THE MOLECULAR DETERMINANTS OF TUMOR CELL MODULATION OF IMMUNE SELECTION

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By

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The Molecular Determinants of Tumor Cell Modulation of Immune Rejection

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ABSTRACT

Over the past century, the identification of molecules that tumor cells utilize to manipulate the activation or survival of immune cells has proven difficult. Expression analyses have helped to discover the downregulation of PD-L1, TGF-β, and FasL in the tumor microenvironment. Mutation analyses have helped identify novel tumor-specific or –associated antigens presented to the cell surface through major histocompatibility (MHC) complexes. Unfortunately, these techniques often fail to recognize the complex cross-talk occurring between cells in the tumor microenvironment to influence the anti-cancer immune response. By employing an RNAi screen in vivo, we have parsed how gene knockdown in tumor cells affects the ability of the immune system to identify and eliminate malignant cells. To identify tumor cell derived immune modulators, the EO771 breast adenocarcinoma cell line derived from a C57Bl/6 mice was transduced via lentiviral vectors with a barcoded genome-wide murine shRNA library and engrafted in immune-competent and immune-deficient mice. By analyzing the relative shRNA representation in tumors grown in the presence of a functional adaptive immune system compared to those grown in immune-deficient mice, we discovered a subset of tumor-based genes whose knock-down affects immune recognition. Pathway analysis identified enrichment of shRNAs targeting previously identified immune regulators, including the
TGF-βR pathway and MHC class I antigen processing. By engrafting shRNA tumor cell lines targeting gene candidates in immune-competent and –deficient mice, we identified that CD47, Tex9, Pex14, and Sgpl1 play putative roles in T cell-dependent recognition and elimination of EO771 tumors. CD47, a known inhibitor of target cell phagocytosis by macrophages, also regulated the adaptive immune response. Two previously understudied molecules, Pex14 and Sgpl1, appear to have immune inhibitory functions, as their respective knockdown suppressed EO771 tumor growth and increased survival of immunocompetent mice. Tex9 serves as a potential tumor antigen or immune stimulant, as reduction of expression enhances tumor growth when EO771 tumors are grown in immunocompetent mice. Therefore, this functional in vivo screening approach enabled the discovery of CD47, Pex14, Sgpl1, and Tex9 as novel tumor-based modulators of anti-tumor adaptive immunity. To the best of our knowledge, we are the first to report the successful utilization of an in vivo functional genomics approach to identify novel tumor-based mediators of immune regulation.

INDEX WORDS: RNAi, Functional screening, Genome-wide, shRNA, Immune editing, In Vivo Screen
Dedication

I dedicate this work first to my family.

To my wife, Sam: Without you this wouldn’t have been possible. It is because of you that I have pushed myself to become the man and scientist that I am today. For this words are not enough, but for now, they will do. Thank you.

To my father and mother, John and Kelly: Your love, support, and encouragement have motivated me beyond compare. Everything I have done and will do is a testament to the quality of your parenting.

To my brother, Chris: Thank you for always being my best friend. Thank you for setting the bar high and showing me the importance of thinking critically.

To the friends and extended family that have helped along the way. Thank you for letting me be a part of our community.

And finally, this work is dedicated to all those who have been touched by cancer. It is for you that I work every day.
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<tr>
<td>APC</td>
<td>Antigen-Presenting Cell</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<td>CD16a</td>
<td>CD16 molecule; also known as FCGR3A, FCG3</td>
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<tr>
<td>CD25</td>
<td>CD25 molecule; also known as IL2RA, TAC Antigen, TCGFR, p55</td>
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<td>CD3 epsilon molecule; also known as CD3e antigen, IMD18, TCRE, T3E</td>
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<td>CD86 molecule; also known as B70, B7-2, B7.2, LAB72, CD28LG2</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>COX-2</td>
<td>COX2 molecule; also known as Cytochrome c oxidase subunit II</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4; also known as CD152, CELIAC3, GSE, GRD4, CD, ALPS5, IDDM12</td>
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<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EMP2</td>
<td>Epithelial membrane protein 2; also known as XMP</td>
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<td>EV</td>
<td>Empty Vector</td>
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<td>F4/80</td>
<td>F4/80 molecule; also known as Adgre1, adhesion G protein-couple receptor E1, Emr1, Ly71, Gpf480</td>
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<tr>
<td>FBS</td>
<td>Fetal-bovine serum</td>
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<tr>
<td>FDR</td>
<td>False discovery rate</td>
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<tr>
<td>FoxP3</td>
<td>forkhead box P3; also known as JM2, AIID, IPEX, PIDX, XPID, DIETER</td>
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<tr>
<td>G418</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>Gr-1</td>
<td>GR-1 molecule; also known as lymphocyte antigen 6 complex, locus G, Ly6g</td>
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HER2  Erb-b2 receptor tyrosine kinase 2; also known as ERBB2, NEU, CD340, NGL, TKR1
ICOSL  Icos Ligand; also known as B7h, Gl50, B7-H2, LICOS
IDO  Indoleamine 2,3-dioxygenase 1; also known as Ido, Indo
IgG  Immunoglobulin G
IHC  Immunohistochemistry
IL-17  Interleukin 17A; also known as CTLA8
IVP  In vivo passage
KD  Knockdown
MDSC  Myeloid-Derived Suppressor Cell
MHC  Major Histocompatibility Complex
MOI  Multiplicity of Infection
mRNA  messenger RNA
NK  Natural Killer
NOD  Non Obese Diabetic
NSG  NOD-SCID il-2 gamma chain
OS  Overall Survival
OX40L  OX40 Ligand; also known as tumor necrosis factor (ligand) superfamily, member 4, TNFSF4, CD252, GP34, CD134L
PBS  Phosphate Buffered Saline
PD-1  Programmed cell death 1; also known as PDCD1, CD279, SLEB2
Pex5  Peroxisomal biogenesis factor 5; also known as PXR1, PBD2A, PTS1R
<table>
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<tr>
<td>Pex14</td>
<td>Peroxisomal biogenesis factor 14; also known as NAPP2, PBD13A</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin molecule</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcript-polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SBI</td>
<td>System Biosciences</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immune Deficiency</td>
</tr>
<tr>
<td>Sec16b</td>
<td>SEC16 homolog B molecule; also known as RGPR, LZTR2, SEC16S</td>
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<tr>
<td>Sgpl1</td>
<td>Sphingosine-1-phosphate lyase 1</td>
</tr>
<tr>
<td>Sphk</td>
<td>Sphingosine kinase</td>
</tr>
<tr>
<td>shRNA</td>
<td>short harpin RNA</td>
</tr>
<tr>
<td>Tex9</td>
<td>Testis-expressed gene 9; also known as tsec-1, Gm19407</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor, beta</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor infiltrating lymphocyte</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
</tr>
<tr>
<td>Yif1a</td>
<td>Yip1 interacting factor homolog A (S. cerevisiae); also known as 54TM, TIF1, TIF1P, FinGER7</td>
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CHAPTER 1

TUMOR IMMUNOLOGY

1.1 INTRODUCTION

The concept of utilizing the host’s immune system to fight cancer has been enticing to researchers and physicians for over a century. It is a field that encompasses the knowledge gained from a wide range of disciplines and is an area of cancer therapeutics that has high potential to procure the ‘magic bullet’ for the treatment of cancer. This opinion is further strengthened by the relative successes of immunotherapy treatments like Trastuzumab, Bevacizumab, and Ipilimumab seen in the past decade. As is the same for all current cancer treatments, immunotherapy is far from perfect and often results in a high percentage of patients who become resistant to treatment. For the field to advance beyond its current state of success, a more in depth understanding of the immune system is required, including determining how cancer induces an immunosuppressive state in the tumor microenvironment and the global effects of this immunosuppression. The classic approach of reductionism will yield a wide breadth of knowledge that will aid in this quest but only through determining the complex interactions of the tumor with the immune system can a true magic bullet be discovered. This review will focus on the current state of immunotherapy with the hope of addressing concerns and problems facing this promising field.

1.2 IMMUNE EDITING THEORY

The field of tumor immunology began in 1901 when Paul Ehrlich hypothesized that the immune system inhibited the formation of cancer in organisms [1]. It wasn’t until
1957, when Burnet and Thomas proposed the idea of cancer immunosurveillance that the field gained prominence. This theory stated that the immune system has a direct regulatory effect on developing tumor cells [11]. This hypothesis was quickly discredited by numerous groups and did not gain any major recognition until 2001, when Robert Schreiber’s group revealed that tumors had different levels of immunogenicity, which he termed ‘edited’ or ‘unedited,’ depending on the immune competence of the host [1]. In this theory, if the host had an intact immune system, then the tumors faced a negative selection pressure by the immune system, which, if the tumors survived, resulted in the evasion from immune attack, hence an ‘edited’ version of the tumor. Likewise, a tumor growing in an immunosuppressed host would not be attacked by the immune system, resulting in no selection pressure and an ‘unedited’ tumor. With the addition of immunogenicity, the immunosurveillance theory shifted to the immunoediting hypothesis, which was further validated through studying the inability of unedited tumors to grow in an immunocompetent host. It was also determined that three phases of immunoediting exists: elimination, where the host’s immune system effectively destroys the tumor; equilibrium, where the tumor is held dormant by the immune system; and escape, where clinical symptoms of cancer would appear [1, 12]. For further evidence and explanation of the immunoediting theory, please refer to references 1 and 12.

Finally, the immune system has been shown to have two major roles in preventing the formation of cancer. First, it inhibits the infection of viruses that have the potential to promote tumorigenesis. Second, the immune system actively destroys newly formed tumors, usually through the recognition of tumor associated antigens (TAAs) or tumor specific antigens (TSAs) on the tumor cells [1]. TSAs are displayed solely by a tumor,
usually resulting from a mutation or from aberrant expression. For example, p53 and bcr-abl appear in their mutated form in cancer cells, which essentially marks these tumors as foreign to the immune system [5,6]. Tumor associated antigens are expressed at high levels by tumors but are also expressed in other cells throughout the body, which could cause the immune system to see the tumor cells as ‘self’ [5,6]. This isn’t always the case and these TAAs can activate the immune system. The best elucidation of this occurs when a patients’ immune system recognizes a TAA that is normally expressed on neurons, resulting in activated CD8+ T cells that attack both the tumor and the central nervous system [1,6]. The most common examples of tumor associated antigens are gp100, HER-2, NYESO, MAGE, MUC1, and PSA, all of which have been shown to elicit an immune response [1,5,6].

1.3 IMMUNE MODULATORY KEY PLAYERS

The complex interplay that occurs between the tumor, stroma, and immune cells in the tumor microenvironment influences the efficacy of an anti-tumor immune response. To help understand their significance, a detailed description of the important immune related cell types and molecules follows.

1.3.1 CYTOTOXIC T CELLS

As mentioned above, the patient’s immune system has the potential to fight off the growth of neoplastic cells. CD8+ T cells, CD4+ T cells, antigen-presenting cells (APCs), and B cells are the main mediators of this effect. CD8+ T cells, also known as Cytotoxic T Lymphocytes (CTLs), are armed with a receptor that can recognize a specific
antigen, and in the case of tumor specific CTLs, they recognize a TAA or a TSA [1,5]. Once an activated CTL recognizes a tumor, it has a direct cytotoxic effect on the cell. To be activated, the T-cell receptor complex (TCR) on the T cell must come directly in to contact with a tumor-specific antigen displayed by a presenting cells’ MHC [5]. Along with this first signal, a second stimulation signal is required for the activation of CTLs, usually done through B7.1 or B7.2 on the APC interacting with CD28 on the T cell [5]. The quantity of activated CTLs that have infiltrated the tumor microenvironment has been shown to be strongly correlated with increased overall survival, indicating the ability of the immune system to eliminate tumor cells [1].

1.3.2 CD4+ T CELLS

As another member of the anti-tumor immune response, CD4+ T cells, also known as CD4+ CD25- Foxp3- T cells, are an important regulator of other immune cells. Naïve CD4+ T cells can differentiate into T helper cells, mainly Th1, Th2, Th17 and Treg cells [9]. Tregs are involved specifically in immunosuppression whereas the other cell types generally mediate immune stimulation [6]. The activated CD4+ T cells stimulate macrophages, induce memory CD8+ T cells, and increase the population of primed CTLs, all of which results in a stronger immune response [6].

1.3.3 ANTIGEN-PRESENTING CELLS (APCS)

As one subtype of APCs, dendritic cells (DCs) are essential for the proper activation of the immune system. By processing cellular material in the body, dendritic cells can travel back into a lymph node to present the discovered peptides to naïve T and B cells, which, depending on the cytokine microenvironment of the lymph node, can

4
result in immune stimulation [6]. Indeed, the specific cytokines that can activate T cells are complex and often depend on the levels of other cytokines to create a certain response. Specifically, GM-CSF, IFN-γ, and IL-2 are some of the best known immune stimulating cytokines. Along with this, other co-stimulatory molecules exist, like CD40, CD209, CD137 and Dectin-1, on the surface of immune cells [1, 6, 10]. As an example, CD40 is found on numerous immune cells including DCs and macrophages and when activated increases the cell membrane expression of pro-immune receptors and peptide presentation [10]. CD137 acts to enhance the function of natural killer (NK) cells, macrophages, dendritic cells, and T cells [5].

1.3.4 REGULATORY T CELLS

With the ability to stimulate the immune system, there are also numerous mechanisms for inhibition, which can result in the prevention of autoimmune diseases or the promotion of tumor progression. The major cells that act to suppress the immune system are Tregs and myeloid derived suppressor cells (MDSCs); the major suppressor molecules are IL-10, IL-4, IL-35, TNF, TGF-β, VEGF, galectin and indoleamine 2,3-dioxygenase (IDO); and some inhibitory surface molecules are programmed death ligand-1 (PD-1), CTLA-4, and Fas ligand (FasL) [1, 2, 4, 5, 6, 7, 8, 9, 11]. As the major cellular regulator of the immune system, Tregs are important in the natural protection from autoimmune disease, but in cancer Tregs are often hijacked in the tumor microenvironment to foster an immunosuppressive state. In numerous cancers, including metastatic breast, gastric, lung, pancreatic, ovarian, and hepatocellular carcinoma, high levels of tumor infiltrating and peripheral Tregs correlate with a worse prognosis [7, 9]. Tregs, which express Foxp3 and IL-2R α-chain (CD25), directly inhibit CTLs, CD4+ T
cells, NK cells, natural killer T cells (NKT) and suppress the antigen processing ability of APCs, either through direct cell to cell inhibitory contact or through the secretion of many of the above immunosuppressive cytokines [9]. Along with this, they express very high levels of CD25, causing a significant depletion of the immunostimulatory cytokine IL-2 in the microenvironment, which results in fewer activated T cells and APCs [1, 9]. Tregs, through direct cell to cell contact with other cells of the immune system, can lyse CD4+ and CD8+ T cells [9]. Expressed on the surface of Tregs, CTLA-4 can directly bind to CD28 on T cells, with a 100-fold higher affinity than B7.1 and B7.2, which sends a potent inhibitory signal to the T cell, negating the secondary stimulus in the T cell [5, 9]. It is believed that CTLA-4 mediated inhibition of T cells is the primary protection from an over stimulated immune system, as mice lacking CTLA-4 develop numerous autoimmune diseases [9].

**1.3.5 MYELOID DERIVED SUPPRESSOR CELLS (MDSCS)**

As the second major cellular mediator of immune suppression, MDSCs are myeloid progenitor cells that normally evolve into APCs [1,8]. In cancer, numerous cytokines block the normal differentiation of these progenitor cells in the bone marrow, inevitably resulting in fewer immune stimulating APCs and a higher population of the suppressive MDSCs [8]. They secrete TGF-β, deplete levels of important T cell amino acids, through arginase (ARG1) and nitric oxide synthase (iNos), and increase the population of tumor-associated macrophages (TAMs) all of which actively suppresses the immune system in the tumor microenvironment [1,8].
1.3.6 INHIBITORY CYTOKINES

As mentioned above, certain cytokines act to inhibit the function of T cells and APCs. IL-10 acts through STAT3 to inhibit differentiation of APCs and expand Tregs, as well as inducing the expression of more IL-10 and TGF-β [11]. IDO stimulates the accumulation of Tregs in the microenvironment [11]. As one of the most commonly seen growth factors in the circulation of cancer patients, VEGF has a profound effect on the stimulation of new blood vessels as well as causing thymic atrophy [4]. It is thought that VEGF shrinks the thymus through inhibiting the proliferation of progenitor cells, eventually resulting in fewer T cells [4].

Although this is not an extensive review on the immune system, this information elucidates the intense complexity of possible interactions between the host protective and tumor suppressive environment. Any of these interactions could be targeted to foster a better anti-tumor immune response. Tumor cells, by releasing these immune suppressive cytokines or expressing ligands for inhibiting receptors on immune cells, can directly influence the lytic capacity of immune cells in the tumor microenvironment. This immune suppression can result in the reduction of proper processing of antigens by APCs, leading to an improperly stimulated anti-cancer specific immune response. The evolution of a nascent tumor to an immune suppressive tumor is incompletely understood but likely involves the clonal expansion of single malignant cells that exhibit immune suppressive capabilities. The underlying mechanisms behind this clonal expansion are also unknown and are an underlying research focus of the results presented in chapter 2.
As this review will go into more detail below, an individual immunotherapy treatment might provide a decent anti-tumor response but will most likely result in a resistant tumor. Only through a better understanding of the complexity of interactions in the immune system and a subsequent revision of our treatment scheme to more complex and combinatorial treatments, can we finally begin to ‘cure’ cancer.

1.4 IMMUNOTHERAPY

With a basic understanding of the key players involved in immunity, it is essential to identify methods to induce a potent anti-cancer immune response. The majority of immunotherapies attempt to stimulate an immune response through the activation of key immune cells. While this has proven effective, understanding and inhibiting the mechanisms utilized by cancer cells to evade lysis could increase survival and reduce recurrence of treated patients. Therefore, to enhance the clinical efficacy of these novel treatments, it is important to identify how tumor cells evade the immune response, which will be discussed in greater detail in Chapter 2. First though, a basic understanding of the current methods to enhance anti-tumor immunity will be discussed.

The three main types of immunotherapy are tumor vaccines, passive immunity through antibodies, and immunomodulation. Tumor vaccine interventions are very similar to the classic vaccine theory, with the purpose of priming an antigen specific immune response that hopefully results in the formation of an adaptive immunity against the cancer antigen [6, 11]. This theory utilizes TSAs and TAAs to elicit a host-protective response through activation of tumor specific T cells, APCs, or other immune cells.

Passive immunotherapy also utilizes tumor antigens but instead of directly targeting the
activation of the hosts’ immune system, it focuses on the administration of antibodies that target the tumor itself [1]. Once the antibodies attach to the surface molecules they can activate or inactivate the signals involved with the surface molecule but more importantly, they mark the tumor cells for destruction [10]. These marked tumor cells can be recognized by the FcR on NK cells which will directly cause lysis of the cell, in a process known as antibody dependent cellular cytotoxicity (ADCC). Along with this, the antibody marked tumor cells can be directly killed by the classic complement system, called complement dependent cytotoxicity (CDC) [10]. In this process, complement component 1q (C1q) binds directly to the Fc domain of the antibody and activates the membrane attack complex (MAC) that actively creates pores in the cell membrane, resulting in the lysis of the cell [10]. The resulting tumor fragments from both cytolytic responses can then be taken up and processed by APCs, especially DCs, whereupon they can migrate to the lymph nodes and promote tumor specific T and B cell priming [10].

The final therapy type, immunomodulation, focuses on manipulating the inherent immune signals to generate a host-protective immune response [1]. This includes the activation of anti-tumor immune stimulating receptors like OX40 and CD137, as well as inhibiting the function of immunosuppressive cells like Tregs through the inhibition of CTLA-4 and MDSCs through VEGF inhibition [8, 10]. These three therapies represent different approaches to delivering an anti-tumor immune response and their effectiveness is limited by the induction of tumor specific T cells and the negation of immune suppression caused by Tregs and MDSCs [1].
1.5 TUMOR VACCINES

1.5.1 ANTIGEN VACCINES

As mentioned above, tumor vaccines are designed to stimulate an immune response to particular antigens and this is done in two different ways, either exposing the host to a high dose of the tumor antigen or injecting the host with pre-primed lymphocytes [6]. In the first approach, a TSA or TAA is determined based on the patients’ cancer and high levels of this antigen are injected into the patient. This causes a direct immune response through the antigen processing capabilities of APCs, while bypassing the immunosuppressive microenvironment in the tumor, where these antigens reside [6]. In addition to the specific antigen, patients are often given an adjuvant that acts to directly stimulate the antigen processing abilities of the immune system [11]. The two main immune stimulators used are granulocyte-macrophage colony-stimulating factor (GM-CSF), which acts to stimulate the proper differentiation of APCs, and IFN-γ, which also stimulates the production of antigen presenting cells, macrophages in particular [11]. As an example of this, one therapy regime introduces the patient to MUC1, a TAA, along with the immune enhancer E6020, a TLR4 agonist. This resulted in the reduction of size and number of existing polyps in preclinical studies and prevented the clinical progression of irritable bowl syndrome to colitis in colon cancer [11]. A creative treatment alternative involves the infusion of antibody fused with a tumor antigen that target DCs [6]. This preclinical study resulted in a large increase in the population of antigen specific T cells [6].
1.5.2 DENDRITIC CELL VACCINES

A second type of tumor vaccine involves the priming of immune cells with the intent of activating a tumor-specific T cell response. This is done by isolating a patient’s own immune cells, mainly DCs, and exposing them to the tumor antigen and a stimulating factor, usually GM-CSF or IFN-γ, with the intent of activating CTLs and immune stimulating CD4+ helper cells against the patient’s own tumor [6]. Approved for the treatment of metastatic prostate cancer by the FDA in 2010, Sipuleucel-T (APC 8015) is the first approved vaccine for the treatment of cancer [6, 11]. Sipuleucel-T is created by isolating APCs from a patient and exposing the cells in vitro to the fusion protein of prostatic acid phosphatase (PAP), which is a common prostate antigen, and GM-CSF [6, 11]. In the phase III clinical trial that lead to its FDA approval, treatment with Sipuleucel-T prolonged the median survival by four months [6]. Along with GM-CSF, numerous phase III trials investigated using IL-2 as the stimulating cytokine, which also resulted in a significant increase in overall patient survival [6].

Although cancer vaccines show large promise, they also face significant problems. As this treatment regime requires the isolation of a patient’s cells, it is the epitome of personalized medicine. This correlates to semi-prohibitive expenses, is very time consuming, and is almost impossible to scale up. Along with this, the choice of antigen is important to the success and potential side effects of treatment. By choosing the more common tumor associated antigens, one runs the risk of inducing a potent autoimmune attack that can result in severe immune related adverse events (irAEs) [6]. One of the major problems facing the immunotherapy field as a whole is the inability to address the efficacy of an immune-stimulating therapy in a clinical trial. The current
parameters do not accurately determine if the immune system has been effectively activated. Therefore, it will be important for the future of immunotherapy to devise an intelligent, accurate and quantifiable parameter for tumor specific immune activation. Finally, as with any immunotherapy that does not address the immunosuppressive tumor microenvironment, these treatments must be potent enough to overcome the strong inhibitory signals evident in the tumor. Otherwise, the clinical applicability of the immunotherapy will be short-lived and resistant tumors will prevail.

1.6 SCREENING METHODS

Since the discovery of an endogenous mechanism of mRNA transcript degradation, researchers have utilized and advanced RNA interference (RNAi) technology, allowing for direct genotype to phenotype analysis. By knocking down the expression of a target transcript, it is possible to identify the significance of the gene in a functional assay. On top of this, by knocking down multiple transcripts across a population of cells, one can identify the effect of novel genes on a functional assay. The work described in Chapter 2 and 3 utilize a genome-wide RNAi library to identify novel genes utilized by tumor cells to manipulate the anti-cancer immune response. Therefore, it is important to understand the mechanisms the underlie target gene knockdown by RNAi.

1.6.1 TARGET KNOCKDOWN

The transcript knockdown is mediated by the delivery of a double-stranded RNA (dsRNA) segment that has partial sequence similarity to the target transcript [19]. Once introduced, the dsRNA is processed by RNA-induced silencing complex (RISC) and
Argonaute (Ago), whereupon the double stranded short interfering RNA (siRNA) sequence is separated and the guide strand binds to the target mRNA sequence. Upon successful linkage, the dsRNA segment is enzymatically degraded by Ago, thereby reducing the concentration of target transcript in host cells [18]. This method can be mediated by direct delivery of siRNA sequences into cells but as there is only a limited supply of siRNAs, the effect is transient. Introduction of a DNA segment that constantly transcribes short-hairpin RNA (shRNA) through lentiviral delivery can provide a constant supply of siRNA sequences, leading to stable knockdown of target transcripts. This method is riddled with issues limiting its utility but through adequate controls and repeats, it is possible to study the effect of target transcript knockdown in target cells.

1.6.2 SHORT-INTERFERING RNA SCREENS

By utilizing the transient but quick mRNA knockdown mediated by transfection of siRNA in a 96-well, arrayed format, it is possible to determine the significance of a transcript on a pathway, assuming that the effect is testable using an in vitro assay, including cell expansion, apoptosis, and autophagy. Depending on the scope of the experiment, the targeted transcripts can range from a few genes to targeted pathways, like EGFR, to the entire genome [16, 23, 21, 17].

1.6.3 SHORT-HAIRPIN RNA SCREENS

To understand the effect of gene knockdown in a more complicated experimental design, shRNAs are an excellent tool. By introducing a stable DNA segment into a host cells’ genome, it is possible to continuously knockdown a targeted transcript. By combining hundreds to thousands of shRNAs targeting different transcripts, it’s possible
to create a population of target cells, where each cell contains a different shRNA. Using this pooled format and applying a selection pressure such as a chemotherapeutic agent, cytotoxic immune cells, etc., it is possible to identify which shRNAs are gained/lost by selection [25, 24, 22]. The shRNAs are barcoded, in that they can be easily identified using sequencing or array technologies. Since both siRNA and shRNA screens produce a high rate of false positives, it is essential to validate each target using independent siRNAs or shRNAs [20].

1.7 CONCLUSION

As the immune system surveys neoplastic cells, highly immunogenic cells are selectively eliminated. Then, through clonal evolution, surviving cells are less recognizable by the immune system. By reducing the expression of the vital immune-related proteins, like major histocompatibility complex I (MHC CI), immune cells become shielded from dendritic cells (DCs) and cytotoxic T cells (CTLs) [26]. The importance of CTL mediated recognition of tumor cells is emphasized by the enhanced prognosis of patients with higher tumor infiltrating T cells for numerous advanced cancers, including advanced ovarian cancer [27]. Therefore, it is essential to discover novel mechanisms utilized by tumor cells to reduce immune recognition, allowing us to shift the balance of escape from the immune system back towards elimination. With that in mind, my research strategy focused on tumor-derived modulation of immune recognition. By discovering the molecular determinants that tumor cells utilize to shield themselves from the immune system, I hoped to uncover previously unknown targets for therapy aimed at enhancing the anti-tumor immune response. An RNAi model system employing the use of a lentivirally delivered pooled shRNA library targeting the entire
murine genome was utilized in order to discover previously unknown functional mediators of immune recognition. With the results identified in this dissertation, it would be possible to enhance the efficacy of immunotherapy by targeting the mechanisms utilized by cancer cells to shield themselves from immune elimination.

What follows next is two recent reviews of monoclonal and bispecific antibody therapy for the treatment of cancer. As material written during the duration of my PhD, they represent important material towards my total body of work, as well as potential therapeutic targeting mechanisms of tumor-associated molecules discovered through my dissertation research.
1.8 BIBLIOGRAPHY


CHAPTER 2

MONOCLONAL ANTIBODIES FOR THE TREATMENT OF CANCER

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2.1 ABSTRACT

Over the past decade, the clinical utility of monoclonal antibodies has been realized and antibodies are now a mainstay for the treatment of cancer. Antibodies have the unique capacity to target and kill tumor cells while simultaneously activating immune effectors to kill tumor cells through the complement cascade or antibody-dependent cellular cytotoxicity (ADCC). This multifaceted mechanism of action combined with target specificity underlies the capacity of antibodies to elicit anti-tumor responses while minimizing the frequency and magnitude of adverse events. This review will focus on mechanisms of action, clinical applications and putative mechanisms of resistance to monoclonal antibody therapy in the context of cancer.

Keywords: Monoclonal Antibody, Cancer, Immunology, Drug Resistance, Antibody-dependent cellular cytotoxicity (ADCC), Complement Dependent Cytotoxicity (CDC), Antibody-like Molecule

2.2 INTRODUCTION

The concept of utilizing immunotherapy for the treatment of cancer has been enticing to researchers and clinicians for over a century. Cancer immunotherapy encompasses knowledge gained from a wide range of disciplines and has the potential to procure the 'magic bullet' for the treatment of cancer. The advent of hybridoma technology in 1975 and the development of chimeric, humanized, and human antibodies have increased the availability and utility of immunotherapy for the treatment of cancer [1]. Currently, eleven antibodies are approved for use in oncology, nine of those occurring in the past decade [2]. By targeting tumors through specific or associated antigens, it is
possible to selectively eliminate tumor cells and maintain an acceptable toxicity profile. Therapeutic antibodies that target immune cells are also being developed with the goal of breaking local tolerance and stimulating the patient’s anti-tumor immune response. As with other treatment modalities, immunotherapy is far from perfect and requires additional study to optimize clinical response and overcome therapeutic resistance. This review will focus on the current state of cancer immunotherapy with the hope of highlighting mechanisms of action, clinical utility, and determinants of resistance of monoclonal antibody therapy.

2.3 STRUCTURE

Antibodies, or immunoglobulins (Igs), exist in five separate forms denoted from differences in their constant region, which gives them unique properties and functions. They are IgA, IgD, IgE, IgG, and IgM, with IgG being the isotype most commonly used in cancer immunotherapy. Antibodies have two antigen binding fragments (Fabs) and one constant fragment (Fc). The Fab confers antigen specificity via complementarity determining regions (CDRs) while the Fc domain connects IgG antibodies to immune effector mechanisms by engaging Fcγ receptors (FcγRs) on natural killer (NK) cells, neutrophils, monocytes, dendritic cells (DCs) and eosinophils [3]. The Fc region also binds neonatal Fc receptors (FcRns), which is thought to protect circulating antibodies from degradation [4].
2.4 MECHANISMS OF ACTION

Antibodies exert an anti-tumor effect through four mechanisms: perturbation of tumor cell signaling, activation of complement dependent cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC), and induction of adaptive immunity [5].

