ADAPTIVE IMMUNE SUPPRESSION IN
THE TUMOR MICROENVIRONMENT
ADAPTIVE EVENTS IN THE TUMOR LIMIT THE SUCCESS OF CANCER IMMUNOTHERAPY

By

ASA JAMES ROBERT MCGRAY, B.Sc, M.Sc

A Thesis Submitted to the
School of Graduate Studies in Partial Fulfillment
of the Requirements for the Degree

Doctor of Philosophy

McMaster University © Copyright by AJ Robert McGray, October 2012
ABSTRACT

INTRODUCTION: Pre-clinical and clinical data strongly support the use of immunotherapies for cancer treatment. Cancer vaccines offer a promising approach, however, the outcomes of clinical vaccine trials have been largely disappointing, prompting a need for further investigation.

METHODS: Using the B16F10 murine melanoma, we have investigated the local events within growing tumors following recombinant adenovirus immunization.

RESULTS: In chapter 2, we investigated the ability of a pre-clinical vaccine to elicit only transient tumor growth suppression. We observed that tumors were initially infiltrated by a small number of highly functional tumor-specific CD8+ T cells following vaccination that instigated a rapid adaptive response in the tumor that suppressed local immune activity.

In chapter 3 we questioned whether increasing the rate and magnitude of early immune attack would result in more robust tumor attack prior to tumor adaptation. Increasing the rate of tumor-specific CD8+ T cell expansion following vaccination resulted in tumor regression and durable cures in approximately 65% of treated mice. Further analysis revealed that tumor regression correlated with an early burst in immune attack that outpaced tumor adaptation.

In chapter 4, we explored whether the same vaccine could be improved when combined with immunomodulatory antibodies. Vaccination combined with α4-1BB and
αPD-1 resulted in complete tumor regression and durable cure of >70% of treated animals and was associated with increased local immune activity. Gene expression profiling revealed a unique gene signature associated with the curative treatment, which was also associated with positive outcome in human melanoma patients.

**CONCLUSIONS:** The described research sheds new light on mechanisms that limit the efficacy of therapeutic cancer vaccines. Namely, rapid tumor adaptation, triggered by early vaccine-induced CD8+ T cells, acts to suppress the local immune response prior to maximal immune attack. Strategies to overcome these adaptive processes should therefore be considered in future vaccine design.
Acknowledgements

In reflecting on my years spent as a PhD student at McMaster University, it is clear that many people have made significant contributions, scientifically or otherwise, to my academic achievements.

First and foremost, I would like to express tremendous gratitude to my supervisor, Dr. Jonathan Bramson. Jonathan, you have been a true scientific mentor in all possible ways and have repeatedly challenged me to achieve my scientific potential. For this, I will be forever grateful. You have worked hard to establish a first-rate research environment both in your lab and at MIRC and I want to thank you for the opportunity to conduct research in your lab over the past four and a half years. I would also like to thank my committee members, Dr. Yonghong Wan, Dr. Manel Jordan, and Dr. Pingchang Yang for insightful conversations regarding my research progress during committee meetings.

I would also like to thank all Bramson Lab members, past and present, for your tremendous help and support during my PhD studies and for creating a professional, enjoyable, and collaborative work environment – the long harvest days never felt so bad with the lab’s “We’re all in this together” philosophy! First, I wish to thank past lab members Dr. Natalie Grinshtein and Mike Vantresca for helping me to “get my hands dirty” on my early mouse experiments – I’ve come along way since those early days! I would also like to thank past lab members Dr. Dannie Bernard, Dr. Jennifer Bassett, Caitlin Gregory, Mayank Jha, Kory Zane, Florentina Teoderascu, and Ryan Kelly as well
as current lab members Carole Eveleigh, Dr. Stephanie Swift, Heather VanSeggelen, Joni Hamill, Galina Denisova, Robin Parsons, Dr. Farzad Habibagahi, Alina Lelic, and Dr. Jamie Millar - Not only for their technical support, but also for their part in building the lab camaraderie that makes the Bramson Lab a great place to work. I would also like to acknowledge our collaborators who have greatly assisted with our research. First, to Dr. Robot Hallett for lending his bioinformatics expertise to our projects, as well as to Dr. Byram Bridle, Dr. Jonathan Pol, and Dr. Jeanette Boudreau for helpful scientific conversations related to our work. Also, thank you to Dr. Arthur Hurwitz and Dr. Ziqiang Zhu at the NCI for providing us with transgenic T cells for our studies. I also wish to acknowledge the excellent technical staff in the Central Animal Facility, who look after our experimental animals, as well as the Terry Fox Research Institute for providing research funding without which this work would not have been possible.

To my wonderful wife, Colleen – Words simply cannot express my infinite gratitude to you for all the patience, understanding, and support you have given me over my years as a graduate student. I love you and will be forever grateful to you for helping me to keep things in perspective through the many challenges that science can present. To my parents, Kevin and Cathy, my brother Brian, as well as my friends – Thank you for your continued support of my academic endeavors. Lastly, I would like to thank all of the talented musicians that I have had the privilege of performing with over the past years. Scientific research is a grueling endeavor - music has provided an important diversion for me during my studies and you are all an important part of what I have accomplished scientifically.
# Table of Contents

**TITLE PAGE.................................................................** i
**DESCRIPTIVE NOTE............................................................** ii
**ABSTRACT..............................................................................** iii
**ACKNOWLEDGEMENTS............................................................** v
**TABLE OF CONTENTS............................................................** vii
**LIST OF FIGURES AND TABLES...............................................** x
**LIST OF ABBREVIATIONS AND SYMBOLS...................................** xiii
**DECLARATION OF ACADEMIC ACHIEVEMENT...........................** xvi

## CHAPTER 1: Introduction

1.0 **Cancer**  
1.1 The biology of cancer  
1.2 Standard cancer therapies  
1.3 The requirement for new cancer treatments  
1.4 The origins of cancer immunotherapy  

2.0 **The immune system and cancer therapy**  
2.1 Innate and adaptive immunity  
2.2 Immune cell subsets  
2.2.1 Lymphocytes  
2.3 CD8+ T cell immunity  
2.4 CD4+ T cell immunity  
2.5 Regulatory T cells (Tregs)  
2.6 Regulation of T cell homeostasis  
2.7 Myeloid cells  
2.7.1 Myeloid-derived suppressor cells (MDSCs)  
2.7.2 TAMs/TIDCs  
2.8 T cells and cancer  
2.9 Tolerance to self antigens  
2.10 Antigen presentation and tumor antigens  
2.10.1 Antigen processing and presentation  
2.10.2 Tumor antigens  

3.0 **Cancer immunotherapy**  
  

3.1 Immune surveillance and immunoediting 33
3.2 Cancer Vaccines 35
3.3 Adoptive T cell transfer 40
3.4 Immunomodulatory antibodies 44
3.5 Autoimmune risks in targeting tumor antigens 47
3.6 Assessing the effectiveness of anti-tumor immunity 48

4.0 Immune suppression in the tumor 50
4.1 Defects in tumor antigen presentation 50
4.2 Immunosuppressive factors within the tumor environment 51
4.3 Immunosuppressive ligands and receptors in the tumor environment 54

5.0 Models used in this thesis 56
5.1 Summary 56
5.2 Melanoma 56
5.3 Recombinant adenovirus vectors 58
5.4 Tumor antigens – DCT and gp100 58
5.5 DCT_{180-188} TCR-transgenic mice (TCR^{hi} mice) 60

6.0 Scope of described research 61
6.1 Previous work by the Bramson group relevant to the described thesis research 61
6.2 Research objectives 62

Chapter 2: Methods 64

Chapter 3: Early infiltration of DCT-specific CD8+ T cells instigates an adaptive response in the tumor that limits the duration of T cell attack 74
Introduction 75
Results 77
Discussion 86
Figures 90

Chapter 4: The rate of CD8+ T cell expansion following therapeutic cancer vaccination is directly related to overcoming the adaptive response within the tumor and to the effectiveness of the vaccine 113
Introduction 114
Results 116
Discussion 124
Figures 128
Chapter 5: Combined vaccination and immunostimulatory antibodies provides durable cure of murine melanoma and induces transcriptional changes associated with positive outcome in human melanoma patients 143

Introduction 144
Results 146
Discussion 155
Figures 160

Chapter 6: Final Discussion 185

1.0 Summary of research findings 186

2.0 Implications of techniques employed in assessing anti-tumor immune activity 189
2.1 Direct intratumoral analysis permits the discrimination of parameters that lead to ineffective anti-tumor immunity 190
2.2 Repeat sampling of tumor tissues to understand dynamic local events 190
2.3 Improved resolution of anti-tumor immunity through the use of complimentary assays 194

3.0 Biological Implications 197
3.1 Immune suppression in the tumor is multifaceted 197
3.2 Can blockade of immune suppression actually result in co-stimulation? 198
3.3 Adaptive immune suppression in the tumor – does the tumor take advantage of conventional homeostatic mechanisms of immune tolerance? 200
3.4 Relevance of T cell polyfunctionality versus activity in the anti-tumor immune response 202
3.5 The role of tumor-infiltrating myeloid cells in the anti-tumor immune response elicited by rHuAd5-hDCT 204
3.6 Can currently available drugs be used to modulate the tumor environment in favor of improved vaccine efficacy? 207

4.0 Are our observations clinically meaningful? 210

5.0 Concluding Remarks 217

References 221
List of Figures and Tables

Chapter 1

Figure 1: MHC I and MHC II antigen presentation 29

Chapter 3

Figure 2: CD8+ T cells and IFN-γ mediate vaccine-induced tumor growth Suppression 90

Figure 3: rHuAd5-hDCT vaccination results in early intratumoral immune activity that diminishes prior to peak infiltration of DCT-specific CD8+ T cells 92

Figure 4: DCT-specific CD8+ T cells develop a progressive loss in intratumoral immune activity 95

Figure 5: DCT-specific CD8+ TIL develop progressive defects in Polyfunctionality 98

Figure 6: Intratumoral activity by DCT-specific CD8+ T cells results in transient induction of genes associated with immune signaling and antigen presentation 100

Figure 7: Upregulation of immunosuppressive genes in the tumor following vaccination correlates with the early activity of DCT-specific CD8+ T cells 103

Figure 8: Local immune activity instigates the adaptive immunosuppressive response in the tumor but is also required to sustain the adaptive response 106

Table 1: Changes in intratumoral gene expression from day 9 to day 11 post rHuAd5-hDCT immunization 107

Figure 9: The presence of CD8+ TIL correlates with the expression of immunosuppressive genes in human melanomas 108

Table 2: Correlation between the expression of CD8α and immunosuppressive genes in human melanomas 109
Figure 10: rHuAd5-hDCT immunization instigates global transcriptional changes in the tumor 110

Table 3: Positively enriched pathways in tumors following treatment with rHuAd5-hDCT 111

Chapter 4

Figure 11: Increasing the rate of DCT-specific CD8+ T cell expansion results in tumor regression and improved survival that correlates with the early CD8+ T cell response 128

Figure 12: Treatment with rHuAd5-hDCT + 10^6 DCT T cells results in early tumor infiltration by a greater number of DCT-specific CD8+ T cells which distribute throughout the tumor 131

Figure 13: High dose DCT T cell transfer results in sustained immune activity by DCT-specific CD8+ TIL, but CD8+ T cells still show defects in polyfunctionality 134

Figure 14: Robust early immune activity is able to outpace the adaptive suppressive response in the tumor 138

Figure 15: Transfer of high dose DCT T cells in combination with rHuAd5-hDCT initiates similar global changes within the tumor as vaccination alone, but of greater magnitude 141

Chapter 5

Figure 16: Stimulation of 4-1BB enhances the DCT-specific immune response following vaccination resulting in transient tumor regression and improved survival 160

Figure 17: PD-1 is upregulated on tumor-specific CD8+ TIL following tumor infiltration in the context of elevated immunosuppressive PD-1 ligand expression in the tumor 163

Figure 18: Vaccination combined with 4-1BB stimulation and PD-1 blockade results in complete tumor regression 166

Figure 19: 4-1BB co-stimulation and PD-1 blockade following vaccination synergize
to increase immune activity within the tumor, despite no increase in the number of DCT-specific CD8+ T cells and only limited improvements in T cell polyfunctionality.

**Figure 20:** Identification of treatment specific probes

**Table 4:** Gene ontology biological process analysis of treatment specific genes

**Table 5:** Top 25 genes associated with each treatment based on PAM analysis

**Figure 21:** rHuAd5-hDCT + α4-1BB + αPD-1 treatment probes are associated with positive outcomes in human melanoma patients

**Table 6:** List of 85 genes uniquely upregulated in tumors following treatment with rHuAd5-hDCT + α4-1BB + αPD-1, defined as the Immune-Index

**Table 7:** Common Genes between the Immune-Index and rHuAd5-hDCT Treatments

**Table 8:** Top 10 GO terms associated with genes upregulated in both the Immune-Index and rHuAd5-hDCT treatments

**Chapter 6**

**Figure 22:** Schematic summary of findings that mediate differential outcomes following treatment with rHuAd5-hDCT

**Table 9:** Myeloid cell signature derived from Immune-Index

**Table 10:** T cell signature derived from Immune-Index
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>δ</td>
<td>delta</td>
</tr>
<tr>
<td>γ</td>
<td>gamma</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
</tr>
<tr>
<td>ζ</td>
<td>zeta</td>
</tr>
<tr>
<td>2B4</td>
<td>CD244 natural killer cell receptor</td>
</tr>
<tr>
<td>ACT</td>
<td>adoptive T cell transfer</td>
</tr>
<tr>
<td>AIDs</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AIRE</td>
<td>autoimmune regulator</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell or allophycocyanin</td>
</tr>
<tr>
<td>Bim</td>
<td>BCL-2 interacting mediator of cell death</td>
</tr>
<tr>
<td>BTLA</td>
<td>B and T lymphocyte associated</td>
</tr>
<tr>
<td>CAR</td>
<td>chimeric antigen receptor</td>
</tr>
<tr>
<td>Cbl-b</td>
<td>casitas B-lineage lymphoma b</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C chemokine ligand</td>
</tr>
<tr>
<td>CCCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CIITA</td>
<td>class II transactivator</td>
</tr>
<tr>
<td>CLIP</td>
<td>class II-associated Ii peptide</td>
</tr>
<tr>
<td>CR</td>
<td>complete response</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T lymphocyte antigen-4</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>C-X-C chemokine receptor</td>
</tr>
<tr>
<td>Cy</td>
<td>cychrome</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DCT</td>
<td>dopachrome tautomerase</td>
</tr>
<tr>
<td>DHICA</td>
<td>dihydroxyindole carboxylic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FNA</td>
<td>fine needle aspirate</td>
</tr>
<tr>
<td>FoxP3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GATA-3</td>
<td>GATA sequence binding transcription factor 3</td>
</tr>
<tr>
<td>GEO</td>
<td>Gene Expression Omnibus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>GIST</td>
<td>GI stromal tumors</td>
</tr>
<tr>
<td>GITR</td>
<td>glucocorticoid-induced TNFR-related protein</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>gp100</td>
<td>glycoprotein 100 kDa</td>
</tr>
<tr>
<td>hDCT</td>
<td>human DCT</td>
</tr>
<tr>
<td>hgp100</td>
<td>human gp100</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HPV</td>
<td>human papillomavirus</td>
</tr>
<tr>
<td>ICOS</td>
<td>inducible T cell co-stimulator</td>
</tr>
<tr>
<td>ICS</td>
<td>intracellular cytokine staining</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine 2,3-dioxygenase 1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>irRC</td>
<td>immune-related response criteria</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LAG-3</td>
<td>lymphocyte activation gene 3</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>mDCT</td>
<td>murine DCT</td>
</tr>
<tr>
<td>MDSC</td>
<td>myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MFI</td>
<td>mean/median fluorescent intensity</td>
</tr>
<tr>
<td>mgp100</td>
<td>murine gp100</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NK Cell</td>
<td>natural killer Cells</td>
</tr>
<tr>
<td>NKT Cell</td>
<td>natural killer T cells</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
</tr>
<tr>
<td>PAM</td>
<td>prediction analysis of microarrays</td>
</tr>
<tr>
<td>PAMPS</td>
<td>pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood lymphocyte</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>progressive disease</td>
</tr>
<tr>
<td>PD-1</td>
<td>programmed death 1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>programmed death ligand 1</td>
</tr>
<tr>
<td>PD-L2</td>
<td>programmed death ligand 2</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque-forming unit</td>
</tr>
<tr>
<td>PGE2</td>
<td>prostaglandin-E2</td>
</tr>
<tr>
<td>PLC</td>
<td>peptide loading complex</td>
</tr>
<tr>
<td>Term</td>
<td>Full Form</td>
</tr>
<tr>
<td>------</td>
<td>-----------</td>
</tr>
<tr>
<td>PR</td>
<td>partial response</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate specific antigen</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>rAd</td>
<td>recombinant adenovirus</td>
</tr>
<tr>
<td>RAG-2</td>
<td>recombination activating gene 2</td>
</tr>
<tr>
<td>RCC</td>
<td>renal cell carcinoma</td>
</tr>
<tr>
<td>RECIST</td>
<td>response evaluation criteria in solid tumors</td>
</tr>
<tr>
<td>rHuAd5</td>
<td>recombinant human adenovirus serotype 5</td>
</tr>
<tr>
<td>RMA</td>
<td>robust multi-array analysis</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>stable disease</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAA</td>
<td>tumor associated antigen</td>
</tr>
<tr>
<td>TAM</td>
<td>tumor associated macrophage</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen presentation</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tg</td>
<td>transgenic</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIDC</td>
<td>tumor infiltrating dendritic cell</td>
</tr>
<tr>
<td>TIM3</td>
<td>T cell immunoglobulin mucin 3</td>
</tr>
<tr>
<td>TIL</td>
<td>tumor infiltrating lymphocyte</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TSA</td>
<td>tumor specific antigen</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Declaration of Academic Achievement

The work presented in this thesis is a culmination of my research efforts over the past 4 and a half years as a PhD student at McMaster University. The experiments described were designed and the resulting data interpreted through a collaborative effort between myself and my supervisor, Dr. Jonathan Bramson. I had a primary role in conducting all described experiments and collaborated on further data analysis or interpretation as described. All viral constructs used for these studies were prepared by Carole Evelegh. Individual technical efforts have been broken down by chapter and described below.

The research presented in chapter 2 resulted from collaborative efforts primarily involving myself, Dr. Robin Hallett, Dr. Dannie Bernard, and Florentina Teoderascu. I planned and carried out all animal studies, isolated tumor RNA samples, and conducted cell-based assays and qRT-PCR analysis as described and analyzed the data. For many of the described experiments, Dr. Dannie Bernard and myself worked in close collaboration to generate the data sets. Microarray data was generated at the Waller Family Gene Array Facility by Dr. Dora Illieva using tumor RNA samples isolated by me and all bioinformatics analysis was done by Dr. Robin Hallett using the resultant data or publicly available data sets. Florentina Teoderascu conducted further qRT-PCR analysis. Additional technical assistance was provided by Dr. Stephanie Swift and Heather VanSeggelen.
In chapter 3, the data presented was collected through experiments conducted primarily by me, with technical assistance provided by Dr. Stephanie Swift and Dr. Dannie Bernard. Microarray data was generated in the laboratory of Dr. Guillaume Pare (McMaster University) by Amanda Hodge using samples prepared by me. Dr. Robin Hallett performed all bioinformatics analysis. Immunohistochemical staining of tumor sections was performed in the Core Histology Facility at MIRC by Mary Jo Smith and tumor sections were imaged by Kyle Stephenson.

The research presented in chapter 4 is the result of a collaboration primarily involving myself, Dr. Dannie Bernard, and Dr. Robin Hallett. I conducted initial transcriptional analysis, analysis of CD8+ TIL, and titration/testing of 4-1BB and PD-1 monoclonal antibodies used for these studies. The remaining experiments were done in collaboration with Dr. Dannie Bernard. Microarray data was generated in the laboratory of Dr. Guillaume Pare (McMaster University) by Amanda Hodge using samples generated by myself and Dr. Dannie Bernard and all bioinformatics analysis was performed by Dr. Robin Hallett using the resultant data or publicly available data sets. Additional technical support was provided by Ryan Kelly, Mayank Jha, Caitlin Gregory, and Dr. Jennifer Bassett. The work presented in chapter 4 was adapted from a manuscript published in Oncoimmunology in 2012 by McGray et al under a creative commons license, allowing for extensive reuse of published material for non-commercial purposes, as described here:

http://www.landesbioscience.com/journals/oncoimmunology/guidelines/#lbsmod1300
Chapter 1:

Introduction
Introduction

1.0 Cancer

Despite improvements in early detection and treatment, cancer remains the leading cause of death worldwide, accounting for approximately 13% of all deaths, a figure that is expected to increase significantly in the coming years (1). Successful treatment modalities will rely heavily on an improved understanding of the underlying mechanisms related to tumor initiation and growth, as well as resistance to conventional therapies. This section will discuss the biology of cancer and will profile standard cancer treatments, as well as the requirement for new treatment options and will introduce cancer immunotherapy.

1.1 The biology of cancer

Tumors arise through a progressive transformation of normal functioning cells and can be broadly classified as either benign or malignant based on their relative degree of invasiveness (2). Although genetic inheritance may predispose an individual to the development of various cancers, exposure to chemical or physical agents through occupation, lifestyle choices, diet, and tobacco use, as well as exposure to infectious agents are more likely to result in the accumulation of the cellular changes that lead to cancer (2). Exposure to such factors promote step-wise genomic alterations that impact upon the regulation and function of two important classes of genes: genes that act to
prevent tumor development (tumor suppressor genes) and genes that promote tumor development (2). This sequence of randomly occurring genetic and epigenetic changes promote loss of tumor suppressor gene function in combination with increased oncogenic signaling, resulting in dysregulated cellular proliferation, growth, and survival processes that, often over a period of decades, can lead to malignancy (2). Thus far, two distinct enabling characteristics of cancer have been described: 1) Genome instability and mutation and 2) an ability to instigate tumor-promoting inflammation (3). These processes act to endow cancer cells with 8 hallmarks that result in tumor formation: 1) sustained proliferative signaling, 2) evasion of growth suppression, 3) activation of invasion and metastasis, 4) enabled replicative immortality, 5) ability to enable angiogenesis, 6) resistance to cell death, 7) deregulated cell energetics, and 8) avoidance of immune destruction (3).

Cancers can arise from numerous specialized cell types and often share characteristics with their respective tissue of origin (2). The majority of human cancers (>80%) arise from epithelial cell layers present in many bodily tissues and are termed carcinomas. The remaining cancers arise from non-epithelial layers of the body and include the sarcomas (origin: connective tissues), hematopoietic cancers (origin: blood cells), and neuroectodermal tumors (origin: central and peripheral nervous system). It is important to note, however, that not all tumors are easily classified within these subgroups. Examples include melanoma and small-cell lung carcinomas (2).
While tumors may arise from the transformation of a single cell, growing tumors are comprised of heterogeneous cell populations, which support tumor growth (2). The tumor stroma, often comprised of fibroblasts, myofibroblasts, and myeloid cells, is recruited by cancerous cells to provide physiological support to the growing tumor (2,4). Of particular importance are recruited cell populations that participate in tumor vascularization, a critical determinant of continued tumor growth and survival (2,4,5).

Even if a cancer lesion is slow growing, primary tumors (under most circumstances) will grow to dimensions that will impact upon biological processes within their site of origin, leading to clinical diagnosis (2). While primary tumors are often treatable, the dispersion of cancer cells to distant sites of the body, termed metastasis, has often occurred by the time of clinical cancer detection (2). Not only are metastases more challenging to treat clinically, they are more likely to impact upon a number of normal physiological functions and are responsible for approximately 90% of cancer-related deaths, with the remaining small frequency of deaths resulting from primary tumor lesions (2).

1.2 Standard cancer therapies

Conventional cancer treatments have historically included surgery, chemotherapy, and radiation therapy. The first proof that tumors could be cured through surgery arose in the early 1800’s (6) and rapid advancements in the new field of surgical oncology
suggested that any organ affected by cancer could benefit from surgical intervention (6). For solid tumors, surgery remains among the best strategies for successful treatment, permitting complete resection of localized tumors, accurate cancer staging, or debulking of tumors when complete resection is not possible (7). Surgery is often combined with additional therapies, such as chemotherapy or radiation therapy, based on the type of cancer and staging at the time of clinical diagnosis (7). Chemotherapeutics vary widely in their mechanisms of inducing cell death and can be broadly classified as either cell cycle-specific or non-specific, based on whether the mechanism of action is dependent on the cell undergoing mitosis (8). Radiation therapy or radiotherapy uses high-energy ionizing radiation to elicit DNA damage in targeted cancer cells, leading to cellular apoptosis (9,10). Both chemotherapy and radiotherapy can be administered in either the neo-adjuvant or adjuvant setting (before or after a primary treatment, such as surgery) and can be tailored for treatment of residual disease at the primary tumor site or for tumor metastases (9-11). Based on the type and stage of a patient’s cancer, any or all of these treatments strategies may be combined.

1.3 The requirement for new cancer treatments

While surgery can be an effective means of eliminating solid tumors, this approach becomes less effective if a patient develops metastases or in cases where cancers develop within the blood, bone marrow, or lymph nodes. While such cancers can be treated with
Chemotherapy or radiation, these therapies are not tumor-specific, causing ‘off target’ effects on normal healthy cells as well as tumor cells, resulting in adverse toxicities following treatment (8-11). Furthermore, sub-populations of cancer cells will often exhibit resistance to such therapies and while some cancer cells may be killed by the administered therapy, this strategy can ultimately lead to the emergence of treatment resistant cancer (12,13). Therefore, identifying systemic treatment modalities that select for tumor-specific killing in the absence of adverse effects to healthy tissues remains an important goal for therapy development. The criteria for selective anti-tumor effects have led investigators to survey the inherent discriminatory capacity of the immune system as a means of developing potent and targeted cancer treatments.

1.4 The origins of cancer immunotherapy

Spontaneous tumor regression is a phenomenon that has been observed for hundreds, if not thousands of years and has often been associated with acute infections (14,15). Although the correlation between tumor regression and infection had been recognized previously, William Coley became the first researcher to formally investigate the ability of the host’s immune system to eradicate growing tumors in the late 1800s (14,15). Coley developed a treatment consisting of extracts of killed Gram-positive *Streptococcus pyogenes* and Gram-negative *Serratia marcescens*, better known as Coley’s toxins (15). This treatment was used to successfully treat diverse cancer subsets
including sarcomas, carcinomas, lymphomas, melanoma, and myelomas (14). Inspired by these early observations, continued research has led to significant advancements toward developing effective immunotherapies for clinical use. Based on strong progress over the past 25 years, cancer treatments harnessing the immune system have joined surgery, chemotherapy, and radiation as important facets of clinical cancer treatment (6).

2.0 The immune system and cancer therapy

New knowledge in the fields of immunology and vaccinology have led to novel approaches for cancer treatment that have attempted to harness the inherent specificity of targeted immune responses. In this section, basic principles of the immune system will be discussed, with a specific emphasis on the role of T cells in the anti-tumor immune response.

2.1 Innate and adaptive immunity

Humans and other living organisms co-exist with numerous pathogens, toxic substances, and allergenic materials that threaten both survival and normal body homeostasis (16). In response to this diverse threat, the immune system has evolved to use a complex array of biological mechanisms that, under normal conditions, act to protect the host by eliminating invading organisms and toxins (16). This is achieved through the orchestration of a complex series of cellular processes which collectively constitute innate
and adaptive immunity.

The initial host response to a foreign pathogen or toxin comprises the innate immune response, which acts to prevent foreign materials from physically invading the host, as well as by mounting a rapid cellular response upon recognition of invading pathogens (16). Innate immune functions are encoded within germ-line genes of the host and are carried out through the presence of physical barriers (including skin, mucosal epithelium, and mucous secretions), release of constitutive or activation-induced soluble proteins or bioactive molecules (such as complement proteins, defensins, cytokines, and chemokines), as well as through broadly-expressed membrane and cytoplasmic receptors able to recognize molecular structures expressed by invading microbes, collectively termed pathogen associated molecular patterns or PAMPs (16,17).

In contrast to innate immunity, adaptive immunity relies on precise specificity for a given molecular target, termed antigen (16). Adaptive immunity is based largely on the ability of T cells and B cells to recognize a specific antigen through the expression of antigen-specific receptors on the cell surface (16). Because only a small number of cells within the host will have specificity for a single invading pathogen, proliferation or expansion of antigen-specific T cells or B cells is required, therefore the adaptive immune response generally follows the initial activation of innate immunity (16). A major feature of the adaptive response is the ability to retain immunological memory. Following initial activation through antigen recognition, the number of activated cells diminishes, leaving
only a small pool of antigen-experienced, albeit non-activated antigen-specific memory T and B cells (16,18). These memory cells are able to rapidly respond upon re-exposure to their cognate antigen, leading to a more robust and effective immune response (18).

2.2 Immune cell subsets

The complete functional capacity of the immune system involves contribution from numerous subsets of circulating leukocytes, which differentiate from hematopoietic stem cells (16). Differentiation of immune cell subsets commences when hematopoietic stem cells differentiate into either common lymphoid or common myeloid progenitor cells (16). In the interest of remaining within the scope of the document, a brief description of specific cell populations within both the lymphoid and myeloid compartments will be included, with further details integrated as they pertain to cancer immunotherapy and the described research.

2.2.1 Lymphocytes

The four major populations of fully differentiated lymphocytes include T cells, B cells, NK cells, and NKT cells. These lymphocyte subsets have diverse functions in both the innate and adaptive immune response and can be classified based on the distinct surface phenotype exhibited by each cell subset.

The majority of T cells (90-95%) can be distinguished based on surface expression
of an αβ T cell receptor (TCR) (16), allowing T cell recognition of peptide antigen bound within major histocompatibility complex (MHC) class I or II (19), as will be discussed in detail in subsequent sections. αβ TCR-expressing T cells can be further classified based on surface expression of either CD8+ or CD4+, with CD8+ T cells making up the major cytotoxic arm of the cellular adaptive immune system (18,20) and CD4+ T cells acting to orchestrate, support, and regulate both innate and adaptive immune responses (18,21). For this reason, CD4+ T cells are often referred to as helper T cells (16). Individual αβ TCRs possess specificity for a single peptide antigen, with the enormous requirement of TCR specificities for any and all potential foreign antigens achieved through recombination of variable (V), diversity (D), and joining (J) gene segments, leading to the generation of a vast number of unique VαJα and VβDβJβ heterodimeric TCR chains (16,19). Although the majority of CD4+ and CD8+ T cells express a single αβ TCR specific for a single peptide antigen, some T cells may express multiple TCRs, resulting in multiple specificities. Dual TCR-expressing T cells have been described in both mice and humans (22-24). A detailed description of CD4+ and CD8+ T cell differentiation, activation, and function has been included elsewhere in this introduction. The remainder of the T cell compartment is made up of γδ T cells, which differ in their TCR composition (variable γ and δ chains), and a subpopulation of αβ T cells that do not express either CD4 or CD8 (16). As these cell populations do not fall within the scope of this document, they will not be discussed in further detail.
As a detailed discussion of the addition lymphoid cell subsets goes beyond the scope of my thesis research, these cell types will only be discussed briefly. B cells develop in the bone marrow and are responsible for generating the humoral immune response against extracellular antigens through the production of antibodies (Ab) (also known as immunoglobulins [Ig]) (16,18). NK cells are cytotoxic cells of the innate immune system that do not express antigen-specific receptors or develop specificity for a single antigen target (18). Instead, NK cell function is regulated via the orchestration of cellular signals through an array of inhibitory and activating receptors (25). Natural killer T (NKT) cells are a heterogeneous cell population phenotypically defined based on co-expression of NK lineage receptors as well as limited subset of semi-invariant αβ TCRs (26,27) and can be further subdivided based on their capacity for antigen recognition in the context of the MHC Class I-like molecule CD1d (26,27).

2.3 CD8+ T cell immunity

CD8+ T cell responses represent an important component of the adaptive immune response. Activation or priming of CD8+ T cells (also referred to as cytotoxic T lymphocytes or CTL) occurs in draining lymph nodes through interactions between CD8+ T cells and APCs (28). Through recognition of cognate antigen in the context of MHC I (as described in section 2.10.1), in combination with co-stimulatory signals through CD28, and the presence of inflammatory cytokines such as IL-12 and type I
IFNs (29), CD8+ T cells become activated, causing them to proliferate and differentiate into effector T cells (30). Naïve CD8+ T cells have been observed to only require a short interval (around 20hrs) of antigen stimulation to become activated (31), however optimal stimulation and activation occurs when antigen stimulation occurs over a longer interval (32). Following differentiation, CD8+ T cells execute effector functions that fall into two distinct categories. First, the major function of activated CD8+ T cells is to initiate contact-mediated cytotoxicity on target cells (33). This can occur through the release of pre-formed cytolytic molecules, such as perforin and granzymes, at the CD8+ T cell/target cell interface, termed the immunological synapse (34). Here, the release of perforin results in the formation of pores in the membrane of the target cell, allowing granzymes to penetrate the target cells and instigate caspase-dependent and independent mechanisms of apoptosis (33,35). Contact-mediated cytotoxicity can also occur through triggering of the Fas receptor on target cells via Fas-L expression by CTL (33,36).

