, 2.5:1, 5:1, 10:1. After incubation, samples were stained with TO-PRO-3, which is a dead cell stain. Finally, we ran the cells through a BD™ LSR II flow cytometer. This cytotoxicity assay was likewise applied when we tested gamma-delta T cells killing of Jurkat cells.

Chimeric Antigen Receptor Immunotherapy Studies:

Anti-CD5 VLR CAR cloning

For our CAR studies, we had to clone the sequence of anti-CD5 VLR-CAR construct (Figure 2). As before, to accomplish this we ordered a gBlock® of the sequence for the construct. Once this was synthesized by IDT, we performed a restriction enzyme digest on both the gBlock® and a FUGW lentiviral expression vector. After digestion, a ligation was performed between the two digests and the mixture was transformed into competent Stbl3™ cells. These bacteria cells were then cultured and allowed to grow for 24hrs after which plasmid DNA was extracted using a Qiagen mini-prep kit. The plasmid samples extracted were then screened to assure the final plasmid was properly ligated. When this was confirmed via restriction enzyme digest and gel electrophoresis, we expanded the culture that screened correctly. After another 24 hours of growth in a 37°C shaker, we isolated plasmids using a Qiagen midi-prep kit. After screening this plasmid, virus was again produced by Andrew Fedanov in our lab. The viral titer for unconcentrated virus was 5.05×10^6 VP/mL and for concentrated virus it was 9.15×10^7VP/mL.

Transduction and qPCR

Once virus was produced, it was used to transduce Jurkat cells. To set up this experiment, we plated 2×10^6 cells in 2mL of RPMI media (10% FBS, 1% Pen/Strep) on a 6-well plate. We transduced 6 wells of cells at a range of volumes of virus using 5µL, 40µL, and 160µL of unconcentrated virus (5.05×10^6 VP/mL) and the same volumes of concentrated virus (9.15×10^7VP/mL). Next we performed qPCR using RRE primers to determine copy number.

This was done so that upon performing activation assays, we could determine the minimal VCN (vector copy number) that would activate T cells.

Additionally, another transduction experiment was set up using a new virus and a range of MOI's based on the titer of the virus ($7.84x10^8$ VP/mL). In a 6-well plate, $1x10^6$ Jurkat cells in 1mL of RPMI Media (10%FBS, 1%Pen/Strep) were transduced at MOI's of 1, 2, 10, and 20 viral particles/mL. One well of plated Jurkat cells was left untransduced as a control. As with previous transductions 1μL of 1000x polybrene was added to each well.

CD69 Activation Assay

To determine the efficacy of the CAR construct we tested for CD69 (activation marker) expression using flow cytometry. For this assay, cells were stained with an anti-CD69 APC conjugated antibody. We then performed flow cytometry using a BD™ LSR II flow cytometer and looked for expression of CD69.

Results

Cloning of Cytidine Deaminase

To amplify copies of cytidine deaminase gene (both K27 and Q27 variant), we first had to clone the synthesized CDA gene fragments into viral expression plasmids (FUGW). When a restriction enzyme digest and gel electrophoresis was performed on the FUGW plasmid, two bands were observed that were consistent with expectations: a larger band corresponding to the FUGW backbone, and a small band corresponding to excised GFP. DNA isolated and purified from the larger band was ligated with either the K27 or Q27 CDA gene fragment, which had been digested to have complementary cohesive ends.

Bacteria transformed with these ligation products showed growth of colonies on ampicillin selective plates. The presence of colonies indicated bacterial expression of an amp-resistant gene. Since an amp-resistant gene is incorporated as a component of the FUGW backbone, this served as a preliminary indicator that transformations had been successful. Moreover, when plasmids from cultures expanded from these colonies were screened, the band lengths observed on the gel corresponded to the band lengths expected for each of the different restriction enzyme digests performed (Table 1, Figure 3). This provided good evidence that the cytidine deaminase had ligated properly into the FUGW backbone.

Functional Results of CDA over-expression

To determine if CDA can confer resistance to a cell line, we chose MV4-11 cells and Jurkat cells as targets, transduced them with CDA encoded lentivirus, and generated survival curves in the presence of Ara-C.

Transduction of MV4-11 Cells

Two viruses were used to transduce MV4-11 cells: one encoded for the K27 variant of CDA and the other for the Q27 variant. To determine if the transduction was successful and that the CDA gene in had been integrated into the MV4-11 cell genome, a real-time PCR assay was performed. All transduced groups showed at least one copy of the CDA gene, which indicated the viruses had successfully infected MV4-11, cells as intended (Figure 4). The two groups of interest were the K27 with 1.94 copies/cell and the Q27 group with 3.20 copies/cell. These were the most closely matching groups of each variant of CDA and as such were used in subsequent studies to compare the efficacy of each.

MV-411 Survival Curves

Survival curves were used to assess whether cells with multiple copies of the CDA gene could exhibit resistance to Ara-C toxicity. Three groups of cells were tested for their survival after exposure to a range of Ara-C concentrations: naïve, K27 transduced, and Q27 transduced. When cells from each group were counted on day 3 post-addition of drug, they all showed similar percent survival on all points of the curve. On day 10 post-addition of drug, significant separation between transduced and non-transduced groups was observed, at concentrations above 1μM Ara-C. Notably, it was observed that at the highest dose of Ara-C (10μM) the naïve MV-411 cells were almost completely eradicated, while both transduced groups showed roughly 40% survival. The IC-50 of the naïve cells was close to 2μM Ara-C while the IC-50 of the transduced groups was between 6μM and 7μM Ara-C. Surprisingly, groups transduced with the K27 and Q27 variants of CDA did not show a significant difference in their ability to confer resistance to Ara-C (Figure 5).

Transduction of Jurkat Cells

Similar to MV4-11 cells, we transduced Jurkat cells with lentivirus encoded with the K27 or Q27 variant of cytidine deaminase. We used real-time PCR to perform copy number analysis on the genomic DNA extracted from transduced cells. Again, it was observed that the virus was successful at integrating the transgene into the host cell genome as multiple copies were seen for both variants of CDA (Figure 6). We proceeded with experimentation in cells that showed an average of 1.56 copies for the K27 variant of CDA and 4.07 copies for Q27.

Jurkat Cell Survival Curves

In this experiment, transduced cells had no appreciable ability to confer resistance compared to naïve cells. This experiment was performed twice and in both instances a similar result was observed. On day 10 post-addition of drug, transduced and naïve cells were observed to have an IC-50 near 2μM Ara-C. At the 10μM Ara-C concentration point, percent survival was nearly 0% for both transduced and untransduced cells (Figure 7).

Cytotoxicity of NK92 cells against MV4-11 Cells

In our study, NK92 cells served as a potential immunocompetent cell line in which it could be desirable to confer resistance. To test their ability to kill a leukemic cell line, a cytotoxicity assay was performed such that death of MV4-11 cells (targets) was monitored in the absence and presence of NK92 cells (effectors). The control group, which contained only target MV4-11 cells showed only 1.2% target cell death when assayed using flow cytometry. When NK92 cells were incubated with MV4-11 cells at effector to target ratios of 2.5:1 and 10:1, it was observed that there was 73% and 80% target cell death, respectively (Figure 8).

CAR Studies

Anti-CD5 VLR-CAR cloning

Although our drug resistant studies are ongoing, the other technology that we are developing is CAR modified T cells. The sequence for anti-CD5 VLR-CAR construct was synthesized by IDT as a codon optimized gBlock®. Stbl3™ cells were transformed with a ligation product comprised of a FUGW backbone with an anti-CD5 VLR-CAR sequence incorporated in the place to GFP. When plasmids extracted from the Stbl3™ bacterial culture were digested with restriction enzymes and subsequently run on an electrophoresis gel, the anticipated fragments were observed (Table 2, Figure 9). This plasmid product was therefore deemed usable for virus production.

Transduction of Jurkat cells

Jurkat cells were transduced with several volumes an anti-CD5 VLR-CAR encoded lentivirus. Real-time PCR was performed on the genomic DNA extracted from transduced cells. For groups transduced with 5μL, 40μL, and 160μL of unconcentrated virus, the copy numbers were determined to be 0.13, 0.54, and 1.84, copies per cell respectively. For groups transduced with 5μL, 40μL, and 160μL of concentrated virus, the copy numbers were determined to be 3.63, 22.80, and 33.92 copies per cell respectively (Figure 10).

