DISSERTATION

PD-L1 EXPRESSION BY TUMOR MACROPHAGES: REGULATION AND SIGNALING

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ABSTRACT

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Immune checkpoint molecules maintain self-tolerance and prevent uncontrolled inflammation. However, expression of these molecules is often dysregulated in the tumor microenvironment (TME), resulting in overexpression of inhibitory checkpoint molecules such as programmed cell death ligand 1 (PD-L1). This overexpression suppresses T cell activation and effector functions, which interferes with T cell control of tumors. PD-1 and its ligand PD-L1 are inhibitory checkpoint molecules that suppress tumor immunity, and PD-L1 also binds CD80 to inhibit T cell responses. The field of tumor immunotherapy has established that interrupting the PD-1/PD-L1 signaling pathway with therapeutic antibodies can potently activate T cell responses to tumors. For example, PD-L1 antibody treatment increases T cell infiltration and IFN-γ production, and decreases tumor growth in mouse models. Blockade of either molecule with specific antibodies has now been shown to induce dramatic anti-tumor responses in patients with many tumor types. Importantly, relatively few adverse effects were observed in these trials.

The excitement generated by these developments in the treatment of human cancers has been dampened by the realization that only a small subset of patients respond to immunotherapy. This has prompted numerous studies attempting to identify biomarkers that can predict response to treatment and has also illuminated the need for animal models that more accurately translate human biology and disease progression. Countless therapies developed in rodents have failed to successfully treat human disease, but pet dogs with spontaneously-occurring disease are emerging as a promising model for many reasons. These include strong similarities in anatomy

and physiology between dogs and humans, as well as vast differences in genetics, living environment, and diet between dogs that parallel variations found between human patients. Furthermore, advances in the care of animals has allowed for longer lifespans for pet dogs, which has led to an increase in the incidence of many age-related diseases such as cancer. Therefore, the field of comparative oncology utilizes the knowledge gained by treating veterinary patients with spontaneously-occurring disease to improve our understanding of human disease while also translating therapies used for human care to veterinary patients.

Very little is known concerning the expression and regulation of canine immune checkpoint molecules in either normal tissues or by tumors. Therefore, in the studies described in Chapter 2, we measured the expression of PD-L1 by a panel of canine tumor cell lines and by primary canine macrophages and further assessed the effects of immune stimuli on PD-L1 expression by these cells. These studies showed PD-L1 to be constitutively expressed by all 14 canine tumor cell lines evaluated, and PD-L1 expression was upregulated upon stimulation by IFN-γ and a TLR3 ligand. In contrast, canine macrophages did not express PD-L1 under basal conditions but expression was induced upon treatment with IFN-γ. These results are consistent with those reported in humans and in mice, where PD-L1 is primarily regulated by IFN-γ produced by activated T cells. In tumors, this is a mechanism of tumor-mediated T cell suppression that is an important target of immunotherapy, and this study suggests that PD-1 and PD-L1 blockade may be beneficial for the treatment of canine tumors.

Lymphoma is one of most commonly diagnosed cancers in dogs, but to-date there has been little progress in improving prognosis. Protocols for the treatment of canine lymphoma were adapted from those used to treat human patients because chemotherapeutic agents are similarly effective between the two patient groups, and at the same time, drugs that are

ineffective in humans are also ineffective for dogs. PD-1-targeted immunotherapy has been found to be highly effective in the treatment of human lymphoma, and thus canine lymphoma may be a disease that benefits from this therapy. Therefore, we characterized the PD-1 and PD-L1 expression profile in canine lymphoma and the effects of chemotherapy resistance on these expression levels, which is detailed in Chapter 3. We first analyzed PD-1 and PD-L1 expression using fine needle aspirates of lymph nodes from healthy dogs and from dogs with untreated B cell lymphoma (BCL) and T cell lymphoma (TCL). We found increased expression of PD-L1 by BCL compared to healthy B cells, but low to negative expression of PD-1 and PD-L1 by both healthy and malignant T cells. Next, we generated chemotherapy-resistant BCL and TCL cell lines and compared their expression of PD-1 and PD-L1 to untreated cells. We found that chemotherapy-resistant BCL cells slightly upregulated PD-1 and PD-L1 while TCL cells more significantly upregulated both molecules. We were unable to find a significant difference in the expression of PD-1 or PD-L1 between pre- and post-relapse samples from patients with BCL, but our *in vitro* data suggest that this phenomenon would be more apparent in the case of TCL. Previous reports show that PD-1 antibodies activate canine T cells in vitro, the first administration of a canine PD-L1 antibody showed evidence of clinical activity. These studies indicate that PD-1/PD-L1-targeted immunotherapy in canine lymphoma should include a pre-treatment evaluation of PD-L1 expression levels for use as inclusion or exclusion criteria.

PD-L1 expression by tumor cells has been the primary focus of numerous studies, but it has recently been shown that expression of PD-L1 by myeloid cells in the tumor is a stronger marker for both prognosis and for response to immunotherapy. Thus, we investigated the regulation of PD-L1 expression by murine monocytes and tumor macrophages in Chapter 4. We found that PD-L1 expression by immature bone marrow increased as the monocytes entered

circulation and further increased as they matured into macrophages in the tissues. Interestingly, tumor macrophages expressed the highest level of PD-L1 expression, implying regulation by the tumor microenvironment. We therefore used a combination of *in vitro* and *in vivo* methods to identify molecules secreted by murine tumor cells that mediate this increased expression of PD-L1 by tumor macrophages. We found that secretion of versican, an extracellular matrix protein, by tumor cells causes monocytes to produce TNF-α, which stimulates the monocytes themselves to upregulate PD-L1 expression. Upregulated expression of PD-L1 is one of the mechanisms by which tumor macrophages suppress immune responses in the tumor environment and, as a result, causes macrophages to be a crucial target for PD-L1-targed immunotherapy.

Evidence suggests that direct signaling by PD-L1 regulates cellular functions, and that treatment with PD-L1 antibodies alters its signaling activity. Because macrophages comprise one of the main PD-L1-expressing cell populations in tumors, it is crucial to elucidate whether PD-L1 signals in macrophages and the effect of PD-L1 antibodies on altering macrophage phenotype in the tumor environment. To this end, we conducted studies described in Chapter 5 characterizing the effects of PD-L1 antibody treatment on macrophage activation. We found that incubation of macrophages with PD-L1 antibody and with soluble CD80 resulted in macrophage proliferation, spreading, and activation. Treatment of macrophages with soluble PD-1 and untreated macrophages from PD-L1$^{-/-}$ mice showed similar changes, but to a lesser extent. We also found that PD-L1 antibody treatment upregulated mTOR pathway signaling in macrophages, and gene expression profiling showed that macrophages treated with PD-L1 antibodies were skewed towards a pro-inflammatory phenotype. We next used *in vivo* tumor models to assess the effect of PD-L1 antibody treatment on tumor macrophages. These studies revealed that PD-L1 antibody treatment increased numbers of tumor macrophages as well as

their activation, and inhibited tumor growth was observed even in the absence of T cells. Furthermore, combined PD-1/PD-L1 antibody treatment of mice with established tumors resulted in complete tumor elimination in half of the animals. Overall, these findings implicate PD-L1 in constitutively suppressing mTOR pathway signaling in macrophages, and PD-L1 antibody treatment removes this signal to produce activated, proliferating macrophages.

In conclusion, we characterized the expression and regulation of PD-L1 by canine tumors and macrophages as well as a novel signaling role for PD-L1 in macrophages. Our studies provide strong rationale for the development of canine-specific PD-L1 antibodies for immunotherapy, and for the evaluation of PD-L1 expression levels in patients prior to the initiation of treatment. Furthermore, we identified a new mechanism for tumor-induced immune suppression mediated by upregulating PD-L1 expression by tumor macrophages in a murine system. This expression of PD-L1 by macrophages not only suppresses T cells responses in the tumor microenvironment, but we found that it also signals constitutively to maintain an anti-inflammatory, pro-tumor macrophage phenotype. We further showed that treatment of macrophages with PD-L1 antibodies reverses this phenotype, and that the resulting population of proliferating, pro-inflammatory tumor macrophages has significant anti-tumor activity.

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CHAPTER 1

Review of the Literature

The first immune checkpoint: CTLA-4.

Tumor immunotherapy is a field that utilizes the ability of the immune system to recognize tumor cells based on their expression of tumor-related antigens. However, immunotherapeutic agents such as vaccines and cytokines have not been widely successful as single agents due to immunosuppression mediated by the exploitation of immune checkpoints in the tumor environment[1]. Under normal conditions, immune checkpoint molecules prevent autoimmunity and protect tissues from damage by downregulating the duration and magnitude of T cell responses. Two signals are required for full activation of T cells: binding of an antigen-MHC complex with the T cell receptor (signal 1) and co-stimulation such as the binding of B7.1 (CD80) or B7.2 (CD86) on an antigen-presenting cell (APC) to CD28 on the T cell (signal 2)[2,3]. TCR recognition of antigen without signal 2 leads to functional inactivation of antigen-specific T cells that cannot respond to further stimulation, a state known as T cell anergy[4]. Successful functioning of the adaptive immune system depends on a network of both stimulatory and inhibitory signals, but inhibitory checkpoint molecules are often overexpressed in the tumor environment. As a result, this system is dysfunctional in tumors, but specific targeting of these signaling pathways using antibodies can release the endogenous anti-tumor functions of tumor-infiltrating immune cells.

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, also known as CD152) was the first immune checkpoint molecule to be clinically targeted with monoclonal antibodies for the treatment of cancers. It is a co-inhibitory molecule that is induced during the early stages of

activation on T cells and competes with CD28 for binding B7 molecules on APC (signal 2)[5]. Not only does CTLA-4 have a higher affinity than CD28 for their ligands[6], it can also sequester CD80 and CD86 from CD28 as well as remove these ligands from the surface of APC[7]. The level of CTLA-4 expression depends on the strength of the TCR signal, so that greater TCR stimulation leads to more CTLA-4 deposition on the surface of the T cell to dampen the signal and maintain a consistent level of T cell activation[3]. CTLA-4 is not only expressed by activated $CD4^+$ and $CD8^+$ T cells, but is also constitutively by regulatory T cells (Tregs) where it enhances their immunosuppressive activity[3,8].

As a strong negative regulator of immune responses, specific antibodies for blocking CTLA-4 were developed for their potent anti-tumor activity[9,10]. It was quickly discovered that full blockade of this receptor resulted in severe autoimmunity, but partial blockade and combinatorial therapies were found to be effective for inducing tumor regression and were safe in mouse models[9,11]. Two humanized CTLA-4 antibodies (ipilimumab and tremelimumab) were first developed, and clinical trials found both to induce immune-related toxicities in many patients[12-14]. However, toxicities related to treatment with ipilimumab were more clinically manageable and an impressive long-term survival benefit was demonstrated for a limited proportion of treated patients (18%)[15]. The FDA approved ipilimumab for the treatment of advanced melanoma in 2010, and a myriad of studies have since been underway to evaluate the effectiveness of combination therapies as well as to define biomarkers that predict clinical response to CTLA-4 antibody therapy[3]. The therapeutic benefits achieved with CTLA-4 antibody blockade re-ignited interest in the field of tumor immunotherapy, and the identification of new immune checkpoints and development of specific antibodies for those immune checkpoints became a top priority.

Programmed cell death 1 (PD-1) and its ligands.

The second immune checkpoint to be targeted for the treatment of cancer consists of programmed cell death 1 (PD-1, also known as CD279) and its ligands PD-L1 (B7-H1, also known as CD274) and PD-L2 (B7-DC, also known as CD273). While CTLA-4 regulates T cell activation at the time of initial response to antigen, PD-1 is crucial in regulating effector T cell activity at the time of an inflammatory response in peripheral tissues[3]. PD-1 is a co-inhibitory molecule that is upregulated on activated immune cells including $CD4^+$ and $CD8^+$ T cells, B cells, natural killer T (NKT) cells, monocytes, macrophages, and dendritic cells (DC)[16-18]. It is also highly expressed on Tregs, which enhances their proliferation to provide physiological homeostasis[19]. Ligation of PD-1 on T cells suppresses T cell activation and proliferation, and under normal conditions, maintains peripheral tolerance by limiting the activity of T cells during inflammatory responses[20-22]. Furthermore, expression of PD-1 on DC inhibits survival and pro-inflammatory cytokine production[23,24], and PD-1-deficient DC are less capable in inducing $CD8^+$ T cell activation and proliferation[25]. Engagement of PD-1 by its ligands also suppresses B cell production of antibodies and natural killer (NK) cell lytic activity[26,27]. These interactions help to avoid tissue damage caused by exuberant or prolonged inflammation[21,28].

PD-L1 and PD-L2 are distinct in both their expression patterns and regulation of the immune system[29]. PD-L1 is constitutively expressed by T and B cells, DC, macrophages, mesenchymal stem cells, bone marrow-derived mast cells, and non-hematopoietic cells[30], and can be further upregulated by many cell types upon activation[17]. PD-L2 expression is more limited but is inducible on DC, macrophages, bone marrow-derived mast cells, and non-hematopoietic tissues[17,29]. The main role of PD-L1 is to maintain T cell tolerance in peripheral tissues while PD-L2 suppresses T cell activation in lymphoid organs[29]. PD-L1 also binds CD80

to inhibit APC[21], and both PD-L1 and PD-L2 appear to bind an unidentified co-stimulatory molecule on T cells[31,32]. Furthermore, T cells have been shown to physically acquire CD80 from APC after T cell activation[33-35], and binding of PD-L1 to CD80 causes CD80 to deliver an inhibitory signal to T cells[21,22,27].

Increased expression of PD-L1 by tumor cells is an example of a phenomenon known as adaptive immune resistance[3]. In the presence of high levels of PD-L1 expression in tumor tissues, tumor-infiltrating T cells (TILs) become functionally inactivated and lose the ability to effectively control tumor growth[36]. Chronic exposure to antigen without co-stimulation leads to T cell anergy and eventually T cell exhaustion, which is characterized by poor effector function, low proliferative activity, and the inability to persist as memory T cells[36]. PD-L1 expression on human tumor cells has been found to be negatively correlated with prognosis and patient survival in colon, cervical, pancreatic, breast, ovarian, renal cell, hepatocellular, non-small cell lung, melanoma, and esophageal cancers[37-46]. In mice, PD-L1 has been found to be expressed by ovarian, myeloma, lung, melanoma, and mammary cancers[47-50], where it is involved with escape of the tumor cells from the immune system. Canine tumors have also been found to express PD-L1, including melanoma, hemangiosarcoma, osteosarcoma, mastocytoma, and renal cell carcinoma[51-53]. In addition, myeloid cells such as tumor-associated macrophages commonly express PD-L1, and this is thought to be one of the mechanisms by which they exert immunosuppressive effects in the tumor environment[54-59]. While most studies focus on PD-L1 in the tumor environment, PD-L2 has also been found to be upregulated in B cell lymphomas[60].

Upregulated expression of PD-L1 in tumors has been found to be mediated primarily by IFN-γ produced by TILs in humans, mice, and dogs[61-64]. Other cytokines have also been found to regulate PD-L1 expression on human cancer cell lines (IL-4 and TNF-α), but none as strongly as

IFN-γ[65]. Furthermore, environmental factors such as hypoxia cause rapid upregulation of PD-L1 expression by tumor cells, macrophages, and DC through HIF-1α[49]. The innate immune response has also been found to play a role in the regulation of PD-L1 expression in tumors. Toll-like receptor (TLR) expression is increased on many human tumors including colon, gastric, prostate, breast, ovary, and brain tumors, where signaling through the TLRs leads to upregulation of PD-L1[66,67]. Furthermore, TLR2 and TLR4 ligands have been shown to protect acute myeloid leukemia blast cells from T cell killing through the induced expression of PD-L1[68]. Finally, some tumor cells possess "innate immune resistance" that does not depend on signals in the tumor environment[1]. For example, constitutive ALK signaling in lung cancer has been found to upregulate PD-L1 expression through STAT-3 signaling[69] and loss of tumor suppressor PTEN results in increased PD-L1 expression by glioma[70]. Upregulation of PD-L2 in lymphomas has been associated with gene amplification or rearrangement with an MHCII locus[71].

PD-1/PD-L1 blockade in tumors.

The presence of TILs has been correlated with higher expression of both PD-1 and PD-L1, which correlates with both poor prognosis and aggressive tumors[72,73]. PD-1 is expressed by most TILs, and these PD-1-expressing TILs are functionally impaired[74,75]. However, interrupting the PD-1/PD-L1 signaling pathway with therapeutic antagonistic antibodies can potently activate T cell responses to tumors. PD-1 antibody blockade in tumors increases the number of effector T cells, increases T cell proliferation and Th1 cytokine production (IFN-γ, IL-2, TNF-α, IL-1 and IL-6), decreases Th2 cytokine production (IL-2 and IL-13), and decreases both the number and the suppressive activity of Tregs[29,76-78]. PD-L1 antibody blockade increases T cell infiltration and IFN-γ production[48,79].

The first PD-1 antibody to be tested in a human clinical trial was nivolumab. The study found up to 31% objective responses (of which 62% were durable greater than 1 year) in patients with advanced melanoma, non-small cell lung cancer, castration-resistant prostate cancer, renal cell cancer, and colorectal cancer[80,81]. Nivolumab has since been shown to be effective for treatment of several solid tumors both alone and in combination with CTLA-4 antibody ipilimumab[82,83]. Clinical trials in 2016 for another PD-1 antibody, pembrolizumab, showed overall response rates of 21-34% for advanced melanoma and up to 25% for non-small cell lung cancer, which led to FDA approval for these cancers[84]. To date, these PD-1 antibodies have been approved for treatment of melanoma, kidney cancer, non-small cell lung cancer, head and neck cancer, Hodgkin's lymphoma, and urothelial cancer in humans[85]. Studies have also demonstrated the activity of PD-L1 antibodies in a variety of different cancer types[86-88], and PD-L1 antibodies (atezolizumab and durvalumab) are now approved for treatment of lung cancer, bladder cancer, and urothelial cancer in humans[85-87]. Antibody blockade of PD-L1 has also been effective in combination with therapies such as radiation therapy, MAP kinase inhibition, immunovirotherapy, and administration of other checkpoint blocking antibodies such as CTLA-4 antibodies[89-93]. Importantly, PD-1 and PD-L1 antibodies have been found to induce few adverse effects, and of those that occur, most are manageable and not severe[80,94].

Future directions for immune checkpoint blockade.

Unfortunately, most immunotherapeutic agents fail in randomized comparisons to other therapies in Phase III trials[95]. For example, an analysis of all clinical trials for PD-1/PD-L1 antibodies to date found an overall pooled response rate of 24% for cancer patients with advanced stage, refractory, or sensitive to previous treatment[96]. However, this was still higher

than the response rates for standard chemotherapy and for treatment with ipilimumab (anti-CTLA-4) for melanoma. This has led to many studies that focus on finding factors that predict response to treatment, with the goal of identifying patients who will benefit the most from these therapies. Potential biomarkers that have been widely studied include PD-1 and PD-L1 expression in the tumor, tumor genomic features, tumor RNA expression signatures, tumor mutational burden, and immune cell infiltrates[97-101]. These markers have been found to be helpful for certain tumor types in predicting response, but none have been found to accurately predict response when applied to multiple tumor types.

