

Adoptive T cell Therapy for Metastatic Colorectal Cancer

Shauna Ebanks and Bo Xiang

Colorectal cancer is the second leading cause of cancer-related death in US (1). Most of the mortality reflects local, regional and distant metastatic tumors. Cancers that are confined within the wall of the colon are often curable with surgery while tumor cells that have spread into other organs like liver and lung has low curable possibility, even though the chemotherapies and monoclonal antibodies can extend the person's life and improve quality of life(2, 3). Surgery is the main treatment for primary colorectal tumors but recurrence can happen and develop into metastasis. In addition, colorectal cancer has been proved resistant to chemotherapy although little success has been achieved using a combination of 5-fluorouracil and levamisole. Survival rate decreases from 90% for primary tumors to 10% for distal metastasis of colorectal cancer (1). More attention has been attracted to immunotherapy against metastatic colon cancer, because of its potential for higher specificity and less side effect(4). Our main aim is to increase the eradication of metastatic colorectal cancer cells by optimizing immunotherapeutic approaches that have been well-underway. Guanylyl cyclase C (GCC) is a mucosal antigen expressed on apical surface of intestinal epithelial cells. It is a potential target for colorectal cancer immunotherapeutic approaches, because of its expression on >95% of colorectal cancer cells and their maintenance through all the stages of colorectal cancer (5-9). This is because GCC is mainly expressed in the intestine so that its expression can be utilized to detect metastasis of colon cancer. The aim of this project is to develop an immunotherapy based on GCC-specific TCR transduced CD8 T cells to attack metastatic cancer cells. The initial step of this project is cloning out CD8 TCR

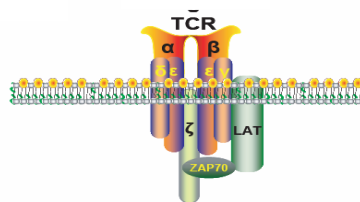
specific to GCC. This can be done by T-hybridoma technology by which we fused T cells from mice immunized by GCC vaccine with fusion partner cells (BWZ/CD8)(10).

Background: Colorectal cancer is the fourth most commonly diagnosed cancer in the world in the US and the 2nd leading cause of cancer-related deaths in US (1). For primary tumors, it is highly curable. This type of cancer occurs when abnormal cells grow in the lining of the large intestine (colon) or rectum. Although there are treatment options such as surgery, radiation, chemical and biological therapies, all treatments present a risk of side effects. Patients in their early state may achieve a cure; however surgery removes the bulk of the tumor leaving behind microscopic residual disease, which ultimately results in recrudescence. Although current radiotherapeutic agents and chemotherapeutic agents and biological toxins are potent cytotoxins, they do not discriminate between normal cells and malignant cells, producing adverse effects and dose limiting toxicity.

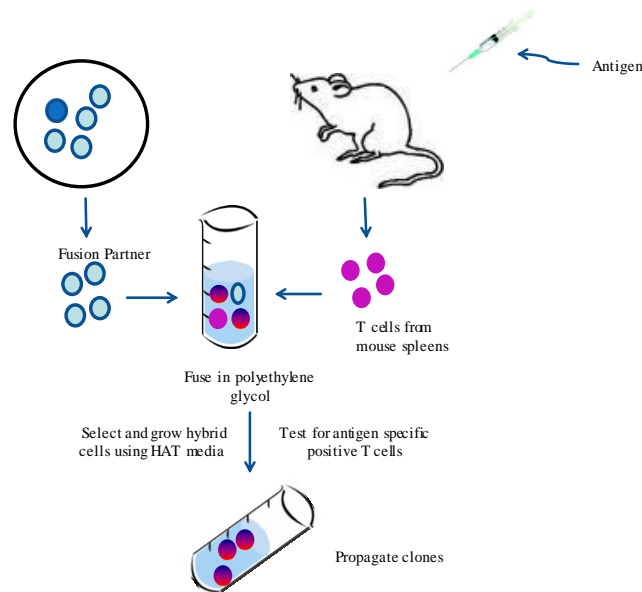
Guanylyl cyclase C, a member of guanylyl cyclase family of receptors, is the receptor for two endogenous ligands guanylin and uroguanylin and for the exogenous ligand heat-stable enterotoxin, ST. ST is produced by enterotoxigenic bacteria causing the infectious diarrhea(11).