2.4.1 SIGNALING PERTURBATION

Antibodies can be designed to target soluble mediators such as cytokines to inhibit their ability to bind to receptors and induce signaling, or they may target membrane bound receptors, where they can act as agonists or antagonists. Growth factor receptors, such as epidermal growth factor receptor (EGFR), are often overexpressed on tumors and antagonistic antibodies inhibit their ability to mediate mitogenic signaling [6–7]. Likewise, antibodies can inhibit immune suppressing receptors, e.g. CTLA-4, or enhance antigen presentation on APCs through the activation of receptors such as CD40 [8–9]. Of the four subsets of IgG antibodies, IgG2 and IgG4 do not activate CDC or ADCC and are designed primarily for signal perturbation. This often results in fewer immune related adverse events (irEVs) as a consequence of non-specific immune activation [10].

2.4.2 COMPLEMENT DEPENDENT CYTOTOXICITY (CDC)

As part of the innate immune system, complement is one of the first mediators of the immune response to pathogens and cells with compromised ‘self’. It is a complex proteolytic cascade comprised of over thirty proteins that act to lyse foreign cells through assembly of the membrane attack complex (MAC), stimulate inflammatory processes through anaphylatoxins, and remove opsonized targets [11–12]. When two or more
antibodies bind to a cell, the classical complement pathway is activated through the binding of the C1 complex, a serine protease consisting of C1q, C1r and C1s, to the antibody’s Fc domains. This activates a proteolytic cascade that leads to the formation of the MAC and the release of potent anaphylatoxins and opsonins resulting in cell lysis and phagocytosis [11, 13].

CDC is an important contributor to the anti-tumor activity of many therapeutic antibodies. Rituximab, a type I chimeric antibody targeting CD20, is approved for treatment of many B-cell malignancies and is a potent activator of CDC. As a type I anti-CD20 antibody, rituximab inhibits internalization and shedding of CD20 and shifts CD20 onto lipid rafts, increasing the likelihood of complement activation through the assembly of rituximab-bound receptors [14]. To emphasize this, an in vivo lymphoma model found that loss of C1q abrogated the protective effects of rituximab therapy [15]. The connection between CDC and efficacy of rituximab is also seen clinically where polymorphisms in the C1qA gene in patients with follicular lymphoma are associated with response to rituximab therapy [16]. The importance of CDC to the clinical efficacy of rituximab is not without controversy. There is evidence to suggest that binding of C3b, an important effector protein in the complement cascade, to the cell surface of tumor cells inhibits rituximab mediated ADCC and that inhibition of C3b enhances efficacy of rituximab in vivo [17–18].

Ofatumumab is another type-I anti-CD20 antibody that binds to a distinct epitope of CD20 and induces greater CDC compared to rituximab [19]. Ofatumumab has been reported to bind C1q with greater avidity than rituximab and efficiently kills rituximab-resistant large B-cell lymphoma cell lines [20]. In addition, ofatumumab is able to lyse
cell lines expressing low levels of CD20, which are not efficiently killed by rituximab [21]. In clinical trials, ofatumumab showed high response rates in patients with refractory chronic lymphocytic leukemia (CLL), leading to its approval by the FDA in 2009 [22].

Activation of the complement cascade may be partially responsible for irEVs associated with antibody therapy. One small clinical study found an association with high circulating levels and rapid accumulation of circulating complement components and severe toxicity following rituximab therapy [23].

2.4.3 ANTIBODY DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC)

The Fc domain of antibodies can activate ADCC through interactions with FcγRs on effector immune cells. The stimulation of immunoreceptor tyrosine-based activation motifs (ITAMs) and immunoreceptor tyrosine-based inhibitory motifs (ITIMs) results in activating or inhibitory signals through FcγRs, respectively. There are three activating FcγRs: FcγRI (CD64), FcγRIIA (CD32A), and FcγRIIIA (CD16A) and one inhibitory receptor, FcγRIIB (CD32B)[24–25]. Natural killer (NK) cells, which predominantly express FcγRIIIA, are the main effector cells of ADCC, although macrophages and granulocytes cells have been shown to mediate ADCC to a lesser extent [25]. These effector cells, through FcγRs, recognize an antibody coated target cell and cause direct lysis of the target cell through release of granzymes and perforin [5]. A seminal study by Clynes et al. showed that the in vivo anti-tumor effect of two clinically useful antibodies, trastuzumab and rituximab, required functional activating FcγRs [26]. In addition, animals lacking expression of FcγRIIB displayed a greater anti-tumor response when treated with therapeutic antibodies [26]. Thus, the balance between expression of activating and
inhibitory FcγRs may be an important determinant of the clinical efficacy of therapeutic antibodies. In support of this hypothesis, one clinical study showed that the FcγRIIA polymorphisms FcγRIIA 131 H/H and FcγRIIIA 158 V/V are associated with increased response rates to patients with follicular non-Hodgkin’s lymphoma (NHL) treated with rituximab [27]. These polymorphisms result in the enhanced affinity of NK cells, monocytes, and granulocytes to the Fc domain of rituximab [25]. These results were further validated in larger clinical studies, which found that the same polymorphisms were independent markers of improved response to rituximab therapy in patients with B-cell non-Hodgkin’s lymphoma [28], to cetuximab therapy in patients with metastatic colorectal cancer [29–30], and to trastuzumab therapy in patients with metastatic breast cancer [31].

High levels of macrophages, which can act as an effector for ADCC, are normally a prognostic factor for poor survival but two separate clinical studies have shown that follicular lymphoma patients with high levels of tumor associated macrophages (TAMs) have an improved response to rituximab [32–33]. This enhanced effect of rituximab in patients with increased levels of macrophages is hypothesized, by the two groups, to be due to an increase in ADCC. More studies are required to test this hypothesis and confirm the clinical effects of ADCC on patient outcome.

2.4.4 INDUCTION OF ADAPTIVE IMMUNITY

Several groups have suggested that maximal benefit of antibody therapy is achieved through induction of adaptive immunity. In support of this hypothesis is the amount of time it takes to see clinical benefits of antibody therapy; clinical responses to rituximab were observed six weeks after initiation of treatment [34]. Mechanistically,
antibodies can generate adaptive immunity through CDC, ADCC, or antibody dependent cell-mediated phagocytosis (ADCP). CDC and ADCC generate tumor cell fragments and release tumor antigens that can be taken up by professional APCs, such as DCs, to initiate tumor directed adaptive immunity. In addition, antibodies can trigger adaptive immunity by acting as an opsonin and triggering Fc dependent phagocytosis of tumor cells by APCs [35]. Tumor antigens are processed by DCs through the endocytic pathway and presented on MHC II to prime CD4+ T cells. In addition, DCs are capable of presenting engulfed tumor antigens on MHC I molecules to generate tumor-specific CD8+ cytotoxic T-cells (CTLs) in a process called cross presentation [35]. Upon activation, CTLs can directly kill tumor cells that express the cognate peptide loaded on MHC I, or further differentiate into tumor specific memory T cells [36]. Numerous pre-clinical studies support the claim that tumor targeted antibodies can elicit adaptive immune responses, and a growing pool of clinical evidence suggests that this mechanism may contribute to the clinical efficacy of antibodies. DCs loaded with antibody coated ovarian and melanoma cells were able to elicit tumor specific CTLs [37]. Moreover, these CTLs were capable of killing the primary ovarian and melanoma cells [37]. Human DC loaded with myeloma cells coated with anti-syndecan-1 antibodies were able to generate CTLs specific for cancer-testis antigens expressed by the tumor cells [36]. These results were confirmed by a recent study showing that colon cancer cell lines coated with cetuximab, an anti-EGFR antibody, were able to generate tumor specific CTLs from autologous DCs [38]. Blockade of inhibitory FcγRIIB signaling by selective antibodies enhanced the antigen processing ability of DCs and suggests a potential mechanism of boosting antibody initiated adaptive immunity [39]. Antibody-Fc interactions allow for the processing and presentation of native tumor
antigens and may explain why DCs loaded with antibody coated tumor cells elicit stronger CTL responses than DCs loaded with peptide [36]. Induction of adaptive immunity has the potential to create and maintain a sustained anti-tumor immune response in patients. However, the tumor microenvironment is a key determinant of whether this adaptive immune response promotes tolerance or an anti-tumor response [40–41]. Thus, approaches to neutralize tolerogenic factors in the tumor microenvironment are a rational approach to enhance antibody initiated adaptive immunity.

2.4.5 Fc ENGINEERING

The capacity of antibodies to activate immune effector mechanisms is highly influenced by post-translational modifications of the Fc domain. Highly fucosylated Fc domains results in abrogated immune activation, therefore, many groups have engineered antibodies lacking Fc fucosylation [5]. One such non-fucosylated antibody, obinutuzumab (GA101, Genentech), targets CD20 and has shown clinical efficacy, even in patients with rituximab-refractory disease [42]. Obinutuzumab is currently undergoing three phase III clinical trials to evaluate its effectiveness compared to rituximab therapy and in rituximab refractory lymphomas (NCT01332968). Two other anti-CD20 antibodies, AME-133 (LY2469298, Mentrik) and PRO131921 (Genentech), both glycoengineered to lack Fc fucosylation, underwent Phase I/II clinical trials in B cell NHL [42]. AME-133 recently successfully completed the dose-escalating trial while trials involving PRO131921 were terminated.
2.4.6 ANTIBODY-LIKE MOLECULES

To further optimize the anti-tumor functions of monoclonal antibodies, numerous antibody-like molecules are in preclinical and clinical trials. These structures adopt certain features of antibody structure, but are structurally distinct from traditional monoclonal antibodies. Antibody-like molecules include bispecific antibodies (bsAbs), DARPinS, adnectins, diabodies, and tandem scFvs. Bispecific antibodies often lack functional Fc domains and have two distinct Fab regions and are thus capable of binding two distinct epitopes. Bispecific antibodies that retain functional Fc domains are termed bispecific tri-functional antibodies (triomabs) due to their capability to bind tumor cells and immune cells while maintaining the capacity to mediate Fc dependent effector functions such as CDC and ADCC. This technology has evolved to overcome the suppressive tumor microenvironment, but due to their complexity, widespread development has been cost prohibitive [43]. Catumaxomab is a triomab that targets the tumor antigen EpCAM and the T-cell stimulatory receptor CD3, which allows for direct stimulation of CTLs in the tumor microenvironment [44]. It is currently undergoing numerous phase I, II, and III trials for a variety of EpCam positive tumors and has been shown to enhance survival in patients with malignant ascites [45]. Other triomabs targeting EGFR, HER2, VEGFA, CD64, and CD30 are currently in preclinical and clinical development, [43,46].

Another type of bsAb are bispecific T cell engagers (BiTEs), which have two single-chain variable fragments (scFvs) fused together and therefore lack an Fc domain. Blinatumomab (MT103, Micromet), a BiTE that targets CD19 and CD3, has been successful in clinical trials for B-precursor ALL and NHL [47]. Another BiTE, MT110
(Micromet), targets both EpCAM and CD3 and is currently undergoing a Phase I study for numerous solid tumors (NCT00635596).

Adnectins are antibody mimetics that consist of the type III domain of fibronectin engineered to contain an antigen binding domain. Since adnectins are created from a fibronectin backbone, they are significantly smaller, allowing for a simpler structure and easier manipulation without substituting the affinity or specificity seen with antibodies [48]. CT-322 (Adnexus) is a novel adnectin targeting VEGFR-2, which is important for tumor angiogenesis [49]. It is currently undergoing numerous phase II trials for the treatment of gliomas (NCT00562419), non-small cell lung cancer (NCT00850577), and NHL (NCT00374179).

2.5 TARGETING IMMUNE CELLS

2.5.1 REGULATORY T-CELLS (TREGS)

One approach to cancer immunotherapy is to target the effector cells that largely contribute to the immune suppressive tumor microenvironment. CD4+ CD25+ FOXP3+ T cells, also known as regulatory T cells, transmit inhibitory signals to immune cells [25]. Evidence of their pro-tumor effects is suggested by the association of high Treg content and a negative overall survival in cancer patients [50]. Daclizumab (Zenapax, Roche), a humanized IgG1 mAb targeting CD25 (IL-2Rα), depletes Tregs in patients with metastatic breast cancer when administered with a cancer vaccine [51]. It is currently undergoing phase I/II trials for the treatment of gliomas, breast cancer, melanoma, lymphoma, and leukemia with and without additional vaccines.
Instead of focusing on reducing the quantity of Tregs, another method is to disrupt the potent immune suppressor molecule CTLA-4 that is expressed by Tregs. By inhibiting the functions of CTLA-4, the capacity of Tregs to inhibit the anti-tumor immune response is diminished, resulting in increased levels of CTLs, CD4+ T cells, and APCs [8]. Ipilimumab (Yevroy, Bristol-Myers Squibb), an antagonistic antibody that targets CTLA-4, is effective in activating an anti-tumor immune response and was FDA approved for the treatment of melanoma in early 2011 [52]. In a landmark phase III study, ipilimumab increased the median overall survival of patients with unresectable stage III or IV melanoma from 6.4 months to 10.1 months [53]. This antibody represents a breakthrough in the treatment of metastatic melanoma, as no promising treatments previously existed.

Another anti-CTLA-4 antibody, tremelimumab (CP-675-206, Pfizer) is an IgG2 that is being studied for the treatment of numerous metastatic carcinomas [2]. A phase III trial with tremelimumab for melanoma patients was terminated due to treatment failure, but further analysis determined that a patient subgroup may benefit from tremelimumab treatment [48]. Tremelimumab is also undergoing two phase I trials for prostate (NCT00702923), and bladder cancer (NCT00880854) and recently completed a phase II trial for colorectal cancer (NCT00313794).

2.5.2 CD40

CD40 is a TNF family receptor whose activation results in release of IL-12 and increased expression of MHC and the co-stimulatory receptor CD86, leading to increased antigen presentation and T cell activation by APCs [10]. CD40 can also be expressed on B-cell malignancies, melanomas, and numerous solid tumors [54]. Ligation of CD40 on tumor cells results in direct cytotoxic effects [55]. As a proinflammatory receptor, CD40
also appears to be vital to the induction of the adaptive immune system [55]. Thus, targeting CD40 can boost anti-tumor immunity and also have a direct cytotoxic effect on tumor cells making it a promising candidate for the treatment of cancer. One CD40 agonist (CP-870.893, Pfizer) was shown to be well tolerated in patients with solid tumors and caused a rapid increase in the percentage of circulating levels of B cells expressing CD86 as well as resulting in one complete and four partial responses [10]. As an IgG2, CP-870.893 does not activate ADCC or CDC, highlighting the efficacy of CD40 ligation in the stimulation of B cells. It is currently undergoing phase I trials for melanoma and advanced pancreatic cancer. Preliminary results show that CP-870.893 in combination with gemcitabine exhibits anti-tumor activity in patients with pancreatic cancer and pre-clinical data suggests that CP-870.893 may function independent of T-cells [56]. In another phase I trial, the CD40 agonist dacetuzumab (SGN-40, Seattle Genetics), resulted in six patients with B-cell non-Hodgkin’s lymphoma displaying an objective response and thirteen patients having stable disease [57]. Unlike CP-870.893, dacetuzumab is an IgG1 antibody whose antitumor response is partially attributed to induction of ADCC by NK cells [54]. It is currently undergoing a phase II trial to study its effect on patients with diffuse large B-cell lymphoma when given in conjunction with R-ICE chemotherapy (NCT00529503).

2.5.3 OTHER PROMISING TARGETS

Many other avenues of targeting immune cells are being evaluated as potential treatments of cancer. CT-011 (CureTech Ltd) is an antagonistic antibody to PD1, whose inhibition can reverse tumor escape from the immune system [58]. It is involved in seven phase II clinical trials for a variety of malignancies. Another target, CD137, has been
shown to stimulate CTLs and APCs upon activation [59]. Two CD137 agonists, PF-05082566 (Pfizer) and BMS-663513 (Bristol-Myers Squibb) are currently in a phase I trial for solid tumors and NHL (NCT01307267) and a phase II trial for melanoma (NCT00612664).

2.6 TARGETING THE TUMOR MICROENVIRONMENT

2.6.1 TGF-β

As mentioned above, the tumor microenvironment is enriched with factors that inhibit the anti-tumor immune response and promote tumor cell growth. Elevated levels of TGF-β, which is produced by Tregs and by some tumor cells, serves to potently inhibit CTLs in the tumor microenvironment[41]. GC-1008 is an IgG4 that targets multiple isoforms of TGF-β [60]. It is currently in a phase I clinical trial for metastatic renal cell carcinoma or malignant melanoma (NCT00356460) and a phase II trial for mesothelioma (NCT01112293).

2.6.2 ANGIOGENESIS

Anti-angiogenic antibodies represent a novel method of reducing tumor growth, by preventing vital nutrients from reaching the tumor. Bevacizumab (Avastin; Genentech) is an anti-VEGFA antibody that sequesters VEGFA, preventing it from binding to its receptors [61]. Due to the development of clinical resistance and recent disappointing clinical trials, other antiangiogenic antibodies have entered clinical trials. Ramucirumab (IMC-1121B, ImClone Systems) is a human IgG1 that blocks VEGFR-2 [62]. It is currently undergoing a phase III clinical trial to compare its utility to docetaxel.
chemotherapy for HER2-negative metastatic breast cancer (NCT00703326) [62].
Similarly, IMC-18F1 (ImClone Systems) is a human IgG1 that blocks VEGFR-1, and due to promising preclinical data, is being pursued in phase II clinical trials for colorectal and breast cancer [63].

In addition to the VEGF/VEGF-R axis, angiogenesis can be stimulated by platelet-derived growth factor receptors (PDGFRs). IMC-2C5 is an antagonist PDGFRβ antibody that reduced tumor growth in preclinical models [64]. Other pre-clinical data suggest that targeting PDGFRα may be a more effective anti-cancer strategy than targeting PDGFRβ. IMC-3G3 (ImClone Systems) is an IgG1 that binds to PDGFRα with high affinity, blocking its activation [65]. In a mouse prostate cancer model, treatment with IMC-3G3 reduced the growth of xenografts as well as inhibiting the growth of metastases [66]. Presently, it is undergoing three phase II clinical trials for the treatment of gliomas (NCT00895180), metastatic gastrointestinal stromal tumors (NCT01316263), and prostate cancer (NCT01316263). These antibodies represent a compelling strategy to inhibit angiogenesis and metastasis and will see the most benefit when used in combination with tumor targeted therapy.

2.6.3 CANCER ASSOCIATED FIBROBLASTS (CAFS)

CAFs constitute 20–50% of cells in the microenvironment of various carcinomas [67]. They contribute to tumor initiation, progression and metastasis, but are not themselves transformed [68]. One potential target, fibroblast activating protein (FAP), appears to be present in CAFs but absent in normal fibroblasts [69]. FAP is a type II membrane bound glycoprotein whose blockade inhibits the ability of CAFs to simulate
proliferation and invasion [68]. Sibrotuzumab (F19, Boehringer Ingelheim Pharma), is a humanized antibody that targets FAP. It was shown to have a good safety profile with metastatic FAP-positive cancer, resulting in two patients with stable disease and twenty one with progressive disease [67]. Another phase I trial for patients with colorectal cancer was recently completed (NCT00004042). Tenascin, another molecule expressed by CAFs, has been associated with colon cancer metastases [68]. 81C6 is an IgG2 chimeric antibody that acts as an antagonist to tenascin and recently completed a phase II trial for the treatment of metastatic brain cancer {NCT00002752}. Results for these studies are expected soon.

2.7 TARGETING SOLID TUMORS

2.7.1 EGFR (ErbB1)

EGFR is overexpressed in many different cancers including colon, head and neck, ovary, lung and malignant gliomas [70]. Signaling via EGFR leads to cell proliferation, migration and invasion primarily via activation of the MAPK and AKT pathways. Cetuximab, the most thoroughly studied anti-EGFR therapeutic antibody, induces cell cycle arrest and apoptosis in tumor cells by blocking ligand binding [71] and receptor dimerization [72]. It has been proposed that cetuximab exerts its in vivo anti-tumor effect in part through induction of ADCC and CDC [73]. However, the clinical relevance of these effector mechanisms in the context of cetuximab therapy is still controversial. Cetuximab has limited impact on progression free survival (PFS) when used as monotherapy for colorectal cancer but increases PFS when combined with FOLFIRI chemotherapy [74]. Recent data form the CRYSTAL trial showed that addition of
cetuximab increased PFS by 1.5 months and improved median overall survival by 3.5 months compared to FOLFIRI alone in patients harboring wild type KRAS [75].

In addition to cetuximab, panitumumab, a fully human anti-EGFR antibody, is also approved for the treatment of metastatic colorectal cancer. Like cetuximab, panitumumab binds to EGFR and inhibits ligand binding and receptor dimerization [76], however, given its IgG2a isotype, panitumumab is not thought to mediate ADCC. Recent work has challenged this hypothesis by demonstrating that panitumumab is capable of inducing ADCC in vitro using myeloid effector cells [77]. Panitumumab monotherapy is well tolerated [78] and significantly improves PFS in patients with refractory metastatic colorectal cancer [79]. When combined with FOFLOX4 chemotherapy, panitumumab increased PFS in patients with previously untreated, wild-type KRAS metastatic colorectal cancer although no improvement in overall survival was reported [80].

Three new anti-EGFR antibodies are currently being evaluated in phase III studies: necitumumab, zalutumumab, and nimotuzumab. Necitumumab binds to a similar epitope compared with cetuximab [81], is well tolerated [82] and is currently in a phase III study with or without cisplatin and pemetrexed in patients with non-small cell lung cancer (NCT00982111). Zalutumumab, in addition to blocking receptor activation, has been shown to induce ADCC in vitro [77]. Phase III studies in patients with refractory squamous carcinoma of the head and neck showed a modest increase in PFS compared to best supportive care, but with no improvement in overall survival [83]. Nimotuzumab is approved in many countries for the treatment of head and neck cancer and malignant gliomas and is currently being investigated as a treatment for pancreatic [84] and non-small cell lung cancer [85]. Unlike other anti-EGFR antibodies, nimotuzumab has not
been associated with development of severe adverse events such as aciniform rash [86]. Talavera and colleagues suggest that the absence of rash could be explained by nimotuzumab’s unique binding properties: nimotuzumab binds to EGFR with a 10-fold lower affinity compared to cetuximab and panitumumab [87], and allows EGFR to adopt an active confirmation, thereby allowing low levels of ligand-independent signaling through EGFR [86]. This low level of signaling may be enough to maintain survival of normal epithelial cells and avoid toxicities associated with destruction of epithelial tissues [86]. This is in contrast to other anti-EGFR antibodies that promote the inactive conformation of EGFR and thus block signaling through the receptor.

2.7.2 HER2 (ErbB2)

HER2 is gene amplified and overexpressed in approximately 30% of breast cancer[88] and overexpressed by some adenocarcinomas of the gastrointestinal tract, lung, ovary and prostate [89]. In contrast to other members of the EGFR family, HER2 has no known ligand and constitutively adopts an open configuration priming it for heterodimerization and increased mitogenic signaling. Currently, trastuzumab is the only FDA approved anti-HER2 antibody. In patients with previously untreated metastatic breast cancer, trastuzumab monotherapy showed a 35% objective response rate [90]. Trastuzumab’s exact mechanisms of action are still unknown, but are thought to include signaling perturbation, inhibition of HER2 shedding, and activation of immune effector mechanisms such as ADCC [91]. Trastuzumab emtansine is a new immunoconjugate in which trastuzumab is conjugated to a derivative of maytansine called DM1. DM1 is a potent anti-mitotic, however, its clinical use is limited by significant toxicity. Trastuzumab-DM1 provides targeted delivery of DM1 and has shown promise in pre-
clinical models [92]. Several clinical trials are currently ongoing involving patients with metastatic breast cancer.

Pertuzumab (Omnitarg), another HER2 directed antibody, is clinically well tolerated [93] and is currently in phase II clinical trials for the treatment of breast and ovarian cancer. Pertuzumab binds to a unique epitope on HER2, but has similar mechanisms of action compared to trastuzumab [94]. Interestingly, unlike trastuzumab, preclinical data suggests that pertuzumab can induce cell death in the absence of HER2 overexpression, possibly by inhibiting ligand-induced HER2 heterodimerization [95–96]; however, no apparent clinical benefit of pertuzumab is seen in patients with HER2 negative metastatic breast cancer [96].

2.7.3 HER3 (ErbB3)

HER3 is overexpressed by certain types of cancers including lung, breast, ovarian and prostate [97]. Although catalytically inactive, HER3 is capable of binding ligand, most notably neuregulin-1, and heterodimerizing with EGFR or HER2 to promote cell proliferation and invasion [97]. MM-121, a fully human IgG2a anti-HER3 antibody, inhibits ligand induced HER3 signaling, HER2-HER3 dimerization, and growth of HER3-expressing xenografts in vivo. MM-121 is currently in phase I clinical trials in patients with advanced solid tumors (NCT00734305).

2.7.4 IGF/IGF-R

Signaling through the insulin-like growth factor receptor (IGF-1R) is reported to play an important role in transformation and cell growth and is overexpressed in a wide range of cancers [98]. Strategies to target IGF-1R and its ligands, IGF-I and IGF-II, using
antibodies are being explored in both pre-clinical models and clinical trials. EM164 is a humanized anti-IGF-1R antibody that has been reported to inhibit signaling through IGF-1R \textit{in vitro} and delays growth of human pancreatic and neuroblastoma xenografts \textit{in vivo} [98–99]. Dalotuzumab is another humanized IGF-1R antibody that shows anti-tumor efficacy against breast and lung tumor xenografts [100] and is currently in clinical trials for the treatment of breast (NCT01234857), lung (NCT00654420) and colon cancer (NCT00614393). Similarly, MEDI-573, a fully human antibody that neutralizes the IGF-1R ligands IGF-I and IGF-II, has shown anti-tumor efficacy in pre-clinical models [101] and is currently being studied in a phase I clinical trial (NCT00816361).

\section*{2.8 COMBINATION APPROACHES}

\subsection*{2.8.1 SURGERY}

The clinical utility of antibodies in the adjuvant setting is the subject of ongoing investigation. A one year follow up of the Herceptin Adjuvant Trial (HERA) demonstrated that trastuzumab increased disease free survival when given for one year following adjuvant chemotherapy [102] and this benefit persisted four years after initial trastuzumab treatment, although significant crossover between treatment groups was observed [103]. Adding trastuzumab after adjuvant chemotherapy improved survival at two years follow up [104], however, this improvement was lost after four years [103]. Despite the biases and confounding variables in the HERA trial, results of the trial support previously published data on the clinical utility of trastuzumab in the adjuvant setting [105]. In the setting of colorectal cancer, addition of cetuximab to mFOLFLOX6 chemotherapy did not
improve outcomes in patients with resected stage III disease [106]. More studies are needed to clearly define the role of tumor targeted antibodies in the adjuvant setting [107].

2.8.2 CYTOTOXIC CHEMOTHERAPY

Cytotoxic chemotherapy has generally been viewed as a modality to induce tumor cell death, with negligible impact on the anti-cancer immune response. However, cell death induced by cytotoxic agents’ results in release of tumor antigens and danger signals, which could lead to activation of immune effectors and development of tumor-targeted adaptive immunity [38, 108]. This putative mechanism of synergy between tumor targeted antibodies and cytotoxic chemotherapy could underlie the clinical benefits seen in numerous clinical trials combining antibodies with chemotherapeutic regimens. Addition of trastuzumab to chemotherapy resulted in a higher objective response rate and longer time to disease progression compared to chemotherapy alone in patients with metastatic breast cancer [109]. One preliminary study showed that breast cancer patients who developed anti-HER2 humoral responses after treatment with trastuzumab and chemotherapy had a more favorable outcome compared to patients who failed to develop a humoral response [110]. Similar findings of synergy between cytotoxic chemotherapy and tumor targeted antibodies have been observed using rituximab in patients with non-Hodgkin’s lymphoma and using cetuximab in patients with advanced colon cancer [111]. However, adding antibodies to chemotherapeutic regiments is not always beneficial. Recently, a study showed that addition of cetuximab to a regimen of oxaliplatin and capecitabine showed no improvement in progression free survival and overall survival in patients with colorectal cancer, possibly due to the toxicity profile of the oxaliplatin and capecitabine regimen [112].
2.8.3 RADIOTHERAPY

Antibody therapy has also been combined successfully with radiotherapy for the treatment of several cancers. Addition of cetuximab to radiotherapy significantly improved five year survival in patients with squamous cell carcinoma of the head and neck [113]. Recently, Blumenschein and colleagues reported the feasibility of adding cetuximab to a regimen of chemotherapy and radiation in patients with non-small cell lung cancer [114]. Mechanistically, similar to chemotherapy, it has been hypothesized that radiotherapy induces release of danger signals and subsequent activation of the anti-tumor immune response. Clinically, there are numerous reports of elimination of non-irradiated tumor cells at a distant site after local radiotherapy, the so-called “abscopal effect” [115]. Although the mechanism of the abscopal effect is currently unknown, it has been hypothesized that local radiotherapy induces an anti-tumor adaptive immune response that is responsible for eliminating the distant metastases [116]. In addition, ionizing radiation has been shown to increase expression of tumor associated antigens and MHC I leading to enhanced CTL mediated tumor cell killing [117] and potentially enhanced ADCC.

