T-Cell Mediated Mechanisms of Autoimmunity, Anti-Tumor Immunity and Tumorigenesis

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UNIVERSITY OF MIAMI

T-CELL MEDIATED MECHANISMS OF AUTOIMMUNITY, ANTI-TUMOR IMMUNITY AND TUMORIGENESIS

By

Jason Miska

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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the requirements for the degree of
Doctor of Philosophy

T-CELL MEDIATED MECHANISMS OF AUTOIMMUNITY, ANTI-TUMOR
IMMUNITY AND TUMORIGENESIS

Jason Miska

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The difficulty in identifying a tumor-specific antigen (TSA) in a majority of tumors poses a clinical problem in treating cancer with immunotherapy. To date, the majority of tumor antigens identified are not tumor-specific, but are rather largely “self” in nature, and the destruction of these tissues appear to be similar to autoimmune damage of normal tissues. Indeed, clinical studies of successful immune checkpoint therapies like such as anti-CTLA4 and anti-PD1/PD-L1 antibody treatment revealed a strong association of autoimmune side effects with therapeutic benefit. In these cases the anti-tumor effect seems to be mediated by, in part, an autoimmune response. The potential interrelatedness of autoimmunity and anti-tumor immunity indicates that the autoimmune process can be used as a potential tool for the destruction of tumor tissue.

Further evidence is derived from genetic polymorphisms that alter CTLA4 expression in humans. CTLA4 polymorphisms that cause a reduction in its expression are associated with both a significant increase in autoimmune risk and a decrease in the risk of certain cancers. However, quite perplexingly, the same CTLA4 polymorphisms also are associated with increased risk of gastrointestinal carcinogenesis.
These previous studies suggest that regulators of autoimmunity have both pro- and anti-tumor capability depending on the context in which it is studied. To address these questions, we examined the mechanisms of the critical immune checkpoint regulator CTLA4 in the context of autoimmunity, anti-tumor immunity, and tumorigenesis.

We first characterized the behavior of autoimmune cells directly in the target tissue utilizing intravital microscopy. By using the anterior chamber of the eye as a “window” to the live tissues, we could visualize the behavior of TCR restricted (BDC2.5) autoimmune T-effector (T_{eff}) and T-regulatory (T_{reg}) cells in protection and rejection of islet grafts. T_{eff} and T_{reg} directly contact one another, and this contact is essential in protecting islet grafts from autoimmune destruction. In a separate model, we demonstrate islet grafts that are directly adjacent to target islets proliferate rapidly as a result of CD8^{+} mediated destruction of the target. Both of these observations are critical to our understanding of autoimmunity.

To study the potential role of autoimmune effectors in anti-tumor immunity, we transplanted tumor cells into mice bearing the same autoimmune targets as the endogenous pancreas. After the injection of autoimmune effectors, both the endogenous pancreas and tumor were completely destroyed. The inclusion of a suboptimal amount of T_{reg} restored protection to the tumor but not the endogenous pancreas. Anti-CTLA4 could overcome this protective effect, suggesting that Anti-CTLA4 antibody therapy works, in part, by inducing an autoimmune mediated antitumor response. The data from these experiments
suggest the autoimmunity can play a force in anti-tumor immunity, and CTLA4 can regulate this response.

Since small changes in CTLA4 expression can dramatically change the outcome of an anti-tumor response, we generated CTLA4 RNAi mice. These mice have reduced CTLA4 expression that is comparable to reductions caused by promoter polymorphisms in humans. Astoundingly, these mice uniformly develop gastric metaplastic pathology by 5 weeks of age; a process initiated by an inflammatory autoimmune response.

This pathology persisted until 17-18 months of age, at which point the gastric metaplasia progressed into adenocarcinoma. An age associated increase in tumor protective 5-hmC epigenetic modification occurs in wildtype mice, which did not occur in the CTLA4 RNAi mice; a finding extended to human adenocarcinoma.

The data from these experiments demonstrate the complicated and sometimes paradoxical role of T-cells in autoimmunity and tumor immunity. A better understanding of these mechanisms will lead to new insights on how to: restore immunological balance in individuals with autoimmunity; harness the immense power of the immune system to eradicate tumors; and prevent inflammation induced tumorigenesis.
DEDICATION

To my friends, family, and one true love; you have been the solid foundation that has allowed me to weather any storm.
ACKNOWLEDGEMENTS

I first want to acknowledge and thank Zhibin Chen for the continuous support and mentorship over these last four years. Without his consistent dedication to my success, we would not have accomplished the incredible work we have produced. I also want to thank my mentors Drs. Rebecca Adkins, Eli Gilboa, Geoffrey Stone, and Balakrishna Lokeshwar for their critical review of my work and insightful suggestions which have both contributed significantly to my development as a scientist. I want to thank Dr. Priyamvada Rai for her time and efforts being my external examiner. My fellow graduate students Priyadharshini Devarajan and Jen Bon Lui have provided constant feedback and scientific insights critical to the success of my studies. I would also like to thank previous lab members for their positivity and making the lab a wonderful place to be.

I also wanted to thank Dr. Midhat Abdulreda, for his expertise with the imaging platform and extensive work on the intra-vital imaging study. With our efforts combined, the imaging study has produced magnificent results I previously thought would be impossible to obtain.

I want to give a special acknowledgement to my family. To Caryn who has been with me every step of this journey. Her love and dedication to me has truly been the greatest gift I’ve ever received. Without her, my life and work would be meaningless. My family Ian, Leah, and Brad Miska have also been instrumental in my success as a scientist. They have been willing to do anything to make sure I had the opportunities to succeed in life.
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<tr>
<td>5-hmC</td>
<td>5-hydroxymethylcytosine</td>
</tr>
<tr>
<td>5-mC</td>
<td>5-methylcytosine</td>
</tr>
<tr>
<td>ACE</td>
<td>anterior chamber of the eye</td>
</tr>
<tr>
<td>AIRE</td>
<td>autoimmune Regulator</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variants</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>APECED</td>
<td>Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>gene encoding B-cell lymphoma oncogene</td>
</tr>
<tr>
<td>BDC2.5</td>
<td>CD4⁺ restricted TCR against chromogranin A</td>
</tr>
<tr>
<td>BG</td>
<td>blood glucose</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromodeoxyuridine</td>
</tr>
<tr>
<td>C57/BL6</td>
<td>Standard black mouse</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>Ccna</td>
<td>cyclin A1</td>
</tr>
<tr>
<td>Cdkn1c</td>
<td>cyclin-dependent kinase inhibitor 1C</td>
</tr>
<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>Ctgf</td>
<td>connective tissue growth factor</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T-Lymphocyte Antigen 4</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DT</td>
<td>diphtheria toxin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DTR</td>
<td>diphtheria toxin receptor</td>
</tr>
<tr>
<td>E.G7</td>
<td>lymphoma line expressing mOVA</td>
</tr>
<tr>
<td>EL-4</td>
<td>lymphoma line</td>
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<tr>
<td>FIR</td>
<td>foxp3-IRES-RFP</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead box P3</td>
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<tr>
<td>GA</td>
<td>gastric adenocarcinoma</td>
</tr>
<tr>
<td>GC</td>
<td>gastric cancer</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal tract</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>Hif</td>
<td>genes encoding hypoxia inducible factors</td>
</tr>
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<td>horseradish peroxidase</td>
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<tr>
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<td>knockdown</td>
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<tr>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
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NKT  natural killer T cells
NOD  non-obese diabetic
OT1  CD8+ restricted TCR against ovalbumin
OVA  ovalbumin
p53  gene encoding tumor suppressor protein size of 53,000 daltons
p73  gene encoding tumor suppressor protein size of 73,000 daltons
PL4  GFP+ transgene+ mice
RAG  recombinase activating gene
RFP  red fluorescent protein
RIP  rat insulin promoter
SCID  mice with severe combined immunodeficiency
SD  standard deviation
SEM  standard error of the mean
Serpine1  gene encoding plasminogen activator inhibitor-1
SPEM  spasmolytic polypeptide-expressing metaplasia
TAA  tumor associated antigens
TCR  T-cell receptor
TEC  thymic epithelial cells
Tet  genes encoding ten eleven translocation family of enzymes
Teff  effector T-cell
TGF-β  transforming growth factor beta
<table>
<thead>
<tr>
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<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>$T_{\text{reg}}$</td>
<td>regulatory T-cell</td>
</tr>
<tr>
<td>TSA</td>
<td>tumor specific antigens</td>
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CHAPTER 1: INTRODUCTION

1.1 The Adaptive Immune System

Adaptive immunity is a specialized branch of the immune system defined by its ability to recognize pathogens with exquisite precision. The adaptive immune system originated approximately 500 million years ago, and has continued to evolve rapidly alongside the pathogens it has been design to protect [1]. The vertebrate immune system we know today arose out of this evolutionary “arms race” and is why it is such a complicated and multifaceted system, requiring complicated and fine-tuned regulation to perform adequately [2].

T-lymphocytes represent the hallmark of this genetic complexity, as individual cells can rearrange their own genomes to produce a virtually limitless array of receptors to recognize pathogens [3]. The rearrangement of their genomes is achieved through the expression of the recombinase activating gene (RAG). RAG genes are expressed exclusively within developing T-cells and B-cells, and their activity is responsible for the incredible diversity of T and B cell receptors found on their surface [4].

This is potentially hazardous, as the surface receptors can recognize anything it could possibly encounter, including our own tissues. Therefore development of T-cells is a tightly regulated process, wherein they undergo a rigorous process known as selection, which is a complicated multi-step process designed to get rid of “self”-reactive T-cells.
The question then becomes, what is the “self”? Broadly defined, the self is our body’s own healthy tissues, and any cellular constituents made within. In order to have a functioning immune system the self must be extensively defined to the developing cells of the adaptive immune system; but also must be able to recognize foreign antigens and clear pathogens before they become lethal to the host. This “us versus them” approach of the immune system is extraordinarily successful considering the constant barrage of foreign invaders to our immune system. Furthermore the adaptive immune system is even able to detect small perturbations in normal cell physiology, as tumors are constantly forming and are eradicated successfully [5].

The immune system has the immense challenge of having its lymphocytes be self-tolerant, but also ready to mount an immune response to virtually any pathogen. T-cells undergo a strict and rigorous education as they develop in the thymus, and even as they exit into the periphery they are regulated to prevent damage to the self.

1.2 Tolerance: Defining the Self

Central and peripheral tolerance represents the two branches of critical immune-regulatory mechanisms found in vertebrates. Central tolerance refers mainly to the mechanisms by which auto-reactive (self-reactive) T-cells are removed during development in the thymus. This thymic education involves multiple complicated steps as the developing thymocytes travels through the thymus.
As progenitors enter the thymus, they are stimulated to proliferate along the T-cell lineage using distinct differentiation pathways [6]. Double negative (meaning without CD4⁺ or CD8⁺ expressed on their surface) thymocytes are the earliest T-cell progenitors. Some of these cells will develop into “minority” lineages of T-cells; these include Natural Killer T (NKT) cells and gamma-delta T-cells [7]. These minority populations express a T-cell receptor (TCR) on their surface, but are very limited in its diversity.

As thymocytes progress through development, they begin to rearrange the \( \beta \) chain of the T cell receptor. If the thymocyte can produce a functional \( \beta \)-chain, it will associate with a surrogate \( \alpha \)-chain to form the pre-TCR [8]. This stimulates proliferation and co-expression of CD4 and CD8 on their surface, termed double positive lymphocytes. If the thymocyte cannot produce a functional pre-TCR they die due to lack of proliferative signaling [8].

At this point, the developing T-cell begins to rearrange the \( \alpha \)-chain and begins another stage of selection. If the developing T-cell cannot produce a functional T-cell receptor it will undergo apoptosis due to lack of survival signals, termed death-by-neglect. Conversely, if a T-cell receptor engages peptide-MHC too strongly, the T-cell will undergo apoptosis via negative selection. The remaining cells that produce a TCR with moderate affinity are selected for, termed positive selection [9].

During this selection process the thymocytes are presented antigens from the periphery, by thymic epithelial cells (TECs) expressing the transcription factor AIRE [10]. AIRE, or the AutoImmune REgulator, is a master transcriptional
regulator that allows TECs to present antigens from specialized cellular subsets (i.e. insulin) that would not otherwise be expressed within the thymus. AIRE regulates the transcription of thousands of genes, and provides a comprehensive supply of peripheral tissue antigens to present to developing thymocytes [10]. If any of the T-cells bind too strongly to these presented antigens they are removed before leaving to the periphery. Its importance is highlighted in patients with a dysfunction in AIRE. These patients develop APECED or (Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy), which causes autoimmunity in many peripheral organs [11].

Central tolerogenic mechanisms are so rigorous that only 1-3% of T-cells generated make it out of the thymus [9]. After these processes, then the mature T-cells can exit the thymus to patrol the periphery. Even though the process of central tolerance is rigorous, it is incomplete. In patients with APECED, autoimmune disease takes time to develop, suggesting that other tolerance mechanisms have evolved to prevent autoimmunity.

Peripheral tolerance mechanisms are also critical to the maintenance of long term tolerance in the host. Peripheral tolerance consists of many different mechanisms. One important mechanism is anergy, or functional inactivation of T-cells. T-cells require costimulation, or a second activating signal through their surface receptors [12]. The absence of these (mainly) innate activating signals, paired with activation of the TCR, sends signals to the T-cell to become anergic [12].
1.3 T-Regulatory Cells and CTLA4 are Masters of Peripheral Tolerance

Of all peripheral tolerogenic mechanisms, T-regulatory cells (T_{reg}) are perhaps the most intensely studied and well understood. Originally defined as CD4^{+}CD25^{+} T-cells, this cell subpopulation was discovered for its ability to suppress T-effector cell (T_{eff}) proliferation [13, 14]. Furthermore, depleting these cells from an animal or adoptively transferring splenocytes without them causes massive autoimmunity and death within weeks [13]. The transcription factor Foxp3 defines this population [15], and dysfunction of this critical transcription factor leads to systemic autoimmunity and death in mice [16].

Despite considerable evidence, how T_{reg} can specifically suppress T-cell mediated autoimmunity is a continuing topic of intense debate. When originally defined with in-vitro systems, T_{reg} functioned via direct contact-mediated suppression [17-22]. The critical interactions of surface receptors on T_{reg} with those on T_{eff} are a highlight of their functionality. Therefore, suppression using ligands on the surface of T_{reg} is one way which T_{reg} might suppress T-cell proliferation. One of the most studied of these mediators is CTLA4 (Cytoxic T-lymphocyte Antigen 4).

CTLA4 is protein with four domains, and four splice variants; each of which has been implicated in augmenting autoimmunity [23-27]. One variant expresses the full length protein, secreted CTLA4 is missing the transmembrane domain and is thusly secreted; ligand independent CTLA4 consists of the
transmembrane and cytoplasmic domain, and 1-4 CTLA4 only has the cytoplasmic domain [23].

CTLA4 can work via multiple modalities. It can bind CD80 and CD86 on antigen presenting cells (APC), changing both the APC and the cell expressing CTLA4 [23, 28, 29]. On effector cells, CTLA4 directly competes with CD28 to prevent costimulation [30]. CTLA4 has even been shown to remove CD80/86 from the cells it contacts with, thus preventing the costimulation needed to activate T-cells [31]. It is such an important molecule that even slight reductions in CTLA4 expression in humans have been linked to autoimmunity [24]. These studies not only demonstrate the role of CTLA4 in preventing autoimmunity, but also highlight the molecule’s important role as a tolerogenic mechanism.

1.4 Imaging Tolerance and Autoimmunity Within Target Tissues

Since its initial discovery, the intravital imaging platform has risen to prominence as a way of assessing cellular interactions in-vivo. These in-vivo imaging experiments demonstrated that, in fact, T\textsubscript{reg} do not directly interact with T\textsubscript{eff} [32, 33]. Evidence that cytokines such as IL-10 and TGF-β act directly [34] and indirectly [35] on autoantigen specific T-cells, suggests that T\textsubscript{reg}-T\textsubscript{eff} contact is not necessary for their function. These observations are clearly in contradiction with the initial in-vitro findings.

The in-vivo studies of T-cell behavior, with few exceptions, been within the lymph nodes of animals [32, 33, 36]. The lymph node is the site of immune activation, and thus provides a limited view of the immune response. The effector phase of the immune response, or when the T-cells actually migrate to their
target site, have been less studied \textit{in-vivo}, and could provide new insights into T\textsubscript{reg} behavior not previously appreciated.

T\textsubscript{reg} based therapies are being developed to treat autoimmunity [37-39], alloimmunity and organ/graft rejection [40, 41]. Therefore understanding the modalities by which T\textsubscript{reg} function is critical to the future success of these endeavors.

We sought to reconcile the differences between the studies of \textit{in-vitro} and \textit{in-vivo} mediated suppression utilizing a newly developed imaging platform. Intravital microscopy of pancreatic islets engrafted in the anterior chamber of the mouse eye (ACE), facilitated high-resolution visualization of immune cells noninvasively and longitudinally [42-44]. In this part of my thesis we took advantage of this imaging platform together with a series of reductionist animal models. We established models of effective immune responses in the ACE imaging site versus the native pancreas, in terms of equivalent kinetics of tissue damage and T\textsubscript{reg}-cell-mediated protection. Using this noninvasive imaging approach, we studied in real-time how self-antigen-specific T cells interacted with target tissue cells \textit{in vivo}. We depicted the behavior of three major T-cell lineages – CD4\textsuperscript{+} T\textsubscript{eff} cells, CD4\textsuperscript{+} T\textsubscript{reg} cells, and CD8\textsuperscript{+} T\textsubscript{eff} cells--, analyzed the regulatory effect of CTLA4 on their behavior, and examined tissue responses in destructive settings.

1.5 The Tumor as the Altered Self

Since the cardinal capacity of the immune system is to differentiate between “self” and “non-self”, malignant tumor tissues present a distinct challenge to the
immune system as “altered self”. Antigenic proteins from mutated genes in cancer cells, or viral products from transformed tumor cells, may trigger the immune system as tumor-specific antigens (TSAs) that are not expressed by non-malignant tissues. However, for the vast majority of tumors, TSA have yet to be identified. Well-studied tumor associated antigens (TAAs) are in fact self-antigens associated with cellular differentiation [45].

The difficulty to identify TSA compels a supposition that cancer cells are largely “self”. The premise of cancer cells as “altered self” would predict the well-recognized association of autoimmune risk with cancer immunotherapy [46]. On the other hand, the “altered self” view could also foretell autoimmunity as a beneficial effector to destroy cancer cells. In other words, although autoimmunity and tumor immunity are often viewed as being on opposite sides of the same coin, they could also be viewed to be on the same side of the coin, serving as overlapping mechanisms for tumor destruction.

Indeed, the remarkable benefits of cancer immunotherapies enjoyed by some patients in recent immunotherapy trials, most notably in anti-CTLA4/PD-1/PD-L1 antibody therapies, often came with the price of autoimmune adverse effects [47-49]. The intricate tangle of autoimmune toxicity and antitumor immunity substantially affects the benefit/risk ratio calculation in immunotherapies [45]. On the other hand, autoimmunity may serve to benefit clinical management of cancers. Evidence gathered from the clinics treating a variety of cancers with immunotherapies based on IL-2 [50], interferon α-2b [51] or CTLA4 [47, 52] suggests that the therapy-induced autoimmunity, at least in part, may actually
mediate the destruction of cancer cells. The clinical observations provoke suggestion of a paradigm shift, to which autoimmunity is not a shunned side effect, but instead an acceptable or even desirable antitumor mechanism [53]. However, it is still not well understood how self-antigen-specific T cells target tumor versus healthy tissues, and how they are regulated in tumor settings.

A variety of immune suppressive mechanisms have been implicated in cancers. In the adaptive branches, Treg cells and CTLA4 are among the most prominent cellular and molecular inhibitors. T_{reg} cells depend on CTLA4 for function [54]. CTLA4 is constitutively expressed on T_{reg} cells but is also induced in activated T_{eff} cells. Conditional knockout experiments indicated that CTLA4 functions predominantly through T_{reg} cells [54]. However, other studies with CTLA4 knockout models or antibody blockade indicate that CTLA4 regulates T_{eff} cells intrinsically and through extrinsic effect by T_{reg} cells [29, 55].

In human populations, no CTLA4 deficiency has been identified, nor is there a qualitative difference in mature CTLA4 protein expression among individuals. Instead, the polymorphisms of the human CTLA4 locus determine modest, quantitative variations in the CTLA4 mRNA and protein expression [23, 56-59]. Genetic studies have associated CTLA4 polymorphisms with autoimmunity [59], as well as antitumor immunity in settings including lymphoma, breast cancer and skin cancer [60-64]. It remains a challenge to elucidate how subtle variations in CTLA4 levels impact autoimmune effector and regulatory mechanisms in antitumor immunity.
Even though clinical observations have strongly suggested that autoimmune effectors are intricately involved in tumor-killing, evidence provided so far from studies with antigen-specific animal models indicates that the immune system selectively targets tumor tissues but spares healthy tissues [65-67]. This apparent disconnection prompted us to examine the role and regulation of autoantigen-specific T cells with well-characterized animal models of robust autoimmunity. A better understanding of the regulatory mechanisms of autoantigen-specific T cells in antitumor immunity could suggest approaches to enhance the efficacy of adoptive T cell therapies.

