

**The**

**Conception of**

**GCC- Specific**

**Chimeric Antigen Receptors**

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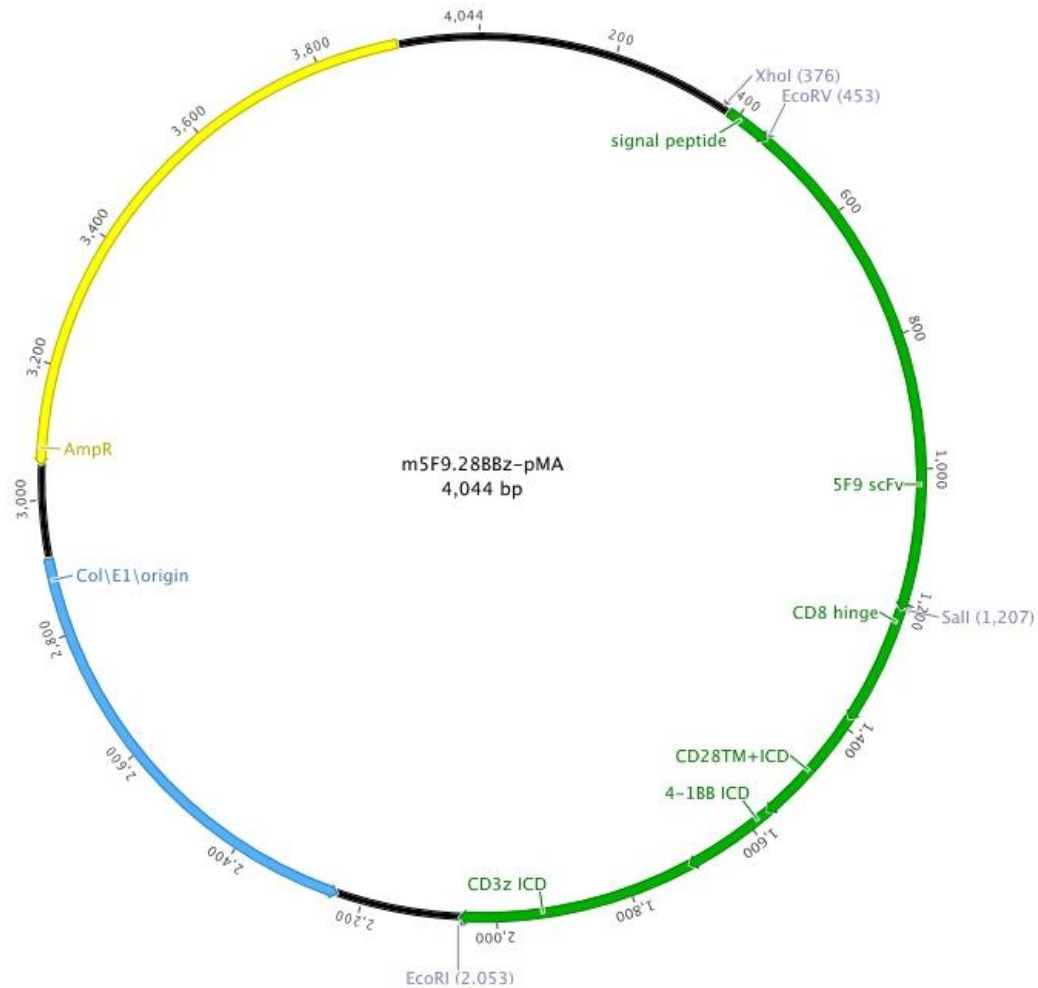
## Abstract

Colorectal Cancer is an aggressive disease that claims the lives of both men and women every year. It is the second leading cause of cancer-related death in the U.S. The overwhelming mortality rate it amasses begs for the discovery of alternative forms of treatment. The nature of cancer requires immunotherapeutic approach that is tumor specific in nature. Adoptive T-cell therapy is an alternative that satisfies these conditions. Guanylyl Cyclase C is a receptor found on the luminal side of the gut that is tissue-specific for the intestinal epithelium. Further, its expression is maintained throughout colorectal tumorigenesis making in an excellent marker of metastatic disease. By employing the use of chimeric antigen receptors we have created twelve different CAR's containing GCC-specificity which we will use to target tumors expressing

Guanyl Cyclase C. We hypothesize that these new CAR's will recognize GCC and induce T cell effector responses.