PD-L1 was studied as a potential biomarker when a correlation was found between PD-L1 expression in the tumor and response to nivolumab[80]. Multiple studies agree that the density of PD-L1-expressing macrophages in tumors is predictive of the efficacy of both PD-1 and PD-L1 antibody therapy[99,102,103]. For example, in some cases, response rates as high as 80% have been observed in patients where macrophages were positive for PD-L1 expression[99]. However, other studies have reported patients with PD-L1-negative tumors experiencing objective responses[104] and a lack of correlation between PD-L1-positivity and improved response to PD-1 therapy[82]. These discrepancies could be addressed by the fluid expression profile of PD-L1 in the tumor, as expression levels can change based on the previously described inflammatory signals. For example, PD-L1 expression can be induced in a tumor that was negative at the time of analysis. Furthermore, some PD-L1^{+} tumors solely express PD-L1 in the cytosol where it is not able to bind PD-1 on T cells[3], leading to PD-L1 positivity that may not correlate to response to PD-1 blockade therapy.

Recently, a study published in *Science* found a correlation that is being seen as a breakthrough in the search for a biomarker that can predict response to immune checkpoint

blockade. Mutation associated neoantigens (MANAs) arise during cellular transformation, and are thus tumor cell-specific. However, little is known concerning the nature of the tumor antigens that are targeted by T cells following activation by immune checkpoint blockade, and whether it is the mutation load or the identity of the mutations that allows tumor cells to be recognized as foreign by the immune system[105,106]. Mismatch repair (MMR)-deficiency in tumors has long been predicted to generate large numbers of MANAs, increasing the odds that some may be recognizable by the immune system[107-109]. The "breakthrough" study found that, indeed, MMR-deficient cancers led to the accumulation of large numbers of MANAs that made patients responsive to PD-1 blockade (pembrolizumab)[110]. Data were collected from a pool of 86 patients with advanced MMR-deficient cancers spanning 12 tumor types, with objective responses found in 53% of patients and complete responses in 21% of patients. Interestingly, deep sequencing showed that all of the MANAs that were recognizable by T cells resulted from frameshift mutations, which is the mutation most characteristic of MMR-deficiency. Further work in this study showed that, in an evaluation of over 12,000 cancers representing 32 tumor types, up to 8% of tumors were MMR-deficient. Fortunately, MMR-deficiency tests are widely available and should be considered for identifying patients that may benefit from PD-1 blockade, as it is a very promising pan-tumor biomarker for predicting response to immune checkpoint blockade.

New immune checkpoint molecules have now been discovered, including T cell membrane protein 3 (TIM3), lymphocyte activation gene 3 (LAG3), and other members of the B7 family such as B7-H3 and B7-H4. TIM3 is overexpressed by Tregs in the tumor and is a marker of $CD8^+$ T cell exhaustion[111]. LAG3 (CD223) is expressed by plasmacytoid DC, B cells, NKT cells, and exhausted CD8+ T cells, where it inhibits signaling in tumor-infiltrating $CD8^+$ T cells upon binding to PD-1[112]. LAG3 is also expressed by Tregs, and antibody blockade of

LAG3 decreases their suppressive function[113]. Ligands B7-H3 and B7-H4 are expressed by both tumor cells and tumor-infiltrating cells, specifically by endothelial cells in the tumor vasculature (B7-H3) and tumor-associated macrophages (B7-H4)[114,115]. The receptors for these ligands are still unidentified, but they appear to be inhibitors of the immune response[115]. Tumor cells often express multiple inhibitory immune checkpoint ligands that bind their cognate receptors on TILs, which may explain the lack of clinical response in many patients to single antibody blockade. Therefore, current studies for immune checkpoint blockade largely focus on combination therapies that may target multiple inhibitory pathways, inhibitory and activating pathways, or multiple activating pathways.

An important aspect of tumor immunotherapy to consider is the unique mechanisms-of-action and kinetics of immune checkpoint antibody blockade. For example, response to CTLA-4 antibody therapy can be delayed up to 6 months after treatment, during which lesions typically increase in size before regressing[3]. A combination of large international studies for ipilimumab detected four response patterns: immediate response, durable stable disease, response after tumor burden increase, and response in the presence of new lesions[116,117]. The delay in treatment response demonstrates the time needed to build a tumor-specific cellular immune response, and the increase in tumor size can reflect the influx of pro-inflammatory cells into the tumor[15]. Therefore, new tools for evaluation and new response criteria for trial endpoints is needed, as the conventional method of measuring time-to-progression or RECIST (Response Evaluation Criteria in Solid Tumors) was developed for chemotherapeutic agents and is not appropriate for immunotherapeutic agents[118].

Several international initiatives have been facilitated by expert panels to systematically evaluate and redefine biological outcomes and clinical endpoints for immunotherapy[119-121].

Based on the biological response to immunotherapy, three main events are being viewed as potential clinical trial endpoints: early immune activation and T cell proliferation, anti-tumor effects mediated by activated immune cells, and delayed effect on patient survival[122]. Therefore, the considerations for these endpoints are as follows: minimize variability in T cell assays to establish reproducible measurements of cellular immune response, adapt RECIST to the clinical pattern of antitumor response for immunotherapeutic agents (to be called irRC, or immune-related response criteria), and use appropriate statistical methods for both trial design and analysis of delayed separation for Kaplan-Meier survival curves.

CHAPTER 2

Immune Regulation of Canine Tumor and Macrophage PD-L1 Expression

Summary.

High expression of programmed cell death receptor ligand 1 (PD-L1) on tumor cells has been associated with immune escape in human and murine cancers, but little is known regarding the expression and regulation of PD-L1 by cancers in dogs. Therefore, 14 canine tumor cell lines were evaluated for constitutive PD-L1 expression and for responsiveness to immune stimuli. We found PD-L1 was expressed constitutively on all canine tumor cell lines evaluated, though the levels of basal expression were variable. Significant upregulation of PD-L1 by all cell lines was observed following IFN-γ and a TLR3 ligand treatment. In contrast, canine macrophages did not constitutively express PD-L1 but were also responsive to IFN-γ. These findings suggest that most canine tumors express PD-L1 constitutively and that both innate and adaptive immune stimuli can further upregulate PD-L1 expression. Therefore, upregulation of PD-L1 expression by cancers in response to cytokines such as IFN-γ may represent an important mechanism of tumor-mediated T cell suppression in dogs as well as in humans.

Background.

In recent years, it has become evident that there is a critical need for animal models that more accurately depict human biology and disease progression. Thus, the field of comparative oncology draws from the premise that veterinary patients with naturally-occurring cancers provide an invaluable source of knowledge that can be applied to human biology and disease[123]. When the canine genome was sequenced and compared to the human genome, the two were

found to have over 80% genetic similarity[124,125]. This leads to genetic drivers for the development of cancers, molecular and clinical presentation of disease, and response to treatment that are often identical between canine and human[126-130]. Furthermore, there is great genetic diversity even among closely related dog breeds[131]. This is crucial for biomedical research, as research mice are inbred to be genetically identical and live in a highly-regulated environment. In contrast, dogs differ greatly in terms of genetics, living environment, diet, and lifestyle, which can more accurately model the differences between humans. These variables allow for naturally occurring disease in both species that can be more directly compared instead of inbred mice that have artificially been injected with tumor cells to initiate tumor growth.

The success of PD-1 and PD-L1 immunotherapy in the treatment of human cancers has prompted the research and development of canine-specific immunotherapies. Recently, canine PD-1 and PD-L1 were molecularly characterized, and the corresponding genes were found to be conserved among dog breeds[51]. We, and others, have reported that PD-1 and PD-L1 antibodies can activate canine T cells, stimulating proliferation and cytokine production[132,133]. In addition, a recent report describes the first use of a canine chimeric PD-L1 antibody in dogs with cancer, with anti-tumor responses observed in several treated animals[133].

However, the expression of PD-L1 by a broad panel of canine tumor types has not been previously investigated, nor has the regulation of canine PD-L1 expression on tumor cells been studied. Therefore, the purpose of the current study was to investigate PD-L1 expression by a series of canine tumor cell lines and to determine how PD-L1 expression on these cells was regulated by IFN-γ and also by TLR ligands. Because PD-L1 is also known to be expressed by tumor-infiltrating macrophages in humans, we also investigated the expression of PD-L1 by *in vitro* generated canine macrophage cultures, and the effects of cytokines on macrophage PD-L1

expression. We hypothesized that both tumor cell and macrophage PD-L1 expression would be primarily regulated by IFN-γ, because this cytokine is known to regulate PD-L1 expression by tumor cells in humans, mice, and dogs[61-64]. A recently developed canine PD-L1 antibody was used in this study, and expression of PD-L1 by canine tumor cell lines was assessed by flow cytometry and by immunocytology. The tumor PD-L1 response to treatment with recombinant canine IFN-γ and to TLR ligands was also assessed, as was the PD-L1 response to cytokine-enriched conditioned medium from activated canine T cells.

Materials and Methods.

Cell lines. Fourteen different canine tumor cell lines were evaluated in this study. All cell lines were validated and screed to be genetically unique[134]. Melanoma (MEL) cells included Talsky, Shadow, and Jones (Colorado State University - CSU). Canine osteosarcoma (OS) cells included Abrams (CSU), D17 (ATCC), and McKinley (CSU). Canine hemangiosarcoma (HSA) cells included DEN-HSA (University of Wisconsin) and SB (University of Minnesota). The Bliley cell line was a transitional cell carcinoma (TCC) from CSU while the Oswald cell line was a T cell lymphoma (LSA) from Ohio State University - OSU. The C2 cell line was a mast cell tumor (MCT) from the University of California, San Francisco. The CTAC cell line was a thyroid cancer (TA) from Auburn University, and canine histiocytic sarcomas (HS) included Nike (CSU) and DH82 (ATCC).

Tumor Cell culture. Tumor cells were grown in MEM medium (Gibco, Grand Island, NY) supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO) and 5% CTM (10,000 ug/mL Pen/Strep, 200 mM L-glutamine, 10 mM essential amino acids without L-glutamine, 10 mM

non-essential amino acids, and 7.5% bicarbonate solution (all from Gibco). The cells were cultured in standard plastic tissue culture flasks (Cell Treat, Shirley, MA) and strongly adherent cells were harvested by treatment with 0.25% Trypsin/1mM EDTA (Gibco) followed by Trypsin/EDTA inactivation with media. Viability of the cells was determined using 0.4% Trypan blue stain (Gibco) for dead cell exclusion.

After the tumor cells were harvested from culture, 1.0×10^5 viable cells were plated in 24-well polystyrene cell culture plates (Falcon, Durham, NC). The cells were then treated with cytokines, supernatants from Concavalin A (ConA) activated peripheral blood mononuclear cells (PBMCs), or TLR ligands for 24 hours at 37°C. The supernatants were generated by incubating primary canine PBMCs in 10 ug/ml conA (Sigma-Aldrich, St. Louis, MO) overnight, collecting the supernatants, and centrifuging to remove the remaining cells out of the supernatants. For analysis, the cells were trypsinized and washed with media before transfer to a round bottom 96-well plate (Falcon) for immunostaining. The wells were washed with FACS buffer and centrifugation to collect the cell pellets, and the cells were stained for flow cytometry.

Macrophage culture. Canine macrophages were derived from peripheral blood monocytes obtained from healthy dogs. Briefly, PBMCs were obtained using LSM Lymphocyte Separation Medium (MP Biomedicals, Solon, OH) and plated on fibronectin-coated wells in 24-well polystyrene cell culture plates (fibronectin was obtained from Sigma-Aldrich). Non-adherent cells were removed after 18 hours in culture and adherent monocytes were cultured for 7 days in 10 ng/ml human M-CSF (Peprotech, Rocky Hill, NJ) in DMEM medium (Gibco) supplemented with 10% FBS. The growth media was changed and fresh huM-CSF (10 ng/ml) was added every 2 days.

Cytokines and TLR reagents. Recombinant canine IFN-γ was obtained from R&D Systems, Minneapolis, MN and titrated at 0.1, 1, 10, and 100 ng/ml in tumor culture medium. Tumor cells were cultured in the presence of IFN-γ in complete medium for 24h prior to analysis of PD-L1 expression. For the majority of studies, IFN-γ was used at 10 ng/ml based on the results of the titration studies. Polyinosinic:polycytidylic acid, Poly(I:C), was purchased from Invivogen (San Diego, CA) and was used at 10 ug/ml. Plasmid DNA (pDNA) was produced by Juvaris Biotherapeutics, Inc (Pleasanton, CA) and used at 10 ug/mL. Lipopolysaccharide, LPS (*E.coli* serotype 0111:B4), was obtained from Sigma-Aldrich and used at 1 ug/ml. R848 from Invivogen was used at 10 ug/ml. These concentrations were chosen based on the working concentration ranges suggested by the manufacturers. The activity of these TLR agonists was verified in mouse studies, and their activity in stimulating canine TLRs was assessed by stimulation of canine monocyte-derived macrophages and measurement of IL-6 secretion with an ELISA (data not shown).

Antibodies. A murine anti-canine PD-L1 monoclonal antibody (clone 4F9) was used in these studies to detect canine PD-L1 expression. This antibody was developed by Merck Animal Health (Millsboro, DE) and was used for flow cytometry, immunofluorescence staining, and Western blotting. An isotype matched, irrelevant antibody was used at the same concentration (eBioscience, San Diego, CA). Mouse mAbs were detected using a donkey anti-mouse secondary (Jackson ImmunoResearch, West Grove, PA). Mouse anti-human CD11b (clone Bear1) was obtained from Immunotech by Beckman Coulter (Marseilles, France) and used for used for flow cytometric evaluation of primary canine macrophages. A polyclonal goat anti-canine IFN-γ antibody from Novus Biologicals (Littleton, CO) was used at 5 ug/ml to neutralize

IFN-γ in activated PBMC-conditioned media for 30 minutes prior to treating the tumor cells, and an irrelevant control was a polyclonal goat IgG antibody from Jackson ImmunoResearch, used at the same concentration.

Western Blot. A standard Western blotting protocol from (Bio-Rad, Hercules, CA) was followed. Briefly, the tumor cells were harvested by scraping, then lysed in the presence of protease inhibitors (Thermo Fisher Scientific, Waltham, MA) and the protein concentration was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific). Samples were prepared under non-reducing, denaturing (boiled) conditions, and 20 ug total protein was loaded into a 4-15% gradient mini PROTEAN TGX BioRad gel. 5% non-fat dry milk was used for blocking and 4F9 mAb was used to probe for PD-L1 protein, followed by a peroxidase-conjugated donkey anti-mouse secondary. The blots were visualized using BioRad Clarity Western ECL Substrate.

Flow cytometry. Tumor cells were harvested from culture using 0.25% Trypsin/1mM EDTA (Gibco), washed with PBS, and incubated 10% normal donkey serum (Jackson ImmunoResearch) to reduce non-specific binding of antibodies. To detect expression of canine PD-L1 on the surface of the cells, appropriately diluted PD-L1 4F9 mAb or concentration matched isotype control MOPC-21 mAb (BioXcell, West Lebanon, NH) were added to tumor cells for 20 minutes at room temperature. The cells were then washed in FACS buffer and incubated with biotin-conjugated donkey-anti-mouse (Jackson ImmunoResearch) antibody followed by washing and then incubation with streptavidin-conjugated PE (eBioscience, San Diego, CA). After a final wash, cells were resuspended in FACS staining buffer and 7-AAD

viability dye (eBioscience) was added for dead cell exclusion. The cells were evaluated for PD-L1 expression using a Beckman Coulter Gallios flow cytometer (Brea, CA), and data was analyzed using FlowJo Software (Ashland, OR).

Monocyte-derived macrophages were detached from tissue culture plastic with 2mM EDTA in ice-cold PBS for 30 minutes on ice followed by gentle pipetting to wash off cells from the plate. The cells were blocked with 5% normal donkey serum and dog serum before incubating with 4F9 (or isotype control) along with CD11b, and were afterwards stained following the same protocol as the tumor cells.

Immunofluorescence imaging. Tumor cells were cultured on glass coverslips overnight, with or without IFN-γ (10 ng/ml). The next day, the coverslips were washed, fixed in ice-cold acetone for 10 minutes, and rehydrated in 1X PBS prior to staining. After blocking with 5% donkey serum and Streptavidin block (Vector Laboratories, Burlingame, CA), 4F9 or isotype antibody with Avidin block (Vector Laboratories) was added in appropriate concentrations. This was followed by biotin-conjugated donkey-anti-mouse IgG and streptavidin-conjugated Cy-3 (Invitrogen). Lastly, the cells were stained with DAPI (Molecular Probes, Eugene, OR) and mounted onto Superfrost slides (VWR, Radnor, PA) with Fluoromount Aqueous Mounting Medium (Sigma-Aldrich).

Primary tumor tissues were frozen in OCT (Optimal Cutting Temperature compound) and cut to a thickness of 5 microns. After fixing in ice-cold acetone for 5 minutes, they were rehydrated with 1X PBS and stained the same way as the tumor cell lines grown on coverslips.

RT-PCR. Total RNA was isolated from canine tumor cell lines with and without IFN-γ treatment using an RNeasy Mini Kit (Qiagen, Frederick, MD), and this was transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen). SYBR Green-based PCR (Bio-Rad, Berkeley, CA) was conducted with the following primer sequences to amplify PD-L1 mRNA (Integrated DNA Technologies, Coralville, IA): 5'-CCG CCA GCA GGT CAC TT-3' (forward) and 5' TCC ATT GTC ACA TTG CCA CC-3' (reverse). This primer pair was validated with an amplification efficiency of 102% and an R^2 value of 0.991. Data analysis was based on the fold change = $2^{-\Delta Ct}$ method, with normalization of the data to the *GAPDH* housekeeping gene. Relative quantification was calculated using the average value of duplicate samples.

Results.

PD-L1 expression and responsiveness to IFN-γ. The expression of PD-L1 was assessed on 14 distinct canine tumor cell lines, using flow cytometric analysis. We found all 14 expressed PD-L1 under basal conditions, with the two histiocytic sarcoma cells lines expressing the highest level **(Figure 2.1)**. The cell lines with the lowest levels of surface PD-L1 expression were the lymphoma and two hemangiosarcoma cell lines. We also assessed the intracellular expression of PD-L1 by fixing and permeabilizing tumor cells on coverslips and assessing PD-L1 expression microscopically **(Figure 2.2)**. We found that all 4 tumor cell lines screened expressed PD-L1 intracellularly as well as extracellularly, when staining intensity was compared to that of the isotype control antibody.