Aided with well-characterized models of robust autoimmunity, we explored if tumor destruction can be promoted by autoimmune mechanisms, and if a quantitative reduction of CTLA4 can overcome tumor protective mechanisms. This was tested by examining how autoantigen-specific T-cells interact with normal or malignant tissues expressing the same antigens. The aim was also to test if T\textsubscript{reg} cells prevent autoimmune destruction of tumor tissue, and if CTLA4 reduction on the effector and/or regulatory compartment will augment the suppression.

1.6 Autoimmune Induced Carcinogenesis

To further complicate our understanding of autoimmunity in cancer; the inflammatory mediators used to promote an autoimmune response can also be positively associated with cancer [68-70]. Inflammation has recently come to light as a major contributor to carcinogenesis, tumor promotion, and metastasis [71-73]. This has been observed particularly in cancers of the GI tract. In the colon,
colitis and IBD have long been associated with risk of colorectal cancer [74]. In patients with chronic liver disease, the consistent inflammatory damage occurring in the liver has been proposed as a promoter of tumorigenesis [75, 76]. Understanding the ignition of such inflammatory signals, and the role they play in tumorigenesis is then critical to prevention and future treatment of cancer.

In our mouse models, CTLA4 modulation triggered gastric pathology in the absence of pathogenic Helicobacter infection. We took this opportunity to uncover mechanisms behind the initiation and progression of gastric pathology. GC is usually diagnosed after 60 years of age. Worldwide, GC is the 2nd most lethal and the 4th most common cancer, causing more than 700,000 deaths per year [77]. GC has poor prognosis [78], underlining the importance of an effective prevention strategy.

Gastric adenocarcinoma (GA) accounts for most GC cases. Its origin remains to be defined. In a classical paradigm, known as the “Correa Cascade”, the etiology of GA has been described as a process through a series of histopathological stages: superficial gastritis, chronic atrophic gastritis, intestinal metaplasia, dysplasia and carcinoma [79]. A new type of gastric metaplasia, Spasmolytic Polypeptide-Expressing Metaplasia (SPEM), has been identified as a second lineage of metaplastic cells in gastric mucosa [80-83], and was associated with human GC [82].

As previously described in other types of cancer, inflammation plays a critical role in gastric tumorigenesis [68, 84-87]. The inflammatory signals could originate from autoimmune responses against gastric mucosa or immune
responses against microbial agents. The most recognized environmental factor for GC is *Helicobacter pylori* (HP) infection [78, 88, 89]. However, substantial variations exist in the association between GC and HP, with population variation patterns dubbed as the “African enigma” [90] or “Asian paradox” [91, 92]. These variations are even more confounding if one considers the extraordinary prevalence of HP in a majority of human beings (58% and 76% in developed and developing countries, respectively) [93]. For most cases, HP colonization in the stomach is believed to have occurred in childhood. Overall, it has been estimated that 0.5%-2% of HP infections lead to GA [88]. Thus, the etiology of GC, as in other tumors, likely implicates complex interactions between environment and host factors, some of which could have been masked by the predominant prevalence of HP.

The host factors that control gastric carcinogenesis are not completely understood. A number of them have been implicated [84-86, 88] in the initiation and progression of mucosal changes associated with GC, and some host factors have been demonstrated to cause GC in animal models in the absence of Helicobacter infection [94, 95]. Recent genetic studies by independent groups [96, 97] have found paradoxical associations of the *CTLA4* gene with human GC. While it has been recognized as a prototypical inhibitor of antitumor immunity [98] the *CTLA4* promoter and exon 1 polymorphisms associated with GC [96, 97] predict reduced CTLA4 expression at the mRNA or protein levels [56-58]. To model the natural variation of CTLA4 expression in the human *CTLA4* locus, we used our CTLA4 RNAi “knockdown” (KD) transgenic mouse lines. Unlike CTLA4
knockout mice [99-101], CTLA4KD mice exhibited MHC-dependent, tissue-specific autoimmune damage without signs of systemic immune perturbation [102, 103].

CTLA4 modulation by antibody-mediated blockade or RNAi transgenesis in mice initiated spontaneous tumorigenesis in the stomach. This effect is consistent with the paradoxical association found between $CTLA4$ polymorphisms and human GC [96, 97] Importantly, the spontaneous cascade from metaplasia to aging-associated transition to neoplasia in the CTLA4KD models occurred without pathogenic Helicobacter infection, providing an opportunity to understand the factors in aging-associated spontaneous GC that could be extrapolated to other solid tumors.

1.7 Summary

These data demonstrate the complicated and often paradoxical role of the immune response in cancer. Understanding how autoimmunity is prevented or promoted at the target tissue; and how its regulation in involved in the tumor response will give great insights to future treatment for a wide array of diseases. Thus the overall goal of my research was to elucidate the role of autoimmune regulators in both pro- and anti-tumor immunity, and how they regulate the responses at the target tissue. Furthermore, we dissect the role of the prototypical immunosuppressive molecule CTLA4 in these settings by both genetic manipulation and antibody therapy.
CHAPTER 2: Real-Time Immune Cell Interactions in Target Tissue During Immune Damage and Protection

2.1 Summary

Real-time imaging studies are reshaping immunological paradigms, but a visual framework is lacking for self-antigen-specific T cells at the effector phase in target tissues. To address this issue, we conducted intravital, longitudinal imaging analyses of cellular behavior in nonlymphoid target tissues, to illustrate some key aspects of T cell biology. We used mouse models of T-cell-mediated damage and protection of pancreatic islet grafts.

2.2 Materials and Methods

2.2.1 Mouse Models

Lines of transgenic mouse models were crossed or backcrossed to generate necessary combinations of specific T cells and target tissue with distinct fluorescence reporters. Detailed descriptions of their genetic makeup, antigen-specificity and fluorescence properties were provided in Table S1. NOD.BDC2.5 [104], NOD.Foxp3DTR [105], and NOD.CTLA4 shRNA (CTLA4KD7) [102], NOD.PL4 [102], OT1 [106], RIP-mOVA [107], MIP-CFP [108] and CAG-CFP [109] transgenic lines were described previously. The CTLA4shRNA and PL4 lines were backcrossed onto B6 background for >10 generations [103]. The CTLA4shRNA transgene cause 2-3-fold reduction in CTLA4 expression. The stability of the RNAi effect in the transgenic lines on different genetic background has been established [102, 103]. FIR (Foxp3-IRIS-RFP “knockin”) mice [110] were backcrossed onto the NOD background for 10 generation to create the NOD.Foxp3FIR line. CAG-CFP, obtained on the C57BL/6 background, were
backcrossed onto the NOD genetic background for 10 generations, and crossed with NOD.BDC2.5 and NOD.Foxp3^{FIR}. All animals were maintained in a specific-pathogen-free barrier facility in the University of Miami and the studies are approved by the Institutional Animal Care and Use Committee at the University of Miami.

2.2.2 Cell Sorting and Adoptive Transfer

Antigen-specific CD4^{+} BDC2.5 T_{eff} and T_{reg} cells were purified from transgenic mice on the NOD genetic background that carries the BDC2.5 transgene and the Foxp3^{FIR} knockin alleles, in combination with either the PL4 transgene for a GFP reporter or the CAG.CFP transgene for a CFP reporter. T_{eff} and T_{reg} cells were sorted by using a FACSaria II flow cytometer (Becton Dickinson, San Jose, CA, USA), using the following parameters: CD4^{+}CD25^{-}CFP(or GFP)^{+}BDC2.5^{+}CD62L^{+}Foxp3^{FIR-} for T_{eff} cells, and CD4^{+}CD25^{+}GFP^{+}BDC2.5^{+}Foxp3^{FIR^{+}} for T_{reg} cells. The RFP expressed by the Foxp3^{FIR} allele is used as a specific lineage marker for flow cytometry purification of T_{reg} cells, but RFP signal is not strong enough for a reliable tracking of live cells in animals. Therefore we used an additional, non-lineage-specific GFP marker to track the cells in animals after the purified GFP^{+}Foxp3^{RFP^{+}} cells were transferred. To enable punctuated, acute removal/depletion of Foxp3^{+} T_{reg} cells after T_{reg}-cell protection is established, an additional transgene, Foxp3^{DTR}, the diphtheria toxin receptor (DTR) driven by a Foxp3 promoter, is crossed with NOD.BDC2.5.Foxp3^{FIR^{+}}.PL4 transgenic mice, to generate NOD.BDC2.5.Foxp3^{FIR^{+}}.PL4.Foxp3^{DTR^{+}} mice or
NOD.BDC2.5.Foxp3^{FLR}.PL4.Foxp3^{DTR-} controls. From these mice, Foxp3^{DTR+} and Foxp3^{DTR-} T_{reg} cells, respectively, were purified with CD4^{+}GFP^{+}BDC2.5^{+}Foxp3^{FLR+}CD25^{+} markers, for adoptive transfer. Purified T_{eff} cells, or a mixture of purified T_{eff} and T_{reg} cells were injected intravenously into NOD.SCID mice, at doses of 5-10 \times 10^4 cells with T_{eff}-T_{reg} ratios at 2:1 or 1:1. Some of the recipients had stable islet grafts established in ACE. In animals bearing islet grafts in ACE, the cervical lymph nodes draining the eyes likely play an important role in activation and differentiation of the antigen-specific T cells. The lymphopenia reconstitution model was necessary for our study, due to current limitation in tools for long-term, simultaneous tracking of different lineages of T cell in vivo. If one uses an immunocompetent animal as a recipient, rather than reconstitute a lymphopenic animal with highly purified, fluorescence-tagged T cell players, an "invisible" T_{reg} cell from host could be interacting with a fluorescence-tagged T_{eff} cell, but we would have to count that T_{eff} cell as if it had no T_{reg} cell interaction. That would prevent us from making meaningful conclusions on the extent of T_{reg} and T_{eff} cell interaction. Flow cytometry analyses of the recipient mice were performed to verify the stability of Foxp3 expression of the adoptive transferred T_{reg} cells about 3 weeks later, using the Foxp3-IRES-RFP reporter [110], and revealed that more than 91% of cells remained positive for the Foxp3 reporter (data not shown).

Antigen-specific CD8^{+} T_{eff} cells were purified from transgenic mice on the C57BL/6 background carrying the OT1 transgene and the Foxp3^{FLR} knockin allele, in combination with the PL4 transgene for a GFP reporter, or
CTLA4shRNA/PL4 transgene for a GFP reporter and CTLA4shRNA for CTLA4 RNAi knockdown. Splenocytes from the mice were stimulated with the OT1 specific ovalbumin peptide (SIINFEKL) for 24 hours. CD8\(^+\) T\(_{\text{eff}}\) cells were purified (>95% purity) with magnetic-bead-based cell sorting, and injected intravenously into B6 mice bearing islet grafts in ACE or under the kidney capsules.

### 2.2.3 CTLA4 Blockade and Acute Depletion of T\(_{\text{reg}}\) Cells

To examine the molecular role of CTLA4 in maintaining T\(_{\text{reg}}\)-T\(_{\text{eff}}\) cell interaction, anti-CTLA4 antibody (clone UC10-4F10-11) were injected intraperitoneally into mice, at 40\(\mu\text{g/g body weight},\) two consecutive doses at 3 days apart, after T\(_{\text{reg}}\) cell established graft protection and stable T\(_{\text{reg}}\)-T\(_{\text{eff}}\) cell interaction in the graft were detectable (typically >15 days post T cell transfer). This monoclonal antibody blocks CTLA4 function without depleting T\(_{\text{reg}}\) cells [111]. To test the effect of CTLA4 blockade on CD8\(^+\) T\(_{\text{eff}}\) cells, one dose of anti-CTLA4 antibody was used at the time of OT1 T cell transfer into the animals. For punctual removal of diphtheria-toxin-receptor-tagged T\(_{\text{reg}}\) cells, mice carrying DTR\(^+\) T\(_{\text{reg}}\) cells or control DTR\(^-\) T\(_{\text{reg}}\) cells were injected (50ng/g body weight) with diphtheria toxin at a schedule of day 0,+1,+3, similar to a regimen described before [105], after graft protection was established and stable T\(_{\text{reg}}\)-T\(_{\text{eff}}\) interactions were detected.

### 2.2.4 Intraocular Injection of Fluorescence Tagged Antibodies for In-Situ Cytolabeling

In some instances, fluorescently conjugated antibodies were injected directly into ACE for in vivo cytolabeling in situ. With our intravital imaging
platform, the injected antibodies can effectively label cells in 50µm depth within the graft tissue, which is at the similar depth capacity for accurately tracking cells marked with genetic tags of fluorescence markers [44]. After mice were adoptively transferred with T\textsubscript{reg} and T\textsubscript{eff} cells, mice were injected with fluochrome-conjugated antibodies directly into the anterior chamber. All antibodies were tested with isotype controls to determine specificity of the \textit{in situ} labeling. The following is a list of the antibodies and their isotype controls. BV605 conjugated anti-CD11b monoclonal antibody (cat#101237, Biolegend, San Diego, CA), and BV605 conjugated RatIg2a (Biolegend #400649) as isotype control; Alexa647 conjugated anti-CD11c monoclonal antibody (Biolegend #117314), Alexa647 conjugated anti-CD80 and anti-CD86 monoclonal antibodies (Biolegend #104717 and #105019, respectively), and Alexa647 conjugated Hamster IgG (Biolegend #400924) as isotype control for the three antibodies. APC conjugated annexin-V (#BMS306APC/100, Ebioscience, San Diego, CA) was used to assess apoptotic cells in ACE grafts. This \textit{in vivo} assay for β cell apoptosis was described in details previously [42, 43]. In addition, no annexin-V signal was detected by injecting APC-conjugated annexin-V into ACE bearing islet grafts before transfer of β-cell-specific T cells.

\subsubsection{2.2.5 Islet Transplantation into ACE and Noninvasive In-Vivo Imaging}

The pancreatic islets were transplanted into ACE by injection through the cornea, as previously described [42-44]. The cornea serves as a natural “window” for noninvasive visualization of transplants. This injection procedure does not entail substantial injury to the cornea or the eye chamber. At least two
weeks of time were allowed for complete engraftment of the islets before any experimentation. Intravital imaging of the transplants was conducted by confocal microscopy, with a Leica upright TCS SP-5 broadband confocal microscope (using Leica 20X/0.5NA HCX APO L U-V-I 12 lens for PBS immersion), as previously described [42-44]. $T_{\text{reg}}$ and $T_{\text{eff}}$ cells were visualized by GFP or CFP fluorescence. Target islet cells were visualized by laser backscatter (reflection) or CFP fluorescence Three-dimensional (xyz; 3-D) or time-lapse (xyzt; 4-D) noninvasive in vivo imaging was acquired longitudinally. In time-lapse recordings, z-stacks were acquired every 2 minutes up to 30 - 75 min and the z-spacing ranged from 5-7µm. A key strength of this intravital imaging platform is noninvasiveness that few other intravital cellular imaging platforms have been afforded. This strength not only avoids inadvertent inflammatory signals caused by surgical exposure for imaging needs, but enables us to monitor the same tissue in the same animal longitudinally at cellular resolution. A baseline measurement serves as a rigorous, “internal” control for all post-intervention measurements of the same live tissue in the same live animal.

Animals were routinely monitored by urine and blood glucose levels. Animals transplanted with islet grafts in ACE were examined 2 weeks post transplantation for engraftment. After T cell transfer, the islet grafts in ACE were examined every 2-3 days with the imaging microscope. Animals with two consecutive readings of BG>250 mg/dL were considered diabetic. With regard to individual islet grafts in ACE in setting of immune responses, a graft maintaining >80% of its original mass was considered protected, and a graft with <20% of its original mass was
considered damaged or failure in immune regulation. In experiments that a relatively small number of islets were transplanted in ACE, the islet grafts served as indicators of immune responses and the endogenous pancreatic islets maintained blood glucose homeostasis of the animals. The immune damage of islet grafts in ACE always correlated with incidence of diabetes that was due to immune destruction of endogenous pancreatic islets.

2.2.6 **BrdU Labeling of Proliferating Cells**

Islets were transplanted under the kidney capsule of B6 mice by the Diabetes Research Institute Preclinical Cell Processing and Translational Models Core facility following standard procedures [112]. The graft-bearing mice were rested for one month post transplantation to ensure engraftment, and then adoptively transferred with activated OT1 CD8 T cells by intravenous injection. Two days later, BrdU was injected at a concentration of 10mg/kg every 12 hours. On day 10, kidneys were removed, fixed in 4% PFA overnight, followed by immersing in 30% sucrose overnight, and then embedded in OCT. Sections were cut and stained for BrdU incorporation with biotinylated antibodies against BrdU, using an *in situ* BrdU detection kit (Cat#550803, Becton Dickinson, San Jose, CA) designed for histology use. We modified the secondary staining procedure for immunofluorescence staining. Although isotype controls were not included in the kit, we established the specificity of the BrdU staining procedure with spleen tissue sections from animals with or without BrdU injection treatment. To expose BrdU epitopes in the nuclear DNA, a covered plastic coplin (filled with 50ml of diluted BD retrievagen buffer) was pre-heated in a water bath at 95-97°C. After
30 minutes, the tissue slides were quickly placed into a pre-heated jar, and incubated for another 30 minutes. The tissue slides in the closed jar were then removed and left at ambient temperature for one hour. Primary anti-insulin antibodies (polyclonal guinea pig anti-insulin, #A056401, DAKO, Carpinteria, CA; titration, 1:1000) and the biotinylated anti-BrdU antibodies were incubated overnight with the tissue sections at 4°C, and then washed according to manufacturer instructions. Secondary staining were done with Alexa488 conjugated streptavidin (#S-11223, Life Technologies, Grand Island, NY; titration, 1:500) and Alexa647-conjugated donkey anti-guinea pig F(ab)_2 (#706-605-148, Jackson ImmunoResearch, West Grove, PA; titration, 1:500). Sections were counterstained with DAPI and mounted for fluorescence microscopy. Images were acquired with a Leica inverted TCS SP-5 broadband confocal microscope (using Leica 40X/1.25-0.75NA HCX PL APO lens for oil immersion).

2.2.7 Image Analysis

Image analysis was performed using the Volocity software (version 6; Perkin Elmer) as previously described [44]. Images were de-noised and contrast-enhanced equally for consistent analysis. Quantitative analysis of cellular movement and T_{reg}-T_{eff} cell interaction dynamics were performed automatically in the Volocity with user feedback on drift-corrected 3-D time-lapse recordings. Drift correction was performed in Volocity based on proprietary algorithms. T cell counting and movement tracking were performed automatically by the software and dynamic parameters (e.g., velocity, displacement) were derived from time-lapse recordings. T_{reg}-T_{eff} cell interaction time and interaction index were
calculated manually. The interaction time between the $T_{\text{reg}}$-$T_{\text{eff}}$ cell interacting pairs was calculated manually using the time stamps embedded in each image frame in a time-lapse series. The interaction index is calculated by dividing the number of $T_{\text{eff}}$ cells interacting with $T_{\text{reg}}$ cells with the number of $T_{\text{eff}}$ cells not interacting with $T_{\text{reg}}$ cells. $\beta$-cell (islet) mass was measured by the software as previously described [44], based on volume detected by either laser backscatter or CFP fluorescence.

Annexin-V labeling in islet grafts in ACE was quantified with Z-stack images that were acquired approximately 10 - 15 min after injection of APC-labeled annexin-V directly into ACE. Using proprietary algorithms in Volocity, we then measured in the 3D images the amount of overlap in the volume the annexin-positive stain with either that of $\beta$ cells (visualized by CFP or backscatter) or CFP/GFP-labeled $T_{\text{eff}}$ cells [113]. Automatic selection, optimized with user feedback, based on fluorescence of annexin-positive cells, $\beta$ cells, and T cells and volume measurements were performed automatically by the software. The overlap (volume) in annexin-V stain with either that of $\beta$ or T cells was also derived by the software, and was expressed as a fraction of the total volume of the $\beta$ and T cells in each islet. Similarly, the number of graft-infiltrating $T_{\text{eff}}$ cells was automatically measured based on fluorescence intensity [44]. Islet cell mass was measured using Volocity software as previously described in details [44], based on islet volume detected either by laser backscatter (reflection) or CFP fluorescence. For example, in Figure 2-4, the volume of CFP-labeled beta cells was measured based on CFP fluorescence which is in this case restricted to the
bystander islets in this case. In brief, a proprietary detection algorithm built into the Volocity software was used to detect CFP signal based on fluorescence intensity. The detection threshold was set with user feedback to restrict the selection to the CFP-labeled beta cells. Once the selection was made, the volume was derived automatically by the software. Longitudinal analyses on the same individual islets were performed using the same approach, and numerical values of islet volumes were expressed as means ± SEM at the different time points under the different conditions.

2.2.8 Statistical Analysis

Unpaired Student’s t-test was used to compare two samples. For multiple group comparisons, One-way ANOVA tests were performed followed up by Tukey’s post-hoc multiple comparisons test. $P \leq 0.05$ was considered significant. Asterisks indicate significance (*$p<0.05$; **$p<0.01$; ***$p<0.001$) and “n.s” is not significant.