Additionally, activated CD8+ T cells secrete cytokines and chemokines, which can modulate the functionality of both immune and non-immune cell subsets and contribute to the local inflammatory response. Two important cytokines produced by activated CD8+ T cells are IFN-γ and TNF-α (37).

IFN-γ exerts diverse biological functions which are primarily involved in the host defence against pathogens. Specifically, IFN-γ signaling leads to enhanced MHC I and II antigen presentation (which can improve antigen recognition and cytolysis function),
reduces viral replication and cell proliferation, and increases cell sensitivity to apoptotic signals (38). Moreover, IFN-γ signaling results in immunomodulatory effects, including Th1 polarization of CD4+ T cells and macrophages, and induces the expression of chemokines involved in recruitment of monocytes/macrophages, and T cells (38). Additional aspects of IFN-γ functionality as they pertain to anti-tumor immunity will be described in subsequent sections. TNF-α plays pleiotropic roles in regulating innate and adaptive immunity, as well as the function of non-immune cells (39). Both IFN-γ and TNF-α are rapidly induced following TCR activation through peptide:MHC interaction (40). However, the production of these cytokines is tightly regulated and requires ongoing TCR engagement (40,41), presumably as a mechanism of protecting host tissues from inflammatory effects.

2.4 CD4+ T cell immunity

CD4+ T cells orchestrate aspects of both the innate and adaptive immune response. Similar to CD8+ T cells, CD4+ T cells become activated and expand following interaction with their cognate peptide antigen, but in the context of MHC II, as discussed in section 2.10.1. Although a detailed description of CD4+ T cells goes beyond the scope of my thesis, the cytokine milieu within the local environment at the time of CD4+ T cell activation influences the differentiation of activated CD4+ T cells, which develop into functionally distinct cell subsets including Th1, Th2, Th17, and T_{reg} (21). As an
example, Th1 CD4+ T cells differentiation is dependent on IL-12/STAT-4/T-bet signaling and leads to the production of IFN-γ, TNF-α, and IL-2 by activated CD4+ T cells. Th1 polarization is further enhanced through IFN-γ signaling, representing a positive feedback loop for CD4+ polarization (21,42). In contrast, CD4+ T cells are polarized towards a Th2 phenotype in response to IL-4/STAT-6/GATA-3 signals and secrete IL-4, IL-5, IL-10, and IL-13, with IL-4 supporting further Th2 differentiation (21,42). Interestingly IFN-γ and IL-4 can potently antagonize CD4+ polarization towards Th2 or Th1, respectively, acting as a counter measure to support continued polarization (21). Of particular significance to my thesis research are Tregs, which represent a heterogeneous subset of immunosuppressive CD4+ T cells, discussed in detail in section 2.5

Conventionally, CD4+ T cells are thought to predominantly play a ‘helper’ role in mediating the immune activity of CD8+ T cells, as well as other immune cells including B cells, macrophages, and NK cells (16,21). Important to the functionality of CD8+ T cells, CD40L expressed on activated CD4+ T cells can activate or license DCs through CD40 (43-45), thus enhancing the ability of DCs to activate CD8+ T cells (45). CD4+ T cells can also directly help activated CD8+ T cells through the production of IL-2 (43), a critical factor required for T cell function and homeostasis (46).

2.5 Regulatory T cells (Tregs)

Tregs have a central role in the maintenance of tolerance and immune homeostasis
Natural Tregs (nTregs) develop in the thymus and comprise 5-10% of the circulating CD4+ T cells, whereas inducible Tregs (iTregs) differentiate in the periphery from naïve conventional CD4+ T cells (47). The four best characterized mechanisms of Tregs-mediated immune suppression are 1) production of secreted or membrane bound factors, 2) direct cytolysis, 3) metabolic disruption, and 4) suppression of DC function (48). Although Tregs naturally possess antigen specificity through their TCR, their immunosuppressive capacity can be antigen non-specific (49), suggesting that Tregs can prevent anti-tumor activity over a broad range of tumor infiltrating immune cells of broad specificity through bystander effects. The ability of Tregs to mediate suppressive functions appears to be largely dependent on the expression of the forkhead box P3 (FoxP3) transcription factor (50). The capacity for Tregs-mediated control of T cell homeostasis is exemplified in humans and mice harboring a loss of function mutation in FoxP3, which results in the development of severe T cell-dependent systemic autoimmunity (51). Increased numbers of Tregs have been associated with poor survival in a number of tumors including breast, gastric, and ovarian cancers, as well as melanoma (52-54). For this reason a number of approaches are currently being evaluated in order to limit the impact of Tregs on anti-tumor immunity that may have important implications for future treatment design (47).
2.6 Regulation of T cell homeostasis

Controlling antigen-specific T cell responses requires the precise regulation of T cell activation, proliferation, effector function(s), as well as the duration of activity. Following TCR ligation, this is achieved through the complex orchestration of positive stimulatory and negative inhibitory signals to the T cell. Robust activation of a T cell requires the co-ordination of at least 3 signaling events: 1) TCR ligation, 2) co-stimulatory signals to the T cell, and 3) stimulation delivered through inflammatory cytokines (29). Co-stimulatory signals delivered through CD28 and the TNF Receptor Superfamily have shown a potent capacity for enhancing T cell responses (55,56). Specifically, CD28 signaling follow TCR stimulation increases the strength of activation, improves T cell proliferation, leads to upregulation of effector cytokines such as IL-2, IFN-\(\gamma\) and IL-4, and increases upregulation of cell surface receptors, including those for chemokines, cytokines, as well as additional co-stimulatory receptors such as 4-1BB and OX40 (57). Signaling through 4-1BB or OX40, as well as other members of the TNFR family, delivers a later second wave of co-stimulatory signals to activated T cells and assist in enhancing T cell proliferation, functionality, survival, and establishment of long term T cell memory (58-63). Additionally, inflammatory cytokines including, type I interferons, IL-12, and IL-1 provide important signals for T cell activation, leading to expansion and increased effector functions, including cytolytic capacity and production of IFN-\(\gamma\) (64,65).
Receptor mediated signaling can also lead to suppression of T cell activation. CTLA-4 expression increases on the surface of T cells following activation (66), and competes with CD28 for binding of the co-stimulatory B7 molecules (CD80/86). Given that the affinity of CD80/86 is much greater for CTLA-4 than for CD28 (56), surface expression of CTLA-4 acts to limit co-stimulation to the T cell and prevents long term T cell activation (56). Additionally PD-1, another member of the CD28 receptor family, is upregulated on activated T cells and through interaction with it’s ligands (PD-L1 and PD-L2), results in impaired TCR signaling, cytokine production, and cell survival (67). T cell activation is a tightly regulated process, exemplified by the natural intrinsic pathways that coordinate T cell activation, but also prevent prolonged immune attack that may result in collateral tissue pathology. Given the implications of co-stimulation and inhibition on the resultant magnitude and duration of T cell responsiveness, targeting pathways that control T cell activity have emerged as an important facet of cancer immunotherapy, as will be discussed in section 3.4.

2.7 Myeloid Cells

Myeloid progenitor cells give rise to numerous cell subsets, many of which assist in important immune functions. Although by no means exhaustive, the role of monocytes, macrophages, and dendritic cells (DCs) is of particular interest to the scope of the work described in chapter 4 and will therefore be highlighted herein.
Monocytes are generated in the bone marrow and traffic between the blood, bone marrow and spleen, migrating into tissues based on the expression of chemokine and adhesion receptors (68,69). Monocytes are comprised of a heterogeneous pool of cells that can be further classified based on surface phenotype and the expression of chemokine receptors. In mice, inflammatory or Ly6C\textsuperscript{hi} monocytes can be defined based on surface expression of Ly6C and CD11b and express high levels of CCR2, but low levels of CX\textsubscript{3}CR1 (70) and rapidly accumulate in the circulation and are recruited into tissues in response to injury or infection (70-73). In contrast, Ly6C\textsuperscript{lo} monocytes express high levels of CX\textsubscript{3}CR1 and low levels of CCR2 and exert their function through the patrolling of blood vessels via adherence and migration to luminal epithelial surfaces (70). Activated monocytes are capable of phagocytosis and the production of cytokines and chemokines upon tissue infiltration and act as part of the initial inflammatory response (74). Monocytes, however, are heavily influenced by the local environment that they encounter upon tissue entry and further differentiate into subsets of macrophages and DCs (70,74). Residing in both lymphatic and non-lymphatic tissues, macrophages are phagocytic cells involved in removal of apoptotic host cells, while also expressing a range of pathogen recognition receptors that facilitate phagocytosis of foreign material and induction of an inflammatory response (69). Macrophages can be classified based on their anatomical location within the body, as well as their functional status (75). Of particular relevance to this thesis are tumor-associated macrophages, which will be detailed in section 2.7.2.
Although macrophages can vary dramatically in their functional phenotype in response to stimuli, two functional states are commonly described for activated macrophages. The first, referred to as classical or M1 activation, can be induced by microbial agents and IFN-γ (76) and results in macrophages that mediate defense against a variety of invading pathogens (bacteria, viruses, protazoa), as well as playing a role in anti-tumor immunity (75). M1 macrophages can be characterized based on the production of inflammatory cytokines such as IL-12, TNF-α, and IL-1 (75,76), production of specific chemokines associated with Th1 cell recruitment (such as CXCL9, CXCL10, and CXCL11) (77), as well as the expression of iNOS (78). In contrast, alternative or M2 activated macrophages play a role in mediating wound healing and have anti-inflammatory functions, producing IL-10, TGF-β (76), and arginase (78), as well as mediating the recruitment of Th2 cells (76) and can be induced through stimulation with IL-4 and IL-13 (76), although steady-state macrophages also possess intrinsic anti-inflammatory functions as a means of maintaining tissue homeostasis (75). It has been speculated that instead of comprising a static functional dichotomy, inherent plasticity may instead allow macrophages to exist within a functional spectrum where cellular adaptations occur under the influence of the local environment and dictate cellular function (79). Importantly, it has been reported that M1 macrophages can convert to an anti-inflammatory M2-like phenotype (80), a cellular plasticity with important implications in the anti-tumor immune response mediated by macrophages.
**DCs** are specialized for antigen capture, processing, and presentation to T cells and are responsible for controlling the balance between T cell tolerance and activation (81). While DC subpopulations comprise a great degree of heterogeneity that goes beyond the scope of this thesis, DCs can be simply subdivided into conventional DCs (cDCs), which possess DC functionality under steady-state conditions, and precursor DCs, which require additional stimuli and/or differentiation to develop full DC-like phenotype and functionality (81).

Upon capture of exogenous material in the context of activating cytokines (such as IFNs and TNF-α) or danger signals, DCs are able to mediate 3 critical signaling events that are required for T cell activation. First, DCs increase antigen presentation on MHC Class I and II (82), facilitating TCR interaction with cognate antigen. Secondly, DCs upregulate a multitude of co-stimulatory molecules, such as CD40, CD80, and CD86 (82), providing critical signals for T cell activation. Additional co-stimulatory molecules expressed by DCs include OX40L and 4-1BBL (63). Lastly, DCs can secrete chemokines (83) and cytokines (such as IL-12, TNF-α, and IL-6) (84) that attract T cells and induce an inflammatory response. These functional and phenotypic changes, referred to as DC maturation, are critical to the induction of an antigen-specific T cell response.

It discussing macrophage and DC populations, it should be noted that at present, distinguishing macrophages from the various DC subsets remains a challenge, as these cell populations express many shared surface markers and carryout similar functions in
response to inflammatory stimuli, further emphasizing that these cell populations exist within a range of differentiation states, generated through the inherent plasticity of myeloid cells (75).

2.7.1 Myeloid-derived suppressor cells (MDSCs)

MDSCs represent a highly immunosuppressive heterogeneous population of myeloid cells that can accumulate under various pathological conditions and are present in both human and mouse tumors (85). Comprised of myeloid cells at various stages of differentiation and possessing a largely immature phenotype, murine MDSCs can be characterized based on the co-expression of CD11b and Gr-1 (Ly6C/G) and can be further subdivided based on the specific expression of Ly6C or Ly6G as granulocytic MDSCs (CD11b+Ly6G+Ly6C\text{lo}) or monocytic MDSCs (CD11b+Ly6G-Ly6C\text{hi}) (85). MDSCs orchestrate suppressive functions through the production of ROS and NO (86), expression of both arginase-1 and NOS2 (87), secretion of immunosuppressive cytokines including IL-10 and TGF-β (88), and may contribute to the expansion of T\text{regs} (89). MDSC expansion occurs in response to numerous stimuli and often involve upregulation of the transcription factor STAT3 (85). MDSCs activity has been observed to lead to a reduced capacity for proliferation by T cells, as well as downregulation of the TCR ζ chain, thus limiting signal transduction (90). Interestingly, blockade of IFN-γ has been shown to reduce the suppression of T cells by MDSCs (91), suggesting that MDSC
activity act as a direct means of regulating sustained T cell activity. As such, overcoming
MDSC-mediated immune suppression has important implications in the design of cancer
immunotherapies. In addition to blockade of MDSC expansion and function (discussed in
detail in chapter 5), promoting maturation of MDSCs into fully differentiated myeloid
cells lacking suppressive characteristics (85) has been considered as potential methods of
improving anti-tumor immunity.

2.7.2 TAMs/TIDCs

Strong tumor infiltration by myeloid cells, such as TAMs, has been associated with
tumor progression and poor survival outcome (92-94). Recruited into the tumor through
the production of chemokines such as CCL2 (95), myeloid populations including
monocytes, differentiating macrophages, and DCs are influenced by local factors, as
discussed in section 4.2, and support tumor immune escape and continued growth by
limiting anti-tumor immune responses and supporting the tumor.

As discussed previously, macrophages show tremendous plasticity and tumor-
associated macrophages (TAMs) are heavily influenced by the immunosuppressive and
hypoxic tumor environment (96). TAMs, although phenotypically and functionally
diverse (97), have been predominantly described as developing an alternatively activated
or M2-like phenotype (discussed in section 2.7) and have poor capacity to present
antigen (98), increase production of the immunosuppressive cytokines IL-10 and TGF-β
and decrease expression of pro-inflammatory IL-12 and TNF-α (99), increase expression of arginase-1 (99), and possess a capacity for increased T\(_{\text{regs}}\) recruitment through production of CCL22 (52), thereby supporting local immune suppression. In addition, TAMs have been reported to support tumor growth through the production of pro-angiogenic factors (76), promote and support metastasis (100), as well as protecting tumor cells from apoptosis (101). It should be noted however, that the M2 phenotype of TAMs can be reversed, allowing TAMs to mediate anti-tumor activity (102), a point which may have important implications in developing effective immunotherapies.

Similarly, tumor infiltrating DCs (TIDCs) display functional impairments, largely through immunosuppressive mechanisms that prevent proper DC maturation and/or immune suppression, as detailed in previous sections of this chapter. Generally speaking, DCs within the tumor have an immature phenotype, express low levels of co-stimulatory molecules (103), and can upregulate surface expression of suppressive ligands including PD-L1 (104). Importantly, \textit{ex vivo} culture and stimulation of TIDCs in the absence of immunosuppressive signals can restore their capacity to produce inflammatory cytokines, upregulate co-stimulatory molecules and become potent APCs capable of stimulating T cell activation (105,106), suggesting that the functional impairments exhibited by tolerogenic TIDCs are not static and that modulation of local signals within the tumor may restore their functionality and improve local anti-tumor immune activity.
2.8 T cells and Cancer

Although improved treatment outcome following delivery of non-specific immunomodulatory agents such as αCTLA-4 or αPD-1 suggests that anti-tumor effects are mediated by tumor-specific T cells (107,108), adoptive transfer of tumor-specific T cells or T cells engineered to express tumor-specific TCRs provides the most direct evidence of the potent capacity of T cells to mediate tumor destruction (109-111). Pre-clinical studies (112,113), as well as the adoptive transfer of enriched CD8+ T cells into patients (114) have firmly established the capacity of CD8+ T cells to elicit robust anti-tumor immunity. Additionally, CD4+ T cells are able to support CD8+ T cell responses (115,116), possess cytotoxic ability (117), as well as the capacity to mediate indirect tumor destruction through cytokine production and recruitment/activation of innate immune cells (118-120). CD4+ T cell subpopulations also have the ability to suppress anti-tumor immune responses in the case of Tregs, as discussed previously. Therefore, intense investigation has focused on identifying treatment modalities that can enhance anti-tumor T cell responses and to understand barriers to anti-tumor T cell function that may limit the impact of cancer immunotherapy. In light of this, the remainder of this thesis will largely focus on critical aspects of T cell biology and the implications of tumor-specific T cell responses in initiating and sustaining anti-tumor immunity.
2.9 Tolerance to self-antigens

The immune system has evolved a precise mechanism by which to initiate immune responses against foreign antigens, while preventing activation against self that may result in tissue destruction and pathology. This intrinsic ability to prevent both immunodeficiency as well as autoimmunity is achieved through mechanisms of tolerance. In this section, I will describe the process of deleting auto-reactive T cells during thymic selection (central tolerance), as well as mechanisms of controlling the activation of auto-reactive T cells that escape thymic selection, termed peripheral tolerance.

Central Tolerance

Selection of T cells carrying functional TCRs specific for non-self antigens occurs in the thymus, where immature double positive (CD4+/CD8+) αβ lymphocytes, termed thymocytes first undergo positive selection within the thymic cortex (16). Through interaction with cortical epithelium (121), cells are selected based on an ability to demonstrate sufficient avidity for self MHC as to allow for antigen recognition in the context of MHC (122). Thymocytes are programmed for apoptosis unless signaling occurs following TCR ligation to a self-peptide:MHC complex (122). Failing to demonstrate specificity for MHC, the majority of developing lymphocyte will undergo apoptosis (123). Thymocytes exhibiting successful peptide:MHC interaction differentiate into single positive (CD4+ or CD8+) cells and next undergo negative
selection within the medullary thymus, where they are tested for reactivity to self-antigens found in peripheral tissues (16,122). This process is partially under the control of the gene AIRE (autoimmune regulator), which ensures the expression of representative self-antigens from all peripheral tissues by medullary epithelial cells (124-126). Cells that recognize self-antigens with high avidity are removed by apoptosis (16), or are diverted to become T_{regs}, which possess TCRs with medium to high avidity for self-antigens (122,127). T cells that survive positive and negative selection (less than 5% of developing cells) (16) continue their maturation and enter the pool of circulating T cells.

**Peripheral Tolerance**

Despite the relatively efficient removal of auto-reactive T cells through central tolerance, a small frequency of T cells specific for self antigens enter the circulation. Therefore intrinsic mechanisms to subvert auto-reactivity by T cells in the periphery are also necessary. The first barrier involves the physical separation between naïve auto-reactive T cells and their cognate antigens expressed in peripheral tissues. As naïve T cells traffic through the blood and secondary lymphoid tissues (128), auto-reactive naïve T cells will remain ignorant of antigens expressed in peripheral tissues. However, since antigen-experienced T cells circulate through most tissues and can preferentially home to sights of inflammation (129), “antigen discovery” by activated auto-reactive T cells in peripheral tissues can occur, resulting in tissue destruction (130,131). Therefore
maintaining peripheral tolerance through additional processes is also necessary.

One mechanism involves the deletion of auto-reactive T cells, whereby T cells that are chronically engaged by peptide:MHC die by apoptosis, also referred to as activation-induced cell death (132). This process occurs through a series of molecular events and includes signaling through death receptors such as Fas/Fas-L interaction (132). A second mechanism of peripheral tolerance involves the induction of T cell anergy, a form of functional non-responsiveness following TCR stimulation in the absence of co-stimulatory signals (133). Anergic T cells remain refractory to additional stimulation (even if co-stimulation is delivered) and most notably display defects in proliferation and IL-2 production (133). T cell anergy occurs through TCR engagement by peptide:MHC presented by either tolerogenic or immature APCs (134), or through interaction with MHC on cells that do not express co-stimulatory molecules (133,135,136). Additionally, activated T cells upregulate surface expression of suppressive receptors including CTLA-4 and PD-1 (137), which act to limit prolonged T cell activation and maintain peripheral tolerance (138,139) as already discussed in section 2.6. Lastly, Tregs play an important role in maintaining peripheral tolerance as previously discussed.

2.10 Antigen presentation and tumor antigens

In discussing anti-tumor immunity, it is important to consider the underlying mechanisms of antigen presentation, as well as potential antigen targets expressed within
growing tumors. In this section, I will describe cellular mechanisms of antigen processing and presentation, as well as the nature of tumor antigens.

2.10.1 Antigen processing and presentation

Recognition of peptide antigen in the context of surface expressed MHC is critical to T cell activation. CD8+ T cell activation requires antigen recognition in the context of MHC Class I molecules, whereas CD4+ activation occurs in the context of MHC Class II (140), as will be detailed below.

MHC Class I (MHC I) presentation

Expressed by all nucleated cells, MHC I molecules present peptide fragments derived from proteins expressed predominantly within the cell (140). Heterodimeric MHC I molecules are assembled in the endoplasmic reticulum (ER), consisting of a polymorphic heavy chain (α chain) and a light chain (β2-microglobulin) (140). The majority of peptide antigens loaded onto MHC I molecules are generated through proteosome-mediated degradation of functional proteins (140) or newly synthesized proteins that contain defects (141). Once generated, peptides are pumped from the cytoplasm into the ER by the transporter associated with antigen presentation (TAP) which, together with the chaperone molecules tapasin, calreticulin, ERp57, as well as MHC I, form the peptide loading complex (PLC) that ensures proper peptide loading.
onto MHC I (140). Upon successful loading, peptide-MHC I complexes are released from the ER for antigen presentation at the plasma membrane to CD8+ T cells (142) (Fig. 1a). Presentation of exogenous antigens on MHC I can also occur through the process of cross-presentation or cross-priming, whereby antigenic material from neighbouring cells is taken up, either through gap junction-mediated movement of antigen (143) or by endocytosis (144). Cross-presentation has been observed to facilitate the presentation of tumor antigens by APCs, thereby inducing anti-tumor immune responses (143,145).

**MHC Class II (MHC II) presentation**

While MHC I is expressed by all nucleated cells, MHC II expression is largely
restricted to APCs, including DCs, macrophages, and B cells and is specialized for the presentation of exogenous antigens degraded through the endocytic pathway (140). MHC II expression is tightly controlled by the MHC class II transactivator (CIITA) (146). Similar to MHC I, MHC II molecules are assembled in the ER, where the α and β chains complex with the invariant chain Ii (CD74), which then translocates to late endosomal compartments, known as MHC II compartments (140). Here, Ii is digested, leaving a small fragment (the class II-associated Ii peptide or CLIP) within the peptide binding groove of the MHC II molecule, which is then removed by the MHC II chaperone HLA-DM (H2-DM in mice) and MHC II loaded with peptides produced through lysosomal degradation (140). Peptide-MHC II complexes can then be transported to the cell surface for antigen presentation to CD4+ T cells (142) (Fig. 1b). Interestingly, endogenous antigens can also be loaded onto MHC II, using either proteosome and TAP-mediated antigen processing (147,148) or through the process of autophagy (142,149), which likely has important implications in presenting self antigens, such as TAA, to CD4+ as well as CD8+ T cells.

2.10.2 Tumor antigens

Now more than 20 years since the identification of the first tumor antigen recognized by T cells (MAGE-1) (150), a diverse collection of more than 130 tumor antigens have now been identified (151-154). Although a number of broad classifications
exist for tumor antigens, as will be discussed, tumor antigens can be more generally considered as either tumor specific or tumor associated antigens.

Tumor specific antigens (TSAs) are non-self antigens and unique to the tumor (155), meaning that targeting these antigens should elicit an immune response not confined by mechanisms of tolerance (152), resulting in restricted immune attack to only the tumor. The first type of TSA arises from somatic mutations (152), creating immunogenic non-self epitopes that can be recognized by T cells and can include B-Raf (melanoma), K-ras (pancreatic cancer), and p53 (head and neck squamous cell carcinoma) (151). It has been reported that 30% (or greater) of the tumor antigens recognized by T cells in cancer patients result from mutations (152), which is not surprising given the high occurrence rate of genetic mutations observed in cancers (156). However, one potential drawback of targeting mutation-derived antigens is the tremendous heterogeneity among mutations within individual patient tumors (157), requiring that individual tumor genomes be sequenced and epitope targets verified in order to develop targeted immunotherapies to these antigens (153), which represents a major technical challenge to implementation. A second type of TSA arise from viral antigen targets originating from oncogenic viruses (152) and include antigens from the E6 and E7 oncoproteins of HPV-16, as well as Epstein-barr viral proteins (151).

In contrast, the majority of tumor antigens that have been targeted in clinical trials are tumor-associated antigens (TAA). TAAs are self-antigens and belong to one of three
distinct classes: 1) Cancer-germline (or cancer testis) antigens, which are not expressed in normal adult cells (with the exception of the testis) (152), but become re-expressed in certain cancers and include MAGE, BAGE, GAGE, and NY-ESO-1 among others (151), 2) Differentiation antigens, which are shared between tumors and their tissue of origin (153) and include gp100, tyrosinase, DCT, PSA, and mammaglobin-A (151) and 3) Over expressed antigens, which are also expressed by normal cells but are found at higher levels on tumors and include Her2/neu, Muc-1, VEGF, and telomerase (151). Note that a comprehensive list of tumor antigens based on these classifications can be found on the cancer immunity peptide database (151).

3.0 Cancer Immunotherapy

Solid modern day evidence that the immune system could be manipulated for the treatment of established cancers came in the 1970’s, when treatment with bacillus Calmette-Guérin (BCG) led to tumor clearance in patients with superficial bladder cancer (158). This was followed by observations in the 1980’s that treating patients with IL-2 resulted in reproducible tumor regressions (159,160). Since then, many approaches to elicit a potent and targeted anti-tumor immune response have been tested using pre-clinical models, with promising strategies translated into the clinic. In this section, the diverse strategies identified and developed as potential cancer immunotherapies will be discussed. Furthermore, strong emphasis will be placed on existing evidence for the
ability of treatment strategies to elicit meaningful clinical benefit to treated patients.

3.1 Immune surveillance and immunoediting

The idea that the immune system can effectively control the growth of cancer goes back to the early 1900’s, when Paul Ehrlich proposed the initial ideas of host immune protection from cancer (161,162). The ‘immune surveillance’ hypothesis, however was not officially proposed until 1957 by Macfarlane Burnet (163), where Burnet proposed that it seemed plausible that an accumulation of tumor cells possessing novel target antigens could elicit an effective immune response, leading to tumor clearance in the absence of clinical detection (163). Subsequent studies utilized various means of inducing immune suppression to investigate whether immunocompromised mice did in fact show greater tumor incidence, however these studies were largely inconclusive. The immune surveillance hypothesis was eventually abandoned when it was observed that immune deficient athymic nude mice developed similar frequencies of chemically induced tumors as wild type mice (164). However, subsequent studies revealed that the nude mice used in these studies, although immune compromised, are not completely immune deficient and do have detectable populations of functional T cells (165).

The concept of immune surveillance returned in the early 1990’s, when improved mouse models allowed for the direct assessment of immune-mediated cancer control. Indeed, the increased frequency of chemically induced tumors observed in the absence of
IFN-γ signaling (166-168) and perforin (169), and, most importantly, in RAG-2−/− mice lacking T and B lymphocytes (168) strongly supported a role for the immune system in preventing tumor growth. Pivotal studies revealed that the immune system cannot only act to eliminate tumors but can also shape their immunogenicity (168), leading to the evolution of the tumor immune surveillance hypothesis towards the concept of cancer immunoediting.

The theory of cancer immunoediting is comprised of 3 distinct phases: Elimination, Equilibrium, and Escape (162). In the elimination phase, developing tumors are destroyed by combined innate and adaptive immune responses. If however, tumor cell variants arise that cannot be destroyed by the anti-tumor immune response, then the tumor enters a state of equilibrium, whereby immunogenic cells continue to be destroyed, while poorly immunogenic cells proliferate. Therefore, the process of immunological editing occurs during the equilibrium phase and may lead to selection of tumor cells that are no longer recognized by immune cells, tumor cells that are no longer sensitive to immune-mediated killing, or tumor cells that are able to induce an immunosuppressive state within the tumor environment. At this point, tumors are able to escape immune control and continue to grow (170,171).

While many of the observations regarding cancer immunoediting have come from studies conducted in mice, increasing evidence suggests that the same principles may apply to human cancers. The elimination phase is exemplified by the increased risk of
developing virally and non-virally induced malignancies (170) among those with immune deficiencies, as in the case of individuals with AIDS or those receiving immunosuppressants following organ transplant (172). Tumor equilibrium may help to explain the improved prognosis for patients exhibiting strong T cell infiltrate and the production of cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\) that can control tumor growth over a prolonged period (170), the observation that patients enter phases of progression free survival or stable disease following immunomodulatory treatments such as \(\alpha\)CTLA-4 (173), as well as the report of two kidney transplant recipients both developing malignant melanoma after both receiving organ transplants from a woman who had been successfully treated for melanoma 16 years previously (174), suggesting that the melanoma metastases had been held in equilibrium within the kidneys for a prolonged period prior to transplant. Lastly clinically detectable tumors are poorly immunogenic and possess intrinsic mechanisms of circumventing or suppressing host immune responses (as will be discussed in section 4), suggesting these tumors have effectively escaped immune control.

### 3.2 Cancer Vaccines

Numerous approaches for the development of vaccines that can elicit an effective anti-tumor immune response have been identified and tested in pre-clinical and clinical settings (175). Cancer vaccines have been developed for either prophylactic (prior to tumor formation) or therapeutic (in the context of an established tumor) administration.
Prophylactic cancer vaccines have been successfully developed that diminish the risk of developing cervical cancer and hepatocellular carcinoma through targeting immune responses against causative agents (human papillomavirus and hepatitis B virus respectively) (176,177). While these medical advances surely serve as a testament to the capacity of the immune system to seek out and eliminate transformed cells, these approaches exploit an inherent ability of the immune system to target foreign antigens. In stark contrast, the majority of human cancers do not express readily identifiable foreign or mutated antigens and vaccination strategies must overcome both central and peripheral tolerance mechanisms in order to generate robust immune responses against poorly immunogenic self antigen targets, as described in section 2.10.2. The development of broadly effective therapeutic cancer vaccines has remained an elusive goal of tumor immunologists (178,179) and ongoing efforts have continued to test new strategies to improve vaccine efficacy.

Built on the successful use of whole (attenuated) pathogens as a vaccine platform, cancer vaccines utilizing irradiated or inactivated whole tumor cells gained early attention (180,181), based on the idea that the tumor itself would provide an abundant source of immunogenic targets from which to generate anti-tumor immune responses. While TAAs were already being identified (150) and characterized for the presence of immunogenic epitopes, advantages of adopting a whole cell vaccine approach included the removal of challenges in targeting individual (and at that time largely undefined) tumor antigens, as
well as an ability to raise immune responses against unique tumor antigens expressed only by an individual’s particular tumor. Challenges in developing individualized whole cell vaccines from individual tumors, based on the inherent challenges in attaining tumor biopsies, led to the development of allogeneic tumor cell lines that could be further engineered to produce cytokines or growth factors and used for broad administration (182). Although vaccination with tumor cells was a source of initial optimism based on pre-clinical observations (183,184), similar approaches have shown only modest efficacy when tested in clinical trials (185,186).

A number of vaccine platforms have also been tested in an attempt to target a single or subgroup of identified tumor antigens. The most straightforward approach to vaccination against cancer has been the delivery of peptide or small epitope vaccines along with an immunological adjuvant in order to stimulate targeted immune responses directed at tumors (187). and was an initial a source of enthusiasm (188-190). While peptide vaccines have shown a poor ability to generate robust anti-tumor effects (191,192), results from a recent phase 3 trial reported that combining a gp100 peptide vaccine with IL-2 treatment improved response rates and overall survival compared to treatment with IL-2 alone (193), perhaps leading to a renewed interest in the development of peptide-based cancer vaccines. To this end, pre-clinical investigation of methods to further improve on peptide vaccine approaches has continued to receive attention (194,195).

