12-2017

Memory Potential, Molecular Characterization, and Translational Applications of the Novel ThEO/TcEO T Cell Phenotype

Todd Bartkowiak

Follow this and additional works at: http://digitalcommons.library.tmc.edu/utgsbs_dissertations

Part of the Immunopathology Commons, Medical Immunology Commons, Oncology Commons, and the Therapeutics Commons

Recommended Citation

http://digitalcommons.library.tmc.edu/utgsbs_dissertations/816

This Dissertation (PhD) is brought to you for free and open access by the Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in UT GSBS Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact laurel.sanders@library.tmc.edu.
MEMORY POTENTIAL, MOLECULAR CHARACTERIZATION, AND TRANSLATIONAL APPLICATIONS OF THE NOVEL ThEO/TcEO T CELL PHENOTYPE

by

Todd Jacob Bartkowiak, M.S.

APPROVED:

____________________________________________
Michael A. Curran, Ph.D
Advisory Professor

____________________________________________
Chantale Bernatchez, Ph.D

____________________________________________
Joya Chandra, Ph.D

____________________________________________
Roza Nurieva, Ph.D

____________________________________________
Kimberly Schluns, Ph.D

APPROVED:

____________________________________________
Dean, The University of Texas
MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences
MEMORY POTENTIAL, MOLECULAR CHARACTERIZATION, AND TRANSLATIONAL APPLICATIONS OF THE NOVEL ThEO/TcEO T CELL PHENOTYPE

A

DISSERTATION

Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

by
Todd Jacob Bartkowiak, M.S.
Houston, Texas

December 2017
COPYRIGHT

The Proceedings of the National Academy of Sciences allows authors and their affiliated institution to retain extensive rights to the use of materials after publication, including, but not limited to “the right to include your article in your thesis or dissertation.” The full list of authorship rights can be found at

http://www.pnas.org/site/aboutpnas/rightpermfaq.xhtml

Frontiers in Oncology is an open access journal wherein articles are “published under the CC-BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original authors and the source are credited.” More information about the publishing policies of Frontiers journals can be found at

http://home.frontiersin.org/about/open-access
DEDICATION

I would like to dedicate this dissertation to my family without whom I would not be here. My father, James, and my mother, Renalda, have been instrumental in guiding my passion for science. My brothers (Patrick and Matthew), my sister-in-law, Nikki, and my nephews Luke and Logan, and my niece, Lily, all of whom encouraged me to pursue my love for science.
ACKNOWLEDGEMENTS

I would like to acknowledge all of the current and past members of the Curran lab and all of my friends and coworkers who helped me succeed in graduate school: Ashvin, Casey, Priya, Chao-Hsien, Renee, Pratha, Krishna, Midan, Matt, Courtney, Brittany, Rachel, Shail, Felix, Scott, Spencer, Stephen, Colm, Lucy, Nana-Ama, and all of the graduate students, faculty, and staff at MD Anderson.

I would also like to thank all of my advisors and collaborators at MD Anderson who have helped me reach this point in my life. Dr. Michael Curran, whose mentorship was vital to helping me succeed in graduate school and grow as a scientist. To my advisory committee members especially Dr. Kimberly Schluns, who was always willing to take the time to help me with manage experiments and provide a helping hand. I would also like to extend my gratitude to collaborators Dr. Jagan Sastry who was always willing to listen and provide valuable advice to help me navigate graduate school and to Dr. Amy Heimberger; all of whom provided guidance and helped me to secure my future in science.

I would like to especially thank Shailbala Singh and Guojun Yang for their assistance with the HPV immunotherapy experiments and Ashvin Jaiswal and Casey Ager for their help with the liver toxicity studies.

Thank you all.
T cells comprise a substantial arm of the immune system and are exquisitely adapted to combat pathogens and tumors. The inflammatory environment largely dictates the nature of T cell response. A hallmark of T cell-mediated immunity is formation of immunological memory; the ability to respond more potently to re-encounter with pathogens. The immune system is also capable of recognizing tumors as foreign, much like viral or bacterial pathogens. Tumors have evolved, though, to generate an immunosuppressive environment to avoid destruction. The field of immunotherapy seeks to overcome immune suppression, in part by targeting T cell co-receptors on the cell surface with either agonist or antagonist antibodies. Targeting the T cell co-stimulatory receptor 4-1BB with agonist antibodies engenders strong antitumor responses in multiple murine tumor models, in part by expanding the proliferative capacity, survival, and cytotoxicity of T cells within the tumor microenvironment. We have previously shown that systemic administration of α4-1BB antibodies induces a novel T cell program typified by expression of the T-box transcription factor Eomesodermin (Eomes) and the co-inhibitory receptor Killer Cell Lectin-like Receptor G1 (KLRG1) which we collectively term ThEO.

Herein, we demonstrate that the ThEO phenotype constitutes a stable T cell polarity capable of recalling to subsequent antigen challenge. Despite expression of terminal differentiation markers, ThEO cells phenotypically resemble discrete memory T cell subsets. We also find that the activation of the Signal Transducer and Activator of Transcription (STAT) pathways, in particular STAT1 and STAT3, is critical to ThEO polarization.

ThEO cells possess clinical relevance. Anti-4-1BB antibodies synergize with
HPV peptide vaccination to eradicate HPV+ murine tumors, due to a honed tumor-specific ThEO response. Further, ThEO phenotype cells infiltrate the livers of α4-1BB treated mice, which may play a role in 4-1BB mediated hepatotoxicity. Finally, we show that melanoma patients enrolled in α4-1BB clinical trials upregulate key markers associated with the ThEO phenotype; hence formation of ThEO cells within patient blood may act as a biomarker for therapeutic outcome.

This body of work demonstrates that the ThEO phenotype constitutes a unique T cell polarity that may prove beneficial in cancer treatment.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approval Signatures</td>
<td>i</td>
</tr>
<tr>
<td>Title Page</td>
<td>ii</td>
</tr>
<tr>
<td>Copyright</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>Abstract</td>
<td>vi</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>viii</td>
</tr>
<tr>
<td>List of Illustrations</td>
<td>xvi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xix</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xx</td>
</tr>
<tr>
<td><strong>Chapter 1: Background</strong></td>
<td>22</td>
</tr>
<tr>
<td>1.1 <em>Tumor Immunology and Immune Evasion</em></td>
<td>22</td>
</tr>
<tr>
<td>1.2 <em>Cancer Immunotherapy</em></td>
<td>23</td>
</tr>
<tr>
<td>1.3 <em>4-1BB</em></td>
<td>25</td>
</tr>
<tr>
<td>1.4 <em>Transcriptional Regulation of T cell subsets</em></td>
<td>32</td>
</tr>
</tbody>
</table>
1.5 T Cell Subsets ................................................................. 37
  1.5.1: Th1 ................................................................. 37
  1.5.2: Th2 ................................................................. 37
  1.5.3: Th17 ............................................................... 38
  1.5.4: Treg ............................................................... 39
  1.5.5: Tfh ................................................................. 40
  1.5.6: ThCTL ........................................................... 41

1.6 Adaptive Immunity and T cell memory ................................. 42
  1.6.1: Central Memory .................................................. 44
  1.6.2: Effector Memory ................................................ 45
  1.6.3: Tissue Resident Memory ....................................... 45
  1.6.4: Stem Cell Memory .............................................. 46

Chapter 2: Specific Aims ........................................................ 48
  2.1: Determine the phenotypic stability and memory potential of the novel
       TcEO/ThEO T cell polarity ............................................ 48
  2.2 Determine potential intrinsic and extrinsic factors that contribute to
       TcEO/ThEO cell development and/or persistence .................. 48
  2.3 Determine the clinical potential of the TcEO/ThEO phenotype in the context
       of therapeutic, pathologic, and prognostic potential .............. 48

Chapter 3: Materials and Methods ......................................... 51
  3.1 Animals .................................................................. 51
  3.2 Cell lines and reagents ............................................. 51
4.2 Results.................................................................................................................66

4.2.1 4-1BB co-stimulation induces ThEO phenotype cells capable of long-term persistence and rapid recall potential upon subsequent antigen exposure........66

4.2.2 ThEO phenotype cells reside within secondary lymphoid organs and recall rapidly to distant antigen challenge..............................................................77

4.2.3 ThEO phenotype cells phenotypically resemble multiple memory T cell subsets.................................................................................................................81

4.2.4 ThEO phenotype cells remain a stable phenotype stemming from an Eomes+KLRG1+ phenotype.............................................................89

4.2.5. Upon rechallenge, ThEO phenotype cells recall with potent effector function.................................................................92

4.3 Discussion.............................................................................................................94

Chapter 5: Intrinsic and extrinsic factors contributing to TcEO/ThEO development.................................................................................................................96

5.1 Introduction...........................................................................................................96

5.2 Results..................................................................................................................99

5.2.1. Runx2 is expressed by both ThEO and TcEO phenotype cells upon 4-1BB stimulation.........................................................................................99

5.2.2. Expression of Runx2 is induced upon antigenic stimulation......101

5.2.3. STAT1/STAT3 activating cytokines and STING signaling aid in polarization towards the ThEO phenotype.........................................................104
5.2.4. STAT1 and STAT3 are key signaling pathways necessary for ThEO polarization .................................................107

5.3 Discussion ..................................................................................................................................................110

Chapter 6: Therapeutic potential of the ThEO phenotype .................................................................113

6.1: 4-1BB agonist therapy in combination with HPV peptide vaccination .........................................................114

6.1.1 Introduction ..............................................................................................................................................114

6.1.2 Results ....................................................................................................................................................117

6.1.2.1 Intranasal vaccination with HPV E6/E7 peptides in combination with αGalCer slows the growth of preimplanted HPV+ tumors ........................................................................................................117

6.1.2.2 Intranasal vaccination in combination with systemic anti-4-1BB immunotherapy promotes regression of established s.c. HPV+ tumors ..........................................................................................120

6.1.2.3 Vaccination and α4-1BB therapy promotes high-density tumor infiltration by cytotoxic CD8 T cells .................................................................128

6.1.2.4 Intranasal HPV peptide vaccination generates Inducible T-cell Costimulator-expressing CD4 and CD8 T cells, which infiltrate HPV+ tumors ...........................................................................................................134

6.1.2.5 Treatment with 4-1BB agonist antibody polarizes tumor-specific T cells to the highly cytotoxic ThEO/TcEO phenotype .................................................................135

6.1.2.6 Vaccination plus α-1BB immunotherapy promotes complete regression of intravaginally implanted HPV E6/E7-driven tumors ..........143

6.1.3 Discussion ..............................................................................................................................................149
6.2: A potential role for ThEO phenotype cells in 4-1BB agonist induced liver pathology

6.2.1 Introduction ............................................................................................................. 152

6.2.2 Results ................................................................................................................. 154

6.2.2.1 Disparate effects of CTLA-4 and PD-1 checkpoint blockade on α4-1BB mediated hepatotoxicity ................................................................. 154

6.2.2.2 4-1BB agonists initiate liver pathology through activation of liver-resident myeloid cells ................................................................. 160

6.2.2.3 Interleukin 27 is a critical regulator of liver inflammation........................................... 166

6.2.2.4 Regulatory T cells restrict 4-1BB agonist antibody induced liver pathology ........................................................................................................... 170

6.2.2.5 CCR2 and CXCR3 are differentially required for liver and tumor T cell trafficking ........................................................................................................... 175

6.2.3 Discussion ........................................................................................................... 180

6.3: Potential for ThEO phenotype cells to act as a biomarker for therapeutic efficacy in the clinic ........................................................................................................... 183

6.3.1 Introduction ........................................................................................................... 183

6.3.2 Results ................................................................................................................. 185

6.3.2.1 Urelumab treated human PMBC upregulate markers of ThEO polarization ................................................................. 185

6.3.3 Discussion ........................................................................................................... 187
Chapter 7: Summary and Future Directions

7.1 Summary

7.2 Effects of 4-1BB co-stimulation on memory potential

7.3 Sca-1 as a novel marker of ThEO/TcEO phenotype memory cell

7.4 Further investigation of the effects of 4-1BB co-stimulation on memory potential

7.5 Runx2 expression and the importance of STAT signaling in ThEO phenotype cells

7.6 Future investigation into the role of Runx2 and STAT signaling in ThEO development

7.7 Use of 4-1BB agonists in treating multiple tumor models

7.8 ICOS expression in the vaccine setting

7.9 4-1BB mediated activation of myeloid cells

7.10 Impact of the gut microbiota on α4-1BB efficacy/induced hepatic damage

7.11 Implications for combination therapy

7.12 Use of the ThEO phenotype as a biomarker of clinical response

7.13 Adoptive transfer of ThEO phenotype cells as a therapeutic strategy

7.14 Role of Eomes as a lineage defining transcription factor

7.15 Role of ThEO phenotype cells in a global immune context

BIBLIOGRAPHY
# List of Illustrations

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>A multi-potent role for 4-1BB targeted immunotherapy</td>
<td>28</td>
</tr>
<tr>
<td>Figure 2</td>
<td>T cell subsets</td>
<td>35</td>
</tr>
<tr>
<td>Figure 3</td>
<td>ThEO phenotype cells are generated in response to 4-1BB agonists in multiple tumor models</td>
<td>69</td>
</tr>
<tr>
<td>Figure 4</td>
<td>ThEO phenotype cells are capable for persisting long-term with potent recall potential</td>
<td>70</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Polarization of OT-I T cells toward the TcEO phenotype</td>
<td>72</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Antigen-specific CD4 T cells develop a delayed and muted ThEO polarization and memory kinetic</td>
<td>73</td>
</tr>
<tr>
<td>Figure 7</td>
<td>HPV E7-antigen-specific recalled TcEO responses</td>
<td>74</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Spas-antigen specific CD8 T cells are induced to become TcEO phenotype cells</td>
<td>75</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Non-bystander induction of ThEO phenotype cells</td>
<td>76</td>
</tr>
<tr>
<td>Figure 10</td>
<td>TcEO phenotype cells reside in the secondary lymphoid organs absent secondary antigen exposure</td>
<td>79</td>
</tr>
<tr>
<td>Figure 11</td>
<td>ThEO phenotype cells are found in the secondary lymphoid organs after recall</td>
<td>80</td>
</tr>
<tr>
<td>Figure 12</td>
<td>ThEO phenotype cells exhibit a memory phenotype consistent with effector memory, central memory, and stem cell memory subsets</td>
<td>86</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Co-inhibitory receptor expression on ThEO/TcEO phenotype cells</td>
<td>88</td>
</tr>
<tr>
<td>Figure 14</td>
<td>The TcEO phenotype remains a stable T cell population</td>
<td>91</td>
</tr>
<tr>
<td>Figure 15</td>
<td>ThEO phenotype cells recall with potent cytotoxicity and effector function</td>
<td>93</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Runx2 is expressed in ThEO and TcEO phenotype cells</td>
<td>100</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Runx2 is induced upon antigen exposure</td>
<td>103</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Cytokines aid in the polarization of ThEO phenotype cells</td>
<td>106</td>
</tr>
</tbody>
</table>
Figure 37. Effects of IL-27 pathway inactivation on CD4 T cells………………………………..169

Figure 38. Regulatory T cells suppress 4-1BB agonist antibody induced liver pathology……172

Figure 39. Depletion of Treg cells in FoxP3-DTR mice…………………………………………..174

Figure 40. The chemokine receptors CCR2 and CXCR3 contribute to 4-1BB agonist-induced liver pathology………………………………………………………………………………….177

Figure 41. Mechanistic model of 4-1BB agonist antibody-mediated hepatotoxicity………179

Figure 42. Induction of ThEO factors in α4-1BB treated patients…………………………….186
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1. Combinations with 4-1BB targeted therapies</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>
ABBREVIATIONS

ACT: Adoptive Cellular Therapy/Adoptive Cell Transfer
ADCC: Antibody-dependent Cellular Cytotoxicity
AICD: Activation-induced Cell Death
APC: Antigen-presenting Cell
BMS: Bristol-Myers Squibb
CD: Cluster of Differentiation
CR: Complete Response
CTLA-4: Cytotoxic T Lymphocyte Antigen-4
DC: Dendritic Cell
DLT: Dose Limiting Toxicity
DPEC: Double Positive Effector Cell
EEC: Early Effector Cell
FACS: Fluorescence Activated Cell Sorting
Flt3L: FMS-like tyrosine kinase 3 Ligand
FVAX: Irradiated B16 melanoma tumor cells transduced to express Flt3 Ligand
GMCSF: Granulocyte Macrophage Colony Stimulating Factor
GVAX: Irradiated B16 melanoma cells transduced to express GMCSF
HPV: Human Papilloma Virus
IDO: Indolamine pyrrole 2, 3-dioxygenase
IEL: Intraepithelial Lymphocyte
IFN: Interferon
I.P: Intraperitoneal
MDSC: Myeloid Derived Suppressor Cell
MFI: Mean Fluorescence Intensity
MHC: Major Histocompatibility Complex
MPEC: Memory Precursor Effector Cell
mTOR: Mammalian Target of Rapamycin
NFκB: Nuclear Factor kappa B
OVA: Chicken Egg Ovalbumin
PD-1: Programmed Death Receptor 1
PR: Partial Response
PTC: Post Tumor Challenge
RECIST: Response Evaluation Criteria In Solid Tumors
Runx: Runt-related transcription factor
S.C.: Subcutaneous
SD: Stable Disease
S.D.: Standard Deviation
SEM: Standard Error of the Mean
SLEC: Short Lived Effector Cell
STAT: Signal Transducer and Activator of Transcription
STING: Stimulator of Interferon Genes
Tc: Cytotoxic T cell
Tcm: Central Memory T cell
Teff: Effector T cell
Tem: Effector Memory T cell
TGFβ: Transforming Growth Factor β
Th: Helper T cell
TIL: Tumor Infiltrating Lymphocyte
TMRM: Tetramethylrhodamine Methyl Ester
TNF: Tumor Necrosis Factor
Treg: regulatory T cell
Trm: Tissue resident memory cell
Tscm: Stem cell memory T cell
Chapter 1: BACKGROUND

*Figures, tables, and much of the text within this section, particularly in regards to 4-1BB were adapted from “Bartkowiak and Curran. 4-1BB agonists: Multipotent potentiators of tumor immunity. Front Onc 2015 Jun 8;5:117. doi: 10.3389/fonc.2015.00117”(1)

1.1 Tumor Immunology and Immune Evasion

Until pioneering work performed by William Coley and Paul Ehrlich demonstrated that the immune system plays a significant role in the prevention of malignant transformation as well as the clearance of established lesions, tumors were thought to avoid immunological detection. Tumors were thought to exist in a state of “altered-self” whereby mature immune cells should not be able to recognize self-antigens lest they be removed from the immune repertoire or elicit autoimmunity (2, 3). In fact, tumors express a variety of antigens that can be recognized by the adaptive arm of the immune system, particularly tumoricidal T cells. These antigens may consist of over-expressed differentiation peptides, over-expressed tissue-restricted peptides, or mutated self-peptides (4, 5). Tumors themselves often present these peptides on the cell surface, and naïve CD8 T cells then recognize specific tumor antigens as foreign and attack the tumors directly. In addition, cell debris from dead or dying tumors may be ingested by the innate immune system, particularly macrophages and dendritic cells (DC), which are then capable of presenting the tumor antigens to both CD8 and CD4 T cells not only within the tumor bed, but also within the local lymph nodes (6). Tumors, however, have developed a multitude of mechanisms to remain hidden from the immune system (7-11). These may include: nutrient deprivation, that is draining metabolites from the nutrient pool, thus restricting immune cell access to available fuel sources (12-16); downregulation of antigen presentation and/or co-stimulatory machinery necessary for T cell recognition of tumor peptides and adequate activation
subsequent to tumor recognition (17, 18); recruitment of immunosuppressive cell populations including regulatory T cells (Tregs) (19, 20), suppressive neutrophils, macrophages, and myeloid derived suppressor cells (MDSC) that produce suppressive cytokines (e.g. TGFβ, IL-10, IDO) and express ligands for co-inhibitory checkpoint receptors (e.g. PD-L1, B7-H3, B7-H4)(21-24); or direct suppression the immune response by production of suppressive factors (TGFβ, arginase, IDO) or checkpoint ligands (PD-L1) (23, 25). The resulting struggle between active anti-tumor immunity attempting to suppress tumor growth and clear malignant lesions, and the aberrant growth of tumor cells often leads to T cell exhaustion or anergy, a state wherein chronically activated T cells can no longer achieve optimal stimulation from repeated antigen exposure (26, 27). These cells demonstrate both qualitative and quantitative defects in effector function after repeated stimulation fails to induce rapid proliferation, and the quality of T cell effector function declines as T cells lose poly-functionality and fail to initiate significant cytokine production (28-30). The end result of this immune exhaustion leads to unrestricted tumor growth, metastasis, and eventually patient death. Current first line therapies (e.g. radiotherapy and chemotherapy) can delay tumor growth, though tumors often develop resistance and continue to grow. Next generation therapies targeting the immune system, however, have shown great potential in treating and even curing established tumors, thus revolutionizing the field of clinical oncology and opening the field of immune-oncology.

1.2 Cancer Immunotherapy

The ultimate goal of immunotherapy seeks to eradicate tumors by enhancing the anti-tumor immune response through a variety of means. This may be through vaccination with whole tumor cells or tumor peptides (31-33), infusion of T cell-stimulating cytokines such is IL-2 (34-36), isolation of tumor infiltrating lymphocytes (TIL) and reinfusion into patients (37, 38), or genetically engineering T cells to express
chimeric antigen receptors (CAR T cells) which can target tumor antigens (39-42). Perhaps the most versatile, and the most effective, immunotherapy involves the use of therapeutic antibodies targeting co-inhibitory and co-stimulatory receptors on the T cell surface (43-46).

Antagonist antibodies, in particular targeting the checkpoint receptor Cytotoxic T Lymphocyte Antigen-4 (CTLA-4; CD152), have proven incredibly effective in unleashing the anti-tumor T cell response, rejuvenating T cell effector function, and leading to durable tumor regression and long-term relapse free survival in melanoma patients; a disease that was once thought to be a death sentence (44, 46-51). Subsequently, additional T cell co-inhibitory receptors have been targeted including the Programmed Death Receptor-1 (PD-1; CD279) as well as its ligand PD-L1 (CD274) (52-56). Targeting the PD-1 pathway has proven even more effective in treating melanoma than αCTLA-4 therapy, and either αCTLA-4 or αPD-1 antibodies have been approved by the Food and Drug Administration (FDA) for the treatment of melanoma, lung, bladder, renal cell, and breast cancer to name a few (57). Further, dual checkpoint blockade therapy has now been approved for the treatment of melanoma with very promising results (58-60).

The incredible success of CTLA-4 and PD-1 checkpoint blockade has piqued interest in researching additional co-inhibitory molecules (e.g. TIM3, LAG3, VISTA, B7H3, B7H4), as well as targeting co-stimulatory receptors expressed by T cells in order to enhance T cell survival and effector function in the tumor microenvironment (61-70). In particular, therapeutic use of agonist antibodies targeting the 4-1BB co-receptor has proven strikingly effective in treating multiple murine cancers (Table 1)(1).
4-1BB is a co-stimulatory receptor in the TNF receptor super family along with members OX-40, CD40, CD27, and GITR which also serve as interesting targets for immunotherapy (71, 72). Unlike other TNF receptor family members which demonstrate more restricted cellular expression patterns, 4-1BB expression has been observed on a variety of immune cells. While most predominantly expressed on cytotoxic CD8 T cells (73-77), 4-1BB is also expressed to a lesser degree on CD4 helper T cells (76, 78, 79), Natural Killer (NK) cells (80-82), Natural Killer T cells (83), γδ T cells (84), B cells (85, 86), macrophages (87), DCs (87-89), and mast cells, as well as various non-immune cells (90-96).

Upon engagement with its ligand (4-1BBL expressed on APCs), 4-1BB stimulates a signaling cascade through the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), AKT/mTOR, and β-catenin pathways ultimately culminating in activation of the master regulator NFκB (97-102). The signaling cascade propagated by 4-1BB ligation leads to the inhibition of activation-induced cell death (AICD) particularly through the upregulation of anti-apoptotic factors (e.g. Bcl-xl, Bcl-2, and Bfl-1) (75, 76).

In addition to enhancing cellular proliferation and prolonging T cell survival crucial for sustaining immunological memory, 4-1BB co-stimulation also augments effector immune responses, making 4-1BB an alluring target for anti-tumor immunotherapy (Figure 1)(73, 79, 103-105). As 4-1BB can be expressed by a variety of immune cell subsets, stimulation of the receptor can have a global impact on the immune response. Within the T cell compartment, 4-1BB activation leads to production of inflammatory cytokines including interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and cytotoxicity molecules such as granzyme B and perforin in both effector CD4 and CD8 T cells (87, 106, 107). In this way, 4-1BB skews the immune
response towards Th1 immunity and away from Th2, Th17, and Treg T cell polarization (87, 108). The exact impact of 4-1BB co-stimulation on Tregs, however, remains controversial. While 4-1BB stimulation restricts conversion of effector CD4 T cells and induction of the Treg molecular program (iTregs), 4-1BB may also lead to the expansion and enhanced suppressive capacity of natural Tregs (nTregs) (109, 110). Moreover, through an unclear mechanism, 4-1BB may also lead to the induction of a cytotoxicity program within Tregs, allowing for Tregs themselves to contribute to anti-tumor responses (111). In a manner similar to the effects on T cells, antibody mediated 4-1BB co-stimulation also leads to elevated effector function of NK cells including increases in IFNγ, TNFα, cytotoxicity, and antibody-dependent cellular cytotoxicity (ADCC) (80, 81, 112). Accompanying enhanced T cell responses, or perhaps prior to these responses, 4-1BB agonists also stimulate maturation and effector function within the myeloid compartment. Both macrophages and DCs increase antigen presentation by expressing elevated levels of major histocompatibility complex molecules (MHC-I, MHC-II) as well as costimulatory molecules B7-1 (CD80) and B7-2 (CD86) (87). In addition, both DCs and macrophages stimulated with 4-1BB agonists increase expression of the suppressive metabolic enzyme indoleamine 2, 3 dioxygenase (IDO) (113). Further, 4-1BB activated DCs express elevated levels of IL-12 family cytokines (e.g. IL-23, IL-27) (87, 114), aiding the polarization of T cells towards the Th1 phenotype.

The uncanny ability of the 4-1BB pathway to stimulate strong effector responses has been applied to anti-tumor immunity ever since its discovery (115). A variety of tactics have been used to stimulate the 4-1BB pathway from 4-1BB targeted aptamers (116-119), to administration of soluble 4-1BBL (120-123), to administration of 4-1BB targeted antibodies (33, 87, 115, 124-127). Each method offers its own array of pros and cons, however, the end results clearly demonstrate a distinct role for 4-1BB in promoting and sustaining anti-tumor responses. While α4-1BB administered as a
monotherapy has proven effective in limiting tumor growth in a number of murine tumor models, perhaps the real impact of 4-1BB agonist therapy lies in the ability of 4-1BB stimulation to synergize with a multitude of anti-tumor therapies (e.g. radiotherapy, chemotherapy, immunotherapy) to completely eradicate established tumors (Table 1) (1). These promising pre-clinical data have helped transition 4-1BB targeted therapies into the clinical setting. Early clinical trials investigating the safety and efficacy of a 4-1BB targeted antibody sponsored by Bristol-Myers Squibb (BMS-663513; Urelumab) demonstrated impressive anti-tumor responses in advanced or metastatic solid tumors (NCT00309023) and stage IV melanoma (NCT00612664) including several cases of stable disease (SD) or partial responses (PR) as defined by RECIST (Response Evaluation Criteria In Solid Tumors) criteria (128, 129). Unfortunately, though, these trials were halted due to the incidence of severe drug-related hepatotoxicity (128). Results in murine models demonstrate that this toxicity may be overcome if 4-1BB agonists are given in combination with checkpoint inhibition, particularly αCTLA-4 (130), although α4-1BB/αPD-1 combinations appear to exacerbate liver pathology (131). These results have indeed hindered the progression of 4-1BB agonists in the clinic, although Pfizer has developed a new agonist antibody (PF-05082566; Utomilumab) administered at a significantly lower dose which appears to limit hepatotoxicity, although perhaps at the cost of efficacy (NCT02179918; NCT01307267). The exact mechanism initiating this toxicity remains elusive, however, results reported here in Chapter 6 may shed light on a potential mechanism of action related to α4-1BB mediated toxicity and I offer potential combination therapies that may synergerize to enhance the antitumor response while limiting hepatogenic T cell responses.
“Figure 1. A multi-potent role for 4-1BB targeted immunotherapy. 4-1BB agonist therapies elicit diverse immune effector responses on both the innate and adaptive immune arms. The most potent of responses stimulate CD8⁺ cytotoxic T cells to proliferate and increase their effector potential through increased interferon gamma production and expression of multiple granzymes. CD4⁺ effector T cells can also be stimulated to expand and produce pro-inflammatory cytokines. The role of 4-1BB stimulation on regulatory T cells, however, is controversial. 4-1BB agonist therapy may either inhibit differentiation of conventional effector cells into Tregs while also inhibiting Treg suppression, or, conversely, maintain Treg expansion and suppressive capacity. NK cells also benefit from 4-1BB agonist therapy. Not only can α4-1BB antibodies stimulate antibody-dependent cell-mediated cytotoxicity through Fc/FcR interactions, but activated NK cells express 4-1BB to become targets of therapy. Additionally, cells of the myeloid lineage upregulate 4-1BB upon activation. 4-1BB agonists targeting dendritic cells induce DC maturation and antigen presentation. In addition, α4-1BB stimulated DCs begin to express IL-12 and IL-27 as well as the enzyme IDO to modulate T cell function. 4-1BB⁺ macrophages can also be stimulated to increase antigen presentation and produce IL-8 as well as IDO (1).” “Bartkowiak and Curran. 4-1BB agonists: Multipotent potentiators of tumor immunity. Front Onc 2015 Jun 8;5:117. doi: 10.3389/fonc.2015.00117”
<table>
<thead>
<tr>
<th>Combination</th>
<th>Tumor Model</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vira Gene Therapy</td>
<td>Metastatic MCA26 colon carcinoma</td>
<td>- ovied hepatic and lung metastases - NK &amp; CD8+ mediated</td>
</tr>
<tr>
<td>Adv/L-12 + Adv/L-15B</td>
<td>Metastatic MCA26 colon carcinoma</td>
<td>- 1 survival - NK &amp; CD8+ mediated</td>
</tr>
<tr>
<td>Metastatic J1 breast carcinoma</td>
<td>- 1 survival</td>
<td></td>
</tr>
<tr>
<td>plKHCh4E/T-15B</td>
<td>AO104A carcinoma</td>
<td>- CTL activity</td>
</tr>
<tr>
<td>P815 mastocytoma</td>
<td>- complete rejection with CD28 stimulation</td>
<td></td>
</tr>
<tr>
<td>Adv(CMV)12-10Ci + wt-4-1BB mAb</td>
<td>CT26 colon adenocarcinoma</td>
<td>- complete rejection - systemic immunity</td>
</tr>
<tr>
<td>MC38 colon adenocarcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adv/L-12</td>
<td>B16-F10 melanoma EL4 lymphoma</td>
<td>- CTL activity - NK &amp; CD8+ mediated</td>
</tr>
<tr>
<td>Adv-mT-12 + Adv/L-4-1BB</td>
<td>MCA26 colon carcinoma</td>
<td>- pulmonary metastases - complete regression - long-term survival</td>
</tr>
<tr>
<td>Adv/L-12/134-1BB</td>
<td>B16-F10 melanoma</td>
<td>- 1 tumor burden - Th1 responses</td>
</tr>
<tr>
<td>Vaccinia virus + wt-1BB mAb</td>
<td>AT-3 breast carcinoma</td>
<td>- 1 tumor burden</td>
</tr>
<tr>
<td>MC38 colon carcinoma</td>
<td>- 1 metastasis in b1b2</td>
<td></td>
</tr>
<tr>
<td>HV-4-188L + lymphodepletion</td>
<td>B16-F10 melanoma</td>
<td>- MHC-I expression - anti-viral antibodies - viral persistence</td>
</tr>
<tr>
<td>HV-4-188L</td>
<td>MC38-OVA+ colon carcinoma</td>
<td>- CD4+ effector memory - 1 tumor burden</td>
</tr>
<tr>
<td>Samki Forest Virus L-12 + 4-1BB mAb</td>
<td>B16 melanoma</td>
<td>- 75% complete regression - tumor-specific CTL - anti-vector humoral response</td>
</tr>
<tr>
<td>Chengple (Blockade)</td>
<td>TG-1 HPI- lung adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>eCTLA4 + wt-1BB</td>
<td>B16 melanoma</td>
<td>- 1 survival with FIVAX - CD8+ T cell infiltrate - 1 Treg infiltrate - 1 T cell function - 1 T cell/MHC-II ratio</td>
</tr>
<tr>
<td>MC38 colon carcinoma</td>
<td>- 1 tumor infiltrate</td>
<td></td>
</tr>
<tr>
<td>eCTLA4 + 4-1BB-tumor vaccine</td>
<td>RM-1 prostate carcinoma</td>
<td>- 1 survival</td>
</tr>
<tr>
<td>ePD-1 + wt-1BB</td>
<td>B16-F10 melanoma</td>
<td>- complete regression - 1 T cell function - 1 T cell/melanoma ratio - 1 T cell effector function - 1 T cell tumor infiltrate - 1 T cell effector function - 1 T cell effector function</td>
</tr>
<tr>
<td>eCTLA4 + 4-1BB</td>
<td>CT26 colon adenocarcinoma</td>
<td>- Complete tumor rejection - 1 T cell effector function - 1 T cell effector function - 1 T cell effector function</td>
</tr>
<tr>
<td>p40PD-1 + wt-1BB</td>
<td>H22 hepatocarcinoma</td>
<td>- anti-tumor immunity - 1 tumor burden</td>
</tr>
<tr>
<td>eCTLA4 + 4-1BB</td>
<td>ID-8 ovarian adenocarcinoma</td>
<td>- 1 survival</td>
</tr>
<tr>
<td>e4-1BB</td>
<td>B16 melanoma</td>
<td>- 1 tumor infiltrate - 1 T cell effector function</td>
</tr>
<tr>
<td>Single dose or fractionated radiation</td>
<td>B16F10 mammary carcinoma</td>
<td>- 1 tumor burden (high dose)</td>
</tr>
<tr>
<td>Focal radiation + e4-1BB + eCTLA4</td>
<td>GL261 gloma</td>
<td>- 1 long term survival - 1 T cell brain - CD4+ T cell dependent - Established protective immunity</td>
</tr>
<tr>
<td>Whole brain irradiation</td>
<td>GL261 gloma</td>
<td>- 1 survival</td>
</tr>
<tr>
<td>e4-1BB + Chemotherapy</td>
<td>CT26 colon adenocarcinoma</td>
<td>- 60% complete regression - 1 lymphopenia - 1 nephrotoxicity - 1 survival - 1 tumor volume - Long lasting immunity - 1 T cell effector function - 1 T cell effector function - 1 T cell effector function - 1 T cell effector function - 1 T cell effector function</td>
</tr>
<tr>
<td>CL18T-15B + APOD-1</td>
<td>CT26 colon adenocarcinoma</td>
<td>- 1 survival - 1 tumor volume</td>
</tr>
<tr>
<td>5-fluorouracil + e4-1BB</td>
<td>Renca renal cell carcinoma</td>
<td>- CD8+ T cell mediated</td>
</tr>
<tr>
<td>Cyclophosphamide + e4-1BB</td>
<td>B16 melanoma</td>
<td>- long-term immunity - CD8+ T cell dependent</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>TC-1 HPI- lung adenocarcinoma</td>
<td>- long-term immunity</td>
</tr>
<tr>
<td>e4-1BB + Gefitinib</td>
<td>EGFR+ SGC4, SGC5, squamous cell carcinoma</td>
<td>-</td>
</tr>
<tr>
<td>EGFR1 PCT pancreatic adenocarcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T84 and HCT116 colorectal carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD20+ B cell lymphoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e4-1BB + Rituximab</td>
<td>HER2+ breast cancer</td>
<td></td>
</tr>
<tr>
<td>e4-1BB + Trastuzumab</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1)
1.4 Transcriptional Regulation of T cell subsets

Transcription factors are key regulators of T cell polarity and function. Importantly, in addition to their defined roles in developing the immune response, T cell lineage defining transcription factors are critical developmental drivers during embryogenesis as well. This section will briefly describe the roles of lineage-defining transcription factors (Tbet, GATA3, Rorγt, Foxp3, Bcl6, and Eomes) in developmental processes as well in the immune response.

