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Novel Imaging-Based Techniques Reveal a Role for PD-1/PD-L1 in Tumor Immune Surveillance in the Lung

Todd Bartkowiak

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Novel Imaging-Based Techniques Reveal a Role for PD-1/PD-L1 in Tumor Immune Surveillance in the Lung

by

Todd Jacob Bartkowiak, B.S.

APPROVED:

____________________________
Tomasz Zal, Ph.D
Supervisory Professor

____________________________
[Francois Claret, Ph.D]

____________________________
[Eugenie Kleinerman, M.D.]

____________________________
[Dean Lee, M.D., Ph.D]

____________________________
[Gregory Lizee, Ph.D]

APPROVED:

________________________________________________________
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston
NOVEL IMAGING-BASED TECHNIQUES REVEAL A ROLE FOR PD-1/PD-L1 IN TUMOR IMMUNE SURVEILLANCE IN THE LUNG

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The University of Texas
Health Science Center at Houston
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for the Degree of
MASTER OF SCIENCE

By
Todd Jacob Bartkowiak, Bachelor of Science
Houston, Texas
May, 2013
The binding of immune inhibitory receptor Programmed Death 1 (PD-1) on T cells to its ligand PD-L1 has been implicated as a major contributor to tumor induced immune suppression. Clinical trials of PD-L1 blockade have proven effective in unleashing therapeutic anti-tumor immune responses in a subset of patients with advanced melanoma, yet current response rates are low for reasons that remain unclear. Hypothesizing that the PD-1/PD-L1 pathway regulates T cell surveillance within the tumor microenvironment, we employed intravital microscopy to investigate the in vivo impact of PD-L1 blocking antibody upon tumor-associated immune cell migration. However, current analytical methods of intravital dynamic microscopy data lack the ability to identify cellular targets of T cell interactions in vivo, a crucial means for discovering which interactions are modulated by therapeutic intervention. By developing novel imaging techniques that allowed us to better analyze tumor progression and T cell dynamics in the microenvironment; we were able to explore the impact of PD-L1 blockade upon the migratory properties of tumor-associated immune cells, including T cells and antigen presenting cells, in lung tumor progression. Our results demonstrate that early changes in tumor morphology may be indicative of responsiveness to anti-PD-L1 therapy. We show that immune cells in the tumor microenvironment as well as tumors themselves express PD-L1, but
immune phenotype alone is not a predictive marker of effective anti-tumor responses. Through a novel method in which we quantify T cell interactions, we show that T cells are largely engaged in interactions with dendritic cells in the tumor microenvironment. Additionally, we show that during PD-L1 blockade, non-activated T cells are recruited in greater numbers into the tumor microenvironment and engage more preferentially with dendritic cells. We further show that during PD-L1 blockade, activated T cells engage in more confined, immune synapse-like interactions with dendritic cells, as opposed to more dynamic, kinapse-like interactions with dendritic cells when PD-L1 is free to bind its receptor. By advancing the contextual analysis of anti-tumor immune surveillance in vivo, this study implicates the interaction between T cells and tumor-associated dendritic cells as a possible modulator in targeting PD-L1 for anti-tumor immunotherapy.
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ABBREVIATIONS

AITP: Average intensity time projection

APC: Antigen-presenting cell

CD: Cluster of differentiation

cDC: Conventional dendritic cell

CFP: Cyan fluorescent protein

DC: Dendritic cell

FACS: Fluorescence activated cell sorting

GFP: Green fluorescent protein

ICOS: Inducible costimulator

IFNγ: Interferon γ

IL6: Interleukin 6

IL10: Interleukin 10

IM: Intravital microscopy

ITIM: Immunoreceptor tyrosine-based inhibitory motif

MCA: Methylcholanthrene

MDSC: Myeloid derived suppressor cell
MFI: Mean fluorescence intensity

MHC: Major histocompatibility complex

MITP: Maximum intensity time projection

PD-1: Programmed cell death receptor -1

PD-L1: Programmed cell death receptor ligand-1

PD-L2: Programmed cell death receptor ligand-2

PI3K: Phosphatidylinositol 3-kinase

RFP: Red fluorescent protein

SHP2: Src-homology region 2 domain-containing phosphatase-2

SMAC: Supramolecular activating cluster

TAA: Tumor-associated antigen

TAM: Tumor-associated macrophage

TCR: T cell receptor

TGF-β: Transforming growth factor β

Treg: Regulatory T cell

YFP: Yellow fluorescent protein

ZAP-70: Zeta-chain-associated protein kinase
1. Introduction

1.1 Immune surveillance

The theory that the immune system plays an important role in inhibiting malignant transformation and growth of the body’s own cells, tumor immune surveillance, was developed by Macfarlane Burnet from the 1950s to the 1970s (1-3). Robert Schreiber’s work (4, 5) further expounded the work of Burnet to uncover mechanisms by which tumors may escape immune surveillance and continue to grow.

In a broad context, immune surveillance (Figure 1) as reviewed by Kupper and Fuhlbrigge (6) is the process by which immune cells in the peripheral tissue recognize and eliminate foreign pathogens that may be deleterious to the organism. The immune system is also able to recognize stress and/or damage signals given off by transformed tumor cells, and engulf altered self-antigens expressed by the tumor (3, 7). Phagocytic cells in the peripheral tissue engulf these tumor-associated antigens (TAAs) and destroy transformed cells. Occasionally, transformed cells can evade phagocytosis and continue to grow. In these instances, an adaptive immune response must be initiated for tumor elimination (8). In order for the adaptive immune system to mount an anti-tumor response, tumor antigens engulfed in the tissue by antigen-presenting cells (APCs) must be presented to naïve T cells in the lymph node. Upon engagement with antigen in the lymph node, T cells receive activation signals and are primed to respond to antigen in the peripheral tissue. Primed, mature T cells then leave the lymph node and enter the circulation in search
of the source of foreign antigen. Following cytokine gradients, mature T cells extravasate into peripheral tissue and migrate to the tumor site. Within the tumor site, T cells must maintain their activation state and begin to perform their effector function. T cells can act in a supportive role to boost the anti-tumor response as is the case for CD4+ T cells, in a cytotoxic response to kill the tumor, as is the case for a variety of cells including CD8+ T cells (9), or in a regulatory role to prevent unrestrained effector responses, as is the role of regulatory T cells (Tregs) in the tumor microenvironment (10).
Figure 1.

1. **Peripheral Tissue**
   - Engulfment of tumor antigen
   - Trafficking to lymph node

2. **Lymph Node**
   - Antigen presentation
   - T cell activation

3. **Peripheral Tissue**
   - Recruitment
   - Activation
   - Migration
   - Effector function
Figure 1. Components of immune surveillance. Tumor immune surveillance is a multi-step process whereby the immune system is able to continuously recognize and eliminate malignant transformed cells in tissues. 1) Surveillance initiates when innate immune cells respond to stress/damage signals given off by tumor cells and engulf tumor antigens. These cells then traffic to the lymph node to present tumor antigens to naïve T cells. 2) Upon recognition of presented antigen, T cells receive activation signals before leaving the lymph node. 3) Once T cells leave the lymph node, they circulate throughout the body. Following chemokine gradients, T cells must be recruited to the tumor site. Maintaining activation at the tumor site as well as migration around the tumor are important aspects of T cell anti-tumor responses. These aspects allow T cells to exert various effector functions in order to eliminate tumor cells.
1.2 Immune synapse formation

Presentation of tumor antigens to T cells in the lymph node is crucial to an adaptive immune response (6). Antigen presentation occurs as T cells, through their T cell receptor (TCR), recognize foreign peptides presented by major histocompatibility complexes (MHC) on antigen-presenting dendritic cells (DC). Each T cell expresses a clonally distinct TCR that recognize specific epitopes on the antigen surface which must be recognized in the context of MHC.

Clusters of TCR-MHC complexes form the immune synapse (11). The immune synapse is a major signaling complex through which T cells interact with DC to receive activation signals. The immune synapse consists of three distinct rings of supramolecular activating clusters (SMACs) on the T cell surface. The central ring, the cSMAC consists of 1) TCR microclusters as well as 2) coreceptors (CD8 or CD4) necessary for stabilization of the TCR-MHC complex, and 3) costimulatory molecules (CD28, ICOS) that are necessary for propagation of a strong activating signal. The peripheral SMAC (pSMAC) encircles the cSMAC and consists of adhesion molecules such as CD2 and leukocyte function associated antigen-1 (LFA-1) which are spatially segregated from the cSMAC based dimension and which provide structural support to the synaptic complex. The distal SMAC (dSMAC) surrounds the pSMAC and consists of very large phosphatase (CD43, CD44 and CD45) molecules that further support signaling at the immune synapse. The clustering of the molecules into discrete regions in the immune synapse allows for strong, stable interactions between T cells and APCs and efficient propagation of molecular signals resulting in T cell activation (12, 13).
Recent in vitro evidence, though, suggests that motile T cells do not always form strong, prolonged immune synapses with APCs. Loosely formed synapses are characterized by stretching of the SMAC regions as the synapse is dragged across the DC surface during T cell movement. This type of interactions is termed the kinetic synapse or kinapse (14, 15). T cells in vitro that make strong receptor/ligand binding interactions at the synapse concomitantly reduce their motility in order to receive a full activation signal (16), however, T cells that form immune kinapses do not reduce their mobility, and as such form less stable, transient receptor/ligand interactions that are easily and quickly broken. These interactions occur as T cells move and slide across the surface of the APC and typically last on the order of only a few minutes as T cells receive weak activation signals (12). T cell engagement in either strong immune synapses or weak immune kinapses suggests a mechanism of activation by which T cells can either form long lasting synaptic signaling complexes with APCs to achieve full activation, or contrarily T cells may form short term kinaptic interactions with multiple APCs over a short time receiving only a partial activation signal from each APC which ultimately results in full T cell activation (12). While evidence suggests that T cells can form both synapses and kinapses in vivo (17), whether T cells makes these interactions in the context of tumors, and the role that PD-1/PD-L1 interactions play in forming these interactions in the tumor microenvironment is yet to be fully elucidated.

1.3 Tumor immunogenicity

Regardless of the means by which T cells becomes activated, the adaptive response is largely dependent on the immunogenicity of the tumor; that is the ability
of the altered tumor antigens expressed by the tumor to elicit an immune response in the body. Tumors can be classified based on how well they can provoke an immune response (18). Table 1 illustrates some of the varying degrees of tumor immunogenicity.

Highly immunogenic tumors elicit strong adaptive immune responses with large T cell infiltration. Tumors in this category are rejected in naïve syngeneic hosts upon primary transplantation of tumors. Subsequent transplantation of the same tumor type also leads to tumor rejection.

In contrast, tumors with intermediate immunogenicity are not rejected upon primary transplantation of tumor cells into naïve syngeneic hosts. However, any subsequent transplantation of the same tumor cell line is rejected. In this case, tumors trigger an adaptive response that is unable to effectively respond to the primary transplantation; however, upon secondary encounter T cells mount a potent anti-tumor response leading to rejection of any secondary transplantation.

Lastly, poorly immunogenic tumors, or tumors with no immunogenicity are not rejected when transplanted into naïve syngeneic hosts, nor are any subsequently transplanted tumor cells rejected. Tumors that have low or no immunogenicity may evoke a small adaptive immune response if any adaptive response at all.

