The Role, Function, and Generation of Eomes^{hi} CD8^{+} T Cells in OX40 and CTLA-4 Targeted Cancer Immunotherapy

Dana Emerson

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Abstract

Monoclonal antibodies targeting key receptors that inhibit T cell function such as CTLA-4 and PD-1 have demonstrated the potency of checkpoint blockade, highlighted by long-term complete responses for metastatic cancers once thought incurable. However, only a subset of patients will respond to checkpoint blockade due to a multitude of factors including an immunosuppressive tumor microenvironment and the mutational burden of the cancer. Novel antibodies that target "co-stimulatory" immune receptors are being developed and tested in clinical trials to further enhance the anti-tumor immune response. CTLA-4 blockade in combination with an agonist OX40-specific mAb synergizes to augment antitumor immunity through enhanced T-cell effector function, leading to increased survival in preclinical cancer models. Treatment with Anti-OX40 /anti-CTLA-4 therapy synergistically enhances the expression of Eomesodermin (Eomes) in $CD8^+$ T cells. Eomes is a critical transcription factor for the differentiation and memory function of $CD8^+$ T cells. However, the role, function, and benefit of $Eomes^{hi}$ $CD8^+$ T cells in anti-OX40/anti-CTLA-4 therapy is poorly understood. I hypothesized that $Eomes^{hi}CD8^+$ T cells were necessary for anti-OX40/anti-CTLA-4 immunotherapy efficacy and that further enhancement of this population would improve tumor-free survival. $CD8^+$ T cell–specific deletion of Eomes abrogated the efficacy of anti-OX40/anti-CTLA-4 therapy. I also found that anti-OX40/anti-CTLA-4-induced $Eomes^{hi}$ $CD8^+$ T cells expressed lower levels of checkpoint receptors (PD1, Tim-3, and Lag-3) and higher levels of effector cytokines (IFNγ and TNFα) than their $Eomes^{lo}$ counterparts. Eomes expression is negatively regulated in T cells through interleukin-2–inducible T-cell kinase (ITK) signaling. I investigated the impact of modulating ITK signaling with ibrutinib, an FDA-approved tyrosine kinase inhibitor, and found that anti-OX40/anti-CTLA-4/ibrutinib therapy further enhanced $CD8^+$ T cell–specific Eomes expression, leading to enhanced tumor regression and improved survival, both of which were associated with increased T-cell effector function across multiple tumor models. Taken together, these data demonstrate the potential of anti-OX40/anti-CTLA-4/ibrutinib as a triple therapy to improve the efficacy of immunotherapy.

Chapter I: Overview of T cells in the immune system

1.1. Antigen recognition in immunology and presentation

The immune system protects the organism from actual and perceived pathogens ranging from viruses and bacteria to parasites and transformed cancerous tissue. In defending against these threats, the immune system evolved a dynamic network of cells to handle both broad and specific tasks involved in this response. Recognizing these threats as foreign or dangerous material different from normal host tissue, is a key first step in this process. While the features that differentiate between virus and self are clearly drawn, the features that discriminate between self and transformed cancer tissue are more difficult for the immune system to distinguish.

Recognizing, finding, and eliminating cancerous and transformed cells relies on recognition of molecular patterns associated with damaged tissue similar to how viruses, bacteria, and parasites are recognized. At the core of this response is the recognition of proteins or genetic material that are different from healthy tissues. In the case of infectious disease, foreign antigens are typically distinct from self-tissue. Common motifs found in bacteria, viruses, and parasites are identified by pattern recognition receptors (PRRs), which can trigger an immune response (1). However, the hallmarks of cancer are more nuanced compared to the explicit motifs expressed by infectious organisms. For example, the immune system recognizes damage-associated molecular patterns (DAMPs) through PRRs. This recognition can lead to chronic inflammation and possible carcinogenesis (2). Many DAMPs, such as heat shock proteins, activate members 2 and 4 of the toll-like receptor (TLR) family (3). Activation of TLRs and other DAMP PRRs triggers nuanced immune responses to investigate and target the damaged tissue.

The initiation and formation of an adaptive immune response against cancer starts with the presentation of antigens derived from the mutated proteins of transformed cells. There are two main pathways of antigen presentation differentiated by the cell's ability to process protein-based antigens and present them on major histocompatibility complex (MHC) proteins on the cell surface. The two main types of MHC molecules are known as MHC class I and MHC class II. All nucleated cells, including most non-immune cells, present antigens on MHC class I (4); however, MHC class II expression is more specialized, and is limited to dendritic cells, monocytes, macrophages, B cells, and thymic epithelial cells (5). These two main classes of MHC molecules differ in the size and source of antigen they present, and what type of immune response they elicit.

MHC class I molecules are made from three alpha chain domains covalently bound to a β-2 microglobulin molecule anchored by a single transmembrane segment. The structure of MHC class I produces an antigen binding cleft that is of sufficient size to bind a peptide antigen of 8 to 10 amino acids in length. The peptide-loaded MHC I molecule is presented on the cell surface to $CD8^+$ T cells, which have T cell receptors (TCR) complimentary to MHC I containing the bound peptide. Alternatively, MHC class II has a structure composed of two alpha and two β-chain domains which are associated through noncovalent interactions and anchored by two transmembrane segments. The structure of MHC class II produces an antigen binding cleft that acts as an open-ended groove, sufficient to bind longer 13 to 18 amino acid peptides, which are presented to $CD4^+$ T helper cells. The two types of MHC molecule are also differentiated based on the source of the antigen they present.

The two main classes of MHC molecules differ in the pathways by which antigen is taken up by the cell, processed into small peptides, and loaded onto the molecule for presentation to T cells. This distinction originates in whether the antigen is found endogenously within the cell or taken up exogenously by the cell. Antigens found endogenously, indicating that foreign material

has made its way into the cell, are processed through the cytosolic pathway to indicate a possible threat within the cell. Normal cellular components are also presented as a means of sampling for damaged or mutated proteins. In this pathway, proteins within the cell are marked for degradation by ubiquitin tagging. Ubiquitin tagging labels proteins to be processed and broken down into peptide segments by the 26S proteasome complex, or the specialized immunoproteasome (6). Trimming of the peptides of optimal length for presentation is achieved through aminopeptidases such as endoplasmic reticulum aminopeptidase 1 (ERAP). The resulting peptides are then transported across the endoplasmic reticulum (ER) membrane by the transporter associated with antigen processing (TAP) (7). Once transported into the rough ER lumen, peptides assemble into class I MHC with the help of chaperone proteins. Peptide binding to MHC I results in a stabilized complex which can be presented on the cell surface to $CD8^+$ T cells. Without sufficiently strong peptide binding, the complex is not stable enough for cell surface presentation. This pathway addresses antigens found within the cell, indicating a possible cellular infection, cellular damage, or cellular transformation.

Antigens that are taken up exogenously by the cell through a variety of mechanisms are processed through the endocytic pathway. Some cells constantly sample their environment looking for signs of infection or cellular damage. Cells such as dendritic cells and macrophages can sample their environment through pinocytosis and phagocytosis, which internalize potential antigens into intracellular vesicles (8). Internalized antigens in vesicles transit though increasingly acidic compartments in the cell, traveling through early endosomes, late endosomes, and eventually lysosomes. These acid compartments break down the proteins into small peptides with the help of cathepsin proteases for loading onto MHC class II. MHC class II is assembled in the rough ER and stabilized by a trimeric protein called the invariant chain which temporarily holds the place of the antigen peptide (9). The lysosome containing broken down peptides fuses with the vesicle containing the MHC class II complex in the cytosol. Once

fused, the remains of the invariant chain are exchanged for a peptide between 13 and 18 amino acids in length. The exchange is facilitated by HLA-DM, a nonclassical class II MHC molecule. As with MHC class I, class II is stabilized and maintained by bound peptide. After peptide has been bound, MHC class II is presented on the cell surface to $CD4^+$ T helper cells. MHC class II expression and presentation of antigen to $CD4^+$ T helper cells is restricted to a subset of immune cells known as professional antigen-presenting cells, which includes dendritic cells, mononuclear phagocytes, thymic epithelial cells, and B cells. However, these two pathways do not account for exogenous antigen presentation on MHC class I to activate $CD8^+$ T cells.

How do naïve $CD8^+$ T cells become activated by antigen presenting cells such as dendritic cells if the dendritic cell does not have endogenous antigen and therefore can't present antigen on MHC I? Some dendritic cells specialize in presenting antigens taken up by phagocytosis and presenting them on MHC I in a process called cross-presentation (10). For example, some antigens are only partially degraded in the endosome and are transferred to the endogenous pathway for MHC I presentation. This subset of cross-presenting dendritic cells is characterized by CD8a expression in mice and BDCA-3 expression in humans and are important for the anti-tumor immune response and in immunotherapy (11). Licensing of dendritic cells for presentation to $CD8^+$ T cells will be discussed in chapter 3.

1.2. The T cell receptor and T cell subtypes

T cells are activated through their T cell receptor (TCR), a highly variable receptor in the immunoglobulin superfamily composed of either alpha-beta or gamma-delta pairs expressed on the cell surface (12). The portion of the TCR that comes into contact with the peptide being presented by the MHC molecule is composed of three hypervariable loops called the complimentary determining region (CDR) (13). To produce a high level of binding variation, the

TCR and hypervariable CDR are generated through DNA recombination and somatic recombination, which are facilitated by recombination activation genes (RAG) 1 and 2 (14). The genome contains variations of the sections that recombine to form the alpha, beta, gamma, and delta components of the TCR which produce unique alpha-beta, and gamma-delta TCR pairs. There are a certain number of each component segments in the genome that recombine to make the TCR consisting of V, D, J, and C segments. For example, the genes used to make the beta chain in humans are composed 67 V segments, 2 D segments, 14 J segments, and 2 C segments (14) The alpha chain is similarly generated, but does not incorporate a D segment. Combination joining of one of each of these gene segments together allows for a large diversity of possible combinations to be formed. For example, 4600 beta chains alone and 1.4 million alpha-beta pairs combinations are possible. Junctional flexibility, and the addition of P-region and N-region nucleotides are mechanisms that even more diversity to the TCR pool. These mechanisms allow for TCRs with a wide variety of CDRs and potential binding partners to be generated. Subsequently, the large diversity of T cells bearing unique TCRs must go through a selection process to ensure two key properties: 1) The TCR is capable of binding to MHC-antigen complexes; and 2) The TCR does not bind to self-antigens, which could result in autoimmunity and tolerance. These processes are known as positive and negative selection, respectively (15). Around 98% of developing thymocytes do not reach maturity, largely due to a high positive selection fail rate. Culling of 98% of the pre-developed T cell population is achieved through activation of the apoptosis (programmed cell death) pathway in these cells.

During thymic development, T cells express the TCR co-receptors $CD4^+$ and CD8, the expression of which will define what type of T cell they will mature into (16). During development, thymocytes express both $CD4^+$ and CD8 simultaneously. At this stage they are referred to as double positive (DP). Double positive cells that interact with MHC class I will mature into $CD8^+$ T cells and lose $CD4^+$ expression, and double positive cells that interact with

MHC class II will mature into $CD4^+$ T cells and lose CD8 expression. $CD8^+$ T cells, also known as cytotoxic killer cells, are responsible for killing cancer cells and cells that have been infected by virus. They are named $CD8^+$ T cells for their CD8 co-receptor molecule, which binds to MHC I and helps stabilize the TCR:MHC I interaction. $CD4^+$ T cells express the CD4 co-receptor, which serves a similar function in stabilizing the TCR:MHC II interaction. The CD8 co-receptor takes the form of either an alpha-alpha homodimer or alpha-beta heterodimer of single immunoglobulin-fold (Ig-fold) domains, and the monomeric CD4 co-receptor is made of four Ig-fold domains.

$CD8^+$ T cells specialize in cytotoxic killing of targeted cells. Once activated and engaged with a target cell, $CD8^+$ T cells release perforin, a pore forming toxin, that forms pores in the target cell's membrane (17). Granzyme serine proteases released into the target cell through the perforin pores activates caspase 3, which triggers cell death via apoptosis (18). Additional effector functions of $CD8^+$ T cells include production and release of cytokines such as interferon gamma (IFN-gamma; IFN-γ) and tumor necrosis factor alpha (TNF-alpha; TNF-α), which have anti-tumor and anti-viral effects.

In contrast to the cytotoxic effects of $CD8^+$ T cells, $CD4^+$ T helper cells can have varying functions in aiding the immune response (19). The most critical $CD4^+$ T cells involved in the anti-cancer immune response are Th1, Th2, and regulatory T cells. Th1 and Th2 cells differ mainly in the cytokines they produce to direct and control the direction of the immune response, and specifically the anti-cancer response. Th1 T helper cells aid in the anti-tumor response through the cytokines they release (20). Th1 cytokines include many pro-inflammatory molecules that lead to tumor elimination and control, such as IFN-γ, TNF-α, and interleukin-2 (IL-2). IFN-γ is a class II interferon released by Th1 $CD4^+$ T cells, $CD8^+$ T cells, and natural killer (NK) cells. Upon binding to the interferon receptor, IFN-γ exerts several anti-tumor properties, such as increasing MHC class I and class II expression and inducing the immunoproteasome, which specializes in

producing hydrophobic c-termini peptides for better antigen presentation (21). IFN-γ receptor activation also induces production of IgG2a by B cells, which play an important role in antibody-dependent cellular cytotoxicity (ADCC). Additional functions of IFN-γ include producing a positive feedback loop on Th1 $CD4^+$ T cells which in turn produce more IFN-γ, and inhibition of Th2 differentiation by naïve $CD4^+$ T cells. TNF-α is a homotrimeric cytokine that signals through tumor necrosis factor receptors to activate several intracellular signaling pathways that lead to activation of NF-kB and AP-1 pathways in T cells. TNF signaling promotes the inflammatory response, proliferation, survival, and generation of anti-apoptotic factors in immune cells that express receptors for TNF-α (22). TNF signaling can also induce cell death through activation of caspase-8 (23). IL-2 binding to its receptor induces signaling of Janus Kinases (JAK) 1 and 3. JAK1 and JAK3 signaling leads to activation of Signal Transducer and Activator of Transcription (STAT) 5. Activated STAT5 phosphorylates and activates NF-kB, NFAT, and AP-1, which all promote T cell proliferation, differentiation, and support T cell survival. All of these cytokines play an important role in the anti-tumor response and reinforce the Th1 T cell phenotype.

Th2 T helper cells, on the other hand, are generally thought to promote tumor growth and inhibit the anti-tumor immune response, in part due to their cytokine profile (24). Cytokines released by Th2 T helper cells include IL-4, IL-5, and IL-10. IL-4 induces B cell class switching to production of IgE, an antibody isotype that triggers eosinophil and mast cell degranulation for allergic diseases. IL-4 also promotes MHC class II expression and inhibits Th1 cell activity and Th1 cytokine production. IL-5 also plays a role in allergic reactions through eosinophil activation and IgA class switching. IL-10 inhibits the production of Th1 cytokines, downregulates MHC expression, blocks NF-kB signaling, and aids in primary tumor growth (25).

Regulatory $CD4^+$ T helper cells play an inhibitory role in the anti-tumor response through the cytokines they release, and through direct suppression of effector cell function (26). Regulatory $CD4^+$ T helper cells are typically defined by expression of the IL-2 receptor alpha

chain and the transcription factor FoxP3, which programs their behavior. In terms of cytokines, regulatory $CD4^+$ T helper cells produce transforming growth factor beta (TGF-beta; TGF-β), IL-10, and IL-35, all of which can contribute to tumor growth and inhibit the anti-tumor immune response. TGF-β, in particular, plays a wide and varying role in immune cell and tumor function. One consequence of TGF-β is supporting Treg expansion, inhibiting effector T cell function, inhibits antigen presentation, and reduces NK cell cytotoxicity (27). Additionally, TGF-β also plays a role in producing an immune suppressive tumor microenvironment. IL-10 has immune suppressive effects by inhibiting Th1 responses and blocking the NF-kB pathway (28). IL-35 suppresses conventional T cell function and promotes further Treg development (29).

1.3. T cell activation

Three signals are required for full T cell activation and determine the fate of T cell differentiation: 1) TCR stimulation (Signal 1); 2) co-stimulation through CD28 and other corresponding receptors (Signal 2); and 3) cytokine signaling (Signal 3).

1.3.1. TCR activation

Upon interacting with peptide-loaded MHC, the TCR changes conformation to activate a signal transduction pathway once binding has crossed past a sufficient threshold (30). The interaction between TCR and peptide-loaded MHC requires the association of several surface membrane proteins to start the signal transduction pathway (30). The cell will be activated through expression of immediate, early, and late genes. Immediate genes consist primarily of transcription factors such as NF-κB and NFAT, which are expressed in the first 30 minutes following activation. Early genes, such as genes that for interleukin receptors and cytokines, are expressed within 2 hours of activation. Late genes encode for adhesion molecule expression

and are expressed beyond 2 days after activation. Upon TCR engagement to the MHC-peptide complex, the tyrosine kinase LCK is recruited from lipid rafts to phosphorylate the immunoreceptor tyrosine-based activation domains (ITAMs) of the associated CD3 zeta chain domains (31). Once phosphorylated, the CD3 zeta chains provide docking sites for another tyrosine kinase, ZAP-70, to bind and become active. Activated ZAP-70 phosphorylates a number of membrane-associated adaptor molecules such as LAT and SLP-76. Activation of these and other associated molecules results in the activation of three main transcription factors: NF-kB, NFAT, and AP-1. AP-1 activation begins through a kinase signaling cascade that results in phosphorylated Fos and Jun transcription factors in the nucleus. In contrast, the activation of NF-kB and NFAT are both facilitated by phospholipase C gamma (PLC-gamma; PLC-γ).

PLC-γ is activated through phosphorylation by the associated kinases LAT and inducible T cell kinase (ITK). Once activated, PLC-γ hydrolyzes the membrane phospholipid PIP2 to form IP3 and diacylglycerol (DAG). IP3, in turn, activates calcium channels in the ER, and DAG activates protein kinase C (PKC). Calcium activates calcineurin-calmodulin-Ca2+, which de-phosphorylates NFAT, allowing it to transit to the nucleus and activate gene transcription, primarily for cytokines that promote T cell growth. DAG activates PKC which in turn leads to the activation and transit of NF-kB into the nucleus.

The AP-1 transcription factor promotes cell growth, proliferation, and apoptosis (32). NF-kB plays a role in T cell development, activation, differentiation, and survival (33). NFAT plays a role in growth through induction of IL-2 and IL-4 cytokine expression (34). These three main transcription factors, NF-kB, AP-1, and NFAT, which are activated by TCR stimulation, signal the cell to activate and prepare to respond to stimulation from signal 2 (co-stimulation) and signal 3 (cytokines).

The strength of TCR stimulation, meaning the strength to which the TCR binds to the MHC presented peptide, partially dictates the extent to which these and other transcription factors will be expressed. Details of these pathways will be discussed in Chapters 4 and 5. The strength of binding plays a role in NF-kB, AP-1, NFAT, and T cell differentiation transcription factor expression, which determines what functional role the T cell will play. Other factors contribute to expression of these transcription factors such as signal 2 and signal 3.

1.3.2. Co-stimulation

Once a naive T cell has been activated through TCR:peptide:MHC interactions, it must then receive further co-stimulatory signals from antigen presenting cells to ensure that the immune response is warranted. The primary co-stimulatory signal involves two related forms of the B7 immunoglobulin superfamily protein: B7-1 and B7-2. These surface proteins are expressed by antigen presenting cells such as dendritic cells, macrophages, and activated B cells. The ligands for both B7-1 and B7-2 (also known as CD80 and CD86, respectively) are CD28 and CTLA-4 (CD152), which are expressed on T cells (35). However, CD28 and CTLA-4 provide opposing signals to the T cell (36). CD28 is expressed by both naïve and activated T cells and binding to B7 ligands provides a co-stimulatory signal that enhances IL-2 production and NF-kB activation. CTLA-4, on the other hand, is an inhibitory checkpoint receptor induced by TCR engagement that downregulates the activation of the T cell. CD28 and CTLA-4 compete for the available B7 ligands, however CTLA-4 has 20 times higher affinity for B7 ligands compared to CD28 and increases expression with continued CD28 co-stimulation. This provides a regulatory brake for the T cell depending upon how much CD28 and CTLA-4 are expressed and whether they are binding to their B7 ligands.

In addition to CD28-B7 signaling, CD40 and CD40 ligand (CD40L) play an important role in the cross-talk between $CD4^+$ and $CD8^+$ T cells to generate $CD8^+$ T cell memory (37). Mice that lack $CD4^+$ T cells can still generate effector $CD8^+$ T cell responses but are unable to generate $CD8^+$ T cell memory (38). This is likely due to a requirement for both IL-2 and CD40 signaling in $CD8^+$ T cells to generate a memory population through IL-2 autocrine signaling, and CD4 licensing of antigen presenting cells though CD40-CD40L interactions (39). Memory T cells are derived from both naïve T cells following activation and from differentiating effector T cells. Memory T cells are generally long-lived and possess a heightened response to the same antigen to generate a secondary response. The function and role of CD40 in memory T cell generation will be discussed in greater detail in Chapter 3.

T cells that receive sufficient activation signals following TCR:MHC binding but do not receive a sufficient signal 2 in the form of CD28 stimulation enter a state of clonal anergy in which they fail to proliferate and have markedly reduced IL-2 production (40). An example of this is self-reactive T cells that have made it out of thymic development and recognize their reactive antigen in the periphery. These cells will often become anergic because non-inflamed peripheral tissues do not express signal 2 ligands. Anergic cells may be transitioned into regulatory T cells or may be clonally deleted. High levels of CTLA-4 signaling can also induce anergy (41). Similarly, tolerance can form in T cells responding to self-antigens when they don't receive any co-stimulation or inflammatory activation either.

Several other types of co-stimulatory receptors exist that can provide signal 2. These are typically members of the tumor necrosis family of receptors and signal through NF-κB in a TRAF-dependent manner. Examples of other co-stimulatory receptors are OX40 (CD134), 4-1BB (CD137), GITR (CD357), and others that will be discussed in detail in Chapter 3, as will their relevance to cancer immunotherapy. The effects of co-stimulation between a T cell and an APC are often bi-directional with both cell types receiving an activating signal.

In addition to several types of co-stimulatory receptors, there are a variety of checkpoint inhibitory receptors that serve the opposing function of shutting down and blocking an immune response. Inhibitory receptors include CTLA-4 and Programmed Death Receptor-1 (PD-1), which will be discussed in Chapters 2 and 3.

1.3.3. Cytokine stimulation

Cytokines influence the differentiation and function of activated T cells depending upon what concentrations of cytokines are present as well as which of their cognate receptors are expressed on the T cell surface. One of the most critical cytokines for primary T cell responses and survival is IL-2. TCR stimulation and additional co-stimulation triggers proliferation, as well as transcription of IL-2 and the high-affinity IL-2 receptor (CD25). The IL-2 receptor is composed of IL-2Rα, IL-2Rβ, and the common gamma chain subunits. The common gamma chain is a component in several cytokine receptors including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (42). IL-2 autocrine signaling maintains T cell proliferation and survival for several generations resulting in subpopulations of effector and memory cells (43). Memory T cells are antigen experienced and have high reactivity to the same antigen to form the secondary response. Memory cells are long-lived and have a lower threshold of activation compared to naïve non-antigen experienced T cells (44). High dose IL-2 therapy is FDA-approved for the treatment of melanoma and renal cell cancer. However, IL-2 therapy has many severe adverse effects (45). IL-15 therapy may give the same benefits of IL-2 therapy while mitigating the adverse effects.

IL-15 is an important cytokine for the development and survival of memory $CD8^+$ T cells and NK cells. IL-15 binds to CD122, which is composed of the common gamma chain, IL-2Rβ, and IL-15Rα. One of the mechanisms by which IL-15 enhances T and NK cell survival is by inducing expression of Bcl-2, an anti-apoptotic molecule, in addition to sharing signaling

pathways with IL-2. Memory $CD8^+$ T cells also express high levels of the IL-2Rβ to respond to IL-15 stimuli. IL-15 has gained interest as a possible immunotherapy for cancer following the success of IL-2 therapy in an interest to limit the toxicity associated with IL-2 therapy (46, 47).

Other critical cytokines for optimal T cell activation and survival are IL-12 and type I interferon-β (48). Lack of these cytokines, or impaired signaling in response to them, results in reduced expansion and activity in response to stimulation.

Many of these cytokines signal though the JAK-STAT pathway, which translates the initial cytokine signal into transcriptional changes and, ultimately, changes in T cell function (49). Cytokine receptors are typically composed of a heterodimer or homodimer that is brought together by the binding of the cytokine. The cytoplasmic domains of the cytokine receptors contain JAKs which cross-phosphorylate each other when cytokine is bound. The phosphorylated JAKs have SH2 binding domains which bind inactive STATs in the cytoplasm. JAKs phosphorylate STAT proteins, which in turn causes them to dimerize, disassociate from JAKs, and translocate into the nucleus. Once in the nucleus, STAT dimers act as transcription factors to induce the expression of specific genes. There are four members of the JAK family, and six members of the STAT family with varying binding partners and specificities. Which JAKs become activated by specific cytokines helps determine STAT activation and subsequent changes in gene expression.

Signal 3 cytokines are immensely critical for $CD4^+$ T cell differentiation. For example, TGF-β induces $CD4^+$ T cells to express the FoxP3 transcription factor, which drives the suppressive function of Treg cells (50). IL-12 and IFN-γ induce expression of the transcription factor T-bet, which produces Th1 T cells. IL-4 induces GATA3 expression, which produces Th2 T cells. IL-6 plays dual roles in inducing Bcl-6 expressing T follicular helper cells, or when paired with TGF-β, IL-6 induces RORγt-expressing Th17 cells (51). As described previously, each of

these cell populations have their own distinct effector functions and roles in response to pathogens and in the regulation of anti-tumor immunity.

1.4. T cell differentiation and function

Activated T cells follow differing paths of functions, epigenetic profiles, and genetic programs that determine how long they live and how they function. Understanding these subsets and what dictates them is critical to understanding the anti-cancer immune response. Determining which populations are critical to the immune response will help direct therapies to target and expand those populations.

Following activation, $CD4^+$ effector T cells are defined by having high CD25 expression, low CD127 expression (IL-7 receptor), and as well as high levels of IFN-γ (52).These effector cells can then shift into memory cells, which are defined by low CD25 expression and high CD127 expression (54).

Effector $CD8^+$ T cells are defined by the cytotoxic molecules they release. Effector $CD8^+$ T cells first bind their target cell through non-specific cell adhesion molecules, including LFA-1, ICAM, and CD2 (55). If the $CD8^+$ T cell does not recognize its cognate antigen on the target cell then the cells separate. However, if the cognate antigen is recognized on the target cell, then an immunological synapse forms to direct the release of effector molecules. Changes to the cytoskeletal protein talin and tighter binding of LFA-1 to ICAM-1 adhesion molecules form an outer seal surrounding TCR:MHC complexes. Cytoskeletal rearrangement polarizes the T cell towards the target cell. Perforin and granzyme are two of the main cytotoxic molecules released in the immunological synapse (56). Perforin is a type of pore forming molecule that binds to the target cell's membrane to form a passive diffusion pore (57). This allows granzyme to pass across the cell membrane and into the target cell. Perforin has structure similarities to

complement component 9 (C9), which is another pore forming protein that forms dimerized structures. Granzymes are a class of serine proteases that induce apoptosis in their targeted cell. Most granzymes induce cell death through activation of caspase-3, which leads to the release of cytochrome c and formation of the apoptosome (58). Granzyme A and B are the most studied granzymes for their cytotoxic properties, however over 10 distinct granzyme exist with lesser-known roles and functions in inflammation and infection (18).

Memory $CD8^+$ T cells are currently divided into three subgroups consisting of effector memory (Tem), central memory (Tcm), and tissue resident memory (Trm) cells (59). All of these populations share common defining qualities, such as being long-lived, antigen-experienced, having a quick and heightened response to antigen encounter, and persisting in the absence of antigen. Tem and Tcm populations are distinguished by several cell surface markers and by where the T cells reside (60). Tcm cells express CCR7 and CD62L, which facilitates their trafficking to secondary lymphoid organs. Alternatively, Tem cells express more integrins and chemokine receptors to traffic to peripheral inflamed tissues such as tumors and sites of tissue damage. The third subset, Trm cells, reside permanently in a specific tissue without recirculation and are further defined by expression of CD69 and the integrin CD103. These subsets of memory each have their own implications in cancer progression and cancer immunotherapy, which will be discussed further in Chapters 4 and 5.

Beyond the subsets of $CD4^+$ and $CD8^+$ T cell already discussed, senescent, exhausted, and tolerant T cells can also be generated, which can limit the efficacy of cancer therapies. Exhausted and senescent T cells share several qualities defined by an overall defect in cellular function. Both have impaired proliferation and proliferative capacity, exhibit cell cycle arrest, and have impaired cytotoxic functions. Exhausted T cells are further defined by expression of inhibitory markers such as PD-1, CTLA-4, LAG-3, and TIM-3, among others (61). Progression towards T cell exhaustion follows a pattern of reduced IL-2 production, and then increased PD-1

expression, followed by LAG-3 expression. Exhausted T cells also have impaired effector cytokine production, as defined by low expression of IL-2, IFN-γ, and TNF-α (62). Specific transcription factors such as Eomesodermin (Eomes), TOX, and GATA3 play important roles in T cell exhaustion in tumor-infiltrating $CD8^+$ T cells. Eomes also plays an important role in $CD8^+$ T cell memory and cytotoxicity. The role and function of Eomes expression in $CD8^+$ T cells will be discussed in great detail in Chapters 4 and 5. Certain epigenetic modifications such as changes to the pdcd1 locus resulting in high, long-tern PD-1 expression are also observed in exhausted T cells (63, 64). Senescent T cells are found in elderly individuals but can also be found in younger people with chronic viral infections and certain cancers (65). Senescent T cells have shortened telomeres, high levels of cellular stress, possible chemotherapy damage, and DNA damage. Additionally, senescent cells lack key co-stimulatory molecule expression such as CD27 and CD28. However, unlike exhausted T cells, senescent T cells can still express inflammatory cytokines and suppressive cytokines, such as IL-10 and TGF-β (66, 67).

Tolerant T cells are defined by a lack of response against their target antigen. In the case of cancer cells peripheral tolerance is often induced by the tumor microenvironment (68). Tolerance can be induced by several factors and ligands expressed in the TME, such as indoleamine 2,3-dioxygenase (IDO) and arginase, both of which suppress T cell activation and proliferation. High expression of FasL, PD-L1, and B7 ligands in the TME also can induce T cell tolerance by shutting down the T cell response. Reversing induced T cell tolerance in the TME is a research area of great interest for cancer therapy. Treatment with checkpoint blocking antibodies (e.g., anti-PD-1, anti-PD-L1, anti-CTLA-4, etc.) and agonist co-stimulatory antibodies (e.g., anti-OX40, anti-4-1BB, etc.) may help reverse cancer-induced tolerance and are discussed in more detail in Chapter 3.

1.5. T cell trafficking and circulation

After lymphocytes have developed in central lymphoid tissues such as the thymus for T cells, and the bone marrow for B cells, they traffic in the blood to peripheral lymphoid tissues such as lymph nodes (69). Lymph nodes have highly specialized lymphoid structures that divide cell types into specific regions. The organization of these lymphoid structures is governed by chemokine gradients, which influence different cell types depending on what receptors are expressed. Lymph nodes are divided into two main regions consisting of an outer region called the cortex, and an inner region called the medulla. The cortex is composed of B cells, and a special lymph node cell called a follicular dendritic cell (FDC). The deeper region of the cortex known as the paracortex consists of T cells and dendritic cells. The medulla is made up of plasma cells and macrophages. Afferent lymph vessels bring lymph into the lymph node where circulating cells encounter the cortex. Alternatively, lymphocytes can directly enter lymph nodes through high endothelial venules (HEVs), which come from post-capillary veins. HEVs enter into the paracortex of the lymph node. These structures provide the opportunity for B cells and T cells to encounter antigen that has been sampled from the tissues and brought into lymph nodes through afferent lymphatic vesicles and HEVs as part of the lymphatic system. Cells exit the lymph node through efferent lymph vessels back into circulation. Naïve T cells are in a constant state of trafficking in and out of lymph nodes and reentering the circulatory system until they encounter their specific antigen or until they die.

Several cell types play key roles in the lymph node for the purpose of lymphocyte activation by antigen. B cells are attracted to FDCs through CXCR5 chemoattraction to CXCL13 produced by FDCs and other stromal cells (70). B cells that encounter antigen in the lymph node form structures called germinal centers (GCs). In GCs, B cells undergo rapid cell growth, proliferation, and somatic hypermutation to produce a large pool of antibody producing cells known as plasma cells, as well as memory B cells. T cells localize into the T cell zone through

expression of CCR7, which responds to both CCL19 and CCL21, predominately produced by stromal cells (71).

T cells typically spend between 6 and 12 hours in a lymph node before exiting through the efferent lymph vessel back into circulation (69). This process is regulated by sphingosine-1-phosphate (S1P), produced by lymphatic endothelial cells, and its receptor (S1PR), which is expressed by T cells (72). When in circulation, T cells express low levels of S1PR, but upon entry into a lymph node, T cells upregulate S1PR expression. S1PR is internalized and degraded upon S1P ligand binding. Once the amount of S1PR expressed on T cells falls below a certain level due to binding S1P in the LN, the T cells can egress out the efferent lymph vessel. Additionally, CCR7 regulates the duration of time spent in the LN by T cells. T cells that lack CCR7 expression egress out of the lymph much more rapidly than WT T cells (73).