2.3 Results

2.3.1 Noninvasive Imaging of T cells in ACE Without Hindrance by the Putative Immunoprivilege

To study CD4$^+$ T-cell responses in target tissue, we used CD4$^+$ Teff and T$_{reg}$ cells from the NOD.BDC2.5 T-cell receptor (TCR) transgenic mice [104], with a specificity against a natural antigen in the pancreatic islet $\beta$ cells. ACE offers the technical advantage of noninvasive access and high resolution in vivo imaging, but studies using ACE could be complicated by a status of immune privilege attributed to this compartment of the eye [114]. To test whether this
impacts on the immune responses of antigen-specific T cells in the islet grafts in ACE, we compared the tempo of immune damage by BDC2.5 CD4$^+$ T$_{eff}$ cells against $\beta$ cells in the ACE graft and that in the native pancreas. Donor pancreatic islets were injected into ACE through the cornea [42-44], at least two weeks before T cell transfer, to ensure stable engraftment of the islets and complete healing of the minor injection wound.

Although previous studies in other settings showed that immune cells in ACE could be impacted by the eye-associated immunoprivilege [114], in our model we found that the $\beta$-cell-specific CD4$^+$ BDC2.5 T$_{eff}$ cells destroyed the islets in the pancreas and the islet grafts in ACE at a similar tempo. Importantly, the protective T$_{reg}$ cells acted with a similar efficacy (~50%) in controlling T$_{eff}$ cell damage in ACE and in the endogenous pancreas (Figure 2-1 A-D). These results are consistent with a previous report that rejection of fully MHC-mismatched islets in ACE occurred similarly to that in a conventional extra-pancreatic implant site, the kidney sub-capsular space [44]. Therefore, the overall kinetics of immune destruction and protection of engrafted islet tissue in ACE was comparable to that in the native pancreas, and thus the islet grafts in ACE could serve as a surrogate in noninvasive and longitudinal imaging studies of basic T cell biology at the effector phase in the nonlymphoid target tissue.
Figure 2-1. Noninvasive imaging in ACE was not complicated by the putative immunoprivilege. The efficacies of β-cell-antigen-specific CD4+ T_{eff} and T_{reg} cells in the endogenous pancreatic islet were compared to that of islet grafts in the anterior chamber of the eye (ACE). (A) For immune responses in native pancreata, NOD.SCID mice were injected with either BDC2.5 T_{eff} cells or T_{reg}-T_{eff} mixture. Damage to pancreatic islets was monitored by reading blood glucose (BG) levels. Animals with consecutive readings of BG > 250 mg/dL were considered diabetic, or failed T_{reg} cell protection if T_{reg} cells were co-transferred with T_{eff} cells. For immune responses in ACE grafts, NOD.SCID mice were rendered diabetic through streptozotocin destruction of pancreatic β cells and then transplanted with pancreatic islets from allogeneic (B6) or syngeneic (NOD.SCID) donors into ACE. After stable engraftment (>2 wks after transplantation), (B) CD4+ T_{eff} cells alone or (C) T_{reg}-T_{eff} mixture were injected into the graft-bearing mice. Graft damage was monitored by BG readings. (D) Summary of results in (A-C). The cumulative incidence of diabetes was calculated for the groups of animals (n=4-26; 2-3 experiments) presented individually in A-C. It is important to note than in B, C (and the ACE data in D) streptozotocin was used to remove the endogenous islets, and normal glycemia was achieved through the islet transplanted into the eye. Therefore protection and/or rejection kinetics reflects what is occurring specifically within the ACE.
A. Islets in pancreas

- $T_{eff}$ alone
- $T_{eff} + T_{reg}$ protected
- $T_{eff} + T_{reg}$ failed to protect

Days post T-cell transfer

B. Islet graft in ACE

- CD4+ $T_{eff}$ to Allo. graft
- CD4+ $T_{eff}$ to Syn. graft

Days post transplant

C. Islet graft in ACE

- $T_{eff} + T_{reg}$ protected
- $T_{eff} + T_{reg}$ failed to protect

Days post T-cell transfer

D. Diabetes-free (%)

Days post T-cell transfer
2.3.2 Direct Contact Between Antigen-Specific CD4\(^+\) Teff Cells and Their Target Cells

CD4\(^+\) T cells are categorized into several helper and regulatory subsets. Their function as killers has also been shown [115]. The \textit{in vivo} capacity of CD4 T\(_{\text{eff}}\) cells killing target \(\beta\) cells was shown in Figure 2-1 (A-D). How CD4\(^+\) T cells kill remains to be fully examined. Most target tissues do not express MHC class II molecules which are necessary for antigen-specific, cognate interaction with CD4\(^+\) T cells. To study tissue destruction by antigen-specific CD4\(^+\) T\(_{\text{eff}}\) cells, pancreatic islets tagged with cyan fluorescence proteins (CFP) [108] were grafted in ACE. CD4\(^+\)Foxp3\(^-\) BDC2.5 T\(_{\text{eff}}\) cells marked with green fluorescence proteins (GFP) were injected into the intraocular graft-bearing animals. The GFP\(^+\)CD4\(^+\) T\(_{\text{eff}}\) cells appeared in the islet grafts and engaged in direct contact with their target \(\beta\) cells. We then used annexin-V \textit{in situ} cytolabeling to visualize apoptosis of \(\beta\) cells, by injecting allopheocyanin (APC)-conjugated annexin-V into ACE. The use of this \textit{in vivo} assay for \(\beta\) cell apoptosis was described in details in previous works [42, 43]. Apoptosis signals were present in either the contact “zone” between T\(_{\text{eff}}\) cells and target islet cells, or on the target islet cells with T\(_{\text{eff}}\) cells in the vicinity but not in direct contact (Figure 2-2; Video 1).
Figure 2-2. Visualization of the interaction between CD4+ Teff cells and their target cells within the anterior chamber of the eye. 3-D rendering of in vivo fluorescence micrographs for apoptotic signal annexin-V in areas of CD4+ BDC2.5 Teff cells engaging in direct contact with β-cell targets. The results represent two experiments with a total of 4 mice analyzed with snapshot imaging of 1-3 islets per animal. Scale bar = 100μm. Also see Video 1.
We also examined the involvement of myeloid cells at the inflammatory site, by *in situ* immunocytolabeling [44] with anti-CD11b antibodies. We could not detect CD11b+ cells in most of the areas wherein T<sub>eff</sub> cells interacted with target β cells. A low frequency of CD11b+ cells were found but usually in the periphery of damaged grafts. Importantly, T<sub>reg</sub> cells co-localized in the protected “clusters” of β cells that persisted amidst areas of immune damage (*Figure 2-3: A-C*). Overall, the majority of annexin-V signals associated with β cells rather than T<sub>eff</sub> cells and the amount of the apoptotic signals on β cells positively correlated with the number of T<sub>eff</sub> cells at the inflammatory site (*Figure 2-3: D,E*). The exact molecular cause of the immunopathology by the CD4 T<sub>eff</sub> cells remains unclear. IFN-γ and IL-17 could be detected by flow cytometry in substantial proportions of the CD4 T<sub>eff</sub> cells in the draining cervical lymph nodes of the eyes (14 ± 2 and 6 ± 1, respectively, mean ± SEM, n = 8 mice). However, further studies are needed to determine whether these or other cytokines have a pathogenic role. The imaging data suggest that, although not exclusive, direct-contact may be involved in CD4<sup>+</sup> T<sub>eff</sub> cells killing of target cells, even in the absence of CD8<sup>+</sup> T<sub>eff</sub> cells. The contact-dependent mode is a hallmark of cytotoxicity by CD8<sup>+</sup> T<sub>eff</sub> cells.
Figure 2-3. Direct contact between CD4⁺ T-eff cells, target tissue cells and T-reg cells, with or without close proximity to myeloid cells. Fluorescence confocal micrographs (z-stacks) acquired within intraocular islet grafts in NOD.SCID recipients *in vivo*. The mice were reconstituted with CFP-labeled CD4⁺ BDC2.5 T-eff cells (blue) and GFP-labeled T-reg cells (green), and later injected directly into ACE with BV605-conjugated anti-CD11b antibodies to label a broad subset of myeloid cells (yellow) and APC-conjugated annexin-V (red) to detect apoptotic cells *in situ*. The target β cells were visualized by laser backscatter (grey). (A-B) The annexin-V apoptotic signals were detected in areas of CD4⁺ BDC2.5 T-eff cells engaging target β cells, in the absence (A) or in the presence (B) of CD11b⁺ myeloid cells. (C) T-reg cells co-localized in the protected “cluster” of β cells, which persisted amidst rejected areas as long as T-reg cells existed. (d) The annexin-V signals on either β cells or T cells were measured as overlap between annexin-V stain volume and that of either β or T cells, normalized to total volume (β and T cells) (mean±SEM). (E) Linear regression analysis correlating β cell apoptosis (annexin-V signal) with the number of intra-graft T cells (n = 10 islet grafts in 4 mice). Scale bars = 50μm. See also **Video 1**.
**Green:** CD4⁺ Treg cells; **Blue:** CD4⁺ T eff cells; **Red:** annexin V; **Yellow:** CD11b; **Grey:** Islet

**d**

Annexin-V signal (ratio to total volume) vs. 
Data: on β cells, on T eff cells

**e**

T eff cell Count vs. Annexin-V signal on β cells (μm²)

R² = 0.7965
2.3.3 Direct Engagement Between CD8\(^+\) \(T_{\text{eff}}\) Cells and Target Cells: Bystander \(\beta\) cells were not Subject to Killing but Grew at the Interface of Immune Damage

We next studied noninvasively CD8\(^+\) \(T_{\text{eff}}\) cell activity in target tissue, by using CD8\(^+\) OT1 transgenic T cells [106], which are specific toward a model antigen, ovalbumin. Donor islets from both the RIP-mOVA transgenic mice [107] which express ovalbumin in \(\beta\) cells and the “MIP-CFP” mice lacking ovalbumin but have CFP-labeled \(\beta\) cells [108] were grafted together in ACE of C57BL/6 (B6) animals. We selected transplanted animals that carried at least one pair of “conjoined” mOVA\(^+\)CFP\(^-\) and mOVA\(^-\)CFP\(^+\) grafts like “Siamese twins”, and transferred them with antigen-activated CD8\(^+\)GFP\(^+\) OT1 \(T_{\text{eff}}\) cells. This was done to examine antigen-specific killing versus “bystander” tissue destruction [116]. The CD8\(^+\) OT1 \(T_{\text{eff}}\) cells selectively destroyed \(\beta\) cells carrying the specific antigen (Figure 2-4: A-C).

Strikingly, the bystander mOVA\(^-\)CFP\(^+\) islets “conjoined” to damaged mOVA\(^+\)CFP\(^-\) islet grafts grew at the interface of immune destruction within days, rather than being subjected to killing (Fig 2-4: A). Increase of the islet mass lacking the specific antigen required close juxtaposition with the site of antigen-specific immune responses. mOVA\(^-\)CFP\(^+\) graft mass remained constant if it was not immediately adjacent (i.e., isolated) to a graft harboring the specific antigen (Figure 2-4: A-C). Importantly, the increased size (i.e., mass) of conjoined mOVA\(^+\)CFP\(^+\) grafts was not due to hypertrophy of the \(\beta\) cells, as the nuclear density of the conjoined and isolated islets was unchanged. (Figure 2-5). Moreover, imaging analysis showed preservation of the 3-dimensional (3-D)
structure of these islets, precluding the possibility of imaging artifacts associated with islet “flattening” over time. Since the CFP expression in these islets is driven by the insulin promoter \[108\] and therefore labels differentiated $\beta$ cells, this direct observation suggests that $\beta$ cells can regenerate by replication at the site of immune damage, with an extraordinary potential of doubling in days.
Figure 2-4. CD8+ $T_{\text{eff}}$ cells damaged antigen-specific target cells but not bystander cells. (A) Longitudinal fluorescence confocal micrographs (z-stacks shown as maximum projection) of islet grafts in ACE, acquired noninvasively in vivo. Visualized were infiltration and damage by ovalbumin (OVA)-specific GFP-labeled CD8+OT1 $T_{\text{eff}}$ cells in OVA+CFP- islet grafts (grey) versus bystander OVA-CFP+ islets (blue). Data represent a total of 6 pairs of antigen-specific OVA+CFP- and non-specific OVA-CFP+ islets conjoined like “Siamese twins” (left panels), total 6 separated OVA-CFP+ islets (right panels) in the same ACE environment but not conjoined with OVA+CFP- grafts, and total 6 separated OVA+CFP- islet grafts, from 5 different mice in three experiments. (B) Changes in relative islet mass (volume) (mean±SEM) of OT1 antigen specific islets (OVA+) compared to bystander CFP islets in non-adjacent positions, after CD8+ OT1 $T_{\text{eff}}$ cells transfer. (C) Changes in relative mass (volume) (mean±SEM) of bystander islet grafts conjoined with the specific islet graft or separated from the specific islet graft in the same ACE. Scale bar = 100 m. **, p<0.01; ***, p<0.001.
(a) Conjoined and Isolated

Day 3
- Iris
  - OVA* islet
  - OVA islet

Post-adoptive transfer
Day 5

Day 10

Grey islet: OVA*CFP, Blue islet: OVA*CFP*, Green: CD8+ OT1 T cells

(b) Islet mass (% of original)

Days post T-cell transfer

(c) Islet mass (% of original)

Days post T-cell transfer
**Figure 2-5. Digital estimation of nuclear density in islet grafts over time in the living animal.** (a) Our noninvasive imaging platform allows monitoring of the same individual islet grafts in the same live animals over time, with cellular resolution, a technical advantage not afforded by other imaging approaches. To address whether the increased volume (i.e., islet mass) of “conjoined” OVA islet grafts was due to increased cell size (hypertrophy) or replication of cells, we used a digital strategy to quantify nuclear density. We used laser backscatter (reflection) to identify cell nuclei as illustrated in the shown scheme: *step 1*: CFP$^+$ islet cells are identified based on CFP fluorescence (blue) to select a 3-D region of interest (ROI) representative of the OVA islet; *step 2*: the image is cropped to the ROI to restrict the analysis to the islet; *step 3*: the reflection signal is revealed by removing the blue (CFP) fluorescence signal; *step 4*: using a random systematic sampling approach, we analyzed nuclear density (nuclei/surface area) in single confocal z slices (“digital” sections) at 15, 30, and 45 μm deep within the islet grafts. Nuclei are identified as “dark” (less-reflective) spots within islet cells (see inset in *step 3*). (b) Quantitative analysis of nuclear density in conjoined versus isolated islet grafts (OVA) measured in images acquired between day 3 and day 10 (also see Figure 2-4) (mean±SEM). The nuclear density of the conjoined islets was similar to that in isolated islet, indicating that the increased volume of the conjoined islets was not due to cellular hypertrophy. Data obtained in 4 islets per mouse from 3 mice in each group. “ns” is not significant.
2.3.4 Proliferation of Bystander β Bells in Islet Grafts Under the Kidney Capsule

To examine β-cell replication under inflammatory conditions in a site other than ACE, we performed our experiments using a conventional islet transplantation model. Recapitulating the settings in ACE using islets with or without specific antigens for CD8⁺ OT1 T_eff cells, B6 recipient mice were transplanted under the kidney capsule with islets from either wildtype B6 or RIP-mOVA⁺ transgenic donors, or a mixture of the two. The kidney subscapular space, although does not allow noninvasive longitudinal analyses of the same graft tissue at a cellular level, enabled the retrieval of a relatively large number of islet grafts for histological analyses.

After engraftment of islets transplanted under the kidney capsule, the recipient animals were injected with activated CD8⁺ OT1 T_eff cells, as done with the animals bearing ACE grafts (Figure 2-4). We then administered 5-bromo-2'-deoxyuridine (BrdU) to the animals to label proliferating cells. The CD8 T_eff cells destroyed the RIP-mOVA⁺ islet grafts. This was confirmed by microscopic examination and insulin-staining of kidney tissue sections at the site of the islet engraftment in animals transplanted with RIP-mOVA⁺ islet alone (not shown). Using tissue-sections of the islet grafts from the mice receiving B6 islets alone or a mixture of B6 and RIP-mOVA⁺ islets, we conducted immunofluorescence staining to detect BrdU incorporation in the nuclei of proliferating β cells. We found that in the group with the mixed engraftment (RIP-mOVA⁺ and B6 islets) and subsequent destruction of RIP-mOVA⁺ islets by OT1 CD8 T_eff cells, there
was a substantial increase in the proportion of BrdU+ β cells in the remaining B6 islets, compared to the group that was transplanted with B6 islets alone (Figure 2-6). These results, obtained with a platform distinct from the ACE model, corroborated the notion that β cells lacking specific antigens are not subject to “by-stander” killing or damage, but replicate in an inflammatory setting, which is consistent with the observations from the imaging analyses of islet grafts in ACE.
Figure 2-6. Analyses of cellular proliferation by BrdU incorporation. (A) B6 islets, OVA⁺ islets, or a mixture of B6 and Ova+ islets were transplanted under the kidney capsules of B6 mice. (B) After one month post transplantation, 2 x 10⁶ activated OT1 cells were injected i.v. into the mice. BrdU was administered by i.p injection beginning from day 2 after OT1 T cell transfer. The animals were sacrificed on day 10 and kidney tissue sections were prepared to analyze islet grafts. (C) Tissue sections of islet graft and surrounding kidney tissues were analyzed by immunostaining with anti-insulin and anti-BrdU antibodies and counterstained for nuclei with DAPI. The Ova⁺ islet graft group had a complete destruction of islet grafts and therefore not presented. Tissue sections of the B6 islet alone group (D) or B6 and OVA⁺ islets mixed engraftment group (E) were compared for insulin⁺ cells with BrdU signals. A serial section of the latter group was also used to show the secondary staining only (F). Inset in (E) is the zoomed-in image of the highlighted area. The BrdU⁺ Insulin⁻ cells were likely the infiltrating lymphocytes that destroyed the Ova⁺ islets in the mixed engraftment group. (G) Data were pooled from two experiments with 4 mice, with 36 and 34 islets analyzed in the B6 islet alone engraftment and the mixed engraftment groups, respectively. Each data point represents one islet graft (mean±SEM). **, p < 0.01. Scale bar = 50μm.
a. Islet Tx
kidney capsule

b. OT1 CD0 Teff, BrdU

c. Histology:

d. B6 islet Tx

Yellow: insulin; Blue: DAPI; Red: BrdU⁺; Pink: DAPI⁺BrdU⁺
2.3.5 T\textsubscript{reg} Cells Interacted with T\textsubscript{eff} Cells Through Direct Cell-Cell Contact in Nonlymphoid Target Tissue

Immune effector function at the target tissue is controlled by various mechanisms coordinated by T\textsubscript{reg} cells [117]. How exactly T\textsubscript{reg} cells suppress immune response in vivo remains a debate. Initial studies with in vitro transwell culture systems showed that T\textsubscript{reg} cell suppression was effective only if T\textsubscript{reg} cells were placed in the same culture chamber with T\textsubscript{eff} cells and antigen-presenting cells [118, 119]. While subsequent studies showed that T\textsubscript{reg} cells could inhibit T\textsubscript{eff} cell activation by modulating antigen-presenting cells [54, 120, 121], a number of in vitro studies also demonstrated that both human and murine T\textsubscript{reg} cells could directly suppress T\textsubscript{eff} cells independent of antigen-presenting cells [17-22]. However, ex vivo and in vivo imaging studies in lymph nodes did not detect stable contact between T\textsubscript{reg} and T\textsubscript{eff} cells [32, 33]. This contradiction between in vitro and in vivo studies has left a doubt about the in vivo relevance of contact-dependent T\textsubscript{reg} suppression. The in vitro “trademark” activity of T\textsubscript{reg} cells remains to be reconciled in vivo.

Here, we examined the pathophysiological relevance of T\textsubscript{reg}-T\textsubscript{eff} contact at the effector phase in the target tissue in vivo. We used the NOD.SCID reconstitution model with antigen-specific T\textsubscript{reg} and T\textsubscript{eff} cells that are genetically tagged with different fluorescence markers for stable labeling and longitudinal study. Adoptive transfer of T cells to lymphopenic animals is followed by homeostatic proliferation and activation of the transferred T cells to fill “empty” niches in the lymphoid organs [122], which could complicate studies of T cell
activation and differentiation. However, lymphopenia-associated activation is likely to impact minimally on our study as we focused on T cell biology at the final effector phase in the nonlymphoid target tissue, i.e., during the effector phase post activation and differentiation. The lymphopenic reconstitution model is also necessary to avoid undercounting invisible interactions (see Methods) and to generate meaningful measurement of the interactive behavior among T cell subsets.