Our lab is interested in studying tumors with intermediate immunogenicity (Figure 2). In particular, we are interested in the possible mechanisms by which an intermediate tumor can be recognized by the immune system and effectively killed, but by unclear mechanism(s) can evade the immune response. To study potential
mechanisms of immune evasion in intermediately immunogenic tumors, we used an experimental model of metastasis which allows for tracking of tumor progression from a single cell stage. Our tumor model is an MCA transformed fibrosarcoma which we show readily metastasizes, engrafts, and grows in the lung (Figure 2A). Evidence from our lab shows that tumors progress until overwhelming tumor burden in the lungs restricts lung function and turns lethal. However, when naïve mice receive first a subcutaneous injection of MCA tumor cells, followed by a second intravenous injection of MCA cells, the majority of tumors are rejected in the lung (Figure 2B). Our evidence of the MCA immunogenicity is in line with other reports of the immunogenicity of MCA induced fibrosarcomas (19). In our model shown in Figure 2B, subcutaneous tumor burden leads to mortality, not tumor burden in the lung. We also see T cell recruitment to MCA tumor nodules after the primary transplant, suggesting a mechanism of immune suppression in the tumor microenvironment.
Table 1. Tumor immunogenicity

<table>
<thead>
<tr>
<th>Immunogenicity</th>
<th>Primary Transplant</th>
<th>Secondary Transplant</th>
<th>Adaptive Immune Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>Rejected</td>
<td>Rejected</td>
<td>Yes</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Not Rejected</td>
<td>Rejected</td>
<td>Yes</td>
</tr>
<tr>
<td>None</td>
<td>Not Rejected</td>
<td>Not Rejected</td>
<td>Maybe</td>
</tr>
</tbody>
</table>
Figure 2.
Figure 2. Methylcholanthrene induced MCA-205 fibrosarcoma represents an intermediately immunogenic cancer model. A) MCA-205 fibrosarcoma cells transduced with the tdimer (12) red fluorescent reporter were injected intravenously (IV), resulting in a robust growth of tumors in the lungs of naïve syngeneic mice, eventually leading to death. B) However, similar IV injection of MCA cells into the mice that were, seven days earlier, implanted with MCA cells subcutaneously resulted in the rejection of cancer cells in the lung despite the continued growth of the subcutaneous tumors.
1.4. Tumor-mediated immune suppression

Tumors employ a host of mechanisms to evade effective anti-tumor immune surveillance. Table 2 lists a few mechanisms by which tumors suppress immune responses, though this is by no means an exhaustive list. One mechanism of suppression involves recruitment of immune cells into the tumor microenvironment that can suppress T cell function. For instance, regulatory T cells recruited to the tumor microenvironment can express IL10 and TGF-β (10, 20) to inhibit T cell proliferation. Myeloid derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs) recruited into tumor lesions can express inhibitory cytokines to inhibit anti-tumor responses as well pro-tumorigenic cytokines that help the tumor grow (21). Recent evidence also suggests that a population of dendritic cells (DC) recruited to the tumor microenvironment acquires a suppressive function (22-24). A second mechanism of immune suppression entails secretion of inhibitory molecules in the tumor microenvironment such as IL6 and IL10 by the tumor itself or by suppressive cells in the tumor microenvironment (25-27).

A new and developing interest in the field of tumor immunology relates to the recent discovery that tumors can express various ligands for inhibitory receptors found on the T cell surface (28). Blockade of these inhibitory molecules may increase T cell activation and promote cancer cell killing by otherwise immune-suppressed T cells. Figure 3 shows several different inhibitory receptors found on T cells as well as their ligands present in the tumor microenvironment, though the exact mechanisms by which immune suppression is facilitated by the receptor/ligand interactions varies between receptor types (28).
<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Example</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressive cells</td>
<td>Tregs</td>
<td>• Produce TGFβ and IL10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Uptake IL2</td>
</tr>
<tr>
<td></td>
<td>MDSC</td>
<td>• Produce IL6, IL10, TGFβ, iNOS and arginase</td>
</tr>
<tr>
<td></td>
<td>TAM</td>
<td>• Produce IL10, iNOS, and arginase</td>
</tr>
<tr>
<td>Cytokines</td>
<td>IL6</td>
<td>• Promotes inflammation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Inhibits Th1 differentiation</td>
</tr>
<tr>
<td></td>
<td>IL10</td>
<td>• Inhibits IFNγ, TNFα, and IL2 production</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Suppresses antigen presentation</td>
</tr>
<tr>
<td></td>
<td>TGFβ</td>
<td>• Converts T&lt;sub&gt;off&lt;/sub&gt; cells to T&lt;sub&gt;reg&lt;/sub&gt; cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Blocks T cell activation</td>
</tr>
<tr>
<td>Inhibitory receptor/ligand</td>
<td>CTLA4/CD80(CD86)</td>
<td>• Suppresses T cell stimulation</td>
</tr>
<tr>
<td>interactions</td>
<td></td>
<td>• Dampens T cell activation</td>
</tr>
<tr>
<td></td>
<td>PD-1/PD-L1(2)</td>
<td>• Dampens T cell activation and effector responses</td>
</tr>
</tbody>
</table>
Figure 3. Inhibitory receptor/ligand interactions in the tumor microenvironment. Ligands for inhibitory receptors expressed by T cells can be highly expressed in the tumor microenvironment and through different mechanisms lead to suppressed T cell responses. For instance, either of the B7 molecules (B7.1/CD80 or B7.2/CD86) bind CTLA4, suppressing activation signals. Similarly, CD80/PD-L1 interactions hamper T cell costimulation. Likewise, PD-1/PD-L1(2) interactions in the tumor microenvironment stifle T cell signaling cascades.
1.5. PD-1/PD-L1 interactions

Programmed Death Receptor-1 (PD-1) is a type I transmembrane protein consisting of an extracellular IgV domain, a transmembrane domain, and a short intracellular domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) (29, 30). PD-1 is upregulated on monocytes (31), dendritic cells (24), regulatory T cells (32), some hematological tumors (33), activated B cells (34) and chronically activated/exhausted T cells (35). Upregulation and subsequent binding of PD-1 to PD-L1 during chronic activation leads to suppressed T cell signaling in the immune synapse. Src-homology region 2-containing phosphates-2 (SHP-2) has been implicated in binding and transportation of PD-1 into the central supramolecular activation cluster (cSMAC) in the immune synapse (36), where phosphorylated signaling complexes including zeta-chain associated protein kinase 70 (ZAP-70) are dephosphorylated (Figure 4). Disruption of the phosphatidylinositol 3-kinase (PI3K) pathway has also been implicated in dampening of T cell activation signal through PD-1 signaling (37).

Two ligands for PD-1 are currently known: Programmed Death Receptor Ligand-1 (PD-L1) and Programmed Death Receptor Ligand-2 (PD-L2). While PD-L2 expression is restricted to dendritic cells (38-40), PD-L1 is more ubiquitously expressed on a variety of cells including antigen-presenting cells (41), T cells (42), endothelial cells (43), and on various tumor types (44, 45).

PD-L1 is a type I transmembrane protein whose expression is regulated through a balance between interferon-gamma (IFNγ) signals (46, 47) and microRNA
activity (47). PD-L1 expressed on the cell surface not only binds to PD-1, but can also bind the costimulatory molecule CD80 which not only sequesters CD80 from making costimulatory interactions with CD28 on T cells, but this CD80/PD-L1 interaction also inhibits T cell proliferation and effector responses (48-51).
Figure 4.
**Figure 4. PD-1 dampens TCR signaling.** Recognition and binding of peptide-MHC complexes by the T cell receptor propagates a weak activation signaling cascade through the T cell. Coreceptor molecules such as CD4 or CD8 stabilize TCR/peptide-MHC binding allowing for stronger, longer signaling. Costimulation provided by CD28/CD80 binding further amplifies the TCR activation cascade. However, ligation of PD-1 on chronically activated T cells to PD-L1 allows for recruitment and binding of SHP-2 the ITIM motif of PD-1. This complex then moves into the immune synapse and dephosphorylates surface level signaling intermediates in the TCR signaling cascade.
1.6. Cancer immunotherapy by immune checkpoint blockade

Immune checkpoint blockade using antibodies to target inhibitory receptors or their ligands (28, 52) has proven effective in the treatment of several cancer types (53-56). Ipilimumab, an anti-CTLA4 blocking antibody has already been approved for the treatment of melanoma (57-59). In mice, anti-PD-1 and anti-PD-L1 antibody blockade has been shown to increase T cell activation in the lymph nodes of mice bearing B16 melanomas, leading to tumor regression (60). Anti-PD-1 (55) and anti-PD-L1 (56) antibodies are currently in Phase II clinical trials showing promising results in the treatment of advanced melanoma. While patients receiving anti-PD-L1 therapy have shown long-lasting clinical responses with few side-effects, response rates remain low. In order to improve the efficacy of PD-L1 targeted immunotherapies, we seek to better understand the exact role of PD-1/PD-L1 interactions within the tumor microenvironment and how these interactions inhibit effective T cell immune surveillance.

1.7. A need for novel methods to analyze tumor progression and anti-tumor immune responses.

Current methods to study tumor progression and anti-tumor immune responses do not accurately reflect true tumor progression in vivo. One current method to analyze tumor progression involves the genetic manipulation of mouse models to promote development of spontaneous tumors. In addition, these models require constant monitoring of tumor development. In these models, tumors growth is typically assessed using calipers to measure tumor dimensions over time. However,
the tumor must be 1) visible and large enough to measure and 2) easily accessible for measurement. Hence, the development of tumors prior to this point is largely unexplored using this method. Microscopy can circumvent this limitation with the ability to image small tumors before they can be seen with the naked eye. However, most microscopists are unconcerned with using microscopy to look at tumor development over time, and instead focus on events occurring at the tumor lesion. Our lab developed a novel imaging technique that allows us to visualize total tumor burden in an entire organ, the lung, whether tumors are microscopic or large enough to be seen. By imaging the full lung tissue, we gain a better insight into the total amount of lung area taken up by the tumor. In line with studying tumor progression, we also developed novel analyses, what we call the “tumor roundness index” to determine the effect of anti-PD-L1 blockade on tumor morphology.

Intravital microscopy (IM) is a valuable tool that has greatly enhanced our understanding of immune cell dynamics in the lymph node (61-63) as well as within the peripheral tissue (64, 65) and tumor microenvironment (66-68). However, how and where T cells interact with other cells in vivo is poorly understood. Davis reviews mechanisms by which lymphocytes interact (69), but apart from molecular interactions, defining dynamic interactions in vivo has proven difficult. Interactions can be easily studied in vitro by looking at cell-cell binding interactions at the molecular level (15, 36, 70). However, cell-cell interactions are more difficult to uncover in vivo. In vivo, an interaction can be defined as a reduction in velocity (70), or by a local proximity to another cell concomitant with expression of effector molecules (64, 67). Yet these methods do not take into account interactions which
may not provoke an effector response. In our lab, we developed a novel method to analyze T cell interactions in the tumor microenvironment based on the longevity and localization of cell persistence, which we derive from the intensity-coded time projections of intravital motility recordings. Using this method, we were able to discern areas where T cells were engaged for long periods with various interacting partners. We could then quantify each interaction and then determine the effect that PD-L1 blockade had on these actions.

1.8 Specific Aims

We hypothesize that PD-1/PD-L1 interactions in the tumor microenvironment inhibit immune-mediated tumor destruction in part by modulating the spatiotemporal dynamics of T cell surveillance behavior. To address this hypothesis, we first needed to develop more efficient imaging-based methods that would allow us to explore the effect of PD-L1 blockade on tumor burden and T cell dynamics in the tumor microenvironment. The intent of my thesis was therefore twofold:

AIM 1: Develop novel imaging-based techniques to study tumor growth and immune cell interactions in the tumor microenvironment.

AIM 2: Determine the effect of PD-L1 blockade on tumor progression and on immune cell dynamics in the tumor microenvironment.

Development of these novel methods showed that tumors regress over a thirty day period when treated with anti-PD-L1 antibody. The beginning of tumor regression appears to be concordant with a change in morphology and reduction in tumor area seen within one week of PD-L1 blockade. Most importantly, using our
novel method to quantify T cell interactions in the tumor microenvironment, we showed that non-activated T cells are recruited in greater numbers during PD-L1 blockade and these T cells make more preferential interactions with dendritic cells in the tumor microenvironment. Additionally, activated T cells are able to make more stable interactions with dendritic cells in the tumor microenvironment during PD-L1 blockade.
2. Materials and Methods

2.1 Mice

C57BL/6 albino mice were purchased from the National Cancer Institute (Bethesda MD). CD2-DsRed reporter mice were a kind gift from Dimitris Kioussis (MRC National Institute for Medical Research, London, UK) (71). IL2p8-GFP mice were a gift from Ellen Rothenberg (California Institute of Technology, Pasadena, CA) (72). CD11c-YFP mice were from Michel Nussenzweig (Rockefeller University, New York City, NY) (61). All transgenic mouse strains were bred on a C57BL/6 background. Strains were interbred to produce a single mouse strain homozygous for all three fluorescent reporter transgenes. Mice were maintained in a specific pathogen free facility, and all procedures carried were out in accordance with the guidelines established by the UT MD Anderson Institutional Animal Care and Use Committee.