The process by which T cell enter into inflamed tissues, in this instance inflamed cancer tissue, is regulated by both cellular adhesion molecules (CAMs) and chemokines (74). To reach inflamed tissues, cell must past through endothelial cells of blood vessels in a process known as extravasation. There are several families of CAMs including mucins, selectins, integrins, and Ig-superfamily CAMs that aid in extravasation. Inflamed tissues express specific CAMs, which bind to their complementary CAM expressed on T cells. Expression of CAMs is induced and regulated by chemokines released from the inflamed tissue. Leukocyte extravasation follows several steps. First, leukocytes roll along the wall of blood vessels mediated by selective binding to mucin-like CAMs at low affinity. Next, chemokine reception triggers a conformational change in integrin molecules to a higher affinity conformation, allowing for binding and arresting to the endothelium. Cells adhere more firmly though interactions between Ig-superfamily CAMs and mucin-like CAMs. Finally, leukocytes follow a chemokine gradient to transverse across tight endothelial junctions into the underlying inflamed tissue. Critical chemokines for homing to inflamed tissues such as cancer tissues, are CXCL9, CXCL10, and CXCL11, which all bind to

CXCR3 expressed on activated T cells (75). The role of CXCR3 will be discussed in greater detail in Chapters 4 and 5.

Activation changes the circulation patterns of T cells. T cells that have become activated enter a shutdown phase in which they spend several days in secondary lymphoid organs, such as the lymph node, instead of the usual 6-12 hours (76).

1.6. Introduction to Cancer

Cancers, and specifically prostate cancer, will be discussed in detail in Chapter 2, however this section will serve as a brief introduction. Dysfunction in cell growth and division as a result of dysregulated control mechanisms has the potential to result in cancer. Cancers that grow beyond their own tissue border and expand aggressively are malignant. When a portion of the malignant tumor can break away from the primary tumor and travel to different tissues through the circulatory and lymph systems to seed new tumors it is metastatic. Tumors that arise from ectodermal or endodermal tissue are known as carcinomas, which makes up the majority of tumors. Tumors that arise from mesodermal tissue are known as sarcomas and only make up a small frequency of cancers. Leukemias and lymphomas are of hematopoietic origin and make up the rest of cancer incidence.

Cancers can arise due to either environmental factors such UV light, ionizing radiation, and carcinogens, or by genetic factors, such as gene mutations in oncogenes (77). Cancers can also arise from viral genetic material because certain viruses carry oncogenes in their viral genome. Oncogenes, also known as cancer genes, can cause cancer when mutated or expressed at high levels. Before oncogenes are mutated, they are known as proto-oncogenes and have the potential to cause cancer if mutated or over expressed. Most proto-oncogenes play roles in regulation of cell growth and division. Cancer genes can be divided into three

categories; genes that induce cellular proliferation, tumor suppressor genes that suppress cellular proliferation, and genes that regulate apoptosis. Commonly mutated or overexpressed genes that induce cellular proliferation are neu (HER2), src, K-ras, N-ras, jun, fos, and myc (78). Commonly mutated, but not over-expressed, tumor suppressor genes are Rb, and p53. BCL2 is an example of a commonly mutated gene that regulates apoptosis.

The development of cancer is usually a multistep process of acquired mutations as a result of previous mutations dysregulating cell division (79). Several cancers have well defined states of progressive mutations that have a snowball effect on cellular proliferation dysregulation. For example, the progression of colon cancer is well defined through loss of function of several key tumor suppressors such as p53 as the normal epithelial tissue progresses towards metastatic carcinoma (80). This progression is also found in mouse models of cancer as demonstrated by mice with mutations in tumor suppressor genes and oncogenes progressing towards metastatic cancer much more quickly compared to mice with a single mutation in either gene.

Tumors utilize a variety of mechanisms to downregulate immune function and enhance tumor growth. Tumor immune suppression and the tumor microenvironment will be discussed in detail in Chapter 2. Chapter 2 will also place an emphasis on prostate cancer, and will cover tumor specific antigens, models of prostate cancer, the immune intersection with prostate cancer, and immunotherapy treatments of prostate cancer.

This chapter has been a brief introduction to the immune system with an emphasis on the function of T cells and cancer. Cancer antigens are processed and presented by antigen presenting cells, primarily through the endocytic and cytosolic pathways, to be recognized by T cells with TCRs capable of binding to the MHC:peptide complex. The TCR undergoes complex rearrangement during T cell development to generate a highly diverse pool of T cells capable of responding to a variety of antigens. T cells are activated by TCR binding to peptide bound MHC,

co-stimulation, and cytokines to develop into effector, memory, or dysfunctional T cells depending on their environment and stimulatory conditions. The following chapters will examine these topics in greater depth and will focus on immune cancer therapy. They will describe my understanding of the role and functions of T cells in cancer and cancer therapy, as well as my contributions to the field of cancer therapy.

Chapter 2: Immunological complexity of the prostate cancer microenvironment influences the response to immunotherapy

Abstract

Prostate cancer is one of the most common cancers in men and a leading cause of cancer-related death. Recent advances in the treatment of advanced prostate cancer, including the use of more potent and selective inhibitors of the androgen signaling pathway, have provided significant clinical benefit for men with metastatic castration-resistant prostate cancer (mCRPC). However, most patients develop progressive lethal disease, highlighting the need for more effective treatments. One such approach is immunotherapy, which harness the power of the patient's immune system to identify and destroy cancer cells through the activation of cytotoxic $CD8^+$ T cells specific for tumor antigens. Although immunotherapy, particularly checkpoint blockade, can induce significant clinical responses in patients with solid tumors or hematological malignancies, minimal efficacy has been observed in men with mCRPC. In the current review, we discuss our current understanding of the immunological complexity of the immunosuppressive prostate cancer microenvironment, preclinical models of prostate cancer, and recent advances in immunotherapy clinical trials to improve outcomes for men with mCRPC.

2.1. Introduction

Current advances in cancer therapeutics have led to the development of immunotherapies that target the body's own immune system to combat cancer. Advances in immunotherapy have led to the development of checkpoint blockade, with anti-programmed cell death 1 (PD-1) and anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) successfully treating many solid tumors. Checkpoint blockade can increase both the proliferative capacity and cytotoxicity of exhausted $CD8^+$ T cells, leading to disease regression (81, 82). Even though these are very effective treatments, not all cancers have a high response rate to immunotherapy. These treatments depend on the presence of tumor-specific $CD8^+$ T cells, which can respond to checkpoint blockade. Therefore, understanding basic tumor immunology may help predict patient survival and response to checkpoint blockade.

2.2. $CD8^+$ T cells in cancer

$CD8^+$ T cells are a crucial part of the immune response against tumors. Many studies have shown that $CD8^+$ T cells found within tumors acquire an exhausted phenotype (83). These exhausted cells lose their ability to proliferate, have increased expression of inhibitory receptors, and have lower effector function, including reduced interferon gamma (IFN-γ) production, granzyme B expression, and/or cytolytic activity (84). Even though these cells have varying degrees of functionality, $CD8^+$ T cell infiltration into tumors predicts disease progression in melanoma, breast cancer, head and neck cancer, ovarian cancer, non-small cell lung cancer, esophageal cancer, small cell lung cancer, hepatocellular carcinoma, and renal cell carcinoma (85-87). Recently, a group developed an "immunoscore" for tumors based on T cell infiltration by looking at $CD8^+$ and $CD45RO^+$ T cells in both the tumor core and invasive margin. Higher immunoscores indicate more $CD8^+$ T cell infiltration, where patients with higher scores have better disease-free survival and overall survival compared to patients with low immunoscores (88, 89). The number of $CD8^+$ T cells in the tumor can also predict response to PD-1 blockade,

making it an important biomarker for both survival and response to current immunotherapies (90). Understanding the mechanism behind the diversity of $CD8^+$ T cell infiltration in different cancers is crucial to improving current immunotherapies. Additionally, recent studies have changed our understanding of $CD8^+$ T cell differentiation and exhaustion in the context of chronic infections and cancer. Here, we will present evidence about the factors controlling the magnitude of an immune response in viral infections, discuss how this response differs in cancer, and why these responses are so variable.

2.3. Immune response to viruses

The primary role of the immune system is to protect against infections. To achieve this, the immune system possesses incredible intricacy and organization of many cell types that co-ordinate responses against perceived threats. In cancer, the immune system fails to function and organize a response in the same way. By understanding the successful immune response to a virus and applying that knowledge to tumor immunology we can better understand how the system fails to eradicate tumors.

Danger sensing

A prototypical viral infection begins when a virus enters the body and infects permissive cells. Infected cells sense viral infections through pattern recognition receptors (PRRs) such as Toll-like receptors (TLR), Nod-like receptors, retinoic acid-inducible gene I (RIG-I)-like receptors, and the stimulator of interferon genes (STING) pathway, which trigger the release of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor (TNF)-α (Fig. 1A) (91, 92). This pro-inflammatory reaction recruits immune cells including antigen presenting cells (APCs) that aid in clearing the infection, phagocytosis of antigen, and migration through the lymphatics to activate T and B cells (Fig. 1B). This is a crucial step that connects

the innate immune system to the adaptive immune system, thus allowing immune-mediated clearance of the infection.

Antigen presentation

Dendritic cells (DCs) are a critical part of the immune response. When DCs are activated by pathogen-associated molecular patterns (PAMPs) and take up antigen, they process it into peptides that are 8-10 amino acids in length and load them onto the major histocompatibility complex (MHC) for presentation to T cells (93). DCs traffic to secondary and tertiary lymphoid organs through the lymphatics via expression of chemokine receptors (e.g., CC chemokine receptor-7; CCR7), wherein they interact with cognate antigen-specific T cells to facilitate T cell activation. In addition to T cell receptor (TCR) recognition of the MHC-peptide complex, T cells also need co-stimulation through the co-stimulatory receptor, CD28, which binds its ligands CD80/86 expressed by APCs (94). Signaling through TCR and CD28 activates T cells and the presence of pro-inflammatory cytokines produced by DCs, such as IL-12 and type I IFN, further promote T cell activation and proliferation (95). Once a T cell is activated, it upregulates effector molecules, chemokine receptors, and proliferates to produce a population of more antigen-specific $CD8^+$ T cells capable of combating the infection.

Lymphoid organization in viral infections

Only naive antigen-specific $CD8^+$ T cells are activated by DCs presenting cognate MHC-peptides, and for this to happen there needs to be an organization of $CD8^+$ T cells and DCs, which typically occurs in draining lymph nodes (dLN) or within tertiary lymphoid structures. The organization of immune cells is crucial to the anti-tumor response since conventional DCs (cDC1s, which are $CD8\alpha^+XCR1^+$) and plasmacytoid DCs (pDCs) help orchestrate $CD8^+$ T cell activation and response to viral antigens. The expression of XCL1 by $CD8^+$ T cells recruits cDC1s, while the production of type I IFN by pDCs acts on DCs and $CD8^+$ T cells to further

activation (96). Thus, for optimal antigen-specific CD8$^+$ T cell activation, there must be collaboration of different DC subsets with each other and CD8$^+$ T cells. This level of organization of DCs and CD8$^+$ T cells is crucial for the immune response to viral infections.

CD8$^+$ T cell effector function

Once activated CD8$^+$ T cells upregulate chemokine receptors such as CXC chemokine receptor-3 (CXCR3), they can leave lymphoid organs and migrate to the site of infection and proliferate to produce many antigen-specific CD8$^+$ T cells (Fig. 1C). Activated CD8$^+$ T cells also upregulate effector molecules such as granzyme B, perforin, and death ligands such as Fas and TNF-related apoptosis inducing ligand (TRAIL). CD8$^+$ T cells selectively kill cells that express the MHC-peptide complex for which they are specific, leaving uninfected cells intact while targeting only infected cells. When activated CD8$^+$ T cells encounter an infected and antigen-specific cell, the engagement of its TCR leads to the release of cytotoxic granules. Perforin polymerizes to form a pore on the target cell, allowing different granzymes to enter the target cell. Granzyme B is a protease that can activate caspase-3 and cleave Bid. This induces an intrinsic apoptotic pathway wherein the activation of caspase-3 leads to DNA fragmentation and eventually apoptosis. The cleaved truncated Bid also interacts with Bax and Bad, leading to the release of cytochrome C from the mitochondria, which also leads to an intrinsic apoptosis. Overall, the selective killing of infected cells by cytotoxic CD8$^+$ T cells prevents unnecessary inflammation and damage as the apoptotic cells are phagocytosed and cleared.

Another pathway by which CD8$^+$ T cells can induce apoptosis is through the Fas pathway. Activated CD8$^+$ T cells can express Fas ligand (FasL), which binds to Fas on the target cell, leading to the trimerization of Fas and caspase-9 activation. Similar to apoptosis through caspase 3, the activation of caspase-9 leads to DNA degradation and apoptosis (97). The primary function of activated CD8$^+$ T cells is to induce apoptosis of antigen-specific infected cells or transformed tumor cells that express tumor-specific antigens. In a viral infection when

the antigen is cleared, some activated CD8⁺ T cells survive and become memory CD8⁺ T cells. This is an important function of CD8⁺ T cells, since having long-lived memory to a pathogen leads to fast recall responses upon reinfection. In the context of progressively growing tumors, CD8⁺ T cells do not become memory cells and instead gain an exhausted phenotype, upregulate expression of numerous inhibitory receptors, and lose their ability to proliferate.

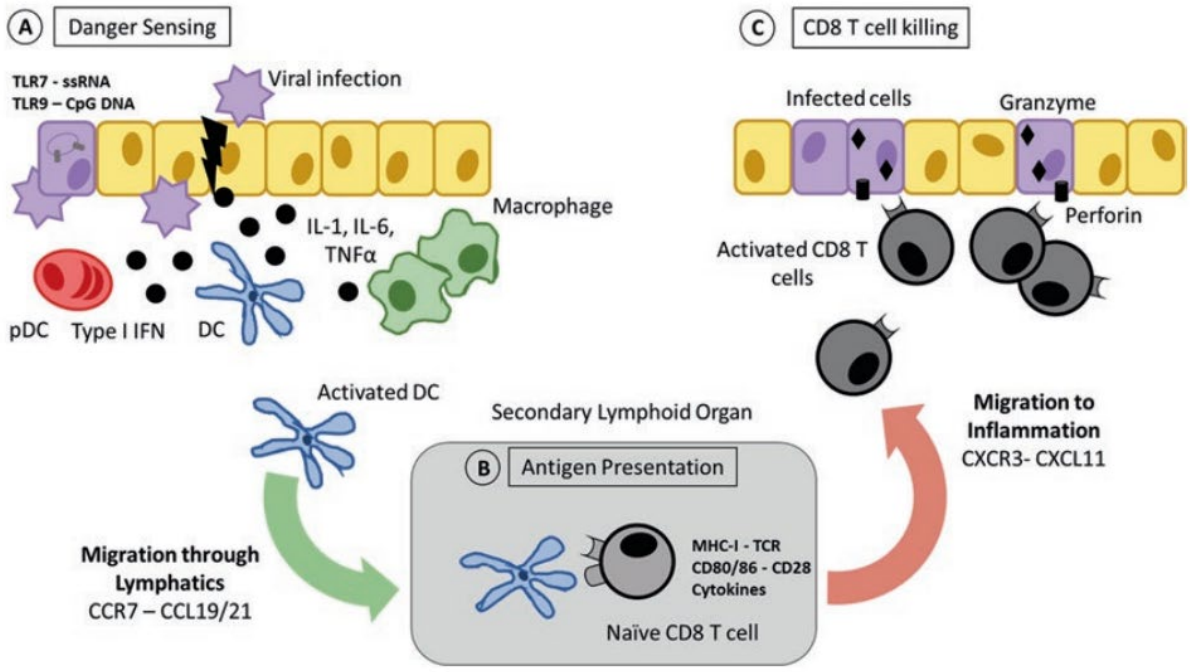


Fig. 1. Immune response to a viral infection. (a) Once a virus has infected cells the viral PAMPs induce the expression of pro-inflammatory cytokines, which can recruit macrophages and DCs. pDCs are activated through TLR7/9 and produce large quantities of type I IFN. The combination of viral PAMPs and pro-inflammatory cytokines leads to the activation of DCs and their migration to secondary lymphoid organs. (b) Once an activated APC finds an antigen-specific CD8⁺ T cell it can activate the CD8⁺ T cells through MHCpeptide interaction with TCR, co-stimulation with CD80/86 interacting with CD28, and through the production of IL-12. (c) Activated CD8⁺ T cells upregulate chemokine receptors, effector molecules, and proliferate. Once the activated CD8⁺ T cells leave the lymphoid organs and migrate to the site of infection, they can exert effector function by causing apoptosis of infected cells through perforin and granzyme B. Figure from Emerson et. al. Adv Exp Med Biol, PMID: 31900908.

2.4. The immune response to cancer

During viral infections there are specific phases of the immune response: danger sensing, antigen presentation and then clearance of the infection. The immune response to

cancer differs in how the immune system is activated and how it responds to the tumor antigens, due to the chronic nature of tumor development as well as differences in the types of antigens to which the immune system responds. Increased T cell infiltration in tumors is associated with improved survival as well as better response to checkpoint blockade (98-100). To generate activated tumor-specific $CD8^+$ T cells, there must be APC activation and antigen presentation. How the $CD8^+$ T cells are supported within the tumor microenvironment (TME) and which cells promote their infiltration is important to understand in the context of clinical outcomes and future immunotherapies.

Danger sensing in tumors

In a viral infection there are numerous pro-inflammatory cytokines and PAMPs that activate different immune cells and recruit APCs to the site of infection. In tumors, the recruitment and activation of immune cells often does not occur in the same pro-inflammatory environment. In tumors, the immune system relies on danger-associated molecular patterns (DAMPs) such as extracellular ATP, heat shock proteins, hydrophobic aggregates, reactive oxygen species, uric acid crystals, and nucleic acids, which signal that there has been tissue damage and cell death, thereby eliciting an immune response (101). The DAMPs released by necrotic cells can activate DCs and induce T cell proliferation, whereas apoptotic cell death does not induce the same type of DC activation and T cell activation (102). This has been shown in both mouse and human DCs that are stimulated and activated by necrotic syngeneic cells or necrotic tumor cells, from melanoma, kidney adenocarcinoma, and thymoma cell lines. DCs activated by necrotic tumor cells are capable of activating antigen-specific $CD8^+$ T cells (103). Necrotic cells derived from prostate cancer cell lines, such as LNCaP and PC3, are also capable of activating DCs, which can then present antigen and activate $CD8^+$ T cells. Studies with DCs from healthy donors and from stage IV prostate cancer patients, demonstrated that there is no intrinsic defect in DCs from prostate cancer patients (104). This is a crucial

component of generating a productive immune response against tumors, as is the composition of APCs within the TME capable of presenting tumor antigens to tumor-specific $CD8^+$ T cells.

2.5. Antigen presentation of tumor antigens

APCs are the interface between antigen and T cell activation. Understanding how this population of cells operates in cancer is key to understanding the initial generation of the anti-tumor T cell response.

DCs in cancer

Several DC subsets have been classified based upon their phenotype and function in mice and humans. DCs can be broadly classified by the high expression of CD11c and MHC class II (MHC II). One crucial subset of DCs for $CD8^+$ T cell activation is the cross-presenting DC, which refers to the processing and presentation of exogenous antigens on MHC class I molecules (105). These cells are of interest in the context of cancer immunology since most tumor antigens are exogenous proteins and must be presented on MHC I in order to activate tumor-specific $CD8^+$ T cells. The cross-presenting DC (cDC1) subset has been thoroughly characterized in mice. These cells are defined by $CD8\alpha$ and XCR1 expression and show increased antigen uptake, processing and presentation on MHC I (106). This DC subset has also been characterized in human tissue, distinguished by the expression of CD141, and has been seen in the lung, liver, skin and blood compartments (107). This DC subset is indispensable in the activation of $CD8^+$ T cells in infection and tumor progression as cDC1-deficient mice do not control influenza infection or immunogenic tumors in a T cell-dependent manner (108, 109). Overall, this is a key DC subset in the immune response that specializes in the activation of viral and tumor-specific $CD8^+$ T cells. Another major DC subset (cDC2) in mice is characterized by the expression of CD11b on $CD11c^+$ MHC II^+ DCs. $CD11b^+$ DC (mice) and CD1c (humans) are less efficient at cross- presentation of exogenous antigens and therefore

thought to mainly activate $CD4^+$ T cells through MHC II (110). Even though there are many similarities between DC subsets in mice and humans the phenotype of the cells is not always translatable, which is an important consideration when analyzing and comparing DCs from mouse and human tissues.

The last major subset of DCs are pDCs, which are a crucial part of the antiviral immune response. In mouse and humans, this highly specialized subset of cells produces the largest quantities of type I IFN early after viral infection. These cells circulate in the periphery and express high levels of TLR7 and TLR9, which activate the pDCs and induce the expression of pro-inflammatory cytokines. pDCs are a crucial bridge between the innate and adaptive immune system during viral infections as they promote the activation of DCs and T cells. Although some studies have shown pDC infiltration in breast cancer predicts poorer overall survival, more studies are needed to determine how pDCs influence the $CD8^+$ T cell response to tumors (111). The activation of pDCs is a rapid response to viral infection that may not occur during tumor growth, and this lack of pDC activation may limit the generation of potent anti-tumor $CD8^+$ T cells.

There have been numerous studies showing the prognostic power of DC infiltration in tumors. For example, one study used data from the cancer genome atlas (TCGA) and analyzed CD103/141-associated genes to determine cross-presenting DC infiltration (cDC1). The ratio of $CD103/141^+$ signature genes to genes not associated with CD103/141 DCs acts as a prognostic marker that predicts overall survival in numerous cancer types including breast cancer, head-neck squamous cell carcinoma, and lung adenocarcinoma (110, 112). This shows how the extent of DC infiltration alone can have prognostic power over a wide variety of cancers. Other studies using TCGA data have also determined that the CD141 gene signature correlated with $CD8^+$ transcript levels (108).This demonstrates the importance of DCs in the TME to promote and support $CD8^+$ T cell infiltration and how DC and $CD8^+$ T cell infiltration can be used to predict survival in patients with cancer.

Studies have also shown in human melanoma tumors the crucial role of CD141+ DCs expressing CCR7, which allows for the migration into lymph nodes to present tumor antigens to tumor-specific $CD8^+$ T cells. The tumors containing higher levels of CCR7 transcripts correlated with more $CD3^+$ T cell infiltration and better survival (112). DCs need to be able to bring tumor antigens into the lymphoid organs to activate tumor-specific $CD8^+$ T cells more efficiently due to the higher concentration of $CD8^+$ T cells and DCs in lymphoid tissues. In prostate cancer after androgen ablative therapy, there is an increase in DC and macrophage infiltration (113), but their phenotype before and after treatment remains poorly understood. Other studies, which also looked at DCs in the blood of prostate cancer patients before and after vaccination (GVAX) and checkpoint blockade (αCTLA-4; ipilimumab) treatment, revealed that increased $CD1c^+$ DCs and $CD11c^+CD14^{lo}$ DCs predicted better survival with the treatment (114). Collectively, these studies show that the presence of DCs, especially $CD141^+$ DCs in tumors, correlates with more T cell infiltration and better prognosis and survival in many tumor types. Understanding why DCs infiltrate certain tumors may help us understand the differences in $CD8^+$ T cell infiltration.

Recent studies have also shown that murine DCs present within the TME are less efficient at presenting antigen to and activating $CD8^+$ T cells. These DCs induce lower $CD8^+$ T cell proliferation, express lower levels of co-stimulatory molecules and produce less IL-12 (115). Additional studies showed an inhibitory effect of IL-10-secreting macrophages in the TME, which suppresses DC activation and IL-12 production (116, 117). Together, these studies show that it is important to not only have good DC infiltration in the tumor, but also to have functional DCs capable of activating T cells and promoting a pro-inflammatory immune response.

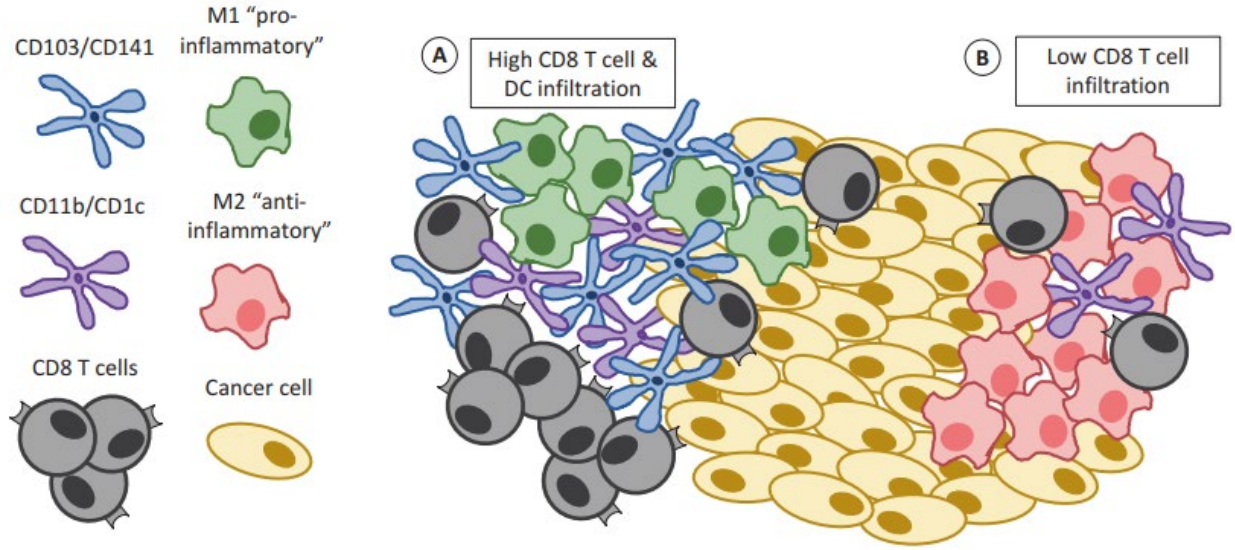


Fig. 2. Differences in immune cell composition in high and low infiltrated tumor. (a) Highly immune-infiltrated tumor with clusters of CD103+/CD141+ and CD11b+/ CD1c+ DCs, M1-skewed macrophages and $CD8^+$ T cells. These tumors have more pro-inflammatory APCs that can promote $CD8^+$ T cell infiltration and effector function. (b) Poorly $CD8^+$ T cell infiltrated tumors with more M2-skewed tumor-associated macrophages, which are immunosuppressive and prevent further $CD8^+$ T cell activation. Figure from Emerson et. al. Adv Exp Med Biol, PMID: 31900908.

Lymphoid organization in tumors

Recent work has shown that in certain instances tertiary lymphoid structures (TLS), which contain $CD8^+$ T cells, DCs, follicular DCs and high endothelial venules, can form near the tumor (118). Tumors containing a TLS were associated with higher T cell infiltration and improved disease-free survival in both breast and colorectal cancer (119, 120). In viral infections, the organization of APCs and $CD8^+$ T cells is crucial for activating and promoting effector differentiation of antigen-specific $CD8^+$ T cells. Interestingly, in non-small cell lung cancer, TLS-associated mature DCs correlate with $CD8^+$ T cell infiltration and improved survival (121, 122). This demonstrates the power of having an organized structure that supports T cells and DCs near the tumor that is comparable to the organization of lymphoid tissues during a viral infection. In prostate cancer, some immune structures contain T regulatory cells and other

immunosuppressive cells (123). In prostate cancer, TLS comprised of more pro-inflammatory Type 1 helper (Th1) and $CD8^+$ T cells are associated with improved tumor regression (124). Understanding how these TLS form and how the composition and organization can affect patient outcomes is an important step towards developing novel therapeutics for patients that are refractory to immunotherapies and may have immunologically "cold" tumors, which are poorly infiltrated with $CD8^+$ T cells and/or DCs.

Macrophages in cancer

Macrophages, which are APCs that are a critical component of the TME, are capable of presenting tumor antigens to T cells, phagocytosing apoptotic cells and secreting various cytokines (125). Due to the plasticity of these cells, they can acquire different phenotypes based on the immune environment that influences them. A classically activated macrophage acquires an M1 pro-inflammatory phenotype, capable of secreting TNF, IL-1, IL-6, IL-8 to promote the activation of T cells (126). M1-skewed macrophages can also secrete reactive oxygen species (ROS) and chemokines to attract more pro-inflammatory immune cells to mediate the destruction of pathogens and tumor cells. The other phenotype of macrophages, that is more common in the TME, is a "wound repair" alternatively-activated M2 macrophage. These are a necessary part of the immune response for tissue homeostasis as well as wound repair but are thought to promote tumorigenesis within the TME. M2 macrophages can secrete anti-inflammatory cytokines such as IL-10 and TGF-β, and act as poor APCs. M2-skewed macrophages can also significantly hinder the $CD8^+$ T cell anti-tumor response through arginase-1 and ROS secretion, which limits $CD8^+$ T cell activation. They can also secrete growth factors to promote tumor growth and metastasis, such as epidermal growth factor (EGF), as well as suppress T cell anti-tumor activity (116, 127). Macrophages can also express programmed cell death ligands 1/2 (PD-L1/2) and further hinder $CD8^+$ T cell anti-tumor activity. Tumor-infiltrating macrophages with an M2 phenotype can express PD-1 and respond to PD-1-

blockade leading to reduced tumor burden in mouse models (128). The location of macrophages in the TME can alter $CD8^+$ T cell responses, for example when macrophages are in the stroma of lung squamous-cell carcinomas they prevent the interaction of $CD8^+$ T cells with DCs that are present in the TME (86). Overall, macrophages are a crucial component of the TME and can directly impact the tumor-specific $CD8^+$ T cell response.

When macrophages are incubated with conditioned media from prostate cancer cells, they are skewed towards an M2 phenotype and produce IL-10 (127). Conditioned media from prostate cancer cells can also re-program M1 macrophages into an M2-like phenotype, demonstrating how the TME can alter the phenotype of immune cells. Especially in tumors that are not highly infiltrated by $CD8^+$ T cells and pro-inflammatory APCs, it is important to understand how the TME can skew the immune environment to promote, rather than inhibit, tumor growth. A higher macrophage density leads to poor prognosis in lung, hepatocellular carcinoma, and renal cell carcinomas (129, 130). Macrophages are very versatile APCs that can promote immune responses as well as the growth and vascularization of tumors. Understanding what role macrophages play in the TME and how they can directly impact $CD8^+$ T cells as well as how they can be used to predict clinical outcomes is crucial for understanding the highly complex TME.

The TME also contains cells broadly classified as myeloid-derived suppressor cells (MDSCs), which can promote tumor growth and down-regulate $CD8^+$ T cell activity. Currently there are two subsets that have been classified, monocytic MDSCs (M-MDSCs, which originate from monocytes) and polymorphonuclear MDSCs (PMN-MDSCs, arising from granulocytic PMN precursors) (131). In prostate cancer, mostly PMN-MDSCs have been identified and suggested to promote castrate-resistant prostate cancer (CRPC). M-MDSCs share many markers with

monocytes and macrophages, making it difficult to distinguish between these cells. Similarly, PMN-MDSCs share many markers with other granulocytes and PMNs.

Understanding how the various APC populations influence $CD8^+$ T cell responses is crucial to determine how to improve immunotherapy in cancers that have not responded to current therapies. In prostate tumors there is a large population of M2 macrophages, which could be the reason for less $CD8^+$ T cell infiltration and reduced activation. Historically, prostate cancer has not responded to T cell-focused therapies. However, when DCs pulsed with tumor antigen are given as a therapeutic intervention, such as with Sipuleucel-T treatment, a form of prostate cancer therapy, $CD8^+$ T cell infiltration increased and clinical outcomes were modestly improved (132, 133). Therapies that focus on enhancing immune infiltration into prostate tumors are a current area of interest and could potentially be combined with T cell-focused therapies in order to achieve better clinical outcomes.

2.5. $CD8^+$ T cells in cancer

The extent of DC infiltration correlates with improved $CD8^+$ T cell infiltration and better overall survival. Even though DCs can predict survival, $CD8^+$ T cells are the effector cells capable of destroying tumors. The connection between DCs and $CD8^+$ T cells is crucial in understanding the immune response to cancer. $CD8^+$ T cells found within tumors typically have an exhausted phenotype, with increased inhibitory receptor expression, like PD-1 or T cell immunoglobulin and mucin domain-containing protein-3 (Tim-3), along with decreased proliferation and effector function (83). This does not explain the necessity of DCs within the TME, but the new stem-like model of $CD8^+$ T cell exhaustion demonstrates the need for a DC-rich nice to support stem-like $CD8^+$ T cells.

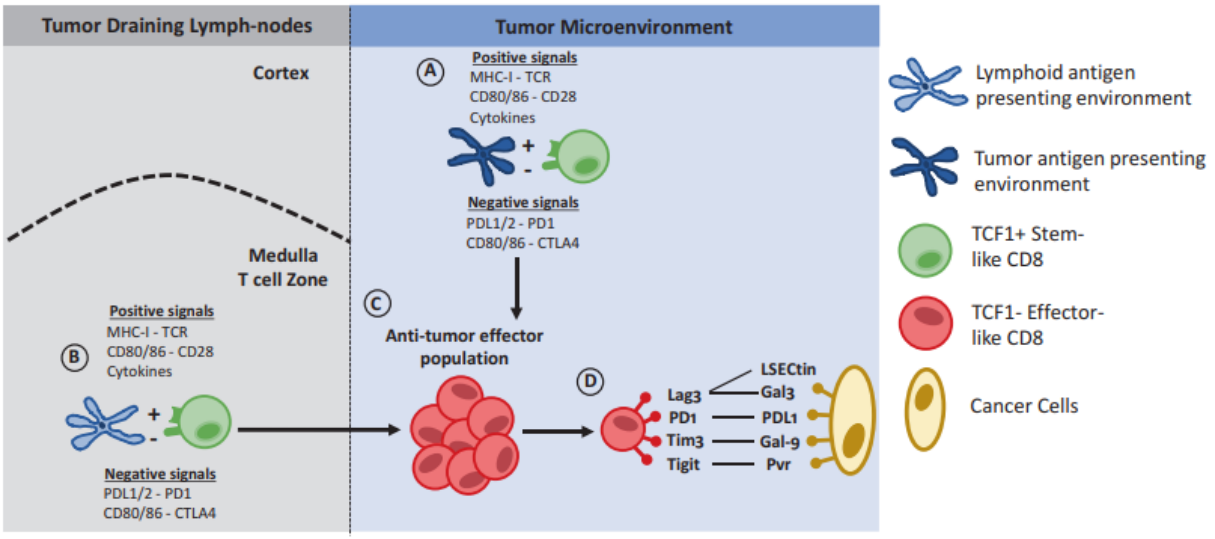


Fig. 3. Generation of an anti-tumor effector response against cancer. (a) Stem-like cells reside within tumors and their presence requires an antigen presenting niche for support: These cells receive their activating and inhibitory signals from a range of APCs including DCs, M1 and M2 macrophages, TAMs, and/or inflammatory monocytes. The rate of generation is an aggregate of many complex positive and negative signals. For example, PD-L1 blockade can increase the rate of effector generation from stemlike cells. Failure to generate an effector population due to lack of the correct signaling results in a tumor with low $CD8^+$ T cell numbers, but no terminally exhausted cells. (b) It is unclear if a stem-like $CD8^+$ T cell in lymphoid tissue gives rise to effector cells that migrate to areas of inflammation to kill target cells. One hypothesis is that these cells will be present in regions where DCs are most dense. (c) The total yield from the stem-like cell is an aggregate of what is produced by cells in the lymph nodes and tumor. The number of cells generated by this process may be critical for controlling tumor growth. High numbers of terminally exhausted $CD8^+$ T cells implies the activation process of the stem-like cell is continuing effectively. (d) The anti-cancer effect caused by $CD8^+$ T cells is proportional to the number of effector $CD8^+$ T cells, the quality of these cells, and the negative signals sent back from tumor cells expressing inhibitory ligands. Inhibitory molecules including Tim-3, Lag-3, and others probably have minimal effect on the number of $CD8^+$ T cells generated, but may have critical effects on the ability of the effector population to kill tumor cells. Figure from Emerson et. al. Adv Exp Med Biol, PMID: 31900908.