Colon Cancer is an aggressive form of cancer that develops in the rectum. Colorectal cancer is the fourth most commonly diagnosed cancer and the second leading cause of cancer death in both men and women combined within the United States. It is estimated that in 2013 over 50,000 people will die from colorectal cancer. As colorectal cancer progresses it becomes metastatic and travels to the lymph nodes and various parts of the body. The abysmal death rate in regards to colorectal cancer begs for alternative forms of cancer treatment.

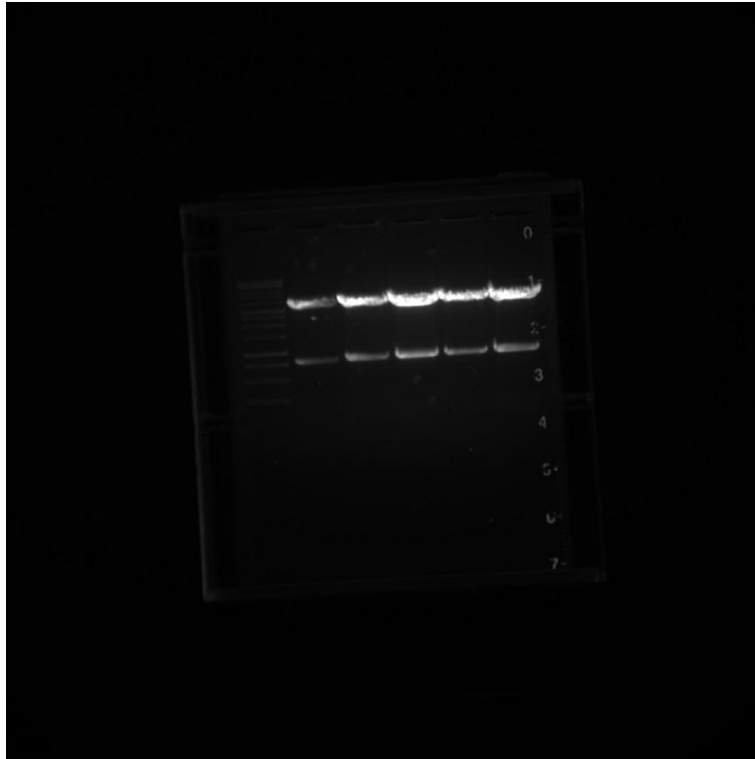
Adoptive T-Cell therapy is a form of cancer treatment that can prove to be very successful. Guanylyl Cyclase C is a gene that codes for a protein found in the intestines and cancers arising from the intestine. Guanylyl Cyclase C has limited expression in extra-intestinal tissues making it an ideal marker for the detection of colorectal cancer metastasis in non-intestinal tissues. Recently, we have engineered T cells to express GCC specific chimeric antigen receptors in the hope that they will attack GCC expressing tumors.



**Figure #1: pMA plasmid containing 5F9.**

Our research began with the use of circular plasmid entitled pMA. The pMA contained a CAR sequence with an antibody sequence derived from the 5F9 antibody. 5F9 is a single chain fv that recognizes human