IFN-γ has been reported to regulate PD-L1 in human and mouse systems[62,64]. Therefore, the effects of canine rIFN-γ on PD-L1 expression on our canine tumor cell lines was assessed. We found that there was a titratable effect of IFN-γ on PD-L1 upregulation **(Figure 2.3A)**. A

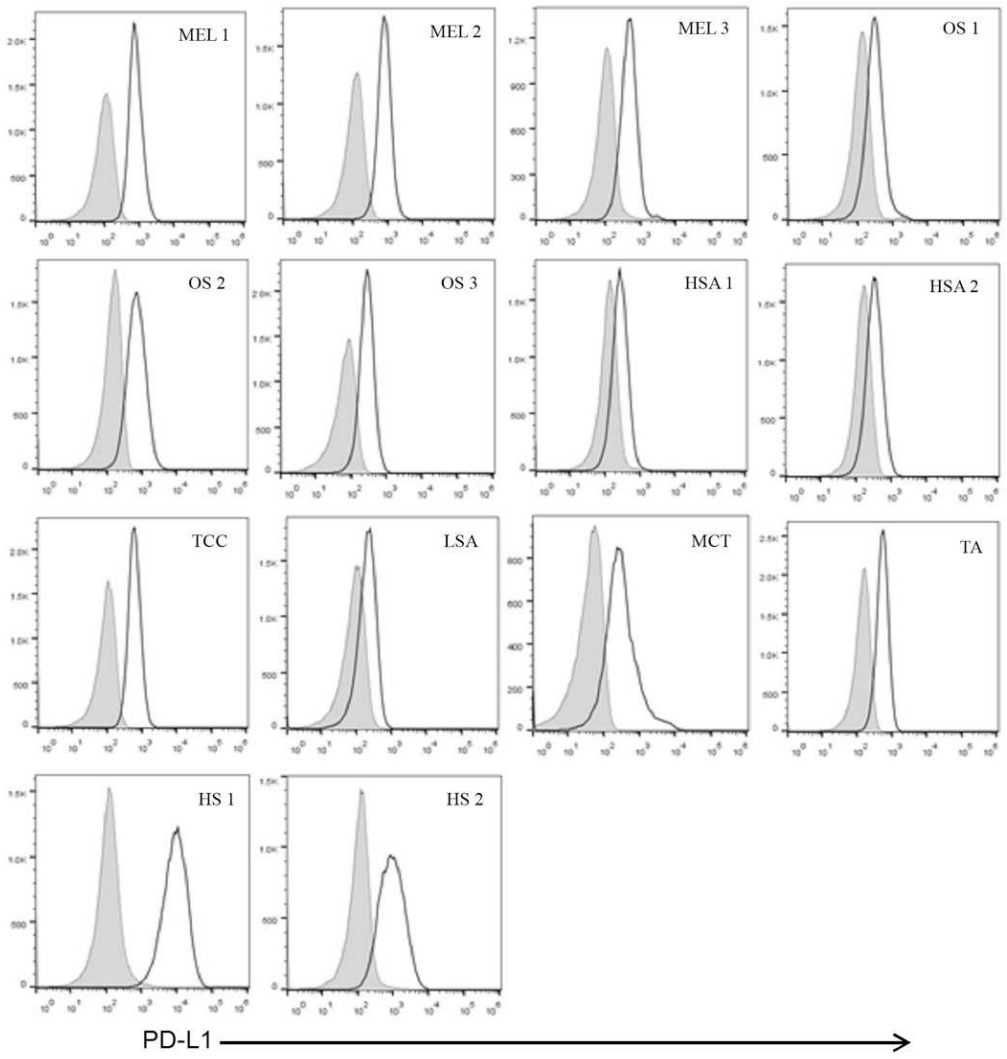

Figure 2.1 PD-L1 expression by 14 canine tumor cell lines. Canine tumor cells were cultured in 24-well plates overnight and stained the following day for PD-L1 expression by flow cytometry. The cells were incubated with the appropriately matched concentration of irrelevant isotype (gray filled line) or PD-L1 antibody (bold white line) and the data were analyzed using FlowJo Software. PD-L1 expression was determined with respect to the mean fluorescence intensity of the isotype. This experiment was repeated 4 times with similar results each time.

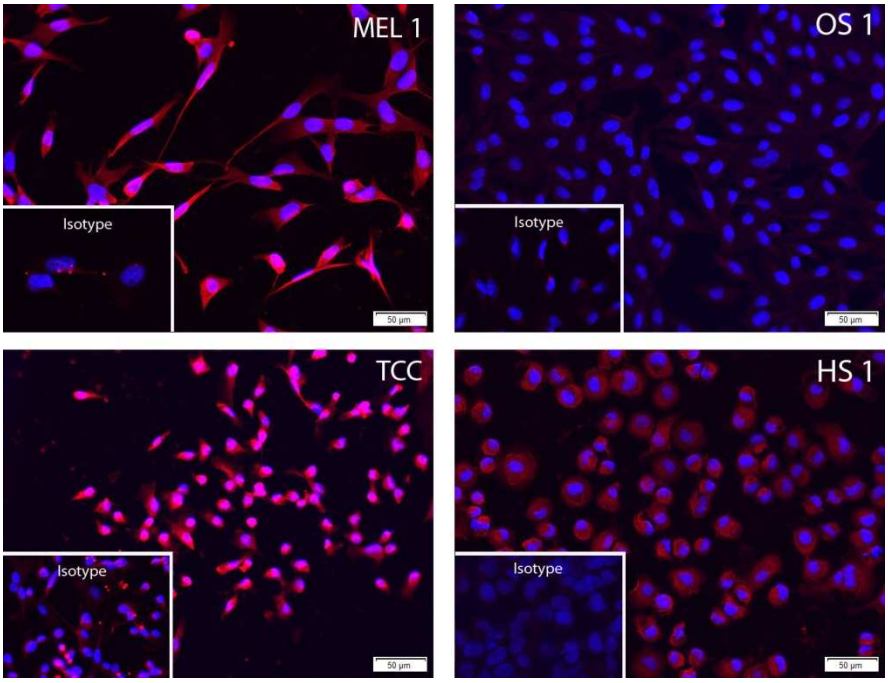

Figure 2.2 PD-L1 expression by canine tumor cell lines as assessed by immunofluorescence imaging. Canine melanoma, osteosarcoma, transitional cell carcinoma, and histiocytic sarcoma cells were grown as monolayers on glass coverslips and stained for immunofluorescence with either PD-L1 or irrelevant isotype antibody (red) and a DAPI nuclear stain (blue). The coverslips were mounted on glass slides and the images were obtained with the same microscope settings to show the relative intensity of PD-L1 staining between tumor cell lines.

mid-range concentration of IFN-γ (10 ng/ml) was then selected for most of the remaining IFN-γ studies. This concentration of IFN-γ was found to upregulate PD-L1 expression by all the tumor cell lines except the B cell lymphoma cell lines, though to differing degrees depending on the cell line **(Figure 2.3B)**. For example, histiocytic sarcoma cells were the most responsive to IFN-γ stimulation, upregulating PD-L1 significantly more than other cell lines when comparing MFI between unstimulated and IFN-γ treated cells (p-value < 0.001). qRT-PCR for 5 different cell lines with and without IFN-γ treatments showed that, in general, *Pdl1* transcript levels increased after exposure of the cells to IFN-γ but do not correspond to the amount of PD-L1 protein expressed by the cells when compared between tumor cell lines **(Figure 2.4)**. These data were also shown using immunocytology, where PD-L1 expression was increased in both locations

after treatment with IFN-γ **(Figure 2.5).** Finally, the 4F9 antibody was shown to bind a protein of the correct molecular weight of glycosylated PD-L1, 50 kDA, and its expression was increased after treatment of histiocytic sarcoma cells with IFN-γ **(Figure 2.6)**.

Cytokine regulation of PD-L1. The effect of other immune-regulatory cytokines on PD-L1 expression was also investigated. Because T cell cytokines other than IFN-γ (eg, IL-4, GM-CSF, TGF- β) have been shown to upregulate PD-L1 expression on different murine cell types[30,65], we were interested to determine the net effect of all cytokines produced by activated T cells on canine tumor PD-L1 expression. A mixture of different T cell cytokines was generated from in vitro activated canine PBMC, using ConA stimulation. The canine IFN-γ concentration in the supernatants of conA stimulated PBMC was found to be 8 ng/ml by ELISA (data not shown). The T cell cytokine mixture was then evaluated for upregulation of tumor PD-L1 expression. We found that the T cell cytokine mixture strongly upregulated PD-L1 expression **(Figure 2.7A)**, above levels observed following incubation with equivalent amounts of 8 ng/ml IFN-γ alone **(Figure 2.7B)**. These findings suggested that IFN-γ in combination with other T cell cytokines can stimulate even greater upregulation of tumor PD-L1 expression. When IFN-γ was neutralized in the supernatants of conA stimulated PBMCs, PD-L1 upregulation by histiocytic sarcoma cells was markedly inhibited **(Figure 2.8)**. These findings suggest that IFN-γ is the primary cytokine produced by activated canine T cells that is responsible for upregulating PD-L1 expression on canine tumor cells and macrophages, though other T cell derived cytokines appear to play a role as well in amplifying the overall PD-L1 stimulatory effect of IFN-γ.

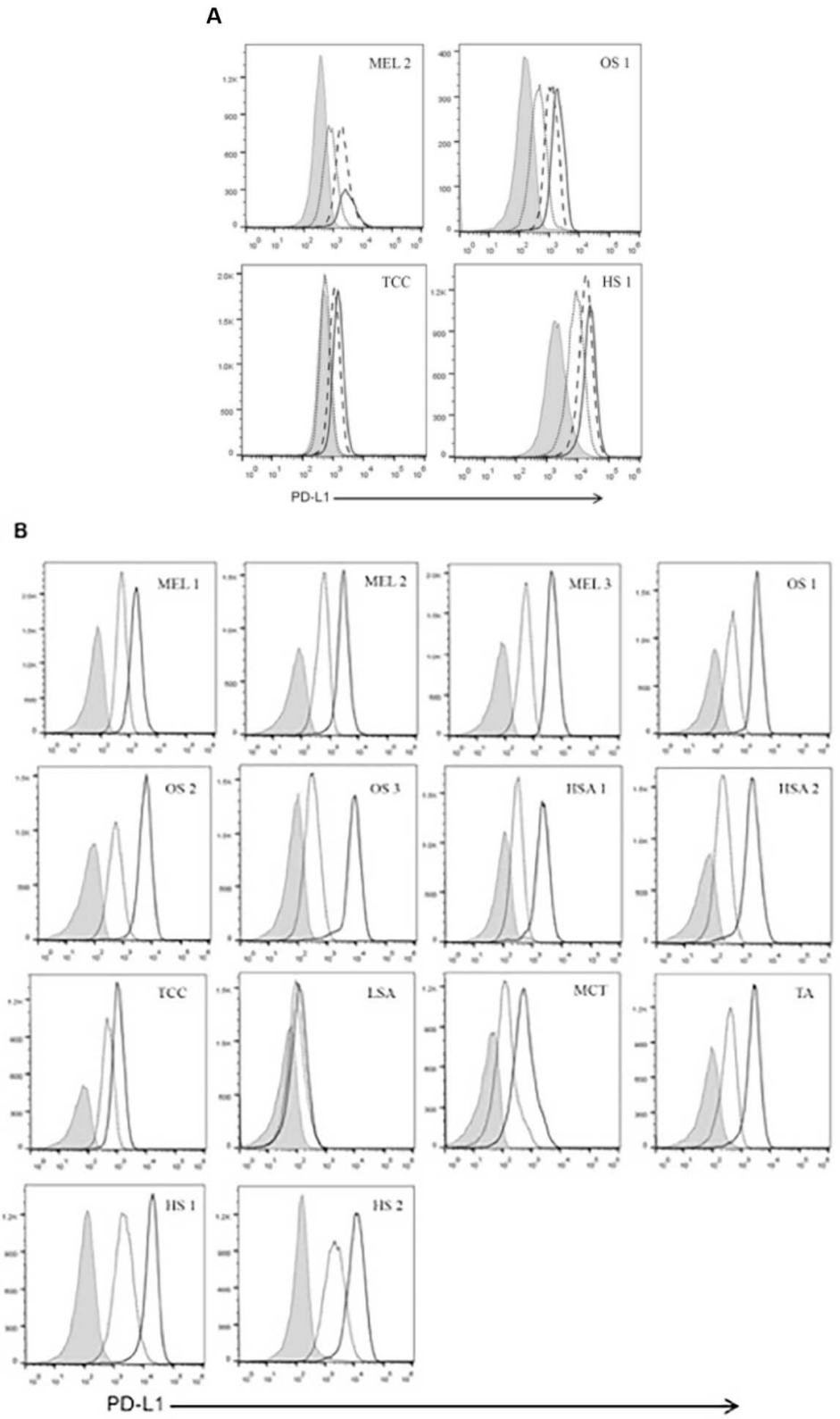

Figure 2.3 Effect of IFN-γ stimulation on PD-L1 expression by canine tumor cells. (A) Titration of IFN- γ. Canine melanoma, osteosarcoma, transitional cell carcinoma, and histiocytic sarcoma cells were cultured with 0 (gray line), 1 (dotted line), 10 (dashed line), or 100 ng/ml

rcIFN-γ (bold line) in media overnight. The cells were then immunostained for PD-L1, and flow cytometry was used to compare the mean fluorescence intensity of each treatment group. This titration experiment was conducted 4 times, with similar results each time. **(B)** Treatment of all canine tumor cell lines with 10 ng/ml IFN- γ. Tumor cell lines were treated with 10 ng/ml rcIFN-γ overnight and stained with an irrelevant isotype antibody (gray filled line) or PD-L1 antibody (unstimulated is the dotted line and IFN-γ treated is the bold line) and analyzed by flow cytometry for PD-L1 expression. These data are representative of 6 different independent experiments.

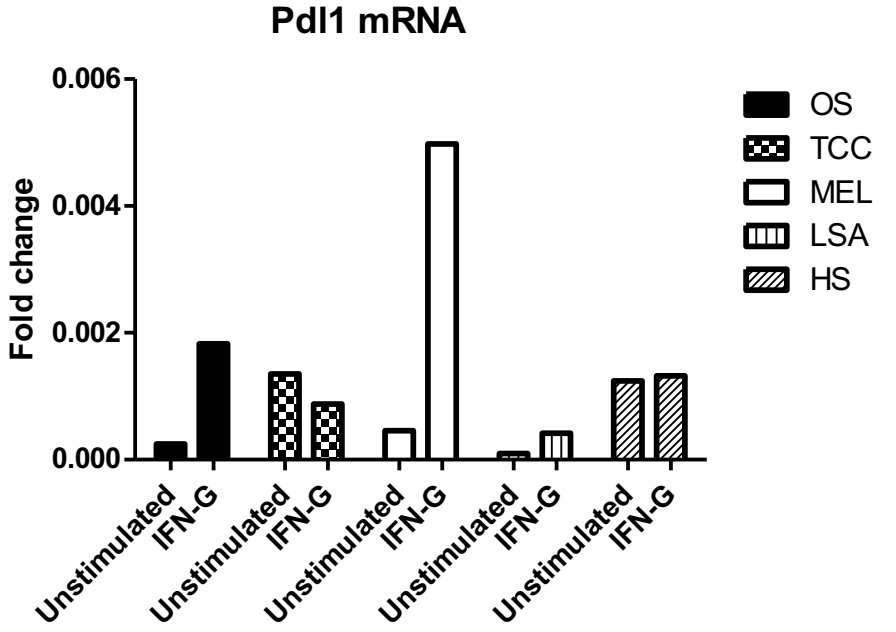

Figure 2.4. qRT-PCR of canine tumor cell lines with and without IFN-γ treatment. 5 canine tumor cell lines were cultured overnight in regular media or media with IFN-γ. RNA was extracted for qRT-PCR, and Ct values were normalized to the housekeeping gene GAPDH. Fold increase was calculated as $2^{-\Delta Ct}$.

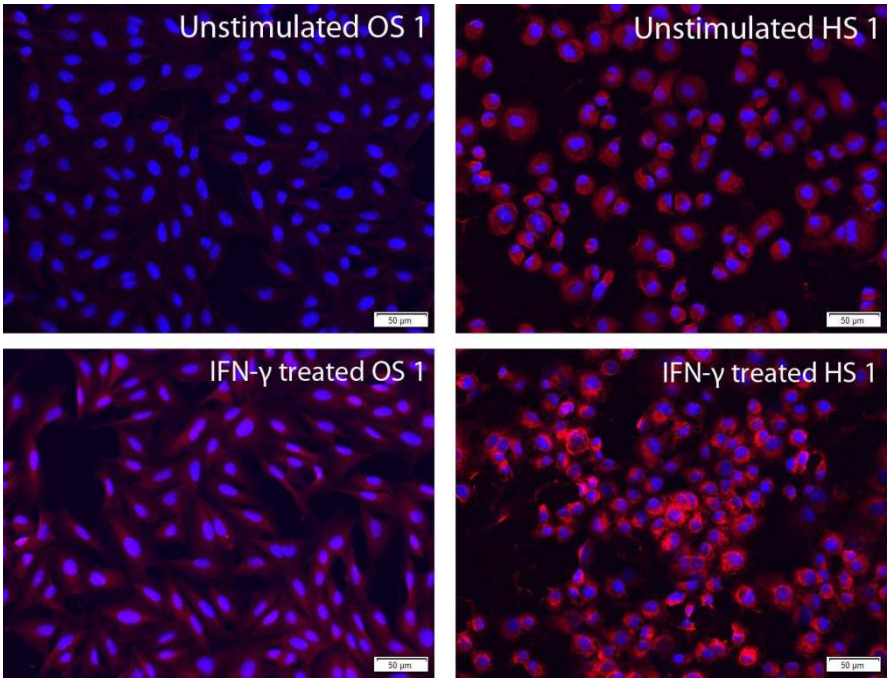

Figure 2.5 Effect of IFN-γ stimulation on PD-L1 expression by canine tumor cell lines, as assessed by immunofluorescence imaging. Canine melanoma, osteosarcoma, transitional cell carcinoma, and histiocytic sarcoma cells were grown as monolayers on glass coverslips in media alone or with10 ng/ml rcIFN-γ overnight. The next day they were stained for immunofluorescence with either PD-L1 or irrelevant isotype antibodies (red) and a DAPI nuclear stain (blue). The coverslips were mounted on glass slides and the images were obtained with the same microscope settings to show the relative intensity of PD-L1 staining between unstimulated and rcIFN-γ treated cells. This experiment repeated 3 times to demonstrate consistency of the assay.