LCMV model of $CD8^+$ T cell exhaustion

The lymphocytic choriomeningitis virus (LCMV) model has been used to discover and understand many immunological phenomena, spanning from $CD8^+$ T cell memory to exhaustion. Two strains of LCMV allow for the study of an acute versus chronic viral infection. LCMV Armstrong is an acute viral infection that is cleared via $CD8^+$ T cells and elicits a strong

memory $CD8^+$ T cell response. The LCMV clone 13 strain models a chronic infection; by depleting $CD4^+$ T cells and then infecting the mice with clone 13, the infection becomes truly chronic and is not cleared by the immune system (134, 135). The early model of LCMV-induced T cell exhaustion described a gradual increase in inhibitory receptor expression and loss of effector function. $CD8^+$ T cells first lose their ability to produce IL-2 and their cytotoxic function, followed by the loss of proliferation and ability to produce IFN-γ and TNF-α (84, 136). PD-L1 blockade was shown to rescue antigen-specific exhausted $CD8^+$ T cells in chronic LCMV clone 13 infected mice, thus restoring their ability to proliferate and produce IFN-γ (82). This was the first example of PD-1 blockade restoring $CD8^+$ T cell functionality in a chronic antigen setting.

Chronically stimulated $CD8^+$ T cells can have the canonical exhausted phenotype. They have up-regulated numerous inhibitory receptors, such as PD-1, CTLA-4, lymphocyte activation gene 3 (Lag-3), and Tim-3. They also have a diminished ability to proliferate and produce effector cytokines (137, 138). This shows that tumor-infiltrating $CD8^+$ T cells have a comparable phenotype to the antigen-specific $CD8^+$ T cells found in the chronic viral model of LCMV clone 13 infected mice. While this model of T cell exhaustion offers an understanding of why T cells in tumors have lost functionality, it does not explain why some tumors have very low T cell numbers, or more importantly why some patients do not respond to checkpoint therapy.

Stem-like model of $CD8^+$ T cell exhaustion

Recent work in the field of $CD8^+$ T cell exhaustion has resulted in a revised model of how T cell exhaustion occurs (139). The new working model of $CD8^+$ T cell exhaustion has implications in how we think about $CD8^+$ T cell exhaustion as well as modern immunotherapy approaches. This work described two populations of $CD8^+$ T cells in a chronic viral infection that express PD-1. One is a stem-like $CD8^+$ T cell that expresses CXCR5, T cell factor 1 (TCF1),

and has higher expression of CD28, while the other is a terminally-differentiated Tim-3$^+$ CD8$^+$ T cell. The stem-like CD8$^+$ T cells reside in a DC-rich location of the spleen (Fig. 3B), while the Tim-3+ subset localizes to sites of infection and is not restricted to lymphoid tissues and acts as effectors, expressing granzyme B and IFN-γ. The CXCR5$^+$ TCF1$^+$ stem-like CD8$^+$ T cell subset can self-renew and give rise to Tim-3$^+$ effector-like cells (Fig. 3C). During viral infection, PD-1 blockade promotes CXCR5$^+$TCF1$^+$ stem-like cell proliferation and differentiation into a large population of antigen-specific effector-like CD8$^+$ T cells capable of killing infected cells. PD-1 blockade also affected Tim-3$^+$ CD8$^+$ T cells by blocking negative signaling and increasing their effector functions at the site of infection (139). This new model of CD8$^+$ T cell exhaustion can help us understand how the immune system responds to chronic antigen as well as how PD-1 blockade works within this model.

Since this model was described, several groups have observed a stem-like model of T cell exhaustion in numerous cancers and murine tumor models. Populations of TCF1$^+$ and terminally-differentiated Tim-3$^+$ CD8$^+$ T cells have been described in MC38 sarcoma, B16 melanoma, Transgenic adenocarcinoma of the mouse prostate (TRAMP)-C1 prostate cancer, Lewis Lung Carcinoma, and CT26 colon tumors (140, 141). These cells closely resemble the originally identified CD8$^+$ T cells from chronic viral infection. TCF1$^+$ CD8$^+$ T cells have higher expression of IL-7R, CCR7 and CD62L, like CXCR5$^+$ cells. The effector TCF1$^-$Tim-3$^+$ CD8$^+$ T cells were also found to express higher levels of effector molecules such as IFN-γ and granzyme B, which parallels their original description. An important part of the stem-like model of CD8$^+$ T cell exhaustion is the ability of TCF1$^+$ CD8$^+$ T cells to proliferate and give rise to Tim-3$^+$ effector cells. While Tim-3$^+$ CD8$^+$ T cells lack the ability to proliferate, they do express more effector molecules, such as IFNγ (139). These studies demonstrate that the stem-like model of CD8$^+$ T cell exhaustion can be applied to numerous mouse tumor models.

The stem-like model of $CD8^+$ T cell exhaustion is also translatable to human cancer. Many recent studies have shown that in different human cancers there are similar stem-like and effector $CD8^+$ T cell populations. In lung cancer patients, a $CXCR5^+$ $CD8^+$ T cell population, and a $CXCR5^-Tim\text{-}3^+$ population was found by using high dimensional mass cytometry (CyTOF) analysis. These two subsets closely resemble what has been described in mouse models, wherein $CXCR5^+$ $CD8^+$ T cells retain proliferative capacity and give rise to $CXCR5^-Tim\text{-}3^+$ effector $CD8^+$ T cells (142). In a single-cell RNA sequencing study of melanoma, a transitional and dysfunctional $CD8^+$ T cell subset was found. Transitional $CD8^+$ T cells present within tumors retain TCF1 expression, while dysfunctional $CD8^+$ T cells have higher expression of inhibitory receptors such as PD-1 and Lag-3 (143). There needs to be both a functional $TCF1^+$ stem-like $CD8^+$ T cell and it must produce Tim-3+ effector-like $CD8^+$ T cells in order to have a productive $CD8^+$ T cell response to the tumor.

The environment in which these $CD8^+$ T cell stem-cells are maintained is a crucial aspect of the stem-model of exhaustion, especially when considering that originally the stem-like $TCF1^+$ $CD8^+$ T cells were discovered in lymphoid tissues surrounded by DCs in a chronic viral infection. The organization of DCs and $CD8^+$ T cells is crucial for the activation of $CD8^+$ T cells in viral infections. These discoveries have led to an increasing interest in the immune environment that best supports stem-like $CD8^+$ T cells and induces their proliferation and differentiation, especially within the TME. Since the amount of $CD8^+$ T cells within tumors has clear positive prognostic power, it is important to understand the immune niche supporting $CD8^+$ T cell infiltration into tumors.

Overall, the immune system is crucial in the response to cancer as $CD8^+$ T cell infiltration into tumors can predict patient outcomes as well as response to immunotherapy. Understanding the stem-like model of $CD8^+$ T cell exhaustion has led to a better understanding of the $CD8^+$ T cell response to PD-1 blockade, which increases the differentiation of $Tim\text{-}3^+$ $CD8^+$ T cells as well as their effector function. This model of $CD8^+$ T cell exhaustion has led to

an interest in other immune cells within the TME that can support stem-like $CD8^+$ T cells, such as DCs. $CD141^+$ DC (cDC1) infiltration has been shown to predict better outcomes in patients as well as correlate with $CD8^+$ T cell infiltration (144). Tumors that cannot support an environment with DCs and T cells do not have high $CD8^+$ T cell infiltration and therefore have worse outcomes and respond poorly to checkpoint blockade. The future of immunotherapy likely needs to focus on rebuilding the tumor immune microenvironment to support DCs and stem-like $CD8^+$ T cells and facilitate their proliferation and differentiation into effector-like $CD8^+$ T cells capable of destroying tumor cells. The exhausted T cell phenotype and the transcription factor TOX will be further discussed in Chapter 5.

2.6. Prostate Cancer Tumor-Associated Antigens

Inducing an immune response against cancer is critical for producing an effective and long-lasting response. Integral to the anti-tumor immune response is the presentation of tumor-associated antigens (TAA) on APCs to $CD8^+$ T cells for priming of tumor-specific cytotoxic T cells. Identifying tumor antigens present in prostate cancer and understanding their role in inducing an adaptive immune response is essential for developing effective vaccine strategies that enhance the generation of tumor-specific $CD8^+$ T cells. Several of the most common prostate cancer tumor antigens being utilized for immune therapy in preclinical models and in ongoing clinical trials are described below.

Prostate-Specific Antigen

Epithelial cells lining the acini and ducts of the prostate gland produce the serine protease prostate-specific antigen (PSA), and secrete it into the prostate lumen. PSA aids in liquefying semen coagulum and is excreted in seminal fluid. In healthy individuals, serum PSA levels are common at low concentrations (0-2.5 ng/ml), with serum concentrations above 2.5 ng/ml indicating a cause for biopsy (145). Serum concentrations of PSA correlate with prostate

cancer disease progression, which makes PSA a useful prognostic marker that aids in the grading and staging of prostate cancer, as well as an indicator of disease recurrence and progression (146, 147). However, increased PSA levels are also associated with benign inflammatory conditions such as benign prostate hyperplasia (BPH) and prostatitis, which may result in a false positive screen that is not indicative of cancer progression (148).

In addition to its value as a prognostic marker, PSA is an immunogenic antigen that can drive immune responses (149). PSA-specific $CD8^+$ T cells are found in both healthy individuals and prostate cancer patients (150). For this reason, PSA can serve as a target for prostate cancer vaccination to elicit an anti-tumor immune response from $CD8^+$ T cells. For example, PROSTVAC is a poxvirus-based (Vaccinia and fowlpox) vaccine that expresses the PSA antigen as well as molecules to aid in T cell stimulation including CD80, intercellular adhesion molecule 1 (ICAM-1), and lymphocyte function-associated antigen 3 (LFA-3) (151, 152). A phase II trial for the treatment of metastatic castration-resistant prostate cancer (mCRPC) showed that patients receiving PROSTVAC had an increase in their median overall survival of 8.5 months compared to control (25.1 vs 16.6 months) (153). However, a recent phase III trial failed to demonstrate improved overall survival compared to controls (NCT01322490). Failures to respond to a PSA-expressing vaccine may be the result of a high frequency of PSA-specific $CD8^+$ T cells expressing Tim-3, a marker of T cell exhaustion (154). This suggests that while patients possess tumor-specific T cells needed to mount an anti-tumor response, those T cells may be unable to respond in a productive and effective manner. Thus, current ongoing studies are assessing the potential benefit of combination immunotherapy, specifically PD-1 checkpoint blockade, to improve vaccine efficacy.

Prostate Acid Phosphatase

The prostatic epithelium synthesizes prostate acid phosphatase (PAP), a prostate-specific, secreted glycoprotein enzyme that is involved in the liquification of semen (155).

Expression of PAP and its serum concentration correlates with testosterone levels, disease progression and the amount of bone metastases (156). Due to these prognostic correlations, PAP serum levels were originally used as a prostate tumor biomarker dating back to the 1940's, prior to the adoption of PSA screening in the 80's. Despite the widespread use of PSA for prostate cancer screening, PAP still has prognostic value as a biomarker for determining and differentiating intermediate- and high-risk patients, and for predicting clinical recurrence and likelihood of developing distant metastases (157).

Like PSA, PAP also has properties as an immunogenic antigen recognized by T cells in humans and mice (158). Evidence for this is provided by one study showing that intratumoral injection of a PAP-expressing vector resulted in decreased tumor growth in a xenograft model (159). Additional studies have shown that PAP expressed by a DNA vaccine successfully induced PAP-specific $CD8^+$ T cells, while also increasing PSA doubling time, suggesting that prostate cancer patients may benefit from targeting PAP using cancer vaccines (160, 161). Sipuleucel-T, the first therapeutic cancer vaccine to receive Food and Drug Administration (FDA) approval works as an immunostimulant to trigger an anti-PAP immune response for the treatment of mCRPC (133, 162). Delivery of Sipuleucel-T is achieved using leukapheresis of patient blood to remove and isolate primary DCs. The patient's DCs are loaded with PAP peptide in an incubation step and are additionally stimulated with granulocyte-macrophage colony stimulating factor (GM-CSF) to promote cell growth and survival in culture. These PAP peptide-loaded DCs are then re-infused back into the patient to promote a $CD8^+$ T cell-mediated anti-tumor response. The IMPACT clinical trial assessing the efficacy of Sipuleucel-T (NCT00065442) showed that patients receiving Sipuleucel-T experienced a statistically significant increase in median survival time of 4.1 months compared to the control arm. However, despite an increase in survival time, patients receiving Sipuleucel-T did not experience a significant decrease in tumor size (133). The following D9901 trial (NCT00005947)

confirmed these findings (163). Sipuleucel-T was FDA-approved for mCRPC in 2010 and is in ongoing clinical trials to evaluate its efficacy when used in combination with other therapies.

Prostate-Specific Membrane Antigen

Prostate-specific membrane antigen (PSMA) is a membrane-bound zinc metalloenzyme expressed primarily on prostate epithelial cells, although low expression can be found in other tissues such as the kidney (164). While PSMA is expressed in the healthy prostate epithelium compared to other tissues, it is also one of the most commonly and highly upregulated genes found in prostate cancers, including high staining on epithelial cells of prostatic intraepithelial neoplasia (PIN) and on malignant carcinomas (164). Interestingly, PSMA expression appears to be linked to androgen deprivation therapy (ADT), with increased expression in response to therapy. (165). Like PSA and PAP, PSMA expression may be a useful prognostic biomarker. It has been shown that expression correlates with disease progression and time to recurrence (166), which could make PSMA useful as a prognostic marker accounting disease progression and tumor cell disease potential; yet, attempts to utilize PSMA serum levels alone as a prognostic marker have not been successful. However, other methods involving PSMA expression have had success, including the ProstaScint scan that combines CT and MRI scans. The ProstaScint technique utilizes PSMA-specific antibodies bound to radioactive indium-111 to bind and identify prostate cancer metastases and has proven to be a valuable tool for identifying distant, remaining, and recurrent disease (167).

PSMA can also be targeted using various forms of monoclonal antibody (mAb) therapy. For example, unconjugated, radiolabeled, and drug-conjugated humanized mAbs against PSMA have all been utilized to induce antibody-dependent cellular cytotoxicity (ADCC) and cell death (168). PSMA contains a cytoplasmic tail internalization signal that induces protein internalization to the endosome upon ligand binding, which leads to trafficking back to the cell surface or targeting to lysosomes for protein degradation (169). This key property of PSMA internalization

is exploited by treatments to introduce toxins specifically into prostate cancer cells. In treatments such as D7-PE40, a targeted immunotoxin consisting of an antibody fragment specific for PSMA is linked to exotoxin A, a dimer protein that blocks protein synthesis through elongation factor-2 inhibition. This conjugated therapy targeted against PSMA has shown efficacy in blocking tumor growth in pre-clinical models (170). Other therapeutic strategies targeting PSMA have been developed, such as chimeric antigen receptor T cells (CAR-T) specific for PSMA. In a pre-clinical xenograft model of human prostate cancer, anti-PSMA CAR-T cells were highly effective at eradicating tumors and inducing tumor-specific lysis (171, 172).

Prostate Stem Cell Antigen

Prostate Stem Cell Antigen (PSCA) is a GPI-anchored surface protein involved in stem cell survival (173). PSCA is highly expressed in prostate cancer, pancreatic cancer, and bladder cancer epithelial cells (174). Expression of PSCA is found in 90% of primary prostate cancers and a high proportion of metastatic sites contain amplification of the PSCA gene (175). Further, PSCA expression is associated with a higher Gleason score, high staging, and cancer progression to bone metastases (176). In addition to these associations, PSCA detection also functions as a marker of response to therapy, as decreased PSCA mRNA levels correlate with response to radiation therapy (177). The PSCA gene is located about 3kb downstream of an androgen-responsive enhancer, resulting in decreased PSCA expression in response to ADT (178). However, despite these correlations, not much is known about the role and function of PSCA in prostate cancer or in normal prostate tissue.

Due to its high expression in primary prostate cancers and metastases, PSCA is being evaluated as a therapeutic target. Preclinical murine studies have examined the efficacy of a PSCA-based DNA vaccine and the use of engineered CAR-T cells specific for PSCA (179). Clinical trials have included examinations of vaccine and mAb therapies targeting PSCA as well. A phase I/II clinical trial assessed the efficacy and safety of a PSCA peptide-loaded DCs

vaccine, which induced an immune response against PSCA and showed promise for patients with mCRPC (180). A six-patient phase I trial for the treatment of mCRPC utilized another DC-based vaccine that employed multiple binding epitopes of PSCA. This trial resulted in a beneficial increase in PSA doubling time and the generation of memory T cell responses against the peptides administered (181). Finally, a phase I clinical trial for the treatment of mCRPC tested AGS-1C4D4, a mAb targeting PSCA (182). In this trial, the therapy was well tolerated by the 13 patients treated, with six patients having stable disease for 24 months. Targeting PSCA through vaccine and antibody-based therapeutics has so far shown promise, which warrants further exploration in pre-clinical and clinical studies based upon its induction of memory T cells. It will be interesting to determine how PSCA-targeted therapeutics will combine and potentially synergize with established prostate cancer therapies and immunotherapies, such as PD-1 blockade.

Mucin-1

Mucin-1 (MUC1), a highly glycosylated protein, is expressed across a wide variety of epithelial cancers including prostate adenocarcinoma, but is not detected in healthy prostate tissue (183). MUC1 is expressed on the apical borders of epithelial cells where it plays a role in cell adhesion and mucosal barrier protection. MUC1 exists in secreted and membrane-bound forms; however, in prostate adenocarcinomas and neoplasms, MUC1 is also expressed in the cytoplasm. Expression of MUC1 correlates with disease progression, tumor volume, and lymph node metastases (184). However, MUC1 is suppressive of androgen receptor (AR) expression, leading to decreased androgen sensitivity and response to ADT (185).

Due to its high expression in adenocarcinomas, MUC1 is a target for antibody-based targeted therapy as well as vaccine therapy. Therapeutic targeting of MUC1 for prostate cancer is currently being tested in clinical trials. Researchers have developed an adenovirus-based vaccine that expresses MUC1 along with two other TAAs and contains specific gene

modifications that decrease viral gene expression and prevent a host immune response against the viral protein components (186). This vaccine, ETBX-061, is currently being tested for clinical trial use. This trial (NCT03481816) is testing the efficacy and safety of the MUC1 viral vaccine alongside other adenovirus-based vaccines expressing PSA and brachyury proteins for patients with mCRPC.

NY-ESO-1

NY-ESO-1 is a well-characterized cancer testis antigen of unknown function expressed in bladder, esophageal, liver, and breast cancer (187). In transitional cell carcinoma, expression of NY-ESO-1 correlates with staging and seropositivity in high grade patients (188). NY-ESO-1 is a MHC class I and class II antigen that induces cellular and humoral immune responses that are associated with anti-tumor immune activity (189) and the presence of these NY-ESO-1-specific T cells correlates with good prognosis. In one study, 10% of prostate cancer patients had autoantibodies against NY-ESO-1 in their serum, while healthy patients had none (190). This supports evidence that NY-ESO-1 is expressed highly in cancerous tissue and lowly in healthy tissue, a property that makes NY-ESO-1 attractive as a potential cancer vaccine antigen. Over 30 clinical trials have been conducted with NY-ESO-1 vaccines or NY-ESO-1-targeted adoptive T cell therapy, several of which focus specifically on prostate cancer. Clinical trial NCT02692976 utilized a DC-based vaccine loaded with NY-ESO-1 and MUC1 peptides, with separate groups assessing the efficacy of plasmacytoid, myeloid, or the combination of both subtypes of DCs. The therapy was well-tolerated, with only 33% of patients experiencing low grade toxicity, and efficacious, with 67% of patients achieving six months of stable disease. To increase response to this vaccine, combinations of NY-ESO-1 vaccine therapy with immunotherapies are being considered. Preliminary work with CTLA-4 blockade/NY-ESO-1 vaccine combination therapy demonstrated an increase in the numbers of NY-ESO-1 specific T cells in metastatic melanoma, which correlated with clinical responses (191).

T-cell receptor alternate reading frame protein

T-cell receptor alternate reading frame protein (TARP) is an MHC class II-restricted protein expressed on prostate and breast cancer epithelial cells (192). TARP expression correlates with cancer progression and is found in primary and metastatic sites (193-195). TARP is a 58-residue sequence product of an alternate reading frame of the TCR locus (196). This alternate reading frame sequence contains two known $CD8^+$ T-cell binding epitopes that are presented on APCs and prostate cancer cells (197). Testosterone upregulates TARP expression, which suggests a role for androgens in promoting its expression (198). However, TARP expression has been observed in androgen-sensitive and androgen-insensitive prostate cancer (199). Its high expression in prostate cancer makes it an ideal target antigen for cancer vaccine therapy.

Due to its immunogenicity, TARP-specific vaccination was explored in a phase I clinical trial. This trial consisted of 41 patients with asymptomatic hormone-sensitive prostate cancer receiving either emulsified TARP peptide with GM-CSF or DCs pulsed with TARP peptide (200). The five-dose vaccination schedule was well tolerated and had an acceptable safety profile in both treatment groups. Clinical outcomes were assessed based upon PSA doubling time, which is a measure of the time it takes for serum concentrations of PSA to double. Both treatment groups showed a decrease in PSA doubling time, with 74.2% of total patients displaying a reduction in PSA doubling time 48 weeks post-treatment. Additionally, the TARP vaccine increased TARP peptide-specific IFN-γ production, with responses recorded for 80% of patients. These data suggest that TARP vaccine therapy may help in controlling micro-metastases and slowing disease progression. A trial with a second generation of this vaccine (NCT02362451) is underway with modifications that include the addition of MHC class II binding sites to the peptide sequence (200).

GRB2-Like Endophilin B2

The GRB2-Like Endophilin B2 (SH3GLB2) peptide was discovered in the TRAMP murine prostate model as a stimulator of prostatic adenocarcinoma specific T cells (SPAS1). TRAMP mice receiving vaccination with the TRAMP-C2 cell line, CTLA-4 blockade, and GM-CSF experienced a reduction in tumor growth and a reduction in spontaneously forming tumors. This treatment method was employed to identify unknown prostate cancer antigens, as had been done previously for melanoma (201, 202). By testing T cell activity from the spleens of treated mice a 395 amino acid sequence was identified that induced T cell activation and shared 96% homology with human SH3GLB2, a protein of unknown function (203). SH3GLB2 is highly expressed in prostate cancer metastases and is orthologous to SPAS1, the immunodominant model antigen in the TRAMP-C1 murine model. Analysis of SH3GLB2 expression in human prostate cancer has shown that it is highly expressed in lymph node metastases in patients with aggressive cancer (204). Because T cell responses can be induced in a mouse vaccination model, it is thought that SH3GLB2 is a viable vaccination target antigen in humans. However, to our knowledge, there have been no attempts at this therapy as of this time.

Six transmembrane epithelial antigen of the prostate 1

Six transmembrane epithelial antigen of the prostate 1 (STEAP1) is a metalloreductase enzyme expressed at cell-cell junctions and is overexpressed in the cell membrane and cytoplasm of prostate cancer cells, with lower expression in healthy tissue (205, 206). STEAP1 is highly expressed in PIN lesions, which suggests that it plays a role in early prostate cancer development and thus might be an attractive biomarker for early disease (207). STEAP1 has been a target antigen for antibody-based therapy as well as vaccine therapy due to its high expression in PIN and more advanced forms of prostate cancer. The CV9103 vaccine utilized STEAP1 mRNA in a phase I/II trial of 44 patients (NCT00831467) (208). This strategy effectively induces cytotoxic $CD8^+$ T lymphocytes with reactivity against STEAP1. Additionally, a

modified vaccinia virus Ankara (MVA) vector delivery system containing STEAP1 has shown promise as a vaccine therapy in murine models (209). Developed mAbs against STEAP1 have also shown promise in murine models by inhibiting xenograft tumor growth. This success has led to the development of an antibody-drug conjugate (ADC). DSTP3086S is an ADC linked to monomethyl auristatin E, which inhibits cell growth by blocking tubulin polymerization. DSTP3086S has shown promise in a phase I clinical trial (NCT01283373) for mCRPC and has shown greater effectiveness at higher concentrations (210).

2.7. Models of Murine Prostate Cancer

TRAMP

The TRAMP murine model was originally developed in 1995, which was followed by further characterization and development of cell lines and modified models of TRAMP (211). The TRAMP model of spontaneous prostate cancer was developed using the simian vacuolating virus 40 (SV40), a virus with oncogenic proteins, to induce cancer. Expression of the early region of the virus which is composed of the large and small T antigen was driven by a modified probasin-ARR2 promoter specific to the prostate. The large-T and small-t antigens of SV40 protein bind to and inhibit the activity of p53 and Rb tumor suppressors. Additionally, the small-t antigen binds to phosphatase PP2A along with several other oncogenic intracellular proteins. Inhibiting p53 and Rb tumor suppressors results in a predictable progression from PIN lesions to highly penetrant metastatic disease by week 28. All of these mice develop lymph node metastases and 67% go on to develop pulmonary metastases. However, these mice exhibit a variable response to ADT, although mice that are resistant to ADT are more likely to develop metastases (212). Additionally, these mice develop phyllodes-like lesions that have a leaf-like structure, similar to that found in human breast cancer. It should also be noted that some studies have demonstrated that TRAMP may not actually be a form of adenocarcinoma

and is instead an atypical epithelial hyperplasia that develops into neuroendocrine carcinoma (213).

Three prostate cancer cell lines were derived from tumor-bearing TRAMP mice (214). Two of these cell lines (TRAMP-C1 and TRAMP-C2) readily form tumors when implanted subcutaneously into syngeneic wild-type (C57/BL6) mice. However, the third cell line (TRAMP-C3) only grows *in vitro*. These tumorigenic cell lines retained AR expression, but lost expression of the T antigen found in the transgenic mouse. TRAMP-C1 and TRAMP-C2 are amenable for studies investigating therapeutic efficacy of various immunotherapies, although it should be noted that TRAMP-C1 is considered poorly immunogenic with a low level of basal immune infiltration, while TRAMP-C2 is moderately immunogenic. TRAMP-C1 is responsive to CTLA-4 blockade in a T and natural killer (NK) cell-dependent manner. TRAMP-C2 cells are also responsive to checkpoint blockade, but can form metastases in the draining lymph nodes and lungs, thus making it a useful model for surgical resection and metastasis. For example, while 95% of mice with surgically resected primary TRAMP-C2 tumors develop metastases, this was reduced to 50% following CTLA-4 blockade (215).

LADY

The LADY model of prostate cancer is designed similarly to the TRAMP model; however, it has modifications in the probasin (PB) promoter region and a mutation to the small-t antigen, which renders it unable to bind phosphatase PP2A and other oncogenic intracellular proteins. Many different lines of the LADY model have been generated with variable tag transgene expression. These lines are named 12t-1 through 11. The 12 in the name indicates the approximate 12kb promoter length, t for the small-t antigen transgene, and 1 – 11 for the 11 lines generated. The mouse line that develops cancer at the fastest rate is 12t-7, which progresses to locally invasive adenocarcinoma at 15-22 weeks, but does not become metastatic

(216). In contrast, Line 12t-10 grows slowly, progressing to invasive neuroendocrine carcinoma at 33 weeks, and develops lymph node and lung metastases at 50 weeks-of-age. Considering the range in variation in metastatic potential seen across the model, these various lines may be helpful for comparing the genetics involved in metastatic progression.

The TRAMP and LADY transgenic mice each carry advantages and disadvantages as models of human prostate cancer. For example, both were generated by driving expression of SV40 T antigens off of a prostate-specific promoter to produce highly penetrant and well characterized disease, which is a mechanism of oncogenic transformation that is not mirrored in humans. Not all models of TRAMP and LADY are capable of progressing to metastatic disease, but both the TRAMP and the 12t-10 LADY models have metastatic potential. Unfortunately, these models do not reciprocate human prostate cancer progression completely, due to being driven by an exogenous oncogene that does not exist in humans. This leads to accumulation of different mutations and subsequently altered disease progression than is found in patients with prostate cancer. This SV40 oncogene driver produces mostly neuroendocrine cancers and similar carcinoma subtypes in mice, which likely represents only 25-30% of cases of men with advanced prostate cancer (217). However, the mice that do develop neuroendocrine prostate cancer may be useful for studying human neuroendocrine prostate cancer.

c-Myc models

c-Myc is a proto-oncogene commonly overexpressed or mutated in human prostate cancer. Furthermore, c-Myc overexpression is also found in PIN, which suggests a role in early cancer progression. Thus, to recapitulate the onset and progression of human prostate cancer, several mouse models utilize mutations and overexpression of c-Myc along with other commonly associated mutations of tumor suppressors, such as p53 and phosphatase and tensin homolog (PTEN). Two models were developed in which c-Myc expression is driven from either the Pb promoter or the ARR2PB promoter, similar to the TRAMP and LADY models.

These two distinct models of c-Myc-driven prostate cancer are categorized as Lo-Myc and Hi-Myc. They are distinct in their responsiveness to androgens and ADT making them good models for studying human prostate cancer disease progression and responsiveness. The Lo-Myc model results in prostate cancer progression that is unresponsive to ADT, which makes this model castration-resistant. In contrast, the Hi-Myc model exhibits androgen sensitivity, which reflects the regulation of cMyc transgene expression by the androgen-regulated Pb promoter. However, in human prostate cancer, castration does not inhibit c-Myc expression (218). Hi-Myc mice progress to adenocarcinoma around 26 weeks-of-age, while the Lo-Myc model progresses more slowly with adenocarcinoma developing at 56 weeks (219). However, neither Hi-Myc nor Lo-Myc models develop spontaneous metastases (219). Similar to what is seen in human prostate cancer, expression of the prostate-specific tumor suppressor NKX3.1 is reduced in Hi-Myc PIN and adenocarcinoma (220). Further studies of this model have coupled it to genetic knockouts that are common in human prostate cancer.

PTEN knockout

PTEN is a major tumor suppressor that functions to dephosphorylate activated AKT and 3-phosphoinositide-dependent protein kinase-1 (PDK1) (221, 222). PTEN is commonly mutated or lost in many cancers, including a high percentage of human prostate cancers (223, 224). Original models of PTEN heterozygous mice were not ideal for studying prostate cancer because many of the mice that survived embryonic development developed other types of cancers due to the non-specific knockout of PTEN. To overcome these limitations, prostate tissue-specific PTEN knockout mice were generated using the Cre recombinase system, driving Cre expression off of a prostate-specific promoter to flox out a section of PTEN flanked by loxP sites. These mice develop progressive prostate cancer from PIN to metastatic adenocarcinoma in a manner similar to human prostate cancer progression.

Two groups have developed PTEN knockout mice using this system. One group deleted exon 5 of PTEN and these mice develop PIN at six weeks and adenocarcinoma by 9 to 29 weeks-of-age with lung and lymph node metastases forming at 12 to 29 weeks in 45% of the mice (225). Additionally, this model may be useful for studying androgen dependency as tumors initially regress in response to androgen ablation, but then become resistant mirroring what is observed in humans. Another group developed a PTEN model by deleting both exon 4 and 5. These mice developed prostate cancer more slowly compared to the exon 5 deleted mice, with observed lesions by 42 weeks (226). An additional model utilizes a tamoxifen-inducible Cre transgene knocked in to the NKX3.1 locus, which simultaneously knocks out one allele of NKX3.1 and brings Cre expression under control of the NKX3.1 promoter (227). This adds temporal control over PTEN deletion. Mice that receive tamoxifen at two months-of-age develop high grade PIN and micro-invasive adenocarcinoma (228). PTEN knockout models have also been combined with targeted deletion of p53 as well. This combination of tumor suppressor knockouts results in an aggressive prostate cancer by week 11 and eventual death by week 29 (229).

2.8. Prostate cancer immunotherapy: vaccines, checkpoint blockade, and combination therapy

Vaccines

As described previously, Sipuleucel-T was the first therapeutic cancer vaccine to garner FDA approval and uses PAP-loaded DCs to direct anti-tumor (PAP) responses in patients (133, 162). In contrast, PROSTVAC is a poxvirus-based vaccine expressing PSA along with the co-stimulatory molecules CD80, ICAM-1, and LFA-3. In addition to these agents, GVAX is another prostate cancer vaccine platform, and instead of focusing on a single target, such as PSA or PAP, to induce an anti-tumor immune response, GVAX is a cellular vaccine that contains irradiated prostate cells from two different human prostate cancer cells lines, LNCaP and PC3 (230). These cell lines have been additionally modified to produce GM-CSF in order to stimulate

DCs for antigen presentation. This vaccine could potentially induce immune responses to multiple prostate cancer antigens at once, which is beneficial to eliminate a heterogeneous population of prostate cancer cells from the body. This vaccination method would bypass the need to human leukocyte antigen (HLA) match patients because it relies on the patient's own DCs to present antigen *in vivo* (231). However, despite this potential for an effective and easier cancer vaccine design, GVAX has not seen success in clinical trials when compared to docetaxel in two separate phase III trials (NCT00089856, NCT00133224).