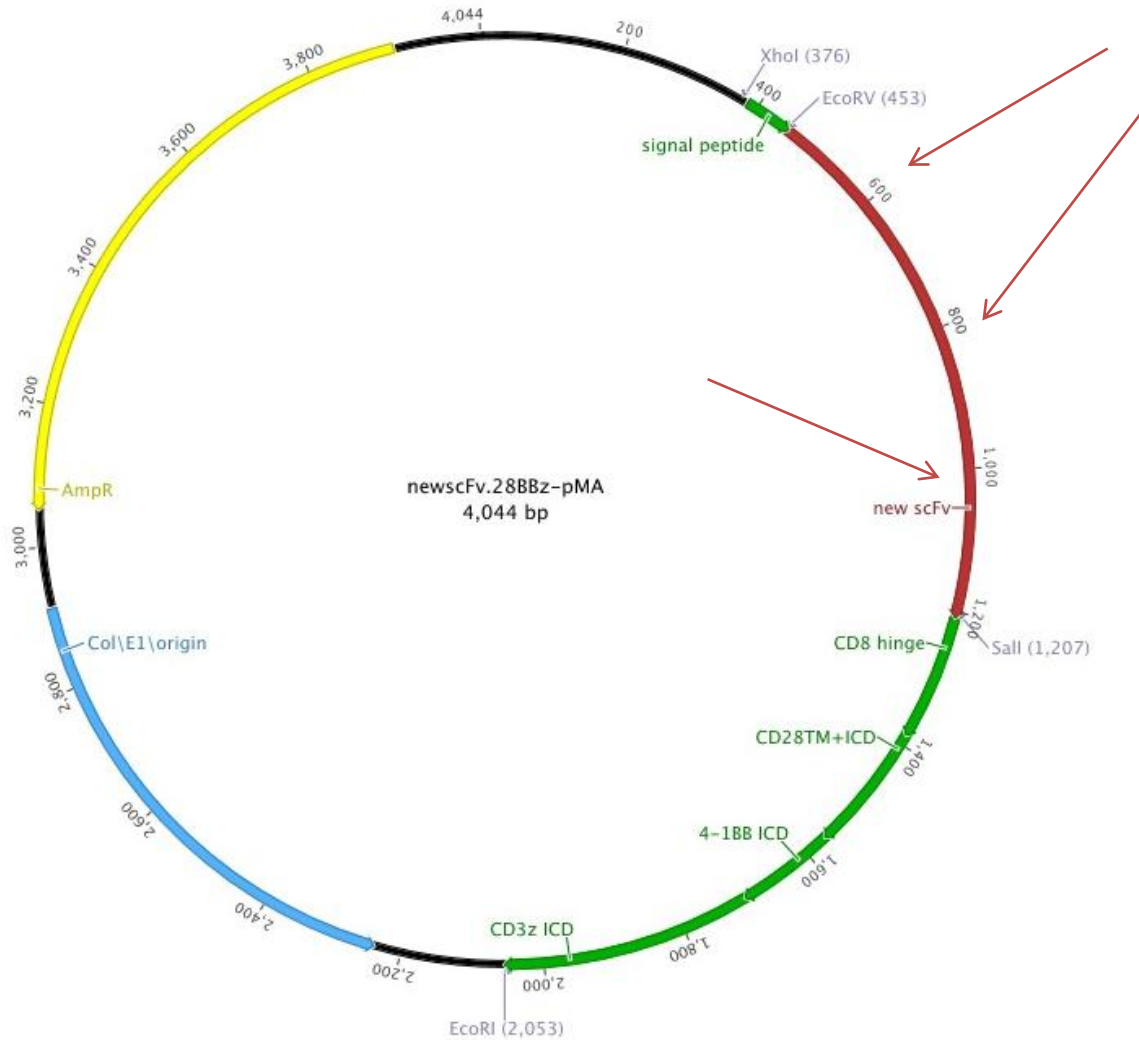
GCC. A digestion reaction was performed in order to remove the 5F9 antibody sequence from the CAR pMA plasmid.



**Figure 2: Digestion reaction containing 5F9 & pMA CAR**

After completing the digestion, a gel extraction was performed to remove the PMA CAR from within the gel itself. After isolating the CAR containing pma plasmid GeneArt assembly was used to connect individual antibodies to the CAR pMA construct. There were twelve antibody sequences used to create twelve new CARS. The antibody

sequences used for the benefit of this experiment are Abx012, Abx020, Abx106, Abx198, Abx221, Abx229, Abx338, Abx393, 6H8, 8C2, 10C10 and 10D3. These antibodies were chosen due to their capabilities to recognize human GCC with hopes that at least some would be cross reactive for mouse GCC. Seeing as any results obtained would be done from a mouse model, the outcome, if favorable, would need to be recreated using a human model.