2.2 Cell lines

The MCA-mCer cell line was generated by lentiviral transduction of the MCA-205 C57BL/6 mouse fibrosarcoma cell line with the mCerulean fluorescent protein gene driven by the CMV promoter. The cDNA encoding mCerulean was a gift from David Piston (Vanderbilt, Nashville TN). Mouse B16 melanoma cell were purchased from ATCC (Manassas, VA) and transduced with mCerulean by nucleoporation. Cells were cultured in IMDM media (Hyclon, ThermoScientific) supplemented with 5% FCS, 50 μM β-mercaptoethanol, 6mM L-glutamine, 100 U/ml penicillin-G, and 100μg/ml streptomycin. Cultures were grown in 5% carbon dioxide at 37°C.
2.3 Tumor injection and anti-PD-L1 therapy

MCA-205 mCerulean cells were trypsinized and washed in HBSS before injection. Single cell suspensions of tumor cells were then injected intravenously into 4-6 month old mice (3X10⁵ cells per mouse). After allowing the tumors to engraft and begin growing in the lung for seven days, PBS (control) or rat anti-mouse PD-L1 blocking antibody (10F.9G2, Bioxcell, West Lebanon, NH) was administered intravenously (100 µg/mouse in PBS) every two days for up to three weeks.

2.4 Cell isolation from mouse lungs

Lungs from tumor bearing mice receiving either anti-PD-L1 immunotherapy PBS as a control were finely chopped and digested in 10% Collagenase A and DNase 1 (10mg/ml) in IMDM supplemented with 5% FCS. Lungs were digested for one hour in a 37°C incubator while rocking at 400 RPM. Intact tissue was then passed through a pipette before being filtered through a 70µm cell strainer (BD Biosciences). Filtrate was then washed three times in IMDM with 5% FCS and centrifuged at 1200RPM/4°C after each wash. Cells were then stained for FACS analysis.

2.5 Antibodies

The following antibodies were used for flow cytometry: CD11b-PerCP/Cy5.5 (Mac-1a), CD11c-FITC (N418), F4/80-Pacific Blue (BM8), Ly6G-AlexaFluor647 (AF647) (RB6-8C5), CD80-PE (16-10A1), and CD86-PE (GL1) were purchased from eBioscience (San Diego, CA). PD-1-AF647 (RPM1-30) and PD-L1-AF647 (MIH6) were purchased from AbD Serotec (Raleigh, NC). CD8-PerCP/Cy5.5 (53-6.7) and
CD4-APC/Cy7 (GK1.5) were purchased from BD Biosciences (San Diego, CA). CD69-AF647 (HI-2F3) and ICOS-AF647 (C398.4A) were purchased from Biolegend (San Diego, CA). Rat anti-mouse IgG2a, IgG2b, or Hamster anti-mouse Ig (eBioscience) were used as isotype matched control antibodies. Rat anti-mouse CD16/CD32 (2.4G2, Fc-block, BD Biosciences) was used in all staining to block non-specific binding of antibody to Fc-receptors.

2.6 Flow cytometry

Two weeks after weaning, FACS analysis was performed on peripheral blood cells to verify fluorescence of the reporter genes. Briefly, blood was drawn from tail veins, and red blood cells were lysed with distilled water for one minute, followed by administration of 10X PBS to stop lysis. Cells were then stained with CD4-AF647 antibody (GK1.5, eBioscience) to confirm fluorescence specificity. Flow cytometry was performed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Cells from digested mouse lungs fifteen days after tumor injection were suspended in sterile IMDM supplemented with 5% FBS and blocked in Fc block (1:100) for 30 minutes before being washed three times. The cells were stained for one hour with various antibody combinations, including isotype-matched control antibodies, in sterile IMDM with 5% FBS, at 4°C. Cells were then thoroughly washed in sterile IMDM with 5% FBS and analyzed on an LSR Fortessa flow cytometer equipped with lasers for 405, 488, 561, and 640 nm wavelengths and controlled by
BD FACSDiva v6.3.1 software (BD Biosciences). All FACS analysis was performed using FlowJo software v10.0.5 (Treestar) on at least 5 mice per group.

2.7 In vitro analysis of PD-L1 and MHC Class I upregulation on cancer cells in response to interferon γ

3×10^5 cells were cultured with or without IFNγ (20U/ml) (from BD OptEIA mouse IFNγ ELISA set, BD Biosciences) for 48 hours before being trypsinized (0.05% trypsin, Fisher Scientific), washed in culture media and stained with anti-PD-L1 antibody conjugated to AlexaFluor 647 (MIH6, AbD Serotec), anti-H2Kb antibody conjugated to FITC (AF6-88.5, Biolegend), or isotype controls. Flow cytometry was then performed using an LSR Fortessa flow cytometer (BD Biosciences, San Jose CA).

2.8 Intravital Microscopy

All imaging was performed using a TCS SP5 RS laser resonant scanning microscope (Leica Microsystems, Wetzlar Germany) equipped with lasers for 405nm, 458nm, 488nm, 514nm, 543nm, and 633nm wavelengths. Intravital imaging was performed on tumor-bearing control mice or mice receiving anti-PD-L1 immunotherapy, all expressing various combinations of the CD2-RFP, IL2p8-GFP, and CD11c-YFP fluorescent reporter genes. Individual mice were anesthetized with pentobarbital (0.1mg/g body weight) (Sigma Aldrich) for the duration of imaging. After one full one-hour-long movie was imaged, mice were sacrificed without recovery before subsequent mice were anesthetized and imaged. Lungs were excised, fixed for at least one hour in 4% formaldehyde, and stored in a 30%
Anesthesia was maintained by administering a sucrose solution for further imaging. Diluted pentobarbital (0.6mg/ml) was administered throughout the entire experiment via an infusion pump (Instech Solomon, Plymouth Meeting, PA). Anesthetized mice were tracheotomized, intubated, and ventilated using a constant volume ventilator set at 0.20 cc injection volume and 129 breaths per minute (BPM) (Inspira Harvard Apparatus, Holliston MA). Mice were thoracotomized to open the chest cavity and a custom designed imaging apparatus was applied to the left lung. The apparatus consisted of an imaging window attached to a thermo-regulator and vacuum suction. Mice were placed on a heated microscope stage maintained at 37°C during the entire acquisition. Intravital images were obtained through sequential excitation scanning using a 20X objective (HCPL APO 20X/0.70 NA, Leica Microsystems) attached to a piezoelectric focusing lens (Piezosystem Jena, Hopedale MA) allowing for optimal z-scanning every 20 seconds. Images were acquired using 8-bit resolution, 512 pixel X 512 pixel dimension, with sequential scanning in x,y,z and time dimensions such that a full z-scan was acquired sequentially in each channel. To minimize bleed-through of fluorescent emission, green fluorescence emission was acquired separately from yellow fluorescence emission. Red and blue fluorescence were acquired concurrently. All images were taken using a zoom = 1.7, z-step size = 2 µm, and pinhole = 106 µm. Single one-hour-long movies were taken per mouse in at least 3 and up to 5 mice per group.

2.9 Ex-vivo imaging

To image entire lung lobes, mice were sacrificed and lung lobes were excised before being gently washed in PBS and mounted to a cover slide. Images were then
taken using a 4X objective (XLFluor 4x/360, 0.28NA, Olympus NDT Inc, Waltham MA).

To image lung tissue after intravital imaging, lungs were harvested from euthanized mice and fixed for a minimum of one hour in 4% formaldehyde (Sigma Aldrich, St. Louis MO), then washed in PBS and soaked in 30% sucrose in PBS before static images were taken using a 20X objective (HCPL APO 20X/0.70 NA, Leica Microsystems).

2.10 Image processing and motility analysis

Images were processed using the Leica Application Suite version 1.7.0 build 1240 (Leica Microsystems). Briefly, maximum intensity projections were created from all images. If spectral bleed-through was detected, linear unmixing was performed in each channel. To reduce noise, median filtering was performed using a filter width of 3.

To obtain percent tumor coverage, whole lung images taken from a minimum of three mice/group/day using a 4X objective (XL Fluor 4X/340 Olympus, NA=0.28) were stitched together in Photoshop (Adobe Photoshop CS4 Extended v11.0.2, Adobe Systems Incorporated). Tumor morphology parameters were obtained in Slidebook version 5.0 (Intelligent Imaging Innovations, Denver CO) using Ridler-Calvard thresholding with manual correction if needed. Total tumor area was compared to total lung area to acquire percent tumor coverage. Intensity-based masks were acquired from at least 15 thresholded images taken from at least 3 mice with a 20X objective (HCPL APO 20X/0.70 NA, Leica Microsystems) to find tumor
area and tumor roundness. Tumor roundness was obtained using the following equation:

\[ R = \frac{4\pi A}{P^2} \]

where \( R \) = tumor roundness, \( A \) = tumor area, and \( P \) = tumor perimeter.

T cell densities were acquired from static images by first enumerating the number of T cells in the image and normalizing to tumor area.

Intravital movies were processed in the Leica Application Suite as above. Tracking analysis was performed in Imaris v 7.5.1 (Bitplane, South Windsor CT). If necessary, movies were corrected for translational drift and smoothed for time using a filter width of 2. Cells were tracked using spot detection with a diameter of 10 \( \mu \)m. Tracks persisting for less than half of the movie time were excluded from analysis. Spider-plots were created by translating cell tracks to a common origin.

2.11 Analysis of T cell interactions

Analysis of T cell interactions was performed using the Leica Application Suite version 1.7.0 build 1240 (Leica Microsystems). An interaction was defined as a cell lacking translational motility within a confined area less than or equal to the cell’s own diameter for at least 10 minutes. To detect the sites of cell immobilization based on three-dimensional intravital motility recordings, we devised the multi-step protocol as follows. The raw 3-D time lapse recordings were first thresholded and binarized such as to convert the variable fluorescence intensities to intensity-independent cell shapes whose pixel intensities were equal to 1 whereas the non-
cell pixel intensities were equal 0. Next, the binarized time lapse data sets were processed to generate two types of time projections: the average intensity time projection multiplied by the constant factor 256 (AITP) and the maximum intensity time projection (MITP). The AITP image has the important property that the pixel intensities represent the duration of cell persistence in the area. For example, an intensity of 256 indicates that a cell spent 100% of its time in a location and an intensity of 128 indicates that a cell spent 50% of its time in said location. The MITP image represents all places visited by cells during intravital motility recording. To detect the sites of cell persistence for 10 min or longer in a 60 min long recording, the AITP image was thresholded at a pixel intensity value equal to 43 in an 8-bit image. This intensity value corresponded to the ten minutes of a one-hour-long movie according to the formula 10 min = 60 min x 43/256. The thresholded AITP and MITP images were then merged into a single two-color image. The areas of overlap represent areas of protracted immobility of 10 or more minutes in relation to all areas of cellular motility. The image representing the sites of T cell persistence were then overlaid over the images of other cells that were visualized during intravital recording, such as tumor cells or dendritic cells, and the overlaid composite images were inspected. Sites of persistence were categorized based on the type of immediately adjacent neighboring cells. Interaction was defined as T cells persisting with thresholded overlaps within one T cell length around a potential target. Analysis of T cell interactions was performed on 4 movies from 4 mice per treatment and control groups.
2.12 Statistical analysis

All statistical analysis was performed in Graphpad Prism version 5.03 (Graphpad Software). Bar graphs show mean ± standard deviation unless otherwise indicated. Bars in scatter plots show median values. Arrest coefficient was defined as the proportion of time a T cell maintained an instantaneous velocity below 1.5 μm/min. Statistical significance was determined using a non-parametric Mann-Whitney U test or unpaired Students’ T to compare differences between groups. P-values less than 0.05 were considered significant.
Results