In addition to regulation of Th1 immune responses, Tbet (Tbx21) plays a critical role in brain development, particularly neuronal growth in the olfactory bulb (132). While the Th2-associated transcription factor GATA3 drives IL-4 expression in T cells, it is also critical for trophoblast development during embryogenesis (133, 134). Further, Rorγt, a driver of Th17 immunity, is also responsible for thymopoiesis, particularly maturation of CD4+CD8+ thymocytes as well as lymphoid tissue inducer cells (LTI) (135, 136). The Fox family of transcription factors have a range of roles in embryogenesis and in driving tumorigenesis, though the role of Foxp3 outside of mediating Treg function has been poorly characterized (137, 138). The transcription factor Bcl6 drives the follicular helper T cell program to mediate B cell maturation; however, Bcl6 has also recently been implicated in promoting memory formation in T cells (139). Further, translocation of Bcl6 has been shown to drive lymphomagenesis (140), and Bcl6 also play a role in right-left patterning and neurogenesis during embryonic development (141-143). Lastly, Eomesodermin (Eomes) plays a role in brain development and limb bud patterning (144, 145). Within the context of the immune system, Eomes drives development of Natural Killer cells, NK cells and T cell cytotoxicity, and instills memory potential within T cells (146-148).

Transcriptional regulation of T cell lineage polarity acts as a critical driver of immunity, allowing the immune system to differentially respond to a variety of
pathological conditions. Lineage-defining transcription factors, however, are not unique to the lymphoid system, and often contribute to developmental biology, embryogenesis, and hematopoiesis while also giving immune cells functional characteristics.

1.5 T Cell Subsets

The immune response against growing tumors is variable. Multiple distinct subtypes of T cells can be found in the tumor microenvironment, some with potent anti-tumor function, and others with immune-suppressive capacity. The overall response to tumors, and the ability of the immune system to control tumors is dictated by factors in the microenvironment that control the effector functions of T cells. The factors that influence T cell function have largely been elucidated in the context of pathological infection and these insights are now being applied to anti-tumor responses.

The immune microenvironment and type of pathogenic infection heavily dictates the subsequent immune response. Depending on the type of infection (e.g. viral, bacterial, fungal) the responding myeloid cells produce cytokines which polarize T cells towards one of several subsets of responding cells that are functionally specialized to eliminate the specific pathogen. This “polarization” initiates a signaling cascade, particularly through a family of cytoplasmic transcription factors (TFs) known as STATs (Signal Transducer and Activator of Transcription), ultimately leading to the transcription of unique, lineage-defining TFs required for subsequent T cell effector function (149, 150).

Currently, there are five well-characterized, functionally unique T cell subsets (Th1, Th2, Th17, Treg, Tfh) each specialized to perform a distinct role within the context of the immune response (Figure 2)(151). Further, these subsets are often mutually exclusive, restricting expression of other transcription factors and effector
cytokines (e.g. Th1/Th2; Th17/Treg). Recent evidence has also shed some light on the ability of conventional helper CD4 T cells to adopt a cytotoxicity profile consistent with a CD8 response (152). These cells, broadly dubbed ThCTL, and their role in the immune response has only recently begun to be investigated. The Curran lab has recently reported on the potential for 4-1BB agonist antibodies to induce a unique population of cytotoxic CD4 T cells characterized by expression of the transcription factor Eomesoderm (Eomes) and the co-inhibitory receptor KLRG1(87). These cells have been dubbed TcEO in regards to a CD8 phenotype and ThEO in regards to the CD4 response (collectively dubbed ThEO) in respect to the TF that drives their function. Whether the ThEO phenotype is initiated through the same pathways as ThCTL and whether ThEO cells pheno-copy ThCTLs remains to be seen. Within Chapter 5 of this report, I uncover potential cytokines, STAT signaling networks, and TFs necessary for ThEO polarization. I present data suggesting that factors that drive the ThEO phenotype are unique among other classically defined T helper phenotypes and I propose that the ThEO phenotype constitutes a novel T cell polarity. It should also be noted that, while most heavily studied within the context of helper CD4 T cells, each T cell subset outlined below can also be found within the CD8 compartment (Tc1, Tc2, Tc17), and hence my work would also suggest the development of a related TcEO T cell phenotype in conjunction with ThEO cells.
Figure 2

IL-12 → Anti-viral/intracellular infection → STAT1, STAT4

TGFβ → IFNγ → Tolerance → STAT5

Foxp3 → Treg → STAT5

IL-10 TGFβ → IL-6 IL-21

IL-12 → STAT1

B cell maturation Antibody production → STAT3

GzmB, GzmK, IFNγ++, TNFα++ → STAT 1?

IL-6 IL-10

STAT 3?

GzmB, GzmK, IFNγ++, TNFα++ → STAT 5?

Allergy/Extracellular infection → IL-4

TGFβ IL-6 → Parasite/Autoimmunity → STAT3

STAT5, STAT6

IL-4 IL-5 IL-13

STAT5, STAT6

IL-17 IL-21 IL-22

Anti-Viral/Anti-tumor Killer Cell

STAT 1?
Figure 2: T cell subsets. T cells are able adopt specialized functions in order to fight a variety of distinct pathogens. Each subset is defined by a unique set of cytokines which induce the response, STAT usage, lineage-defining transcription factors, and effector function. Th1 cells specialize in fighting intracellular infections. They are driven by IL-12 and IFNγ signaling predominantly through STAT4. The defining transcription factor of a Th1 cell is Tbet, which induces expression of IFNγ. Th2 cells specialize in fighting extracellular pathogens and play a role in development of allergies. They are driven predominantly by IL-4 signaling through STAT6. The lineage defining transcription factor associated with Th2 cells is GATA3, which induces expression of IL-4, IL-5, and IL-13. The role of Tregs lies in maintaining peripheral tolerance. Tregs are driven by TGFβ and STAT5 signaling which promotes Foxp3 expression. Follicular helper T cells (Tfh) play critical roles in the production of class-switched antibodies and in B cell maturation. Tfh cells are driven by IL-6 and IL-21 signaling through STAT3 leading to expression of the lineage defining transcription factor Bcl6, which in turn leads to expression of IL-6 and IL-10. The novel ThEO T cell phenotype may play a role in chronic viral infection and cancer. Evidence suggests that IL-27 and type I interferons trigger STAT1 activation and to some extent STAT3. In turn, this leads to expression of the lineage defining transcription factor Eomes, which then drives expression of cytotoxicity molecules in the granzyme family.
1.5.1: Th1

T helper 1 T cells (Th1) are specialized to combat viral infections as well as infection with other intracellular pathogens (151, 153, 154). Initiation of Th1 polarization begins upon infection. Myeloid cells respond to intracellular infection by increasing expression of MHC and by producing the inflammatory cytokine IL-12. Upon ligation to its receptor (IL12R) expressed on T cells, IL-12 initiates the JAK/STAT pathway, particularly recruitment and signaling through STAT4 (150). Upon phosphorylation and subsequent nuclear translocation, STAT4 activates transcription of several interferon related genes; however, the most significant are Tbet and IFNγ. Once secreted, IFNγ acts to activate macrophage phagocytic activity and antigen presentation as well as to directly inhibit viral replication (155, 156). IFNγ can also act in an autocrine manner to initiate and maintain transcription of the T box transcription factor Tbet while suppressing transcription of the Th2 TF GATA3 (153). Tbet, in turn, acts to promote IFNγ production and presentation of the IFNγ receptor on the cell surface in order to sustain the Th1 response. Agonism through the 4-1BB receptor drives Th1-like anti-tumor responses that act as the primary drivers of anti-tumor immunity and are the major cell type into which the majority of tumor immunology is focused (106, 107).

1.4.2: Th2

In contrast to Th1 cells, Th2 cells specialize in fighting extracellular bacterial infection (151, 153). In response to extracellular infection, macrophages and polymorphonuclear cells (e.g. eosinophils, mast cells, basophils) begin producing IL-4 critical for Th2 polarization (157). Upon binding to its cytokine receptor, IL-4 activates the STAT6 pathway (150). Upon phosphorylation, STAT6 forms homodimers necessary for nuclear translocation and activation of the Th2 response, including production of the Th2 lineage defining transcription factor GATA3 as well as IL-4 (158).
In contrast to Tbet, GATA3 can suppress the expression of Th1 defining factors including IL-12, IFNγ, and Tbet, while promoting the expression of Th2 cytokines. IL-4 in particular acts in an autocrine manner to promote Th2 immunity as well as to induce B cell class switching to produce IgE isotypes. Moreover, overproduction of IL-4 can lead to a hyper activated inflammatory response characterized by excessive mucous production, and airway inflammation consistent with allergy. Th2 cells promote a wound-healing response and, in doing so support a pro-tumorigenic environment (159). Signaling through the 4-1BB receptor suppresses Th2 immunity to promote tumor rejection (160).

1.4.3: Th17

Th17 cells specialize in fighting parasitic and fungal infections and also play a role in the exacerbation of several autoimmune conditions (161-167). Th17 cells are so named due to their production of IL-17 in addition to IL-22 and IL-23 (151, 168-171). The induction of the Th17 response begins as TGFβ, and IL-6 produced by macrophages and dendritic cells, initiates activation of STAT3 (150, 172-174) within responsive T cells. Activation of the STAT3 pathway subsequently leads to expression of the retinoic acid related orphan receptor family of TFs, particularly RORγt-the dominant driver of Th17 polarization (175). Production of RORγt, along with IL-23 stabilizes the Th17 phenotype, suppresses Foxp3 expression and Treg polarization, and induces the production of multiple cytokines including IL-17, IL-6, IL-1, and TGFβ (175). IL17 acts to promote inflammatory responses in part by recruitment of neutrophils (176, 177). Th17 cells also play key roles in promoting autoimmune diseases including inflammatory bowel disease (178, 179), psoriasis (161, 162), rheumatoid arthritis (163, 164), and multiple sclerosis (167, 180). Th17 cells also have controversial roles in anti-tumor immunity. In some contexts, Th17 have proven detrimental to effective antitumor responses through recruitment of suppressive
myeloid populations and expression of cytokines and chemokines that promote tumor growth (181-183). In other cases, however, Th17 (and Tc17) polarized T cells has proven quite efficacious in eliciting anti-tumor immunity (184-186). Within the context of α4-1BB antibody administration, the Th17 response is suppressed in favor a Th1-like response (108).

1.4.4: Treg

The fundamental role of regulatory T cells (Tregs) lies in maintaining peripheral tolerance and immune homeostasis (151, 187, 188). Two subsets of Tregs have been described; natural Tregs (nTregs) develop during thymic selection whereas induced Tregs (iTregs) derive from cytokine polarized CD4 helper cells (189-191). Production of TGFβ by suppressive myeloid cells triggers T cell expression of the Forkhead Box transcriptional family member Foxp3 (192-194). Foxp3 is a critical defining characteristic, phenotypically separating suppressive Tregs from other conventionally defined effector CD4 subsets (195). In fact, Foxp3 can bind to regulatory elements in the RORγt gene, suppressing polarization towards the Th17 phenotype (196, 197). In addition to TGFβ, common gamma chain cytokines (γc) are necessary to maintain Foxp3 expression, particularly IL-2 and to a lesser extent IL-15 (198), reflected in the higher expression levels of IL-2 receptor alpha subunit (CD25) on Tregs compared to Teff (199, 200). Signaling through these cytokines induces a STAT5 cascade which aids in the maintenance of Foxp3 expression (150). Within the context of tumor immunology, Tregs play a fundamental role in promoting tumor growth and suppressing effective anti-tumor responses (201, 202). Tumor- macrophage- and fibroblast-derived TGFβ abound within the tumor microenvironment and aid in the polarization of recruited effector cells towards the Treg polarity (203-205). In fact, great effort has been taken to understand Treg biology in order to develop therapies (e.g. α4-1BB therapy) that can overcome the suppressive effects of these cells (206).
1.4.5: Tfh

Follicular helper T cells (Tfh) are key regulators of B cell maturation and antibody-mediated immunity (151, 207). While T cells with a Tfh phenotype have been found in the blood under certain conditions (208-210), Tfh cells mainly reside within secondary lymphoid organs, particularly adjacent to the B cell zone of the germinal centers. It is within the germinal centers that Tfh cells perform their dominant role in supporting B cell survival and antibody class switching within the B cell compartment. Production of IL-6 and IL-21 by B cells within the germinal center initiates T cell signaling through the STAT3 pathway (207, 211). Activation of the STAT3 pathway culminates in the expression of a core set of Tfh genes, with the transcription factor Bcl6 acting as a master regulator of the Tfh phenotype (150, 212). Tfh cells then produce the cytokines IL-6 and IL-10 which act to suppress overactive B cell responses, contribute to B cell proliferation, and mature antibody production (207). The exact role of Tfh cells in the antitumor response, particularly to solid tumors, is poorly understood. Tfh cells themselves, however, may develop into lymphomas and may also contribute to survival and proliferation of B cell malignancies (213). Scant evidence correlates increased Tfh infiltration and survival in breast cancer (214). There is also some evidence that Tfh cells may participate in the formation of tertiary lymphoid structures within the microenvironment of solid tumors, thus establishing a local immune microenvironment (215), though their exact roles within multiple cancers must still be investigated. The exact role of 4-1BB signaling on Tfh cells or the Tfh response to tumors is largely unexplored.
1.4.6: ThCTL

Although CD4 T cells are thought to play a supportive function in mediating CD8 lysis of virally infected cells or in activating myeloid cells to control pathogens, recent evidence suggests that subsets of CD4 T cells may acquire a cytotoxicity profile and become killer cells. These cells fall broadly into three categories: cytotoxic CD4 T cells (ThCTL) that include CD4+ intraepithelial lymphocytes (216, 217), cytotoxic CD4s induced during pathogenic infection (152, 218), and therapy-induced cytotoxic CD4 subsets which includes the ThEO phenotype (33, 58, 87, 219, 220).

The first class of cytotoxic CD4 T cells include CD4+ intraepithelial lymphocytes (IELs). These cells play critical roles in maintaining homeostasis in the gut and in fighting enteric infections (221). They are thought to derive from mature thymic emigrants fully committed to the CD4 T cell lineage. Once these cells take up residence within the gut lumen, through an undetermined mechanism, the CD8 lineage defining transcription factor Runx3 becomes de-repressed and then contributes to development of cytotoxicity within this CD4 subset (216, 217).

The second class of cytotoxic CD4 T cells arise during infection. In cases of chronic infection including Chagas Disease, HIV, and hepatitis, a small subset of MHC-II restricted cytotoxic CD4+CD8+ T cells has been found within the blood of affected patients (222-225). While the exact roles of these cells in disease progression and their molecular and cellular origins remains unclear, it should be recognized that these double positive cells have also been found within the tumor bed and may play a role in anti-tumor immunity (226, 227). The most well characterized ThCTL cells are a subset of CD4 single positive cells found in the lung in response to influenza infection. These cells express the markers NKG2A/C/E and produce elevated levels of IFNγ, TNFα and granzyme family members B, C, and F. ThCTL also express CD27, PD-1, and Ly6C as well as the transcription factors Blimp-1 and Eomes (152).
The final class of cytotoxic CD4 T cells arises during the course of anti-tumor immunotherapy. Cytotoxic CD4 T cells expressing the transcription factor Eomes have been witnessed in tumor-bearing lympho-depleted mice treated with OX-40 agonist antibodies (220), or in tumor-bearing mice treated with OX-40/4-1BB dual co-stimulation therapy (219). Importantly, CD4 T cells from tumor challenged mice treated with 4-1BB agonist antibodies express Eomes and KLRG1 (ThEO cells) (33, 58, 87). Moreover, cells with the ThEO phenotype are also found in the livers and spleens of mice infected with Lymphocytic Choriomeningitis Virus (LCMV) or Listeria monocytogenes (87). While ThEO cells bear a striking resemblance to ThCTL, several questions remain: are these cells in fact the same cell type? What is the exact role of ThEO cells in the anti-tumor response? How does 4-1BB activation lead to ThEO development? What factors aid in the polarization of this phenotype? and Is the ThEO phenotype induced by 4-1BB even a stable T cell population? Results presented here in the following chapters will provide insight into many of these questions.

1.6 Adaptive Immunity and T cell memory

Development of long-lasting immunological memory is a critical component of adaptive immunity (228-230). When T (or B) cells recognize their cognate antigen upon a secondary and subsequent encounter, they proliferate much more quickly and elicit a much stronger effector response than upon primary antigen exposure. In fact, upon initial antigen exposure, the T cell response may take days or weeks to develop. Antigen must first be phagocytosed by APCs residing in the tissue. These APCs must then traffic to the local lymph node wherein they must present peptide antigens to T cells. Only T cells that recognize their cognate antigen in the context of appropriate TCR-peptide-MHC pairing will become activated (6). A secondary response, however, occurs much more rapidly (within several hours to days), eliciting much more robust effector function in order to quickly limit pathology.
Proper T cell activation during the primary response must include three distinct processes in order to overcome the signaling threshold necessary to fully activate a T cell’s effector function and lead to downstream formation of immunological memory. The first signal derives directly from the TCR-peptide-MHC interaction. The affinity and avidity of the TCR for the cognate antigen defines the strength of the initial TCR signaling cascade. The second signal, co-stimulation, serves to amplify the initial TCR signal. Costimulatory molecules (e.g. CD28, 4-1BB) form complexes with the TCR, aiding in the recruitment of signaling complexes required for initiation of downstream cascades. Finally, the third signal (the cytokine milieu) acts to polarize T cells towards one of the five classically defined T cell polarities specialized to combat certain infections (231).

Once activated, antigen-experienced T cells begin to rapidly proliferate, initiating the clonal expansion phase of the T cell response where a single T cell clone may expand up to one thousand fold (232). Upon expansion, T cells traffic to the site of infection in sufficient numbers to control the spread of disease. Upon resolution of infection (or tumor clearance), without the presence of antigen to sustain the T cell response, many of the responding cells undergo apoptosis and die off through lack of signaling and the absence of supportive cytokines. While 90-95% of responding T cell clones die during the contraction phase, up to 5-10% of antigen-exposed T cells survive the contraction phase and enter the memory pool where they circulate throughout the body (233-235).

Upon secondary encounter with their cognate antigen, memory T cells require a lower threshold of activation through their TCR, they respond more rapidly, proliferate more extensively, and generate more robust effector responses, thus limiting disease progression and subsequent pathology. Factors such as TCR signal strength and duration and cytokine responsiveness dictate whether a cell will persist into the memory phase (230, 233).
Traditionally, expression of certain transcription factors and surface receptors are associated with heightened memory potential (Eomes, Bcl-6, ID3, STAT3, IL-7Rα, IL-15Rβ, CXCR3, CD62L) and yet others are associated with terminal differentiation (Tbet, Blimp-1, ID2, STAT4, KLRG1, PD-1) (230, 233, 236). Those cells that are able to successfully transition into the memory pool may form one of four well-characterized subsets of T cell memory (central, effector, tissue resident, and stem cell), each with its own unique phenotype, tissue localization, and functional properties (233, 237-239). Moreover, while memory phenotypes and functional capabilities have been extensively studied within the CD8 T cell compartment, less is known about the precise phenotypic and functional characteristics of CD4 T cell memory (240).

1.6.1: Central Memory

Central memory T cells (Tcm) are one of the best described, and longest-lived memory T cell populations (241). While memory phenotypes vary between mouse models and humans, Tcm are largely characterized as CD44+CD62LhiCCR7hi (CD44+CD45RA−CD45RO+CCR7hi in humans) (233). Tcm may also express high levels of the chemokine receptor CXCR3 and low levels of CX3CR1(242). Expression of these chemokines receptors and adhesion molecules allows recirculation and retention of Tcm cells back into secondary lymphoid organs (i.e. lymph nodes and spleen) from the peripheral tissue. Functionally, Tcm are more quiescent than Tem or Teff cells, demonstrating homeostatic proliferation until reencounter with antigen. Upon re-stimulation, these cells produce significant amounts of IL-2 in order to maintain their proliferative capacity and survival. Aside from IL-2, though, Tcm have limited effector capacity post-recall. Upon secondary challenge, central memory T cells respond more slowly than effector memory cells, but are thought to act as a long term reservoir of antigen-specific memory cells (233). Within the context of tumor immunology, generation of long-lived central memory T cells remains a desired
outcome of immunotherapy, as therapies that generate a long-lasting pool of tumor-specific T cells that could respond to tumor regrowth would be an ideal outcome.

1.6.2: Effector Memory

Effector memory T cells (Tem) were first described in conjunction with Tcm. Tem cells, in contrast to Tcm, are characterized phenotypically as CD44+CD62LlowCCR7low (CD44+CD45RA−CD45RO−CCR7low in humans) (230, 233). Phenotypically speaking, Tem resemble effector T cells, and so, in some instances, it may be difficult to distinguish an effector T cell from an effector memory T cell based on phenotype alone. As Tem lack chemokine receptors that allow for retention within secondary lymphoid organs, these cells are predominantly found circulating throughout the blood, as well as within splenic and hepatic circulation. Functionally, Tem rapidly cycle, more so than Tcm after re-stimulation. They also produce less IL-2 upon recall than Tcm, though unlike Tcm, they have heightened effector capacity, able to more quickly transition into fully functional effector cells upon antigen encounter (230, 233). Within the context of tumor immunotherapy, Tem cells would be ideal for rapid clearance of metastatic tumor cells, or newly transformed recurrent tumors.

1.6.3: Tissue Resident Memory

Tissue resident memory T cells (Trm) are a distinct population of memory cells, potentially deriving from Tem (243, 244). Although not definitive, both human and murine Trm are defined phenotypically by high expression of the tissue homing receptor CD103 and the early activation marker CD69. These cells (CD44+CD69hiCD103hi) do not express any homing receptors for secondary lymphoid organs (CD62L−CCR7−) (243, 245-247). Phenotypic markers are not definitive, however, as Trm may lack either CD69 (C44+CD69−CD103+) or CD103
(CD44+CD69+CD103-), making these cells indistinguishable from Teff cells. A more conclusive definition of Trm is their tissue localization. Trm are located within the peripheral tissues and do not circulate throughout the body, but remain tissue resident (248, 249). Trm are also dependent on the cytokines TGFβ and IL-15 in order to maintain tissue residency and persist outside of cytokine-rich lymphoid organs (250). Functionally, Trm resemble Tem. Upon re-stimulation, both produce little IL-2, yet have tremendous effector capacity (230, 233). In addition, a large role for Trm cells lies in the ability to react quickly to subsequent infection within the tissue. Trm act as “sentinels” of reinfection. They are able to respond much more quickly since they reside in the tissue, and more importantly, they act to recruit additional T cell support from the circulation to help control infection (251). This function makes Trm ideal for sensing early events involved in tumor metastasis or tumor recurrence.

1.6.4: Stem Cell Memory

Stem cell memory (Tscm) is the most recently described subset of immune memory. Tscm cells are thought to closely resemble central memory cells, though they possess more “stem cell-like” qualities than Tcm (239). In fact, evidence suggests that Tscm may be the least differentiated form of memory, and subsequently gives rise to all other memory subsets (239, 252). Phenotypic characterization of this subset differs greatly between mouse and man. Within mice, Tscm is largely defined by expression of the stem cell antigen (Sca-1) and loss of CD44 expression (CD44lowSca-1+) (252). The phenotype has been more extensively studied in humans where additional markers have been used to describe the phenotype (CD44+CD45RA-CD45RO-CD62L-CCR7-CD27+CD28+IL15Rβ+Fas+CD11a+Bcl2+) (238, 252). Similar to Tcm, this phenotype can also be found in the secondary lymphoid organs. Unlike Tcm, though, Tscm have a high proliferative capacity upon re-challenge, produce a significant amount of IL-2, and also have the capacity to generate a stronger effector
response than Tcm more in line with Tem (239, 253, 254). Further, Tscm are defined by an increased mitochondrial membrane potential and reliance on oxidative phosphorylation, and, in fact, are superior to either Tcm or Tem in controlling tumor growth in the adoptive T cell therapy setting (255, 256).

Lastly, while not typically thought of as a memory-homing lymphoid organ, in recent years the bone marrow has become accepted as a niche for residency of memory cell populations. In particular, CD8 Tcm and CD4 Tem cells home to the bone marrow using a distinct set of homing receptors (257-259). Whether this is a desired niche for Trm or Tscm is unknown, as is the relative impact of bone marrow residency on tumor progression or relapse.
Chapter 2: Specific Aims

The ThEO phenotype (Eomes\(^+\)KLRG1\(^+\)), induced by \(\alpha_4\)-1BB agonist antibody immunotherapy poses a paradox in conventional T cell biology. Expression of Eomes and induction of a strong cytotoxicity profile in CD4 T cells does not fit within the context of conventionally described helper T cell subsets. Moreover, the factors and signaling pathways through which 4-1BB drives polarization of the ThEO phenotype remain largely unexplored. Further, the long term stability of \(\alpha_4\)-1BB-induced ThEO polarized T cells remains untested. Whether the ThEO phenotype constitutes a long-lived T cell population and contributes to long-term tumor regression, or is only transiently induced during the course of therapy remains to be seen. Paradoxically, ThEO phenotype cells express markers of both long-term memory potential (Eomes), and terminally differentiated cells (KLRG1, PD1).

I hypothesize that the ThEO T cell polarity constitutes a novel and distinct T cell phenotype, driven by unique factors, and capable of long-term persistence and durable anti-tumor effector function.

I have addressed this hypothesis by investigating the following aims:

**Aim 1:** Determine the potential for ThEO cells to maintain their phenotype long-term and transition into the immunological memory phase of the immune response.

**Aim 2:** Determine what T cell intrinsic and extrinsic factors contribute to ThEO development and/or persistence.

**Aim 3:** Determine the clinical potential of the ThEO phenotype in the context of therapeutic benefit, role in mediating liver pathology, and in acting as a diagnostic marker for clinical outcome in patients treated with 4-1BB agonists.

The outcome of this investigation gives valuable insight into the long-term persistence, development, and clinical potential of ThEO phenotype cells. Herein, I
provide data demonstrating that ThEO/TcEO phenotype cells are indeed capable of generating long-lasting immunological memory, and are able to recall to secondary and subsequent antigen exposure for over one year after initial tumor challenge with no additional administration of α4-1BB therapy.

Further, I show that development of the ThEO phenotype stems from the most differentiated Eomes^+KLRG1^+ and/or Eomes^−KLRG1^+ T cell populations. Once initiated, ThEO phenotype cells undergo rapid expansion and contraction phases consistent with cells that enter the memory pool. Phenotypically, I demonstrate that ThEO cells bear markers of memory potential, specifically stem cell memory (Tscm).

I go on to demonstrate that STAT1, and to a lesser degree STAT3, are critical mediators of ThEO development. Further, signaling through IL-27, IFNα, and the STING pathway also influence polarization towards the ThEO phenotype as well as regulation of key ThEO phenotypic markers (e.g. Runx2) which may drive cytotoxicity and development of a more terminally differentiated phenotype.

Lastly, I demonstrate the clinical potential of ThEO phenotype cells. I show that 4-1BB agonist antibodies synergize with intranasal HPV peptide vaccination to cure 100% of cervically implanted HPV^+ tumors. I further demonstrate that only when α4-1BB is co-administered with the peptide vaccine do ThEO phenotype cells upregulate the inducible costimulatory molecule (ICOS). These ICOS^+ TcEO/ThEO cells have greater effector capacity than their ICOS^− counterparts, which may account for greater tumor control seen in the vaccine/α4-1BB combination. I further demonstrate a potential mechanism by which α4-1BB agonist antibodies induce hepatotoxicity. I show that activation of the myeloid compartment is critical for the initiation of liver damage. Interleukin-27, produced by activated CD11b^+ Kupffer cells appears to be a critical driver of hyper-inflammation in the liver. I go on to show that the liver damage, while initiated at the myeloid level, is propagated through subsequent CD8 responses. Control of these CD8 responses is mediated by Tregs, without which, liver damage is
drastically aggravated. Finally, we show that T cell trafficking into the liver can be uncoupled from anti-tumor responses. Particularly, the chemokine receptors CCR2 and CXCR3 appear to influence liver pathology without negating anti-tumor responses.

I also demonstrate that patients receiving α4-1BB therapy (Urelumab; BMS) demonstrate markers of ThEO polarization which may be used as a response evaluation criteria for those on 4-1BB targeted therapy.
Chapter 3: Materials and Methods

3.1 Animals

Six to eight week old male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Taconic Biosciences (Hudson, NY). Six to eight week old female mice used in the vaccination study were purchased from the National Cancer Institute (NCI) or Charles River. OT-I, DO11.10, Balb/c and congenically marked (CD45.1) C57BL/6.SJL mice were purchased from Jackson Laboratories. Mice bearing the YFP fluorescent transgene under control of the Eomesodermin promoter (Eomes-YFP) were a kind gift from E. John Wherry at the University of Pennsylvania (Philadelphia, PA). OT-II, 4-1BB\(^+\), EBI3\(^+\), IL27 receptor alpha\(^+\), β2M\(^+\), MHCII\(^+\), Foxp3-DTR, CXCR3\(^+\), CCR2\(^+\), and CCR5\(^+\) were purchased from the Jackson Laboratory (Bar Harbor, ME). CD4\(^{Cre}\) STAT1\(^{flox/flox}\) mice were a kind gift from Roza Nurieva (MD Anderson Cancer Center, Houston, TX). STAT3\(^{flox/flox}\) mice were a gift from Stephanie Watowich (MD Anderson Cancer Center, Houston, TX) and were bred to CD4\(^{Cre}\) mice to generate CD4\(^{Cre}\) STAT3\(^{flox/flox}\) conditional knockout mice. STING knockout (STINGKO) mice were a gift from Kimberly Schluns (MD Anderson Cancer Center; Houston, TX). IFNαR\(^+\) were a gift from Willem Overwijk (MD Anderson Cancer Center; Houston, TX).

Animals were maintained in a specific pathogen-free environment at the institutional animal facility. All procedures were conducted in accordance with the guidelines established by the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee. All manipulations were performed on animals anesthetized with isofluorane. At different time points, post-tumor challenge and/or immunization, animals were sacrificed according to institutional guidelines.

3.2 Cell lines and reagents

The murine melanoma cell line B16-BL6, pancreatic tumor cell line Panc02, and prostate tumor cell line TRAMPC2 were purchased from American Type Culture Collection (ATCC;
Manassas, VA). The TC1 cell line of lung epithelial origin, transformed with the HPV E6 and E7 oncogenes as well as the Ras oncogene, were a kind gift from Jagannadha K. Sastry at MD Anderson Cancer Center (Houston, TX) (33). The murine glioma cell line GL261 was a gift from Amy Heimberger (MD Anderson Cancer Center, Houston, TX). The mEEERL HPV transformed oropharyngeal epithelial cells line was a gift from John Lee (University of Iowa, Iowa City, IA). The B16 melanoma cell lines expressing GM-CSF (GVAX), FLT3L (FVAX), and chicken ovalbumin (B16-Ova) were previously generated (58, 260). Cell lines were passaged as previously described (33, 87).

The TC-1-luciferase (TC-1–Luc) tumor cell line is of lung epithelial origin from C57BL/6 mice that was transformed with E6 and E7 oncogenes of HPV-16 as well as the Ras oncogene. This cell line additionally expresses firefly luciferase. This cell line was a kind gift from T.-C. Wu and C. Hung (Johns Hopkins School of Medicine, Baltimore). TC-1 cells were maintained in RPMI 1640 (Thermo Scientific HyClone), supplemented with 10% (vol/vol) heat inactivated FBS (Atlanta Biologicals), 50 units/mL of penicillin–streptomycin (Thermo Scientific HyClone), and 50 µg/mL gentamycin (Lonza BioWhittaker).

“The E7^{44–62} peptide, Q19D (QAEPDRAHVYNIVTFCCCKCD); E7^{49–57} peptide, R9F (RAHVYNIVTF); E6^{43–57} peptide, Q15L (QLLRREVYDFAFRDL); and E6^{49–58} peptide, V10C (VYDFAFRDLC), which represent murine H2^b-restricted epitopes, were purchased from Elim Biopharma and dissolved in 1× PBS at a concentration of 10 mg/mL. αGalCer was purchased from DiagnoCine and dissolved in dimethyl sulfoxide (Sigma) at a concentration of 1 mg/mL. APC-labeled H-2D^b epitope E7^{49–57} (RAHVYNIVTF)-containing tetramer was procured from the MHC tetramer production facility at Baylor College of Medicine (Houston) and was used for the detection and analysis of peptide-specific CD8 immunity in different tissues by flow cytometry (33).”

“Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+

The PE-labeled H-2D<sup>b</sup> epitope Spas-1.STH tetramer was procured from the MHC tetramer production facility at Baylor College of Medicine (Houston, TX) and the BV421-labeled H-2Kb epitope OVA<sup>257-264</sup> (SIINFEKL) tetramer was provided by the NIH Tetramer Core Facility (Emory University, Atlanta GA), and each was used for the detection and analysis of peptide-specific CD8 immunity in different tissues by flow cytometry.

The OVA<sup>257-264</sup> and OVA<sup>323-339</sup> peptides were purchased from Genscript (Piscataway, NJ). GP100 was purchased from AnaSpec (Fremont, CA). All peptides were diluted in PBS before further in vivo use. Biochemical agonists of the STING protein (ML-RS-CDA) were custom synthesized at the Institute for Applied Cancer Research (IACS) at the MD Anderson Cancer Center. The murine B16 melanoma cell lines expressing GM-CSF (GVAX), FLT3L (FVAX), and chicken ovalbumin (B16-Ova) were previously generated (58, 260). Cell lines were passaged as previously described (33). The OVA<sup>323-339</sup> peptide were purchased from Genscript (Piscataway, NJ). Murine cytokines including IL-2, IL-10, IL-15, IL-27, TGFβ, Wnt3 agonist, and IFNγ were purchased from R&D Systems (Minneapolis, MN) or PeproTech (Rocky Hill, NJ). The STING agonist cGAMP was purchased from Invivogen (San Diego, CA). All peptides and cytokines were diluted in PBS before being used at a concentration ranging from 500X-2000X.

3.3 Therapeutic antibodies

“The following agonistic and antagonistic antibodies to various T-cell costimulatory modulating receptors were purchased from BioXcell at <1 endotoxin unit/mg of LPS and tested with and without the E6/E7 peptide vaccine: antibody to CTLA-4 (9H10 at 100 μg per dose), to PD-1 (RMP1-14 at 250 μg per dose), to OX-40 (OX-86 at 100 μg per dose), to GITR (DTA-1 at 350 μg per dose), to 4–1BB (LOB12.3 at 350 μg per dose; 3H3 at 250 ug per dose) and to CD40 (FGK4.5 at 100 μg per dose)(33).”

“Bartkowiak et al. Unique potential of 4–1BB agonist antibody to promote

3.4 Tumor challenge and therapeutic antibody administration

Mice received a primary tumor challenge s.c on the right flank delivered in PBS with 30% matrigel (B16-Ova; 7.5 X10⁴ cells, B16-BL6; 1.5X10⁵ cells, Panc02; 7.5 X10⁵ cells, TRAMPC2 1X10⁶ cells; mEERL; 1X10⁶ cells, TC-1; 2 X10⁵ cells, GL261; 2X10⁶ cells). Mice were either left untreated, or were treated with α4-1BB or αCTLA-4 i.p. for three rounds of therapy administered at three day intervals. Mice receiving a primary challenge of B16 melanoma also received an irradiated FVAX/B16-Ova vaccine (15k rads; 1X10⁶ cells/ea) administered s.c. on the left flank or GVAX (15k rads 1X10⁶ cells i.p) in conjunction with antibody therapy. At various time points following cessation of therapy, (D45, D90 etc) mice received a secondary challenge of either live tumor cells (B16-Ova; 1X10⁶ in 30% matrigel) administered on the right flank, or an irradiated cellular vaccine FVAX/B16-Ova (1X10⁶/ea).

For HPV vaccine studies:

“Mice were injected with a single-cell suspension of 2 × 10⁵ TC-1 cells per animal s.c. on the right flank as described previously (261). Tumor growth was measured using a caliper to determine the diameter: longest surface length (a) and width (b), and the tumor size were expressed as area (a × b). Mice were killed when the area of the tumor reached 300 mm². For characterization of tumor infiltrating lymphocytes, TC-1 tumor cells were mixed in a 2:1 ratio with Matrigel (BD Biosciences) and injected at a final volume of 200 μL per animal.

For intravaginal tumor challenge, 2 × 10⁴ TC-1 cells expressing firefly luciferase were implanted in the vaginal tract of diestrus synchronized 6- to 8-wk-old female
C57BL/6 mice according to a previously described protocol (262). Tumor growth was monitored semiweekly using an Xenogen IVIS imaging system.

Five days post-tumor challenge, mice were immunized with the HPV E6/E7 peptide vaccine via the i.n. route of immunization. Mice were first anesthetized by i.p. injection of ketamine and xylazine hydrochloride and then administered 100 µg of each peptide with 2 µg of αGalCer intranasally as described previously (263, 264). The animals were maintained with their heads in anteflexion until they regained consciousness. Mice received two immunizations at 5-d intervals (as depicted in Fig. 20 by the vertical arrows pointing downward) and adaptive immune responses in different tissues were determined at various times post-immunization. Immunized animals also received i.p. injection of therapeutic antibodies on days 5, 8, and 11 post-tumor challenge. Control animals received i.n. immunization with either PBS or peptides alone or i.p. injection with the therapeutic antibodies alone. (33).” “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112”

In order to assess liver infiltration during tumor challenge, wild type, CCR2−/−, CXCR3−/−, or CCR5−/− mice were implanted s.c. with 3X10⁵ B16-Ova cells on the flank as described. On days 3, 6, and 9 mice received α4-1BB i.p, and a mixture of irradiated FVAX and B16-Ova s.c. on the opposite flank as described (12). On day 19, mice were sacrificed and tumors and perfused livers were harvested for analysis of immune infiltrates.

3.5 Tracking endogenous antigen-specific T cell responses

In order to assess endogenous antigen-specific T cell responses, tumor-challenged mice were bled routinely throughout the course of experimentation. In order to remove red blood cells, whole blood was lysed using HybriMax™ RBC Lysing Buffer (Sigma Aldrich). Cells were then
stained for one hour at room temperature with various tetramers before subsequent downstream processing.

3.6 Adoptive T cell transfer

In order to track antigen-specific CD8 T cell responses during the course of immunotherapy, congenically marked (CD45.2) OT-I mice bearing T cells with Ova-antigen-specific TCRs were sacrificed. Lymph nodes were harvested, and cells were adoptively transferred into naïve C57BL/6 (CD45.1) mice (2X10⁵ cells/mouse). Twenty-four hours later, mice were challenged with B16-Ova and received cellular vaccination and antibody immunotherapy as above. Antigen-specific responses were tracked over time as above using the congenic marker (CD45.2) to delineate antigen-specific T cell populations.

In order to assess antigen-specific CD4 T cell responses, Balb/c mice were sublethally irradiated (300 rad). Twenty-four hours later, lymphocytes from DO11.10 mice in which CD4 T cells bear antigen receptors specific for the Ovalbumin 323-339 peptide were adoptively transferred into irradiated recipient mice (2X10⁶ cells/mouse). Cells were allowed one week to engraft before mice were challenged with a peptide/adjuvant vaccine (25 ug Ova323-339/ 5ug ML-RS-CDA) delivered on both flanks in 30% matrigel. Mice received vaccine alone or in combination with α4-1BB for three rounds of treatment spaced three days apart. Blood was collected and processed routinely throughout the experiment as noted above. Antigen-specific CD4 responses were assessed using an antibody specific for the DO11.10 TCR (Thermo).

3.7 Cell Isolation

“On day 16 post-s.c.-tumor challenge, mice were killed, and spleens, tumor draining lymph nodes, and tumors were harvested to characterize cell-mediated antitumor responses. Briefly, tumors were digested in X-Vivo-15 (Lonza) supplemented with Collagenase H (Sigma) and DNase (Roche) and incubated at 37 °C, 5% CO₂ for 30 min before being filtered through a 70-μm cell strainer. T cells were enriched through density

3.8 Flow cytometry analysis

“Samples were fixed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and then stained with up to 16 antibodies at a time from Biolegend, BD Biosciences, eBioscience, and Life Technologies. Flow data were collected on a five-laser, 18-color BD Biosciences LSR II cytometer and analyzed using FlowJo version 7.6.5 (Treestar) (33)”. “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112”

3.9 TMRM staining and sorting

In order to assess T cell mitochondrial membrane potential during the memory phase, mice were tumor challenged with B16-Ova (7.5X10⁶) and were treated with cellular vaccine in combination with either α4-1BB or αCTLA-4 as above. Forty-five days after primary challenge, mice were sacrificed, spleens were harvested, digested as above, and stained with Tetramethylrhodamine methyl ester (TMRM; Thermo) as previously reported (255). Briefly, TMRM was diluted to a concentration of 25 nM in PBS. Splenocytes were stained for 30 minutes at 37°C before quenching with excess PBS. Cells were then sorted based on the top 10% highest and bottom 10% lowest staining for TMRM dye. Cells were then tetramer stained and fixed using the Foxp3 Transcription Factor Staining Buffer Kit as above before staining with a cocktail of antibodies and subsequent flow cytometric analysis.
3.10 *Eomes-YFP sorting and adoptive transfer*

In order to investigate the stability of the ThEO phenotype, Eomes-YFP transgenic mice were challenged with B16-Ova and treated with α4-1BB as above. On day 12 post-tumor challenge, mice were sacrificed and spleens and lymph nodes were harvested. CD8 T cells were sorted as above (Eomes-YFP^−KLRG1^−, Eomes-YFP^+KLRG1^−, Eomes-YFP^+KLRG1^+, Eomes-YFP^KLRG1^+) before adoptive transfer (2x10^5 cells) into congenically marked C57BL/6.SJL mice. Mice were bled at regular intervals and the ability for transferred cells (CD45.2^+) to maintain their phenotype was analyzed by flow cytometry. Mice were then challenged with a cellular vaccine (FVAX/B16-Ova) as above before further flow cytometric analysis.

3.11 *In vitro polarization*

In order to test the effects of various cytokines on T cell polarization, naïve OT-II mice were sacrificed and splenocytes were harvested as above. Bulk splenocytes were then plated at a concentration of 5x10^6 cells/ml in complete RPMI. Cells were pulsed with OVA^{323-339} antigen at a final concentration of 5 nM for 48 hours. Cells were then rigorously washed before culture in a cocktail containing various cytokines. Cytokine mixes were refreshed every two days for up to eight days. Cells were then fixed, stained, and analyzed by flow cytometry.

3.12 *Cytokine production assays*

“Tumor draining lymph nodes were isolated at day 16 as described above. Lymph node cells were pooled from five animals per group and then sorted using TCRβ PE-Cy7 and CD4 eFluor 450 (eBioscience) and CD8 Alexa 488 (Biolegend) antibodies into CD4 and CD8 T-cell pools. A total of 200,000 sorted CD4 or CD8 T cells were restimulated with 40,000 CD11c^+ dendritic cells (DCs) pulsed with Q19D, Q15L, R9F, and V10C peptides per well for 48 h. Cytokine production was determined from supernatants using the BD Biosciences TH1/TH2/TH17 cytometric bead array kit”(33). “Bartkowiak et al. *Unique potential of 4-1BB agonist antibody to promote durable regression of*
3.13 **Immune ablation and reconstitution**

For liver toxicity studies, C57BL/6 mice or 4-1BB− mice were sub-lethally irradiated at 500 rads using a Cesium-137 irradiator. One day later, splenic lymphocytes from either wildtype or 4-1BB− mice were isolated using CD90.2 magnetic beads (Miltenyi Biotec, San Diego, CA) and injected i.v. at 2X10⁶ cells/mouse into irradiated hosts.

3.14 **Liver enzyme analysis**

For liver toxicity studies, antibodies were given i.p. for 3 doses every 3 days. On day 16, mice were bled and serum levels of aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (AP) were measured by the MDACC Veterinary Diagnostic Laboratory. Mice were sacrificed, livers were perfused with PBS and harvested for immune infiltrates.

3.15 **T cell depletion**

For HPV vaccination studies:

“Mice were challenged with TC-1 and treated as above. One day before tumor challenge, mice were treated i.p. with depleting αCD8 (2.43; 350 μg) or αCD4 (GK1.5; 350 μg) Mice were re-administered depleting antibodies 1 d after challenge, and depletion was maintained by administration every 3 days until mice were killed(33).” “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112”
For liver toxicity studies, mice bearing the diphtheria toxin (DT) receptor driven by the Foxp3 promoter (Foxp3-DTR) were administered DT at 10 μg/kg one day prior to α4-1BB and every 3 days thereafter until sacrifice. Alternately, CD4+CD25+CD3+ cells were FACS sorted from naïve spleens and then 5X10^5 cells were injected into host mice one day prior to immunotherapy.

3.16 Immunohistochemistry

For liver pathology studies, each liver lobe was collected and formalin fixed separately for ≥ 24 hours. Tissues were then paraffin embedded (FFPE), sectioned and stained for H&E and IHC for CD8 and F4/80, at the MDACC Research Histology, Pathology, and Imaging Core at Sciencepark. Two sections were generated from the left lateral lobe at the widest dimension, and stained by H&E. H&E sections were evaluated by semi-quantitative scoring based on the number of inflammatory and necrotic cells in the portal triad, central vein, or parenchyma. A score of 0 or nil indicates no inflammation; Score 1, minimal inflammation, <15 inflammatory cells around portal triad, central vein, or in parenchyma; Score 2, mild inflammation, > 15 inflammatory cells around portal triad, central vein, or in parenchyma; Score 3, moderate inflammation, > 30, inflammatory cells around portal triad, central vein, or in parenchyma; and Score 4, severe inflammation, approximately > 50 cells around portal triad, central vein, or in the parenchyma. Two sections per animal per group were stained with the following immunohistochemical stains: CD8 and F4/80. The number of CD8+, and F4/80+, cells in the liver, both at the perivascular zones (central vein or portal area) and in the parenchyma, were counted separately in a microscopic field at 20X magnification. Four areas with the most abundant infiltration were selected for both areas and the average number per animal was calculated as described in Peng et.al. 2015.

3.17 Real time PCR

In order to assess cytokine production by liver resident myeloid cell populations, liver myeloid subpopulations were sorted as shown (Figure 33) at the MD Anderson Flow Cytometry and Cellular Imaging Core Facility (FCCIF). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, MD) and reverse transcribed using the SuperScript IV Reverse Transcriptase kit.
(Thermo). Taqman real-time PCR was performed on a Via 7 Real Time PCR System (Applied Biosystem, CA) as previously described (87). Levels of *il27-p28*, *ifng*, and *tnfa* were expressed as the fold change using the ΔΔCt method.

3.18 Acquisition of patient samples

Peripheral blood mononuclear cells (PBMC) were obtained from patients with stage III/IV melanoma enrolled in a Phase II trial investigating the efficacy of α4-1BB agonist antibodies (Urelumab; Bristol Myers Squibb; NCT00612664). Urelumab was dosed at 0.1 mg/kg, 1 mg/kg or 5 mg/kg every 3 weeks or 1 mg/kg every 6 weeks. Blood was sampled prior to infusion of antibody at the time points indicated.

3.19 Patient sample processing

Approximately 30 ml of blood was collected/time point in a sterile heparin tube. Blood was pooled and lymphocytes were isolated by Ficoll centrifugation using Histopaque 1119 (Sigma). Cells were then counted using a Vi-cell cell counter (Beckman Coulter) before being frozen down at 2×10^7 cells/ml in human AB serum supplemented with 10% DMSO. Upon collection of a full series of patient samples, cells were thawed and processed for flow cytometry analysis. Briefly, frozen patient PBMC were thawed and stained with a cell viability dye (Molecular Probes) before fixation using the Foxp3 Fixation Kit (eBiosciences). Cells were then stained with a panel of up to 16 antibodies or their corresponding isotype controls from Biolegend, BD Biosciences, and Thermo. Flow cytometry data was collected on an 18-color BD LSR II cytometer and analyzed in FlowJo (Treestar).

3.20 Statistical Analysis.

“All statistics were calculated using Graphpad Prism version 6 for Windows. Statistical significance was determined using a one-way ANOVA to test for differences between multiple groups or a Mann–Whitney *U* Test to test for differences between two groups
unless otherwise indicated. Statistical significance for survival analysis was analyzed using the Mantel–Cox or log rank test where indicated. Graphs show mean ± SD unless otherwise indicated. P values of <0.05 were considered significant” (33). “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112”
Chapter 4: Memory Potential of the ThEO Phenotype

4.1 Introduction

Establishment of long-lasting immunological memory remains a cardinal feature of the adaptive immune response. The ability of antigen-experienced T cells to mount a strong secondary recall response upon antigenic re-stimulation defines the memory response and the potential for the immune system to limit ensuing pathology associated with infection. Further, multiple environmental cues, many of which have yet to be elaborated, drive the conversion of T cells into one of many possible memory subsets each of which maintains its own phenotypic and functional properties and particular tissue distribution (230). Effector memory (Tem) and central memory (Tcm) were initially described as two subsets that preferentially circulate through the blood and tissue, or home to the secondary lymphoid organs respectively (241). Since the description of Tem and Tcm as unique memory subsets, new subsets with specialized properties have come to light. Tissue resident memory cells (Trm) have become a well characterized subset of memory cells demonstrating long-term persistence and maintenance within the peripheral tissue without recirculation within the blood (243, 244). Moreover, newly described stem cell memory cells (Tscm) are thought to persist as an undifferentiated phenotype that gives rise to both Tcm and Tem and is thought to exhibit both exquisite long-term persistence and effector potential (239, 252, 265).

Transformed tumor cells induce a persistent immune response characterized by the establishment of chronic antigen exposure, recruitment of suppressive immune infiltrates, and persistence of weak inflammatory signals which drive inefficient T cell responses. These weak signals, in combination with chronic exposure to tumor antigens, renders T cells unresponsive to further antigenic stimulation, a state known as “exhaustion” which may eventually lead to T cell anergy (28, 29, 266-271). Ineffective anti-tumor responses elicited by exhausted T cells remains a critical driver of tumor immune escape, reversal of which may be accomplished through targeting various immune checkpoint receptors.
The advent of antibody-mediated checkpoint blockade immunotherapy for cancer, targeting the cytotoxic T lymphocyte antigen-4 (CTLA-4) or programmed death receptor-1 (PD-1) pathways has paved the way for the validation of additional immune-targeted tumor therapies (54, 56, 60, 272-275). While anti-tumor T cells possess a variety of both co-stimulatory and co-inhibitory receptors that could act as enticing potential targets, targeting the costimulatory tumor necrosis factor family receptor 4-1BB (CD137), expressed on activated T cells, has proven to have a phenomenal capacity to generate robust anti-tumor responses (1). Stimulation through the 4-1BB receptor potentiates strong effector function in both CD4 and CD8 T cells in the tumor microenvironment and enhances proliferative capacity offering enhanced anti-tumor potential (73, 79, 103, 106, 107). As a consequence, 4-1BB activation has proven to be an exquisitely effective therapy, alone or combined with an array of other therapeutic modalities, in a vast range of murine cancer models. Co-stimulation through 4-1BB is capable of achieving complete long-term tumor eradication with minimal recurrence (1). Given the evidence for the role of 4-1BB in generating long-term T cell memory in conjunction with the paucity of data demonstrating tumor recurrences after administration of 4-1BB agonists (105, 276-278), experimental evidence suggests a role for 4-1BB in shaping and potentially enhancing the memory response toward tumors.

We and others have demonstrated a unique capacity for 4-1BB co-stimulation to induce a novel, highly cytotoxic phenotype in both the CD8 and CD4 T cell compartments which are both highly proliferative and capable of expressing multiple granzyme family members (33, 87). We have previously demonstrated that this cytotoxic phenotype is marked by expression of the co-inhibitory natural killer cell receptor killer cell lectin-like receptor G1 (KLRG1) and is driven by the Tbox transcription factor Eomesodermin (Eomes) which we define as ThEO cells to demarcate Eomes+KLRG1+ cells in the CD4 compartment or TcEO cells for those cells in the CD8 compartment (87). Eomes is an established driver of cytotoxicity(279), while also acting as a critical component that helps promote T cell entry into the memory phase of the immune response (148, 280). KLRG1, on the other hand, is thought to act in an inhibitory capacity to limit
cellular proliferation and effector function while also acting as a marker of terminal differentiation and cellular senescence (281-285). It remains to be seen, however, whether ThEO phenotype cells bearing both markers of long-term memory potential (Eomes) and terminal differentiation (KLRG1) are capable of generating long-lasting immune protection against tumors or are marked for terminal senescence.

In this study, I have determined that the ThEO phenotype constitutes a unique and stable molecular program. Antigen-specific T cells adopt the ThEO phenotype early during the anti-tumor response, undergoing a rapid expansion phase, followed by contraction and re-expansion subsequent to secondary recall. I also find that these cells localize to niches within the secondary lymphoid organs associated with retention of memory cells (i.e. lymph nodes and spleen). Further, ThEO/TcEO phenotype cells bear markers of memory potential, being closely related both phenotypically and transcriptionally with recently characterized stem cell memory T cells (Tscm). I go on to demonstrate that, along with potent recall potential, both ThEO and TcEO phenotype cells are capable of maintaining enhanced effector potential and tumor killing capacity. My findings indicate that 4-1BB agonism induces a novel, and stable T cell phenotype while also playing a pivotal role in the generation of stem cell memory.
4.2 Results

4.2.1 4-1BB co-stimulation induces ThEO phenotype cells capable of long-term persistence and rapid recall potential upon subsequent antigen exposure.

Recently, we and others have demonstrated the ability of systemically administered 4-1BB agonist antibodies to induce a unique Eomes⁺KLRG1⁺ TcEO or ThEO T cell phenotype (87), which appears to be an intrinsic property of 4-1BB co-stimulation, as the response does not appear to be tumor-type specific nor is tumor challenge required for ThEO/TcEO polarization (Fig. 3). In order to further assess the long-term memory potential of the 4-1BB agonist induced TcEO or ThEO phenotype, I first investigated the ability of the TcEO/ThEO phenotype to persist long-term. Upon secondary tumor challenge with the murine B16 melanoma cell line expressing the ovalbumin antigen (B16-ova), CD4⁺ ThEO and CD8⁺ TcEO T cells were present within the spleens of α4-1BB treated, but not αCTLA-4 treated mice, with a frequency of approximately 40% of both the CD4 and CD8 effector T cell populations bearing the ThEO/TcEO phenotype respectively. Moreover, within the fraction of tumor-infiltrating lymphocytes (TIL), approximately 40% of effector CD4 T cells and 50% of CD8 effector T cells bore the Eomes⁺KLRG1⁺ phenotype (Fig. 4 a,b).

During the course of tumor establishment, exhausted T cells develop a pseudo-memory state characterized by an inflated expansion phase followed by partial or incomplete contraction (230, 286, 287). In order to determine whether the TcEO/ThEO phenotype present after secondary challenge constituted an inflated exhaustion phenotype or whether the phenotype was truly capable of contracting and re-expanding, I bled mice and monitored endogenous OVA tetramer-specific CD8 responses every ten days throughout the course of treatment. Administration of α4-1BB therapy induced expansion of antigen-specific T cells in the blood beginning ten days after initial tumor challenge, peaking approximately 20 days post-challenge, before undergoing a slow contraction until day 45 ptc, in the same fashion as tumor-bearing mice treated with αCTLA-4 checkpoint blockade therapy. Upon rechallenge with irradiated B16-Ova
tumor cells, antigen-specific CD8 T cells re-expanded in the blood 2-3 fold as soon as five days post-secondary challenge. Within the antigen-specific CD8 population of α4-1BB treated mice, those cells bearing the Eomes^+^KLRG1^+^ TcEO phenotype peaked ten days ptc, with approximately 40% of antigen-specific T cells bearing the phenotype. This was followed by a rapid contraction of the TcEO phenotype, reaching a nadir of ~4% of antigen-specific cells 40 days ptc. Upon rechallenge at day 45, antigen-specific T cells in the blood bearing the Eomes^+^KLRG1^+^ TcEO phenotype re-expanded to approximately 40% of total tetramer^+^ cells (Fig. 4c). This suggested that the TcEO phenotype represented a true memory T cell population capable of expanding and contracting in the same fashion as traditional memory T cells and was not indicative of persistent pseudo-memory.

I next sought to determine to what extent and duration the antigen-specific TcEO T cell population was able to persist long-term. In order to address TcEO longevity, tumor-bearing mice receiving either α4-1BB or αCTLA-4 therapy were rechallenged with irradiated B16-Ova every 45 days for up to one year. The frequency of antigen-specific TcEO T cells was measured in the blood 5 days prior to, and 5 days subsequent to rechallenge. While the TcEO phenotype had not yet developed as early as five days after tumor challenge, interestingly, the majority of antigen-specific cells already expressed high levels of Eomes. As early as 10 days after tumor challenge, the majority of antigen-specific cells from α4-1BB treated mice had developed the TcEO phenotype, with the remainder exhibiting an Eomes^+^KLRG1^+^ phenotype. This is in contrast to antigen-specific cells in the blood of αCTLA-4 treated mice which bore only a small fraction (<15%) of TcEO phenotype cells, with the majority bearing an Eomes^lo/neg^KLRG1^+^ phenotype. By day 20, the Eomes^KLRG1^+^ TcEO phenotype had disappeared and an Eomes^lo^KLRG1^+^ phenotype remained as the dominant population in the blood of both α4-1BB and αCTLA-4 treated mice. This trend continued throughout the contraction phase of the immune response through D45. Upon restimulation, the TcEO phenotype reappeared within the antigen-specific population in the blood of α4-1BB treated mice, making up roughly one-quarter to one third of antigen-specific cells; however, the Eomes^lo^KLRG1^+^ population remained the dominant
population in αCTLA-4 treated mice. The disappearance and reappearance of the Eomes$^{hi}$KLRG1$^{+}$ population within α4-1BB treated mice continued upon each subsequent boost for up to one year after the primary challenge (Fig. 4d, e). This kinetic of the TcEO response also bore out in adoptively transferred OT-I mice (Fig. 5). Using the Ova-specific D011.10 CD4 T cell model, I also demonstrated that Ova-specific CD4 T cells develop a similar kinetic upon peptide vaccination, though the response was much more muted than the CD8 response, with only 10% of CD4s developing the ThEO phenotype at the peak of expansion, which was notably delayed until day 20 post-challenge, consistent with significant lag time of a CD4 response behind a CD8 response (Fig 6) (76, 104, 288). I also investigated the ability of the endogenous TcEO response to recall in response to the viral HPV E7 antigen (Fig. 7), and toward the Spas self-antigen in the context of TRAMPC2 prostate tumor challenge (Fig. 8). Both of these models further demonstrated similar recall kinetics to the OVA system, though with differing magnitudes of response, further supporting the ability of TcEO/ThEO phenotype cells to develop memory potential. Moreover, this response appears to be antigen-specific, as lack of cognate antigen recognition failed to significantly polarize cells towards a TcEO phenotype (Fig. 9).

Taken together, my data demonstrates that TcEO/ThEO phenotype cells are not only present after secondary antigen exposure, but exhibit an expansion, contraction, and re-expansion kinetic consistent with formation of immunological memory.
Figure 3. ThEO phenotype cells are generated in response to 4-1BB agonists in multiple tumor models. Mice were challenged s.c. with various murine tumor cell lines and were either left untreated or treated with α4-1BB therapy (D3, 6, 9) before tumors were harvested on D16 and analyzed by flow cytometry. A plurality of both CD4 (top) and CD8 (bottom) T cells bearing the Eomes*KLRG1* ThEO or TcEO T cell phenotype, respectively, can be found within the microenvironment of multiple tumor models within α4-1BB treated mice compared to untreated controls. FACS plots are representative of 5-10 mice in each group from n = 2-3 independent experiments.
Figure 4.
Figure 4. ThEO phenotype cells are capable for persisting long-term with potent recall potential.  (a) Frequency of CD4 effector T cells (top) and CD8 effector cells (bottom) in the spleens and TIL fraction of α4-1BB or αCTLA-4 treated mice bearing the Eomes⁺KLRG1⁺ ThEO or TcEO T cell phenotype respectively five days after B16-Ova rechallenge.  (b) Quantification of the percent of CD4 or CD8 T cells bearing the ThEO/TcEO phenotype in the spleen and TIL fraction of α4-1BB or αCTLA-4 treated mice as in (a).  (c) Frequency of total endogenous tetramer positive CD8 T cells (left) or Eomes⁺KLRG1⁺ T cells amongst the tetramer positive fraction (right) within the blood of α4-1BB or αCTLA-4 treated mice throughout the course of immunotherapy.  Black arrow; time of tumor rechallenge.  (d) Frequency of endogenous antigen-specific CD8 T cells in the blood of α4-1BB treated or αCTLA-4 treated mice bearing an EomeshiKLRG1⁺ TcEO phenotype (blue) or EomesloKLRG1⁺ phenotype (red).  Black arrows; Rechallenge with irradiated FVAX/B16-Ova tumor vaccine.  (e) Quantification of the contraction of EomeshiKLRG1⁺ and expansion of EomesloKLRG1⁺ phenotype within antigen-specific cells through the course of the first boost at D45 (black arrow) (far left).  Frequency of antigen-specific CD8 T cells pre- and post-boost and of EomeshiKLRG1⁺ cells within the antigen-specific fraction after tumor rechallenge at D45 (middle) and D90 (far right).  Quantification in b, c, and e are presented as mean ± SEM (n= 5-15 mice) from 2-3 independent experiments.  ns, not statistically significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
Figure 5. Polarization of OT-I T cells toward the TcEO phenotype. Representative FACS plots of OT-I (CD45.2) T cells expressing the Eomes hi KLRG1 + phenotype (blue) or the Eomes lo KLRG1 + phenotype (red) within the blood of α4-1BB or αCTLA-4 treated mice. Plots are representative (5-10 mice per group) of n = 3 independent experiments.
Figure 6. Antigen-specific CD4 T cells develop a delayed and muted ThEO polarization and memory kinetic. (a) Frequency of Eomes$^{hi}$KLRG1$^+$ and Eomes$^{lo}$KLRG1$^+$ antigen-specific DO11.10 CD4 T effector cells in the blood of vaccinated mice over time. (b) Quantification of antigen-specific CD4 cells in the blood of treated mice (left) or of antigen-specific cells bearing the Eomes$^{hi}$KLRG1$^+$ phenotype (right) from a. (c) Comparison of Eomes$^{hi}$KLRG1$^+$ and Eomes$^{lo}$KLRG1$^+$ antigen-specific cells within the blood of α4-1BB treated mice. Black arrows in b indicate timepoint of rechallenge. Quantification in b and c are presented as mean ± SEM (n=15 mice) from 3 independent experiments (Student's T). ns, not statistically significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
Figure 7. HPV E7-antigen-specific recalled TcEO responses. Antigen-specific CD8 responses in the blood of mice challenged with HPV positive mEERL cells and later rechallenged with HPV positive TC-1 tumor cells. FACS plots are representative (3 mice) from one experiment.
Figure 8. Spas-antigen specific CD8 T cells are induced to become TcEO phenotype cells.

(a) Frequency of Eomes$^{hi}$KLRG1$^+$ and Eomes$^{lo}$KLRG1$^+$ cells within the Spas-antigen specific T cell population found in the blood of either α4-1BB or αCTLA-4 treated mice. (b) Quantification of the total antigen-specific population (left) or of the Eomes$^+$KLRG1$^+$ fraction within the antigen-specific population (right). (c) Comparison of antigen-specific Eomes$^{hi}$KLRG1$^+$ and Eomes$^{lo}$KLRG1$^+$ within the blood of α4-1BB treated mice. Quantification in b and c are presented as mean ± SEM (n= 15 mice) from 3 independent experiments (Student’s T). ns, not statistically significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
Figure 9. Non-bystander induction of ThEO phenotype cells. Frequency of Eomes+KLRG1+ CD8 T cells in the blood of tumor challenged, α4-1BB treated mice WT Eomes-YFP reporter mice or mice expressing the gp33-antigen-specific TCR (P14 Eomes-YFP), ten and twenty days after initial challenge. FACS plots are representative of 2-3 mice/group. Images are representative of tumor growth before sacrifice due to excessive tumor burden approximately 40 days after tumor challenge.
4.2.2 ThEO phenotype cells reside within secondary lymphoid organs and recall rapidly to distant antigen challenge.

In order to gain contextual insight the TcEO/ThEO recall response, I next investigated the tissue distribution of the TcEO/ThEO population before and after secondary tumor challenge. After three rounds of α4-1BB immunotherapy, I found that the TcEO phenotype made up approximately one quarter of antigen-specific T cells in the spleen and bone marrow and over thirty percent of antigen-specific T cells in the lymph nodes 45 days after primary tumor challenge, prior to antigenic re-stimulation. Antigen-specific TcEO cells, however, were largely absent from the blood and peripheral tissue (i.e. liver) (Fig. 10.) Further, ninety days after intraperitoneal (i.p.) tumor challenge (GVAX), mice treated with α4-1BB therapy received a second subcutaneous (s.c.) tumor challenge of B16-BL6 melanoma cells. After rechallenge, approximately 5% of CD4 effector cells and 5% of CD8 cells bore the Eomes×KLRG1 phenotype in the spleen, whereas the phenotype was largely absent in the tumor draining lymph node (dLN). Interestingly, a larger portion of both CD4 and CD8 T cells in the bone marrow (B.M.) exhibited the TcEO/ThEO phenotype compared to the spleen, suggesting that this site may act as a selective reservoir for ThEO phenotype cells in the memory phase. Roughly, ten percent of both CD4 and CD8 T cells also bore the ThEO or TcEO phenotype respectively with the tumor (TIL), suggesting that this phenotype is capable of recalling to distant tumor sites, affording systemic protection against secondary metastasis (Fig 11a). I also found distinct enrichment of TcEO or ThEO phenotype cells within each tissue, excluding the lymph node, compared to tissue taken from mice five days after primary tumor challenge (Fig 11b), suggesting that the TcEO/ThEO phenotype evolves over time and α4-1BB therapy is not merely releasing cells bearing the phenotype from a particular niche.