Indeed, in the T\textsubscript{reg}-cell-protected grafts, a majority of T\textsubscript{eff} cells were in direct contact with T\textsubscript{reg} cells (Figure 2-7: A); they displayed a dynamic and contact-featured choreography. The interaction between the T\textsubscript{reg} and T\textsubscript{eff} cells usually persisted for the entire length of the imaging sessions (≥ 30min) and was characterized by reduced motility (Figure 2-7: A-E and Video 2). This direct contact between T\textsubscript{reg} and T\textsubscript{eff} cells was not due to mere crowdedness; in tissue areas that were only sparsely infiltrated, long-lasting contact between T\textsubscript{reg} and T\textsubscript{eff} cells was still evident (Figure 2-8).
Figure 2-7. Noninvasive intravital visualization of T$_{\text{reg}}$-T$_{\text{eff}}$ cell contact in target tissue. (A) Snapshots from representative time-lapse recordings demonstrating stable, long-lasting interaction between CD4$^{+}$ T$_{\text{reg}}$-T$_{\text{eff}}$ interactions within the islet tissue. The majority of interactions lasted for the entire time of the recordings (≥30 min) (see Video 2). (B) Absolute and (C) relative interaction time between the CD4$^{+}$BDC2.5 T$_{\text{eff}}$ and T$_{\text{reg}}$ cells (n=50 cell pairs, mean±SEM). Relative interaction time is calculated by dividing absolute interaction time with total imaging session length. Of note, many of the interactions were already established at the beginning of imaging; hence, actual interaction times are likely longer than those measured during the in vivo imaging timeframe which is limited by the animals' tolerance of general anesthesia. Data points represent one T$_{\text{reg}}$-T$_{\text{eff}}$ pair and lines represent the mean±SEM. (D) The interaction index and (E) velocity of both T$_{\text{eff}}$ and T$_{\text{reg}}$ cells in a mode of contact interaction or not (n=55-100 cells, mean±SEM). Data represent experiments with 6 mice. Scale bar = 30µm.
Green: CD4+ Treg cells; Blue: CD4+ T eff cells; Grey: Islet graft
Figure 2-8. Long-lasting contact between $T_{\text{reg}}$-$T_{\text{eff}}$ in the target tissue – not due to mere crowdedness. To examine whether stable long-lasting $T_{\text{reg}}$-$T_{\text{eff}}$ interactions are influenced by spatial constraints and T-cell crowding within target tissue, we performed *in vivo* imaging studies in intraocular islet grafts with sparse T cell infiltration. Shown are snapshots (z-stacks shown as maximum projection) of a time-lapse recording in ACE islet graft of NOD.SCID recipient mouse which was reconstituted with CFP-labeled CD4$^+$ BDC2.5 $T_{\text{eff}}$ cells (blue) and GFP-labeled $T_{\text{reg}}$ cells (green). Highlighted in circles are $T_{\text{reg}}$-$T_{\text{eff}}$ cell pairs which maintained stable interaction for the entire imaging time. Data represent imaging sessions (≥ 60 min) in 4 mice acquired especially for this purpose. In these and all other experiments (typically lasing ≥ 30 min), the majority of $T_{\text{reg}}$-$T_{\text{eff}}$ interactions were consistently observed for the entirety of the imaging sessions.
2.3.6 Contact Interaction Between T_{reg} and T_{eff} Cells with or without CD11c^{+} Dendritic Cells

T_{reg} cells can dampen the expression of co-stimulatory molecules CD80 and CD86 on the surface of dendritic cells (DCs) and thus inhibit T-cell activation \[54, \ 123\]. Whether the function of T_{reg} cells in the target tissue depends on DCs during the effector phase remains unclear. To examine T_{reg}-T_{eff} interaction in a context of DCs in protected target tissues, we injected fluorescence-conjugated anti-CD11c antibodies to visualize DCs in addition to GFP- and CFP-labeled T_{reg} and T_{eff} cells, respectively (Figure 2-9: A-B). CD11c^{+} DCs could be detected in the islet graft, mostly at the periphery (Figure 2-9: B). Consequently, the majority of T_{reg}-T_{eff} cell interactions within the graft occurred in the absence of DCs, and it was also the most abundant among the various types of interactions of T_{reg}, T_{eff} and/or CD11c^{+} DCs. Clusters of the three types of cells, T_{reg}-T_{eff}-DC, could be detected, but at a much lower frequency than that of T_{reg}-T_{eff} cell interaction without DCs. T_{eff}-DC or T_{reg}-DC interactions could be found at minor frequencies (Figure 2-9: C). The interactions between T_{reg}-T_{eff} cells were also stable, with or without CD11c^{+} DCs (Figure 2-9: D-E). Of note, the interactions between CD11c^{+} cells and T_{eff} or T_{reg} cells, although occurring to only a minor proportion of the T cells, were also mainly long-lasting, with T_{reg}-CD11c^{+} cells interactions somewhat less stable (Figure 2-9: D-E). Overall, these results shows that direct-contact-based interactions between T_{reg} and T_{eff} cells persisted with or without CD11c^{+} DCs, which could reflect distinct subsets of T cells or distinct stages of the T cell function in the target tissues.
Figure 2-9. Stable T\textsubscript{reg}-T\textsubscript{eff} cell interaction with or without CD11c\textsuperscript{+} dendritic cells. (A and B) Alexa-647-labeled-anti-CD11c antibodies or isotype controls were injected into ACE to visualize DCs at the site of T\textsubscript{reg}-T\textsubscript{eff} cell interaction in islet grafts. (C) The total count for each type of the interactions was calculated from the images in this experiment (mean±SEM). (D) The interaction times of the different subsets in time-lapse recordings. (E) The relative interaction time is calculated by dividing absolute interaction time with total imaging session length. The data was pooled from 6 videos from experiments with 5 mice. *, p<0.05; ***, p<0.001. Scale bar = 100µm.
2.3.7 T<sub>reg</sub> Cells Persistently Interacted with T<sub>eff</sub> Cells Even When Outnumbered by T<sub>eff</sub> Cells in Damaged Target Tissues

Next, we examined the behavior of T<sub>reg</sub> cells in a setting of failed immune regulation. We found that most T<sub>reg</sub> cells at the site of extensive tissue damage were still persistently interacting with T<sub>eff</sub> cells, with durations (interaction time) comparable to those in the protected tissues (Figure 2-10: A-B). However, T<sub>reg</sub> cells were largely outnumbered by T<sub>eff</sub> cells; as a result, most T<sub>eff</sub> cells were without T<sub>reg</sub> cell interactions (Figure 2-10: C-D). Thus, regardless of success or failure in protecting the target tissue, T<sub>reg</sub> cells persistently interacted with T<sub>eff</sub> cells, but an imbalance in the numbers of T<sub>reg</sub> versus T<sub>eff</sub> cells characterized the outcome, that is, immune damage versus protection.

To further examine the behavior of T<sub>reg</sub> cells in a setting of severe imbalance with T<sub>eff</sub> cells, we examined the impact of acute removal of T<sub>reg</sub> cells after establishment of target tissue protection. This approach also tested whether persistent contact with T<sub>reg</sub> cells might impinge a lasting change of migratory behavior of T<sub>eff</sub> cells. We utilized a Foxp<sup>3<sub>DTR</sub></sup> transgenic model, which carries a diphtheria toxin (DT) receptor transgene under the control of Foxp3 promoter, enabling acute depletion of 80-90% of T<sub>reg</sub> cells with a low dose of DT [105]. In ACE islet grafts, the T<sub>eff</sub>-cell-mediated islet damage was suppressed by using either Foxp<sup>3<sub>DTR+</sub></sup> T<sub>reg</sub> cells or Foxp<sup>3<sub>DTR-</sub></sup> T<sub>reg</sub> controls. After stable protection of the islet grafts by the T<sub>reg</sub> cells was established, the animals were treated with DT. This treatment led to an acute removal of the T<sub>reg</sub> cells and a precipitous reduction in the T<sub>reg</sub>/T<sub>eff</sub> cell ratio in the tissue (Figure 2-11: A). The efficacy of
GFP<sup>+</sup> cell removal in the islet graft indicated that the adoptively transferred GFP<sup>+</sup> cells maintained Foxp3 expression even at the effector phase in the target tissues. Depletion of T<sub>reg</sub> cells led to extensive tissue damage (Figure 2-11: B), and increased motility of T<sub>eff</sub> cells (Figure 2-11: C-E). While the residual T<sub>reg</sub> cells remained in stable contact with T<sub>eff</sub> cells, the T<sub>reg</sub>/T<sub>eff</sub> cell disproportion caused by the T<sub>reg</sub>-cell depletion treatment resulted in most of the T<sub>eff</sub> cells in the target tissue no longer having T<sub>reg</sub>-cell partners. Thus, intimate T<sub>reg</sub>-T<sub>eff</sub> interaction did not irreversibly alter the aggressiveness of the T<sub>eff</sub> cells.
Figure 2-10. **T\textsubscript{reg} cells persistently interacted with T\textsubscript{eff} cells in damaged tissue despite an imbalance in T\textsubscript{reg} versus T\textsubscript{eff} numbers.** CD4\textsuperscript{+} T cell behavior was analyzed in the graft tissue that was being destroyed due to failed immune regulation, or protected by successful T\textsubscript{reg}-cell regulation. Protected islet grafts had at least 80% of original islet mass, whereas rejected islet graft had 20% or less. (A) Representative fluorescence micrographs (3D rendering) showing protected (left) and damaged (right) islet grafts (grey; visualized by laser backscatter) in NOD.SCID mice reconstituted with GFP-labeled CD4\textsuperscript{+} BDC2.5 T\textsubscript{reg} cells and CFP-labeled CD4\textsuperscript{+} BDC2.5 T\textsubscript{eff} cells. (B) T\textsubscript{reg}-T\textsubscript{eff} interaction time in protected vs. damaged grafts (mean±SEM). (C) Ratios of T\textsubscript{reg}-T\textsubscript{eff} cell pairs within protected vs. damaged grafts. The T\textsubscript{reg}-T\textsubscript{eff} cell pairs outside of the target graft tissue, in the iris in the same ACE, were analyzed as controls for the target tissue (mean±SEM). (D) Interaction index (ratio of T\textsubscript{eff} cells with or without T\textsubscript{reg} interactions during the entire imaging sessions) in protected vs. damaged grafts (mean±SEM). Results represent 3 experiments in a total of 5-6 mice in each group (protected vs. damaged). Scale bar = 50\textmu m.
Figure 2-11. Acute removal of T_{reg} cells after T_{reg}-T_{eff} interaction establishment altered T_{eff} cell motility but residual T_{reg} cells maintained contact with T_{eff} cells. NOD.SCID mice with Islet grafts established at the ACE site were injected with CD4^{+} BDC2.5 T_{eff} cells, together with CD4^{+} BDC2.5 T_{reg} cells (blue) with or without the Diphtheria Toxin Receptor (green) at a ratio of 1:1. After establishment of protection, all mice were injected with Diphtheria Toxin (50ng/g body tissue). (A-B) The effect of acute T_{reg}-cell depletion (with an efficacy of ~80%-90%) on T_{reg}/T_{eff} ratio (A) and tissue damage (B) after acute T_{reg} depletion (mean±SEM). (C) The effect of T_{reg} cell removal on T_{eff}-cell motility (physical displacement, flower plot of individual cell tracks), to test if T_{eff} cells exhibit altered motility after disengagement from T_{reg} cells. (D) T_{eff} cell displacement rate (µm/min) (mean±SEM). (E) The average T_{reg}-T_{eff} interaction time after acute removal of the majority of the T_{reg} cells by diphtheria toxin treatment (mean±SEM). Data represent 4 mice per group from two experiments. It should be noted that the intravital imaging platform enabled us to perform noninvasive imaging at the same tissue spot in the same animal longitudinally, so the pre-treatment measurements (day 0) also serve as “internal” controls for post-treatment measurements within each group.
2.3.8 A Role of CTLA4 in T_{reg}\cdot T_{eff} Cell Interaction Likely Through Motility Regulation

The function of T_{reg} cells depends on CTLA4 [54], which also regulate T_{eff} cell function [23]. We tested here the role of CTLA4 in maintaining T_{reg}\cdot T_{eff} cell interaction by administering anti-CTLA4-antibody blockade after stable T_{reg}\cdot T_{eff} cell interactions and T_{reg}-cell protection were established. The anti-CTLA4 treatment under this condition did not cause diabetes (data not shown). It increased T_{eff} cell numbers in the target tissue, more so than in T_{reg} cell numbers (Figure 2-12: A-B), and resulted in decreased T_{reg}/T_{eff} ratios (Figure 2-12: C-D). The treatment did not immediately disrupt the interaction between the CD4^{+} T_{reg}\cdot T_{eff} pairs. However, the proportion of interacting T_{reg}\cdot T_{eff} pairs declined over time after CTLA4 blockade, and their interaction time was shortened (Figure 2-12: E-F). While CTLA4 blockade led to increased motility of CD4^{+} BDC2.5 T_{eff} and T_{reg} cells in T_{reg}-cell-protected grafts, it decreased the motility of CD8^{+} OT1 T_{eff} cells. Moreover, in a model wherein CTLA4 in CD8^{+} OT1 T_{eff} cells were modulated with RNAi [102, 103], CTLA4 reduction decreased motility of T_{eff} cells, suggesting an intrinsic effect of CTLA4 in T_{eff} cell motility (Figure 2-13; Videos 3a-b). Taken together, these results suggest that CTLA4 might influence T_{reg}\cdot T_{eff} interaction through motility regulation, but the exact effect depends on the nature of the immune settings and cell types.
Figure 2-12. The effect of CTLA4 modulation on $T_{reg}$-$T_{eff}$ cell interaction. NOD.SCID mice carrying islet grafts in ACE were injected with CD4$^+$ BDC2.5 $T_{eff}$ and $T_{reg}$ cell mixture. After $T_{reg}$-cell-protection in the tissue is established (~30 days post T cell transfer), mice were treated with either PBS control or anti-CTLA4 monoclonal antibody (20 $\mu$g/g body weight, n = 3 different mice in each group). Of note, the intravital imaging platform enable us to noninvasive imaging the same tissue spot in the same animal longitudinally, as in this experiments and throughout of the study. Therefore, the pre-treatment measurements also serve as “internal” controls for post-treatment measurements. (A) $T_{eff}$ cell numbers and (B) $T_{reg}$ cells numbers in the target tissues (mean±SEM). (C) Intra-islet-graft $T_{reg}/T_{eff}$ cell ratio over time after anti-CTLA4 antibody blockade (n=10-11 islet grafts per group per time points, mean±SEM). (D) Interaction index, calculated as the ratio of $T_{eff}$ cells with or without $T_{reg}$ cell interaction, after anti-CTLA4 or control treatment (mean±SEM). Arrow heads in a-d indicated injection of anti-CTLA4 antibodies. (E-F) The duration of $T_{reg}$-$T_{eff}$ cell interactions after CTLA4 blockade, in actual imaging time (E) and relative to the length of whole imaging session (F) (n=24-40 cell pairs, mean±SEM). There was significant difference among pre-treatment controls and post-treatment measurements within the anti-CTLA4 treatment group. *, p <0.05, one way ANOVA was performed with a Tukey’s multiple comparison’s post-hoc analysis, compared to both pre-treatment measurement of the same animal or control treated animals.
Figure 2-13. The effect of CTLA4 on T cell motility may depend on the type of T cells and their settings. (A) The velocity of CD8$^+$ OT1 T$_{eff}$ cells (n = 3 mice, 40 cells, mean ± SEM) in ACE islet grafts in B6 mice is compared to that of CD4$^+$ BDC2.5 T cells in ACE islet grafts in NOD.SCID mice. For CD4$^+$ BDC2.5 T cell analysis, the CD4$^+$ T$_{eff}$ cells (n = 5 mice, 110 cells, mean ± SEM) and CD4$^+$ BDC2.5 T$_{reg}$ cells (n = 5 mice, 101 cells, mean ± SEM) were from animals injected either with T$_{eff}$ and T$_{reg}$ cell mixture (the second and third groups), or CD4$^+$ BDC2.5 T$_{eff}$ cells only (fourth group, n = 3 mice and 107 cells). Each dot represents one cell and the lines in plots represent mean ± SEM). (B and C) Transient change of intra-graft CD4$^+$ BDC2.5 T-cell velocities after anti-CTLA4 treatment (n = 30-90 cells per group for each time point, mean ± SEM). (D and E) The effect of CTLA4 modulation on displacement (D) and velocity (E), respectively, on intra-graft CD8$^+$ OT1 T$_{eff}$ cells. Animals were treated with anti-CTLA4 antibodies (n = 3 mice), or PBS or hamster Ig control (n = 4 mice), or carried a CTLA4 RNAi transgene (n= 3 mice). Data represent two experiments and are from analyses of 40 cells (mean ± SEM) in each group and each dot in (E) represents one cell. Also see Videos 3a-b.
2.4 Discussion

Control of immune damage at the effector phase is a crucial and perhaps the most realistic therapeutic target in clinical intervention of immune-mediated diseases [124]. Improvement of therapeutic interventions will require in-depth understanding of the immune cell behavior in target tissues and of the reaction of target tissue cells in response to insult. The current study suggests that the contact-dependent mode of immune cell interaction in the target tissue is a critical part of pathophysiology at the effector phase of immune responses, and immune tolerance induction may be facilitated by promoting intimacy between pathogenic and protective immune cells. In this regard, it is highly relevant that tissue antigen-specificity, as opposed to bystander killing [116], shapes tissue fate in the effector phase.

With the tools currently available for longitudinal imaging of antigen-specific T cells in target tissues, we uncovered some basic behaviors of different lineages of T cells during the effector phase. CD8 T cells are well known for contact-dependent killing. CD4 T cells, on the other hand, are better known as various “helper” subsets, although increasing attention has been put on their potential “cytotoxicity” function. Although our current models do not allow us to compare the biology of CD4 and CD8 T cells in an ideally analogous setting, our studies provides in vivo evidence for contact-based killing of target cells. The observation adds to efforts to understand the behaviors of these two distinct lineages of T cells at various stages of their activation, differentiation and functioning [125]. Our results, however, do not exclude indirect mechanisms of
target killing by CD4 $T_{eff}$ cells, and development of new tools should enable further studies to investigate such mechanisms *in vivo*.

It is important to make the distinction between our findings of stable $T_{reg}$-$T_{eff}$ contact interaction in target tissue and those in previous reports on lack of direct $T_{reg}$-$T_{eff}$ contact in lymph nodes [32, 33]. $T_{reg}$ cells play a major role in peripheral immune tolerance, likely through a variety of mechanisms [117]. However, key features of $T_{reg}$ cell biology *in vivo* remain to be clarified, including whether $T_{reg}$ cells interact with $T_{eff}$ cells through direct contact. Two groups independently reported that $T_{reg}$ cells could not suppress *in vitro* proliferation responses of $T_{eff}$ cells if they were placed in a different chamber in a trans-well culture system [118, 119]. Although this could be attributed to an effect of $T_{reg}$ cells on antigen-presenting cells [54, 120, 121], robust evidence has also been presented for direct suppression $T_{eff}$ cells by $T_{reg}$ cells independent of antigen-presenting cells [17-22], such that contact-dependent suppression has been regarded as an *in vitro* “trademark” of $T_{reg}$ cell activity [126]. On the other hand, imaging analyses of explanted lymph nodes in an autoimmune diabetes animal model did not detect stable interaction between CD4$^+$ BDC2.5 $T_{eff}$ cells and $T_{reg}$ cells [33]. Absence of stable $T_{eff}$-$T_{reg}$ cell contact in draining lymph nodes was also reported independently by another group using a different model system [32].

By contrast, our *in vivo* studies focused on the effector phase in the nonlymphoid target tissue. We found that $T_{reg}$ cells persistently interacted with $T_{eff}$ cells through direct cell-cell contact. Importantly, The contact-based interactions
between T\textsubscript{reg} and T\textsubscript{eff} cells in the target tissues were observed both in the presence and in the absence CD11c\textsuperscript{+} DCs, although more often for the latter. In this regard, it should be noted that a previous study [33] showed that, in draining lymph nodes, although both T\textsubscript{eff} cells and T\textsubscript{reg} cells had stable interactions with DCs, such interactions did not lead to stable T\textsubscript{reg}-T\textsubscript{eff} contact. Therefore, a platform of antigen-presenting cells does not obligate direct T\textsubscript{reg}-T\textsubscript{eff} cell interaction. In addition, recent evidence also suggested that niche-filling homeostasis of T\textsubscript{reg} cells may occur independently of DCs [127]. Nevertheless, our observations do not necessarily underscore the role of DCs in initiation and progression of immune damage. Homeostasis of DCs was shown to play a critical role in autoimmune damage of pancreatic islets [128]. In BDC2.5 TCR-transgenic model and the standard NOD model of autoimmune diabetes, the role of DCs in initiating autoimmune diseases and their potential in tolerogenic therapies have been clearly demonstrated [38, 129, 130]. We believe that the visual evidence of direct T\textsubscript{reg}-T\textsubscript{eff} interaction in target tissue \textit{in vivo}, with or without the involvement of CD11c\textsuperscript{+} DCs, reconciles the discrepancy between \textit{in vitro} and \textit{in vivo} observations on a basic aspect of T\textsubscript{reg} cell biology.

The molecular basis of T\textsubscript{reg}-T\textsubscript{eff} cell direct engagement remains to be elucidated. A previous study [131] reported that CD80/CD86-deficient T\textsubscript{eff} cells were resistant to T\textsubscript{reg} cells suppression. We explored along this line but did not detect expression of CD80 or CD86 on T\textsubscript{reg} cells or T\textsubscript{eff} cells in the target tissues by cytolabling \textit{in situ} with specific antibodies, nor did we found disruptive effect on T\textsubscript{reg}-T\textsubscript{eff} cell interaction by injecting the anti-CD80 or anti-CD86 antibodies
directly into ACE (Miska, Abdulreda and Chen, unpublished data). However, our observation in this regard is preliminary in scope and limited to the target tissue, and thus does not invalidate the hypothesis that CTLA4 expressed by T\textsubscript{reg} cells may bind to CD80/CD86 on T\textsubscript{eff} cells to facilitate direct cellular interactions [131]. On the other hand, the data from our studies suggest that CTLA4 might affect T\textsubscript{reg}-T\textsubscript{eff} cell interaction through other mechanisms. A study with a conditional “knockout” of CTLA4 showed that T\textsubscript{reg} cells require CTLA4 for functioning \textit{in vivo} [54]. We showed here that CTLA4 played a role in regulating T\textsubscript{reg}-T\textsubscript{eff} cell interaction in target tissue. This role may be related to motility regulation of both T\textsubscript{reg} and T\textsubscript{eff} cells. Of note, the role of CTLA4 in T-cell motility control has been debated. Schneider \textit{et al} reported that CTLA4 enhanced the motility of T cells and thus reversed the “stop signal” originated from engaging the TCR [132]. This evidence suggested that manipulating CTLA4-based motility control could lead to therapeutic advance. Indeed, a recent study found that anti-CTLA4 antibody treatment inhibited CD8 T-cell motility and promoted antitumor immunity [133]. However, another study by Fife \textit{et al} showed that anti-CTLA4 treatment did not alter the motility of autoimmune CD4\textsuperscript{+} BDC2.5 T cells in draining lymph nodes [134]. Our imaging analyses of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells during the effector phase within target tissue indicate that the exact effect of CTLA4 on T-cell motility may vary in different T cell subsets and may be influenced by distinct circumstance of cellular interactions.