With a better idea of how efficiently Jurkat cells were transduced, we performed a follow up experiment using newly produced virus encoded with the same anti-CD5 VLR CAR gene. In this experiment, the multiplicities of infection (MOI) used to set up the experimental groups were 1,

2, 10, and 20 viral particles/cell. Jurkat cells transduced at these ratios showed corresponding copy numbers of 1.51, 2.16, 7.84, and 15.16 copies/cell respectively (Figure 11).

CD69 Activation in CAR Modified Jurkat Cells

Jurkat cell activation was measured by assaying for CD69 expression on cells using flow cytometry. The population of naïve Jurkat cells showed 0.9% activation. Cells transduced with 5μL, 40μL, and 160μL of unconcentrated virus, showed activation at levels close to the naïve group. A non-negligible amount of activation (4.6%) was observed in the population of cells transduced with 5μL of concentrated virus. Since activation was observed between groups transduced with 160μL unconcentrated virus 5μL concentrated virus, corresponding to copy numbers of 1.84 and 3.63 copies respectively, the minimum viral copy number that results in activation is somewhere in this range. Most notably, groups transduced with 40μL and 160μL of concentrated virus showed 50.3% and 78.1% activation respectively (Figure 12).

When this assay was performed on cells from the second transduction experiment, activation was again observed in a viral dose dependent manner. Naïve cells showed activation of only 0.4% and the cells from groups transduced at an MOI of 1 VP/cell and MOI of 2 VP/cell showed 1.8% and 2.6% activation respectively. The extent of activation was even greater in cells at higher MOI's of 10 and 20 VP/cell. These populations showed 7.6% and 18.5% activation respectively (Figure 13). In both experiments, the amount of activation observed was proportional to the average number of copies determined to be in those cells from qPCR (Figure 14).

Transduced Cell Growth

Cell growth was monitored for transduced Jurkat cells to observe if there were any adverse effects resulting from viral transduction. Interestingly, over the span of 6 days, cells transduced with 40μL and 160μL of concentrated virus showed counts that persisted at around $1x10^6$ cells. In contrast, the groups transduced with fewer virus showed more consistent growth during that period of time, with as much as a 3-fold increase in number of total viable cells (Figure 15).

Gamma-Delta T cell cytotoxicity against Jurkat cells

As immunocompetent cells of interest for CAR modification, gamma-delta T cells were tested for their ability to Jurkat cells as a target. As in the previous cytotoxicity assay, target cell death was measured with and without incubation alongside an effector cell line. Jurkat cells assayed alone showed low cell death at only 0.9%. When gamma delta T cells were incubated with Jurkat cells at a target to effector ratio of 5:1, target cell death was observed to be 11.0%. Increasing the effector to target ratio to 10:1 yielded 24.1% target cell death (Figure 16).

Discussion

In this study, one of the aims was to confer resistance to the chemotherapeutic Ara-C by way of transducing cells with lentivirus encoded with a cytidine deaminase gene. If properly expressed, cytidine deaminase would function to inactivate Ara-C into Ara-U and thereby protect a cell from the cytotoxicity of the chemotherapeutic. To test this, we experimented with two variants of cytidine deaminase, K27 and Q27, the later of which was expected to have higher efficiency. The two cell lines we used for this chemotherapy resistance study were MV4-11 cells and Jurkat cells.

We first experimented, with MV4-11 cells transduced with either K27 or Q27 encoded lentivirus. We set up survival curve studies to see if transduced cells had a greater capacity to survive in the presence of Ara-C. On day 3 post-addition of drug, both naïve and transduced groups showed similar percent survival across each concentrations tested. This result was expected because it takes several days for Ara-C to take noticeable effect in the cells. It is likely that at this time point, cells in the naïve group that were dying (but not visibly dead) were counted as living, thus inflating the percent survival. This notion is supported by the data gathered on day 10 post-addition of drug which showed a greater separation in the percent survival between transduced and untransduced groups. The naive cells were observed to have an IC-50 at roughly 2μM Ara-C while both transduced groups had an IC-50 at around 6μM Ara-C. Of particular note is the extent of cell survival at the highest concentration of Ara-C (10μM). The transduced groups had close to 40% survival while the naïve group was nearly entirely killed by the drug. This is good evidence that the transduced groups are actually expressing the cytidine deaminase genes that were observed to be integrated into the MV4-11 cell genome and that the enzyme is functioning properly in the cells.

In comparing the efficacy of the K27 variant of cytidine deaminase to the Q27, there was not a significant difference observed in resistance conferred between the two groups. It was expected that the Q27 variant would be more successful because compared to K27, as it had previously been shown to have a lower K_m and a greater V_{max} for Ara-C as a substrate. A possible explanation for the lack of discrepancy between the two variants could be attributed to the copy number associated with each. The cells transduced with the K27 CDA had a copy number of 4.04 copies/cell while those transduced with Q27 CDA had an average copy number of 3.2 copies/cell. As such, although the Q27 variant was expected to be more efficient, there were more copies of the K27 that could have offset the advantage. Another factor to consider in comparing these variants is where the CDA gene copies may have inserted in the genome. Although lentiviral vectors tend to target active transcription units, they integrate somewhat randomly throughout the gene.[22] Accordingly, a gene inserted in an unfavorable place can offset the effects that would otherwise result from its expression. It is possible that this may have occurred with the integration of the Q27 CDA gene into the target cell genome.

Following studies with MV4-11 cells, Jurkat cells were investigated to try to achieve similar results in a T cell line since T cells are utilized in the engineering and implementation of CARs. Transduction of Jurkat cells was successful since several copies of the CDA gene were observed from qPCR of genomic DNA. However, while the CDA gene was integrated in to the Jurkat cell genome, transduced groups did not show any resistance to Ara-C. In both trials of the experiment, transduced and non-transduced groups alike had an IC-50 at around 2μM Ara-C and showed near complete death at a concentration of 10μM Ara-C. This data suggests that T-cells transduced with CDA encoded lentivirus garner no advantage against cytotoxicity from Ara-C. It appears that while the CDA gene was incorporated in the Jurkat cell genome, it is not being

expressed. As mentioned, this could possibly be due to insertion at a location unfavorable to its expression. Alternatively, other studies have shown that Jurkat cells demonstrate greater accumulation of nucleoside analogs compared to other cell types such a B-cells.[24] Although the mechanism for this is not yet clear, increased accumulation of Ara-C in cells would lead to increased integration of this cytotoxic analogue into DNA. It is therefore possible that in the Jurkat cells, CDA may have been successfully expressed, but the amount of accumulated Ara-C was too large to be effectively inactivated by CDA. To overcome this shortcoming in Jurkat cells, a cassette with a stronger promoter might need to be incorporated which would result in enhanced expression and could confer resistance at these higher effective concentrations of Ara-C.

From these studies, it was observed that resistance could be conferred in MV4-11 cells but not Jurkat cells. One of the reasons that we performed experiments using Jurkat cells was that they are T cell line and with our CAR studies, we were aiming to modify T cells. As such they served as a good bridge between both resistance and immunotherapy studies. However, not observing resistance in Jurkat cells redirected our resistance studies towards finding another immunocompetent cell line in which to confer resistance. Before transducing another cell line with CDA encoded lentivirus, we sought to verify that our new cell line of interest (NK92 cells) could demonstrate a functional response against MV4-11 cells (human AML cell line). These cells resemble wildtype natural killer cells, and if capable of targeting and killing MV4-11 AML tumor cells, it would make NK cells a particularly advantageous population of cells to keep alive in the presence of chemotherapy. The cytotoxicity assay that we performed tested for NK92 cell killing of MV4-11 cells. At an effector to target ratio of just 2.5:1, 73% of target cells were killed. This number went up to 80% at an E:T ratio of 10:1. The significance of this result is that

NK92 cells have an innate ability to be cytotoxic against AML cells and are thus good candidates for drug resistant modification. We are continuing our resistance studies in the direction of modifying NK92 cells to be resistant to Ara-C.

In our other study of immunocompetent cells, we found that naive gamma-delta T cells are capable of killing Jurkat cells. We observed that at effector to target ratios of 5:1 there was 11% cell death in the target population. When this ratio was increased to 10:1, we observed 24.1% target cell death. An explanation for why gamma-delta T cells are able to recognize and kill Jurkat cells is their ability to interact with stress ligands. In particular NKG92 ligands like ULBP-1,-2, and MICAB which are expressed on Jurkat cells. Furthermore, the importance of the cytotoxic ability demonstrated by the gamma delta T cells is two fold. First, with respect to CAR studies, to further enhance the ability of gamma delta T cells to target Jurkat cells, they can be modified to express an anti-CD5 VLR-CAR. Upon doing so, it is anticipated that gamma-delta T cells will demonstrate improved killing due to improved targeting and activation. Second, because of their innate ability to recognize and be cytotoxic against cells expressing the aforementioned stress antigens, these cells (like NK92 cells) are strong candidates for modification to confer resistance to a chemotherapeutic agent.