A second approach for vaccine design has been the use of recombinant plasmids or
viral vectors as a means of immunogenic tumor antigen delivery systems. In the case of plasmid technologies, circular DNA can encode tumor antigens, exogenous adjuvants, and immune stimulatory molecules (196), with naked plasmid delivered through diverse techniques ranging from simple intramuscular injection to electroporation to enhance cellular entry of the plasmid DNA (196). While showing some degree of promise in early animal studies, DNA vaccines have been relatively unimpressive when tested in clinical cancer patients (175). Alternatively, a diverse set of viral vectors has been tested for vaccine application, where tumor antigens are inserted as into the viral genome. Most viruses are immunogenic by nature and can infect host cells, allowing for robust expression (and presentation) of tumor antigens in the context of an inflammatory response (197). Importantly, this immunogenicity is able to enhance the immune response to poorly immunogenic antigens, such as TAAs which, as discussed previously, are inherently tolerogenic. In most cases, vectors have been manipulated to retain their immunogenicity, while removing or limiting undesired pathological effects (197). Numerous virus-based platforms have been developed for vaccine delivery, including but not limited to poxviruses, adenoviruses, alphaviruses, herpes simplex viruses, and vesicular stomatitis virus (197). Although overall success rates have been low, recent clinical benefits of administering viral vaccines using either a single vector or a heterologous prime/boost approach to treat diverse cancers has been observed (197-202), emphasizing that viral vaccines can elicit effective anti-tumor immune responses, but that
successful development and administration is perhaps not as straightforward as initially anticipated.

A final promising approach for cancer vaccine design that will be discussed is the use of dendritic cell (DC)-based vaccines. Recognized as a strong candidate for cancer vaccines based on their potent capacity for antigen presentation and T cell activation, DC-based cancer vaccines have been a major focus of experimental vaccine development. Preparation of DC vaccines can involve either the *ex vivo* differentiation, expansion, and loading of DCs from autologous pre-cursor cells or can employ *in vivo* delivery of antigen specifically to DC populations by fusing DC-specific antibodies to a selected antigen (203). Recently, Sipuleucel-T (Provenge), an APC-based cancer vaccine targeting the TAA prostatic acid phosphatase (PAP) (204) showed a 4.1-month median survival benefit in a phase 3 trial for men with metastatic castration-resistant prostate cancer (205) and became the first FDA-approved therapeutic cancer vaccine (204,206), highlighting the capacity of APCs to elicit anti-tumor immunity.

Although DC vaccines have shown some clinical promise, many questions remain regarding the optimal preparation of DCs to maximize the consequent anti-tumor immune response. Aside from the obvious challenge of preparing personalized vaccines from autologous PBMCs, numerous methods have been described for the *ex vivo* differentiation of DCs, effective antigen loading, and suitable DC maturation that will elicit an inflammatory and not tolerogenic response (207), making optimizing vaccine
preparation a challenge. Additionally, ex vivo DC manipulation may benefit from further genetic modifications to enhance antigen presentation, co-stimulation, cytokine secretion or recruitment of T cells via chemokines, which continues to be an ongoing area of study (82). Lastly, the most favorable location for DC infusion has been heavily questioned, with successful reports based on delivery via intravenous, subcutaneous, intradermal, intranodal, and intratumoral injection (207,208). While it is clear that DC-based therapies remain a strong contender for cancer vaccine development, this approach includes an inherent complexity in vaccine preparation and many questions remain as to how best to prepare and deliver these potent APCs to cancer patients.

3.3 Adoptive T cell transfer

Adoptive T cell transfer (ACT) involves the identification of autologous tumor-reactive T cells, using in vitro assays, that are then expanded in vitro to high numbers for infusion into cancer patients (160). One major advantage of ACT is the capacity to generate very high numbers of tumor-reactive cells, in some cases as high as $10^{11}$ cells, that can be pre-conditioned or activated prior to injection in order to confer superior anti-tumor T cell effector functions (160). To date, two major approaches have been used for the generation of large numbers of tumor-reactive T cells. The first involves the isolation of TILs, which are then selected for tumor-reactivity, grown to large numbers in culture, and are then infused back into the host (160). When combined with an aggressive
lymphodepleting treatment prior to infusion, ACT of tumor-reactive TIL has produced objective response rates as high as 72% in metastatic melanoma patients, with a high proportion of treated patients exhibiting durable complete responses (109). Further analysis of TIL from patients in this study demonstrated that infusion of TIL with longer telomere length, greater proportion of CD8$^+$CD27$^+$ cell phenotype, and longer in vivo persistence strongly correlated with outcome, although no single factor was predictive of treatment success (109). These observations further emphasize that while ex vivo generation of large numbers of TIL from patients is likely possible in most cases, the age/proliferative potential, activation status, and survival capacity of the transferred cells are critical parameters in determining treatment success.

While TIL therapy offers a promising treatment for cancer, this therapy requires that tumor-reactive TIL be successfully isolated from tumors, making the presence of resectable lesion(s) an absolute requirement (160). Additionally, while the majority of melanomas show high rates of T cell infiltration from which to isolate tumor-reactive cells, this is not universally true of all cancers (160), creating an inherent challenge to broadly expanding this therapy to diverse tumor types. Furthermore, patients must be able to tolerate the aggressive lymphodepleting pre-treatments, as well as the time required for ex vivo expansion of the TIL for infusion, although recent clinical protocols have improved upon the time required for T cell expansion (114). Based on these challenges, a second approach to the generation of large numbers of tumor-reactive T cells
has involved genetically engineering circulating T cells in order to endow them with anti-tumor reactivity (160). This has been achieved by transducing isolated circulating poly-specific T cells with constructs expressing either a tumor-specific TCR or a chimeric antigen-receptor.

Pre-clinical studies in mice showed convincingly that infusion of large numbers of TAA-specific transgenic or TCR-transduced T cells repeatedly resulted in rapid clearance of large growing tumors (112,209-211). Studies revealed that transduction of human T cells with a gene transfer vector expressing a high avidity TAA-specific TCR could also produce a pool of redirected tumor-specific T cells (212), making the application of “transgenic T cell-like” cancer treatments also a clinical reality. The approach of engineering TCR specificity has been tested in a number of recent clinical trials, with objective responses observed against melanoma (213,214), colorectal cancer (215), as well as synovial sarcoma (213).

One shortcoming of TCR engineering to confer tumor specificity is the constraint of HLA/MHC restriction, limiting the TCR-based therapy to patients who express a particular HLA type (216). One way to overcome this restriction is through the use of chimeric antigen receptors (CARs), which are engineered to allow tumor recognition by T cells through antibody specificity, which is not HLA/MHC restricted. First generation CARs were comprised of a tumor antigen-specific single chain antibody fused to the intracellular signaling components involved in T cell activation (216). Although beyond
the scope of this document, second and third generation CARs have been engineered to include co-stimulatory signaling domains to further enhance T cell proliferation, effector function, and survival following CAR ligation (216,217). One potential disadvantage of CARs is the requirement for the targeted antigen to be expressed on the cell surface, while TCRs can recognize all potential antigens expressed by the cell. However, given the broad range of tumor antigen-specific antibodies currently known (216), CAR-engineered T cell therapies offers a promising platform for current and future T cell-based therapies. Encouraging clinical response rates have been reported from recent trials testing CAR-engineered T cells (110,217-219). While this is true, clinical trials have also reported a lack of persistence and/or no anti-tumor activity by CAR-engineered T cells (220,221), while two additional independent studies have reported adverse events as a result of CAR-based therapies targeting different tumor antigen targets (CD19 and HER2) (222,223). This spectrum of anti-tumor effects suggests that a very precise fine-tuning of tumor-specific T cells is required for successful CAR-based therapies.

While therapies involving ACT of tumor-reactive T cells have proven to be the most effective treatment for patients with metastatic melanoma (160) and are quickly becoming strong candidate therapies for additional cancers, the requirement for isolating, growing, and engineering a patient’s own T cells for cancer treatment represent size-able financial and technical challenges to the broad therapeutic administration of these technologies. It remains to be determined whether these and other emerging technologies
for adoptive T cell transfer can effectively transition out of academic settings and be tailored to commercial application.

3.4 Immunomodulatory antibodies

Over the past two decades, the use of antibody-based therapies has proven to be a potent method of treating a number of cancers, acting through mechanisms of direct tumor cell killing, immune-mediated killing, and disruption of the tumor cell stroma/vasculature through targeted antibody/antigen interactions (224). To date, a broad range of therapeutic tumor targets have been identified, including growth/differentiation molecules, glycosylated proteins and lipids, tumor-expressed carbohydrates, as well as angiogenic/stromal targets, resulting in a number of clinically approved antibodies for treating diverse cancers (224). Additionally the availability of antibodies that can either act as agonists or antagonists of immunological pathways have led to the emergence of new insights into the use of antibody-based therapies as a means of modulating the anti-tumor immune response.

The utility of immunomodulatory monoclonal antibodies (mAb) as a cancer therapy is largely based on ideas related to immune suppression and restoring the functionality of suppressed or exhausted T cells in order to induce a more potent and lasting anti-tumor immune response. Indeed, upon tumor infiltration, T cells encounter a myriad of suppressive signals that overshadow immunostimulatory mechanisms (detailed in section
4), acting to limit the local activity of anti-tumor T cells (187,225). In this regard, immunomodulatory therapies have been developed as a means of either enhancing stimulatory signals on T cells or to block suppressive or checkpoint signals that a T cell may encounter.

The most well characterized immune regulatory pathway targeted by mAbs involves blockade of inhibitory signaling through CTLA-4. Knockout studies have revealed the importance of CTLA-4 in the control of T cell activation, as CTLA-4<sup>−/−</sup> mice display marked lymphoproliferative disorder and rapidly succumb to fatal autoimmune tissue destruction (139). However, transient blockade of CTLA-4 using mAbs have shown a remarkable ability to enhance immunotherapies in pre-clinical models of cancer (226-228). Based on the successful of clinical translation of αCTLA-4 therapy, ipilimumab (αCTLA-4, Bristol Myers-Squibb) (229) was approved by the FDA for the treatment of unresectable metastatic melanoma (230).

Blockade of inhibitory PD-1 signals has also emerged as a promising approach to enhancing the functionality of immune cells, and specifically T cells, infiltrating the tumor. PD-1 is expressed on a number of immune cell subsets (231) and interacts with two known ligands, PD-L1 and PD-L2, which are widely expressed on both immune and non-immune cell subsets, including tumors (231-235). Upregulated in response to TCR-stimulation, PD-1 signaling results in the functional exhaustion of activated T cells, which show diminished functional capacity (236,237). Studies blocking PD-1 signals, through
delivery of either \( \alpha \text{PD-1} \) or \( \alpha \text{PD-L1} \), have shown an ability to restore T cell functionality and have improved treatment efficacy in murine tumor studies (227,238,239). Recent clinical trials for \( \alpha \text{PD-1} \) and \( \alpha \text{PD-L1} \) treatment have independently exhibited objective response rates in patients with advanced cancer (108,240). While these studies did not combine mAb treatment with a specific targeted immunotherapy, these findings strongly suggest that PD-1 inhibition represents a second promising strategy for enhancing anti-tumor immunity by blocking immune regulation and may be used as a means of further improving existing immunotherapies.

While inhibition of immunosuppressive signals has exhibited great potential to improve anti-tumor immunity in patients, the development of agonist antibodies that can induce signaling through immune stimulatory pathways offers additional treatment approaches to enhance the anti-tumor immune response. Of particular interest in this approach are members of the TNF receptor superfamily, including 4-1BB, OX40, GITR, and CD40, as immunomodulatory antibodies directed at these receptors have displayed an impressive ability to enhance anti-tumor immunity (187,226,241-243), with early clinical trials exhibiting meaningful response rates (187).

It is important to note that the majority of clinical studies where mAb therapies have been tested, either as a means of blocking immunosuppressive signals or strengthening immune stimulatory signals, have reported the onset of a broad range of immune-related adverse events associated with treatment (107,108,187,240,244). This
striking observation highlights the importance of controlling consequent autoimmune pathology when attempting to override intrinsic mechanisms of immune regulation in developing cancer immunotherapies.

3.5 Autoimmune risks in targeting tumor antigens

As discussed previously, while some tumor-specific antigens are limited to expression only in the tumor, the majority of tumor antigens are also expressed to some degree in normal tissues. In such cases, while a targeted immune response may elicit the desired anti-tumor effect, a strong likelihood for accompanying autoimmunity pathology also exists (245,246). Autoimmunity in the context of anti-tumor immunity can be broadly classified into two categories. First, self-reactive immune responses may mimic genuine autoimmune pathologies, where an immune response is raised against an intended antigen target on the tumor, however immune attack is also initiated against unintended target cells within normal tissue. This is commonly observed following treatment with TIL-based ACT, as well as the transfer of genetically modified T cells targeting self-antigens to treat a broad range of cancers (245). Secondly, autoimmunity may be instigated through administration of immunomodulatory agents, such as αCTLA-4 (107) or IL-2 (247), where activation of poly-specific auto-reactive T cells may result in a broad range of immune-mediated toxicities (245). Autoimmune sequellae has been observed to accompany tumor regression (248) and has been reported to be a requirement of effective
long-lived anti-tumor immunity to self-antigens (249) and a positive prognostic indicator in patients (250,251). Further, it is likely that as cancer immunotherapies become more potent, controlling of more severe auto-immunity may be an unavoidable requisite, exemplified by reports of patient deaths due to autoimmune complications following administration of potent ACT-based T cell therapies (222,223). However, we and others have previously reported that anti-tumor immunity can be successfully uncoupled from accompanying auto-immune pathology using murine models (252-254). Furthermore, sustained anti-tumor immunity has been observed to persist in patients following treatment with corticosteroids to control autoimmunity (248), suggesting that it may be possible to elicit potent anti-tumor immune responses while avoiding the onset of accompanying pathologies in the clinic.

3.6 Assessing effectiveness of anti-tumor immunity

With the exception of ACT therapies, a robust anti-tumor immune response is not elicited immediately following administration of cancer immunotherapies and may take weeks or more to reach maximal levels of activity. Therefore, measurable impacts of treatment may be delayed, occurring after a period of disease progression following treatment (108,205,255,256). Given the differing response patterns observed for immunotherapies compared to more acute-acting cytotoxic therapies (173), it is likely that the clinical benefits of such therapies need to be evaluated using different principles.
For this reason, the immune-related response criteria (irRC) has been proposed as an improved method of assessing clinical benefit in immunotherapy trials (257). While some research groups have advocated for the continued use of WHO guidelines and the Response Evaluation Criteria in Solid Tumors (RECIST) to evaluate the clinical benefit of immunotherapies (178, 179), the irRC allows for the evaluation of changes in tumor status that are more in line with response patterns observed following immunotherapy (173). Specifically, the irRC encompasses 4 possible positive responses to treatment: (1) Immediate response, (2) durable stable disease, (3) response following initial increase in tumor size, and (4) response despite the presence of new tumor lesions, defining complete response (irCR), partial response (irPR), stable disease (irSD), and progressive disease (irPD) using standard WHO criteria (173, 257). Importantly, each of these response patterns has been reported to correlate with favorable outcome (compared to patients with progressive disease) despite demonstrating a delayed clinical effect (107, 173, 201, 257), suggesting that these new criteria may more accurately capture meaningful treatment benefit that would otherwise be overlooked in clinical trials using standard guidelines. Based on the success of αCTLA-4 in demonstrating the important implications of employing these criteria (257), the continued use of the irRC to evaluate the efficacy of new immunotherapies in the clinic will undoubtedly improve the identification of effective treatments.
4.0 Immune Suppression in the tumor

Growing tumors represent an immune refractory microenvironment that limits attack by infiltrating immune cells, presenting a major hurdle to the generation of successful immunotherapies. This section will focus on underlying mechanisms of immune evasion or immune suppression within the local tumor environment which contribute to limited anti-tumor immunity within the local tumor environment.

4.1 Defects in tumor antigen presentation

Abnormalities in MHC I antigen presentation have now been documented in a diverse set of solid and hematological tumors (258), representing an important mechanism through which tumors can escape recognition by CD8+ T cells. Loss of MHC Class I expression ranging from selective loss or down regulation to complete absence has been reported (259-261) and can be associated with disease progression (261). Defects in antigen presentation are often mediated by impaired expression of components of the antigen processing machinery by tumor cells. In particular, defects can be associated with irreversible alterations, such as mutations in β2-microglobulin (262) or components of the antigen processing machinery (260), as well as loss of heterozygosity at the MHC I loci on chromosome 6 (260,263,264). On the other hand, reversible defects may be simply due to diminished gene transcription, where MHC Class I presentation may be restored through the use of DNA de-methylating agents (265) or through treatment with
immunostimulatory cytokines, such as IFN-γ (265-267). Loss of antigen presentation has been reported in both murine and human tumors (267,268) and has been associated with poor survival prognosis in cancer patients (269). Importantly, MHC class I expression has been observed to correlate with tumor regression or progression within individual metastatic lesions (270), suggesting that restoring antigen presentation is likely an important determinant in the success of immunotherapy. While treatment with IFN-γ/IFN-α has been observed to restore in vitro antigen presentation in a number of melanoma cell lines (267), similar effects in vivo are likely dependent on in situ cytokine production by CD8+ T cells following antigen recognition and may ultimately limit the anti-tumor impact of tumor-specific CD8+ T cells.

4.2 Immunosuppressive factors within the tumor environment

While high frequency tumor-specific T cell responses can be readily achieved following delivery of immunotherapies, a number of soluble immunosuppressive factors have been characterized within tumors that augment the function of infiltrating immune cells and limit anti-tumor immunity.

First and foremost, although not strictly considered to be immunosuppressive, growing tumors secrete chemokines that act to recruit immune cells into the tumor environment in order to support continued tumor growth. Chemokine expression by diverse tumor types has been well characterized and promotes tumor infiltration by
TAMs (CCL2, CCL5, CCL7), DCs (CCL5, CCL19, CCL20), T\textsubscript{regs} (CCL22), and TIL (CXCL9/10/16, CX3CL1), based on cognate receptor expression by these cell types (271). As discussed previously,, a number of these immune cell subsets are heavily influenced by the local tumor environment and play important roles in promoting tumor growth and suppressing anti-tumor immune responses in situ. In addition to recruiting immune cells into the tumor via chemokine expression, tumors also express a diverse set of chemokine receptors, which aid in dissemination and metastases (271), as well as improving proliferation and survival of tumor cells (272).

Additionally, tumor cells, as well as immune infiltrating cells, can secrete a range of factors that act to suppress the anti-tumor activity of infiltrating immune cells including T cells (273). Specifically, the cytokines IL-10 and TGF-β are produced by the tumor, as well as immune infiltrating cells (274) and promote T\textsubscript{regs} function, inhibit the pro-inflammatory function of APCs by preventing suitable maturation, and prevent T cell proliferation, cytotoxicity, and the production of cytokines including IFN-γ and IL-2 (275-277). PGE\textsubscript{2}, present at high levels in tumor tissue (278), acts to inhibit DC maturation, limits T cell proliferation and function, increases immunosupression by myeloid cells, and enhances the suppressive effects of T\textsubscript{regs} (274,279). VEGF, an important angiogenic factor required for tumor growth, has also been reported to promote recruitment of MDSCs and macrophages to the tumor (274) and prevents immunostimulatory functioning of APCs (280). Adenosine, a purine nucleoside derived
through the catabolism of adenine nucleotides by the enzymatic activity of CD39 and CD73, is often present at high levels within the tumor (281). Produced through the activity of both the tumor (282) and Tregs (283), adenosine has both pro-angiogenic as well as immunosuppressive functions and limits the function of T cells (281,283).

Lastly, the intratumoral production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can contribute to T cell suppression and tolerance by preventing TCR/MHC interactions and restricting T cell responsiveness to stimuli (284-286), and limiting tumor infiltration by T cells (287).

Additionally, the availability of amino acids important for immune function are often reduced by local enzymatic reactions within the tumor, consequently reducing the functionality of infiltrating immune cells. L-arginine is an essential amino acid required for T cell function that is catabolized by arginase and NOS, enzymes often implicated in tumor-induced immune suppression and expressed at high levels within the tumor (274,285). A reduction in available L-arginine results in downregulation of the TCR ζ chain and inhibits the activity of T cells (288,289). Additionally, the enzyme IDO, expressed at high levels by the tumor, tumor stroma, as well as immune cells including DCs and macrophages (274), acts to degrade the essential amino acid tryptophan, thereby limiting local immune activity.

Together, the localized activity mediated by these factors acts to prevent sustained local anti-tumor immune responses. While effective inhibitors for many of these factors
have been identified and observed to correlate with improved treatment outcome in pre-clinical cancer studies (274), the breadth of immunosuppressive processes within the tumor represents a major hurdle to the production of effective and prolonged anti-tumor immunity.

4.3 Immunosuppressive ligands and receptors in the tumor environment

In addition to locally produced immunosuppressive factors within the tumor, numerous receptor/ligand interactions can also act to promote immune evasion and tumor progression. The finding that apoptosis-inducing FasL and TRAIL are expressed by tumors (290,291) suggests a mechanism by which the tumor can eliminate infiltrating immune cells expressing cognate receptors and underscores active measures by the tumor to evade host immune attack. Similarly, while ICOS-L is expressed on tumor cells (292) and could provide a source of co-stimulation for activated tumor-specific T cells, stimulation of ICOS-expressing regulatory T cells results in increased expansion of Foxp3 hi IL-10-producing Tregs (292), which may suppress local immune activity within the tumor. Moreover, constitutive expression of CTLA-4 by Tregs can lead to upregulation of IDO by APCs through interaction with CD80/86 (293,294), which can further inhibit T cell responses (47). As already detailed in section 2.6, the interaction between PD-1 and PD-L1/PD-L2 can drastically influence the activation status of immune cells within the tumor. PD-L1 and PD-L2 can be expressed on the surface of
tumor cells (232-235), as well as by immune cells (295,296) and PD-1 expression has been shown to correlate with reduced functionality of TIL (236). Furthermore, additional inhibitory or checkpoint receptors have been identified as having important immunoregulatory properties within the tumor. TIM3, a receptor expressed on CD4+ and CD8+ T cells, as well as DCs, monocytes, and other lymphocyte populations (297), has been shown to negatively impact T cell responses through interaction with its ligand galectin-9 (298), while LAG-3, through interaction with MHC II (299,300), negatively regulates TCR signaling in T cells, leading to functional impairment (301). Additionally, recent evidence suggests that LAG-3/MHC II interaction can initiate reverse signals that act to protect MHC II+ tumor cells from apoptosis (302), while LAG-3 expressed on T_{regs} can interact with MHC II on DCs to inhibit their immunostimulatory capacity (303). Inhibition of TIM3 activity has been shown to improve T cell proliferation and cytokine production (304) and blockade can enhance T cell-dependent anti-tumor immunity when administered alone and in combination with αCTLA-4 and/or αPD-1 (239,305,306). Similarly, LAG-3 blockade is able to enhance cytokine production by T cells and shows a synergistic improvement in anti-tumor immunity when combined with PD-1 blockade (307), suggesting that blocking multiple checkpoint pathways simultaneously may further improve anti-tumor immunity.
5.0 Models used in this thesis

5.1 Summary

The research carried out and presented in this thesis has focused on the treatment of established B16F10 murine melanomas as a relevant animal model with which to test and investigate candidate immunotherapies, namely vaccines. The prototypic cancer vaccines we have studied are replication-deficient human adenovirus serotype 5 (rHuAd5) vectors engineered to express the full-length melanoma antigens dopachrome tautomerase (DCT; rHuAd5-hDCT) or gp100 (rHuAd5-hgp100). In studies involving the adoptive transfer of naïve DCT-specific T cells, T cells were isolated from the spleens and lymph nodes of DCT-transgenic mice, where all T cells express a high avidity TCR specific for the immunodominant epitope of DCT. For further clarification, each of these models has been described in greater detail in the following sections.

5.2 Melanoma

The incidence of malignant melanoma continues to increase worldwide each year, with a growing number of patients developing advanced disease with poor prognosis (308,309). Primary tumors develop at cutaneous sites from transformed melanocytes, but can invade both local and regional lymph nodes and will eventually metastasize to distant organs (309). Melanoma is curable by surgical resection when confined to the
primary site and may be combined with adjuvant therapies including chemotherapy, radiation, or immunotherapy (309) to prevent relapse or treat metastatic disease. Metastatic melanoma, with patients developing stage IV melanoma typically surviving less than one year (308). Melanoma has been considered a good candidate for immunotherapies, as tumor lesions are often infiltrated by TIL with reactivity to TAAs expressed by melanomas (310,311). In line with this, recent advancements in immunomodulatory agents (such as monoclonal antibodies), as well as adoptive cell therapies have shown impressive rates of clinical response in advanced stage melanoma patients, as discussed previously. The poorly immunogenic B16F10 murine melanoma cell line was derived from a subcutaneous tumor in a C57BL/6 mouse (312) and isolated through repeated serial in vivo passage of tumor cells, based on the ability of cells to form pulmonary tumor nodules following IV injection (312,313). The potential of the isolated cell lines to form tumor nodules was observed to increase with repeated in vivo passage and re-isolation with later isolates, such B16F10, exhibiting increased invasiveness and metastatic potential (313) and therefore more closely mimicking human disease. Importantly, B16F10 cells express a number of clinically relevant TAAs identified in human melanomas, providing a relevant pre-clinical implantable tumor model for testing immunotherapies. Studies by our group have used B16F10 extensively to test a variety of immunotherapies in the context of primary skin tumor models (314-318), as well as pulmonary and brain metastases models (319-321).
5.3 Recombinant adenovirus vectors

The vaccine vectors that we have investigated in the described research are comprised of recombinant human adenovirus type 5 (rHuAd5) vectors expressing full-length tumor antigens. The rHuAd5 vectors we have utilized are replication-defective first generation recombinant adenovirus (rAd) vectors lacking the E1 and E3 regions of their genome. The E1 genes play a key role in viral replication (322), therefore vectors are propagated *in vitro* using cell lines expressing the E1 proteins necessary for viral replication (323). E3 genes are important in permitting the adenovirus to evade host defense mechanisms and are dispensable for *in vitro* viral production (322). Importantly, first generation rAd vectors can elicit a significant immune response *in vivo*, through the synthesis of viral proteins (323). rAd vectors can be grown to high titres, can infect a wide variety of both dividing and non-dividing cells (323), and rarely integrate their genomic material into the genome of infected cells (324), making this a strong candidate platform for cancer vaccine development.

5.4 Tumor antigens – DCT and gp100

**DCT**  
Also known as tyrosinase-related protein (TRP)-2, DCT is an immunogenic TAA that we have extensively targeted in order to investigate underlying mechanisms of improving anti-tumor immunity against growing B16F10 tumors. Expressed by both melanocytes and melanoma cells, DCT is a melanogenic enzyme which catalyzes the
rearrangement of DOPAchrome to the carboxylated derivative DHICA (325). DCT was first identified as a TAA based on recognition by a T cell clone isolated from the TIL of a melanoma patient (326) and soon after was also shown to be a TAA recognized by T cells in mice that could mediate rejection of B16 pulmonary metastases (327). Human DCT (hDCT) shares 83% amino acid identity with murine DCT (328), making DCT a strong candidate target antigen for xenoimmunization as a means of breaking self-tolerance. Importantly, the immunodominant CD8+ epitope DCT\textsubscript{180-188} (SVYDFFVWL) is completely conserved between the 2 species (310,329). We and others (314,317,328,330) have previously shown that immunization with hDCT elicits improved DCT-specific immune reactivity in mice compared to immunization with mDCT, resulting in superior protection against high dose B16 challenge.

**gp100** Expressed by both melanocytes and melanoma cells, gp100 is an intracellular glycoprotein implicated in the biogenesis and restructuring of early melanosomes (331,332). The importance of gp100 as a melanoma TAA was first realized following the observation that TIL recognizing gp100 were able to mediate tumor regression when adoptively transferred into a melanoma patient (333). Cloning of the murine gp100 homologue from a B16 cDNA library revealed a 75.5% amino acid identity with human gp100 (334). Xenoimmunization strategies in the pre-clinical setting using hgp100 resulted in some protection from tumor challenge (113,335,336), due to the
presence of a heteroclitic immunodominant CD8+ T cell epitope (hgp100_{25-33}: KVPRNQDWL, mgp100_{25-33}: EGSRNQDWL) which possesses higher avidity for MHC I than the murine epitope (113,335). Studies by our group have demonstrated that the protection elicited by rHuAd5-hgp100 is relatively weak compared to rHuAd5-hDCT, owing to inadequate presentation of mgp100 epitopes by B16F10 (337).

### 5.5 DCT_{180-188} TCR transgenic mice (TCR^{hi} mice)

TCR-transgenic (TCR-Tg) mouse models provide a useful platform for studies involving T cells, as they provide a means of isolating large numbers of T cells of a single known antigen specificity. Studies involving the use of DCT TCR-Tg T cells were carried out in collaboration with Dr. Arthur Hurwitz (NCI, Maryland), who constructed this transgenic mouse model. Briefly, DCT TCR-Tg mice were generated by cloning the Vα and Vβ TCR chains from a DCT (TRP-2)-specific T cell clone following peptide sensitization of mice to the immunodominant epitope DCT_{180-188}. The cDNAs for these genes were inserted into a human CD2 promoter-regulated expression vector and used to generate transgenic mice on the C57BL/6 background, as detailed in (338). This technique was used to generate the low avidity DCT TCR-Tg mice reported in (338), and was similarly used to generate the high avidity DCT TCR-Tg mice (unpublished data) that were used as a source of DCT-specific T cells for the studies presented herein.
6.0 Scope of described research

The overarching goal of the described PhD thesis research was to investigate intratumoral mechanisms leading to the limited therapeutic efficacy of the prototypic rHuAd5-hDCT vaccine against growing tumors. The research objectives set forth for investigation were based on previous findings from the Bramson laboratory and have focused largely on mechanisms within the local tumor environment that limit the activity of tumor infiltrating CD8+ T cells following vaccination.

6.1 Previous work by the Bramson group relevant to the described thesis research

Initial studies revealed that immunization with rHuAd5-hDCT provided robust prophylactic protection from subsequent tumor challenge, which could be mediated by both DCT-specific CD4+ and CD8+ T cells (314,317,318). Strikingly, as the time interval between vaccination and tumor challenge was reduced, rHuAd5-hDCT immunization displayed a similarly reduced capacity to control tumor formation and growth, with the vaccine exhibiting a limited ability to slow the growth of established tumors (315). Evaluation of the DCT-specific CD8+ TIL following therapeutic vaccination revealed a reduced functional capacity not observed for DCT-specific T cells in peripheral sites, suggestive of local immune suppression within the tumor (315). Importantly, neo-adjuvant immunization with rHuAd5-hDCT prior to surgical resection of the primary tumor revealed that vaccination could protect from subsequent tumor
relapse (316), further demonstrating that immunosuppressive mechanisms present within the tumor microenvironment present a true hurdle to the generation of effective anti-tumor T cell responses.

My thesis research has focused on evaluating the dynamic events within the tumor following immunization, with an emphasis on mechanisms present within the tumor microenvironment that can promote immune suppression. Importantly, a strong focus has been placed on developing treatment modalities with the capacity to overcome immune suppression within the tumor, improving the duration and quality of immune attack following vaccination.

6.2 Research objectives

The research conducted during my PhD studies has been subdivided into three (3) research objectives, which have been described in chapters 2-4 of this thesis, as outlined below:

**Objective 1:** Investigate the limited therapeutic efficacy of rHuAd5-hDCT against growing tumors by exploring local events within the tumor following vaccination. Results pertaining to this objective have been presented in **Chapter 3**.

**Objective 2:** Examine the rate and magnitude of DCT-specific CD8+ T cell expansion in
overcoming adaptive immune suppression within the tumor environment. Results pertaining to this objective have been presented in Chapter 4.

**Objective 3:** Evaluate the use of immunomodulatory monoclonal antibodies as a means of improving the anti-tumor immune response following rHuAd5-hDCT immunization. Results pertaining to this objective have been presented in Chapter 5 and have been adapted from the following published manuscript:

Chapter 2:
Methods
Methods

Mice
Female C57BL/6 mice were purchased from Charles River Breeding Laboratory (Wilmington, MA). IFN-γ-deficient mice were purchased from Jackson Laboratories (Bar Harbor, ME). TCR transgenic mice bearing a high-avidity TCR transgene specific for the H-2Kb-restricted epitope DCT180-188 were kindly provided by Dr. Arthur Hurwitz (National Cancer Institute, Frederick, MD). All of our investigations have been approved by the McMaster Animal Research Ethics Board.

Recombinant adenoviruses
The E1,E3-deleted recombinant human adenovirus serotype 5 (rHuAd5) vectors (339) used in this study have been described previously (318,337). rHuAd5-hDCT expresses the full-length human dopachrome tautomerase (DCT) gene. rHuAd5-hgp100 expresses the full-length human gp100 gene. rHuAd5-LCMV-GP encodes the dominant CD8+ and CD4+ T cell epitopes of the lymphocytic choriomeningitis virus glycoprotein.