2.8.4 IMMUNOMODULATORS

Many studies have investigated the rationale of combining immunostimulatory agents with antibody therapy to boost immune effector mechanisms such as ADCC and development of adaptive immunity. IL-2 has been shown to enhance the efficacy of rituximab against non-Hodgkin’s lymphoma xenografts in pre-clinical models [118]. This enhancement of tumor cell killing was Fc dependent, suggesting that IL-2 enhanced the capacity of rituximab to mediate ADCC or CDC [118]. However, a phase II study found
no clinical benefit in adding rIL-2 to rituximab in patients with indolent non-Hodgkin’s lymphoma [119]. Similarly, GM-CSF is capable of enhancing the capacity of monocytes and macrophages to mediate ADCC and was tested in a phase II study in combination with rituximab in patients with follicular lymphoma. Patients receiving GM-CSF and rituximab showed a high response rate and an increased complete response rate compared to rituximab monotherapy [120]. In addition, patients receiving combination therapy had more circulating monocytes, granulocytes and dendritic cells, although no difference in cell counts was observed between the complete responders and non-complete responders, confounding the link between effector cell recruitment and efficacy [120–121]. Studies are ongoing to evaluate the ability of peptide vaccination in combination with antibody therapy to boost tumor-specific adaptive immune responses. Pre-clinical studies using a HER2 peptide vaccine in combination with an anti-HER2 antibody showed generation of HER2 specific T-cells and tumor regression in mice [122]. A phase I/II study showed that combination therapy of HER2 peptide vaccine with trastuzumab was well tolerated and resulted in generation of long-lived HER2 specific T cells [123]. Another phase I study showed similar results using a HER2 plasmid-DNA vaccine and trastuzumab [124]. The true impact of combining a tumor-targeted antibody with vaccination regimens on survival has yet to be determined but early studies are promising.
2.9 MECHANISMS OF RESISTANCE TO ANTIBODY THERAPY

2.9.1 AMPLIFICATION OF DOWNSTREAM SIGNALING AND ALTERNATIVE SIGNALING PATHWAYS

The most thoroughly described example of a molecular determinant of primary resistance to antibody therapy is KRAS mutational status and sensitivity to the anti-EGFR antibodies cetuximab and panitumumab. Numerous clinical trials have demonstrated that response to cetuximab and panitumumab requires colorectal cancer patients to harbor wild type KRAS. Patients with activating KRAS mutations, most frequently in codons 12 and 13, fail to benefit from anti-EGFR antibody therapy [75, 125–126]. Interestingly, the link between KRAS mutational status and response to anti-EGFR antibodies may not apply to all type of cancers. Initial reports show that KRAS mutational status is not a predictor of clinical response to cetuximab in patients with non-small cell lung cancer [127].

Since KRAS mutations only account for 35–45% of patients who do not respond to anti-EGFR antibody therapy [126], recent studies have focused on the mutational status of BRAF and PIK3CA/PTEN, both of which are activated following engagement of EGFR. Results from the CRYSTAL trial showed that patients harboring V600E BRAF mutations had worse clinical outcomes at all end points [75]. However, due to a small sample size, this trial could not determine the clinical utility of adding cetuximab to FOLFIRI chemotherapy [75]. Indeed, retrospective analysis suggests that BRAF mutations confers resistance to cetuximab therapy, however, definitive prospective studies are needed to confirm this trend [126, 128]. If the trend between BRAF mutations and cetuximab resistance is confirmed, adding a BRAF inhibitor to a regimen of cetuximab and FOLFIRI
Chemotherapy would be a rational approach to overcome resistance to cetuximab in patients harboring mutated BRAF and wild type KRAS. Vemurafenib, a small molecule inhibitor of mutated BRAF, is currently in clinical trials and has been shown to prolong survival in patients with advanced melanoma [129].

Studies on PIK3CA/PTEN and cetuximab resistance have been conflicting, but suggest that PIK3CA mutations and PTEN loss are associated with resistance to cetuximab [126, 130–131]. It has been difficult to directly implicate PIK3CA/PTEN alterations in cetuximab resistance since PIK3CA mutations and PTEN loss/inactivation can co-exist, unlike KRAS and BRAF mutations which are mutually exclusive [126, 132]. In contrast, activating PIK3CA mutations and PTEN loss are thought to be mutually exclusive in breast cancer [133] and mediate resistance to trastuzumab [134–135]. Additionally, PI3K inhibitors reversed trastuzumab resistance mediated by PTEN loss in vitro and patients with trastuzumab resistant breast cancer who were treated with an mTOR inhibitor, which is downstream of PI3K, showed promising clinical responses [136].

IGF-IR has been implicated in resistance to trastuzumab in many pre-clinical studies, as it is capable of heterodimerizing with members of the EGFR family [137–138]. In support of the pre-clinical data, a small study showed that overexpression of IGF-IR correlates with a poor response to trastuzumab plus chemotherapy [139]. Clinical trials evaluating the efficacy of combination therapy of IGF-1R inhibition in the setting of trastuzumab resistance are currently underway (NCT00788333).
Tumor cells often express immune inhibitory molecules that limit the efficacy of antibody therapy and promote resistance. Pre-clinical data demonstrated that HLA-G, a non-classical MHC molecule, is robustly expressed on human ovarian cancer cell lines and is capable of inhibiting NK mediated lysis \textit{in vitro} [140]. Similarly, Levy et al. demonstrated that HLA-E, another non-classical MHC molecule, is expressed by primary colon cancer tissue and showed preliminary data suggesting that high HLA-E levels could correlate with shorter disease free survival [141]. Further study revealed that HLA-E expression by colon cancer cell lines inhibited the capacity of cetuximab to mediate ADCC \textit{in vitro} [142].

Tumors also actively inhibit CDC by expression of membrane bound complement regulatory proteins (mCRPs) such as CD46, CD55, and CD59. mCRPs control the activation of C3 and are expressed by a wide range of cancers including breast, colon, lung and hematological malignancies [143]. Neutralization of mCRPs has been reported to enhance the capability of trastuzumab [144] and rituximab [145] to mediate CDC \textit{in vitro}. A small study showed that low expression of CD46 and CD55 were associated with response to rituximab. Similarly, CD55 and CD59 expression were correlated with bulky disease in patients with non-Hodgkin’s lymphoma [146]. This is in contrast to a previous study that showed no correlation between mCRP expression and response to rituximab [147]. Furthermore, there is marked variation in expression of mCRPs in tumor samples and the clinical utility of targeting mCRPs in combination with antibody therapy has not yet been justified using appropriate \textit{in vivo} models [143].
Impairment of proper antigen presentation is another putative mechanism of resistance to antibody therapy. Tumor cells often down regulate expression of MHC I and as a result evade destruction by MHC-restricted CTLs [148]. A study by Watson and colleagues showed that high MHC I expression, as determined by immunohistochemistry, was associated with longer disease-specific survival (DSS) and that down regulation of MHC I was associated with a worse prognosis in patients with colorectal cancer [149]. Down-regulation of MHC may have a clinically relevant impact on the efficacy of antibody therapy given the evidence that therapeutic antibodies can elicit adaptive immune responses [110].

As mentioned above, the tumor microenvironment is heavily infiltrated by Tregs and MDSCs that serve to suppress the anti-cancer immune response. Tregs inhibit anti-tumor immunity by causing apoptosis of immune effectors via perforin and granzymes, by expression of indolamine 2,3 dioxygenase and by production of soluble mediators such as TGF-β and IL-10 [150]. In addition to its negative effect on immune effectors, TGF-β in the setting of HER2 amplification and overexpression has been reported to potentiate oncogenic signaling mediated by HER2 and promote resistance to trastuzumab [151]. IL-10 could limit the induction of tumor directed adaptive immunity due to its capacity to downregulate MHC II and B7 expression on antigen presenting cells. MDSCs are capable of recruiting Tregs to the tumor microenvironment and have the capacity to directly inhibit T-cell signaling through nitrosylation of the T-cell receptor [152]. Taken together, cellular and soluble factors in the tumor microenvironment have the capacity to limit the efficacy of antibody therapy by inhibiting immune effectors and promoting oncogenic signaling.
2.10 CONCLUSION

Monoclonal antibody therapy has revolutionized the treatment of cancer and will continue to be an important treatment modality for cancer in the decades to come. Clinical success of antibody therapy is dependent on understanding the effects of antibody therapy on tumor biology and the anti-cancer immune response. Rational combinations of tumor targeted antibodies with other anti-cancer drugs and agents that target the immunosuppressive tumor microenvironment offer the best hope of maximizing the clinical benefit of antibodies.
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2.12 Primary Figures

2.12.1 Figure Legends

Figure 2.1. Proposed mechanisms of resistance to antibody therapy. The most thoroughly characterized mechanism of resistance to antibody therapy is amplification of downstream signaling. In the case of EGFR, resistance to anti-EGFR antibodies can be mediated by mutations (stars) in K-Ras, B-Raf, or PI3K/PTEN that allow tumor cells to bypass receptor activation to activate pro-survival genes (A). Tumor cells may upregulate expression of inhibitory receptors, such as HLA-E and HLA-G, which engage inhibitor receptors on NK cells to inhibit ADCC (B). Similarly, tumor cells may express membrane bound complement regulatory proteins (mCRP), such as CD55 and CD46, which act to inhibit cleavage of C3 by C3 convertase and inhibit generation of the membrane attack complex (MAC) (C). Tumor cells that express MHC I can be recognized by activated CD8+ cytotoxic T lymphocytes (CTL), which release perforin and granzyme to induce tumor cell apoptosis. However, tumor cells often downregulate expression of MHC I and display aberrant antigen processing machinery, resulting in loss of MHC I expression on the cell surface and protection from CTL mediated killing (D). The tumor microenvironment is enriched with myeloid-derived suppressor cells (MDSC) and T regulatory cells (Treg) that produce IL-10, TGF-β and reactive oxygen species (ROS) to inhibit tumor cell killing by CTL (E).
Figure 2.1. Proposed Mechanisms of Resistance to Antibody Therapy.
CHAPTER 3

BIFUNCTIONAL ANTIBODIES: PRECLINICAL AND CLINICAL APPLICATIONS

Adapted from Publication:


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Abstract Treating malignancies with antibody-based immunotherapy has revolutionized the concept of targeted therapy. Rituximab and Trastuzumab, two monoclonal antibodies approved in the 1990s by the FDA, elucidated the potential of harnessing the immune system to eliminate transformed cells. As with any cancer therapy, a significant proportion of patients relapse, driving the development of non-traditional antibody-based therapies. Therefore, in an effort to enhance the ability of antibodies to retarget immune cells toward cancer cells, bispecific antibodies were born. Created through a variety of techniques they conform to an assortment of structures, recapitulating the basic structure of an antibody or deconvoluting the antigen-binding domains into unique designs. The European Union’s approval in 2009 of Catumaxomab, a bispecific antibody that links cells of the innate and adaptive immune system to EpCAM + cells for the treatment of malignant ascites, marks the first clinically approved dual-targeting antibody. Blinatumomab, a bispecific T-cell engager (BiTE), links T-cells directly to malignant cells, activating target-cell apoptosis through perforin-granzyme release. Early clinical results of Blinatumomab show a remarkable 80 % response rate in a heavily pretreated ALL patient sub-group. These enticing clinical results represent the forefront of the bispecific antibody field but evidence exists that point to the clinical success of numerous bispecific antibody formats. Although it is unknown which format will exhibit the highest clinical efficacy, it is clear that dual-targeting antibodies represent the future of immunotherapy for the treatment of cancer.

Keywords Bispecific antibody • Triomab • Diabody • Tandab • Tetrabody • Triplebody • Bispecific T-cell engager • Quadroma • Dock-and-lock technique • CovX Body
3.2 INTRODUCTION

The clinical and commercial success of monoclonal antibodies has proven the hypothesis established by Paul Erhlich in the 1900s that the immune system could provide a magic bullet for the treatment of cancer. While these single target antibodies have vastly improved survival for patients with HER2+ breast cancer, CD20+ lymphoma, and CD33 lymphoma, inevitably a significant proportion of patients relapse. With the proof of principle for immunotherapies, a revolution in the tumor immunology world began, with the goal of designing and developing techniques to overcome the current limitations of cancer targeting monoclonal antibodies (mAbs). This has led to the enhancement of effector functions through Fc region modifications, as well as the direct delivery of toxins to transformed cells through toxin–mAb conjugation (trastuzumab-DM1) [68]. A viable alternative method to enhance the efficacy of tumor immunotherapy is the design of antibodies or recombinant proteins that target multiple antigens and/or induce cancer cell destruction through the redirection of lytic immune cells. These dual-targeting antibodies are created through chemical conjugation, fusion of two mAb-producing hybridomas, and genetic recombination. The resulting bispecific antibody (bsAb) field has expanded immensely, with dozens currently undergoing various phases of clinical trials for the treatment of cancer and many more in preclinical studies. Of particular interest are trifunctional antibodies (Triomabs) developed by Trion Pharma and bispecific T-cell engagers (BiTEs) developed by Micromet. Other less clinically developed bsAbs include single-chain Fv (scFv) fusion proteins, diabodies, tribodies, bispecific CovX bodies, and random site mutation bsAbs. Although the structure and function of these bsAbs differ, they share antigen targets. On the tumor
cell, they recognize members of the EGFR family, CD19, CD20, CD33, MCSPs, and EpCAM. For those that redirect effector cells, the antigen targets include CD3 on T-cells, CD16 on NK cells, monocytes, macrophages, and neutrophils, and CD64 on macrophages and monocytes. In this chapter, we describe the development of bispecific antibodies for the treatment of cancer in a historical perspective, highlighting the bsAbs that have entered clinical trials.

3.3 FIRST GENERATION BSABS

Before the development of advanced genetic recombination, bsAbs were created by the direct chemical crosslinking of antibody domains or through the fusion of two mAb producing hybridomas, called quadromas (Fig. 1.1) [56, 82]. Both methods produce a full-length antibody that can effectively bind to multiple antigens, either manipulating the downstream signals of the target or conjugating immune effector cells directly to the target cell. In the 1990s and early 2000s, several of these first generation bsAbs entered phase I clinical trials with limited success [26, 27, 92, 94, 116, 119]. For chemical crosslinking, the production of large quantities of purified bsAbs was cost prohibitive, as each reaction created numerous inert and unusable proteins. For the quadroma bsAbs, which were of murine or rat origin, the development of human anti-mouse antibodies (HAMA) or human anti-rat antibodies (HARA) precluded multiple dosing regimes in patients, severely limiting their clinical applicability. Also, quadromas created from the same species secrete ten possible combinations of light-chain and heavy-chain antibodies, with only one having the correct dual-targeting functionality [69]. A novel discovery was made in 1995 by Horst Lindhofer, that the fusion of a rat hybridoma with a murine hybridoma resulted in the preferential creation of the correct
bsAb formation, increasing the yield from quadromas by 3.5-fold [69].

3.3.1 CHEMICAL CONJUGATION

The first chemically cross-linked bsAb to enter clinical trials targeted HER2 and FcγRI (CD64), in an attempt to utilize macrophages and monocytes to lyse cancer cells. HER2 is one of the most targeted antigens by immunotherapy, as ~30% of all breast cancers overexpress this potent tyrosine kinase receptor and is the target for the FDA approved mAb trastuzumab [11]. The murine bsAb, called MDX-210, was formed through the reduction of the two murine parental mAbs into Fab’ fragments, chemically cross-linked using o-phenylenedimaleimide, acetylated with the alkylation agent iodoacetamide, and then purified through chromatography. Therefore, this molecule mimics the structure of a HER2 mAb, with two antigen-binding arms recognizing HER2 and one region recognizing an FcγR. The major difference compared to standard mAbs is the unique antigen-binding target of CD64, instead of FcγRIII (CD16), which IgG1 molecules recognize through their Fc region. MDX-210 was delivered intravenously to ten patients with HER2+ advanced breast or ovarian cancer, at a dose ranging from 0.35 to 10.0 mg/m². The bsAb was well tolerated in the ten evaluable patients, never reaching the maximum tolerated dose (MTD). Most patients only experienced grade 1 and 2 adverse events but two experienced the grade 3 adverse event hypotension. Although the goal was only to test the toxicity of the molecule, MDX-210 treatment resulted in one partial and one mixed tumor response. As expected, HAMA was detectable in six of the ten patients [116]. This observed limiting factor prompted the development of a humanized version of the bsAb, MDX-H210, which was tested in patients with metastatic HER2+ breast cancer in two phase I trials. To enhance the proportion of immune cells
with lytic capabilities, these investigators then coadministered MDX-H210 with granulocyte-colony-stimulating factor (G-CSF), which has been shown to induce the expression of CD64 on neutrophils. In the first clinical trial, of the 23 patients enrolled, the majority of patients experienced fevers or diarrhea, while no MTD was reached. No objective clinical responses were observed and all patients had circulating antibodies that recognized the bsAb [92]. The second phase I trial of MDX-H210 was delivered to 30 stage IV HER2+ breast cancer patients in 2003. Similar to the previous trials, no MTD was reached, the molecule was well tolerated, no objective responses were seen, and a majority of patients developed anti-bsAb anti-bodies [94]. Another chemically conjugated bsAb, MDX-447, which targets EGFR and CD64, was evaluated with and without G-CSF in 64 patients with advanced solid tumors. Although the addition of G-CSF caused dose-limiting toxicities, MDX-447 was relatively tolerated and resulted in no objective clinical responses, halting its further development [34]. The lack of clinical activity in the patients treated with these conjugated molecules might be explained by the strong pre-stimulation of polymorph nuclear neutrophils (PMN) cells with IFN-γ and G-CSF, and the high effector to target ratio of PMNs to tumors cells (50–200:1) that is required for in vitro lysis by MDX-210 and MDX-H210 [57]. Moreover, the development of neutralizing antibodies or the lower lytic capability of CD64 expressing immune cells might have attributed to the lack of clinical efficacy.

### 3.3.2 Quadrormas

The first generation of quadrorna bsAbs recognized CD16 or CD3 to link lytic immune cells directly to tumor cells. The resulting bsAbs use one Fab arm to recognize the tumor antigen, another Fab to bind to CD16 or CD3, and use an intact Fc
domain to bind to CD16 and FcRn. Therefore, CD16 × tumor antigen bsAbs have two unique binding domains for CD16 on innate immune cells, theoretically enhancing their ability to mediate antibody-dependent cellular cytotoxicity (ADCC). This design was utilized for two murine bsAbs that entered phase I clinical trials in the 1990s, 2B1 and HRS-3/A9. 2B1, which targets HER2/neu and CD16, was utilized in two clinical trials for patients with HER2+ tumors. Preclinical data suggested that the 2B1 bsAb derived from the 520C9 and 3G8 fusion quadroma was effective in eliminating HER2+ target cells in vitro and potentiating their growth in vivo [117, 118]. Similar to MDX-210, the effector cells required significant stimulation and high E:T ratios (25–50:1). Minimal clinical activity was observed following 2B1 therapy, with a total of 33 out of 34 patients developing HAMA [15, 119]. Of particular interest was the induction of an adaptive immune response to HER2/neu, suggesting that 2B1 enhanced antigen presentation for HER2. However, this adaptive immunity did not translate to clinical responses. HRS-3/A9 was created through the fusion of the murine hybridomas HRS-3, which produces a mAb for CD30, and A9, which produces a mAb against CD16 [52]. CD30, a marker for Hodgkin lymphomas, represents a valid target for selective targeting of lymphoma cells [111]. Preclinical studies with HRS-3/A9 were promising, as it was shown to cure mice with CD30+ Hodgkin’s lymphomas after only one injection [52]. In 1997, HRS-3/A9 was given to 15 patients with refractory Hodgkin’s disease, was well tolerated, and resulted in the first clinical responses seen with a bispecific antibody, with one complete response (CR), one partial response (PR), and three minor responses [43]. Similar results were seen in a second phase I trial, with one CR, three PRs, and four patients with stable disease (SD) [44]. While these results elucidated the potential of bsAbs for the treatment
of cancer, 15 of 31 patients developed HAMAs, prohibiting subsequent clinical use [43, 44].

In an effort to enhance the effector functions of antibody therapy, many groups sought to link the most powerful arm of the adaptive immune system to cancer cells. By conjugating CTLs through CD3, directly to a tumor cell through a tumor-associated or -specific antigen, effective lysis can occur. After being primed in the lymph nodes through ligation of CD3 and a second activating signal, CTLs disseminate throughout the body. Through the T-cell receptor (TCR), educated CTLs recognize their “primed antigen” presented on the surface of a cell through major histocompatibility complex (MHC) class I. Upon conjugation, CTLs release perforin and granzymes, activating the apoptotic pathway inside the target cell [81]. Therefore, the creation of a second antigen-binding domain of an antibody to recognize the activating T-cell receptor CD3 could effectively utilize the cytotoxic arm of the adaptive immune system to eliminate cancer cells. Unfortunately, when given to patients, T-cell retargeting bsAbs resulted in a systemic cytokine release induced by T-cell activation. This, on top of the development of HAMA, significantly limited the bsAb dose, reducing their clinical efficacy. The first T-cell retargeting quadroma was SHR-1, a rat/murine hybrid that recognized CD3 and the B-cell lymphoma marker, CD19. Preclinical studies found that SHR-1 could lyse CD19+ B-cell lines and B-cells taken directly from lymphoma patients with activated T-cells [39, 40]. SHR-1 was capable of mediating significant lysis of transformed B-cells with T-cells isolated from patients with lymphoma, proving that CD3 could serve as a potent activator of previously tolerant T-cells. Further in vitro studies demonstrated complete cell growth inhibition at a relatively low E:T ratio of 9:1 when SHR-1 was combined with Il-2.
stimulated peripheral blood mono- nuclear cells (PMBCs) [41]. These promising results led to a phase I clinical trial in 1995, where three non-Hodgkin’s lymphoma (NHL) patients were exposed to increasing doses of SHR-1. Although the bsAb was well tolerated with little observed toxicity, clinical activity was minimal [26, 27]. HEA125 × OKT3, another first generation quadroma bsAb of murine origin, retargeted T-cells to EpCAM (CD326) positive tumors. EpCAM expression is commonly associated with poor prognosis, is expressed on a broad range of carcinomas, and is often used as a marker for stem cell-like properties [83, 86, 109]. EpCAM is also expressed on normal cells but is generally confined to intracellular spaces, making it an attractive target for anticancer therapy. In 2002 HEA125 × OKT3 was evaluated in ten EpCAM + ovarian carcinoma patients with malignant ascites. At the time, patients with malignant ascites, which are tumor cells that metastasize to the peritoneal cavity causing pain and swelling, had limited options for palliative treatment. Diuretics or direct fluid drainage through paracentesis were the only viable options to improve quality of life. Since the malignant epithelial cells in ascites caused by ovarian carcinoma are often EpCAM +, treatment with HEA125 × OKT3 was considered to potentially fill a significant unmet need in cancer palliative care. When injected intraperitoneally, HEA125 × OKT3 resulted in the complete inhibition of ascites formation in eight patients and reduced ascites formation in two patients. However, 80 % of the patients developed HAMAs, seemingly slowing its further clinical development [77].
3.4 TRIOMABS

3.4.1 Early Generation Triomabs

Triomabs, the most successful bsAb created using the quadroma technique, are the only type of bispecific antibody used to treat cancer. Since first generation quadromas produced ten antibody formations randomly, it was difficult to purify the correct dual-targeting antibody. Utilizing the discovery of preferential pairing of bsAbs in mouse IgG2a/rat IgG2b quadromas and a single step pH elution on protein A, the desired bsAb could be isolated with previously unimagined ease [69]. Surprisingly, the Fc region of these triomabs bound only to activating FcγRs (Fcγ RI and Fcγ RIII) on effector cells and not to inhibitory receptors (Fcγ RIIB) [123]. This prompted the development of numerous commercially viable bsAbs, targeting EpCAM, HER2, CD20, melanoma-associated proteoglycans, and the melanoma-associated gangliosides GD2 and GD3, all of which utilize CD3 as their secondary binding target [70]. The first proof of principle triomab targeted against EpCAM, called BiUII, could mediate lysis of PCL-1 and FaDu, squamous carcinoma cells of the head and neck, with unstimulated PBMCs [122]. This was evident at relatively low concentrations of BiUII (5 ng/ml) and could mediate higher lysis of target cells than a combination of both parental CD3 and EpCAM antibodies. Therefore, the direct linkage of T cells, NK cells, and tumor cells appears to impart a cytotoxic advantage, possibly due to the secondary activation of T cells by NK cells or macrophages (Fig. 1.2). Although a high E:T ratio of 20:1 was required to reach specific lysis near 90 %, BiUII was capable of mediating 60 % and 25 % lysis at the conservative E:T ratios of 5:1 and 1:1, respectively [122]. Other preclinical EpCAM triomabs were shown to mediate significant lysis of EpCAM positive prostate cancer cell lines as well
as protect 100 % of the mice from secondary challenge with B16 melanoma cells 144 days after initial inoculation [96, 98]. The induction of an adaptive immune response was likely due to the triomab’s ability to activate dendritic cells, T cells, and macrophages [123]. These trifunctional antibodies overcame the limitations of large-scale purification and were able to link the innate and adaptive together in a common anticancer goal, prompting their clinical development.

3.4.2 Catumaxomab (Removab)

Learning from the design of previous EpCAM triomabs, catumaxomab (Removab®) was developed specifically for the clinic and similar to HEA125 × OKT3, was utilized for the treatment of malignant ascites. Initial phase I results demonstrated limited toxicity of catumaxomab therapy when delivered intraperitoneally to seven patients in 2005, prompting a phase I/II trial with 23 women presenting with malignant ascites [46]. Patients received 4–5 regimens of catumaxomab at increasing doses starting from 10 µg and ending at 200 µg. Although a significant proportion of patients experienced grade 3 adverse events, the majority of events were reversible. Remarkably after the 37 day trial, only one of the twenty-three patients required paracentesis, a significant improvement from the median time to paracentesis of 7–11 days commonly seen in this patient subgroup [21]. After the final treatment, those treated with catumaxomab saw a 99.9 % reduction in the mean EpCAM + tumor cell numbers in the peritoneal fluid, clearly demonstrating the antitumor ability of catumaxomab [21]. Using the dose established in this trial, a second phase II trial was conducted for 13 patients with malignant ascites to determine the pharmacokinetics and efficacy of catumaxomab. At days 0, 3, 6 or 7, and 10, patients received doses of 10, 20,
50, and 150 μg of the triomab intraperitoneally. The half-life was determined to be 2.13 days and although peritoneal concentrations were consistently high ranging from 552 to 6,121 pg/ml, the systemic levels in the serum remained extremely low, with a maximum concentration of 403 pg/ml [100]. While the low systemic concentration could limit the efficacy of Catumaxomab, it likely reduced the toxicity of this therapy. After a single 10 μg dose of Catumaxomab, the median EpCAM + tumor cell count reduced from 9,362 tumor cells per million ascites cells to only 49 [100].

A landmark phase II/III trial (NCT 00836654) with 258 patients with recurrent symptomatic malignant ascites caused by ovarian or non-ovarian cancer proved that intraperitoneal treatment of catumaxomab leads to a significant enhancement in puncture-free survival, median time to next paracentesis, and higher overall survival in gastric cancer patients. Utilizing the same increasing dose regimen as the previous phase II trial, patients were separated into two groups: paracentesis and catumaxomab (treatment) or paracentesis alone (control). Catumaxomab’s addition resulted in an increase in puncture-free survival from 11 days in the control group to 46 days and enhanced the median time to paracentesis from 13 days to 77 days [47]. Catumaxomab also completely eliminated the EpCAM + tumor cell count in the ascites fluid in 95 of the 115 evaluable samples. The adverse events associated with catumaxomab were widespread, affecting 98 % of the patients. Toxicities from cytokine release were well controlled and reversible. Eight days after the last infusion, 76 % of all patients (85/112) presented with HAMA even though only 5 % (6/124) of patients had detectable HAMAs before the last infusion of 150 μg [47]. In a post hoc analysis, the induction of HAMA did not adversely influence clinical benefits, but instead suggested that the development
of HAMA could effectively be used as a biomarker for responders to catumaxomab treatment [89]. A second post hoc analysis demonstrated that peritoneal samples from catumaxomab-treated patients had significantly lower tumor cell counts, no stem cell-like tumor cells (CD133+/EpCAM+), lower VEGF levels, and double the number of activated CD4+ and CD8+ T-cells compared to the control group [54]. Early results of this Phase II/III trial prompted the European Union to approve the use of catumaxomab for the treatment of malignant ascites in 2009, as no effective therapeutic existed for this patient group. Catumaxomab is the first and only bispecific antibody to receive approval for clinical use as a cancer therapeutic.

However, as EpCAM is expressed on such a broad range of carcinomas, it could be utilized for the treatment of numerous different cancers. As the most similar indication to malignant ascites, catumaxomab has been used in a phase II trial to treat patients with malignant pleural effusions caused by breast cancer or NSCLC. Three escalating intra-pleural doses of 5–200 µg in the 24 patients led to significant adverse events, with two dose limiting toxicities (DLTs). Although the trial was stopped early due to the high number of serious adverse events (23) and dropouts, one CR and four PRs associated with pleural effusion, occurred in the breast cancer group. Not surprisingly, most patients developed HAMA [107]. Since catumaxomab has the potential to eliminate solid tumors, one phase I and one phase II trial have been performed for NSCLC and epithelial ovarian cancer, respectively. The phase I trial represents the only instance where catumaxomab was delivered intravenously to patients. Utilizing an increasing dosing regimen of 2–7.5 µg, the maximal tolerable dose was determined to be 5 µg when administered with 40 mg of dexamethasone, a potent anti-
inflammatory agent [106]. Surprisingly, no patients developed HAMA or HARA when exposed to catumaxomab, although one patient, who had preexisting HARA before treatment, had elevated levels by the end of the trial. In an effort to directly treat patients with epithelial ovarian cancer, catumaxomab was delivered intraperitoneally to 45 patients in 2011. Stratified into two groups, the low dose group of four infusions of 10 µg resulted in two patients with stable disease. The high dose group received infusions of 10, 20, 50, and 100 µg of catumaxomab, leading to one partial response and five patients with stable disease. Every patient exhibited a treatment-related adverse event, 17 of 45 patients developed HAMA or HARA, and no difference in progression-free survival was seen between the two groups [12]. Additional trials are being performed to determine the efficacy of catumaxomab as an anticancer therapeutic for indications different from malignant ascites. As of 2012, catumaxomab is being utilized in close to a dozen clinical trials with the most notable being a phase III trial for malignant ascites with or without the anti-inflammatory drug prednisolone (NCT 00822809).