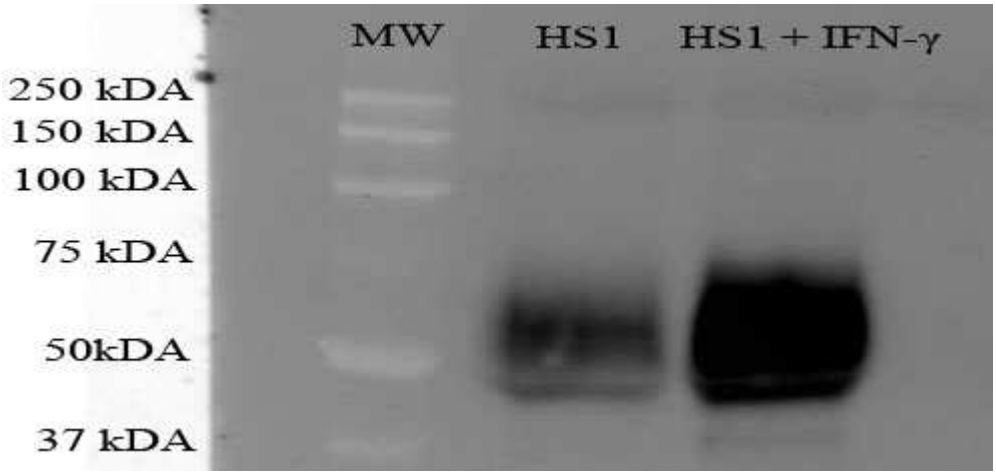

Figure 2.6 Western Blot of PD-L1 expression by HS1 cells with and without IFN-γ treatment. Equal numbers of canine histiocytic sarcoma cells were cultured overnight with and without IFN-γ treatment. They were collected, lysed, and 20 ug total protein was loaded into a boiled gel for electrophoresis under non-reducing, denaturing conditions. The gel was blotted with 4F9 and a donkey anti-mouse secondary antibody.

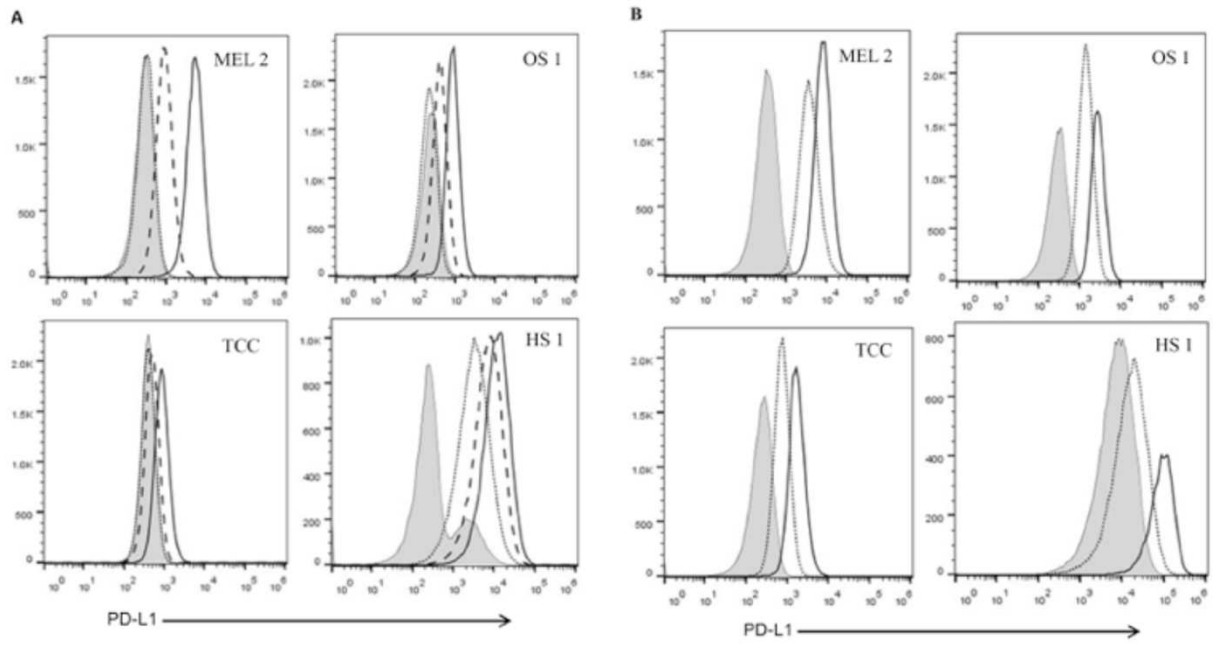

Figure 2.7 Effect of cytokines produced by concanavalin A activated canine T cells on tumor PD-L1 expression. (A) Dilutions of supernatants from conA-stimulated PBMCs. Canine melanoma, osteosarcoma, transitional cell carcinoma, and histiocytic sarcoma cells were exposed to 4 dilutions of supernatants from ConA stimulated PBMC overnight. Groups include no ConA supernatant (light gray filled line), 1:100 dilution (dotted line), 1:10 dilution (dashed line), and 1:1 dilution (bold line). The cells were then harvested and stained with PD-L1 for flow cytometric analysis as previously described. This experiment was repeated 4 times. **(B)** Supernatants from conA-stimulated PBMCs and IFN-γ. The same tumor cell lines tested in (A) were incubated overnight with media only (gray filled line), 4 ng/ml rcIFN-γ (dotted line), or a 1:1 dilution of these supernatants (bold line) and stained for PD-L1 expression. These data are representative of 3 independent experiments.

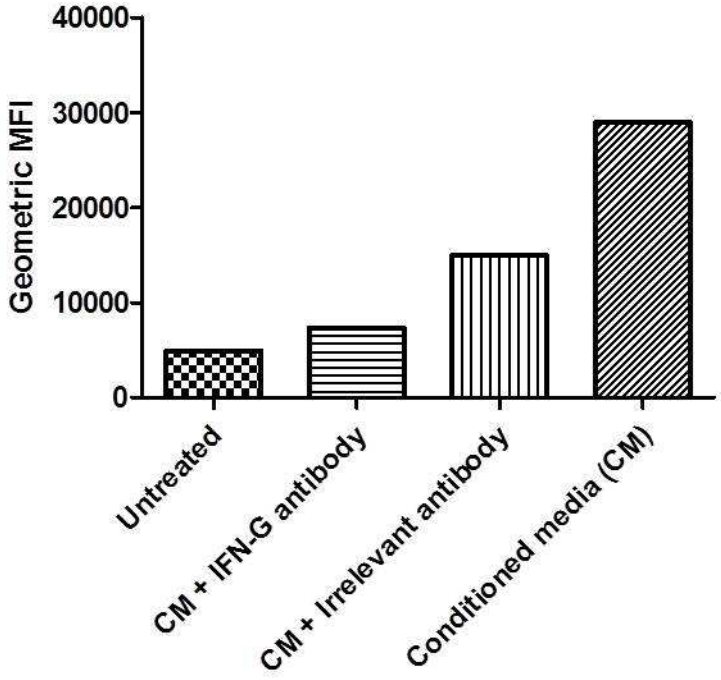

Figure 2.8 IFN-γ neutralization in supernatants from conA-stimulated PBMCs. Canine histiocytic sarcoma cells were cultured overnight in regular media, conditioned supernatants from conA-stimulated PBMCs, and conditioned supernatants with an IFN-γ neutralizing antibody or its corresponding isotype antibody. They were then harvested and stained for PD-L1 upregulation by flow cytometric analysis.

Regulation of PD-L1 by innate immune stimuli. We also tested the effects of TLR activation on tumor expression of PD-L1. The canine tumor cell lines were treated with ligands for TLR3 (pIC), TLR9 (pDNA), TLR4 (LPS), and TLR7/8 (R848) for 24 hours and their PD-L1 expression level was assessed by flow cytometry. As shown in **Figure 2.9**, we found that treatment with pIC consistently upregulated PD-L1 on all 4 cell lines screened (p-value = 0.048). However, only histiocytic sarcoma was responsive to all 4 TLR ligands with respect to upregulation of PD-L1 expression (p-value < 0.001).

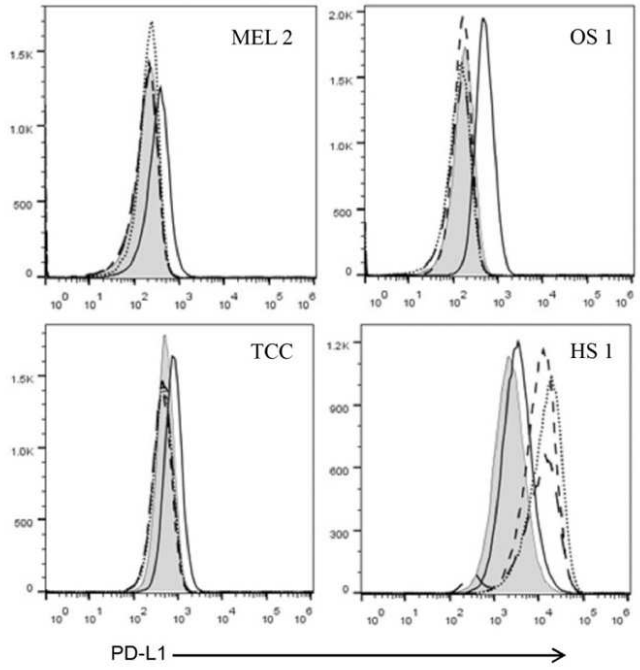

Figure 2.9 PD-L1 expression response to tumor cell activation with TLR ligands. Canine melanoma, osteosarcoma, transitional cell carcinoma, and histiocytic sarcoma cells were treated with media only (gray filled line), pDNA (long dashed line), LPS (dotted line), poly(I:C) (bold line), or R848 (dashed line) overnight before analysis for PD-L1 expression by flow cytometry. This experiment was repeated 6 times.

PD-L1 expression by monocyte-derived macrophages. Studies in rodents and humans have found that PD-L1 is also expressed by myeloid cells, especially macrophages, in addition to tumor cells[56,135]. Therefore, canine monocyte-derived macrophages were evaluated for PD-L1

expression after overnight incubation in media to select for adherent cells **(Figure 2.10A)** and after 7-day culture in human M-CSF **(Figure 2.10B)**. Macrophages were found to express low to negative levels of PD-L1 after overnight culture. Moreover, the macrophages did not express PD-L1 after 1 week in culture with human M-CSF. However, the macrophages did respond to IFN-γ stimulation, as their PD-L1 expression increased significantly (P value = 0.001) after overnight treatment with IFN-γ.

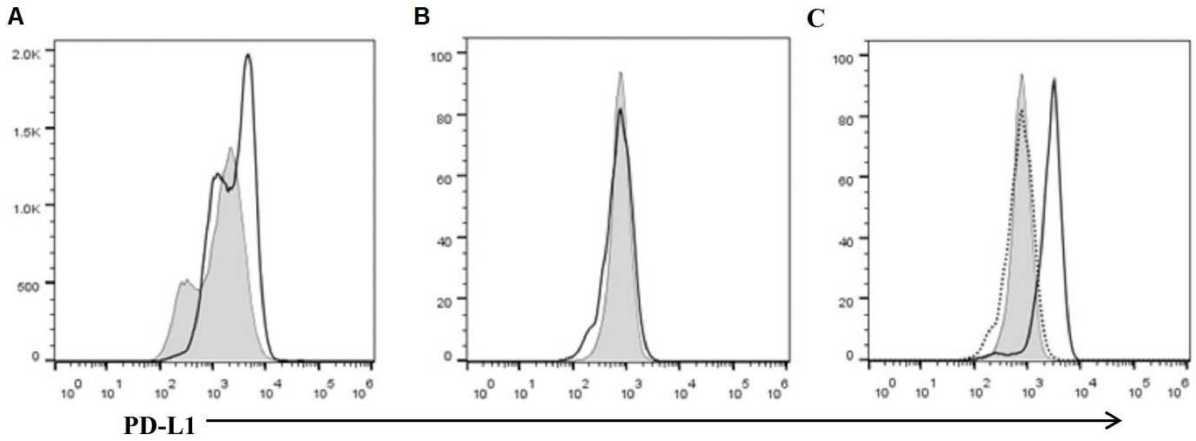

Figure 2.10 PD-L1 expression by canine monocyte-derived macrophages. Primary canine monocytes were cultured as described in Methods, then detached and immunostained with PD-L1 antibody for analysis by flow cytometry for PD-L1 expression. **(A)** Primary Canine monocytes plated overnight. **(B)** Macrophage enrichment with huM-CSF. Primary canine monocytes were cultured in huM-CSF for one week to enrich for macrophages (Isotype is light gray filled and PD-L1 is bold). **(C)** Monocyte-derived macrophages stimulated with IFN-γ. Cultured cells from (B) were treated overnight with rcIFN-γ (Isotype is light gray filled, baseline PD-L1 is dotted, and IFN-γ treated is bold). These data are representative of staining from 3 different dogs.

PD-L1 expression on primary tumors. Expression of PD-L1 by a canine histiocytic sarcoma tumor biopsy was assessed to compare the staining intensity to *in vitro* histiocytic sarcoma cell lines. These tumor biopsy studies are being extended by a larger study currently in progress, where we are currently assessing PD-L1 expression by a panel of canine tumor biopsies (Faulhaber, *et al*., manuscript in preparation). Fresh frozen tumor samples were immunostained

for PD-L1 expression. PD-L1 was found to be highly expressed in the histiocytic sarcoma tumor tissues, with expression located on both the cell membrane and within the cytoplasm **(Figure 2.11)**.

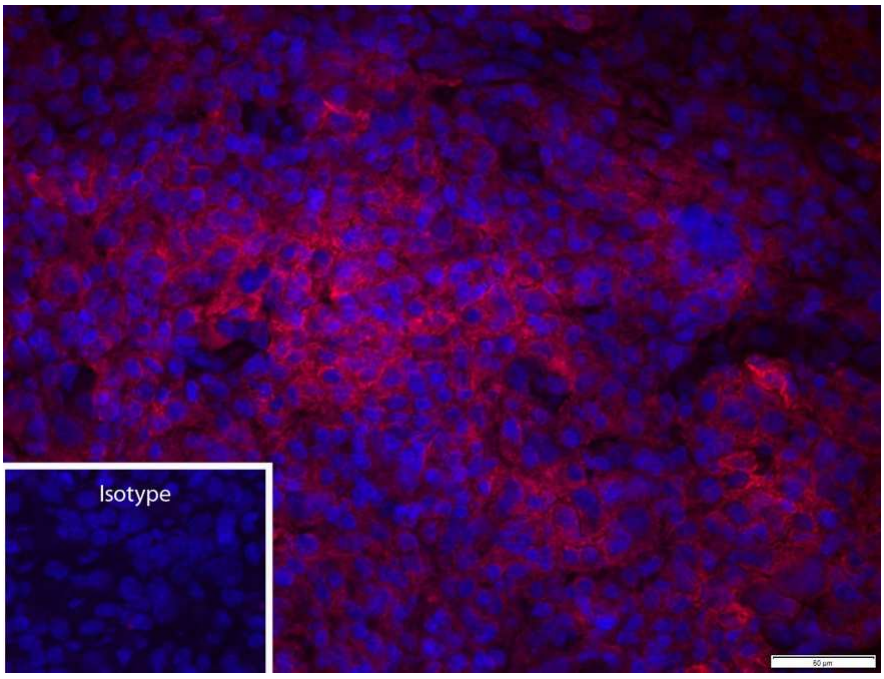

Figure 2.11 PD-L1 expression by canine histiocytic sarcoma tissue biopsy. Tumor biopsy was obtained from a dog with malignant histiosarcoma and imbedded in OCT and cryosectioned. Tumor tissues were immunostained as described in Methods and imaged by confocal microscopy. Intense expression of PD-L1 (red) was observed throughout the tumor section. Cell nuclei were stained with DAPI (blue).

Discussion.

In this study we observed that all the canine tumor cell lines evaluated expressed detectable levels of PD-L1 under basal (ie, unstimulated) conditions. Moreover, all the canine tumor cell lines were highly responsive to treatment with IFN-γ with the histiocytic sarcoma lines being the most responsive. In addition, the histiocytic sarcoma cell lines were also the most responsive to stimulation with a variety of TLR agonists. The increased expression of PD-L1 and increased overall sensitivity to activation with immune stimuli may be related to the origin of the

histiocytic sarcoma cell lines from cells of the macrophage/dendritic cell lineage, which are normally very sensitive to activation by TLR ligands[136,137].

IFN-γ is recognized as the principle cytokine that upregulates PD-L1 expression on many tumor types in human and mouse models[62,64]. Recently, it was reported that IFN-γ also upregulates PD-L1 expression on certain canine tumor lines when used at a high concentration[51].We observed that all of the canine tumor cell lines evaluated upregulated PD-L1 expression in response to IFN-γ in a dose-dependent manner. Moreover, we also found that cytokine-rich supernatants from activated canine T cells also induced a significant increase in PD-L1 expression over that induced by treatment with IFN-γ alone. This finding suggests the presence of other immune-modulating cytokines may further upregulate PD-L1 expression by canine tumor cells, or cytokines that may increase the activity of IFN-γ in inducing PD-L1 upregulation. It has been reported that IL-4 and TNF-α together can synergistically induce PD-L1 expression by human renal cell carcinoma cells[65]. Thus, IL-4 and TNF-α from T cells may potentially be active in upregulating PD-L1 expression on canine tumor cells.

Human tumor cells have been found to express various TLRs and to upregulate PD-L1 following activation by ligands for TLR2, TLR3 and TLR4[66,68]. We found that the TLR3 agonist poly(I:C) induced upregulation of PD-L1 on melanoma, osteosarcoma, transitional cell carcinoma, and histiocytic sarcoma cells, while all 4 TLR agonists evaluated (ie, agonists for TLR3, TLR4, TLR7/8, and TLR9) triggered upregulated PD-L1 expression on canine histiocytic sarcoma cells. These data suggest that the innate immune responses may also play a role in regulating canine tumor PD-L1 expression.

For the cell lines evaluated in this study, PD-L1 expression was observed be located on both the cell membrane and within the cytoplasm. This is somewhat different than the location of

PD-L1 expression on human tumor cells, which has been reported to be expressed solely on the plasma membrane of some tumor cells and in the cytoplasmic compartment of others, even within the same tumor sample[138]. However, other studies have demonstrated both cytoplasmic and surface staining in the same cells[139,140]. Our findings also add additional tumor cell lines and mechanistic insights to a previous study that evaluated the presence or absence of PD-L1 expression on canine tumor cell lines[51].

Tumor-associated macrophages (TAM) comprise a large population of tumor stromal cells[135]. We did not observe constitutive PD-L1 expression on early cultures of canine macrophages, which is in contrast to findings from a prior study in mice[30]. However, we did find that treatment with IFN-γ significantly upregulated PD-L1 expression by cultured canine macrophages, which is in agreement with the previous rodent study. It should also be noted that the source of macrophages was different in our study (blood) versus the rodent studies (peritoneal cavity), which may also account for the differences in constitutive PD-L1 expression. IFN-γ is widely accepted to be a pro-inflammatory cytokine. However, it has been shown that it also plays a role in inducing the production of anti-inflammatory cytokines such as IL-1Ra and IL-18BP[141]. These counter-regulatory, immune-suppressive activities allow IFN-γ to protect the host from tissue damage caused by uncontrolled inflammation. Based on these data, the induction of PD-L1 expression may be another way by which IFN-γ acts to control the duration and intensity of host inflammatory responses.

Upregulated expression of PD-L1 by tumor cells is an example of a phenomenon known as adaptive immune resistance[3]. PD-L1 expression by tumor cells may therefore be useful as a marker of the level of adaptive immune resistance exhibited by tumors[48,142], as well as a predictor of the effectiveness of PD-1/PD-L1 blockade for cancer immunotherapy[55,80]. It has

recently been shown that the use of PD-L1 blocking antibodies enhances cytokine secretion by peripheral blood mononuclear cells and tumor-infiltrating cells *in vitro* in dogs[51], suggesting that PD-L1 blockade may be an effectiveness means of immunotherapy for certain canine tumors. Studies are currently underway in our laboratory assessing the patterns of PD-L1 expression by canine tumor cells in vivo (using tumor biopsies) to determine the degree of PD-L1 expression heterogeneity and tumor-type association (Faulhaber E, manuscript in preparation). The tremendous potential for checkpoint molecule blockade, based on the results of recent human clinical trials[97,143], suggests that such an approach also has considerable merit for treatment of canine cancer.