For our CAR studies, to test for the expression of our anti-CD5 VLR-CAR construct, we looked for CD69 expression on the surface of transduced Jurkat cells using flow cytometry. CD69 is an activation antigen and its expression on the Jurkat cell surface is induced upon activation. It is expected that Jurkat cells expressing an anti-CD5 VLR-CAR should become activated in the presence of other Jurkat cells, as they are naturally CD5+. As such, cells expressing a properly functioning CAR construct against CD5 should show CD69 expression as a result of interacting with CD5 on the surface of Jurkat cells and becoming activated. Our data

showed that following transduction, Jurkat cells with average of 2.16 copies of the anti-CD5 VLR-CAR gene, showed activation in 2.6% of cells, compared to naïve Jurkat cells which showed only 0.4% activation. Although this improvement was modest, at higher copy numbers better results were observed, with up to 78.1% of transduced cells showing activation. This suggests that the CAR construct was not only being expressed on the surface of transduced cells, but also recognizing antigen and signaling properly. This result was very encouraging since it showed that the novel approach of using a VLR as the antigen recognition portion of a CAR is compatible with the rest of the construct and can result in proper antigen recognition and signaling.

An interesting observation in this study was the stagnation of growth for cells transduced with higher volumes of concentrated virus. It is possible that being exposed to more virus had a deleterious effect on these cells, however the stagnation was persistent over time, and no recovery towards consistent growth was observed when virus had been washed from the cells. Alternatively a possible explanation for this is that the cells that received the more virus and showed higher copy numbers for the CAR gene, were also exhibiting the most CAR expression on their surface. Because Jurkat cells are CD5+, the presence of Jurkat cells expressing an anti-CD5 VLR-CAR can result in cell-to-cell interaction. If the CAR functioned properly, it is possible that the cells cannot physically divide properly because they are binding to each other. Another possible explanation is that such an interaction leading to Jurkat cell activation could consequently result in activation induced cell death if over-stimulated.[25]

Overall, this study helped propel the potential applications of drug resistance immunotherapy. We have shown that overexpression of cytidine deaminase can in fact confer

resistance to Ara-C as demonstrated with our studies of MV4-11 cells. Moving forward, we hope to confer resistance to Ara-C in the NK92 cell line.

With regards to immunocompetent cells and potential targets for both resistance and CAR modification studies, we confirmed that NK92 cells and gamma-delta T cells are worthy candidates. Both of these cytotoxic effector cells were able to successfully kill target cell lines. We anticipate that engineering both of these cells types to express a CAR would further enhance their cytotoxicity.

Most significantly, we were able to construct and effectively test a chimeric antigen receptor using a VLR. This is the first time that T cells have been successfully activated by a CAR utilizing a variable lymphocyte receptor against a therapeutically relevant antigen. Using a VLR for antigen recognition in CAR constructs greatly broadens and even strengthens the potential of CAR T cell therapy. The ease of isolation and cloning of VLR sequences makes them conducive to gene therapy protocols. VLRs are single chain structures and as such they require less bioengineering to be integrated into a CAR construct than single chain variable fragments derived and modified from antibodies. Furthermore, VLRs evolved independently from immunoglobulins and have an extensive structural diversity with distinctive binding site geometry. This gives VLRs the capacity to recognize unique epitopes that mammalian antibodies cannot bind to due to structural limitations. As such, there is great potential in the therapeutic use of variable lymphocyte receptors, and in this study we were able to affirm at least one aspect of their broad range of applications by showing they can successfully be integrated in chimeric antigen receptors to activate T cells.

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Tables and Figures

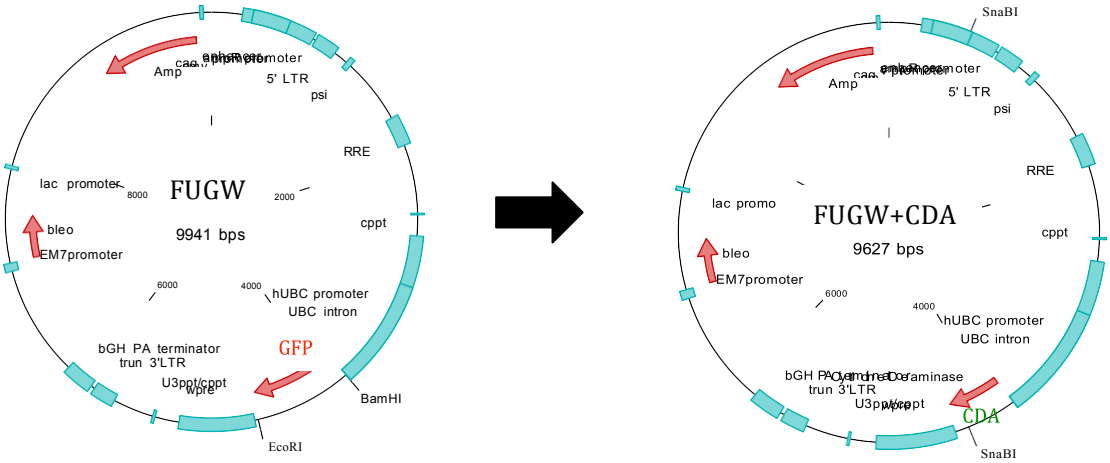


Figure 1: (A) FUGW backbone (B) FUGW+CDA (cytidine deaminase). GFP was excised from the FUGW vector using the restriction enzymes EcoRI and BamHI. Cytidine deaminase flanked by EcoRI and BamHI sequences was likewise digested by those enzymes and subsequently ligated in the place of GFP.

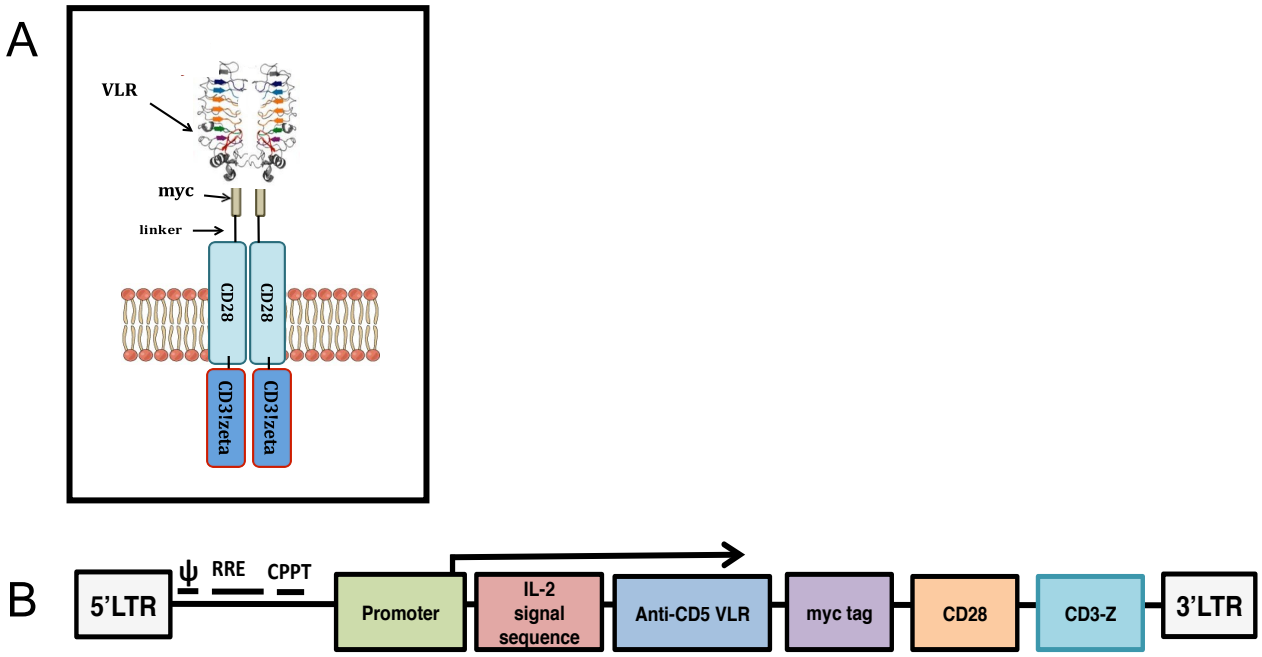


Figure 2: (A) Schematic of anti-CD5 VLR construct as it would appear in membrane. **(B)** Anti- CD5 VLR chimeric antigen receptor construct. From left to right: An IL-2 signal peptide sequence is incorporated to facilitate protein transport to the cell membrane. Next, is the anti- CD5 variable lymphocyte receptor sequence, which gives the CAR construct its specific antigen recognition. Following the VLR sequence is a myc tag sequence. This is incorporated as a proxy for CAR expression as it can be identified using a myc antibody in flow cytometry. CD28 functions as the trans-membrane domain for the CAR as well as a co-stimulatory signal provider. Finally, a CD3-zeta domain comprises the intra-cytoplasmic portion of the CAR and functions as an intercellular signal provider for activation.