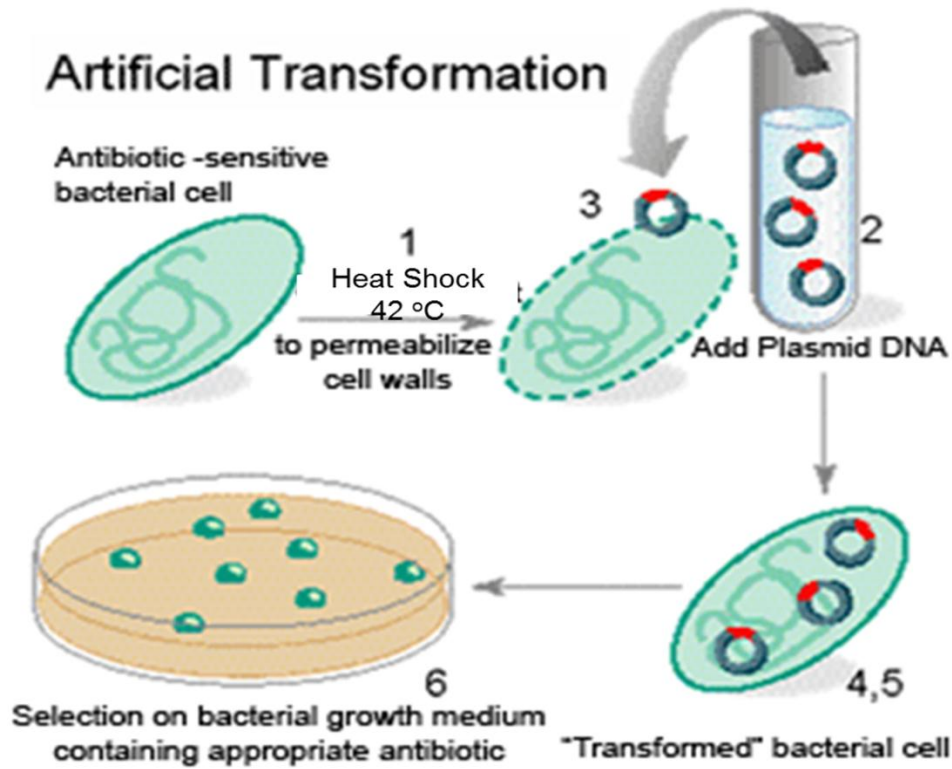


**Figure 3: pMA plasmid containing new antibody sequence (outlined in red).**

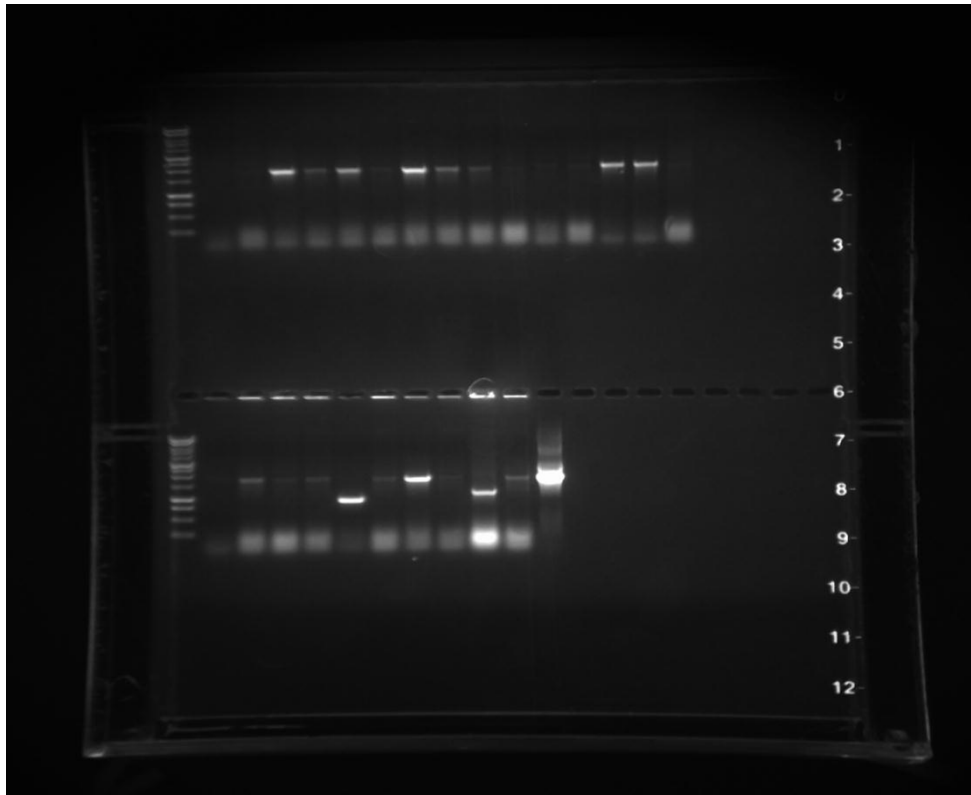
Next, a transformation was performed inserting the GeneArt reactions into bacteria. This allowed the assembled CAR pMA plasmids to get into the bacteria. This was made possible by heat shocking the bacteria cells. Heat shocking allows for the mild disruption of the cell which permits the DNA to enter. The bacteria was then plated out on agarose