3. Effect of PD-L1 blockade on tumor growth and immune phenotype

3.1 Whole-lung image analysis revealed therapeutic antibody blockade of PD-L1 reduces MCA tumor burden in mouse lungs.

To determine the effect elicited by anti-PD-L1 blockade therapy, four to six month old C57BL/6 mice were intravenously injected with $3 \times 10^5$ MCA-mcer fibrosarcoma tumor cells. One week was given for tumors to engraft in the lung before anti-PD-L1 blocking antibody (10F.9G2) was administered (100ug/mouse, given intravenously every other day), for up to one month post tumor injection (Figure 5A). Tumor burden was then assessed in excised lungs using image-based analysis (Figure 5B and C) to determine the percent of total lung area covered by the fluorescent tumors. A few small tumors were visible in the lung after 8 days post tumor injection (arrows in figure 5B, day 8) in both anti-PD-L1 treated mice and untreated controls. Percent lung coverage by tumors then increased up to day 15 post tumor injection (8 days of treatment in the experimental group). Tumor burden continued to increase in control mice, however, after 15 days post injection tumor burden in the lungs of treated mice leveled off and began declining after 23 days post tumor injection ($p = 0.0544$) (Figure 5B and 5C). At this point, tumors were visible to the naked eye in untreated mice, but were microscopic in treated mice (Figure 5C, inset). Twenty nine to thirty days post injection, tumors from untreated mice covered an average 14.04% of the entire lung. These mice were under severe respiratory distress as evidenced by heavy breathing and lethargy (data not shown).
However, mice treated with anti-PD-L1 blocking antibody had only one or two small tumors covering 0.369% of the lung (p = 0.0004). These mice were active and showed normal breathing patterns (data not shown). Together these data demonstrate that anti-PD-L1 blockade therapy is effective in reducing MCA fibrosarcoma tumor burden in mouse lungs.
Figure 5.

A

MCA/mcer
IV 3 X10^5 cells/mouse
0
7
9
11
13
15
17
19
21
23
25
27
29

α PD-L1
100 μg IV

Tumor Imaging

B

Day 8
Day 15
Day 23
Day 30

Control

αPD-L1

C

Percent Tumor Coverage

***

Tumor Treat

Days Post Tumor Injection

Control
α PD-L1
Figure 5. Anti-PD-L1 antibody blockade reduced MCA tumor burden in the mouse lung. **A)** Four to six month old mice were injected intravenously with $3 \times 10^5$ MCA-205 mCerulean tumor cells. After seven days, mice were treated with anti-PD-L1 blocking antibody (10F.9G2) or PBS (control) every other day for up to three weeks (top arrows). Lungs from tumor-bearing mice were excised on a weekly basis (bottom arrows) to ascertain tumor burden with or without treatment. **B)** Micrographs were taken using a 4X (0.28 NA) objective, $z$-step = 10 μm. Lung sections were compiled in Adobe Photoshop to image the full lung (outlined). Bright green fluorescence indicates tumor nodules in the lung. White arrows indicate small tumor nodules. **C)** Tumors from untreated mice were clearly visible as large macroscopic nodules (inset) on the lungs (black arrow heads) as early as 23 days post tumor injection, whereas no large nodules were visible in mice treated with PD-L1 blocking antibody. Tumor burden was quantified as percent of total lung area covered by tumors. Tumor area and lung area were calculated using intensity-based masks in Slidebook software. Arrows indicate time of tumor injection and treatment respectively. Comparisons of tumor coverage between untreated (Control, blue line) and treated (αPD-L1, red line) were made using an unpaired Students’ T Test. N=3 mice/group/day. Error bars represent mean +/- standard deviation. ***= p<0.001.
3.2 A change in tumor morphology after 8 days of PD-L1 blockade.

We were interested in probing deeper into anti-tumor events occurring around fifteen days post tumor injection (eight days after the initiation of anti-PD-L1 therapy), as this appeared to be a critical time point before tumors began to regress in mice treated with anti-PD-L1 blockade therapy. To further study this time point, we first looked more closely to see if PD-L1 blockade had a noticeable effect on tumor morphology at this time point in the treatment protocol.

Visualizing tumor cells under a higher magnification objective (20X/0.70 NA) allowed for detection of finer details of individual tumors. Using a higher power objective, we could see a subtle difference in tumor morphology after 8 days of anti-PD-L1 blockade. Tumors in control mice had more jagged edges that extended into the lung tissue, consistent with the invasive phenotype of progressing fibrosarcoma tumors (Figure 6A, left). In contrast, tumors in the lungs of mice treated with anti-PD-L1 therapy for 8 days had smoother edges and fewer extensions into the surrounding lung tissue (Figure 6A, right).

First quantifying the tumor area using higher magnification, we found that median tumor area was significantly lower after PD-L1 therapy (p = 0.0075) (Figure 6B). This difference in tumor area, but not total lung coverage (Figure 6C), was not due to an increase in the number of tumors present in the lung (Figure 6C), but was most likely due to an increase in image resolution due to use of a higher power objective. Inserting tumor area into the formula for roundness (see methods), we found that after 8 days of therapy, tumors were significantly more round than tumors
from untreated mice ($p = 0.046$) (Figure 6D). Together, these data suggest that PD-L1 blockade is affecting tumor growth in the lung within eight days of treatment by containing tumor nodules and limiting tumor invasiveness.
Figure 6.
Figure 6: Effects of anti-PD-L1 blockade on tumor morphology were seen after 8 days of immunotherapy.  A) Tumors from mice treated for 8 days with anti-PD-L1 antibody (right) had a different morphology than untreated mice (left). This morphology was characterized by smoother edges and fewer protrusions into the surrounding lung tissue. Representative micrographs from n=15 tumors/group from either untreated (left) or treated (right) mice fifteen days post tumor injection. Scale bar = 100 μm. Micrographs were taken using a 20X objective. B) After 8 days of anti-PD-L1 antibody blockade, enhanced resolution imaging showed tumors from treated mice were significantly reduced in area compared to tumors from untreated mice. C) However, the number of tumors engrafted in the lung within 15 days of tumor injection was not significantly different. D) Tumors from mice receiving anti-PD-L1 antibody had a significantly more round morphology than tumors from untreated mice. Statistical significance was determined using a Mann-Whitney U Test. Data collected from at least 15 tumors from a minimum of 3 mice per group. Bars represent median values. * = p < 0.05, ** = p< 0.01.
3.3. PD-L1 was expressed by MCA fibrosarcoma tumor cells and tumor-associated stromal cells.

Next, we sought to determine potential cellular targets expressing PD-L1 that may be affected by anti-PD-L1 blockade. To determine which cells in the tumor microenvironment express PD-L1, we harvested lungs from mice 15 days post tumor injection, digested the lungs according to established protocols (see methods) and stained for fluorescent markers. Cells were gated based on their expression of cyan fluorescent protein (CFP) expressed by all tumors and PD-L1. Roughly 9% of non-tumor stromal cells express PD-L1 in the lung, whereas less than 2% of tumor cells express PD-L1 (Figure 7A). This finding was in contrast to experiments in vitro which showed that PD-L1 is expressed at very low levels on MCA fibrosarcoma cells compared to a B16 melanoma model that expresses high levels of PD-L1 (Figure 7B). However, in agreement with several reports that demonstrate upregulation of both MHC (73) and PD-L1 (46, 74) in the presence of interferon gamma (IFNγ), MCA fibrosarcoma cells upregulated both MHC-I (H2-kβ) and PD-L1 when stimulated with IFNγ in vitro (Figure 7C).

It is of great interest to note that PD-L1 was expressed to a much greater degree on non-tumor cells than tumors themselves in the lung. To determine whether these PD-L1+ stromal cells had an impact in the local tumor microenvironment we stained lungs with anti-PD-L1 staining antibody (MIH6, conjugated to Alexa Fluor 647). PD-L1+ stromal cells densely populated the tumor microenvironment, and a subset of PD-L1+ cells were also positive for the CD11c-YFP transgenic reporter construct (Figure 7D, yellow arrows). Together, these
data show that MCA fibrosarcoma cells can upregulate PD-L1 in the presence of IFNγ, suggesting that IFNγ secreting T cell and NK cells in the tumor microenvironment may act as a mechanism by which PD-L1 is upregulated on tumors in vivo (75). This data also shows that MCA fibrosarcomas are not the only cell type in the tumor microenvironment that may express PD-L1 indicating that PD-L1+ cells, potentially of myeloid origin, may play a role in inhibiting effective anti-tumor immune responses.
Figure 7.
**Figure 7: PD-L1 is expressed both on tumor cells and tumor-associated stromal cells.**  

**A)** C57BL/6 mice were injected with $3 \times 10^5$ MCA fibrosarcoma cells. Fifteen days later, lungs were harvested and digested according to established methods to release cells for further analysis. Cell suspensions were then stained with PD-L1-AF647 antibody to determine PD-L1 expression. Tumor cells were gated based on CFP fluorescence expression (y axis) and PD-L1 expression (x axis). A small subset of CFP+ tumor cells expressed PD-L1; however, a much larger percent of non-tumor stromal cells expressed PD-L1 compared to tumor. This PD-L1+ stromal cell subset is present in the normal lung without the presence of tumors.  

**B)** In vitro cultured MCA fibrosarcoma or B16 melanoma were stained with anti-PD-L1 fluorescent antibody. Compared to B16 (orange curve), MCA (red curve) expresses very low levels of PD-L1. (Isotype control in blue).  

**C)** When cultured for 48 hours with IFNγ (20U/ml), MCA cells were able to upregulate MHC-I (H2-Kb) and PD-L1. Blue curve = isotype, red curve = MCA cells, green curve = MCA cultured with IFNγ.  

**D)** Lung tissue stained with anti-PD-L1 antibody showed an abundance of PD-L1+ stromal cells within the tumor microenvironment, some of which were dendritic cells (yellow arrows). (Blue = tumor, red = T cells, green = dendritic cells, white = PD-L1). In vivo PD-L1 expression was performed on a minimum of 2 mice. In vitro MHC and PD-L1 expression was performed on three independent experiments with at least 2 replicates.
3.4. Anti-PD-L1 antibody therapy reduced available PD-L1 but did not alter expression of costimulatory molecules on myeloid cells.

We were next interested in the effect PD-L1 antibody blockade had on PD-L1+ stromal cells in the tumor microenvironment. Several different myeloid cell populations express PD-L1 including myeloid derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs)\(^{(21)}\), inflammatory dendritic cells, and conventional dendritic cells (cDCs) \(^{(76)}\). All four of these cell types have been shown to suppress the immune response in the tumor microenvironment and aid cancer progression \(^{(23, 24, 77, 78)}\).

Of primary interest was the effect of PD-L1 blockade on the ability of these myeloid populations to suppress T cell function in the tumor microenvironment. We first tested how PD-L1 blockade influenced the proportion of these suppressive cells in the lung tissue. To ascertain the role of PD-L1 on myeloid cells, we harvested and digested lungs from tumor bearing mice 15 days post tumor injection. First, gating on large cells, \((\text{Figure 8A, left})\), we next investigated the proportions of myeloid cells in the lung based on expression of CD11b and CD11c. Using these markers, we found three distinctly different cell populations: CD11b+CD11c-, CD11b+CD11c+, and CD11b-CD11c+. CD11b+CD11c- cells were differentiated by expression of F4/80 and Gr-1 (Ly6G). Specifically, CD11b+CD11c-F4/80+Gr1high cells were consistent with the phenotype for MDSC \(^{(77)}\), and CD11b+CD11c-F4/80+Gr1low was a phenotype for TAMs \(^{(78)}\). Expression both CD11b and CD11c was phenotype consistent with inflammatory DC \(^{(22, 76)}\) and a CD11b-CD11c+ phenotype was consistent with cDCs \(^{(76)}\) \((\text{Figure 8A})\).
We found that while 70% of CD11b+CD11c- cells were MDSCs, eight days of anti-PD-L1 blockade did not alter the proportion of MDSCs in the lung (p = 0.0688). Tumor-associated macrophages made up 28% of the CD11b+CD11c- cells, and PD-L1 blockade again did not affect the proportion of these cells in the lung (p = 0.1057). Inflammatory dendritic cells made up less than 1% of myeloid cells in the lung and conventional DC made up 2% of myeloid cells. Again, anti-PD-L1 blockade did not significantly impact proportions of either of these populations into the lung (p = 0.9938 and 0.4407 respectively) (Figure 8B).