Restriction Enzymes Used To Digest	Expected Band Lengths With Correct Ligation (in base pairs)
PvuII, NotI	3514, 2379, 1748, 1167, 818
Bsu36I, BssHII	3884, 2339, 1798, 835, 768
ScaI, draIII	2838, 2314, 2035, 1005, 824, 610
BamHI, EcoRI	9180, 446

Table 1: Restriction enzymes used for digestion of ligated FUGW+CDA plasmids and the respective expected band lengths with successful ligation.

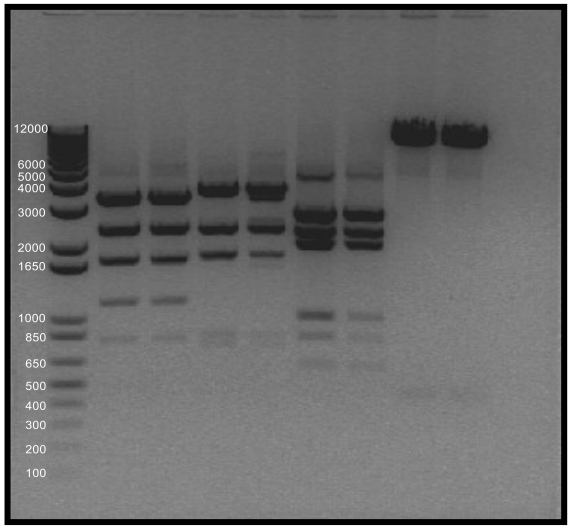


Figure 3: Electrophoresis gel from restriction enzyme screening digest of cytidine deaminase (CDA) constructs. Lane 1 is the ladder. Alternating lanes with K27 (even) and Q27 (odd) variants of CDA, samples were digested with the following enzymes: PvuII/NotI (lanes 2,3), Bsu36I/BssHII (lanes 4,5), DraIII/ScaI (lanes 6,7), and BamHI/EcoRI (lanes 8,9).

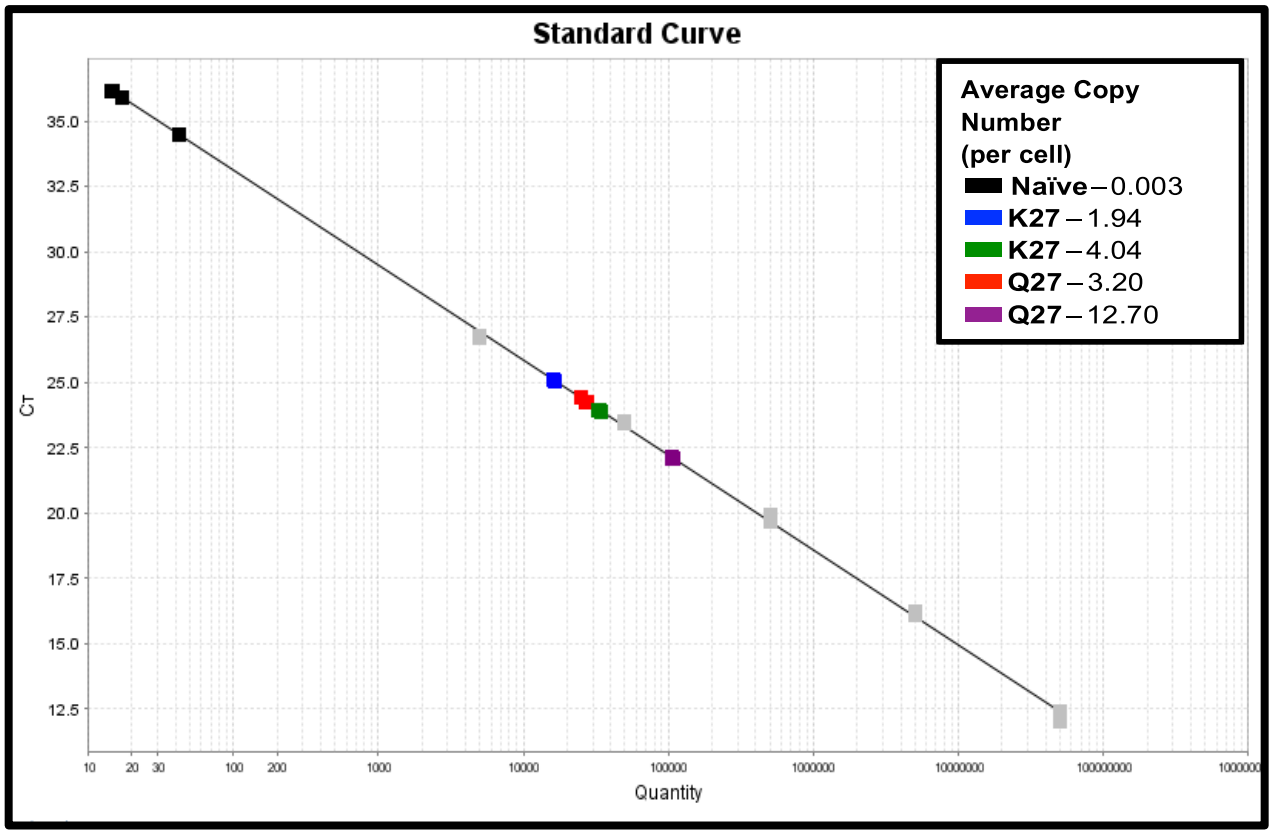


Figure 4: Real-time PCR data used to determine copy number of MV-411 cells transduced with CDA encoded lentivirus. Transductions were performed with both the K27 and the Q27 variants of CDA. These data show that viruses encoded with each variant were successful at integrating the CDA gene in MV4-11 host cell genomes.

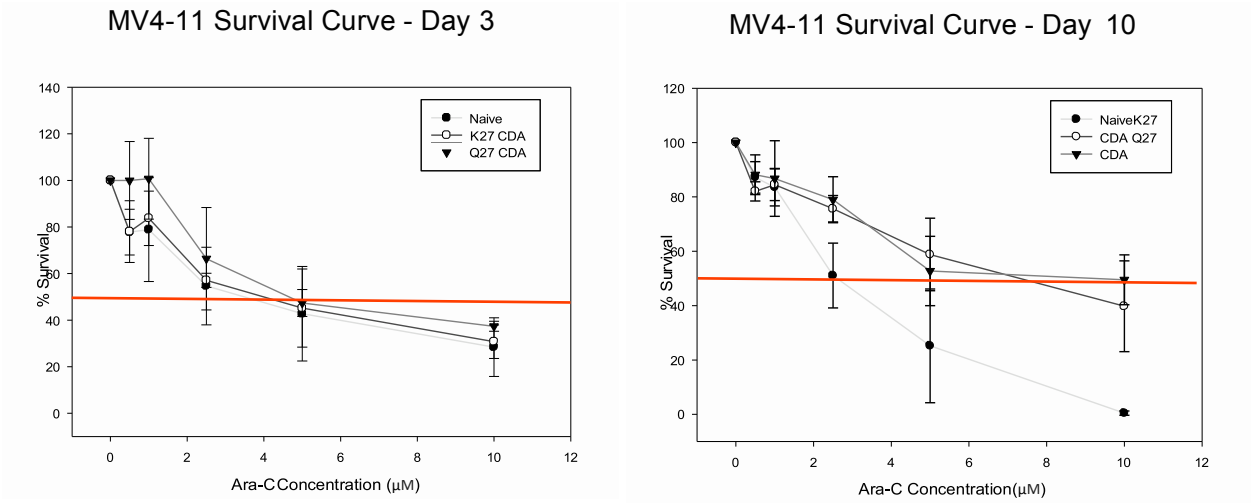


Figure 5: MV4-11 survival curves. MV4-11 cells were plated in a 6-well plate at a density of $1x10^6$ cells/mL in 1mL of media. The concentrations of Ara-C added to the wells were 0uM, 0.5μM, 1μM, 2.5μM, 5μM, 10μM. This experiment was carried out in triplicate. Percent survival was calculated against the population of the 0μM Ara-C control for each group. (A) Percent survival on day 3 of naïve, K27 transduced, and Q27 transduced cells. On day 3, all the groups have similar percent survivals. This was expected because it takes several days for Ara-C to take noticeable effect in the cells (B) Percent survival of cells on day 10. By day 10, it is observed that untransduced cells have an IC-50 at roughly 2μM Ara-C while the transduced groups have an IC-50 at around 6μM Ara-C. This is good evidence of resistance to Ara-C in transduced groups, which appear to be successfully overexpressing cytidine deaminase. Moreover, at a 10μM dose of Ara-C, the transduced groups retained about 40% of their initial population, while naïve cells are almost entirely dead. With regards to comparing the chemo-resistant action of the two forms of cytidine deaminase (K27 and Q27), the data show that both exhibit a similar capacity to confer resistance to Ara-C. This is contrary to our expectation that the Q27 variant would be more effective.