In our experiments, CTLA4 blockade caused only modest changes on cellular interaction. It did not substantially break tolerance under the conditions
we tested. The small effect on T<sub>reg</sub>-T<sub>eff</sub> cell interaction could be contributed by altered motility controls. It remains to be determined how CTLA4 blockade led to an increase of T<sub>eff</sub> cells, rather than T<sub>reg</sub> cells, in the target tissue. The resulting imbalance of T<sub>reg</sub>/T<sub>eff</sub> ratios, however, unlikely accounted for the changes in durations of T<sub>reg</sub>-T<sub>eff</sub> cell interactions, since reduced T<sub>reg</sub>/T<sub>eff</sub> ratios did not lead to reduced T<sub>reg</sub>-T<sub>eff</sub> interactions in the other settings of our studies. These results, while suggesting novel facets of CTLA4 function beyond the scope of this study, may on the other hand lend reconciliation to the debate on whether CTLA4 controls T cell motility [132, 134]. Importantly, in addition to its impact on the TCR stop signals, dysregulation of CTLA4-based motility may lead to disruption of the T<sub>reg</sub>-T<sub>eff</sub> cell interaction in target tissues, which may in turn lead to exacerbated tissue damage.

Our longitudinal and noninvasive observation of live tissue in animals documented growth of healthy tissue that avoided immune cell recognition. For most target tissue cells, unlike immune cells, motility is not typically in their nature. However, they may not be a mere “sitting duck” in a setting of immune destruction. Rather, they may be capable of a resilient response at the inflammatory front, as illustrated by the dramatic growth of the β cells in bystander islets that were not recognized by antigen-specific T<sub>eff</sub> cells. The insulin-producing β cells can regenerate through various mechanisms, which is a topic of debate. Notably, an association between microenvironment inflammation and increased β-cell proliferation was recognized during insulitis and pancreatitis [112, 135, 136]. A mode of β-cell regeneration through replication was
demonstrated in animal models, but it was evident only after months of follow-up [137]. A recent study [138] showed that fast replication of pancreatic β cells could be induced by treatment with an antagonist compound of insulin receptors which stimulates production of betatrophin. The intravital evidence from our study is consistent with the notion that β cells can regenerate in vivo by replication from differentiated cells, yet with a surprising rate of doubling in mass within days. The fast regeneration could be contributed to by inflammatory signals released at the interface of immune damage. Future studies are needed to uncover the molecular signals and contextual cues that led to this surprising potential of β-cell growth under antigenic incognito. These studies may ultimately aid tissue regenerative therapies in type 1 diabetes and other disorders caused by immune damage.
CHAPTER 3: Autoimmunity-Mediated Antitumor Immunity: Tumor as an Immunoprivileged Self

3.1 Summary

The association of autoimmunity with antitumor immunity challenges a paradigm of selective surveillance against tumors. Aided with well-characterized models of robust autoimmunity, we show that self-antigen-specific effector T (Teff) cell clones could eradicate tumor cells. However, a tumor microenvironment reinforced by Treg cells and myeloid-derived suppressor cells (MDSCs) presented a barrier to the autoimmune effectors, more so in tumors than in healthy tissues. This barrier required optimal CTLA4 expression in Teff cells. In a spontaneous model of breast cancer, subtle reductions in CTLA4 expression impeded tumor onset and progression, providing the first direct evidence that CTLA4 inhibits spontaneous tumor development. In an adoptive therapy model of lymphoma, self-antigen-specific Teff cells were potentiated by even a modest reduction of CTLA4. A subtle reduction of CTLA4 did not curtail Treg-cell suppression. Thus, Teff cells had an exquisite sensitivity to physiological levels of CTLA4 variations. However, both Treg and Teff cells were impacted by anti-CTLA4 antibody blockade. Therefore, whether CTLA4 impacts through Treg cells or Teff cells depends on its expression level. Overall, the results suggest that the tumor microenvironment represents an “immunoprivileged self” that could be overcome practically and at least partially by RNAi silencing of CTLA4 in Teff cells.
3.2 Materials and Methods

3.2.1 Mouse Models

Transgenic and knockout mouse models constructed for autoimmunity studies were transitioned to study autoimmune mechanisms in antitumor immunity. A detailed description of the use of these models in the current study is provided in a supplementary table (Supporting information Table 1). BDC2.5/NOD, Foxp3-deficient C57BL/6 (B6) and NOD, NOD.Foxp3<sup>DTR</sup>, Rag-deficient-BDC2.5/NOD, and CTLA4 shRNA (CTLA4KD7) and PL4 transgenic mice were described previously [102, 104, 105, 139-141]. CTLA4KD7 and PL4 mice were backcrossed onto B6 background for >10 generations, and then crossed with BALB-neuT [142], FIR (Foxp3-IRIS-RFP “knockin”) mice [110] or OT1 transgenic line [106]. All animals were maintained in a specific-pathogen-free barrier facility and the studies are approved by the Institutional Animal Care and Use Committee at the University of Miami.

3.2.2 Tumor Cell Implantation, Tumor Growth Monitoring and Histological Analysis of Tumor and Healthy Tissue

The NIT-1 insulinoma, EL4 lymphoma and E.G7-OVA lymphoma cell lines were obtained from ATCC (Manassas, VA) and implanted subcutaneously at 5x10<sup>6</sup>/mouse for insulinoma and 5x10<sup>5</sup> for lymphoma. For the NIT-1 model, tumor burden was quantified by measuring blood glucose levels and tumor mass. The tumor and pancreas samples were fixed in formalin solution. Paraffin embedded sections were stained with hematoxylin and eosin (H-E) and examined by microscopy. Scoring for pancreas pathology was determined as
follows: 0, intact islet with no lymphocytes in the islet area; 1, lymphocytes within
the vicinity of the islet, but no infiltration; 2, peripheral insulitic lesion; 3, near or
complete destruction of the islet.

3.2.3 Flow Cytometry, Cell Sorting and Adoptive Transfer of T cells

Flow cytometry analyses were conducted with a standard procedure [140]. The cells were stained with fluorescent-antibody conjugates to determine cells phenotype. The following antibody conjugates were used: PE-Cy7 conjugated
anti-CD44, anti-CD8, anti-Cd11b, anti-CD11c, anti-CD69, anti-Ter119, anti-B220; APC conjugated anti-CD62L; Alexa-Flour 405 conjugated anti-CD4; PerCP-
Cy5.5 conjugated anti-CD25 (eBioscience, San Diego, CA). Mice that either
received or were part of any PL4 or KD 7 line had intrinsic GFP expression. For
experiments involving T$_{reg}$ transfer, all donor lines have a Foxp3$^{FIR}$ knockin that
expresses RFP in only Foxp3 producing cells. Samples were analyzed with flow
cytometers (LSR-II and Fortessa, Becton Dickinson, San Jose, CA). Naïve T$_{reg}$
cells (CD4$^{+}$CD62L$^{+}$CD25$^{+}$Foxp3$^{FIR^{+}}$CD69$^{+}$CD11b$^{-}$CD11c$^{-}$CD49b$^{-}$Ter119$^{-}$B220$^{-}$) and T$_{eff}$ cells (CD4$^{+}$CD62L$^{+}$CD25$^{+}$Foxp3$^{FIR}$-CD69$^{+}$CD11b$^{+}$CD11c$^{+}$CD49b$^{+}$Ter119$^{+}$
B220$^{+}$) were sorted (purity >95%) and transferred into recipient mice. OT1 T cells
were stimulated in vitro with specific ovalbumin peptides (SIINFEKL) and purified
by magnetic-bead sorting of CD8$^{+}$ cells.

3.2.4 Blood Glucose Measurements

Blood glucose was used as measure to assess both pancreatic β-cell
destruction as well as insulinoma growth. In cases of rejection of both insulinoma
and β-cell mass, blood glucose reading will increase as a reflection of destruction.
When the insulinoma grows, it produces insulin constitutively which causes hypoglycemia. Therefore tumor growth will be inversely correlated with blood glucose levels in the NIT-1 model.

3.2.5 Statistics

Log-rank (Mantel-Cox) test was used for cumulative cancer incidence. Student’s t tests were used for single comparisons. One-way ANOVA was used for multiple comparisons followed by Tukey’s post-hoc test. Longitudinal data from multiple groups were analyzed with two-way ANOVA followed with Bonferroni’s multiple sample post-hoc test. P ≤ 0.05 were considered significant. * P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.

3.3 Results

3.3.1 Efficacy of Self-Antigen-Specific T eff Cells in Tumor Destruction.

To address the role of self-antigen-specific T cell clones in antitumor immunity, we did initial experiments with a well-characterized model of T-cell-mediated autoimmunity, the BDC2.5 T-cell receptor (TCR) transgenic mouse [104]. The BDC2.5 TCR transgenic line expresses the TCR of a CD4+ T-cell clone that recognizes a physiological antigen, chromogranin A [143], in the pancreatic β cells. Chromogranin A has also been reported as a tumor-associated antigen [144]. We used the NIT-1 insulinoma model. The NIT-1 cells are a mouse tumor cell line derived from a spontaneously developed pancreatic β-cell adenoma (insulinoma) in the NOD mice that carried a hybrid rat insulin-promoter/SV40 large T-antigen transgene [145]. When implanted into mice, these cells can establish fatal insulinoma in the animals [146].
NOD.SCID mice were rendered diabetic by chemical destruction of endogenous β cells with streptozotocin, and then implanted with NIT-1 insulinoma cells, which secrete insulin and reduce blood glucose levels. To test if an autoimmune T cell clone is sufficient to reject the tumor, the animals received CD4+ Teff cells from Rag-deficient BDC2.5 mice. The Rag deficiency precludes the generation of other T cell clones from the endogenous TCR locus, so the animals harbor a monoclonal of the self-antigen-specific BDC2.5 Teff cells. Alternatively, purified CD4+ naïve Teff cells from BDC2.5/NOD mice were used. We transferred 5-10 x10^4 BDC2.5 Teff cells into the animals at the time of tumor cell implantation (Figure 3-1: A) or 3-7 days after tumor cells injection (Figure 3-1: B). The implanted tumor cells established a palpable subcutaneous tumor and effectively reduced the blood glucose level of the tumor-bearing animals, which enables an objective assessment of tumor burdens regardless of the location of tumors. Adoptively transferred autoimmune Teff cells eradicated palpable insulinoma. Complete killing of insulinoma cells in the animals was reflected by the rise in blood glucose levels (Figure 3-1: A,B). To examine the efficacy of autoimmune Teff cells without having to adoptively transfer T cells, we implanted NIT-1 tumor cells into Foxp3-deficient BDC2.5 mice (the BDC2.5/NOD.Foxp3^sf congenic line) [140], in which autoimmune Teff cells are free of T reg cell suppression. In Foxp3-deficient BDC2.5 mice, the implanted NIT cells initially established an insulinoma but the tumor was effectively rejected, whereas fatal insulinoma developed in all control BDC2.5 mice that harbor natural T reg cells (Figure 3-1: C,D).
Figure 3-1. Eradication of insulinoma cells by T_{eff}-cell clones specific to a physiological self-antigen, chromogranin A. (A) NOD.SCID mice were rendered diabetic by chemical destruction of their endogenous β cells. The animals were injected with NIT-1 insulinoma cells subcutaneously and treated with PBS control (broken lines), or 5x10^4 CD4^+ T_{eff} cells (solid lines) from Rag-deficient BDC2.5/NOD transgenic mice. Animals were monitored for blood glucose levels, which over a range inversely reflected the burden of the insulin-secreting NIT-1 tumor. A high blood glucose level (>300mg/dL) indicated destruction of both healthy pancreatic β cells and implanted insulinoma. Each line represents one mouse; data shown are from one experiment representative of two (n=6-7 per group total). (B) NOD.SCID mice were injected with NIT-1 cells. Three to seven days later, animals received 1x10^5 purified CD4^+ T_{eff} cells from BDC2.5/NOD transgenic mice. The results are shown as mean ± SEM of n=3 and represent one of two independent experiments. (C-D) NIT-1 cells were injected into either BDC2.5/NOD or Foxp3-deficient BDC2.5/NOD mice. Animals were then monitored for (C) blood glucose levels and (D) tumor weight. Data are shown as mean ± SEM of 3-4 mice per group and are representative of three experiments. *P<0.05; **P<0.01; ***P<0.001; Student’s t test (B, D) and ANOVA (C).
3.3.2 Local Suppression of Self-Antigen-Specific CD4+ T eff Cells in the Insulinoma Microenvironment

A prominent role for T_{reg} cells has been established in suppressing antitumor immunity. We examined the function of T_{reg} cells in suppressing tumor-killing capacity of self-antigen-specific T_{eff} cells. NIT-1 tumor-bearing NOD.SCID mice were treated with the self-antigen-specific CD4^+ T_{eff} cells alone, T_{eff}:T_{reg} mixture at a 10:1 ratio, or no-T-cell control. Blood glucose readings indicated that autoantigen-specific T_{reg} cells efficiently suppressed insulinoma-killing by the autoimmune T_{eff} cells (Figure 3-2: A). In the group of animals that received autoimmune T_{eff} cell alone, only a residual tumor was recovered. Pathological analyses of residual insulinoma and healthy pancreatic β cells revealed virtually complete destruction of both malignant and non-malignant tissues (Figure 3-2: B). In the presence of T_{reg} cells, the tumor was preserved. However, this relatively low ratio of T_{reg} cells did not substantially suppress autoimmune T_{eff} cells in healthy pancreatic islets (Figure 3-2: B-D). Flow-cytometry analyses revealed a substantially increased ratio of CD4^+Foxp3^+ T_{reg} cells to T_{eff} cells at the tumor site (Figure 3-2: E-F).

In addition, given the generally established, prominent role of CD11b^+Gr-1^+ MDSC in tumor microenvironment [147], we analyzed CD11b^+Gr1^+ cells in insulinoma versus healthy pancreata. Four-week-old BDC2.5/NOD mice (n=5) were inoculated with NIT-1 cells. After insulinoma is established (approximately 3wk post tumor cell inoculation), the tumor and healthy pancreata in the same mouse were analyzed by flow cytometry. The ratio of T_{eff} cell counts versus
CD11b⁺Gr-1⁺ cell counts are increased about 5-fold (53 ± 10, mean ± SEM) in the pancreas versus that in the tumor (9 ± 3, mean ± SEM) (Figure 3-3). Moreover, the profile of the populations differs in the healthy versus malignant tissues, in that the CD11b⁺Gr1⁺ cells in tumors had a much higher expression of CD11b.

T<sub>reg</sub>-cell reconstitution did modestly increase circulating TGF-β1 levels in the tumor-bearing mice compared with that of control groups (Figure 3-4: A). The elevated TGF-β1 level in blood circulation, however, had no apparent suppression on immunopathology in the pancreas, even though the increase in TGF-β1 was detectable before onset of immune damage in pancreas. Taken together, these results indicate that the insulinoma microenvironment, in combination with T<sub>reg</sub> cells and MDSC, effectively suppressed progression of autoimmunity-mediated damage of tumors by self-antigen-specific CD4⁺ T<sub>eff</sub> cells. This suppressive effect was local at the tumor site, with negligible systemic inhibition on the self-antigen-specific cells, as they retained their capacity in destroying non-malignant target cells in the same animals.
Figure 3-2. Local suppression of self-antigen-specific CD4$^+$ T$_{eff}$ cells in the tumor microenvironment, without distal impairment of T$_{eff}$ cells of the same specificity in nonmalignant tissues.

(A) NOD.SCID mice were injected with NIT-1 insulinoma cells. Five days later, animals received either 1x10$^5$ BDC2.5 T$_{eff}$ cells alone or a mixture of 1x10$^4$ BDC2.5 T$_{reg}$ cells (T$_{eff}$:T$_{reg}$ = 10:1). Animals were monitored for blood glucose levels for immune destruction of tumor cells as well as healthy cells. 

(B) Hematoxylin-eosin staining of tumor and healthy pancreas tissue sections from the same animals (original magnification, 250×) is shown. In the T$_{eff}$-cell alone group, only a residual tumor mass was recovered and sectioned. 

(C) Pathology score of autoimmune damage in pancreatic islets is shown. 

(D) Tumor area at the endpoint (Day 18-19) of the experiment in (A) is shown. 

(E-F) Flow cytometry analysis of the tumor-bearing animals is shown. PLN, pancreatic draining lymph nodes; TLN, tumor draining lymph nodes. Data are shown as mean ± SEM of n=4-7 per group (A, C, D, F) and are representative of three independent experiments. *P<0.05; ***P<0.001; ANOVA.
Figure 3-3. Flow cytometry analysis of CD11b^+Gr-1^+ cells in the normal pancreatic tissue versus insulinoma tissue. Flow cytometry analyses were conducted with BDC2.5/NOD mice bearing the NIT-1 insulinoma. (A) Plots were derived from CD4^−CD8^−CD11c^− cells. The numbers in the plots are percentages of the gated populations (mean ± SEM). (B) Ratio of total CD4^+ T_{eff} cell counts versus CD11b^+Gr-1^+ cell counts in the pancreas or the tumor (insulinoma) from the same mouse (Mean ± SEM). Data shown represent two experiments (n=4 per group total, Student’s t test ).
**Figure 3-4. Analysis of circulating TGF-β levels in the tumor-bearing animals.** Blood samples were collected from animals bearing insulinoma and lymphoma (see Figure 2 and 3). TGF-β1 in plasma was quantified by ELISA, with a TGF-β Sandwich ELISA kit from R&D Systems (Minneapolis, MN) and by a procedure according to the manufacture’s protocol. **A**, TGF-β1 levels in the plasma of insulinoma-bearing mice. Data are pooled from two independent experiments with 2-3 mice / group for each experiment (mean ± SEM). **B**, TGF-β1 levels in the plasma from lymphoma-bearing mice treated with CTLA4KD OT1 or PL4 control OT1 cells. Data are pooled from two independent experiments with 3-4 mice per group for each experiment (mean ± SEM). * P<0.05, the group receiving T_{reg} + T_{eff} cell injection and the tumor bearing group that did not receive T cells, analyzed by one-way ANOVA with Tukey’s post-hoc test.
3.3.3  **Local Suppression of CD8\(^+\) T\(_{\text{eff}}\) Cells Specific to a Surrogate Self Antigen at the Lymphoma Site**

CD8\(^+\) T cells are potent effectors in antitumor immunity. Prompted by the observation of local suppression of autoimmune CD4\(^+\) T\(_{\text{eff}}\) cells at the tumor site, we tested whether tumor microenvironment, as opposed to healthy tissues, also suppress self-antigen-specific CD8\(^+\) T\(_{\text{eff}}\) cells. The RIP-mOVA transgenic mice express an ovalbumin transgene in healthy pancreatic \(\beta\) cells [107]. Transgenic ovalbumin expression serves as a surrogate self-antigen. These mice were used as a recipient for implanting E.G7-OVA lymphoma cells, which were stably transfected with the ovalbumin gene [148]. Adoptive transfer of activated CD8\(^+\) T\(_{\text{eff}}\) cells from the OT1 transgenic mice [106], which are specific to the ovalbumin antigen, completely destroyed the ovalbumin-expressing \(\beta\) cells and caused overt diabetes in the animals. However, lymphoma mass was only partially reduced, with limited inflammatory infiltration in the tumor tissue (**Figure 3-5**). Thus, the CD8\(^+\) T\(_{\text{eff}}\) cells were inhibited at the tumor site in the lymphoma-bearing animals, without being substantially curtailed at the healthy tissue site expressing the same self-antigens.
Figure 3-5. Local suppression of CD8⁺ Teff cells specific to a surrogate self-antigen in the lymphoma microenvironment. RIP-mOVA transgenic B6 mice, expressing ovalbumin (OVA) in the pancreatic islet β cells as a surrogate self-antigen, were implanted with E.G7-OVA lymphoma cells which express ovalbumin. Five days after the tumor cell implant, the mice were injected with 2x10⁶ activated OT1 CD8⁺ T cells specific to ovalbumin. (A) Blood glucose levels were monitored for destruction of healthy β cells by the CD8⁺ Teff cells. Data are shown as mean ± SEM of n=3 from 1 experiment representative of three. (B) Pancreatic islet damage was assessed by pathological analysis and shown as mean ± SEM of n=4-5. (C) Lymphoma weight was measured in the Teff-cell-treated mice normalized to controls (sacrificed at day 18-27, total n=3 controls and 8 OT1 treated, data shown as mean ± SEM). (D) Hematoxylin-eosin stained sections of healthy pancreas and lymphoma tissue expressing the same target antigen (original magnification, 250×). Data shown are representative of n=4-5.
3.3.4 Partial Ablation of T\textsubscript{reg} Cells Minimally Impacted Tumor but Severely Damaged Self-Tissue