CTLA-4 blockade

T cell costimulation by CD28 is critical for their activation and induction of cytotoxic activity. This pathway is negatively regulated by CTLA-4, which competes for the CD28 ligand. Ipilimumab is a CTLA-4 blocking mAb that prevents its activity and to increase CD28 signaling on T cells (232). Initial studies of Ipilimumab in prostate cancer patients led to unacceptable adverse effects, including death among several patients being treated for CRPC, and unfavorable outcomes, with no increase in overall survival (233). However, several patients did have a complete remission in response to this therapy (234). Improvement in patient and biomarker selection, as well as new and improved CTLA-4 blocking antibodies may limit severe adverse effects in the future. Ipilimumab is being evaluated for its ability to elicit T cell responses against tumor-specific neoantigens as part of a phase II trial for CRPC (NCT02113657) and as neoadjuvant therapy prior to radical prostatectomy (NCT01194271).

While initial trials testing the efficacy of ipilimumab have not resulted in increased overall survival, ipilimumab may still be beneficial when applied in combination with other therapies. A phase I clinical trial of GVAX in combination with ipilimumab for patients with CRPC showed that the treatment had an acceptable safety profile. Additionally, the therapy led to favorable tumor responses and prolonged survival, especially among patients with higher peripheral blood

expression of CTLA-4 and PD-1 on their $CD4^+$ T cells and lower frequencies of regulatory T cells prior to therapy. Overall, the best predictor of favorable outcome to this therapy was CTLA-4 expression by $CD4^+$ T cells, which might suggest CTLA-4 as a biomarker for selection of patients that would benefit from this therapy. A phase II trial is ongoing to determine the efficacy of the PROSTVAC vaccine in combination with ipilimumab (NCT02506114) as a neoadjuvant therapy for patients with localized prostatic neoplasia.

Ipilimumab is also being tested in an ongoing phase I trial in combination with GM-CSF for the treatment of recurrent prostate carcinomas and stage IV prostate cancer (NCT00064129). It was found that patients with immune-related adverse events (IRAEs) had an increase in T cell clonality two weeks after initial ipilimumab treatment, and an increase in PSA-specific T cells was associated with increased T cell diversity (235). Additional trials of ipilimumab include its use in combination with ADT for CRPC (NCT01377389, NCT02703623), stage IV and recurrent prostate cancer (NCT01498978, NCT00170157), for chemotherapy-naïve CRPC (NCT01688492), and for use prior to radical prostatectomy (NCT02020070).

PD-1/PD-L1 blockade

PD-1 is a glycoprotein expressed on T cells that interacts with PD-L1 expressed in cancer cells and a range of other immune cells, to inhibit T cell activation. Targeting the PD-1/PD-L1 axis with blocking mAbs is an area of great focus in cancer therapy, having received FDA approval for several cancer types (236, 237). However, PD-1-targeted therapy in prostate cancer may have a minimal impact due to lower PD-L1 expression in prostate cancer compared to other cancers (238). Low PD-L1 expression may explain the minimal response seen in early clinical trials of nivolumab, which is a PD-1 blocking antibody, for mCRPC. Other studies looking at pembrolizumab (anti-PD-1 mAb) therapy in combination with enzalutamide for mCRPC have demonstrated meaningful clinical benefit for a subset of patients (239). This may be a possible therapeutic avenue to follow after patients' progress towards androgen insensitivity because

during the progression to androgen insensitivity, DCs increase expression of PD-L1 and $CD8^+$ T cells increase expression of PD-1. A phase III trial is underway to determine the efficacy of atezolizumab (a PD-L1 blocking antibody) in combination with enzalutamide compared to enzalutamide alone (NCT03016312). Additionally, the use of ipilimumab is being investigated in combination with nivolumab in mCRPC (NCT03061539, NCT02985957, NCT02601014).

A phase I/II trial is ongoing to investigate the efficacy of ADXS31-142, a PSA based vaccine, as a monotherapy or in combination with pembrolizumab for mCRPC (NCT02325557). The pTVG-HP PAP antigen-based DNA vaccine is being investigated in combination with pembrolizumab (NCT02499835). An ongoing phase II trial is looking at the efficacy of pembrolizumab in combination with Radium-223, which targets bone metastases (NCT03093428) and has shown efficacy in pre-clinical models (240). Another phase II trial is investigating the efficacy of combination immunotherapy using durvalumab, an anti-PD-L1 antibody, in combination with tremelimumab, and anti-CTLA-4 antibody for the treatment of mCRPC (NCT03204812), as well as in combination with a TLR3 agonist (NCT02643303). Nivolumab is being looked at in combination with a poxvirus-based PSA expressing cancer vaccine (NCT02933255). An ongoing phase II trial is investigating the safety and efficacy of nivolumab in combination with multiple ADTs and poly-ADP ribose polymerase (PARP) inhibitors (NCT03338790, NCT02484404). Nivolumab is being investigated as part of a phase I trial looking at the safety of AM0010, a pegylated IL-10 immune stimulating agent (NCT02009449). Atezolizumab is being investigated as part of a phase I trial looking at the safety of CPI-444, an adenosine A2A receptor agonist for the treatment of mCRPC (NCT02655822).

<u>Other therapies</u>

An alternative immunotherapy strategy is using bi-specific antibodies to link T cells to their cancer cell target (241). A therapeutic method utilizing this strategy targeted PSMA and CD3 simultaneously to bring T cells in contact with their target cell to induce cytotoxicity. This therapy has shown promise in xenograft models with a reduction in PSA levels, and reduced tumor growth (242). These promising findings have led to two phase I clinical trials (NCT01723475, NCT02262910). CAR-T cells have also been developed to target prostate cancer antigens. These CAR-T cells are modified by lentiviral- or retroviral-induced expression of an engineered TCR specific for a tumor surface antigen (243). CAR-T targeting PSMA has shown promise in pre-clinical murine studies, as well as in a phase I clinical trial (NCT01140373). CAR-T therapies targeting other prostate tumor antigens such as MUC-1 and TARP have also shown promise in pre-clinical models (244).

Table 1 Clinical trials for prostate cancer immunotherapy

Therapy name	Description	NCT identifier
PROSTVAC	PSA vaccine	NCT01322490
Sipuleucel-T	PAP vaccine	NCT00065442 NCT00005947
AGS-1C4D4	PSCA targeted mAb	NCT00519233
ETBX-061	MUC1 vaccine	NCT03481816
	NY-ESO-1 and MUC1 vaccine	NCT02692976
	TARP vaccination	NCT02362451
CV9103	STEAP1 vaccination	NCT00831467
DSTP3086S	STEAP1 antibody-drug conjugate	NCT01283373
GVAX	Prostate cell line vaccination	NCT00089856 NCT00133224
Ipilimumab	CTLA-4 blocking mAb	NCT02113657 NCT01194271
Ipilimumab	In combination with GVAX	NCT01510288
Ipilimumab	In combination with PROSTVAC	NCT02506114
Ipilimumab	In combination with GM-CSF	NCT00064129
Ipilimumab	In combination with ADT	NCT01377389 NCT02703623 NCT01498978 NCT00170157 NCT01688492 NCT02020070
Atezolizumab	PD-L1 blocking mAb	NCT03016312
Atezolizumab CPI-444	In combination with an A2A receptor agonist	NCT02655822
Nivolumab	PD-1 blocking mAb, used in combination with Ipilimumab	NCT03061539 NCT02985957 NCT02601014
Nivolumab	In combination with a PSA vaccine	NCT02933255
Nivolumab	In combination with ADT	NCT03338790 NCT02484404
Nivolumab AM0010	In combination with pegylated IL-10	NCT02009449
Pembrolizumab ADXS31-142	PD-1 blocking mAb, used in combination with a PSA vaccine	NCT02325557
Pembrolizumab pTVG-HP	PD-1 blocking mAb, used in combination with a PAP vaccine	NCT02499835
Pembrolizumab	PD-1 blocking mAb, used in combination with a radium-223	NCT03093428
Durvalumab Tremelimumab	PD-1 blocking mAb, used in combination with a CTLA-4 blocking mAb	NCT03204812
Durvalumab	In combination with a TLR3 agonist	NCT02643303
	PSA and CD3 Bi-specific antibody	NCT01723475 NCT02262910
	CAR-T cell targeting PSMA	NCT01140373

2.9. Conclusions

Inducing immune responses against TAAs is a critical step in producing an effective and long-lasting anti-cancer response. Multiple prostate cancer antigens have been identified with varying degrees of expression in prostate cancer as well as other cancers and healthy tissues. Many of these associated antigens are capable of being identified by $CD8^+$ T cells and inducing an effective immune response. However, translating these findings into effective vaccine therapies has been challenging. The FDA approval of Sipuleucel-T has shown that vaccine therapy has efficacy in prostate cancer patients. The development of immune checkpoint blocking antibodies such as ipilimumab, nivolumab, and pembrolizumab have also shown promise as effective therapeutic strategies in treating prostate cancer. Ongoing and future approaches are looking at combinations of tumor antigen-specific vaccines along with checkpoint blockade to activate tumor-specific $CD8^+$ T cells and thereby enhance ant-tumor immunity.

Chapter 3: Immuno-Oncology Drugs: From relieving inhibition to providing co-stimulation with T cell agonists

Abstract

Recent advancements in T cell biology and antibody engineering have opened the door to significant improvements in cancer immunotherapy. Initial success with monoclonal antibodies targeting key receptors that inhibit T cell function such as CTLA-4 and PD-1 have demonstrated the potency of this new class of therapy, highlighted by long-term complete responses for metastatic cancers once thought incurable. However, only a subset of patients will respond to checkpoint blockade due to a multitude of factors including an immunosuppressive tumor microenvironment and the mutational burden of the cancer. Novel antibodies as well as ligand-immunoglobulin fusion proteins that target co-stimulatory immune receptors are being developed and tested in clinical trials to further enhance the anti-tumor immune response. Many of these co-stimulatory receptors are in the tumor necrosis factor receptor superfamily (TNFRSF) and are expressed on multiple immune cell types, including inhibitory cells. While TNFRSFs signal through common pathways, the outcome of targeting different receptors is dependent on the functional status of the cell types expressing the relevant receptors. In this review, we will discuss the current state of targeted co-stimulatory immunotherapy.

3.1. Introduction

The generation of potent T cell-mediated anti-tumor immunity relies on the provision of several critical signals including T cell receptor (TCR)-mediated recognition of peptide-MHC molecules on antigen-presenting cells (APCs) along with appropriate co-stimulatory signals (245, 246). Even in the presence of these optimal conditions, tumors utilize a myriad of mechanisms to evade and suppress the immune response. For example, tumors suppress recognition and visibility to the immune system by changing their microenvironment (TME) and reducing MHC class I presentation of tumor-associated antigens (TAAs) (247). Changes to the TME can occur through secretion of immunosuppressive cytokines such as IL-10 and TGF-β, which inhibit effector T cell responses and promote regulatory FoxP3$^+$ CD4$^+$ T cell (Treg) function (248) or through up-regulation of programmed death-ligand 1 (PD-L1), which inhibits the TCR signaling pathway. PD-L1 binds to programed cell death protein 1 (PD-1) expressed by activated or exhausted T cells, resulting in phosphatase activity that inhibits T cell receptor (TCR) kinase signaling (249, 250). Importantly, tumors that are immunologically "silent", which refers to low MHC I and high PD-L1 expression, often have little or no response to immunotherapy (251-254).

Thus, there is an urgent need to develop therapies that enhance tumor antigen presentation along with T cell priming, activation, and differentiation in order to counteract tumor-induced immune evasion and suppression. One such approach is to enhance T cell recognition of TAAs presented on MHC molecules through the provision of exogenous tumor-specific vaccines, known as therapeutic vaccination (255, 256). However, therapeutic vaccines have achieved limited clinical success thus far. Another approach is the provision of monoclonal antibodies (mAb) that block inhibitory checkpoint molecules such as CTLA-4, PD-1, and PD-L1. In contrast to therapeutic vaccines, checkpoint blockade immunotherapy has demonstrated significant therapeutic benefit for patients with metastatic cancer, which led to their FDA-

approval for a variety of tumor types (251, 252). A third approach is the use of agonist mAbs that boost T cell function by engaging co-stimulatory molecules such as OX40, 4-1BB, and CD40. In this Chapter, we review checkpoint inhibitors and their impact on the current landscape of cancer immunotherapy, followed by a discussion of the current state of therapies that target T cell co-stimulatory receptors with a focus on OX40, 4-1BB, GITR, CD40, and ICOS and their use in combination with checkpoint inhibitors.

3.2. Checkpoint inhibitors (anti-CTLA-4, anti-PD-1) and the first bispecific antibody

The first T cell-targeted immunotherapies came after the discovery of immune checkpoints that regulate activation and inhibition following TCR stimulation. One of the first inhibitory regulators of T cell function to be cloned was Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) in 1987 (257). CTLA-4 and the homologous receptor CD28 both bind to B7-1 and B7-2 ligands expressed on APCs (258-260). When CD28 engages B7 ligands it enhances the TCR signaling pathway through enhanced PI3K activity. However, activated T cells downregulate expression of CD28 and upregulate CTLA-4, which inhibits T cell signaling by engaging B7 ligands with 20-fold higher affinity than CD28, thereby blocking CD28-mediated costimulation. Additionally, CTLA-4 is constitutively expressed on Tregs. In mouse models of cancer, CTLA-4 blockade was shown to boost anti-tumor immunity by inhibiting Tregs and enhancing T cell effector function (261, 262).

These data led to the clinical development of two blocking antibodies targeting human CTLA-4 (αCTLA-4): ipilimumab (263), and tremelimumab (264). Clinical trials of ipilimumab for the treatment of metastatic melanoma showed promising results, which lead to a successful phase III trial in 2009 for patients with high grade unresectable melanoma showed a 10.1 month overall median survival when receiving ipilimumab alone, compared to 6.4 months survival for

patients receiving a gp100 vaccine alone. The addition of the gp100 vaccine to ipilimumab therapy had no additional impact on overall survival in these patients (265). Tremelimumab, however, was not as successful perhaps due to its staggered treatment schedule of infusions every 3 months compared to every 3 weeks for ipilimumab. Ipilimumab was subsequently approved for metastatic melanoma in 2011. Tremelimumab is still being investigated for several other indications, but has not seen clinical success for melanoma, small cell lung cancer, and mesothelioma (266).

The success of immunotherapies such as ipilimumab in 2011 and the vaccine-based therapy Sipuleucel-T for hormone refractory prostate cancer in 2010 led to the development of multiple T cell-targeted immunotherapy agents. Leading candidates to be targeted by the next wave of immunotherapies were T cell checkpoints PD-1 and PD-L1, molecules that inhibit T cell activity and function when engaged (267). The PD-1/PD-L1 pathway is upregulated in many cancers and is an important mechanism of inhibiting activated tumor-reactive T cells (268). Two PD-1 blocking antibodies (αPD-1), pembrolizumab and nivolumab, have demonstrated clinical success and received FDA approval. Pembrolizumab was approved in 2014 following a successful phase III trial looking at the use of pembrolizumab following failed ipilimumab therapy for advanced metastatic melanoma (269). Pembrolizumab was approved for metastatic non-small cell lung cancer in 2015 (270), metastatic head and neck squamous cell carcinoma in 2016 (271), and DNA mismatch repair deficient solid tumors in 2017. Nivolumab is currently FDA approved for metastatic melanoma, renal cell carcinoma, lung cancer, bladder cancer, and Hodgkin's lymphoma (272). Additionally, three antibodies targeting PD-L1 (αPD-L1) have also seen success in clinical trials including FDA approval of atezolizumab for the treatment of metastatic non-small cell lung cancer (273), durvalumab for the treatment of metastatic urothelial cancer (274), and avelumab for the treatment of non-small cell lung cancer (275).

Among this next wave of agents was an alternate class of immunotherapeutic antibodies that simultaneously target two different receptors, known as Bispecific T cell Engagers (BiTE) (276). BiTEs are engineered antibodies that contain Fab (Fragment, antigen-binding) regions against different targets to link two different surface proteins together. If the targeted surface proteins are expressed on different cells, then the BiTE will link the two cells together. This rationale was used to develop one of the first BiTEs, blinatumomab (277). This BiTE targets CD19 overexpressed on B cell malignancies and CD3 expressed by T cells in order to bring the cancer cell and the T cell together with the goal of simultaneously enhancing T cell activity through CD3 binding (277). Blinatumomab was FDA approved for acute lymphoblastic leukemia in 2014.

While these various immunotherapeutic agents have demonstrated marked success, particularly in a subset of cancers (melanoma, lung), several important issues remain. One of the primary challenges is that immunotherapy typically only benefits a subset of patients. Other issues include the onset of potentially severe adverse events (SAE) associated with therapy such as colitis, diarrhea, and increase alanine aminotransferase, that can be enhanced when these agents are given in combination, such as in the case of αCTLA-4/αPD-1 therapy (278). Additional questions remain about treatment timing, dosage, route of administration, and identification of patients that are most likely to respond to therapy. While ipilimumab, pembrolizumab, and nivolumab have proven that immune checkpoint blockade can lead to long-term responses in metastatic disease, immunotherapy agents in development are aimed at engaging T cell costimulatory receptors to activate and enhance the T cell-mediated anti-tumor response.

3.3. Targeting the TNFRSF, ICOS, and combination immunotherapy

Recent studies have increasingly focused on therapies targeting costimulatory receptors expressed on activated T cells and antigen presenting cells. Tumor necrosis factor receptor superfamily (TNFRSF) members are a promising class of immune-modulating molecules that are under rapid development for cancer immunotherapy. TNFRSF members are typically homotrimeric transmembrane proteins with a cysteine rich extracellular domain (279). Intracellular signaling domains of TNFRSFs can induce pro-apoptotic or pro-survival and pro-inflammatory programs depending on the cell type and signaling domains expressed. TNFRSFs signal through tumor necrosis factor receptor (TNFR) associated factor (TRAF) adaptor proteins. Activating TRAF proteins (TRAF1, TRAF2, and TRAF5) signal to activate the canonical NF-κB and JNK pathway and activate the inhibitor of apoptosis (IAP) (280). These signaling pathways enhance T cell function and survival following TNFRSF engagement. However, some TNFRSFs, such as TNF-related apoptosis-inducing ligand (TRAIL) receptor and apoptosis antigen 1 (FAS) receptor, signal through death domains to initiate a caspase-mediated apoptosis program (281). Multiple pro-survival and pro-inflammatory TNFRSFs are expressed on T cells following TCR activation, making them promising targets as both monotherapies and as complimentary targets to the established checkpoint inhibitor immunotherapy agents. Thus, targeting TNFRSFs with agonistic antibodies and ligand-Fc fusion proteins has the potential to potently activate and enhance the anti-tumor T cell response.

Antibodies that target receptors can be either inhibitory (antagonist) or activating (agonist) and must be designed and engineered specifically to accomplish those signals. The most commonly used antibody isotype for immunotherapy is IgG, however there are several subtypes of IgG that influence its immune function depending on how they engage Fcγ receptors or activate the complement system cascade (282). IgG1 strongly induces antibody-dependent cellular cytotoxicity (ADCC) by engaging Fcγ receptors which lends itself well to

targeting tumor specific antigens (e.g., Herceptin which targets the Her2 receptor on breast cancer), or potentially depleting inhibitory immune cells, such as Tregs. Nivolumab and pembrolizumab, however, are IgG4 isotype antibodies, which only weakly engage Fcγ receptors and makes them amenable for receptor blockade without inducing ADCC.

Antibodies used for clinical applications are typically produced in 3 forms: murine, humanized, and fully human. Murine antibodies produced by mouse hybridomas are seen as foreign, thus they can only be dosed for a short time before the human immune system mounts an adaptive immune response to clear the foreign (murine) antibody. Humanized antibodies are modified murine antibodies engineered such that the Fc regions of the Fab domain are comprised of the human antibody sequence (283). Humanized antibodies can be dosed without inducing an adaptive immune response (284). An alternative to antibody targeting is to generate ligand-Fc fusion proteins, which express the natural ligand bound to the Fc region of antibody rather than engineering a human antibody with different binding kinetics and regions of interaction compared to the natural ligand (285). This may provide an advantage over conventional antibodies for targeting TNFRSFs which are homotrimeric and bind homotrimeric ligands. The natural homotrimeric TNFRSF ligand binds the TNFRSF molecules in a way that may be different than the complimentary binding region of an antibody to produce superior engagement.

3.3.1. OX40

OX40 (CD134; TNFRSF4) is expressed by $CD4^+$ and $CD8^+$ T cells following TCR ligation (286, 287). Murine regulatory T cells (Tregs) also express OX40, although high OX40 expression on human Tregs is only seen following activation (288-290). OX40 is transiently expressed 24 to 72 hours after T cell activation, which creates a critical window for engagement

with its ligand, OX40 ligand (OX40L; CD252). OX40L is also transiently expressed on APCs, with particularly high expression on CD40-licensed dendritic cells, which are important for priming $CD8^+$ T cell responses (291). The costimulatory activity of OX40 was initially discovered in 1987, when it was shown that agonistic antibody targeting of OX40 enhanced $CD4^+$ T cell proliferation *in vitro* (292). Following this, the ligand for OX40 was discovered to be a previously known glycoprotein expressed in human T cell lymphoma/leukemia virus-1 infected cells, formerly known as gp34 (293).

Early studies on the role of OX40-OX40L interaction in experimental autoimmune encephalomyelitis (EAE) and murine models of arthritis demonstrated that blocking antibodies against either OX40 or OX40L effectively decreased autoimmunity (294). Building upon these data, it was shown that T cells isolated from the TME expressed OX40 and subsequently hypothesized that these were tumor-reactive T cells. Additional studies revealed that treatment with an agonist αOX40 mAb or OX40L-IgG fusion protein significantly enhanced tumor-free survival across four different murine tumor models (295). Depletion of $CD4^+$ or $CD8^+$ T cells demonstrated that the efficacy of αOX40 therapy was T cell-dependent (296). To investigate if OX40 expression on $CD8^+$ T cells was necessary for treatment efficacy, OT-I TCR transgenic mice, which express a T cell receptor specific for the SIINFEKL peptide of ovalbumin protein, were crossed with OX40-deficient mice, to create $OX40^{-/-}$ OT-I's. In this antigen-specific model, $OX40^{-/-}$ OT-I T cells exhibited reduced $CD8^+$ T cell expansion and survival as compared to wild-type OT-I T cells, highlighting the critical role that OX40 signaling plays in regulating $CD8^+$ T cell activation and survival (297). Moreover, direct ligation of an OX40 agonist to antigen-specific $CD8^+$ T cells significantly enhanced expression of the effector molecule, granzyme B, leading to enhanced tumor-regression and long-term survival of tumor-bearing mice (298).

These preclinical studies showed the efficacy of agonist αOX40 therapy and formed a rationale for further evaluation in clinical trials. An initial phase I clinical trial used a murine IgG

anti-human OX40 mAb for the treatment of patients with metastatic carcinoma, lymphoma, or sarcoma (NCT01644968). After three doses of αOX40, 12 out of 30 patients saw a reduction in at least one metastatic lesion (299). However, the study was limited to three sequential doses given within 5 days due to the use of a murine antibody. Despite the use of a murine antibody, the trial still demonstrated that αOX40 therapy led to immunological effects (T cell proliferation) and supported further clinical development. Currently, several phase I and phase II clinical trials are ongoing to evaluate αOX40 therapy across multiple cancer types including head and neck (MEDI6469; NCT02274155), colorectal neoplasia (MEDI6469; NCT02559024), metastatic prostate (MEDI6469; NCT01303705), renal cell carcinoma (PF-04518600; NCT03092856), and solid tumors (INCAGN01949; NCT02923349). Additionally, there are multiple phase I clinical trials exploring αOX40 in combination with other immunotherapies including tremelimumab (αCTLA-4) and durvalumab (αPD-L1) for the treatment of advanced solid tumors (MEDI0562; NCT02705482, and NCT02205333), in combination with pembrolizumab alone (GSK3174998; NCT02528357), or the combination of pembrolizumab and nivolumab for advanced cancers (BMS-986178; NCT02737475), and in combination with a TLR9 agonist for lymphomas (BMS-986178; NCT03410901). Results from these clinical trials are still pending, however the results from a phase 1 dose escalation trial for the treatment of advanced solid malignancies using a combination of αOX40 and αPD-L1 (MOXR0916; NCT02410512) showed no highgrade adverse effects at the higher dosage, indicating that this combination was well tolerated. Agonist αOX40 therapy is a promising and exciting treatment that may lend itself well to combinations with checkpoint inhibitors such as αPD-1 and αCTLA-4.

3.3.2. CD40

CD40L (CD154; TNFSF5) is expressed primarily on activated $CD4^+$ T cells, along with B cells, monocytes, NK cells, basophils, and mast cells (300). This ligand plays an important role

in binding CD40 (TNFRSF5) expressed on antigen presenting cells and B cells and acting as an important signal to induce activation (301). The interaction between CD40 and CD40L is critical for developing an adaptive immune response that is highly context dependent on the cell types and cytokines involved. Activated $CD4^+$ T cell CD40L engagement of CD40 on B cells results in TRAF adaptor protein induction of NF-κB, MAPK, PI3K, and PLCγ pathways that activate B cells (302, 303). Activated B cells form germinal centers, undergo antibody isotype switching, and differentiate into plasma cells to produce antibodies (304). Signaling through CD40 on dendritic cells is a critical step for dendritic cell "licensing" which enables dendritic cells to prime $CD8^+$ T cell responses effectively through cross-presentation (305-308). Enhancing APC activation and DC licensing is the main rationale behind agonist aCD40 therapy and the rationale behind its use in combination therapy (309). Studies of agonistic aCD40 antibodies in murine models of cancer have shown significant therapeutic efficacy. In a mesothelioma model, aCD40 was effective in inhibiting tumor growth in a dose-dependent manner (310). Interestingly, one group discovered that aCD40 therapy was more effective at clearing tumors in a lymphoma model when treatment was delayed (311). It was speculated that the larger tumor burden of delayed treatment may increase the available tumor antigens that can be taken up and cross-presented by dendritic cells.

An initial clinical trial using recombinant human CD40L for non-Hodgkin's lymphoma showed some promise when one patient out of 32 had a complete response and another had a partial response in the absence of major toxicity (312). Several CD40 agonists have moved forward into clinical trials including the humanized aCD40 CP-870,893, which led to a partial response in 14% of melanoma patients (NCT01103635) (313). Studies investigating SGN-40 have not induced any clinical responses to date, however SGN-40 is being used in a clinical trial for lymphomas (NCT00435916) and in combination with rituximab (NCT00655837). Other agents in clinical trials include HCD122 (lucatumumab) for lymphomas (NCT01275209,

NCT00670592) (314), CDX-1140 for solid cancers (NCT03329950), APX005M for solid tumors (NCT02482168), central nervous system tumors (NCT03389802), and esophageal cancer (NCT03165994). Additional monotherapy trials include ADC-1013 for solid tumors and pancreatic cancer (NCT02379741, NCT03214250), and Chi Lob 4/7 for advanced malignancies (NCT01561911). Combination therapy trials include APX005M in combination with pembrolizumab for metastatic melanoma (NCT02706353) and in combination with nivolumab for metastatic lung cancer, melanoma, and pancreatic cancer (NCT03123783, NCT03214250).

While initial clinical trial data suggests that agonistic CD40 agents may not be effective as monotherapies, CD40 agonists may be effective agents in combination with other immunotherapies. Agonistic CD40 agents may also synergize well with chemotherapies and radiation, which release tumor antigens to aid in the antigen presentation process. Different antibodies have seen varying levels of SAEs, which suggests that a clear understanding of the optimal timing and dosage for these drugs will be critical to their development and future success (315).

3.3.3. 4-1BB

4-1BB (CD137; TNFRSF9) is a glycosylated costimulatory molecule expressed transiently on activated T cells, NK cells, dendritic cells, as well as expressed constitutively on Tregs (316). Engagement of 4-1BB by its ligand 4-1BBL (CD137L; TNFSF9) induces strong T cell activation and survival, particularly in activated $CD8^+$ T cells (317, 318). The role of 4-1BB in cancer immunology has been evaluated in 4-1BBL-deficient mice, which develop spontaneous B cell lymphomas, and in 4-$1BB^{-/-}$ mice, which develop systemic lupus erythematosus likely due to tumor suppressive activity of 4-1BBL for B cells (319, 320). Initial studies of 4-1BB agonists in cancer models of sarcoma and mastocytoma showed enhanced numbers of tumor-specific

$CD8^+$ T cells and improved T cell memory against tumor re-challenge (321). 4-1BB agonists have since been shown to have efficacy across multiple tumor models by inducing a population of tumor-specific cytotoxic $CD8^+$ T cells that produce potent pro-inflammatory cytokines and effector molecules, such as granzymes (322-325).

4-1BB also plays an important role in activating and enhancing NK cell function through Fcγ receptors (326). Based on this finding, it was hypothesized that 4-1BB stimulation of NK cells aids in NK mediated antibody-dependent cell-mediated cytotoxicity (ADCC). This would make agonistic 4-1BB therapy a candidate for use in combination with tumor antigen-targeted antibodies such as rituximab, which targets CD20 for the treatment of non-Hodgkin's lymphoma and chronic lymphocytic leukemia. In murine models of lymphoma, an agonist a4-1BB showed strong efficacy when given after rituximab treatment (327).

A phase I clinical trial of utomilumab (α4-1BB) in combination with rituximab for patients with B cell lymphomas resulted in two complete responses in follicular lymphoma that lasted beyond 2 years (NCT01307267). Additionally, an increase in memory T cells and activated NK cells was observed. No significant severe adverse effects were reported with no patients stopping treatment due to toxicity in the utomilumab study (328). Utomilumab is also being tested in multiple clinical trials with other agents such as avelumab (αPD-L1; NCT02554812), αOX40 (NCT02315066), mogamulizumab (an aCCR4 antibody; NCT02444793), and pembrolizumab (NCT02179918). Another phase I/II study of an a4-1BB agonist (urelumab; NCT02253992) resulted in dose-dependent adverse effects and a 50% objective response rate in advanced metastatic melanoma patients when given with αPD-1 (nivolumab), and is also being tested in combination with nivolumab for bladder cancer and malignant tumors (NCT02845323, NCT02534506). Clearly, 4-1BB agonists are an agent of great interest as both a monotherapy and in combination with other immunotherapies.

3.3.4. GITR

Glucocorticoid-induced TNFR-related protein (GITR; CD357; TNFRSF18) is expressed on activated T cells, constitutively expressed on Tregs, and moderately expressed on memory T cells (329, 330). Once activated, T cells transiently express GITR 24 hours after stimulation. GITR expression is regulated by the FoxP3 transcription factor in Tregs and by canonical NF-κB signaling in activated T cells (331). GITR signaling in activated T cells lowers the threshold for CD28 co-stimulation and results in NF-κB, MAPK, and JNK signal pathway activation through TRAF adaptor proteins (332, 333). However, the rationale for targeting GITR with monoclonal antibodies relies on its high constitutive expression on Tregs and costimulatory signaling in $CD4^+$ and $CD8^+$ T cells. GITR was originally thought to be a unique marker of Tregs before later being found on other cell types (334). Anti-GITR antibodies seem to be well tolerated without inducing significant autoimmunity. Targeting GITR with IgG1 antibodies has been effective at depleting tumor-infiltrating Tregs from the tumor microenvironment, but not in the periphery in a B16 mouse model of melanoma (335). Using a FoxP3-GFP transgenic mouse, it was discovered that αGITR was effective in depleting Tregs in B16 tumor-bearing mice, resulting in tumor clearance (336). On activated $CD4^+$ and $CD8^+$ T cells, GITR is upregulated following TCR stimulation and signals through activating TRAF molecules to enhance proliferation, pro-inflammatory cytokine production, and resistance to Treg-mediated suppression (337-339).

Based on the high expression of GITR on tumor infiltrating Tregs, αGITR therapy is expected to be more effective in cancers with high levels of infiltrating Treg such as cervical, renal cell carcinoma, hepatocellular carcinoma, lung, and melanoma (340, 341). Therefore, it is hypothesized that αGITR therapy would synergize well with other immunotherapies, however the potential for more severe SAEs resulting from Treg depletion will need to be taken into consideration. Multiple clinical trials are underway testing both αGITR mAbs and GITRL-Fc fusions including trials as monotherapy (INCAGN0187; NCT02697591), (GWN323,

NCT02740270), (TRX518; NCT01239134), and (OMP-336B11; NCT03295942). Anti-GITR mAbs are also being tested in combination with pembrolizumab (MK-4166; NCT02132754, NCT02553499), (INCAGN0187; NCT03277352, NCT03126110) and nivolumab (BMS-986156; NCT02598960, NCT03335540). MEDI1873 is a novel hexameric GITRL-Fc fusion protein aimed at enhancing activated T cell function, rather than depleting Tregs, and has shown superior activity compared to αGITR mAbs *in vivo* (NCT02583165) (342).

3.3.5. ICOS

Inducible T-cell COStimulator (ICOS; CD278) is an immunoglobulin superfamily receptor expressed on activated T cells (343). Its ligand, ICOSL (CD275; B7-H2) is expressed on both B cells and dendritic cells (344). ICOS, CD28, and CTLA-4 all share a homologous proline-rich motif that facilitates their binding to B7 ligands. While CD28 and CTLA-4 can bind B7-1, B7-2, and B7-H2, ICOS is only known to bind B7-H2, although in a different position from CD28 and CTLA-4 and at much higher affinity (345, 346). ICOS stimulation by ICOSL induces PI3K and AKT pathway signaling, which aides in $CD4^+$ T cell differentiation into follicular T helper cells (T_{FH}), Th1, and Th2 T cells (347). The effect of ICOS signaling on activated $CD4^+$ T cells appears to be context dependent, although signaling likely drives IL-4 and IL-10 production for Th2 differentiation in the absence of additional stimulation (348).