CHAPTER 3

Checkpoint Molecule Expression by B and T cell Lymphomas in Dogs

Summary.

Immunotherapies targeting checkpoint molecule programmed death receptor-1 (PD-1) protein were shown to be effective for treatment of Hodgkin lymphoma in people, but little is known about the expression of PD-1 or its ligand PD-L1 by canine lymphoma. Therefore, flow cytometry was used to analyze expression of PD-1 and PD-L1 in canine lymphoma, using fine-needle aspirates of lymph nodes from 34 dogs with B cell lymphoma (BCL), 6 dogs with T cell lymphoma (TCL), and 11 dogs that had relapsed. Furthermore, fine-needle aspirates were obtained from 17 healthy dogs for comparison. Lastly, the impact of chemotherapy resistance on expression of PD-1 and PD-L1 was assessed *in vitro*. These studies revealed increased expression of PD-L1 by malignant B cells compared to normal B cells. In the case of TCL, tumor cells and normal T cells both showed low to negative expression of PD-1 and PD-L1. In addition, tumor infiltrating lymphocytes from both BCL and TCL had increased expression of both PD-1 and PD-L1 expression compared to B and T cells from lymph nodes of healthy animals. In vitro, chemotherapy-resistant BCL and TCL cell lines exhibited increases in both PD-1 and PD-L1 expression, compared to non-chemotherapy selected tumor cells. These findings indicate that canine lymphomas exhibit upregulated checkpoint molecule expression, though the impact of checkpoint molecule expression on tumor biological behavior remains unclear.

Background.

Lymphoma is one of the most common malignancies in dogs, accounting for up to 24% of all diagnosed canine neoplasms[144]. Furthermore, evidence suggests that the incidence of naturally-occurring lymphomas in dogs is increasing[145]. The most widely used cytotoxic chemotherapy protocols to date generate remission times of 1-1.5 years, but virtually all dogs eventually relapse and require treatment with rescue cytotoxic chemotherapy protocols[146-148]. Thus, an immunotherapy that could be combined with chemotherapy, or administered after remission induced by chemotherapy, could have a significant impact on survival in dogs with lymphoma. There have been several trials evaluating cancer vaccines in dogs with lymphoma, but to date improvement in disease-free interval or overall survival time has not been observed[149-151].

The goal of our study was to gain a better understanding of the biology of two key checkpoint molecules (PD-1 and PD-L1) in dogs with lymphoma, and determine how chemotherapy resistance may affect expression levels. To address these questions, we obtained lymph node aspirates from 17 healthy dogs and from 51 dogs with lymphoma. Samples were evaluated by flow cytometry to compare PD-1 and PD-L1 expression by malignant and healthy B and T cells. In addition, the effects of chemotherapy resistance on expression of checkpoint molecules by canine lymphoma cells was assessed. Our hypothesis was that expression of both PD-1 and PD-L1 would be increased on malignant B and T cells compared to healthy B and T cells, and that chemotherapy resistance would lead to further increases in expression of these checkpoint molecules. The findings reported here indicate overall upregulation of checkpoint molecule expression by B cell lymphomas and by tumor-infiltrating lymphocytes in dogs, and suggest a connection with chemotherapy resistance and increased PD-1 and PD-L1 expression.

Materials and Methods.

Clinical samples and cell lines. Fine needle aspirates (FNAs) were obtained from the lymph nodes of 17 healthy dogs and from lymph nodes of 34 dogs with untreated B cell lymphoma (BCL), 6 dogs with untreated T cell lymphoma (TCL), 9 dogs with relapsed BCL, and 2 dogs with relapsed TCL. Control lymph node FNAs were obtained from dogs owned by hospital staff, and the animals were deemed healthy based on a normal physical exam and normal blood work conducted in the last year. Tissue sampling procedures were kept consistent for all samples and were obtained by the same hospital personnel. All applicable international, national, and institutional guidelines for the care and use of animals were followed. Ages for healthy dogs ranged from 1 to 12 years and for lymphoma dogs ranged from 3 to 14 years (the mean age difference between the two groups was not statistically significant, as shown in **Figure 3.1**).

Validated canine lymphoma cell lines CLBL1 (BCL[152]) and Oswald (TCL[153]) were maintained in MEM medium (Gibco, Grand Island, NY) supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO) and 5% CTM (10,000 ug/mL Pen/Strep, 200 mM L-glutamine, 10 mM essential amino acids without L-glutamine, 10 mM non-essential amino acids, and 7.5% bicarbonate solution (all from Gibco)[154].

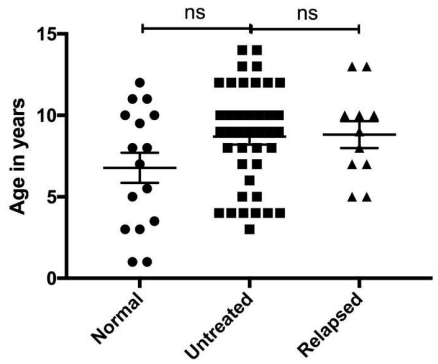

Figure 3.1. Fine needle aspirates were obtained from the lymph nodes of 17 healthy dogs, 41 untreated lymphoma patients, and 10 relapsed lymphoma patients. Ages of the dogs were

graphed and data are presented as mean ± SEM of the dog ages. Statistical analysis was performed using one-tailed ANOVA.

Antibodies. Murine anti-canine PD-1 and PD-L1 monoclonal antibodies, described recently[63,132], were used for flow cytometry and immunofluorescence staining. An isotype matched, irrelevant antibody was used at the same concentrations for each study (eBioscience, San Diego, CA). Mouse mAbs were detected using a donkey anti-mouse secondary, conjugated to Alexa Fluor 488 (Jackson ImmunoResearch, West Grove, PA). Mouse anti-canine CD21 (clone CA2.1D6), rat anti-canine CD5 (clone YKIX322.3), rat anti-canine CD8 (clone YCATE55.9), and rat anti-canine CD4 (clone YKIX302.9) were obtained from Bio-Rad Laboratories (Hercules, CA) and used to identify lymphocyte populations.

Flow cytometry. Fine needle aspirate samples for immunostaining were incubated with ammonium-chloride-potassium (ACK) lysis buffer (0.5% Phenol Red solution, 8% NH_4Cl, 1% $KHCO_3$, 0.037% Na_2EDTA in distilled water) to lyse red blood cells and next washed with fluorescence-activated cell sorting (FACS) buffer (1% BSA in PBS with 0.05% sodium azide) before single cells in suspension were added to a 96 well round bottom plate (Falcon, Durham, NC) for immunostaining. Lymphoma cell lines were harvested and washed with PBS prior to plating and immunostaining. Addition of 10% normal dog serum (Jackson ImmunoResearch) was used with primary antibodies to minimize non-specific binding, and the samples were immunostained with antibodies diluted in FACS buffer. To exclude dead cells from analysis, 7-AAD viability dye (eBioscience) was added prior to analysis, and 7-AAD^+ cells were not analyzed. Cells were analyzed for fluorescence expression using a Beckman Coulter Gallios flow cytometer (Brea, CA). Data were analyzed using FlowJo Software (Ashland, OR).

Generation of chemotherapy-resistant canine cell lines. To create chemotherapy-resistant cell lines, we used a combination of doxorubicin, vincristine, and dexamethasone (all from Sigma-Aldrich, St. Louis, MO) at 4 times the IC50 for each drug, as determined by *in vitro* assays (data not shown, 4 times the IC50s are as follows: CLBL1 cells: 845 ng/ml Dexamethasone, 3 ng/ml vincristine, and 80 ug/ml dexamethasone and Oswald cells: 30 ng/ml doxorubicin, 125 ng/ml vincristine, and 80 ug/ml dexamethasone). The cells were passaged and treated with a fresh dose of chemo-selection drugs every 3 days for 2 months. Cell death was measured by flow cytometric staining for 7-AAD and Annexin V (eBiosciences, San Diego, CA), and a minimum of 80% cell death was achieved after the first chemo-selection treatment **(Figure 3.2)**. The chemo-selected cells were maintained in drugs until the resistant population expanded, at which point they were used for flow cytometric analysis of checkpoint molecule expression.

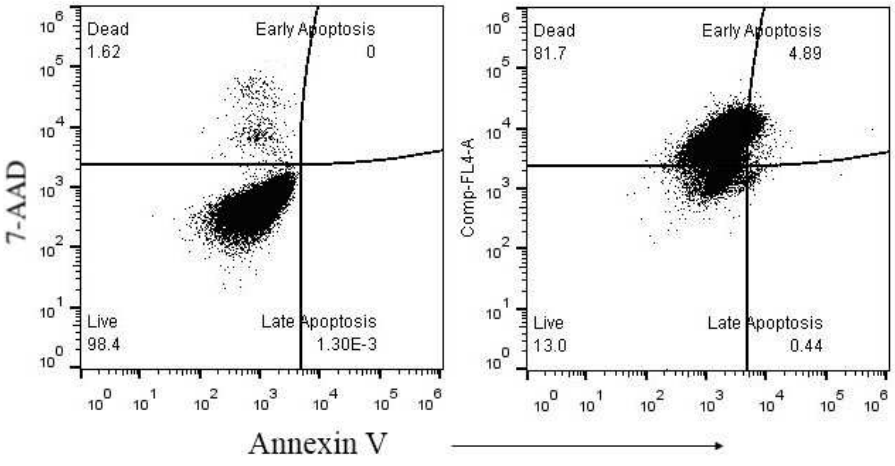

Figure 3.2. Untreated (left) and chemo-resistant canine lymphoma cell lines (right) were stained for 7-AAD and Annexin V to measure cell death after 1 treatment of chemotherapy (Doxorubicin, Vincristine, and Dexamethasone at 4 times the IC50 for each drug). Shown is a representative flow cytometry plot of CLBL1 cells.

Statistical analysis. Statistical comparisons between those data sets with two sample groups were done using non-parametric *t*-tests (Mann-Whitney test). Comparisons between 3 or more groups

were done using ANOVA, followed by Tukey multiple means post-test. Analyses were done using Prism7 software (GraphPad, La Jolla, CA) and statistical significance was determined for $p < 0.05$.

Results.

PD-1 and PD-L1 expression by normal and malignant lymphocytes. Using flow cytometry, malignant canine lymphocytes in blood and lymph nodes by can be distinguished from normal lymphocytes, neutrophils, and monocytes by their larger size (high forward scatter) and medium granularity (medium side scatter)[155]. We therefore used these criteria for lymph node aspirate cells to distinguish malignant lymphocytes from tumor-infiltrating T cells and B cells. Live lymphocytes were identified by negative staining for 7-AAD and classified based on their expression of CD5 or CD21 prior to assessing the percentage of PD-1 and PD-L1 positive staining **(Figure 3.3A)**. CD5+ cells were further divided into $CD8^+$ and $CD4^+$ T cells, based on positive staining for these markers.

Comparison of PD-1 expression by malignant B cells in dogs with BCL and normal B cells from lymph nodes of healthy dogs showed no significant difference in expression, though malignant B cells from several dogs with BCL did express detectable PD-1 **(Figure 3.3B)**. In dogs with TCL, we observed that malignant T cells from 6 dogs with $CD4^+$ TCL were largely negative for expression of PD-1, whereas 50% of normal T cells expressed PD-1 **(Figure 3.3C)**.

PD-L1 expression by malignant lymphocytes was also evaluated in dogs with BCL and TCL **(Figure 3.4A)**. The percentage of malignant B cells that were PD-$L1^+$ (mean = 20%) was significantly higher than the percentage of normal B cells expressing PD-L1 (mean = 2%)

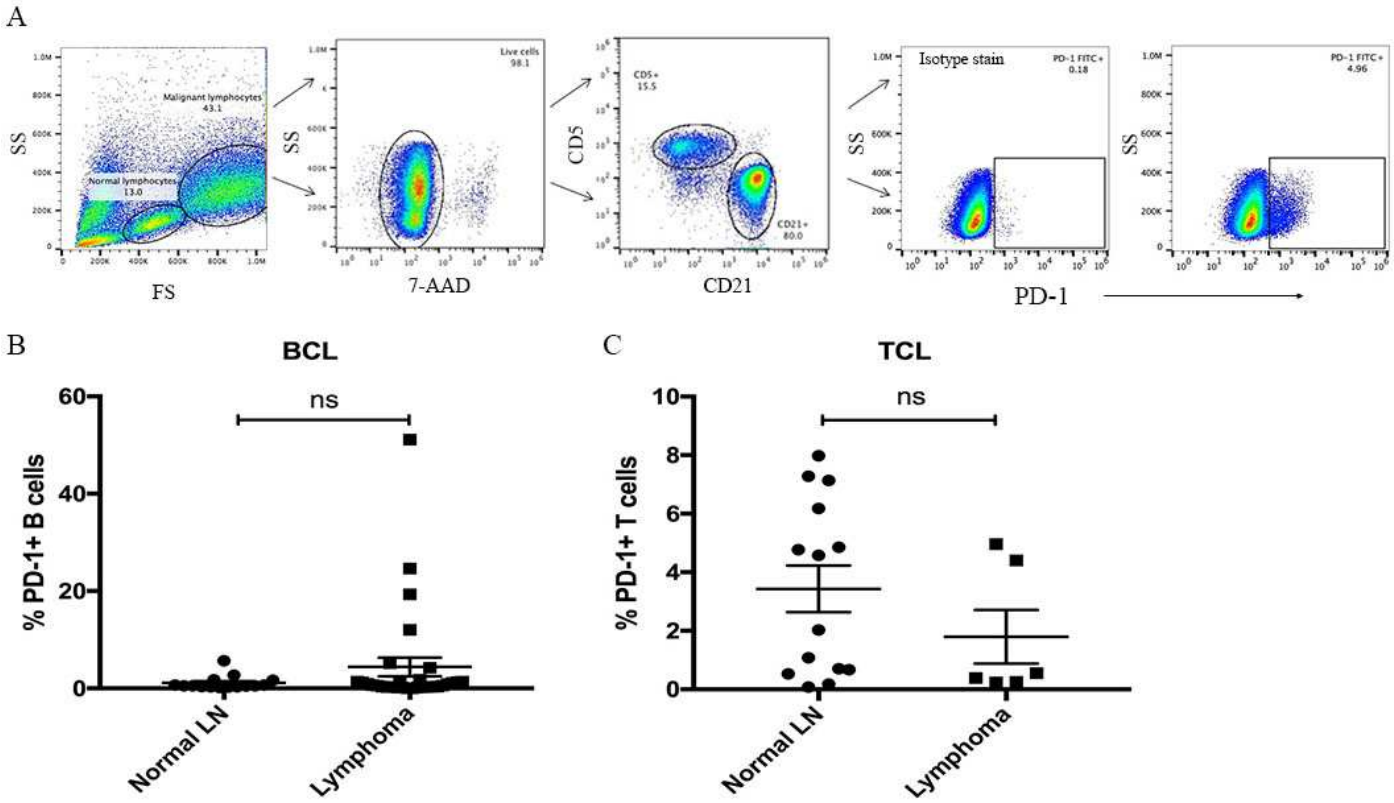

Figure 3.3 PD-1 expression by malignant B cells and T cells from dogs with BCL and TCL, respectively. Fine needle aspirates were obtained from lymph nodes of normal dogs and from lymph nodes of dogs with lymphoma (BCL or TCL) for flow cytometric analysis. The cells were stained for CD21 and CD5 to distinguish between B and T cells, and positive staining for PD-1 was determined with respect to cells stained with an irrelevant isotype antibody. The data were analyzed using FlowJo Software and the gating strategy is shown in **(A)** where PD-1 gating is based on isotype-stained malignant $CD5^+$ lymphocytes**.** Data shown represents **(B)** malignant $CD21^+$ B cells from dogs with BCL, while the plots in **(C)** represent malignant $CD5^+$ T cells from dogs with TCL, both compared to corresponding normal B and T cells from lymph nodes of healthy dogs. Data are presented as mean ± SEM of the percentage of marker expression. Statistical analysis was performed using non-parametric t-test.

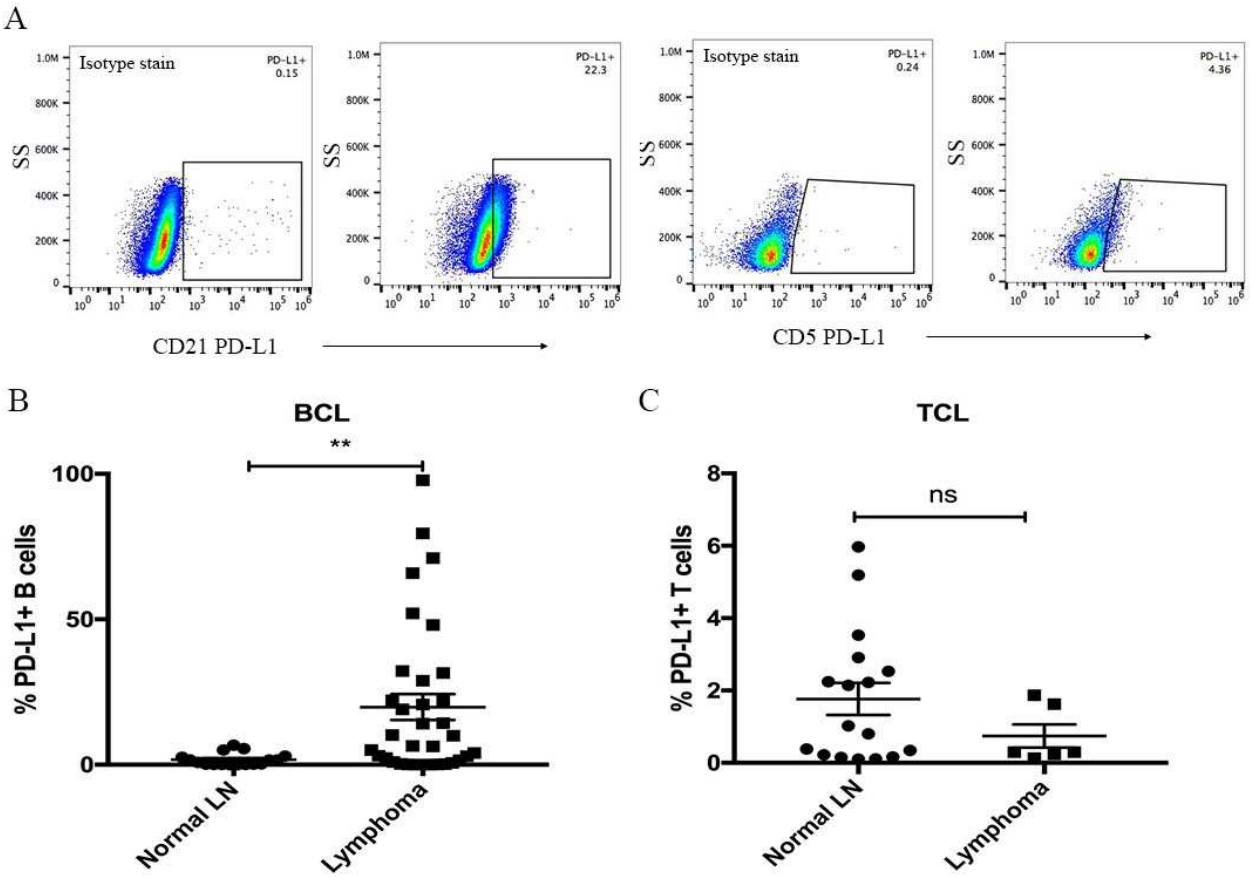

Figure 3.4 Expression of PD-L1 by malignant cells from dogs with BCL and TCL.
Fine needle aspirates were obtained from lymph nodes of normal dogs and from dogs with BCL or TCL for flow cytometric analysis. The cells were stained for CD21 and CD5 to identify B and T cells as shown in Figure 1, and positive staining for PD-L1 was determined with respect to cells stained with an irrelevant isotype antibody **(A)**. The data were analyzed using FlowJo Software and data shown is **(B)** $CD21^+$ tumor cells from lymph nodes of dogs with BCL and **(C)** $CD5^+$ tumor cells from dogs with TCL compared to corresponding B and T cells from lymph nodes of normal dogs. Data are presented as mean ± SEM of the percentage of marker expression. Statistical analysis was performed using non-parametric t-test and statistically significant differences were denoted as ** = $p < 0.005$.