plates for an overnight incubation. Following incubation, colonies were picked and grown in order to test for positive insertions.



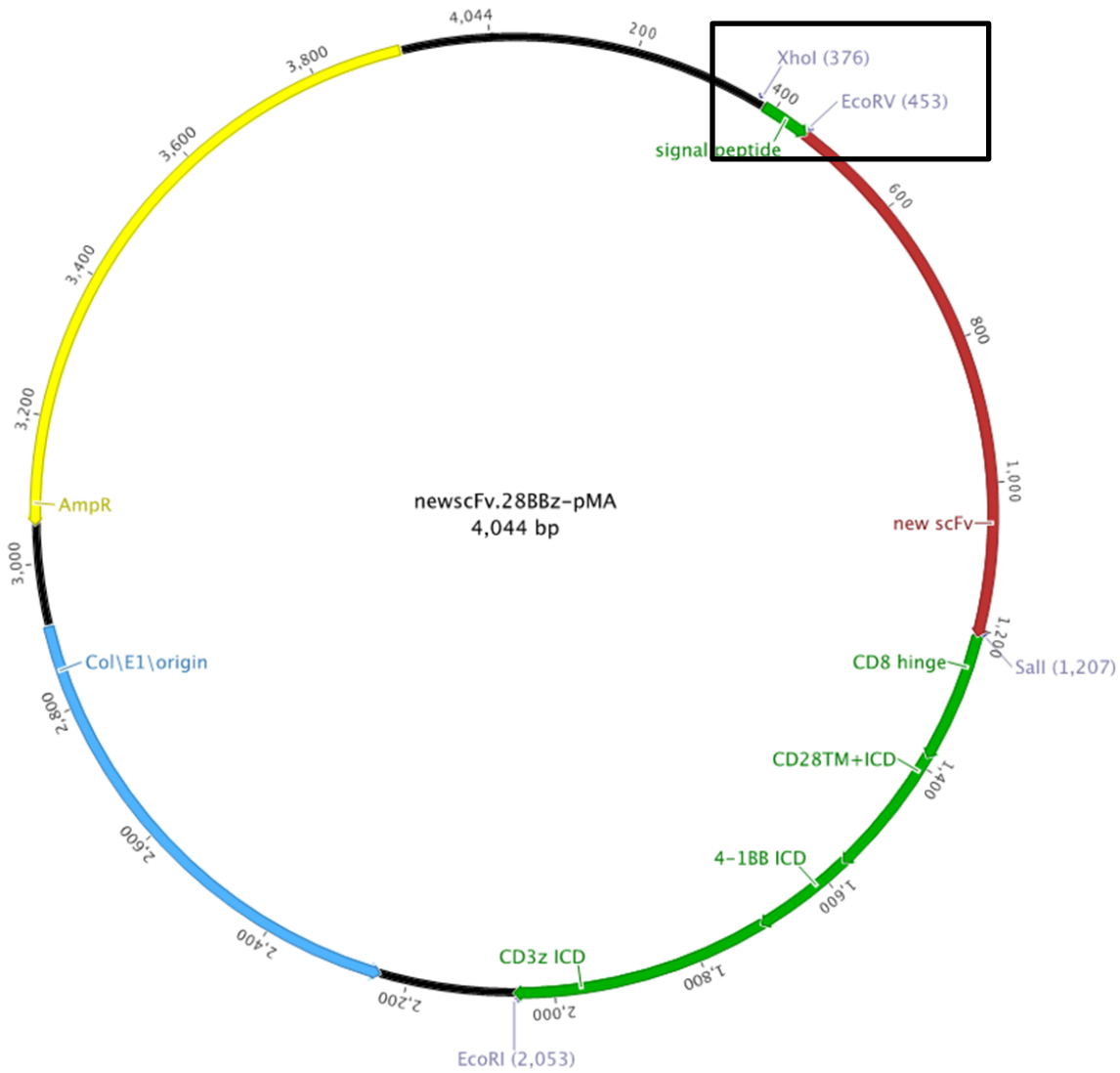
**Figure 4: Model depicting a transformation reaction.**