To further examine the pathophysiology of autoimmune mechanisms in antitumor immunity, we investigated the role of T\textsubscript{reg}-cell-mediated suppression of self-antigen-specific T\textsubscript{eff} cells at tumor site in a setting that necessitated neither adoptive transfer of T cells nor lymphopenic conditions. The BDC2.5/NOD.Foxp3\textsuperscript{DTR} model [105] was used. It carries a diphtheria toxin (DT) receptor transgene under the control of a Foxp3 promoter, enabling timed removal of 80-90% of T\textsubscript{reg} cells with a low dose of DT. NIT-1 tumor cells were injected into BDC2.5\textsuperscript{+} Foxp3\textsuperscript{DTR\textsuperscript{+}} mice or littermate BDC2.5\textsuperscript{+} Foxp3\textsuperscript{DTR\textsuperscript{-}} controls. After tumor establishment, the animals were treated with DT [105], resulting in ~80% reduction of T\textsubscript{reg} cells in the BDC2.5\textsuperscript{+} Foxp3\textsuperscript{DTR\textsuperscript{+}} mice compared with the controls. The partial ablation of T\textsubscript{reg} cells did not inhibit the progressive growth of the NIT-1 tumor (Figure 3-6: A-C). However, as reported before [105] and consistent with the adoptive transfer studies in Figure 2A-D, the residual T\textsubscript{reg} cells were not sufficient to restrain autoimmune damage in the pancreatic islets [105, 140]; instead, partial T\textsubscript{reg} depletion caused complete destruction of the tissue. At the tumor site, partial depletion of T\textsubscript{reg} cells did not cause progression of autoimmune damage, as the inflammatory infiltrates remained at the periphery of tumor mass in both BDC2.5\textsuperscript{+} Foxp3\textsuperscript{DTR\textsuperscript{+}} mice or littermate BDC2.5\textsuperscript{+} Foxp3\textsuperscript{DTR\textsuperscript{-}} controls after DT treatment (Figure 3-6: D-E).
Figure 3-6. Partial depletion of T_{reg} cells provoked self-antigen-specific T_{eff} cells to severely damage healthy tissues but not the tumor target in the same animals. BDC2.5 mice were crossed with Foxp3^{DTR+} mice. BDC2.5^{+}Foxp3^{DTR+} mice and BDC2.5^{+}Foxp3^{DTR-} controls were injected with NIT-1 tumor cells. After a palpable tumor was established, partial ablation of T_{reg} cells (~80%) was done by injecting diphtheria toxin (50µg/gram body weight) at days 0, 1 and 3. (A) Blood glucose levels were measured to monitor tumor burden and are shown as mean ± SEM of n=4. (B) Tumor growth was measured and shown as mean ± SEM of n=4. (C) Tumor weights at the endpoint are also shown as mean ± SEM of n=6-7 (day 17-19). (D) Representative histological sections of pancreas and tumor tissues are shown (arrows showing lymphocytic infiltration at the periphery of tumors, original magnification, 250×). (E) Pancreatic pathology scores are shown as mean ± SEM of n=7. All data shown are pooled from three independent experiments. ***P<0.001, Student’s t test.
3.3.5 *A Modest Reduction of CTLA4 Promoted Autoreactive T_{eff} Cells Intrinsically in Antitumor Immunity*

The studies with insulinoma and lymphoma models identified a suppressive milieu against self-antigen-specific T_{eff} cells, formed by the tumor microenvironment in combination with T_{reg} cells and MDSC. T_{reg} cells depend on CTLA4 for suppressive function [54]. CTLA4 is a prototypical inhibitor in antitumor immunity. In humans, expression of CTLA4 varies subtly due to polymorphisms in the *CTLA4* locus.

To examine how modest variation of CTLA4 impacts tumor destruction by self-antigen-specific T_{eff} cells, we utilized a model of subtle CTLA4 reduction (~60% in both mRNA and protein) constructed by shRNA transgenesis, CTLA4KD7 [102], that mimics a natural reduction due to genetic variations. The CTLA4KD7 or PL4 vector control line [102] were crossed with the OT1 transgenic mice. E.G7-OVA lymphoma cells were implanted into RIP-mOVA mice. The lymphoma-bearing mice were treated with activated CD8^{+} T_{eff} cells from OT1.CTLA4KD7/B6 or OT1.PL4/B6 mice. Both CTLA4KD and PL4 control CD8^{+} T_{eff} effectively destroyed healthy pancreatic β cells expressing the OVA antigen, as evidenced by the severe hyperglycemia (*Figure 3-7: A*). However, the transgenic CTLA4 shRNA significantly promoted the destruction of lymphoma cells expressing the OVA antigen in the same mice by the OT1 T_{eff} cells (*Figure 3-7: B*). We did not detect any difference in circulating TGF-β1 levels between the groups receiving either CTLA4KD7 or control OT1 cells (*Figure 3-4: B*)
To examine if a subtle reduction in CTLA4 also affects $T_{\text{reg}}$ cell potency, we reconstituted neonatal Foxp3-deficient B6 mice with $T_{\text{reg}}$ cells from either CTLA4KD7 or PL4 controls, and injected them with syngeneic EL4 lymphoma cells. There was no significant difference in lymphoma cell growth in the two groups of animals (Figure 3-7: C), indicating that CTLA4 reduction did not impair $T_{\text{reg}}$ cell functions in tumor bearing mice. To further test this observation, we used a Foxp3-deficient BDC2.5 model. As shown in Figure 3-1, the absence of $T_{\text{reg}}$ cells enabled the animals to reject NIT-1 tumor cells. The $T_{\text{reg}}$-cell-deficient mice were reconstituted with self-antigen-specific $T_{\text{reg}}$ cells from BDC2.5/NOD.CTLA4KD mice or BDC2.5/NOD.PL4 controls. The animals were then challenged with NIT-1 tumor cells. As shown in Figure 3-7 (D-E), CTLA4 reduction in $T_{\text{reg}}$ cells did not compromise its efficacy in protecting the tumor cells from destruction by self-antigen-specific $T_{\text{eff}}$ cells.
Figure 3-7. A modest reduction in CTLA4 intrinsically, rather than extrinsically, enhanced autoimmune T<sub>eff</sub> cells in tumors. (A,B) RIP-mOVA mice were injected with E.G7-OVA lymphoma cells. Five days later, the mice were injected with 2x10<sup>6</sup> activated OVA-specific CD8<sup>+</sup> T<sub>eff</sub> cells from OT1 transgenic mice carrying either the CTLA4KD7 shRNA transgene or PL4 vector control. The destruction of (A) healthy β cells was monitored by blood glucose levels (shown as mean ± SEM of n=4) and (B) tumor by weight (shown as mean ± SEM, n=7-10, each symbol representing a single mouse). (C) 2-4 day-old Foxp3-deficient B6 mice were reconstituted with 1-2x10<sup>5</sup> T<sub>reg</sub> cells from either CTLA4KD7/B6 transgenic mice or PL4/B6 controls. Seven days later, they were injected with 1x10<sup>5</sup> syngeneic EL-4 lymphoma cells. Tumor weights at day 24-25 were normalized to paired controls in each experiment. (D and E) 2-4 day old Foxp3-deficient BDC2.5 mice were reconstituted with 1-2x10<sup>5</sup> antigen-specific-T<sub>reg</sub> cells from either CTLA4KD7.BDC2.5 mice or PL4.BDC2.5 controls. Seven days later, the recipient mice were injected with NIT-1 cells and monitored for blood glucose levels to assess tumor growth. (C-E) Data shown are pooled from three experiments with 1 mouse per group in each experiment. *P<0.05; ***P<0.001; ANOVA.
3.3.6 A Subtle Reduction in CTLA4 Substantially Broke Tolerance in a Spontaneous Model of Breast Cancer

Our studies with 3 different tumor cell lines for two types of cancers, insulinoma and lymphoma, illustrated a quantitative impact by CTLA4 on autoimmune Teff cells. These implanted tumor models enabled the studies in an antigen-specific manner. It would be desirable to validate the key finding in naturally developed tumors. We used a spontaneous breast cancer model, BALB-neuT mice [142], to test the impact of subtle CTLA4 reduction on self-tolerance of tumors. In this model, it was shown that over-expression of a self-antigen in tumors promoted a dominant self-tolerance in the tumor microenvironment which facilitated breast cancer development [149]. In humans, genetic studies have associated breast cancer with polymorphisms of the CTLA4 locus [63, 64].

The CTLA4KD7 or PL4 transgenic lines were crossed with BALB-neuT transgenic mice. The CTLA4KD7+neuT+ mice, compared with CTLA4KD7-neuT+ littermate or PL4+neuT+ controls, had a delayed incidence of breast cancer (Figure 3-8: A). Among the animals that had breast tumors, the age of tumor onset was significantly delayed in CTLA4KD7+neuT+ mice than in controls (Figure 3-8: B), and the tumor grew at a slower pace (Figure 3-8: C) and with a significantly smaller mass (Figure 3-8: D). A histopathological analysis of the breast tumors revealed that whereas control neuT+ mice exhibited minimal sign of immune destruction of the tumors, substantial lymphocytic infiltration and inflammatory damage were evident in the tumors from CTLA4KD7+neuT+ mice.
(Figure 6E). This difference in the tumor pathology was consistent with increased activation of both CD4\(^+\) and CD8\(^+\) Teff cells in the CTLA4KD7\(^+\)neuT\(^+\) mice versus controls (Figure 3-9). Taken together with the critical role of dominant peripheral self-tolerance in breast cancer development demonstrated by a previous study [149], the results suggest that genetically relevant, physiological levels of CTLA4 quantitative variations can play a critical role in unmasking self-antigen-specific, antitumor immunity, perhaps by diminishing local tolerance at the tumor site. Furthermore, the CTLA4KD model enabled us to provide the first experimental evidence for a role of CTLA4 in spontaneous tumor onset and progression. Further studies are needed to understand the exact mechanisms by which CTLA4 reduction impacts spontaneous breast cancer development.
Figure 3-8. A subtle reduction in CTLA4 substantially inhibited spontaneous breast cancer development facilitated by self-tolerance. To examine the effect of CTLA4 reduction on immune tolerance of tumor cells in spontaneous cancer development, BALB-neuT mice were crossed with CTLA4KD7 mice or PL4 controls. (A) The cumulative incidence of spontaneous breast cancer in the neuT⁺ transgenic mice harboring the CTLA4KD7 transgene (n=6), and control neuT⁺ mice (n=29) that were CTLA4KD-transgene-negative littermates or carrying PL4 vector transgene was analyzed by the Mantel-Cox test. (B) The average age of breast cancer onset and (C) the breast tumor growth kinetic in the animals that had developed breast cancer are shown as mean ± SEM of n=3-4 animals pooled from three experiments, with 1-2 mice in each group for each experiment. (D) The weight of excised tumor at experimental endpoint (week 16-34), normalized to paired controls. Data shown are pooled from three experiments (n=6 controls, n=4 CTLA4KD7 mice) shown as mean ± SEM. (E) Hematoxylin-eosin staining of breast tumor tissues. Arrow indicates increased lymphocytic infiltration into the tumor tissues and destruction of tumor tissues in the CTLA4KD group (original magnification, 100×). Data shown in (E) are representative of n=3 in each group. *P<0.05; **P<0.01; ***P<0.001; Student’s t test.
Figure 3-9. Flow cytometry analysis of T-cell activation phenotypes in breast-tumor-bearing CTLA4KD NeuT$^+$ versus control NeuT$^+$ mice. Activation status of CD4$^+$ (A) and CD8$^+$ T$_{eff}$ (B) in the tumor and tumor draining lymph nodes (TLN) of the tumor bearing mice (Figure 6) were assessed by expression of CD44 and CD62L markers. The numbers in the flow cytometry plot are the percentages (mean ± SEM) of CD44$^{hi}$CD62L$^{lo}$ (activated) and CD44$^{lo}$CD62L$^{hi}$ (naïve) population of the T cells. Data are pooled from two experiments, n = 4-5 per group. Student’s $t$ test was used to compare CD44$^{hi}$CD62L$^{lo}$ populations in CTLA4KD NeuT$^+$ versus control NeuT$^+$ mice. *, P<0.05; **, P<0.01, Student’s $t$ test.
3.3.7 Anti-CTLA4 Antibody Blockade Impact on Both Teff and Treg Cells

Clinical trials with anti-CTLA4 antibody blockade has produced remarkable antitumor benefit but also suggested that autoimmunity, at least in part, actually mediated the tumor destruction. We sought to characterize how autoimmune Teff and Treg cells were implicated and impacted by CTLA4 blockade in tumor-bearing animals.

NOD.SCID mice bearing the NIT-1 tumor were reconstituted with self-antigen-specific Teff cell alone, or together with Treg cells at a 10:1 ratio. As shown in blood glucose reading (Figure 3-10: A) and tumor weight (Figure 3-10: B), anti-CTLA4 treatment effectively promoted the antitumor activity of the self-antigen-specific Teff cells by overcoming Treg-cell-mediated suppression. Flow cytometry analysis of the anti-CTLA4-treated and control animals demonstrated that CTLA4 blockade had impacted both Teff and Treg cells in various lymphoid organs, resulting in a substantially skewed ratio of Treg:Teff cells (Figure 3-10: C-E). This dual effect of anti-CTLA4 antibody blockade was distinct from that by a subtle CTLA4 reduction (Figure 3-7). Nonetheless, the results collectively establish a predominant role of CTLA4 in suppressing autoimmunity-mediated antitumor immunity at the tumor site.
Figure 3-10. Anti-CTLA4-antibody blockade increased self-antigen-specific T_{eff} cell proportions and overcame immunosuppression by tumors. NOD.SCID mice were injected with NIT-1 insulinoma cells. After 5 days, the animals were treated with 1x10^5 autoantigen-specific CD4^+ T_{eff} cells, with or without 1x10^4 autoantigen-specific T_{reg} cells from BDC2.5 mice. Anti-CTLA4 antibody treatment (200 μg each) was done on day 0 and 4 after T-cell injection. (A) Blood glucose levels for assessing tumor burden (inverse correlation) as well as destruction of healthy pancreatic β cells are shown. (B) Tumor weight was determined at the day 20 endpoint are shown. (C) A representative flow cytometry analysis of T_{reg} vs. T_{eff} profiles, gated on CD4^+ cells, is shown (D-E) Summary of total T_{eff} and T_{reg} cell numbers and ratios are shown. All data are shown as mean ± SEM of 3-4 mice per group and are pooled from two experiments. *P<0.05; **P<0.01; ***P<0.001; ANOVA.
3.4 Discussion

Evidence from previous studies with animal models has suggested that immune tolerance can preferentially distinguish healthy tissues from malignant cells expressing the same antigens [65-67]. Those results are consistent with the hypothesis of cancer immune surveillance. However, recent clinical trials of immunotherapies in general have not demonstrated a therapeutic effect against cancers in the absence of substantial off-target autoimmune toxicity [47, 50-52]. Instead, observations from the clinical trials ostensibly highlighted autoimmunity as a potential “double-edged sword” against tumors as well as healthy cells. Mechanistic studies with animal models are needed to dissect the autoimmune implications identified in the clinical setting.

In a melanoma model, the efficacies of self-antigen-specific T cells in antitumor immunity has been well-studied by using CD4- and CD8-restricted TCR-transgenic models [150, 151]. Perhaps due to the clonal nature of the antigen-specific T cells, those transgenic models did not develop spontaneous autoimmunity. Our study, aided with a battery of well-characterized models of autoimmunity, aimed to understand how T cell clones with a potential of spontaneous autoimmunity function in tumor settings versus healthy tissues. Indeed, self-antigen-specific T$_{\text{eff}}$ could eradicate tumor cells. However, findings with the self-antigen-specific T cells also revealed a tumor microenvironment that is more tolerogenic than healthy tissues, i.e., the tumor sites akin to an “immunoprivileged” environment that effectively inactivates autoimmune effectors. This is not merely because tumor cells might proliferate faster than
healthy cells. Activated T cells can multiply at a rate on par with even a highly proliferative tumor cell. Indeed, as our study demonstrated, in the absence of \( T_{\text{reg}} \) cells, \( T_{\text{eff}} \) cells completely destroyed both tumor and healthy cells in the same animals.

Tumor-mediated immunosuppression is a generally recognized obstacle for antitumor immunity. It has been debated whether and to what extent the suppression is systemic or limited to the site of tumor. This study, evaluating the destruction of tumor versus healthy cells in the same animal by the same specific \( T_{\text{eff}} \) cells, unequivocally demonstrated that tumors suppressed autoimmune \( T_{\text{eff}} \) cells locally without a distal impairment. A hallmark cytokine associated with tumor-induced immunosuppression is TGF-\( \beta \)1. Although we detected increased circulation of TGF-\( \beta \)1 in tumor-bearing animals in some experiments, it did not exert an apparent inhibition on the autoimmune \( T_{\text{eff}} \) at a distal site in healthy tissues.

At cellular levels, \( T_{\text{reg}} \) cells and MDSCs have long been recognized as critical mediators of immunosuppression in cancer. Our studies with self-antigen-specific T cells highlighted an increased potency of these regulatory mechanisms in tumors versus healthy tissues. The molecular mechanisms responsible for the local immunosuppression remain to be elucidated. Possibly, a suppressive cytokine milieu, directly or indirectly related to \( T_{\text{reg}} \) cells and MDSC, inactivates \( T_{\text{eff}} \) cells at the tumor site, which could be re-activated by an agonistic cytokine stimulation [152] or a global alteration of tumor gene expression profiles [153]. This study implicates CTLA4. Suggestive of the intertwining between
autoimmunity and antitumor immunity, protection from cancer is often associated with the same polymorphisms of the CTLA4 locus that are linked to autoimmune susceptibility [23, 62-64]. A conditional knockout model established an essential role for CTLA4 in T_{reg} cells [54]. Its intrinsic role in T_{eff} cells has also been well-documented [29, 55]. Our study with a CTLA4 shRNA model indicates a distinction between quantitative variation in CTLA4 and the “all-or-nothing” model of CTLA4 knockouts. A subtle reduction of CTLA4 did not impair T_{reg} function, but substantially promoted T_{eff} capacity in tumor settings.

An expansion of immunotherapy trials has generated a plethora of novel ideas in cancer immunology. The entangling of autoimmunity toxicity with antitumor benefit has provoked a shift of perspective whereby autoimmune side effects are considered not only a welcome marker but actual effectors for antitumor immunity [53]. A direct comparison of cancerous versus healthy tissue in interaction with self-antigen-specific T_{eff} cells revealed their intrinsic potential in tumor eradication. However, they were subjected to regulatory mechanisms that have been evolved to induce tolerance to nonmalignant self-tissue, even more so in the tumor microenvironment. Therefore, self-antigen can be effectively targeted for antitumor immunity, but harnessing the tumor-destruction capacity of self-antigen-specific T cells requires effective strategies to overcome the suppressive microenvironment at the tumor site. CTLA4 blockade therapies can abrogate suppressive tumor milieu by reverting the local predominance of T_{reg} cells over self-antigen-specific T_{eff} cells. On the other hand, a subtle reduction of
CTLA4 reflecting genetic variations may substantially alter an immunoprivileged environment evolved in a solid tumor through an intrinsic impact on $T_{eff}$ cells.

Adoptive cell therapies with antigen-specific T cells are emerging as a promising clinical intervention in cancer treatment [154, 155]. Results gathered in this study suggest that a status of “immunoprivileged self” in tumors barricade specific $T_{eff}$ cells. This suggestion portends that it might be very difficult, if possible, to circumvent autoimmunity toxicity in a systemic immunotherapy against cancer, unless a substantial antigenic difference is identified between the tumor target and healthy tissue. Therefore, targeting immunoregulatory elements at the tumor site would be desirable. Indeed, local delivery of engineered dendritic cells secreting anti-CTLA4 antibodies promoted immunity against melanoma in mice without eliciting autoimmunity [156]. A nexus of immunosuppressive elements evolved at the tumor site likely suppress self-antigen-specific T lymphocytes as well as bona fide tumor-specific T cells. A subtle reduction of CTLA4 in $T_{eff}$ cells by RNAi silencing could substantially overcome the tumor barrier, suggesting a practical approach to enhance the efficacies of antigen-specific T cells for cancer therapies.
CHAPTER 4: Gastric Cancer Initiation by CTLA4 Deregulation and Progression With Age

4.1 Summary

Gastric cancer is a common lethal cancer whose incidence increases with age and is due to a complex interaction between host and environmental factors leading to chronic inflammation. Among the host factors implicated are the polymorphisms of the CTLA4 gene, which encodes an adaptive immune regulator. Here we show that anti-CTLA4-antibody treatment or transgenic RNAi knockdown of CTLA4 initiated gastric metaplasia in mice in the absence of pathogenic Helicobacter infection. The metaplasia depended on T-cell repertoire and genetic background. Aging promoted malignancy development such that all CTLA4 knockdown animals over 70 weeks of age had metaplastic lesions replaced by neoplasia. The tumorigenesis in mice was associated with decreased expression of isocitrate dehydrogenase 2 and reduced content of 5-hydroxymethylcytosine in DNA, the latter detected also in human gastric adenocarcinoma. These findings suggest distinct roles played by a genetic risk followed by an epigenetic defect leading to age-associated gastric carcinogenesis.