GCC-specific CD8 T cells largely limits the tumor eradication. To overcome the limitation, we would transduce GCC-specific TCR gene into activated T cells to increase the frequency of GCC-specific T cells in the entire population. Therefore, the first step of this project is to clone out the GCC-specific TCR.

The process of identifying GCC-specific TCR was done by using T hybridoma technology to make hybrid cell lines (called hybridomas) by fusing T cells that have been exposed to an antigen with fusion partner cells which are thymomas caused by irradiation. For biological studies, T-cell hybridomas have several advantages over normal T cells and T-cell lines. T-cell hybridomas grow rapidly in tissue culture. Their proliferation does not require stimulations because they divide spontaneously. Normal resting T cells do not divide in culture and must be freshly isolated from animals immediately before use. Splenocytes can divide in vitro, however they require stimulation in the form of antigen and antigen presenting cells and/or cytokines such as interleukin 2 (IL-2).



T-hybridoma Technology Diagram



The results of selecting GCC-specific T hybridoma are usually tested by using an assay called MUG assay. MUG assay is a sensitive fluorescence assay in which activated T hybridoma can express β -galactosidase. In the presence of β -galactosidase, the colorless 4-methylumbelliferyl β -D-galactopyranoside (MUG) substrate is hydrolyzed into a product that has a bright blue fluorescence. Fluorescence can then be detected at Excitation/Emission = 360/460 nm. Thymoma/fusion partner cells have been engineered to have β -Galactosidase genes with no TCR expression on surface. However, β -Galactosidase expression requires thymoma/hybridoma activation. T cells have T cell receptors but no β -Galactosidase genes. When they fuse into T-hybridoma, this T-hybridoma has both T cell receptors and β -Galactosidase genes. If

they are stimulated with GCC epitope (GCC254-262) and they also can express the β -Galactosidase, then they are GCC-specific T hybridoma.(5)

The main aim of such a process (T cell hybridoma technology) in this experiment is to clone out GCC-specific T cell receptors and transduce normal T cells with this T cell receptor then introduced it back into the mice system to target cancer. This process is known as adoptive T cell therapy. Adoptive T cell therapy involves the isolation and ex vivo expansion of tumor specific T cells to achieve greater number of T cells than what could be obtained by vaccination alone. The tumor-specific T cells are then injected into patients with cancer in an attempt to give their immune system the ability to overwhelm remaining tumor with T cells, which can attack cancer cells.