While PD-L1 blockade did not affect the proportion of myeloid cells in the lung, we hypothesized that blockade would affect the ability of myeloid cells to stimulate T cells in the tumor microenvironment particularly in the expression of costimulatory molecules (CD80 and CD86) and coinhibitory molecules (PD-1 and PD-L1). A small portion of MDSCs (Figure 8C), expressed the costimulatory molecules CD80 and CD86 at low levels. Anti-PD-L1 therapy did not significantly alter the CD80+ nor CD86+ populations within the lung (p = 0.2145 and p = 0.4567 respectively) nor did therapy alter the expression level of CD80 or 86 at the cellular level as indicated using mean fluorescence intensity values (MFI) (p = 0.7304 and 0.7879 respectively). Roughly 20% of MDSCs in the lung expressed PD-1, however, anti-PD-L1 blockade did not significantly alter the proportion of PD-1+ MDSCs (p = 0.5588) nor PD-1 expression on a per cell basis (p = 0.6891). On the other hand, PD-L1 was expressed on 34% of MDSCs in the lung. During PD-L1 blockade, however, only 11% of MDSCs expressed PD-L1 (p = 0.0005). However, PD-L1 expression was not significantly altered on a per cell basis (p = 0.5588).
Much like MDSCs, tumor associated macrophages in the lung did not significantly alter CD80, CD86 or PD-1 expression either. Less than 10% of TAMs expressed CD80 or CD86, and the percent of TAMs expressing either CD80 or CD86 was not significantly altered by therapy (p = 0.142 and 0.7546 respectively). TAM expression of neither CD80 nor CD86 was altered on a per cell basis either (p = 0.5476 and 0.5728 respectively). The TAMs expressing PD-1 were similarly unaltered during PD-L1 therapy (p = 0.1949), nor was PD-1 expression significantly altered on a cell to cell basis (p = 0.0946). Roughly 43% of TAMs expressed PD-L1 within the lung. This number was significantly reduced after 8 days of anti-PD-L1 blocking therapy to 12% of TAMs expressing PD-L1 (p = 0.0019). However, MFI values showed that PD-L1 were not significantly reduced by therapy on a per cell basis (p = 0.0632).

Next, we analyzed the effect of anti-PD-L1 blockade on the ability of the inflammatory DC subset of myeloid cells to provide costimulation to T cells (Figure 8E). While roughly half of inflammatory DC expressed CD80 and CD86 (50.56% and 47.57% of cells respectively), eight days of anti-PD-L1 therapy did not significantly alter the proportion of the CD80+ (p = 0.5495) nor the proportion of CD86+ (p = 0.3357) inflammatory DCs in the lung. Looking at MFI values, the expression of CD80 and CD86 on a per cell basis was also not significantly altered by anti-PD-L1 therapy (p = 0.9476 and p=0.4061 respectively). Over 70% of inflammatory DCs expressed PD-1, but the levels of PD-1+ inflammatory DC were not significantly altered when mice were treated with anti-PD-L1 blocking antibody for eight days (p = 0.2587). The PD-1 mean fluorescence intensity was also not
significantly altered by therapy (p = 0.4061) indicating that PD-1 was not significantly downregulated due to therapy. PD-L1+ inflammatory DC were prevalent within the lung tissue with 90% of inflammatory DC expressing PD-L1. After eight days of PD-L1 blockade, the PD-L1+ inflammatory DC population significantly dropped to only 63% of inflammatory DC expressing PD-L1 (p = 0.0014). The MFI of PD-L1 was also significantly lower in the treated group p = 0.0028 indicating that PD-L1 was being blocked on a per cell basis.

Half of conventional dendritic cells (cDCs) expressed CD80 and 30% expressed CD86 (Figure 8F). Similar to other myeloid populations, the CD80+ cDC population was not significantly altered by PD-L1 therapy p = 0.2247. This was also evident on at the cellular expression level (p = 0.7349). Akin to the effect of PD-L1 blockade on CD80 expression, the CD86 cDC population was not significantly altered as a percent of the total population (p = 0.4597) or on a cell by cell basis (p = 0.6649) as a result of eight days of treatment. PD-1 was expressed on an average of 58% of cDCs, but the proportion of cDCs expressing PD-1 was not significantly altered within eight days of therapy (p = 0.5362, not was PD-1 expression altered on a per cell basis (p = 0.2799). The majority of conventional DC (over 70%) expressed PD-L1. Anti-PD-L1 blockade significantly reduced the PD-L1+ cDC population in the lung to 42% of cDC (p = 0.0051). Looking at mean fluorescence intensity, PD-L1 blockade also significantly reduced PD-L1 expression on cDCs on a cell-by-cell basis (p <0.0001). Taken together, our data shows that PD-L1 blockade effectively reduced PD-L1 on myeloid cell populations in the lung without altering expression of B7 costimulatory molecules or the coinhibitory molecule PD-1. We also showed
limited expression of B7 costimulatory molecules on MDSCs and TAMs consistent with poor antigen presentation capability, which was unaffected by PD-L1 blockade. However, a higher proportion of both inflammatory DCs and cDCs expressed the B7 costimulatory molecules consistent with elevated antigen presentation capability by dendritic cells. These data suggest that eight days of therapy has limited effects on the capacity of myeloid cells in the lung to stimulate T cells, though at least one mechanism of T cell suppression was abated.
Figure 8.

A

SSC

FSC

CD11b

CD11c

Gr-1

F480

B

<table>
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<th>Macrophages</th>
<th>Inflammatory DC</th>
<th>cDC</th>
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Percent of CD11b+CD11c+ Population

Percent of Myeloid Cells
Figure 8.

C

MDSC

CD80

CD86

PD-1

PD-L1
Figure 8.
Figure 8.

E  Inflammatory DC
Figure 8.
Figure 8. Anti-PD-L1 antibody blockade reduced available PD-L1 in the tumor microenvironment. 

A) Lung tissue was taken from tumor-bearing mice treated with either PBS or anti-PD-L1 antibody for 7 days. Cell suspensions were then FACS sorted based on CD11b and CD11c expression. CD11b+CD11c- cells were then further sorted based on expression of F4/80 and Ly6G (Gr-1). A CD11b+CD11c-F4/80+Gr-1high phenotype was consistent with myeloid derived suppressor cells (MDSC). A phenotype of CD11b+CD11c-F4/80+Gr-1low was consistent with tumor-associated macrophages (TAMs). A phenotype of CD11b+CD11c+ was classified as inflammatory dendritic cells, whereas a CD11b-CD11c+ phenotype classified cells as conventional DCs (cDCs). 

B) Eight days of PD-L1 treatment did not significantly alter the proportion of the MDSC, macrophage, inflammatory DC or cDC populations in the lung. Eight days of anti-PD-L1 blockade therapy did not significantly alter CD80 expression, CD86 expression nor PD-1 expression on MDSC (C) TAM (D) Inflammatory DC (E) or conventional DC (F). However PD-L1 expression was significantly reduced on all four populations tested. Analysis was performed on n=6 mice per group. Significance was determined using a Mann-Whitney U-Test. Error bars indicate mean +/- standard deviation. ** = p<0.01, ***= p<0.001, ns= not significant.
3.5 Anti-PD-L1 blockade increased the presence of T cells in the tumor microenvironment, but did not affect T cell activation in the lung.

Considering that PD-L1 blockade had little effect on the expression of costimulatory molecules by myeloid cells, we next hypothesized that a reduction of available PD-L1 in the tumor microenvironment during therapy would tip the balance between positive and negative signaling and modulate T cell activation in the tumor microenvironment. To deduce any effect, we first looked at whether PD-L1 blockade therapy affected recruitment of T cells into the tumor microenvironment. T cells formed distinct halo patterns around tumors regardless of treatment (Figure 9A), however, eight days of anti-PD-L1 therapy significantly increased the average density of non-activated (CD2-RFP+) T cells surrounding the tumor (12 cells/2500 sq. μm in untreated mice vs. 27 cells/ 2500 sq. μm in treated mice, p = 0.0011) without significantly altering the density of activated (IL2p8-GFP+) T cells (8 cells/2500 sq. μm in untreated mice vs. 6 cells/2500 sq. μm in treated mice, p = 0.5478). The ratio of activated to non-activated T cells in the tumor microenvironment remained unchanged during therapy (p = 0.5932) (Figure 9A).

We next sought to determine the phenotype of the non-activated (CD2-RFP+) during PD-L1 blockade, particularly if these cells were CD8+ cytotoxic T cells or CD4+ helper T cells. To determine if PD-L1 blockade had any effect on the presence of CD8+ or CD4+ T cells in the lung fluorescent MCAmcer tumor cells were injected into transgenic mice expressing the CD2-RFP reporter transgene. Fifteen days post tumor injection (and after 8 days of therapy) we performed FACS analysis by gating on RFP-high cells before analyzing the CD4+ and CD8+...
populations (Figure 9B). Twenty percent of RFP-high cells in the lung were CD4+, and 22% of RFP-high cells were CD8+. After 8 days of anti-PD-L1 blockade, neither the percent of CD4+ nor CD8+ RFP-high populations in the lung dramatically changed (p = 0.6543, and p = 0.3892, respectively) nor was the ratio of CD8+ to CD4+ T cells in the lung altered by treatment (p = 0.1092).

In order to better understand the effects of anti-PD-L1 therapy upon the states of activation of tumor-associated T cells, besides using the IL2-promoter-GFP reporter of T cell activation, we also assessed lung T cells for the levels of expression of other markers of activation. In particular, using flow cytometry, we measured expression of the CD69 C-type lectin as an early activation marker (79), inducible T cell costimulator (ICOS) as a marker of intermediate activation (80), and PD-1 as a marker of chronic activation. CD4+CD69+ T cells made up 29% of the total CD4+ T cell population, but the proportion of CD4+CD69+ T cells was not significantly altered after eight days of therapy (p = 0.9169), nor was CD69 expression altered on a per cell basis based on MFI (p = 0.8182). ICOS was expressed on 48% of CD4+ T cells in the lung, but ICOS was not significantly altered after eight days of therapy either as a portion of total CD4+ T cells expressing ICOS (p = 0.3429) or on a per cell basis as indicated by mean fluorescence intensity (p = 0.1143). We found that 32% of CD4+ T cells in the lung expressed PD-1. The percent of CD4+ T cells expressing PD-1 was unchanged by anti-PD-L1 therapy (p = 0.4206) (Figure 9C). MFI was also not significantly different after administering therapy (p = 0.9372) indicating that therapy had no effect on the expression of PD-1 on a per cell basis.
Subsequently, we investigated the effect of PD-L1 therapy on expression of these activation markers on CD8+ T cells *(Figure 9D)*. CD8+CD69+ T cells accounted for 25% of CD8+ T cells in tumor bearing mice. During PD-L1 treatment, the percent of CD8+CD69+ T cells in the lung remained unchanged *(p = 1.00)*, as did CD69 expression on a cell-by-cell basis *(p = 0.5368)*. We found that 29% of CD8+ T cells expressed ICOS in the lungs of tumor bearing mice. Anti-PD-L1 blockade therapy did not affect the proportion of CD8+ T cells expressing ICOS *(p = 0.4857)*, nor ICOS expression on a cell by cell basis when analyzing MFI *(p = 0.400)*. Analyzing PD-1 expression, we found that 23% of CD8+ T cells in the lung expressed PD-1 fifteen days after tumor injection. The percent of CD8+PD-1+ T cells in the lungs of tumor bearing mice remained unchanged during PD-L1 blockade therapy *(p = 0.4206)*, as was PD-1 expression on per cell basis *(p = 0.6991)*.