4.2 Materials and Methods

4.2.1 Mouse Models

CTLA4 RNAi (CTLA4KD7) and PL4 transgenic mice were described previously =. CTLA4KD7 and PL4 mice were backcrossed onto the C57BL/6 (B6) background for >10 generations [103] and crossed with Foxp3FIR (Foxp3-IRES-RFP “knockin”) mice on the B6 background [110], the OT1 transgenic line on the
B6 genetic background, the B6.H-2\(^{g7}\) (B6.NOD-(D17Mit21-D17Mit10)/LtJ) congenic line, or intercrossed with the Balb/c genetic background to generate F1 animals called CB6F1 [157, 158]. All animals were maintained in a specific-pathogen-free barrier facility and the studies are approved by the Institutional Animal Care and Use Committee at the University of Miami.

### 4.2.2 Antibody Treatment

CB6F1 mice [157, 158], non-inbred but with a homogeneous genetic background, were treated with monoclonal anti-CTLA4 antibodies (clone UC10-4F10-11), isotype controls, or PBS (vehicle) controls. The treatment was done by intraperitoneal injection, at a dose of 30 µg per gram body weight, twice every week for four weeks. The animals were analyzed one week after the last treatment.

### 4.2.3 Histopathology

Stomach samples were collected from sacrificed animals and were flattened by cutting through the greater curvature. After washing away the food, the samples were fixed in 10% formalin solution. Paraffin-embedded sections and H-E staining were done by the Pathology Research Resource core at the University of Miami. H-E images were taken with a dry 4x Olympus, and 25x and 40x Vistavision lens. Some images were taken with a Vistavision microscope (VWR, Radnor, Pennsylvania) or with an Olympus BH-2 microscope (Olympus, Tokyo, Japan) that has an additional 1.25x magnification within the light path of the scope.
4.2.4 Adoptive Transfer of Spleen Cells to Neonate Recipients

Single cell solutions were prepared with spleen cells from CTLA4KD/CB6F1 and littermate control donors. Neonate Rag-deficient CB6F1 mice were injected, i.p., with $15 \times 10^6$ donor spleen cells. The animals were sacrificed at 5 weeks of age and analyzed for gastric histopathology and immune cell phenotypes.

4.2.5 Quantitative RT-PCR

The stomach sample was cut along the greater curvature and was flattened. The exposed lumenal side was washed with sterile PBS. The clean stomach samples were transferred to a disposable grinder unit, with lumenal side remains exposed. Trizol reagents (Life Technologies Corporation, San Diego, CA) were added immediately to the lumenal side to preserve the RNA quality. A grinder plunger was then inserted into the tube with stomach samples and Trizol. The rough surface of the plunger was used to grind the mucosal tissues directly into the Trizol solution, by gentle friction until the mucosal layer was visibly removed. This method was chosen for its consistency with animals at different ages and better quality of RNA preparation, especially when experimenting with very young animals, because technically it was difficult to avoid contamination when dissecting the mucosa layer from the stomach samples of very young animals using instruments.
4.2.6 Quantification of Gastric Normal Flora Bacteria by Quantitative PCR for Helicobacter or all Bacteria

DNA was isolated from stomach tissue samples. CYBR Green quantitative PCR were conducted with primers specific to the 16S RNA gene of all Helicobacter spp., or all bacterial species, or the eukaryotic gene beta-casein. The relative levels of 16S RNA gene were normalized with the level of beta-casein DNA in each sample.

4.2.7 Quantification of 5hmC in DNA

DNA was purified from gastric mucosal tissue samples, and were analyzed via dot-blot as described in previous studies [159, 160], with specific antibodies for 5hmC, and counterstained with methylene blue to normalize for total quantity. Briefly, DNA was loaded onto a Hybond N+ nylon membrane (GE Healthcare Life Sciences, Pittsburgh, PA), and cross-linked by heating at 80°C for 30 minutes. The membrane was then blocked for 1 hour, and then incubated with polyclonal rabbit anti-5hmC antibody (Active Motif #39769, titration: 1:10,000) for overnight at 4°C. Horse radish peroxidase (HRP)-conjugated anti-rabbit antibodies (Jackson ImmunoResearch Laboratories 1:5,000) were then added and developed using enhanced chemiluminescence (ECL) to determine quantity of 5-hmC signal.

4.2.8 Immunofluorescence Staining of 5hmC in Cell Nuclei of Tissue Sections

Human gastric adenocarcinoma samples were obtained from the Tissue Bank Core of the Sylvester Cancer Center at the University of Miami. For three of the samples, normal stomach mucosal tissue from the same patient was also
obtained as matched controls. The tissues were embedded in OCT compound and cryosections of the tumors and normal tissues at 7μM thickness were prepared. The tissue sections were fixed with paraformaldehyde, and stained with specific antibodies against 5hmC, as described in previous studies [159, 160]. Briefly, cryosections were fixed in 4% PFA for 15 minutes. After 3x PBS washes, slides were treated with pre-warmed 1N HCl for 30 minutes at 37°C. Sections were then blocked with 3% normal donkey serum / 0.4% Triton X-100 in PBS for 1 hr at room temperature. Rabbit anti-5hmC antibody (#39769, Active Motif, Carlsbad, CA; titration 1:10,000) was then added and incubated for overnight at 4°C, and followed with Cy3-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories; titration, 1:500) in 2% NDS/0.1% Triton X-100 in PBS for 45mins at room temperature. After 3x washes in PBS, the slides were counterstained with DAPI mounting medium and imaged with a Leica inverted TCS SP-5 broadband confocal microscope (Leica 40x/1.25-0.75NA HCX PL APO oil immersion lens). Images were then linearly contrasted and noise was removed using ImageJ software.

4.2.9 Flow Cytometry

Flow cytometry analyses were conducted with a standard procedure [161]. Cells were then blocked from non-specific binding using a cocktail anti-CD16/32 (2.4G2) and normal mouse sera (Jackson ImmunoResearch, West Grove, PA). The cells were then stained with fluorescent-antibody conjugates to determine cell phenotype. The following antibody conjugates were used: PE-TR anti-CD8 (Life Technologies Corporation, San Diego, CA); Efluor450 conjugated anti-
CD44; APC-efluor780 conjugated anti-CD62L; PE-cy7 and APC-efluor 780 conjugated anti-CD4; PerCP-Cy5.5 conjugated anti-CD25 (eBioscience, San Diego, CA). Mice that either received or were part of any PL4 or KD7 line had intrinsic GFP expression. For intracellular cytokine staining, cells were stimulated for 7 hours in PMA and ionomycin (Sigma, St. Louis, MO), with brefeldin A (eBioscience) added 1 hour after stimulation. Cells were then stained for surface makers, then fixed using proprietary buffer for 30mins. Cells were permeabilized and blocked for 15 minutes, followed up by addition of both PE-cy7 conjugated anti-IL17A and APC conjugated anti-IFN-γ (eBioscience) for 20 minutes at 4°C. Cells were then washed and re-suspended in 2% FBS/PBS for analysis with flow cytometers (LSR-II and Fortessa, Becton Dickinson, San Jose, CA).

4.2.10 Statistics

Chi-square test was used for incidence of tumorigenesis. Student’s t tests were used when appropriate for single comparisons. For multiple groups, one-way ANOVA analyses followed up with Tukey’s post-hoc test were performed to test for significance. For longitudinal observations among multiple groups, two way ANOVA analyses were performed, and significance was calculated using the Bonferroni’s multiple samples Post-hoc test. P values equal to or less than 0.05 were considered significant. * P<0.05; **, P<0.01; ***, P<0.001; ns, not statistically significant.
4.3 Results

4.3.1 Anti-CTLA4 Treatment Induces Gastric Metaplasia

To examine the effect of CTLA4 modulation on gastric inflammation and tumorigenesis, we used anti-CTLA4 antibody or RNAi transgenesis in animals on the Balb/c(C) x C57BL/6 (B6) F1 mixed genetic background, called CB6F1 [157, 158]. CB6F1 mice have a non-inbred but homogenous genetic makeup [157, 158], which facilitates studies of epigenetics and other factors. The initial anti-CTLA4 antibody blockade experiments were started with animals at 3-4 days of age. The treatment in the young CB6F1 mice caused SPEM-like metaplasia, in 5 of 7 treated animals (Figure 4-1). The induction of metaplasia by CTLA4 blockade depended on the age of the animals when the treatment began: Anti-CTLA4 antibody did not induce gastric metaplasia in young adults or older mice (11-20 weeks of age, n=6; 68-98 weeks, n=4). The results suggest that CTLA4 modulation has the potential to induce de novo tumorigenesis, which is consistent with the paradoxical association between CTLA4 polymorphisms and human GC.
Figure 4-1. Blocking the CTLA4-mediated immune checkpoint with anti-CTLA4 monoclonal antibodies induced metaplasia in wildtype mice.

Wildtype mice on the mixed Balb/c x B6 background (CB6F1) were treated with monoclonal antibodies against CTLA4 or control Ig, from 3-4 days of age for 4 weeks. The animals were analyzed at 5 weeks of age for gastric histology by H-E staining of tissue sections. (A) External macroscopic appearance of the stomach. (B) The lumenal side of the stomach. (C) H-E sections of the stomach from treated mice and controls, images with a 5x objective. (D) “Zoomed-in” images (30x) from highlighted areas in (C). The metaplasia pathology represents 5 of the 7 mice treated with anti-CTLA4 antibodies, in comparison to the gastric histology of 4 mice treated with hamster Ig controls (X^2 test, p=0.02). Data represent two experiments with independent cohorts of mice.
4.3.2 CTLA4 RNAi Mice Develop Pathology That Progresses to Neoplasia with Age

To mimic the functional effect of subtle reduction in CTLA4 expression associated with CTLA4 polymorphisms in GC [96, 97], we used CTLA4KD transgenic mouse models [102, 103]. The CTLA4KD and PL4 vector control transgenic lines on the B6 genetic background (CTLA4KD/B6 or PL4/B6) [103] were crossed with Balb/c mice to generate CTLA4KD mice and PL4 controls on the CB6F1 genetic background. Transgene-negative littermates were also used as controls. Consistent with results from anti-CTLA4 antibody treatment (Figure 1), and genetic associations of the CTLA4 locus with human GC [96, 97], CTLA4KD mice exhibited tumorigenesis in the stomach. As shown in Figure 4-2, inflammatory infiltration in the stomach mucosa of CTLA4KD mice began at 3 weeks of age, and metaplasia started to develop at 4 weeks of age. At 16-17 weeks of age, all CTLA4KD mice exhibited extensive pathology (Figure 4-2). Gross examination of the stomach could identify a “wrinkled” appearance of the external surface (Figure 4-3: A,B). At the lumenal surface, enlarged rugae were readily visible; histological examination revealed massive metaplasia (Figure 4-3: C-G). We did not detect tumorigenesis in the small and large intestines, liver, lung and pancreas by histological examination and by visual inspection throughout the animal body.
Figure 4-2. Aging-associated transition from metaplasia to neoplasia in mice with deregulated CTLA4 checkpoint by CTLA4 RNAi transgenesis. CTLA4 RNAi KD mice and controls from ~3 to ~78 weeks of age were analyzed for gastric histopathology by H-E staining of tissue sections. Broken arrow (top right) indicates the inflammatory infiltrates in 3wk-old (n=5) CTLA4KD mice. Gastric histology of controls mice (left) was compared to that of CTLA4KD animals, analyzed at the age of 4wks (second row, n=4), ~20wks (third row, n=5), ~52wks (fourth row, n=4) and ~78wks (fifth row, n=6). The neoplastic pathology in aging CTLA4KD is heterogeneous types of neoplastic cells existed. Images at 4x of sections are followed by images at 25x magnification of highlighted areas.
Figure 4-3. Modest reduction of CTLA4 expression in mice by RNAi transgenesis caused extensive metaplasia in gastric mucosa. In 3 experiments, 16-20-week-old CTLA4 RNAi KD mice, transgene-negative littermates and PL4 vector transgenic controls, all on Balb/c x B6 F1 (CB6F1) genetic background, were examined for stomach pathology. (A) Image of abdominal cavity highlighting stomach before dissection. (B) Dissected stomach showing macroscopic difference between CTLA4KD and controls. (C) The lumen of the stomach were exposed and washed with PBS before images were taken. (D) and (E) Histological sections of the WT littermate control and CTLA4KD mice, respectively, stained with H-E. Image taken with a 4x objective. (F) and (G) Images taken with 40x objectives from highlighted area in (D) and (E).
4.3.3 CTLA4 RNAi Pathology Progresses Into Metastatic Mancer

GC development perhaps represents one of the clearest examples of aging-associated carcinogenesis. We examined the impact of age on GC development in CTLA4KD mice. Metaplasia in this model persisted until ~52 weeks of age. However, by ~78 weeks of age, the mucosa exhibited extensive adenomatous lesions, but a heterogeneous tumor pathology could also be identified (Figure 4-2). Two of the six CTLA4KD animals analyzed after 70 weeks of age had tumor lesions in the liver or lungs, with histopathology (Figure 4-4) resembling signet cell metastases in human GC [162].
Figure 4-4. Likely metastasis of gastric adenocarcinoma in the liver.

Pathology analyses of the liver from CTLA4 RNAi KD mice on the CB6F1 background at 18 months of age. The animal had gastric adenocarcinoma. The tumor cells in the liver of the animal resembled signet cell metastasis in human gastric adenocarcinoma. Images were taken at the interface between healthy and cancerous tissue in the same liver, to serve as an internal control. Two of the six CTLA4KD animals analyzed after 78 weeks of age had the liver tumor pathology likely due to metastasis of GC.
4.3.4 Pathology is Dependent on Immune System and is Controlled by MHC, TCR, and Genetic Background

We next examined the effect of genetic modifiers on gastric tumorigenesis in CTLA4KD models. CTLA4KD/B6 mice had a much milder and later-onset metaplasia compared to CTLA4KD/CB6F1 animals. Metaplasia was absent in CTLA4KD/B6.H-2\textsuperscript{g7} MHC congenic strains, or in CTLA4KD/B6 mice carrying the OT1 T-cell-receptor (TCR) transgene which largely limits the T cell repertoire to a T cell clone (Table 4-1). To corroborate that the effect of CTLA4KD on gastric tumorigenesis indeed originated from immune cells, we conducted adoptive transfer experiments with spleen cells from CTLA4KD/CB6F1 mice or littermate controls. Indeed, CTLA4KD donor cells, but not the controls, caused metaplasia in reconstituted Rag\textsuperscript{1\textsuperscript{o/o}} recipients (Figure 4-5). Thus, the effect of CTLA4 modulation on initiating gastric tumorigenesis depended on T-cell repertoire and genetic modifiers including MHC. Previous studies showed that Th1 [163] and Th17 [164] could play a role in triggering inflammatory tumorigenesis. We analyzed the Th subsets in the CTLA4 modulation models. We found that CTLA4 modulation, by RNAi or antibody, promoted Th1 and Th17 differentiation (Figure 4-6).
<table>
<thead>
<tr>
<th>Genetic background</th>
<th>B6</th>
<th>CB6F1</th>
<th>CB6F1</th>
<th>NOD</th>
<th>B6. H-2\textsuperscript{b}</th>
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<td>A\c{e}</td>
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<td>Gastric metaplasia</td>
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**Table 4-1. Summary of CTLA4 RNAi effect on different strains of mice.** As an initial assessment of the effect of MHC, other genetic modifiers and T cell repertoire on the metaplastic pathology in CTLA4KD mice, we analyzed the histopathology of gastric mucosa of CTLA4KD mice on different genetic background. B6 (MHC H-2\textsuperscript{b}); CB6F1 (MHC H-2\textsuperscript{b/d}); NOD (MHC H-2\textsuperscript{g7}); B6.H-2\textsuperscript{g7} (congenic of B6 carrying NOD MHC, H-2\textsuperscript{g7}); B6.OT1 (OT1 CD8-restricted T-cell-receptor transgenic on B6 genetic background). Scoring criteria for gastric pathology as follows: -, no metaplasia; +, has detectable metaplastic lesions in the stomach; ++, \leq 50\% of basolateral mucosae has lesions; +++ is \ (> 50\% of basolateral mucosae has lesions; ++++ is 100\% of basolateral mucosae has lesions and the lesions extend up into the lumenal side of the stomach. In CTLA4KD B6, +/- signifies only a fraction of the mice examined had pathology.
Figure 4-5. Gastric metaplasia caused by adoptive transfer of spleen cells from CTLA4KD mice. To test whether the effect of CTLA4 RNAi on gastric tumorigenesis is indeed mediated by immune cells, adoptive transfer experiments were performed with splenocytes from CTLA4KD and control mice. 1.5 x 10^7 splenocytes from CTLA4KD mice, or their transgene-negative littermates, were injected into recipient Rag0/0 mice on the CB6F1 genetic background. The histology represents 6-7 mice in each group from two experiments. (A) Stomachs were sectioned and stained via H&E staining. (B) Gastric luminal pH (mean ± SEM) was measured in the fluid contents of the excised stomach, which reflected the destruction of parietal cells by immune cells. Student’s t test, ***p<0.001.
Figure 4-6. CTLA4 modulation by antibody blockade or RNAi transgenesis led to increased Th1- and Th17-immunity. Lymph node cells from the 4-5-wk-old animals on the CB6F1 genetic background were re-stimulated for 6 hours and analyzed by intracellular flow cytometry staining for cytokines. (A) representative flow cytometry plots with percentage of gated cells (n=4-7, mean ± SEM ). (B) Total cell counts of activated populations and Th subsets. One way ANOVA with a Tukey's post-hoc for multiple comparisons; *p<0.05, **p<0.01, ***p<0.001.
4.3.5 Gene Expression Profiles of Afflicted Stomachs

To gain a molecular profile of the gastric mucosa from metaplasia to neoplasia stages, we analyzed the mRNA expression of a set of genes that are associated with tumorigenesis (Figure 4-7), in animals from one to ~78 weeks of age. For proto-oncogenes, there is significant increase in Bcl2 expression in the old CTLA4KD mice versus controls, but not in Myc expression. For tumor suppressor genes, p53 expression had an unexpected increase in old CTLA4KD mice versus controls. The cell cycle control gene cyclin A1 (Ccna1) had an increased expression in CTLA4KD mice from 20 weeks of age; conversely, cyclin-dependent kinase inhibitor 1c (Cdkn1c) expression was reduced. Mmp9, Serpine1 and connective tissue growth factor (Ctgf), three genes associated with tumor growth and/or metastasis, had a consistently increased expression from metaplasia in young adults to neoplasia in old animals (Figure 4-7).
Figure 4-7. Tumorigenic gene expressions in gastric mucosa of CTLA4KD mice from metaplastic to neoplastic stages. Differential age-related gene expression in gastric tissues from CTLA4 RNAi KD mice or transgene-negative controls on the CB6F1 background was assessed with qRT-PCR. Relative (Rel.) mRNA levels were calculated against beta-actin as the housekeeping gene, then the data was normalized to the average of 1wk-old controls (n=4-6 mice per group, for each time point except for 1wk age group (n=3 for the 1wk group); mean ± SEM). Student’s t test, *p<0.05, **p<0.01, ***p<0.001.
4.3.6 Pathology is Independent of Helicobacterae Spp

The SPEM-like pathology is similar to that in B6 mice infected with H. felis (HF) [165], which prompted us to examine potential Helicobacter infection. As in other facilities [166], our specific-pathogen-free facility does not exclude Helicobacter spp. that are not considered pathogens. To investigate if the metaplasia in CTLA4KD mice was caused by undetected infection, we purified DNA from stomach samples and conducted a group-specific PCR assay for the 16S rRNA gene of all known Helicobacter species [166], or for all bacteria. As shown in Figure 4-8, a low level of Helicobacter 16S rRNA DNA was detected in normal control mice. Compared to the normal controls, the levels of Helicobacter 16S rRNA gene, as well as the 16S rRNA genes for all bacteria, was much reduced in stomach samples from CTLA4KD mice. The results indicate that the gastric tumorigenesis in CTLA4KD mice is independent of pathogenic Helicobacter infections.
Figure 4-8. Gastric mucosal metaplasia in CTLA4KD mice was NOT associated with pathogenic Helicobacter infection. (A) Quantitative PCR for all Helicobacter spp., using primers specific to the 16S rRNA gene of Helicobacter species, relative to eukaryotic genomic DNA of stomach mucosal samples assayed by primers specific to the beta-casein gene. (B) Quantitative PCR for all bacteria, using primers that are homologous to the 16S RNA gene of all bacterial species, relative to eukaryotic genomic DNA of stomach mucosal samples assayed by primers specific to the beta-casein gene. (C) Group-specific PCR to detect all known helicobacter spp., in gastric mucosa tissue (top panel), using primers specific to the 16S rRNA gene of Helicobacter species. PCR for the Rag1 gene was used as a genomic DNA loading control (bottom panel). M, molecular weight. 1, H. felis (positive control); 2,3: normal mouse gastric tissue; 4,5: CTLA4KD mouse gastric tissue. 6: negative control. Represents one of two experiments, sampling two different cohorts.
4.3.7 CTLA4 RNAi Pathology Alters Epigenetic Regulation of 5-hmC with Aging