I also examined localization of the TcEO/ThEO phenotype upon multiple restimulations, one year after receiving a primary challenge (Fig 11c). Similar to a D90 rechallenge, after 365 days, TcEO/ThEO phenotype cells could still be found within these secondary lymphoid organs.
Particularly interesting, when I analyzed antigen-specific cells based on SIINFEKL-tetramer staining, irrespective of the total percent of Eomes^+^KLRG1^+^ cells found within each tissue, between 20-30% of all antigen specific cells bore the TcEO phenotype. This further suggests that TcEO/ThEO polarization remains a stable systemic response, maintaining residence within the primary and secondary lymphoid organs. Moreover, this data suggests that the TcEO phenotype is able to maintain a global presence in order to respond rapidly to secondary antigen exposure.
Figure 10. TcEO phenotype cells reside in the secondary lymphoid organs absent secondary antigen exposure. Frequency of antigen-specific Eomes$^{hi}$KLRG1$^+$ TcEO phenotype cells within the spleen, lymph node, blood, bone marrow, and liver of either α4-1BB-treated or αCTLA-4 treated mice. FACS plots are representative of (n=5 mice per group) from one experiment. Quantitation represents the percent of cells within the gated population. dLN = draining lymph node. BM = bone marrow.
Figure 11. ThEO phenotype cells are found in the secondary lymphoid organs after recall.

(a) Frequency of bulk CD4 (top) or CD8 (bottom) T cells bearing the Eomes\textsuperscript{+}KLRG1\textsuperscript{+} ThEO phenotype found within various tissues of naïve or α4-1BB treated mice after rechallenge at D95. 

(b) Quantification of the frequency of bulk CD4 effector cells (left) or CD8 cells (right) five days after rechallenge at D90 (black bars) or five days after primary challenge (white bars) bearing the Eomes\textsuperscript{+}KLRG1\textsuperscript{+} phenotype within various tissues. (c) Frequency of bulk CD4 (top) or CD8 (middle) or antigen-specific (bottom) T cells bearing the Eomes\textsuperscript{+}KLRG1\textsuperscript{+} ThEO phenotype found within various tissues of αCTLA-4 or α4-1BB treated mice after rechallenge at D365. FACS plots in a are representative of n= 15 mice. Quantification in b is presented as mean ± SEM (n= 15 mice) from 3 independent experiments (Student’s T). FACS plots in c are representative of 10 mice from 2 independent experiments. ns, not statistically significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
4.2.3. ThEO phenotype cells phenotypically resemble multiple memory T cell subsets.

I next sought to characterize the memory phenotype associated with the TcEO/ThEO T cell population. Multiple different methods as well as nomenclature have been used to describe memory T cells. In order to enrich our understanding of the ability of TcEO/ThEO phenotype cells to transition into the memory phase, I used multiple definitions to aid in the characterization of TcEO/ThEO phenotype cells. I first analyzed the potential for immunotherapy to modulate the memory precursor populations based on expression of the IL-7 receptor alpha (CD127) and KLRG1 (235, 285, 289). CD127 and KLRG1 are well defined markers of memory precursor cells or terminally differentiated cells respectively (290). Further, CD127\(^+\)KLRG1\(^+\) cells may persist as a memory population with potent effector potential (291). The frequency of these populations formed in response to infection is largely dictated by the inflammatory microenvironment, though the frequency of these populations generated in response to tumors is poorly understood.

In order to investigate the memory precursor populations generated in response to growing tumors in the context of immunotherapy, mice were challenged with B16-Ova and received three doses of immunotherapy as described. Mice were bled routinely and the frequency of antigen-specific T cells bearing the phenotypic identity of various memory precursor subsets was analyzed. The relative abundance of memory precursor effector cells (MPECs; CD127\(^+\)KLRG1\(^-\)), double-positive effector cells (DPECs; CD127\(^+\)KLRG1\(^+\)), short-lived effector cells (SLECs; CD127\(^-\)KLRG1\(^+\)), and early effector cells (EECs; CD127\(^-\)KLRG1\(^-\)) was not significantly altered during the course of \(\alpha\)4-1BB immunotherapy compared to checkpoint blockade, nor were the frequencies of these populations significantly altered after rechallenge (Fig. 12a). This suggested that the relative frequency of memory precursors may be a function of tumor rejection, independent of the mechanism of rejection.

Within \(\alpha\)4-1BB treated mice, I next analyzed the ability of various subsets to express phenotypic markers of immunological memory. In order to accomplish this, I divided antigen specific cells into memory or terminally differentiated subsets based on expression of Eomes and
Traditionally, Eomes\textsuperscript{+}KLRG1\textsuperscript{−} cells are defined as true memory precursors, whereas Eomes\textsuperscript{−}KLRG1\textsuperscript{+} cells are defined as terminally differentiated cells (148, 284). I further classified Eomes\textsuperscript{−}KLRG1\textsuperscript{−} cells as undifferentiated precursor cells. In this way, I found that Eomes\textsuperscript{+}KLRG1\textsuperscript{+} TcEO cells lose CD127 as they convert to effectors and undergo contraction. Well into the memory phase, however, the remaining TcEO phenotype cells express the IL-7R\(\alpha\), similarly to Eomes\textsuperscript{−}KLRG1\textsuperscript{−} traditional memory cells, persisting as DPECs to a larger degree than “differentiated” Eomes\textsuperscript{+}KLRG1\textsuperscript{+} cells. Further, after recall, the majority of TcEO cells maintained expression of the IL-7R\(\alpha\) (Fig. 12b), suggesting that these cells are capable of responding to homeostatic cytokines necessary for memory formation.

I next characterized ThEO phenotype cells based on classical markers of effector memory (Tem), central memory (Tcm) (CD44 and CD62L), and tissue resident memory (Trm; CD69 and CD103) (241, 244, 251). I analyzed the blood of mice treated with \(\alpha_4\)-1BB therapy 40 days after tumor challenge for the presence of central and effector memory cells or the TIL fraction five days after mice were rechallenged with B16-Ova tumor cells in order to investigate tissue resident memory populations (Fig 12c). Within \(\alpha_4\)-1BB treated mice, the majority of CD4 ThEO and CD8 TcEO cells expressed an effector memory phenotype (CD44\textsuperscript{+}CD62L\textsuperscript{−}), however, a sizable fraction (~15% of ThEOs and ~30% of TcEOs) did in fact bear a central memory phenotype. Cells expressing markers of tissue resident memory in the tumor microenvironment on the other hand, were scarce in \(\alpha_4\)-1BB treated mice. In fact, minimal CD69 expression was measured in any subset investigated, whereas CD103 expression was highest in Eomes\textsuperscript{−}KLRG1\textsuperscript{−} CD8 T cells and Eomes\textsuperscript{−}KLRG1\textsuperscript{+} CD4 T cells, but not in ThEO or TcEO phenotype cells. These data suggest that TcEO/ThEO cells largely generate effector memory phenotype cells in the blood and further, do not maintain their phenotype within the tissue to generate tissue resident memory cells.

I next investigated the expression CD27, CD28, and the chemokine receptor CX3CR1 as markers of memory potential within the ThEO phenotype. Activation through 4-1BB increases expression of CD28 on T cells (292), and co-expression of both CD27 and CD28 demarcates
cells with enhanced memory potential such that CD28^+CD27^- > CD28^+CD27^-> CD28^+CD27^-> CD28^+CD27^- (293). I investigated the expression of these markers on antigen-specific CD8 T cells in various tissue 16 days after tumor challenge. Immunotherapy with 4-1BB agonists largely generated CD28^+CD27^- cells in the spleen. Of these, CD28^+CD27^- cells made up the majority (>60%) of Eomes^hiKLRG1^+TcEO cells, followed by CD28^+CD27^- effector cells. This is in contrast to Eomes^loKLRG1^+ cells, which were more heavily dominated by a CD28^-CD27^- and CD28^-CD27^- phenotype, suggesting that these two KLRG1^+ phenotypes are distinct, and further that the Eomes^lo phenotype is more terminally differentiated than the Eomes^hi phenotype (Fig 12d). Further, in the tumor microenvironment, bulk antigen-specific cells, as well as Eomes^hiKLRG1^+ and Eomes^lo KLRG1^+ cells, exhibited a more differentiated phenotype characterized by absence of CD28, suggesting that splenic TcEO cells may generate long-term immunological memory, and the TIL fraction may only contribute a small fraction to the memory pool if any.

Memory potential has also recently been characterized using the inflammatory chemokine receptor CX3CR1 (fractalkine receptor) (242). Gerlach et al. presented convincing data in an LCMV model suggesting that CX3CR1^low cells (CXCR3^hiCD27^hi) are the most efficient at generating central memory T cell populations. Further, CX3CR1 intermediate cells generated effector memory populations and CX3CR1^high cells generated terminal effectors cells with the greatest tissue penetrance. Using this approach, I found that α4-1BB therapy generated antigen-specific CX3CR1^low central memory cells and CX3CR1^int effector memory cells, with only a small fraction (<20%) exhibiting a CX3CR1^high terminal effector phenotype in the spleen sixteen days after tumor challenge (Fig 12e). Further, Eomes^hiKLRG1^+ cells within the antigen-specific T cell pool bore a CX3CR1^int effector memory phenotype as well as a CX3CR1^low central memory phenotype consistent with CD44 and CD62L expression and CD27/CD28 expression demarcating these cells bearing memory potential (Fig 12c). In contrast, a majority of splenic Eomes^loKLRG1^+ cells bore a CX3CR1^high terminal effector phenotype, and very few exhibited a central memory (CX3CR1^low) phenotype. Further, within the TIL fraction, all antigen-specific cells
bore a CX3CR1\textsuperscript{hi} terminal effector phenotype consistent with terminal differentiation and loss of memory potential in the tumor microenvironment.

I continued to characterize the ThEO/TcEO memory phenotype in the secondary lymphoid organs of α4-1BB treated mice (Fig 12f). Forty days after primary tumor challenge, all Eomes/KLRG1 subsets expressed Ly6C, CCR7, and CD127, consistent with generation of memory and potential to traffic into secondary lymphoid organs. Moreover, Eomes\textsuperscript{*}KLRG1\textsuperscript{*} “conventional memory” cells also expressed high levels of CXCR3 and IL15Rβ (CD122), consistent with their designation as memory cells, whereas Eomes\textsuperscript{lo/l}-KLRG1\textsuperscript{*} and Eomes\textsuperscript{-} KLRG1\textsuperscript{*} cells expressed low levels of CXCR3 and IL15Rβ, consistent with their designation as terminal and undifferentiated cells respectively. Further, Eomes\textsuperscript{*}KLRG1\textsuperscript{*} ThEO/TcEO cells expressed these receptors at similar levels to conventional memory cells. This suggested that not only do ThEO/TcEO cells possess receptors allowing for residency within secondary lymphoid organs, but also that these cells are capable of responding to homeostatic cytokines necessary for maintenance in the memory phase. Interestingly, TcEO/ThEO phenotype cells also express high levels of the stem cell antigen Sca-1, a marker of the recently characterized stem cell memory subset (Tscm) (290, 294). Tscm constitutes a novel precursor memory population that gives rise to central memory as well as effector memory and subsequent effectors (239). While Tscm cells bear a striking resemblance to Tcm, Sukumar et al. have demonstrated that these cells can be distinguished based on mitochondrial membrane potential based on uptake of the mitochondrial membrane permeable dye tetramethylrhodamine methyl ester (TMRM) (256). Using a similar approach, splenocytes from α4-1BB treated mice (45 days post tumor challenge) were stained with TMRM before sorting the 10% brightest and 10% dimmest splenocytes capable of TMRM uptake. ThEO and TcEO phenotype cells were predominantly found in the TMRM\textsuperscript{low} fraction (Fig 12g), suggesting that ThEO/TcEO cells bear a phenotype and mitochondrial membrane potential consistent with generation of Tscm.

Taken together, these data suggest that ThEO phenotype cells express markers of memory potential. Based on the markers used, ThEO/TcEO cells are phenotypically similar to
Tscm and Tem, despite expression of the terminal marker KLRG1 and the exhaustion marker PD-1 (Fig 13). Moreover, differences in expression of these markers within Eomes\textsuperscript{hi} and Eomes\textsuperscript{lo} KLRG1\textsuperscript{+} subsets suggest that these two subsets are distinct and TcEO/ThEO cells possess greater memory potential than Eomes\textsuperscript{lo/neg} cells. Further, cells infiltrating the tumor lacked markers of long-term memory potential and exhibit phenotypes consistent with more terminal effector function, suggesting that the tumor may serve as a site of terminal differentiation. Expression of the stem cell markers Sca-1 as well as limited uptake of TMRM dye would further suggest that ThEO/TcEO phenotype cells bear Tscm characteristics, which may be consistent with their localization to the bone marrow stem cell niche.
Figure 12.
Figure 12. ThEO phenotype cells exhibit a memory phenotype consistent with effector memory, central memory, and stem cell memory subsets. (a) Antigen-specific CD8 cells in the blood of α4-1BB treated (top) or αCTLA4 treated (bottom) mice bearing markers of precursor memory (MPEC: CD127⁺KLRG1⁻, DPEC: CD127⁺KLRG1⁺, SLEC: CD127⁻KLRG1⁺, EEC: CD127⁻KLRG1⁻). (b) Antigen-specific CD8 cells in the blood of α4-1BB treated mice categorized by expression patterns of Eomes and KLRG1 were further divided into memory precursor categories as in a. (c) Left: The percentage of CD4 or CD8 effector cells within the blood of α4-1BB treated mice (left) or ThEO/TcEO phenotype cells (right) 45 days after primary tumor challenge expressing markers of effector memory (CD44⁺CD62L⁻) or central memory (CD44⁺CD62L⁺). Right: mean fluorescence intensity of tissue resident memory markers CD69 and CD103 within subsets of Eomes and KLRG1 expressing cells within the TIL fraction after tumor rechallenge. (d) Percentage of total antigen specific CD8 T cells or within the Eomes high or Eomes low fraction of KLRG1⁺ antigen specific cells expressing CD27 and CD28 within the spleens and TIL fraction of α4-1BB treated mice. (e) Percentage of total antigen-specific CD8 T cells within the spleen (left) and TIL fraction (right) or as a fraction of Eomes high or Eomes low KLRG1⁺ cells expressing either CD27 (top) or CXCR3 (bottom) and the fractalkine receptor CX3CR1. (f) Mean fluorescence intensity denoting expression of memory markers on CD4 effector cells (top) or CD8 cells (bottom) within memory cells (Eomes⁺KLRG1⁺), ThEO/TcEO cells (Eomes⁺KLRG1⁺), terminally differentiated cells (Eomes⁻KLRG1⁺) or undifferentiated cells (Eomes⁻KLRG1⁻). (g) Frequency (left) and quantification (right) of ThEO or TcEO (Eomes⁺KLRG1⁺) cells amongst TMRMhi and TMRMlo cells within the spleen. MPEC = memory-precursor effector cell. DPEC = Double-positive effector cell. SLEC = Short-lived effector cell. EEC = Early effector cell. Numbers in graphs and FACS plots indicate percentages. Numbers in histogram plots indicate mean fluorescence intensity. FACS plots in d-g are representative of n=15 mice. Quantification in a, b, and g is presented as mean ± SEM (n= 15 mice) from 3 independent experiments (Student’s T). ns, not statistically significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
Figure 13. Co-inhibitory receptor expression on ThEO/TcEO phenotype cells. Expression of PD-1 within subsets of memory (Eomes*KLRG1−), ThEO/TcEO (Eomes*KLRG1+) terminally differentiated (Eomes−KLRG1+) or undifferentiated (Eomes−KLRG1−) populations in the spleen. Numbers represent mean fluorescence intensity. Histogram plots are representative of (n =5-10 mice) from 2 independent experiments.
4.2.4 ThEO phenotype cells remain a stable phenotype stemming from an Eomes∗KLRG1+ phenotype.

I next sought to further investigate the stability of the ThEO phenotype and the potential precursor populations that may give rise to ThEO cells. ThEO/TcEO phenotype cells resemble conventional memory populations (Eomes∗KLRG1−) in some ways (CXCR3, CD44/CD62L, IL15Rβ) and terminally differentiated cells (Eomes KLRG1+) in others (Sca-1, KLRG1, PD-1) (Fig 12, Fig 13) and yet bear more memory potential than those same terminal cells. Further, T cells are thought to lose memory potential over time, suggesting these cells may stem from Eomes∗KLRG1− cells as they transition into a more terminal phenotype, leading to eventual loss of Eomes to become Eomes lo/neg (Fig 4c) (230). In order to investigate the cellular origin of ThEO/TcEO phenotype cells, mice expressing an YFP reporter gene under control of the Eomes promoter (Eomes-YFP; CD45.2) were challenged with B16-Ova and subsequently administered three rounds of α4-1BB therapy. Twelve days after tumor challenge, CD8 cells were then sorted based on YFP and KLRG1 expression before adoptive transfer into congenically marked naïve mice (CD45.1). Mice were then bled routinely in order to measure the conversion of transferred T cells into other phenotypes. Interestingly, transferred Eomes∗KLRG1+ cells initially gave rise to all four subsets in the blood, though over time, the Eomes KLRG1− and Eomes KLRG1+ terminal phenotypes were lost in favor of memory Eomes∗KLRG1− and Eomes∗KLRG1+ TcEO phenotype cells (Fig 14). Unexpectedly, as early as three days after sorting (D15 ptc), transferred Eomes∗KLRG1+ cells had converted to a large extent into Eomes∗KLRG1+ TcEO phenotype cells. Similarly to transferred TcEO phenotype cells, transferred Eomes KLRG1+ continued to transition into Eomes∗KLRG1− memory cells as the more terminal Eomes∗KLRG1+ and Eomes KLRG1− cells were lost in the blood. Transferred Eomes∗KLRG1− cells, on the other hand, maintained their phenotype throughout the transition into the memory phase and any Eomes∗KLRG1− were lost. Further, transferred Eomes KLRG1− cells transitioned directly into Eomes∗KLRG1− memory cells, failing to induce KLRG1 expression or cycle through a transitory TcEO phenotype.
Taken together, these data suggest that ThEO/TcEO phenotype cells maintain phenotypic stability, with some ability to transition into an Eomes<sup>+</sup>KLRG1<sup>-</sup> memory phase. Further, this phenotype appears to stem from an Eomes<sup>-</sup>KLRG1<sup>+</sup> terminal phenotype, not from an Eomes<sup>+</sup>KLRG1<sup>-</sup> memory phenotype.
Figure 14. The TcEO phenotype remains a stable T cell population. Frequency of adoptively transferred CD8 T cell subsets within CD45.2+ cells. Cells were pooled from n = 15 mice before adoptive transfer. FACS plots are representative of n = 5-10 mice/group. Pie charts represent quantification of the percent of adoptively transferred cells bearing various phenotypes.
4.2.5. Upon rechallenge, ThEO phenotype cells recall with effector function

I next sought to investigate the effector potential of recalled ThEO/TcEO phenotype cells. Tumor challenged mice were treated with three rounds of α4-1BB immunotherapy and bled five days prior to, and five days subsequent to, rechallenge. Memory, ThEO/TcEO, terminal, and undifferentiated cells were analyzed for markers of effector function (Ki67 and Granzyme B) at each time point. Prior to rechallenge at D90, all four T cell populations exhibited little if any proliferative capacity as indicated by Ki67 expression, or cytotoxicity as evidenced by little granzyme B expression in either CD4 or CD8 T cells (Fig 15a). After tumor rechallenge, however, Eomes+KLRG1- memory, Eomes+KLRG1- ThEO, and Eomes KLRG1+ differentiated CD4 T cells and Eomes+KLRG1- memory, Eomes+KLRG1+ TcEO, and Eomes KLRG1+ undifferentiated CD8 T cells began to rapidly proliferate as noted by increased Ki67 expression. Moreover, TcEO and differentiated cells gained cytotoxic capacity after rechallenge as examined by granzyme B expression. Interestingly, ThEO phenotype cells were the only CD4 subset to reacquire cytotoxic effector function, suggesting that this phenotype is uniquely programmed to maintain cytotoxic effector potential. Further, effector capacity (proliferation and cytotoxicity) were maintained long-term for up to one year after initial challenge, with both CD4 ThEO and CD8 TcEO subsets capable of responding to subsequent tumor challenge (Fig. 15b).

Taken together, this data suggests that both CD4 ThEO and CD8 TcEO cells maintain effector capacity well into the memory phase, and are capable of recalling with potent cytotoxicity to secondary and subsequent tumor challenge.
Figure 15. Figure 15. ThEO phenotype cells recall with potent cytotoxicity and effector function. (a) Histogram plots indicating the fluorescence intensity of the proliferation marker Ki67 and the cytotoxic effector molecule Granzyme B (GzmB) in both CD4 and CD8 T cells before and after secondary tumor challenge at D90. (b) Histograms indicating Ki67 and granzyme B expression as in a, though, one year after primary tumor challenge (45 days after last antigen exposure).
4.3 Discussion

Development of persistent immunological memory in the tumor setting remains the ultimate goal of anti-tumor therapies in order to prevent relapse and engender long lasting clinical responses. Administration of checkpoint blockade antibodies has demonstrated some efficacy in achieving this goal, providing a 20-40% progression-free survival rate against melanoma (295). Targeting costimulatory receptors in the tumor necrosis factor superfamily has proven equally effective at treating a variety of solid and hematological malignancies (1, 33, 115, 296-298). Targeting the 4-1BB co-receptor in particular has proven equally effective as checkpoint blockade at prolonging survival in B16 melanoma-bearing mice (58).

We have recently demonstrated one potential mechanism through which α4-1BB therapy induces robust anti-tumor responses, namely induction of a potently cytotoxic Eomes+KLRG1+ T cell phenotype in both the CD8 subset (TcEO) as well as the CD4 subset (ThEO)(87). This study sought to uncover the ability of the ThEO phenotype to mediate long-term tumor protection through acquisition of memory potential. Eomes is traditionally defined as a marker of immunological memory, whereas KLRG1 expression is typical of terminally differentiated T cell populations (230). I found that, despite expression of terminal differentiation markers (KLRG1, PD-1), ThEO phenotype cells demonstrated an antigen-specific immune response (i.e. expansion, contraction, and re-expansion) consistent with a recalled memory response to both foreign antigens (Ova, E7 peptide) and mutated self-antigens (Spas). I further demonstrated that antigen-specific CD4 cells also acquired the Eomes+KLRG1+ phenotype, albeit at a delayed, and muted response kinetic. I further demonstrated that the TcEO/ThEO phenotype resided in the secondary lymphoid tissue both prior to, and after secondary recall, up to one year after tumor challenge without a requirement for additional α4-1BB administration.
I went on to demonstrate that the ThEO population closely resembled effector memory, but also maintained characteristics of central and stem cell memory. While ThEO cells did not express markers of tissue resident memory, I cannot rule out the possibility that an “ex-ThEO” cell lost its ThEO phenotype to contribute to the Trm pool, nor can I rule out the possibility that some ThEO cells maintain tissue residency without acquiring a traditionally defined Trm phenotype. Further, as Tscm is thought to contribute to Tcm and Tem populations (239), it remains to be defined whether ThEO phenotype cells receive different signals polarizing them towards three different subsets of Tscm, Tcm, and Tem, or whether ThEO cells transition into Tscm and subsequently give rise to Tcm and Tem. Both TcEO and ThEO cells recalled potently with discernable proliferative potential and cytotoxic capacity. Interestingly, upon recall, the Eomes+KLRG1+ ThEO phenotype recall with potent cytotoxicity, as opposed to the Eomes+KLRG1− memory cells or Eomes−KLRG1+ terminal cells. This suggests that α4-1BB instills a cytotoxicity program specifically in the Eomes+KLRG1+ phenotype that is maintained long-term. What factors mediate this long-term cytotoxic potential remain unexplored, but may involve epigenetic factors that leave the transcriptional machinery open at the cytotoxicity/effector genes (299-301).

Overall, data presented here suggest a role for 4-1BB co-stimulation in generating novel cytotoxic ThEO/TcEO T cell phenotypes capable of persisting long-term as a stable population, which may afford long-term anti-tumor surveillance and aid in the prevention of tumor relapse. Further, the stability of this phenotype suggests that 4-1BB agonism, through as of yet undefined mechanisms, may polarize a novel T cell phenotype distinct from existing, conventionally defined T helper subsets.
Chapter 5: Intrinsic and Extrinsic Factors that influence ThEO development

5.1 Introduction

T cells exhibit a mutable plasticity in the range of their effector functions, allowing for rapid adaptability to a multitude of distinct pathologic conditions. These defined T cell polarities (e.g. Th1, Th2) are influenced by extrinsic factors that initiate downstream signaling cascades resulting in expression of lineage-defining transcriptional programming. This programming, in turn, leads to the induction of particular T cell effector functions intended to precipitate rapid clearance of infection.

Helper T cell subsets closely follow this mechanism of effector programming. Th1 cells, for example, respond to interferon gamma (IFNγ) and interleukin-12 (IL-12) to initiate a program resulting in expression the T-box transcription factor Tbet. Expression of T-bet, in turn, reinforces IFNγ production necessary for adequate viral clearance, while suppressing the expression of other lineage-defining transcription factors and subsequent cytokine production superfluous to viral clearance (151). Five unique helper T cell subsets, each with corresponding subsets in the CD8 lineage, have been identified. Each of these subsets is defined by unique transcriptional activity that drives distinct effector functions (Th1, Th2, Th17, Tfh, Treg)(302).

The signal transducer and activator of transcription (STAT) family of proteins consists of seven members (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6) which provide critical regulatory machinery necessary to maintain T cell survival and to drive polarization toward distinct T helper subsets. Each T helper subset is driven by a unique STAT signaling cascade. For instance, STAT4 is critical in driving Th1 immunity, whereas STAT6 drives Th2 polarity (150).

While CD4 T cells are generally thought to provide a helper function to myeloid cells and CD8 cells, the generation of cytotoxic CD4 T cells under certain conditions has recently been identified (33, 87, 152, 219). These cells are characterized by
expression of the T-box transcription factor Eomesodermin (Eomes) and the zinc-finger transcription factor Blimp-1 (PRDM1) (152). We have recently demonstrated that CD4 cytotoxicity can be induced through co-stimulation through the 4-1BB receptor. This, in turn, induces expression of Eomes which drives expression of a range of cytotoxic granzyme serine proteases. We have designated these 4-1BB agonist induced cytotoxic CD4 T cells “ThEO” and their analogous CD8 subset “TcEO” (87).

In addition to STAT signaling, a myriad of factors controls T cell effector function. While STAT3, for instance, controls CD8 cytotoxic effector capacity (303, 304), cytotoxicity is regulated by additional factors, particularly members of the Runt-related transcription factor family (Runx). The Runx family consists of three canonical family members (Runx1, Runx2, Runx3). While Runx1 and Runx3 have defined roles in T cell immunity, thymic selection, and development of cytotoxicity (305-307), Runx2 has an established role in osteogenesis (308-310), but no known role has been ascertained for Runx2 in the function of mature T cells. In collaboration with the Hwu lab (Mbofung et al, unpublished), we have demonstrated that Runx2 is a critical driver of T cell cytotoxicity; however, Runx2 also drives T cell apoptosis, preventing adequate tumor infiltration and tumor clearance. Interestingly, T cells engineered to overexpress Runx2 upregulate expression of several ThEO/TcEO related genes (e.g. Eomes, KLRG1, Granzyme B, Granzyme D, Granyzme K, perforin).

Within this chapter, I seek to define signaling mechanisms induced by 4-1BB co-stimulation that control ThEO/TcEO T cell polarization and regulate ThEO cell cytotoxicity and effector function. I focus on dissecting Runx2 expression patterns in ThEO/TcEO phenotype cells and STAT signaling mechanisms that may influence ThEO/TcEO T cell polarization. I find that Runx2 is induced by 4-1BB co-stimulation, and further, that virtually all ThEO/TcEO phenotype cells express this transcription factor. Runx2 expression is not unique to 4-1BB co-stimulation, as stimulation through
multiple TNF receptor family members can induce Runx2. I go on to demonstrate a key role for several cytokines and STAT signaling pathways, in particular STAT1, and to a lesser degree STAT3, in the induction of ThEO phenotype cells.
5.2 Results

5.2.1. Runx2 is expressed by both ThEO and TcEO phenotype cells upon 4-1BB stimulation.

As Runx2 overexpression has been shown to induce several ThEO/TcEO phenotype genes (e.g. Eomes, granzyme B) (Mbofung et al, unpublished), I next investigated the potential for α4-1BB therapy to induce Runx2 expression. Mice were challenged with 1.5X10^5 B16-BL6 murine melanoma tumor cells before administration of three rounds of tumor vaccine (FVAX) in combination with either αCTLA-4 or α4-1BB antibody. Mice were then sacrificed at day 16 post tumor challenge and immune infiltrates were analyzed by flow cytometry. Interestingly, α4-1BB therapy induced Runx2 in approximately 20% of splenic CD4 T cells and 40% of splenic CD8 T cells. This corresponded to a 3-fold increase in the percentage of Runx2^+ CD4 T cells and a 2-fold increase in Runx2^+ CD8 T cells compared to αCTLA-4 treated mice. Further, over 90% of Eomes^+KLRG1^+ ThEO CD4 or TcEO CD8 phenotype cells expressed Runx2 (Figure 16a). I then compared Runx2 expression in ThEO phenotype cells to memory phenotype cells (Eomes^+KLRG1^-), terminally differentiated cells (Eomes^- KLRG1^+), or undifferentiated cells (Eomes^-KLRG1^-). Consistent with findings that Runx2 induces apoptosis (Mbofung et al, unpublished), terminally differentiated cells in the spleen expressed the highest levels of Runx2. ThEO/TcEO phenotype cells demonstrated reduced Runx2, though higher levels than memory or undifferentiated cells (Figure 16b).

Taken together, these data suggest that α4-1BB immunotherapy induces Runx2 expression, which correlates phenotypically with increased terminal differentiation. Consistent with data presented in Chapter 4, ThEO phenotype cells have similar levels of Runx2 expression as classically defined terminally differentiated Eomes^-KLRG1^+ cells, consistent with differentiation of ThEO cells from this phenotype.
Figure 16. Runx2 is expressed in ThEO and TcEO phenotype cells. Mice were challenged with 1.5X10^5 B16-BL6 melanoma cells on the right flank. Mice then received three doses (D3, 6, 9) of an irradiated tumor vaccine s.c. in combination with either i.p. α4-1BB (350 ug/inj) or αCTLA-4 (100ug/inj). Sixteen days after tumor challenge, mice were sacrificed for tissue analysis. (a) Frequency of Runx2+ cells within splenic CD4 effector T cells (top) or CD8 T cells (bottom) of αCTLA-4 or α4-1BB treated mice, or within Eomes+KLRG1+ ThEO/TcEO phenotype cells within α4-1BB treated mice. (Right) Representative histogram plots of Runx2 expression in splenic CD4 or CD8 T cells. (b) Mean fluorescence intensity of Runx2 expression within undifferentiated (Eomes−KLRG1−), memory (Eomes−KLRG1+) ThEO (Eomes+KLRG1+) and terminally differentiated (Eomes−KLRG1+) T cell subsets within α4-1BB treated mice. Numbers in histogram plots indicate mean fluorescence intensity. FACS plots are representative of n= 5 mice. Quantification is presented as mean ± SD (n= 5 mice) from 1 independent experiment (Student’s T). ns, not statistically significant; *p<0.05; **p<0.01; ***p<0.001;****p<0.0001.
5.2.2. Expression of Runx2 is induced upon antigenic stimulation

I next sought to determine whether Runx2 expression was unique to 4-1BB agonism and/or the ability of 4-1BB to induce ThEO phenotype cells, or whether other members of the TNF receptor superfamily could also induce Runx2 independent of ThEO cell polarization. Mice were challenged with B16-Ova before treatment with either α4-1BB, αCTLA-4, α4-1BB/αCTLA-4, α4-1BB/αPD-1, αGITR, αOX-40, or αCD40 therapies. Mice were then bled at routine intervals to assess ThEO/TcEO polarization and Runx2 expression. As expected, α4-1BB or combinations with α4-1BB therapy were the only treatments that induced a sustainable ThEO/TcEO T cell population in the blood (Figure 17a). As previously reported (87), treatment with αGITR or αOX-40 failed to induce a ThEO phenotype. Interestingly, αCD40 induced an Eomes+KLRG1+ phenotype during the early effector phase, however, this population was only transiently induced and was not stably maintained over time. Further, Runx2 was expressed to some degree in every immunotherapy tested. While α4-1BB therapy appeared to be the most efficient at inducing high levels of long-term Runx2 expression in both CD4 and CD8 T cells, the other therapies tested were also capable of regulating Runx2 expression, although to a lesser degree. Again, next to α4-1BB therapies, CD40 agonist therapy appeared to be one of the most potent inducers of Runx2 (Figure 17b). Strikingly, though, Runx2 elevation increased after antigen exposure, whether after the primary tumor challenge, secondary challenge (D45) or tertiary challenge (D90) independent of the immunotherapy administered.

Taken together, these data suggest that Runx2 induction is not unique to α4-1BB co-stimulation, nor does it appear to be an effect of ThEO polarization, as all TNFR co-stimulation induced Runx2, but not a ThEO phenotype. Further, CD40 agonists were able to induce a transient TcEO-like phenotype concurrently with high levels of Runx2. Given that CD40 is predominantly expressed on antigen-presenting
cells, this data points towards a T cell extrinsic role for ThEO polarization as well as a T cell intrinsic role for maintenance of the ThEO phenotype. Importantly, data presented herein suggests antigenic stimulation to some degree induces Runx2 expression. Given that α4-1BB, and TNF receptors in general, enhance TCR signaling and promote T cell activation, enhanced NFκB, of NFAT signaling induced through these receptors may explain elevations in Runx2.
Figure 17. Runx2 is induced upon antigen exposure. Mice were challenged s.c. with $7.5 \times 10^4$ B16-Ova tumor cells before administration of three rounds (D3,6,9) of tumor vaccine (FVAX) plus indicated immunotherapy. The frequency of Eomes$^+$KLRG1$^+$ cells in the blood was tracked over time (a) as well as the percentage of cells expressing Runx2 (b). The fold change in Runx2 MFI (b, bottom) was calculated by dividing the MFI of Runx2 within each treatment group by the MFI of Runx2 in naïve blood at each time point. Quantitation indicates mean ± SD from (n= 5 mice) one experiment. Dashed lines indicate day of tumor rechallenge (D45, D90).
5.2.3. STAT1/STAT3 activating cytokines and STING signaling aid in polarization towards the ThEO phenotype.

