Creating a Model Antigen System to Study the Mechanisms of GCC-Specific Tolerance

Patrick Ihejirika, Adam Snook, Laurence Eisenlohr.

Abstract: Immunotherapeutics for colorectal cancer have been under investigation for decades. Unfortunately, a recent meta-analysis has revealed only a 1% response rate for all immunotherapeutic trials in colorectal cancer, highlighting the need for improved immunotherapeutic target antigens which are tumor-specific, immunogenic, and shared by patients. However, to produce immunological responses targeting tumor/self antigens, one has to overcome tolerance, which has constituted an obstacle to previous studies. Guanylyl cyclase C is the index cancer-mucosa antigen, an emerging class of tumor antigens in colorectal cancer. As a mucosa-restricted antigen, GCC is amenable to immunological targeting and uniquely suited for immunotherapy of colorectal cancers. GCC is only expressed in the intestine and it is so highly restricted in its expression that it can be utilized to detect metastases in non-intestinal tissues. However, previous studies have defined tolerance as a key mechanism limiting efficacy of GCC-targeted vaccines. A tissue-specific model-self antigen expressed in the intestine is required to define tolerance mechanisms to intestinal antigens. Here, we created a model self-antigen comprised of the OT-II CD4+ T cell epitope, OT-I CD8+ T cell epitope, and HA tag B cell epitope that can be employed to create transgenic mice for studying mechanisms of GCC-specific tolerance.
Background: Colorectal cancer is a leading cause of cancer and morbidity around the world. In fact, it is the 3rd most frequent cause of cancer in both men and women in the United States and it is the 2nd leading cause of cancer-related deaths in the United States. The 5 year survival rate for this disease is 60%, resulting in about 500,000 worldwide deaths per year. Although there are treatment options such as surgery, radiation, chemical and biological therapies, all treatments present a risk of side effects. More importantly, the standards of care are chemotherapeutic combinations known as FOLFOX and FOLFIRI, some components of which were developed as far back as 1980, but these regimens have minimal benefit for patients. With these numbers, one certainly can develop a sense of the danger colorectal cancer possesses, which makes it a major public health concern requiring immediate, but effective, immunotherapy.

For decades, efforts have been channeled toward developing immunotherapy for colorectal cancer but a dearth of tumor specific antigens which are immunogenic and universally associated with colorectal cancer, continues to hamper such efforts. This was confirmed by recent meta-analysis which revealed that there is only 1% response rate for all immunotherapeutic trials involving colorectal cancer. Already examined target antigens include mutated proteins, oncofetal and cancer testis antigens, and overexpressed self-antigens, leaving a fourth class of antigens (tissue-specific differentiation antigens) unexplored. Data show that this class of antigens are promising and may serve as the most effective targets for cancer immunotherapy by their tumor-specificity, immunogenicity, and common association with the disease.

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 infectious diarrhea in developing countries. As a mucosa-specific protein, which is so highly restricted in its expression, GCC can be utilized to detect
metastases in non-intestinal tissues. Moreover, its mucosa-restricted expression makes it ideally suited as a novel target antigen in colorectal cancer. To that effect, a recombinant GCC-expressing adenovirus vaccine (Ad5-GCC) has been generated and tested in mice. Vaccination of mice with Ad5-GCC showed split immunological responses - one out of three effector arms responded. Specifically, vaccination produced GCC-specific CD8+ T cell responses, but failed to generate GCC-specific antibodies or GCC-specific CD4+ T cell responses. However, the mechanism by which B cell and CD4+ T cell responses were eliminated is unknown.