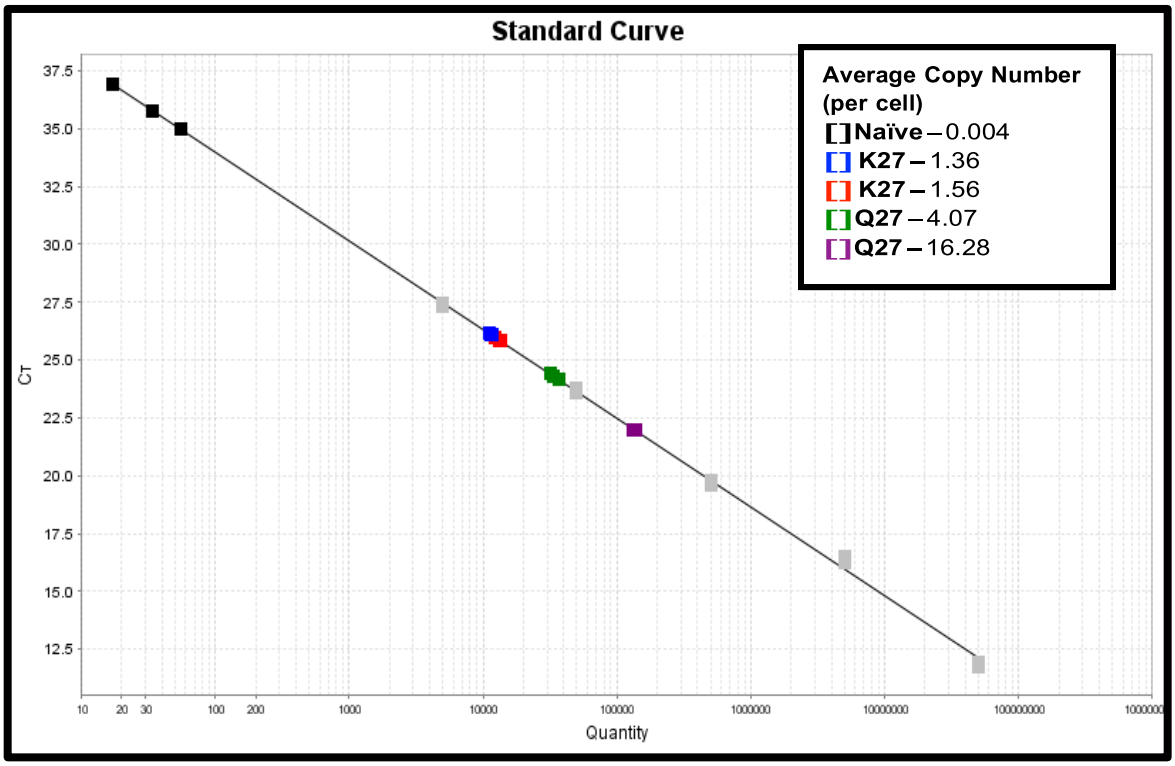
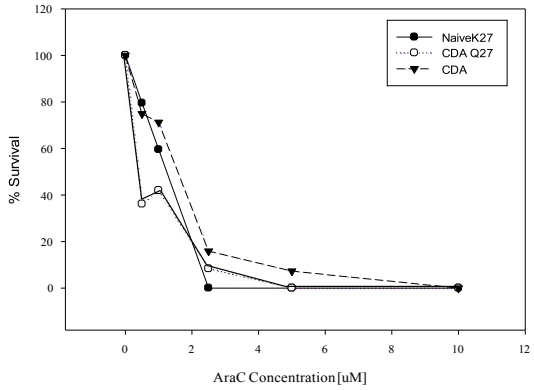


Figure 6: Real-time PCR was used to determine copy number of Jurkat cells transduced with CDA encoded lentivirus. This data showed that integration of the CDA gene into Jurkat cell genome was successful and thus these groups could be used for further study testing Ara-C resistance.

Experiment 1 Jurkat Survival Curve:
Day 10



Experiment 2 Jurkat Survival Curve :
Day 10

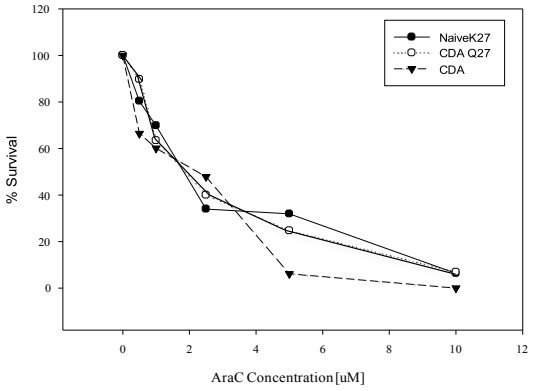


Figure 7: Jurkat survival curves measuring percent survival of two experiments on day 10 post-addition of Ara-C. Jurkat cells were transduced with cytidine deaminase encoded lentivirus. As with the MV4-11 survival curve, the concentrations of Ara-C added to an initial population of $1x10^6$ Jurkat cells were 0μM, 0.5μM, 1μM, 2.5μM, 5μM, 10μM. In both trials of this experiment, it was observed that Jurkat cells transduced with both variants of CDA (K27 and Q27) were unsuccessful in conferring resistance to Ara-C. Rather, at all points along the survival curve on day 10, they showed percent survival that was similar to naïve Jurkat cells. At 10μM Ara-C, all the groups showed nearly zero percent survival.

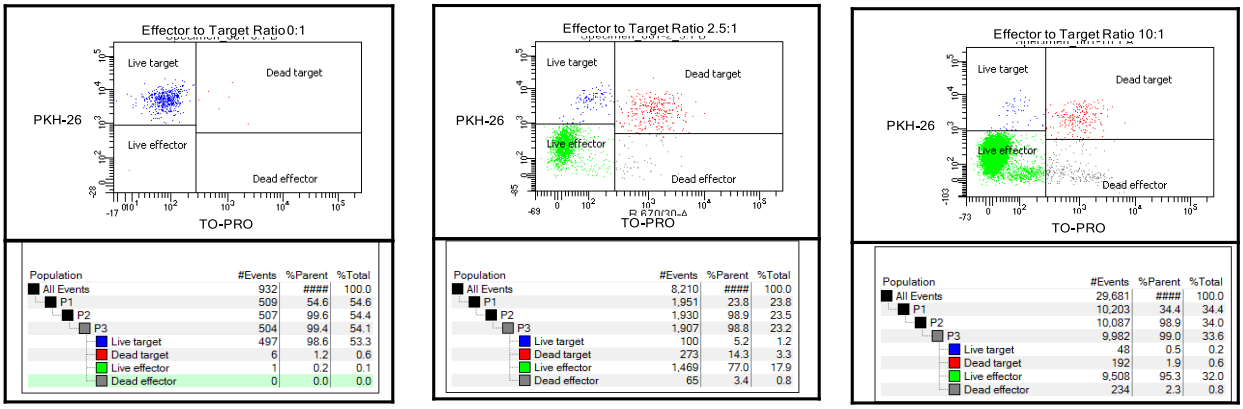


Figure 8: Flow cytometry data from cytotoxicity assay of NK92 versus MV4-11 cells. MV4-11 cells were stained with the cell membrane dye PKH27. They were then incubated with NK92 cells for 4 hours. Prior to analysis, the incubated mixture was stained with the live/dead cell stain TO-PRO 3. This assay was performed to see if unmodified NK92 cells have the capacity to kill an AML cell line. An effector to target ratio of 0:1 showed only 1.2% target cell death, ratios of 2.5:1 and 10:1 showed 73% and 80% of target cell death respectively. This demonstrates that NK92 cells have a natural cytotoxic capacity against MV4-11 cells. As such, NK92 cells are an attractive population to render Ara-C resistant since sustaining this population in the presence of chemotherapy could have an additive effect.