I next sought to investigate the cytokine signaling networks responsible for inducing Runx2 expression and ThEO T cell polarization. We have previously demonstrated a role for interleukin-10, IL-15, and IL-27 in mediating ThEO polarization. We further demonstrated that IL-12/23, IFNγ, and Tbet are not required for ThEO polarization (87). Hypothesizing that ThEO phenotype cells are developmentally similar, yet distinct from Th1 polarized cells, I stimulated OT-II CD4 T cells for 48 hours with cognate antigen (Ova\textsuperscript{323-339}) before culture in a mixture of various cytokines consisting of various concentration of IL-2,-10, -15, and -27 as well as IFNγ, Wnt3 agonist, and the STING agonist 2′3′-cyclic GMP/AMP (cGAMP) with or without α4-1BB (100ug/ml) for one week (87, 101). The STING pathway is a potent activator of type I interferons (311-313), which I hypothesized may act to induce Eomes expression and ThEO polarization (314). Further, the STING pathway has a role in IL-15 trans-presentation which may also support ThEO polarization (315, 316). After one week of culture, I found that the cytomix induced a modest ThEO phenotype (approximately 10% of CD4 T cells expressed THEO phenotypic markers (Figure 18)). While not affecting the total percent of ThEO phenotype cells polarized, addition of the cytomix, in particular in combination with 5ug/ml of cGAMP, increased Eomes and granzyme B expression in these cells. Moreover, the cytomix increased Runx2 expression in \textit{in vitro} polarized cells. Given prior data suggesting that there exists a limit in Runx2 expression before induction of apoptosis (Mbofung et al, unpublished), these cells may have already reached this limit.

Taken together, these data suggest that, while 4-1BB signaling may provide T cell intrinsic signals necessary for ThEO polarization, the cytokine milieu also plays an important role in ThEO polarization. Much like the role for IFNγ in inducing Th1
cells, or IL-4 in the induction of Th2 immunity, I demonstrated a role of cytokines in inducing ThEO phenotype cells, though I was unable to clearly define a single cytokine necessary for ThEO induction.
Figure 18. Cytokines aid in the polarization of ThEO phenotype cells. Splenocytes were harvested from OT-II transgenic mice whose T cells express TCRs specific for the Ova$^{323-339}$ antigen. Cells were cultured with 5 μM peptide plus IL-2 for 48 hours before addition of cytomix (IL-2/IL-10/IL-15/IL-27/IFNγ/Wnt3a agonist). After culture in the cytomix for one week, cells were analyzed for acquisition of ThEO phenotype markers by flow cytometry.
5.2.4. STAT1 and STAT3 are key signaling pathways necessary for ThEO polarization.

Given the cytokines required for ThEO polarization, I next tested the requirement for STAT signaling in ThEO development. As many of the cytokines used in \textit{in vitro} cultures involved activation of signaling cascades through either STAT1 (IFNγ, IL-27, and potentially STING through IFNα), STAT3 (IL-10) or STAT5 (IL-2, IL-15) and the similarities between ThEO cells and Th1 cells, I focused on these STATs and the effects of their upstream inducers (IL-27, IFNα) on ThEO polarization. As STAT5 is also critical for T cell development and survival (317), I did not choose to further pursue the effects of this STAT on ThEO development.

In order to investigate the potential role of STAT signaling on ThEO polarization, either wildtype (WT) C57BL/6 mice or mice bearing conditional knockout of STAT1 or STAT3 in their T cell compartments (CD4\textsuperscript{Cre}/STAT1\textsuperscript{f/f} or CD4\textsuperscript{Cre}/STAT3\textsuperscript{f/f} respectively), or lacking systemic STING (STINGKO), IL-27 (Ebi3KO), or interferon alpha receptor expression (IFNαRKO) were challenged s.c. with B16-Ova before vaccination and administration of three rounds of α4-1BB agonist therapy. Mice were bled routinely and blood was analyzed for polarization towards the ThEO phenotype. As early as D10 after tumor challenge, defects in ThEO polarization were evident in the various knockout models (\textbf{Figure 19a}). As early as 24 hours after cessation of treatment (D10), three percent of CD4 T cells already bore the ThEO phenotype in the blood of WT treated mice, whereas no ThEO phenotype cells were found in the blood of any of the knockout mice, except Ebi3KO in which ThEO cells were reduced three-fold. Moreover, the presence of the CD8 TcEO phenotype was reduced by >4-fold in the blood of CD4\textsuperscript{Cre}/STAT1\textsuperscript{f/f}, CD4\textsuperscript{Cre}/STAT3\textsuperscript{f/f} and STINGKO mice. Ebi3KO mice, on the other hand demonstrated a mildly impaired TcEO response, with an increased population of Eomes\textsuperscript{lo/neg}KLRG1\textsuperscript{+} cells, while ablation of the IFNα pathway reduced the
TcEO population by half. This data suggested that STAT1 and STAT3 regulate ThEO/TcEO development and or persistence, and IL-27 and type I interferon may also regulate the ThEO polarity.

I next wished to uncover which aspects of the ThEO phenotype were being impacted by abrogation of these signaling pathways (Figure 19b). As expected, abolishing the STAT1 pathway reduced Sca-1 expression in CD8 cells (318), as well as proliferation and granzyme B expression to some degree. Further, defects in STAT3 signaling reduced expression of Runx2, Ki67, granzyme B, Eomes and KLRG1, consistent with the published literature (303, 319-321). By knocking out the STING pathway, Sca-1, Runx2, Ki67, granzyme B, and Eomes levels were also reduced. Abrogation of IL-27 signaling reduced the expression of granzyme B and Eomes without significantly impacting expression of other ThEO phenotype markers. Lastly, intact IFNα signaling appeared necessary for proper Sca-1 expression, as well as expression of Ki67, granzyme B, Eomes, and to a lesser extent KLRG1.

Together, this data suggests a dependence on STAT1 and STAT3 signaling, potentially through type I interferons and IL-27, for the proper polarization of the ThEO phenotype. Further, both STAT1 and STAT3 appeared to have differential roles in ThEO polarization. STAT1 predominantly appeared to control Sca-1 and granzyme B expression, whereas STAT3 appeared to regulate Eomes, KLRG1, and Runx2 expression.
Figure 19. Signaling pathways necessary for efficient ThEO polarization. Mice were challenged s.c. with 7.5×10^4 B16-Ova tumor cells before administration of three rounds (D3,6,9) of tumor vaccine (FVAX) plus indicated immunotherapy. Mice were then bled on D10 and cells were analyzed for conversion toward the ThEO/TcEO T cell phenotype. (a) Frequency of Eomes^hi^KLRG1^+^ ThEO phenotype (top) or TcEO phenotype (bottom) cells within the blood of α4-1BB treated WT or various conditional or total knockout mice as indicated. (b) Mean fluorescence intensity of various phenotypic markers within WT or knockout CD8 T cells 10 days post tumor challenge. FACS plots in a are representative of n= 5-15 mice from 3 independent experiments.
5.3 Discussion

The STAT transcription factors critically regulate T cell phenotypic and functional capacity (150). Signaling through the six STAT family members aids in the polarization of unique T cell subsets giving T cells the plasticity to adapt to environmental conditions and clear pathogen infection. Interferon gamma secreted by STAT4 driven Th1 polarized T cells is necessary for adequate anti-tumor responses leading to tumor clearance, and thus Th1 polarized T cells remain the predominant anti-tumor phenotype. I have recently demonstrated that administration of 4-1BB agonist antibodies induces a potently cytotoxic Th1-like T cell phenotype characterized by expression of both Eomesodermin and KLRG1 (TcEO/ThEO). In addition, we have demonstrated that signaling through several cytokine pathways (IL10, IL15, IL27) is critical for the formation of the ThEO phenotype (87). Results presented here in Chapter 5 have further uncovered the cytokines and STAT signaling pathways necessary for efficient ThEO polarization.

I demonstrated that the IL-27 and interferon alpha signaling pathways help to regulate ThEO T cell polarization. As early as ten days after antigen challenge, abrogation of IL-27 signaling (Ebi3KO) induced a modest decrease in the ThEO and TcEO cell populations in the blood of α4-1BB treated mice, while increasing a population of Eomes<sup>-lo</sup>KLRG1<sup>+</sup> T cells. This effect most likely stems from a defect in Eomes expression brought on by impaired IL-27 signaling (322). I also demonstrated that impaired IL-27 signaling blunted granzyme B expression. Whether this is a direct effect of IL-27 or is a function of impaired Eomes expression is unclear. I further provided evidence that type I interferons play a role in TcEO/ThEO polarization. Abolishing the interferon alpha signaling pathway in IFNαR knockout mice reduced the frequency of TcEO phenotype cells in the blood by approximately 3-fold. Interferon alpha (and potentially interferon beta) are critical regulators of Sca-1 and Eomes expression (314, 323), in agreement with my data demonstrating a role in ThEO
polarization. IFNα signals predominantly through STAT1, and IL-27 through STAT3 (324-326). T cell intrinsic abrogation of the STAT1 and STAT3 pathways significantly reduced ThEO populations in the blood by approximately 4-fold, suggesting that these two pathways, potentially through IFNα and IL-27, regulate ThEO polarization. Interestingly, while impaired STAT3 responses dysregulated “canonical” ThEO phenotypic markers (i.e. Eomes, KLRG1, Granzyme B, Ki67), STAT1 ablation impacted ThEO development without significantly impacting specific ThEO markers. This suggests that STAT1 may act instead in coordinately co-regulating multiple genes involved in ThEO polarization, through an unknown pathway. Further, STAT1 ablation did significantly decrease Sca-1 expression, suggesting that Sca-1 may play a role early on in ThEO cell polarization or maintenance. I further demonstrated a role for the stimulator of interferon genes (STING) pathway in mediating ThEO cell polarization. Global abrogation of the STING pathway (STINGKO) significantly reduced the frequency of the ThEO population in the blood of treated mice by reducing expression of Eomes and KLRG1 as well as Sca-1. STING has defined roles in IFNα induction (327), as well as IL15 presentation (316), a critical regulator of ThEO polarization (87). Whether STING is critical in activating one or both of these pathways for proper ThEO polarization remains to be seen. Further, STING is traditionally thought of as an activator of myeloid function; however, murine STING is present in both myeloid and lymphoid compartments (328, 329). Whether STING mediates T cell intrinsic functions in ThEO development is, as of yet, are unknown.

Data presented here accounts for a role of STAT1 and STAT3 in ThEO development, however, a role for other STAT family members or non-canonical STAT signaling in ThEO development has not been addressed. For instance interferon signaling can induce STAT1 homodimerization as well as STAT1:STAT2 heterodimers and to a lesser degree can also induce STAT3 and STAT4 (330), while STAT1:STAT4 and STAT1:STAT3 heterodimers have also been reported (331-333). The impact of
STAT heterodimerization has yet to be validated in the induction of a ThEO response and remains an area of future interest.

I also demonstrated that ablation of these signaling pathways, particularly STAT3 and STING, reduced expression of the Runt-related transcription factor Runx2. Runx2 transcriptional activity is required for bone formation (334), and only recently has a role for Runx2 been elucidated in the function of mature T cells (Mbofung et al., unpublished). Over-expression Runx2 induces candidate ThEO genes (Eomes, KLRG1, Granzyme-A,-B,-K) which drives cytotoxicity and T cell apoptosis. I have demonstrated that, in fact, administration of α4-1BB therapy induces Runx2 expression, particularly in ThEO and TcEO phenotype cells. This effect appears to be an effect of antigen exposure, as therapeutic administration of other TNF receptor family members were also able to induce Runx2 expression despite an inability to polarize ThEO phenotype cells. I further demonstrated that ThEO phenotype cells exhibit high Runx2 expression, only slightly lower than that of terminally differentiated Eomes*KLRG1+ cells, supporting my findings in Chapter 4 that ThEO cells may be more closely related to terminal effectors and in fact stem from this population. The precise effects of Runx2 ablation on ThEO polarization have yet to be determined; however, Runx2 may act as a critical factor regulating ThEO cell cytotoxicity, potentially regulated by STAT3 and/or STING activation.
Chapter 6. *Therapeutic potential of the ThEO phenotype*

The potent anti-tumor potential of 4-1BB targeted therapy is well documented, as are the complications evidenced during the course of therapy (1, 128). How 4-1BB co-stimulation mediates these effects, and the contribution of the ThEO phenotype towards the anti-tumor efficacy or pathology associated with α4-1BB therapy remains largely unexplored. In this chapter, I explore the clinical potential of the ThEO phenotype by analyzing the therapeutic, pathologic, and prognostic potential that ThEO development affords to the anti-tumor immune response. In Section 1, I demonstrate a potent synergy between an intranasal HPV peptide vaccination strategy when combined with α4-1BB therapy and further show the ability of vaccination to hone and augment the ThEO phenotype. In Section 2, I provide data suggesting a role for myeloid activation and IL-27 production in the pathogenesis of α4-1BB induced liver toxicity. Finally, in Section 3 of this chapter, I provide evidence for development of a ThEO-like phenotype in melanoma patients treated with α4-1BB therapy (Urelumab) and a potential correlation between induction of a ThEO phenotype and clinical outcome.
6.1: 4-1BB agonist therapy in combination with HPV peptide vaccination

* Figures and results reported in this section have been previously published in “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112”

6.1.1 Introduction

“Cervical cancer is the second most common malignancy in women worldwide and continues to cause significant morbidity and mortality in the developing world, where screening and prevention programs remain rudimentary (335). The E6 and E7 oncoproteins of human papilloma virus (HPV) drive cervical cancer formation and are critical for maintenance of the transformed state (336). Beyond cervical cancer, HPV infection underlies 40% or greater of cases of oropharyngeal, anal, penile, vaginal, and vulvar cancers (337).

The immunologically foreign nature of the HPV E6 and E7 proteins, coupled with their critical role in maintaining the oncogenic state, makes them ideal target antigens for therapeutic cancer vaccination. Whereas peptide-, protein-, viral- and DNA-based vaccines targeting E6 and E7 have been studied both pre-clinically and in clinical trials, most fail to induce regression of established HPV+ tumors (338). To some degree, all of these vaccines succeed in eliciting peripheral E6/E7-specific T-cell responses; however, not all are proven to generate T cells capable of trafficking to the genital mucosa where cervical cancer develops. Whereas a number of these vaccines extend survival, few can induce regression of established cervical cancer, suggesting that the T cells they elicit lack the capacity to overcome the suppressive tumor microenvironment and eradicate bulky disease (339).

Within the tumor microenvironment, tumor and stromal cells engage coinhibitory receptors on T cells to attenuate potentially tumoricidal immunity (340). We have previously shown that combination blockade of two of these T-cell checkpoint receptors, CTLA-4 and PD-1, promotes synergistic tumor rejection of murine melanoma (59). In a
recently completed phase I clinical trial, more than 50% of metastatic melanoma patients receiving this combination therapy achieved objective clinical responses with the majority experiencing more than 85% reduction in tumor burden (341). Activation of T-cell costimulatory receptors using agonist antibodies, alone or in combination with checkpoint blockade, can also extend T-cell survival and effector function within the microenvironment and promote tumor rejection (71). Among costimulatory receptors, we have found that the tumor necrosis factor receptor superfamily member 4-1BB has a unique capacity to activate both T cells and antigen presenting cells (APCs), which fosters generation of an exquisitely cytotoxic T-cell phenotype termed ThEO/TcEO (T helper/cytotoxic Eomesodermin) (87). We hypothesized that through antibody modulation of T-cell costimulation, the E6/E7-specific T cells generated by therapeutic HPV vaccination could be empowered to expand in magnitude, infiltrate the tumor, and maintain effector function in the suppressive microenvironment.

Previously, we identified 15–19 amino acid peptides derived from HPV-16 E6 and E7, which were the target of measurable T-cell responses in 19/22 patients who remained recurrence-free following treatment for high grade cervical intraepithelial neoplasia (CIN) versus in 0/10 patients who relapsed (342). Subsequently, we found these peptides are also presented on the C57BL/6 mouse background. We have formulated these peptides and the adjuvant alpha-galactosylceramide (αGalCer) into an intranasal (i.n.) vaccine, which evokes both mucosal and systemic E6/E7 T-cell responses (261). In the syngeneic C57BL/6 HPV E6/E7-positive TC-1 tumor model, this vaccine alone significantly delayed the growth of 6-d established tumors and extended survival by more than 20 d. Addition of 4-1BB agonist antibodies, but not blockade of either CTLA-4 or PD-1, converted this therapeutic E6/E7 peptide vaccine into a curative therapy capable of inducing tumor regression, and complete elimination of the majority of s.c. implanted and all vaginally implanted TC-1 tumors. Multiple mechanisms underlie this therapeutic cooperativity including profound enhancement of tumor infiltration by antigen-specific effector T cells.
and generation of highly cytotoxic ThEO/TcEO phenotype T cells. These observations suggest an optimal T-cell costimulatory antibody partner for therapeutic HPV vaccination capable of converting weakly therapeutic responses into potentially curative ones. Further, the therapeutic and mechanistic insights gained may illuminate future studies seeking to combine other therapeutic vaccines for cancer and infectious disease with T-cell co-stimulatory modulation.
6.1.2 Results

6.1.2.1 Intranasal vaccination with HPV E6/E7 peptides in combination with αGalCer slows the growth of preimplanted HPV+ tumors.

HPV E6/E7-driven TC-1 tumors are an established murine model of cervical cancer(343). To test the efficacy of i.n. vaccination with the E6/E7 peptides identified in our prior study coupled with αGalCer adjuvant, we s.c. implanted 2 × 10^5 TC-1 cells and waited 6 d for tumors to establish before beginning vaccine administration(342). Mice were vaccinated on days 6, 12, 24, and 32 and followed for tumor growth and survival over 45 d. Compared with untreated animals, αGalCer adjuvant or E6/E7 peptides alone failed to extend median survival as has previously been reported (Fig. 20A) (344, 345); however, the intranasal vaccination of peptides in combination with αGalCer significantly delayed outgrowth of TC-1 tumors and extended overall survival in this system (Fig. 20 B and C).
Figure 20. Intranasal vaccination with HPV E6/E7 peptides in combination with α-galactosylceramide (αGalCer) slows the growth of preimplanted HPV+ TC-1 tumors. Mice were challenged s.c. with $2 \times 10^5$ TC-1 tumor cells and immunized via the intranasal route with either HPV peptides and αGalCer (vaccine), HPV peptides alone, αGalCer alone, or PBS on days 6, 12, 24, and 32 postimplantation (vertical arrows). The survival (A) and tumor growth (B) of the mice were monitored over time. (A) Survival curves of tumor-bearing mice given different therapeutic treatments. Significance in survival proportions was measured using the log-rank test. $P < 0.05$. (B) The average tumor size is shown as mean area ± SD for each group of mice. Statistical analyses between different groups were performed using Student’s t test between different treatments and the different levels of significance are indicated by * ($P \leq 0.05$). (C) Tumor progression of s.c.-implanted TC-1 tumors in mice given the indicated treatment. Each line indicates an individual mouse. Experiments were performed on four to six mice per group (33). “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112” This figure was generated with the assistance of Drs. Shailbala Singh and Guojun Yang.
6.1.2.2 Intranasal Vaccination in Combination with Systemic Anti-4-1BB Immunotherapy Promotes Regression of Established s.c. HPV+ Tumors.

To evaluate whether the therapeutic efficacy of the HPV peptide vaccine could be enhanced with coadministration of immunotherapeutic antibodies, we combined the intranasal administration of HPV peptide/αGalCer vaccine with i.p. administration of either immune checkpoint blocking (αCTLA and αPD-1) or costimulatory TNF-receptor agonist (α4-1BB) antibodies. We implanted $2 \times 10^5$ TC-1 cells s.c. on the right flank and vaccinated mice with E6/E7 peptide + αGalCer i.n. on days 5 and 11 and the indicated antibody i.p. on days 5, 8, and 11. Antibody therapy alone modestly extended survival relative to untreated animals; however, these differences did not reach statistical significance (Fig. 21A). Whereas αCTLA-4 and α4-1BB monotherapy was able to delay tumor growth in some animals, PD-1 blockade was largely ineffective (Fig. 21B).

Significant therapeutic responses, however, were observed when checkpoint modulating antibodies were administered in combination with the HPV peptide vaccine. Unique among the therapies tested, the combination of 4-1BB agonist antibodies and vaccination induced tumor regressions in all animals with 5/8 surviving tumor free and 3/8 relapsing (Fig. 21B). The three relapsing vaccine + α4-1BB–treated mice were killed at day 38 and RT-PCR analysis confirmed that their relapses were not due to loss of E6 or E7 expression, which are essential for maintenance of the transformed state in human cervical cancer (Fig. 22) (346). CTLA-4 blockade with vaccination promoted tumor regression in 2/10 mice; however, none of these regressions were durable. Surprisingly, PD-1 blockade failed to significantly improve the efficacy of this vaccine. Of the other TNF-receptor agonist antibodies tested with the peptide vaccine, αOX-40 and αCD40 produced outcomes similar to αCTLA-4, whereas responses to GITR agonist antibodies resembled those to αPD-1 (Fig. 23). Aside from α4-1BB, however, no other checkpoint modulating antibody tested proved capable of eliciting any durable TC-1 tumor
regression. Together, these data reveal a unique and unexpected potential for intranasal vaccination with HPV E6/E7 peptides in combination with α4-1BB immunotherapy to promote complete and sustained immune rejection of established HPV+ tumors. We further validated the durability of the immune response generated by vaccine and α4-1BB combination therapy by rechallenging the mice that had achieved complete tumor regression with an additional 2 × 10⁵TC-1 cells at 3 wk posttumor regression. All of these animals remained tumor-free for 60 d postrechallenge and maintained detectable, E7-specific CD8 T-cell memory responses (Fig. 21C).

To uncover the relative contribution of the T-cell response in mediating regression in mice administered the vaccine in combination with α4-1BB, animals were challenged s.c. with 2.5 × 10⁵ TC-1 tumors and depleted of either CD8 or CD4 T cells starting 1 d before challenge and concurrently with α-41BB immunotherapy. Depletion of CD4 T cells had little effect on the ability of 4-1BB stimulation to boost the antitumor effect of the peptide vaccine. In line with recently published literature (347), depletion of CD8 T cells, however, completely abrogated any therapeutic benefit imparted by the dual therapies (Fig. 21D). This loss of tumor control following CD8 depletion suggested a critical role for cytotoxic antitumor T-cell responses in mediating regression of HPV+ tumors that we wished to further examine.
Figure 21. Combination therapy with an HPV E6/E7 peptide vaccine and T-cell costimulatory modulating antibodies. Mice were challenged s.c. with $2 \times 10^5$ TC-1 tumor cells before being immunized intranasally twice (days 5 and 11 posttumor challenge) with the HPV peptide vaccine (HPV peptides and αGalCer) and αCTLA-4, αPD-1, or α4-1BB (days 5, 8, and 11 posttumor challenge). Control animals received PBS, vaccine, or monotherapy alone. Tumor growth (measured in square millimeters) and animal survival were monitored over time. (A) Survival curves of tumor-bearing mice receiving the indicated treatment. (B) Tumor progression in mice receiving various monotherapies or in combination with the HPV peptide vaccine. (C) Three weeks post–complete-tumor regression, mice ($n = 3$) treated with vaccine in combination with α4-1BB monotherapy (from B) were rechallenged with $2 \times 10^5$ TC-1 cells. Tumor growth is presented as an average area ± SD for untreated ($n = 5$), vaccine-treated ($n = 5$), and vaccine + α4-1BB monotherapy-treated ($n = 3$) animals. Mice were monitored for 60 d post-rechallenge for evidence of tumor growth. The persistence of E7 antigen-specific CD8$^+$ T lymphocytes was determined in the peripheral blood mononuclear cells of mice receiving the vaccine either alone (the three longest-surviving vaccine-alone animals were used) or with α4-1BB monotherapy by staining with fluorescently labeled E7$^{49-57}/K^b$ tetramer and antibodies to CD44 and CD8, and expressed as a percentage of CD8$^+$ T lymphocytes from different time points (Inset). (D) In a different set of experiments, mice treated i.n. with peptide vaccine with i.p. α4-1BB were depleted of CD8 or CD4 T cells 1 d pre- and 1 d post-tumor challenge ($2.5 \times 10^5$ TC-1 s.c.). Depletion was maintained every 3 d until mice were killed. Tumor growth was measured in each group and the average tumor area for each group was plotted over time ($n = 5$ mice per group). Data in B were pooled from two independent experiments ($n = 5$–10 mice per group). Significance in survival proportions in A was determined using a Mantel–Cox test ($P < 0.001$). Each line in B represents an individual mouse. The average tumor growth in B is shown as mean area ± SD. Circles represent mean ± SD. Statistical significance in D was calculated using
a Student’s $T$ test to compare the vaccine + α4-1BB group to the CD8-depleted group. ns, not significant; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$(33). "Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112" This figure was generated with the assistance of Drs. Shailbala Singh and Guojun Yang.
Figure 22. Tumor relapse in vaccine and α4-1BB–treated mice is not due to E6/E7 antigen loss. Tumors were isolated from mice relapsing after initial response to combo therapy (progressor) or in the process of marked regression, suggesting a likely complete response (regressor). Tumor RNA was purified using Trireagent (Sigma), reverse transcribed into DNA using the SuperScript II reverse transcriptase kit (Life Technologies), and then PCR amplified for the full-length E6 and E7 reading frames using HotStar Taq Mastermix (Qiagen). Separate gels were run for E6 and E7 and amplified DNA was visualized using ethidium bromide staining (33). “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112” This figure was generated with the assistance of Dhwani Haria.
Figure 23.

A. Vaccine + αCD40

B. Vaccine + αOX40

C. Vaccine + αGITR
Figure 23. HPV E6/E7 peptide vaccine in combination with TNF-receptor agonist antibody therapy. Mice were s.c. challenged with $2 \times 10^5$ TC-1 tumor cells and immunized intranasally twice (days 5 and 11 posttumor challenge) with the HPV peptide vaccine (HPV peptides and αGalCer) in addition to (A) αCD-40 (FGK4.5), (B) αOX-40 (OX-86), or (C) αGITR (DTA-1) agonist antibodies (days 5, 8, and 11 posttumor challenge). Tumor growth (expressed in square millimeters) was monitored over time. Each line represents an individual mouse ($n = 5$ mice per group). Two tumor-bearing animals in $B$ were killed before day 30 for nontumor, nontreatment-related causes at the request of the facility veterinarians (33). “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112” This figure was generated with the assistance of Drs. Shailbala Singh and Guojun Yang.
6.1.2.3 Vaccination and α4-1BB therapy promotes high-density tumor infiltration by cytotoxic CD8 T cells.

To further explore the mechanisms driving the synergy between intranasal vaccination and α4-1BB immunotherapy, we vaccinated mice with pre-established TC-1 tumors and administered immunotherapy, harvested tumors on day 16, and analyzed tumor infiltrating lymphocytes (TILs) by flow cytometry. We have previously shown that improved intratumoral ratios of effector T cells relative to suppressive CD4+ FoxP3+ regulatory T cells (Tregs) correlate with greater therapeutic efficacy for T-cell immune checkpoint modulation (59). Combination of the peptide vaccine with α4-1BB therapy synergized in promoting proinflammatory ratios of CD8 versus Tregs within TC-1 tumors (17:1) relative to 4-1BB antibody alone (2.8:1) or vaccine alone (6.5:1; Fig. 24A). The combination of peptide vaccine with CTLA-4 blockade also resulted in elevated CD8 to Treg ratios (10:1), but not to the level achieved with the 4-1BB agonist in combination with peptide vaccine. Relative to vaccine alone, the 4-1BB combination resulted in significantly elevated CD8 frequencies coupled with profoundly diminished Treg frequencies. Whereas the absolute density of Tregs in the tumor did not decrease, the numbers of effector CD8 T cells increased so dramatically that the representation of Tregs as a fraction of TILs fell (Fig. 25C).

Beyond its efficiency at evoking overall tumor infiltration by CD8 T cells, this therapy also increased the fraction of those CD8 T cells composed of HPV E7-specific CTL (Fig. 24B and Fig. 25A). In fact, intranasal vaccination in combination with α4-1BB, but not αCTLA-4, greatly increased the density of antigen-specific CD8 T cells within the tumor microenvironment to over 160 CD8 T cells per square millimeter of tumor (Fig. 24B and Fig. 25C). From a functional standpoint, the fraction of antigen-specific CD8 T cells expressing granzyme B also increased with 4-1BB agonist combination therapy (Fig. 24B and Fig. 25B). Underlying these advantageous CD8-to-Treg ratios, we found
that co-administration of α4-1BB and the vaccine drove the highest frequency of both effector CD8 and CD4 T-cell proliferation in the tumor without augmenting Treg expansion compared with the vaccine alone (Fig. 24C).

The vaccine and α4-1BB combination also modestly increased CD4 effector T-cell proliferation resulting in elevated effector-to-Treg ratios in the combination (3:1) versus either vaccine (0.8:1) or antibody (2:1) alone (Fig. 24C and Fig. 25D). The capacity of CTLA-4 antibodies to deplete Tregs from the microenvironment while expanding CD4 effectors promoted the highest CD4 effector-to-Treg ratios (5:1) in combination with the vaccine (Fig. 25D and E) (348, 349). We found that both vaccine alone and the combination drove T cells to robustly infiltrate the tumor tissue itself, whereas in untreated tumors, CD8 T cells were scant and often appeared contained within, or tethered to blood vessels (Fig. 25E). Taken together these data suggest that the impressive therapeutic responses evidenced during α4-1BB therapy are due, in part, to robust vaccine-induced antigen-specific CD8 T-cell infiltration into the tumor microenvironment, absent a proportional increase in the suppressive Treg population.
**Figure 24. Immune correlates of protection.** Antitumor T-cell responses in the tumor microenvironment of mice treated with vaccine, αCTLA-4, α4-1BB monotherapy, or in combination were characterized by flow cytometry. (A) The CD8/Treg ratio was calculated by dividing the total number of CD8+ CTLs infiltrating the tumor by the total number of CD4+Foxp3+ T-cell infiltrate. Percent of CD8+ T-cell infiltrate was calculated as a percent of total CD3+ T-cell infiltrate. The percent of Treg infiltrate was calculated as a percent of total CD4+ T cells in the tumor fraction. (B) Infiltration (*Left* and *Center*) and cytotoxic effector function (*Right*) of E7-specific CD8 T cells in the tumor infiltrate was also determined by staining lymphocytes with fluorescently labeled E749-57/Kb tetramer and antibodies to CD8 and granzyme B. Data are expressed as a percentage of CD8+ T cells (*Left*), a population of CD8 T cells in the tumor (*Middle*), or as a percentage of antigen-specific CD8+ T cells expressing granzyme B (*Right*). (C) T-cell proliferation (as indicated by percent of cells expressing Ki67) and (D) ICOS expression was also determined for CD8, CD4, Teff, and CD4 Treg cells in the tumor microenvironment and is represented as percent of T cells expressing ICOS and fold increase in %ICOS-positive cells over untreated mice. Circles represent individual mice (3–10 mice per group from two experiments). Bars represent mean ± SD. Statistical significance was calculated using a one-way ANOVA. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001(33). “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112” This figure was generated with the assistance of Drs. Shailbala Singh and Guojun Yang.
Figure 25.
Figure 25. Gating strategy and representative FACS analysis of tumor infiltrating T-cell populations. (A) Representative gating strategy to analyze CD8⁺, CD4 Teff, and CD4 Treg populations in the TILs as well as tetramer-specific CD8 responses. (B) Representative gating strategy for analysis of cytotoxic antigen-specific CD8 T-cell responses in TILs. (C) Quantitation of CD8⁺ T-cell density (Left) CD4 Teff density (Middle) and Treg density (Right) in the tumor microenvironment. (D) Quantitation of CD4 Teff/Treg ratio in the tumor microenvironment calculated by dividing the total number of CD4⁺Foxp3⁻ T cells by the total number of CD4⁺Foxp3⁺ T cells recovered from the TIL fraction. Experiments were performed in duplicate with at least three mice per group. Bars in C and D represent mean ± SD. Statistical significance was calculated using a one-way ANOVA. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (E) TC-1 tumors were treated as indicated, excised at day 16, frozen in OCT, sectioned, and stained for CD8 (Alexa 488), CD4 (V450), FoxP3 (eFluor 570), and CD31 (Alexa 647). Images were acquired using a TCS SP8 laser scanning confocal microscope (Leica) equipped with lasers for fluorescence excitation at 405, 442, 458, 488, 514, 561, and 633 nm. Images were analyzed using ImageJ image analysis software (33). “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112” This figure was generated with the assistance of Drs. Shailbala Singh and Guojun Yang and Midan Ai.
6.1.2.4 Intranasal HPV peptide vaccination generates Inducible T-Cell Costimulator-expressing CD4 and CD8 T cells, which infiltrate HPV\(^+\) tumors.

We next investigated the phenotypic characteristics of T cells, which may account for the enhanced qualitative and quantitative antitumor effector responses. Whereas expression of the checkpoint receptor CTLA-4 was largely unaltered during the course of treatment, PD-1 expression was significantly elevated in CD8 T cells during the course of vaccination therapy (Fig. 26 A and B). We also observed an unexpected increase in the frequency of inducible T-cell costimulator (ICOS\(^+\)) T cells infiltrating the tumor environment (Fig. 24D). Administration of peptide vaccine alone increased the frequency of ICOS\(^+\) CD8 T cells greater than fourfold relative to untreated mice. In accordance with recent publications, αCTLA-4 therapy also increased the infiltration of tumors by ICOS\(^+\) T cells, although not to a level that reached statistical significance (350, 351). Addition of the peptide vaccine to αCTLA-4 promoted a threefold higher frequency of ICOS\(^+\) CD8 T-cell infiltration (28% versus 9%) relative to antibody monotherapy. Additionally, whereas α4-1BB alone failed to induce ICOS on T cells, administration of α4-1BB in combination with the intranasal vaccine increased the percentage of ICOS\(^+\) CD8 T cells to \(\sim\)20% (Fig. 24D). As observed for CD8 T cells, CD4 T effector cells also expressed ICOS at a higher frequency in mice treated with the intranasal vaccine. In much the same way that αCTLA-4 increased ICOS\(^+\) CD8 T-cell infiltrates alone or in combination with vaccination, ICOS\(^+\) CD4 effector infiltration also increased, though the magnitude of these increases was less than for CD8 T cells. HPV peptide vaccination also drove substantially increased expression of ICOS by Tregs in the tumor microenvironment. Vaccination, alone or in combination with immunotherapy, induced ICOS expression on \(\sim\)80% of Tregs, though the fold increase in ICOS\(^+\) Tregs was greatly minor compared with CD8 or effector CD4 T cells (Fig. 24D). Taken together, our results show that intranasal vaccination induces ICOS expression on tumor infiltrating lymphocytes, suggesting that these T cells have adopted a more robust effector phenotype.
6.1.2.5 Treatment with 4-1BB agonist antibody polarizes tumor-specific T cells to the highly cytotoxic ThEO/TcEO phenotype.