Tumor challenge and immunization
Mice were challenged intradermally with 10⁵ B16F10 cells in 30μl PBS as previously described (315). 10⁶ pfu of Ad vector was prepared in 100μl PBS and injected in both rear thighs (50μl/thigh) 5 days after tumor challenge. Tumor growth was monitored daily and measured with calipers every other day or daily as tumors approached endpoint. Tumor volume was calculated as width x length x depth. For experiments involving
adoptive transfer of naïve transgenic DCT-specific T cells (referred to as $DCT^T$ cells), cells were isolated from the spleen and lymph nodes of naïve mice and subjected to ACK-mediated lysis of red blood cells. Cells were then enumerated and viability confirmed to be greater than 80% by trypan blue exclusion. Following this, cells were prepared as to allow for delivery of the indicated number of viable cells in 200µl PBS. $DCT^T$ cells were administered within 2-3 hours of vaccination by intravenous injection.

**Isolation of tumor infiltrating lymphocytes**

TIL were isolated as previously described (315). Briefly, tumors were digested in a mixture of 0.5 mg/mL collagenase type I (Gibco), 0.2 mg/mL DNase (Roche) and 0.02 mg/mL hyaluronidase (Sigma) prepared in Hank’s Buffered Saline (10ml/250mg of tumor). The digested material was passed successively through 70µm and 40µm nylon cell strainers and lymphocytes were purified using either mouse CD90.2 or CD45.2 positive selection by magnetic separation (EasySep, Stemcell Technologies). Prior to staining of TIL samples for analysis by flow cytometry, cells were stained using LIVE/DEAD-fixable dead cell stain (Invitrogen) to permit the discrimination of viable cells.

**Monoclonal antibodies**

In all cases, monoclonal antibodies were delivered to mice by intraperitoneal injection in 500µl PBS. Anti-PD-1 (clone RMP1-14) was purchased from BioXcell and administered 3 days following vaccination using a schedule of 250µg/mouse every 3 days (227) for a
total of 4 injections. Anti-4-1BB was produced at McMaster University from the 3H3 hybridoma (kindly provided by Robert Mittler, Emory University) and administered to mice 5 days after vaccination at a dose of 100-500µg/mouse. Total rat IgG (Sigma) was used as control. For depletion and neutralization experiments, CD4 (clone GK1.5), CD8 (clone 2.43), and IFN-γ (clone R4-6A2) antibodies were produced in our laboratory from hybridomas obtained from the American Type and Culture Collection. Depletion or neutralization was commenced 3 days following vaccination. For CD4+ and CD8+ T cell depletion, 200µg of the indicated antibody was delivered on two consecutive days, then every second (GK1.5) or third (2.43) day. Depletion of the desired T cell population(s) was confirmed to be greater than 98% in the spleen by flow cytometry (data not shown). For IFN-γ neutralization, 250µg-1mg of antibody was delivered every other day. Successful neutralization of IFN-γ signaling at administered doses was confirmed based on the ability of antibody treatment to successfully block PD-L1 induction in the tumor following vaccination, as observed in IFN-γ−/− mice (data not shown). All flow cytometry antibodies (anti-CD16/CD32, anti-CD28, anti-CD4-PE-Cy7, anti-CD8α-PerCP-Cy5.5, anti-PD-1-PE, anti-Thy1.1-PE, anti-CD107a-FITC, anti-IFN-γ-APC and anti-TNF-α-FITC) were purchased from BD Biosciences.

**Intracellular cytokine staining (ICS)**

CD8+ T cell epitope peptides (DCT_{180-188}, hDCT_{342-351}, hDCT_{363-371}, LCMV-GP_{31-43} and LCMV-GP_{34-41}) were purchased from Biomer Technologies, dissolved in DMSO
and stored at -20°C. CD8+ T cell epitope peptides specific for gp100 were identified from epitope mapping assays (unpublished data) and pooled for ICS analysis. The ICS method has been described previously (315). Briefly, lymphocytes were stimulated with pooled DCT, LCMV-GP, or gp100 peptides (1µg/mL) for 5 hrs at 37°C in the presence of 8µg/mL anti-CD28 and GolgiPlug™ protein transport inhibitor (1:1000 v/v, BD Pharmingen). The CD107a mobilization assay was performed by adding anti-CD107a-FITC at the beginning of the peptide stimulation as described (315). Data were acquired on a FACSCanto (BD Biosciences) and analyzed using FlowJo software (TreeStar).

**Immunohistochemistry**

Tumors were excised from mice and fixed in 10% Neutral Buffered Formalin for a period of 5 days and then paraffin embedded, sectioned and stained at the Core Histology Facility, McMaster Immunology Research Centre (MIRC). Tumors were sectioned at a thickness of 3 µm and stained for CD3 using an anti-CD3 rabbit monoclonal antibody (Clone SP7, Fisher Scientific) using a pre-treatment of EDTA buffer pH=9 and Envision plus-rabbit detection (Dako Cytomation). Slides were developed using AEC chromogen and visualized with a Leica DMRA microscope using the 5x optical lens. Images were captured using a QImaging MicroPublisher 5.0 RTV camera and Openlab imaging software (PerkinElmer).

**RNA extraction from solid tumors and quantitative real-time PCR**

Tumors were excised, snap-frozen in liquid nitrogen and stored at -80°C. Tumors were homogenized in Trizol (Invitrogen) using a Polytron PT 1200C (Kinematica) and total
RNA was extracted according to the manufacturer’s specifications. RNA samples were further purified using an RNeasy mini kit (Qiagen) and treated with Ambion’s DNA-free kit. Reverse transcription was performed with Superscript III First-Strand (Invitrogen) according to the manufacturer’s instructions. Quantitative PCR was carried out on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using Perfecta SYBR Green SuperMix, ROX (Quanta Biosciences). Reaction efficiency was determined for individual primer sets using a minimum of 5 serial dilutions to ensure similar efficiency between target and endogenous control reactions. Data for target genes of interest were analyzed via the delta/delta CT method using GAPDH as an endogenous control. Analysis was performed using Sequence Detector Software version 2.2 (Applied Biosystems). Primer sequences used for the detection of all genes are as follows:

Table 1. List of primer sequences used for the analysis of genes by qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
</thead>
</table>
| IFN-γ  | FWD: CTTGAAAGACAATCAGCCATC  
         | REV: CAGCAGCGACTCTCTTTCC  | PD-1   | FWD: TAGGGCAATAAAGGGAACCA  
         |                               |        | REV: GGTGTGAAGGAGAGCCAGAA  |
| TNF-α  | FWD: AATAGCTCCCAGAAAAGCAAG  
         | REV: CTGCCACAAGCAGAATGAG     | Arginase-1 | FWD: GGGTTAACCTTGCTGC  
         |                             |        | REV: GAGGGCCTTGTAGTTCTGT  |
| STAT-1 | FWD: AGAAAAACGCTGGGAACAGA  
         | REV: GGGTTCAAGAAGAGGAGAGA    | iNOS    | FWD: ACGAGACGGATAGGCAGAGA  
         |                             |        | REV: GGGAGGACGGTCAGTGGAGTAG |
Gene expression analysis by microarray

RNA from B16F10 melanoma tumors was isolated (as described above) 5, 7, 9, or 11 days post-vaccination for each treatment as indicated and prepared for profiling on either MouseRef-8_V2 beadchips (Illumina) (day 5 and 9 tumors), Affymetrix MoGene 1.0 ST gene chips (day 7 tumors) or Affymetrix MoEx 1.0 ST-v1 Exon Chips (day 9 and day 11 tumors) according to the manufacturer’s protocol. Affymetrix array expression files were created from raw .CEL files and normalized using Robust Multi-Array Analysis (RMA) (340). Illumina expression files were created using the IlluminaExpressionFileCreator module available on Gene Pattern (http://genepattern.broadinstitute.org/gp/pages/index.jsf), similar to Illumina BeadStudio, from raw .IDAT files. Genes were considered differentially expressed if the fold-change was > 1.5. Gene ontology analysis was carried out using the DAVID Functional
Annotation Resource (341), and pathway analysis was carried out using the NCI-Nature pathway interaction database resource (342). For analysis of human melanoma samples presented in chapters 2, we downloaded the gene expression profiles of 101 melanoma tumors from the Gene Expression Omnibus (GEO, GSE22153 [n=57, Illumina human-6 v2.0 expression beadchips], and GSE19234 [n=44, Affymetrix HG-U133 Plus 2.0 arrays]. All datasets were filtered such that when multiple probes recognized the same gene transcripts, only the probe with the highest mean intensity was used. For cross-platform comparisons, genes were mapped by Unigene IDs to either Affymetrix HG-U133 Plus 2.0 arrays or Illumina human-6 v2.0 expression beadchips. In chapter 4, treatment specific genes were determined using PAM analysis and the top 25 genes for each treatment group were used to complete a gene ontology analysis (343).

**Comparison of Immune Index with clinical melanoma samples**

We calculated all Illumina probes which were consistently differentially expressed between the rHuAd5-hDCT + α4-1BB + αPD-1 treated tumors and all other treatment groups (rHuAd5-hDCT, rHuAd5-hDCT + α4-1BB, rHuAd5-hDCT + αPD-1). Genes were considered differentially expressed if the fold-change was > 1.5 and the p-value < 0.05 in each comparison (Two-tailed, unpaired Student’s t test) (344). Gene expression profiles of 123 melanoma tumors for which clinical outcome data was available were downloaded from the Gene Expression Omnibus (GEO, GSE22153 [n=57, Illumina
human-6 v2.0 expression beadchips] (345), GSE22154 [n=22, Illumina HumanHT-12 V3.0 expression beadchips] (345), and GSE19234 [n=44, Affymetrix HG-U133 Plus 2.0 arrays] (346). All datasets were filtered such that when multiple probes recognized the same gene transcripts, only the probe with the highest mean intensity was used. For cross-platform comparisons, genes were mapped by Unigene IDs to either Affymetrix HG-U133 Plus 2.0 arrays, Illumina human-6 v2.0 expression beadchips, or Illumina HumanHT-12 V3.0 expression beadchips. The expression values for each gene were transformed such that the mean was 0 and the standard deviation was 1 within each individual dataset. An immune index was calculated for each patient as follows:

$$\frac{\sum_{i \in P} x_i}{n_p} - \frac{\sum_{j \in N} x_j}{n_N}$$

Where \( x \) is the transformed expression, \( n \) is the number of genes that could be mapped between platforms, \( P \) is the set of probes with higher expression in rHuAd-hDCT + α4-1BB + αPD-1 treated tumors, and \( N \) is the set of probes with lower expression in rHuAd-hDCT + α4-1BB + αPD-1 treated tumors (347). The median immune index value was used as the cut-point between high and low immune index values. Kaplan-Meier analysis was used to compare survival characteristics between patients with high and low immune indices. The gene signature analysis for myeloid cells and T cells derived from the Immune-Index was completed using gene functional annotations from the GeneCards database version 3 (www.genecards.org) (348) and from published literature.
**Statistical analysis**
Two-tailed, unpaired Student’s *t* tests were used to compare two treatment groups. One and two way Analysis of Variances (ANOVA) were used for data analysis of more than two groups and a Bonferroni post test was utilized to determine significant differences between treatment groups. Survival data was compared using a logrank test. Results were generated using GraphPad Prism 4.0b software. Differences between means were considered significant at *p*<0.05: * p<0.05, ** p<0.01, *** p<0.001. NS: not significant.
Chapter 3:

Early infiltration of DCT-specific CD8+ T cells instigates an adaptive response in the tumor that limits the duration of T cell attack
Chapter 3: Early infiltration of DCT-specific CD8+ T cells instigates an adaptive response in the tumor that limits the duration of T cell attack

Introduction

The proven immunogenicity of cancer vaccine platforms suggest that active immunization may be an ideal approach for the treatment of metastatic cancers. However, numerous vaccination strategies targeting a diverse array of tumor antigens have now been tested in large numbers of clinical trials (175), where candidate treatments have yielded few promising results (111,178). In all, the result of years of effort towards the development of therapeutic vaccines has yielded a single clinically-approved vaccine therapy (204,205). While successes in several recent clinical trials (193,199,201,349) has led to continued enthusiasm towards the potential of vaccine therapies, broadly effective and curative cancer vaccines may still be a distant goal.

Continued research in the field of tumor immunology has revealed many important mechanisms that may help to explain the limited success of therapeutic vaccination. It is now widely accepted that the tumor presents an immunosuppressive environment capable of limiting effective immune attack by infiltrating cells (206,350,351). Investigation of local tumor processes, as well as tumor infiltrating T cells, has yielded new insights into the suppressive nature of the tumor microenvironment, which must be overcome to improve the anti-tumor activity of tumor-infiltrating immune cells.
(206,236,350). The collective contributions of suppressive ligands, checkpoint receptors, suppressive factors, as well as infiltrating suppressive or tolerogenic immune cell populations all contribute to the complex immunosuppressive network within the tumor, as detailed in the introductory chapter. The consequent role of immune suppression in limiting local anti-tumor immune activity has been further demonstrated based on improvements in clinical response rates following administration of monoclonal antibodies directed against the well characterized immunosuppressive targets CTLA-4 (107,352-354) and PD-1/PD-L1 (108,240,355), although the mechanism(s) of action for these agents remains to be determined.

In this chapter, we have investigated the limited efficacy of a recombinant adenovirus vaccine (rAd) against growing tumors. By examining local events within the tumor, we have identified an ‘adaptive response’ to immune attack by a small number of tumor-specific CD8+ T cells within the first few days following vaccination. This adaptive response results in the induction of numerous suppressive processes within the local tumor environment that prevent sustained immune attack, despite infiltration by increasing numbers of tumor-specific TIL over time. These findings emphasize that low-level tumor attack can induce a local immunosuppressive response within the tumor and suggest that the development of effective cancer vaccines is strongly limited by the rate of T cell expansion following immunization.
Results

Vaccination produces modest suppression of tumor growth despite a robust expansion of tumor-specific CD8+ T cells

In accordance with our previous reports (314-317,337), immunization of mice with recombinant adenovirus vaccines expressing either dopachrome tautomerase (rHuAd5-hDCT) or gp100 (rHuAd5-hgp100) yielded a robust antigen-specific CD8+ T cell response, which peaked around 2 weeks after immunization (Fig. 2a). While vaccination with rHuAd5-hDCT resulted in a modest, yet significant, suppression of tumor growth, treatment with rHuAd5-hgp100 had no significant impact on tumor growth (Fig. 2b), consistent with our previous observations. To investigate the relative contribution of CD8+ and CD4+ T cell subsets in mediating the growth suppression produced by rHuAd5-hDCT administration, we conducted depletion studies following immunization. Using antibodies to deplete specific T cell subsets, we determined that CD8+ T cells, but not CD4+ T cells, were required for the growth inhibition produced by the rHuAd5-hDCT vaccine (Fig. 2c).

Vaccine-induced T cells are initially functional within the tumor but display progressive loss of function within a few days.

The modest effect on tumor growth resulting from immunization with rHuAd5-hDCT was inconsistent with the level of tumor-specific CD8+ T cells in the periphery,
which is reminiscent of clinical observations following treatment with cancer vaccines where robust tumor-specific T cell responses can be measured in the peripheral blood yet tumors continue to grow. Reports from our lab, and others, have demonstrated that tumor-specific T cells within the tumor display significant functional impairments that are not observed in the peripheral T cells (236,315,356). Since the ex vivo assays for polyfunctionality cannot confirm whether the DCT-specific T cells are active within the tumor, we employed transcriptional analysis of whole tumor tissue as a tool to monitor intratumoral T cell activity. Using a combination of antibody depletion studies and knock-out mice, we determined that IFN-\(\gamma\) was critical to the modest anti-tumor effect produced by the rHuAd5-hDCT vaccination (Fig. 3a). We reasoned that monitoring IFN-\(\gamma\) transcripts in the tumor following vaccination would provide a direct measure of the activity of vaccine-induced T cells based on evidence that IFN-\(\gamma\) is rapidly upregulated following TCR stimulation and extinguished with equal rapidity when contact with the MHC/peptide complex is disrupted (40). Whole tumor RNA was prepared from mice that were vaccinated with rHuAd5-hDCT, rHuAd5-hgp100, or left untreated and IFN-\(\gamma\) expression was measured by qRT-PCR. Expression of IFN-\(\gamma\) was relatively low in untreated tumors (Fig. 3b, left panel) whereas immunization with rHuAd5-hDCT led to a rapid (peak at day 5), but transient, increase in intratumoral IFN-\(\gamma\) (Fig. 3b, left panel). Little expression of IFN-\(\gamma\) was measured in tumors from mice immunized with rHuAd5-hgp100, despite a robust gp100-specific CD8\(^+\) T cell response (Fig. 2a),
although expression was significantly elevated relative to untreated mice at days 9 - 11, indicating some activation of gp100-specific T cells within these tumors. To confirm that IFN-γ expression in the tumor was due to T cell activation, mice bearing B16F10 tumors were immunized with rHuAd5-hDCT and depleted of CD8+ or CD4+ T cells. CD8+ T cell-depletion reduced IFN-γ expression to the level of untreated mice while CD4+ T cell-depletion had no impact on cytokine expression within the tumor (Fig. 3b, right panel).

TNF-α is another pro-inflammatory cytokine that is produced by effector CD8+ T cells and is associated with anti-tumor immunity (357,358). Following rHuAd5-hDCT immunization, TNF-α expression displayed kinetics similar to IFN-γ, albeit delayed (Fig. 3c, left panel); however, the relative increase in expression of TNF-α did not achieve the same magnitude as IFN-γ. Similar to IFN-γ, the expression of TNF-α was wholly dependent upon CD8+ T cells (Fig. 3c, right panel). Again, TNF-α expression in tumors from mice immunized with rHuAd5-hgp100 was only measurable at late time points and did not achieve peak levels comparable to the expression in tumors from mice immunized with rHuAd5-hDCT. It is notable that the period of heightened IFN-γ and TNF-α expression (Fig. 3b & c) within the tumor corresponded to the period of rHuAd5-hDCT-mediated tumor growth suppression (Fig. 2b).

The early peak in immune activity within the tumor was not consistent with the expansion kinetics observed for DCT-specific CD8+ T cells in the periphery. To gain a
better understanding of the tumor infiltration and functionality of vaccine-induced CD8+ T cells, we assayed the DCT-specific CD8+ T cells within the tumor at an early time point (5 days) and later time point (10 days) post rHuAd5-hDCT immunization. Consistent with the expansion of DCT-specific CD8+ T cells (Fig. 2a), there was a greater number of DCT-specific CD8+ TIL at day 10 than at day 5 (Fig. 3d). In line with our previous observations (315), we observed that tumor infiltrating CD8+ T cells (TILs) displayed reduced functionality compared to the peripheral T cells (PBLs) (Fig. 3e). However, the functionality of the DCT-specific CD8+ TIL, based on cytokine production and capacity for degranulation, showed further impairment at day 10 compared to day 5 (Fig. 3e, left panels).

The transcriptional studies combined with the ex vivo polyfunctional analysis suggested that a small number of DCT-specific T cells within the tumor were initially functional but were becoming progressively dysfunctional over time. To directly address this possibility, we employed DCT-specific TCR-transgenic T cells to allow direct monitoring of DCT-specific T cells in the tumor and the periphery over time following vaccination. For these experiments, we employed congenically marked (Thy1.1+) T cells from DCT TCR-transgenic mice (^{DCT}T cells), where all T cells express a high avidity TCR specific for the immunodominant epitope of DCT (unpublished data), allowing us to efficiently track DCT-specific CD8+ T cells. For these experiments, naïve ^{DCT}T cells were transferred into mice immediately following immunization with rHuAd5-hDCT.
Titration experiments revealed that delivery of $10^4$ DCT$^+$ T cells following immunization with rHuAd5-hDCT did not effect either the overall expansion of DCT-specific CD8$^+$ T cells or the kinetics of tumor growth relative to mice that did not receive any DCT$^+$ T cells (Fig. 4a). We also noted that the transfer of the DCT$^+$ T cells did not abrogate the endogenous T cell response (ie. Thy1.1 $-$ve cells; Fig. 4c). Monitoring the accumulation of the Thy1.1$^+$ CD8$^+$ T cells in the tumor revealed that few cells had infiltrated by day 5 (the time of peak IFN-$\gamma$ transcription), with cell number peaking in the tumor between days 8 and 10 post transfer (Fig. 4b). Functional analysis of the Thy1.1$^+$ CD8$^+$ T cells in both the blood and tumor revealed a progressive loss of function in CD8$^+$ TIL, as measured by the production of IFN-$\gamma$. The endogenous DCT-specific CD8$^+$ T cells (Thy1.1-ve) within the tumor displayed similar functional impairment, confirming that this effect was not an artifact of the TCR transgenic T cells (Fig. 4c & d).

Additional analysis of the polyfunctionality of the transferred CD8$^+$ DCT$^+$ T cells within the tumor revealed progressive defects in the ability of CD8$^+$ T cells to produce multiple cytokines (IFN-$\gamma$ and TNF-$\alpha$) (Fig. 5) and in their ability to degranulate (based on CD107a mobilization) ($p<0.05$, unpaired t test) (Fig. 5) when compared to the same cells in the blood, confirming our previous observations for endogenous DCT-specific CD8$^+$ TIL. These data support the idea that infiltrating DCT-specific CD8$^+$ T cells are initially functional within the tumor, but that these cells are progressively suppressed by factors within the local environment.
Early intratumoral activity by DCT-specific CD8+ T cells induces upregulation of genes associated with immune signaling and antigen presentation

As a follow up to assaying the effector cytokines IFN-γ and TNF-α, we performed kinetic analyses on a subset of key immunological genes in the tumor to better understand the temporal relationships between activated immune processes. As described previously, whole tumor transcriptional analysis following vaccination revealed that immune attack, based on intratumoral IFN-γ and TNF-α expression, peaked early in the tumor followed by rapid decline (Fig. 3b & c). This early immune attack was also accompanied by early induction of genes associated with IFN-γ signaling (STAT 1), and antigen presentation (CIITA, MHC I, MHC II) in the tumor (Fig. 6a). In contrast to the early induction of these genes, TCR-α expression continued to increase over time, consistent with a gradual increase in T cell infiltration (Fig 6a), despite diminishing immune attack at later time points (Fig. 3b & c). Immunization with rHuAd5-hgp100 yielded minimal increases in STAT 1 and CIITA expression compared to untreated tumors and did not enhance MHC I or MHC II expression beyond levels observed in untreated tumors (Fig. 6a). In line with the extinguished immune attack (IFN-γ and TNF-α) observed at later time points following rHuAd5-hDCT treatment, we observed a reduction in the intratumoral expression of STAT1, CIITA, MHC I, and MHC II, suggesting that sustained immune attack is also required to drive these processes. Depletion studies and the use of IFN-γ−/− animals confirmed expression of STAT1,
CIITA, MHC I, and MHC II within the tumor to be dependent on CD8+ T cells and IFN-γ following rHuAd5-hDCT treatment (Fig. 6b & c). These data provide further evidence that DCT-specific CD8+ T cells infiltrate the tumor and instigate early inflammatory processes, but that these processes are not sustained over time.

**Early intratumoral immune attack elicits a rapid adaptive response in the tumor involving numerous immunosuppressive genes**

Our observations that the activity of later infiltrating CD8+ TIL was being extinguished compared to early infiltrating CD8+ TIL suggested that changes within the local tumor environment were mediating a progressive decline in local immune activity. To investigate if known immunosuppressive processes might be mediating suppressive effects on the DCT-specific CD8+ TIL following rHuAd5-hDCT vaccination, we interrogated the transcriptional profiles of whole tumors by qRT-PCR following immunization with rHuAd5-hDCT compared to untreated control tumors. Transcriptional analyses revealed rapid induction of genes associated with immune suppression following rHuAd5-hDCT immunization, including PD-L1, galectin-9, arginase 1, PD-L2, iNOS, as well as the checkpoint receptors PD-1, LAG3, and TIM-3 beginning only 5 days post vaccination (Fig. 7a), corresponding to the initial point of immune attack within the tumor, but well before peak T cell infiltration (Fig. 3b-d). TGF-β1 induction was delayed compared to the other measured genes and was not upregulated.
until day 7 post vaccination (Fig. 7a). Depletion studies and the use of knockout animals confirmed that CD8+ T cells were critical for the upregulation of all suppressive genes analyzed with the exception of Galectin 9, which was not T cell dependent (Fig. 7b). Interestingly, IFN-γ was required for many, but not all, of these transcriptional changes, suggesting a key role for IFN-γ in the generation of this adaptive immune suppression (Fig 8a). Blockade of IFN-γ signaling using a monoclonal antibody resulted in sustained T cell activity within the tumor, based on prolonged IFN-γ transcription, confirming IFN-γ to be a key component in triggering immune suppression in the tumor (Fig. 8b). Unfortunately, as demonstrated in Fig. 3a, anti-tumor immunity is attenuated in the absence of IFN-γ, highlighting the dual role of this cytokine as both a potent anti-tumor effector and regulator of anti-tumor immunity. Importantly, the sustained expression of immunosuppressive genes in the tumor was directly dependent on continual local immune attack, as transcriptional analysis at day 11-post vaccination, when immune attack had greatly diminished (Fig. 3b & c), revealed a rapid reduction in the expression of all measured immunosuppressive genes (Table 1), closely resembling expression levels in control tumors. Taken together, this data suggests that early immune attack by CD8+ T cells results in dynamic and transient upregulation of numerous immunosuppressive processes in the tumor, which are sustained only under conditions of local immune attack.

To learn whether immune suppression might also accompany CD8+ T cell activity in human tumors, we investigated whether the presence of T cells in human melanomas
was associated with the expression of immunosuppressive genes. To this end, we used CD8\(^a\) transcript expression as a surrogate marker for TIL and examined the expression of the same well-characterized suppressive genes as we had interrogated in murine tumors. In two independent cohorts of melanoma patients, PD-L1, PD-L2, LAG3, and TIM3 expression all correlated with CD8 expression (p<0.05, Spearman’s rank correlation), whereas PD-1, IDO, and TGF\(\beta\)1 showed positive correlation with CD8 expression in one of the two datasets (Fig. 9a and Table 2), suggesting a strong likelihood that CD8+ T cell infiltration is also directly linked to the expression of suppressive factors in human tumors.

**rHuAd5-hDCT immunization elicits global changes within the tumor**

In order to better understand the full breadth of intratumoral changes elicited by rHuAd5-hDCT, we performed microarray analysis comparing rHuAd5-hDCT to rHuAd5-hgp100 and untreated control tumors. We observed 379 probes to be differentially expressed (FC > 1.5, q-value <0.1), of which 303 were upregulated and 76 were downregulated in the rHuAd5-hDCT treatment group (Fig. 10a). Gene ontology analysis revealed that genes over expressed after rHuAd5-hDCT treatment were enriched in immune system processes including antigen processing and presentation as well as regulation of T cell-mediated cytotoxicity (Fig. 10b). To examine what signaling pathways might mediate these immune system processes, we employed the NCI-Nature
pathway interaction database to determine if differentially expressed genes corresponded to specific signaling pathways following rHuAd5-hDCT immunization. Interestingly, the majority of the pathways showing highest enrichment corresponded to immune processes or pathways, highlighting the breadth of immunological changes elicited by rHuAd5-hDCT (Table 3). Surprisingly, the top differentially expressed genes following rHuAd5-hDCT treatment were largely comprised of genes that we would not have anticipated to be influenced by the vaccine (Fig. 10c), highlighting the overall breadth of the changes initiated within the tumor following vaccination.

**Discussion**

In this chapter, we have investigated the limited ability of a prototypic cancer vaccine, rHuAd5-hDCT, to slow the growth of established B16F10 melanomas. Our findings suggest that the limited anti-tumor effect generated through vaccination is due to rapid adaptation events within the tumor in response to early immune attack, which subsequently act to prevent persistent local immune activity despite continued CD8+ T cell expansion and tumor infiltration.

Although the overall treatment success rate for cancer vaccines in the clinic has been disappointing, our findings have strong implications towards elucidating the underlying mechanisms that may be limiting observable clinical benefit. While it has been argued that the tumor-targeting immune responses measurable in the peripheral blood of patients may
not actually manifest as productive anti-tumor immunity at the tumor site (236,359-361), our data argue that a lack of measurable immune activity within the tumor may, in fact, be due to the local induction of suppressive processes that are driven by the vaccine-induced T cells themselves. A recent report by Taube et al (232) described a positive association of PD-L1 (B7-H1) expression in human melanomas with T cell infiltration and the local expression of IFN-γ. These data directly support our observations that tumor-specific T cells act locally to initiate immunosuppressive processes in the tumor and, in doing so, limit the duration of productive anti-tumor activity. Our data further suggest that tumor-specific CD8+ TIL act to initiate numerous immunosuppressive processes within the tumor and that targeting a single immunosuppressive pathway may not be sufficient to overcome local immune suppression.

In the current study, we have shown that vaccination with rHuAd5-hDCT leads to induction of suppressive mechanisms within the tumor that impact on the anti-tumor response mediated by the vaccine. Perhaps more striking, however, is the observation that the level of immune attack following treatment correlated directly with the level of local immune suppression within the tumor, with the expression of immunosuppressive genes returning back to baseline as immune attack within the tumor was extinguished. This observation suggests that induction of suppressive events within the tumor is a dynamic process and that tumors may not remain in a static suppressive state once initial adaptive events have been instigated. It is tempting to speculate that the dynamics of this
adaptive process may help to partially explain the treatment benefit reported for recent phase II trials testing a fowlpox prime/boost treatment (199). It is possible that the prime vaccine initiated local immune attack and subsequent upregulation of suppressive processes in the tumor, followed by a decline in local immune activity and down regulation of local suppressive processes, similar to our observations described herein. Assuming this, the rapid re-instigation of immune attack generated through delivery of the boost vaccine would occur in the context of limited immune suppression in the tumor, resulting in a more favorable setting for robust immune attack on the tumor. Direct analysis of local events within the tumor following prime/boost vaccinations that have shown improved efficacy against growing B16F10 tumors (321) and unpublished data are currently underway by our group to better understand the dynamics between transient tumor adaptation and the improved anti-tumor effects achieved through boosting agents.

Overall, our findings highlight a critical limitation of cancer vaccines, namely the ability of the local tumor environment to rapidly adapt to early low-level immune attack prior to maximal induction of the targeted immune response. Future cancer vaccine trials should make strong attempts to find true surrogates of treatment impact within the local tumor environment, as sampling of peripheral blood or scoring rates of overall survival may not provide an adequate assessment of the overall effects of vaccination within the local tumor. Our results demonstrate that transcriptional analysis can provide a direct
measure of vaccine-induced CD8+ T cell activity within the tumor microenvironment and demonstrate that tumor-specific CD8+ T cells are only transiently activated within the tumor following immunization. Importantly, profiling of the tumor environment may allow for a better understanding of suppressive mechanisms within the tumor that lead to poor vaccine performance and may assist in addressing likely hurdles for future cancer vaccine treatment.
Figure 2: CD8+ T cells and IFN-γ mediate vaccine-induced tumor growth suppression. (a) DCT (□) and gp100 (△) -specific CD8+ PBL were measured post-rHuAd5-hDCT or rHuAd5-hgp100 immunization (n=5-12) (b) Tumor-bearing mice were immunized with either rHuAd5-hDCT (□), rHuAd5-hgp100 (△), or left untreated (○). (c) Tumor-bearing mice were treated with rHuAd5-hDCT and depleted of CD4+ (■), CD8+ (▼), or both cell subsets (◆) or left non-depleted (NT) (□). Tumor volumes in b & c reflect individual representative experiments (n = 4-5). Data presented as mean +/- SEM.
Figure 2

PhD Thesis - AJ R McGray
McMaster University - Medical Sciences
Figure 3: rHuAd5-hDCT vaccination results in early intratumoral immune activity that diminishes prior to peak infiltration of DCT-specific CD8+ T cells. (a) Left Panel: Tumor-bearing mice were treated with rHuAd5-hDCT (□) or the vaccine in combination with IFN-γ neutralization (△). Right Panel: WT (□) or IFN-γ−/− (◇) mice were treated with rHuAd5-hDCT. (b) Left Panel: Expression of IFN-γ in tumors from mice treated with either rHuAd5-hDCT (□), rHuAd5-hgp100 (△) or left untreated (○) (n=4). Right Panel: Expression of IFN-γ in tumors from mice treated with rHuAd5-hDCT and depleted of CD4+ (αCD4), CD8+ (αCD8), both cell subsets (αCD8/αCD4) or left non-depleted (NT) (n =4). Data point corresponds to peak expression of IFN-γ. (c) Intratumoral TNF-α expression as described in (b). (d) DCT-specific CD8+ TIL were enumerated following rHuAd5-hDCT immunization (n=8-19). (e) Representative FACs plots showing the frequency of DCT-specific CD8+ TIL or PBL capable of producing multiple cytokines (IFN-γ/TNF-α, upper panels) or degranulating (IFN-γ/CD107a, lower panels) at the indicated time points post vaccination in response to peptide stimulation (n=13-19). Tumor volumes in a reflect individual representative experiments (n = 4-6). The grey area in the right panels of b & c correspond to the mean +/- SEM of untreated tumors. Data presented as mean +/- SEM.
Figure 3 (p1)
Figure 3 (p2)
**Figure 4:** DCT-specific CD8+ T cells develop a progressive loss in intratumoral immune activity.  
(a) Left Panel: DCT-specific CD8+ PBL were measured following treatment with rHuAd5-hDCT (black) or rHuAd5-hDCT + 10^4 DCT T cells (grey) (n=5-13). Right Panel: Tumor growth in mice following treatment as described in a.  
(b) Transferred DCT-specific CD8+ TIL (Thy1.1+CD8+) were enumerated following vaccination with rHuAd5-hDCT (n=5).  
(c) Representative FACs plots showing IFN-γ production by transferred (Thy1.1+) and endogenous (Thy1.1-) DCT-specific CD8+ T cells in response to peptide stimulation following rHuAd5-hDCT immunization.  
(d) Graphical representation of MFI calculations for IFN-γ production by transferred (Thy1.1+) and endogenous (Thy1.2+) CD8+ T cells as shown in (c) (n=5-9). Tumor volumes in a reflect measurements from an individual representative experiment (n = 5). Data presented as mean +/- SEM.
Figure 4 (p1)
c

**PBL**  
Day 5  
![Graph showing IFN-γ MFI](image)

Day 8  
![Graph showing IFN-γ MFI](image)

Day 10  
![Graph showing IFN-γ MFI](image)

**TIL**

![Graph showing IFN-γ MFI](image)

(Gated on CD8+ T cells)

d

**PBL**

![Bar graph showing IFN-γ MFI](image)

**TIL**

![Bar graph showing IFN-γ MFI](image)

*Figure 4 (p2)*
Figure 5: DCT-specific CD8+ TIL develop progressive defects in polyfunctionality.