3.4.3 Ertumaxomab (Rexomum)

The second triomab, ertumaxomab, was developed with the same CD3 binding arm of catumaxomab, to retarget T cells and innate killer cells to HER2+ tumor cells. When compared to trastuzumab at a high E:T ratio of 20:1, ertumaxomab-mediated maximum lysis of high HER2 expressing cells, classified as HER2 3+, at a fivefold lower concentration (1 ng/ml vs. 5 ng/ml). Reducing the E:T ratio to 5:1 to closer reflect in vivo conditions, trastuzumab only mediated 40 % lysis of tumor cells at maximum concentrations of 1–5 µg, while ertumaxomab was able to mediate total tumor lysis at 25 ng/ml [53]. One major limitation of trastuzumab therapy is its inability to lyse
tumor cells that express low levels of HER2. In the same preclinical study, trastuzumab only lysed 10% of 1+ HER2 cells at a concentration of 5 µg/ml, while ertumaxomab mediated 100% tumor cell killing at 5 ng/ml, at an E:T ratio of 20:1. This potency was maintained when the effector cells were significantly reduced [53]. Therefore, ertumaxomab mediates significantly improved lysis of HER2+ cancer cells at low E:T ratios and can promote the lysis of cells that are typically resistant to trastuzumab-promoted ADCC. As of 2012, ertumaxomab has been studied in two phase I clinical trials, in order to determine its toxicity profile. The first phase I trial, with four malignant ascites patients, demonstrated that ertumaxomab was well tolerated, resulting mainly in flu-like symptoms, including fever, chills, and fatigue [46]. This prompted a second phase I trial for 15 patients with HER2 expressing metastatic breast cancer. Utilizing a similar dosing regimen as catumaxomab, ertumaxomab was administered i.v. on days 1.7±1 and 13±1 with an initial dose of 10 µg and a final dose of 200 µg. Again, most adverse events were associated with flu-like symptoms. A few adverse events were considered serious, based on grade 3 and 4 lymphocytopenia and an increase in liver enzymes. All adverse events were reversible and the MTD was determined to be 100 µg. Of the 15 evaluable patients, one patient experienced a CR, two had PR, and two had SD [59]. Measurement of cytokine levels revealed that ertumaxomab elicited a strong Th1 cytokine response, as IL-6, IL-2, TNF-α, and IFN-γ were all elevated. Also, surprisingly, only five of the fifteen patients developed HAMA or HARA. Currently, ertumaxomab is being studied in one phase I/II clinical trial for the treatment of HER2+ solid tumors (NCT 01569412).
3.4.4 Bi20 (FBTA20)

A third Triomab, Bi20 or FBTA20, targets CD20 on lymphoma cells, linking T-cells and innate immune cells through the same CD3 domain and the intact Fc domain, respectively, as the other two triomabs. Preclinical data demonstrated the potent ability of Bi20 to mediate cytotoxicity of CD20+ B cell lines at E:T ratios of 5:1. At a concentration of 50 ng/ml, Bi20 killed 95–100 % of B-cells, while rituximab, the FDA approved mAb to treat CD20 cancers, only mediated 65 % of B-cell lysis at 50 µg/ml [110]. In contrast to rituximab, Bi20 mediated enhanced T-cell and monocyte/macrophage activation and proliferation, a strong Th1 cytokine response, and lysed low expressing CD20 B-cells. These findings were replicated using B-cells isolated from patients with chronic lymphocytic leukemia, where Bi20 mediated efficient lysis even when CD20 expression was extremely low [14]. A phase I trial of i.v. delivered Bi20 for CLL and NHL patients in combination with donor lymphocytes, demonstrated mild adverse events of fever, chills, and bone pain. None of the six patients developed HAMA with Bi20 treatment and survival ranged from 38 to 486 days [20]. Bi20 is currently not being tested in any clinical trials but could prove to be efficacious in B cell malignancies.

Also of interest are two triomabs created in 2004 that target melanoma-associated proteoglycans (TRBs02) and the melanoma-associated gangliosides GD2 and GD3 (TRBs07). The parental hybridomas B5 and Me361 were each fused with the CD3 hybridoma 26II6 to create TRBs02 and TRBs07, respectively. Both triomabs were capable of inducing a Th1 cytokine response when exposed to tumor cells and PBMCs, associated with TNF-α, IL-2, IL-6, and IFN-γ, as well as the immunosuppressive
cytokines IL-10. TRBs02 and TRBs07 induced the proliferation of CD8+ and CD4+ T cells but reduced the number of NK cells and monocytes [99]. Lysis of antigen presenting cells was modest compared to other triomabs but combining both bsAbs was especially potent against cells expressing both tumor antigen targets.

3.5 ALTERNATIVE FULL-LENGTH BSABS

With the first clinical approval of a bsAb as well as extremely promising preclinical and clinical results of other bsAbs, there has been a renewed interest in developing methods to produce non-immunogenic, production-scalable bsAbs. These include two novel redox reactions, a knobs-into-holes method, a CDR mutational method, bispecific CovX bodies, and a mAb with an extra Fv domain. Both redox reactions take existing mAbs and expose them to reducing agents, 2-mercaptoethane-sulfonic acid sodium salt (MESNA) or glutathione (GSH), respectively [22, 112]. These methods result in the relatively quick formation of a bsAb with one binding domain for each antigen. Strop and colleagues mutated the hinge region with oppositely charged amino acids and the CH3 domain of each separately expressed mAb. This method facilitates the stabilization of the bispecific antibody format. As a proof of principle, using this method, they created a HER2 × EGFR and a CD20 × CD3 bsAb, which have hybrid IgG1/IgG2 Fc domains [112]. The CD20/CD3 bsAb mediated 80 % lysis of the CD20 positive cell line, A20, at 1 µg/ml with a low E:T ratio of 5:1 and could mediate a depletion of CD19+ A20 cells in vivo. The proof of principle bsAbs created from Heath’s group demonstrated that the redox method could create bsAbs in 6–10 days of same species or cross species fusions [22].
Another unique method utilizes the knobs-into-holes technology originally hypothesized by Watson and Crick and developed in the 1990s. By replacing a small amino acid (AA) side-chain with a large AA side-chain, or “knob,” in the CH3 domain of one antibody and replacing a large AA side-chain with a small AA side-chain, or “hole,” in the CH3 domain of another antibody, the yield of bsAbs can be significantly increased [95]. This discovery enhances the heterodimerization of dual-targeting antibodies and reduces the different types of possible isomers to four. As one example, they created a bsAb targeted against VEGF-A and Ang-2, showing that the bsAb bound to both anti-gens with the same affinity as the parental mAbs. This bsAb also inhibited in vivo growth of Colo205 cells by 92 %, while the combination of both mAbs were somewhat less efficient, inhibiting growth by 78 % [101, 102]. Therefore, targeting two growth factors with one antibody may impart an enhanced antitumor effect.

One group, in an effort to approach the bi-specificity from a different angle, developed an intriguing method that results in two different antigen-binding domains on each Fab arm. Starting with a full-length mAb, this group creates a library of variants with mutated light-chain (LC) complementarity determining regions (CDRs). All variants are then analyzed for binding to the two antigens of interest, with the goal of isolating a variant with strong affinity for two targets. The first of these “two-in-one” bsAbs was created from trastuzumab and mutated to also bind to VEGF. The best dual-targeting variant bH1 underwent a second round of mutations to enhance the affinity for each antigen. The resulting high affinity variant, hB1-44, was able to inhibit the growth of Colo205 cells in vivo better than either parental antibody alone [16]. Another two-in-one antibody, MEHD7945A, was created using a phage display library of mutated Fab
domains to target HER3 and EGFR. As before, this bsAb has identical Fab arms and is capable of binding either antigen with high affinity. MEHD7945A was capable of inactivating the EGFR and ERK pathway at an IC50 = 0.03 and 0.16 µg/ml, respectively, inhibiting growth of EGFR+ lines in vitro, and stunting the growth of EGFR+/HER3+ cells in vivo. This was compared to the combination of the two parental mAbs [101, 102]. Also, this bsAb could mediate ADCC and when tested in cynomolgous monkeys, elicited significantly less dermatological toxicity than the FDA approved EGFR parental antibody, cetuximab. The clinical dose was determined to be 8–12 mg/kg of MEHD7945A and is currently being evaluated in a phase I trial for patients with epithelial tumors (NCT01577173) [55].

Other promising chemical conjugation techniques are bispecific CovX bodies and mAb-Fv fusions. The bispecific CovX body is a unique method that utilizes the same Fc backbone, a so-called scaffold antibody CVX-2000, with interchangeable antigen-binding domains, allowing for rapid creation of mAbs or bsAbs. The antigen binding domains are chemically linked using an azetidione linker to the scaffold antibody after the antigen peptides are fused together using maleimidethiol ligation. The first of these bsCovX bodies, CVX-241 targeted Ang-2 and VEGF-A, with bivalent binding on each Fab arm, as each arm of the antibody can bind to either antigen [30]. CVX-241 was capable of inhibiting the growth of Colo205 cells in a xenograft model, compared to the combination of Ang-2 and VEGF-A monoclonal CovX bodies. Of particular interest, the bispecific antibody synergized with the common chemotherapeutic agent irinotecan to significantly inhibit in vivo growth. The first phase I trial with CVX-241 was terminated due to poor pharmacological properties at the highest dose of 25 mg/kg.
Instead of trying to manipulate the basic binding structure of mAbs to allow for a second binding site, another method fuses an Fv region to the Fc domain of a mAb. If the Fv domain targets CD3, then the resulting bispecific antibody, called a mAb-Fv, can mediate antigen binding, ADCC, and retargeting of T-cells, similar to triomabs. The first set of these antibodies targeted HER2 × CD3 × CD16 and HM 1.24 × CD3 × CD16. Surprisingly the HER2 mAb-Fv variant bound to CD16 with higher affinity and mediated greater than sevenfold higher lysis via ADCC than the parental mAb trastuzumab [85]. Moreover, the mAb-Fvs still bound to FcRn with similar affinity as the parental antibodies, suggesting that serum half-life of the fusion antibodies will not be compromised.

### 3.6 RECOMBINANT BISPECIFIC ANTIBODIES

#### 3.6.1 TANDEM SCFV

As an alternative approach to creating full-length bsAbs, many groups have chosen to manipulate the inherent structure of the antibody itself, creating a plethora of unique molecules. The basic design of these recombinant proteins involves deconvoluting the structure of an antibody down to the elements that are vital for antigen recognition and binding. With two binding sites for antigens and no Fc domain, these proteins retarget immune cells to tumor cells through CD3 or CD16. Utilizing T cells or innate killer cells, they can mediate significant lysis of antigen expressing cells (Fig. 1.3). Expressed in bacterial or mammalian cells, the recombinant proteins are purified using a variety of techniques. The first developed technique fused two single-chain variable regions (scFvs) together using a peptide linker usually between 15 and 20 AA in length,
creating a tandem scFv (TaFv). The AA linker length imparted flexibility for the scFvs, allowing for the correct domains to form together. Early designs demonstrated the lytic capability of these immune cell retargeting antibodies, as they were more cytotoxic than their parental mAbs [28, 37]. One TaFv, developed in an effort to circumvent the lack of success seen by the quadroma 2B1, targeted HER2 and CD16. The protein-mediated significant lysis of HER2 overexpressing cells and demonstrated good tumor retention in vivo [79]. rM28, a unique TaFv, targeted the melanoma-associated proteoglycan (NG2) and redirected T-cells through CD28 activation. Therefore, rM28 links tumor cells to T-cells while simultaneously providing them with a potent activating signal, resulting in “targeted supra-agonistic CD28 stimulation” directly at the tumor site [36]. Exciting preclinical results moved this bsAb to a phase I/II clinical trial in 2005 (NCT 00204594). Unfortunately, this trial was terminated due to the significant adverse events seen in a phase I trial of the CD28 mAb TGN1412, which caused life-threatening cytokine storm in treated volunteers [113]. A successor TaFv that targets CD20 and CD28 was then created using the same CD28 scFv. r2820 eliminated CD20+ lymphoma cells at modest antibody concentrations (0.5 µg/ml) [90]. E3Bi, another TaFv created using a 14 AA linker sequence, targeted EpCAM and CD3. This molecule was capable of eliminating EpCAM+ cells both in vitro at extremely low E:T ratios of 1–2.5:1, and in vivo, where it significantly inhibited tumor growth [93]. An alternative approach to the retargeting of immune cells is the inhibition of growth factor receptor signaling. One example of this method is the scFv fusion protein, MM-111, which recognizes HER2 and HER3, inhibiting the intracellular signaling of both tyrosine kinase receptors [80]. This fusion protein differs in the basic structure from other TaFvs, as it is also bound to modified
human serum albumin, in an effort to enhance MM-111 serum stability. Further studies need to be done to determine if this type of scFv fusion has antitumor capabilities, as it lacks any effector cell mediated lytic potential. It is currently involved in three phase I clinical trials for HER2-amplified cancers.

3.6.2 DIABODY

Diabodies (Db) are another recombinant bsAb that have been intensively studied. These proteins consist of the same scFv domains as TaFvs but utilize a shorter linker sequence. This results in a reduced yield of homodimers, essentially driving the formation of the bispecific format. Specifically, the heavy-chain variable domain (VH) and the light-chain variable domain (VL) of each antigen binding chain are forced to connect with respective domains on the second antigen chain. In other words, for antigens A and B, TaFvs read VH(A)—VL(A) (linker) VH(B)—VL(B), while Dbs read VH(A)—VL(B) (linker) VH(B)—VL(A). This allows for bivalent antigen binding on each half of the Db. As of 2012, over 40 different types of bispecific Dbs have been developed in preclinical studies, but none have entered human trials. This is mainly due to the short half-life seen with these small molecules, as continuous infusion appears to be necessary for adequate serum concentrations. The first Db design targeted the hapten phenyloxazone and hen egg lysozyme, was expressed in Escherichia coli (E. coli), and purified using affinity chromatography, yielding 0.3–1 mg/L [49]. The first Db to be tested in vivo targeted HER2, was almost cleared from the blood within 4 h but was retained in the tumor for close to a day [1]. Holliger and colleagues also developed the first Db that retargeted T-cells to tumor cells, using CD3 to link T-cells to BCL-1 + lymphoma cells. At a molecular weight of 50 kDa, this Db was capable of mediating 80
% lysis of lymphoma cells at the extremely low concentration of 63 ng/ml [50]. It also induced significantly higher lysis than a CD3 × BCL-1 quadroma bsAb, showing for the first time that the Db format could mediate higher tumor lysis than a similar quadroma bsAb. Of the CD3 T-cell retargeting Dbs CD19, CEA, EGFR, Endoglin, and PSMA Dbs are of particular interest. All five of these Dbs were expressed in E. coli, with a yield ranging from 0.1 to 3 mg/L [33, 45, 51, 60, 65]. The CD19 × CD3 Db-mediated lysis of CD19+ lymphoma cells at a tenfold lower concentration than a CD19 × CD3 quadroma bsAb, although the E:T ratios and maximum lysis were modest [60]. Targeting CEA expressing colon carcinoma cells with IL-2 stimulated PBMCs and 100 ng/ml of the respective Db, resulted in 50 % lysis at the moderate E:T ratio of 10:1 [51]. The EGFR Db, Ex3, only mediated 60 % lysis at 1 µg/ml with 100 times more lymphokine-activated killer cells than EGFR expressing bile duct carcinoma cells [45]. Therefore, the group attempted to enhance the lytic capacity by humanizing the diabody, creating hEx3. This Db was able to mediate 100 % tumor inhibition at 0.5 pmol/ml at an E:T of 5:1 and enhanced in vivo survival from 10 weeks (control) to 23 weeks [6]. The endoglin targeting Db-mediated 50–60 % maximum lysis of endothelial cells at high LAK levels [65]. Lastly, the PSMA single-chain Db (scDb), which conforms to a slightly different shape with the binding domain for PSMA and CD3 existing on opposite sides of the molecule, was able to significantly inhibit tumor formation of prostate carcinoma cells in mice [33]. The remaining immune retargeting Dbs bind to CD16 and CD19, CD30, or EGFR. Not surprisingly, these Dbs required significantly high E:T ratios to mediate effective lysis of target cells [5, 8, 62].
3.6.3 MULTI-TARGETING ANTIBODIES

In an effort to enhance the serum stability of these recombinant proteins, increase the avidity for an antigen, or increase the number of targets, groups added additional antigen binding domains. Similar to the CD3 x CD19 Db, Kipriyanov et al. created a Tandab, or tandem diabody, that targeted the same antigens. This format results in a protein that’s double the size of Dbs (~114 kDa), increasing half-life, with four Vh–Vl domains instead of two [61]. This Tandab was able to stimulate T-cells, elicit higher lysis, and significantly inhibit the growth of in vivo lymphoma cells compared to the CD19 x CD3 Db [24, 61]. Another group developed a similar molecule, called a tetrabody, from the humanized Ex3 Db. Although this molecule has four binding domains, similar to Tandabs, it is arranged in a circular format instead of a linear one. This hEx3 tetrabody was 113 kDa and could mediate the same lysis as the Db but at a 100-fold lower concentration, resulting in an extremely low EC50 of 0.5 fmol/ml at an E:T of 5:1 with LAK cells [7]. The triplebody, another bsAb format which binds to three antigens, is created by linking three scFvs together. Four promising candidates, CD19 x CD16 x CD19, CD33 x CD123 x CD16, CD123 x CD16 x CD123, CD33 x CD19 x CD16, exhibited enhanced stability, were expressed in mammalian cells, and induced half-maximum lysis in the low picomolar range [58, 67, 105]. As these molecules have the potential to form aggregates when in suspension, it remains to be seen if their potent lytic capabilities will translate safely into humans.
3.7 BISPECIFIC T-CELL ENGAGERS

3.7.1 EARLY GENERATION BITES

Even though BiTEs, or bispecific T cell engagers, have not been evaluated in phase III clinical trials, they may represent the future of the bsAb field. The concentrations required to induce significant lysis in vitro and in vivo are some of the lowest seen for any anticancer therapy. By redirecting T cells to antigen positive tumor cells, this approach is not unique. What is unique, is the ability to eliminate cancer cells at E:T ratios that are orders of magnitude lower than other bsAbs (1:1 or 1:10) with PBMCs that do not require pre-stimulation with IL-2 or CD28 activation [66, 121]. As two scFvs bound together with a short gly4-ser1 linker, the bispecific molecules are only approximately 55 kDa and are capable of binding only one antigen on each arm (Fig. 1.4). Currently, Micromet is in various stages of preclinical and clinical tests involving ten BiTEs that target eight different antigens. They are CD19, EpCAM, HER2/neu, EGFR, CEA, CD33, EphA2, and MCSPs [9]. One of the most surprising features of these bispecific molecules is their ability to mediate potent lysis without the need for MHC Class I [87]. Therefore, these molecules can utilize T-cells to target any type of cell, regardless of the T-cell’s ability to inherently recognize the target cell. Importantly, BiTEs address previous toxicity limitations seen with earlier CD3-based bsAbs by utilizing a lower binding affinity to CD3, allowing for T-cell activation only at tumor sites. Therefore, BiTEs lead to specific antitumor effects without potentially life-threatening host toxicity caused by excessive cytokine release.

MT103 and MT110, which target CD19 and EpCAM, respectively, are the only
two BiTEs in clinical trials but many more will follow soon, as all published results for these molecules are promising. The EpCAM-targeted BiTE is only undergoing a phase I clinical trial, when it was the first developed. In 1995, Mack et al. developed the scFv fusion protein targeting CD3 and EpCAM. This molecule was capable of only being expressed in CHO cells, as *E. coli* expression resulted in inert protein [75]. Fortunately, the harvest of the EpCAM scFv fusion was significantly higher than other recombinant bsAbs, yielding 12–15 mg of protein per liter of media. This early generation BiTE was capable of inducing significant lysis at only 8 ng/ml and was stable in PBS for 6 months and serum for 56 h [66, 75]. Not surprisingly, CD8+ and CD4+ T-cells were required for the antitumor lysis [66]. The early generation EpCAM BiTEs could induce substantial lysis at concentrations near or below 1 ng/ml, all utilizing unstimulated PBMCs from healthy and cancer patients [76, 103, 120]. One in vivo study found that 1 µg of the EpCAM BiTE could protect all six mice from challenge with SW480 colon carcinoma cells and even eliminate growth in some mice with established tumors. This same study discovered that the bsAb could utilize tumor infiltrating T cells to eliminate tumors engrafted in mice when isolated directly from patients at only 5 µg/mouse [103]. This experiment, where three of six mice treated with the BiTE survived challenge, proved that this molecule could utilize a patients’ own T-cells to modulate an antitumor effect. Early generation CD19 BiTEs showed similar promising results. First created in 2000, Loffler et al. demonstrated that the CD19 BiTE could retarget T-cells to lymphoma cells, eliciting significant lysis at low E:T ratios (2:1 or 4:1) at concentrations between 10 and 100 pg/ml, a 10–100-fold lower concentration than even the EpCAM BiTE and 1–10,000-fold lower than a quadroma CD19 × CD3
bsAb [71]. Interestingly, short-term addition of IL-2 to PBMCs did not drastically enhance the lysis of B-cells by the CD19 BiTEs, although long-term exposure of IL-2 resulting in LAK effector cells enhanced the EC50 by tenfold [31, 72]. Even at E:T ratios of 1:4, the CD19 BiTE could eliminate all CD19+ B-cells at a low concentration of 5 ng/ml [72]. An in vivo study found that, similar to the EpCAM BiTE, the CD19 BiTE could protect mice from tumor challenge and even eliminate large establish tumors [32]. The BiTEs appear to require T-cells to release perforin to mediate lysis, as cells treated with the perforin inhibitor Oncanamycin A (OMA), had significantly reduced lysis [38].

3.7.2 MT103 (BLINATUMOMAB)

Learning from previous designs, Micromet designed and created MT103, the CD19 × CD3 BiTE currently in clinical trials. MT103 exhibited significant preclinical potency to CD19+ autologous B-cells, with an EC50 of 130 pg/ml with human PBMCs and 150 pg/ml with chimpanzee PBMCs [104]. When the BiTE was given to chimpanzees, the molecule was well tolerated, with an increase in Th1 cytokines and T-cell activation. After a dose of 0.1 µg/kg in chimpanzees, MT103 peaked around 1 ng/ml and reduced to 50 ρg/ml within 10–24 h after treatment [104]. As MT103 can induce the release of the Th1 cytokines IFN-γ, TNF-α, and IL-2, it would most likely require co-administration with an anti-inflammatory agent. Therefore, Micromet analyzed the functionality of MT103 with the clinically used anti-inflammatory agent dexamethasone [17, 19]. Fortunately, the anti-inflammatory agent did not reduce lysis or T-cell activation but did attenuate cytokine release [17]. When compared to a CD19 × CD3 tandab, MT103 was 1,000 times more potent, when unstimulated T-cells were used,
highlighting for the first time, that the BiTE format was more potent than similarly designed recombinant bsAbs [84]. Another comparison study found that MT103 mediated 150–450 times more efficient lysis than rituximab to CD19+ CD20+ cells but found that even in optimal conditions for rituximab, MT103’s addition could still enhance tumor cell apoptosis [25]. Even with extremely low E:T ratios, MT103 was capable of inducing significant cell death, implying that T-cells can mediate serial killing of target cells [25]. These encouraging results led to a phase I trial for MT103 for patients with non- Hodgkin’s B-cell lymphoma. Of the 38 patients treated, 11 exhibited objective responses, with four CRs and seven PRs. All six patients treated at the highest dose of 0.06 mg/m² showed a response to treatment. Overall, the adverse events were mild, including chills, pyrexia, lymphopenia, and leukopenia and occurred in the first week of treatment. No antidrug antibodies were seen in any of the 38 patients [10]. A phase II trial, published in 2011, demonstrated an 80 % response rate in 21 patients with adult B-cell lineage ALL, treated with a dose of 0.015 mg/ m². After a single cycle of MT103, 16 of 20 evaluable patients were switched from minimum residual disease (MRD) positive status to negative. As MRD is a common indicator of relapse and these patients were heavily pretreated, these results are promising. Overall, the estimate of relapse-free survival was 78 % [114]. As in the phase I trial, MT103 was well tolerated, with similar adverse events. A secondary analysis of the immunological responses to MT103 revealed that most patients responded with similar trends after treatment. Within a single day, the number of circulating B-cells dropped to one B-cell/µL of blood, and did not increase through-out the treatment cycles. The percentage of activated CD8+ and CD4+ T-cells increased from 19.47 to 48.78 % and 12.32 to
35.63 %, respectively, elucidating MT103’s potent effects on T-cells. Fortunately, no patient developed HAMA [64]. With these results, MT103 is currently being evaluated in six phase I or phase II clinical trials, and phase III trials results are awaited with interest.

3.7.3 MT110

Similar to MT103, Micromet used previous EpCAM BiTE designs to enhance the potency of the bispecific molecule being tested in clinical trials. The resulting BiTE, MT110, is ~55 kDa, can redirect PBMCs to tumor cells with an EC50 of 230 µg/ml, can eliminate tumor initiating cells and is not affected by circulating levels of EpCAMs ectodomain, EpEx [18, 19, 48, 91]. MT110 was also capable of killing primary pancreatic cells at concentrations of 1–100 ng/ml [23]. In vivo treatment of 1 µg MT110 resulted in the complete prevention of tumor growth in both challenge and established tumors [18]. Interestingly, MT110 was able to induce tumor elimination in mice utilizing only the T-cells that infiltrated the tumor before removal from patients, again elucidating the potency of retargeting T-cells to fight cancer. In an attempt to determine if an EpCAM BiTE would be toxic to normal EpCAM expressing cells, Brischwein and colleagues performed a series of pre-clinical studies using a murine EpCAM BiTE analog in immune-competent mice. MuS110 could recognize murine CD3 and murine EpCAM and has an EC50 similar to MT110, albeit slightly worse [2, 19]. At doses of 15 µg/kg, MuS110 was well tolerated in mice and was capable of inhibiting syngeneic tumor growth. Increasing the dose to 50 µg/kg or 500 µg/kg resulted in cytokine release syndrome or death in mice, respectively [2, 3]. In the 15 µg/kg treated mice, MuS110 caused a fivefold drop in B-cell numbers and an eightfold drop in T-cell numbers [3]. Unlike humans, mice have EpCAM + lymphocytes, which might be
contributing to side effects such as lymphopenia. Therefore, it is possible that initially eliminating this subpopulation of cells could enhance the tolerability of MuS110. Initial low doses of 0.4–10 µg/kg tolerized mice to doses of MuS110 up to 500 µg/ kg [3, 4]. MuS110 did not damage EpCAM + tissues or organs and long-term dosing did not result in T-cell anergy, strengthening the clinical utility of an EpCAM BiTE. All of these results led to an ongoing phase I clinical trial in patients with EpCAM + solid tumors.

3.7.4 OTHER BITES

The remaining BiTEs in clinical trials target CEA, EphA2, EGFR, CD33, and MCSPs. The CEA BiTE was found from a panel of CEA BiTEs created from three CEA mAbs and thirteen scFvs directed against CEA, all with the same CD3 binding domain. The most potent protein, MT111, had an EC50 of 200–500 pg/ml with unstimulated PBMCs and an EC50 of 2.3 pg/ml using stimulated human T cells. It inhibited tumor xenograft growth and had similar lysis across 12 different CEA expressing tumor lines [73]. As many patients with CEA + tumors have circulating soluble CEA (sCEA), it was important to discover that sCEA did not inhibit tumor cell lysis by MT111 [73, 88]. On top of this, a murine analog of MT111 was capable of inhibiting metastatic lung lesions in a syngeneic mouse model [73]. Both of these findings underscore the clinical potential of this BiTE.

EphA2 is a tyrosine kinase receptor that is often overexpressed in aggressive epithelial cancers. The EphA2 BiTE developed by Micromet is unique from others in its class, as it only recognizes EphA2 on malignant cells, sparing any T cell elimination of normal cells. The BiTE was capable of eliminating tumor cells at EC50 concentrations
of about 6 ng/ml and could mediate maximal lysis at an E:T ratio of 1:5 with 7 ng/ml of the bsAb. Confirmatory results were seen in vivo [42]. The remaining BiTEs targeting EGFR, CD33, and MCSPs offer promising further pre-clinical and clinical results. C-BiTE and P-BiTE, developed from the binding domains of cetuximab and panitumumab, two FDA approved EGFR mAbs, were able to lyse KRAS- and BRAF-mutated colorectal cells [74]. The MCSP BiTE recognizes the D3 domain of the heavily glycosylated human melanoma chondroitin sulfate proteoglycan (MCSP) [13]. The bsAb-mediated significant lysis of 33 MCSP+ melanoma cell lines with PBMCs isolated from healthy and melanoma patients, albeit reduced with the latter cell sources [115]. The CD33 BiTE, MT114, exhibits similar potent lysis, with EC50 values as low as 5 pg/ml and mediates lysis across numerous CD33+ AML cell lines [29, 63].

3.8 IMAGING

There is an evolving area of the tumor immunology field that is utilizing bsAbs for cancer imaging. Since the second target can be engineered to bind to a radiolabeled agent, these bsAbs are simpler to create than radiolabeled mAbs. One of the most common methods for creating radiolabeled bsAbs is the dock-and-lock (DNL) technique. Originally developed in 2006, the DNL method can be used to efficiently create bsAbs for any target but as of 2012 was entirely devoted to the creation of radiolabeled bsAbs [97]. TF2, which targets CEA and the hapten histamine- succinyl-glycine (HSG), is currently undergoing three phase I trials and one phase I/II trial for imaging colorectal or lung cancers [78]. After injection and clearance of the bsAb, 99mTc-labeled hapten is infused into the patient, resulting in radioactive labeling of only bsAb bound cells. TF4 and TF10 are two other DNL bsAbs directed against CD20 × HSG hapten and MUC1 ×
HSG hapten, respectively [35, 108].

3.9 CONCLUSIONS

The clinical efficacy of catumaxomab and blinatumomab highlight the potential of bispecific antibody therapy modalities. The progress seen 25 years following the production of the first such molecules augurs well for the future of the field (Table 1). Learning from previous designs and trials, researchers have developed molecules that overcome problems like the development of HAMA, by creating bsAbs that work at increasingly low concentrations, precluding the need for excessive doses or humanization. First generation bsAbs required such high ratios of effector to tumor cells that could never translate into clinical efficacy. The efficiency of newer compounds permits them to be used at low concentrations that require potentially physiological effector: target ratios. Moreover, by targeting hematologic malignancies, the potential barriers of access of effector cells to tumor targets may be minimized. Challenges remain; the need for prolonged continuous infusion makes BiTEs inconvenient and expensive for patients. Additional modifications to promote prolonged half-lives may be required for these reagents to achieve their full potential. Moreover, the ability of BiTEs to effectively treat solid human tumors in the clinical setting remains unproven. However, the presence of these challenges should not obscure the fact that bispecific antibodies can work to effectively treat cancer. The challenge to the field is to build upon the exciting findings for the benefit of patients with diverse forms of cancer.
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3.11 PRIMARY FIGURES

3.11.1 FIGURE LEGENDS

Figure 3.1. First generation bispecific antibodies. *Dark regions* correspond to heavy chains, while *light regions* correspond to light chains. A monoclonal antibody is depicted in, with the Fab domain binding to an antigen and the Fc domain binding to FcγRs. *Red domains* represent regions with a different antigen specificity. The *green antibody domain* symbolizes the scaffold antibody for a bs-CovX body. *Black lines* represent linker domains. Bispecific antibodies of the quadroma, chemical crosslinking, bs-CovX body, and mAb-Fv formats are shown.