Restriction Enzymes Used To	Expected Band Lengths With Correct Ligation (in base pairs)
AscI/NheI	9285, 584
AleI/SnaBI	9023, 2485, 1361
AhdI/XhoI	5335, 2557, 1531, 446
SpeI/XhoI	4956, 3519, 1394

Table 2: Restriction enzymes used for digestion of ligated FUGW+CD5-VLR-CAR plasmids and the respective expected band lengths with successful ligation.

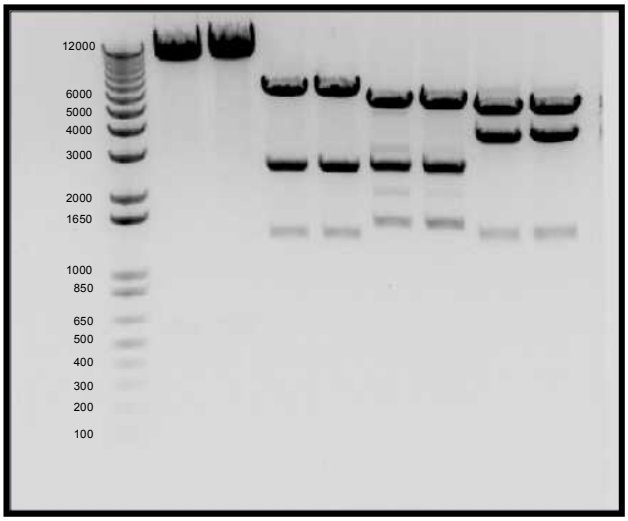


Figure 9: Electrophoresis gel from restriction enzyme digest of Anti-CD5 VLR CAR plasmid construct. Two plasmid samples were screened and both screened correctly based on the expected band lengths. The restriction enzymes used in this digest were AscI/NheI (lanes 2 and 3), AleI/SnaBI (lanes 4 and 5), AhdI/PmlI (lanes 6 and 7), and SpeI/XhoI (lanes 8 and 9).

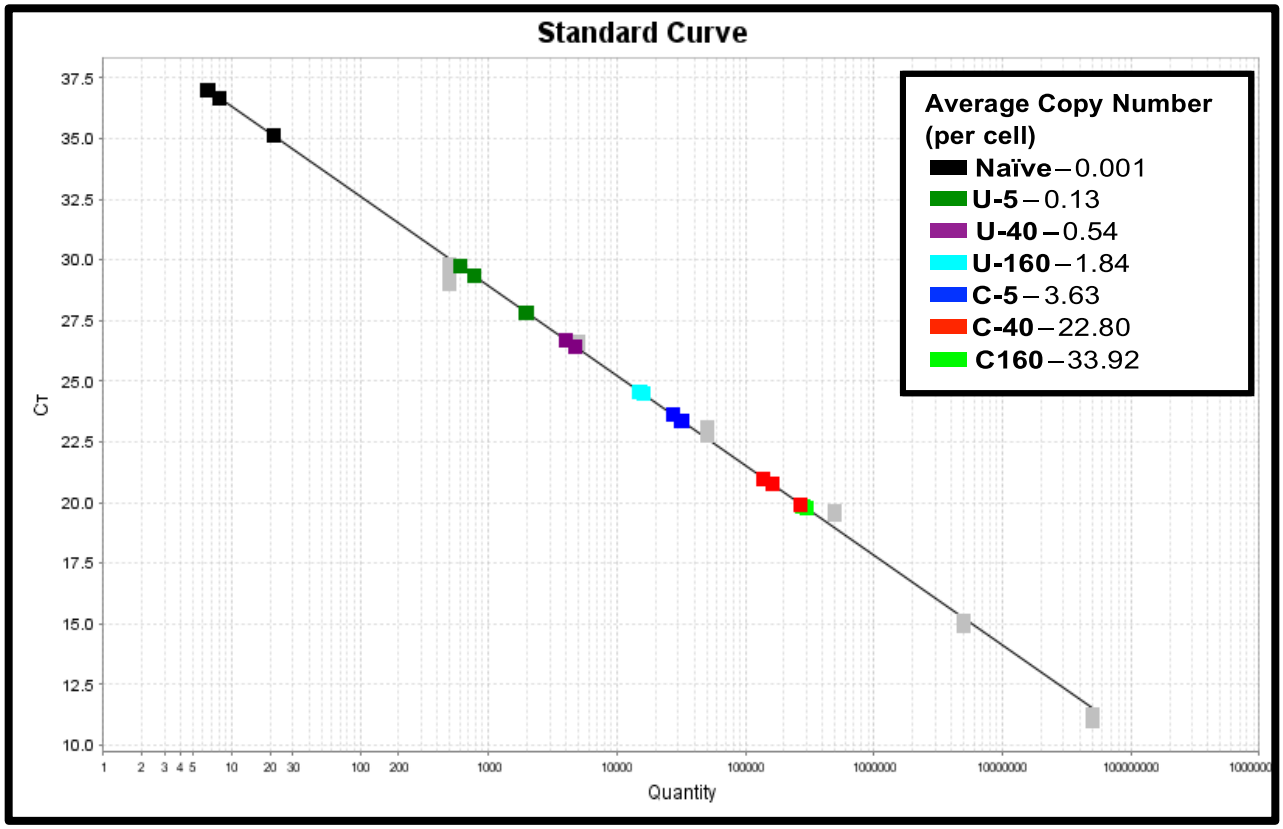


Figure 10: Anti-CD5 VLR-CAR copy number analysis from qPCR. Transduced Jurkat cells showed more than one copy of the Anti-CD5 VLR CAR sequence in all groups transduced with concentrated virus as well as the group transduced with 160µL of un-concentrated virus. Successful integration of the CAR construct sequence into these groups made them suitable for subsequent studies testing for CAR expression.