Flow cytometric analysis of IFN-γ and TNF-α production (upper panels) and CD107a mobilization (lower panels) by transferred (Thy1.1+) DCT-specific CD8+ T cells following rHuAd5-hDCT immunization. Values correspond to mean values calculated from compiled data (n=5-9).
Figure 5

TIL

Day 5

IFN-γ

71.6% 28.5%

Day 8

96.1% 3.9%

Day 10

92.3% 7.7%

IFN-γ

71.6% 28.5%

CD107a

44.8% 55.2%

Day 8

69.5% 30.5%

Day 10

66.8% 33.2%

IFN-γ

63.1% 36.9%

PBL (Day 10)

TNF-α

6.3% 93.7%

CD107a
Figure 6: Intratumoral activity by DCT-specific CD8+ T cells results in transient induction of genes associated with immune signaling and antigen presentation. (a) Expression of STAT-1, CIITA, MHC I, MHC II, and TCR-α in tumors from mice treated with either rHuAd5-hDCT (□) or rHuAd5-hgp100 (△) or left untreated (○) (n=4). (b) Expression of STAT-1, CIITA, MHC I, and MHC II in tumors from mice treated with rHuAd5-hDCT and depleted of CD4+ (αCD4), CD8+ (αCD8), both cell subsets (αCD8/αCD4) or left non-depleted (NT) (n =4). Data points correspond to peak expression of individual genes and grey areas correspond to the mean +/- SEM in untreated tumors. (c) Expression of STAT1, CIITA, MHC I, MHC II in tumors from WT (day 5 or 7, corresponding to peak expression) or IFN-γ−/−(day 5 & 8) mice treated with rHuAd5-hDCT (n=4). Data presented as mean +/- SEM.
Figure 6 (p1)
Figure 5

Gene Expression

0.0 0.5 1.0 1.5 2.0 2.5 3.0

IFN$\gamma$ -/- (day 5)

IFN$\gamma$ -/- (day 8)

WT (day 7)

STAT-1 CIITA MHC I MHC II

Days Post Vaccination

Figure 6 (p2)
Figure 7: Upregulation of immunosuppressive genes in the tumor following vaccination correlates with the early activity of DCT-specific CD8+ T cells. (a) Hierarchical clustering of the relative expression of immunosuppressive genes in rHuAd5-hDCT or Untreated tumors (n=4). (b) Expression of PD-L1, PD-L2, PD-1, Arginase 1, iNOS, TGF-β1, Galectin 9, Tim3, and LAG-3 in tumors from mice treated with rHuAd5-hDCT and depleted of CD4+ (αCD4), CD8+ (αCD8), both cell subsets (αCD8/αCD4) or left non depleted (NT) (n =4). Data points correspond to peak expression of individual genes and grey areas correspond to the mean +/- SEM in untreated tumors. Data presented as means +/- SEM.
Figure 7 (p2)
Figure 8: Local immune activity instigates the adaptive immunosuppressive response in the tumor but is also required to sustain the adaptive response. (a) Expression of PD-L1, PD-L2, Arginase 1, iNOS, TGF-β1, and Galectin 9 in tumors from WT (day 5 or 9, corresponding to peak expression) or IFN-γ−/− (day 5 & 8) mice treated with rHuAd5-hDCT (n=4). (b) Expression of IFN-γ in tumors following treatment with rHuAd5-hDCT or rHuAd-5hDCT in combination with IFN-γ neutralization (n=4-5). Data presented as mean +/- SEM.
Table 1. Changes in intratumoral gene expression from day 9 to day 11 post rHuAd5-hDCT vaccination

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-L1</td>
<td>-2.26</td>
</tr>
<tr>
<td>PD-L2</td>
<td>-1.45</td>
</tr>
<tr>
<td>PD-1</td>
<td>-1.38</td>
</tr>
<tr>
<td>Arg1</td>
<td>-1.59</td>
</tr>
<tr>
<td>iNOS</td>
<td>-1.73</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>-1.67</td>
</tr>
<tr>
<td>Gal-9</td>
<td>-1.75</td>
</tr>
<tr>
<td>TIM3</td>
<td>-2.04</td>
</tr>
<tr>
<td>LAG3</td>
<td>-1.55</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>-1.56</td>
</tr>
<tr>
<td>TNF-α</td>
<td>-1.99</td>
</tr>
</tbody>
</table>

Figure 8
Figure 9: The presence of CD8+ TIL correlates with the expression of immunosuppressive genes in human melanomas. (a) Heat maps showing the relative expression of CD8α and immunosuppressive genes in tumors from two independent cohorts of human melanoma patients (n=44-57).
Table 2. Correlation between the expression of CD8α and immunosuppressive genes in human melanomas

<table>
<thead>
<tr>
<th>Gene</th>
<th>GSE22153 Correlation</th>
<th>GSE19234 Correlation</th>
<th>p</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-L1</td>
<td>0.56</td>
<td>0.61</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PD-L2</td>
<td>0.57</td>
<td>0.52</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PD-1</td>
<td>0.93</td>
<td>0.21</td>
<td>0.00</td>
<td>0.18</td>
</tr>
<tr>
<td>Arg1</td>
<td>-0.32</td>
<td>0.03</td>
<td>0.02</td>
<td>0.84</td>
</tr>
<tr>
<td>iNOS</td>
<td>-0.12</td>
<td>0.11</td>
<td>0.38</td>
<td>0.48</td>
</tr>
<tr>
<td>TGFB1</td>
<td>0.08</td>
<td>0.38</td>
<td>0.53</td>
<td>0.01</td>
</tr>
<tr>
<td>TIM3</td>
<td>0.69</td>
<td>0.68</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>LAG-3</td>
<td>0.88</td>
<td>0.83</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>INDO</td>
<td>0.88</td>
<td>-0.10</td>
<td>0.00</td>
<td>0.53</td>
</tr>
</tbody>
</table>
Figure 10: rHuAd5-hDCT immunization instigates global transcriptional changes in the tumor. (a) Hierarchical clustering of differentially expressed genes in tumors following treatment with rHuAd5-hDCT, rHuAd5-hgp100, or untreated tumors (n=3). (b) Graphical representation of gene ontology (GO) analysis of genes differentially expressed in rHuAd5-hDCT treated tumors. (c) Graphical representation of top 20 differentially expressed genes (up and down regulated) between rHuAd5-hDCT and untreated control tumors.
Table 3. Positively enriched pathways in tumors following treatment with rHuAd5-hDCT

<table>
<thead>
<tr>
<th>Pathway Name</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL12-mediated signaling events</td>
<td>2.08E-06</td>
</tr>
<tr>
<td>IL23-mediated signaling events</td>
<td>2.90E-06</td>
</tr>
<tr>
<td>amb2 Integrin signaling</td>
<td>5.93E-06</td>
</tr>
<tr>
<td>Signaling events mediated by TCPTP</td>
<td>8.22E-06</td>
</tr>
<tr>
<td>Beta2 integrin cell surface interactions</td>
<td>8.97E-06</td>
</tr>
<tr>
<td>Notch-mediated HES/HEY network</td>
<td>1.98E-05</td>
</tr>
<tr>
<td>GMCSF-mediated signaling events</td>
<td>3.27E-05</td>
</tr>
<tr>
<td>Regulation of RAC1 activity</td>
<td>4.47E-05</td>
</tr>
<tr>
<td>CXCR4-mediated signaling events</td>
<td>6.74E-05</td>
</tr>
<tr>
<td>IL27-mediated signaling events</td>
<td>7.56E-05</td>
</tr>
<tr>
<td>IL5-mediated signaling events</td>
<td>8.10E-05</td>
</tr>
<tr>
<td>CXCR3-mediated signaling events</td>
<td>1.03E-04</td>
</tr>
<tr>
<td>Regulation of RhoA activity</td>
<td>1.33E-04</td>
</tr>
<tr>
<td>TNF receptor signaling pathway</td>
<td>1.68E-04</td>
</tr>
<tr>
<td>IFN-gamma pathway</td>
<td>8.26E-04</td>
</tr>
<tr>
<td>IL3-mediated signaling events</td>
<td>9.82E-04</td>
</tr>
</tbody>
</table>

Figure 10 (p1)
Figure 10 (p2)
Chapter 4:

The rate of CD8+ T cell expansion following therapeutic cancer vaccination is directly related to overcoming the adaptive response within the tumor and to the effectiveness of the vaccine.
Chapter 4: The rate of CD8+ T cell expansion following therapeutic cancer vaccination is directly related to overcoming the adaptive response within the tumor and to the effectiveness of the vaccine

Introduction

The development of effective cancer vaccine platforms able to elicit curative anti-tumor immune responses remains an elusive goal. While the generation of measurable tumor-specific T cell responses following vaccination confirms that vaccination can lead to the engagement of TAA-specific T cells, it does not appear that the resulting anti-tumor immune responses can elicit sufficient tumor attack to produce meaningful clinical responses (178).

It is well established that the tumor represents a complex immunosuppressive environment that limits the activity of infiltrating immune cells (274,350). As detailed in the introductory chapter, the tumor, tumor stroma, and infiltrating immune cell populations combine to initiate an array of local suppressive processes that act to prevent sustained immune attack on the tumor. While these mechanisms are often thought of as inherent properties of the tumor that must be overcome in developing successful immunotherapies, our data suggests that this is not the case. In the previous chapter, I described our observations regarding an intratumoral adaptive response to early immune attack by vaccine-induced CD8+ T cells, which resulted in a progressive decline
in local immune activity by DCT-specific CD8+ TIL. Importantly, this adaptive response occurred well before peak expansion or tumor infiltration by vaccine-induced CD8+ T cells. Overall, these observations present a major barrier to the generation of effective cancer vaccines, whereby early vaccine-induced T cells are insufficient to mount robust anti-tumor immunity, but are able to induce immunosuppressive mechanisms that ultimately limit the activity of tumor-infiltrating T cells. Put simply, the slow expansion of tumor-specific CD8+ T cells following vaccination instigates a suppressive tumor environment before the anti-tumor T cell response can be fully engaged. Therefore, increasing the rate of CD8+ T cell expansion following vaccination is likely critical to improving the impact of tumor-specific CD8+ T cells within the local tumor environment.

In the current chapter, we have investigated the rate and magnitude of CD8+ T cell expansion following rHuAd5-hDCT immunization and its implications in overcoming the adaptive response to immune attack in the tumor. Increasing the precursor frequency of DCT-specific CD8+ T cells, through adoptive transfer of naïve DCT T cells following rHuAd5-hDCT immunization, revealed that the rate and magnitude of DCT-specific CD8+ T cell expansion could be greatly increased and correlated with rapid tumor regression. Examination of the local tumor environment following treatment revealed rapid infiltration by high numbers of DCT-specific CD8+ T cells and the onset of early and robust immune attack that was able to outpace the adaptive immunosuppressive response within the tumor. These findings suggest that the slow rate of T cell expansion
following vaccination limits the true anti-tumor potential of vaccine therapies and may explain the limited clinical efficacy observed in therapeutic vaccine trials.

Results

Increasing the rate of CD8+ T cell expansion following vaccination results in tumor regression and improved survival

Our data presented in Chapter 2 suggest that the peak of the immune attack in the tumor, revealed by cytokine transcription, occurs several days before peak T cell infiltration. Further, the early immune attack triggers an adaptive response by the tumor which leads to elevated immune suppression and impaired functionality of the T cells that infiltrate the tumor at later time points. We hypothesized that augmenting the rate of T cell expansion following vaccination would lead to an enhanced level of immune attack at day 5 that would overcome the early adaptive response by the tumor. Badovinac et al. observed that precursor CD8+ T cell numbers at the time of vaccination defined the rate and magnitude of primary expansion (362). Therefore, increasing numbers of \(10^4\), \(10^5\) and \(10^6\) \(\text{DCT}^+\) T cells were delivered to mice immediately following immunization with rHuAd5-hDCT as a strategy to enhance the rate and magnitude of the primary expansion. Transfer of \(10^5\) \(\text{DCT}^+\) T cells resulted in a small increase in the overall frequency of DCT-specific CD8+ T cells (endogenous and exogenous CD8+ T cells) compared to vaccination alone or vaccination combined with \(10^4\) \(\text{DCT}^+\) T cells (Fig. 11a). However, transfer of \(10^6\)
DCT-T cells resulted in a robust expansion of circulating DCT-specific CD8+ T cells, resulting in a 5.2-fold increase in the overall frequency of DCT-specific CD8+ T cells 5 days following vaccination (Fig. 11a). Interestingly, despite initial differences in the magnitude of the vaccine-induced T cell response at day 5, we observed no differences among any of the groups in the frequency of DCT-specific CD8+ T cells by day 10 post vaccination, regardless of the number of transferred cells (Fig. 11a). The expansion of DCT-specific CD8+ T cells following transfer of DCT-T cells absolutely required rHuAd5-hDCT immunization, as treatment with the irrelevant vaccine rHuAd5-LCMV-GP did not result in expansion of DCT-specific CD8+ T cells (Fig. 11a).

The rHuAd-hDCT + 10^6 DCT-T cell treatment resulted in rapid tumor regression in all treated mice (Fig. 11b) and durable cures in approximately 65% of treated animals (Fig. 11c); the remaining mice ultimately experienced tumor relapse, despite complete clearance of detectable tumor lesions over a prolonged period. Interestingly, although all cured mice developed at least some auto-immune vitiligo at the tumor site, only a proportion exhibited disseminated vitiligo (Fig. 11d), suggesting variations in the severity of the autoimmune pathology accompanying the robust anti-tumor effects. A small improvement in tumor growth control and survival was observed following transfer of 10^5 DCT-T cells in combination with rHuAd5-hDCT compared to rHuAd5-hDCT +/- 10^4 DCT-T cells, although this treatment was not curative in any mice (Fig. 11b & c). The robust anti-tumor effect observed upon transfer of 10^6 DCT-T cells was again dependent on
rHuAd5-hDCT immunization (Fig. 11b & c).

**Tumor regression or growth correlates with the magnitude of the early CD8+ T cell response following vaccination but regression requires sustained anti-tumor immunity**

Based on the observation that robust CD8+ T cell expansion seemed to be important in eliciting tumor regression following vaccination, we next sought to determine if the early (day 5) or late (day 10) DCT-specific CD8+ T cell response correlated with tumor growth or regression following immunization. CD8+ T cell responses were clustered based on whether tumors initially grew or regressed following treatment with rHuAd5-hDCT +/- DCT-T cells (Fig. 11e). Tumor regression was associated with a high level T cell response early (day 5) post-vaccination whereas the response at a later time point (day 10) was no different between the mice where tumors regressed versus those that continued to grow (Fig. 11e). Complete regression, however, was dependent upon sustained anti-tumor immunity as depletion of CD8+ T cells on day 10 post vaccination/T cell transfer resulted in complete relapse in all treated animals (Fig. 11f).

**Heightened and more rapid expansion of DCT-specific CD8+ T cells following**
vaccination leads to more rapid tumor infiltration and sustained functionality

We next set out to determine if rHuAd5-hDCT immunization combined with transfer of $10^6$ DCT T cells was also leading to increased tumor infiltration by DCT-specific CD8+ T cells and how the functionality of these cells compared to cells at the lower transferred dose of $10^4$ DCT T cells. To improve resolution, the DCT-specific CD8+ T cell population within tumors was enumerated based on Thy1.1 expression. We observed a greater than 11-fold increase in the number of tumor infiltrating DCT-specific CD8+ T cells 5 days following immunization combined with the high dose of DCT T cells ($10^6$) compared to the low dose ($10^4$) (Fig. 12a). Transferred CD8+ TIL cell numbers were only enumerated until 8 days post treatment with rHuAd5-hDCT + $10^6$ DCT T cells, as tumors had regressed to the point that TIL could not be consistently quantified beyond this time point. Immunohistochemical staining of tumor sections revealed that treatment with rHuAd5-hDCT + $10^6$ DCT T cells resulted in not only a greater number of TIL, but also a more uniform distribution of T cells throughout the entire tumor than was observed at the $10^4$ T cell dose (Fig. 12b), where infiltrating T cells were located more within the peripheral margins of the tumor.

Further, DCT-specific CD8+ T cells in the tumors of mice receiving high dose transfer displayed a sustained capacity for IFN-γ production in response to ex vivo peptide stimulation, but were functionally impaired compared to the same CD8+ T cells in the peripheral blood (Fig. 13a). Additionally, transferred CD8+ TIL at the $10^6$ dose
displayed evidence of progressive functional defects in their ability to produce multiple cytokines (p<0.01, unpaired t test), although this observation was also made for transferred CD8+ T cells in the peripheral blood between day 5 and day 8 post treatment (p<0.001, unpaired t test) (Fig. 13b & c). The transferred CD8+ TIL showed a reduced capacity for degranulation compared to the PBL at both day 5 and day 8 (p<0.01, unpaired t test), but in contrast to cytokine production, this defect did not appear to be progressive (p>0.05, unpaired t test) (Fig. 13b & c).

**Rapid tumor infiltration by CD8+ T cells following T cell transfer results in robust immune attack that is able to outpace the adaptive response of the tumor**

To further investigate the local events within the tumor, we again utilized whole tumor transcriptional analysis. Consistent with early and robust immune attack, rHuAd5-hDCT combined with transfer of $10^6$ DCT T cells resulted in a considerable burst in intratumoral IFN-γ expression at day 5, increasing over levels following vaccination alone by greater than 5-fold (Fig. 14a). As observed following vaccination alone, TNF-α expression in the tumor was again delayed compared to IFN-γ, peaking at day 7, with expression increasing over vaccine alone by greater than 19-fold (Fig. 14a). As expected based on our cellular data, TCR-α expression in the tumor also increased following high-dose T cell transfer, showing a nearly 12-fold increase over rHuAd5-hDCT alone (Fig. 14a). Expression of genes associated with immune signaling (STAT1) and antigen
presentation (CIITA, MHC I, and MHC II) in the tumor were induced with similar kinetics to those observed following rHuAd5-hDCT alone, however the overall magnitude of expression was increased for all genes following the combination of rHuAd5-hDCT + $10^6 \text{DCT}$ T cells (Fig. 14b).

Perhaps more striking was that the robust early immune attack in the tumor, based on IFN-$\gamma$ expression, was instigated prior to the ability of the tumor to fully adapt to immune attack. PD-L1 expression, which increased in step with IFN-$\gamma$ following rHuAd5-hDCT vaccination (compare Fig. 14a with Fig. 14c), was outpaced by the rapid upregulation of IFN-$\gamma$ within the tumor following the combination treatment and did not peak until day 7 post treatment (Fig. 14c). Similarly, a number of other well-characterized immunosuppressive factors (PD-L2, Gal-9, iNOS, Arg-1, TGF-$\beta 1$) were also observed to exhibit slow induction kinetics relative to the rapid burst in IFN-$\gamma$ (Fig. 14c & d). Importantly, the robust IFN-$\gamma$ expression observed following vaccination + T cell transfer began to decline by day 7 post treatment, coinciding with induction of the previously described immunosuppressive factors (Fig. 14c & d), as well as immune checkpoint receptors PD-1, TIM3, and LAG-3 in the tumor (Fig. 14e), emphasizing the multitude of immunosuppressive factors acting within the tumor microenvironment. The transcriptional analysis following rHuAd5-hDCT + $10^6 \text{DCT}$ T cell treatment could not be extended beyond day 7 because the tumors had become too small to reliably retrieve good quality RNA for analysis. While we observed that the robust local activity following
transfer of $10^6$ DCT-T cells was absolutely dependent on rHuAd5-hDCT immunization, vaccination with rHuAd5-LCMV-GP + $10^6$ DCT-T cells did lead to small, but significant increases in the expression of TCR-α, STAT1, CIITA, PD-L1, PD-L2, Galectin 9, and LAG-3 at day 7 and TGF-β1 and iNOS from days 7-9 post treatment when compared to untreated control tumors, suggesting that the control vaccine + high dose DCT-T cell treatment was not completely immunological inert and could instigate small but measurable changes within the tumor (Fig. 14a-e).

**rHuAd5-hDCT + high dose DCT-T cell treatment results in similar global intratumoral changes as vaccination alone, but of greater magnitude**

To further investigate the breadth of changes induced by rHuAd5-hDCT + $10^6$ DCT-T cell treatment compared to rHuAd5-hDCT alone, we completed global gene expression profiling of tumors 5 days following treatment with rHuAd5-hDCT, rHuAd5-hDCT + $10^6$ DCT-T cells, or untreated control tumors to correspond to the point of maximal local immune activity as measured by IFN-γ expression (Fig. 14a). When compared to untreated tumors, we observed a large number of differentially expressed genes associated with both rHuAd5-hDCT-based therapies (Fig. 15a). Treatment with rHuAd5hDCT resulted in differential expression of 329 genes (316 upregulated, 13 downregulated), while rHuAd5-hDCT + $10^6$ DCT-T cells resulted in differential expression of 990 genes (880 upregulated, 110 downregulated) (Fig. 15b). Interestingly, almost all
genes differentially expressed following rHuAd5-hDCT treatment were also differentially expressed when vaccination was combined with DCT-T cell transfer (Fig. 15b), with a large number of genes uniquely changing following treatment with rHuAd5-hDCT + $10^6$DCT-T cells. Despite the greater breadth of local changes instigated by vaccination in combination with DCT-specific T cell transfer, GO analysis of the differentially expressed genes for each treatment revealed that 8 of the top 10 categories were common between the two vaccine treatments (Fig. 15c, left panel) and were associated with local inflammatory processes (Fig. 15c, right panel), suggesting that the two rHuAd5-hDCT-based therapies induced similar immunological changes within the tumor. However, comparison of the average relative expression for all common differentially expressed genes between rHuAd5-hDCT and rHuAd5-hDCT + $10^6$DCT-T cells revealed that the transfer of DCT-specific T cells instigated intratumoral changes of a greater magnitude than did vaccination alone, with co-induced genes increasing by a greater average magnitude and co-repressed genes decreasing by a greater average magnitude (Fig. 15d). These data suggest that although the rHuAd5-hDCT and rHuAd5-hDCT + $10^6$DCT-T cell treatments initiate similar immunological processes within tumors, the rHuAd5-hDCT + $10^6$DCT-T cell treatment induced response is considerably more robust.
Discussion

In this chapter, we have investigated the implications of increased early immune attack in overcoming the adaptive response in the tumor following vaccination as described in chapter 2. While increased pre-cursor frequencies of tumor-specific CD8+ T cells has been previously reported to improve vaccine efficacy against established tumors (363), we have determined, through analysis of local events within the tumor, that increasing the precursor frequency of DCT-specific CD8+ T cells in combination with rHuAd5-hDCT immunization results in a substantial burst in early intratumoral immune activity by a greater number of DCT-specific CD8+ TIL. Despite heightened induction of the same immunosuppressive mechanisms in the tumor as were observed following vaccination alone, this rapid and robust immune attack is able to outpace the adaptive response within the tumor, leading to tumor regression and durable cures in the majority of mice, suggesting a general enhancement in the local immune response within the tumor by this combination treatment. This is further supported by global tumor analysis following vaccination +/- high dose T cell transfer, where we observed similar immunological processes to be upregulated as were observed following vaccination alone, but of a greater relative magnitude, suggesting that the transferred DCT-specific T cells initiate comparable vaccine-induced changes within the tumor, but are able to greatly increase local immune attack compared to the endogenous DCT-specific CD8+ TIL following vaccination.
We have identified the magnitude of the early CD8+ T cell attack on the tumor to be a critical determinant in generating successful tumor regression against growing tumors. Using titrated doses of transferred DCT-specific T cells, we observed that tumors were eradicated only when the initial expansion of vaccine-induced CD8+ T cells occurred at a sufficient rate. When considering vaccine modalities that target self antigens, an important consideration is that most auto-reactive T cells are eliminated through central and peripheral tolerance and it has been reported from mouse studies that pre-cursor T cell frequency for tumor antigens may be as low as 1 cell/million naïve T cells (363). Therefore, generating even modest anti-tumor T cell frequencies following immunization requires vigorous T cell proliferation, with maximal T cell expansion occurring over a prolonged period. When considered in the context of our data, this point emphasizes that treatment modalities that can generate immediate immune attack are likely advantageous compared to treatments that elicit slower immune responses, such as vaccination, as slower acting treatments may give the tumor time to adapt prior to the generation of maximal anti-tumor immunity. This is further illustrated when considering ACT therapies, where high doses of ex vivo expanded and pre-conditioned T cells are infused into patients and have shown promising clinical response rates (109,111,213,219,364,365). It is tempting to speculate that the rapid immune attack potentiated in clinical ACT treatments may be effective in outstripping the ability of the tumor to upregulate adaptive immunosuppressive mechanisms, similar to observations
made herein, and resulting in a more pronounced anti-tumor effect than has been observed following vaccine delivery.

Importantly, the swift burst in immune attack generated when combining rHuAd5-hDCT with high dose DCT\(^{+}\) T cell transfer did not prevent the adaptive response from occurring within the tumor and actually led to greater expression of immunosuppressive genes over time compared to vaccination alone. Consistent with the observed induction of immunosuppressive genes, we observed a decline in intratumoral IFN-\(\gamma\) expression at the last sampled time point. While this may suggest that the CD8\(^{+}\) TIL were again being influenced by the local suppressive environment and were becoming less immunologically active within the tumor, it is equally possible that tumor destruction by activated T cells led to a reduction in available target antigen, resulting in engagement of fewer DCT-specific CD8\(^{+}\) T cells within the tumor, leading to lower global IFN-\(\gamma\) expression. This latter possibility is strengthened by our observation that activated DCT-specific CD8\(^{+}\) TIL (defined as IFN-\(\gamma^{+}\)) at the high transfer dose did not exhibit a reduced ability to produce IFN-\(\gamma\) over time and while these TIL did develop a diminished capacity to produce multiple cytokines, this was paralleled in the transferred DCT-specific CD8\(^{+}\) PBL, suggesting that the local tumor environment did not mediate this functional defect. Furthermore, our data argue in favor of a requirement for sustained local attack and not simply a short-lived burst in immune activity, as depletion of CD8\(^{+}\) T cells at the point when tumors had almost completely regressed resulted in rapid tumor relapse. When
considered together, our data indicate that the intratumoral activity of transferred DCT-specific CD8+ TIL, although declining globally at the last sampled time point, remained elevated compared to vaccination alone and was sustained at a sufficient level over a prolonged period as to mediate complete tumor clearance.

Overall, our findings point to a fundamental limitation of cancer vaccines. Namely, the ability of the tumor to rapidly respond to early immune attack by a small number of early tumor-specific CD8+ TIL appears to be the limiting factor in generating robust anti-tumor immunity and tumor regression. However, by increasing the rate and magnitude of T cell expansion following vaccination, it is possible to “turn the tables” on the tumor through generation of an early burst of immune attack to which the tumor cannot adequately respond prior to the onset of tumor destruction. These findings have important implications in the future design of vaccination strategies for clinical use and provide important rationale for investigating local events within the tumor as a means of understanding the impact of immunotherapeutic treatments.
Figure 11: Increasing the rate of DCT-specific CD8+ T cell expansion results in tumor regression and improved survival that correlates with the early CD8+ T cell response.  (a) DCT-specific CD8+ PBL were measured following treatment with rHuAd5-hDCT, rHuAd5-hDCT + 10^4-10^6 DCT T cells, or rHuAd5-LCMV-GP + 10^6 DCT T cells (n=5-14). (b) Tumor-bearing mice were immunized with rHuAd5-hDCT + 10^4 (□), 10^5 (△), or 10^6 (○) DCT T cells, rHuAd5-hDCT alone (■) or rHuAd5-LCMV-GP + 10^6 DCT T cells (◇). (c) Survival data corresponding to the treatments outlined in b. (d) Auto-immune vitiligo observed in mice following curative treatment with rHuAd5-hDCT +10^6 DCT T cells. (e) Analysis of the early (day 5) and late (day 10) frequencies of DCT-specific CD8+ PBL clustered based on tumor regression versus tumor growth (n=14-35). (f) Tumor bearing mice were immunized with rHuAd5-hDCT + 10^6 DCT T cells and depleted of CD8+ T cells 10 days following treatment. Tumor volumes were calculated from a single representative experiment (n=4-5) and survival data was compiled from independent experiments (n=7-14). Data presented as mean +/- SEM.
Figure 11 (p1)
Figure 11 (p2)
Figure 12: Treatment with rHuAd5-hDCT + 10^6_{DCT} T cells results in early tumor infiltration by a greater number of DCT-specific CD8+ T cells which distribute throughout the tumor. (a) Transferred DCT-specific CD8+ TIL (Thy1.1+CD8+) were enumerated following vaccination with rHuAd5-hDCT (n=5). rHuAd5-hDCT + 10^4_{DCT} T cells data reproduced from chapter 2 for reference. (b) Immunohistochemical staining of CD3 (red-brown AEC chromogen) in tumors following treatment with rHuAd5-hDCT + 10^4 or 10^6_{DCT} T cells. Data presented as mean +/- SEM.
Figure 12 (p1)
Figure 12 (p2)
Figure 13: High dose $^{DCT}T$ cell transfer results in sustained immune activity by DCT-specific CD8+ TIL, but CD8+ T cells still show defects in polyfunctionality. (a) Left Panels: Representative FACs plots showing IFN-γ production by transferred (Thy1.1+) DCT-specific CD8+ T cells in response to peptide stimulation following rHuAd5-hDCT + $10^6^{DCT}T$ cell treatment. Right Panel: Graphical representation of MFI calculations for IFN-γ production by transferred (Thy1.1+) DCT-specific CD8+ T cells as shown in the left panel (n=5). (b & c) Flow cytometric analysis of IFN-γ and TNF-α production (upper panels) and CD107a mobilization (lower panels) by transferred (Thy1.1+) DCT-specific CD8+ T cells following rHuAd5-hDCT + $10^6$ $^{DCT}T$ cell treatment. Values correspond to mean values calculated from compiled data (n=5). Data presented as mean +/- SEM.
Figure 13 (p1)
b

Figure 13 (p2)
c

PBL

Day 5

IFN-γ

10^0

10^1

10^2

10^3

10^4

10^5

0

58.8%

Day 8

IFN-γ

10^0

10^1

10^2

10^3

10^4

10^5

0

73.2%

26.8%

IFN-γ

10^0

10^1

10^2

10^3

10^4

10^5

0

82.7%

8.6%

CD107a

17.3%

8.6%

58.8%

91.4%

Figure 13 (p3)
Figure 14: Robust early immune activity is able to outpace the adaptive suppressive response in the tumor. Intratumoral expression of genes associated with T cell effector function (a), Immune signaling and antigen presentation (b), Immunosuppressive ligands (c), Checkpoint receptors (d), and Miscellaneous immunosuppressive processes (e) (n = 4). Gene expression data following rHuAd5-hDCT vaccination has been reproduced from figures in Chapter 2 for reference.
Figure 14 (p1)
**Figure 14 (p2)**

**d**

**Misc. Immune Suppression**

- Green: hDCT
- Red: hDCT + $10^6$ DCT T cells
- Blue: LCMV + $10^6$ DCT T cells

**e**

**Checkpoint Receptors**

- Green: hDCT
- Red: hDCT + $10^6$ DCT T cells
- Blue: LCMV + $10^6$ DCT T cells
Figure 15: Transfer of high dose $^{\text{DCT}}$T cells in combination with rHuAd5-hDCT initiates similar global changes within the tumor as vaccination alone, but of greater magnitude. (a) Hierarchical clustering of differentially expressed genes in untreated tumors compared to tumors treated with rHuAd5-hDCT (left) or rHuAd5-hDCT + $10^6$ $^{\text{DCT}}$T cells (right) (n=4). (b) Comparison of differentially expressed genes shown in (a) using Venn diagrams. (c) Comparison of top 10 GO terms associated with the differentially expressed genes following treatment with rHuAd5-hDCT +/- $10^6$ $^{\text{DCT}}$T cells. (d) Comparison of average relative expression for all co-induced genes following treatment with rHuAd5-hDCT +/- $10^6$ $^{\text{DCT}}$T cells. Data presented as mean +/- SEM.
antigen processing and presentation of exogenous peptide antigen
positive regulation of T cell mediated immunity
antigen processing and presentation of exogenous peptide antigen via MHC class II
antigen processing and presentation of peptide antigen via MHC class I
antigen processing and presentation of peptide antigen
positive regulation of T cell mediated cytotoxicity
antigen processing and presentation of exogenous antigen
positive regulation of T cell mediated cytotoxicity
antigen processing and presentation of exogenous peptide antigen

Figure 15
Chapter 5:

Combined vaccination and immunostimulatory antibodies provides durable cure of murine melanoma and induces transcriptional changes associated with positive outcome in human melanoma patients
Chapter 5: Combined vaccination and immunostimulatory antibodies provides
durable cure of murine melanoma and induces transcriptional changes associated
with positive outcome in human melanoma patients

Introduction

In the previous chapters, we have demonstrated that the anti-tumor immune
response elicited following rHuAd5-hDCT vaccination is only minimally effective in
slowing the growth of established tumors due to a rapid intratumoral adaptive response to
early immune attack by vaccine-induced CD8+ T cells. However, we have observed that
this adaptive response can be overcome by increasing the magnitude of early intratumoral
immune attack through adoptive transfer of DCT-specific CD8+ T cells at the time of
vaccination, resulting in rapid tumor regression and durable cures. While the studies
described in Chapter 2 and 3 provide key insights into the limitations of cancer vaccines
and the importance of the early immune attack in anti-tumor immune response, it is
equally important to consider the context in which therapeutic cancer vaccines are likely
to be administered. Specifically, it is unlikely that cancer vaccines will always be
administered in combination with adoptive transfer of a high dose of tumor-specific T
cells. Therefore, it is necessary to consider strategies of improving the endogenous anti-
tumor immune response following vaccination as a means of improving the utility of
cancer vaccines.