Interestingly, ICOS is upregulated on $CD4^+$ T cells following αCTLA-4 therapy, suggesting that ICOS expression is linked to CTLA-4 and may play a compensatory role to CTLA-4 when CTLA-4 is blocked therapeutically (349). Additionally, therapeutic synergy was observed when both pathways were targeted (349-351). In the B16 melanoma model, CTLA-4 blockade and an agonist αICOS mAb synergized to provide protection that the monotherapies did not (349). Clinical trials for αICOS include MEDI-570 for various lymphomas

(NCT02520791), GSK3359609 in combination with pembrolizumab for solid tumors (NCT02723955), and JTX-2001 in combination with nivolumab for solid tumors (NCT02904226). Future clinical trials may focus on αICOS in combination with αCTLA-4 agents to determine if this combination induces additional therapeutic benefit over either therapy alone.

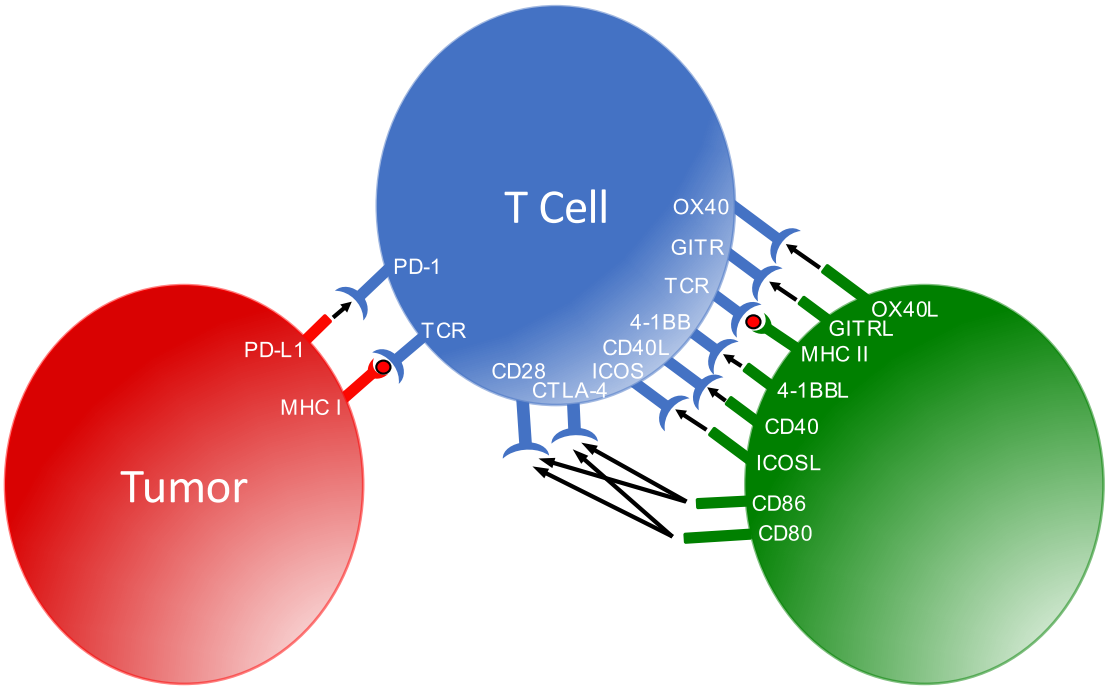


Figure 1. Inhibitory checkpoint and costimulatory receptors with their respective ligands expressed by antigen presenting cells (APC), T cells, and tumor cells. Figure from Emerson et al. Biodrugs. 2018. PMID: 29637478.

3.4. Conclusions

Immunotherapy has shown great promise in recent years, leading to durable responses and even cures for a subset of patients with metastatic cancer. While targeting immune checkpoints such as CTLA-4 and PD-1 has proven to be a viable therapy, it has not been a universal success. Targeting inhibitory receptors with a single agent may not be enough to augment the anti-cancer-mediated immune suppression. New antibodies and ligand-Fc fusion proteins targeting co-stimulatory receptors such as OX40, 4-1BB, GITR, and ICOS might provide T cells with a stimulus that is otherwise lacking in the TME. Other costimulatory

therapies are targeting alternative (non-T cell) cell types such as dendritic cells (aCD40 therapy) and NK cells (a4-1BB). Moving forward, there is a critical need to understand how to rationally combine these agents, including balancing increased efficacy with the potential for increased toxicity. As the field moves into combination therapies of inhibitory and activating antibodies, sequencing of these agents will also be critical for eliciting potent anti-cancer responses. However, synergistic combination therapy may have the added benefit of working effectively at lower doses, leading to less severe adverse events. A great deal of effort is also focusing on identifying predictive biomarkers of response. For example, tumor-specific PD-L1 expression has already proven to be a predictive marker for αPD-1 therapy in some instances, however additional immunological analysis may be critical for deciding which immunotherapies to use in combination. In conclusion, immunotherapy targeting of co-stimulatory receptors, particularly of the tumor necrosis factor receptor superfamily, are a promising addition to the growing list of immunotherapy agents being tested in clinical trials.

Chapter 4: Enhancing the generation of Eomes^{hi} CD8^{+} T cells augment the efficacy of OX40- and CTLA-4–targeted immunotherapy

Abstract

CTLA-4 blockade in combination with an agonist OX40-specific mAb synergizes to augment antitumor immunity through enhanced T-cell effector function, leading to increased survival in preclinical cancer models. We have shown previously that anti-OX40/anti-CTLA-4 combination therapy synergistically enhances the expression of Eomesodermin (Eomes) in CD8^{+} T cells. Eomes is a critical transcription factor for the differentiation and memory function of CD8^{+} T cells. We hypothesized that Eomes^{hi}CD8^{+} T cells were necessary for anti-OX40/anti-CTLA-4 immunotherapy efficacy and that further enhancement of this population would improve tumor-free survival. Indeed, CD8^{+} T cell–specific deletion of Eomes abrogated the efficacy of anti-OX40/anti-CTLA-4 therapy. We also found that anti-OX40/anti-CTLA-4-induced Eomes^{hi} CD8^{+} T cells expressed lower levels of checkpoint receptors (PD1, Tim-3, and Lag-3) and higher levels of effector cytokines (IFNγ and TNFα) than their Eomes^{lo} counterparts. Eomes expression is negatively regulated in T cells through interleukin-2–inducible T-cell kinase (ITK) signaling. We investigated the impact of modulating ITK signaling with ibrutinib, an FDA-approved tyrosine kinase inhibitor designed to inhibit BTK, and found that anti-OX40/anti-CTLA-4/ibrutinib therapy further enhanced CD8^{+} T cell–specific Eomes expression, leading to enhanced tumor regression and improved survival, both of which were associated with increased T-cell effector function across multiple tumor models. Taken together, these data demonstrate the potential of anti-OX40/anti-CTLA-4/ibrutinib as a triple therapy to improve the efficacy of immunotherapy.

4.1. Introduction

Generating effective $CD8^+$ T-cell responses is critical to support the efficacy of cancer immunotherapies, such as immune checkpoint blockade (ICB) (352). ICB can generate robust tumor-specific immunity in patients, leading to improved long-term survival (265). However, the therapeutic efficacy of ICB has been limited to a subset of patients, highlighting the need to understand the underlying mechanisms by which they function to inform the design of rational combinations. Blockade of the inhibitory checkpoint receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) effectively releases the brakes on T cells through enhanced priming and inhibition of regulatory $FoxP3^+$ $CD4^+$ T cells (Treg) (353). A CTLA-4-specific mAb, ipilimumab, was the first checkpoint inhibitor to garner FDA approval and markedly improves 5-year survival in patients with metastatic melanoma (265, 354, 355). Immune checkpoint inhibitors targeting the negative regulatory molecule programmed cell death protein 1 (PD1, also known as CD279) and its ligand PD-L1 (also known as B7-H1 and CD274) are approved for the treatment of a variety of cancer types including melanoma, non-small cell lung cancer, Hodgkin's Lymphoma, renal cell carcinoma, and others (356, 357). Alternatively, agonist mAbs targeting co-stimulatory members of the tumor necrosis factor receptor (TNFR) family, including OX40 (also known as CD134), 4-1BB (also known as CD137) and CD27, can boost T-cell responses to augment antitumor immunity (358). Specifically, agonist OX40-specific mAb therapy enhances T-cell proliferation, survival, cytokine production, and the generation of long-lived memory T cells (359, 360).

Combination therapy with anti-CTLA-4 and anti-PD1 elicited robust tumor regression in preclinical and clinical studies leading to its FDA approval for treating metastatic melanoma and renal cell carcinoma (361), highlighting the potential of combination immunotherapy. Alternatively, combining ICB with co-stimulatory receptor stimulation enhances antitumor immunity in numerous preclinical models. For example, we found that anti-OX40/anti-CTLA-4 combination therapy significantly enhances overall treatment efficacy compared to either

monotherapy through the generation of effector T cells with increased proliferation and expression of effector cytokines (granzyme B/IFNγ) and the T-box transcription factor Eomesodermin (Eomes) (296, 362). Eomes and T-bet both drive the generation of effector cytokines and cytotoxic molecules, but T-bet preferentially drives the formation of short-lived effector cells (SLEC) and is sufficient for the effector function of T cells, whereas Eomes is required for the generation of longer-lived memory precursor effector cells (MPEC) through increased expression of anti-apoptotic molecules and survival (363-365). Increased Eomes expression in $CD8^+$ T cells is also associated with better clinical responses to immunotherapy (366, 367), suggesting that inducing Eomes may further improve outcomes.

Eomes expression in $CD8^+$ T cells is regulated by multiple signaling pathways triggered by T-cell activation and differentiation, including TCR signaling, NF-κB signaling, and interleukin receptor signaling (368, 369). TCR signaling through Zap70 and Lck phosphorylates interleukin-2–inducible T-cell kinase (ITK) to induce phospho-IRF4 expression, which in turn represses Eomes (370). ITK/IRF4-mediated repression of Eomes is dictated by the strength of TCR signaling since high affinity TCR binding leads to greater Eomes repression (371). We hypothesized that $Eomes^{hi}$ $CD8^+$ T cells were a critical component of anti-OX40/anti-CTLA-4 therapy and that the efficacy of anti-OX40/anti-CTLA-4 therapy could be enhanced by pharmacological blockade of ITK, which would enhance $CD8^+$ T cell–specific Eomes expression.

In the current study, we demonstrated that combined anti-OX40/anti-CTLA-4 therapy induced a unique population of $Eomes^{hi}$ $CD8^+$ T cells, which resembled central memory cells with high levels of proliferation and effector function, as defined by RNA and protein expression. Furthermore, ITK inhibition with the FDA-approved drug ibrutinib further enhanced Eomes expression in the presence of anti-OX40/anti-CTLA-4 therapy, resulting in significantly improved effector T-cell function, tumor regression, and survival in comparison to anti-OX40/anti-CTLA-4

alone. These data suggest that ITK inhibition enhances the efficacy of combination immunotherapy for the treatment of cancer.

4.2. Materials and Methods

Mice

Wild-type BALB/c, C57BL/6, Eomes$^{fl/fl}$, CD8^{cre}, and Nur77^{GFP} reporter mice were purchased from Jackson Laboratory. Eomes-GFP mice (C57BL/6 background) were kindly provided by Dr. John Wherry, University of Pennsylvania and Dr. Joseph Sun, Memorial Sloan Kettering Cancer Center. Eomes conditional knockout mice were bred in our facility by crossing Eomes$^{fl/fl}$ mice with CD8^{cre} mice (all on the C57BL/6 background). All mice were housed under specific pathogen-free conditions in the Providence Portland Medical Center (Portland, OR) vivarium animal facility. All experimental procedures were approved by the Providence Portland Medical Center Institutional Animal Care and Use Committee (IACUC) and were performed under the NIH Guide for the Care and Use of Laboratory Animals.

Cell lines and Cell-culture Conditions and Reagents

TRAMP-C1 prostate adenocarcinoma, MCA-205 fibrosarcoma, and 4T1 mammary carcinoma cell lines were obtained between 2009 and 2011 from Dr. Andrew Weinberg (TRAMP-C1, MCA-205) and Dr. Emmanuel Akporiaye (4T1). Cell lines have not been re-authenticated within the past year. All cell lines were maintained in 10% cRPMI and verified *Mycoplasm*a-free (MycoAlert Mycoplasma Testing Kit; Lonza) within 6 months of use.

Tumor Challenge and Treatments

TRAMP-C1 prostate adenocarcinoma cells (1×10^6) were injected subcutaneously into the right flank of naïve male C57BL/6 mice. MCA-205 fibrosarcoma cells (1×10^6) were injected subcutaneously into the right flank of naïve female C57BL/6 mice. 4T1 mammary carcinoma cells (5×10^4) were injected into the mammary fat pad of naïve BALB/c mice. Tumor-bearing mice were measured for tumor growth every 2 to 3 days by tumor area using microcalipers and were sacrificed when tumor area exceeded 175 mm^2. Tumor-bearing animals were treated with

200 μg rat IgG (Sigma; I4131), and/or 250 μg anti-OX40 (clone OX86; BioXCell; BE0031), and/or 200 μg anti-CTLA-4 (clone 9D9; BioXCell; BE0164), and/or 200 μg anti-PD-1 (clone RMP1-14, BioXCell; BE0146) (all ip). All mAbs were verified to be endotoxin-free. Ibrutinib (Cat S2680 SelleckChem) treatments were administered at a dose of 6 mg/kg (ip) as previously described (372). For tumor growth and survival experiments, tumor-bearing mice were treated with anti-OX40 on days 8, and 12; and anti-CTLA-4 was given on days 8, 10, and 12. Control mice were treated with rat IgG on days 8, and 12. Ibrutinib was dosed on days 8, 10, 12, 15, 17, and 19 for tumor growth and survival experiments. For tissue harvest experiments, treatments were initiated when average tumor size exceeded 60mm^2, following the same schedule as the survival experiments. FTY720 (Sigma; SML0700) was dosed at 1 mg/kg (ip) every three days starting one week prior to immunotherapy.

Tissue Isolation

Tumors and lymph nodes (inguinal, axillary, and brachial) were harvested from tumor-bearing mice one week after the initiation of treatment. Lymph nodes were mechanically fragmented between two frosted slides and filtered through nylon mesh for flow cytometry staining. Tumors were mechanically fragmented into small pieces and digested with 5 mg/ml DNase (Sigma; 4536282001) and 1 mg/ml collagenase (Sigma; C8051) in serum-free RPMI (Lonza; 12-702Q). Digested tumor suspension was filtered through nylon mesh and stained for flow cytometry as described below.

Flow Cytometry/FACS Analysis

Single-cell suspensions of blood, lymph node, and tumor-infiltrating lymphocytes (TIL) were stained with surface antibodies in flow cytometry wash buffer for 30 minutes at 4°C with surface antibodies: CD45 BV421(Biolegend; Clone 30-F11; 103134), CD4 BV605 (Biolegend; Clone

RM4-5; 100548), CD8 BV785 (Biolegend; Clone 53-6.3; 100750), CXCR3 AF488 (Biolegend; Clone CXCR3-173; 126542, PD-1 PE-Cy7 (Biolegend; Clone 29F.1A12; 135216), LAG-3 PerCP-Cy5.5 (Biolegend; Clone C9B7W; 125212), TIM-3 PE (R&D Systems; Clone NA; FAB1529P), CD11b BV785 (Biolegend; Clone M1/70; 101243), Ly6C PerCP-Cy5.5 (Biolegend; Clone HK1.4; 128012), Ly6G FITC (BD Biosciences; Clone 1A8; 551460), MHC II eF450 (eBioscience; Clone M5/114.15.2; 48-5321-82), PD-L1 BV711 (Biolegend; Clone 10F.9G2; 124319), CD11c APC (eBioscience; Clone N418; 17-0114-82), CD86 PE-Cy7 (Biolegend; Clone PO3; 105116), CD44 APC (eBioscience; Clone IM7; 17-0441-83), CD62L AF700 (Biolegend; Clone MEL-14; 104426). Cells were fixed and permeabilized using the Thermofisher FoxP3 intracellular staining buffer kit (00-5523-00) for intracellular staining as per the manufacturer's instructions. Cells were stained intracellularly for 30 minutes at 4°C in accordance with the manufacturer's protocol. For intracellular cytokine staining, cells were incubated in 96-well U-bottom plates in 10% complete RPMIand 2.5 μl/ml Golgi Block/Brefeldin A (BD Biosciences; 51-2301KZ). 10% complete RPMI consists of RMPI (Lonza; 12-702Q), 10% Fetal Bovine Serum (Lonza;14-501F), 1% HEPES Bummer from 1M stock (Lonza; 17-737E), 1% Sodium Pyruvate Solution from 100mM stock (Lonza; 13-115E), 1% Non-essential amino acids (Lonza; 13-114E), 1% Pen/Strep/Glutamine (Invitrogen; 10378016), 0.005% 2-Mercaptoethanol (Sigma Aldrich; M3148-100ml). Cells were stimulated by 2 μg/ml plate bound anti-CD3 (BioXcell; BE0001-1-5MG) and 5 ug/ml anti-CD28 (BioXcell; BE000150105MG) in suspension. Following a 4hr stimulation at 37 °C in a 5% CO_2 incubator, cells were stained at 4 °C for 30 minutes with: Eomes eF660 (eBioscience; Clone Dan11mag; 50-4875-82), Granzyme A PE-Cy7 (eBioscience; Clone GzA-3G8.5; 25-5831-82), IFN-γ PE (eBioscience; Clone XMG1.2; 12-7311-82), TNF-α PE-Cy7 (eBioscience; Clone TN3-19.12; 25-7423-82), Ki-67 FITC (BD Biosciences; Clone B56; 556026), T-bet eF660 (eBioscience; Clone eBio4B10; 50-5825-82) , pITK PE (Biolegend; Clone A16064A; 646904), IRF4 PE-Cy7 (Biolegend; Clone IRF4.3E4; 646414),

CXCL9 AF647 (Biolegend; Clone MIG-2F5.5; 515606), CCL5 PE (Biolegned; Clone 2E9/CCL5; 149104). Stained cells were analyzed using an LSR II flow cytometer running Diva (BD Biosciences) software and the data was processed using FlowJo 10 software (BD Biosciences). For lymph node and TIL, cells were counted and quantified for viability using the Guava cell counter as per the manufacturer protocol.

Tetramer Staining

PE-conjugated H-2D^{b} tetramer to SPAS-1 (STHVNHLHC) peptide and APC-conjugated H-2L^{d} tetramer to AH1-A5 (SPSYAYHQF) peptide was provided by the NIH Tetramer Core Facility. Cells were surface stained with tetramer (1μl per 10^6 cells) in flow cytometry wash buffer for 15 minutes at room temperature prior to surface antibody staining, as described above. Flowcytometry wash buffer consists of PBS, 0.5% FBS (Peak Serum; PS-FB4), 0.4% EDTA (Alfa Aesar; 60-00-4, and 0.05% NaN_3 Sigma Aldrich; 26628-22-8).

CTL Assay

MCA-205 tumor-bearing mice were treated with anti-OX40/anti-CTLA-4 therapy starting on day 12 and then tumors were harvested 7 days later. Tumors were pooled and processed for surface cell staining and sorting of TIL. Eomes-GFP^{+} and Eomes-GFP^{-} CD8^{+} T cells were sorted from the LN and co-cultured with MCA-205 tumor cells in a 96-well plate at an effector:target ratio of 30:1 along with Caspase-3/7 green dye (Essen Bioscience; 4440) following the manufacturer's protocol and then incubated in an Essen Biosciences Incucyte imager within a CO_2 incubator. Images were acquired 6 hours later and used to determine the percent cell lysis as a calculation of Cas3/7^{+} MCA-205 cells/total MCA-205 cells (*100).

In vitro Chemokine Assay

TRAMP-C1 and 4T1 cells were incubated for 24 hours +/- 0.3 μM ibrutinib (SelleckChem; S2680) and stained intracellularly forCXCL9 AF647 (BioLegend; Clone MIG-2F5.5; 515606), and CCL5 PE (BioLegend; Clone 2E9/CCL5; 149104). As a control, WT C57BL/6 splenocytes were incubated for 24 hours +/- 5 μg/ml anti-CD3 (BioXcell; BE0001-1-5MG) and stained intracellularly for CXCL9 and CCL5.

RNA Expression Analysis

MCA-205 tumor-bearing mice were treated with anti-OX40/anti-CTLA-4 therapy starting day 12 and tumors were harvested on day 19 post-implant and then pooled and processed for surface cell staining. Eomes-GFP$^+$ and Eomes-GFP$^-$ CD8$^+$ T cells were sorted from the tumor using a FACS Aria II flow cytometry cell sorter into RNA preserving TRIzol buffer (Qiagen). RNA was isolated from the sorted cells using a Qiagen RNeasy mini kit (Qiagen; 74104). Three replicate samples were hybridized to the Affymetrix Mouse 430 2.0 GeneChip array. Expression analysis was performed using Affymetrix Transcriptome Analysis Console (TAC) software (ThermoFisher). To identify cell types with similar transcriptional profiles as Eomes+ cells in an unbiased manner, we performed gene set enrichment analysis (GSEA) using GSEA v3.0 (Broad Institute). We compared our gene sets from Eomes- or Eomes+ cells against all immunologic signature genes sets from the molecular signatures database, the C7 collection (MSigDB v6.2). We found significant associations with genes down in effector vs. memory CD8$^+$ T cells (GSE9650_EFFECTOR_VS_MEMORY_CD8_TCELL_DN).

TEC Kinase Inhibitor Assay

CD8$^+$ T cells from the spleens of Nur77-GFP mice were isolated using a CD8 bead isolation kit (ThermoFisher; 11417D) from a single-cell suspension of splenocytes. Isolated CD8$^+$ T cells were stimulated with plate-bound anti-CD3 in 96-well U-bottom plates for 24 hrs. 10% complete

RPMI media was added containing no drug, ibrutinib (Selleckchem; S2680), acalabrutinib (Selleckchem; S8116), or BMS-509733 (Sigma; 419820) at concentrations ranging from 0.03 to 10 μM. Cells were stained 24 hours post-stimulation for flow cytometry analysis, as described above.

Statistical Analysis

Statistical significance was determined using unpaired t-test (for comparisons between 2 groups, two-tailed), one-way ANOVA (for comparisons of 3 or more groups), and Kaplan-Meier survival where appropriate using GraphPad Prism software (GraphPad); *p* value less than 0.05 was considered significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

4.3. Results

4.3.1. Combining anti-OX40 and anti-CTLA-4 increases CD8⁺ T cell–specific Eomes expression.

MCA-205 (sarcoma) or TRAMP-C1 (prostate adenocarcinoma) cells were implanted into cohorts of wild-type C57BL/6 mice. Eight days following tumor implant, mice were treated with rat IgG (control), agonist anti-OX40, CTLA-4 blockade, or combined anti-OX40/anti-CTLA-4 therapy (Fig. 1A). Monotherapy did not significantly reduce tumor growth or increase survival. However, combined anti-OX40/anti-CTLA-4 therapy led to complete tumor regression in 63% of MCA-205 tumor–bearing mice (Fig. 2A-B, 1B), confirming our previous finding (296). Combined anti-OX40/anti-CTLA-4 therapy significantly increased $CD8^+$ T cell–specific Eomes expression in the lymph nodes in MCA-205 (Fig. 2C-E) and TRAMP-C1 (Fig. 2F) tumor–bearing mice as compared with monotherapy-treated controls. Eomes expression was not induced in the $CD4^+$ T-cell compartment (Fig. 2D) and no changes were observed in NK cells (Fig. 1C). The strong synergistic induction of $Eomes^{hi}$ $CD8^+$ T cells that we observed following anti-OX40/anti-CTLA-4 therapy occurred primarily in the lymph nodes; the same effect was not observed in the tumor (Fig. 1D-E). Although OX40-mediated therapy has demonstrated therapeutic potential in combination with anti-PD-1 (373, 374), combined anti-PD-1/anti-OX40 therapy did not induce the same robust Eomes expression as anti-OX40/anti-CTLA-4 (Fig. 2G).

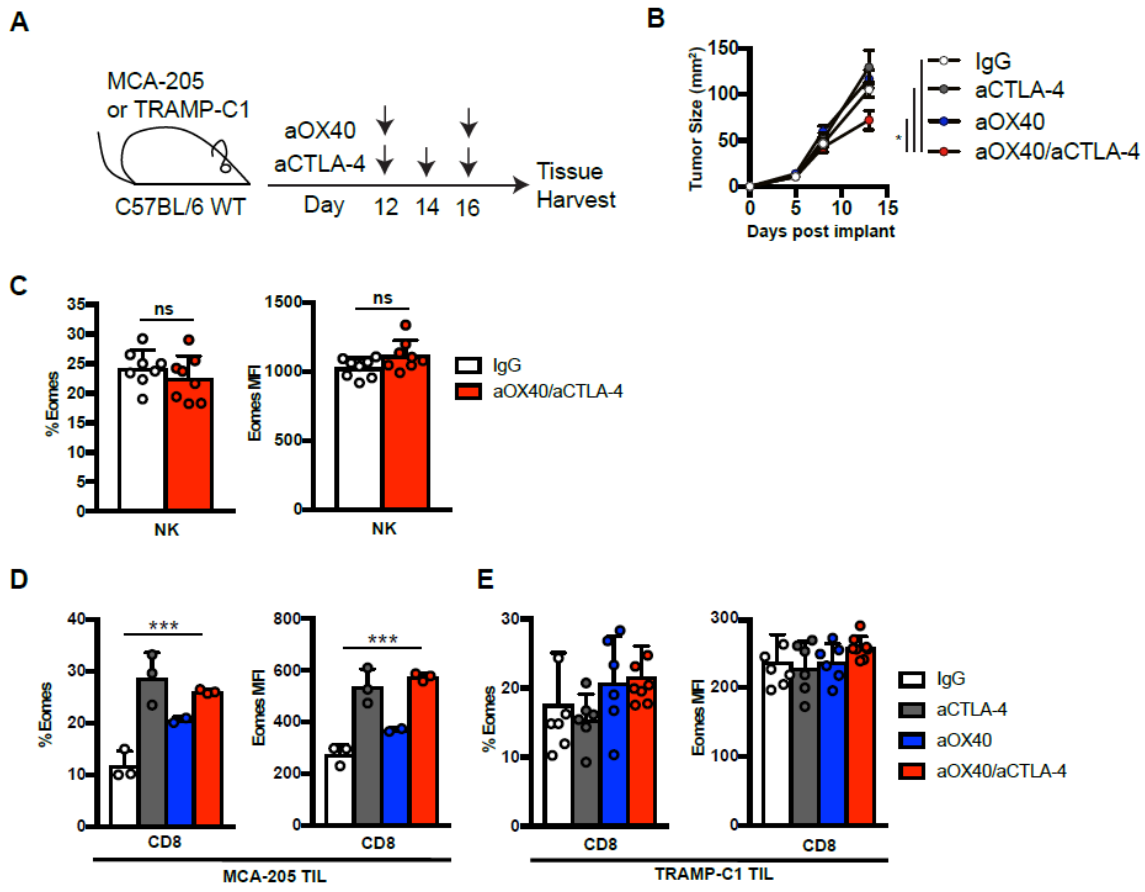


Figure 1. CD8⁺ T cell-specific Eomes expression in the tumor following combination αOX40/αCTLA-4 therapy. A) Treatment schema (tumor-bearing mice were treated as in Fig. 1). B) Average tumor growth C) The extent of Eomes^{hi} NK Cells (%) and MFI within the lymph node was determined in MCA-205. D-E) The extent of Eomes^{hi} CD8^{+} T cells (% and MFI) within the tumor was determined in (C) MCA-205 and (D) TRAMP-C1 tumor-bearing mice. Graphs represent the mean+/-SD from n=2-6/group. ***P<0.001

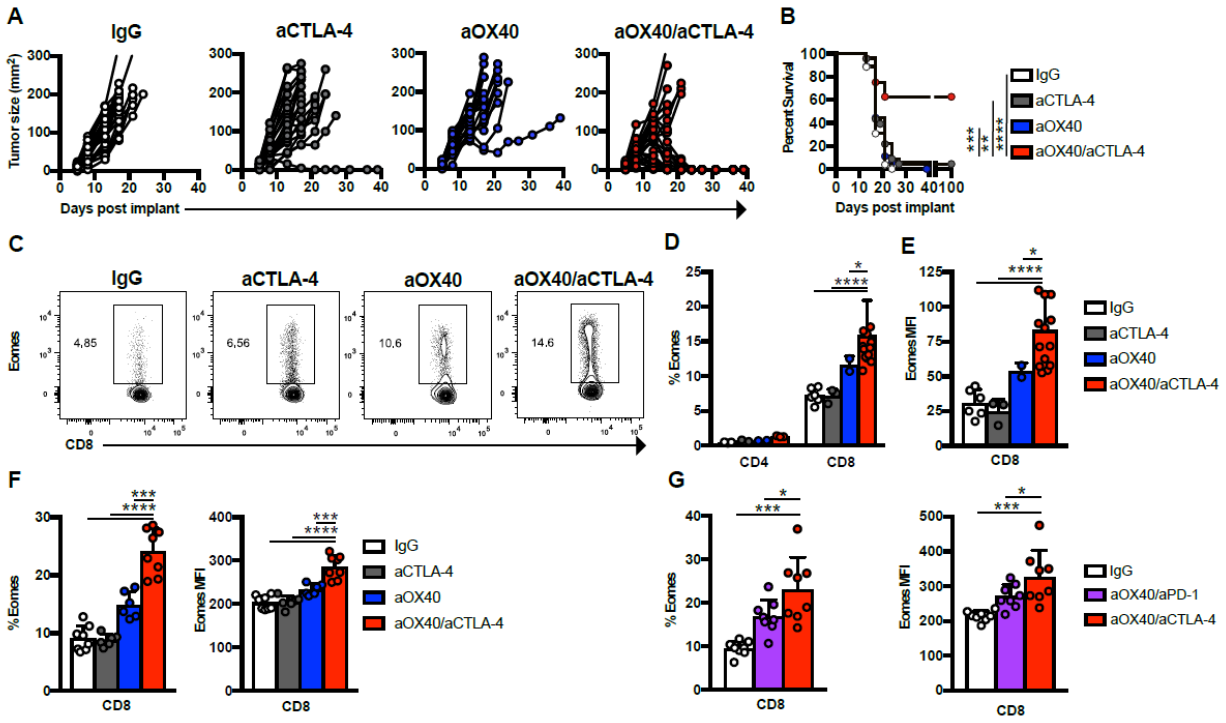


Figure 2. Combining agonist anti-OX40 therapy with CTLA-4 blockade enhances antitumor immunity and CD8⁺ T cell–specific Eomes expression. A-B) MCA-205 tumor cells were implanted subcutaneously in wild-type or Eomes-GFP mice (C57BL/6). Eight days later, tumor-bearing mice were treated with control (IgG; days 8, 12), anti-OX40 (days 8, 12), anti-CTLA-4 (days 8, 10, 12), or the combination of anti-OX40 and anti-CTLA-4. A) Tumor growth (area; mm^2) and B) survival of tumor-bearing mice was determined. Mice were sacrificed when tumor area $\geq$175mm^2. C-G) For lymph node and tumor harvest, treatment was initiated on day 12 using the same treatment schedule as described. Tissues were harvested on day 19. C-D) The % and (E) MFI of Eomes expression in the lymph nodes (LN) of Eomes-GFP mice was determined by flow cytometry 7 days post-treatment. F) The extent of Eomes expression (% and MFI) in LN of TRAMP-C1 tumor-bearing mice was determined as in (A). G) % and MFI of Eomes expression of CD8⁺ T cells in the LN of MCA-205 tumor bearing mice following the same schedule as on (A) with additional anti-OX40/anti-αPD-1 cohort treatment on the same days as anti-CTLA-4 treatment. Graphs represent the mean+/-SD from n=5-12/group from 2 independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by one-way ANOVA or Kaplan-Meier survival test.

4.3.2. Effective anti-OX40/anti-CTLA-4 therapy requires CD8⁺ T-cell Eomes expression

Because the levels of Eomes^{hi} CD8⁺ T cells correlated with tumor regression following anti-OX40/anti-CTLA-4 and have been associated with improved clinical responses in patients (366, 367), we investigated whether Eomes^{hi} CD8⁺ T cells were necessary for the efficacy of

anti-OX40/anti-CTLA-4 therapy. We crossed CD8^{cre} mice with Eomes$^{fl/fl}$ transgenic mice to generate CD8^{+} T cell–specific Eomes-deficient conditional knockout mice (Eomes^{CKO}) (375, 376). Eomes expression was ablated in the CD8^{+} T-cell compartment without changes to the frequency of CD8^{+} T cells in the Eomes^{CKO} mice (Fig. 3A). There was no impairment of Eomes expression in NK cells in Eomes^{CKO} mice (Fig. 3B, 3C). Next, we implanted MCA-205 tumors into Eomes^{CKO} and WT mice and assessed tumor growth and response to immunotherapy. There was no difference in tumor growth between Eomes^{CKO} and WT mice treated with IgG (Fig. 3D). However, the efficacy of anti-OX40/anti-CTLA-4 therapy was significantly abrogated in Eomes^{CKO} mice compared with WT mice (Fig. 3E-F). Similar results were obtained in the TRAMP-C1 model (Fig. 4A-B). Anti-OX40/anti-CTLA-4 treated Eomes^{CKO} mice were found to exhibit reduced granzyme A expression compared to WT mice (Fig. 3G). Together, these data indicate that Eomes^{hi} CD8^{+} T cells are required for the full therapeutic efficacy of anti-OX40/anti-CTLA-4 therapy.