Previously, we and others have described the capacity of 4-1BB agonist antibodies to polarize T cells to a potently cytotoxic T-cell phenotype in both the CD4 (ThEO) and CD8 (TcEO) T-cell pools driven by high expression of the T-box transcription factor eomesodermin and typified by surface expression of the co-inhibitory receptor KLRG1(87, 352). Analysis of mice treated with 4-1BB agonist antibody, with or without i.n. HPV E6/E7 peptide vaccination, revealed that ThEO and TcEO cells are present within the tumor microenvironment (Fig. 27A and Fig. 28A), as well as in the spleen (Fig. 28B) and lymph nodes (LN) (Fig. 28C). Whereas the percentages of ThEO/TcEO cells appear reduced in mice treated with vaccine and α4-1BB compared with α4-1BB alone, the combination treatment dramatically increased the numerical infiltration of CD4 ThEO cells (>17-fold) and CD8 TcEO cells (>70-fold) into TC-1 tumors. Moreover, whereas the ThEO/Treg ratio during vaccine and α4-1BB therapy remained unchanged relative to α4-1BB therapy alone, the TcEO/Treg ratio increased 4-fold with combination therapy (Fig. 27B). Additionally, we confirmed that nearly a quarter of TcEO cells are specific for the dominant E7 peptide incorporated in the vaccine versus <5% with 4-1BB antibody alone (Fig. 27C). These eomesodermin-driven T
cells, which have been shown to kill tumors more efficiently than their Tc1/Th1 counterparts, may acquire even higher levels of effector function through coexpression of ICOS, which has been correlated with increased IFN-γ production and cytotoxicity (Fig. 29B)(87, 350).
Figure 26. Phenotypic analysis of tumor infiltrating lymphocytes. The percent of CTLA-4–expressing (A) and PD-1–expressing (B) CD8 T cells (Top), CD4 T effector cells (Middle), and regulatory T cells (Bottom) within the tumor microenvironment. Experiments were performed in duplicate with at least three mice per group. Bars represent mean ± SD. Statistical significance was calculated using a one-way ANOVA. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (33). “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112.” This figure was generated with the assistance of Drs. Shailbala Singh and Guojun Yang.
Figure 27. Anti–4-1BB agonist antibody therapy induces potent ThEO/TcEO T-cell responses against HPV+ tumors. Induction of a ThEO/TcEO T-cell response in the tumor microenvironment was determined by staining isolated lymphocytes with fluorescently labeled antibodies toward eomesodermin and KLRG1. (A) Percent of total CD4 (Left) and CD8 (Right) effector T-cell populations in TIL composed of Eomes+KLRG1+ ThEO or TcEO T cells. Density of ThEO (Left) and TcEO (Right) T cells expressed as number of cells per square millimeter of tumor. (B) The ThEO/Treg ratio and TcEO/Treg ratio was calculated by dividing the total number of effector CD4+Eomes+KLRG1+ or CD8+Eomes+KLRG1+ cells, respectively, by the total number of CD4+Foxp3+ T cells collected from the TIL fraction. (C) The antigen-specific CD8+ TcEO cells were analyzed by calculating the fraction of CD8+Eomes+KLRG1+ T cells bound to fluorescently labeled E749-57/Kb tetramer. Experiments were performed in duplicate with at least three mice per group. Bars represent mean ± SD. Statistical significance was calculated using a one-way ANOVA (A) or Mann–Whitney U test (B and C). ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001(33). “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112” This figure was generated with the assistance of Drs. Shailbala Singh and Guojun Yang.
Figure 28. Gating strategy and analysis of ThEO/TcEO cells in the spleens and draining lymph nodes of tumor-bearing mice. (A) Representative FACS plots gating on Eomes+KLRG1+ CD4 T cells (Top) or CD8 T cells (Bottom) of mice receiving various immunomodulatory antibody therapies alone or in combination with the HPV peptide vaccine. Quantitation of Eomes+KLRG1+ ThEO (Left) and TcEO (Right) cells in the spleen (B) and draining lymph nodes (C) of tumor-bearing mice. Experiments were performed in duplicate by pooling spleens or lymph nodes from at least three mice per group. Bars represent mean ± SD. Statistical significance was calculated using a one-way ANOVA(33). “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112.” This figure was generated with the assistance of Drs. Shailbala Singh and Guojun Yang.
Figure 29.

(A) TcEO

- %ICOS+ (% of ICOS+ cells)
- Comparison of %ICOS+ between α4-1BB and Vax + α4-1BB

(B) ThEO

- %ICOS+ (% of ICOS+ cells)
- Comparison of %ICOS+ between α4-1BB and Vax + α4-1BB

(B) TcEO

- GzmB MFI (mean Fluo-3 intensity for GzmB)
- Comparison of GzmB MFI between α4-1BB and Vax + α4-1BB

(B) ThEO

- GzmB MFI (mean Fluo-3 intensity for GzmB)
- Comparison of GzmB MFI between α4-1BB and Vax + α4-1BB
Figure 29. Intranasal vaccination with HPV E6/E7 induces infiltration of ICOS<sup>+</sup> ThEO and TcEO cells into the tumor microenvironment. (A) The percent of ICOS expression on Eomes<sup>+</sup>KLRG1<sup>+</sup> TcEO (Left) and ThEO (Right) infiltrating s.c. implanted TC-1 tumors. (B) Cytotoxic potential of ICOS-expressing cells was measured by comparing the mean fluorescence intensity (MFI) of granzyme B in ICOS<sup>+</sup> versus ICOS<sup>-</sup> TcEO or ThEO cells isolated from the TIL fraction. Experiments were performed on at least three mice per group. Bars represent mean ± SD. Statistical significance was calculated using a Student’s *T* test. ns, not significant; *P* < 0.05, **P* < 0.01, ***P* < 0.001, ****P* < 0.0001(33). “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112” This figure was generated with the assistance of Drs. Shailbala Singh and Guojun Yang.
6.1.2.6 Vaccination plus α4-1BB immunotherapy promotes complete regression of intravaginally implanted HPV E6/E7-driven tumors.

To confirm the efficacy of our peptide vaccine in an HPV-genital tumor challenge model, we implanted $2 \times 10^4$ TC-1 tumor cells expressing firefly luciferase (TC-1–Luc) intravaginally (262). Mice received i.n. vaccination on days 5 and 11 with i.p. antibody injections on days 5, 8, and 11, and were imaged for luciferase expression weekly throughout the study to assess tumor growth. In this system, all vaginally implanted TC-1 tumors were cured in mice receiving intranasal vaccination in combination with α4-1BB immunotherapy (Fig. 30A and B). All mice in this group showed only background luciferase signal out to day 60 and had no observable tumor upon dissection. As in the s.c. system, CTLA-4 blockade augmented the therapeutic effect of the vaccine, but to a significantly lesser degree than the 4-1BB agonist antibody curing 2/5 mice (Fig. 30A and B). Within the tumor-bearing female reproductive tracts (FRTs) of these animals, combination therapy with vaccine and α4-1BB evoked the highest level of infiltration by E7-specific CTL (>1.5-fold over vaccine alone) (Fig. 30C). Further, the highly cytotoxic CD4 ThEO and CD8 TcEO cells, which likely support the unique capacity of 4-1BB to promote curative responses in this system, comprised 20% of the tumor-infiltrating CD4 population and ~30% of the CD8 population (Fig. 30D). CD8 T cells isolated from the draining lymphatics of mice receiving the vaccine and α4-1BB combination produced 10- to 20-fold higher levels of the effector cytokines IFN-γ and TNF-α compared with those from mice receiving either vaccine or α4-1BB alone (Fig. 30E). These combination therapy-generated CD8 T cells were also capable of producing more of their own IL-2 than those isolated from mice receiving either monotherapy. CD4 T cells from the nodes also produced IL-2 and low amounts of TNF-α and IFN-γ in response to restimulation with the vaccine peptides (Fig. 31). IFN-γ levels were elevated relative to mice receiving vaccine alone for all animals receiving either αCTLA-4 or α4-1BB. CTLA-4 blockade alone also evoked IL-17 production, which can be tumor
supportive in some contexts (353); however, no mice receiving vaccine and/or α4-1BB exhibited Th17 phenotype T-cell responses. Overall, these results demonstrate that curative immune responses to HPV⁺ tumors implanted in the genital tract closely mimic those observed in the s.c. model with combination therapy.
Figure 30.
Figure 30. Vaccination with HPV E6/E7 peptides in combination with α4-1BB leads to regression of intravaginally implanted tumors. Mice were challenged intravaginally with 2 × 10⁴ TC-1 tumor cells expressing firefly luciferase and then immunized intranasally twice (days 5 and 11) with HPV peptides and αGalCer either alone or in combination with αCTLA-4 or α4-1BB therapy (days 5, 8, and 11). Control animals received either PBS or monotherapy alone. Tumor growth and survival of the mice was monitored over time. (A) Representative images of luciferase⁺ tumor progression over time in mice assigned to different treatment groups. (B) Survival curve (Left) and average tumor growth (Right) as measured by average radiance of luciferase activity of mice treated with peptide vaccine alone or in combination with immunotherapy. Tumor measurements ceased upon the first tumor-related mortality in each group. Survival and tumor growth are from a representative experiment of two independent experiments with three to five mice per group. (C) In a separate experiment, the percent of E7 antigen-specific CD8 T cells infiltrating into vaginal tumors and the associated genital tract was analyzed by isolating TILs and staining with fluorescently labeled E7<sup>49-57</sup>/K<sup>b</sup> tetramer and antibodies to CD8. (D) The percent of ThEO/TcEO cells capable of infiltrating intravaginally implanted TC-1 tumors was measured by gating on Eomes⁺KLRG1⁺ CD4 Teff or CD8 T cells, respectively. (E) CD8 T cells from the draining lymph node were restimulated with E6/E7 peptide-pulsed DCs and their cytokine production measured by cytometric bead array (BD Biosciences). Experiments were performed in duplicate on at least three mice per group except cytokine production, which was performed with quintuplicate wells derived from five pooled LNs per group. Bars represent mean ± SD. Statistical significance was calculated using a one-way ANOVA. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (33). “Bartkowiak et al. Unique
potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112” This figure was generated with the assistance of Drs. Shailbala Singh and Guojun Yang.
Figure 31. Cytokine production from restimulated CD4 T cells isolated from female reproductive tract implanted TC-1 tumor-draining lymph nodes. CD4 T cells from the draining lymph node were restimulated with E6/E7 peptide-pulsed DCs and their cytokine production was measured by cytometric bead array (BD Biosciences). Experiments were performed with quintuplicate wells derived from five pooled LNs per group. Bars represent mean ± SD. Statistical significance was calculated using a one-way ANOVA. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001(33). “Bartkowiak et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine. Proc Natl Acad Sci USA. 2015 Sep 22; 112(38):E5290-9. doi: 10.1073/pnas.1514418112” This figure was generated with the assistance of Drs. Shailbala Singh and Guojun Yang.
6.1.3. Discussion

Whereas many therapeutic cancer vaccines have proven capable of slowing growth of established tumors, few, if any, can fully overcome the suppressive tumor microenvironment and achieve durable regressions. Here we show that an intranasal HPV E6/E7 peptide vaccine formulated with αGalCer as an adjuvant can promote durable regression of HPV+ TC-1 tumors residing in the skin or in the female reproductive tract when combined with systemic administration of 4-1BB agonist antibodies. Neither antibodies blocking the T-cell immune checkpoint receptors CTLA-4 or PD-1, nor agonist antibodies targeting the TNF receptor family members OX-40, CD40, and GITR could recapitulate the effect of 4-1BB activation in this setting. Reflecting the therapeutic outcome, we found the highest CD8-to-Treg ratios in animals treated with vaccine in combination with 4-1BB agonists. The density of HPV E7-specific T cells in the tumors of these mice was 10-fold greater in animals receiving the vaccine with α4-1BB versus αCTLA-4. The capacity of 4-1BB agonist antibodies to foster such dramatic expansion of the vaccine-induced, tumor-specific CD8 T cells, which efficiently infiltrate and proliferate within these HPV+ tumors, partially explains the ability to drive regression of these tumors in a setting where other types of checkpoint modulation did not.

Previously, we described a potently cytotoxic T-cell phenotype termed ThEO/TcEO to which tumor-infiltrating effector T cells are polarized following treatment with 4-1BB agonist antibodies (87). In combination with the HPV E6/E7 peptide vaccination, 4-1BB agonist antibody treatment promoted accumulation of >17-fold higher densities of ThEO CD4 and >70-fold higher densities of TcEO CD8 T cells within these HPV+ tumors relative to all other therapies tested. Tumors are known to express serine protease inhibitors, which blunt the efficacy of the primary CD8 T-cell lytic effector molecules granzymes A and B; however, TcEO-polarized CD8 cells express high levels of additional granzymes normally limited to natural killer (NK) cells, including granzymes E, D, G, and K, which may account for their heightened killing potential (354). We also observed an unexpected capacity of the vaccine to drive up-regulation of ICOS on T cells,
which then infiltrate TC-1 tumors. Whereas CTLA-4 and PD-1 blockade are known to promote ICOS up-regulation, 4-1BB agonist does not (59). Given that ICOS expression has been associated with enhanced T-cell effector function and prolonged survival, we hypothesize that the ICOS+ ThEO and TcEO T cells formed by the combination of vaccination and α4-1BB may have an unparalleled potential to eliminate established tumors (350, 351). Our data suggest that either direct or indirect action of the αGalCer adjuvant at the vaccine site must contribute to ICOS induction as peptides alone or α4-1BB antibody alone have not been reported to have this effect. Among the adjuvant effects of αGalCer, we find that it increases 4-1BB expression by NK T cells raising the possibility of synergy between the antibody and the vaccine during T-cell priming. Both the mechanism of ICOS induction by this vaccine and the details of its subsequent effect on ThEO phenotype T cells will be the focus of future studies.

As most HPV-driven malignancies occur in mucosal tissues, we sought to augment the efficiency of generation of T-cell responses programmed to traffic to and enter these tumor sites by using a mucosally-focused intranasal vaccination strategy consisting of HPV E6 and E7 peptides coupled with the NK T-cell adjuvant αGalCer. In this context, we find that, of a battery of clinically relevant T-cell modulating antibodies, only 4-1BB agonist treatment was capable of converting this vaccine into a curative therapy. Whereas we have yet to experimentally address the breadth of these findings across diverse antigen types, adjuvants, and routes of delivery, recent literature suggests that 4-1BB activation may engage uniquely advantageous biologic pathways for augmenting therapeutic vaccination. Using recombinant 4-1BB ligand and a TLR4 agonist as adjuvant delivered by s.c. injection, Srivastava et al. demonstrated profound enhancement of both therapeutic HPV E7 protein vaccines against cervical cancer, as well as of a survivin-based vaccine targeting lung adenocarcinoma (355).

We expected that PD-1–mediated T-cell suppression might play a role in limiting the efficacy of the vaccine; however, we observed no significant ability of PD-1 blockade to augment vaccine efficacy. The most effective antibodies in our studies were those capable of augmenting T-cell priming and/or amplifying T-cell proliferation either through relieving repression or directly
stimulating division. The blockade of PD-1, in contrast, functions primarily through restoration of T-cell effector function and induction of metabolic programs, which increase fitness in the tumor microenvironment (32). The capacity of PD-L1 blockade to augment HPV peptide vaccine responses against TC-1 tumors, although not to a curative degree, has been reported previously by Badou et al. (356). Differences in the T cells generated by vaccination at disparate sites could explain the differences in our findings, as their vaccine was injected into the peritoneum rather than delivered via the nasal mucosa. In terms of their capacity to augment numbers of CD8 T cells with high level effector function, α4-1BB is generally more potent than αCTLA-4, which is generally more potent than αPD-1. In this setting, this suggests that, in the context of vaccination, CD8 numbers are the most limiting factor. PD-1 blockade may have been capable of restoring effector function to exhausted cells, but too few CD8 cells were present, even with blockade, to effect regressions. Similarly, CTLA-4 blockade excels at augmenting effector CD4s and depressing Treg numbers and function, but those benefits appear to be less impactful in this setting compared with CD8 expansion. Human tumors develop over much longer time spans compared with murine transplantable cancer cell lines such as TC-1; however it is possible that in the context of years of tumor development with extended chronic antigen exposure, PD-1 blockade might be more efficacious.

Multiple therapeutic vaccines targeting HPV-driven malignancies, including peptide-based vaccines, are advancing through clinical trials (357). Our preclinical findings from both s.c. and vaginally implanted HPV+ tumors suggest that the efficacy of these ongoing clinical studies could be profoundly enhanced through combination with 4-1BB (CD137) agonist antibodies. Beyond the impact on cervical cancer and other HPV-driven malignancies, these data may also provide insight for designing effective combination vaccine and checkpoint blockade trials for non-HPV cancers.”(33)
6.2: A potential role for ThEO phenotype cells in 4-1BB agonist induced liver pathology

6.2.1 Introduction

The transformative efficacy of checkpoint blockade immunotherapy for the treatment of melanoma has revolutionized the field of oncology and initiated a new era of immune-targeted therapeutics (340, 358). Beyond blockade of T cell co-inhibitory receptors, agonist antibodies which activate tumor necrosis factor superfamily receptors have demonstrated significant therapeutic potential both in pre-clinical models and clinical trials (359). Among these agonists, activators of the co-stimulatory receptor 4-1BB (CD137) have demonstrated exceptional potency across multiple pre-clinical tumor models, as well as the capacity to elicit objective clinical responses in patients with diverse cancers (1, 360).

In addition to mediating tumor regressions, releasing the “brakes” on T cell responses with checkpoint blockade can also trigger T cell responses targeting normal self-tissues known as Immune Related Adverse Events (IRAE). These IRAE can be severe and even life-threatening, but are readily managed with timely steroid intervention (361). 4-1BB agonist antibodies, by contrast, can effectively treat autoimmunity in a variety of murine models and may even ameliorate CTLA-4 antagonist antibody induced IRAE (130, 362). Despite this, these agents induce a unique spectrum of on-target adverse events ranging from mild to moderate hematologic perturbations, up to high grade transaminitis and potentially fatal hepatotoxicity (363, 364).

I sought to elucidate the underlying mechanisms by which α4-1BB antibody therapy promotes liver damage, as well as to explore potential avenues to uncouple augmentation of anti-tumor immunity from hepatitis. Results presented here demonstrate that 4-1BB agonist-induced hepatotoxicity initiates at the myeloid level through activation of liver-resident Kupffer cells. Moreover, I find that the inflammatory cytokine interleukin 27 (IL-27), released from these cells in response to activation, is critically required for hepatic damage. I further show that, in contrast to CD40 agonist induced acute hepatotoxicity, 4-1BB agonist antibody therapy induces
a chronic hepatotoxicity characterized by dense and persistent T cell infiltration in the hepatic portal zones. This infiltrate is dominated by CD8+ cytotoxic T cells which are the primary effectors of liver tissue injury. CD4+Foxp3+ regulatory T cells (Treg), on the other hand, act to maintain tissue tolerance and limit α4-1BB-induced hepatic damage. Treg ablation severely exacerbates 4-1BB agonist liver inflammation and abrogates the capacity of CTLA-4 blockade to ameliorate transaminitis. Finally, I show that chemotaxis of immune cells into the liver is a critical step in the progression of liver injury. While hepatogenic immune responses following 4-1BB agonist therapy rely heavily on the chemokine receptors CCR2 and, to a lesser degree, CXCR3, these receptors appear to be largely dispensable for anti-melanoma immunity in the same animals. These data suggest that differential trafficking requirements for the liver and tumor microenvironments could be exploited to increase the tumor selectivity of 4-1BB agonist antibody immunotherapy.
6.2.2 Results

6.2.2.1 Disparate effects of CTLA-4 and PD-1 checkpoint blockade on α4-1BB-mediated hepatotoxicity

In order to determine the potential for currently approved checkpoint blockade antibodies (αCTLA-4, αPD-1) to ameliorate 4-1BB agonist antibody induced liver pathology, mice were treated with three administrations of checkpoint blockade alone, α4-1BB alone, α4-1BB in combination with αCTLA-4 or αPD-1, or triple combination therapy. At the peak of hepatic injury, sixteen days after the initiation of treatment, mice were bled and serum was analyzed for liver transaminases including alanine aminotransferase (ALT) and aspartate aminotransferase (AST). As noted previously, co-administration of αCTLA-4 significantly decreased serum transaminase levels compared to α4-1BB monotherapy (130), whereas dual therapy with α4-1BB and αPD-1 significantly increased transaminase levels (Fig. 32A) (131). The protective effect of αCTLA-4 therapy was lost when given in combination with both α4-1BB and αPD-1, showing that exacerbation of hepatitis by αPD-1 dominates over the capacity of αCTLA-4 to limit it. As triple combination therapy failed to alleviate hepatic damage, I sought to define the cellular mechanisms by which CTLA-4 blockade acted to limit 4-1BB agonist hepatotoxicity.

4-1BB agonist administration drove robust CD3+ T cell infiltration of the liver including >2-fold increases in cytotoxic CD8 T cells relative to untreated animals or those receiving CTLA-4 blockade (Fig. 32B, 33A), but did not significantly impact infiltration of CD4+ effectors (Fig 33B). Functionally, the majority of these infiltrating T cells bore the recently defined Eomesodermin+KLRG1+ signature of the cytotoxic ThEO (CD4) and TcEO (CD8) phenotype which has been described as critical for 4-1BB agonist-induced tumor immunity (Fig 33C,D)(87, 111, 219, 352). These cells exhibit elevated per cell cytotoxicity compared to their Th1/Tc1 counterparts and likely play a significant role in mediating the damage to the liver. The addition of CTLA-4 blockade to α4-1BB treatment reduced the frequency of T cell infiltration into the liver versus α4-1BB alone (Fig. 32B). Whereas the overall CD3 density was reduced in α4-
1BB/αCTLA-4 combination treated animals, no changes in the CD4 and CD8 frequencies within the infiltrating T cell pool, nor in the percentage of cells adopting the ThEO/TcEO phenotype were observed (Fig. 32B, Fig. 33B,D). Moreover, inflammatory foci (Fig. 32C) and clusters of CD8 T cells around the liver vasculature (Fig. 32D, E) decreased when αCTLA-4 was co-administered with α4-1BB, but were exacerbated by triple combination therapy. Overall, αCTLA-4 co-administration with α4-1BB significantly decreased the severity of inflammation, necrotic regions, and CD8 T cell infiltration in liver parenchyma as indicated by a significantly reduced pathology score (Fig. 32F).

To test if the ability of CTLA-4 blockade to reduce liver pathology was specific for 4-1BB agonist therapy, I also tested αCTLA-4 in combination with antibodies targeting the TNF receptor CD40. Co-stimulation through CD40 induces an acute and transient hepatic injury that peaks within a week of antibody administration and declines thereafter, whereas 4-1BB agonists induced a chronic, and persistent hepatic pathology as measured by maintained elevation of serum transaminases over the 16-day study period (Fig. 32G). Further, in contrast to α4-1BB, αCD40-induced liver damage could not be ameliorated by co-administration with αCTLA-4 (Fig. 32H).

These data suggest that 4-1BB agonist antibodies mediate chronic liver pathology through a mechanism distinct from that of CD40 activation. Although CTLA-4 blockade can ameliorate 4-1BB agonist induced hepatitis through reduction of T cell infiltration; this mechanism fails to impact liver injury resulting from αCD40 or α4-1BBα/PD-1 combination therapy.
Figure 32.
**Figure 32:** Combination immunotherapy augments α4-1BB mediated hepatotoxicity. Mice were administered α4-1BB, αCTLA-4, or αPD-1 antibodies alone or in combination within 3 day intervals (days 0, 3, and 6). Mice were bled 16 days after initiation of therapy and sacrificed to measure liver immune infiltrates by flow cytometry. **A)** Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured upon sacrifice as units of enzyme/liter of blood. **B)** Immune infiltrates within perfused livers of treated mice were measured by flow cytometry. Frequency of CD3+ cells within perfused livers was calculated as a fraction of liver CD45+ cells. Frequency of CD8+ T cells was calculated as a percent of CD3+ cells. **C)** Hematoxylin and Eosin (H&E) staining or immunohistochemistry (IHC) targeting CD8 (D) was performed on sectioned liver tissues from treated mice 16 days after initiation of therapy. **E)** Total CD8+ infiltrates were enumerated per section, and (F) sections were assigned a clinical score by a pathologist based on the number of inflammatory cells in the portal triad, central vein, or parenchyma. **G)** Mice administered either α4-1BB or αCD40 agonist antibodies were bled 8 or 16 days after initiation of therapy. Serum levels of ALT and AST were measured. **H)** Mice were administered either CD40 agonist antibodies alone or in combination with CTLA-4 blockade. Mice were then bled at the peak of αCD40-mediated liver damage (D8) in order to assess serum transaminase levels. Each point in A, and B represent individual mice. Micrographs in C and D were imaged at 20X magnification. Data were pooled from ≥ 2 experiments with 5 mice per group. Bars represent mean ± SD. Statistical significance was calculated using a two-sided Student’s T test applying Welch’s correction for unequal variance. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. This figure was generated with the assistance of Ashvin Jaiswal and Casey Ager.
Figure 33.
Figure 33: Gating strategy and representative flow cytometry analysis of liver immune infiltrates. A) Representative gating strategy to analyze CD8⁺, CD4⁺ Teff, and CD4⁺ Treg T cell populations as well as F4/80⁺CD11b⁺CD68⁻, F4/80⁺CD11b⁻CD68⁺, and F4/80⁺CD11b⁻CD68⁺ myeloid populations within perfused livers. B) Quantification of CD4 Teff infiltrate into the perfused livers of treated mice 16 days after initiation of therapy. Percent of Teff was calculated as percent of total CD4⁺CD3⁺ cells lacking Foxp3 expression (CD4⁺Foxp3⁻). C) Representative gating strategy for analysis of Eomes⁺KLRG1⁺ TcEO (top) or ThEO (bottom) phenotype cells infiltrating the livers of treated mice. D) Quantification of TcEO (left) and ThEO (right) phenotype cells enumerated at the percent of CD3⁺CD8⁺ or CD3⁺CD4⁺ Foxp3⁻ cells respectively that infiltrated perfused livers. Data were pooled from ≥ 2 experiments with 5 mice per group. Bars represent mean ± SD. Statistical significance was calculated using a two-sided Student’s T test applying Welch’s correction for unequal variance. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. This figure was generated with the assistance of Ashvin Jaiswal and Casey Ager.
6.2.2.2 4-1BB agonists initiate liver pathology through activation of liver-resident myeloid cells.

Given the differential liver toxicities associated with 4-1BB agonists and CD40 agonists, I sought to uncover the relative contribution of the myeloid and T cell pools to 4-1BB agonist-induced liver damage. Whereas CD40 is exclusively expressed by myeloid cells (365), 4-1BB can be expressed on both T cell, NK cell, and myeloid populations (1, 87, 366, 367), and the relative contribution of each of these to liver pathology has remained undefined.

To reveal the relative contribution of the myeloid versus lymphocyte compartments to α4-1BB induced hepatotoxicity, wildtype or 4-1BB−/− mice were administered a sub-lethal dose of radiation sufficient to eliminate their endogenous lymphocytes. Twenty-four hours after irradiation, splenic lymphocytes from wildtype or 4-1BB−/− mice were magnetically sorted and adoptively transferred into irradiated wildtype or 4-1BB−/− hosts. In this way, ablation of the lymphoid pool, but not the radio-resistant myeloid pool, allowed me to specifically target 4-1BB on either T cells or myeloid cells. Mice then received 4-1BB agonist therapy as previously described. Mice receiving WT to WT splenocyte transfers (myeloid:4-1BB+, lymphocyte:4-1BB+) clearly manifested ALT elevation in response to 4-1BB agonist antibody treatment (Fig. 34A), while AST elevation, which is always less affected by α4-1BB, showed modest elevation as well (Fig. 35). Wildtype mice that received splenocytes from 4-1BB−/− mice (myeloid: 4-1BB+, lymphocyte: 4-1BB+) were not significantly protected against ALT elevation, but did show reduced elevation of AST. On the other hand, 4-1BB−/− mice receiving splenocytes from wildtype mice (myeloid: 4-1BB−, lymphocyte: 4-1BB+), were fully protected from ALT elevation and showed no significant elevation of AST relative to mice lacking 4-1BB only on T cells. Thus, when 4-1BB was absent from the myeloid compartment α4-1BB could no longer trigger hepatotoxicity suggesting a requirement for myeloid 4-1BB activation to initiate a liver inflammatory cascade. The absence of 4-1BB on T cells did not appear deterministic for liver inflammation, but the modest reductions in transaminases relative to WT mice suggested a contributory role for 4-1BB.
on T cells as well.

Given my prior data, I investigated the role of myeloid cells in initiating α4-1BB-induced liver pathology. I found that, in comparison to untreated livers, α4-1BB therapy induced an expansion of F4/80+ macrophages within the liver parenchyma which was significantly reduced by combining αCTLA-4 with α4-1BB (Fig. 34B, C, D). Interestingly, combination therapy favored accumulation of F4/80+ cells within the perivascular space compared to infiltration into the tissue parenchyma (Fig. 34D). Macrophages in the liver consist of F4/80+ tissue-resident Kupffer cells that remain relatively quiescent within healthy liver, and are replenished by bone marrow-derived myeloid precursors or via low-level homeostatic proliferation (368). Further, Kupffer cells can be sub-classified into populations of CD11b+CD68+ myeloid cells specialized for cytokine production, CD11b+CD68+ phagocytic macrophages and CD11b+CD68+ cells with intermediate phagocytic activity and cytokine expression (369). These populations are distinct from CD11b+F4/80+ circulating monocytes.

All three Kupffer cell subsets showed signs of activation in response to 4-1BB agonist antibody (Fig. 34E). Increases in infiltration of CCR5+CD11b+CD68− and CD11b+CD68+ cells by approximately 2-fold suggested that not only that these cells are responding to inflammatory chemokines, but also remains consistent with infiltration into the perivascular space (370, 371). CCR5 expression decreased, however, on the CD11b+CD68+ subset, which may be a result of receptor downregulation by recent emigrants from the bone marrow as we observed no evidence of elevated in situ proliferative expansion by Ki67. Moreover, all three subsets of F4/80+ cells increased MHC-II expression, further suggesting that these populations are becoming activated by 4-1BB antibody consistent with published literature demonstrating that this activation leads to enhanced co-stimulatory capacity (87, 367).

I sought to confirm the ability of the cytokine-producing myeloid populations to mediate liver damage during the course of α4-1BB therapy, as well as to determine what effector molecules these populations produce to mobilize immune responses leading to hepatic damage.

Within the F4/80 positive population, CD68+ (F4/80+CD11b−CD68+), CD11b+ (F4/80+
CD11b-CD68+), and CD68+CD11b+ (F4/80+CD11b+CD68+) cells as well as CD11b+F4/80- monocytes were FACS sorted on day 7 from the livers of treated mice (Fig. 33), and RNA was extracted for quantitative real time PCR. I found that the F4/80+CD11b+CD68- and F4/80+CD11b+CD68+ myeloid cells were the predominant cytokine producers with little or no contribution from the CD11b- subset within the livers of α4-1BB treated mice. Within the two CD11b-CD68- subsets, I observed approximately 20-fold increased expression of IL-27-p28 following 4-1BB agonist therapy compared to treatment-naïve mice. In contrast, the CD11b-CD68+ subset was the primary source of interferon-γ (Fig. 34F). Moreover, both CD11b+ subsets of Kupffer cells produced the majority of TNFα. Notably, the cytokine-producing subsets of myeloid cells produced less IL-27 and TNFα in mice receiving the α4-1BB/αCTLA-4 combination therapy compared to mice receiving α4-1BB monotherapy.

Together, these data suggest that α4-1BB inflammatory hepatotoxicity is induced at the myeloid level via activation of tissue-resident Kupffer cells. All three subsets of Kupffer cells show signs of activated antigen presentation, and both CD11b+ cytokine-producing subsets increase production of IL-27. Co-administration of CTLA-4 blockade reduced inflammatory cytokine production in these subsets, consistent with the reduced transaminase elevation observed in those mice.
Figure 34.