Maximizing the activity of cancer vaccines necessitates an appreciation of the
complex regulatory pathways that control the T cell response. Following ligation of the antigen receptor, T cell activation and function is regulated by costimulatory receptors of the TNFR and CD28 families (55,56). Of particular interest to the studies described in this chapter is the TNFR family member, 4-1BB, that plays a key role in T cell proliferation (59,366), effector function (367), and memory formation (62). Agonist 4-1BB monoclonal antibodies, used alone or with cancer vaccines, can improve T cell immunity against poorly immunogenic tumors (366,368-370). Of equal interest is PD-1, a CD28 family member that negatively regulates T cell function. PD-1 plays a role in limiting immune pathology (231) and is upregulated on T cells exposed to high antigen levels, such as tumor infiltrating lymphocytes (236,371). Antagonists of PD-1 signaling can partially reverse T cell exhaustion and improve T cell-mediated control of tumor growth (233,237,372).

In this chapter, we have employed immunomodulatory antibodies in order to enhance the efficacy of rHuAd5-hDCT without the requirement for adoptive transfer of DCT-specific CD8+ T cells. Treatment with a 4-1BB agonist following vaccination markedly increased the frequency of DCT-specific CD8+ T cells, but only produced transient tumor regression. Blockade of PD-1 signaling also enhanced vaccine efficacy but complete tumor regression was only achieved when 4-1BB co-stimulation was combined with PD-1 blockade. Strikingly, the benefit of the combined immunomodulatory antibodies did not manifest as a dramatic alteration in T cell polyfunctionality despite
evidence of a synergistic enhancement of immune activity within the tumor. In fact, global transcriptional analysis of the tumor following curative vaccination revealed significant upregulation of a gene signature that extended beyond T cells, indicating that successful tumor rejection following vaccination requires more than simply vaccine-induced T cells.

**Results**

**Increased 4-1BB signaling can improve immune attack resulting in enhanced rHuAd5-hDCT efficacy**

We hypothesized that the limited anti-tumor efficacy of rHuAd5-hDCT could be improved by employing an agonist monoclonal antibody against 4-1BB (α4-1BB), based on reports that this agonist could enhance genetic vaccines (368,373-375) and recover TIL function (376). Similar improvements in anti-tumor immunity were observed with 100µg – 500µg of α4-1BB, therefore we employed a dose of 200µg delivered on day 5 post-immunization for our experiments. Immunization of tumor-bearing mice with rHuAd5-hDCT + α4-1BB elicited a DCT-specific CD8+ T cell response that was 8.7-fold greater than rHuAd5-hDCT alone in the peripheral blood (Fig. 16a) which was associated with transient tumor regression (Fig. 16b) and improved overall survival (Fig. 16c); however, most tumors ultimately relapsed. It is notable that progressive autoimmune vitiligo was observed in mice that experienced complete tumor regression (Fig. 16d). Treatment with
α4-1BB in combination with an irrelevant vaccine, rHuAd5-LCMV-GP, did not elicit DCT-specific CD8+ T cells (Fig. 16b) and had no impact on tumor growth or survival relative to untreated mice (Fig. 16c and data not shown). To gain further insight into the events within the tumor, we measured the expression of the T cell-associated cytokines, IFN-γ and TNF-α, within the treated tumors. Whole tumor RNA was prepared from mice that were vaccinated with rHuAd5-hDCT +/- α4-1BB or the control vaccine rHuAd5-LCMV-GP + α4-1BB and cytokine expression was measured by qRT-PCR. Whereas cytokine expression in the tumors from mice immunized with rHuAd5-hDCT alone peaked 5-7 days post-vaccination and subsequently declined (Fig. 16e, broken line), treatment of tumor-bearing mice with the vaccine and α4-1BB significantly enhanced immune attack within the tumor (Fig. 16e, solid line). Interestingly, TNF-α production was increased to a higher level than IFN-γ. The elevation in cytokine expression was not due to the α4-1BB alone since tumors from mice treated with rHuAd5-LCMV-GP in combination with α4-1BB revealed no change in cytokine expression compared to untreated tumors (Fig. 16f). Expression of both IFN-γ and TNF-α persisted for a prolonged period when rHuAd5-hDCT was combined with α4-1BB, but began to decline around the same time point that the tumors relapsed.

Therapeutic vaccination with rHuAd5-hDCT promotes upregulation of PD-1 and its ligands within treated tumors
The immunosuppressive receptor PD-1 is often upregulated on CD8+ T cells faced with a high antigen burden, as in the case of the tumor microenvironment (236,371), so PD-1 expression was measured on vaccine-induced CD8+ T cells in the peripheral blood (PBL) and within the tumor (TIL). While PD-1 expression was largely absent on DCT-specific CD8+ PBL (Fig. 17a, upper left panels, grey histograms), PD-1 was significantly upregulated on DCT-specific CD8+ TIL, irrespective of treatment with α4-1BB (Fig. 17a, upper right panels, grey histograms, rHuAd5-hDCT CD8+ TIL MFI = 879.5 +/- 74.8, rHuAd5-hDCT + α4-1BB CD8+ TIL MFI = 838.0 +/- 87.4). PD-1 expression by CD8+ TIL required cognate interaction with tumor-associated antigen because LCMV-GP-specific CD8+ PBL and TIL, which do not recognize antigen in B16F10 tumors, were both largely PD-1-negative, in the presence or absence of α4-1BB (Fig. 17a, lower panels, grey histograms, rHuAd5-LCMV-GP CD8+ TIL MFI = 202.7 +/- 47.6, rHuAd5-LCMV-GP + α4-1BB CD8+ TIL MFI = 238.8 +/- 41.7).

We also investigated expression of the PD-1 ligands, PD-L1 and PD-L2, in the tumor following immunization. Quantitative RT-PCR revealed that expression of PD-L1 and PD-L2 was significantly upregulated in the tumor following treatment with rHuAd5-hDCT +/- 4-1BB, but not in tumors from mice immunized with rHuAd5-LCMV GP +/- 4-1BB or left untreated (Fig. 17b), confirming the likelihood that PD-1 ligand/PD-1 interactions were limiting the anti-tumor function of tumor infiltrating CD8+ T cells.
PD-1 blockade acts synergistically with 4-1BB co-stimulation to enhance immune attack within the tumor leading to complete tumor regression

Our data indicated that blockade of the PD-1 signaling pathway would be required to obtain the full benefit of the enhanced immunogenicity of the rHuAd5-hDCT + α4-1BB combination. Therefore, we combined the vaccination protocol with an antagonist monoclonal antibody to block PD-1 signaling (αPD-1), delivered every 3rd day beginning 3 days post vaccination. PD-1 blockade alone had no impact on the DCT-specific T cell response produced by rHuAd5-hDCT (Fig. 18a), but the blockade did promote transient tumor regression (Fig. 18b), confirming the utility of the antibody to reverse local immune defects within the tumor. Strikingly, the combination of αPD-1 and α4-1BB acted synergistically to enhance the efficacy of rHuAd5-hDCT, leading to complete regression of most tumors (Fig. 18b) despite no enhancement in the magnitude of the DCT-specific CD8+ T cell response (Fig. 18a). This synergistic benefit manifested as a durable cure in >70% of the mice, who remained tumor-free (Fig. 18c, p<0.0001 compared to all other treatments); the cured mice subsequently developed progressive autoimmune vitiligo (Fig. 18d). Mice immunized with rHuAd5-LCMV-GP + α4-1BB + αPD-1 displayed no change in tumor growth (data not shown) or long-term survival (Fig. 18c, closed diamonds).
The therapeutic benefit of the combination therapy is not reflected in either the magnitude or functionality of the DCT-specific CD8+ TIL

To understand the benefit of the mAb combination, we investigated the DCT-specific CD8+ TIL following the various treatments. Strikingly, the combination treatments did not result in a significant change in the number of DCT-specific TIL compared to vaccination alone (Fig. 19a), suggesting that the observed differences in tumor growth were not due to increased infiltration of tumor-reactive T cells. The polyfunctionality of the DCT-specific CD8+ PBL and TIL was examined to determine whether 4-1BB co-stimulation and/or PD-1 blockade reversed the previously described functional defects manifest in DCT-specific TIL (315). Similar to our previous report (315), the DCT-specific CD8+ T cells in the PBL were capable of producing multiple cytokines (IFN-γ and TNF-α) and undergoing degranulation (measured by mobilization of CD107a), while the DCT-specific CD8+ TIL were compromised in their ability to produce TNF-α and degranulate (Fig. 19b). We noted small, but significant, increases in TIL functionality in groups receiving the PD-1 mAb, as measured by increased frequencies of IFN-γ+/TNF-α+ and IFN-γ+/CD107a+ CD8+ TIL, suggesting that PD-1 blockade can recover some functionality in the vaccine-induced TIL. However, the change in polyfunctionality was not significantly different between mice who received αPD-1 alone and those that received α4-1BB + αPD-1, indicating that this modest enhancement in polyfunctionality could not explain the dramatic therapeutic effect.
Intratumoral transcriptional analysis reveals synergistic enhancement of local T cell activity upon inclusion of α4-1BB/αPD-1 treatment

We previously observed that intratumoral production of IFN-γ and TNF-α correlated with therapeutic outcome (Fig. 16e), therefore, to determine whether the combination therapy was associated with enhanced immune attack within the tumor, whole tumor RNA was isolated at discrete time intervals following treatment with rHuAd5-hDCT or rHuAd5-hDCT in combination with α4-1BB and/or αPD-1. Whereas treatment with rHuAd5-hDCT + α4-1BB or αPD-1 alone only resulted in transient elevation of IFN-γ and TNF-α expression within the tumor relative to treatment with rHuAd5-hDCT alone (Fig. 19c i and ii and data not shown), the combination of α4-1BB and αPD-1 produced a synergistic enhancement of cytokine expression relative to treatment with the individual mAbs (Fig. 19c i and ii). Further, the cytokine expression produced by the combination treatment continued to escalate until the tumors were too small to successfully retrieve RNA (day 14), while the cytokine expression in the mice receiving single mAbs plateaued and, ultimately, declined as the tumors relapsed. We also observed a synergistic enhancement in the expression of the PD-1 ligands PD-L1 and PD-L2, reinforcing the reciprocity between immune attack and upregulation of immune suppressive pathways in the tumor (Fig. 19c iii and iv).
Gene expression profiling of treated tumors reveals molecular differences between vaccine treatment groups.

Our results thus far demonstrated that the combination therapy produces complete tumor regression and a profound immune attack within the tumor (as measured by IFN-γ and TNF-α production). Yet, this enhanced intratumoral immunity was not associated with a remarkable change in the DCT-specific CD8+ TIL. To gain further insight into the mechanisms underlying the synergistic enhancements achieved through the combination treatment, we evaluated global transcriptional differences among tumors comprising each of the 4 treatment groups (rHuAd5-hDCT +/- α4-1BB and/or αPD-1). RNA was isolated from whole tumors 9 days post-vaccination and gene expression analyses were conducted using 3 biological replicates for each treatment group (n=12). To gain insight into the biological differences between the treatment groups, we first identified the top 25 genes associated with each treatment using prediction analysis of microarrays (PAM), and completed a Gene Ontology (GO) analysis (343) (Fig. 20a, supplementary table 1). GO analysis of identified genes (Table 4 & 5) revealed that several immune related processes were enriched in rHuAd5-hDCT + α4-1BB + αPD-1 treated tumors, whereas cell survival programs such as the negative regulation of apoptosis or JNK signaling were enriched in the rHuAd5-hDCT treated tumors. Interestingly, this suggests that treatment with the rHuAd5-hDCT vaccine alone did not induce strong immunity against the tumor, but rather resulted in activation of tumor cell
survival processes. Conversely, inclusion of $+\alpha 4$-1BB + $\alpha$PD-1 with the vaccine was sufficient to induce tumor immunity and overcame the activation of survival processes.

Taken together, these transcriptional analyses suggest that the combination of rHuAd5-hDCT with $+\alpha 4$-1BB + $\alpha$PD-1 not only results in induction of strong anti-tumor immunity, but also overcomes the activation of tumor cell survival processes associated with rHuAd5-hDCT treatment alone.

**Treatment-induced changes in gene expression are associated with good clinical outcome in human melanoma patients.**

Our data suggest that rHuAd5-hDCT + $\alpha 4$-1BB + $\alpha$PD-1 treatment elicited durable cures through complex immunological mechanisms which seem to involve both T cell-dependent and independent processes. We hypothesized that these same processes may be involved in the clinical course of human melanoma. To this end, we identified differentially expressed genes between rHuAd5-hDCT + $\alpha 4$-1BB +PD-1 treated tumors and all other treatment groups (Fig. 21a). We identified 94 differentially expressed Illumina probes, representing 85 unique genes, which we defined as the *immune-index* (Fig. 21b & c and Table 6).

To determine whether the biological changes embodied in our *immune-index* gene signature were consistent with observations in human melanoma patients, we interrogated the gene expression profiles of 123 metastatic melanoma samples (GSE19234,
GSE22155) for which patient survival data was also available. Briefly, GSE19234 comprised 39 stage III and 5 stage IV metastatic melanomas, whereas GSE22155 comprised 79 stage IV metastatic melanomas. Clinical outcome data was available for all 44 GSE19234 patients and 76 of 79 GSE22155 patients. Within the GSE19234 (Fig. 21d) cohort, patients with high immune-index scores experienced superior overall survival relative to those patients with lower immune index scores, and overall survival between these two groups was statistically different (HR: 0.38, p = 0.018) (Fig. 21e). We observed a similar improvement in survival between immune-index high and immune-index low patients within the GSE22155 cohort (HR: 0.59, p = 0.035) (Fig. 21f & g). Overall, these observations demonstrate that the unique intratumoral biological processes induced by rHuAd5-hDCT + α4-1BB + αPD-1 treatment are associated with improved survival in 2 independent cohorts of human melanoma patients. Notably, these data suggest that cancer immunotherapies that elicit similar changes within human tumors may be beneficial in the treatment of melanoma patients.

**Overlap in differentially expressed genes following treatment with two curative vaccine therapies suggests similar local changes associated with anti-tumor immunity.**

To determine if similar intratumoral changes might be mediating tumor clearance following treatment with rHuAd5-hDCT + α4-1BB/αPD-1 and the previously described
rHuAd5-hDCT + 10^6 DCT T cell therapy, we examined whether the differentially expressed genes comprising the *Immune-Index* where also differentially expressed in the tumor following treatment with the vaccine + high dose T cell transfer therapy. Interestingly, more than half of the genes found in the *Immune Index* (49 of the 85 genes or 57.6%) were also upregulated following rHuAd5-hDCT + 10^6 DCT T cell treatment, but not vaccination alone, compared to untreated tumors (*Table 7*). GO analysis revealed strong enrichment for immune processes among the 49 overlapping genes between the two curative treatments (*Table 8*). Notably, 20 additional genes present within the *Immune-Index* were upregulated in both rHuAd5-hDCT + 10^6 DCT T cell and rHuAd5-hDCT alone compared to untreated control tumors and were also associated with GO terms related to immune processes (*Table 7 & 8*).

**Discussion**

In this chapter, we have addressed the limited efficacy of rHuAd5-hDCT against growing B16F10 melanomas by combining vaccination with delivery of immunomodulatory antibodies. While the data presented in chapter 2 and 3 suggest that the kinetics of the immune response elicited by vaccination may be too slow to overcome the adaptive response within the tumor and significantly impact upon growing tumors, our observations in this chapter suggest that the true hurdle to effective anti-tumor immunity is the limited duration of intratumoral immune activity elicited by the vaccine.
It is notable that under circumstances where we combined α4-1BB and αPD-1, we measured a synergistic increase in the production of IFN-γ and TNF-α within the tumor compared to treatment with either mAb on its own, despite no increase in tumor-specific TIL numbers or remarkable change in TIL polyfunctionality. This is in contrast to our data presented in chapter 3, where we observed tumor regression to correlate with increased numbers of DCT-specific CD8+ TIL, although the TIL also exhibited reduced polyfunctionality. Our observations in this chapter further demonstrate that the true measure of vaccine activity, and ultimately efficacy, requires analysis of immunological events within the tumor and may not be apparent from ex vivo analysis of circulating T cells or TIL. This issue is of primary importance for extending vaccine strategies to humans as most studies rely upon sampling peripheral blood due to limited access to tumor tissues. Indeed, it is clear from our data and reports from others (236,315,356) that T cells in the peripheral blood do not accurately reflect the cells in the tumor. Our data goes further to demonstrate that ex vivo analysis of TIL may not provide an accurate measure of the events within the tumor either. Transcriptional analysis, however, provides an accurate and important measure of these events.

We have interpreted the expression of IFN-γ and TNF-α as evidence of T cell activity; however, it is equally possible that other cell types, such as NK cells and macrophages, also contributed to the expression of these cytokines and, thus, the synergistic increase in local expression following treatment with combined α4-1BB and
αPD-1 may be the result of activation of infiltrating populations other than T cells. Indeed monocytes, macrophages and NK cells can express both 4-1BB and PD-1 receptors (377-379), supporting the possibility that the ultimate anti-tumor effect is due to the combined actions of these mAbs on T cells as well as non-T cells. Further investigation is required to fully understand these mechanisms. As a step towards the elucidation of non-T cell-dependent mechanisms, we examined global transcriptional changes in tumors that regressed and did not regress. Strikingly, the majority of the most highly over expressed genes in the tumors from mice treated with the curative therapy were consistent with T cell, monocyte/macrophage/DC, and NK cell infiltration, supporting a potential role for these cells in tumor clearance.

Using global transcriptional data, we defined a set of immune genes associated with tumors that undergo complete regression and applied this gene set to transcriptome data from metastatic melanoma samples taken from 2 natural history cohorts. Strikingly, our Immune-Index gene signature was found to be predictive of improved survival in human melanoma patients. It has previously been reported that tumors displaying an inflammatory phenotype are associated with improved prognosis in human melanoma patients through the use of similar transcriptional profiling approaches (345,346,350). In the present study, we have identified a unique immune signature generated through the delivery of a pre-clinical immunotherapy in the context of a growing tumor that promotes tumor clearance. The observation that this immune signature is predictive of survival
outcome in two independent cohorts of melanoma patients suggests that development of therapeutic interventions that produce similar changes of immune status in human melanoma tumor is worthy of further investigation.

Interestingly, we observed that a subset of genes found within the *Immune-Index* is also upregulated by a second curative therapy, rHuAd5-hDCT + 10^6 DCT T cells. Additionally, a second subset of genes from the immune-index was upregulated in both curative (rHuAd5-hDCT + 10^6 DCT T) and modestly effective (rHuAd5-hDCT alone) therapies. While the latter gene subset may be reflective of the small degree of tumor growth control achieved through rHAd5-hDCT treatment, this observation suggests that not all genes present within the immune-index are exclusively associated with curative outcome. Therefore, it may be possible to further refine the *immune-index* to improve its predictive value through comparison to additional pre-clinical treatments. In spite of this, our identification of a subset of genes associated with improved clinical outcome indicates that global transcriptional analysis is a useful tool to bridge the gap between preclinical discoveries and clinical challenges in humans. Further, this study supports the inclusion of transcriptional signatures derived from efficacious pre-clinical immunotherapy models as useful secondary clinical endpoints for cancer immunotherapy trials.

Overall, our findings further highlight the limitations of cancer vaccines and reinforce the concept that optimal delivery of cancer vaccines will require maximizing vaccine immunogenicity and suppressing negative regulators of T cell function (380).
data also indicate that *ex vivo* analyses of PBL and TIL should be interpreted with caution since they do not accurately reflect the true immunological events within the tumor. Lastly, global analysis of vaccine treatment resulting in regression of murine tumors has revealed that similar immune signatures within human tumors are associated with good clinical outcome, further emphasizing the importance of understanding immunological changes within pre-clinical tumors as a means of improving the treatment of human cancer.
Figure 16: Stimulation of 4-1BB enhances the DCT-specific immune response following vaccination resulting in transient tumor regression and improved survival. (a) Tumor-bearing mice were immunized with rHuAd5-hDCT or rHuAd5-LCMV-GP and treated 5 days later with α4-1BB or Rat IgG. DCT-specific CD8+ PBL were quantified 10 days post-vaccination (n=5-20). (b,c) Tumor-bearing mice were immunized with rHuAd5-hDCT and treated with α4-1BB (△; n=20) or Rat IgG (□; n=9). As controls, mice were immunized with rHuAd5-LCMV-GP + α4-1BB (◇; n =8). (d) Example of progressive disseminated autoimmune vitiligo observed in cured mice following rHuAd5-hDCT + α4-1BB treatment. (e) Expression of IFN-γ and TNF-α in tumors from mice immunized with rHuAd5-hDCT + α4-1BB (△; n=4-8). (f) Expression of IFN-γ and TNF-α in tumors 9 days after treatment with rHuAd5-hDCT + α4-1BB (n=7-9), rHuAd5-LCMV-GP + α4-1BB (n=4) or untreated (n=4). Tumor volumes were calculated from a single representative experiment (n=4-5) and survival data was compiled from independent experiments. Data presented as mean +/- SEM.
Figure 16 (p2)
Figure 17: PD-1 is upregulated on tumor-specific CD8+ TIL following tumor infiltration in the context of elevated immunosuppressive PD-1 ligand expression in the tumor. (a) PD-1 was measured on antigen-specific CD8+ PBL and TIL (grey histograms) 10 days following immunization with rHuAd5-hDCT +/- α4-1BB or rHuAd5-LCMV-GP +/- α4-1BB. Dashed lines correspond to controls without PD-1 staining. Data presented from a single representative sample (n=5-8). (b) Expression of PD-L1 and PD-L2 in tumors from mice left untreated (n=4), treated rHuAd5-hDCT +/- α4-1BB (n=4-8), or rHuAd5-LCMV-GP + α4-1BB (n=4). Data presented as mean +/- SEM.
a

DCT-Specific CD8+ PBL

rHuAd5-DCT

DCT-Specific CD8+ TIL

rHuAd5-hDCT + α4-1BB

LCMV-GP- Specific CD8+ PBL

rHuAd5-LCMV-GP

LCMV-GP- Specific CD8+ TIL

rHuAd5-LCMV-GP + α41BB

Figure 17 (p1)
Figure 17 (p2)
Figure 18: Vaccination combined with 4-1BB stimulation and PD-1 blockade results in complete tumor regression. (a) Tumor-bearing mice were immunized with rHuAd5-hDCT or rHuAd5-LCMV-GP and treated with α4-1BB and/or αPD-1 as indicated. DCT-specific CD8+ T cells were quantified 10 days post vaccination (n=3-20). rHuAd5-hDCT +/- α4-1BB data reproduced from figure 1 for reference. (b,c) Tumor-bearing mice were immunized with rHuAd5-hDCT + α4-1BB + αPD-1 (▲; n=10), rHuAd5-hDCT + αPD-1 (■; n=10) or rHuAd5-LCMV-GP + α4-1BB + αPD-1 (◆; n=5) and rHuAd5-LCMV-GP + αPD-1 (○) (n=5) as controls. rHuAd5-hDCT +/- α4-1BB survival data reproduced from figure 1 for reference. (d) Example of progressive disseminated autoimmune vitiligo observed in cured mice following rHuAd5-hDCT + α4-1BB + αPD-1 treatment. Tumor volumes were calculated from a single representative experiment (n=4-5) and survival data was compiled from independent experiments. Data presented as mean +/- SEM.
**Figure 18**

(a) % DCT-specific CD8+ T cells of total lymphocytes

(b) Tumor Volume (mm$^3$)

(c) Percent Survival

(d) Image of a mouse, 10mm scale

```
- rHuAd5-hDCT + Rat IgG
- rHuAd5-hDCT + α4-1BB
- rHuAd5-hDCT + α4-1BB + αPD-1
- rHuAd5-hDCT + αPD-1
- rHuAd5-LCMV-GP + α4-1BB + αPD-1
- rHuAd5-LCMV-GP + αPD-1
```
Figure 19: 4-1BB co-stimulation and PD-1 blockade following vaccination synergize to increase immune activity within the tumor, despite no increase in the number of DCT-specific CD8+ T cells and only limited improvements in T cell polyfunctionality (a) Tumor-bearing mice were immunized with rHuAd5-hDCT and treated with α4-1BB, α4-1BB + αPD1, αPD-1 alone, or were given no additional treatment. DCT-specific CD8+ TIL were quantified 10 days post-vaccination (n=5 per treatment group). (b) Representative flow cytometric analysis of IFN-γ and TNF-α production and CD107a mobilization from DCT-specific CD8+ PBL following rHuAd5hDCT + α4-1BB + αPD-1 treatment as described in (a). Values correspond to mean values calculated from compiled data (n=5-19). (c) Expression of IFN-γ, TNF-α, PD-L1, and PD-L2 in tumors from mice treated with rHuAd5-hDCT in combination with α4-1BB (△), α4-1BB + αPD-1 (▲), or αPD-1 (■) (n=4-8). α4-1BB data reproduced from figure 1 for reference. Data presented as mean +/- SEM.
Figure 19 (p1)
Figure 19 (p2)
Figure 20. Identification of treatment specific probes. (a) PAM analysis was used to identify the top 25 probes associated with (a) rHuAd5-hDCT + α4-1BB + αPD-1, (b) rHuAd5-hDCT, and (c) rHuAd5-hDCT + α4-1BB. There were no probes which were specifically associated with the rHuAd5-hDCT + αPD-1 treatment (d).
### Table 4. Gene Ontology biological process analysis of treatment specific genes

**Gene Ontology - Biological Processes**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdhDCT + 4-1BB + PD-1</td>
<td>Positive regulation of immune response</td>
<td>2.6 E-7</td>
</tr>
<tr>
<td></td>
<td>Positive regulation of response to stimulus</td>
<td>1.2 E-6</td>
</tr>
<tr>
<td></td>
<td>Activation of immune response</td>
<td>1.9 E-6</td>
</tr>
<tr>
<td></td>
<td>Positive regulation of immune system process</td>
<td>2.0 E-6</td>
</tr>
<tr>
<td></td>
<td>Leukocyte mediated immunity</td>
<td>1.2 E-4</td>
</tr>
<tr>
<td><strong>AdhDCT</strong></td>
<td>Negative regulation of apoptosis</td>
<td>2.8 E-2</td>
</tr>
<tr>
<td></td>
<td>Negative regulation of programmed cell death</td>
<td>2.9 E-2</td>
</tr>
<tr>
<td></td>
<td>Negative regulation of cell death</td>
<td>2.9 E-2</td>
</tr>
<tr>
<td></td>
<td>Regulation of JNK cascade</td>
<td>4.9 E-2</td>
</tr>
<tr>
<td></td>
<td>Regulation of stress-activated protein kinase signaling pathway</td>
<td>5.0 E-2</td>
</tr>
<tr>
<td><strong>AdhDCT + 4-1BB</strong></td>
<td>Immune response</td>
<td>3.8 E-4</td>
</tr>
<tr>
<td></td>
<td>Regulation of actin filament polymerization</td>
<td>1.8 E-3</td>
</tr>
<tr>
<td></td>
<td>Regulation of actin polymerization of depolymerization</td>
<td>2.2 E-3</td>
</tr>
<tr>
<td></td>
<td>Regulation of actin filament length</td>
<td>2.3 E-3</td>
</tr>
<tr>
<td></td>
<td>Regulation of protein polymerization</td>
<td>2.8 E-3</td>
</tr>
<tr>
<td><strong>AdhDCT + PD-1</strong> (Negative association genes, no positives)</td>
<td>Immune response</td>
<td>1.6 E-4</td>
</tr>
<tr>
<td></td>
<td>Antigen processing and presentation of peptide antigen</td>
<td>7.6 E-4</td>
</tr>
<tr>
<td></td>
<td>Immunoglobulin mediated immune response</td>
<td>2.4 E-3</td>
</tr>
<tr>
<td></td>
<td>B cell mediated immunity</td>
<td>2.6 E-3</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte mediated immunity</td>
<td>3.5 E-3</td>
</tr>
</tbody>
</table>
Table 5. Top 25 genes associated with each treatment based on PAM analysis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Probe ID</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_1255416</td>
<td>LY6A</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2967266</td>
<td>FXYD5</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2863837</td>
<td>ALOX5AP</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2715840</td>
<td>C1QC</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2872058</td>
<td>CTSH</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2983516</td>
<td>SLC11A1</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2619620</td>
<td>C1QB</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2705119</td>
<td>LOC100047353</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2630993</td>
<td>PPAP2B</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2795078</td>
<td>UNC93B1</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2715042</td>
<td>SDC3</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2692797</td>
<td>SLC11A1</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_1217855</td>
<td>NKG7</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_1217849</td>
<td>LAPTM5</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2694179</td>
<td>CCL6</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_1235493</td>
<td>FXYD5</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2598622</td>
<td>SLAMF8</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_3143483</td>
<td>LAT2</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2606162</td>
<td>PDLIM4</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2597868</td>
<td>LGMN</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2675811</td>
<td>SLC11A1</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2792868</td>
<td>BLVRB</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_3001914</td>
<td>NFKBIA</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_3162407</td>
<td>ZFP36</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB + αPD-1</td>
<td>ILMN_2984828</td>
<td>LY6E</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_1241965</td>
<td>MITF</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_1231420</td>
<td>GOLGA2</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_1214163</td>
<td>B230380D07RIK</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2747381</td>
<td>DDX24</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2511355</td>
<td>TRIM2</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2760485</td>
<td>MAPK8IP1</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2491589</td>
<td>VGLL4</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2631143</td>
<td>SOX5</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2617425</td>
<td>CLPTM1</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_1254409</td>
<td>ATXN1</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2606667</td>
<td>DDX6</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_3009685</td>
<td>PTPRS</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2664224</td>
<td>EPHX1</td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------</td>
<td>-------------</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2707207</td>
<td>HHAATL</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2883016</td>
<td>NAV1</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2650732</td>
<td>TAF15</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_1254031</td>
<td>KLF9</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_1256171</td>
<td>TMEM63B</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2994779</td>
<td>GPSN2</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2621148</td>
<td>KIF3A</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2736153</td>
<td>MAP3K11</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2655058</td>
<td>FSTL4</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2752940</td>
<td>DHCR24</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_2698799</td>
<td>ACTB</td>
</tr>
<tr>
<td>rHuAd5-hDCT</td>
<td>ILMN_1214394</td>
<td>SPNB2</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_2771766</td>
<td>LOC100048556</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_1215862</td>
<td>CXCL9</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_2762944</td>
<td>IFI27</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_2988143</td>
<td>PLAC8</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_2426853</td>
<td>UBD</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_3083163</td>
<td>CP</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_1223179</td>
<td>H2-T23</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_3122961</td>
<td>GBP2</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_3008859</td>
<td>CTSC</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_1235499</td>
<td>PROS1</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_3104349</td>
<td>CAPZB</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_2944666</td>
<td>IFIT3</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_3139875</td>
<td>ACOT1</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_1215810</td>
<td>MCT51</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_1244866</td>
<td>GBP5</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_1244513</td>
<td>GBP3</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_2652857</td>
<td>IFI47</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_1227836</td>
<td>ARPC1B</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_2644415</td>
<td>PACSIN1</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_2603725</td>
<td>ARPC3</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_2827780</td>
<td>D14ERTD668E</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_3161805</td>
<td>LOC100048556</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_2893605</td>
<td>EIF4E3</td>
</tr>
<tr>
<td>rHuAd5-hDCT + α41BB</td>
<td>ILMN_1228913</td>
<td>PARP12</td>
</tr>
</tbody>
</table>