Taken together, our data suggested that while eight days of anti-PD-L1 antibody blockade may correlate with recruitment of more non-activated T cells into the tumor microenvironment, therapy had no significant effect on activation of T cells within the affected tissue as assessed by expression of the IL2 transgene, as well as the expression of CD69, ICOS and PD-1. This data suggested that T cell activation in the lung is not an accurate predictive marker for responsiveness to anti-PD-L1 blockade.
Figure 9.

A

Untreated  αPD-L1

Activated T cells  Non-Activated T cells  Activated/Non-activated T cell ratio

ns  **  ns

Activated T cells

Cell Density (cells/2500 μm²)

Control  αPD-L1

B

CD2-RFP  FSC  CD4  CD8

Percentage of CD2-RFP + T cells

ns  ns  ns

Percentage of CD8 + T cells

ns  ns  ns

Ratio CD8/CD4

ns  ns  ns
Figure 9. Eight days of anti-PD-L1 blockade increased T cell density around tumors, but did not affect expression of T cell activation markers. A) Activated (IL2p8-GFP, green) and non-activated (CD2-RFP, red) T cell densities were enumerated from micrographs taken of MCA fibrosarcoma tumor nodules (blue) from untreated mice (left) or mice receiving αPD-L1 antibody (right). Micrographs show characteristic swarming of T cells around tumor nodules. Quantification of T cell density around tumors reveals significantly higher densities of non-activated T cells (red in micrographs) around tumor nodules from treated mice compared to tumor nodules from untreated mice. No significant difference in activated T cell density (green in micrograph) nor the ratio of activated to non-activated T cells around tumor nodules could be seen. Images were acquired using a 20X objective (0.70 NA) Z-step = 2 µm. B) To look for the presence of CD4+ and CD8+ T cells within the lungs, FACS analysis was performed on CD2-RFP mice. RFP^high cells were gated and analyzed for CD4 and CD8 expression (far left plots). Eight days of anti-PD-L1 therapy did not significantly alter the presence of CD4 or CD8 T cells, nor the ratio of CD8 to CD4 T cells within the lung. C) Eight days of antibody therapy did not significantly alter expression of PD-1, CD69, or ICOS on CD 4+ T cells. D) CD8+ T cell expression of PD-1, CD69, and ICOS were also not significantly altered by 8 days of anti-PD-L1 blockade therapy. Analysis was performed on a total of n=6 mice per group using a Mann-Whitney U-Test. Error bars represent mean +/- standard deviation. ** = p< 0.01, ns= not significant.
4. T cell dynamics in the tumor microenvironment

4.1 Assessment of dynamic behavior patterns in the tumor microenvironment.

Taking into consideration that the activation state of T cells in the tumor microenvironment did not correlate with the responsiveness of tumors to PD-L1 blockade therapy, we next hypothesized that PD-L1 immunotherapy in fact altered T cell behavior patterns in the tumor microenvironment which would account in part for tumor rejection. In order to test this hypothesis MCA/mcer fluorescent fibrosarcoma tumors were injected into mice expressing the transgenes for activated T cells (IL2p8-GFP+), non-activated T cells (CD2-RFP+), and dendritic cells (CD11c-YFP+) (see methods) in order to explore the dynamic interplay between these immune cells and the tumor. Immune cell interactions in the tumor microenvironment were very heterogeneous. (Supplementary Movie 1, see Appendix). Dendritic cells exhibited little translational motion and tended to cluster around the tumor while both activated and non-activated T cells exhibited an amalgam of different behavioral modalities. Some T cells clustering around dendritic cells, while other T cells moved around the periphery of the tumor and yet others did not interact at all with the tumor nodule. After eight days of anti-PD-L1 blockade, immune cells in the tumor microenvironment moved in dynamic behavior patterns similar to those seen in untreated mice (Supplementary Movie 2, see Appendix). Tracking software, used to probe deeper into motility parameter analysis (81) (Figure 10A), revealed that the mean velocity (Figure 10B) of pooled activated T cells stood at 1.26 μm/min., matching the mean velocity of pooled non-activated T cells from tumor bearing mice (p = 0.9355). However, after eight days of anti-PD-L1 immunotherapy, pooled
activated T cells exhibited a median velocity of 1.08 μm/min and pooled non-activated T cells showed a median velocity of 1.32 μm/min (p = 0.0011). Our data suggested that PD-L1 blockade may have small effects on T cell behaviors in the tumor microenvironment which require deeper analysis of T cell interactions in order to elucidate mechanisms by blockade influence tumor immune surveillance.
Figure 10.
Figure 10. Analysis of T cell migration velocities in lung tumors in vivo in response to anti-PD-L1 antibody. A) Fluorescent lung tumors from anesthetized mice bearing the CD2-DsRed, IL2p8-GFP, and CD11c-YFP transgenes were imaged for one full hour (20X objective, NA 0.70, sequential laser scanning, z-step = 2 μm) after receiving one week of anti-PD-L1 antibody or PBS as a control. Individual cells were tracked over time and several different motility parameters were derived. B) Pooled median velocity was similar between activated (IL2-p8-GFP+) T cells and non-activated (CD2-DsRed) T cells (left graph). However, after one week of anti-PD-L1 therapy, a significant difference in mean velocity between activated and non-activated T cells was seen (right graph). Analysis was performed on a minimum of three movies per group. Statistical significance was determined using a Mann-Whitney U-Test. Circles indicate individual cells. Bars indicate median values. **= p<0.01, ns = not significant.
4.2 T cells remained confined in the tumor microenvironment during PD-L1 blockade

Next, we investigated the effect of PD-L1 blockade on the populations of activated and non-activated T cells. After eight days of therapy, the pooled median velocity of activated T cells was similar to activated T cells from control mice ($p = 0.1246$) as were arrest coefficients ($p = 0.7242$) (Figure 11A). Spider-plots of T cell tracks (Figure 11B, D) revealed that activated T cell tracks from either treated or untreated mice were densely confined around a point of origin, traveling no more than a few T cell lengths (Figure 11B).

Similar to activated T cells, the median velocity of pooled non-activated T cells after eight days of therapy was comparable to the median velocity of untreated controls ($p = 0.4647$) (Figure 11C). However, the arrest coefficients of non-activated T cells were reduced after eight days of anti-PD-L1 therapy ($p = 0.0321$) (Figure 11C). Analogous to activated T cells, spider-plots of non-activated T cell tracks revealed heavy confinement (Figure 11D). However, low median velocities (less than 2 $\mu$m/min) and high median arrest coefficients (greater than 0.75) along with evidence of limited motility from spider plots led us to hypothesize that T cells were heavily engaged in interactions in the tumor microenvironment. Upon further investigation of potential targets of engagement, we found that T cell tracks overlapped in large part with DC in the tumor microenvironment (Figure 11E). Together these data suggested that both activated and non-activated T cells in the tumor microenvironment engage in tightly confined motility patterns, with DC being a potential focus of confinement.
Figure 11.
Figure 11.
Figure 11. T cells were confined in the tumor microenvironment. A) The median velocity (left) and arrest coefficients (right) of pooled activated T cell were unchanged after 8 days of anti-PD-L1 therapy. B) Activated T cells from both treated and untreated mice were densely confined in the tumor microenvironment as indicated from spider-plots which indicated that T cells travel no further than a few T cell lengths. C) The median velocity (left) of pooled non-activated T cells was not significantly altered by therapy, however, arrest coefficients (right) from the pool of non-activated T cells were significantly lower after 8 days of PD-L1 blockade therapy. D) Non-activated T cells traveled only short confined distances as indicated by spider-plot analysis. E) Confined T cell motility patterns overlapped with CD11c-YFP+ dendritic cells within the tumor microenvironment (white arrows). Images taken using a 20X objective (NA 0.70, z-step = 2 µm) Motility analysis performed on a minimum of 3 independent experiments. Statistical significance was determined using a Mann-Whitney U-Test. Bars indicate median values. ** = p<0.01, ns = not significant.
4.3 Non-activated T cells interacted preferentially with dendritic cells during PD-L1 blockade.

We next hypothesized that confined T cell motility patterns correlate to interactions with various types of cells in the tumor microenvironment. Furthermore, PD-L1 blockade would modulate these interactions, leading to enhanced tumor surveillance and tumor killing. To explore this hypothesis, we developed an image-analysis based method which allowed us to define T cell interactions and quantify what types of cells with which T cells interacted.

In order to quantify T cell interactions in the tumor microenvironment using an imaging-based approach, we employed the Leica Application Suite to process images (see methods). Maximum intensity time projections (MITPs) were thresholded to represent all areas where a cell visited during a motility recording. (Figure 12A, left micrograph) Average intensity time projections (AITPs) were thresholded to an intensity value of 43; representing sites were cells persisted for 10 or more minutes (Figure 12A, right micrograph). MITPs and AITPs were then merged into a single image (Figure 12A, bottom micrograph). Areas of fluorescence overlap (yellow) indicate sites of cellular persistence. Each cell in the micrograph was then carefully enumerated (Figure 12B). Large areas of overlapping fluorescence were carefully analyzed in the original MITP to find the exact number of cells contained in the overlapping section (Figure 12B, left micrograph).
Next, each merged image was compared to the original movie file in order to correlate areas of persistent confinement with potential cellular targets in close proximity. Cellular persistence was classified as an interaction in close proximity with either: DC only, with tumor only, with DC and tumor, or with neither DC nor tumor. To reiterate, an interaction was defined as a T cell with overlapping fluorescence in the merged image (yellow) in close proximity (within one T cell length) of an interacting partner. T cell interactions were then categorized and enumerated for both activated and non-activated T cells taken from one hour recordings of T cell motility around tumor nodules from either untreated mice or mice treated for 8 days with PD-L1 blocking antibody (Figure 12C). T cells with no overlapping fluorescence (1, 2, 3 in 12C) did not make stable engagements and left the viewing area (Supplementary movie 3, see appendix) and did not count toward T cell interactions. T cells also made stable interactions with non-DC/non-tumor cells (6, 7, 8 in 12C), with DC only (26, 27, 31, 35), or with both DC and tumor (19, 40 in 12C) (see Supplementary Movie 3 in appendix for clarification).

By quantifying the percent of T cells in each image sequence with persistent interactions in each target category, we found that 40-50% of both activated and non-activated T cell interactions occurred with both DCs and the tumor (Figure 12D). Non-activated T cells appeared to interact slightly more than activated T cells with neither tumor nor DC.

Next we sought to determine whether eight days of anti-PD-L1 blockade therapy had any effect on the T cells making stable interactions with either dendritic cells or the tumor itself. While activated T cells made similar preferential interactions
with DC and tumor targets during PD-L1 treatment compared to controls, (Figure 12E, left panel), non-activated T cells made more preferential interactions with DC partners when treated with anti-PD-L1 blocking antibody, (19% vs. 35% p = 0.0286) (Figure 12E right panel).

While it appeared that non-activated T cells made more preferential interactions with DC during anti-PD-L1 therapy, this affect could simply have been caused by an effect of PD-L1 blockade the ability of DC to engage the tumor. To explore this possibility, we analyzed DC contacting the tumor in both untreated and anti-PD-L1 treated mice. On average, 58% of DC were in contact with the tumor, and anti-PD-L1 blockade therapy did not significantly release DC from tumor engagements (p = 0.3429) (Figure 12F). Our data show that while activated T cells had no significant preference for interacting partner during anti-PD-L1 blockade, non-activated T cells greatly preferred DC-only interactions in the tumor microenvironment during blockade. This suggested that non-activated T cells may be making more positive engagements with DC, perhaps to gain activation signals as opposed to tumor-killing interactions at the tumor site.
Figure 12.
Figure 12.
Figure 12. Quantification of T cell interactions in the tumor microenvironment.  

A) T cell interactions in the tumor microenvironment were quantified using the Leica Application Suite image processing software. Maximum intensity time projections (MITPs) represent areas where T cells visited throughout the entire image sequence. Average intensity time projections (AITPs) were thresholded to represent areas where T cells persisted for 10 minutes or longer. These two images were then merged to create areas of overlapping intensities indicating areas of T cell persistence.  