A tumorigenic cascade is typically marked by aberrant epigenetic modifications [167]. One of the prominent changes is an altered profile of methylation in genomic DNA at the 5 position of cytosine (5mC) [168]. However, the relative abundance of 5mC in the genomes of both healthy and cancer cells made 5mC content an insensitive parameter for quantifying change in cancer cell development [169]. Prompted by a recent report of melanoma-associated loss of 5-hydroxymethylcytosine (5hmC) [169], hydroxylated 5mC, we quantified 5hmC content in genomic DNA of gastric mucosa of CTLA4KD mice vs. controls (Figure 4-9). Consistent with the finding in melanoma [169], there was a reduction of global 5hmC content in GC samples that were collected from 74- to 78-week-old animals, in comparison to that of healthy controls. There was no difference in 5hmC quantity between samples with metaplastic lesions and controls in young animals. Surprisingly, the relative decrease of 5hmC content in neoplastic lesions vs. controls in this model was not due to a decrease in GC, but an increase in the normal tissue during aging. Given that enhancing 5hmC led to protection against cancer development [169], the observation suggests that overall 5hmC levels might reflect a protective mechanism in aging cells against cancer development in certain organs.
**Figure 4-9. Aging-associated increase in 5hmC in normal stomach mucosa but not in that of CTLA4KD mice.** (A) Experiment 1: Relative quantification of 5hmC content in DNA from stomach mucosal tissue of CTLA4 RNAi KD mice and littermate controls of 4 week old mice (n=3 in each group, mean ± SEM). Experiment 2: Relative quantification of 5hmC content in DNA from stomach mucosal tissue of CTLA4KD mice and littermate controls, from 4 to 78 weeks of age. Each bar represents one animal. Exp 3: 5hmC content in DNA in cohorts of CTLA4KD and normal controls from ~20 to ~78 weeks of age (n=3 in each group for each time point, mean ± SEM). (B) Representative dot-plot of 5hmC staining of genomic DNA (top), compared to methylene blue staining of DNA (loading control).
4.3.8 Tet2 and Idh2 are Differentially Regulated in Mice with Gastric Pathology

Hydroxylation of 5mC to 5hmC is catalyzed by the ten eleven translocation (TET) enzymes [159, 169], which use 2-oxoglutarate as a co-substrate. 2-oxoglutarate, an intermediate in the Krebs cycle, is produced by isocitrate dehydrogenase (IDH)-mediated metabolism of isocitrate. We tested whether dysregulation of 5hmC in stomach mucosa of aging CTLA4KD mice was associated with altered expression of Tet and Idh genes. We did not detect significant differences between CTLA4KD mice and controls in Tet2 and Idh1 (cytoplasmic Idh). There was an age-associated increase of Tet2 mRNA after 52 week of age versus younger adults, for both CTLAKD and controls (Figure 4-10; Figure 4-11). For Tet1 and Tet3, there was a modest decrease in CTLA4KD samples at 4wk of age when metaplasia was initiated, but not in other age groups. The expression of Idh2 (mitochondrial Idh homolog) in CTLA4KD and control mice was comparable at 1 week of age. However, its expression in CTLA4KD mice was substantially reduced through 4 to 78 weeks of age, after metaplasia initiation (Figure 4-10). It is not known what caused Idh2 dysregulation even in non-malignancy (metaplasia). Given the complex relationship among tissue oxygen supply, metabolism, and epigenetic regulation, we searched for evidence of hypoxia-induced responses. Although it is now generally accepted that established tumors create and thrive in a hypoxic microenvironment [170], it remains unclear when hypoxia develops and how it affects progression of pre-neoplastic lesions. We analyzed the expression of
hypoxia-induced factors (HIFs) from metaplasia to neoplasia stages. Hif1a and Hif2b were induced throughout metaplasia to neoplasia (Figure 4-10). Therefore, the hypoxic response was associated with decreased expression of Idh2, which might contribute to 5hmC dysregulation.

4.3.9 5-hmC Reduction also Occurs in Human Gastric Adenocarcinoma

We next examined 5hmC dysregulation in human GC. We obtained GA samples and healthy control tissues and conducted 5hmC staining as described [160, 169]. As shown in Figure 4-12, the proportion of 5hmC+ cell nuclei was substantially reduced in GA cell nuclei, compared to healthy tissue controls. These results on human GC, taken together with the evidence from CTLA4KD mice, suggest that 5hmC dysregulation may play a role in aging-associated GC development. Such epigenetic abnormity may account for, at least in part, the perturbation of global gene expression profiles documented in previous studies [171, 172].
Figure 4-10. Age-associated profiles of Tet, Idh, and Hif mRNA expression in gastric mucosa of CTLA4KD and control mice. Differential age-related gene expression in gastric tissues from CTLA4 RNAi KD mice or transgene-negative controls on the CB6F1 background was assessed with qRT-PCR. Relative (Rel.) mRNA levels were calculated against beta-actin as the housekeeping gene, then the data was normalized to the average of 1wk-old controls (n=4-6 mice per group, for each time point except for 1wk age group (n=3 for the 1wk group; mean ± SEM). Student’s t test, *p<0.05, **p<0.01, ***p<0.001.
Figure 4-11. Age-associated increase in Tet2 expression in both CTLA4KD mice and controls. 4-20 week old mice were compared to older mice (52-78 weeks) for Tet2 mRNA levels, quantified by qRT-PCR, and normalized to Beta-actin expression. Each Dot represents one mouse (n=8). *p<0.05, One way ANOVA was performed, with a Tukey’s post-hoc analysis for intra group comparison.
Figure 4-12. 5hmC reduction in human gastric adenocarcinoma (GA). GA samples and normal tissues were sectioned and stained for 5hmC. Results are representative of 5 normal gastric tissue samples and 6 GA samples. (A) Representative immunofluorescence staining (top panels, no 1o Ab staining control of normal tissues; middle and bottom panels, 5hmC staining of healthy and tumor sections, respectively). Left, DAPI counterstain showing cell nuclei; middle, 5hmC staining; right, the merged images of DAPI and 5hmC staining. (B) Percentages of 5hmC+ nuclei in the normal vs. tumor group (n=5-6 per group, mean ± SEM, p<0.001, Student’s t test). Total 7230 and 7434 nuclei were randomly sampled in the normal and tumor group, respectively. Each data point represents one sample.
4.4 **Discussion**

Chronic inflammatory signals from innate immune cells have been shown to promote progression of nascent tumors [147, 173, 174]. However, the role of inflammation in initiation of tumorigenesis remains to be characterized. Advances in human genetics have offered a number of novel insights into cancer biology. Relevant to the work described herein, polymorphisms in CTLA4 that result in down-regulation of its expression are more common in GC patients compared with controls [96, 97]. On the other hand, anti-CTLA4 treatment is a recently approved, prototypical antitumor therapy which blocks immune checkpoints, and a commonly detected side effect of anti-CTLA4 treatment includes gastritis [47]. It is therefore critical to understand the biological function of CTLA4 down-regulation in the initiation of GC and also the potential impact of anti-CTLA4 therapy in the context of patients with gastric pathology.

In this study, anti-CTLA4 antibody treatment, or modest reduction of CTLA4 by RNAi, caused mild inflammatory damage to the stomach mucosa and initiated metaplasia that spontaneously led to neoplasia. Carcinogenesis is generally thought to be caused by accumulation and selection of multiple mutational events in individual cell clones, according to the well-supported “multiple hits” hypothesis [175]. In our models, metaplasia was initiated with antibody blockade or RNAi inhibition of the CTLA4 immune checkpoint, without intentional mutagenesis. In addition, the extensive metaplasia across the mucosal area does not suggest a single clonal origin of the mutant lineage. We explored the possibility that Lgr5+ gastric stem cell lineage [176] that distributes
in the crypt along gastric mucosa might be the origin of cancer cells, but so far we have not found solid evidence in that regard (JM and ZC, unpublished data). The triggers of metaplasia development, including the antigenic resource and the nature of the inflammatory signals, have yet to be identified. Autoimmune damage to gastric mucosa, such as in pernicious anemia, has been associated with risk of human GC [177]. It is possible that CTLA4 modulation led to a breakdown of immune tolerance to self-antigens in gastric mucosa and thus initiated a cascade of inflammatory carcinogenesis. Alternatively or in addition, dysregulation of CTLA4-mediated host immune control could lead to aberrant responses to the normal gastric microflora (also called microbiota). Normal flora has been suspected to play an important role in inflammatory carcinogenesis in the gastrointestinal tract in general [178] and has been implicated in HP-induced GC in particular [179]. Furthermore, although gastric carcinogenesis induced by CTLA4 modulation did not require pathogenic Helicobacter infection, there could be synergy between CTLA4 modulation and HP. Anti-CTLA4 trials have been expanded to various cancer patient populations including children. Since HP colonizes the stomach of a majority of humans, it might be clinically pertinent to understand how the immune therapy interacts with HP in gastric inflammation and carcinogenesis.

As in most cancer cases, the exact cause of human GC remains elusive. An oft-repeated observation is to the effect that cancer, particularly that of solid organs, is largely a problem of aging. How aging affects cancer development has long been a subject of intense interest. The relationships between cancer and
aging are complex and even paradoxical, perhaps reflecting multiple pathways that underlie the two processes [180, 181]. Tumor suppressor genes such as \textit{p53} can inhibit tumor development by promoting senescence [182], although such effect can also be pro-tumor [183]. Deficiency of TA\textit{p73}, another tumor suppressor in the p53 family, causes premature aging [184]. While these experiments and many other studies unequivocally demonstrate the intricacy between cancer and aging, the studies of the exact effect of aging on cancer development has been impeded by the lack of a robust model with spontaneous cancer development associated with aging. The CTLA4KD mice exhibited synchronized initiation of metaplasia at a young age and spontaneous progression from metaplasia (non-malignant growth) to neoplasia at an older age. Of note, non-malignant growth can be readily detected in a typical elderly individual. While the exact mechanisms remain to be defined for the prevention of malignant transition, we have detected an epigenetic abnormality in our animal models that might suggest how, on the other hand, aging could also protect against carcinogenic development.

Aberrant epigenetic modifications are essential in cancer development [167]. One of the well-characterized epigenetic marks is the methylation in genomic DNA at the 5 position of cytosine (5mC) [168]. The conversion of 5mC to 5hmC is so far the most consistent mechanism underlying active DNA demethylation. It has been shown as a marker and a protective mechanism against cancer [169]. A global loss of 5hmC was also evident in human GC. How, and at what stage, this epigenetic difference arises remains unclear. In the
CTLA4KD models of gastric carcinogenesis, there was no difference in the overall quantity of 5hmC content in DNA between the metaplastic tissue and normal controls. Interestingly, in the healthy tissue there was an age-associated increase of 5hmC in DNA. The relatively lower content of 5hmC in DNA of cancerous tissue was not due to an absolute loss during malignant transition, but a recalcitrance of metaplastic cells to age-associated increase. The relatively lower content of 5hmC in the mucosal cells of the aging mice during transition from metaplasia to neoplasia might reflect a decelerated dynamics of DNA modification, which could eventually lead to genomic instability. Therefore, the studies of aging-associated spontaneous development of GC in CTLA4KD models uncovered two opposite effects of aging on GC development: promoting transition from metaplasia to neoplasia by yet-to-be identified factors, and potential protection by 5hmC-based epigenetic regulation.

Similar to other highly lethal cancers, stomach cancer has a poor prognosis [78]. Therefore, the key to lowering the burden of this disease may lie in prevention of malignant development. This study demonstrates distinct roles played by a genetic risk followed by an epigenetic abnormality in the gastric carcinogenic cascade. First, subtle genetic variations in an immune checkpoint gene predispose individuals to initiation of tumorigenesis through inflammation. Second, a nonmalignant lesion can be kept in check for a long time, but yet-to-be-identified factors associated with aging promote its progression to cancer; this progression occurs in the absence of sufficient epigenetic control mediated by 5hmC, which by itself may be a protective mechanism against cancer.
development during healthy aging. Together with recent breakthroughs in understanding the 5hmC epigenetic pathways [159, 169], and potential druggability of this pathway [160], the insight gained from this study may help develop strategies for early detection in high-risk populations and prevention of transition from non-malignant lesions to neoplasia.
CHAPTER 5: CONCLUSIONS

5.1 Immunology at the Target Tissue: the Next Frontier

Our studies within the immunological target tissue provide key answers and new exciting questions into immunological research. Our data shows for the first time that $T_{\text{eff}}$ and $T_{\text{reg}}$ have long lasting, meaningful contacts in-vivo. What was once considered an in-vitro trademark of $T_{\text{reg}}$ function [126] now have rejuvenated this once discarded philosophy. This suggests that promoting intimacy between $T_{\text{eff}}$ and $T_{\text{reg}}$ can have therapeutic outcomes in preventing autoimmunity; and perturbing these interactions could lead to more effective anti-tumor immunity.

Furthermore, our data also demonstrates islet cell proliferation within a matter of days; another observation that could be visualized within the target tissue. Only recently have other groups demonstrated the quick growth of $\beta$-cells in-vitro [138]. Our study is the 1st to demonstrate this in-vivo, providing promise for future research and treatment for diabetes.

The drastic difference in T-cell behavior that we see compared previous studies that have looked into the lymph nodes [32, 33] suggests that the target tissue is a dynamic environment that is regulated very differently than the lymph node. This includes tissue specific signals [185], tissue resident T-cell populations [186], and the architecture of the tissue being studied.

Recently the function of T-cells in these target tissues has been highlighted, and some very important differences have been discovered. Recently it has been shown that CD4$^+$ have cytotoxic functions in the target
tissues [187], a function traditionally thought to exist exclusively within CD8+ T-cells. Tissue resident T-cells behave much differently than their circulating counterparts; in many models tissue resident memory cells perform significantly better than phenotypically similar cells in the periphery [188].

These studies and our data highlight the drastic difference between T-cell biology in the peripheral tissues versus traditional research within lymphoid tissues. This paradigm shift has been critical to our understanding of immunobiology and will continue to produce insights for years to come.

5.2 The Immunological Identity of Tumor

Recent clinical trials of cancer immunotherapies have generated exciting progress [47-49]. The ultimate success of such interventions, however, will likely pin on the immunological identity of the tumor. Adaptive immunity is characterized by fine specificities, with a repertoire of lymphocyte clones capable of discriminating the body’s “self” tissues from “non-self”, foreign substance. Tumor presents a dilemma to this dichotomy. Tumors cells are derived from transformation of healthy cells in the body, i.e., with a “self” origin. However, they are also characterized by genome instability [175], and thus presumably generate a composition of new antigens (neoantigens) that may not be “seen” as “self” by the immune system. A long-standing premise of tumor as altered “self” posits that there are sufficient antigenic changes in tumors for an effective immunosurveillance against tumors [189]. However, searching for tumor-specific antigens (TSAs) in human cancers remains a struggle, and clinical benefits of cancer immunotherapy were often intricately tangled with autoimmune toxicity.
[45], leading to a supposition that tumor cells, no matter how malignant there are, are perhaps largely “self”.

To examine how self-antigen-specific immune effectors deal with tumors, we used CD4$^+$ or CD8$^+$ effector T ($T_{\text{eff}}$) cell clones that are fully capable of spontaneous autoimmune destruction [103]. The CD4$^+$ and CD8$^+$ autoimmune $T_{\text{eff}}$ cells were tested for their efficacy in animals against insulinoma or lymphoma cancer cells, versus healthy tissue cells that express the same antigens. A few observations from this study stood out with its implications to cancer immunotherapies. First, an autoimmune $T_{\text{eff}}$ cell clone could eradicate an established tumor, if there were no adaptive immune suppressor cells, despite the presence of innate immune suppressors including myeloid-derived suppressor cells. Second, a suboptimal fraction of self-antigen-specific, Foxp3$^+$ regulatory T ($T_{\text{reg}}$) cells that were ineffective in healthy tissue were nonetheless sufficient to protect tumors, in both adoptive T-cell transfer settings and acute $T_{\text{reg}}$-cell depletion experiments. Third, in an adoptive T-cell therapy setting, intrinsic modulation of cytotoxic lymphocyte antigen 4 (CTLA4) by RNAi could substantially boost the efficacy of autoimmune $T_{\text{eff}}$ cells against tumors [103].

We concluded that tumor represents an immunoprivileged “self”, based on the result that tumors could more efficiently utilize self-tolerance mechanisms to avoid autoimmune attack than their healthy tissue counterpart [103]. The immunoprivilege concept has long been used to explain a status heightened protection from adverse immune damage, for a few critical organs, such as the brain, eye and testis. Traditional view of immunoprivilege has been centered on
exclusion of immune cells from the “privileged” cites. However, recent studies has shift the focus more on active immune regulation in immunoprivileged tissue [114]. Along this vein, one can extrapolate exclusion-based immunoprivilege to some types of cancer, e.g., lung cancer, and immunoprivilege mainly mediated by in situ regulation to other cancers, such as melanoma.

It should be noted that there is a large body of evidence from experimental tumor models that tumor-specific immunity can be readily achieved, and antitumor immunity can kill tumors without autoimmunity toxicity (for review see [46]). Our study [103] does not contradict those previous findings. The focus of our study [103] was to test how potent autoimmune T cells respond to an established tumor, beginning when the tumor size was very small. The study was not purported to test potential surveillance by the autoimmune T eff cells at tumor initiating stages. The study was not an “attack” on the immune surveillance hypothesis, nor a direct refutation of the widely-accepted view of tumor as altered “self” [189]. Likely, both altered “self” and immunoprivileged “self” could be represented in the natural history of a spontaneous tumor.

Nonetheless, the premises of tumor as an altered “self” or an immunoprivileged “self” project distinct implications for cancer immunity and immunotherapies (Figure 5-1). In the altered “self” view, genetic changes in the tumor initiating cell (TIC) generate an array of neoantigenic epitopes. Tumors evade immune attack with a suppressive microenvironment constituted with many immune inhibitors. Targeting tumor-specific antigens, coupled with blocking the suppressive factors, can reduce the tumor burden and eventually eradicate
the tumor. On the other hand, in the immunoprivileged “self” view, despite substantial genetic and epigenetic changes in TIC, neoantigens only account for a minimal fraction, compared to the large array of self-antigens. The established tumor is largely “self” in its immunological identity. Furthermore, immunosuppressive elements orchestrated by self-antigen-specific T_{reg} cells forms a local environment in tumors that can inhibit even potent autoimmune effectors. In this view, neoantigen-specific antitumor immunity has only a limited impact on editing the tumor identity, leaving behind an immunoprivileged “self” core of tumor burden.

Is tumor an immunology problem or oncology one? [190]. The “immunoprivileged self” hypothesis would suggest that tumor is an immunology problem at its root, yet eradication of this problem would be beyond the reach of immunology, without synergy from oncological intervention. “But the worst enemy you can meet will always be yourself…”, as a nineteen-century German philosopher, Friedrich Nietzsche, put it in Thus Spoke Zarathustra, “you must be ready to burn yourself in your own flame…”. Tumor as an immunoprivileged “self” could be the worst for the immune system to face. “Your own flame”, autoimmunity-based inflammatory destruction, can be effective, but only if the “burn” is not life-threatening. Therefore, the impact immune therapies alone may often be limited, unless the tumor “self” is substantially altered or the tumor immunoprivilege is eliminated, by physical intervention such as surgical removal and radiation therapy, or chemical targeting.
Figure 5-1. The altered “self” or immunoprivileged “self” premises of tumor identity and their Implications. Blue dashes depict immunosuppressive microenvironments associated with tumors. Oval areas reflect overall tumor burdens and do not necessarily represent individual tumor sites. TIC: tumor initiating cell.
5.3 Understanding the Complex Interplay Between Inflammation, Aging and Cancer

The connection between inflammation and cancer was originally theorized over 150 years ago by the physician Rudolf Virchow [191]. Only recently has his theory not only been validated, but proven to be a crucial promoter the tumorigenic response. It is then no surprise that autoimmunity plays a force not only in antitumor immunity (see above) but also in tumorigenesis. Indeed our stomach model is line with previous studies of autoimmune induced gastritis [34, 99]. These models utilized TCR transgenic animals, and highly artificial scenarios to produce their results.

The strength of our model, is that our mice exhibit CTLA4 reduction comparable to human polymorphisms [102], and this reduction causes gastritis. This provides direct comparison to humans, and allows us to study how this subtle reduction in CTLA4 eventually leads to gastric cancer as previous association studies have shown [96, 97].

Our studies have uncovered that autoimmune gastritis reliably precedes gastric cancer, and age-associated molecular “switches” govern this transition. These switches are genes that govern epigenetic regulation of 5-hmC, a further modification of 5-mC. This hydroxymethylation is emerging as a critical hallmark of cancer [169]. Our data extends this notion to gastric cancer. Perhaps more importantly, our data suggests that genomic 5-hmC content increases with age, to serve a possible protective mechanism against aging. As CTLA4 RNAi mice age, they are unable to upregulate 5-hmC as aging progress. This process is
mediated by the hypoxic cancer environment which has already been linked to reduction in TET (and IDH) enzymes [169].

Therefore our model provides unique and far reaching insights into the complex interplay between cancer and aging. Also because of the similarities between our models and humans, we can learn new insights on how to potentially alleviate or prevent gastric cancer in humans. Also our data also suggests the possibility that Anti-CTLA4 treatment can, in the long term, lead to gastric carcinogenesis. Since Ipilumimab has been recently cleared by the FDA for the treatment of melanoma; our data suggests caution with its use.

The data presented in this thesis demonstrates the necessity for further understanding of the extraordinarily complex mechanisms underpinning autoimmunity. Elucidating why we attack ourselves, how we prevent it, and how (and when) to promote it; will be critical to future successes in treating human diseases.
REFERENCES