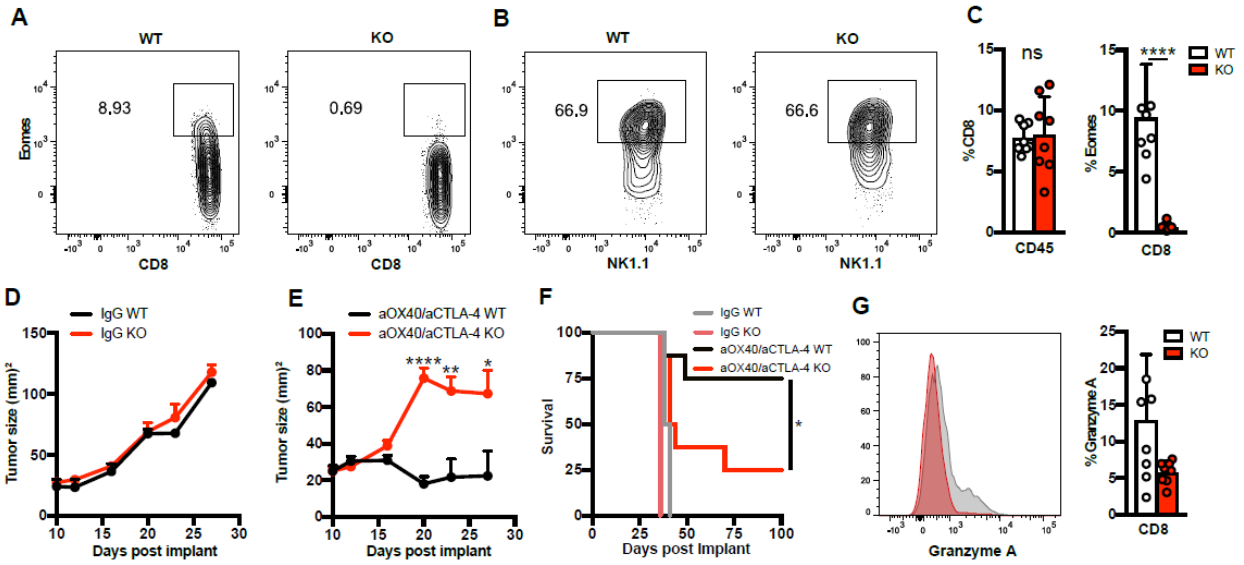


Figure 3. CD8^{+} T cell–specific Eomes-deletion abrogates the efficacy of anti-OX40/anti-CTLA-4 therapy. A-G) MCA-205 tumor-bearing wild-type or CD8^{cre}Eomes$^{fl/fl}$ mice (C57BL/6) were treated with control (IgG; days 8, 12), anti-OX40 (days 8, 12), anti-CTLA-4 (days 8, 10, 12), or the combination of anti-OX40/anti-CTLA-4 mAbs. A-B) The extent of Eomes expression in (A) CD8^{+} T cells and (B) NK cells was determined by flow cytometry. C) Cumulative frequency of CD8^{+}CD45+ T cells and CD8^{+} T cell–specific Eomes expression in the peripheral

blood one-week post-treatment. D-F) Tumor area (mm^2) of (D) control (IgG) or (E) anti-OX40/anti-CTLA-4-treated mice and F) survival were determined at the indicated time points. G) $CD8^+$ T cell–specific granzyme A expression in the peripheral blood was determined one-week after the initiation of therapy. Graphs represent the mean+/-SD from n=5-8/group from 2 independent experiments. *P<0.05, **P<0.01, ****P<0.0001 by one-way ANOVA (A-E) or Kaplan-Meier survival test (F).

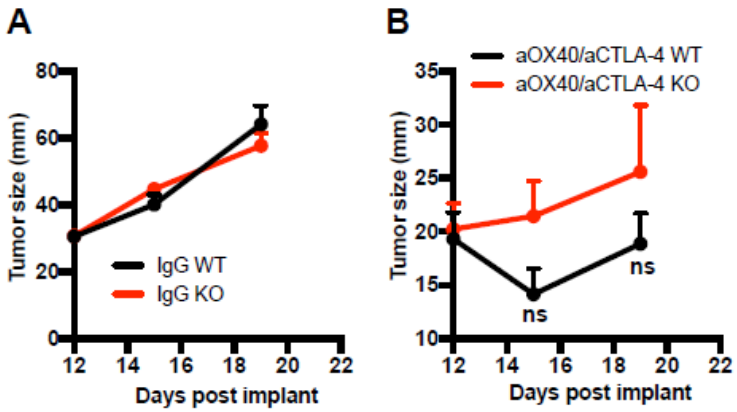


Figure 4. Tumor growth kinetics of TRAMP-C1 in WT and $Eomes^{CKO}$ mice following combination αOX40/αCTLA-4 therapy. A-B) MCA-205 tumor-bearing wild-type or $CD8^{cre}Eomes^{fl/fl}$ mice (C57BL/6) were treated with control (IgG; days 8, 12), αOX40 (days 8, 12), αCTLA-4 (days 8, 10, 12), or the combination αOX40/αCTLA-4 mAbs. Tumor area (mm^2) of (A) control (IgG) or (B) αOX40/αCTLA-4-treated mice was determined at the indicated time points.

4.3.3. $Eomes^{hi}CD8^+$ T cells are induced in the lymph node prior to trafficking to the tumor

The expansion of $Eomes^{hi}$ $CD8^+$ T cells by anti-OX40/anti-CTLA-4 was primarily observed in the lymph nodes, suggesting an important role for T-cell priming in this process. TRAMP-C1 tumor–bearing mice were treated starting at day 3 with FTY720, a small molecule agonist of sphingosine-1-phosphate receptor (S1PR) that blocks lymph node egress, anti-OX40/anti-CTLA-4 (starting at day 12), or the combination of FTY720 and anti-OX40/anti-CTLA-4. Tumors and lymph nodes were harvested on day 19 (Fig. 5A). As expected, circulating numbers of $CD8^+$ T cells were significantly reduced following FTY720 treatment (Fig. 5B). The frequencies of $Eomes^{hi}CD8^+$ T cells were increased in the lymph nodes but reduced in the tumors post-FTY720 and anti-OX40/anti-CTLA-4 treatment (Fig. 5C). Similar changes were

observed in GzmA^{+}CD8^{+} T cells (Fig. 5D). Together, these data suggest that anti-OX40/anti-CTLA-4 therapy drives the priming of effector Eomes^{hi} CD8^{+} T cells within the lymph nodes of tumor-bearing mice.

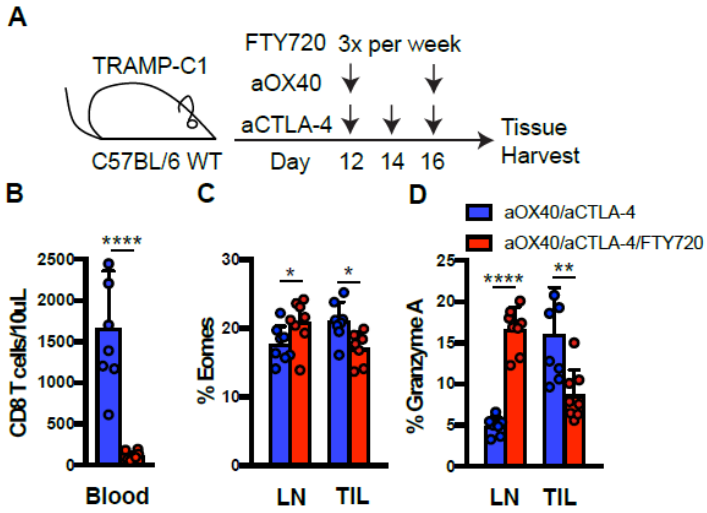


Figure 5. FTY720 treatment sequesters effector Eomes^{hi} CD8^{+} T cells within the lymph nodes and reduces their trafficking to the tumor. A) Treatment schema. B) TRAMP-C1 tumor–bearing mice were treated with anti-OX40 (days 12, 16) and anti-CTLA-4 (days 12, 14, 16) +/- FTY720 (3 times per week for 2 weeks). Total number of CD8^{+} T cells (blood) were quantified by flow cytometry one-week post-treatment. C-D) The extent of (C) Eomes and (D) granzyme A expression in the lymph nodes and tumors was determined one week after the initiation of therapy. Graphs represent the mean+/-SD from n=7-8/group from 2 independent experiments. *P<0.05, **P<0.01, ****P<0.0001 by unpaired t-test

4.3.4. Eomes^{hi} CD8^{+} T cells exhibit a distinct transcriptional profile.

To understand the functional role of Eomes^{hi} CD8^{+} T cells in regulating anti-OX40/anti-CTLA-4 responses, we compared the transcriptional profile of Eomes^{hi} vs. Eomes^{lo} CD8^{+} T cells following anti-OX40/anti-CTLA-4 therapy. Tumor-infiltrating Eomes-GFP^{+} and GFP^{-} CD8^{+} T cells were sorted for mRNA analysis following anti-OX40/anti-CTLA-4 therapy of Eomes-GFP mice (Fig. 6A). Hierarchical clustering revealed distinct phenotypic populations with 378 (P<0.05) differentially expressed transcripts (Fig. 6B). We also employed GSEA to compare Eomes^{hi} CD8^{+} T cells to known cell types. The Eomes^{hi} CD8^{+} T-cell profile correlated most closely with

that of memory T cells, suggesting that Eomes^{hi} CD8^{+} T cells have a more memory-like than effector-like phenotype (Fig. 6C).

We next compared the relative expression of Eomes^{hi} CD8^{+} T cell–associated genes to naïve, effector, and effector memory CD8^{+} T-cell populations responding to acute LCMV infection available in the Gene Expression Omnibus (GEO) database (Fig. 6D-G) (377). We found that, with the exception of PD1 expression, our Eomes^{hi} CD8^{+} T-cell phenotype most closely resembled effector memory cells (Fig. 6D). For example, TCF1 was increased in Eomes^{hi} CD8^{+} T cells to similar levels as observed in effector memory cells, suggesting self-renewal memory formation (378) (Fig. 6E). We also observed increased CD62L and granzyme A expression, which was similar to the levels seen in the effector memory population (Fig. 6F-G). Analysis of CD62L and CD44 showed that Eomes was most highly expressed in the central memory (CD44^{hi}, CD62L^{hi}) and effector/effector memory (CD44^{hi}, CD62L^{lo}) CD8^{+} T-cell population in the lymph nodes and tumors following anti-OX40/anti-CTLA-4 therapy (Fig. 7A-B). Thus, Eomes^{hi} CD8^{+} T cells induced by anti-OX40/anti-CTLA-4 therapy have a phenotypic profile similar to central memory CD8^{+} T cells with strong effector potential. In addition, Eomes^{hi} CD8^{+} T cells expressed significantly lower levels of critical inhibitory and exhaustion markers including PD1, LAG-3, and TIM-3 compared with Eomes^{lo} CD8^{+} T cells (Fig. 6H). We confirmed these markers by protein expression, including verification of checkpoint inhibitors (PD1, TIM-3, LAG-3), proliferation (Ki-67), and effector cytokine production (granzyme A, polyfunctional IFNγ/TNFα population) (Fig. 6I).

High granzyme A expression among Eomes^{hi} compared with Eomes^{lo} CD8^{+} T cells following anti-OX40/anti-CTLA-4 therapy raised concern over whether they might be functionally exhausted, as increased Eomes expression has been associated with the development of CD8^{+} T-cell exhaustion (61, 379). Therefore, the cytolytic activity of Eomes^{hi} and Eomes^{lo} CD8^{+} T cells isolated from anti-OX40/anti-CTLA-4-treated tumor-bearing mice was determined. Eomes^{hi} CD8^{+} T cells trended toward lysing more target cells (tumor) compared with Eomes^{lo} cells (Fig.

8), demonstrating that Eomes^{hi} CD8^{+} T cells retain comparable or slightly increased cytotoxic function along with producing higher levels of granzyme A.

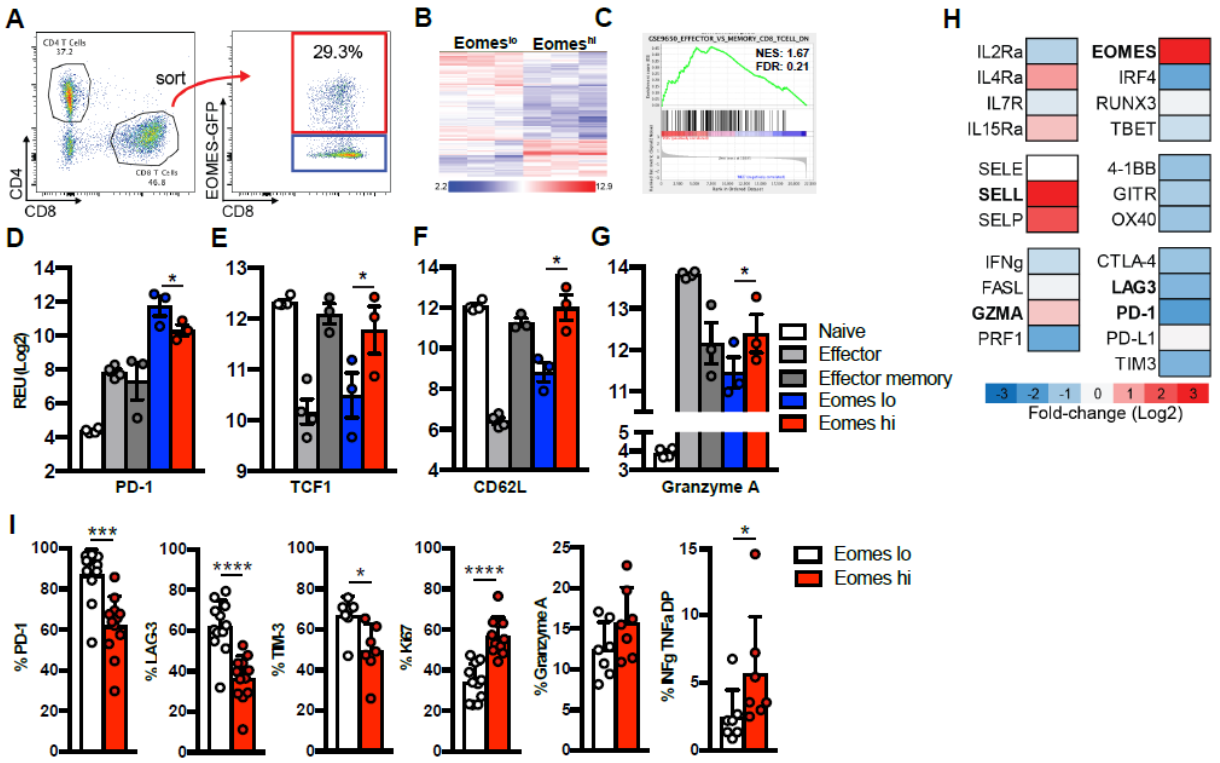


Figure 6. Transcriptional profiling of Eomes^{hi} CD8^{+} T cells following anti-OX40/anti-CTLA-4 therapy. A) MCA-205 tumor–bearing wild-type or Eomes-GFP mice (C57BL/6) were treated with control (IgG; days 8, 12), anti-OX40 (days 8, 12), anti-CTLA-4 (days 8, 10, 12), or anti-OX40/anti-CTLA-4 mAbs and then eight days later, Eomes^{hi} and Eomes^{lo} CD8^{+} T cells were sorted from the tumor for mRNA isolation. B) Heatmap of differentially expressed transcripts. C) GSEA analysis comparing Eomes-associated genes (increased-left; decreased-right) against immunologic signature gene sets from the molecular signatures database (MSigDB), an online repository of annotated gene sets. D-G) Comparison of selected effector/memory transcripts in Eomes^{hi} and Eomes^{lo} CD8^{+} T cells to those in naïve, effector, and effector memory CD8^{+} T cells isolated following LCMV infection from GEO dataset GSE61927. H) Heatmap depicting fold-change (mRNA) between Eomes^{hi} / Eomes^{lo} for selected genes. I) Expression of the indicated proteins on Eomes^{hi} and Eomes^{lo} CD8^{+} T cells was determined by flow cytometry. D-G) Graphs represent the mean+/-SD from n=3-4/group from one experiment. I) Graphs represent the mean+/-SD from n=7-10/group. *P<0.05, ***P<0.001, ****P<0.0001 by one-way ANOVA (D-G), or unpaired t-test (I)

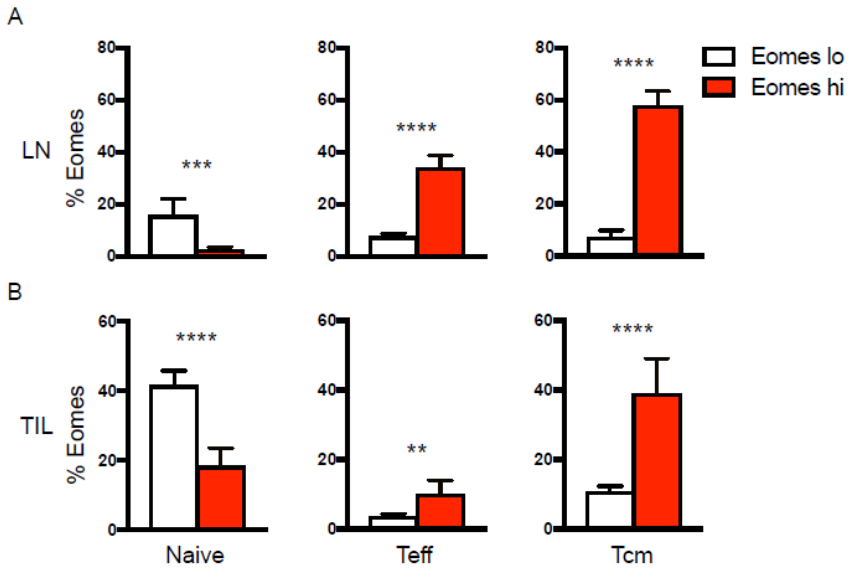


Figure 7. Eomes expression in CD8⁺ T cell phenotypes following combination αOX40/αCTLA-4 therapy. A-B) TRAMP-C1 tumor-bearing wild-type mice (C57BL/6) were treated with αOX40 (days 12, 16) and αCTLA-4 (days 12, 14, 16) mAbs. LNs and tumors were harvest on day 19 for flow cytometry analysis. Eomes expression was determined in naïve ($CD44^-$ $CD62L^+$), Teff ($CD44^+$ $CD62L^-$), and Tcm ($CD44^+$, $CD62L^+$) populations in the A) LN, and B) TIL. Graphs represent the mean+/-SD from n=7/group. **P<0.01, ***P<0.001, ****P<0.0001.

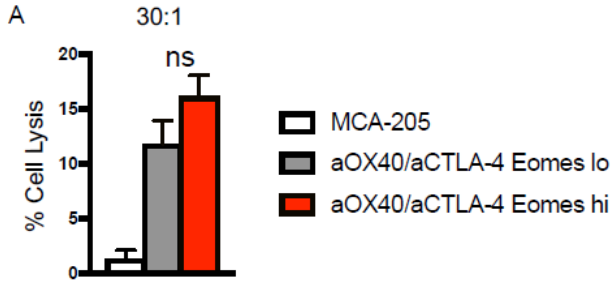


Figure 8. $Eomes^{hi}$ $CD8^+$ T cells are effective tumor killers following combination αOX40/αCTLA-4 therapy. A) MCA-205 tumor-bearing Eomes-GFP mice (C57BL/6) were treated with αOX40 (days 12, 16) and αCTLA-4 (days 12, 14, 16) mAbs. LNs were harvested on day 19 for sorting Eomes-GFP^+ and Eomes-GFP^- populations. Sorted cells were plated onto MCA-205 cells at an effector:target ratio of 30:1. Percent cell lysis was determined at 6 hours as a calculation of Cas3/7 positive MCA-205 cells/total MCA-205 cells (*100). Graphs represent the mean+/-SEM from n=6/group.

4.3.5. Use of an ITK inhibitor does not impair Eomes expression in CD8⁺ T cells.

Considering that CD8⁺ T cell–specific deletion of Eomes abrogated the efficacy of anti-OX40/anti-CTLA-4 therapy, we next asked whether enhancing CD8⁺ T-cell Eomes expression would augment treatment efficacy. Since CD8⁺ T cells deficient in ITK or IRF4 express high levels of Eomes, even in the absence of stimulation (371), we hypothesized that pharmacological inhibition of ITK may further increase Eomes expression following anti-OX40/anti-CTLA-4 therapy. Ibrutinib is the only FDA-approved drug with activity against Bruton's tyrosine kinase (BTK), as well as ITK (380). Because ITK plays an important role in TCR signaling and the downstream regulation of Eomes, we investigated whether ITK inhibition could impair TCR signaling in CD8⁺ T cells *in vitro*. CD8⁺ T cells are not completely dependent on ITK because they express a redundant kinase (RLK) that activates NFAT, AP-1, and NF-κB in the absence of ITK (371, 381). To investigate the impact of ibrutinib on TCR signaling, we utilized the Nur77-GFP mouse model, which expresses GFP in proportion to the extent of TCR stimulation, independent of inflammatory stimuli (382). Naive Nur77-GFP CD8⁺ T cells were stimulated in the presence of ibrutinib (ITK/BTK-specific; ITK/BTKi), BMS-509733 (ITK-specific; ITKi), or acalabrutinib (BTK-specific; BTKi). Ibrutinib did not inhibit Nur77-GFP expression at or below clinical concentrations (0.3 μM), indicating that the clinical does not disrupt TCR signaling (Fig. 9A-B). However, we did observe a reduction in Nur77-GFP expression at ibrutinib concentrations higher than 0.3 μM (Fig. 9C). BTK inhibition facilitated by acalabrutinib had no effect on Nur77 expression, however the ITK-specific inhibitor (BMS-509744) inhibited Nur77 expression above 0.3 μM (Fig. 9D). Neither BTKi nor ITKi affected cell viability following 24-hours of co-culture (0.3 μM) (Fig. 9E). For these reasons, we sought to investigate whether ITK inhibition by ibrutinib in combination with anti-OX40/anti-CTLA-4 therapy would further enhance Eomes expression and therapeutic efficacy *in vivo*.

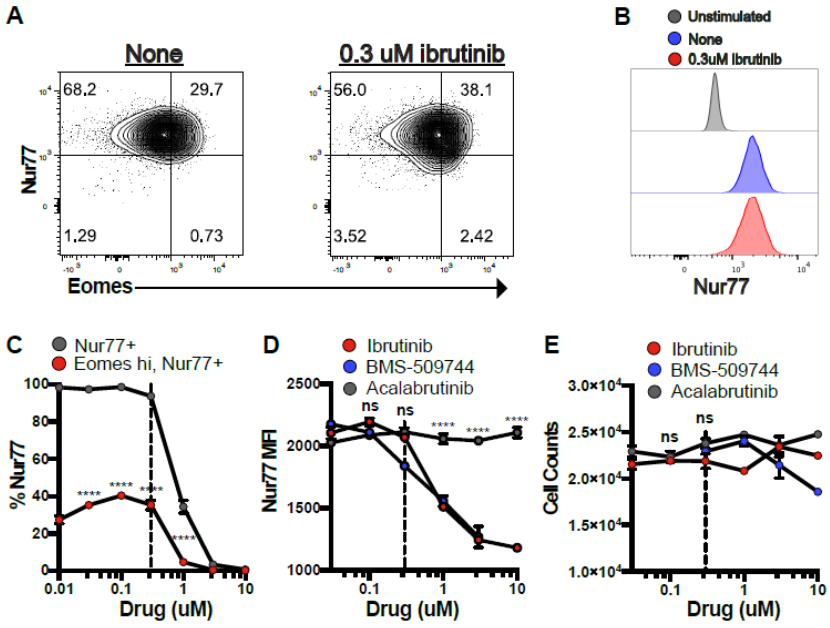


Figure 9. ITK inhibition (ibrutinib) does not inhibit TCR signaling at clinically relevant concentrations. Purified CD8⁺ T cells were purified from Nur77-GFP mice and then stimulated with anti-CD3 +/- ibrutinib (BTK/ITKi), BMS-509744 (ITKi), or acalabrutinib (BTKi) at the indicated concentrations. After 24 hours, cells were collected and the extent of Nur77 and Eomes expression and cell survival were determined. A-B) Representative A) plots and B) histogram of Nur77-GFP expression following CD8⁺ T cell stimulation +/- ibrutinib. C) The frequency of Nur77-GFP expression in all CD8⁺ T cells (gray) and Eomes^{hi} CD8⁺ T cells (red) following stimulation. C-E) Note: dotted line indicates clinically active concentration of ibrutinib (0.3 μM). D) Nur77-GFP MFI and E) total live CD8⁺ T-cell counts were determined following activation. Note: dotted line indicates clinically active concentration of ibrutinib (0.3 μM). Graphs represent the mean+/-SEM from triplicate wells from two independent experiments. ****P<0.0001 by unpaired t-test (C), or one-way ANOVA (D-E).

4.3.6. ITK inhibition synergizes with anti-OX40/anti-CTLA-4-therapy

TRAMP-C1 and 4T1 tumor–bearing mice were treated with rat IgG (control), ibrutinib, anti-OX40/anti-CTLA-4, or the combination of ibrutinib and anti-OX40/anti-CTLA-4 (referred to as triple therapy). Eomes expression was unaffected by ibrutinib monotherapy, but it was enhanced by anti-OX40/anti-CTLA-4 therapy. Notably, triple therapy significantly increased Eomes expression (% and MFI) compared with anti-OX40/anti-CTLA-4, suggesting that there is synergy between ibrutinib and anti-OX40/anti-CTLA-4 (Fig. 10A-B). Similar CD8⁺ T-cell Eomes

expression was also observed in the 4T1 model (Fig. 11A-B), suggesting that this is a general phenomenon of triple therapy in different models of cancer.

The Eomes^{hi} population induced by triple therapy exhibited significantly increased proliferation (Ki-67^{+}), reduced inhibitory receptor expression (PD1), and more potent effector function (granzyme A and IFNγ) within the lymph nodes (Fig. 10C, 11C) and tumors (Fig. 10D, 11D) as compared to Eomes^{lo} CD8^{+} T cells, similar to the phenotype we observed following combination therapy (Fig. 6). Eomes^{hi} CD8^{+} T cells generated by triple therapy also expressed higher levels of CXCR3 in the lymph nodes compared with Eomes^{lo} CD8^{+} T cells, supporting increased trafficking to inflamed tissues (Fig. 10C). Triple therapy did not significantly impact CD8^{+} T cell frequency or T-bet expression in lymph nodes or TIL compared with anti-OX40/anti-CTLA-4 therapy (Fig. 11E-F). Ibrutinib alone also had no effect on CXCL9 or CCL5 expression in TRAMP-C1 or 4T1 tumor cells (Fig. 12A-B).

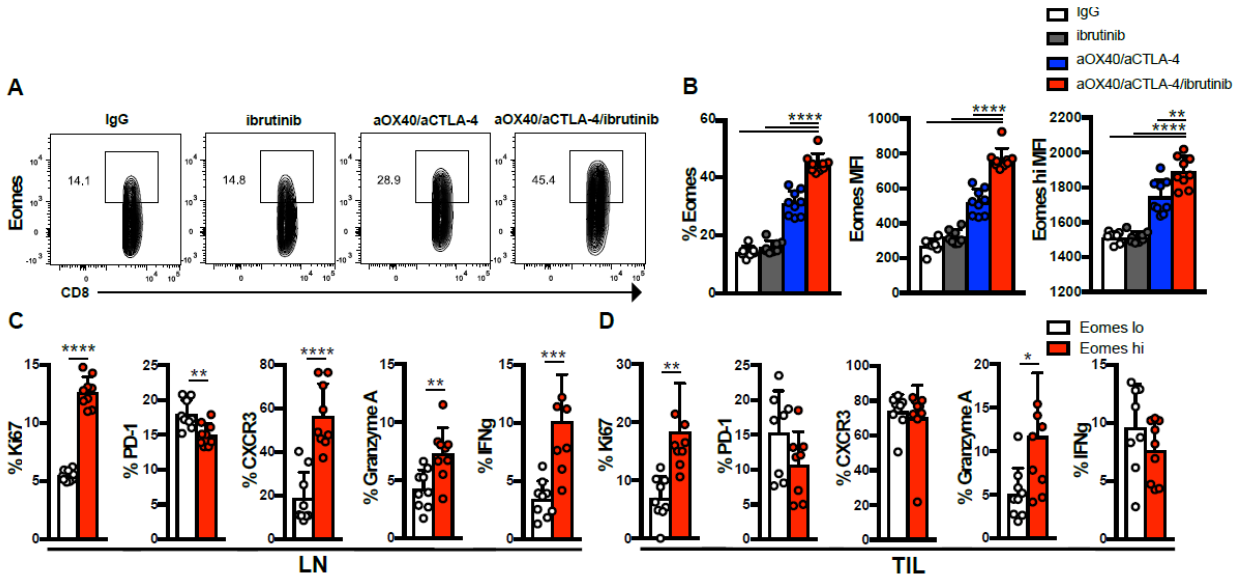


Figure 10. The addition of ibrutinib to anti-OX40/anti-CTLA-4 therapy significantly enhances CD8^{+} T cell–specific Eomes expression. TRAMP-C1 tumor–bearing mice were treated with control (IgG; days 12, 14, 16), anti-OX40/anti-CTLA-4 (days 12, 14, 16), and/or ibrutinib (days 12, 14, 16) and then tissues were harvested on day 19 for analysis. A-B) Eomes

expression (% and MFI) within CD8⁺ T cells from the lymph nodes of TRAMP-C1 tumor–bearing mice. C-D) Expression of the indicated activation and effector proteins were determined in Eomes^{lo} and Eomes^{hi} CD8⁺ T cells from the (C) lymph nodes and (D) tumors. Graphs represent the mean+/-SD from n=8-9/group from two independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by one-way ANOVA (B), or unpaired t-test (C-D).

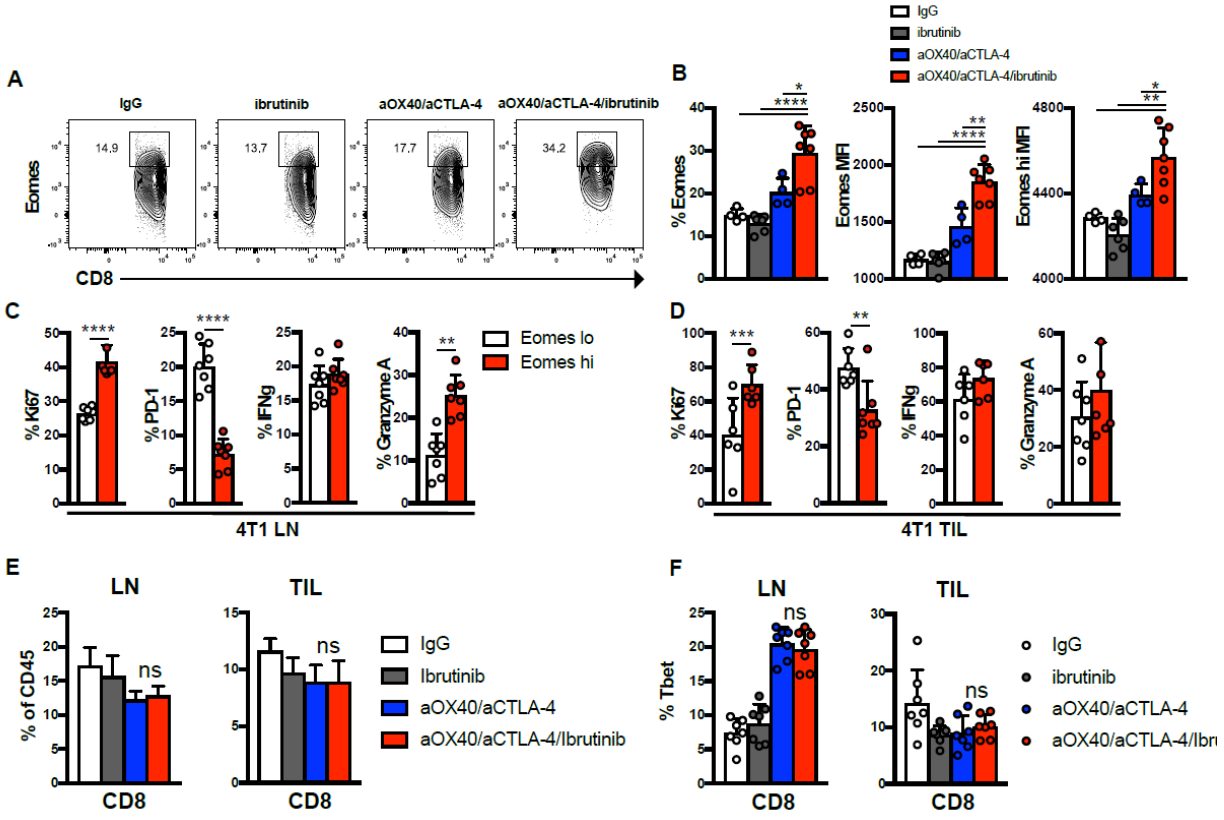


Figure 11. The addition of ibrutinib to αOX40/αCTLA-4 therapy significantly enhances CD8⁺ T cell-specific Eomes expression in 4T1. 4T1 tumor-bearing mice were treated with control (IgG; days 12, 14, 16), αOX40/αCTLA-4 (days 12, 14, 16), and/or ibrutinib (days 12, 14, 16) and then tissues were harvested on day 19 for analysis. A-B) Eomes expression (% and MFI) within CD8⁺ T cells from the lymph nodes of 4T1 tumor-bearing mice. C-D) Expression of the indicated activation and effector proteins were determined in Eomes^{lo} and Eomes^{hi} CD8⁺ T cells from the (C) lymph nodes and (D) tumor. E) Frequency of CD8⁺ T cells as a percent of CD45. F) Expression of Tbet. Graphs represent the mean+/-SD from n=4-7/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

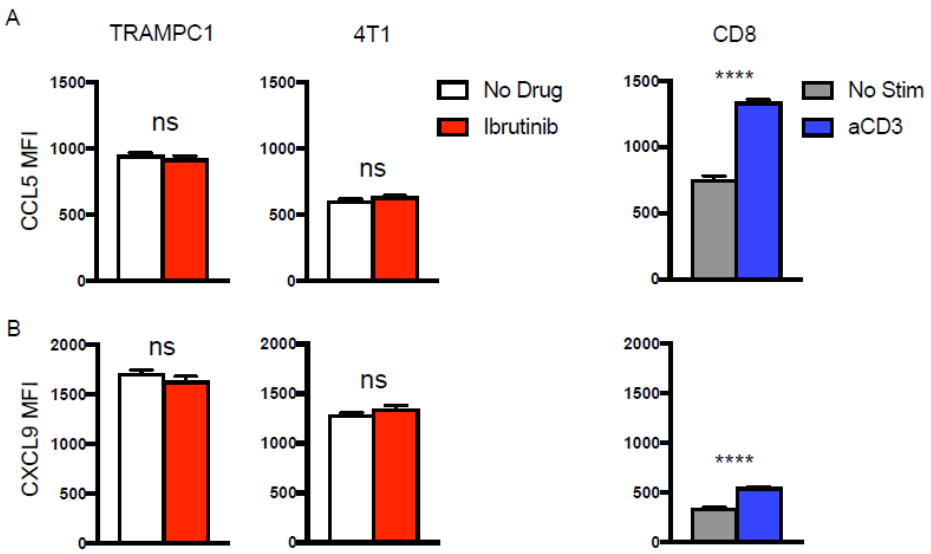


Figure 12. Tumor cell line CXCL9 and CCL5 expression is unaltered by ibrutinib. A-B) TRAMP-C1 and 4T1 cells were incubated for 24 hours with, or without, 0.3 μM ibrutinib and stained intracellularly for CXCL9 and CCL5. As a control, WT C57BL/6 splenocytes were incubated for 24 hours with, or without, 5.0 μg/ml αCD3 and stained intracellularly for CXCL9 and CCL5. Graphs represent the mean+/-SEM from n=6/group. ****P<0.0001.