B) Areas of T cell persistence were then individually enumerated and compared to the original hour long movie to determine with which cell types T cells were in close proximity. These areas were then classified as interactions.  

D) T cell persistent interactions were then categorized as interactions with DC, with tumor, with tumor and DC, or with neither tumor nor DC, and quantified for both activated and non-activated T cells. Both T cell types interacted heavily with DCs, whether in DC only interactions or DC and tumor interactions.  

E) During PD-L1 therapy, activated T cells made similar types of interactions compared to controls. However, non-activated T cells showed a significantly greater preference for DC only interactions in the tumor microenvironment compared to non-activated T cells from untreated mice.  

F) This increased preference was not due to an effect of PD-L1 blockade on the ability of DC to contact the tumor as the total number as well as percent of DC contacting the tumor was similar in treated and untreated mice. Analysis was from n=4 movies per group. Statistical significance was determined using a Mann-Whitney U-Test to compare percent of interactions within each category. Bars represent median values. * = p<0.05, ns = not significant.
4.4 Activated T cells formed more stable contacts with DC during anti-PD-L1 blockade therapy.

The vast majority of T cell interactions with dendritic cells in the tumor microenvironment (with DC contacting the tumor or with DC alone) suggested that T cells were receiving signals from dendritic cells. We hypothesized that PD-L1 blockade was modulating these T cell/DC interactions. To explore this hypothesis, we sought to evaluate whether the sites of T cell confinement correlate with the localization of DC in lung tumors. By plotting the mean displacement of a large pool of T cells vs. the square root of time, three distinctly different T cell motility patterns can be deciphered \((81)\) (Figure 13A). A linear function with positive correlation would indicate T cell motion in a random fashion, as T cells move indiscriminately around a target (Figure 13A, left top and bottom). A quadratic function of T cell displacement suggests T cells move in a directional pattern toward a target in a chemokine driven manner (Figure 13A, middle top and bottom). A logarithmic function would indicate that T cells are engaging in confined motility patterns and are being kept confined in a chemokine-driven manner (Figure 13A, right top and bottom). If the scale of displacement is sufficiently small, T cell confinement would indicate stable cell-cell engagement with the target. Larger displacements would indicate that T cells may be confined to larger areas around their targets, being kept in a broader area in a chemokine-dependent fashion.

Logarithmic functions of displacement graphs indicated that both activated and non-activated T cells exhibited confined motility in the tumor microenvironment, lending further support to previous findings of T cell confinement (Figure 13B, C).
Displacement on the scale of tens of micrometers indicated tight cell-cell interactions as opposed to broad patrolling behavior. After 8 days of anti-PD-L1 therapy, activated T cells exhibited smaller displacements over time than activated T cells from control mice (Figure 13B, left). T cell track straightness (Figure 13B, right), a measure of the ratio between a T cell’s displacement and its track length, confirm reduced T cell displacements, as T cells from treated mice displayed median track straightness of 0.12 units compared to T cells from untreated mice which displayed median track straightness of 0.26 units, p < 0.001 (Figure 13B, right).

Non-activated T cells also displayed logarithmic displacement curves indicating T cell confinement on the scale of tens of micrometers, indicating cell-cell interactions which were on the order seen in activated T cell from anti-PD-L1 treated mice (Figure 13C, left). T cell track straightness (0.17 units) was not significantly altered by anti-PD-L1 therapy, p = 0.1241 (Figure 13C, right).

We next sought to correlate the higher confinement of activated T cells from mice treated anti-PD-L1 antibody with actual motility in vivo. Activated T cells interacted in close proximity to DC in the tumor microenvironment in untreated mice (Supplementary movie 4, see Appendix). These interactions were very loose sliding motions as T cells moved around and over DC. However, activated T cells from anti-PD-L1 treated mice engaged in tighter, more stable interactions with limited movement around DC (Supplementary movie 5, see Appendix). These results were in line with displacement graphs (Figure 13B) demonstrating higher T cell displacements in untreated mice and lower T cell displacements in mice treated with anti-PD-L1 antibody.
Our data showed that, as a consequence of PD-L1 blockade, activated T cells engaged in more tightly confined, stable interactions, particularly with DC in the tumor microenvironment, whereas activated T cells engaged with DC in looser, less stable interactions when PD-L1 was able to engage its receptors. This data suggested that PD-L1 interactions with PD-1 on T cells may prohibit T cells from fully engaging DC and may prevent T cells from receiving long term activation signals.
Figure 13.
Figure 13. Activated T cells engaged in stable interactions with DC in the tumor microenvironment in response to PD-L1 blockade. A) Graphs of T cell mean displacement indicate distinct T cell motility patterns. Linear functions indicate random motility around a target cell. Quadratic functions indicate directional movement of T cells toward a target in a chemokine-dependent manner. Logarithmic functions indicate confined motility around a target that may indicate tight cell-cell interactions if displacements are low or larger patrolling behaviors if displacements are sufficiently large. B) Activated T cells displayed confined displacements on a small scale, indicating tightly confined cell-cell interactions. Activated T cells during anti-PD-L1 treatment displayed reduced displacements (left), and exhibited reduced track straightness (right) compared to activated T cells from controls. C) Non-activated T cells displayed confined displacements on a small scale, indicating tight cell-cell interactions similar to activated T cells during PD-L1 blockade. Displacements (left) nor track straightness (right) were not significantly altered by anti-PD-L1 therapy. Analysis from n= 3 movies per group. Displacement graphs represent mean +/- SEM. Bars in track straightness plots represent median values. *** = p < 0.001, ns = not significant.
5. Discussion

5.1 Anti-PD-L1 therapy

Anti-PD-L1 blocking antibody is already being used in Phase II clinical trials for the treatment of late stage cancers including melanoma, non-small-cell lung cancer, and renal cell carcinoma. Patients with objective responses to this immunotherapy have shown at the very least stable disease progression and in the most promising cases, complete tumor regression lasting for months or even years (56). While patients receiving the treatment exhibited minimal immune-mediated adverse reactions (imAR) (82), and responses are impressive, only a fraction of patients receiving anti-PD-L1 therapy alone responded to treatment (56). Work has also tried to elucidate any benefit of combinatorial therapies with the anti-CTLA4 antibody Ipilimumab for melanoma (60, 83, 84), however, a better understanding of the biology of PD-1/PD-L1 interactions is key to improving the response rates of patients to anti-PD-L1 therapy.

To study the role of PD-L1 in immune suppression in the tumor microenvironment, our lab used a commercially available rat anti-mouse blocking antibody that blocked both the binding epitope for PD-1 as well as CD80 on the PD-L1 molecule (51). Using a novel approach to image tumor in the lungs, we analyzed the total amount of tumors engrafted on the lung surface at various time points during tumor progression and found that MCA induced fibrosarcomas from mice receiving anti-PD-L1 antibody regressed after three weeks of treatment. This method had its advantages over conventional methods (e.g. calipers) which require

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that tumors be 1) visible to the human eye, 2) of a large enough size to measure, and 3) easily accessible to measurement of tumor size. These methods typically do not take into account smaller, unseen tumors and may be subject to selection bias. Our novel method, allowed visualization of a broad picture of engraftment of even microscopic tumors throughout the entire lung as well as a broader sense of the entire scope of tumor coverage in an organ. One key drawback, though, was the depth of tissue penetration as only surface nodules within 200 μm of the surface could be visualized. However, current microscopy-based techniques are seeking to improve the depth and resolution of tissue imaging (85, 86).

Using a novel imaging-based approach to elucidate the “tumor roundness index”, changes in tumor morphology as a result of anti-PD-L1 therapy were easily visualized and quantified. Increased tumor roundness during PD-L1 blockade suggested a potential mechanism by which tumors are first contained early during PD-L1 blockade before later tumor clearance. It would be interesting to see if this observed increase in tumor roundness after 8 days of anti-PD-L1 therapy continued throughout the entire course of treatment, which would solidify our hypothesis. Furthermore, it remains to be seen whether tumors completely regress during PD-L1 blockade or return after cessation of treatment. If this were indeed the case, then it would be interesting to see whether mice were immunized against the tumor after receiving the therapy and would therefore be able to effectively eliminate tumors upon re-challenge.

We also showed that some, but not all, tumors expressed PD-L1 in vivo 15 days after tumor injection (Figure 5). It would be of interest to determine whether
tumors further upregulate PD-L1 as tumors continued to grow or if there is a selective advantage of PD-L1+ tumor survival in the lung over time. We also observed heterogeneity in recruitment of T cells to tumor nodules. Thus, it would be of value to determine the mechanism by which some T cells were recruited in abundance to some tumor nodules while other nodules recruited fewer T cells, and whether PD-L1 expression played a role in this heterogeneity. In addition, we found that PD-L1 is expressed at low levels on MCA fibrosarcomas in vitro, but much greater in vivo. We showed that IFN-γ, in line with other reports (46), upregulated PD-L1 in vitro. Our findings that PD-L1 was expressed in vivo but not in vitro suggested that immune cells in the tumor microenvironment may produce IFNγ in response to the tumor. We would like to confirm this, as well as look at other effector responses by T cells in vivo.

5.2 Immune cell characterization

Strikingly, a majority of PD-L1 was expressed on stromal cells in the tumor microenvironment, including myeloid derived suppressor cells (MDSCs), tumor associated macrophages (TAMs), inflammatory DCs and conventional DCs (cDCs). To our knowledge this is the first report that has begun phenotyping PD-L1+ immune cells in the tumor microenvironment and the effect of PD-L1 blockade on these immune populations. We showed that the majority of dendritic cells (both inflammatory DC and cDC) express PD-L1. The expression of PD-L1 and the inhibitory receptor PD-1 on a large portion of inflammatory DC further support their function as suppressors (22, 23). The large percentage of PD-1+ and PD-L1+ conventional DC would also suggest that these DC may be suppressive as well (24).
though their function remains to be determined. We found that anti-PD-L1 therapy A) did not significantly alter recruitment of any of the myeloid cells tested into the peripheral lung tissue and B) blocked available PD-L1 on all of the myeloid cell populations tested without significantly altering the expression of the costimulatory B7 molecules or the inhibitory receptor PD-1. We found a trend in the reduction of the percent of MDSC in the lungs after 8 days of therapy ($p = 0.0688$), as well as a trend in lower MFI of the expression of both PD-1 ($p = 0.0688$) as well PD-L1 on TAMs ($p = 0.0632$). It would be interesting to determine whether these trends become significantly different over the treatment period, and would thus suggest another mechanism by which anti-PD-L1 therapy effectively led to tumor regression. Moreover, each of these myeloid cell populations need further phenotypic characterization in order to further differentiate each population as well as to determine if the expression of other markers could be affected by anti-PD-L1 therapy. Plasmacytoid dendritic cells (pDCs) which have been shown to express PD-L1 and play an important role in immune regulation (87) need also be characterized to determine how their role in immune surveillance is altered, if at all, during PD-L1 blockade. A relatively new marker added to further characterize the B220+CD11c+ plasmacytoid dendritic cell population is the marker Gr-1 (88) which has generally been used to characterize MDSC (77). While we observed a Gr-1+ population within the CD11b-CD11c+ conventional DC population, it would be premature to call these cells plasmacytoid DC before further phenotypic analyses were conducted.
Several groups have noted the effect of anti-PD-1 or anti-PD-L1 blockade on T cell activation and motility in the lymph nodes (60) (70), however, little has been done to assess the effect of PD-L1 blockade on T cells within peripheral tissue, especially in response to tumors. In agreement with one report (89), we showed that during blockade/inhibition of PD-L1, T cells more heavily infiltrated into the microenvironment. Within 8 days of PD-L1 blockade therapy, non-activated T cells swarmed more densely around tumors than non-activated T cells from controls. This additional recruitment of non-activated T cells as early as eight days of treatment may contribute to tumor regression. A similar increase in density for activated T cells was not observed; however, this may be due to the relative paucity of activated T cells in the tumor microenvironment compared to non-activated T cells. It would be of interest to ascertain whether T cells could be activated in the tumor microenvironment or if activated T cells are instead recruited from the lymph nodes. Of similar interest would be to determine whether PD-1/PD-L1 interactions in the tumor microenvironment prevent non-activated T cells from being activated and/or maintaining activation. Moreover, how the total numbers and densities of activated and non-activated T cells changed over the entire course of treatment should be investigated particularly whether T cells were recruited later in controls or not at all. For example, if response time were a factor the ability of the immune system to control tumor growth, T cells may in fact form similar dense clusters around tumors in control mice; however T cell recruitment into the tumor microenvironment may be delayed until tumor progression is beyond control. Therefore, PD-L1 blockade may disable a mechanism that would prevent T cells
from infiltrating the tumor microenvironment at higher densities around nodules much earlier the time course of tumor progression when the tumors can still be controlled and eliminated. Future experiments would be needed to determine if earlier T cell recruitment would in fact contribute to tumor elimination.