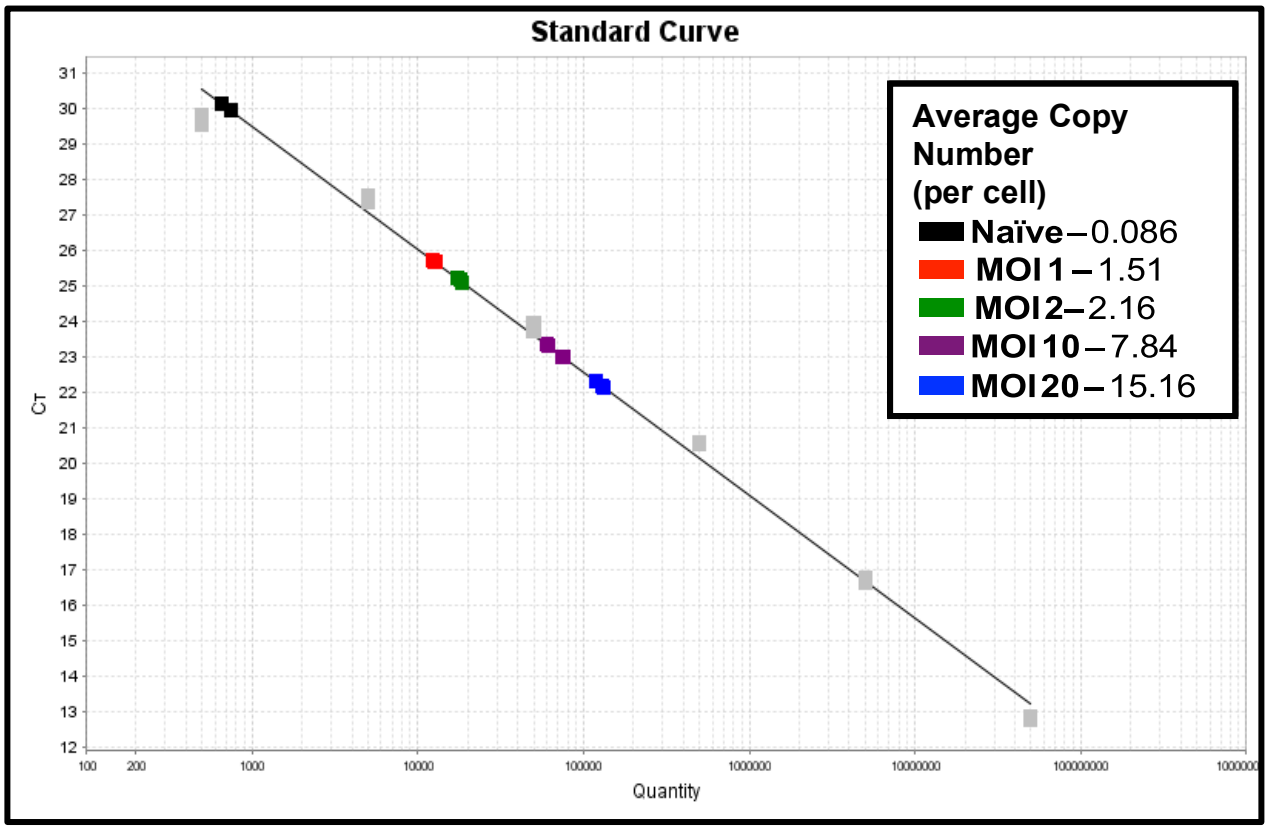


Figure 11: Real-time PCR data from transduction of Jurkat cells with anti-CD5 VLR CAR encoded lentivirus. Using a viral titer of 7.84×10^8 VP/mL, transductions were set up such that groups of 1×10^6 cells were transduced at MOI's of 1, 2, 10, or 20 viral particles/cell. Since the copy numbers closely parallel the MOI used in the transduction, it can be inferred that the virus is working efficiently.

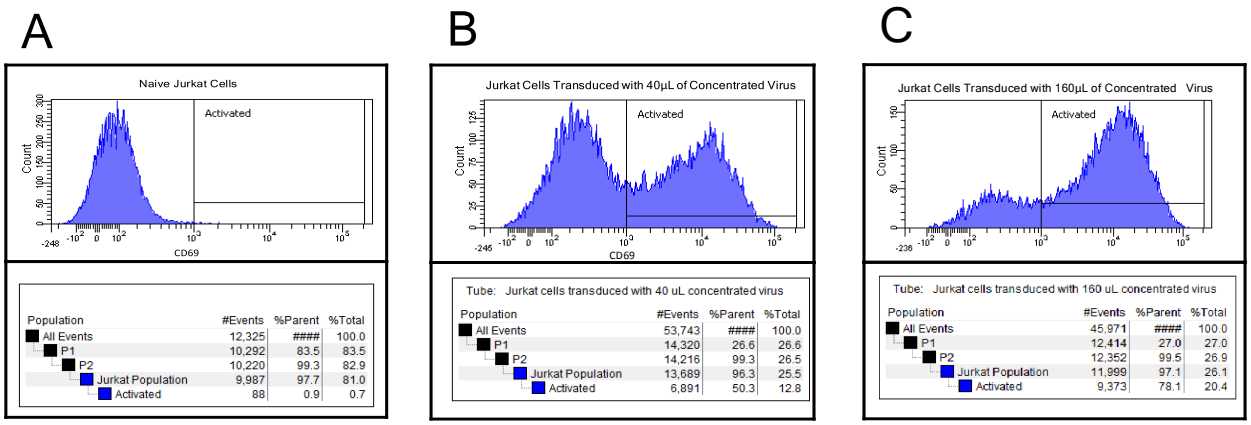


Figure 12: Flow cytometry data for CD69 Activation. Jurkat cells that were transduced with an anti-CD5 VLR CAR encoded lentivirus were stained with an anti-CD69 antibody (APC). CD69 is an activation antigen and it's expression on the Jurkat cell surface is induced upon activation. These data show that un-transduced (naïve) Jurkat cells show negligible CD69 expression (0.9%). In contrast, cells that were transduced with 40µL and 160µL of concentrated virus showed 50.3% and 78.1% activation respectively. This result is a good indicator that the CAR construct is working properly.

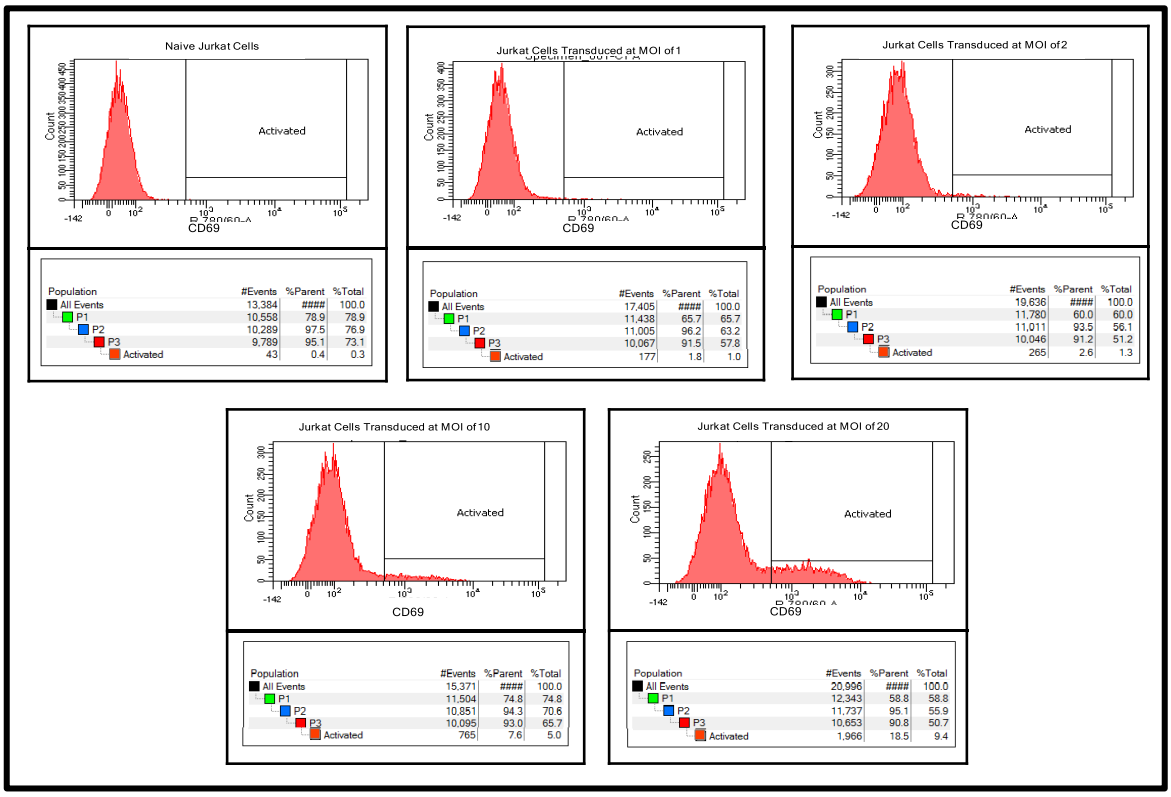


Figure 13: Activation assay using Jurkat cells transduced at MOI's of 1, 2, 10, 20. As before, cells were stained with an anti-CD69 APC conjugated antibody to test for CD69 expression on activated T cells. All transduced groups showed more activation than naïve groups. Groups transduced at higher MOI's, that had been shown to also have higher copy numbers, exhibited more CD69 expression and thereby activation.

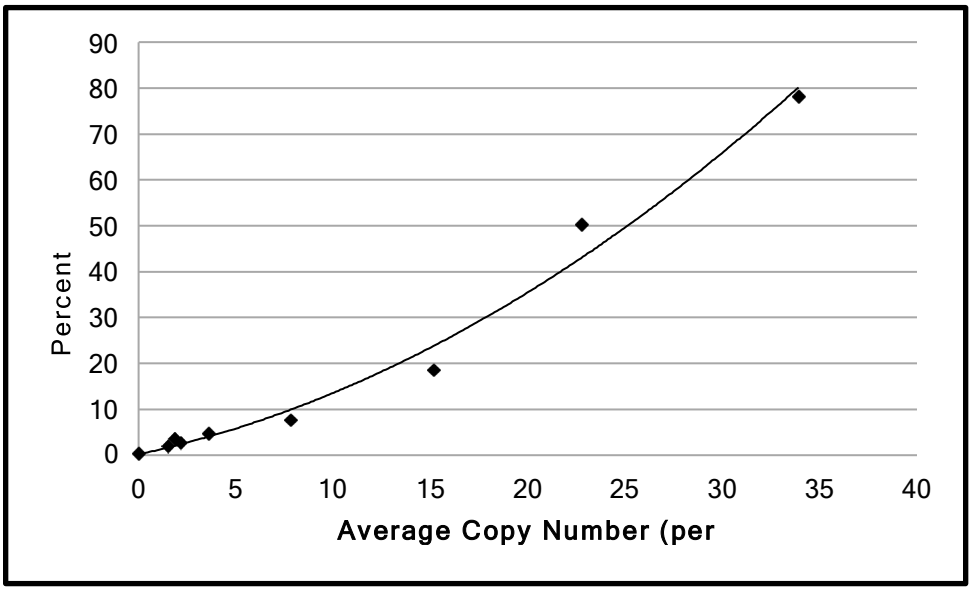


Figure 14: Data for copy number and activation were consolidated from both experiments of Jurkat cells transduced with anti-CD5 VLR-CAR encoded virus. There is a positive correlation between the number of copies of the CAR construct sequence and the percent activation observed in cells. The relationship indicates a dose-dependent response of activation to the average number of copies per cell for a population. A second order polynomial line was used to fit the points.