Figure 3.2. Triomab’s mechanism of action. Trifunctional antibodies, such as catumaxomab, ertumaxomab, and Bi20, lyse tumor cells by retargeting T-cells to antigen expressing tumor cells. The Fc region of the triomab binds to cells of the innate immune system, such as NK cells, which can induce ADCC of the tumor cell as well as supplying stimulatory signals to the retargeted T-cells. Once conjugated, T-cells or NK cells release perforin and granzymes to induce apoptosis of the tumor cell.

Figure 3.3. Recombinant bsAbs. The antigen binding components of a mAb are isolated to create smaller antibodies, reducing their stability and immunogenicity. *Black lines* represent linker domains. Bispecific antibodies of the diabody, single-chain diabody, BiTE, tandab, triplebody, and tetrabody formats are shown.

Figure 3.4. BiTE’s mechanism of action. Bispecific T-cell engager’s function by linking T-cells directly to tumor cells through CD3 on T-cells. Conjugation of T-cells to tumor
cells results in the release of perforin and granzyme, inducing apoptosis in the target cells. BiTEs exist that target CD19, EpCAM, EGFR, CD33, MCSPs, CEA, and EphA2
FIGURE 3.1. First Generation Bispecific Antibodies
Figure 3.2. Triomab’s Mechanism of Action.
Figure 3.3. Recombinant bsAbs.
Figure 3.4. BiTE's Mechanism of Action.
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Citations represent the most recently published trial. If a reference isn’t cited, the clinical trial is currently in progress.
CHAPTER 4

FUNCTIONAL GENOMICS SCREENING APPROACH TO IDENTIFY NOVEL TUMOR-BASED MODULATORS OF IMMUNE REJECTION

4.1 AIM, HYPOTHESIS, & GOAL

The overall aim of this study was to identify some of the molecular mechanisms utilized by cancer cells to manipulate the immune response in the tumor microenvironment. We hypothesized that since immune-edited tumor cells modulate recognition and elimination by the immune system through diverse mechanisms, it would be possible to identify gene knockdowns that affect survival when exposed to a functional immune system. By utilizing a genome-wide shRNA library and exposing library transduced tumor cells to differing components of immunity would select for tumor clones that have an integrated shRNA that modulates the anti-tumor immunity. The goal was to identify novel tumor antigens or targets for immune-stimulatory therapy.

4.1.1 SPECIFIC AIMS

1. Establish a genome-wide shRNA library in EO771 murine mammary carcinoma cells
2. Screen the EO771-library cells in an *in vivo* setting with immune-competent and –deficient mice to identify shRNAs that are differentially enriched or lost when exposed to the adaptive immune system

3. Perform an *in vivo* screen with EO771-library cells in mice with an immune system primed to eliminate EO771 tumor cells

4. Validate the identified gene candidates from the *in vivo* screens as functional mediators of immune modulation

5. Characterize the mechanisms behind successful immune modulation of validate targets

6. Generalize the validated findings across multiple syngeneic murine model systems

### 4.2 SIGNIFICANCE AND CONTEXT

The tumor microenvironment is composed of a heterogeneous population of cell types, including, but not limited to, malignant cells, fibroblasts, T-cells, dendritic cells (DCs), and macrophages. The cross-talk occurring among these cell populations to influence the anti-cancer immune response is exceedingly complex and challenging to decipher in traditional models (Dunn et al. 2002). The immune system’s ability to recognize and eliminate malignant cells, a process termed immune surveillance, can determine the fate of the neoplasm and ultimately, the host. Efficient elimination of neoplastic cells is mediated by a slew of factors regulated by a multitude of cells types. For the adaptive immune system, proper antigen
processing, dendritic cell (DC) and cytotoxic T lymphocyte (CTL) activation, and target cell lysis mediate one side of the anti-cancer immune response. Tumor cells modulate surface receptors that directly affect immune activation and recognition, such as MHC class I, PD-L1, B7 family members, CD40, ICOSL, and OX40L, to avoid immune surveillance (Driessens, Kline, and Gajewski 2009; Pardoll 2012; Dunn et al. 2002). In the tumor microenvironment, the local levels of immune-related soluble factors such as IL-4, Indoleamine-pyrrole 2,3 dioxygenase (IDO) and TGF-β, can disrupt the recruitment, activation, and survival of cytotoxic immune cells, thereby limiting the magnitude and effectiveness of the anti-tumor immune response (Shuptrine, Surana, and Weiner 2012) (Surana et al. 2014). Therefore, even if the immune system is capable of inducing an effective adaptive immune response – by processing tumor antigens, activating a Th1 immune response, and infiltrating CD8+ T cells into the tumor – neoplastic cells can degrade their cytotoxic potency.

While the mechanisms utilized to modulate this anti-cancer immune response are being revealed, the clinical success of antagonistic CTLA-4 and PD-1 monoclonal antibodies (mAbs) highlight the potential of immune-stimulatory therapy in treating cancer (Wolchok et al. 2013), (Hodi et al. 2010), (Lynch et al. 2012), (Topalian et al. 2012). When PD-1 and CTLA-4 receptors on CTLs bind to their ligands, B7-H1 and CD80/86, respectively, an inhibitory cascade is triggered, resulting in T cell anergy (Gruber et al. 1994; Hodi et al. 2010; de Jonge et al. 1998; Duraiswamy et al. 2013). Importantly though, the majority of patients treated with a single non-tumor antigen-targeted immune stimulating antibody
develop resistance, indicating that other tumor-specific evasion mechanisms remain in play. While certain mechanisms are known, it is clear that neoplastic cell populations utilize a plethora of mechanisms to survive in immune competent hosts. Therefore, it is essential to identify these mechanisms with the ultimate objective of identifying combinatorial approaches that can overcome immune evasion. The potential of combination immunotherapy is underscored by successes in treating advanced melanoma with the combination of PD-1 and CTLA4 antibody antagonists [(Wolchok et al. 2013)].

In vitro models incompletely replicate the complexity of tumor: host immune-related interactions. Several groups have utilized in vivo screening approaches to examine determinants of resistance to small molecules (Seyhan et al. 2012; Berns et al. 2007; Brummelkamp et al. 2006). Recently, Zhou et al. used such an approach to deconvolute the T cell mechanisms involved in an anti-cancer immune response (Zhou et al. 2014). That study modulated T cell gene expression by shRNA knockdown to identify genes that are relevant to the in vivo anti-cancer immune response. We hypothesized that a similar in vivo RNAi screen utilizing an shRNA-transduced syngeneic cancer cell line in immune competent mice would facilitate the discovery of novel immune regulators in tumor cells.

Here, to our knowledge for the first time, we describe an in vivo screening approach to identify novel tumor-based mediators of the anti-tumor adaptive immune response. We developed a model system comprising of whole-genome shRNA library-transduced EO771 syngeneic breast cancer cells (EO771-library) engrafted in immune-competent or immune-compromised C57Bl/6 mice. We demonstrate that,
when grown in immune-competent or -deficient mice, EO771-library cells that evade functional adaptive immunity are selectively over-represented in surviving tumor cell populations. Knockdown of testis-expressed gene 9 (Tex9) led to selective survival in wild type (WT), but not immunodeficient mice. In contrast, EO771-library cells that are selectively targeted by functional adaptive immunity are relatively under-represented in surviving tumor cell populations in WT mice. Knockdown of CD47 and TGF-β as well as novel targets such as Sgpl1 and Pex14 led to the selective under-representation of the targeted cells in the immune-competent mice. Beyond these gene targets identified by in vivo screening and additional validation, other over- and under-represented gene targets were identified. This approach thus identifies potential targets that regulate adaptive anti-tumor immune immunity.

4.3 MATERIALS & METHODS

4.3.1 CELL LINES AND CULTURE

EO771 (gift from Dr. Peter Goedegeburre, Washington University in St. Louis, MO) and HEK293T (Georgetown Tissue Culture Shared Resource (TCSR)) cells were cultured at 37°C with 5% CO2 in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1x L-glutamine, and 100 units/ml of penicillin and streptomycin. All cell lines were periodically tested and determined to be free of Mycoplasma and other rodent pathogens.
4.3.2 VIABILITY AND PROLIFERATION ASSAYS

Crystal violet (CV) was used as a surrogate measure for viability. Briefly, EO771 cells were exposed to 50 μl 0.25% CV for 10 minutes, washed, allowed to dry overnight, exposed to 100 μl sodium acetate for 5 minutes, and luminescence (570 nm) was read by a multi-plate reader (Envision; Waltham, MA).

4.3.3 RNAi LIBRARY TRANSDUCTION

EO771 cells were transduced with lentivirus containing the pre-packaged shRNA library and Transdux (SBI) in 5 separate T-75 flasks. Infection was performed at an MOI of 3.76:1 in order to integrate shRNAs into 45% of cells (according to a positive-control empty vector infection assay), reducing the probability of multiple shRNAs per cell. After 72 hrs, the cells were selected using 5 μg/ml of puromycin for 10 days, whereupon they were cultured without puromycin. Library cells were cultured in vitro for 21 days, to ensure loss of cells containing shRNAs that inhibited fitness.

4.3.4 ANIMAL XENOGRAFT STUDIES

2 x 10⁶ EO771 cells were engrafted in the right flank of female C57Bl/6 (Jackson), C57Bl/6-SCID (Jackson), and NSG (Jackson) mice all aged 6-8 weeks. Tumors were measured twice-weekly using calipers. Once tumors reached 1 cm³, using the formula volume = length x width²/2, the mouse was sacrificed using CO2 inhalation, and the tumors were excised under sterile conditions. If the cells were to
be cultured, 50 μg/μl gentamycin was added. After one or two passages cells were cryopreserved and RNA was isolated. If the cells were to be stained for IHC, a section of the tumor was placed in 10% formalin for 48 hrs, whereupon it was placed in 70% ethanol (molecular grade). If the cells were to utilized for immune infiltrate analysis, a section of the tumor was digested in 1% Collagenase D (Sigma) at 37°C for 30 minutes while shaking vigorously.

4.3.5 LIBRARY shRNA AMPLIFICATION AND HYBRIDIZATION

Tumor shRNAs were amplified and biotinylated according to SBI’s instruction manual. Briefly, mRNA was reverse transcribed into cDNA, shRNAs were amplified and biotinylated using nested primers (SI691B-1), and non-shRNA DNA was degraded using a lambda exonuclease (NEB M0262L). Resulting amplified shRNA biotinylated barcodes were hybridized to an Affymetrix GeneChip Mouse Genome 430 2.0 array. For the first two in vivo screens, two tumor samples were combined (4.5 μg of each) and hybridized to an Affymetrix GeneChip Mouse Genome 430 2.0 array at the Genomics Core at George Washington University Medical Center. For the serial passaging screen, one tumor sample (9 μg of amplified DNA) was hybridized to one Affymetrix array at the Children’s Hospital in Washington, DC. Each array was analyzed using the software R.
4.3.6 DIFFERENTIAL shRNA ANALYSIS

Expression values for each array were batched together based on strain of mice and log2 transformed. A linear fit model, using the Limma Bioconductor package, was created and analysis was adjusted for multiple corrections (FDR). shRNAs with a log2 fold change $> 1$ and a q value $< 0.05$ were considered significant.

4.3.7 REAL-TIME PCR

RNA was isolated from tumor cells using an RNA isolation kit (Ambion; Catalog 12183018A). RNA concentration was identified spectrophotometrically using NanoDrop 1000 (Thermo Scientific, Waltham, MA). RNA was reverse-transcribed in 25 μl using Omniscript RT (Qiagen). According to the manufacturer’s recommendations, quantitative PCR was performed using Quantitect SYBR Green (Qiagen) with predesigned primer sets for CD47, Pex14, Sgpl1, Tex9, and GAPDH (Quantitect Primer Assay; Qiagen). The reactions were performed using the Mastercycler RealPlex² Real-Time PCR system (Eppendorf). Analysis was performed by using the $\Delta \Delta C_T$ method, with GAPDH as the endogenous loading control.
4.3.8 WESTERN BLOTTING

Protein expression confirmation was performed by flow cytometry or immunoblotting. Protein was isolated using a modified boiling technique and protein concentration was determined by the BCA assay (Bio-Rad). Cell pellets were lysed by heating to 100°C for 10 minutes in 100 μl of Boiling Buffer (1% SDS, 10 mM Tris, 1 mM Na3NO4, ddH2O, and 1 protease inhibitor tablet (Sigma)). Approximately 30 μg of protein was run on tris-glycine gels under reducing conditions. After being transferred to a nitrocellulose membrane (Bio-Rad), the membrane was blocked with 5% milk for 1 hour. Primary blotting for proteins was performed overnight at 4°C in PBS-T with 5% milk using the following antibodies: Tex9 (Sigma; SAB2103771; 1:750), Sgpl1 (Abcam; ab56183; 1:2000), Pex14 (Sigma; SAB4502176; 1:500), and beta-actin (Sigma; clone AC-74, 1:2500). Membranes were exposed to a secondary antibody in PBS-T for 1 hour at room temperature (horseradish peroxidase (HRP)-labeled anti-rabbit or anti-mouse IgG; 1:5000; GE Healthcare; Little Chalfont, Buckinghamshire, United Kingdom).

Supersignal West Femto high-sensitivity substrate (Thermo Scientific) and Supersignal West Pico (Thermo Scientific) were utilized for visualization of the western blots. A phycoerythrin (PE) conjugated antibody was used for flow cytometry analysis of CD47 (Biolegend; clone Miap301).
4.3.9 shRNA KNOCKDOWN

Individual pLKO shRNA plasmids, validated through the RNAi consortium (TRC), were purchased from Sigma or Open Biosystems. Lentivirus was created by transfecting HEK293T cells at 60% confluence in 10 cm plates with 6 μg shRNA DNA, 3.0 μg psPAX2 (Addgene plasmid 12260), 0.3 μg pCMV-VSV-G (gift from Dr. Todd Waldman, Georgetown University, Washington, DC), and 18 μl of Fugene 6 (Promega). 24 hours after transfection, media was replaced with normal growth medium. 48 hours after transfection, virus-containing media was isolated, centrifuged at 250g, and filtered using a 0.45-um syringe filter. Virus media was frozen at -80°C or exposed to target cells immediately. To generate shRNA infected cells, EO771 cells were transduced, at 50% confluence in a T-25 flask, with 3.5 ml virus media, 0.5 ml fresh media, and 3.2 μg polybrene (Sigma; St. Louis, MO). Cells were selected after 48 hours with 7.5 μg/ml of Puromycin (Sigma). After 10 days of selection, cells were assessed for protein knockdown.

4.3.10 FORCED OVEREXPRESSION

EO771 cells were transfected at 50% confluence in a 12 well dish with 1ug cDNA plasmids (Origene) and Fugene 6 (Promega; E2691) to induce expression of the desired transcript. After 48 hrs of exposure, EO771 cells were selected using 0.5 μg/ml neomycin (G418) for 10 days. Long-term culturing was performed with 0.1 μg/ml of G418.
4.3.11 IMMUNE INFILTRATE

Tumors isolated from mice were separated into a single cell suspension using 1% collagenase D in 10% FBS media (Sigma). Cells were stained with a live-dead dye (Life Technologies Catalog: L-3224) and various fluorescently tagged antibodies for murine immunological markers (CD45, Gr-1, F4/80, CD8, CD3, CD8a, CD11b, CD11c, NK1.1, CD4, CD25, CD80, CD86, MHC Class II) to identify macrophages, MDSCs, DCs, CTLs, CD4+ T cells, NK cells, and Tregs. Flow cytometry was utilized to quantify the components of the immune system evident in the tumors. Fc-receptors were blocked using 1mg anti-CD16/CD32 (Biolegend; TruStain FcX). Staining for surface antigens was done in PBS+1%BSA using the following antibodies: CD45 (Biolegend; clone 30-F11), F4/80 (Biolegend; clone BM8), MHCII (Biolegend; clone M5/114.15.2), CD80 (BD Bioscience; clone 16-10A1), CD86 (BD Bioscience; clone GL1), Gr-1 (Biolegend; clone RB6-8C5), CD11b (Biolegend; clone M1/70), NK1.1 (eBioscience; clone PK136), CD3 (Biolegend; clone 145-2C11), CD4 (eBioscience; clone RM4-5), and CD8a (Biolegend; clone 53-6.7). Results were analyzed using Prism 5 (GraphPad) and the total number of each cell population was normalized to the number of living CD45+ cells. Cell populations were considered significant if a two-way ANOVA resulted in a p < 0.05.
4.3.12 IMMUNOHISTOCHEMISTRY

Excised tumors from mice were separated into segments and a section was placed in 10% formalin for 48 hours, whereupon the tumors were placed in 70% molecular grade ethanol. Fixed tumor segments were sectioned and stained for various murine immunological markers by Georgetown’s Histopathology and Tissue Shared Resource (HTSR). Antibodies used are as follows: F4/80 (eBioscience; clone BM8), CD4 (eBioscience; clone 4SM95), CD68 (Abcam; clone KP1), FoxP3 (eBioscience; clone FJK-116s), CD8a (Santa Cruz; clone H-160), Gr-1 (eBioscience; clone RB6-8C5), and CD11c (Abcam; clone N418).

4.3.13 SURVIVAL ANALYSIS

Human survival analysis of breast cancer patients, stratified by gene expression, was performed using the online toolkit KMPlot.com, which includes Affymetrix HG-U133A, HG-U133 Plus 2.0, and HG-U133A 2.0 array analysis on 4,142 breast cancer patients. Analysis was performed by selecting probes corresponding to gene targets and best cutoff threshold was automatically selected. Biomarkers were considered significant with an FDR < 0.1 at a log-rank p value ≤ 0.027. Analysis was performed on the entire patient subset, as well as those with a triple negative basal phenotype. Animal survival analysis was performed using Prism 5 (GraphPad). Survival plots were considered significantly different if the Log-Rank (Mantel-Cox) test resulted in a p ≤ 0.05.
4.3.14 PATHWAY ANALYSIS

Pathway analysis of significant genes targeted by shRNAs was performed using Pathway Studio. Briefly, all statistically significant targeted genes, and their aliases, were uploaded to Pathway Studio for pathway creation. Selecting for direct connections between gene candidates identified enriched pathways. Statistically significant pathways were identified if the Jaccard index was $\leq 0.05$ or the $p \leq 0.05$.

4.3.15 STATISTICAL ANALYSIS

All statistical analysis, unless otherwise noted, was performed using GraphPad Prism version 5 (GraphPad Software; La Jolla, CA). * Indicates a $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.4 RESULTS

4.4.1 DEVELOPMENT OF A FUNCTIONAL GENOMICS SCREENING APPROACH FOR THE IDENTIFICATION OF TUMOR-DERIVED IMMUNE REGULATORS

Pooled shRNA screening approaches have proven to be useful in novel gene discovery (Zhou et al. 2014; Zender et al. 2008; Chen et al. 2015; Wolf et al. 2013; Andrysik et al. 2013; Yeung et al. 2009; Hattori et al. 2007). Individual barcoded shRNAs expressed in a heterogeneous population of transduced and non-transduced cells are readily identified, allowing for efficient screening in complex biological systems. We hypothesized that using an in vivo screening approach with a carcinoma cell line transduced
with a barcoded shRNA library, it would be possible to identify gene knockdowns that affect survival when those cells are exposed to a functional immune system. By engrafting these library cells in mice with varying immune phenotypes (e.g., wild-type (WT), severe-combined immunodeficient (SCID) and NOD SCID Gamma (NSG)), it thus is possible to parse the effect of gene knockdown on the immune system’s ability to identify and eliminate malignant cells (Fig. 4.1A). Hybridizing the shRNA barcodes to an Affymetrix array can identify the relative expression of individual shRNAs within a tumor (Fig. 4.1A).

The lentivirally-delivered shRNA library from System Biosciences used in these studies contains 150,000 unique shRNA constructs targeting 39,000 unique mRNA transcripts in the murine genome (3-5 shRNAs per target).

We chose to transduce the pooled shRNA library into EO771, a syngeneic breast adenocarcinoma cell line that spontaneously arose in a C57Bl/6 mouse (SUGIURA and STOCK 1952). As it is syngeneic to C57Bl/6, the tumor cell line thus grows in an immune-tolerant host, mimicking the analogous human tumor: host interaction. EO771 cells resemble human triple-negative/basal breast cancer, as they are estrogen-insensitive, HER2-negative, and have a mutated p53 (Johnstone et al. 2015).

Initial experiments showed that EO771 cells grow aggressively when engrafted subcutaneously and in the mammary fat pad of C57Bl/6 mice (data not shown). Preliminary multiplicity of infection
(MOI) experiments were conducted to achieve an average of one shRNA integration per transduced cell (data not shown.) The relative expression of each shRNA across two representative libraries was well preserved, as evidence by a strong linear fit (Fig. 4.1B). Transduced cells were cultured in vitro for 21 days, thereby creating an initial library containing shRNAs with limited effect on in vitro cell fitness. As a result, it was possible that an shRNA that enhanced the growth rate of EO771 cells could become overrepresented in the library. Therefore, we analyzed the shRNA representation of four independent freeze-downs of the same initial EO771-library cells 21 days post-transduction and did not find an overrepresentation of a few shRNAs (Fig. 4.1C). Occurring across all four batches, 20,408 shRNAs targeting 14,679 unique genes existed, with at least 26,160 shRNAs targeting 17,281 unique genes in each library batch. Although a large repertoire of shRNAs are lost, most likely due to poor transduction efficiency and synthetic lethality, the cells contain a large enough gene target list to warrant screening approaches.

4.4.2 PRIMARY SCREENS FOR IMMUNE MODULATORS

Having produced a suitable shRNA pooled library, a primary and secondary in vivo screen were performed. Three separate strains of mice were utilized: wild-type C57Bl/6, which possess intact adaptive and innate immune systems, C57Bl/6-SCID, which lack functional components of the adaptive immune system, and NOD-SCID II-2γ−/− knockout (NSG), which lack components of both the innate and adaptive
immune system. SCID and NSG mice serve as in vivo controls for the adaptive and innate immune system, as well as the effects of gene knockdown on inherent in vivo growth. A schematic describing the primary in vivo screen is shown in Figure 4.2, which clarifies the identification and prioritization for secondary screening. When the library cells were engrafted in each strain of mice, differential growth response corresponded inversely to intensity of immune selection pressure. Median survival was 17 days in NSG mice, 21 days in SCID mice and 31 days in wild-type mice (Fig. 4.3A). Wild-type, empty vector (EV), and library cells grew at equivalent rates in C57Bl/6 mice, implying that lentiviral vector transduction had little effect on the induction of an immune response (Supp. Fig. 4.1). The secondary screen was performed using C57Bl/6-SCID and C57Bl/6 mice, with median survival of 20 and 36 days, respectively. For both screens, as expected, the combined shRNA repertoire derived after in vivo growth was significantly reduced compared to the initial EO771-library cells, suggesting the presence of in vivo selection pressure (Fig. 4.3B).

Differential analysis of the represented shRNAs derived from each mouse strain resulted in the identification of shRNAs selectively and significantly over- or under-represented in C57Bl/6 versus immune-deficient mice (SCID and/or NSG strains) (Fig. 4.3C, Fig. 4.3D). As the most well described differences between C57Bl/6 mice and the immune deficient strains is the constitution of the immune system, we consider these hits to be immune response regulators. The results of both screens were
combined to facilitate analysis and significant hits are presented (Table 4.2). Significant hits met the dual criteria of an adjusted p-value < 0.05 and at least a log<sub>2</sub> change in representation in the surviving tumor cell population in any pairwise comparison. A full listing of the pairwise comparison hits is included in Supplemental Tables 4.1 and 4.2. Of the over-represented shRNAs, testis expressed gene 9 (Tex9), Yip1 interacting factor homolog A (Yif1a), and epithelial membrane protein 2 (EMP2), were of particular interest, as they were targeted by multiple shRNAs or have previously been shown to be involved in immune modulation. More than 600 significantly contracted shRNAs were identified across both screens but sphingosine phosphate lyase 1 (Sgpl1), peroxisomal biogenesis factor 14 (Pex14), protein transport protein Sec16b, and CD47 were identified in both screens or through multiple unique shRNAs (Fig. 4.3E). Figure 4.3F describes the relative expression of four selected shRNAs across the different strains of mice.

### 4.4.3 ENHANCED IMMUNITY SCREENS FOR IMMUNE MODULATORS

Although the *in vivo* screen utilized was able to identify functional mediators of the adaptive immune response, it is possible that given the short duration of selection other important mediators of immune selection were lost in the noise. The relatively high number of shRNAs in the tumors (Table 4.2) suggests this indeed was true, as this potentially indicates poor elimination of cells containing shRNAs that are less important for immune selection. Therefore, we hypothesized that prolonging the duration of
adaptive immune selection pressure could help select for immune-shielded clones. To do this, we serially passed EO771-library cells in vivo in both WT and SCID mice (Fig. 4.4A). After each in vivo passage, EO771-library cells exhibited enhanced growth kinetics, suggesting a progressive selection for previously immune-shielded or quicker dividing cells. When EO771-library cells were serially transplanted three times in WT C57Bl/6 mice median survival was 31 days after one passage, 16 after two passages, and 13 days after three passages. Similarly, the median survival of SCID mice challenged similarly declined from 21, to 18, to 13 days. By the third successive C57Bl/6 in vivo passage, EO771-library cells grew equally rapidly in WT (Fig. 4.4B, Supp. Fig. 4.2) and SCID (Fig. 4.4C, Supp. Fig. 4.2) mice. When EO771-library cells that were passaged twice in SCID mice were then re-exposed to an adaptive immune system, tumor growth was significantly retarded (Supp. Fig. 4.3B, 4.3D). This did not occur for EO771-library cells passaged twice in C57Bl/6 mice, as they grew at the same rates in both C57Bl/6 and SCID mice (Supp. Fig. 4.3A, 4.3C). The shRNA repertoire in each of the third passage tumors (n=5) was determined to identify shRNAs influenced by prolonged immune selection pressure.

Three in vivo serial passages resulted in a reduction of shRNA representation, compared to Fig 3B, in the EO771-library cell line, with about 3000 and 4000 shRNAs present in the C57BL/6 and C57Bl/6-SCID tumors, respectively (Fig. 4.4D). ShRNAs targeting Sgpl1 and Sec16b were significantly reduced in the EO771 cells grown in WT as compared with SCID mice, further highlighting the significance of these genes in EO771-related tumor immunity, as they appear in all screens. Newly
identified hits with reduced representation in immune-competent C57Bl/6 mice included Cxcl1 and cathepsin b, both of which are proteins that mediate immune modulation (Fig. 4.4E) (Dhawan and Richmond; Acharyya et al. 2012; Nouh et al. 2011). Only two shRNAs from the initial screens were significantly over-represented in the serially passaged tumors in C57Bl/6-WT mice, suggesting that using this screening method in the context of sustained immune selection is more likely to identify factors that block immune recognition and cytotoxicity. A full list of significant shRNAs identified in this screen is included in Supplemental Table 4.3.

4.4.4 VALIDATION OF IDENTIFIED GENE CANDIDATES

To validate the findings from our in vivo screens, EO771 cells were transduced with lentivirus independently encoding a single shRNA for each target gene. After protein knockdown was validated (Supp. Fig. 4.7), EO771 shRNA cells were engrafted in C57Bl/6 or C57Bl/6-SCID mice. Tumor growth rates and surviving cell populations were analyzed for each shRNA knockdown-validated cell line. Of the enriched gene candidates, Tex9 knockdown cells exhibited growth enhancement when exposed to an intact adaptive immune system, as compared with a scrambled shRNA control, suggesting that Tex9 serves as a tumor antigen of immune-stimulator (Fig. 4.5).
Of the contracted gene candidates, EO771 tumor cells containing CD47, Pex14, and Sgp11 knockdowns exhibited substantially reduced growth compared with cells containing scrambled shRNA control when grown in WT C57Bl/6 mice (Figure 4.5). The targets Yif1a and EMP2 failed to validate in these studies, as attempted gene knockdown did not enhance growth in C57Bl/6 mice. This failure was likely due to poor protein knockdown, as only a modest reduction in protein expression was observed (data not shown). It should also be noted that the shRNAs utilized for individual validation were different than those used in the genome-wide library.

CD47, also known as integrin-associated protein, is an important receptor involved in numerous physiological processes, most importantly as an inhibitor of phagocytosis (Willingham et al. 2012). To further clarify the role of CD47 in our model system, we forced over-expression of CD47 on the surface of EO771 cells and found an accelerated growth rate of engrafted tumors in C57Bl/6 mice (data not shown). The differential effects on survival were only evident when CD47 overexpressing cells or knockdown cells were grown in immune-competent mice, suggesting that CD47 manipulation affects more than the shielding of target cells from macrophage phagocytosis, since SCID mice have functional macrophages (Supp. Fig. 4.4b). The Weismann group made a similar discovery, as CD47 abrogation enhanced antigen specific T cells, resulting in tumor growth inhibition (Tseng et al.).
Sgpl1, or sphingosine-1 phosphate lyase, is a member of the sphingosine metabolism pathway that directly and irreversibly degrades sphingosine-1 phosphate (S1P) (Spiegel and Milstien 2011). Sgpl1, by regulating the intracellular and secreted levels of S1P, can affect immune cell migration, induction of apoptosis, activation of MAPK, and resistance to chemotherapy (Brizuela et al. 2012; Oskouian et al. 2006; Colie et al. 2009). Also, even though shRNA transduced cells showed a significant reduction in survival in C57Bl/6 mice, the cell line exhibited a reduction in growth in SCID mice (Supp. Fig. 4.4C). Manipulation of Sgpl1 in EO771 cells appears to enhance the anti-tumor immune response in both WT and SCID mouse strains. This may occur through the complex regulation of S1P by SGPl1 and the S1P kinases (SPHK1 and SPHK2) (Spiegel and Milstien 2011).