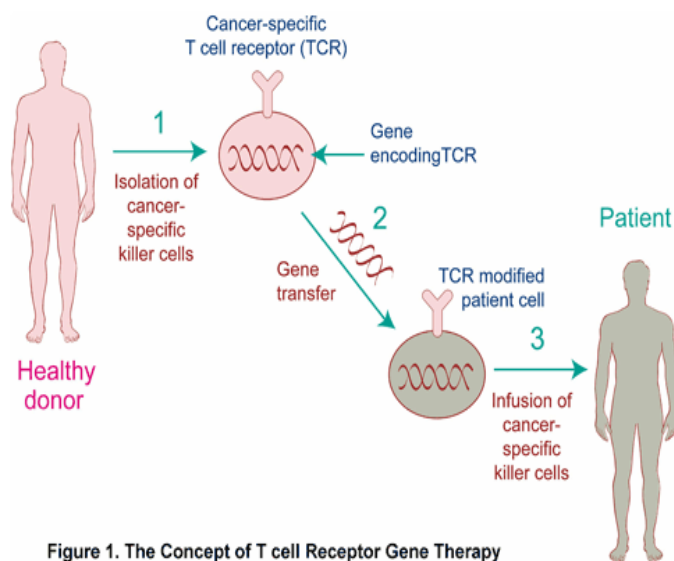
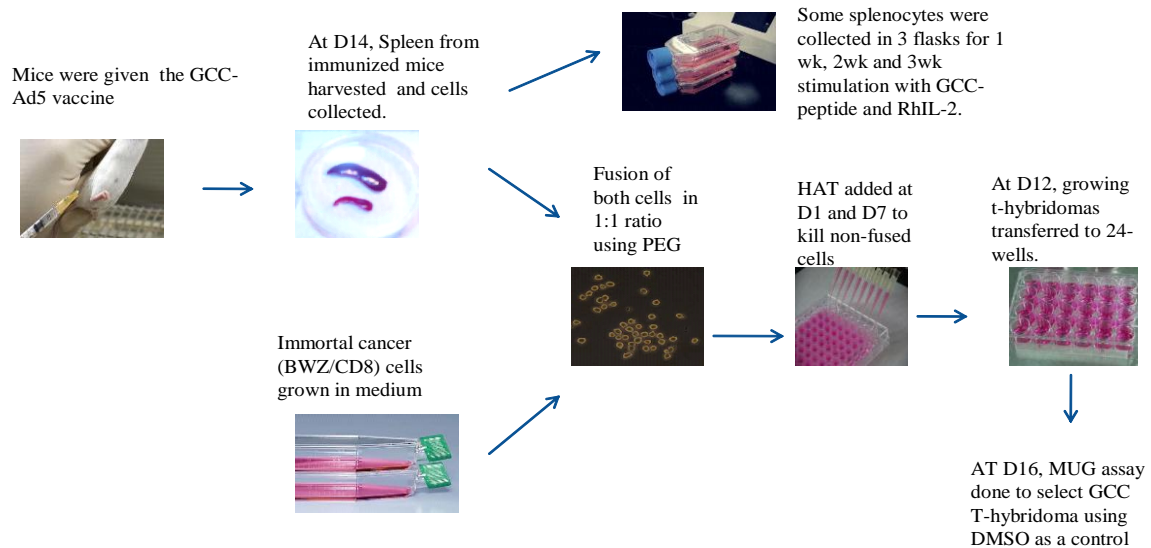


Figure 1. The Concept of T cell Receptor Gene Therapy

Aim: Cloning of GCC-specific T cell Receptor

Procedure:

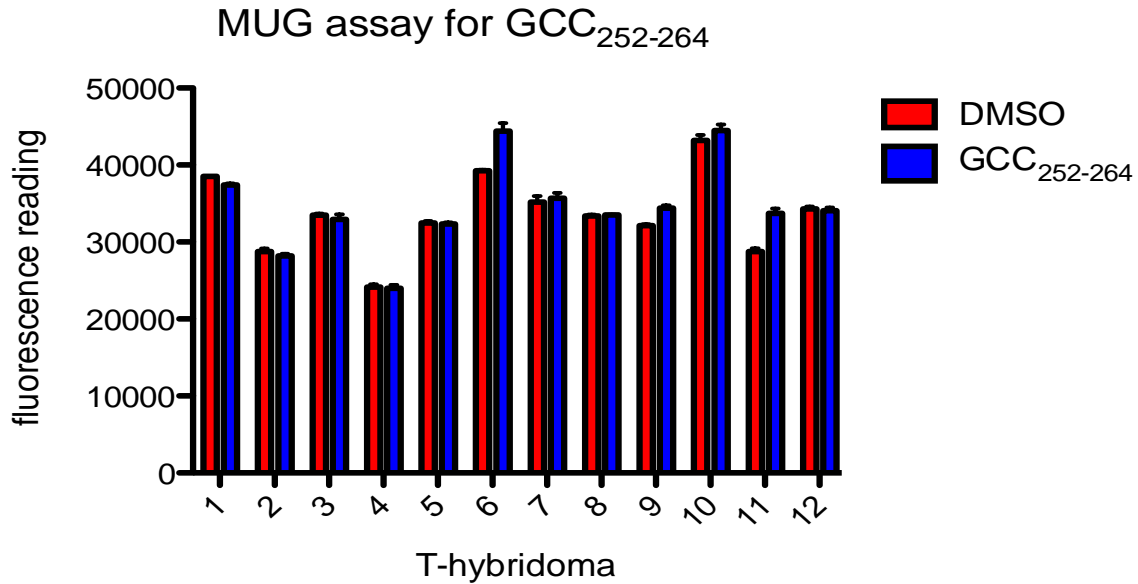


1. BALB/c mice were immunized with recombinant GCC-expressing viral vectors (GCC-Ad).
2. At day 14, the time of the optimal immune response, the spleens are harvested and the splenocytes are then isolated. Lysis buffer is used to kill all the red blood cells thus leaving only the white blood lymphocytes.
3. Once the splenocytes are isolated, they are then fused with the immortalized thymoma/fusion partner cells in a 1:1 ratio and then placed in 96-well plates to grow. The rest of the splenocytes are then placed into three flasks for 1, 2 and 3 weeks of

stimulation respectively using rhIL-2 and GCC peptide for more T hybridoma fusions. Antigen presenting cells are added periodically as well, this is to increase the frequency of GCC specific T cells for further fusions by T cell proliferation. The fusion is accomplished by using polyethylene glycol (PEG).

4. Hypoxanthine aminopterin thymidine medium is then added 1 day and 1 week after fusion. Aminopterin blocks the *de novo* pathway that allows for nucleotide synthesis. Hence, unfused thymoma cells die, as they cannot produce nucleotides by the *de novo* or salvage pathways because their HGPRT gene (hypoxanthine-guanine phosphoribosyltransferase) is mutated. However, T-hybridoma can produce HGPRT by genes from T cells and survive the HAT selection. Removal of the unfused thymoma cells is necessary because they have the potential to outgrow other cells, especially weakly established hybridomas by competing for resources from medium. Unfused T cells die as they have a short life span without stimulation. In this way, only the T cell hybridomas survive.
5. At day 12, 96-well plates are then screened for growing T-hybridomas using a microscope. Growing T-hybridomas are identified by the presence of cell colonies.
6. Growing T hybridomas are then transferred to 24-well plates to allow further growth for 5 days. MUG assay is then done to test for selecting GCC- specific T cells.

Results:



Graph #1 showing results obtained where DMSO (blue) was used as the control and GCC₂₅₂₋₂₆₄ peptide (red) was used to stimulate GCC-specific T hybridoma. The results show that growing T-hybridomas were none that were GCC-specific. In order to identify GCC-specific T-hybridomas, the GCC peptide (blue) must have at least 10000 points increase compared to the control (DMSO) in fluorescence reading; however the results show that there was no difference.

Summary and Future Directions:

T hybridoma technology is an effective way to identify antigen specific T cell receptors (TCRs). In order to increase the chance of amplifying the frequency of antigen-specific T cells, we have to make them proliferate by stimulating them with antigen. However, in order to increase the fusion efficiency for tests, critical measures have to be revised: During the fusion reaction, maintaining the temperature at 37°C is very important for the membrane events that occur during and immediately after the PEG mediated fusion event. A major limitation is because of the small volume, the cells can rapidly come down to room temperature. In addition, the thymoma/fusion partner cells could also be tested using super antigens to see if they are still potent for the method.

Future directions for this project are: To clone out the T-cell receptor of GCC-specific CD8 T cells and test their affinity. In addition, we also want to give the mice colon cancer cell lines-CT26 modified to express GCC and then treat them with GCC-specific TCR transduced CD8 T cells.

References:

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