Phospho-ITK and IRF4 suppress Eomes expression downstream of TCR stimulation in $CD8^+$ T cells. Since ibrutinib acts as an ITK inhibitor, we measured pITK and IRF4 expression in the tumor following anti-OX40/anti-CTLA-4 and triple therapy. Triple therapy significantly reduced expression of both pITK and IRF4 (Fig. 13A-B). The reduction in Eomes repressors likely led to the observed increase in Eomes with triple therapy. Interestingly, triple therapy did not significantly impact myeloid cell frequency in the tumors (Fig. 14).

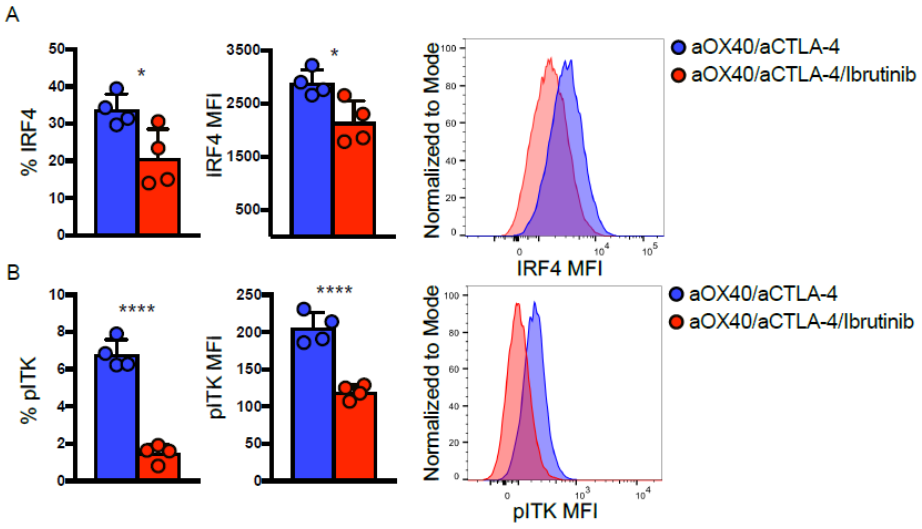


Figure 13. Ibrutinib inhibits IRF4 and pITK when used in combination with αOX40/αCTLA-4 therapy. A-B) MCA-205 tumor-bearing mice were treated with αOX40/αCTLA-4 (days 12, 14, 16), and/or ibrutinib (days 12, 14, 16) and then tissues were harvested on day 19 for flow cytometry analysis. A) IRF4 expression. B) pITK expression. Graphs represent the mean+/-SD from n=4/group. *P<0.05, ****P<0.0001.

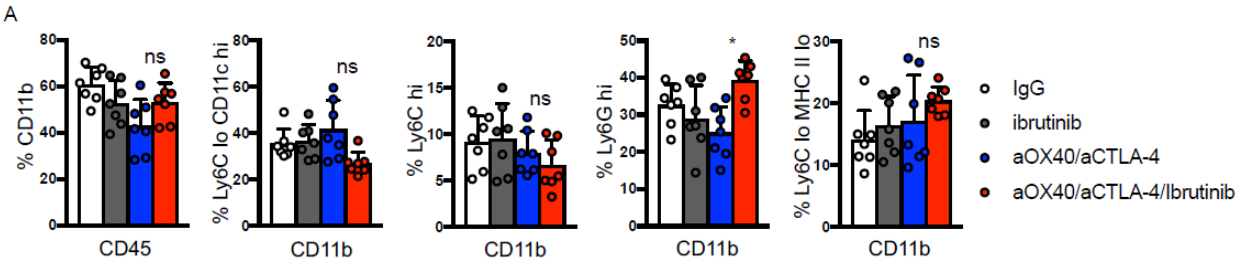


Figure 14. Changes in myeloid frequencies in response to triple therapy. TRAMP-C1 tumor-bearing mice were treated with control (IgG; days 12, 14, 16), αOX40/αCTLA-4 (days 12, 14, 16), and/or ibrutinib (days 12, 14, 16) and then tissues were harvested on day 19 for analysis. Total myeloid cell frequencies are represented by %CD11b expression. Dendritic cell frequency is represented by % Ly6C lo CD11c hi expression. Monocyte frequency is represented by % Ly6C hi expression. Neutrophil frequency is represented by % Ly6G hi expression. Macrophage frequency is represented by % Ly6C lo MHCII hi expression. Graphs represent the mean+/-SD from n=7/group. *P<0.05.

We also investigated the impact of triple therapy on T-cell differentiation. TRAMP-C1 or 4T1 tumor–bearing mice were treated with rat IgG (control), ibrutinib, anti-OX40/anti-CTLA-4, or

triple therapy and then 1 week later, lymph nodes and tumors were harvested for flow cytometry analysis. Proliferation (Ki-67) of effector (FoxP3$^-$) CD4$^+$ T cells and CD8$^+$ T cells in the lymph nodes was enhanced by triple therapy compared with either anti-OX40/anti-CTLA-4 therapy or ibrutinib alone (Fig. 15A). However, Treg frequencies and proliferation in the lymph nodes and TIL were unchanged (Fig. 15A-B). In addition, ibrutinib monotherapy had minimal impact on CXCR3 expression, whereas triple therapy significantly enhanced the generation of CXCR3$^+$ cells within the CD4$^+$ and CD8$^+$ T cell compartment in the lymph nodes and the tumors (Figs. 16A-B, 15A-B). Triple therapy also resulted in increased granzyme A$^+$ and IFNγ^+ CD8$^+$ T cells, and IFNγ^+CD4$^+$ T cells (Fig. 16A-B,15A-B), further indicating the generation of potent effector-cell responses. TRAMP-C1 tumors endogenously express the MHC class I–restricted antigen SPAS-1, which is an ortholog to the human prostate cancer antigen SH3GLB2 (203). We utilized SPAS-1–specific MHC I tetramers to determine the impact of triple therapy on these tumor antigen–specific CD8$^+$ T cells. Neither ibrutinib nor anti-OX40/anti-CTLA-4 therapy increased the frequency of SPAS-1$^+$CD8$^+$ T cells. However, triple therapy significantly increased the frequency of these cells in the lymph nodes and tumors (Fig. 16A-B).

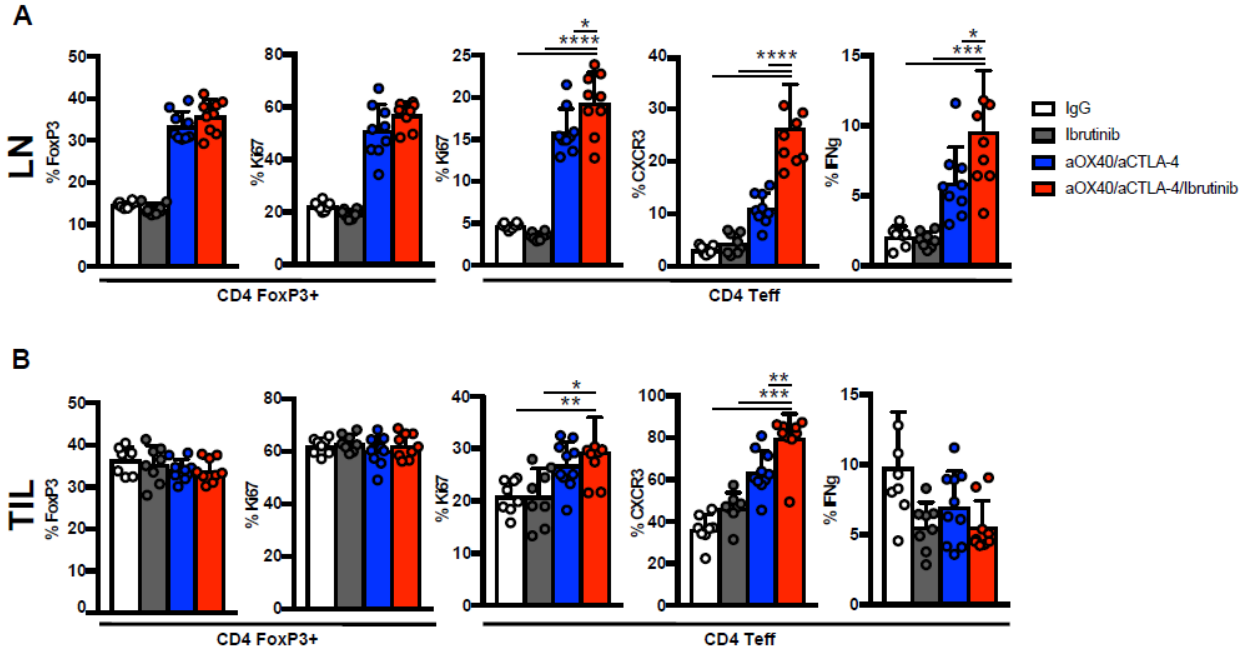


Figure 15. Triple therapy (αOX40/αCTLA-4/ibrutinib) enhances $CD4^+$ effector T cell proliferation and CXCR3 expression. TRAMP-C1 tumor-bearing mice were treated with control (IgG), αOX40/αCTLA-4, and/or ibrutinib (days 12, 14, 16). A-B) Seven days after the initiation of treatment (day 19 post-tumor implant) the (A) lymph nodes and (B) tumor were harvested and the extent of $CD4^+$ T cell activation and differentiation was determined by flow cytometry. Graphs represent the mean+/-SD from n=8-10/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Given that triple therapy induced the expansion of granzyme $A^+PD\text{-}1^{lo}CXCR3^+Eomes^{hi}$ $CD8^+$ T cells, we asked whether it also improved tumor control and survival over anti-OX40/anti-CTLA-4 combination therapy. Indeed, triple therapy led to a significant reduction in tumor growth and increased survival in the TRAMP-C1 model, highlighting the therapeutic efficacy of this approach (Fig. 16C, 17A). We also evaluated triple therapy in the 4T1 model, which revealed an initial strong antitumor effect within the first 2 weeks post-treatment, but minimal improvement in long-term survival (Fig. 17B-C). Furthermore, all tumor-free mice following triple combination therapy rejected autologous tumor rechallenge, demonstrating the formation of a durable memory response (Fig. 17D). The inability to maintain complete tumor clearance in 4T1 is likely the result of the myeloid-cell infiltration into the tumor that is associated with the 4T1 model (383). These results indicate that the addition of ibrutinib to anti-OX40/anti-CTLA-4 therapy not only expands the critical $Eomes^{hi}$ $CD8^+$ T cell population, but also leads to decreased tumor growth and improved survival.

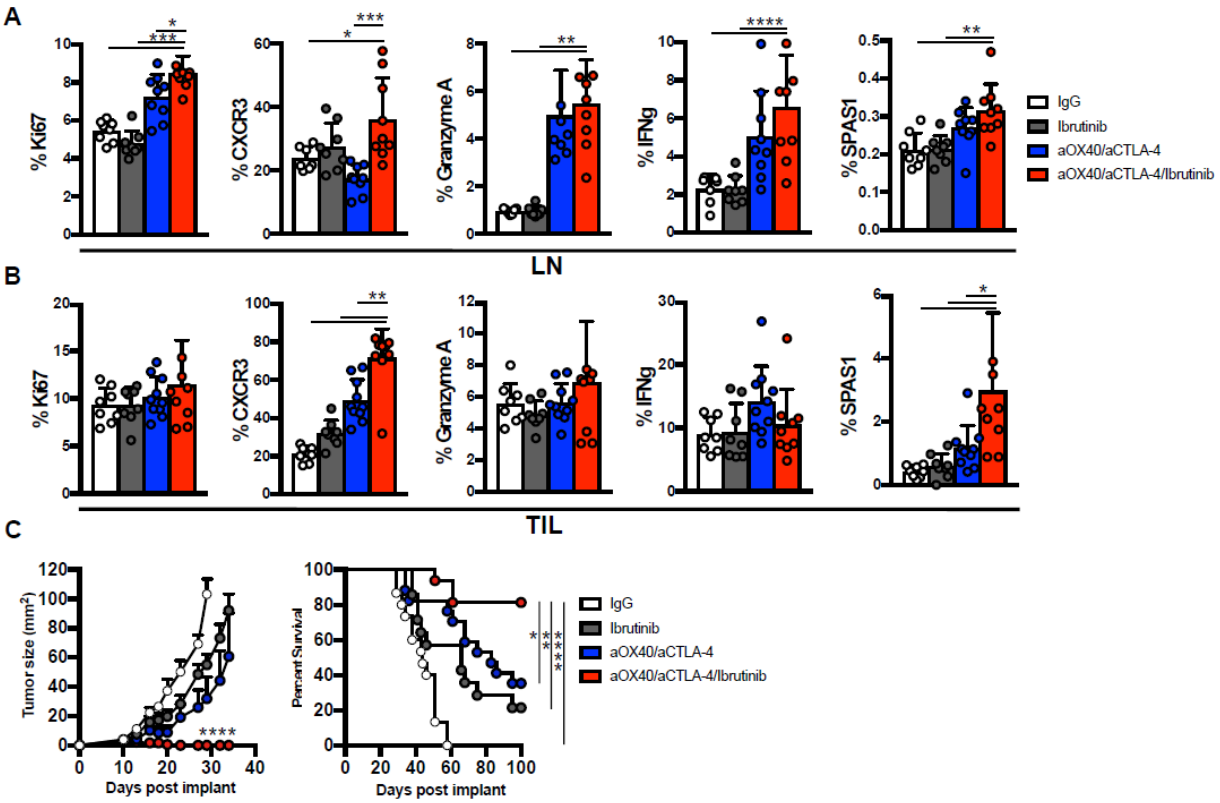


Figure 16. Triple therapy (anti-OX40/anti-CTLA-4/ibrutinib) enhances CD8⁺ T-cell proliferation and CXCR3 expression, leading to significantly improved tumor regression and survival. TRAMP-C1 tumor–bearing mice were treated with control (IgG), anti-OX40/anti-CTLA-4, and/or ibrutinib (days 12, 14, 16). A-B) Seven days after the initiation of treatment (day 19 post-tumor implant) the (A) lymph nodes and (B) tumor were harvested and the extent of CD8⁺ T-cell activation and differentiation was determined by flow cytometry. C) tumor growth and survival of tumor-bearing mice were determined. Graphs represent the mean+/-SD from n=8-10/group from two independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by one-way ANOVA (A-B), or Kaplan-Meier survival test (C).

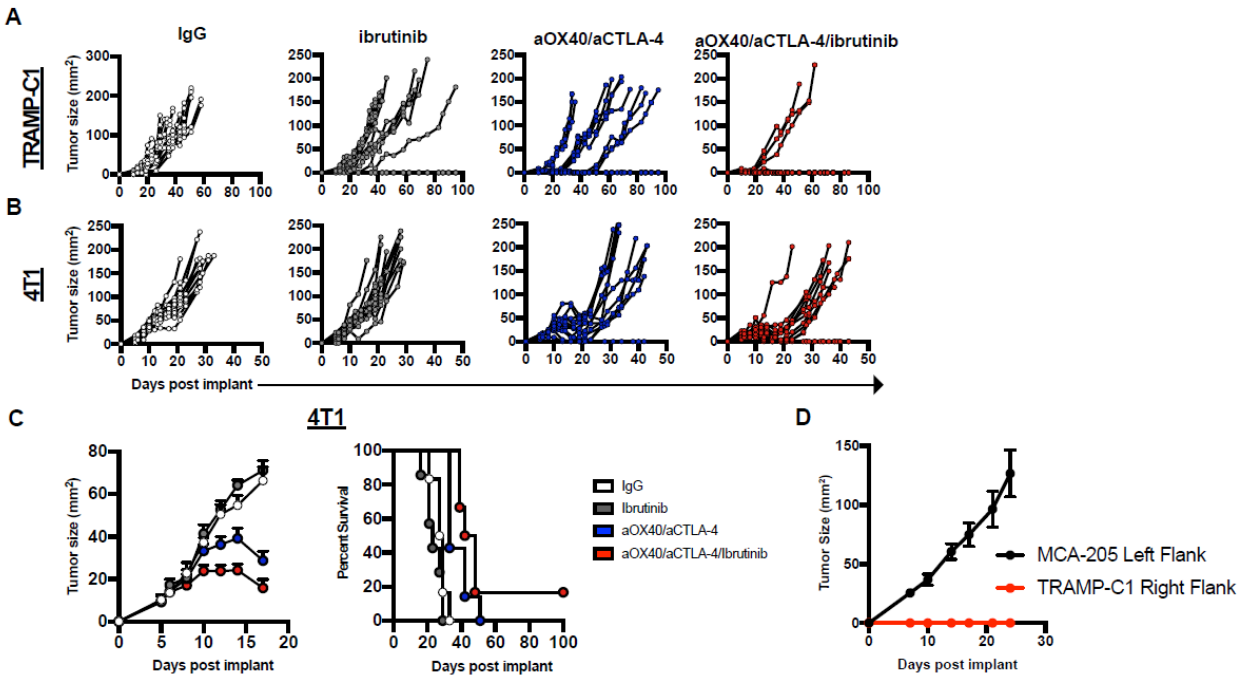


Figure 17. Triple therapy (αOX40/αCTLA-4/ibrutinib) improves tumor regression and survival in both TRAMP-C1 and 4T1 tumor bearing mice. TRAMP-C1 and 4T1tumor-bearing mice were treated with control (IgG), αOX40/αCTLA-4, and/or ibrutinib (days 8, 10, 12, 15, 17, 19). A) TRAMP-C1 and B) 4T1 tumor growth kinetics. C) 4T1 Average tumor growth (mm^2) and survival. n=13-16/group. D) tumor growth averages for re-challenge. $1x10^6$ MCA-205 cells were injected subcutaneously into the left flank, and $1x10^6$ TRAMP-C1 cells were injected subcutaneously into the right flank of mice that had cured TRAMP-C1 following triple combination therapy. (n=7).

4.4. Discussion

Developing rational combinatorial immunotherapy treatments with synergistic effects on antitumor immunity is critical to provide clinical benefit for a greater percentage of patients. In the current study, we demonstrated that anti-OX40/anti-CTLA-4 therapy synergized to enhance the survival of tumor-bearing mice across multiple tumor models. This increased efficacy corresponded with the generation of $Eomes^{hi}$ $CD8^+$ T cells and was abrogated in the absence of $CD8^+$ T cell–specific Eomes expression, which correlated with significantly decreased effector function (granzyme A) in $Eomes^{CKO}$ as compared to WT mice, highlighting the critical role of these cells in mediating antitumor responses. Several questions were raised by these findings such as what is the expression profile and function of $Eomes^{hi}$ $CD8^+$ T cells and does a further increase in $Eomes^{hi}$ $CD8^+$ T cells improve therapeutic efficacy? The increased expression of Eomes in $CD8^+$ T cell may be a unique property of the anti-OX40/anti-CTLA-4 combination, as anti-OX40/anti-PD1 did not elicit the same response, indicating that anti-CTLA-4 has unique properties when used concurrently with anti-OX40. This may reflect PD1 blockade functioning through the rescue of exhausted T cells, whereas anti-OX40 and anti-CTLA-4-mediated therapies promote effector T-cell priming and, in some cases, Treg depletion (353, 384, 385). Thus, it seems likely that T-cell priming and TNFR stimulation may be critical for the generation of $Eomes^{hi}$ $CD8^+$ T cells. Future studies will investigate whether ligation of other TNFR family members in the presence of ICB similarly induces $Eomes^{hi}$ $CD8^+$ T cells. Our findings suggest that the benefit of anti-CTLA-4 therapy may be enhanced through anti-OX40 stimulation and the generation of $Eomes^{hi}$ $CD8^+$ T cells, which may be an effective alternative approach for treating PD1 refractory patients.

The observed synergy between these two therapies may be a unique property of how they signal and influence co-stimulatory receptor expression. CTLA-4 blockade aids in T-cell stimulation through enhanced CD28 co-stimulatory receptor signaling by blocking CTLA-4 from binding to the ligands of CD28 (B7-1, B7-2). The CD28–B7 ligand interaction directly regulates

OX40 receptor expression through enhanced IL2 production, while also promoting OX40 receptor expression (296, 386). OX40 signaling, in turn, sensitizes tumor-reactive $CD8^+$ T cells to the direct effects of CTLA-4 blockade by increasing CTLA-4 expression on tumor-reactive $CD8^+$ T cells (385, 387). Our data demonstrated that when T-cell lymph node egress was inhibited by FTY720 treatment, $Eomes^{hi}$ $CD8^+$ T cells accumulated in the lymph node and were diminished in number in the tumor, further suggesting that $Eomes^{hi}$ $CD8^+$ T cells were driven by T-cell activation and priming in the lymph nodes prior to traffic to the tumor site (Fig. 5C). This finding may have implications for lymphadenectomy, common in surgery for many types of cancer, which may be removing critical sites of $Eomes^{hi}$ $CD8^+$ T-cell generation.

Compared with their $Eomes^{lo}$ counterparts, $Eomes^{hi}$ $CD8^+$ T cells had lower RNA and protein expression of several key inhibitory receptors including PD1, LAG-3, and TIM-3, as well as higher expression of several key effector markers such as Ki-67, granzyme A, TNFα, and IFNγ, indicating a unique effector memory phenotype. IRF4, a key negative regulator of Eomes expression in the TCR signaling pathway (371), was also reduced in $Eomes^{hi}$ $CD8^+$ T cells, suggesting that Eomes expression may be regulated by TCR signaling as a result of anti-OX40/anti-CTLA-4 therapy. Additionally, we observed increased expression of TCF1 in $Eomes^{hi}$ $CD8^+$ T cells. TCF1 is a transcription factor that identifies $CD8^+$ T cells that are stimulated by antigen. TCF1 maintains lymphoid recirculation and self-renewal potential of T cells and positively regulates the transcription of Eomes (388). TCF1 has also been linked to responsiveness to PD1 blockade immunotherapy through the development of memory T-cell responses (140). This was supported by our finding of high Eomes expression in central memory $CD8^+$ T cells and the ability of these cells to kill tumor cells *in vitro*, which indicates that this subset is not exhausted.

TCR ligation signals downstream through ITK and inhibits Eomes through IRF4. Importantly, ITK is redundant to RLK signaling in $CD8^+$ T cells. Ibrutinib covalently binds to BTK

and ITK, but does not bind to RLK in $CD8^+$ T cells (389). Ibrutinib's effects on treating B-cell malignancies through BTK inhibition are well documented, however its effects mediated by ITK blockade, including enhancing T-cell function, have only recently been appreciated (390, 391). For example, ITK inhibition was shown to improve T-cell function, number, and TCR diversity in chronic lymphocytic leukemia (CLL) patients receiving long-term ibrutinib therapy (392, 393). This effect was the result of the ITK-specific effects of ibrutinib (but not the BTK-specific inhibitor acalabrutinib) to increase T-cell numbers in CLL patients, suggesting a specific role for ITK inhibition in enhancing T-cell function in cancer therapy (372).

Ibrutinib has primarily been utilized in trials focused on BTK-expressing cancers such as CLL, diffuse large B-cell lymphoma, follicular lymphoma, and mantle cell lymphoma. However, the immune-modulating activity of ibrutinib provides a strong rationale for its combination with immunotherapies. In preclinical murine models, ibrutinib plus anti-PD-L1 leads to a greater frequency of antigen-experienced $CD8^+$ T cells (372). Our studies revealed that ibrutinib plus anti-OX40/anti-CTLA-4 therapy led to a synergistic increase in Eomes expression, reduced pITK and IRF4 expression, enhanced therapeutic efficacy, and markedly enhanced tumor regression.

Triple therapy also enhanced several aspects of T-cell effector function including proliferation of $CD8^+$ T cells and effector ($FoxP3^-$) $CD4^+$ T cells, which was associated with a striking increase in CXCR3 expression. The induction of CXCR3 is typically associated with type 1 helper T cell (Th1)-polarized immune responses, which are critical to an effective anticancer response through enhanced tumor trafficking via the CXCR3 ligands CXCL9 and CXCL10 (20). Ibrutinib alone had a small, but significant, impact on CXCR3 expression in $FoxP3^-$ $CD4^+$ T cells and $CD8^+$ T cells within the tumor, which likely stems from ITK-mediated regulation of CXCR3 expression, as *Itk*$^{-/-}$ mice are characterized by high expression of CXCR3 and Eomes (371). The generation of a Th1-polarized population by triple therapy was further supported by increased expression of IFNγ, TNFα, and granzyme A by effector $CD4^+$ T cells and $CD8^+$ T cells. ITK inhibition likely plays a role in the generation of Th1 immunity beyond its role in regulating

Eomes. For Th2-polarized $CD4^+$ T cells, ITK is a required TEC kinase as Th2 cells do not express the redundant RLK found in Th1 $CD4^+$ and $CD8^+$ T cells. As a result, inhibition of ITK by ibrutinib may inhibit the formation of Th2 $CD4^+$ T cells while leaving Th1-polarized $CD4^+$ and $CD8^+$ T cell populations intact.

ITK signaling acts as a rheostat for T-cell function and differentiation. The quantity of phosphorylated ITK (pITK) is determined by LCK and Zap70-mediated phosphorylation through stable TCR complexes based on antigen binding affinity. The downstream effects of pITK can be divided into digital and graded responses (394). The activation of the NFAT and NF-κB pathways are digital responses, meaning that any activity of pITK triggers these pathways. AP-1 activation, however, is a graded response, meaning the amount of upstream pITK determines the extent to which AP-1 is activated. By inhibiting ITK with ibrutinib, we are likely shifting $CD8^+$ T cells receiving strong TCR stimulation to expresses higher levels of Eomes. This functional shift would occur without inhibiting NF-κB and NFAT signaling. Recently, it has been shown that submaximal TCR affinity stimulation leads to a survival benefit through Eomes expression (395). This in turn allows for $Eomes^{hi}$ $CD8^+$ T cells to compete for a memory niche that would otherwise be occupied by high affinity cells in the absence of Eomes. We are currently investigating the role of TCR stimulation in the Eomes response to understand how the strength of TCR signal determines and influences $CD8^+$ T cell function and response to immunotherapy.

As new cancer immunotherapies continue to emerge, opportunities for novel combinations of therapies have expanded. While a greater percentage of patients may benefit from combination immunotherapy, particular attention needs to be given to the development of adverse events that may result from new combinations. Adverse effects may be ameliorated, in part, by finding combinations that have synergistic, rather than additive or antagonist effects. Our studies revealed unique synergy between ibrutinib and anti-OX40/anti-CTLA-4 immunotherapy, through a $CD8^+$ T cell Eomes-specific mechanism. How ibrutinib may combine

and possibly synergize with the plethora of other immunotherapies currently being evaluated clinically (e.g., vaccines, oncolytic viruses, checkpoint inhibitors, T cell agonists, cytokines, and myeloid-targeted therapies) remains unclear. However, our data demonstrates that ibrutinib/anti-OX40/anti-CTLA-4 therapy warrants further investigation and evaluation in the clinic.

Chapter 5: Future Directions

5.1. Summary of my research and overview of questions raised

Treating cancer patients by utilizing the immune system to eliminate cancer has been groundbreaking in its advancement of cancer therapy in the recent decades. Immunotherapy, a class of treatments that utilize and enhance immune function in patients, consists of several different strategies to augment the immune response, as covered in previous chapters. One of these strategies enhances the function of T cells through targeting specific T cell receptors using monoclonal antibodies. There are multiple strategies regarding the targeting of stimulatory and inhibitory receptors, typically through immune checkpoint blockade or agonist binding to co-stimulatory receptors (396, 397). Immune checkpoint blockade works by releasing the inhibitory "brakes" on T cells by preventing ligation of inhibitory receptors by their binding partners. Clinically, immune checkpoint blockade has had success in the treatment of several cancers including melanoma, head and neck squamous cell carcinoma, Hodgkin's lymphoma, bladder, kidney, and lung, as well as cancers with microsatellite instability or mismatch repair defects (265, 354, 355, 398). Three immune checkpoint blockade therapies have found clinical success (αCTLA-4, αPD-1, αPD-L1). However, the efficacy of these therapies alone has been limited to a subset of patients, and further augmentation of the response has required combining immune checkpoint blockade with additional therapies (399). One combination therapy approach is to pair immune checkpoint blockade with agonist co-stimulation. Co-stimulating monoclonal antibodies effectively "step on the gas" of T cells to augment and enhance their survival and cytotoxicity by binding to a co-stimulatory receptor in a way that simulates binding by the native ligand (358, 400). One target for agonist co-stimulatory therapies is members of the tumor necrosis factor receptor (TNFR) family. For example, targeting the TNFR OX40, with αOX40 has shown success pre-clinically but has had limited success thus far in the clinic (401, 402). As

monotherapies of either checkpoint blockade or agonist co-stimulation have had limited success, the development of rational immunotherapy combinations is critical to providing clinical benefit to the highest number of patients.

My research revealed a novel mechanism driving improved anti-cancer immunity through the combination of αOX40 and αCTLA-4 therapies, which together enhance a population of $CD8^+$ T cells expressing the transcription factor, Eomesodermin. An understanding of the mechanisms by which an immunotherapy functions is critical to further enhance that combination therapy; for example, a mechanistic understanding of how a therapy works might influence changes to the timing or dosage of the therapy, its utilization against specific cancers, and the development of novel therapies that build on the functional properties of previous therapies. My research demonstrated that αOX40/αCTLA-4 therapy synergized to enhanced survival in tumor-bearing mice, whereas αOX40 or αCTLA-4 alone had very limited efficacy against several cancers, as shown in MCA-205 sarcoma, TRAMP-C1 prostate adenocarcinoma, 4T1 mammary carcinoma (403). I worked out both the mechanism by which these two therapies synergize and how that mechanism can be enhanced. While αOX40/αCTLA-4 therapy had many effects on T cell function in terms of cytokines produced and receptors expressed, one of the more interesting findings was a change in which master transcription factors were expressed. The expression of Eomes in $CD8^+$ T cells correlated closely with outcomes. Both αOX40 or αCTLA-4 alone had little effect of the expression of Eomes, however, αOX40/αCTLA-4 therapy led to a synergistic increase in $CD8^+$ T cell-specific Eomes expression (increase in $Eomes^{hi}$ cells). We then hypothesized that Eomes expression in $CD8^+$ T cells could explain the increased efficacy of this combination.

Investigations into the role and function of $Eomes^{hi}$ $CD8^+$ T cells generated by αOX40/αCTLA-4 therapy demonstrated they may play a unique role in the anti-tumor response in the context of this therapy. In the absence of Eomes expression in $CD8^+$ T cells, the efficacy

of αOX40/αCTLA-4 therapy was abrogated, suggesting that this population of $CD8^+$ T cells is critical to the functionality of this therapy. $Eomes^{hi}$ $CD8^+$ T cells generated following αOX40/αCTLA-4 therapy had a unique transcriptional profile characterized by lower checkpoint receptor expression, central memory characteristics, and increased cytotoxicity. These findings were verified by flow cytometry and suggest that $Eomes^{hi}$ $CD8^+$ T cells may play a critical role in prolonged survival, resistance to tumor and immune suppression, and tumor cytotoxicity.

With an understanding of the functional role of $Eomes^{hi}$ $CD8^+$ T cells in the anti-tumor immune response induced by αOX40/αCTLA-4 therapy, I next investigated whether Eomes expression could be further enhanced, and if this increased expression would lead to better outcomes in tumor-bearing mice. While the generation of Eomes expression is complex and multi-faceted, I sought to find molecules that could be targeted by existing drugs. TCR ligation to peptide-bound MHC signals downstream through Inducible T cell Kinase (ITK). ITK phosphorylation/activation inhibits Eomes expression, mediated by interferon regulatory factor 4 (IRF4). Because ITK plays such an important role in Eomes expression, it suggests a potential therapeutic target that can be blocked, which might enhance Eomes expression by reducing suppression of Eomes. Ibrutinib, a small molecule kinase inhibitor that is FDA approved for the treatment of several B cell malignancies such as chronic lymphocytic leukemia, mantle cell lymphoma, and Waldenstrom's macroglobulinemia, works through covalently modifying Bruton's tyrosine kinase (BTK) (404). Additionally, ibrutinib has similar activity against ITK since ITK and BTK share a homologous ATP binding structure (389). The combination of ibrutinib with immunotherapy checkpoint blockade has previously been explored in the form of ibrutinib and PD-L1, however the mechanism describing how ibrutinib synergizes with immunotherapy has not previously been defined (372). My studies revealed that ibrutinib plus αOX40/αCTLA-4 therapy led to a synergistic increase in $CD8^+$ T cell Eomes expression, characterized by reduced pITK and IRF4 expression. This novel triple combination therapy also had enhanced

therapeutic efficacy across multiple tumor models, leading to enhanced tumor regression and improved survival.