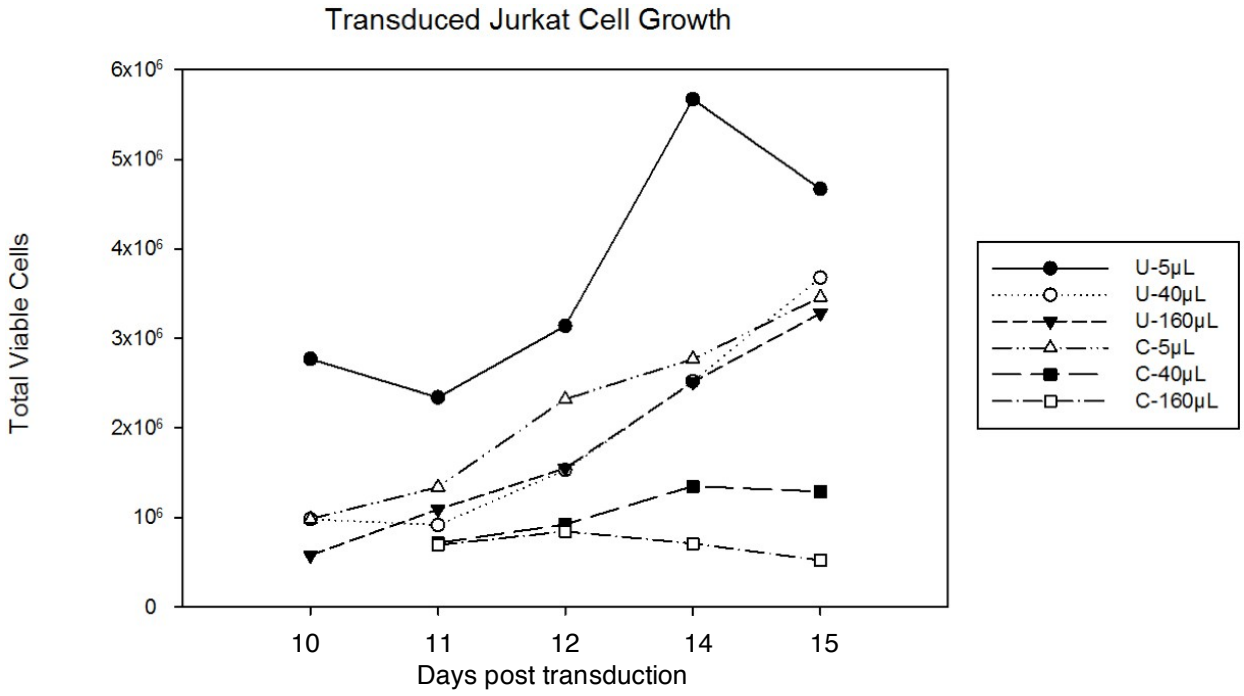
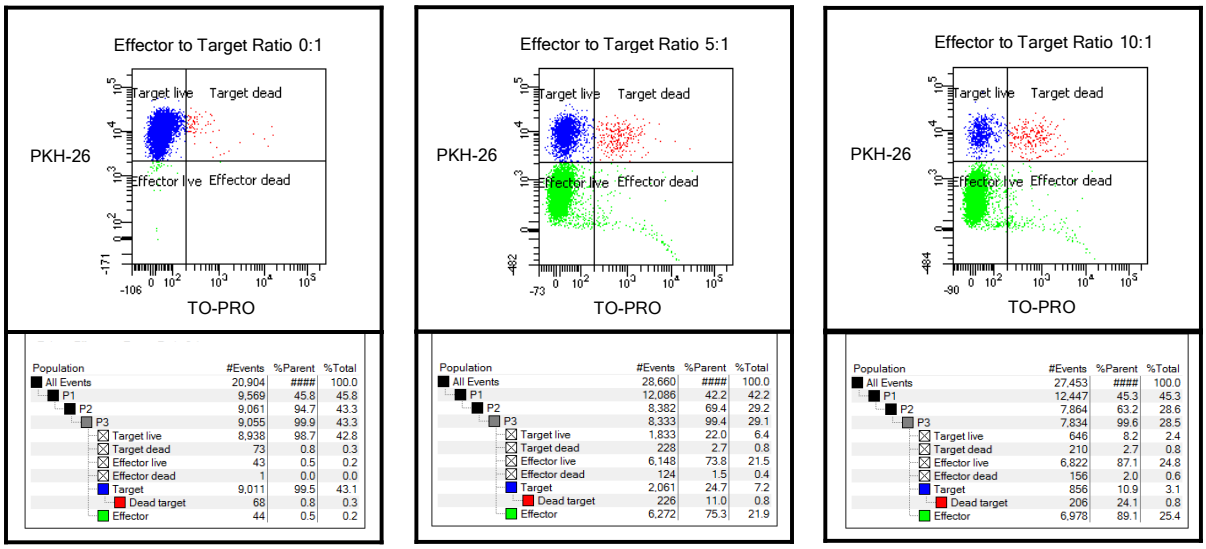


Figure 15: Growth of Jurkat cells transduced with anti-CD5 VLR CAR encoded lentivirus. Transductions were set up using a range of volumes of unconcentrated virus (designated U) and concentrated virus (designated C). Growth of the unconcentrated groups and the C-5uL were steady and essentially uninhibited. In contrast the, groups transduced with the greater concentrations of virus, C-40µL and C-160µL, showed stagnation in their population.



Population	#Events	%Parent	%Total
All Events	20,904	####	100.0
P1	9,569	45.8	45.8
P2	9,061	94.7	43.3
P3	9,055	99.9	43.3
Target live	8,938	98.7	42.8
Target dead	73	0.8	0.3
Effector live	43	0.5	0.2
Effector dead	1	0.0	0.0
Target	9,011	99.5	43.1
Dead target	68	0.8	0.3
Effector	44	0.5	0.2

Population	#Events	%Parent	%Total
All Events	28,660	####	100.0
P1	12,086	42.2	42.2
P2	8,382	69.4	29.2
P3	8,333	99.4	29.1
Target live	1,833	22.0	6.4
Target dead	228	2.7	0.8
Effector live	6,148	73.8	21.5
Effector dead	124	1.5	0.4
Target	2,061	24.7	7.2
Dead target	226	11.0	0.8
Effector	6,272	75.3	21.9

Population	#Events	%Parent	%Total
All Events	27,453	####	100.0
P1	12,447	45.3	45.3
P2	7,864	63.2	28.6
P3	7,834	99.6	28.5
Target live	646	8.2	2.4
Target dead	210	2.7	0.8
Effector live	6,822	87.1	24.8
Effector dead	156	2.0	0.6
Target	856	10.9	3.1
Dead target	206	24.1	0.8
Effector	6,978	89.1	25.4

Figure 16: Flow cytometry data of cytotoxicity assay with gamma delta T cells versus Jurkat cells. Jurkat cells were stained with the cell membrane dye PKH27. They were then incubated with gamma delta T cells for 4 hours. Prior to analysis, the incubated mixture was stained with the live/dead cell stain TO-PRO 3. This assay was performed to see if unmodified gamma delta T cells could effectively kill a lymphoblastic cell line (Jurkat). While an effector to target ratio of 0:1 showed only 0.8% target cell death, ratios of 5:1 and 10:1 showed 11.0% and 24.1% of target cell death respectively. This demonstrated that gamma delta T cells have a natural cytotoxic capacity against Jurkat cells.