(Table 5 Continued)
Figure 21. rHuAd5-hDCT + α4-1BB + αPD-1 treatment probes are associated with positive outcomes in human melanoma patients. (a) Summary of treatment comparisons to identify probes associated with treatment induced B16F10 tumor regression. (b) Probes that were differentially expressed in all comparisons are highlighted with Venn diagrams. 94 probes were consistently over-expressed in the rHuAd5-hDCT + α4-1BB + αPD-1 treatment group relative to other treatments, whereas 0 probes were consistently under-expressed in the rHuAd5-hDCT + α4-1BB + αPD-1 treatment group relative to other treatments (immune index). (c) Heatmap displaying expression levels of the 94 probes in each treatment group, (a) rHuAd5-hDCT + α4-1BB + αPD-1, (b) rHuAd5-hDCT, (c) rHuAd5-hDCT + α4-1BB, (d) rHuAd5-hDCT + αPD-1. (d-g) Kaplan-Meier survival curves for human patients with metastatic melanoma, d) overall survival for 44 patients comprising the GSE19234 dataset, e) overall survival for the GSE19234 patient cohort divided into immune-index high and immune-index low groups, f) overall survival for 76 patients comprising the GSE22155 dataset, g) overall survival for the GSE22155 patient cohort divided into immune-index high and immune-index low groups.
Figure 21 (p1)
Figure 21 (p2)
Table 6. List of 85 genes uniquely upregulated with rHuAd5-hDCT + anti-4-1BB + Immune-Index

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILMN_2598622</td>
<td>SLAMF8</td>
</tr>
<tr>
<td>ILMN_1217855</td>
<td>NKG7</td>
</tr>
<tr>
<td>ILMN_1228404</td>
<td>SH2D2A</td>
</tr>
<tr>
<td>ILMN_2692797</td>
<td>SLC11A1</td>
</tr>
<tr>
<td>ILMN_2705119</td>
<td>LOC100047353</td>
</tr>
<tr>
<td>ILMN_2788073</td>
<td>HMOX1</td>
</tr>
<tr>
<td>ILMN_2689785</td>
<td>CD68</td>
</tr>
<tr>
<td>ILMN_2983516</td>
<td>SLC11A1</td>
</tr>
<tr>
<td>ILMN_2820893</td>
<td>SELPLG</td>
</tr>
<tr>
<td>ILMN_2759484</td>
<td>C3</td>
</tr>
<tr>
<td>ILMN_2688912</td>
<td>F10</td>
</tr>
<tr>
<td>ILMN_1219820</td>
<td>1200002N14RIK</td>
</tr>
<tr>
<td>ILMN_2687586</td>
<td>CXCL16</td>
</tr>
<tr>
<td>ILMN_2792868</td>
<td>BLVRB</td>
</tr>
<tr>
<td>ILMN_2715840</td>
<td>C1QC</td>
</tr>
<tr>
<td>ILMN_2606162</td>
<td>PDLIM4</td>
</tr>
<tr>
<td>ILMN_2675811</td>
<td>SLC11A1</td>
</tr>
<tr>
<td>ILMN_2784580</td>
<td>CD3E</td>
</tr>
<tr>
<td>ILMN_3143483</td>
<td>LAT2</td>
</tr>
<tr>
<td>ILMN_2967266</td>
<td>FXYD5</td>
</tr>
<tr>
<td>ILMN_2987709</td>
<td>SLC15A3</td>
</tr>
<tr>
<td>ILMN_2748875</td>
<td>FCER1G</td>
</tr>
<tr>
<td>ILMN_2863837</td>
<td>ALOX5AP</td>
</tr>
<tr>
<td>ILMN_1239055</td>
<td>XDH</td>
</tr>
<tr>
<td>ILMN_3001914</td>
<td>NFKBIA</td>
</tr>
<tr>
<td>ILMN_2866189</td>
<td>BTG1</td>
</tr>
<tr>
<td>ILMN_2630993</td>
<td>PPAP2B</td>
</tr>
<tr>
<td>ILMN_2720836</td>
<td>RBM47</td>
</tr>
<tr>
<td>ILMN_2795078</td>
<td>UNC93B1</td>
</tr>
<tr>
<td>ILMN_1259400</td>
<td>ENSMUSG000000-</td>
</tr>
<tr>
<td>ILMN_1224472</td>
<td>CCL4</td>
</tr>
<tr>
<td>ILMN_2984828</td>
<td>LY6E</td>
</tr>
<tr>
<td>ILMN_2776431</td>
<td>C1QA</td>
</tr>
<tr>
<td>ILMN_2687403</td>
<td>FCGR3</td>
</tr>
<tr>
<td>ILMN_1239765</td>
<td>PRDX5</td>
</tr>
<tr>
<td>ILMN_2715042</td>
<td>SDC3</td>
</tr>
<tr>
<td>ILMN_1260073</td>
<td>D16ERTD472E</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ILMN_1247811</td>
<td>Ass1</td>
</tr>
<tr>
<td>ILMN_2872058</td>
<td>CtsH</td>
</tr>
<tr>
<td>ILMN_2787257</td>
<td>Coro1a</td>
</tr>
<tr>
<td>ILMN_1253813</td>
<td>Nckap1l</td>
</tr>
<tr>
<td>ILMN_1215120</td>
<td>SemA4a</td>
</tr>
<tr>
<td>ILMN_1224077</td>
<td>Vav1</td>
</tr>
<tr>
<td>ILMN_1213954</td>
<td>Sgk1</td>
</tr>
<tr>
<td>ILMN_2663374</td>
<td>Nr1h3</td>
</tr>
<tr>
<td>ILMN_2663130</td>
<td>P2ry6</td>
</tr>
<tr>
<td>ILMN_2749448</td>
<td>Plekho2</td>
</tr>
<tr>
<td>ILMN_2719811</td>
<td>Lag3</td>
</tr>
<tr>
<td>ILMN_2733793</td>
<td>Coro1a</td>
</tr>
<tr>
<td>ILMN_2680038</td>
<td>Nuak2</td>
</tr>
<tr>
<td>ILMN_2619620</td>
<td>C1qb</td>
</tr>
<tr>
<td>ILMN_2721399</td>
<td>CtsW</td>
</tr>
<tr>
<td>ILMN_1235493</td>
<td>FxdY5</td>
</tr>
<tr>
<td>ILMN_2600678</td>
<td>Rac2</td>
</tr>
<tr>
<td>ILMN_2742928</td>
<td>FxdY5</td>
</tr>
<tr>
<td>ILMN_2714796</td>
<td>Coro1a</td>
</tr>
<tr>
<td>ILMN_1227434</td>
<td>Itgb7</td>
</tr>
<tr>
<td>ILMN_2769330</td>
<td>Cd6</td>
</tr>
<tr>
<td>ILMN_1234223</td>
<td>Pld4</td>
</tr>
<tr>
<td>ILMN_1213645</td>
<td>A1467606</td>
</tr>
<tr>
<td>ILMN_2866185</td>
<td>Btg1</td>
</tr>
<tr>
<td>ILMN_2634235</td>
<td>Lrrc33</td>
</tr>
<tr>
<td>ILMN_2513870</td>
<td>Zap70</td>
</tr>
<tr>
<td>ILMN_2685516</td>
<td>Hcls1</td>
</tr>
<tr>
<td>ILMN_2621752</td>
<td>Irf5</td>
</tr>
<tr>
<td>ILMN_2619528</td>
<td>Klhl6</td>
</tr>
<tr>
<td>ILMN_2747060</td>
<td>Coro1a</td>
</tr>
<tr>
<td>ILMN_2806700</td>
<td>Cyba</td>
</tr>
<tr>
<td>ILMN_1217849</td>
<td>Laptm5</td>
</tr>
<tr>
<td>ILMN_2743013</td>
<td>Ncf4</td>
</tr>
<tr>
<td>ILMN_2644350</td>
<td>Thy1</td>
</tr>
<tr>
<td>ILMN_2645662</td>
<td>Tmem86a</td>
</tr>
<tr>
<td>ILMN_3160659</td>
<td>Dok2</td>
</tr>
<tr>
<td>ILMN_2604029</td>
<td>Klf2</td>
</tr>
<tr>
<td>ILMN_2753697</td>
<td>Cd2</td>
</tr>
<tr>
<td>ILMN_1228213</td>
<td>Ifi30</td>
</tr>
<tr>
<td>ILMN_2607675</td>
<td>Loc641240</td>
</tr>
<tr>
<td>ILMN_1239963</td>
<td>Snx20</td>
</tr>
<tr>
<td>ILMN_1230587</td>
<td>Lpxn</td>
</tr>
<tr>
<td>ILMN_1258578</td>
<td>Ahnak</td>
</tr>
<tr>
<td>ILMN_2992709</td>
<td>Trem2</td>
</tr>
</tbody>
</table>

(Table 6 Continued)
<table>
<thead>
<tr>
<th>Genbank Accession</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILMN_2840514</td>
<td>KLHL6</td>
</tr>
<tr>
<td>ILMN_2735961</td>
<td>TLR13</td>
</tr>
<tr>
<td>ILMN_1251390</td>
<td>TBXAS1</td>
</tr>
<tr>
<td>ILMN_1220418</td>
<td>HCST</td>
</tr>
<tr>
<td>ILMN_1219946</td>
<td>IL10RA</td>
</tr>
<tr>
<td>ILMN_1257060</td>
<td>PLA2G15</td>
</tr>
<tr>
<td>ILMN_2611022</td>
<td>BCL11B</td>
</tr>
<tr>
<td>ILMN_2960700</td>
<td>PRF1</td>
</tr>
<tr>
<td>ILMN_2769191</td>
<td>TBXAS1</td>
</tr>
<tr>
<td>ILMN_2961091</td>
<td>SLC29A3</td>
</tr>
<tr>
<td>ILMN_2695793</td>
<td>FES</td>
</tr>
<tr>
<td>ILMN_2597868</td>
<td>LGMN</td>
</tr>
<tr>
<td>ILMN_2714638</td>
<td>MERTK</td>
</tr>
</tbody>
</table>

(Table 6 Continued)
### Table 7. Common genes between the Immune-Index and rHuAD5-hDCT Treatments

<table>
<thead>
<tr>
<th><strong>DCT + $10^6$ DCT T cells</strong></th>
<th><strong>DCT alone and DCT + $10^6$ DCT T cells</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>AHNAK</td>
<td>1200002N14RIK</td>
</tr>
<tr>
<td>ALOX5AP</td>
<td>C3</td>
</tr>
<tr>
<td>ASS1</td>
<td>CCL4</td>
</tr>
<tr>
<td>BCL11B</td>
<td>CORO1A</td>
</tr>
<tr>
<td>BLVRB</td>
<td>CTSH</td>
</tr>
<tr>
<td>BTG1</td>
<td>DOK2</td>
</tr>
<tr>
<td>C1QA</td>
<td>FCER1G</td>
</tr>
<tr>
<td>C1QB</td>
<td>LAPTM5</td>
</tr>
<tr>
<td>C1QC</td>
<td>LAT2</td>
</tr>
<tr>
<td>CD2</td>
<td>LOC641240</td>
</tr>
<tr>
<td>CD3E</td>
<td>NKG7</td>
</tr>
<tr>
<td>CD6</td>
<td>NUAK2</td>
</tr>
<tr>
<td>CD68</td>
<td>SDC3</td>
</tr>
<tr>
<td>CTSW</td>
<td>SELPLG</td>
</tr>
<tr>
<td>CXCL16</td>
<td>SEMA4A</td>
</tr>
<tr>
<td>CYBA</td>
<td>SH2D2A</td>
</tr>
<tr>
<td>D16ERTD472E</td>
<td>SLAMF8</td>
</tr>
<tr>
<td>ENSMUSG00000043795</td>
<td>SLC11A1</td>
</tr>
<tr>
<td>F10</td>
<td>SLC15A3</td>
</tr>
<tr>
<td>FCGR3</td>
<td>UNC93B1</td>
</tr>
<tr>
<td>FXYD5</td>
<td></td>
</tr>
<tr>
<td>HCLS1</td>
<td></td>
</tr>
<tr>
<td>HMOX1</td>
<td></td>
</tr>
<tr>
<td>IL10RA</td>
<td></td>
</tr>
<tr>
<td>IRF5</td>
<td></td>
</tr>
<tr>
<td>ITGB7</td>
<td></td>
</tr>
<tr>
<td>LAG3</td>
<td></td>
</tr>
<tr>
<td>LGMN</td>
<td></td>
</tr>
<tr>
<td>LOC100047353</td>
<td></td>
</tr>
<tr>
<td>LPXN</td>
<td></td>
</tr>
<tr>
<td>LY6E</td>
<td></td>
</tr>
<tr>
<td>NCF4</td>
<td></td>
</tr>
<tr>
<td>NCKAP1L</td>
<td></td>
</tr>
</tbody>
</table>
### DCT + $10^6$ DCT cells Cont.

<table>
<thead>
<tr>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFKBIA</td>
</tr>
<tr>
<td>NR1H3</td>
</tr>
<tr>
<td>P2RY6</td>
</tr>
<tr>
<td>PDLIM4</td>
</tr>
<tr>
<td>PLD4</td>
</tr>
<tr>
<td>PLEKHO2</td>
</tr>
<tr>
<td>PRDX5</td>
</tr>
<tr>
<td>PRF1</td>
</tr>
<tr>
<td>RAC2</td>
</tr>
<tr>
<td>RBM47</td>
</tr>
<tr>
<td>SNX20</td>
</tr>
<tr>
<td>THY1</td>
</tr>
<tr>
<td>TLR13</td>
</tr>
<tr>
<td>VAV1</td>
</tr>
<tr>
<td>XDH</td>
</tr>
<tr>
<td>ZAP70</td>
</tr>
</tbody>
</table>

(Table 7 Continued)
Table 8. Top 10 GO terms associated with genes upregulated in both the Imune-Index and rHuAd5-hDCT treatments

**Genes common in Immune Index and rHuAd5-hDCT + 10^6 DCT T cells**

<table>
<thead>
<tr>
<th>Gene Ontology - Biological Process</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Positive regulation of immune response</td>
<td>2.48E-09</td>
</tr>
<tr>
<td>2. Positive regulation of immune system process</td>
<td>3.06E-09</td>
</tr>
<tr>
<td>3. Positive regulation of response to stimulus</td>
<td>2.92E-08</td>
</tr>
<tr>
<td>4. Activation of immune response</td>
<td>1.07E-07</td>
</tr>
<tr>
<td>5. Regulation of leukocyte activation</td>
<td>5.70E-05</td>
</tr>
<tr>
<td>6. Regulation of cell activation</td>
<td>6.06E-05</td>
</tr>
<tr>
<td>7. Regulation of cell proliferation</td>
<td>8.01E-05</td>
</tr>
<tr>
<td>8. Regulation of T cell activation</td>
<td>2.04E-04</td>
</tr>
<tr>
<td>9. Immune response-activating signal transduction</td>
<td>2.95E-04</td>
</tr>
<tr>
<td>10. Immune response-regulating signal transduction</td>
<td>3.75E-04</td>
</tr>
</tbody>
</table>

**Genes common in Immune-Index and rHuAd5-hDCT +/- 10^6 DCT T cells**

<table>
<thead>
<tr>
<th>Gene Ontology - Biological Process</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Positive regulation of immune system process</td>
<td>8.90E-07</td>
</tr>
<tr>
<td>2. Antigen processing and presentation of peptide antigen</td>
<td>4.40E-06</td>
</tr>
<tr>
<td>3. Positive regulation of immune response</td>
<td>6.40E-06</td>
</tr>
<tr>
<td>4. Positive regulation of response to stimulus</td>
<td>2.21E-05</td>
</tr>
<tr>
<td>5. Immune response</td>
<td>5.01E-05</td>
</tr>
<tr>
<td>6. Activation of immune response</td>
<td>6.69E-05</td>
</tr>
<tr>
<td>7. Antigen processing and presentation</td>
<td>6.92E-05</td>
</tr>
<tr>
<td>8. Leukocyte mediated immunity</td>
<td>7.41E-05</td>
</tr>
<tr>
<td>9. Antigen processing and presentation of exogenous peptide antigen via MHC II</td>
<td>1.01E-04</td>
</tr>
<tr>
<td>10. Antigen processing and presentation of peptide antigen via MHC II</td>
<td>1.01E-04</td>
</tr>
</tbody>
</table>
Chapter 6:

Final Discussion
Final Discussion

In this final chapter, I will first summarize my research findings, as detailed in chapters 3-5. Next, I will focus on key concepts that have come to light in generating these data. I will emphasize that employing accurate surrogate readouts of local intratumoral immune activity is an absolute requirement for improving the design of effective immunotherapies. Secondly, I will detail the biological implications of our data, discussing how our findings can be integrated into the design of current and future treatments. I will conclude by discussing common mechanisms and hurdles that have emerged in conducting the described research that, I believe, also represent key obstacles to successful cancer vaccination in the clinic.

1.0 Summary of research findings

In the work described in chapter 3, we observed that a prototypic adenoviral vaccine, rHuAd5-hDCT, could elicit some growth suppression of established tumors, through mechanisms involving CD8+ T cells and the cytokine IFN-γ. Using a combination of whole tumor transcriptional analysis and cellular assays, we found that tumors were initially infiltrated by a small number of highly functional DCT-specific CD8+ T cells following vaccination. The DCT-specific CD8+ TIL, however, exhibited a progressive loss in immune activity and effector function over time, despite continued infiltration by greater numbers of DCT-specific T cells. Whole tumor transcriptional
analysis revealed that while rHuAd5-hDCT immunization was able to elicit potent inflammatory changes within the tumor environment, vaccination also resulted in rapid tumor adaptation, involving upregulation of an array of immunosuppressive genes in the tumor in response to attack by vaccine-induced CD8+ T cells. This adaptive response in turn acted to thwart prolonged local activity by DCT-specific CD8+ TIL and prevented the generation of robust anti-tumor immunity.

Given that the adaptive tumor response was instigated by a small number of early infiltrating DCT-specific CD8+ T cells, we hypothesized that increasing the rate of CD8+ T cell expansion following vaccination would result in more robust local immune attack prior to these adaptive events, thus enhancing the impact of vaccination. As described in Chapter 4, increasing the rate of DCT-specific CD8+ T cell expansion following vaccination through adoptive transfer of naïve DCT-specific T cells resulted in rapid tumor regression and durable cures in approximately 65% of treated mice. Importantly, tumor regression or growth correlated with the early CD8+ T cell response, highlighting early immunological changes following vaccination as critical determinants of treatment outcome. Cellular analysis revealed that the curative therapy correlated with an early increase in DCT-specific CD8+ TIL number and that the CD8+ TIL did not display the same level of functional suppression as had been observed previously. Whole tumor transcriptional analysis revealed that the rapid CD8+ T cell expansion associated with tumor regression was the result of a swift and forceful immune attack on the tumor that
was able to outpace the adaptive immunosuppressive response by the tumor and instigated similar global transcriptional changes as vaccination alone, but of a greater overall magnitude. Collectively, our data suggest that the rate of T cell expansion following vaccination is too slow and allows adaptive events within the tumor to suppress immune attack prior to full engagement of the anti-tumor immune response. Rapid expansion of tumor-specific T cells, however, results in a burst in local immune attack, which is able to surmount the adaptive tumor response, resulting in complete tumor regression and cure.

In chapter 5, I described our ability to enhance the DCT-specific CD8+ T cell response by combining rHuAd5-hDCT with an agonist 4-1BB mAb. This combination treatment resulted in transient tumor regression prior to relapse and was associated with an improved duration of intratumoral immune activity (compared to vaccination alone) that again declined as tumors relapsed. Analysis of DCT-specific CD8+ T cells in the peripheral blood and tumor revealed that the immunosuppressive receptor PD-1 was upregulated on DCT-specific CD8+ T cells following tumor infiltration. Whole tumor transcriptional analysis revealed that expression of the PD-1 ligands, PD-L1 and PD-L2, correlated directly with the level of immune attack following treatment with rHuAd5-hDCT +/- α4-1BB, suggesting the presence of a potent suppressive mechanism limiting the local activity of DCT-specific CD8+ T cells. Blockade of PD-1 in combination with rHuAd5-hDCT + α4-1BB resulted in complete tumor regression and durable cure of
>70% of treated animals. Analysis of DCT-specific CD8+ TIL did not reveal meaningful changes in TIL number or polyfunctionality associated with the curative treatment, although transcriptional analysis revealed an increased magnitude and duration of local immune activity for the curative treatment. Gene expression profiling revealed a unique 85-gene signature associated with the rHuAd5-hDCT + α4-1BB/αPD-1 treatment, termed the *immune-index* and comprising signatures from a number of immune cell subsets. This immune index was associated with a positive outcome in two independent cohorts of human metastatic melanoma patients, underscoring the relevance of immunological changes measured in pre-clinical models to those observed in human patients. Overall, these findings further highlight the limitation of vaccination against growing tumors and emphasize the requirement for improved vaccine immunogenicity, while also limiting the negative impact of relevant immunological checkpoints on anti-tumor immunity.

2.0 Implications of techniques employed in assessing anti-tumor immune activity

In carrying out the described research, we have explored the limited therapeutic success generated through administration of cancer vaccines. By combining cellular analyses with time course whole tumor transcriptional assays in the setting of both minimally effective and curative therapies, we have gained tremendous insight into the immunological determinants of treatment success or failure. While our overall findings will undoubtedly have a direct impact on the design and development of new therapies for
cancer, the significance of the techniques we have used to measure vaccine-induced immune attack and to further our understanding of events within the local tumor also warrant further discussion.

2.1 Direct intratumoral analysis permits the discrimination of parameters that lead to ineffective anti-tumor immunity

In the clinical setting, numerous approaches have been identified that can elicit moderate to robust anti-tumor immune responses (111,175,230). In most cases, the objective of these approaches has been to generate high frequency anti-tumor T cell responses, typically CD8+, that will initiate vigorous tumor attack on a growing tumor. Our observations in chapter 3 regarding tumor adaptation in response to early immune attack that ultimately extinguishes local immune activity provide a plausible explanation for the numerous failed attempts to successfully vaccinate against a growing tumor. Importantly, our findings emphasize that it is necessary to consider not only the ultimate magnitude of the T cell response following vaccination, but also the rate at which immune attack is elicited on the tumor and points to the need to investigate local events within the tumor to fully understand the global impact of vaccination and its role in limiting anti-tumor immunity.

2.2 Repeat sampling of tumor tissues to understand dynamic local events
To understand the global impact of vaccinating with rHuAd5-hDCT against established tumors, we performed gene expression profiling of tumors at a fixed time following treatment (Chapters 3-5). Similarly, a number of investigators have used clinical tumor biopsies to examine the tumor microenvironment using transcriptomics (345,346,350,381,382), gaining meaningful expression data regarding the inflammatory status of tumors, as well as the presence of suppressive mechanisms that may impact upon immunotherapeutic success.

While this approach can lead to the generation of insightful data, it became readily apparent to us that the conclusions we could draw regarding the local expression of both inflammatory and immunosuppressive processes in the tumor from a single data sampling was insufficient for a complete understanding of how vaccination was impacting on the tumor microenvironment. This idea was similarly explored by Wang and Marincola (383), where they describe the value of using fine needle aspirates (FNAs) to repeatedly sample and re-analyze tumors before and after immunotherapy in order to better understand local changes in the tumor. Using serial excision of whole tumors, we conducted time course analyses following treatment as a way to capture additional information regarding gene expression in the tumor over time in response to vaccination. Through collection of an extensive bank of serially-isolated RNA samples from tumors in the context of control, minimally effective, and curative therapies, as well as under knockout, antibody depletion, and neutralization conditions, we have been able to
effectively correlate intratumoral changes in gene expression with treatment outcome, identify key mediators of intratumoral immune activity and suppressive events, while also gaining an appreciation for the complexity of vaccine-mediated changes within the tumor.

Given the obvious limitations for human studies in obtaining relevant tumor biopsies for analysis at multiple time points, such approaches to immune monitoring may go beyond the scope of typical clinical practice. However, Kammula et al (384) reported on the feasibility of using sequential FNAs measure local immune activity (based on IFN-γ expression) within subcutaneous melanoma metastases following vaccination and recent reports have detailed the feasibility of obtaining adequate tumor material from CT-compatible needle biopsies to perform multiple assays (385). In the latter study, repeat biopsy sampling and analysis of T cell-related genes from a single metastatic deposit for each patient revealed reproducible gene expression levels by both IHC and qRT-PCR and gene expression profiling revealed a high degree of intratumoral homogeneity, suggesting that data acquired following biopsy collection is truly representative of the local tumor environment. The use of sequential biopsies could therefore facilitate repeat immune analysis of tumors during clinical treatment as tumors grow or regress, which has been suggested as a necessary requisite to understanding the full impact of treatment (386). Based on our findings, it is clear that a complete appreciation of the dynamics of the immune response within the tumor will depend on a capacity to sample the tumor
environment over the duration of treatment. Transcriptional analysis prior to intervention can provide invaluable information regarding the baseline inflammatory or suppressive status of the tumor environment and may impart important insights regarding the optimal choice of immunotherapy to be used (230,350,387). This is demonstrated by the observation that PD-1 blockade in a recent clinical trial only led to objective responses when the patient’s tumor was also PD-L1 positive (240), suggesting that knowledge of the local tumor environment is necessary prior to the selection of therapeutic interventions. Additionally, subsets of differentially expressed immune-related genes from pre-treatment biopsy samples were observed to correlate with positive clinical outcome in 3 reported melanoma vaccine trials employing different vaccine strategies (peptide + IL-12, DC-based vaccine, or recombinant antigen + adjuvant) (387), suggesting that analysis of the tumor environment prior to treatment may allow for the selection of patients that will be most likely to benefit from vaccine therapies. In addition to pre-treatment analysis, given the need to understand how the tumor environment is being impacted by treatment, it seems that repeat analysis post-treatment will be required to determine underlying mechanisms of treatment success or failure as we have described herein. This may be particularly advantageous in early phase clinical trials testing new therapies, where tumors are likely more advanced and may be more accessible for repeat sampling from which to gain additional information.
2.3 Improved resolution of anti-tumor immunity through the use of complementary assays

The parameters related to treatment efficacy that can be obtained as part of current cancer immunotherapy clinical trials are often limited by existing approaches of data collection. In most cases, investigators are limited to assaying blood samples as a means of determining if a given treatment has elicited the desired anti-tumor immune response (230), while monitoring the status of tumor lesions for evidence of clinical response in an attempt to correlate the two parameters. Given that immune responses in the blood have been shown to be poor surrogates of true anti-tumor immunity at the tumor site (356), it is clear that better assays and experimental readouts will be required in order to fully elucidate clinical observations and to advance the field beyond our current understanding. While clinical researchers are now more routinely gaining access to tumor tissue samples that can be used to generate meaningful data using any number of experimental assays, I believe that using a combination of these approaches to evaluate the local tumor environment is an important step towards understanding the biological events within the tumor.

In conducting pre-clinical studies in mice, investigators are presented with the opportunity to perform more invasive studies that go well beyond inherent limitations in obtaining clinical samples. To this end, we have used complementary approaches to understand how vaccination impacts upon the anti-tumor immune response both in the
periphery as well as the tumor and to determine how this response is altered over time following vaccination. First and foremost, the use of cell-based assays have been essential in evaluating the kinetics of the DCT-specific CD8+ T cell response following vaccination and were pivotal in our observation that CD8+ TIL undergo progressive functional exhaustion despite the polyfunctional phenotype of the same DCT-specific T cells in the peripheral blood. Secondly, the use of whole tumor transcriptional analyses afforded us the opportunity to investigate the local events within the tumor on a global level, while removing the requirement for *ex vivo* cellular manipulations, as well as removing the inherent bias of confining our studies to specific cell populations or target molecules of interest.

While both of these approaches can be used to effectively monitor anti-tumor immunity, the true benefit of their utility was achieved through the pairing of these complementary approaches, a point which simply cannot be overstated. Our experiments were often carried out in an iterative fashion, where ‘black box’ observations from the whole tumor could be followed up with *ex vivo* assays to corroborate the initial transcriptional observations. This approach is exemplified through the transcriptional analysis of the CD8+ T cell-derived effector cytokines IFN-γ and TNF-α within the tumor, which provided the first indication that DCT-specific CD8+ TIL were initially highly functional, but were being progressively suppressed over time. Whereas our previous data had suggested that the DCT-specific CD8+ TIL possessed presumably
static functional defects (315), this new data prompted us to re-evaluate the TIL at multiple time points and we observed that a small number of highly functional DCT-specific CD8+ TIL are in fact present at early time points, but become progressively dysfunctional, prompting us to further evaluate local events within the tumor that were mediating this effect.

The use of whole tumor analysis, by transcription or otherwise, may have limitations due to its lack of specificity for resolving the distinct cell populations underpinning observed changes of interest. While this may be true, this approach offers several advantages over assaying individual cell populations within the tumor. First and foremost, it is likely that capturing the global intensity of a particular event from all contributing cell populations in the tumor will more accurately reflect the true nature of a given parameter within the tumor, despite minor or major contributions from individual cells or cell populations. Secondly, the use of such approaches removes the inherent bias in focusing on individual cells or processes of interest that may unduly influence the interpretation of the data or the design of follow up experiments. Third, this approach allows tissue capture without the need for additional ex vivo manipulations that may alter or impede upon processes that were occurring in the tumor at the time of resection. Lastly, if additional resolution of observed changes at the cellular level is an absolute requirement, cellular assays (as previously described) or cell sorting techniques can be employed. In the latter case, we have had preliminary success in employing FACs
sorting of T cells (Thy1.2+), myeloid cells (CD11b+CD11c+), or tumor/tumor stroma (CD45-) prior to RNA isolation as a means of determining the relative contribution of each cellular compartment to transcriptional changes observed within the tumor following rHuAd5-hDCT treatment (data not shown). It is likely that similar approaches will become invaluable in pinpointing cellular compartments and/or processes that may act as barriers to successful immunotherapy, allowing for appropriate targeted interventions to modulate or overcome necessary pathways or mechanisms.