Pex14 is an essential component of the peroxisomal machinery, whose function is vital for the degradation of uric, amino, and fatty acids as well as hydrogen peroxide (Dammai and Subramani 2001; Purrington et al. 2014; Albertini et al. 1997; Duve 1969). Pex14 is a member of the peroxin machinery, which includes 18 other components. Pex14 knockdown in engrafted EO771 cells exhibit reduced growth in the presence of adaptive immune selection pressure (Fig. 4.5D). On top of this, Pex14 knockdown also increased the survival of SCID mice with engrafted tumors; Pex14 plays a role in innate immune system recognition as well.
4.4.5 CHARACTERIZATION OF GENE KD ON TUMOR IMMUNE MILEU

To interrogate the immune consequences elicited by modulation of Pex14, Sgpl1, and Tex9 in tumor cells, we examined the immune cell infiltrates following engraftment of individual shRNA-transduced EO771 cell lines. Tumors were processed and stained with a panel of markers to identify myeloid derived suppressor cells (MDSCs), macrophages, dendritic cells, T cells populations (CTL, helper and regulatory), and NK/NKT cells. There was no difference in the immune cell infiltrate between EO771-WT and -scramble shRNA cells, suggesting that virus infection and shRNA integration had limited effects on local immune composition (Supp. Fig. 4.5). As shown in Figure 4.6, Pex14 knockdown correlated with an increase in tumor infiltrating lymphocytes (TILs), as well as non-significant increases in CD4+ T cells and CD4+ CD25+ T cells and a decrease in NK cells, activated macrophages, and total and activated dendritic cells. Therefore, knockdown of Pex14 increased survival of engrafted mice, which was associated with a decrease in innate cell infiltration to the tumor microenvironment and an increase in T cell infiltration. Knockdown of Sgpl1 resulted in a significant decrease only in the percentage of NK cells that infiltrate the tumor, with a trend toward a slight decrease in MDSCs. Interestingly, even though there is a reduction in the total amount of infiltrated dendritic cells, there is a modest increase in activated dendritic cells, suggesting a potential impact on antigen processing.
4.4.6 SIGNIFICANCE OF VALIDATED GENE CANDIDATES IN HUMAN BREAST CANCER

As each of the four validated genes –CD47, Pex14, Sgpl1, and Tex9 – was identified as a potential functional regulator of tumor cell survival in a syngeneic model of breast cancer in mice, we aimed to establish the significance of these genes in humans with breast cancer. We identified the role of each orthologous gene in human breast cancer patients using the KM Plotter (http://kmplot.com), which holds Affymetrix array expression analysis and survival on 4,142 breast cancer patients. This allowed us to identify the correlation of CD47, Pex14, and Sgpl1 to breast cancer relapse-free survival (RFS) and overall survival (OS). Tex9 was not an annotated protein on the Affymetrix arrays utilized. The expression stratification for all Kaplan-Meier plots was auto-selected for the best possible threshold.

CD47 expression was identified as potentially significant of RFS and OS in all and in basal-like breast cancer patients (Fig. 4.7A & 4.7B; Supp. Fig. 4.6A). Patients with relatively high transcript expression were more likely to recur and less likely to survive, as has been shown in numerous other cancers (Willingham et al. 2012; Wang et al. 2015; Chao et al. 2010; Majeti et al. 2009). Similarly, higher Sgpl1 expression correlated with an enhanced chance of relapse in the total patient group as well as patients with basal-like breast cancer (Fig. 4.7C & 4.7D). Expression of Pex14 did not appear to influence survival in the total breast cancer population. However, when patients were further stratified to a basal phenotype, which better reflects the EO771 model system, Pex14 was identified as a significant biomarker for both RFS and OS (Fig. 4.7E & 4.7F; Supp. Fig. 4.6C). Therefore, the tumor immunity
regulators identified in this murine model system appear to represent prognostic biomarkers in human breast cancer patients.

Using the Human Protein Atlas it was possible to identify the protein levels of each gene candidate in human samples compared to normal tissue, as well as the cellular location of each protein. Tex9 was detected at moderate levels in all breast cancer and normal samples as well as in 93% of the 20 cancers tested. Tex9 appears to be restricted to the cytoplasm in the human cell lines U-2 OS, A-431, and U-251 MG. CD47 protein expression is localized to the plasma membrane, as well as the golgi apparatus and vesicles, and was detected at moderate to low levels in 50% of the breast cancer patient samples. Pex14 appears to be localized to vesicles but also appears in the nucleoli. Protein expression of Pex14 was found to be very high in all cancers tested (98%), with every breast cancer sample (n=22) exhibiting high or moderate expression of the protein and normal tissue exhibiting moderate expression. Lastly, Sgpl1 protein is expressed in moderate amounts across all breast cancer samples and low amounts in normal tissue. The differential expression of Pex14 and Sgpl1 protein levels in breast cancer versus normal tissue suggests an enrichment of overexpression in the neoplastic cells.

4.5 DISCUSSION

This work, to the best of our knowledge, is the first utilization of an RNAi screening approach in a murine setting to identify tumor cell-based mechanisms that regulate the cancer-directed adaptive
immune response. We discovered that modulation of a single gene with little known immune-regulatory function can have profound effects on the in vivo survival of cancer cells in an immune competent environment. This set of findings has important implications for understanding the complex interplay of tumor cells with their immune microenvironment and identifying new targets for immunotherapy.

Identifying novel mediators of the anti-cancer immune response can provide new tools to overcome the eventual development of resistance to immunotherapy. The ability of the immune system to eliminate cancers is rapidly becoming important in the treatment of cancer, as immune-stimulatory therapies are increasingly effective (Wolchok et al. 2013; Topalian et al. 2012; Hodi et al. 2010). While reductionist approaches are effective in understanding the mechanisms of immune signaling, the significance of the interplay of malignant cells with the stroma and the immune system has been understudied (Khandelwal et al. 2015). By utilizing an RNAi screening approach in an in vivo setting, it is possible to identify the mechanisms behind tumor-based immune evasion, without introducing bias towards an individual cell type or interaction. In this work, we report an unbiased, reproducible genome-wide screening strategy for the identification of tumor-based immune modulators, with a particular emphasis on immune inhibitory molecules. The identification of known immune regulators like CD47, TGF-β, and IL-17 highlight the biological significance of the candidates identified from the screening approach. Employing a genome-wide shRNA library in a pooled format also reduces any bias associated
with the genes studied, thereby increasing the likelihood of novel gene discovery. As we sought to identify novel mediators of immune rejection, TGF-β1 and IL-17 were not followed up for functional validation in the current study, though validation of these potentially important targets in this system is planned. Not surprisingly, a Pathways Studio analysis suggested the central importance of TGF-β signaling in immune rejection of EO771 tumors \((\text{Supp. Fig. 4.8})\). This observation is corroborated by the extensive literature regarding the relevance of TGF-β to immune rejection and immunosuppression (Biswas et al. 2014; Swati Biswas 2007).

Since EO771 cells arose spontaneously in a C57Bl/6 mouse, the isolated malignant cells likely underwent immune editing and, thus, have reduced \(\textit{in vivo}\) immunogenicity (Koebel et al. 2007). Since they have been extensively \(\textit{ex vivo}\) cultured since their isolation in the 1950s some reversion from this phenotype may have occurred. This is compatible with the difference in growth kinetics of EO771 cells in NSG, SCID, and WT C57Bl/6 mice. It is also possible that stromal differences between the strains of mice could account for the differential growth observed. Therefore, a screen with EO771 library cells in mice has the potential to identify shRNAs that block (i.e., are enriched in immune-competent tumors) or amplify (i.e., are contracted in immune-competent tumors) the anti-cancer immune response.
By utilizing three independent *in vivo* screens and stringent cutoff criteria, it was possible to identify a short list of genes either targeted by multiple shRNAs or occurring in all screens. Creation and *in vivo* validation of each target – 4 of 6 genes tested to date including CD47, Pex14, Sgpl1, and Tex9 – identified shRNA-containing cells that were preferentially selected for immune elimination.

CD47 has previously been shown to inhibit macrophage-mediated lysis, by binding to SIRPα on macrophages. Antagonism of this axis leads to enhancement in tumor cell lysis by macrophages as well as an increase in CD8⁺ T cells in a syngeneic murine model (Tseng et al.), which our model system recapitulates, further validating the role of tumor-expressed CD47 in adaptive immunity. These results are not solely due to the effects of known actions of CD47 on macrophage function, since knockdown or forced overexpression of CD47 did not alter the growth of EO771 tumors in C57Bl/6-SCID mice. We are encouraged by these results, which demonstrate that the screening method is able to identify known mediators of the adaptive immune response.

Pex14 is an essential component of the peroxisomal machinery and has been shown to bind to Pex5 and can regulate movement of peroxisomes through attachment to tubulin. In our model system, loss of Pex14 resulted in abrogation of tumor growth only in immunocompetent mice. Moreover, high expression of Pex14 is a negative prognostic indicator in human basal-like breast cancer patients. Our
findings demonstrate that knockdown of Pex14 enhanced survival and increased TILs, which is a positive prognostic indicator in solid cancers (Zhang et al. 2003; Bachmayr-Heyda et al. 2013; Gao et al. 2007; Nosho et al. 2010; Gavin P Dunn 2007; Lohr et al. 2011).

Sgpl1 is one of the more interesting and significant targets identified in our *in vivo* screens. Sgp1 irreversibly degrades the protein S1P (sphingosine-1-phosphate), which is also regulated by the sphingosine kinases (Sphk-1 and -2) (Olivera et al. 2013). Sphk1 has been shown to increase cytokine-induced COX-2 and PGE expression, which are often up regulated in colorectal cancers, and S1P has proven important in the prevention of autoimmune disease through the regulation of immune cell trafficking (Oskouian et al. 2006; Olivera et al. 2013; Takabe et al. 2008; Aoyagi et al. 2012). Our results highlight the significance of the sphingosine pathway on immune surveillance of tumor cells, as knockdown of Sgp1 results in a significant increase in animal survival, as well as a decrease in NK cell infiltration. Correlating to human breast cancer patients, Sgp1 expression appears to serve as a prognostic indicator, as patients with high Sgp1 in tumors have a worse relapse-free survival.

This screening method identified only one validated gene that, when knocked down, increased the growth of cells in the presence of the adaptive immune response. Tex9 is a poorly studied gene with little prior known functionality. Our results demonstrated that reduced Tex9 expression effectively shielded tumor cells from immune destruction, allowing them to proliferate more rapidly in immune competent
mice. Therefore, Tex9 may encode for a tumor associated antigen or an immune-stimulatory agent and it will be important to identify if Tex9 is mutated in our model system. Therefore, eliminating Tex9 expression in a tumor cell could shield that cell from anti-Tex9 CTL mediated lysis. Moreover, Tex9 protein expression is detected in a large majority (93%) of human cancers in the Human Protein Atlas. However, normal human tissues do express detectable levels of each of the four validated proteins, which could complicate pharmacological targeting. This is highlighted by the expression of CD47 on human red blood cells (RBCs) (Oldenborg 2000). In one animal study, when genetically depleted CD47 RBCs were infused into WT host mice, the CD47<sup>−/−</sup> cells were quickly cleared from the circulation, apparently by macrophages and dendritic cells (Bruce R Blazar 2001). Therefore, the effect of inhibition of these targets on normal tissues would need to be assessed.

4.5.2 LIMITATIONS OF SHRNAS

When utilizing shRNAs to reduce the expression of a target transcript, it is possible to elicit off-target effects. On top of this, it is possible that even a moderate reduction in RNA or protein expression might not be adequate to produce the desired phenotype. Therefore, to mitigate these issues, we could utilize Cas9-Crispr to remove the DNA segment of interest from our target cells. Although this system comes with its own set of limitations and issues, removal of the specific DNA segment from host cells would allow us to assess the role of the target gene on immune recognition.
4.5.3 LIMITATIONS OF POOLED SCREENING FORMAT

While pooled RNAi screening methods allow for the discovery of novel gene candidates, the extensive time constraints and high noise are significant issues. To validate the candidates discovered in a pooled screen requires the generation of individual shRNA containing tumor cells. This process is also expensive and time consuming, thereby limiting the number of potential gene candidates for validation. By utilizing arrayed siRNA screening approaches, it is possible to identify and validate a large subset of gene candidates in a relatively quick and inexpensive manner. This method requires the generation of focused libraries that significantly limits novel gene discovery, as well as requiring the assay to be in vitro; as siRNA induced gene knockdown is transient. If a large set of gene candidates is validated, then it is possible to perform a pathway analysis of validated hits to better inform which pathways are involved when the target cells respond to the applied selection pressure.

4.5.4 LIMITATIONS OF THE GENENET LIBRARY

The genome-wide shRNA library offered from system biosciences is one of two commercially available library formats targeting the entire mouse genomes. Other companies offer focused libraries that target around 5000 mRNA transcripts, which limits the likelihood of novel gene discovery, as each gene target in the library has already been annotated. Since the purpose of our screens was the identification of novel gene targets of immune recognition in a syngeneic model system, the GeneNet library offered the
highest chance of novel gene discovery. For each mRNA target in the library, there are 3-5 unique shRNA transcripts. Unfortunately, the transduction efficiency in our EO771-library cells was very low, as only 20,408 shRNAs targeting 14769 unique genes integrated into the genome. Therefore, there are rarely going to be more than 1 or 2 shRNAs targeting the same gene target. This limits our confidence over significant results, as there are rarely going to be multiple significant shRNAs targeting the same gene. One method to correct for this is the use of high redundancy next-generation shRNA libraries, which include over 25 independent shRNAs for each gene candidate, as well as thousands of negative control hairpins. (Kampmann et al. 2015). Therefore, even with low transduction efficiencies, there are likely to be at least 5+ shRNAs targeting each transcript.

4.5.5 LIMITATIONS OF AFFYMETRIX ARRAYS FOR IDENTIFICATION OF SHRNAS

In the three screens utilized in this dissertation, all shRNAs were quantified by hybridizing amplified shRNA barcodes to an Affymetrix array. Arrays have proven to be riddled with normalization, background, and quality issues, as well as being prohibitively expensive. Thereby requiring a significant level of normalization and filtering to properly analyze data. Fortunately, next generation sequencing methods have become relatively inexpensive over the past decade. Therefore, as a method to enhance confidence in screening results, sequencing the shRNA barcodes in each tumor cell population should be performed.
4.5.6 LIMITATIONS OF KM PLOTTER

While identification of the impact of target genes in human breast cancer is useful, there are significant issues associated with the KM plotter tool. First and most importantly, the Affymetrix data utilized in the KM plotter database were collected at different times and under different conditions but are pooled together for analysis. Since minor changes in how arrays are hybridized and performed can have significant effects on the resulting data, also known as batch effects, it is possible that the end results are not statistically sound. Therefore, it would be beneficial to utilize the METABRIC database of human breast cancer to perform this analysis (Curtis et al. 2012). METABRIC contains gene expression and copy number analysis of 2,000 breast tumors, as well as long-term clinical follow-up. Since the experiments were performed under similar conditions, the concern over batch effects is limited. Once access has been granted to the database, the significance of each validated gene candidate will be assessed.

4.5.7 LIMITATIONS OF IMMUNE INFILTRATE

Analysis of the immune cells that have infiltrated into shRNA containing tumors is an essential strategy to discover the mechanisms behind target knockdown. Unfortunately though, it is often difficult to adequately select the correct parameters of a positive or negative cell population. On top of this, tumors analyzed have reached 1 cm³ in volume and it is possible the effects of target gene knockdown have already occurred. By performing the same analysis on tumors at multiple time points, it might be possible to parse exactly which immune cell types are being manipulated by target gene knockdown.
4.6 PRIMARY FIGURES

4.6.1 FIGURE LEGENDS

Figure 4.1: Schematic of *in vivo* RNAi screening approach to identify tumor-based modulators of the immune response. A) EO771 cells were infected with a lentivirus delivered genome-wide shRNA library at an MOI of 3.75 to ensure only 1 shRNA integration per cell. After selection with puromycin and *in vitro* culturing for 21 days, EO771-library cells were engrafted in mice with varying levels of immunity; C57Bl/6 (functional adaptive and innate immunity), C57Bl/6-SCID (functional innate immunity and dysfunctional adaptive immunity), and NSG (dysfunctional innate and adaptive immunity) mice. Differential analysis of barcoded shRNAs that exist in each tumor between the different strains of mice is used to identify potential mediators of the anti-tumor immune response. B) Raw expression values from two separate batches of EO771-library cells. The similarity of relative expression values for each shRNA between the two batches is represented by the identity line $y=x$, represented in red. C) Integration of shRNA and gene targets from four separate batches of EO771-library cells describes the overlap of gene targets found in each batch. Therefore, EO771-library cells possess successful integration of shRNAs targeting 14,679 unique genes.
Figure 4.2. Schematic of Differential shRNA Analysis. The *in vivo* screen was designed to identify shRNAs that affect survival of tumor cells when exposed to the adaptive immune system. EO771-library cells that contain an shRNA that enhances survival in immune competent mice but not in immune-deficient mice are represented as red containing cells. In this example, cells containing this shRNA (red) are preferentially expanded in the immune-competent mice; whereas, expression in immune-deficient mice is much lower. This suggests that the shRNA is targeting an immune-stimulatory molecule, as elimination of it allows cells to survive immune selection. Blue shRNA containing cells are preferentially eliminated in immune-competent mice, suggesting that it encodes an immune-inhibitory molecule. ShRNAs with no effect on survival from the immune system will expand at similar rates in both mice (green).

Figure 4.3: *In vivo* RNAi screen identifies shRNAs enriched or lost when shRNA library-infected tumor cells are grown in an immune competent host. A) Survival of mice engrafted with EO771-library cells. Statistical analysis performed using Prism 5. * = Mantel-Cox test with p <0.05. n = 10. B) Total number of genes targeted by shRNAs across each tumor strain. C) Waterfall plot of relative log2 fold change of each shRNA in the C57Bl/6 vs. NSG analysis. Green circles represent those shRNAs with a q < 0.05. More shRNAs are lost than enriched in this comparison. D) Volcano plot of each shRNA by log2 fold change and adjusted p value (q
value). Green circles represent those hits with a q < 0.05. E) Combined results of significant shRNAs from the two independent RNAi screens. F) Relative levels of shRNAs from the first screen in each individual tumor. B8 initial represents the starting shRNA repertoire. The red line represents background array expression. The blue line is considered to be the detection limit for an shRNA in the tumor. WT = wild-type mouse, SCID = severe combined immunodeficient mouse, NSG = NOD SCID Gamma mouse. * Indicates a p < 0.05; ** indicates a p < 0.01; *** indicates a p < 0.001.

Figure 4.4. Sustained immune selection in vivo screen identifies Sgpl1, CXCL1, and Cathepsin b as inhibitors of the anti-tumor immune response. A) EO771-library cells were serially passaged (three times) in C57Bl/6 and SCID mice to select for shRNAs that are preferentially lost or gained in tumors grown in the immunocompetent mice. N = 5. Comparison of shRNAs between C57Bl/6 and SCID mice can isolate shRNAs involved in immune recognition. B, C) Survival of immunocompetent and SCID mice undergoing serial passaging in vivo. D) The number of targeted genes in each strain in C57Bl/6 and SCID mouse tumors, based on Affymetrix array of shRNA barcodes as described in Methods. E) The significant shRNAs identified from the in vivo passaging screen. * Indicates a p<0.05; ** indicates a p<0.01; *** indicates a p<0.001. Survival analysis was performed using PRISM 5 and significance was identified using Mantel-Cox
analysis. ShRNAs were considered significant if they had a log2 fold change $\geq 1$ and a q value $< 0.05$.

Figure 4.5. *In vivo* analysis of C57Bl/6 mice engrafted with EO771 cells that have successful knockdown of Tex9 (A), CD47 (B), Pex14 (C), and Sgpl1 (D). Four gene candidates identified in the *in vivo* screens were validated by engrafting the indicated shRNA containing EO771 cells in C57Bl/6 and C57Bl/6-SCID mice. ShScramble control EO771 cells were transduced with virus containing an shRNA that targets no known mammalian candidates. N = 10 mice per group. Each panel is a representative survival and growth kinetics analysis (panel insert) from one animal study and was repeated three times with equivalent results. Survival was considered significant when the Mantel-Cox test resulted in a p < 0.05. The black lines and markers indicate cells expressing the shScramble, while red lines and markers represent the cells with the indicated shRNA knockdown.

Figure 4.6. Immune infiltrate analysis of Pex14 and Sgpl1 shRNA tumors. EO771-shPex14 and EO771-shSgpl1 tumors were excised from immunocompetent C57Bl/6 mice once they reached 1cm$^3$, digested into single cells, stained with a live/dead dye, Fc blocked and exposed to fluorophore conjugated antibodies for various immunological markers and visualized using a
flow cytometer. Results were analyzed by a two-way ANOVA using PRISM 5. * Indicates \( p < 0.05 \). All cells were gated by low inclusion of the live/dead dye, indicating intact cell membranes. The number of each cell type was normalized by the total number of CD45+ cells to obtain the indicated percentage. Results from the indicated shRNA-knockdown tumors were compared to scramble shRNA control cells.

Figure 4.7. Expression of human CD47, Pex14, and Sgpl1 are significant indicators for relapse-free survival (RFS) of breast cancer patients. The murine proteins are considered to be orthologs to their human counterparts. All results were obtained using the interactive software KMPlot. The data set consisted of 4142 total breast cancer patients followed for periods of up to 25 years, at a variety of stages, as described (Győrffy et al.; Győrffy et al. 2013). Relapse-free survival (RFS) of all breast cancer patients and basal-like patients was stratified on CD47 expression (A, B). Sgpl1 expression (C, D) and Pex14 (E, F). Results were adjusted for multiple comparisons and were considered significant with a \( q \) value < 0.1 if the log rank test identified a \( p \leq 0.027 \).
Figure 4.1. Schematic of in vivo RNAi Screening Approach to Identify Tumor-Based Modulators of the Immune Response

A) Selection Pressure

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B) siRNA Expression

C) Unique Gene Targets

Library 1: 863 genes
Library 2: 459 genes
Library 3: 549 genes
Library 4: 408 genes
Library 1, 2: 270 genes
Library 1, 3: 286 genes
Library 1, 4: 293 genes
Library 2, 3: 297 genes
Library 2, 4: 307 genes
Library 3, 4: 266 genes

D) Library Characterization

Library 1: 1.5x more expression than Library 2
Figure 4.2. Schematic of Differential shRNA Analysis
Figure 4.3. In Vivo RNAi Screen Identifies shRNAs Enriched or Lost when shRNA Library-Transfected Tumor Cells are Grown in an Immune Competent Host

A) Percent survival over days for NSG, SCID, and C57BL/6 groups.

B) Box plot showing the number of targeted genes for B6 initial, C57BL/6, NSG, and SCID groups.

C) Log2 Fold Change vs. Fold Change graph for C57BL/6 vs Immune-deficient.

D) Scatter plot showing p-values for adaptive + innate immunity.

E) Log2 Fold Change bar chart for shCD47, shPex14, shSec16b, shSgpl1, shPlas3, shActg2, shDrd2, shCD47, shYif1a, shEMP2, shYif1a, shTex9, shCSNK1D.

F) Comparative analysis of Sgpl1, Tex9, Pex14, and CD47 shRNA expression.
Figure 4.4. Sustained Immune Selection In Vivo Identifies Sgpl1, CXCL1, and Cathepsin b as inhibitors of the anti-tumor immune response.
Figure 4.5. In Vivo Analysis of C57Bl/6 mice engrafted with EO771 cells that have successful KD of Tex9, CD47, Pex14, and Sgpl1

A) Percent survival

- Black: shScramble
- Red: shTex9

**B) Percent survival**

- Black: EO771-WT
- Red: shCD47

C) Percent survival

- Red: shSgpl1
- Black: shScramble

D) Percent survival

- Red: shPex14
- Black: shScramble
Figure 4.6. Immune Infiltrate Analysis of Pex14 and Sgpl1 shRNA Tumors
Figure 4.7. Expression of Human CD47, Pex14, and Sgpl1 are Significant Indicators for Relapse-Free Survival (RFS) of Breast Cancer Patients

A) Relapse-Free Survival for All Breast Cancer Patients

- CD47
  - Expression: low vs. high
  - HR = 1.39 (1.23 - 1.56)
  - Logrank P = 1.3e-07

B) Relapse-Free Survival for Basal-like Breast Cancer Patients

- CD47
  - Expression: low vs. high
  - HR = 0.84 (0.62 - 1.13)
  - Logrank P = 0.24

C) Relapse-Free Survival for All Breast Cancer Patients

- Sgpl1
  - Expression: low vs. high
  - HR = 1.18 (1.16 - 1.46)
  - Logrank P = 4.9e-06

D) Relapse-Free Survival for Basal-like Breast Cancer Patients

- Sgpl1
  - Expression: low vs. high
  - HR = 1.34 (1.03 - 1.75)
  - Logrank P = 0.027

E) Relapse-Free Survival for All Breast Cancer Patients

- Pex14
  - Expression: low vs. high
  - HR = 1.15 (1.01 - 1.31)
  - Logrank P = 0.032

F) Relapse-Free Survival for Basal-like Breast Cancer Patients

- Pex14
  - Expression: low vs. high
  - HR = 1.88 (1.25 - 2.25)
  - Logrank P = 0.00043
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Table 4.1: Combined results of significant shRNAs from two independent in vivo screens. Genes highlighted by a * were significant in both screens. A (2) following the gene name indicates two unique shRNAs were identified as significant.
4.7 SUPPLEMENTARY MATERIAL

4.7.1 SUPPLEMENTARY FIGURE LEGENDS

Supplemental Figure 4.1: Virus infection of EO771 cells does not influence survival of engrafted mice. Survival of mice bearing EO771 tumor cells infected with empty vector (red line), library (black line), and WT cells (blue line). N= 10 mice per group. Survival differences were considered significant when the Mantel-Cox test resulted in a p < 0.05.

Supplemental Figure 4.2. In vivo analysis of C57Bl/6 and SCID mice engrafted with serial passages of EO771-library cells. EO771-library cells were engrafted in 10 C57Bl/6 wild-type and SCID mice. At time of sacrifice (tumor volume = 1 cm³), tumors were excised, digested, and cultured in vitro. After one or two subsequent in vitro passages, cells were engrafted in 5 wild-type or 5 SCID mice, depending on which animal they were originally grown. This was repeated through three in vivo passages. Survival was considered significant when the Mantel-Cox test resulted in p < 0.05.

Supplemental Figure 4.3. In vivo survival analysis of EO771-library cells passaged twice in C57Bl/6 (Bl6-1 and -2) and SCID (SCID-1 and -2) mice, when grown in C57Bl/6 and SCID mice. Each group represents one of the 5 in vivo passaged cell lines from the enhanced immune
selection screen. C57Bl/6 passaged cells grew equivalently in C57Bl/6 SCID and wild-type mice (Panels A and C). Interestingly, SCID passaged cells were unable to grow when grown in C57Bl/6 wild-type mice (Panels B and D). N=5. Survival was considered significant when the Mantel-Cox test resulted in p < 0.05. ** indicates a p < 0.01.

Supplemental Figure 4.4. In vivo analysis of C57Bl/6-SCID mice engrafted with EO771 cells that have successful knockdown of Sgpl1 (A), Pex14 (B), CD47 (C), and Tex9 (D). shScramble control EO771 cells were transduced with virus containing an shRNA that targets no known mammalian candidates. N = 10 mice per group. Each panel is a representative survival and growth kinetics analysis from one animal study. Each was repeated three times with equivalent results. Survival was considered significant when the Mantel-Cox test resulted in a p < 0.05. ** indicates a p < 0.01.

Supplemental Figure 4.5. shRNA integration does not alter the composition of immune cells infiltrating into tumors grown in C57Bl/6 mice. EO771-WT and -shScramble tumors were excised from C57Bl/6 mice once they reached 1cm³, digested into single cells, stained with a live/dead dye, Fc blocked and exposed to fluorophore conjugated antibodies for various immunological markers and visualized using flow cytometry. Results were analyzed by a two-
way ANOVA using PRISM 5. * indicates p < 0.05. All cells were gated by low inclusion of the live/dead dye, indicating intact cell membranes. The number of each cell type was normalized by the total number of CD45+ cells to obtain a percentage. N = 5.

Supplemental Figure 4.6. Overall survival (OS) analysis of total breast cancer patients (left column) and patients with basal-like breast cancer (right column), stratified by CD47 (A and B), Sgpl1 (C and D), and Pex14 (E and F) expression. CD47 expression is a prognostic indicator of OS for all breast cancer patients (A). Sgpl1 expression is not prognostic for OS for either group of patients. The expression of Pex14 inversely correlates with OS in basal-like breast cancer patients. All results were obtained using the interactive software KMPlot. Results were adjusted for multiple comparisons and were considered significant with a q value < 0.1 if the log rank test identified a p ≤ 0.027.

Supplemental Figure 4.7. Protein knockdown of Tex9 (A), Pex14 (B), Sgpl1 (C), and CD47 (D) across multiple passages. D) Representative histogram of CD47 expression on shRNA transduced cells. E) Quantification of CD47 protein expression across three consecutive in vitro passages.
Supplemental Figure 4.8. Pathway analysis identifies centralized hubs surrounding TGF-β and IL-17. Significant gene candidates identified from the first two in vivo screens were combined and pathway enrichment was performed. Connecting lines indicate evidence for direct regulation. Each protein candidate is targeted by at least one statistically significant shRNA. All analysis was performed using Pathway Studio.
Supplemental Figure 4.1. Virus Infection of EO771 Cells does not Influence Survival of Engrafted Mice.
Supplemental Figure 4.2. In Vivo Analysis of C57Bl/6 and SCID Mice Engrafted with Serial Passages of EO771-Library Cells.