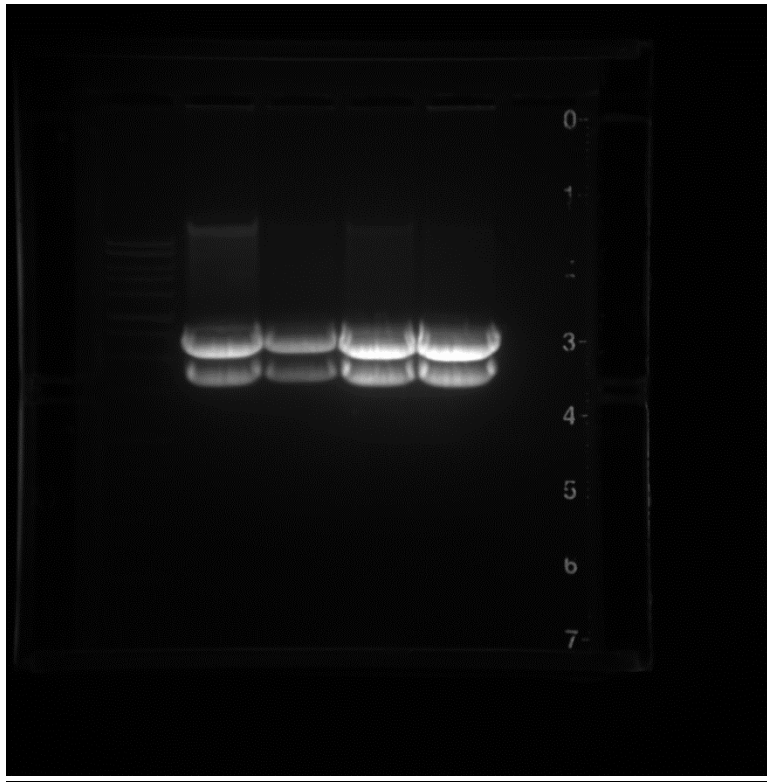
**Figure 5: PCR screen of clones**

After identifying positive clones, 10ML cultures of the cells were grown and placed through the plasmid purification process. Next, the purified plasmids were sent out for sequencing to confirm correct assembly of the new CARs. Once the plasmids were confirmed to be correct, the CARs were then digested out of the pMA plasmid. This was done with the help of two enzymes entitled XhoI and EcoRI. These enzymes used were chosen specifically because they can be found

within the new retroviral plasmid that would be used to further the experiments. The use of these enzymes would ensure a smooth transition of the various CARS from one plasmid to the other.



**Figure #6: pMA plasmid containing XhoI & EcoRI sites.**



**Figure 7: pMA CAR digestion**

A separate digestion reaction was completed in order to disrupt pMIG, our retroviral plasmid. This disruption allowed for the integration of the CAR DNA into the retroviral plasmid. Next, using a ligation reaction the various CAR's were attached to the pMIG plasmid. Following the ligation, bacteria was transformed with the ligation reactions and plated out on agarose plates. Colonies were then grown;

PCR screened to identify positives, and sequenced to confirm correct insertion into pMIG before proceeding to the next step.