Initially we are focusing on the mechanisms of CD4+ T cell tolerance because these cells are central regulators of immunity and tolerance within this cell type may be the most important. T cell development occurs in the thymus where bone marrow-derived T cell precursors undergo T cell receptor rearrangement (Figure 1). Within the thymus, these cells may encounter presented GCC and undergo negative selection through either deletion or differentiation into Tregs, a type of suppressor cell. Tolerance occurring within the thymus is known as central tolerance. Alternatively, if some cells escape the thymus and enter the periphery as mature T cells, they may undergo peripheral tolerance by deletion, Treg development or anergy upon engagement with presented GCC. Only cells escaping both central and peripheral tolerance can become naïve T cells ready to produce effector responses upon vaccination. Because there are no CD4+ T cell effector responses produced by vaccination, we know that one or more of these processes are occurring to produce GCC-specific CD4+ T cell tolerance.
Figure 1: A schematic depicting possible mechanisms of CD4⁺ tolerance.

Unfortunately, we cannot examine GCC-specific T cell tolerance mechanisms because it is an extraordinarily rare event. The frequency of developing T cells to any given antigen are likely in the 1 in 10 million range making it impossible to detect the tolerance event in only GCC-specific cells. One solution would be generating mice in which EVERY T cell is GCC-specific via a TCR transgene. In these mice, the tolerance event would be occurring in EVERY T cell, making it easily detectable. However, a GCC-specific TCR does not currently exist. Alternatively, already in existence are transgenic mice containing the OT-II TCR specific for the ovalbumin323-339 epitope. If we could combine those mice with mice expressing an intestine-specific antigen transgene, it should model the GCC system and reveal mechanisms of tolerance that can be extended to GCC. The purpose of my project was to create a model antigen possessing the OT-II epitope, in addition to the OT-I CD8⁺ T cell epitope, and HA tag B cell epitope. The model antigen would be employed to create transgenic mice expressing it specifically in
the intestine. We have selected GCC as the “backbone” in which to embed the model epitopes creating a chimeric epitope-GCC model antigen.

**Figure 2: Generation of a chimeric antigen by overhang PCR.**

In PCR #1, two PCR products (AB and CD) were generated in which the 3’ end AB and 5’ of CD are complimentary. In PCR #2, the two PCR products were hybridized and amplified with a forward primer that binds to the 5’ end of AB and a reverse primer that binds to the 3’ end of CD, allowing for the amplification of the fused products, resulting in a chimeric molecule.
Using Bip or PrP cDNA template (AB), we PCR amplified the Bip and PrP signal sequences to possess two different versions of our epitopes, known as S and T. In addition, we also PCR amplified GCC (CD) with 5' ends that overlapped with the epitope portions of the signal sequence segments. We then purified these DNA fragments.
After combining the Bip-S and S-GCC fragments from PCR #1 in an overhang PCR reaction, three PCR reactions were performed (here we are showing only Bip-S-GCC, representative of the other reactions). In the first reaction, we used Bip-S specific primers and in the second reaction, we used S-GCC specific primers. These reactions served as positive controls to show that we had good template mixture. The third reaction, which is our reaction of interest included Bip-S forward primer and S-GCC reverse primer to amplify our chimeric DNA fragment.
Figure 5: Screening of chimeric antigen-pENTR/D-TOPO clones.

Having generated a chimeric DNA fragment, we then cloned our chimeric DNA fragments into an intermediate vector known as pENTR/D-TOPO and screened colonies for the correct inserts, identifying several positive clones. Positive clones were then sequenced to confirm that gene assembly occurred correctly and no mutations were inadvertently created.
Having identified correct chimeric antigen fragments in the pENTR intermediate, we subcloned our chimeric DNA fragments into a mammalian retroviral expression vector known as pMSCV-Puro and screened colonies for the correct inserts, identifying several positive clones. Positive clones were then sequenced to confirm their integrity.

Summary/Future Goals:

Here, we have successfully created several chimeric antigen constructs containing a GCC backbone possessing model B cell and CD4+ and CD8+ T cell epitopes. Future steps will involve creating stable cell lines expressing the constructs by transducing cell lines with retrovirus containing our constructs. This will be followed by examination of the new cell lines for 1) chimeric antigen expression, 2) subcellular location or our antigen, 3) and quantity of epitope presentation. Finally, we intend to define how different signal sequences on our model antigen affect antigen processing and presentation.
REFERENCES CITED