Serial Passage 1

- Percent survival vs. Days
- SCID: ●
- C57BL/6: ■
- ***

Serial Passage 2

- Percent survival vs. Days
- SCID: ●
- C57BL/6: ■

Serial Passage 3

- Percent survival vs. Days
- SCID: ●
- C57BL/6: ■
Supplemental Figure 4.3. In Vivo survival Analysis of EO771-Library cells Passaged Twice in C57Bl/6 and SCID mice, when grown in C57Bl/6 and SCID mice.
Supplemental Figure 4.4. In Vivo Analysis of C57Bl/6-SCID mice engrafted with EO771 Cells that have Successful KD of Sgpl1, Pex14, CD47, and Tex9.
Supplemental Figure 4.5. shRNA Integration Does Not Alter the Composition of Immune Cell Infiltrating into Tumors Grown in C57Bl/6 mice.
Supplemental Figure 4.6. Overall Survival (OS) analysis of total breast cancer patients and patients with basal-like breast cancer, stratified by CD47, Sgpl1, and Pex14 expression.
Supplemental Figure 4.7. Protein knockdown of Tex9, Pex14, Sgpl1, and CD47 across multiple passages.
Supplemental Figure 4.8. Pathway analysis identifies centralized hubs surrounding TGF-β and IL-17.
Supplementary Table 4.1: Results from In Vivo Screen 1
GeneBank
shRNA Sequence
Bl6 vs NSG Log2 FC Bl6 vs NSG q Value Bl6 vs SCID Log 2 FC Bl6 vs SCID q Value
AI882525
ACTGAATGTTTCCATGTGCCTCTCGTA
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0.00526
-3.72
0.139
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0.0612
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0.787
BE950866
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0.354
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0.82
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BC027183
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BB471576
TAGAATCCCAACTGCTCTATATTTCTT
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### Supplementary Table 4.1: Results from In Vivo Screen 1

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### Supplementary Table 4.1: Results from In Vivo Screen 1

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### Supplemental Table 4.2: Results from In Vivo Screen 2

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<th>Bl6 vs SCID Log2 FC</th>
<th>Bl6 vs SCID q Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_007798</td>
<td>TTTTCATCTTCTCTTAAGCTGTACATADA</td>
<td>-6.93</td>
<td>1.90E-07</td>
</tr>
<tr>
<td>BM933463</td>
<td>AGTTTGCGTGTTTCTCTGTATCTGTAATTA</td>
<td>-5.85</td>
<td>0.0296</td>
</tr>
<tr>
<td>BB554288</td>
<td>TACATTCTTACTCCGCTCCTCTTCTCCTGTG</td>
<td>-5.28</td>
<td>0.00206</td>
</tr>
<tr>
<td>NM_009163</td>
<td>ACAATTTCATCTCAGCTGGCTGCTCCGT</td>
<td>-4.94</td>
<td>0.000523</td>
</tr>
<tr>
<td>BC022639</td>
<td>ATTCACGTTCTGGTTCTCTAACTGAA</td>
<td>-4.84</td>
<td>0.00145</td>
</tr>
<tr>
<td>AF234179</td>
<td>TTTGACATGTAGACTGCTTTCTTTCTCAT</td>
<td>-4.76</td>
<td>0.0421</td>
</tr>
<tr>
<td>BG068705</td>
<td>TAAATATCTATATTGCTGACAGACAT</td>
<td>-4.6</td>
<td>0.00687</td>
</tr>
<tr>
<td>AF234179</td>
<td>GTTGGACATGTAGACTGCTTTCTTTCTCTCA</td>
<td>-4.41</td>
<td>0.0133</td>
</tr>
<tr>
<td>BB462088</td>
<td>TCAATACATTTGACATCCTCGGTGCTGACA</td>
<td>-4.17</td>
<td>0.0184</td>
</tr>
<tr>
<td>NM_033354</td>
<td>TCACATTCTGACATCTGGCTATCAAGT</td>
<td>-4.11</td>
<td>0.00687</td>
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<tr>
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<td>-3.81</td>
<td>0.0396</td>
</tr>
<tr>
<td>BM211445</td>
<td>TGTGACATCTCATGAGTCATCTGACAAAGGA</td>
<td>-3.12</td>
<td>0.0394</td>
</tr>
<tr>
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<td>-2.55</td>
<td>7.55E-05</td>
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<td>AGGACATGCTGGCTGGACATTTTACTT</td>
<td>-2.34</td>
<td>0.0138</td>
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<td>-1.83</td>
<td>0.0396</td>
</tr>
<tr>
<td>BB016623</td>
<td>TATACCTATGCACTCCATTCCTACCTA</td>
<td>-1.83</td>
<td>0.00129</td>
</tr>
<tr>
<td>AF166486</td>
<td>GACGAAAGGGCCAGGCTCTATCACA</td>
<td>-1.8</td>
<td>0.038</td>
</tr>
<tr>
<td>BE132758</td>
<td>TTTACGAAGGGCCCAGGACCCCAGGCTG</td>
<td>-1.7</td>
<td>0.0138</td>
</tr>
<tr>
<td>BF461585</td>
<td>GAGCTGCTCGATGAGGTGACATGCGTTCA</td>
<td>-1.52</td>
<td>0.0212</td>
</tr>
<tr>
<td>BB487752</td>
<td>GTTCCATACCGGCTCTGACAGTCTTAAG</td>
<td>-1.3</td>
<td>0.0421</td>
</tr>
<tr>
<td>BB308170</td>
<td>GAACACAAGCCGACCACATGCACCTGCTCC</td>
<td>1.1</td>
<td>0.0285</td>
</tr>
<tr>
<td>BG092516</td>
<td>TATACCTACAGACACACCTGGATGCTA</td>
<td>1.54</td>
<td>0.0394</td>
</tr>
</tbody>
</table>
### Bioconductor packages
library('simpleaffy')
library('limma')
library('reactome.db')
library('org.Mm.eg.db')
library('ggplot2')
require('reshape')

### Oompa packages from MD Anderson (for helpful plotting)
### Install using the following commands:
# source('http://bioinformatics.mdanderson.org/OOMPA/oompaLite.R')
# oompaLite()
library('ClassDiscovery')
require('lattice')

### CRAN packages
library('gplots')

# directory structure for analysis#
figDir <- function (x = "", path = getwd()){
  fDir <- file.path(path, "Documents", Sys.Date())
  if (!file.exists(fDir)) {
    dir.create(fDir, recursive = T)
  }
  fDir <- file.path(fDir, x)
  return(fDir)
}

# support functions#

#### Creates a function that isolates the ith variable of a vector v and returns it as a string
geti <- function(l, i) {
  return(sapply(l, function(v) { return(v[[i]]) }))
}

#### Splits a string into its component parts
strspliti <- function(x, split, i = 1, ...)
{

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return(geti(strsplit(x, split = split, ...), i))
}

###############################################################
# Read in array data for basic QC #
###############################################################

rawDat <- ReadAffy(celfile.path='~/Documents/In Vivo Screen/In Vivo Screen 1 Raw Data/CEL/')
samples <- row.names(pData(rawDat))

### output raw images for each sample for quality control
for (s in samples) {
  ### no background correction
  pdf(figDir(paste('RawData',s,'.pdf', sep='.')))
  image(rawDat[,s])
  dev.off()
}

### Note spot on NSG 2 and scrape on SCID 3-1
#### Creates a list that's as long as the number of samples labeling them all as 'GOOD'
arrayQuality <- rep('good', length(samples))
#### Adds an extra label for each index based on the name of the .Cel file
names(arrayQuality) <- samples

#### Labels NSG2 and SCID 3-1 and SCID 3 as badly labeled.
arrayQuality['NSG 2.CEL'] <- 'moderate'
arrayQuality['SCID 3-1.CEL'] <- 'poor'
arrayQuality['SCID 3.CEL'] <- 'poor'

#### Add the arrayQuality index to each array
pData(rawDat)$quality <- arrayQuality

### get the name of the mouse
#### Sets a string with the names of the type of mice
mouse <- sapply(strsplit(samples,'\ '), function(x){x[[1]]})
#### assigns the mouse type in the affy data expression set of pData
pData(rawDat)$mouse <- mouse

### define the baseline file
pData(rawDat)$baseline <- is.na(mouse)

### determine the replicate number of each sample
#### Creates and then assigns the replicate number for each sample. Eg. NSG 1 will be '1', SCID 4 will be '4'. The pData file assigns the replicate number in the affymetrix data file
repID <- sapply(strsplit(samples,'\ '), function(x){
  if (length(x)>1) {
    return(sub('\..','',x[2]))
  }
  return(NA)
})
pData(rawDat)$replicate <- repID

### export to workspace
### Creates a variable 'pheno' that is a 5 column x 16 row data.frame
pheno <- pData(rawDat)

### check scale of each array
pdf(figDir('RawDatBoxplot.pdf'))
boxplot(rawDat, las=2)
dev.off()

### check relationships between samples
pdf(figDir('RawCluster.pdf'))
plot(standard.pearson(exprs(rawDat)))
dev.off()

pdf(figDir('GoodQualityRawCluster.pdf'))
plot(standard.pearson(exprs(rawDat)[,which(pData(rawDat)$quality=='good')]))
dev.off()

### if mappings between array locations and barcodes were available
### would normalize array data using the following code
# bgData <- bg.correct.rma(rawDat[,which(pheno$quality=='good')])
# normData <- normalize.AffyBatch.quantiles(bgData,type='separate')
# pmData <- pmcorrect.pmonly(normData)
# pmData <- log2(pmData)
### extract barcode data for probe index i and sample s by
# exprs(pmData)[i,s]

####################################################################
# read in the raw gene expression data processed by SBI            #
#                                                                  #
# Note: if we have an annotation onto the index on the probe      #
# for each barcode, we could create these files automatically from #
# the CEL files for a more reproducible analysis                 #
#                                                                  #
# This would also enable us to use normalized, background        #
# corrected data, which may yield more accurate results         #
####################################################################
shRNAFiles <- list.files('~/Documents/In Vivo Screen/In Vivo Screen 1 Raw Data/shRNA/',
full.names=T)
names(shRNAFiles) <- sub('.TXT',"",list.files('~/Documents/In Vivo Screen/In Vivo Screen 1 Raw Data/
shRNA/'))

### initialization for shRNA expression data - Inputs just B8 into it's own data frame
tmp <- read.table(shRNAFiles[1],
                    header=T, comment.char="", sep="\t", quote="",
                    fill=T)
tmp <- tmp[which(tmp$siRNA.sense.sequence != ""),]
#### Removes the expression values of B8 from the previous tmp data frame, essentially creating an index data frame for the gene, the genes index, siRNA sequence.

```
shRNAData <- tmp[,c('GeneBank.','Gene.name','siRNA.sense.sequence')]
```

#### For loop that cycles through and adds only the expression values to the end of the shRNAData data frame with an appropriate header

```
for (s in names(shRNAFiles)) {
### read in shRNA data
  tmp <- read.table(shRNAFiles[s],
  header=T, comment.char="", sep="\t", quote="",
  fill=T)
  tmp <- tmp[which(tmp$siRNA.sense.sequence != ""),]
  eval(parse(text=paste(gsub(' ', '.', s), '<- tmp')))
### create combined data
  shRNAData[,s] <- as.numeric(tmp$Value)
}
```

#### subset to high quality arrays and log scale
```
names(shRNAFiles)[which(pData(rawDat)[paste(names(shRNAFiles),
  'CEL',sep=''),
  'quality']=='good')] -> validSamples
```

#### Excludes NSG 2 and SCID 3 - I will write this differently since I can't run the ValidSamples code from above. It also gets rid of the annotations for each gene/probe/siRNA sequence

```
shRNADataSbst <- shRNAData[,validSamples]
shRNADataV <- shRNAData[,validSamples]
```

#### My attempt to manually add in the values
```
#shRNADataSbst<-data.frame(shRNAData[,5:9],shRNAData[,11:15],shRNAData[,17:18])
```

### can optionally quantile normalize samples using limma
### for now, excluding this portion as it was not found to
### significantly improve clustering between replicate samples
### Note: background correction can only be performed from raw
### the raw imaging data as outlined above
```
#shRNADataSbst <- normalizeQuantiles(shRNADataSbst)
```

### store as an expression set to facilitate limma analysis
```
shRNADataSbst <- ExpressionSet(log2(data.matrix(shRNADataSbst)))
```

# plot relationships between samples via clustering
```
pdf(figDir('BarcodeSubsetDataClusterTEST.pdf'))
plot(standard.pearson(exprs(shRNADataSbst)))
dev.off()
```

# heatmap showing correlation structure
```
pdf(figDir('BarcodeSubsetDataCorrelationHeatmapDendrogram.pdf'))
```
### add in gene annotations
#### Adds the gene annotations back into the data frame
```
featureData(shRNADataSbst)$idx <- row.names(shRNAData)
featureData(shRNADataSbst)$GeneBank <- shRNAData[,'GeneBank.']
featureData(shRNADataSbst)$Gene.name <- shRNAData[,'Gene.name']
featureData(shRNADataSbst)$sequence <- shRNAData[,'siRNA.sense.sequence']
```

```
shRNADataV$Gene.name <- shRNADataSbst$Gene.name
shRNADataV$GeneBank <- shRNADataSbst$GeneBank
shRNADataV$idx <- as.numeric(rownames(shRNAData))
shRNADataV$siRNA.sense.sequence <- shRNAData[,'siRNA.sense.sequence']
```

### add in information about samples
#### Utilizes 2 functions defined earlier to isolate on the mouse type index.
```
pData(shRNADataSbst)$mouse <- strspliti(sampleNames(shRNADataSbst),split=" ")
```

# Number of shRNA per Mouse #

# Determine the number of unique genes targeted in each mouse
# To do this, I will perform the same analysis as my 1st draft R code
# Select out the shRNAs that are >2 x the background of each array, then
# I will perform the unique command to pull out the unique genes. The final
# number will just be length (unique(...))

```
shRNAB8 <-shRNADataV[which(shRNADataV$B8>2*shRNADataV$B8[nrow(shRNADataV)]),c(1,14,15,16,17)]
shRNABL6.1 <-shRNADataV[which(shRNADataV$"BL6 1">2*shRNADataV$"BL6 1"[nrow(shRNADataV)]),c(2,14,15,16,17)]
shRNABL6.2 <-shRNADataV[which(shRNADataV$"BL6 2">2*shRNADataV$"BL6 2"[nrow(shRNADataV)]),c(3,14,15,16,17)]
shRNABL6.3 <-shRNADataV[which(shRNADataV$"BL6 3">2*shRNADataV$"BL6 3"[nrow(shRNADataV)]),c(4,14,15,16,17)]
shRNABL6.4 <-shRNADataV[which(shRNADataV$"BL6 4">2*shRNADataV$"BL6 4"[nrow(shRNADataV)]),c(5,14,15,16,17)]
shRNANS.G.1 <-shRNADataV[which(shRNADataV$"NSG 1">2*shRNADataV$"NSG 1"[nrow(shRNADataV)]),c(6,14,15,16,17)]
shRNANS.G.3 <-shRNADataV[which(shRNADataV$"NSG 3">2*shRNADataV$"NSG 3"[nrow(shRNADataV)]),c(7,14,15,16,17)]
```
shRNANSG.4 <- shRNADataV[which(shRNADataV$"NSG 4" > 2 * shRNADataV$"NSG 4"[nrow(shRNADataV)])], c(8,14,15,16,17)]
shRNANSG.5 <- shRNADataV[which(shRNADataV$"NSG 5" > 2 * shRNADataV$"NSG 5"[nrow(shRNADataV)])], c(9,14,15,16,17)]
shRNASCID.1 <- shRNADataV[which(shRNADataV$"SCID 1" > 2 * shRNADataV$"SCID 1"[nrow(shRNADataV)])], c(10,14,15,16,17)]
shRNASCID.2 <- shRNADataV[which(shRNADataV$"SCID 2" > 2 * shRNADataV$"SCID 2"[nrow(shRNADataV)])], c(11,14,15,16,17)]
shRNASCID.4 <- shRNADataV[which(shRNADataV$"SCID 4" > 2 * shRNADataV$"SCID 4"[nrow(shRNADataV)])], c(12,14,15,16,17)]
shRNASCID.5 <- shRNADataV[which(shRNADataV$"SCID 5" > 2 * shRNADataV$"SCID 5"[nrow(shRNADataV)])], c(13,14,15,16,17)]

labels <- c('B8', 'BL6.1', 'BL6.2', 'BL6.3', 'BL6.4', 'NSG.1', 'NSG.3', 'NSG.4', 'NSG.5', 'SCID.1', 'SCID.2', 'SCID.4', 'SCID.5')

Number.shRNA <- c(nrow(shRNAB8), nrow(shRNABL6.1), nrow(shRNABL6.2), nrow(shRNABL6.3), nrow(shRNABL6.4), nrow(shRNANSG.1), nrow(shRNANSG.3), nrow(shRNANSG.4), nrow(shRNANSG.5), nrow(shRNASCID.1), nrow(shRNASCID.2), nrow(shRNASCID.4), nrow(shRNASCID.5))

Number.genes <- c(length(unique(shRNAB8$GeneBank)), length(unique(shRNABL6.1$GeneBank)), length(unique(shRNABL6.2$GeneBank)), length(unique(shRNABL6.3$GeneBank)), length(unique(shRNABL6.4$GeneBank)), length(unique(shRNANSG.1$GeneBank)), length(unique(shRNANSG.3$GeneBank)), length(unique(shRNANSG.4$GeneBank)), length(unique(shRNANSG.5$GeneBank)), length(unique(shRNASCID.1$GeneBank)), length(unique(shRNASCID.4$GeneBank)), length(unique(shRNASCID.5$GeneBank)))

Number.Information <- data.frame(labels, Number.shRNA, Number.genes)

Average.genes.BL6 <- mean(Number.genes[2:5])
Average.genes.SCID <- mean(Number.genes[10:13])
Average.genes.NSG <- mean(Number.genes[6:9])

Average.genes <- c(Average.genes.BL6, Average.genes.SCID)

Average.shRNA.BL6 <- mean(Number.shRNA[2:5])
Average.shRNA.SCID <- mean(Number.shRNA[10:13])
Average.shRNA.NSG <- mean(Number.shRNA[6:9])

labels <- c("BL6", "SCID")
Average.genes <- data.frame(labels, Average.genes)
plot(Average.genes)

df(figDir("IVS-1.NumberofGenes.pdf"))
boxplot(Number.Information$Number.genes ~ mouse, Number.Information, ylab="Number of Unique Targeted Genes")
dev.off()

## Run differential analysis

### specify the design of the experiment for the linear model
#### Returns the individual index for each sample in terms of mouse type.
mouse <- pData(shRNADataSbst)$mouse

#### Creates a matrix with 4 columns of mouse type and then places a 0 or a 1 in the index, where 0 indicates that it is not that mouse and a 1 indicates that it is
mouse.model <- model.matrix(~0 + mouse)

### fit the linear model to the data

#### Creates a linear fit according to the data for each probe data.
#### lmfit requires log-ratios or log-values of expression for a series of microarrays
#### log-ratios are for 2-color arrays, log-values of expression are for 1-color arrays - these are 1-color
#### mouse.fit.sbst <- lmFit(shRNADataSbst, design=mouse.model)

### formulate contrasts to compare to baseline
#### Creates the contrasts necessary for the t-test on each probe below
#### This outputs a numeric vector, in this case a 4X4 vector, which indicates where each comparison should be done
mouse.contrasts <- makeContrasts(mouseBL6 - mouseB8,
                                  mouseNSG - mouseB8,
                                  mouseSCID - mouseB8,
                                  mouseBL6 - mouseSCID,
                                  mouseSCID - mouseNSG,
                                  mouseBL6 - mouseNSG,
                                  levels = mouse.model)

### fit the contrasts (get a t-stat for each comparison)
#### Creates a contrast.fit output from the linear fit data found earlier and performs a t-test for each of the comparisons specified in the contrasts variable.
mouse.contrasts.fit.sbst <- contrasts.fit(fit=mouse.fit.sbst,
                                          contrasts=mouse.contrasts)

### moderate the statistics by empirical Bayes
#### borrows information about variability from other genes
#### to get better estimate for the standard deviation in the t-statistics
#### Creates a rank of genes in order of evidence for differential expression.
mouse.contrasts.fit.sbst <- eBayes(mouse.contrasts.fit.sbst)

#### Need to create a mouse.contrasts with only the indeces in B8.
BL6vsNSGSbst <- topTable(mouse.contrasts.fit.sbst, coef="mouseBL6 - mouseNSG",
                          adjust.method='BH', number=Inf,
                          sort.by='none')

#### Creates a vector with labels of "BL6", "NSG", or ".". If the adj.p.val <0.05 and the logFoldChange is >1, then it labels it as BL6
#### If the adj.p.val <0.05 and the logFoldChange is <-1 then it labels it as NSG. If the adj.p.valu <0.05 and the logFoldChange is between -1 and 1,
#### then it labels it as ".". Likewise, if the adj.p.val is >0.05 then it is labeled as ".". The length of the vector spans all of the probes

```r
BL6NSGStbst <- ifelse(BL6vsNSGStbst$adj.P.Val < 0.05, ifelse(BL6vsNSGStbst$logFC > 1, 'BL6',
ifelse(BL6vsNSGStbst$logFC < -1 ,'NSG', "")), "")
```

# BL6 vs SCID

```r
BL6vsSCIDSbst <- topTable(mouse.contrasts.fit.sbst, coef="mouseBL6 - mouseSCID",
adjust.method='BH', number=Inf,
sort.by='none')
```

```r
BL6SCIDSbst <- ifelse(BL6vsSCIDSbst$adj.P.Val < 0.05,
ifelse(BL6vsSCIDSbst$logFC > 1, 'BL6',
ifelse(BL6vsSCIDSbst$logFC < -1 , 'SCID', "")), "")
```

# SCID vs NSG

```r
SCIDvsNSGSbst <- topTable(mouse.contrasts.fit.sbst, coef="mouseSCID - mouseNSG",
adjust.method='BH', number=Inf,
sort.by='none')
```

```r
SCIDNSGSbst <- ifelse(SCIDvsNSGSbst$adj.P.Val < 0.05,
ifelse(SCIDvsNSGSbst$logFC > 1, 'SCID',
ifelse(SCIDvsNSGSbst$logFC < -1 , 'NSG', "")), "")
```

# Re-initialize some of the vectors needed for the MouseTableTotal Data.frame since they were changed when the Non-EST Section was performed

```r
**************
BL6vsB8 <- topTable(mouse.contrasts.fit.sbst, coef="mouseBL6 - mouseB8",
adjust.method='BH', number=Inf,
sort.by='none')
```

```r
BL6Up <- BL6vsB8$adj.P.Val < 0.05 & BL6vsB8$logFC > 1
BL6Up <- factor(as.character(BL6Up), levels=c('TRUE','FALSE'),
labels=c('BL6',""))
```

```r
BL6Down <- BL6vsB8$adj.P.Val < 0.05 & BL6vsB8$logFC < -1
BL6Down <- factor(as.character(BL6Down), levels=c('TRUE','FALSE'),
labels=c('BL6',""))
```

```r
SCIDvsB8 <- topTable(mouse.contrasts.fit.sbst, coef="mouseSCID - mouseB8",
adjust.method='BH', number=Inf,
sort.by='none')
```

```r
SCIDUp <- SCIDvsB8$adj.P.Val < 0.05 & SCIDvsB8$logFC > 1
SCIDUp <- factor(as.character(SCIDUp), levels=c('TRUE','FALSE'),
labels=c('SCID',""))
```

```r
SCIDDDown <- SCIDvsB8$adj.P.Val < 0.05 & SCIDvsB8$logFC < -1
SCIDDDown <- factor(as.character(SCIDDDown), levels=c('TRUE','FALSE'),
labels=c('SCID',""))
```

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NSGvsB8 <- topTable(mouse.contrasts.fit.sbst, coef="mouseNSG - mouseB8", 
adjust.method='BH', number=Inf, 
sort.by='none')
# significant genes
NSGUp <- NSGvsB8$adj.P.Val < 0.05 & NSGvsB8$logFC > 1
NSGUp <- factor(as.character(NSGUp), levels=c('TRUE','FALSE'), 
labels=c('NSG',''))
NSGDown <- NSGvsB8$adj.P.Val < 0.05 & NSGvsB8$logFC < -1
NSGDown <- factor(as.character(NSGDown), levels=c('TRUE','FALSE'), 
labels=c('NSG',''))

# End re-initialization

# final table
#### Perform the exact same table creation procedure as above
MouseTableSbst <- BL6vsNSGSbst[,c('idx', 'GeneBank', 'Gene.name','sequence')]
MouseTableSbst$Higher.In.BL6.Vs.NSG <- BL6NSGSbst
MouseTableSbst$Higher.In.BL6.Vs.SCID <- BL6SCIDSbst
MouseTableSbst$Higher.In.SCID.Vs.NSG <- SCIDNSGSbst

MouseTableSbst$Higher.Relative.To.B8 <- paste(BL6Up, SCIDUp, NSGUp)
MouseTableSbst$Lower.Relative.To.B8 <- paste(BL6Down, SCIDDown, NSGDown)
MouseTableSbst$BL6.Vs.NSG.logFC <- signif(BL6vsNSGSbst$logFC,3)
MouseTableSbst$BL6.Vs.NSG.adj.P.Val <- signif(BL6vsNSGSbst$adj.P.Val,3)
MouseTableSbst$BL6.Vs.SCID.logFC <- signif(BL6vsSCIDSbst$logFC,3)
MouseTableSbst$BL6.Vs.SCID.adj.P.Val <- signif(BL6vsSCIDSbst$adj.P.Val,3)
MouseTableSbst$SCID.Vs.NSG.logFC <- signif(SCIDvsNSGSbst$logFC,3)
MouseTableSbst$SCID.Vs.NSG.adj.P.Val <- signif(SCIDvsNSGSbst$adj.P.Val,3)

#### Currently I have the total list of shRNAs with the respective FCs and adj.p.values
#### Output results subsetted to significant genes as formatted tables

#### This initializes the data.frame and includes the identifies (of gene and strain differences) as well as the
#### logFC and P value for each strain comparison.
#### The end is an output of a csv file with only the genes that are significantly different between the
#### strains
MouseTableSigSbst <- MouseTableSbst[which(MouseTableSbst$Higher.In.BL6.Vs.NSG!="" | MouseTableSbst$Higher.In.BL6.Vs.SCID!="" | MouseTableSbst$Higher.In.SCID.Vs.NSG != ", ], ]
write.table(MouseTableSigSbst, file=figDir('MouseResultsSbstSigSbst.csv'), 
sep="", row.names=F)
#### Initialize B8Background, which is used in the next line of code
B8Background <- shRNAData$B8[nrow(shRNAData)]
BL6VsNSGSbstidx <- MouseTableSigSbst$idx [which(MouseTableSigSbst$Higher.In.BL6.Vs.NSG ! ="" )]
BL6VsSCIDSbstidx <- MouseTableSigSbst$idx [which(MouseTableSigSbst$Higher.In.BL6.Vs.SCID ! ="" )]
SCIDVsNSGSbstidx <- MouseTableSigSbst$idx [which(MouseTableSigSbst$Higher.In.SCID.Vs.NSG ! ="" )]

B8IndexForBl6VsNSGSbst <- as.numeric(BL6VsNSGSbstidx)
B8IndexForBl6VsSCIDSbst <- as.numeric(BL6VsSCIDSbstidx)
B8IndexForSCIDVsNSGSbst <- as.numeric(SCIDVsNSGSbstidx)

B8InitialForBl6VsNSGSbst <- shRNAData$B8[B8IndexForBl6VsNSGSbst]
B8InitialForBl6VsSCIDSbst <- shRNAData$B8[B8IndexForBl6VsSCIDSbst]
B8InitialForSCIDVsNSGSbst <- shRNAData$B8[B8IndexForSCIDVsNSGSbst]

## Above, I found the B8 values individually for each gene depending on the strain
## Instead, I will source the idx values for the entire significant set and source that back
## to the original values
#B8InitialForSigSubidx <- as.numeric(MouseTableSigSub$Idx)
#B8InitialForSigSub <- shRNAData$B8[B8InitialForSigSubidx]

#### Initialize the data.frame
#MouseTableSigWithB8Sub <- MouseTableSigSub
#### Add in the B8 values for each shRNA
#MouseTableSigWithB8Sub$Initial <- B8InitialForSigSub

#Initialize the background value of the B8 array
B8Background <- shRNAData$B8[nrow(shRNAData)]

#### Isolate only the significant shRNAs that exist in the initial library, which are defined as 2x the background
MouseTableSigWithInitialSubRep <- MouseTableSigSbst

#### Isolate the significant shRNAs that do NOT exist in the initial library
# MouseTableSigWithInitialRepSubLOST <- MouseTableSigWithB8Sub
[which(MouseTableSigWithB8Sub$Initial<2*B8Background),]

BL6NSGSUp  <-MouseTableSigWithInitialSubRep[which(MouseTableSigWithInitialSubRep $Higher.In.BL6.Vs.NSG == "BL6" ),]
BL6NSGDown <- MouseTableSigWithInitialSubRep[which(MouseTableSigWithInitialSubRep $Higher.In.BL6.Vs.NSG == "NSG" ),]

BL6SCIDUp  <-MouseTableSigWithInitialSubRep[which(MouseTableSigWithInitialSubRep $Higher.In.BL6.Vs.SCID == "BL6" ),]
BL6SCIDDown <- MouseTableSigWithInitialSubRep[which(MouseTableSigWithInitialSubRep $Higher.In.BL6.Vs.SCID == "SCID" ),]

SCIDNSGUp <- MouseTableSigWithInitialSubRep[which(MouseTableSigWithInitialSubRep $Higher.In.SCID.Vs.NSG == "SCID" ),]
SCIDNSGSubDown <- MouseTableSigWithInitialSubRep[which(MouseTableSigWithInitialSubRep $Higher.In.SCID.Vs.NSG == "NSG"),]


dir.create(figDir('DifferentialGenesSub'))
dir.create(figDir('DifferentialGenesSub/Filtered'))
dir.create(figDir('DifferentialGenesSub/Filtered/BL6VsNSG'))
dir.create(figDir('DifferentialGenesSub/Filtered/BL6VsSCID'))
dir.create(figDir('DifferentialGenesSub/Filtered/SCIDVsNSG'))

dir.create(figDir('DifferentialGenesSub/Filtered/BL6VsNSG/Up'))
dir.create(figDir('DifferentialGenesSub/Filtered/BL6VsSCID/Up'))
dir.create(figDir('DifferentialGenesSub/Filtered/SCIDVsNSG/Up'))

dir.create(figDir('DifferentialGenesSub/Filtered/BL6VsNSG/Down'))
dir.create(figDir('DifferentialGenesSub/Filtered/BL6VsSCID/Down'))
dir.create(figDir('DifferentialGenesSub/Filtered/SCIDVsNSG/Down'))

### print a barplot of the data for each significant gene
#### Do this for each strain comparison separately

##### BL6 Vs NSG - UP
for (g in which(MouseTableSigWithInitialSubRep$Higher.In.BL6.Vs.NSG=="BL6")) {
  idx <- MouseTableSigWithInitialSubRep$idx[g]
  pdf(figDir(paste('DifferentialGenesSub/Filtered/BL6VsNSG/Up/Log2-', gsub(' ','.', MouseTableSigWithInitialSubRep$Gene.name[g]), idx, 'pdf', sep='.')))
  barplot(exprs(shRNADataSbst)[idx,],las=2, ylab= 'Log2 Expression'
  abline (h=log2(B8Background), col = 'red')
  abline (h=log2(B8Background*2), col= 'blue')
  title(sprintf('%s (%s)', MouseTableSigWithInitialSubRep$Gene.name[g], idx),las=2)
  legend('topright', legend=c('Background Expression', 'Detection Limit'),fill=c('red','blue'))
  dev.off()
}