(Figure 3.4B). In contrast, in animals with TCL, PD-L1 expression by both malignant $CD5^+$ T cells and normal $CD5^+$ T cells was either negative or very low, and the levels of expression were not statistically different between malignant and healthy cells **(Figure 3.4C)**. These analyses revealed therefore that malignant B-lymphocytes primarily upregulated expression of PD-L1, and not PD-1.

PD-1 and PD-L1 expression by tumor-infiltrating lymphocytes. Checkpoint molecule expression by tumor-infiltrating lymphocytes (T cells from BCL and B cells from TCL) was evaluated next, and levels of expression were compared to those on T cells and B cells in normal lymph nodes. Comparison of PD-1 expression by $CD4^+$ and $CD8^+$ T cells in lymph nodes of 17 healthy dogs with 34 dogs with treatment-naïve BCL revealed significantly upregulated expression of PD-1 by T cells infiltrating lymph nodes in animals with BCL, compared to expression by PD-1 by T cells in normal lymph nodes **(Figures 3.5A and 3.5B)**. In addition, we found that $CD5^+$ T cells infiltrating BCL lymph nodes also expressed PD-L1, whereas T cells from healthy lymph nodes were PD-L1 negative **(Figure 3.5C)**. This finding has been reported in human tumors, but to our knowledge, this is the first time this has been shown in dogs[156,157].We also found increased PD-1 and PD-L1 expression by B cells infiltrating lymph nodes in animals with TCL, compared to expression by normal B cells **(Figure 3.5D and 3.5E)**. No correlation was found between tumor cell PD-L1 expression and tumor-infiltrating lymphocyte PD-L1 expression (data not shown).

Effect of chemotherapy on PD-1 and PD-L1 expression by malignant lymphoma. Previous studies have suggested that chemotherapy may select for cells with upregulated checkpoint molecule expression, or may actually induce upregulated expression directly[158,159]. To address

whether the same thing occurs in canine lymphoma cells, CLBL1 (canine BCL) and Oswald (canine TCL) cells were treated with high-dose chemotherapy to generate chemotherapy resistant cell lines. We found that chemotherapy resistant cells (both CLBL1 and Oswald) exhibited significantly upregulated expression of both PD-1 **(Figure 3.6A and 3.6B)** and PD-L1 **(Figure 3.6C and 3.6D)** compared to non-selected tumor cells. These findings indicated that

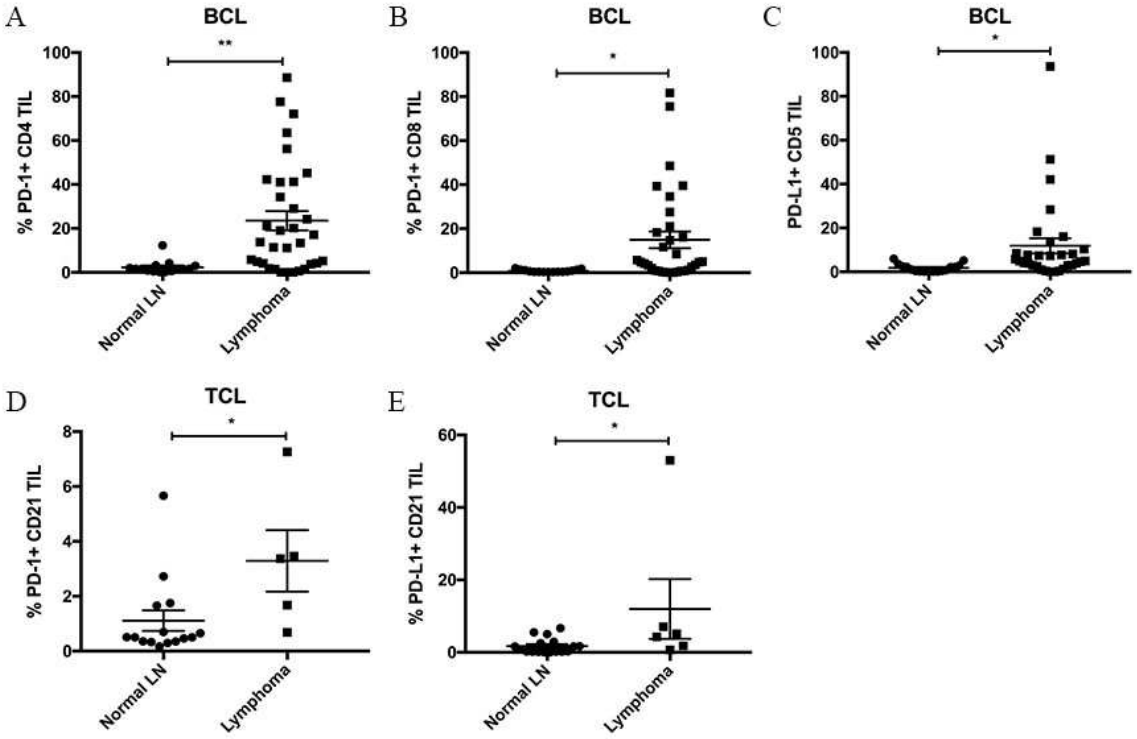

Figure 3.5 PD-1 and PD-L1 expression by tumor-infiltrating lymphocytes in dogs with BCL and TCL. Flow cytometric analysis of tumor-infiltrating lymphocyte expression of PD-1 and PD-L1 was conducted using the same fine needle aspirates obtained from lymph nodes of normal dogs and from dogs with BCL and TCL used in the prior studies. The cells were stained for CD21 and CD5 to distinguish between B and T cells, and T cells were further identified by staining for CD4 and CD8. Positive staining for PD-1 and PD-L1 was determined with respect to cells stained with an irrelevant isotype antibody and the data were analyzed using FlowJo Software. Data shown is **(A)** PD-1 expression by $CD4^+$ T cells, **(B)** PD-1 expression by $CD8^+$ T cells and **(C)** PD-L1 expression by $CD5^+$ T cells all from dogs with BCL, compared to corresponding cells from lymph nodes of normal dogs. In **(D)** is depicted PD-1 expression by $CD21^+$ B cells and in **(E)** is expression of PD-L1 on $CD21^+$ B cells from dogs with TCL. Data are presented as mean ± SEM of the percentage of marker expression. Statistical analysis was performed using non-parametric t-test and statistically significant differences were denoted as * = $p < 0.05$ and ** = $p < 0.005$.

chemotherapy resistance is associated with upregulation of checkpoint molecule expression. However, these studies did not determine whether selection induced upregulated checkpoint molecule expression, or whether high-dose chemotherapy was able to select for and expand a small subpopulation of cells that already expressed high levels of PD-1 and PD-L1. Interestingly, the BCL cell line showed only a slight increase in PD-L1 expression whereas the TCL cells showed both a PD-L1^{+} and a PD-L1^{-} population. These differences point to possible variations in mechanisms for upregulation of PD-L1 expression by canine lymphoma cells, i.e. induction of PD-L1 expression (BCL) versus selection of a subpopulation (TCL).

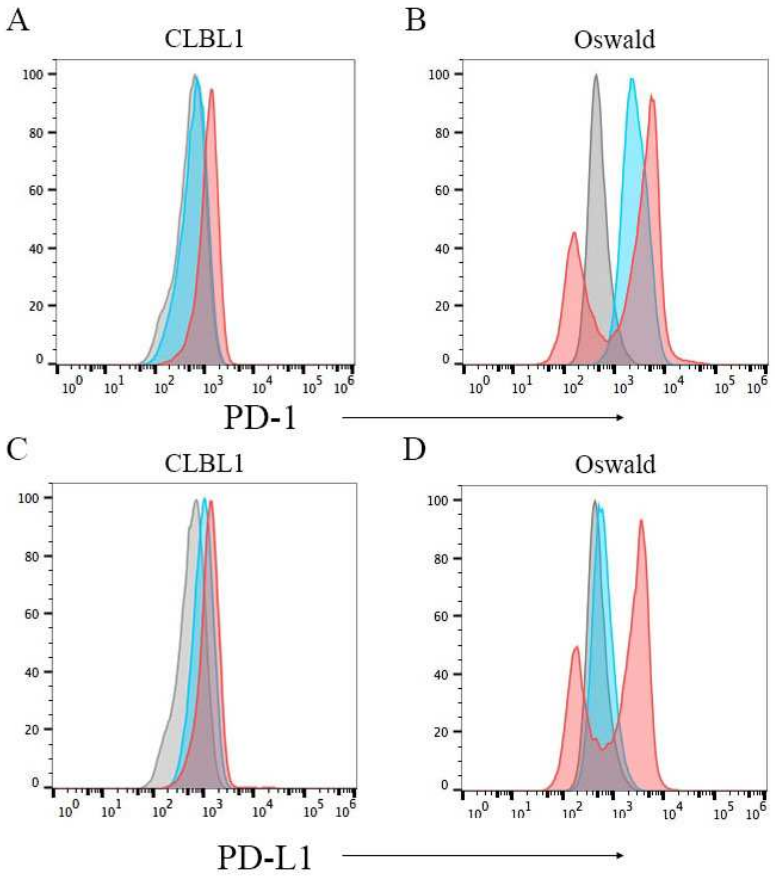

Figure 3.6 Effect of chemotherapy selection on PD-1 and PD-L1 expression by lymphoma cell lines. The canine B cell lymphoma cell line CLBL1 and the T cell lymphoma cell line Oswald were treated with 4-times the IC50 dose of doxorubicin, vincristine, and dexamethasone for 2 months to select for chemotherapy-resistant cells. Untreated and chemotherapy-resistant cells were immunostained for expression of PD-1 and PD-L1, and positive staining was

determined with respect to irrelevant isotype antibody staining. PD-1 geometric MFI is depicted as histograms for CLBL1 cells **(A)** and Oswald cells **(B)**, and histograms for PD-L1 are shown for CLBL1 cells in **(C)** and for Oswald cells in **(D)**. Gray = isotype stain, blue = untreated cells, and red = chemotherapy-resistant line. These experiments were conducted with 3 separately selected chemo-resistant cell lines, with similar results each time.

Comparison of PD-1 and PD-L1 expression by untreated versus relapsed lymphoma. The preceding *in vitro* studies indicated that malignant B cells and T cells exhibited higher levels of PD-1 and PD-L1 expression following exposure to cytotoxic chemotherapy. To determine whether a similar response occurred *in vivo*, PD-1 and PD-L1 expression by malignant B cells from dogs with treatment-naïve BCL was compared to expression by dogs with BCL following their first relapse after cytotoxic chemotherapy. Surprisingly, no difference in PD-1 or PD-L1 expression was observed, though the number of relapsed animals evaluated (eight dogs) was relatively low **(Figure 3.7A and 3.7B)**. Thus, at present it remains unclear to what degree chemotherapy exposure alters PD-1 and PD-L1 expression by lymphoma cells in dogs with BCL.

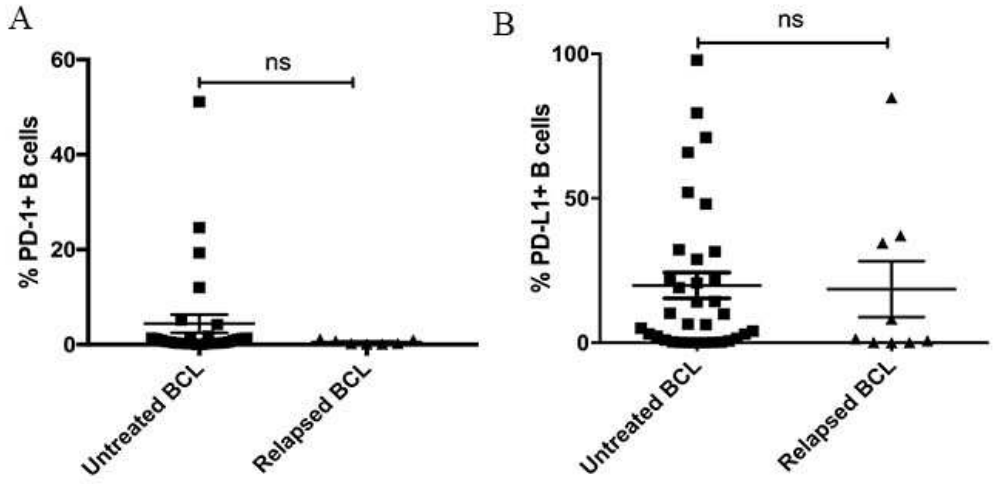

Figure 3.7 PD-1 and PD-L1 expression by tumor cells from dogs with relapsed BCL, compared to expression by tumor cells from untreated BCL. Fine needle aspirates were obtained from lymph nodes of untreated dogs and from dogs with relapsed BCL for flow cytometric analysis. The tumor cells were stained for CD21 and CD5 to distinguish between malignant B and T cells, and positive staining for PD-1 and PD-L1 was determined with respect to cells stained with an irrelevant isotype antibody. PD-1 expression by malignant $CD21^+$ B cells is shown in **(A)** and PD-L1 by malignant $CD21^+$ B cells is shown in **(B)**. The data were analyzed

using FlowJo Software and presented as mean ± SEM of the percentage of marker expression on lymphocytes. Statistical analysis was performed using two-tailed ANOVA, followed by Tukey's multiple means comparison.

Two dogs in this study (both with BCL) had pre-treatment and post-relapse samples available for comparison. In one animal, there was no difference in pre-and post-relapse PD-L1 expression by malignant B cells, whereas in the other dog, PD-L1 expression by malignant B cells was higher in samples obtained from the relapsed tumors (data not shown). We found that the dog with increased PD-L1 expression by malignant B cells post-relapse received standard CHOP induction therapy whereas the dog with no change in PD-L1 expression received doxorubicin induction chemotherapy. Thus, in these very preliminary studies, there was agreement with the in vitro finding of upregulated PD-L1 expression by CHOP chemotherapy. These results indicate that additional analysis of paired pre- and post-relapse samples, followed for longer periods of time, and treated with different chemotherapy protocols is warranted.

Discussion.

Treatment with PD-1 targeted antibodies (eg, Keytruda™ and Opdiva™) in human patients with diffuse large B-cell, follicular, and Hodgkin lymphoma has generated considerable enthusiasm based on overall response rates of up to 50%, with durable responses in up to 90% of responding patients[160-162]. Furthermore, gene expression profiles of several subtypes of canine and human B cell lymphomas have even been found to be similar, possibly with the same mutational drivers[163]. These findings have, not surprisingly, stimulated considerable interest in similar immunotherapeutic approaches for treatment of lymphoma in dogs.

Two previous reports have evaluated PD-1 and PD-L1 expression in dogs with lymphoma[52,53]. One report indicated that none of 5 canine BCL lymphomas biopsies evaluated revealed PD-L1 expression, as assessed by immunohistochemistry[52]. In contrast, a second report by Shosu et al. observed PD-L1 expression by all 15 canine BCL tumors evaluated by

immunohistochemistry[53]. An explanation for these very discordant results is not immediately apparent, though differences in methodology (eg, different PD-L1 antibodies were utilized) is one likely explanation. Certainly our results are much more in agreement with those of Shosu et al.[53], and suggest that PD-L1 expression is highly upregulated in all or nearly all dogs with BCL. Moreover, we recently found that PD-L1 expression could be detected on all of 14 canine tumor cell lines evaluated, and that PD-L1 expression was strongly upregulated by exposure to cytokines such as IFN-γ[63].

We and others have reported that PD-1 blocking antibodies can enhance activation of canine T cells in vitro[132,133]. We also found that PD-1 expression was significantly upregulated by T cells from dogs with cancer compared to healthy age-matched animals[132]. Therefore, there is strong rationale for concluding that PD-1 and PD-L1 targeted immunotherapy might be effective in treatment of canine lymphoma. Indeed, a recent report describes the first administration of fully canine PD-L1 antibody immunotherapy in dogs (though not in dogs with BCL), with some evidence of clinical activity[133].

In our studies, we did not observe increased PD-1 expression by malignant cells in dogs with BCL or TCL. However, expression of PD-L1 by malignant B cells was significantly increased relative to expression by normal B cells from healthy dog lymph nodes. In addition, expression of both PD-1 and PD-L1 was increased on tumor infiltrating T cells in dogs with BCL and on tumor infiltrating B cells in dogs with TCL. Studies of human cancers have found somewhat paradoxically that increased PD-L1 expression by tumor infiltrating immune cells, including T cells, correlates with better survival and more response to PD-L1 antibody therapy[99,157].

We also evaluated the impact of chemotherapy treatment on expression of the PD-1 and PD-L1 by canine lymphoma cells, as previous studies have reported upregulated expression in human cancers following chemotherapy[158,159]. We found that continuous exposure to a partial high dose CHOP combination (doxorubicin, vincristine, and dexamethasone) resulted in significantly higher expression of both PD-1 and PD-L1 by canine BCL and TCL cell lines, especially by a TCL cell line. When tumors from 9 dogs with relapsed BCL were examined, expression of PD-1 and PD-L1 by malignant B cells were not significantly higher than on malignant B cells from treatment-naïve tumors. However, our in vitro data suggests that malignant T cells from CHOP-relapsed TCL patients would show more significantly upregulated immune checkpoint molecules compared to untreated dogs. It is also likely that the *in vitro* models of chemotherapy selection we used our studies do not fully recapitulate the events regulating PD-L1 expression in vivo following chemotherapy and development of relapse. The variability in PD-L1 expression observed in treatment-naïve samples from animals with BCL suggests that clinical trials to evaluate PD-1 or PD-L1 targeted immunotherapy in dogs with lymphoma should include a pre-treatment evaluation of PD-L1 expression levels for use as inclusion or exclusion criteria.