A

ALT

WT\(\times\)4-1BB\(\times\)4-1BB\(\times\)WT \(\Rightarrow\) WT\(\Rightarrow\) WT

B

\% F4/80\(^+\) (of CD45\(^+\))

\(\alpha\)4-1BB

\(\alpha\)CTLA-4

\(\Rightarrow\) = \(\Rightarrow\) +

C

Untreated

\(\alpha\)4-1BB

\(\alpha\)4-1BB/\(\alpha\)CTLA-4

\(\alpha\)4-1BB/\(\alpha\)CTLA-4/\(\alpha\)PD-1

D

F4/80 Cells (Count)

Parenchymal Perivascular

E

Naive

\(\alpha\)4-1BB

\% Positive

CD11b\(^+\)CD68\(^-\)

CD11b\(^+\)CD68\(^+\)

CD11b\(^+\)CD68\(^+\)

F

\(\text{i27-p28}\)

\(\text{ifng}\)

\(\text{tnfa}\)

Fold Change
Figure 34: Administration of 4-1BB agonist antibodies initiates liver pathology through activation of liver-resident myeloid cells. A) Mice were sublethally irradiated (500 rads) before administration of 2×10⁶ CD90⁺ splenocytes. Wildtype mice either received splenocytes from wildtype mice (WT → WT) or from 4-1BB⁻/⁻ mice (4-1BB⁻/⁻ → WT) or 4-1BB⁻/⁻ mice received splenocytes from wildtype mice (WT → 4-1BB⁻/⁻). Mice were subsequently treated with three rounds of α4-1BB immunotherapy. Treated mice were then bled 16 days after first administration of therapy and serum ALT was measured. B) Quantification of myeloid infiltration into perfused livers based on flow cytometry of lymphoid-replete wildtype mice administered either α4-1BB therapy alone or in combination with αCTLA-4 checkpoint blockade. Myeloid infiltration is shown as the frequency of F4/80⁺ cells as a fraction of total CD45⁺ cells. C) Immunohistochemistry staining for F4/80 was performed on sectioned liver tissues from treated mice 16 days after initiation of therapy. D) Quantification F4/80⁺ cellular infiltrates based on IHC staining of liver sections. Individual F4/80⁺ cells were enumerated within the liver parenchyma or perivascular space. E) Quantification of inflammatory/activation markers based on flow cytometry of perfused livers from treated mice based on three subsets of liver-resident macrophages: CD11b⁺CD68⁻ cytokine-producing Kupffer cells, CD11b⁺CD68⁺ cytokine-producing/phagocytic Kupffer cells, and CD11b⁻CD68⁺ phagocytic Kupffer cells. F) Gene expression from individual myeloid populations was calculated using real-time PCR analysis with gapdh as the endogenous control. Each point in A and B represent individual mice. Micrographs in C were imaged at 20X magnification. Gene expression was calculated using Taqman primers via the ΔΔCt method. Data were pooled from ≥ 2 experiments with 5 mice per group. Bars represent mean ± SEM. Statistical significance was calculated using a two-sided Student’s T test applying Welch’s correction for unequal variance. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. This figure was generated with the assistance of Ashvin Jaiswal and Casey Ager.
Figure 35: Effects of selective targeting of 4-1BB costimulation in lymphoid and myeloid compartments on serum transaminases. Mice were sub-lethally irradiated (500 rads) before administration of 2X10⁶ CD90⁺ splenocytes. Wildtype mice either received splenocytes from wildtype mice (WT→WT) or from 4-1BB⁻ mice (4-1BB⁻→WT) or 4-1BB⁻ mice received splenocytes from wildtype mice (WT→4-1BB⁻). Mice were subsequently treated with three round of α4-1BB immunotherapy. Treated mice were then bled 16 days after first administration of therapy and serum AST was measured. Each point within each graph represents an individual mouse. Data were pooled from ≥ 2 experiments with 5 mice per group. Bars represent mean ± SD. Statistical significance was calculated using a two-sided Student’s T test applying Welch’s correction for unequal variance. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. This figure was generated with the assistance of Ashvin Jaiswal and Casey Ager.
6.2.2.3 Interleukin 27 is a critical regulator of liver inflammation.

In addition to the above findings, we previously reported that IL-27 acts as a mediator in polarizing T cells to the cytotoxic ThEO/TcEO phenotype (87), and therefore hypothesized that this inflammatory cytokine may play a role in triggering α4-1BB induced hepatic damage. To evaluate the contribution of IL-27 to immune mediated hepatic damage, mice lacking the Ebi3 subunit of IL-27 (EBI3−/−) or mice lacking the IL-27 receptor alpha subunit (IL27Rα−/−) were treated with α4-1BB therapy followed by analysis of serum transaminase levels. Compared to wildtype mice, EBI3−/− and IL27Rα−/− mice treated with 4-1BB agonists failed to develop liver damage as measured by ALT and AST (Fig. 36A). Remarkably, the high-grade elevation of liver transaminases resulting from triple combination α4-1BB/αCTLA-4/αPD-1 therapy was also nearly completely abrogated in EBI3−/− mice.

In mice lacking the IL-27/IL-27R pathway, CD3+ T cell infiltration of the liver was reduced (Fig. 36B) as were both the frequency and density of cytotoxic CD8+ cells (Fig. 36C). Further, CD4 effector T cells appeared minimally affected by knockout of the IL-27 pathway (Fig. 37A). While the percent of CD4+Eomes*KLRG1+ ThEO phenotype cells (Fig. 37B), and CD8+ TcEO phenotype T cells were unaffected by loss of IL-27, the total numbers of the highly inflammatory TcEO population within the liver parenchyma were significantly diminished absent functional IL-27 signaling (Fig. 36D). Taken together, these data demonstrate a critical requirement for the inflammatory cytokine IL-27 in mediating 4-1BB agonist antibody-induced hepatotoxicity as well as for recruitment and/or expansion of hepatogenic T cells into the liver.
Figure 36.
**Figure 36: Interleukin 27 is a critical regulator of 4-1BB agonist-induced liver inflammation.** Wildtype mice or mice lacking the Ebi3 subunit of the IL-27 cytokine complex (EBI3<sup>-/-</sup>) or the IL-27 receptor alpha subunit (IL27Rα<sup>-/-</sup>) were treated for three rounds of 4-1BB agonist immunotherapy before analysis of serum transaminase levels and hepatic immune infiltrates 16 days after initiation of treatment. **A)** Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured upon sacrifice as units of enzyme/liter of blood volume. **B)** Quantification of immune infiltrates within perfused livers of treated mice was measured by flow cytometry. Percent of CD3<sup>+</sup> cells was calculated as a percent of CD45<sup>+</sup> cells in the liver. **C)** Percent of CD8<sup>+</sup> T cells was calculated as a percent of CD3<sup>+</sup> cells. Total numbers of cells were calculated as total number of CD3<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> cells within perfused livers. **D)** Quantification of percent and total numbers of TcEO T cell infiltration within the livers of treated mice. Percent of TcEO cells was calculated based on the percent of CD3<sup>+</sup>CD8<sup>+</sup> T cells expressing Eomesodermin (Eomes) and KLRG1. Each point within each graph represents an individual mouse. Data were pooled from ≥ 2 experiments with 5 mice per group. Bars represent mean ± SD. Statistical significance was calculated using a two-sided Student’s T test applying Welch’s correction for unequal variance. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. This figure was generated with the assistance of Ashvin Jaiswal and Casey Ager.
Figure 37: Effects of IL-27 pathway inactivation on CD4 T cells. A) Quantification of effector CD4 T cells (CD3^+CD4^+Foxp3^-) infiltrating the perfused livers of α4-1BB treated wildtype (WT), EBI3^-/-, or IL27Rα^-/- mice. B) ThEO phenotype cells (Eomes^+KLRG1^-) enumerated as the percent CD3^+CD4^+ Foxp3^- cells that infiltrated perfused livers. Data were pooled from ≥ 2 experiments with 5 mice per group. Bars represent mean ± SD. Statistical significance was calculated using a two-sided Student's T test applying Welch's correction for unequal variance. ns, not significant; ^P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. This figure was generated with the assistance of Ashvin Jaiswal and Casey Ager.
6.2.2.4 Regulatory T cells restrict 4-1BB agonist antibody induced liver pathology

Given the ability of myeloid cells to activate T cell responses, coupled with the capacity of IL-27 to act as an inflammatory mediator of hepatic damage with pleotropic effects on helper T cell polarization, Treg suppression, and T cell trafficking (372-374), I next investigated the role of T cells in propagating α4-1BB mediated liver damage. To assess the relative contribution of the T cell pool in mediating hepatotoxicity, I treated mice lacking the β2 microglobulin subunit of the major histocompatibility (MHC) I complex (β2M−/−) or mice lacking all H2-A and H2-E MHC genes (MHCII−/−). These mice are deficient in antigen presentation capacity to CD8 and CD4 T cells respectively, causing a failure of these cells to complete thymic positive selection and enter the periphery. Elevation of liver ALT and AST levels in the serum was completely abrogated in α4-1BB-treated β2M−/− mice, confirming the role of CD8+ T cells in mediating the bulk of the liver damage (Fig. 38A). To separate the possibilities that this effect may be due to absent CD8 T cell responses and/or to defective antigen presentation, mice were sub-lethally irradiated and CD8+ splenocytes from wildtype mice were transferred into β2M−/− mice. I hypothesized that if the lack of CD8 T cells in these mice was the sole cause of the abrogated hepatotoxicity, then supplying wildtype CD8+ T cells into β2M−/− mice did not abrogate the resistance of these animals to liver damage when challenged with 4-1BB antibody (Fig. 38B). This suggests that not only are CD8 T cells required to effect 4-1BB agonist-induced liver injury, but that antigen presentation on MHC Class I is also necessary. This further indicates that hepatitis-inducing CD8 T cells are being activated by 4-1BB-activated myeloid cells in an antigen-specific manner. Intriguingly, impairing the CD4 response in MHCII−/− mice significantly escalated liver damage, denoted by approximately 1.5-2-fold increases in serum AST and ALT levels in MHCII−/− mice compared to α4-1BB treated wildtype mice (Figure 38A).

I next hypothesized that exacerbation of hepatotoxicity in MHCII−/− mice stemmed, not from dysregulation of effector T cells responses, but from elimination of Treg cells leading to loss
of immune control in the liver. I also made the related observation that there was a 2-fold increase in the percent of Foxp3+ regulatory T cells in the livers of α4-1BB compared to untreated mice (Fig. 38C) suggesting that Treg expansion might be acting to limit hepatitis. To validate a role for Tregs in limiting 4-1BB agonist-induced liver toxicity, I treated mice expressing the diphtheria toxin (DT) receptor (DTR) under control of the Foxp3 promoter (Foxp3-DTR) in which Foxp3+ regulatory T cells can be depleted upon administration of DT. Briefly, DT was administered 2 days before α4-1BB therapy, and continued until the end of treatment for complete and sustained Treg depletion. Treg depletion was successful based on analysis of blood three days before serum analysis (Fig. 39). Consistent with my hypothesis, depletion of Tregs significantly aggravated α4-1BB induced liver damage, increasing AST and ALT levels 5-6-fold, and eliminating the ability of αCTLA-4 to dampen liver damage (Fig. 38D). This effect was not due to administration of DT, as DT alone did not significantly impact transaminase levels. Moreover, Treg adoptive transfer prior to therapy limited transaminase elevation, suggesting that Treg cells are critical suppressors of inflammation during α4-1BB treatment.

Taken together this data suggests a critical role of CD8 T cell activation in mediating α4-1BB liver damage. Antigen presentation was also required suggesting hepatogenic CD8 T cells are liver tissue antigen specific. Further, Treg cells play a critical role in protecting the liver from CD8-mediated injury downstream of 4-1BB activation.
Figure 38.
Figure 38: Regulatory T cells suppress 4-1BB agonist antibody induced liver pathology.

A) Wildtype mice or mice lacking MHC Class I expression (β2M−/−) or all MHC Class II alleles (MHC-II−/−) were treated for three rounds with 4-1BB agonist antibody (days 0, 3, and 6) before mice were bled for serum liver enzyme analysis 16 days after beginning treatment. Serum ALT and AST were measured upon sacrifice as units of enzyme/liter of blood. B) Mice were sub-lethally irradiated (500 rads) before administration of 2×10⁶ CD8+ splenocytes. Wildtype mice or β2M−/− mice received splenocytes from wildtype mice (WT CD8→WT) or (WT CD8→β2M−/−) respectively. Mice were subsequently treated with three rounds of α4-1BB immunotherapy. Treated mice were then bled 16 days after first administration of therapy and serum ALT and AST were measured. C) Quantification of regulatory T cell (Treg) infiltration into the perfused livers of treated mice 16 days after initiation of therapy. Frequency of Tregs was quantified by flow cytometric analysis as the percent of Foxp3+CD4+ cells as a fraction of total CD4+ T cells. D) Mice received 5×10⁵ CD3+CD4+CD25+ splenocytes FACS-sorted from naïve mice one day prior to treatment. Concurrently, mice expressing the diphtheria toxin receptor under control of the Foxp3 promoter (Foxp3-DTR) were administered 10µg/kg body weight of diphtheria toxin one day prior to initiation of therapy and every three days thereafter until completion of the experiment. Data were pooled from ≥ 2 experiments with 5 mice per group. Bars represent mean ± SD. Statistical significance was calculated using a two-sided Student’s T test applying Welch’s correction for unequal variance. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. This figure was generated with the assistance of Ashvin Jaiswal and Casey Ager.
Figure 39: Depletion of Treg cells in FoxP3-DTR mice. Mice expressing the diphtheria toxin receptor under control of the Foxp3 promoter (Foxp3-DTR) were administered 10µg/kg body weight of diphtheria toxin one day prior to initiation of therapy and every three days thereafter until completion of the experiment. FACS plots are representative of one mouse bled at day 13, prior to sacrifice. This figure was generated with the assistance of Ashvin Jaiswal and Casey Ager.
6.2.2.5 CCR2 and CXCR3 are differentially required for liver and tumor T cell trafficking

Given the ability of IL-27 to induce chemokine receptor expression (375, 376), the reduced immune infiltrate in the liver in the absence of IL-27, and the reduced myeloid presence in mice treated with α4-1BB/αCTLA-4 co-therapy, I hypothesized that 4-1BB agonist therapy might alter T cell trafficking patterns into the tissue via chemokine modulation. Given the differential expression patterns of chemokine receptors on T cells capable of homing into tumor tissue versus liver parenchyma (371, 377), I sought to determine whether anti-tumor immunity could be separated from hepatitis based on differential homing. I challenged either wildtype, CCR2<sup>−/−</sup>, CXCR3<sup>−/−</sup>, or CCR5<sup>−/−</sup> mice subcutaneously with 3X10<sup>5</sup> murine B16 melanoma cells expressing the ovalbumin antigen (B16-Ova). Mice were then treated with 4-1BB agonist and assessed for transaminase elevation and infiltration as previously described. CXCR3 is considered critical for driving T cell trafficking into tumors in response to IFN-γ signaling, while CCR5 has been suggested to be the predominant trafficking mechanism into the liver; however, CXCR3 can also play an important role in liver chemotaxis in response to injury (378). CCR2, in contrast, is thought to minimally impact T cell trafficking to liver even in the context of viral infection. Intriguingly, following 4-1BB agonist antibody therapy, CCR2<sup>−/−</sup> mice exhibited significantly reduced AST and ALT serum levels, while CXCR3<sup>−/−</sup> mice showed significantly reduced ALT levels and a trend towards lower AST levels (p=0.08) (Fig. 40A). In contrast, CCR5<sup>−/−</sup> mice showed no reduction in the liver damage induced by 4-1BB agonists. Ablation of any of these chemokine receptors individually failed to impact the ability of 4-1BB agonist therapy to mediate rejection of subcutaneous melanoma (Fig. 40B), implying either that they are not required, or that sufficient redundancy exists to preserve responses in the tumor setting. Moreover, removing these chemokine receptor pathways did not significantly affect recruitment of antigen-specific T cells into the tumor (Fig. 40C). It has been demonstrated across multiple tumor microenvironments that increased CD8/Treg ratios correlate with more successful responses to immune-based therapies (58, 59, 379). I found that the magnitude of elevation of
CD8/Treg ratios in wildtype, CCR2\textsuperscript{−/−}, CXCR3\textsuperscript{−/−}, and CCR5\textsuperscript{−/−} mice were not significantly different providing additional evidence that loss of a single chemokine receptor pathway does not impact anti-tumor immune responses. Interestingly, within the liver, abrogation of CCR5 significantly increased the CD8/Treg ratio (Figure 40D). While this may be beneficial in the tumor setting, an increased ratio within the liver may account for the maintenance of elevated transaminase elevation in the CCR5 knockout mice.

Taken together, these data suggest that immune infiltration into the liver and tumor can be uncoupled through abrogation of chemokine receptor signaling (Figure 41). Further, CCR2 and CXCR3 appear to be critical mediators of α4-1BB induced hepatotoxicity-mediating T cell trafficking, while disengaging these pathways does not significantly impact the ability of α4-1BB therapy to generate potent anti-tumor immunity.
Figure 40: The chemokine receptors CCR2 and CXCR3 contribute to 4-1BB agonist-induced liver pathology. Wildtype mice or mice lacking specific chemokine receptors (CCR2<sup>−/−</sup>, CXCR3<sup>−/−</sup>, or CCR5<sup>−/−</sup>) were subcutaneously implanted on the right flank with 3×10⁵ B16 melanoma tumor cells expressing the ovalbumin antigen (B16-Ova). At three-day intervals after initial tumor challenge (days 3, 6, and 9) mice were treated with antibody immunotherapy delivered i.p. in combination with an irradiated tumor vaccine (FVAX) administered subcutaneously on the left flank. Mice were bled for serum liver enzyme analysis 16 days after beginning treatment. Mice were then sacrificed and perfused livers and tumors were extracted, weighed, and processed for FACS analysis. A) Serum ALT and AST were measured upon sacrifice as units of enzyme/liter of blood volume. B) Upon sacrifice, tumors were harvested and weighed. C) Tumor infiltration of Ova-specific CD8<sup>+</sup> T cells was determined by staining tumor infiltrating lymphocytes (TIL) with fluorescently labeled Ova<sub>257-264</sub>/K<sub>b</sub> (SIINFEKL) tetramer and antibodies to CD8. Data are expressed as the total number of tetramer positive cells per milligram of tumor. D) Quantification of CD8/Treg ratios within the tumor and liver were calculated by dividing the number of CD8<sup>+</sup>CD3<sup>+</sup> cells by the number of CD4<sup>+</sup>Foxp3<sup>+</sup> cells found within the tissue infiltrate. Data were pooled from ≥2 experiments with 5 mice per group. Bars represent mean ± SD. Statistical significance was calculated using a two-sided Student’s T test applying Welch’s correction for unequal variance. ns, not significant; *<i>P</i> < 0.05, **<i>P</i> < 0.01, ***<i>P</i> < 0.001, ****<i>P</i> < 0.0001. This figure was generated with the assistance of Ashvin Jaiswal and Casey Ager.
Figure 41: Mechanistic model of 4-1BB agonist antibody-mediated hepatotoxicity. Given data presented in this section of my dissertation, I propose that IL-27 production by liver-resident myeloid cells is a key initiating step in the pathogenesis of α4-1BB-mediated liver toxicity. Agonist antibodies first target 4-1BB on myeloid cells, activating them to produce inflammatory IL-27 which then in turn leads to the recruitment and activation of hepatogenic CD8 T cells into the liver parenchyma exacerbating disease. I also demonstrate that regulatory T cells are critical suppressors of inflammatory hepatic damage through an undefined mechanism. This figure was generated with the assistance Casey Ager.
6.2.3 Discussion

While the field of immunotherapy has experienced unprecedented growth due to the success of T cell immune checkpoint blockade, clinical translation of the most efficacious mono- and combination therapies from pre-clinical models has been limited by immune toxicities. 4-1BB agonist antibodies are among the most effective immunotherapeutics across a broad range of pre-clinical models of cancer (380). Severe off-target liver damage in early Phase I trials, however, has limited the clinical progression of highly active 4-1BB antibodies (364). Effective prophylaxis, biomarker prediction or management of this liver toxicity, except through highly attenuated dosing, has proven challenging due to lack of mechanistic understanding of the cellular and molecular mechanisms driving it. Efforts at development of 4-1BB agonist antibodies with limited toxicity are ongoing; however, no 4-1BB agonist has advanced beyond early Phase II trials. In this section, I sought to uncover the mechanisms driving 4-1BB agonist mediated liver pathology so that this knowledge would be available to inform both antibody engineering and combination 4-1BB agonist trial design in the future.

The capacity of 4-1BB activation to potentiate CD8 T cell responses is well appreciated; however, I have found that activation of liver myeloid cells, not T cells, is a critical initiating step that triggers hepatotoxicity. Following α4-1BB administration, antigen presentation capacity increased in multiple Kupffer cell populations based on MHC II upregulation. In addition, the cytokine-producing CD11b+ subsets increased production of IL-27 more than 20-fold. I found that this augmented IL-27 production is essential for the progression of liver inflammation as neither $EBI3^{-/-}$ nor $IL27Ra^{-/-}$ mice showed any evidence of transaminase elevation in response to 4-1BB activation. Despite the requirement for myeloid initiation, CD8 T cells mediated the actual liver injury, as mice lacking them fail to develop transaminase elevation. A previous report indicated that mice expressing only CD8 T cells specific for an Ovalbumin-peptide/H2-Kb complex were also resistant to α4-1BB liver toxicity (363). This observation, coupled with my own $β2M^{-/-}$ data, led me to question whether CD8 T cell activation downstream of myeloid 4-1BB
activation was occurring via an antigen-specific or non-specific mechanism. Mice with wildtype CD8 T cells but incapable of MHC Class I antigen presentation failed to develop liver injury in response to α4-1BB, suggesting that hepatotoxic CD8 T cells recognize liver-specific autoantigens. It is likely then, that 4-1BB activation of myeloid cells leads to enhanced presentation of liver tissue antigens and secreted IL-27 provides a critical signal for liver auto-reactive CD8 T cell activation. The role of IL-27, in this context, could be direct co-stimulation of effector CD8 and/or inhibition of Treg suppressive activity. These mechanistic insights suggest IL-27 blockade as a means to reduce to 4-1BB agonist liver toxicity; however, we have previously found IL-27 to play a critical role in effector T cell polarization downstream of α4-1BB as well as in anti-tumor responses (87, 381, 382).

Currently the only described mechanism to abrogate or reduce 4-1BB agonist liver toxicity involves combination therapy with CTLA-4 blockade (130). I have confirmed the capacity of this combination to block 4-1BB agonist transaminase elevation. Given this combination also shows therapeutic synergy and the capacity to limit αCTLA-4 IRAE (58, 130), it remains unfortunate that no trials have yet tested α4-1BB/αCTLA-4 in patients. In contrast, the α4-1BB/αPD-1 combination has been tested in patients, but with very limited dosing regimens due to the capacity of αPD-1 to worsen α4-1BB-mediated hepatitis – an effect I also validated herein (131). I hypothesized that the liver protective effect of CTLA-4 blockade might also extend to α4-1BB/αPD-1 combination therapy; however, I found that, in fact, the effect of PD-1 blockade was dominant and that triple combination treatment engendered severe transaminitis. In the context of my model (Fig. 41), CTLA-4 blockade limited the accumulation of T cells in the liver following 4-1BB agonist administration and thus attenuated resulting hepatotoxicity. While I was unable to identify a distinct cellular mechanism underlying this effect, I was able to demonstrate an impact of αCTLA-4 co-administration on myeloid infiltration and effector function in the liver; however, this mechanism may also involve a qualitative change in Treg phenotype (no quantitative changes were detected).

I next considered whether the chemokine receptors governing entry of hepatitis-inducing
T cells into the liver, versus migration of tumor-specific T cells into melanoma tumors might be sufficiently different to separate tumor immunity from hepatotoxicity. I found that CCR2 knockout mice, and to a lesser extent CXCR3 knockout mice, were protected from 4-1BB agonist induced liver toxicity but were still capable of effectively combating B16-Ova tumors growing on the flank. The impact of CCR2 knockout in abrogating liver toxicity remains enticing, as both small molecule (CCX872, ChemoCentryx; PF-04136309, Pfizer) and antibody (MLN1202, Millennium) antagonists for CCR2 are currently in clinical trials. Given my findings, 4-1BB agonist antibodies administered in combination with CCR2 inhibitors may prove to be a potent combination in promoting tumor regression while inhibiting off-target liver toxicity.
6.3 *Potential for ThEO phenotype cells to act as a biomarker for therapeutic efficacy in the clinic.*

**6.3.1 Introduction**

Immune-targeted therapies have demonstrated an unprecedented capacity to treat, and even cure a multitude of both solid and hematological malignancies. FDA approved checkpoint blockade therapies, in particular, targeting either the CTLA-4 (Ipilimumab) or PD-1 pathways (Nivolumab, Pembrolizumab, Atezolizumab, Durvalumab), have proven extraordinarily effective in treating melanoma, lung cancer, renal cell carcinoma, ovarian, bladder, and colon cancer (57, 383). The approval of these drugs has opened up the field of immune-oncology to the investigation of additional immune-targeted therapies for cancer care. A promising class of immune targets lies in the tumor necrosis factor receptor superfamily. Agonist antibodies targeting CD27 (Varilimumab: Celldex), OX-40 (PF-04518600: Pfizer, GSK-3174998: GlaxoSmithKline, MEDI0562: Medimmune), and 4-1BB (Urelumab: BMS, Utomilumab: Pfizer) are currently in clinical trials for the treatment of solid malignancies with promising results. The potency of 4-1BB agonist therapies in a wide variety of preclinical tumor models makes this co-receptor a leading target for the next phase of immunotherapies. Response rates among all forms of therapy, however, are limited. Overall response rates to Ipilimumab only reach approximately 10% as a monotherapy and responses to Nivolumab or Pembrolizumab reach 20-30% (60).

A lack of understanding of the mechanisms that correlate with response rate or long-term outcome has left the field lacking in definitive biomarkers and makes completely curative therapy an elusive target. In the case of 4-1BB therapy, however, development of an Eomes+KLRG1+ ThEO/TcEO phenotype may act as a novel biomarker for response rate and duration of response. In this section, I investigated
the ability of a ThEO-like phenotype to develop and endure in patients treated with Urelumab monotherapy.
6.3.2 Results

6.3.2.1 Urelumab treated human PBMC upregulate markers of ThEO polarization.

In order to investigate the ability of α4-1BB to induce a ThEO phenotype in patients, peripheral blood was collected from stage III/IV melanoma patients at regular intervals during the course of therapy (NCT00612664). I then screened these samples for markers of ThEO polarization (Figure 42A). Over multiple rounds of treatment with 4-1BB agonists, patients demonstrated increased Eomes expression, as well as granzyme A and granzyme B. While too few patient samples were collected to make a definitive conclusion regarding the true potential for ThEO markers to be used as a prognostic tool to evaluate clinical outcomes, I did uncover a range of responses in multiple patients which, given increased sample size, may correlate with outcomes (Figure 42B). I uncovered four potential responses to Urelumab therapy. One patient (Patient #8) demonstrated increased Eomes expression in CD8 T cells after initiation of therapy, which was maintained throughout the therapeutic regimen. Patient #10, however, maintained lower Eomes expression within the CD8 T cell compartment, only showing elevated Eomes later in the treatment cycle. Patient #24 initially showed elevated Eomes levels which slowly declined during the course of treatment. Lastly, Patient #27 failed to elevate Eomes to any significant degree. I propose that these different patterns of Eomes expression correlate the ThEO polarization and sustained ThEO phenotype cells in the blood, which may correlate with a sustained response, a late response, a relapsed/refractory tumor, and a non-responder respectively.

Taken together, this data suggests that the ThEO phenotype may be clinically relevant to cancer patients treated with α4-1BB therapy and may correspond to clinical outcome. These results, however, should be taken with caution, as more samples should be evaluated before definitive conclusions should be made.
Figure 42. Induction of ThEO factors in α4-1BB treated patients. Peripheral blood from patients diagnosed with Stage III/IV melanoma enrolled in a clinical trial (NCT00612664) to evaluate the efficacy of α4-1BB therapy (Urelumab) was collected at various intervals. Markers of ThEO phenotype polarization were evaluated within the PBMC throughout the course of treatment. (A) Mean fluorescence intensity of Eomes expression (left) or granzyme expression (right) in a single patient throughout the treatment regimen. (B) Mean fluorescence intensity of Eomes over the course of therapy in four patients exhibiting four distinct response patterns to treatment.
6.3.3 Discussion

Administration of agonist antibodies targeting the 4-1BB T cell costimulatory receptor has proven remarkably effective in treating an array of preclinical murine tumor models (1, 33, 58, 124, 384-386). While toxicities have hampered progression of α4-1BB agonists into the clinical realm, early efficacy studies of Urelumab (BMS) as a monotherapy in a cohort of melanoma, renal cell carcinoma, ovarian, and prostate cancer patients (83 patients total) demonstrated an overall response rate of 8% (3 PRs; 4 SDs)(128). The only biomarkers found in this study correlated induction of IFNγ-inducible genes and circulating activated T cells (HLA-DR+CD69+) with response rates. A second study evaluating the safety and efficacy of Utomilumab (Pfizer) found that in a cohort of predominantly colorectal cancer, Merkel cell carcinoma, and pancreatic adenocarcinoma patients, there were minimal incidence of DLTs and an overall response rate (SD) of 22%. This study, however, did not attempt to correlate biomarkers with outcome. A third study evaluated the combination of Utomilumab with Pembrolizumab (αPD-1) for the treatment of advanced solid tumors, and found that the combination offered a tolerable safety profile and a 26% overall response rate (CR or PR).

While these response rates warrant further investigation of these agents either alone or in combination in the clinic setting, further biomarker analysis is necessary in order to evaluate responses, stratify patients, and design relevant combination therapies, and, ultimately, improve response rates. I demonstrate in this section of Chapter 6 that development of a ThEO-like phenotype in Urelumab-treated patients may act as such a biomarker. Patients receiving α4-1BB therapy (NCT00612664) demonstrated increased expression of ThEO phenotype markers (Eomes, Granzyme-A,-B). I further demonstrated the potential of using ThEO phenotypic markers as a correlative marker of outcome, as the degree to which ThEO markers are induced, or
the longevity of induction may correspond to outcome. It should be emphasized here that these data are only suggestive and evaluation of an increased patient cohort size will give further validity to these findings, though the prognostic value of the ThEO phenotype cannot be undervalued.

Further comparisons between Urelumab and Utomilumab are also warranted, as these antibodies bear different isotypes (IgG4 vs IgG2 respectively) which may impact therapeutic efficacy or ThEO polarization. In addition, whether Utomilumab, administered at a lower dose than Urelumab (0.006-5 mg/kg vs 0.3-15 mg/kg) limited related IRAE at the cost of anti-tumor immunity or ThEO polarization must also be considered. Moreover, as mentioned within this chapter, α4-1BB/αPD-1 therapy leads to increased toxicity and does not appear to synergize to increase response rates compared to either therapy alone (387). In addition, in Chapter 5, I demonstrated that while the α4-1BB/αPD-1 combination extended the effector phase of the ThEO response, ThEO/TcEO phenotype cells exhibit poorer recall responses than α4-1BB monotherapy and may lead ThEO cells towards terminal exhaustion (Fig. 17a).

Overall, data presented here suggests that a ThEO-like T cell polarity can be induced in patients treated with fully human 4-1BB-targeted monoclonal antibodies. Induction and persistence of this phenotype may act as a biomarker of response rates in these patients, although in my hands, small sample size restricted validation of this hypothesis. Future studies, however, may use development of a ThEO cell polarity to assess response rates to 4-1BB targeted therapy.
7. Summary and Future Directions

7.1 Summary

Throughout this dissertation work, I have demonstrated a potential mechanism by which 4-1BB agonist antibodies, used for the treatment of a variety of different cancers, induce a potently cytotoxic T cell phenotype characterized by expression of the T-box transcription factor Eomesodermin as well as the co-inhibitory receptor KLRG1 (TcEO/ThEO). My dissertation work has extensively characterized this phenotype, the phenotypic stability of ThEO/TcEO cells, molecular drivers and signaling pathways necessary for adequate ThEO polarization, as well as clinical aspects related to the use of α4-1BB as an immunotherapeutic strategy to treat cancer.

I have demonstrated that this phenotype is not merely transiently induced, but is capable of residing long-term within the secondary lymphoid organs and is further able to potently recall with cytotoxic effector function long after primary tumor challenge and cessation of therapy. I have further demonstrated that the TcEO/ThEO phenotype is not unique to any single tumor model, as Eomes+KLRG1+ cells can be found in multiple additional tumor models treated with α4-1BB therapy (e.g. pancreatic, prostate, glioma). I went on to show that antigen-specific TcEO responses were generated and recalled in response to foreign antigen (Ova, HPV E7 peptide) as well as mutated self-antigen (Spas), suggesting that TcEO phenotype cells are generated against multiple antigen-specificities. In fact, I demonstrated that significant induction and persistence of ThEO phenotype relies on antigen recognition, as P14 cells specific for the unrelated LCMV gp33 antigen failed to induce a persistent TcEO phenotype. In addition, I have provided evidence which suggests that TcEO/ThEO phenotype cells adopt a memory phenotype characterized by expression of the IL7 receptor alpha (CD127) as well as the IL15 receptor beta (CD122) and the lymphoid homing chemokine receptor CXCR3. Interestingly, 4-1BB co-stimulation also induced
expression of stem cell antigen-1 (Sca-1) a murine marker of the recently characterized population of stem cell memory (Tscm).

I further investigated signaling pathways that may regulate polarization of cells towards the ThEO phenotype. In collaboration with the Hwu lab, I found that overexpression of the runt-related transcription factor Runx2 induced ThEO phenotype genes including Eomes, KLRG1, and several granzyme family members (Mbofung et al, unpublished). Work presented here demonstrates that administration of α4-1BB agonist antibodies in fact induced Runx2 in both CD8 and CD4 T cell subsets. Further, virtually all of the TcEO and ThEO populations expressed high levels of Runx2. While alternative tumor necrosis factor receptor stimulation (GITR, OX-40, CD40) failed to induce a ThEO polarity, co-stimulation through these receptors did induce some degree of Runx2 expression in each case. Moreover, it should be noted that αCD40 agonists were able to induce a transient TcEO phenotype during the effector phase that failed to recall after secondary rechallenge.

I also demonstrated a requirement for STAT signaling, in particular STAT1 and STAT3 in mediating ThEO T cell polarization as early as ten days after primary tumor challenge. Further, abrogation of cytokine signaling pathways that induce these STAT pathways also impacted ThEO polarization. In particular, ablation of IL-27 signaling (Ebi3KO) and type I IFN (IFNαRKO, STINGKO) elicited defects in ThEO cell polarization.

Finally, I demonstrated the clinical potential in using 4-1BB agonist antibodies to treat tumors. Firstly, I demonstrated potent synergy between systemic administration of α4-1BB in combination with an intranasal HPV peptide vaccination strategy for the treatment of a murine HPV+ tumor model (TC-1) (33). In fact, the vaccine/α4-1BB combination was the most efficacious therapy tested, capable of completely curing cervically implanted tumors. The combination therapy was able to expand the CD8 T cell population within the tumor microenvironment, significantly
increasing the CD8/Treg ratio, a validated prognostic indicator of therapeutic response (58, 59), as well as increasing the antigen-specific infiltrate into the tumor. Further, while α4-1BB therapy was capable of generating a TcEO/ThEO response, antibodies in combination with intranasal vaccine increased the total numbers of both TcEO and ThEO phenotype cells within the tumor bed. Intriguingly, the vaccine induced expression of the inducible costimulatory molecule (ICOS) on both CD8 and CD4 T cells. Within α4-1BB treated mice, both ThEO and TcEO phenotype cells also gained expression of ICOS, making TcEO cells more potently cytotoxic effectors than their ICOS negative counterparts.