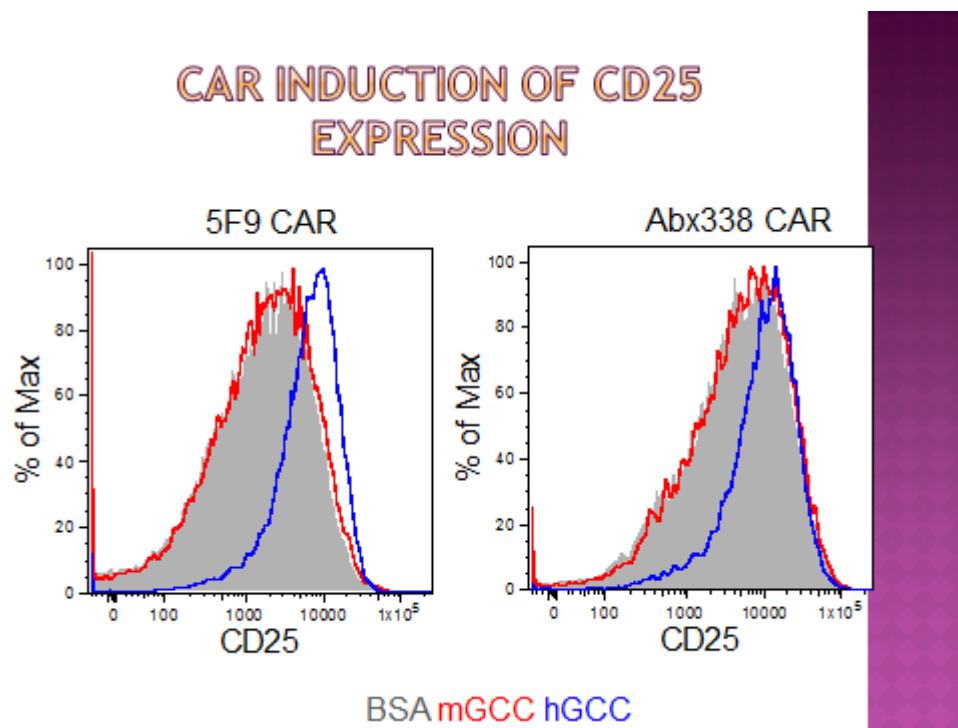
Next, we made retrovirus from the CAR-pMIG plasmids. In order to achieve this, the retroviral packaging cell line referred to as Phoenix were used. Phoenix cells are easy to use where DNA integration and virus collection is concerned. Phoenix Cells create retrovirus when the retrovirus buds off of the Phoenix cell membrane and into the easily accessed cell culture supernatant. 48 hours after the transfection, supernatant containing the virus was collected.

Next we purified CD8<sup>+</sup> T cells from mice. The purification process began with the removal of spleens from mice. The removal was followed by a disruption. The disruption allowed for the isolation of white blood cells (B cells, T cells, dendritic cells, monocytes, macrophages, etc.). The cells were then labeled with an antibody cocktail containing biotin-labeled antibodies specific for all white blood cell types excluding CD8<sup>+</sup> T cells.

The white blood cells were then labeled with anti-biotin magnetic beads so that they will only bind to cells coated with biotin-labeled antibodies (B, CD4 T, DC, macrophages monocytes etc.) again excluding the CD8 T cells. Afterwards the cells were placed through a magnetic column. Any cells labeled with beads were stuck to the magnetic column allowing the unlabeled cells CD8+ T cells to flow through.

Retrovirus require actively dividing cells in order to be infected, therefore we stimulated purified CD8+ T cells with beads coated with antibodies specific for CD3 and CD28. CD3 is the major signaling component of the T cell receptor and CD28 is the classic secondary costimulatory signal that is required for optimal T cell activation which results in T cell proliferation. Immediately after T cell purification, the CD8 T cells were mixed with these beads along with the cytokine interleukin-2 (IL2 ) to activate them. Interleukin- 2 is a T cell growth factor required for T cell growth.

They day after T cell purification and stimulation (with anti-CD3/CD28 beads), the T cells are actively dividing. They are mixed in with the retroviral supernatant. Afterwards the retrovirus then infects the T cells and the CAR DNA is then stably inserted into the genome of T cells. The T cells were monitored and counted on a daily basis after this point and constantly fed with more media and IL2 as they continue to expand.



The graph pictured above shows the results garnered when we tested one out of twelve of our CAR's. Our findings were based on the

increased expression of CD25. The CAR entitled ABX338 showed an increase in CD25 expression where human GCC was concerned but did not mirror the same affinity for mouse GCC. Our findings have led us to believe that further testing is required for a CAR satisfying our requirements may lie within the remaining eleven untested CARs.



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