In summary, PD-L1 expression was found to be significantly higher on malignant B cells in dogs with BCL, compared to normal B cells from lymph nodes. Numerous studies in humans and mice have found that increased PD-L1 expression by tumor cells correlates with a worse prognosis[38,39,46,164]. Of note, PD-L1 expression by non-Hodgkin lymphoma patients was reported to correlate with a worse prognosis[165]. Malignant T cells in animals with TCL did not, however, exhibit upregulated expression of either PD-1 or PD-L1. In tumor-infiltrating T cells in dogs with BCL, there was significant upregulation of PD-1 expression on both $CD4^+$ and $CD8^+$ T

cells, consistent with tumor-induced T cell exhaustion or immune suppression[36,166]. Upregulated expression of PD-1 was not observed on either malignant B cells or on malignant T cells. Interestingly, a population of dual-positive PD-1/PD-L1^{+} tumor infiltrating T cells was also observed in dogs with BCL, whereas similar dual-positive cells have not been observed in the peripheral blood of dogs with cancer[132], or in the lymph nodes of healthy dogs. The significance of these dual expressing T cells is currently not known, but may suggest strong immune activation with concurrent checkpoint molecule expression upregulation. Correlations between checkpoint molecule expression by BCL and TCL and overall treatment outcomes, as well as the effects of chemotherapy selection and relapse, continues to be areas of strong clinical interest. The eventual approval of a canine PD-1 antibody for clinical use will undoubtedly drive new interest in checkpoint molecule expression and the predictive utility of examining these pathways in lymphoma and other canine tumors.

CHAPTER 4

Regulation of PD-L1 Expression on Murine Tumor-associated Monocytes and Macrophages by Locally Produced TNF-α

Summary.

PD-L1 is an immune checkpoint protein that has emerged as a major signaling molecule involved with tumor escape from T cell immune responses. Studies have shown that intra-tumoral expression of PD-L1 can inhibit antitumor immune responses. However, it has recently been shown that expression of PD-L1 on myeloid cells from the tumor is a stronger indicator of prognosis than tumor cell PD-L1 expression. Therefore, it is important to understand the factors that govern the regulation of PD-L1 expression on tumor-infiltrating myeloid cells. We found that immature bone marrow monocytes in tumor-bearing mice had low levels of PD-L1 expression, while higher levels of expression were observed on monocytes in circulation. In contrast, macrophages found in tumor tissues expressed much higher levels of PD-L1 than circulating monocytes, implying upregulation by the tumor microenvironment. We demonstrated that tumor-conditioned media strongly induced increased PD-L1 expression by bone marrow-derived monocytes and TNF-α to be a cytokine that causes an upregulation of PD-L1 expression by the monocytes. Furthermore, we found production of TNF-α by the monocytes themselves to be a TLR2-dependent response to versican secreted by tumor cells. Thus, PD-L1 expression by tumor macrophages appears to be regulated in a different manner than by tumor cells themselves.

Background.

The tumor microenvironment (TME) plays a critical role in the ability of tumor cells to successfully establish themselves in tissues[167]. Several cell types comprise the TME stroma, including fibroblasts, endothelial cells, macrophages, neutrophils, and B and T cells[168]. Interactions between stromal cells and tumor cells support tumor growth and metastasis[169]. Macrophages are one of the key cellular constituents of the TME, and serve to suppress T cell immune responses, stimulate tumor angiogenesis, promote tumor metastasis, and generate additional genomic instability[170,171]. The recruitment of monocytes to tumors in response to tumor-derived chemokines, in particular CCL2, is associated with aggressive tumor growth and metastasis[172,173]. Immature monocytes are also precursors of tumor-associated macrophages (TAM), which have an immune-suppressive phenotype[174].

How TAM suppress tumor immunity is currently an area of intense research interest, and a number of investigations have focused on checkpoint molecules expressed by TAM as a means of negative regulation of T cell function[99,102]. Studies have reported that PD-L1-expressing macrophages were more abundant than PD-L1 expressing tumor cells in tumor biopsy specimens from patients with melanoma, renal cell carcinoma, non-small cell lung, head and neck carcinoma, and colorectal tumors[99,102]. One recent study reported that hypoxic conditions selectively upregulated PD-L1 expression by myeloid-derived suppressor cells and macrophages through HIF-1α[49]. However, little more is known about how PD-L1 expression by macrophages is regulated, and specifically how the tumor microenvironment influences PD-L1 expression by TAM.

In the present study, we addressed the questions of how PD-L1 expression changed over time during macrophage differentiation from monocytes to mature macrophages, and which

cytokines produced in the local tumor microenvironment were responsible for maintaining high levels of PD-L1 expression by TAM. Therefore, we utilized *in vitro* assays to identify cytokines that regulate myeloid cell PD-L1 expression as well as the source of these cytokines. We hypothesized that PD-L1 expression by macrophages is primarily regulated by IFN-γ, similar to the regulation of PD-L1 expression by tumor cells in humans, mice, and dogs[61-64]. We also used monocytes and macrophages from genetic knockout mice to identify a novel signaling loop that regulates myeloid cell PD-L1 expression in the tumor environment.

Materials & Methods.

Mice and tumor models. Wild-type (WT), IFN-$\gamma^{-/-}$ mice (strain B6.129S7-*Ifng*^{tm1Ts}/J), TLR2$^{-/-}$ mice (strain B6.129-Tlr2^{tm1Kir}/J), and TNFR$^{-/-}$ mice (strainB6.129S-Tnfrsf1a^{tm1Imx} Tnfrsf1b^{tm1Imx}/J) bred on the C57Bl/6 background were acquired from Jackson Laboratories (Sacramento, CA), as were BALB/c mice. C57Bl/6 were inoculated s.c in the flank with 1 x 10^6 B16.F10 melanoma cells in 100 ul PBS (Gibco, Grand Island, NY). BALB/c mice were inoculated similarly with 4T1 mammary carcinoma cells. According to the study IACUC protocols, the mice were humanely euthanized when the tumor diameters reached 10 mm or less. Tumor area was determined the day the mice were euthanized, with tumor area calculated as length x width in mm.

Murine cell lines. B16.F10 melanoma cells and 4T1 mammary carcinoma cells were obtained from ATCC (Manassas, VA). These cell lines were screened by PCR to ascertain that they were of murine origin[134].

Tissue preparation for flow cytometry. Mice were euthanized before spleens and tumor tissues were harvested. Tumor tissues were minced with fine scissors, then digested with collagenase (Sigma Aldrich, St. Louis, MO) for 30 minutes at 37°C and filtered to obtain a single cell suspension. Spleen cells were collected by forcing cells through 70 micron filters with cell culture medium. Tissue and blood samples were lysed with ammonium-chloride-potassium (ACK) lysis buffer (0.5% Phenol Red solution, 150mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM disodium salt dihydrate) and the remaining cells were washed with cell culture medium in preparation for flow cytometric staining.

Bone marrow collection and monocyte enrichment. Bone marrow was collected from tibias and femurs by forcing cells out of the marrow cavity, using tissue culture medium and a 25# needle. The bone marrow cells were then lysed with ACK lysis buffer and washed with medium before plating in 24-well polystyrene cell culture plates (Falcon, Durham, NC) for the selection of adherent monocytes. Adherent bone marrow-derived monocytes were then harvested by pipetting with ice-cold 2mM EDTA in PBS (Gibco).

Generation of conditioned medium. Tumor cells were cultured in the same cell culture medium described previously. To generate tumor-conditioned medium (CM), tumor cells were seeded into 24-well plates at 75,000 cells/ml and CM was collected 24 hours later. The CM was then centrifuged to remove any remaining cells.

Co-culture studies and conditioned medium effects on monocyte PD-L1 expression. Bone marrow-derived monocytes were selected by adherence as described above and washed with

PBS. Tumor cells at 50,000 cells/ml or tumor CM at 50% were added to cultures of bone marrow-derived monocytes. After 18 hours of culture, monocytes were harvested as described above and processed for flow cytometry. These culture conditions routinely yielded monocyte cultures that were at least 80-90% pure.

Flow cytometry. Cells prepared from tumor and liver tissues, as well as bone marrow-derived monocytes, blood monocytes, and TAM were obtained as described above and placed in FACS buffer for immunostaining. Nonspecific antibody binding was blocked by addition of normal mouse serum and 0.01% Fc-specific antiserum (CD16/32, clone 93, eBioscience, San Diego, CA).

Cells were immunostained with the following antibodies: CD45 Pacific Orange (Clone 30-F11) from Invitrogen (Grand Island, NY) to identify hematopoietic cells, and with PD-L1 PE (clone MIH5), CD11b Pacific blue (clone M1/70), F4-80 APC (clone Cl:A3-1), Ly6C biotin (AL-21), Ly6G Alexa 488 (clone 1A8), CCR2 APC (clone 475301), and CD11c FITC (clone N418). Cells were also stained with appropriately matched isotype antibodies to assure specificity of immunostaining. All antibodies were purchased from eBioscience unless otherwise noted.

Prior to analysis, 7-AAD viability dye (eBioscience) was added to flow samples to exclude dead cells. Cells were analyzed using a Beckman Coulter Gallios flow cytometer (Brea, CA) and FlowJo Software (Ashland, OR).

In other experiments, intracellular cytokine expression was quantitated. To measure intracellular expression of TNF-α, the cells were initially stained for surface markers before

fixation with paraformaldehyde (PFA), and permeabilization with 0.25% saponin. Cells were then washed and immunostained with an TNF-α PE antibody (clone MP6-XT22, eBioscience).

Cytokines for PD-L1 upregulation by monocytes and macrophages. The following recombinant murine cytokines were purchased from Peprotech (Rocky Hill, NJ): TNF-α, TGF-β, IL-10, MCP-1, IFN-γ, GM-CSF, and IL-3. Each cytokine was used at 10 ng/ml, according to the manufacturer's suggested working concentrations and based on titration studies done in our laboratory (data not shown). Bone marrow-derived monocytes were stimulated with these cytokines for 24 hours prior to analysis of PD-L1 expression by flow cytometry.

Neutralizing antibodies. A TNF-α neutralizing antibody (clone NF-7, Abcam, Cambridge, UK) was used at 5 ug/ml in tumor CM prior to treatment of monocytes with tumor CM. A rabbit polyclonal versican antibody (Santa Cruz, Santa Cruz, CA) was used at 200 ug/ml in tumor CM for 4 hours at 4C. This was followed by incubation of CM and anti-versican antibodies with protein A sepharose beads (Abcam) overnight according to manufacturer's protocol for immunoprecipitation of antibodies. The beads were removed from the CM by centrifugation prior to CM being added to monocyte cultures.

ELISA. Cell culture supernatants were collected and centrifuged for the removal of cells. Cytokines were measured using specific ELISA kits for murine IFN-γ and TNF-α (R&D Systems, Minneapolis, MN), and assays were performed according to manufacturer directions.

Tissue immunofluorescence staining and imaging. Tumor tissues were harvested and fixed in periodate-lysine-paraformaldehyde (PLP) for 24 hours before transferring to a 30% glucose

solution for another 24 hours, all at 4C. Afterwards, tissues were embedded in OCT (Optimal Cutting Temperature compound), frozen at -80C, and cryosectioned to a thickness of 5 microns. The tissues were mounted on glass slides and blocked with 5% donkey serum for 30 minutes prior to staining.

For analysis of PD-L1 expression, we used an unlabeled antibody (clone 10F.9G2, BioXcell, West Lebanon, NH). For analysis of intracellular TNF-α expression, a directly conjugated antibody (clone MP6-XT22) and a matched irrelevant isotype control antibody from eBioscience were used. For detection of macrophages, an unconjugated F4-80 antibody (clone BM8) was used.

Antibodies for PD-L1 and F4-80 and their matched isotype controls were used overnight at 4C, followed by a donkey anti-rat antibody (Jackson Immunoresearch, West Grove, PA) for 30 minute to detect primary antibody binding. For dual staining, antibodies for intracellular cytokines were diluted in 0.25% Saponin diluted in PBST overnight at 4C. Finally, the tissues were stained with DAPI to identify nucleated cells and coverslipped with Prolong Diamond mounting media (LifeTech, Carlsbad, CA) before imaging. Controls included immunostaining with appropriate concentrations of irrelevant isotype-matched antibodies. Figures were then compiled using Adobe Photoshop.

Statistical analysis. Statistical comparisons between those data sets with two treatment groups were done using non-parametric t-tests (Mann-Whitney test). Comparisons between 3 or more groups were done using ANOVA, followed by Dunnet's or Tukey multiple means post-test. Analyses were done using Prism6 software (GraphPad, La Jolla, CA). For all analyses, statistical significance was determined for $p < 0.05$.

Results.

Role of endogenous IFN-γ regulation of PD-L1 expression by monocytes and tumor macrophages. Our studies and those of others have found that IFN-γ can significantly upregulate PD-L1 expression by both tumor cells and macrophages[62,175]. Using bone marrow-derived macrophages and monocytes, we confirmed that exposure to IFN-γ resulted in significant upregulation of PD-L1 expression by monocytes, as well as by tumor cells (data not shown). Moreover, a previous investigation evaluated the role of endogenous cytokines in regulating PD-L1 expression by tumor cells and myeloid cells *in vivo,* and concluded that IFN-γ produced by inflammatory cells stimulated tumor cells to increase their PD-L1 expression[176]. However, this previous study did not conclusively assess the role of IFN-γ in regulating PD-L1 expression by tumor-associated macrophages.

Therefore, we used mice lacking expression of IFN-γ to address more fully the role of endogenous IFN-γ in regulating both tumor and TAM PD-L1 expression *in vivo.* B16.F10 tumors were grown in both WT and IFN-$\gamma^{-/-}$ mice (n = 5 per group), and tumor tissues were processed for flow cytometry for assessment of PD-L1 expression by tumor cells and TAM. We found that $CD45^-$ tumor cells in IFN-$\gamma^{-/-}$ mice expressed significantly less PD-L1 than tumor cells obtained from WT animals **(Figure 4.1A)**. However, macrophages in tumor tissues from WT and IFN-$\gamma^{-/-}$ mice expressed similar levels of PD-L1, based on both MFI and % positive analysis **(Figure 4.1B)**. While these data confirm previous studies with respect to the essential role for IFN-γ in regulating tumor cell PD-L1 expression, the new findings suggested that PD-L1 expression by TAM was regulated in an IFN-γ-independent fashion.

Effect of monocyte maturation into macrophages on PD-L1 expression. We next sought to determine the role of monocyte differentiation into tissue macrophages on regulation of PD-L1 expression. For example, it is possible that macrophages in tumor tissues express higher levels of PD-L1 simply as a result of maturation-related changes. Therefore, we used flow cytometry to examine the level of PD-L1 expression by immature monocytes in bone marrow, circulating monocytes, and macrophages in normal and tumor tissues.

We found that indeed, the level of PD-L1 expression by monocytes increased as the cells age, with the lowest levels of expression in the bone marrow and the highest levels of expression observed in spleen and tumor tissues **(Figure 4.1C and 4.1D)**. PD-L1 expression was measured on immature bone marrow monocytes ($CD11b^+/Ly6C^+/Ly6G^-$), circulating monocytes ($CD11b^+/Ly6C^+/Ly6G^-$), and either tissue macrophages from the spleen or TAM from melanoma-bearing mice ($CD11b^+/F4\text{-}80^+$). Importantly, in tumor tissues the more recently emigrated inflammatory monocytes (as assessed by higher levels of expression of CCR2[172,173]) expressed higher levels of PD-L1 than resident macrophages, suggesting that the newly arrived monocytes further upregulated their PD-L1 expression once they reached tumor tissues.

In vitro regulation of monocyte PD-L1 expression by tumor-conditioned medium. These findings suggested that factors produced locally in tumor tissues were responsible for upregulation of PD-L1 expression. To address this question, we developed a co-culture system in which immature bone marrow-derived monocytes were co-cultured with tumor cell conditioned medium (CM). We found that culture of monocytes with tumor CM resulted in rapid and significant upregulation of PD-L1 expression **(Figure 4.2A)**. For example, exposure to as little as 25% CM triggered significant PD-L1 upregulation, and that the effect occurred within 18h (data

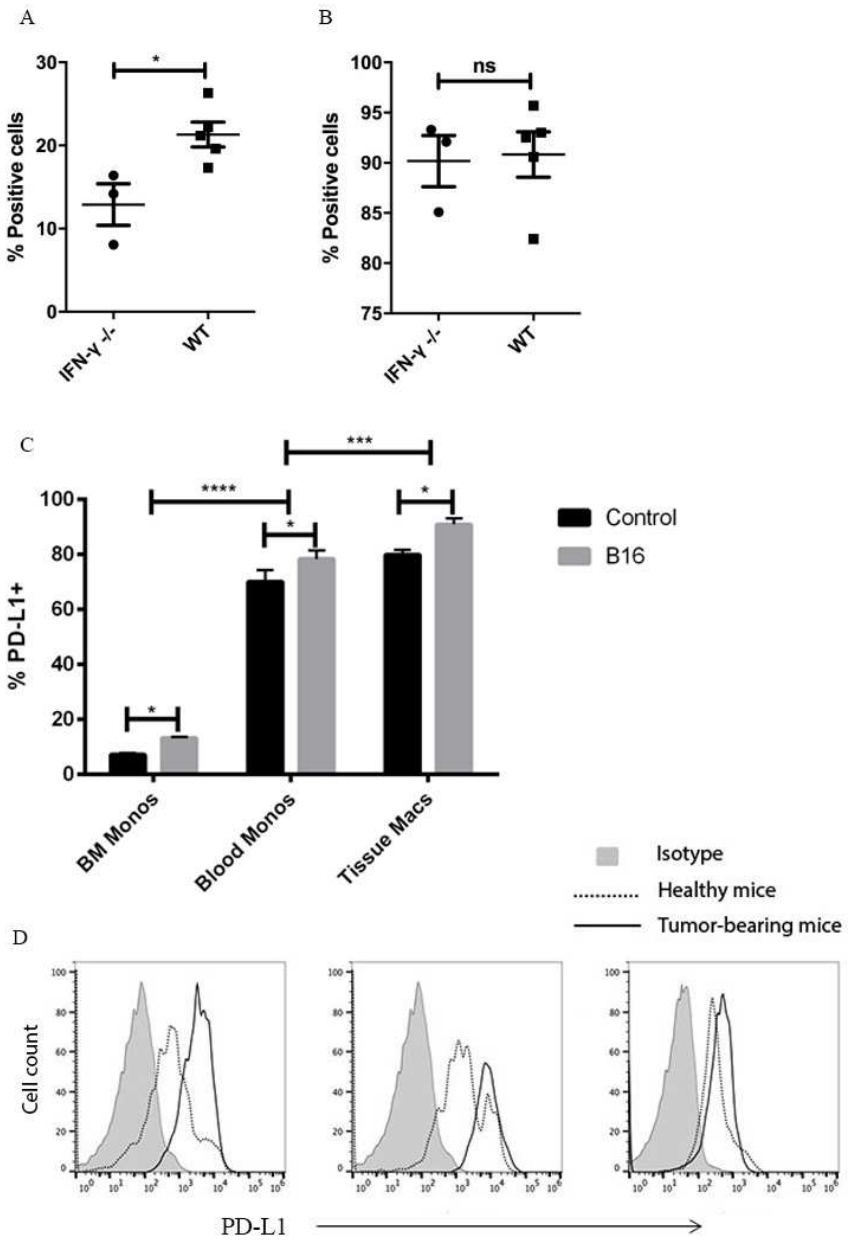

Figure 4.1 PD-L1 expression by tumor cells, monocytes, and macrophages *in vivo*. B16 tumors cells were established s.c. in WT and IFN-$\gamma^{-/-}$ mice (n = 3-5 animals per group), as noted in Methods. Single cell suspensions were prepared from excised tumor tissues and flow cytometry was used to compare PD-L1 expression by $CD45^-$ tumor cells in **(A)** and by tumor-associated macrophages ($CD45^+/CD11b^+/F4\text{-}80^+$) in **(B)** obtained from the two groups of mice. The mean percentage of $PD\text{-}L1^+$ cells present in tumor tissues from WT and IFN-$\gamma^{-/-}$ are depicted and the mean percentages were compared statistically using a non-parametric t-test. In **(C)**, bone marrow monocytes ($CD11b^+/Ly6C^+/Ly6G$-), circulating monocytes ($CD11b^+/Ly6C^+/Ly6G^-$), and tissue macrophages ($CD45^+$/ $CD11b^+$) were harvested from the spleens of healthy mice and from tumors of mice with established s.c. B16 tumors (n = 4-5 mice per group) and PD-L1 expression was quantitated by flow cytometry. The level of expression of PD-L1 on the cells is shown as histograms of geometric MFI in **(D)** where gray filled = isotype stain, dotted line = cells from

healthy mice, and solid line = cells from tumor-bearing mice from bone marrow, blood, and tissues. The mean percentages of PD-L1$^+$ cells in healthy mice and mice with tumors were compared statistically using a non-parametric t-test, and mean percentages of PD-L1$^+$ cells in different tissues from healthy and tumor-bearing mice were compared using two-tailed ANOVA, with Tukey post-test. Groups means with statistically significant differences were denoted as * = $p < 0.05$, *** = $p < 0.0005$, and **** = $p < 0.0001$. Similar results were obtained in 2 additional, independent experiments.

not shown). It was also observed that CM from certain tumor cell lines led to greater upregulation of PD-L1 expression by monocytes than CM from others **(Figure 4.2A)**.