3.0 Biological implications

3.1 Immune suppression in the tumor is multifaceted

In analyzing events within the tumor following vaccination, we have identified a set of immunosuppressive genes that are upregulated in response to rHuAd5-hDCT that, presumably, cooperatively contribute to the observed local suppression of DCT-specific CD8+ T cells. As noted in the introductory chapter, investigators have previously characterized the functional implications of all suppressive genes that we have identified as contributing to the local adaptive immunosuppressive process. While current reports reveal a clear benefit to single immunomodulatory agents, highlighted by the inspiring effects of delivering blocking antibodies directed at CTLA-4 and PD-1/PD-L1 in recent clinical trials (107,108,240,352-355), our data suggest a unison induction of numerous
suppressive processes within the tumor that all likely contribute to local immune suppression. In line with this, studies by Curran et al (227) have demonstrated additional benefit to blocking multiple immunosuppressive pathways simultaneously. Furthermore, studies using human T cells have reported on the ability of tumor-specific T cells infiltrating tumor-positive lymph nodes to simultaneously upregulate a host of suppressive receptors including LAG-3, TIM-3, PD-1, BTLA, 2B4, and CTLA-4 (388), which infiltrate a tumor environment rich in the expression of cognate ligands (388). Paired with data suggesting that T cells receiving multiple suppressive signals possess greater functional impairments (239,389,390), this idea underscores the potential added benefit of a combinatorial approach to blocking immunosuppressive processes to improve local immune activation.

3.2 Can blockade of immune suppression actually result in co-stimulation?

While data presented in chapter 3 and 4 suggest that combining multiple treatment modalities to block immune suppression within the tumor is likely a requirement to improving immunotherapies, this view is complicated by our observation that PD-1 blockade alone was able to enhance the anti-tumor effect of rHuAd5-hDCT. We have interpreted the outcome of the anti-PD-1 treatment as blockade of an immune checkpoint. However, our data are actually more consistent with a situation of enhanced co-stimulation. We have seen that the combined anti-4-1BB/anti-PD-1 treatment produced a
synergistic increase in PD-L1 and PD-L2 expression and a similar increase in other immune checkpoint receptors/ligands (LAG-3/MHCII, TIM3/Galectin9) was also observed following vaccination. As such, it is difficult to imagine that blockade of a single immune checkpoint receptor could manifest such a profound effect on local T cell activity in the presence of additional suppressive signals. Both PD-L1 and PD-L2 can manifest co-stimulatory activity that enhances T cell function (391-394). Although the exact mechanism of this costimulation remains unclear, it is believed to be PD-1-independent and evidence exists to support binding of these ligands to an undefined, costimulatory receptor (395,396). Therefore, it is tempting to speculate that blockade of PD-1 binding may actually increase the availability of PD-L1 and PD-L2 for binding to the putative co-stimulatory receptor resulting in a synergistic enhancement of local T cell activity following stimulation with tumor antigen in the presence of anti-4-1BB. Our findings that checkpoint blockade can be combined with a co-stimulatory signal to produce a synergistic and lasting anti-tumor effect following vaccination is in line with other observations on the administration of combined agonist and antagonist immunomodulatory antibodies (226), which has important implications in the design of future vaccine trials. Put simply, the local activity of a tumor-reactive T cell within the tumor may, in fact, be the consequence of the combined stimulatory and inhibitory signals received by the T cell in situ, meaning that differing approaches of stimulation/blockade may endow tumor-specific T cells with similar improvements in their ability to attack
targeted tumor cells. In further developing such therapeutic strategies, however, it is important to note that treatment methodologies that attempt to completely abrogate or over-ride the restraints placed on self-reactive T cells will likely require careful consideration and may be hampered by agent-associated toxicities, especially in the context of combination therapies where the proposed agents have already displayed clinical toxicities when used as standalone agents (225,355,397,398).

3.3 Adaptive immune suppression in the tumor – does the tumor take advantage of conventional homeostatic mechanisms of immune tolerance?

In the work presented, we have identified a network of immunosuppressive mechanisms that prevent long-lasting anti-tumor immunity, thereby limiting the effectiveness of a therapeutic cancer vaccine. While chronic inflammation has previously been implicated in driving immunosuppressive mechanisms within the tumor that limit the anti-tumor immune response (399-402), we believe that we are first to report on the broad network of suppressive factors that are induced not upon chronic inflammation, but simply through the initiation of an anti-tumor immune response. We have described this occurrence as an adaptive response to immune attack within the tumor microenvironment, however this response is not necessarily unique to the tumor, as many of the same suppressive mechanisms have also been implicated in the maintenance of tolerance under normal homeostatic conditions or as a means of controlling autoimmune tissue destruction.
under circumstances of chronic inflammation.

Similar to our observations in the tumor, PD-L1 expression has been observed to increase with pancreatic inflammation in a mouse model of diabetes (403) and is expressed in the placenta (404) and by T\textsubscript{regs} (405) as a means of preventing immune attack on the semi-allogeneic fetus by infiltrating T cells. Furthermore, PD-L2 expression has been implicated in the maintenance of oral tolerance to ingested antigens (406) and has been shown to aid in controlling airway asthmatic responses (407). Not surprisingly, the checkpoint receptors PD-1, LAG-3, and TIM-3 play a role in the suppression of inflammatory processes to control autoimmune pathologies or tissue homeostasis (408-410), as do arginase (289), iNOS (411), TGF\textsubscript{β1} (412,413), IDO (414,415), and Galectin-9 (298). Therefore, it is clear that the tumor is not unique in its ability to evade inflammatory attack, but instead appears to respond through normal mechanisms of controlling inflammation. We have identified IFN-\textgamma as a critical effector molecule promoting anti-tumor immunity, while also leading to the induction of the many of the intratumoral suppressive factors analyzed. The production of IFN-\textgamma therefore acts as a double-edged sword, regulating both inflammation and immune regulation (416), underscored by the emergence of severe pathology in autoimmune disease models using mice deficient in IFN-\textgamma or the IFN-\textgamma receptor (417-421). Therefore, mechanisms of balancing the wanted and unwanted actions of IFN-\textgamma in order to generate robust local immune activity may be an essential component of effective cancer vaccine design.
We have reported on the induction of numerous suppressive genes in response to vaccination, however low-level expression of these genes was also detected in tumors where mice received control treatments or were left untreated, suggesting that these factors are not absolutely absent prior to immunological intervention. While it is possible that the low level expression of suppressive genes in control tumors is due to constitutive gene expression by tumor cells, it is equally possible that this low-level expression is occurring in response to spontaneous local activity by a small number of tumor-reactive immune cells, which is supported by the strong correlation between CD8α expression and the expression of suppressive factors in human melanoma patients as presented in Chapter 3, despite the fact that only a small number of these patients received immunotherapy (345,346).

3.4 Relevance of T cell polyfunctionality versus activity in the anti-tumor immune response

The polyfunctionality of tumor-specific T cells has been established as an important metric in assessing the tumor-specific T cells elicited by a candidate therapy. It has been reported repeatedly that tumor-specific TIL possess defects that are not observed in the same cell populations in peripheral tissues (236,315,356,422), suggesting that efforts to maintain or restore TIL polyfunctionality may be a key component to improving treatment outcome. However, in testing different therapeutic approaches in
the context of rHuAd5-hDCT vaccination, we did not observe a substantial improvement in DCT-specific CD8+ TIL polyfunctionality when comparing curative treatments to minimally effective treatments, suggesting that tumor regression was not simply the result of restoring CD8+ TIL functionality. Instead, we observed variations in the level and/or duration of expression for CD8+ T cell-dependent effector cytokines within the tumor, which we have interpreted as surrogate markers of local T cell activity.

In conducting our analyses, we have made a careful distinction between T cell activity and T cell functionality, defining activity as the expression of inflammatory molecules within the local tumor and functionality as the ability to produce cytokines or degranulate in response to ex vivo stimulation. Following vaccination, we observed that DCT-specific CD8+ T cells in the tumor were initially polyfunctional, but that over time, these cells began to show a reduced capacity for the production of cytokines and for degranulation. Importantly, we observed similar defects in the DCT-specific CD8+ TIL following delivery of both curative and non-curative therapies. However, in both curative treatments that we have identified, we observed a significant increase, either in magnitude or prolongation, of local immune activity within the tumor. Our data, therefore, suggests that the destructive effects elicited by tumor-reactive T cells may not require that TIL retain (or regain) the polyfunctional phenotype observed in circulating T cells. Instead, it appears that local T cell activity of sufficient magnitude and/or duration can promote tumor regression in the absence of increased or sustained functionality.
In interpreting our data, it is important to consider that we have not assayed a complete array of cytokines or cytotoxic molecules/receptors employed by CD8+ T cells in mediating cell destruction. It is entirely possible that our restricted analysis has not managed to capture a particular functional characteristic of tumor-specific CD8+ TIL that may further explain our observations and future studies may benefit from an expanded analysis. However, by adapting simple criteria, we have developed a means of resolving meaningful differences in CD8+ T cell activity within the tumor, which we have interpreted to be a more predictive of treatment outcome than the polyfunctionality of the CD8+ TIL. The discrimination of T cell functionality versus activity therefore represents an important principle for assessing the response elicited by cancer immunotherapies and warrants consideration by future investigators.

3.5: The role of tumor-infiltrating myeloid cells in the anti-tumor immune response elicited by rHuAd5-hDCT

Although our vaccination strategies have focused on enhancing and sustaining the anti-tumor T cell response, we were somewhat surprised to observe both strong T cell and myeloid cell gene signatures within the Immune Index associated with the curative treatment combining rHuAd5-hDCT with α4-1BB/αPD-1 (Chapter 5). We also observed gene signatures consistent with myeloid cell populations in the tumor following treatment with rHuAd5-hDCT alone (data not shown), however, given the induction of a
suppressive tumor environment associated with rHuAd5-hDCT treatment and the poor ability of the vaccine to elicit productive anti-tumor immunity, it is likely that this identified gene signature is related to immunosuppressive TAMs, MDSCs, or tolerogenic TIDC populations that may be collectively acting to limit local immune attack. In attempting to circumvent the suppressive effects potentially instigated by these cell populations, we immunized CCR2-deficient mice, a chemokine receptor involved in myeloid cell recruitment, (73,74,95), with rHuAd5-hDCT following tumor implantation. Somewhat surprisingly, however, any tumor growth control resulting from immunization was completely abrogated in CCR2-deficient mice (data not shown), suggesting that the recruitment of myeloid cell populations to the tumor is an absolute requirement for effective anti-tumor immunity following rHuAd5-hDCT immunization. In line with this, based on the known cellular functions of the myeloid-associated genes found within the curative Immune Index signature (Table 9), it appears that rHuAd5-hDCT + α4-1BB/αPD-1 may instigate functional changes within the myeloid infiltrate and is not necessarily associated with either an overall increase or differential increase in infiltration by a specific myeloid cell population(s) (based on a limited presence of cell surface marker genes within the signature). Due to the high degree of phenotypic and functional overlap between monocytes, macrophages, MDSCs, and DCs, it is not possible to determine which single myeloid cell population, if any, can be connected to this unique signature without conducting extensive cell-based assays to accurately characterize the
myeloid cell tumor infiltrate. However, it is possible to speculate on how the rHuAd5-
hDCT + α4-1BB/αPD-1 treatment may be altering the functionality of myeloid cells within the tumor.

While our transcriptional analysis suggests that CD8+ T cell activity within the tumor is absolutely required for the initiation and sustain of the anti-tumor immune response following vaccination, the emergence of this curative myeloid signature suggests one of 2 possibilities. First, it is possible that by mounting a higher and more sustained immune attack by vaccine-induced CD8+ T cells, through the combination of 4-1BB co-stimulation and PD-1 blockade, that a reprogramming of myeloid cell populations within the tumor has occurred, enhancing tumor destructive mechanisms over immune suppression. This is in line with a previous report where IL-12 secretion by CD8+ T cells was able to alter the functional capacity of tumor-infiltrating/resident MDSCs, macrophages, and DCs such that these myeloid populations promoted the activation and proliferation of tumor-specific CD8+ T cells, leading to pronounced enhancement of the anti-tumor immune response (423). Secondly, as alluded to in the discussion section of Chapter 5, it is equally probable that the functionality of myeloid populations in the tumor was influenced by the treatment with α4-1BB or αPD-1, as myeloid populations, as well as lymphocytes, have been reported to express these receptors (377,378). Interestingly, PD-1 signaling has been shown to lead to IL-10 secretion by monocytes and is able to inhibit T cell proliferation and function (424). In contrast, disruption of PD-
L1/PD-1 signaling was able to decrease IL-10 secretion (425) and restored the capacity of monocytes/macrophages to produce IL-12 (378,426). Similarly, 4-1BB signaling has been reported to enhance the activation of DCs and promote monocyte/macrophage differentiation (427,428). Based on these observations and the identified myeloid gene signature, it appears that mechanisms of modulating myeloid cell functions in favor of immune activation versus immune suppression represents a potent means of enhancing or sustaining T cell-mediated anti-tumor immunity. Therefore, modulating tumor infiltrating myeloid cell function may represent an important facet of improving future vaccine design as a means of enhancing local immune attack on the tumor.

3.6 Can currently available drugs be used to modulate the tumor environment in favor of improved vaccine efficacy?

In studying the gene signatures present in the *Immune Index* together with the other data presented in Chapter 5, we can hypothesize that the successful tumor clearance associated with rHuAd5-hDCT + α4-1BB/αPD-1 treatment is more inline with the modulation of immune cell function within the tumor than it is with greater infiltration of the tumor by immune cells. This hypothesis is based on two important observations. First, DCT-specific CD8+ TIL, despite not increasing in cell number, appear to persist in an active state for a prolonged period within the tumor and are associated with a gene signature (from the *Immune Index*) suggestive of increased cellular activation (Table 10).
Secondly, as previously stated, the myeloid cell signature found within the Immune Index appears to be more associated with changes in cellular function than changes in cellular infiltrate. If these assumptions are in fact true, then it becomes tempting to speculate that combining vaccination with previously approved clinically-available drugs or compounds that can appropriately modulate the local tumor environment may represent an attractive approach to enhancing the local inflammatory response within the tumor following vaccination. Given the prominent nature of the T cell and myeloid cell signatures within the Immune Index, proposed strategies will emphasize targeting these cellular compartments in particular.

It is interesting to note that a number of currently employed anti-cancer drugs possess immunomodulatory capacities capable of enhancing cancer immunotherapy. We have previously reported on the ability of cyclophosphamide, a commonly used chemotherapy, to enhance the DCT-specific CD8+ T cell response and improve treatment outcome when combined with rHuAd5-hDCT (315). Similarly, recent studies have shown that exposure to clinically relevant doses of platinum-based chemotherapies resulted in downregulation of the suppressive ligands PD-L1/PD-L2 on cultured DCs, as well as PD-L2 on tumor cells, resulting in improved T cell activation (235). Furthermore, the tyrosine kinase inhibitor sunitinib, approved for the treatment of GI stromal tumors (GIST) and metastatic renal cell carcinoma (RCC) (429,430), can prevent MDSC and T_{regs} accumulation, resulting in restored T cell activation through alterations in the
immune cell infiltrate within tumors (431). Anti-angiogenic therapies have also been reported to improve the extravasation of immune cells, and specifically T cells, into tumors by increasing the expression of endothelial cell adhesion molecules within the tumor (432).

Drugs not commonly prescribed for cancer treatment, such as phosphodiesterase-5 inhibitors (including sildenafil, vardenafil, and tadalafil) have also been shown to increase tumor infiltration and activation of CD8+ T cells through mechanisms of downregulating iNOS and arginase activity in intratumoral MDSCs, thereby reversing their suppressive functions (90,401,433). Furthermore, an array of non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to generate pleiotropic effects in enhancing anti-tumor immunity through mechanisms of reversing Tregs-mediated immune suppression, inhibiting TAM-mediated pro-tumor pathways while boosting anti-tumor functions, as well as improving tumor infiltration, proliferation, and function of CD4+ and CD8+ T cells (434).

Although many of these treatments were initially identified as being inherently immunosuppressive, it has become clear that appropriate dosing and timing of administration can drastically alter the immunomodulatory capacity of a particular agent. Our data points to a clear requirement for altering the inflammatory status of the tumor environment in order to enhance the anti-tumor effect of vaccine-induced T cells and the underlying ability of these agents suggest that drug repurposing may represent an
important means of enhancing the anti-tumor immune response, either through preventing the accumulation of immunosuppressive cell populations or by re-establishing their potential for immune attack.

4.0 Are our observations clinically meaningful?

In the presented research, we have interrogated therapeutic cancer treatments that run the spectrum from ineffective or minimally effective to those that result in complete tumor regression and cure. While clinical reports of cancer immunotherapy occupy a similar outcome spectrum, our data is unique in that we have been able to take a singularly ineffective cancer vaccine (rHuAd5-hDCT) and, through treatment modification, have generated two curative treatments with distinct mechanisms of action. In this section, I will organize our general observations regarding vaccine efficacy in the pre-clinical B16F10 melanoma model and will attempt to integrate these findings within the current setting of cancer immunology.

As depicted in Figure 22, Panel B, immunization with rHuAd5-hDCT results in the generation of a DCT-specific immune response, with DCT-specific CD8+ T cells comprising 3-4% of the circulating CD8+ T cells. This type of observation is akin to those made in numerous clinical trials, where tumor antigen-specific CD8+ responses are readily detectable in the peripheral blood following vaccination. However, the expansion kinetics of the DCT-specific CD8+ T cells is quite slow, taking nearly 2 weeks to reach
peak levels following vaccination and, in the end, instigates relatively low-level attack on the tumor. In contrast, we have observed a rapid capacity of the tumor to adapt to local immune attack, through upregulation of a number of immunosuppressive processes. Our analysis of clinical samples strongly supports upregulation of similar intratumoral suppressive processes in response to CD8+ T cell infiltration as we have observed in our murine model. It is therefore possible that human vaccine trials have failed to initiate potent anti-tumor immune responses for the same reason as delivery of rHuAd5-hDCT. More specifically, despite the generation of seemingly high frequencies of tumor-reactive T cells, the ability of the tumor to swiftly adapt to the early low-level vaccine response by upregulating a network of suppressive processes limits the nascent vaccine response well before it reaches full magnitude, resulting in a minimal impact on tumor growth. Not surprisingly, this has manifest as clinical responses to cancer vaccines in less than 4% of treated patients (178), leading many to question the utility of vaccine therapies for cancer.
The first described successful modification to our vaccine protocol involved combining adoptive transfer of naïve DCT-specific CD8+ T cells with vaccination (Fig. 22, Panel A). ACT instigated rapid expansion of DCT-specific CD8+ T cells, resulting in very high frequencies of tumor-reactive CD8+ T cells early following vaccination. I
believe that this approach initiates a very similar immunological scenario to that which has been described in clinical adoptive T cell transfer studies, where large numbers of tumor-specific T cells are infused into patients (111). Furthermore, while our approach involves the infusion of transgenic T cells, current protocols for the genetic-engineering of human poly-specific T cells with tumor-specific TCRs have shown a high degree of success and are currently being evaluated clinically (213-215), suggesting that this approach has direct clinical implications. While clinical studies of ACT have not focused extensively on the dynamic events within the tumor leading to tumor destruction, our data suggest that the high rates of objective and complete responses reported in clinical trials of adoptive T cell transfer are likely the result of a rapid and robust burst in local immune activity. Herein, we have described this process as the capacity of the local immune attack to ‘outpace’ the adaptive response by the tumor, but it is likely that we have exposed a signaling threshold within the tumor, whereby receptors in the tumor have been fully saturated by the activity of infiltrating T cells (or other activated cells) and simply cannot respond at a greater rate. This idea is supported by a continued induction of genes associated with immune signaling, antigen presentation, as well as immune suppression at later time points relative to the initial burst in immune attack (based on IFN-γ expression) following rHuAd5-hDCT + 10^6 DCT T cell treatment, suggesting that the limited capacity of the tumor to respond to inflammatory signals is being exploited, permitting a more efficacious and destructive immune assault on the tumor. Evaluation of clinical tumor
biopsies following ACT treatments would provide invaluable insights towards determining if the same mechanisms are, in fact, leading to tumor destruction in clinical patients.

Although highly effective, the preparation of autologous tumor-reactive T cells for anti-tumor therapies is not a trivial process, in terms of technical or financial requirements (160). Therefore, finding a means of improving on vaccine therapies with the potential for broad application, such as vaccines, that can be readily administered to cancer patients is likely a more attractive and feasible approach. To this end, we have assessed the ability of immunomodulatory monoclonal antibodies to augment the anti-tumor effect of administering rHuAd5-hDCT and observed that combining vaccination with 4-1BB co-stimulation and PD-1 blockade resulted in tumor regression and cure in the majority of treated mice (Fig. 22, Panel C). Although the development of monoclonal antibody-based therapies for cancer is not a new idea, the use of antibodies that can enhance or restore the activity of tumor-reactive immune cells, especially T cells, has become an attractive clinical approach to improving anti-tumor immunity, with the delivery of antibodies such as αCTLA-4 and αPD-1/αPD-L1 demonstrating impressive clinical responses (107,352-354) and PD-1/PD-L1 (108,240,355). Interestingly, in the described studies, combined treatment with α4-1BB and αPD-1 treatments was required for complete tumor regression when combined with rHuAd5-hDCT, whereas singular antibody treatments could not sustain local immune attack and tumors relapsed under
most circumstances. This observation is in line with clinical data, where the majority of patients displaying objective responses following antibody treatment ultimately relapse, with only a small rate of complete response. Interestingly, additional analysis of the unique Immune-Index associated with this therapy indicates that tumor regression may be mediated by alterations in the functionality of a number of immune cell subsets, suggesting that additional investigation of the anti-tumor potential of tumor immune infiltrate beyond the T cell compartment may be warranted.

What has become apparent to us in assessing the local intratumoral events during tumor growth or regression has been the common theme of tumor adaptation in response to immune attack. Although employing very different mechanisms of action, the two curative therapies presented herein share a mutual mechanism of action in their ability to overcome or evade local tumor adaptation, either through rapid and robust immune attack, or by inhibiting the suppressive capacity of the tumor microenvironment and permitting prolonged tumor attack. When considered in the context of clinical cancer vaccine data, it is tempting to speculate that the ability of the tumor to adapt to and suppress local immune attack is likely the singular, albeit multifaceted, mechanism limiting vaccine efficacy and objective clinical responses.

It is important to consider that our immune interventions have been instigated very early following tumor implantation, when tumors are only beginning to become detectable, which is in stark contrast to the majority of immunotherapeutic trials, which
are conducted in the context of advanced metastatic diseases that are refractory to conventional therapies. In attempting to recapitulate this in the B16F10 model, we have tested our curative therapies in the context of more advanced tumors. However, we observed only a small window for effective treatment, with even the robust and rapid tumor attack instigated by rHuAd5-hDCT + 10^6 DCT T cells producing only a negligible effect on the growth of large established tumors (data not shown). This observation may represent a limitation to the use of fast growing transplantable tumors such as B16F10 to develop clinically relevant models or may hint at more complex or complete local immune suppression as tumors become more established. In attempting to extend our observations using models that closely resemble human disease, it may be advantageous to direct future focus towards the treatment of metastatic cancers. Our lab has established technical expertise in combining immunotherapy with excision of primary tumors as a mechanism of evaluating immune control of residual or metastatic disease (316). Therefore, the use of metastatic tumor models (either with or without combination surgery) may provide a more direct means of evaluating treatment efficacy in a clinically relevant model of disease burden and efforts in our lab are currently being directed as such.
Concluding Remarks

Although therapeutic cancer vaccines have been a source of much promise and research effort, the current rate of treatment success remains low, despite reports of modest improvements in objective response rates and/or patient survival benefit. However, the end goal of cancer immunotherapies should be to develop curative treatments and not simply agents capable of slowing disease progression. While personalized therapeutic approaches using ACT-based T cell therapies have certainly provided evidence of the immunological capacity for destruction of even advanced tumors, such therapies possess inherent financial and technical limitations that will undoubtedly stall their broad application. Cancer vaccines on the other hand, while designed for broad and affordable administration, need to be improved as to elicit vigorous immunological attack on the tumor as observed for comparative ACT strategies.

We have identified a major hurdle to vaccine strategies, namely the ability of the tumor to upregulate and exploit immunoregulatory mechanisms to limit immune attack. A number of mechanisms designed to overcome such pathways are currently under investigation and future vaccine design will likely depend on a combinatorial approach to preserve or enhance anti-tumor immune activity within the tumor while at the same time minimizing or controlling the risk of accompanying autoimmune pathology. Furthermore, in attempting to improve the localized inflammatory response instigated through vaccination, investigators must implement improved methods of resolving immunological
events within the tumor as a means of understanding the true impact of treatment and to recognize how targeted effects can be further improved. The development of therapeutic cancer vaccines remains an area of tremendous investigation and development. By implementing more immunogenic approaches to vaccination that can enhance local immune activity in the tumor, I believe that the clinical response rate to vaccine therapies will drastically improve, resulting in the broad use of immunotherapies as first-line treatments for advanced and metastatic cancers.
Table 9. Myeloid cell gene signature from *Immune-Index*

<table>
<thead>
<tr>
<th>Gene</th>
<th>References</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1QA</td>
<td>435</td>
<td>Complement component largely restricted to macrophages/dendritic cells</td>
</tr>
<tr>
<td>C1QB</td>
<td>435</td>
<td>Complement component largely restricted to macrophages/dendritic cells</td>
</tr>
<tr>
<td>C3</td>
<td>436</td>
<td>Complement component, expressed constitutively by bone marrow derived macs and upregulated upon activation</td>
</tr>
<tr>
<td>C1QC</td>
<td>435</td>
<td>Complement component largely restricted to macrophages/dendritic cells</td>
</tr>
<tr>
<td>CD68</td>
<td>437</td>
<td>Monocyte and macrophage-specific protein, but also can be expressed by dendritic cells</td>
</tr>
<tr>
<td>CXCL16</td>
<td>438, 439</td>
<td>Scavenger Receptor expressed by monocyte-derived macrophages, immature myeloid cells. Splice variant expressed by DCs. Inflammatory Chemokine</td>
</tr>
<tr>
<td>CYBA</td>
<td>440, 441</td>
<td>Component of the microbicidal oxidase system of phagocytes</td>
</tr>
<tr>
<td>FCGR3</td>
<td>442, 443</td>
<td>Receptor for the Fc region of IgG</td>
</tr>
<tr>
<td>HCST</td>
<td>444</td>
<td>Transmembrane protein - associates with NKG2D to form an activation receptor</td>
</tr>
<tr>
<td>IFI30</td>
<td>445</td>
<td>Expressed in lysosomes of antigen presenting cells</td>
</tr>
<tr>
<td>LAPTM5</td>
<td>446, 447</td>
<td>Expressed by immature DCs, downregulated following DC maturation?</td>
</tr>
<tr>
<td>IL10Ra</td>
<td>448</td>
<td>Pleiotropic Cytokine Receptor. Implicated in regulatory or suppressive functions</td>
</tr>
<tr>
<td>IRF5</td>
<td>449, 450</td>
<td>Transcription factor involve in Type I interferon signaling and the induction of inflammatory cytokines in response to viral infection and TLR activation</td>
</tr>
<tr>
<td>LAT2</td>
<td>451</td>
<td>High affinity IgG Fc receptor I-mediated signaling in myeloid cells</td>
</tr>
<tr>
<td>LGMN</td>
<td>452</td>
<td>May be involved in the processing of proteins for MHC class II antigen presentation in the lysosomal/endosomal system</td>
</tr>
<tr>
<td>MERTK</td>
<td>453</td>
<td>Plays a role in various cellular processes such as macrophage clearance of apoptotic cells</td>
</tr>
<tr>
<td>NCF4</td>
<td>454</td>
<td>Component of the NADPH-oxidase</td>
</tr>
<tr>
<td>SELPLG</td>
<td>455</td>
<td>mediates rapid rolling of leukocytes over vascular surfaces during the initial steps in inflammation</td>
</tr>
<tr>
<td>SLAMF8</td>
<td>456</td>
<td>Broadly expressed immunomodulatory receptor</td>
</tr>
<tr>
<td>SLC11A1</td>
<td>457, 458</td>
<td>Macrophage-specific membrane transport function</td>
</tr>
<tr>
<td>TLR13</td>
<td>459</td>
<td>Innate immune receptor for sensing pathogens. Strongly expressed by macrophages and DCs, especially in the spleen</td>
</tr>
<tr>
<td>TREM2</td>
<td>460, 461</td>
<td>Forms a receptor signaling complex with TYROBP and triggers activation of the immune responses in macrophages and dendritic cells</td>
</tr>
<tr>
<td>UNC93B1</td>
<td>462</td>
<td>Plays an important role in innate and adaptive immunity by regulating nucleotide-sensing Toll-like receptor (TLR) signaling</td>
</tr>
<tr>
<td>NCKAPIL</td>
<td>463</td>
<td>Transmembrane protein expressed only in hematopoietic cells, involved in cell function and homeostasis</td>
</tr>
</tbody>
</table>

Note: Listed references serve as rationale for gene inclusion as part of cell signature
### Table 10. T cell gene signature from Immune-Index

<table>
<thead>
<tr>
<th>Gene</th>
<th>References</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL11B</td>
<td>464, 465</td>
<td>Differentiation, survival, proliferation of T cells</td>
</tr>
<tr>
<td>CCL4</td>
<td>466</td>
<td>T cell recruitment</td>
</tr>
<tr>
<td>CD2</td>
<td>467, 468</td>
<td>T cell adhesion/activation/proliferation</td>
</tr>
<tr>
<td>CD3e</td>
<td>469, 470</td>
<td>TCR signaling</td>
</tr>
<tr>
<td>CD6</td>
<td>471, 472</td>
<td>T lineage glycoprotein. Implicated in both co-stimulation and T cell suppression</td>
</tr>
<tr>
<td>HCST</td>
<td>473</td>
<td>Transmembrane protein - associates with NKG2D to form an activation receptor</td>
</tr>
<tr>
<td>CTSW</td>
<td>474, 475</td>
<td>Restricted expression to CD8+ T cells and NK cells. Induced upon cell activation</td>
</tr>
<tr>
<td>IL10Ra</td>
<td>476</td>
<td>Pleiotropic Cytokine Receptor. Implicated in regulatory functions, but may have activating role in T cells</td>
</tr>
<tr>
<td>ITGB7</td>
<td>477</td>
<td>Implicated in gut homing of lymphocytes</td>
</tr>
<tr>
<td>KLF2</td>
<td>478</td>
<td>Expressed in mature thymocytes. Role in mediating T cell Trafficking</td>
</tr>
<tr>
<td>LAG3</td>
<td>307, 479</td>
<td>Inhibitory Checkpoint Receptor expressed on T cells. Interacts with MHC II. associated with immune-suppression by iTregs</td>
</tr>
<tr>
<td>LGMN</td>
<td>480</td>
<td>Induced following TCR stimulation. Implicated in the induction of a Treg phenotype</td>
</tr>
<tr>
<td>LAPTM5</td>
<td>481</td>
<td>Negative regulator of surface TCR expression</td>
</tr>
<tr>
<td>PRF1</td>
<td>482</td>
<td>Important role in T cell cytotoxicity, facilitating cellular entry of granzyme molecules</td>
</tr>
<tr>
<td>Rac2</td>
<td>483, 484</td>
<td>Involved in T cell development and activation</td>
</tr>
<tr>
<td>SELPLG</td>
<td>485</td>
<td>T Cell migration and chemotaxis into inflamed tissues and migration/proliferation under homeostatic conditions</td>
</tr>
<tr>
<td>SEMA4a</td>
<td>486</td>
<td>Co-stimulatory effects on activated T cells, Th1/Th2 regulation</td>
</tr>
<tr>
<td>SH2D2A</td>
<td>487</td>
<td>Encodes T cell-specific adapter protein (TSAd)</td>
</tr>
<tr>
<td>SLAMF8</td>
<td>456</td>
<td>Broadly expressed immunomodulatory receptor</td>
</tr>
<tr>
<td>Thy1</td>
<td>488</td>
<td>Surface marker on mouse thymocytes and T cells, may provide stimulatory or activating signals</td>
</tr>
<tr>
<td>VAV1</td>
<td>489</td>
<td>Critical role in propagating TCR signals, important for T cell development and activation</td>
</tr>
<tr>
<td>ZAP70</td>
<td>490</td>
<td>Tyrosine kinase playing an essential role in TCR signaling</td>
</tr>
<tr>
<td>NCKAPIL</td>
<td>463</td>
<td>Transmembrane protein expressed only in hematopoietic cells, involved in cell function and homeostasis. Required for T cell development past double negative stage</td>
</tr>
</tbody>
</table>

Note: Listed references serve as rationale for gene inclusion as part of cell signature
References
References


64. Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Type I IFNs


75.Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets.


152. Seremet T, Brasseur F, Coulie PG. Tumor-specific antigens and immunologic


express ICOS ligand to promote the activation and expansion of T-regulatory cells. Cancer Research. 2010Dec.1;70(23):9581–90.


324. Harui AA, Suzuki SS, Kochanek SS, Mitani KK. Frequency and stability of


334. Zhai YY, Yang JCI, Spiess PP, Nishimura MIM, Overwijk WWW, Roberts BB, et


344. Shi. The balance of reproducibility, sensitivity, and specificity of lists of


Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cells. International Immunology. 2002Mar.1;14(3):275–86.