Secondly, I provided evidence as to a potential mechanism by which liver toxicity associated with α4-1BB therapy may be ascribed (129, 363). I confirmed that α4-1BB in combination with αCTLA-4 alleviated liver pathology (130), signified by decreased levels of serum ALT; whereas αPD-1 exacerbated toxicity (131). Further, I showed that the effects of αPD-1 therapy dominated over that of αCTLA-4, as triple combination therapy induced severe hepatic damage. I further demonstrated that liver toxicity is initiated at the myeloid level, absent of 4-1BB expression on T cells. I went on to show that three distinct subsets of myeloid cells in the liver (Kuppfer cells) demonstrated signs of activation including increased CCR5 expression and increased MHC-II. These cells also produced inflammatory cytokines including IL-27, IFNγ, and TNFα, of which abrogation of IL-27 completely eliminated the potential for α4-1BB administration to induce hepatic damage. Moreover, ablation of IL-27 significantly impaired subsequent T cell infiltration into the liver including CD8 and TcEO phenotype cells. I further demonstrated a role for Tregs in maintenance of liver homeostasis and suppression of hepatotoxic T cell responses, as ablation of Tregs increased the severity of liver damage. I then went on to show that chemokine-initiated immune cell homing into the liver could be uncoupled from anti-tumor responses. Of significance, knockout of the chemokine receptor CCR2, and to a lesser extent CXCR3, abrogated
liver toxicity, while maintaining anti-tumor efficacy associated with α4-1BB therapy.

Finally, I demonstrated that α4-1BB therapies used in the clinic (Urelumab, Utomilumab) induce a ThEO/TcEO-like phenotype in the blood of patients treated with these agents. I demonstrated four potential outcomes related to ThEO induction that may act as a biomarker for clinical outcome to therapy.

The results presented here may have profound implications on current paradigms of T cell biology as well as our collective understanding of the mechanisms underlying α4-1BB co-stimulation.

7.2 Effects of 4-1BB co-stimulation on memory potential

While the necessity of 4-1BB co-stimulation in generating adequate T cell memory responses is established (105, 276, 278, 388), the exact mechanism by which the co-receptor does so remains ill-defined. My results presented here may give insight into not only how 4-1BB acts to engender memory responses, but also how ThEO/TcEO phenotype cells maintain memory potential. Within the field, there are four prevailing models related to the potential for T cells to generate immunological memory that may shed light on 4-1BB agonism and ThEO cell memory potential: The Separate Precursor Model, the Decreasing Potential Model, the Signal Strength Model, and the Asymmetric Cell Fate Model (230).

The Separate Precursor Model dictates that from initial generation, newly formed mature naive T cells are predestined to either a terminal effector fate, or to become long-lived memory. It is certainly possible that unique populations of naïve cells may be in fact be more responsive to α4-1BB therapy and thus more inherently able to transform into ThEO phenotype cells with memory potential. Upon adoptive transfer of unique T cell populations (Eomes⁺KLRG1⁺, Eomes⁺KLRG1⁻, Eomes⁻KLRG1⁺, Eomes⁻KLRG1⁻), however, I found that the terminal Eomes⁻KLRG1⁺ phenotype gave rise to Eomes⁺KLRG1⁺ cells, and subsequently memory
Eomes$^+$KLRG1$^-$ cells. Further, Eomes$^+$KLRG1$^-$ cells directly gave rise to Eomes$^+$KLRG1$^-$ memory cells without a transition into KLRG1 expressing phenotypes. The argument could be made that these cells were “pre-destined” to become memory cells, however, given the fluidity of conversion between these populations, and the fact that multiple populations generated memory, I find that the Separate Precursor Model does not adequately explain my results or the ability of ThEO phenotype cells to persist.

The Decreasing Potential Model suggests that all naïve cells have memory potential, though, it is through multiple signaling processes that memory formation is lost or degraded. For example, repeated antigenic stimulation or prolonged exposure to inflammatory cytokines leads to steady terminal differentiation. In this model, strong TCR stimulation over a short period increases memory potential, and in this case T cells would be more likely to form Tcm. Cells that integrate more signals, whether they are prolonged antigenic stimulation or increased exposure to cytokines, would thereby gain a more terminally differentiated phenotype (i.e. Tem, Teff). While some aspects of 4-1BB agonist-induced memory potential, and in particular ThEO/TcEO cell differentiation, support this hypothesis, other data does not. The fact that T cells in general, and in particular antigen-specific TcEO cells in the tumor microenvironment possess a more terminally differentiated phenotype (based on CD27/CD28 expression as well as CX3CR1), than splenic TcEO phenotype cells would support this hypothesis. Adoptive transfer of Eomes-YFP$^+$ T cells, on the other hand, would suggest something else entirely. The fact that circulating populations of traditionally defined terminally differentiated Eomes$^+$KLRG1$^+$ cells convert to a ThEO phenotype, and from there to an Eomes$^+$KLRG1$^+$ traditional memory phenotype would suggest that terminally differentiated cells are, in fact, gaining memory potential and not losing it. Multiple caveats, however, exist in this setting. For instance, antigen exposure would be much higher in the tumor than in the spleen, as would the complexity of the cytokine milieu
in the tumor microenvironment. Therefore, TcEO/ThEO phenotype TIL may be predisposed to become terminal effectors, whereas splenic TcEO/ThEO cells may be more likely generate true memory. Moreover, it should be noted that in my model, Eomes-YFP+ populations were adoptively transferred into naïve mice. Without persistent antigen exposure, and minimal exogenous cytokine production, these cells may not have converted into memory in a system of prolonged antigen exposure.

The third model, the Signal Strength Model, posits that the strength of TCR signaling, co-stimulation, and cytokine exposure can impact memory potential. Cells that receive very strong TCR signaling are more likely to terminally differentiate. This model diverges from the Decreasing Potential model, as early signaling events are more likely in the Signal Strength Model to lead to divergence in memory potential. The emergence of the ThEO phenotype, and the ability of 4-1BB agonists to support memory formation does not strongly support this hypothesis. Since 4-1BB is known to act as a strong costimulatory signal, supporting stimulation through the TCR, amplifying downstream signaling through the MAPK, NFκB, and NFAT pathways (99-102), abundant signal strength leading to decreased memory potential does not support the role of 4-1BB signaling in generation of immunological memory. Further, the increased effector potential and proliferative capacity of ThEO phenotype cells would suggest that they, too, are receiving strong activation signals and are still capable of persisting.

The fourth model of memory potential, the Asymmetric Cell Fate Model, suggests that the T cell position in the context of activating antigen-presenting population aids in memory differentiation. In this model, upon contact with an APC, a T cell will receive strong antigenic stimulation, co-stimulation, and cytokine output from the neighboring APC would then lead the T cell to adopt a more terminal effector phenotype. This stimulation would lead to subsequent proliferation. Daughter cells generated from this initiating T cell, dividing more distally from the APC, would receive
diminished signaling, and thus be more likely to form true memory. This model is neither fully supported nor contradicted by data presented here, although myeloid intrinsic 4-1BB activation outlined in Chapter 6 may support a role for APC-mediated memory generation within the context of the Asymmetric Cell Fate Model.

Overall, the Decreasing Potential Model most closely aligns with data presented here. It is possible that 4-1BB co-stimulation is capable of subverting some of the signaling processes that lead to terminal differentiation, halting complete differentiation in favor of effector memory formation. This is supported by my data which suggests that the majority of TcEO/ThEO phenotype cells are in fact Tem.

Given the data presented within this body of work, in particular the adoptive transfer of the Eomes-YFP subsets, it is also possible that 4-1BB co-stimulation alters the paradigm of memory potential. A new Gain of Memory Potential Model in the context of 4-1BB, and potentially all, co-stimulation may also help to explain why cells with a terminally differentiated phenotype transition into a ThEO and conventional memory phenotype. It is also likely, however, that cells at the “edges” of these populations (e.g. Eomes$^{lo/med}$KLRG1$^+$; Eomes$^{hi}$KLRG1$^{lo}$) are in fact the cell populations that are transitioning into other subsets and not the entire population as a whole. Nevertheless, work presented in Chapter 4 demonstrates that α4-1BB-generated ThEO phenotype cells definitively transition into the memory phase of the immune response and subsequently recall with potent cytotoxic potential.

7.3 Sca-1 as a novel marker of ThEO/TcEO phenotype memory cells

I also demonstrated that Sca-1 is a significant phenotypic marker of the ThEO/TcEO phenotype. While in mice Sca-1 acts as a marker for Tscm, prior studies demonstrated marked loss of CD44 expression in Tscm (389). My studies, however, demonstrated no loss of CD44 expression in ThEO phenotype cells or any Sca1$^+$ cell. Moreover, 4-1BB induced TcEO/ThEO phenotype cells with Tscm, Tcm, and Tem
phenotypes. While Tscm can give rise to both Tcm and Tem populations, it is still unclear whether this is the case within the context of ThEO polarization, or whether these three populations are distinct populations. Future single cell sorting, followed by RNA sequencing (single cell RNA-Seq) may help provide insight into these scenarios. Additionally, using a TMRM uptake assay, I showed that ThEO phenotype cells are TMRM\textsuperscript{low}, suggesting that memory ThEO cells are not highly metabolically active, in support of literature demonstrating minimal TMRM uptake in metabolically inactive Tscm cells (256). While TMRM indicates mitochondrial membrane permeability, it does not take into account total mitochondrial mass or cellular viability. Additional experiments using JC-1, a more selective membrane potential dye, or Mitotracker d.ye which enters the mitochondria regardless of membrane potential, may further provide insight into mitochondrial bioenergetics. Further, using the Seahorse assay system ThEO glycolytic and oxidative capacity could be measured. As suggested before, 4-1BB may induce Sca-1 expression, while blocking signaling leading to complete acquisition of a Tscm phenotype. Alternatively, Sca-1 may in fact serve as a bystander molecule upregulated by 4-1BB co-stimulation (possibly by increased STAT1 activation) which indirectly serves to increase T cell memory potential. It is interesting, though, that I found that α4-1BB induced Sca-1 expression (D5) before development of a true ThEO phenotype (D10) (data not shown). Given that STAT1 ablation impairs Sca-1 expression and ThEO formation without impacting Eomes or KLRG1 expression, one potential hypothesis could be that Sca-1\textsuperscript{+} cells may be a precursor population of ThEO development, and that without Sca-1 signaling, T cells may not be able to co-regulate Eomes and KLRG1 expression. This is but one avenue of future experimentation.
7.4 Further investigation of the effects of 4-1BB co-stimulation on memory potential

The ability of 4-1BB agonism to induce Sca-1 expression offers potential for future investigation. In one context, understanding what mechanisms are inducing Sca-1 in α4-1BB treated mice remains an open area of investigation. 4-1BB is known to induce other markers of stemness (Wnt signaling, TCF) (101), and Sca-1 may also act downstream of the 4-1BB signaling cascade. Moreover, Sca-1 is typically a marker of hematopoetic stem cells (390), and is rarely found on activated T cells (391). The signaling pathways initiated by Sca-1 are imprecisely understood, and the ligand for Sca-1 is still unknown. Induction of Sca-1 expression on an easily defined T cell population (ThEO) would allow for further investigation of this signaling pathway and perhaps determination of a ligand or ligands which activate Sca-1.

Further, while I suggest that Eomes⁺KLRG1⁺ ThEO cells originate from Eomes⁻ KLRG1⁺ cells and have a unique Tscm-like phenotype, isolation of RNA and further microarray analysis would confirm, on the transcriptional level, how similar the TcEO/ThEO phenotype was to each of these populations.

Lastly, I demonstrate that ThEO phenotype cells are metabolically homeostatic during the memory phase using a TMRM assay. This supports future investigation into the metabolic demands of ThEO phenotype cells, how this metabolic demand is maintained in the hostile tumor microenvironment, and potentially even how metabolism controls genetic and epigenetic regulation of the ThEO phenotype throughout the memory phase.

7.5 Runx2 expression and the importance of STAT signaling in ThEO phenotype cells.

In Chapter 5 of this dissertation I found that TcEO/ThEO phenotype cells express the runt-related transcription factor Runx2. While stimulation of other TNF receptor family members also induced Runx2, most likely due to increased TCR stimulation/NFkB activation, agonists targeting these receptors failed to induce a
strong, persistent ThEO/TcEO response. Further, collaborators in the Hwu lab found that Runx2 overexpression lead to heightened cytotoxicity at the expense of survival (Mbofung et al, unpublished). I demonstrated that, through an unclear mechanism, TcEO/ThEO phenotype cells were able to maintain Runx2 expression while sustaining longevity, although the mechanism driving this process must still be worked out.

I then demonstrated that, through ablation of STAT signaling, specifically in T cells, both STAT1 and STAT3 play a role in TcEO/ThEO development, as do type I interferons and IL-27. Future work need yet uncover the particular signaling processes driven through STAT activation that regulate Eomes and Runx2 expression as well as governance of other ThEO phenotypic markers.

7.6 Future investigation into the role of Runx2 and STAT signaling in ThEO development

Much work remains in order to uncover the exact roles of Runx2 and STAT signaling in ThEO cell development. For instance CD4^{Cre}/Runx2^{f/f} or CD4^{Cre}/Eomes^{f/f} mice could be used to investigate the impact of Runx2 on Eomes expression (or vice versa) and/or the long-term persistence of the ThEO phenotype.

Further, Co-immunoprecipitation (Co-IP) and chromatin-immunoprecipitation (Ch-IP) would provide further insight into the transcriptional activity of these transcription factor binding associations with each other, as well as with the DNA template. For instance, cell lines (e.g. Jurkat; THP1) could be transfected to express 4-1BB and then co-stimulated for varying lengths of time. Cells could then be processed and analyzed by reverse phase protein array (RPPA) to validate which transcription factors and signaling pathways 4-1BB is capable of inducing. Co-IP analysis could then investigate unique protein-protein binding interactions induced by 4-1BB stimulation. For instance, Runx2/Eomes binding interactions or interactions between distinct STAT family members may be elucidated. These novel binding
interactions may subsequently lead to different interactions between transcription factors and the chromosomal structure.

Intriguingly, within the Runx family, Runx3 acts as an epigenetic modifier, able to maintain DNA in an open reading state, or an inaccessible closed state (392, 393). In fact, in some instances, the Runx factors act as a transcriptional tumor suppressors by maintaining closed chromatin (393-396). Runx2 as well, has been shown to have “bookmarking” potential, leaving chromosomal loci in “poised” states ready for future transcription (397, 398). Runx2 may act in this fashion to keep certain gene loci open and ready for STAT and/or Eomes binding in the context of ThEO polarization or memory generation. A much more critical question remains, however: How is Runx2 being regulated by α4-1BB, and how are ThEO cells able to persist if Runx2 functions as a pro-apoptotic factor? Insight into the first question may be given by the ability of 4-1BB co-stimulation to induce Wnt signaling/TCF1 (101), which induces Runx2 expression (399), that acts as a co-factor for TCF family members (400, 401). Insight into the second question may be addressed given the necessity of STAT1 to initiate development of the ThEO phenotype. In the context of osteogenesis, STAT1 plays a role in sequestering Runx2 in the cytoplasm, preventing DNA binding and transcriptional control by Runx2 (402) and so STAT1 may in fact act to suppress the pro-apoptotic effects of Runx2 on the context of ThEO development. Whether these hypotheses bear out in the case of ThEO polarization remain to be tested, however, nuclear extraction and Co-IP would help to shed light on these insights.

Each distinct subset of helper and cytotoxic T cells (Th1/Tc1; Th2/Tc2 etc) uses a unique set of transcriptional regulators to maintain its function. Further, some subsets, particularly newly discovered subsets, demonstrate similarity to established T cell phenotypes. For instance, a recently described Th22 helper T cell subset bears similarity to Th17 cells (both make IL22 and IL23, and are regulated by ROR) (403-405). Further, Th9 cells are similar to Th2 cells as both play a role in allergy and are
induced by IL-4 (406-409). Similarly, ThEO cells appear distinct, but closely related to Th1 cells. Th1 cells are driven by IFNγ and IL-12 through STAT1 and STAT4 to induce Tbet, thus reinforcing Th1 responses. In the same vein, ThEO cells appear to be driven by type I interferons (IFNα) and IL-27 through STAT1 and STAT3 to induce Eomes. These cytokines and transcription factors are within the same families, but appear to drive distinct responses and may account for both the similarities and differences in polarization.

Further, new evidence suggests that for each helper subset there exists a Treg subset with similar transcription factor usage that selectively suppresses individual subsets (410, 411). For example, Tbet+Foxp3+ Tregs selectively suppress Th1 cells and Tfr cells suppress Tfhl cells. We and others have shown that α4-1BB agonists induce a population of Eomes+KLRG1+Foxp3+ Treg cells with cytotoxic capacity (111). While it is still unclear whether 4-1BB agonism inhibits or expands Treg polarization and function (412-415), ThEO-like Tregs (TrEOs) may act as an analogous ThEO phenotype to selectively suppress ThEO cells through either indirect (TGFβ, IL-10) or direct suppression/killing (Granzymes, FasL)(416, 417).

Lastly, many of the helper T cell subsets appear to exist as mutually exclusive pairs. For instance, Th1 and Th2 cells exist as a polar dichotomy; IFNγ and Tbet inhibit GATA3 expression and DNA binding, and conversely, IL-4 inhibits Tbet and IFNγ production (153, 154). This Th1/Th2 distinction is also apparent in the dichotomy between Th17/Treg cells as well (418). Anti-4-1BB therapy promotes a Th1-like phenotype while suppressing Th2 and Th17 responses (108, 419-422), and Eomes inhibits RORγt and Foxp3 (423, 424); however, the “polar opposite” of a ThEO phenotype cell is not clearly defined. The “opposite” of the ThEO cell may be one of the defined T cell populations, or a novel, undiscovered phenotype.
7.7 *Use of 4-1BB agonists in treating multiple tumor models*

I next demonstrated that 4-1BB therapy synergizes with an HPV peptide vaccination strategy to treat an HPV E7-transformed lung epithelial cell line. This method proved extraordinarily efficacious and has, in fact, lead to a clinical trial at MD Anderson investigating the use of an FDA approved HPV synthetic long peptide vaccine in combination with 4-1BB agonists for the treatment of HPV-related malignancies (425, 426). Further research is needed to investigate the applicability of peptide vaccination strategies in combination with α4-1BB or other immune-modulatory drugs for other HPV-related cancers (e.g. head and neck, anal, penile). This strategy may be further extrapolated to include vaccination against other known tumor antigens (e.g. NY-ESO, mesothelin, Muc1)(5). Interestingly, this strategy is already being tested in a similar manner using chimeric antigen receptor (CAR) T cells. By transfecting T cells to express an antibody Fab fragment (e.g. against CD19) linked to the TCR zeta chain for signaling and the 4-1BB endodomain for co-stimulation, the 4-1BB infused CARs have proven to be efficacious tumor killers, though these 19-4-z CAR cells do not persist long-term (41, 427-429). The exquisite ability of 4-1BB to enhance multiple forms of anti-tumor therapy, though, should not be overlooked in the treatment of multiple tumor models (1).

7.8 *ICOS expression in the vaccine setting*

Within the context of HPV vaccination in combination with α4-1BB therapy, I also demonstrated an ability for the HPV peptide vaccine to induce ICOS expression on T cells, be they CD8, CD4, or ThEO cells (33). The exact mechanism through which intranasal peptide vaccination elicits ICOS expression remains elusive. The vaccination strategy may in fact activate unique mucosal populations of antigen presenting cells or NKT cells (using αGalCer as an adjuvant) which in turn stimulate T cells through unclear mechanisms. This remains to be determined and warrants further
research. One exciting finding from this research demonstrates that while α4-1BB therapy can induce a cytotoxic ThEO phenotype, selection of distinct therapeutic agents may further modulate the ThEO phenotype to become more effective tumor killers. It should also be noted, however, that ICOS expression has been linked to terminal differentiation (430), and the highly activated ICOS⁺ ThEO phenotype cells may be marked for terminal differentiation and fail to elicit long-term immune protection. Whether this is indeed the case has yet to be determined.

7.9 4-1BB mediated activation of myeloid cells

In understanding the mechanisms that underlie 4-1BB agonist induced hepatotoxicity, I found a role for 4-1BB mediated myeloid activation and subsequent initiation of liver pathology through expression of IL-27. While multiple populations of myeloid cells express 4-1BB (1, 87), the exact role that 4-1BB plays in myeloid activation remains incompletely understood. Whether 4-1BB agonists induce a ThEO-like phenotype in myeloid populations, as well as the role of Eomes as a marker of activation/effector function within myeloid cells, remain areas of future interest. We and others, though, have begun to unravel these questions demonstrating that liver-resident myeloid cells are activated throughout the course of α4-1BB therapy, and subsequently produce IL-27 and TNFα. Further, it should be noted that distinct myeloid subsets influence polarization of helper T cell subsets. For instance, DCs and M1 macrophages polarize Th1 responses, whereas M2 macrophages, basophils and eosinophils aid in the polarization of Th2 cells. Presumably, though not definitively, I show that IL-27 producing CD11b⁺CD68⁻F4/80⁺ Kupffer cells in the liver may be responsible for ThEO polarization. The relative roles of each liver resident myeloid subset (CD11b⁺CD68⁻, CD11b⁺CD68⁺, CD11b⁻CD68⁺) in regards to liver toxicity and ThEO polarization needs yet to be worked out. Further, the phenotype of the corresponding myeloid populations within the secondary lymph nodes and tumors that
may be responsible for ThEO polarization must be confirmed. It would be of interest, though, to determine in what way T cells are interacting with myeloid cells in the secondary lymphoid organs and peripheral tissue in order to generate a ThEO phenotype.

7.10 **Impact of the gut microbiota on α4-1BB efficacy/induced hepatic damage.**

Within the liver toxicity study, several interesting new developments came to light regarding the role of the gut microbiota in mediating α4-1BB induced liver pathology. During early tests attempting to induce liver toxicity, I noticed that I was generating lower levels of serum transaminases than previously reported, or previously induced at facilities at Memorial Sloan Kettering (105). In order to achieve the most severe liver pathology, I had to administer a supra-clinical dose of α4-1BB (250ug/inj instead of 150ug/inj). I also found that Taconic mice, as opposed to mice from Jackson Laboratories, more easily developed pathology. It is well established that Taconic mice and Jackson mice have different microbiomes (431, 432). Further, the longer I waited after delivery of the mice to MD Anderson before treatment, the less pronounced the liver toxicity became, most likely due to a change of gut flora within the animal facility. These observational data suggest that microbiota may have a role in α4-1BB induced liver toxicity. The role of the microbiome in the clinical efficacy of immunotherapy is not without precedent. The anti-tumor potential of both αCTLA-4 and αPD-1 checkpoint blockade therapy rely, to some degree, on the gut flora (433-435). Anti-4-1BB therapy, and potentially all immunotherapy, may be affected by the patient’s gut flora. How the gut microbiome changes during α4-1BB therapy, and what microbes are critical for liver pathology and/or anti-tumor potential, remains an open area of investigation.
7.11 Implications for combination therapy

The liver toxicity study brings up intriguing implications for future combination with 4-1BB targeted therapy. I showed that α4-1BB/αCTLA-4 therapy decreased liver toxicity, while increasing anti-tumor immune responses (130). Further, α4-1BB/αPD-1 therapy may increase anti-tumor responses at the cost of increased liver damage (131). The triple combination also severely exacerbates hepatic damage. This further emphasizes that combination therapies, especially in the clinic, need to be carefully designed. The fact that Ipilimumab and Nivolumab work well as monotherapies, does not necessarily indicate that they will work well with other therapies without consequences.

I also provided data suggesting that α4-1BB/αCCR2 combination therapy may prove effective at increasing anti-tumor responses while decreasing liver pathology. While I did not intricately examine the method by which ablation of CCR2 decreases α4-1BB mediated liver pathology, it is clear that CCR2 inhibition disrupts Treg recruitment to the tumor microenvironment and may impair monocytes in the tumor bed as well(436-438). Further, small molecule inhibitors of CCR2 are already in the clinic and may offer exciting potential synergism with α4-1BB. How α4-1BB/αCTLA-4/αCCR2 triple combination therapy would impact clinical responses remains to be seen; however, there were no inherent signs of overt toxicity in either individual combination with α4-1BB. Further, whether a triple combination would provide any significant overall benefit over dual combination therapy remains to be seen. As mentioned before, combination therapies must be well thought out before implementation in clinical trials. This is further illustrated in the initiation and maintenance of ThEO polarization. For example, STAT3 inhibitors appear to be potent anti-tumor agents in the context of glioblastoma and diffuse large B cell lymphoma (DLBCL) amongst other tumors (439-446). However, as the ThEO phenotype relies on
STAT3 for polarization, combination of 4-1BB agonists with STAT3 inhibitors may prove counterintuitive. This further emphasizes the need to understand the biology driving anti-tumor responses and the mechanisms by which anti-tumor agents work before adequate combinations are put in place.

7.12 Use of the ThEO phenotype as a biomarker of clinical response

Lastly, I demonstrated that T cells in the peripheral blood of patients receiving α4-1BB therapy (Urelumab) demonstrated signs of ThEO polarization and may serve as a biomarker of clinical outcome. While expression of 4-1BB acts as a biomarker of tumor reactive T cells (447), it is unclear whether these 4-1BB+ cells are in fact ThEO-like cells or are more likely to generate ThEO populations. Perhaps greater numbers of ThEO polarized cells with greater persistence will lead to better clinical outcomes with fewer recurrences. While the sample size was small in this initial screen, and the ThEO phenotype was not was definitively characterized, these data suggest that the ThEO phenotype is clinically relevant and warrants further investigation. I plan to expand the number of patient samples analyzed including patients on Urelumab (BMS) as well as Utomilumab (Pfizer) as monotherapies as well as in various clinically relevant combinations. This will expand the sample pool, and allow us to make valid conclusions based on these findings. Further, I will be able to extend our phenotypic characterization to investigate human T cell memory markers and effector potential within responding human tissue.

7.13 Adoptive transfer of ThEO phenotype cells as a therapeutic strategy

Given the inherent toxicity in the administration of α4-1BB antibodies, one enticing therapeutic strategy involves the ex vivo polarization and expansion of either patient blood or TIL towards ThEO phenotype cells, before reinfusion into patients as a means of adoptive cellular therapy (ACT). This strategy would bypass potential in
vivo toxicity, and, additionally, liver myeloid cells would not be activated in addition to T cell populations. Excitingly, the use of 4-1BB agonists are already in use at MD Anderson for the rapid expansion of extracted TIL pre-infusion (448-450). Given my preliminary work in converting naïve T cells into ThEO-like effectors cells through in vitro polarization, it may be possible in the future to eliminate α4-1BB altogether, and simply use a small cocktail of cytokines to polarize patient TIL into a ThEO phenotype before reinfusion. Further, given that adoptive transfer of Tscm cells have superior longevity and anti-tumor potential (256, 451, 452), ThEO phenotype Tscm may be optimal candidate cells for ACT.

7.14 Role of Eomes as a lineage defining transcription factor

Throughout this dissertation work I have demonstrated that the TcEO/ThEO phenotype stable, persisting well into the memory phase. I have also demonstrated that ThEO polarization requires unique STAT signaling (STAT1, STAT3) in order to elicit a unique function (cytotoxicity). I, therefore, suggest that ThEO phenotype cells be included in the list of unique T cell subsets alongside Th1 and Th2 cells. The role of Eomes as a lineage defining transcription factor is not without precedent. Eomes+ NK cells are phenotypically and functionally distinct from Tbet+ NK cells (279, 453). Similarly, Eomes also delineates a unique population of γδ T cells with similar functions to ThEO cells (454, 455). Further, Eomes+ cytotoxic CD4 T cells (ThCTL) have been characterized in the context of flu infection in the lung. While ThCTL are characterized as Eomes+Blimp1+ and express NKG2A, NKG2C, and NKG2E, I find that ThEO cells are in fact largely Blimp1 negative and lack expression of these NK receptors (152). Interestingly, tumor infiltrating ThEO phenotype cells are in fact Blimp1+, possibly suggesting a requirement for Blimp1 in tissue infiltration and/or initiation of terminal differentiation (456, 457). This suggests that these populations may be two different forms of the same cell population, as ThEO cells induced at
mucosal barrier sites may receive different signals than within the secondary lymphoid tissue, leading to acquisition of distinct phenotypic characteristics.

What signals are inducing Eomes expression also remains unclear. On the one hand, Eomes expression may be a direct consequence of the signaling cascade induced by 4-1BB. On the other hand, cytokines induced by 4-1BB co-stimulation may act to reinforce Eomes expression. Two candidate cytokines are IFNα and IL-27, both of which are known to induce Eomes (314, 458). Early results testing the ability of ThEO phenotype cells to produce these cytokines in an autocrine manner remain inconclusive and need further validation. Further, the mechanism by which α4-1BB therapy, through intrinsic signaling or extrinsic cytokine production, favors Eomes over Tbet expression, especially in the context of ThEO polarization, remains to be investigated.

7.15 Role of ThEO phenotype cells in a global immune context

In addition to anti-tumor responses (33, 87, 219, 220), ThEO-like cells have been described in the flu setting (152) as well as LCMV and Listeria (87). The question remains though: What is the ultimate purpose of a ThEO phenotype cell? Why should ThEOs be classified as a distinct T cell polarity? While these questions need significant future investigation, the immune system may have developed methods to eliminate pathogens that were difficult to clear from the body. Initiation of a killer T cell program to combat pathogens that efficiently evade the immune system would be beneficial in the long-run. For instance, if a pathogen developed a protease inhibitor to eliminate the cytotoxic potential of granzyme B, a ThEO phenotype cell could produce additional granzymes (e.g. granzyme D, granzyme K) to overwhelm the pathogen’s defenses. Moreover, a cytotoxic CD4 subset would be able to recognize, through MHC-II, and kill pathogenically infected APCs or transformed hematological malignancies. While these questions have yet to be addressed, answers would provide
key insight into the role of ThEO phenotype cells in the overall immune response. For instance, during the course of tuberculosis, mycobacteria infected alveolar macrophages form granulomas which subvert effective T cell mediated elimination (459). While IL-27 signaling is detrimental to bacterial control in this setting due to reduced TNFα and IFNγ production (460, 461), induction of IL-27 in conjunction with additional factors that induce ThEO polarization may be beneficial in clearing bacterial infection. Additionally, ThEO polarized cells produce tremendous amounts of IFNγ and TNFα, as well as multiple granzymes which may be able to overcome mechanisms used by macrophages to inhibit granzyme mediated bystander killing.

In conclusion, this dissertation work has expanded our knowledge of the mechanisms by which 4-1BB co-stimulation induces robust and durable anti-tumor responses. I have also demonstrated a potential mechanism for the efficacy of α4-1BB therapy in the tumor setting (i.e. generation of a ThEO phenotype) which may translate well into the clinical setting as a biomarker of therapeutic efficacy. I have extensively characterized the ThEO phenotype, which I believe to be a novel T cell polarity, and advanced our overall understanding of how 4-1BB induces memory formation, in particular stem-cell memory. I have uncovered transcriptional regulators of the ThEO polarity and in particular uncovered a novel transcription factor (Runx2) which may play a role in ThEO development and/or α4-1BB efficacy. My work has also advanced the use of α4-1BB in clinical oncology. I have shown a synergy between peptide vaccination and α4-1BB administration, as well as multiple potential therapeutic combinations which may limit hepatic damage while increasing anti-tumor immune responses. I further advanced our understanding of the mechanisms that drive 4-1BB agonist induced liver toxicity through myeloid activation in the liver. Overall, we have gained significant insight into a novel ThEO polarity, though much
work remains to be done, and many new and exciting questions remain to be answered.
BIBLIOGRAPHY

20. Whiteside TL. 2015. The role of regulatory T cells in cancer immunology. Immunotargets Ther 4: 159-71


74. Cooper D, Bansal-Pakala P, Croft M. 2002. 4-1BB (CD137) controls the clonal expansion and survival of CD8 T cells in vivo but does not contribute to the development of cytotoxicity. *Eur J Immunol* 32: 521-9

75. Lee HW, Park SJ, Choi BK, Kim HH, Nam KO, Kwon BS. 2002. 4-1BB promotes the survival of CD8+ T lymphocytes by increasing expression of Bcl-xL and Bfl-1. *J Immunol* 169: 4882-8


80. Melero I, Johnston JV, Shufford WW, Mittler RS, Chen L. 1998. NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies. *Cell Immunol* 190: 167-72


multifunctional immunomodulator and antigen delivery vehicle for the development of therapeutic cancer vaccines. *Cancer Res* 70: 3945-54


226


177. Abi Abdallah DS, Egan CE, Butcher BA, Denkers EY. 2011. Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation. *Int Immunol* 23: 317-26

upregulated colonic IL-17F expression in active Crohn's disease and analysis of the IL17F p.His161Arg polymorphism in IBD. Inflamm Bowel Dis 14: 437-45


230


a progressive increase in TGF-beta susceptibility of anti-tumor CD4+ T cell function. 

*Jpn J Cancer Res* 84: 315-25


257. Di Rosa F, Watts TH. 2016. Editorial: Bone Marrow T Cells at the Center Stage in Immunological Memory. *Front Immunol* 7: 596

major reservoir and site of recruitment for central memory CD8+ T cells. *Immunity* 22: 259-70


238


KLRG1 signaling induces defective Akt (ser473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells. *Blood* 113: 6619-28


252


Li J, Chen S, Xiao X, Zhao Y, Ding W, Li XC. 2017. IL-9 and Th9 cells in health and diseases-From tolerance to immunopathology. Cytokine Growth Factor Rev


419. Vinay DS, Kwon BS. 1999. Relative abilities of 4-1BB (CD137) and CD28 to co-stimulate the response of cytokine deflected Th1 and Th2 cells. *Immunobiology* 200: 246-63


patients with advanced or recurrent HPV16-induced gynecological carcinoma, a phase II trial. *J Transl Med* 11: 88


257


VITA

Todd Jacob Bartkowiak was born in Houston, Texas on September 19, 1986, the son of Renalda Marie Bartkowiak and James Patrick Bartkowiak. After completing his work at Klein High School, Klein, Texas in 2005, he entered Sam Houston State University in Huntsville, Texas. He received the degree of Bachelor of Science with a major in Biology from Sam Houston State University in May, 2008. For the next two years, he worked as a research technician in the Department of Diagnostic Sciences at the University of Texas Health Science Center. In August of 2011, he then entered the University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences. He received the degree of Master of Science with a major in Immunology in May, 2013. In August of 2013, he entered the University of Texas MD Anderson Cancer Center School of Biomedical Sciences. Todd completed this dissertation under the mentorship of Dr. Michael A. Curran in the Immunology Department at the University of Texas MD Anderson Cancer Center.

Permanent address:

9711 Halkirk St.

Spring, Texas 77379