A characterization of the response to triple therapy led to several significant findings and raised important questions regarding T cell signaling and function in response to immunotherapy. Triple therapy led to enhanced proliferation of $CD8^+$ and effector $CD4^+$ T cell beyond αOX40/αCTLA-4 but did not enhance Treg proliferation beyond αOX40/αCTLA-4 without ibrutinib. The enhanced $CD8^+$ and effector $CD4^+$ T cells were characterized by high expression of CXCR3, an important chemokine receptor for trafficking to inflamed tissues and tumor sites, as well as high expression of effector cytokines such as IFN-γ, TNF-α, and granzyme A. However, many questions still remain regarding the application of triple therapy and the use of ibrutinib in immunotherapy in general. As ibrutinib is designed as a BTK inhibitor, how does it impact cells expressing BTK such as B cells, specific myeloid cells, and possibly cancer cells? How does ibrutinib impact $CD4^+$ T cells that also express ITK, and how does inhibiting an important TCR signaling kinase not negatively impact T cell activation and function? What would be the applications of a pure ITK inhibitor, and are there other points of intervention for enhancing Eomes expression beyond ITK inhibition? How does ibrutinib in triple combination therapy impact and augment the development of antigen specificity in T cells?

Beyond these questions regarding the role of ibrutinib in αOX40/αCTLA-4 therapy, many other questions remain about the function and role of $Eomes^{hi}$ $CD8^+$ T cells in the context of immunotherapy and general immunology. What is the mechanism by which αOX40/αCTLA-4 leads to Eomes expression? We have seen that so far this combination may be unique in its ability to induce Eomes expression, as αPD-1/αOX40 had no effect. However, targeting other TNFRs like 4-1BB might induce Eomes expression if used in combination with αCTLA-4. What exactly is Eomes expression doing in terms of the genes that it activates, and is this gene set different dependent on how Eomes expression is stimulated? What role does Eomes play in

CD8$^+$ T cell priming considering its role and activation/induction in the lymph node? How do my findings fit into, and contribute to, the field of cancer immunotherapy, and the broader field of immunology? All these questions and more will be addressed in the following sections.

5.2. Future directions

5.2.1. αOX40 and αCTLA-4 signaling pathways elicit CD8$^+$ T cell Eomes expression in the lymph node

For T cells to achieve optimal activation and cytotoxic response they need to be stimulated through the T cell receptor, followed by co-stimulation, and finally cytokines binding to cytokine receptors, which tailor and direct the T cell response. Several TNFRs are expressed following TCR stimulation, including OX40, 4-1BB, CD27, and GITR, which augments T cell cytotoxicity, survival, proliferation, and differentiation to varying degrees (358). Additionally, αOX40 therapy has shown promise in the clinic and in pre-clinical models. In a phase I clinical trial for the treatment of patients with metastatic melanoma, αOX40 mAb therapy led to a regression of at least one metastatic site in 12 out of 30 patients, leading to enhanced T cell functions in those patients (402). Thus, αOX40 monotherapy has been shown to have potential in modulating and enhancing the immune response, however it is lacking in potency to fully eliminate cancer on its own.

No matter how potent αOX40 or other TNFR targeting mAbs may be, activated T cells are still susceptible to tumor and immune suppression through CTLA-4 and PD-1 signaling. αCTLA-4, αPD-1, and αPD-L1 mAbs have all been FDA approved for the treatment of a variety of cancers (397). These mAbs function by blocking T cell inhibition. Additionally, αCTLA-4 may not only prevent CTLA-4 inhibition but may also lead to enhanced CD28 and B7 ligand binding. CD28-B7 binding is a critical connection that must be made to achieve proper T cell activation.

αCTLA-4 is a potent therapy to combine with αOX40 due to the unique mechanistic interactions that occur between how they signal and affect cell function. Further, αOX40 has been shown to uniquely sensitize tumor-reactive $CD8^+$ T cells to the effects of CTLA-4 blockade. Expression of OX40 and CTLA-4 on $CD8^+$ T cells is necessary to promote optimal $CD8^+$ T cell activation following αOX40/αCTLA-4 therapy (362). However, currently the full mechanism by which αOX40 synergizes with αCTLA-4 is not fully understood.

My research aimed to investigate one aspect of the synergy between αOX40 and αCTLA-4 therapy; the synergistic development of $Eomes^{hi}$ $CD8^+$ T cells. In my research, the effects of αOX40/αCTLA-4 on transcription factors besides Eomes, such as T-bet, were far less pronounced and less significant in both $CD4^+$ and $CD8^+$ T cells compared to the effect on Eomes. Why are $Eomes^{hi}$ $CD8^+$ T cells induced preferentially in the lymph node by αOX40/αCTLA-4? It should be noted that while increases to $CD8^+$ T cell Eomes expression in the tumor were generally not observed under these conditions, it is possible that the $Eomes^{hi}$ $CD8^+$ T cells had not yet trafficked to the tumor at the time of tissue harvest. This could make sense considering that murine tumor tissues were harvested one week following the start of treatment, which might be too soon to observe the full and lasting effects of the therapy in the tumor. Unfortunately, probing at later timepoints becomes more difficult with tumor-bearing mice. Typically, either the tumor responds to therapy and regresses, leaving no tumor tissue to harvest, or the tumor does not respond and is no longer representative of a productive immune response to the therapy. My experiments into the timing and tracking of induced $Eomes^{hi}$ $CD8^+$ T cells would suggest that more $Eomes^{hi}$ cells are produced in the lymph node compared to the tumor, and that they likely traffic to the tumor from the lymph node. When lymph node egress was blocked using FTY720, a SP1R agonist, in concurrence with αOX40/αCTLA-4 therapy in tumor-bearing mice, mice receiving FTY720 had significantly more $Eomes^{hi}$ $CD8^+$ T cell in the lymph nodes, and significantly fewer $Eomes^{hi}$ $CD8^+$ T cells in the tumor compared to mice not

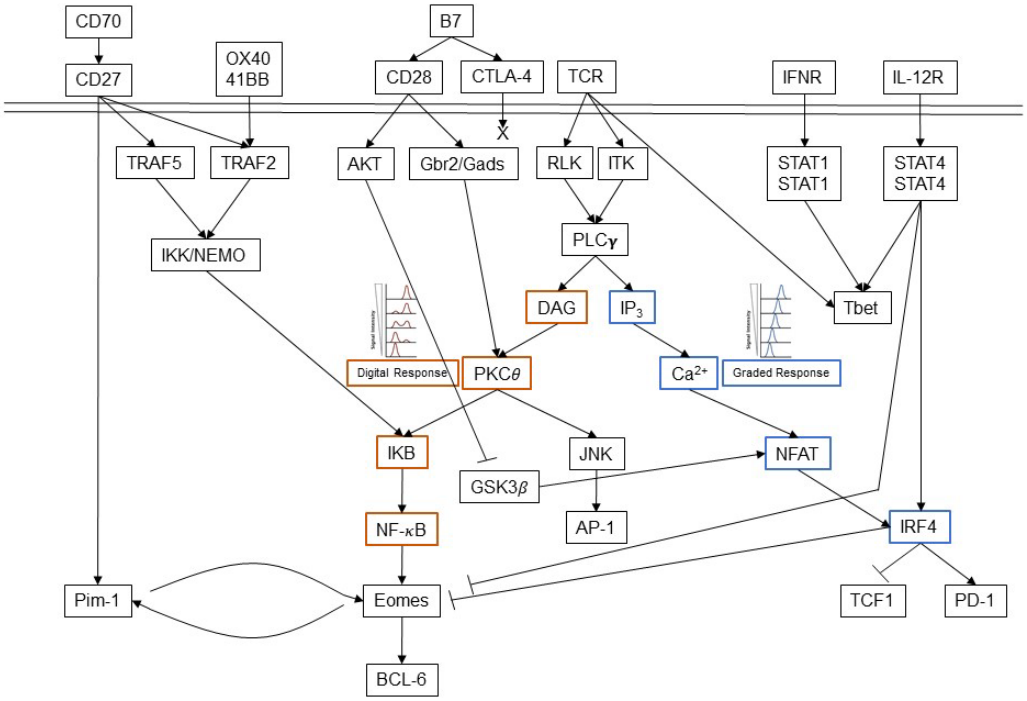
receiving FTY720. We have yet to address what Eomes^{hi} CD8^{+} T cells are doing in the lymph node. Are Eomes^{hi} CD8^{+} T cells important for priming other cells? To address this question, I would treat WT and Eomes KO MCA-205-OVA tumor bearing mice with αOX40/αCTLA-4. The use of an OVA expressing tumor would allow for tracking of MHC I and MHC II OVA specific cells by tetramer staining. One week after treatment I would look in the tumor for tetramer+ CD4^{+} and CD8^{+} T cells indicating tumor specificity. If Eomes^{hi} CD8^{+} T cells are critical for priming other cells, then I would expect the Eomes KO mouse to have reduced tetramer+ cells in the tumor. If Eomes^{hi} cells are not critical for priming in the lymph node, then I would expect similar numbers of tetramer+ cells between the two groups.

How does αOX40/αCTLA-4 specifically synergize to produce Eomes^{hi} CD8^{+} T cells in the lymph nodes? OX40 receptor activation, and to some extent other TNFR activation, likely contributes through which signaling molecules they activate. OX40, as well as 4-1BB, signal through TNF receptor-associated factor 2 (TRAF2), which in turn activates canonical NF-kB signaling (405). NF-kB signaling is critical for Eomes activation (369). As discussed in earlier chapters, TCR stimulation is two sided in its role in Eomes activation as it can activate or inhibit Eomes expression through PKC and eventually NF-kB, or NFAT and IRF4, respectively (394). Whether the TCR activates or inhibits Eomes is determined by the strength of TCR ligation and mediated through ITK. When the TCR binds strongly to its cognate antigen expressed on MHC, the complex is stabilized for longer, leading to more phosphorylated ITK. pITK in turn inhibits Eomes expression through activating IRF4. However, at lower levels of TCR stimulation by antigens of slightly lower binding affinity, NF-kB is still activated at the small levels as with a high affinity antigen, but pITK activation is significantly reduced (394). This shifts the scale in favor of Eomes expression. The addition of OX40 receptor stimulation to TCR stimulation may further shift the scale in favor of Eomes expression. However, this only partially explains our *in-vivo* observations of αOX40 monotherapy. Typically, we observe a small (and only sometimes

significant) increase in $CD8^+$ T cell Eomes expression as a result of αOX40 monotherapy. Perhaps the role that αCTLA-4 plays in contribution to Eomes expression is more critical and key to understanding Eomes induction.

The exact signaling pathways of CTLA-4 are still being debated. It is known how CTLA-4 plays a role in binding to B7 ligands with high affinity, which prevents B7 ligands from binding to CD28. However, any cell intrinsic effects of CTLA-4 are controversial. Is the contribution of αCTLA-4 to Eomes expression simply that it allows for more CD28 signaling? This may partially explain the synergy observed by αOX40/αCTLA-4 therapy. αCD28 does add to NF-kB signaling through PCK signaling (406). Perhaps more importantly, CD28 signaling may indirectly inhibit NFAT expression. CD28 signals through AKT, AKT inhibits GSK3b, and GSK3b activated NFAT. This would make sense that T cell activation through CD28 would contribute to Eomes, a transcription factor that drives survival, memory, and cytotoxicity. While this is a possible explanation for cell intrinsic Eomes expression generated by αOX40/αCTLA-4, other immune cells and signaling pathways are involved, as well as $CD4^+$ T cells and antigen presenting cells.

Figure. 1. Major signaling pathways regulating Eomes expression



I observed that CD8⁺ T cell Eomes expression is most strongly induced in the lymph nodes of tumor-bearing mice. Based on this observation, Eomes^{hi} CD8⁺ T cells may be playing an important role in T cell priming in the lymph node. The signaling pathways involved with Eomes expression induction such as TCR stimulation, TNFR stimulation, and inhibition of CTLA-4 all occur within the lymph and are mediated by antigen presenting cells. Additionally, I observed that Eomes^{hi} CD8⁺ T cells express high levels of the CXCR3 chemokine receptor, which responds to CXCL9 and CXCL10 chemokines produced by inflamed tissues and tumors. This would suggest that Eomes^{hi} CD8⁺ T cells are specifically induced in the lymph node with the intent of trafficking to tissues like the tumor. I could address this question in several different ways. TRAMP-C1 tumor bearing mice would be treated with αOX40/αCTLA-4 +/- an αCXCR3 blocking antibody. One week after treatment, tumors and lymph nodes would be harvested to evaluate the numbers and frequencies of Eomes^{hi} CD8⁺ T cells. If CXCR3 expression is critical

for Eomes^{hi} CD8^{+} T cells to traffic to the tumor, then the αCXCR3 treated cohort would have reduced Eomes in the tumor and possibly increased Eomes in the lymph node or peripheral blood. If CXCR3 is insignificant to tumor trafficking by Eomes^{hi} CD8^{+} T cells then no change in Eomes expression in the tumor would be observed between the two cohorts. Additionally, the TME could be evaluated for CXCL9 and CXCL10 expression by cytokine bead array to determine if the TRAMP-C1 TME, or other tumor lines, are producing these chemokines.

5.2.2. CD8^{+} T cell Eomes expression drives a beneficial immune cancer response

Eomes, and the related molecule T-bet, are members of the T-box transcription factor family. Many of the proteins in this family play important roles in embryonic and neurological development with several being embryonically lethal if missing (407). Eomes and T-bet play important roles in immunological cellular development. Eomes, also known as T-box brain protein 2 (TBR2), binds to a specific genetic sequence known as the T-box. The DNA consensus binding sequence consists of a minimum of 8 core nucleic acids, also called the T-box half site or T-box binding element (TBE). T-box binding proteins have larger binding regions than just the core consensus sequence, adding to variability in the genes activated by the transcription factor. Additionally, T-box transcription factor expression is highly specific to cell types and developmental regions. For example, the related T-box transcription factor Brachyury is expressed in the posterior mesoderm during gastrulation development. Deletion of Eomes is embryonically lethal as it disrupts both trophoblast and cardiac mesoderm development. The exact outcomes of Eomes DNA binding in immune cells however is not well defined.

Studies looking at the effects of T cell specific deletions of Eomes and/or T-bet have provided insight into how these two transcription factors regulate T cell function and development. Through these knockout experiments, Eomes has been linked with several

important CD8$^+$ T cell functions. Eomes has been linked with CD8$^+$ T cell cytotoxicity, such as production of IFN-g, TNF-a, and a variety of granzymes (408). Eomes has been linked to cell trafficking through expression of CD44, CD62L, and CXCR3 (376). Additionally, these trafficking proteins have helped to define Eomes CD8$^+$ T cells as having memory like properties (409). Fitting in with T cell memory is the expression of several pro-survival Bcl molecules (395). Conversely, T-bet has overlapping and differing functions to Eomes in CD8$^+$ T cells. T-bet also plays an important role in the production of cytotoxic molecules such as IFN-γ, TNF-α, and granzymes (410). However, T-bet expressing CD8$^+$ T cells are often referred to as short lived effector cells (363).

Fitting into the role of Eomes expression in CD8$^+$ T cells is the related role of several other transcription factors. The transcription factors T-cell factor 1 (TCF1), Tox, and Pim-1 all play important roles in the regulation of Eomes and in how they effect Eomes-related cellular function. TCF1 shares a lot of similarities with Eomes. TCF1 expression marks antigen experienced CD8$^+$ T cells that have properties of self-renewal and re-circulation (411). Expression of TCF1 is also most frequently found in memory T cells, not effector CD8$^+$ T cells. Eomes and TCF1 also share similar upstream regulators. IRF4, which is a negative regulator of Eomes expression and often determines Eomes expression, is also a negative regulator of TCF1. On the other hand, Tox is a transcription factor that marks the exhausted T cell phenotype in cancer and chronic viral infections (412). Tox expression is driven by NFAT, similarly to expression of IRF4. Expression of Tox leads to upregulation of inhibitory receptors and downregulation of cytotoxicity and cytokine production. In my studies, we observed that Eomes^{hi} CD8$^+$ T cells induced by αOX40/αCTLA-4 therapy have lower Tox expression compared to Eomes^{lo} cells. Pim-1, while not a transcription factor, also plays an important role in Eomes regulation and expression (369). Pim-1 is serine/threonine kinase expressed and activated following CD27 ligation. CD27 itself is a TNF receptor expressed on most T cells and

contributes to NF-κB signaling through TRAF2. What makes the addition of Pim-1 signaling downstream of CD27 different compared to other TNFRs is that Pim-1 and Eomes positively signal back and forth on each other. Pim-1 inhibition results in reduced Eomes expression, and reduced expression of BCL anti-apoptotic molecules downstream of Eomes such as Bcl-2 and Bcl-xL. All of these signaling pathways point to the TCR and subsequent pITK/IRF4 expression as being critical to the expression of Eomes.

A closer investigation of the mechanism by which Eomes expression is induced following αOX40/αCTLA-4 therapy could be looked at *in-vitro* using a model of αOX40/αCTLA-4 stimulation, and inhibitors of specific signaling molecules. I have seen that Eomes expression can be induced by αOX40/αCTLA-4 when lymph node derived lymphocytes are stimulated by plate bound αCD3 Figure 1. To test the mechanism of induced Eomes expression I would use inhibitors for NF-κB (IKKb), ITK, IRF4, and PIM-1 to probe the significance of each of these signaling molecules to Eomes expression. I would hypothesize the chemical inhibition of ITK and IRF4 will lead to enhanced Eomes expression, and inhibition of NF-κB or PIM-1 will lead to decreased Eomes expression in αCD3/αOX40/αCTLA-4 stimulated $CD8^+$ T cells from lymphocytes.

Previous work looking at the role of Eomes expression in $CD8^+$ T cells has suggested that it contributes to overall T cell exhaustion. Li et al. looked at exhausted $CD8^+$ T cells in thymic lymphoma tumors and observed high expression of Eomes to accompany PD-1 and Tim-3 expression (379). However, they found that $CD8^+$ T cells lost cytotoxicity when Eomes was deleted. Additionally, these experiments were in non-treated, tumor bearing WT mice and are not a direct comparison to $Eomes^{hi}$ $CD8^+$ T cells induced by αOX40/αCTLA-4 therapy. In their study, not only was Eomes expressed in exhausted cells but was also highly expressed in memory and effector populations. In the context of their experiments, it is possible that in the absence of T cell stimulation, which would be provided by αOX40/αCTLA-4 therapy, $CD8^+$ T cell

populations progress towards exhaustion in the tumor at a faster rate than I observed. In my observations, the Eomes^{hi} CD8^{+} T cells were cytotoxic and did not express high levels of exhaustion markers. Other studies that suggest a role of Eomes in CD8^{+} T cell exhaustion are examining the response to chronic viral infection such as LCMV (413). In these studies, it was also found that Eomes is expressed in the effector and memory populations as well, with increasing expression in the exhausted population increasing over time. Perhaps if Eomes^{hi} cells are subjected to a chronic infection without additional stimulation, they will transition into an exhausted phenotype. However, relevant studies looking at the role of Eomes expression in clinical cancer patients paints a different picture. Eomes^{hi} CD8^{+} T cells have been shown to be clinically relevant, as demonstrated by the presence of Eomes^{hi} T cells in biopsies associated with better clinical outcomes following αPD-1 or αPD-1/αCTLA-4 immunotherapy (366), and the presence of Eomes^{hi} CD8^{+} T cells within the tumor correlated with long-term survival in patients with pancreatic cancer (414, 415).

The Eomes^{hi} CD8^{+} T cells induced by αOX40/αCTLA-4 therapy had high expression of CD44 and CD62L, placing them in the central memory phenotype. This finding was supported by Eomes^{hi} CD8^{+} T cell transcriptome GSEA analysis. What is the significance of Eomes^{hi} CD8^{+} T cells resembling Tcm, and is this be a benefit to the overall anti-cancer response? Nicholas Restifo has performed extensive research on the role of central memory and effector memory T cells in cancer (416). Adoptive transfer of in-vitro generated of Tcm CD8^{+} T cell led to eradication of large established tumors. However, adoptive transfer of in-vitro generated Tem CD8^{+} T cells were far less effective at eliminating tumors. Their findings indicated that homing to secondary lymphoid tissues prior to tumor trafficking was required for optimal treatment and efficacy. The impact of the memory role that Eomes^{hi} CD8^{+} T cells may be playing in αOX40/αCTLA-4 therapy has yet to be determined. To test the hypothesis that Eomes^{hi} CD8^{+} T cells following αOX40/αCTLA-4 exhibit memory potential and secondary memory responses, we

could utilize the Eomes-GFP model for adoptive transfer experiments. Eomes-GFP+ or Eomes-GFP- $CD8^+$ T cells would be harvested from the lymph nodes of TRAMP-C1 tumor bearing αOX40/αCTLA-4 mice, one week after treatment. These cells on the Thy1.2 C57BL/6 background would be adoptively transferred into WT C57BL/6 Thy1.1 mice. Four weeks after adoptive transfer, the memory phenotype and frequency of Eomes expression would be evaluated by flow cytometry from harvested secondary lymphoid organs as tracked by Thy1.2. We would hypothesize that that Eomes-GFP+ transfer will have greater persistence compared to the Eomes-GFP- transfer, as well as stability of Eomes-GFP expression. Additionally, using this same experimental set up, four weeks after adoptive transfer mice will be challenged with TRAMP-C1 or B16 tumor cells as a negative control. Effector function and memory phenotype of Thy1.2 cells will be evaluated one week after tumor challenge. We would hypothesize that the Eomes-GFP+ Thy1.2 cells to have greater effector function and memory phenotype compared to the Eomes-GFP- Thy1.2 transferred cells.

5.2.3. Multifaceted roles of ibrutinib in αOX40/αCTLA-4 therapy

As the evidence mounted that $Eomes^{hi}$ $CD8^+$ T cells were critical to the anti-tumor efficacy of αOX40/αCTLA-4 therapy, it became important to investigate if $CD8^+$ T cell Eomes expression could be further enhanced. An analysis of the pathways involved in Eomes expression suggested that the TCR signaling pathway, and specifically ITK, could be a point of therapeutic intervention. As described previously in this chapter and in chapter 4, ITK plays a critical role as a rheostat for how a T cell is going to react to TCR stimulation. Specifically, in the case of $CD8^+$ T cells, pITK results in reduced/inhibited Eomes expression as mediated by IRF4. We chose ITK as a potential therapeutic target for a number of reasons. ITK is a signaling kinase and could be targeted for inhibition by a small molecule kinase inhibitor. ITK expression is conserved among T cells and not highly expressed in other tissues. There already is an FDA-

approved ITK inhibitor in the form of ibrutinib, and other drugs exist that target ITK as well. For these reasons I investigated if ibrutinib used in combination with αOX40/αCTLA-4 therapy would enhance $CD8^+$ T cell Eomes expression, and what the effects this novel triple therapy would be. The triple therapy had a synergistic effect with αOX40/αCTLA-4 on the expression of Eomes in $CD8^+$ T cells, while ibrutinib alone had no effect on Eomes expression. This is likely due to the pathways leading to high Eomes expression such as strong T cell activation and blockade of CTLA-4 were not present in these T cells. However, when ibrutinib was given to tumor-bearing mice along with αOX40/αCTLA-4 it led to further increased Eomes expression. Both pITK and IRF4 expression was significantly reduced in mice receiving triple therapy compared to αOX40/αCTLA-4, suggesting that the effect of ibrutinib functioned through ITK inhibition.

I observed that triple therapy led to an increase in tumor antigen-specific $CD8^+$ T cells as determined by SPAS1 tetramer staining compared to αOX40/αCTLA-4 or control treated mice. Similarly, Sagiv-Barfi et al showed an increase in tetramer+ $CD8^+$ T cells in ibrutinib/αPD-L1 treated mice (372). Is ibrutinib therapy in combination with checkpoint blockade/co-stim agonists enhancing the frequency and/or diversity of antigen specific $CD8^+$ T cells? We could use TCR-seq to test the hypothesis that ibrutinib is enhancing the clonality and tumor reactivity of $CD8^+$ T cells for αOX40/αCTLA-4 treatment. $CD8^+$ T cells would be isolated from the blood and tumor one week after treatment from TRAMP-C1 tumor bearing mice for TCR sequencing. Groups would be compared between WT, ibrutinib, αOX40/αCTLA-4, and αOX40/αCTLA-4/ibrutinib cohorts. This approach will allow us to determine TCR diversity and clonal abundance in $CD8^+$ T cells responding to each therapy. We would hypothesize that triple therapy treated mice would have expanded clonal diversity, and enhanced abundance of specific tumor reactive clones.

However, triple therapy had wider effects than just enhancing Eomes expression. There was also an overall increase in both $CD8^+$ and $CD4^+$ T cell function. Some of the effects on the $CD8^+$ T cell compartment can be explained by enhanced Eomes expression, such as higher

proliferation, TNF-α, IFN-γ, and CXCR3 expression. However, this does not explain the similar increased function seen in $CD4^+$ T cells. There are a few important differences between how $CD8^+$ and $CD4^+$ T cells might respond to ITK inhibition. Both $CD8^+$ and Th1 $CD4^+$ T cells have redundant signaling kinases in the TCR signaling pathway that can compensate for the loss of ITK signaling. This redundant kinase is RLK, which is not inhibited by ibrutinib at the concentrations used. Additionally, RLK does not signal through IRF4 in $CD8^+$ T cells, and thus does not inhibit Eomes expression. Th2 $CD4^+$ T cells on the other hand do not express RLK and are dependent on ITK signaling for T cell activation. Byrd et al. investigated the effects of ibrutinib on $CD4^+$ T cell polarization in patients receiving ibrutinib for the treatments of CLL (389). They found that patients receiving ibrutinib had inhibited the activation of Th2 $CD4^+$ T cells and that they skewed towards a Th1 dominant phenotype. Additionally, Levy et al. investigated the effects of ibrutinib on T cells in the context of αPD-L1 cancer therapy (372) and found that this combination had therapeutic efficacy across multiple tumor models, leading to enhanced numbers of tumor-specific T cells. However, a mechanism of action was not fully explained. The effects of αOX40/αCTLA-4/ibrutinib triple therapy may be multi-faceted and work through a combination of enhancing Eomes expression in $CD8^+$ T cells and inhibiting Th2 $CD4^+$ T cell polarization.

While ibrutinib has thus far been discussed in the context of its effect on ITK expression and function in T cells, it has to be noted that ibrutinib is a drug designed to inhibit BTK expression in B cells. A concern of using ibrutinib as a means of inhibiting ITK would be that it could potentially inhibit BTK expression as well, the consequences of which could be detrimental to the therapy. In the case of inhibition of B cells, there appears to be mounting evidence that ibrutinib does not have a significant effect on mature antigen experienced B cells (417). Additionally, I found that B cells were dispensable to the efficacy of triple therapy. Another concern of using ibrutinib is its effect on the myeloid compartment as some myeloid cells

express BTK, such as monocytes and macrophages (418). While this may be a concern, I saw no significant change to overall myeloid cells or their function in mice receiving triple therapy compared to αOX40/αCTLA-4. One further concern with ibrutinib therapy is if BTK inhibition has a direct effect on the tumor growth or the way the tumor tailors the TME. However, if this is the case it would likely lead to further tumor inhibition. Some groups have even proposed BTK as a potential therapeutic target in prostate cancer (419). In my experiments looking at the effect of ibrutinib on any of the tumor cell lines I used for *in-vivo* experiments, ibrutinib had no effect on tumor cell growth, even at concentrations 100X higher than used *in-vivo*. These concerns might further be abrogated with the use of an ITK specific inhibitor such as the BMS compound 509744, however further research would be needed. Regardless, ITK specific inhibitors have great therapeutic potential.

To further evaluate global changes and unique signatures associated with αOX40/aCLTA-4 therapy, and the impact of ibrutinib, we could utilize single-cell RNA sequencing. TRAMP-C1 tumor bearing mice would be treated with control IgG, αOX40/αCTLA-4/ or triple therapy. One week after treatment, CD45+ cells would be sorted out of tumors by FACS for scRNAseq analysis. Unique cell types will be clustered using UMAP. We will determine differentially expressed genes associated with therapy, and unique pathways associated with Eomes regulation.

I have shown that ibrutinib has strong anti-cancer efficacy when used in combination with αOX40/αCTLA-4 therapy. However, ibrutinib may synergize with other therapies, as was shown with PD-L1 (372). Considering that ibrutinib enhanced $CD8^+$ T cell Eomes expression under strong T cell activating conditions and is known to skew $CD4^+$ T cells towards a Th1 phenotype, perhaps we should look at therapies that would drive Eomes expression or be limited by Th2 polarization. For example, other anti-TNFR mAbs, such as a4-1BB, have been suggested to play a role in the induction of Eomes (420). However, targeting other TNFRs might

run into similar issues as αOX40 in that Eomes was not highly expressed without the addition of αCTLA-4. While one report showed that αPD-L1 therapy synergized with ibrutinib to reduce tumor growth, my studies revealed that αPD-1 or αOX40/αPD-1 did not induce Eomes expression, which strongly suggested an important role for αCTLA-4 (372). Targeting ICOS may also be efficacious because it plays a role to $CD4^+$ T cell polarization and skewing towards Th2. Additionally, therapies that enhance TCR signaling like cancer vaccines and therapies that enhance NF-kB signaling, like TLR agonists, may also work well with ibrutinib to further $CD8^+$ T cell Eomes expression.

There are several ongoing clinical trials relevant to my research. One trial is looking at the efficacy of αOX40 and αCTLA-4 in combination. A phase I/II trial is investigating the combination of BMS-986178 by itself or in combination with pembrolizumab and/or ipilimumab for patients with advanced solid tumors (NCT02737475). There is an ongoing OX40 trail looking at the combination of a hexavalent OX40 agonist in combination with pembrolizumab for the treatment of advanced metastatic solid tumors (NCT04198766).

IRF4 inhibition in the form of an antisense oligonucleotide against IRF4 has shown efficacy in the treatment of myeloma (421). Myeloma tumor bearing mice treated with the antisense oligonucleotide against IRF4 had decreased tumor burden and increased overall survival. No analysis was done on the direct effects on the immune cell populations. The use of a drug to inhibit IRF4 rather than upstream ITK may be a cleaner way of tailoring the T cell response but may lose the Th2 inhibitor effects of ITK inhibition (422).

Ibrutinib (Imbruvica) is being investigated in clinical trials for use in combination with immunotherapeutic drugs. Ibrutinib and pembrolizumab are being evaluated in a phase II trial for high-risk lymphocytic leukemia with a focus on evaluating improved immune function (NCT03514017). A phase I trial is looking at the combination of ibrutinib and nivolumab for patients with metastatic solid tumors (NCT03525925). Preliminary results from this phase I trials

indicated that T cell function improved throughout the study for patients receiving combination ibrutinib and nivolumab. Four of the 16 patients had a partial response and another 4 of 16 patients had stable disease. The combination was well tolerated. A Phase Ib/II trial looking at the combination of ibrutinib and durvalumab, a PD-L1 blocking antibody, has been evaluated for patients with pre-treated relapsed/refractory solid tumors (NCT02401048). The combination was well tolerated but had limited anti-tumor activity in the cohort. Other clinical trials looking at the combination of ibrutinib with αPD-1 are ongoing. Many of these trials are for the treatment of refractory lymphomas and leukemias (NCT02332980, NCT03204188). Some attention has been given towards the use of ibrutinib for the treatment of prostate cancer, however ibrutinib has yet to be evaluated in combination with immunotherapy. A phase II trial examined the effects of ibrutinib given pre-surgery to prostate cancer patients, with the intention of inhibiting tumor cell growth and enhancing the patient's anti-tumor immune response prior to surgery (NCT02643667).

5.3. Conclusions

As has been iterated many times in this body of work, checkpoint blockade has great potential but limited success in treating a variety of cancers. From murine studies and clinical trials, it has been determined that monotherapy checkpoint blockade has only limited therapeutic efficacy and that combination immunotherapy may be an answer to better clinical outcomes as demonstrated by the enhanced efficacy of αPD-1/αCTLA-4 therapy. However, there still is a lack of knowledge regarding mechanisms of how certain immunotherapies function and the mechanisms by which combination immunotherapies may synergize. With that knowledge from pre-clinical murine studies, we can inform upon and rationally design novel immunotherapy combinations.

My research aimed to advance immunotherapy design through investigating the combination of a checkpoint inhibitor (αCTLA-4) and a co-stimulatory agonist (αOX40) in order to better understand how they these two specifically interact, what aspects of this combination drives anti-tumor immunity, and how this therapy might be enhanced for clinical use. An important finding from my research was the critical nature of Eomes^{hi} CD8^{+} T cells following αOX40/αCTLA-4 therapy. This raises the question as to whether Eomes^{hi} CD8^{+} T cells are as critical following other cancer therapies or if this is a unique property of αOX40/αCTLA-4. My research profiling the function of Eomes^{hi} CD8^{+} T cells demonstrated they play an important memory T cell role. As evidenced by my research and studies investigating Eomes expression in human cancers, it would seem the generation of Eomes expression is beneficial and may be critical to the anti-tumor immune response. I used this novel understanding of Eomes^{hi} CD8^{+} T cells to inform therapy design by investigating the role of ITK inhibition on Eomes^{hi} generation. The combination of αOX40/αCTLA-4/ibrutinib therapy was investigated to test the hypothesis that if the generation of Eomes^{hi} T cells enhances αOX40/αCTLA-4 therapy, then further enhancing Eomes expression with ibrutinib would improve the overall therapy. My novel finding was that the ITK inhibitor effects of ibrutinib further enhanced Eomes^{hi} CD8^{+} T cells in αOX40/αCTLA-4 therapy leading to reduced tumor growth and enhanced survival in tumor bearing mice compared to αOX40/αCTLA-4 alone. This is a significant and impactful finding, as ibrutinib is FDA-approved, orally available, and has limited side effects. Ongoing clinical trials investigating ibrutinib in combination with checkpoint blockade have shown an acceptable safety profile. The knowledge generated by my research on the generation and function of Eomes^{hi} CD8^{+} T cells, as well as the use of ibrutinib as an ITK inhibitor, has strong potential for therapeutic design and translation to the clinic.

My research also raised several important questions that would be valuable for the field to address in the future, such as a need for further investigation into the mechanism of Eomes

expression in $CD8^+$ T cells, which may inform upon other therapeutic points of intervention for better therapy design. The use of a pure ITK inhibitor such a BMS-509744 may be a more specific way of inhibiting ITK to generate Eomes expression, or perhaps targeting IRF4 may be a better option as it has potential for direct αtumor effects as well. Further studies are needed regarding the expression and function of $Eomes^{hi}$ $CD8^+$ T cells in humans. Overall, my research added valuable knowledge to the field of translational immunotherapy, and suggests the use of ibrutinib and modulating of Eomes for future research and clinical therapy design.

6. References

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