##### BL6 Vs NSG - DOWN
for (g in which(MouseTableSigWithInitialSubRep$Higher.In.BL6.Vs.NSG=="NSG")) {
  idx <- MouseTableSigWithInitialSubRep$idx[g]
  pdf(figDir(paste('DifferentialGenesSub/Filtered/BL6VsNSG/Down/Log2-', gsub(' ','.', MouseTableSigWithInitialSubRep$Gene.name[g]), idx, 'pdf', sep='.')))
  barplot(exprs(shRNADataSbst)[idx,],las=2, ylab= 'Log2 Expression'
  abline (h=log2(B8Background), col = 'red')
  abline (h=log2(B8Background*2), col= 'blue')
  title(sprintf('%s (%s)', MouseTableSigWithInitialSubRep$Gene.name[g], idx),las=2)
  legend('topright', legend=c('Background Expression', 'Detection Limit'),fill=c('red','blue'))
  dev.off()
}

##### BL6 Vs SCID - UP
for (g in which(MouseTableSigWithInitialSubRep$Higher.In.BL6.Vs.SCID=="BL6")) {
  idx <- MouseTableSigWithInitialSubRep$idx[g]
  pdf(figDir(paste('DifferentialGenesSub/Filtered/BL6VsSCID/Up/Log2-', gsub(' ','.', MouseTableSigWithInitialSubRep$Gene.name[g]), idx, 'pdf', sep='.')))
  barplot(exprs(shRNADataSbst)[idx,],las=2, ylab= 'Log2 Expression'
  abline (h=log2(B8Background), col = 'red')
  abline (h=log2(B8Background*2), col= 'blue')
  title(sprintf('%s (%s)', MouseTableSigWithInitialSubRep$Gene.name[g], idx),las=2)
  legend('topright', legend=c('Background Expression', 'Detection Limit'),fill=c('red','blue'))
  dev.off()
}

##### BL6 Vs SCID - DOWN

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for (g in which(MouseTableSigWithInitialSubRep$Higher.In.BL6.Vs.SCID=="SCID")) {
  idx <- MouseTableSigWithInitialSubRep$idx[g]
  pdf(figDir(paste('DifferentialGenesSub/Filtered/BL6vsSCID/Down/Log2-',
                    MouseTableSigWithInitialSubRep$Gene.name[g], idx, 'pdf',
                    sep='.')))
  barplot(exprs(shRNADataSbst)[idx,],las=2, ylab= 'Log2 Expression')
  abline (h=log2(B8Background), col = 'red')
  abline (h=log2(B8Background*2), col= 'blue')
  title(sprintf('%s (%s)', MouseTableSigWithInitialSubRep$Gene.name[g], idx),las=2)
  dev.off()
}

#### SCID Vs NSG - UP
for (g in which(MouseTableSigWithInitialSubRep$Higher.In.SCID.Vs.NSG == "SCID")) {
  idx <- MouseTableSigWithInitialSubRep$idx[g]
  pdf(figDir(paste('DifferentialGenesSub/Filtered/SCIDVsNSG/Up/Log2-',
                    MouseTableSigWithInitialSubRep$Gene.name[g], idx, 'pdf',
                    sep='.')))
  barplot(exprs(shRNADataSbst)[idx,],las=2, ylab= 'Log2 Expression')
  abline (h=log2(B8Background), col = 'red')
  abline (h=log2(B8Background*2), col= 'blue')
  title(sprintf('%s (%s)', MouseTableSigWithInitialSubRep$Gene.name[g], idx),las=2)
  dev.off()
}

#### SCID Vs NSG - Down
for (g in which(MouseTableSigWithInitialSubRep$Higher.In.SCID.Vs.NSG == "NSG")) {
  idx <- MouseTableSigWithInitialSubRep$idx[g]
  pdf(figDir(paste('DifferentialGenesSub/Filtered/SCIDVsNSG/Down/Log2-',
                    MouseTableSigWithInitialSubRep$Gene.name[g], idx, 'pdf',
                    sep='.')))
  barplot(exprs(shRNADataSbst)[idx,],las=2, ylab= 'Log2 Expression')
  abline (h=log2(B8Background), col = 'red')
  abline (h=log2(B8Background*2), col= 'blue')
  title(sprintf('%s (%s)', MouseTableSigWithInitialSubRep$Gene.name[g], idx),las=2)
  dev.off()
}

#### PLOT all barplots together
#quartz()

shRNADataV.melt <- melt(shRNADataV.id=
  c("Gene.name", "GeneBank", "idx", "siRNA.sense.sequence"))
names(shRNADataV.melt)[6] <- "Expr"
names(shRNADataV.melt)[5] <- "Mouse"
shRNADataV.melt.coolio <- shRNADataV.melt[ shRNADataV.melt$idx %in% BL6NSGSubUp$idx, ]
pdf(figDir('Barchart-BL6vsNSG-Upregulated-Subselected.pdf'))
barchart(log2(Expr) ~ Mouse | paste(GeneBank,idx), shRNADataV.melt.coolio,layout=c(3,5),ylab =
  "Log2 (Expression)",
  panel=function(x,y,...){
    panel.abline(h=log2(B8Background), col.line="blue")
  })
panel.abline(h=log2(2*B8Background), col.line="red")
panel.barchart(x,y,...)
}
main="shRNAs Upregulated Between BL6 and NSG", scales=list(x=list(rot=45,cex=.5))

dev.off()
#quartz()
shRNADataV.melt.coolio <- shRNADataV.melt[ shRNADataV.melt$idx %in% BL6NSGSubDown$idx, ]
pdf(figDir('Barchart-BL6vsNSG-Down-Regulated-Subselected.pdf'))
barchart(log2(Expr) ~ Mouse | paste(GeneBank,idx), shRNADataV.melt.coolio,layout=c(12,12),ylab = "Log2 (Expression)",
panel=function(x,y,...){
  panel.abline(h=log2(B8Background), col.line="blue")
  panel.abline(h=log2(2*B8Background), col.line="red")
  panel.barchart(x,y,...)
}
main="shRNAs Down-regulated Between BL6 and NSG", scales=list(x=list(rot=45,cex=.5))

dev.off()
#quartz()
shRNADataV.melt.coolio <- shRNADataV.melt[ shRNADataV.melt$idx %in% BL6SCIDSubUp$idx, ]
pdf(figDir('Barchart-BL6vsSCID-Upregulated-Subselected.pdf'))
barchart(log2(Expr) ~ Mouse | paste(GeneBank,idx), shRNADataV.melt.coolio,layout=c(2,4),ylab = "Log2 (Expression)",
panel=function(x,y,...){
  panel.abline(h=log2(B8Background), col.line="blue")
  panel.abline(h=log2(2*B8Background), col.line="red")
  panel.barchart(x,y,...)
}
main="shRNAs Upregulated Between BL6 and SCID", scales=list(x=list(rot=45,cex=.5))

dev.off()
#quartz()
shRNADataV.melt.coolio <- shRNADataV.melt[ shRNADataV.melt$idx %in% BL6SCIDSubDown $idx, ]
pdf(figDir('Barchart-BL6vsSCID-DownRegulated-Subselected.pdf'))
barchart(log2(Expr) ~ Mouse | paste(GeneBank,idx), shRNADataV.melt.coolio,layout=c(5,4),ylab = "Log2 (Expression)",
panel=function(x,y,...){
  panel.abline(h=log2(B8Background), col.line="blue")
  panel.abline(h=log2(2*B8Background), col.line="red")
  panel.barchart(x,y,...)
}
main="shRNAs Down-regulated Between BL6 and SCID", scales=list(x=list(rot=45,cex=.5))

dev.off()
#quartz()
shRNADataV.melt.coolio <- shRNADataV.melt[ shRNADataV.melt$idx %in% SCIDNSGSubUp$idx, ]
pdf(figDir('Barchart-SCIDvsNSG-Upregulated-Subselected.pdf'))
barchart(log2(Expr) ~ Mouse | paste(GeneBank,idx), shRNADataV.melt.coolio,layout=c(2,1),ylab = "Log2 (Expression)",
panel=function(x,y,...){
  panel.abline(h=log2(B8Background), col.line="blue")
  panel.abline(h=log2(2*B8Background), col.line="red")
  panel.barchart(x,y,...)
}
main="shRNAs Upregulated Between SCID and NSG", scales=list(x=list(rot=45,cex=.5))

dev.off()
#quartz()
shRNADataV.melt.coolio <- shRNADataV.melt[ shRNADataV.melt$idx %in% SCIDNSGSubDown$idx, ]
pdf(figDir('Barchart-SCIDvsNSG-Downregulated-Subselected.pdf'))
barchart(log2Expr ~ Mouse | paste(GeneBank,idx), shRNADataV.melt.coolio,layout=c(11,7),ylab = "Log2 (Expression)",
panel=function(x,y,...){
  panel.abline(h=log2(B8Background), col.line="blue")
  panel.abline(h=log2(2*B8Background), col.line="red")
  panel.barchart(x,y,...)
}, main="shRNAs Down-regulated Between SCID and NSG",scales=list(x=list(rot=45,cex=.5)))
dev.off()

#### Create barcharts of only those shRNAs that are Up in both SCID and NSG (vs BL6) and those
# that are down in both (vs BL6)

# First, shRNAs down in both BL6 vs SCID and BL6 Vs NSG
shRNADataV.melt.coolio <- shRNADataV.melt[ shRNADataV.melt$idx %in% BL6SCIDSubDown$idx, ]
shRNADataV.melt.coolio <- shRNADataV.melt.coolio[ shRNADataV.melt.coolio$idx %in%
BL6NSGSubDown$idx, ]
pdf(figDir('Barchart-BL6vsNSGandSCID-Downregulated-Subselected.pdf'))
barchart(log2Expr ~ Mouse | paste(GeneBank,idx), shRNADataV.melt.coolio,layout=c(2,7),ylab = "Log2 (Expression)",
panel=function(x,y,...){
  panel.abline(h=log2(B8Background), col.line="blue")
  panel.abline(h=log2(2*B8Background), col.line="red")
  panel.barchart(x,y,...)
}, main="shRNAs Contracted Between BL6 vs SCID and NSG",scales=list(x=list(rot=45,cex=.5)))
dev.off()

# Second, shRNAs up in both BL6 vs SCID and BL6 Vs NSG
shRNADataV.melt.coolio <- shRNADataV.melt[ shRNADataV.melt$idx %in% BL6NSGSubUp$idx, ]
shRNADataV.melt.coolio <- shRNADataV.melt.coolio[ shRNADataV.melt.coolio$idx %in%
BL6SCIDSubUp$idx, ]
pdf(figDir('Barchart-BL6vsNSGandSCID-Upregulated-Subselected.pdf'))
barchart(log2Expr ~ Mouse | paste(GeneBank,idx), shRNADataV.melt.coolio,layout=c(2,3),ylab = "Log2 (Expression)",
panel=function(x,y,...){
  panel.abline(h=log2(B8Background), col.line="blue")
  panel.abline(h=log2(2*B8Background), col.line="red")
  panel.barchart(x,y,...)
}, main="shRNAs Expanded Between BL6 vs SCID and NSG",scales=list(x=list(rot=45,cex=.5)))
dev.off()

# Third, shRNAs down in both BL6 vs NSG and SCID Vs NSG
shRNADataV.melt.coolio <- shRNADataV.melt[ shRNADataV.melt$idx %in% BL6NSGSubDown$idx, ]
shRNADataV.melt.coolio <- shRNADataV.melt.coolio[ shRNADataV.melt.coolio$idx %in%
SCIDNSGSubDown$idx, ]

pdf(figDir('Barchart-BL6vsNSGandSCIDvsNSG-Downregulated-Subselected.pdf'))
barchart(log2(Expr) ~ Mouse | paste(GeneBank,idx), shRNADataV.melt.coolio,layout=c(6,10),ylab = "Log2 (Expression)",
panel=function(x,y,...){
  panel.abline(h=log2(B8Background), col.line="blue")
  panel.abline(h=log2(2*B8Background), col.line="red")
  panel.barchart(x,y,...)
}, main="shRNAs Contracted Between BL6 vs NSG and SCID vs NSG",scales=list(x=list(rot=45,cex=.5))
dev.off()

# Fourth, shRNAs Up in both BL6 vs NSG and SCID Vs NSG
shRNADataV.melt.coolio <- shRNADataV.melt[ shRNADataV.melt$idx %in% BL6NSGSubUp$idx, ]
shRNADataV.melt.coolio <- shRNADataV.melt.coolio[ shRNADataV.melt.coolio$idx %in% SCIDNSGSubUp$idx, ]

pdf(figDir('Barchart-BL6vsNSGandSCIDvsNSG-Upregulated-Subselected.pdf'))
barchart(log2(Expr) ~ Mouse | paste(GeneBank,idx), shRNADataV.melt.coolio,layout=c(1,1),ylab = "Log2 (Expression)",
panel=function(x,y,...){
  panel.abline(h=log2(B8Background), col.line="blue")
  panel.abline(h=log2(2*B8Background), col.line="red")
  panel.barchart(x,y,...)
}, main="shRNAs Expanded Between BL6 vs NSG and SCID vs NSG",scales=list(x=list(rot=45,cex=.5))
dev.off()

#### Create a waterfall plot of all of the data
# Sort for BL6 to NSG
Sort.LogFC.BL6.NSG.Sub <-
MouseTableSbst[with(MouseTableSbst,order(BL6.Vs.NSG.logFC,decreasing=TRUE,GeneBank)),]
Sort.LogFC.BL6.SCID.Sub<-
MouseTableSbst[with(MouseTableSbst,order(BL6.Vs.SCID.logFC,decreasing=TRUE,GeneBank)),]
Sort.LogFC.SCID.NSG.Sub<-
MouseTableSbst[with(MouseTableSbst,order(SCID.Vs.NSG.logFC,decreasing=TRUE,GeneBank)),]

#quartz()
index <- 1:nrow(Sort.LogFC.BL6.NSG.Sub)

pdf(figDir('WaterfallPlot-BL6vsNSG-Subselected.pdf'))
plot(Sort.LogFC.BL6.NSG.Sub$BL6.Vs.NSG.logFC, index,
  abline (v=c(-1,1),col='red'),
  ylab = 'shRNA Index',
  xlab = 'Log2 Fold Change',
  main = 'C57BL/6 Vs NSG')
points(Sort.LogFC.BL6.NSG.Sub$BL6.Vs.NSG.logFC[Sort.LogFC.BL6.NSG.Sub$idx%in
%BL6NSGSubUp$idx],
    index[Sort.LogFC.BL6.NSG.Sub$idx%in%BL6NSGSubUp$idx],
    col='green')
points(Sort.LogFC.BL6.NSG.Sub$BL6.Vs.NSG.logFC[Sort.LogFC.BL6.NSG.Sub$idx%in
%BL6NSGSubDown$idx],
    index[Sort.LogFC.BL6.NSG.Sub$idx%in%BL6NSGSubDown$idx],
    col='green')
legend('topright', fill=c('red','green'), legend=c('Detection Limit','Significant Hit'))

dev.off()

dev.off()

dev.off()

dev.off()

dev.off()

Sort.Sig.LogFC.BL6.NSG.Sub <-
MouseTableSigWithInitialSubRep[with(MouseTableSigWithInitialSubRep,order(BL6.Vs.NSG.logFC,dec
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```r
reasing=TRUE,GeneBank)),]
Sort.Sig.LogFC.BL6.SCID.Sub <-
MouseTableSigWithInitialSubRep[with(MouseTableSigWithInitialSubRep,order(BL6.Vs.SCID.logFC,de
creasing=TRUE,GeneBank)),]
Sort.Sig.LogFC.SCID.NSG.Sub <-
MouseTableSigWithInitialSubRep[with(MouseTableSigWithInitialSubRep,order(SCID.Vs.NSG.logFC,de
creasing=TRUE,GeneBank)),]
index = 1:nrow(Sort.Sig.LogFC.BL6.SCID.Sub)

quartz()
pdf(figDir('WaterfallPlot-Significant-BL6vsNSG-Subselected.pdf'))
plot(Sort.Sig.LogFC.BL6.NSG.Sub$BL6.Vs.NSG.logFC, index,
    abline (v=c(-1,1),col='red'),
    ylab = 'shRNA Index',
    xlab = 'Log2 Fold Change',
    main = 'C57BL/6 Vs NSG')
legend('topright', fill='red', legend='Detection Limit')
dev.off()

pdf(figDir('WaterfallPlot-Significant-BL6vsSCID-Subselected.pdf'))
plot(Sort.Sig.LogFC.BL6.SCID.Sub$BL6.Vs.SCID.logFC, index,
    abline (v=c(-1,1),col='red'),
    ylab = 'shRNA Index',
    xlab = 'Log2 Fold Change',
    main = 'C57BL/6 - SCID Vs C57BL/6 - SCID')
legend('topright', fill='red', legend='Detection Limit')
dev.off()

pdf(figDir('WaterfallPlot-Significant-SCIDvsNSG-Subselected.pdf'))
plot(Sort.Sig.LogFC.SCID.NSG.Sub$SCID.Vs.NSG.logFC, index,
    abline (v=c(-1,1),col='red'),
    ylab = 'shRNA Index',
    xlab = 'Log2 Fold Change',
    main = 'C57BL/6 - SCID Vs NSG')
legend('topright', fill='red', legend='Detection Limit')
dev.off()

#plot(MouseTableSig$BL6.Vs.NSG.logFC,-log10(MouseTableSig$BL6.Vs.NSG.adj.P.Val))
# Have to do a volcano plot of the entire MouseTable set and then plot the data points that MATCH to the
MouseTableSig set OR which have the same idx as the MouseTableSig points

##### BL6 Vs NSG
# Creates the idx values that are significantly differently between BL6 and NSG mice
BL6VsNSGSubidx <- MouseTableSigSbstSidx [which(MouseTableSigSbst$Higher.In.BL6.Vs.NSG !="")]
```

# Need to find the actual indexes of MouseTable which have the same idx values as MouseTableSig
BL6VsNSGSubMatchIndex <- match(BL6VsNSGSubidx, MouseTableSbst[, 'idx'])

##### BL6 Vs SCID
# Creates the idx values that are significantly differently between BL6 and NSG mice
BL6VsSCIDSubidx <- MouseTableSigSbst$idx [which(MouseTableSigSbst$Higher.In.BL6.Vs.SCID != "")]
# Need to find the actual indexes of MouseTable which have the same idx values as MouseTableSig
BL6VsSCIDSubMatchIndex <- match(BL6VsSCIDSubidx, MouseTableSbst[, 'idx'])

##### SCID Vs NSG
# Creates the idx values that are significantly differently between BL6 and NSG mice
SCIDVsNSGSubidx <- MouseTableSigSbst$idx [which(MouseTableSigSbst$Higher.In.SCID.Vs.NSG != "")]
# Need to find the actual indexes of MouseTable which have the same idx values as MouseTableSig
SCIDVsNSGSubMatchIndex <- match(SCIDVsNSGSubidx, MouseTableSbst[, 'idx'])

##### Create the volcano plot for BL6 vs NSG
pdf(figDir('VolcanoPlotBL6vsNSG-Sig-Subselected.pdf'))
plot(MouseTableSbst$BL6.Vs.NSG.logFC, -log10(MouseTableSbst$BL6.Vs.NSG.adj.P.Val))
points(MouseTableSbst$BL6.Vs.NSG.logFC[BL6VsNSGSubMatchIndex],
       -log10(MouseTableSbst$BL6.Vs.NSG.adj.P.Val[BL6VsNSGSubMatchIndex]),
col='green')
legend('topright', legend=c('Non-Significant', 'BL6 vs NSG'),
       fill=c('black', 'green'))
dev.off()

##### Create the volcano plot for BL6 vs SCID
pdf(figDir('VolcanoPlotBL6vsSCID-Sig-Subselected.pdf'))
plot(MouseTableSbst$BL6.Vs.SCID.logFC, -log10(MouseTableSbst$BL6.Vs.SCID.adj.P.Val))
points(MouseTableSbst$BL6.Vs.SCID.logFC[BL6VsSCIDSubMatchIndex],
       -log10(MouseTableSbst$BL6.Vs.SCID.adj.P.Val[BL6VsSCIDSubMatchIndex]),
col='blue')
legend('topright', legend=c('Non-Significant', 'BL6 vs SCID'),
       fill=c('black', 'blue'))
dev.off()

##### Create the volcano plot for BL6 vs SCID
pdf(figDir('VolcanoPlotSCIDvsNSG-Sig-Subselected.pdf'))
plot(MouseTableSbst$SCID.Vs.NSG.logFC, -log10(MouseTableSbst$SCID.Vs.NSG.adj.P.Val))
points(MouseTableSbst$SCID.Vs.NSG.logFC[SCIDVsNSGSubMatchIndex],
       -log10(MouseTableSbst$SCID.Vs.NSG.adj.P.Val[SCIDVsNSGSubMatchIndex]),
col='red')
legend('topright', legend=c('Non-Significant', 'SCID vs NSG'),
       fill=c('black', 'red'))
dev.off()
### Summarize comparisons of each strain as Venn diagram
#### Include those that are up and down regulated compared to the initial library

```r
decideTests(mouse.contrasts.fit.sbst, adjust.method = 'BH', p.value = 0.05, lfc = 1) -> mcDT
pdf(figDir('VennStrainComparison-Significant-Sbst-Decreased.pdf'))
vennDiagram(mcDT[,c('mouseBL6 - mouseSCID',
                     'mouseBL6 - mouseNSG',
                     'mouseSCID - mouseNSG')],
            names = c('BL6 vs SCID','BL6 vs NSG','SCID vs NSG'), include = 'down',
            main = "Significant Down-regulated shRNAs")
dev.off()

pdf(figDir('VennStrainComparison-Significant-Sbst-Increased.pdf'))
vennDiagram(mcDT[,c('mouseBL6 - mouseSCID',
                     'mouseBL6 - mouseNSG',
                     'mouseSCID - mouseNSG')],
            names = c('BL6 vs SCID','BL6 vs NSG','SCID vs NSG'), include = 'up',
            main = "Significant Up-regulated shRNAs")
title = "Up-regulated Significant shRNAs"
dev.off()
```

capture.output(sessionInfo(), file = figDir('RSessionInfo.txt'))
save(list = ls(), file = figDir('RWorkspace.Rda'))

# make an extra copy of the script to ensure that figures
# match the script used
file.copy('R/ShuptrineAnalysis.R', figDir('RAnalysisScript.R'))
<table>
<thead>
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<th>Gene Target</th>
<th>Backbone</th>
<th>shRNA Sequence</th>
<th>Target Region</th>
<th>TRCN #</th>
<th>Target Position</th>
</tr>
</thead>
<tbody>
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<td>pLKO.1</td>
<td>CCGGGCAGAAACTTTGGATTAGTTTCGGAACCTAAATCCAAGTAGTCTCCTTTTG</td>
<td>CDS</td>
<td>65434</td>
<td>1031</td>
</tr>
<tr>
<td>CD47</td>
<td>pLKO.1</td>
<td>CCGGCACCCAGAAAGTTGTTGGAACCTCGAGTTCACAAACATTTCTCCTGCTTTTTG</td>
<td>CDS</td>
<td>65436</td>
<td>283</td>
</tr>
<tr>
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CHAPTER 5
CONCLUSIONS & FUTURE WORK

Here, we summarize the findings and conclusions from our study of the tumor-based molecular determinants of adaptive immune rejection. The significance and context of the findings will also be addressed.

5.1 SCREENING FOR MEDIATORS OF IMMUNE REJECTION

A functional genomics screening platform to identify tumor-derived mediators of immune rejection was developed. The spontaneously arising breast adenocarcinoma cell line EO771, was transduced with a lentivirally delivered genome-wide shRNA library. The cell line contained a successful integration of at least 26,160 shRNAs targeting 17,281 unique genes. Three independent in vivo screens were performed in mice with varying components of the immune response, in an effort to identify shRNAs that increase or decrease a cells’ likelihood of survival when exposed to the adaptive immune response. The first in vivo screen identified a set of 13-targeted genes whose shRNAs were over-represented in the WT versus immune-deficient mice. This screen also identified 184-targeted genes that were under-represented in the same comparison. The second in vivo screen identified 3 over-represented and 541 under-represented genes when tumors are exposed to the adaptive immune system. The large proportion of under-represented genes implies a selective elimination of previous immune-shielded clones as opposed to the enrichment of cells that contain an shRNA that results in increased immune shielding. As it was hypothesized that immune-shielded cells might need more time to preferentially expand in vivo, EO771-library cells were serially passaged in mice. After three successive passages in WT and immune-deficient mice, EO771-library cells had enhanced growth kinetics, suggesting enrichment of immune-shielded or of quicker dividing clones.
Out of these three screens, four genes were identified and individually validated to be important for immune-mediated selection of EO771 cells: CD47, Tex9, Pex14, and Sgpl1. By engrafting shRNA-containing cells in WT and immune-deficient mice, it was possible to identify the significance of protein knockdown on tumor growth and survival. Knockdown of CD47, Pex14, and Sgpl1 increased survival of engrafted mice by at least 50% and in some cases completed abrogated tumor growth. Knockdown of Tex9 decreased median survival of engrafted WT mice from 31 days to 20 days, in one representative study. Therefore, we were able to identify four validated targets of immune modulation from multiple independent in vivo shRNA screens.

Next we sought to identify the mechanisms behind the manipulation of immune recognition caused by the knockdown of protein expression. To do this, we isolated shRNA-containing tumors from WT mice and quantified the immune infiltrate into the tumor. Pex14 knockdown elicited a significant increase in CD3+ T cells into tumors, as well as a non-significant increase in CD4+ T cell infiltrate. Knockdown of Sgpl1 resulted in a significant decrease in NK cell infiltrate, as well as a non-significant increase in activated dendritic cells and decrease in MDSCs. Therefore, Pex14 modulation appears to increase adaptive immune infiltrate and Sgpl1 modulation results in increased antigen processing.

To further extrapolate our findings to human cancer, the prognostic indications of each validated candidate was determined using KMPlot. CD47 and Sgpl1 expression was found to correlate with relapse-free survival the 4,142 breast cancer patients analyzed. When further stratified to include only basal-like breast cancer patients, Pex14 was found to be a significant prognostic indicator for survival. Therefore, it appears that the findings from our studies have applicability in human breast cancer.
5.2 FUTURE WORK

5.2.1 IDENTIFICATION OF IMMUNE CELLS REQUIRED FOR IMMUNE MANIPULATION

In our model system, we identified CD47, Tex9, Pex14, and Sgpl1 as important tumor-based mediators of immune rejection. Knockdown of CD47, Pex14, and Sgpl1 resulted in a significant enhancement in survival of immune-competent mice with engrafted tumors. Conversely, knockdown of Tex9 resulted in reduced survival of engrafted immune-competent mice. The main difference between WT and SCID mice is the existence of a functional adaptive immune system in WT mice. Therefore, SCID mice do not contain functional B or T lymphocytes, which have varying but important roles on tumor growth. To begin to determine which immune cells are manipulated by the abrogation of protein expression for our four validated gene candidates, we will pharmacologically deplete specific immune cells in engrafted mice. Mice will be treated with clodronate to deplete macrophages (Lehenkari 2002), an anti-CD4 depleting antibody to eliminate T helper cells (Pohlers et al. 2004), and an anti-CD8 depleting antibody to eliminate CTLs (Reynolds et al., 2002). Upon inhibition of each of these cell types, tumor growth and mouse survival will be determined. Identifying the immune cell types that can rescue the phenotype will elucidate the potential mechanisms of action behind each validated genes’ immune modulatory function. This is especially important, considering that observed effects from Tex9, CD47, Pex14, and Sgpl1 knockdown could be due to changes in angiogenesis and not direct immune manipulation.

5.2.2 INHIBITION OF GENE CANDIDATES IN MULTIPLE SYNGENEIC MURINE MODEL SYSTEMS

Identifying the importance of Tex9, CD47, Pex14, and Sgpl1 across multiple cancer types is essential if we are to understand the significance of pharmacological inhibition in human cancers. To determine the broader applicability of our findings, we
are expanding our validation studies to include the syngeneic cell lines 4T1 (to Balb/c mice) and D5 (to C57Bl/6 mice) (Shangzi Wang 2012; Pulaski and Ostrand-Rosenberg 2001), as model systems for poorly immunogenic mammary carcinoma and melanoma, respectively. Therefore, to begin this process, we have infected the syngeneic cell lines 4T1 and D5 with lentivirus containing shRNAs against Tex9, CD47, Pex14, and Sgp11. The shRNAs that resulted in the highest protein knockdown in EO771 cells were utilized for the two cell lines. Virus packaging and target cell transduction were performed under the same conditions as previously described for EO771 cells. Infected cells were selected with 5 μg/ml of puromycin for 10 days, whereupon they were frozen and cell pellets were collected. Once protein knockdown is assessed and validated, shRNA containing 4T1 cells will be engrafted into the flank of Balb/c-WT and –SCID mice, thereby determining if protein knockdown has an effect on survival in mice. Similarly, shRNA containing D5 cells will be engrafted into the flank of C57Bl/6-WT and –SCID mice. To assess the effects on the tumor immune milieu, tumors will be digested and stained for various immunological markers.

5.3 INTEGRATION & CONTEXT OF OUR FINDINGS

The approach presented here can be used to identify tumor-based determinants of resistance to any selection pressure. Our methodology and validation suggest that it is possible to identify determinants that modulate the in vivo anti-tumor immune response in an efficient, unbiased, and genome-wide approach. Incorporation of an immune-stimulatory treatment, such as anti-CTLA-4 therapy, has the potential to identify novel resistance mechanisms in vivo, and such studies are ongoing in our laboratory. Combining inhibition of discovered under-represented targets with primary anti-CTLA-4 or anti-PD1 or anti-PDL-1 treatment has the potential for a synergistic immune response. Alternatively, identification of the genes associated
with a reduction in adaptive immune control of cancer could be used as biomarkers to identify those patients who might benefit from immunotherapy. Therefore, the preclinical and clinical utility of our screening method could have important implications for the discovery and development of novel immune therapeutic targets.
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