We next sought to determine whether anti-PD-L1 therapy affected recruitment of cytotoxic CD8+ T cells as a means to lyse tumors or CD4+ helper T cells as a means to stimulate T cell anti-tumor responses and likewise whether therapy altered the stage of activation (early, intermediate or late stage) of these cells the lung tissue. Eight days of therapy did not significantly alter the portions of CD8+ or CD4+ T cells in the lung, nor were indicators of early (CD69), intermediate (ICOS), or late stage (PD-1) activation significantly affected in either T cell population. Future experiments need shed light on any possible temporal aspect of T cell recruitment and activation. For instance, it would be interesting to assay for T cell recruitment at other time points during the treatment protocol as cytotoxic or helper T cells may arrive earlier during PD-L1 therapy or increase in the peripheral tissue following eight days of PD-L1 blockade and likewise, T cell may stay active longer in the lung of mice treated with PD-L1 blockade. Future experiments are needed to determine if this is the case. Along with T cell activation, it would be worthwhile to look at how PD-L1 blockade affected the production of effector molecules by T cells (granzymes, perforins, interferon-γ, etc). Insight could then be gained into the relative roles and importance of tumor killing by cytotoxic T cells as well as T cell support offered by a variety of cells including helper T cells, B cells, and dendritic cells in the tumor microenvironment. It should also be noted that CD8 T cells are not the only killers
in the tumor microenvironment; NK cells (90) as well as NKT cells (91, 92) also contribute to tumor killing.

5.3 T cell motility and interactions in the tumor microenvironment

Our data suggested that phenotypic analysis of immune cells in the peripheral tissue was not a sufficient indicator of response to anti-PD-L1 therapy, hence we hypothesized that a change in T cell dynamics in the tumor microenvironment due to PD-L1 blockade contributed to effective tumor clearance. The heart of my thesis revolved around this hypothesis and in the analysis of T cell motility in the tumor microenvironment. T cells, both activated and non-activated, exhibited slow, confined motility patterns in the tumor microenvironment. Contrary to findings in the lymph where T cells were shown to reduce their velocity during anti-PD-L1 blockade (70), or in the spleen where T cells were shown to increase their velocity during viral infection (93), we found that PD-L1 blockade did not significantly impact T cell velocity in the tumor microenvironment compared to controls. This was most likely on account of low T cell mobility (as indicated by low mean velocities and high arrest coefficients) in the tumor microenvironment prior to and including 15 days after tumor injection which suggested that T cells were engaged in interactions in the tumor microenvironment.

However, current methods poorly define a T cell “interaction”. Often times “interaction” is simply defined in an in vivo system as contact with a second cell type (67), or exhibiting an effector function (63) with little progress in truly defining or quantifying T cell interactions in the tumor microenvironment.
To better understand T cell interactions in the tumor microenvironment, we developed a novel, imaging-based approach to quantify T cell interactions in the tumor microenvironment. Using this method, both activated and non-activated T cells were revealed to engage heavily with dendritic cells in the tumor microenvironment, whether with dendritic cells alone or with dendritic cells and the tumor itself. When mice were treated with anti-PD-L1 antibody for eight days, non-activated T cells made more preferential DC only interactions, whereas activated T cells showed no such preference for any interacting partner, but activated T cells were more tightly confined in stable immune synapse-like interactions with dendritic cells in the tumor microenvironment. Our data suggested that while PD-L1 blockade appeared to have affected both activated and non-activated T cells at a microscopic level, therapy appeared to have more influence on non-activated T cells. It is tempting to speculate on the implications of PD-L1 blockade on the dynamics of each of these populations in the tumor microenvironment. It appeared that non-activated T cells were being recruited in larger numbers during therapy (as indicated by higher T cell densities), were making more transient interactions (indicated by reduced arrest coefficients), and more of these interactions were occurred preferentially with DC in the tumor microenvironment. Further experiments would be needed to verify whether non-activated T cells were quickly scanning for antigen as our results suggested by reduced arrest coefficient and whether these cells were gaining activation signals from dendritic cells in the tumor microenvironment. If this were found to be the case, it would be beneficial to identify these activation signals.
Activated T cells made more stable immune-synapse-like interactions with dendritic cells in the tumor microenvironment during PD-L1 blockade as opposed to activated T cells from control mice which appeared to engage in brief immune-kinapse-like interactions with dendritic cells (14). Taking all of our dynamic T cell motility data into account we developed a model (Figure 14) by which 1) non-activated T cells were recruited in higher numbers during PD-L1 blockade, engaged DC more preferentially (potentially to receive activation signals), and arrested for less time (potentially to scan for antigen); and 2) activated T cells were recruited in the same number into the tumor microenvironment but were able to make more stable synapses with DC during PD-L1 blockade (potentially receiving a stronger activation signal) (16). Further experiments would need to determine whether signaling complexes were recruited to form a synapse and to determine the strength of TCR signaling. It would also be interesting to further phenotypically characterize the activated T cells as well as the non-activated T cells making contacts with DC, as well as further characterization of the DC making contacts in the tumor microenvironment.

Taken together, we showed that anti-PD-L1 therapy worked to 1) allow activated T cells to make more stable interactions with dendritic cells in the tumor microenvironment, 2) recruit more non-activated T cells into the tumor microenvironment which make more preferential interactions with dendritic cells. A combination of these two factors may act synergistically to contribute to tumor regression.
Figure 14.
**Figure 14. Model for T cell interactions with dendritic cells in the tumor microenvironment.** PD-L1 may have differential effects on activated and non-activated T cells in the tumor microenvironment. During tumor progression (left), activated T cells (green) and nonactivated T cells (red) were recruited into the tumor microenvironment and engaged in heterogeneous interactions with either the tumor (blue) or dendritic cells (DC) (yellow) in the tumor microenvironment. Activated T cells (T cell “A” in left inset) formed unstable immune kinapse-like interactions characterized by mobile interactions (arrow) with DC. Both activated and non-activated T cells may be receiving activation signals (+ in figure), though this is yet to be determined. However, during PD-L1 blockade (right), non activated T cells were recruited in higher densities and engaged preferentially with dendritic cells (T cell “B” in right inset). Activated T cells (T cell “A” in right inset) were not preferentially recruited, but were able to maintain stronger, more stable immune synapse-like interactions with DC in the tumor microenvironment. Both activated and non-activated T cells potentially received stronger activation signals (+ in right inset), though this still needs to be determined.
5.4 Implications of our novel method on future research

Our novel method of imaging-based quantification of immune cell interactions was useful in elucidating T cell interactions, but this method can be broadly applied to other fields. Study of interactions now need not be solely studied in vitro or ex vivo; interactions which may not be truly applicable to true in vivo dynamic behaviors. With this novel method, true interactions can be observed and quantified in vivo. Of course, this method would need to be combined with further analysis of adhesion molecules and cellular signaling to ensure that interacting cells were stably engaged, but our method is a novel, unique tool that can be added to the tool-bag of image analysis.

5.5 Implications on immune therapy

Our findings have broad implications on cancer immunotherapy. We showed that early tumor biopsies may not clearly indicate patient response rate. T cells in the tumor microenvironment showed no significant difference in the particular activation phenotypes tested. Nor were the phenotypes of myeloid cells significantly different at early stages. This finding reveals the importance of looking at the bigger picture of immune cell dynamics in the tumor microenvironment in order to ascertain whether a patient will respond to therapy. Potentially, tools need to be developed which would include immune cells dynamics as a prognostic indicator of response rates.
6. Appendix

Supplementary Movie 1. Dynamic immune cell motility patterns in tumor bearing mice. Activated T cells (green) as well as non-activated T cells (red) engaged in a heterogeneous mixture behavior patterns with both dendritic cells (white) and tumor nodules (blue) in tumor bearing mice. Some T cells engage the tumor. Other T cells engaged dendritic cells, while others move quickly in and out of frame without engaging any targets. This movie is one representative 45-minute-long movie from a total of four movies taken from four different mice ranging from 45 minutes to one hour long. This movie was taken using 20X objective (0.70 NA), Z-step size = 2 μm, utilizing sequential scanning with a 20 second scan time.

Supplementary Movie 2. Dynamic immune cell behaviors in tumor bearing mice receiving anti-PD-L1 antibody blockade. After 8 days of anti-PD-L1 antibody therapy, activated T cells (green) and non-activated T cells (red) exhibit complex behavior patterns with dendritic cells (white) and tumor nodules (blue). T cells demonstrate a mixture of behaviors ranging from contacting the tumor nodule, dendritic cells, other T cells, or no other visible cell type. Movie is one representative one-hour-long movie from a total of four movies taken from four different mice, taken using a 20X objective (0.70 NA), Z-step size = 2 μm, utilizing sequential scanning with a 20-second scan time.

Supplementary Movie 3. Interactions in the tumor microenvironment. Complex interactions in the tumor microenvironment were broken down using a novel imaging-based analysis technique (Figure 7B,C). Cells with persistent motility
characterized by overlapping binarized fluorescence were enumerated and interactions, defined as persistent motility within one T cell length of a second cell type, were quantified. Cells were categorized as interacting with dendritic cells alone (middle bottom), tumor alone, dendritic cell and tumor together (left middle), or neither dendritic cell nor tumor (upper right corner). Cells without overlap (upper left) were motile and did not stably interact with a partner. This movie is one representative one-hour-long movie from a total of four movies taken from four different mice. The movie was taken using a 20X objective (0.70 NA), Z-step size = 2 μm, utilizing sequential scanning with a 20 second scan time.

**Supplementary Movie 4. Activated T cells engaged in unstable interactions with dendritic cells in the tumor microenvironment.** Activated T cells (green) from control mice made unstable, sliding interactions with dendritic cells (red) surrounding the tumor (blue). One representative 45-minute-long movie taken of four one hour long movies from four different mice is shown. Movies were taken using an 20X objective (0.70 NA), Z-step size = 2 μm, utilizing sequential scanning with a 20 second scan time.

**Supplementary Movie 5. Activated T cells engaged in stable interactions with dendritic cells in the tumor microenvironment during PD-L1 blockade.** Activated T cells (green) from mice receiving one week of anti-PD-L1 antibody therapy made strong, stable engagement with dendritic cells (red) in surrounding the tumor (blue). One representative one-hour-long movie taken of four from four different mice. Movies were taken using an HCPL APO 20X/0.70 NA objective, Z-step size = 2 μm, sequential scanning using a 20-second scan time.
7. Bibliography


8. Vita

Todd Jacob Bartkowiak was born on September 19, 1986 in Houston, Texas to James and Renalda Bartkowiak. After completing high school at Klein High School in Klein, Texas in 2005, he entered Sam Houston State University in Hunstville Texas. In May of 2008, he received his Bachelor of Science degree with a major in Biology and a minor in Chemistry. For two and a half years, Todd worked as a research assistant in the Diagnostic Sciences department at the University of Texas Health Science Center, Houston Texas. In August of 2011, Todd entered the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. Todd completed this thesis under the mentorship of Dr. Tomasz Zal in the Immunology Department at the University of Texas, MD Anderson Cancer Center.