Cytokine production in tumor and monocyte co-cultures. Several secreted cytokines or growth factors were considered candidates for the secreted PD-L1 upregulation, based on previous work[65,177-180], including GM-CSF, MCP-1, IL-10, IL-3, TGF-β, or TNF-α. To help identify the relevant factors, we first screened recombinant cytokines to identify those that most strongly upregulated PD-L1 expression in the bone marrow monocyte system **(Figure 4.2B)**. Using this screen, we identified IFN-γ and TNF-α as potential candidate cytokines **(Figure 4.2C)**. TNF-α is a strong regulator of monocyte PD-L1 expression[181], and CCR2$^+$ inflammatory monocytes are especially responsive to this cytokine **(Figure 4.3A)**[182,183].

To directly address the role of each cytokine, neutralization studies were done. Here we found that neutralization of GM-CSF and IL-10 did not result in significant reduction in PD-L1 upregulation in response to tumor CM (data not shown). However, when tumor CM was treated with TNF-α neutralizing antibody, the upregulation of PD-L1 was significantly abrogated **(Figure 4.3B)**. Furthermore, CCR2$^+$ monocytes showed significantly lower expression of PD-L1 in comparison to CCR2$^-$ monocytes, supporting the previous experiment using exogenous recombinant TNF-α in that CCR2$^+$ inflammatory monocytes were more sensitive to regulation

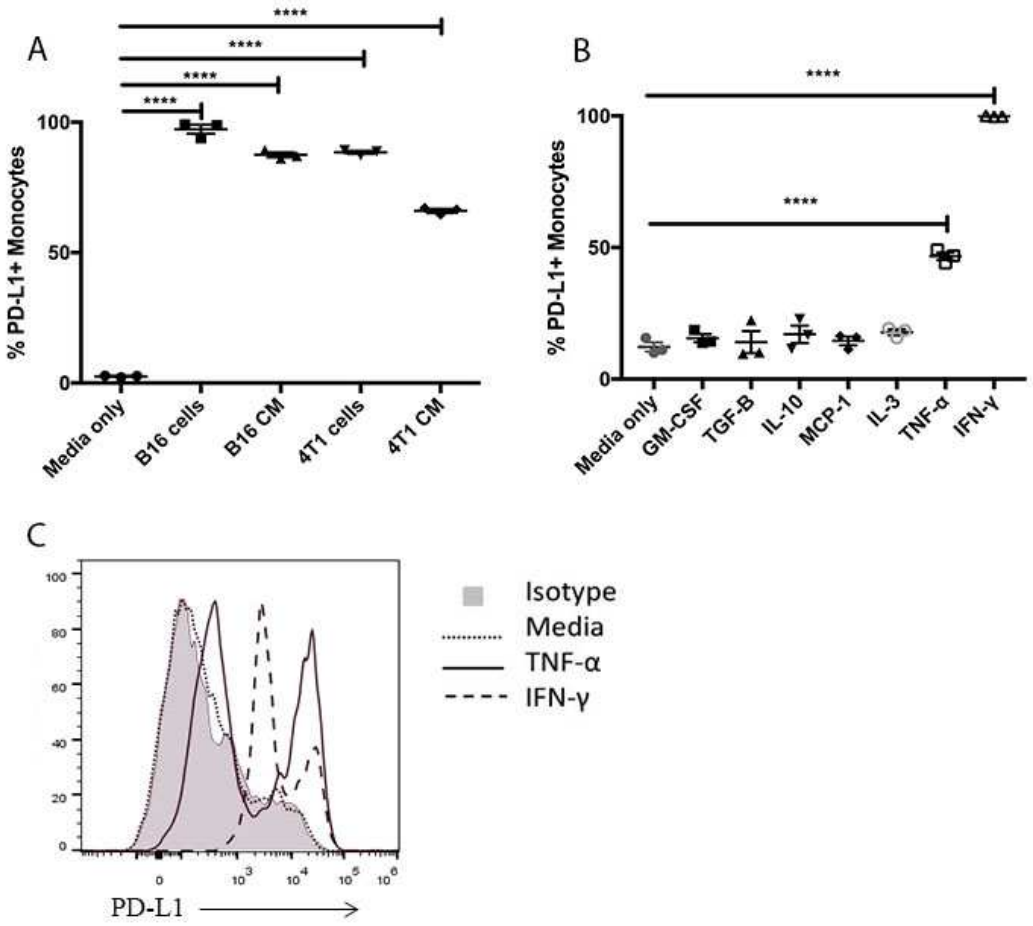

Figure 4.2 Effects of tumor cells, tumor CM, and cytokines on monocyte PD-L1 expression. (**A)** B16 and 4T1 tumor cells were grown as monolayers, then co-cultured with bone marrow-derived monocytes for 24h, as described in Methods. In other studies, bone marrow-derived monocytes were cultured with CM from tumor cells. The effects of co-culture with live tumor cells or with tumor CM on monocyte PD-L1 expression was determined by flow cytometry. **(B)** Bone marrow-derived monocytes were prepared as described in Methods and incubated with the following murine recombinant cytokines (TNF-α, TGF-β, IL-10, MCP-1, IFN-γ, GM-CSF, and IL-3) at a concentration of 10 ng/ml. After overnight culture, the monocytes were collected and immunostained for flow cytometric analysis of PD-L1 expression. **(C)** Histograms of monocyte PD-L1 expression following exposure to TNF-α or IFN-γ. The percentage of PD-L1$^+$ cells was compared between monocytes cultured in medium only and monocytes exposed to tumor cells, tumor CM, or cytokines using one-tailed ANOVA, followed by Dunnet's multiple means comparison. Statistically significant differences were denoted as ** = $p < 0.005$, *** = $p < 0.0005$, and **** = $p < 0.0001$. These data are representative of 4 repeated experiments with similar results.

by TNF-α **(Figure 4.3B)**. These findings suggested therefore that TNF-α might be the more important cytokine in the tumor environment for upregulation of PD-L1 expression.

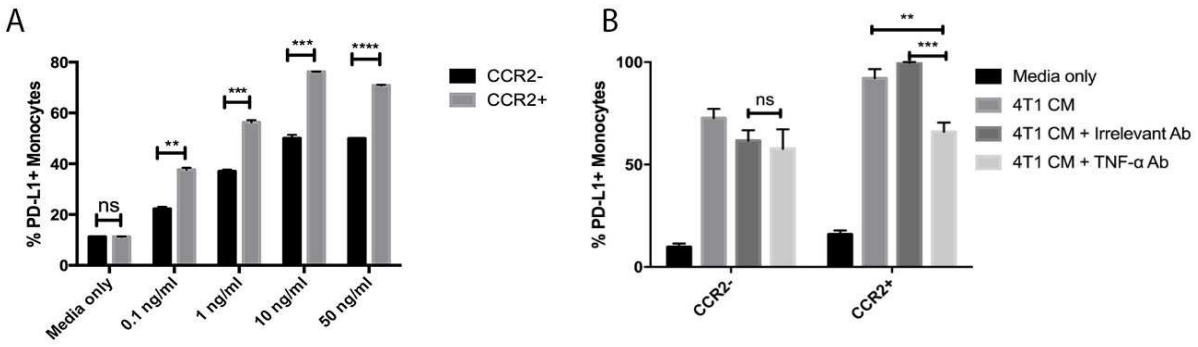

Figure 4.3 Neutralization of TNF-α significantly blocks upregulation of monocyte PD-L1 expression by tumors. (A) Bone marrow-derived monocytes were treated with increasing concentrations of recombinant TNF-α (0.1-50 ng/ml), and PD-L1 expression was compared between inflammatory monocytes ($CD11b^+/Ly6C^+/Ly6G^-/CCR2^+$) and non-inflammatory monocytes ($CD11b^+/Ly6C^+/Ly6G^-/CCR2^-$). **(B)** Conditioned medium from 4T1 cells was treated with a TNF-α neutralizing antibody (or isotype control antibody), then incubated with triplicate wells of bone marrow-derived monocytes for 24h. Effects of tumor CM on monocyte PD-L1 expression was assessed on both inflammatory monocytes ($CD11b^+/Ly6C^+/Ly6G^-/CCR2^+$) and non-inflammatory monocytes ($CD11b^+/Ly6C^+/Ly6G^-/CCR2^-$). Similar results were obtained in 3 additional independent experiments. Statistical comparison of TNF-α concentrations was done by two-tailed ANOVA, followed by Tukey's multiple means comparison. Statistically significant differences were denoted as * = $p < 0.05$ and ** = $p < 0.005$.

The concentrations of TNF-α in tumor and monocyte CM were then measured by ELISA, and these concentrations correlated with the degree of observed PD-L1 upregulation by co-cultured monocytes **(Figure 4.4A)**. First, we found that CM from any of the cells alone contained undetectable concentrations of TNF-α, which indicates that tumor cells were not the source of TNF-α production. Therefore, we examined TNF-α concentrations in the CM of monocytes that had been co-cultured with tumor CM. We observed high concentrations of TNF-α in the monocyte CM following incubation with tumor CM, suggesting production by the monocytes. We also used intracellular staining and found TNF-α production by monocytes cultured with tumor CM **(Figure 4.4B)**. In contrast, IFN-γ concentrations in the same CM were

undetectable (data not shown). Thus, it appeared that tumor cells in culture spontaneously secreted a factor that stimulated monocytes to produce TNF-α, which in turn led to greater upregulation of PD-L1 expression by the monocytes themselves.

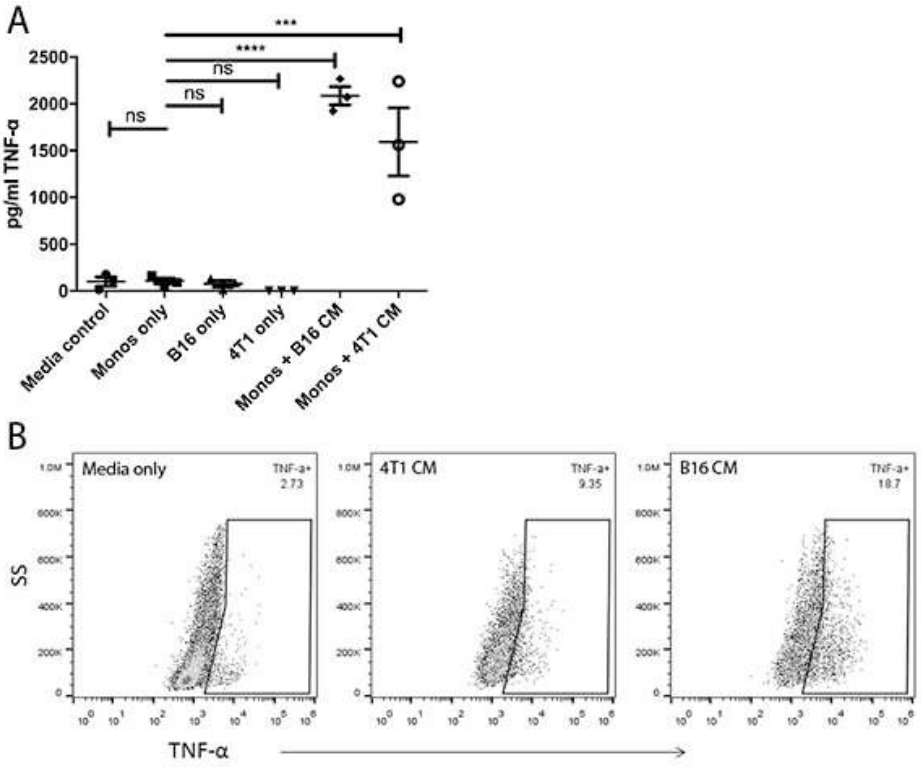

Figure 4.4 Tumor cells and tumor-secreted factors stimulate TNF-α production by monocytes. (A) Bone marrow monocytes were cultured overnight with B16 cells or 4T1 cells, or with tumor CM derived from these cells. Medium from monocyte cultures was collected 24h after co-culture with live tumor cells or with tumor CM, and IFN-γ and TNF-α concentrations were determined by ELISA. **(B)** Bone marrow-derived monocytes were cultured overnight with tumor CM then incubated with Brefeldin A for 4h and immunostained for detection of intracellular TNF-α expression by flow cytometry. Appropriate isotype control antibodies were used to assess the specificity of TNF-α staining. Similar results were obtained in 3 additional independent experiments. Statistical comparison of TNF-α concentrations was done by one-tailed ANOVA, with Dunnet's post-test. Statistically significant differences were denoted as *** = $p < 0.0005$ and **** = $p < 0.0001$.

Regulation of monocyte TNF-α production by tumor-secreted factors. Previous studies have described factors spontaneously secreted by tumor cells that led to TNF-α production by TAM[184,185]. These factors were also reported to stimulate macrophages to produce TNF-α in a

TLR2-dependent manner. Therefore, we first used TLR2$^{-/-}$ bone marrow monocytes to elucidate the role of TLR2-dependent signaling in monocyte upregulation of TNF-α production, and ultimately PD-L1 upregulation. We found that monocytes unable to signal via the TLR2 pathway failed to secrete TNF-α and to upregulate PD-L1 **(Figure 4.5A)**. Thus, this finding was consistent with a signaling pathway similar to that described previously for tumor-induced macrophage TNF-α production, through stimulation of TLR2 by extracellular matrix protein versican[184]. To this end, we found that removal of extracellular matrix protein versican from CM led to an abrogated upregulation of TNF-α production and PD-L1 expression compared to monocytes cultured with tumor CM alone **(Figure 4.5B)**. These data suggest that versican is a primary mediator of TNF-α production and PD-L1 expression upregulation by monocytes.

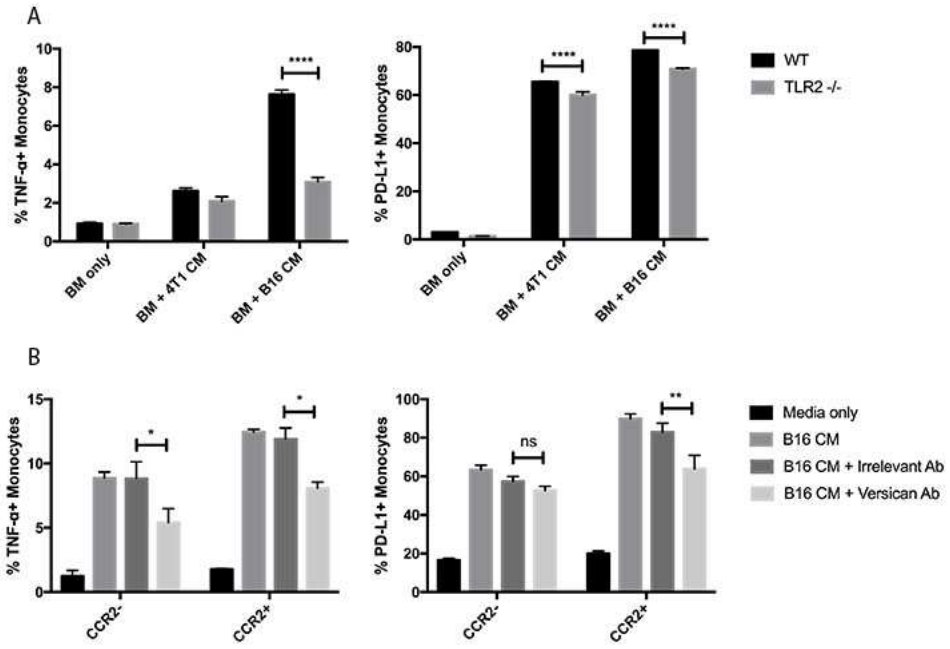

Figure 4.5 Versican secreted by tumor cells induces monocyte TNF-α production via TLR2 signaling pathway. Triplicate wells of bone marrow monocytes generated from WT or TLR2$^{-/-}$ mice were cultured with tumor CM overnight. The monocytes were then detached and processed for detection of intracellular TNF-α expression and surface PD-L1 expression using flow cytometry in **(A)**. In **(B),** versican was immunoprecipitated out of tumor CM using versican antibodies and protein A beads prior to culture with bone marrow-derived monocytes. The

monocytes were immunostained for surface PD-L1 expression and incubated with Brefeldin A for 4h and immunostained for detection of intracellular TNF-α expression by flow cytometry. Statistical comparison of TNF-α and PD-L1 expression was done by two-tailed ANOVA, with Tukey post-test. Statistically significant differences were denoted as * = $p < 0.05$, ** = $p < 0.005$, and *** = $p < 0.0005$. This experiment was repeated once, with similar results.

Cellular source of TNF-α production in tumor tissues and role of TNF-α in vivo. Finally, experiments were done to determine the *in vivo* source of TNF-α production within tumor tissues. Using tissues from subcutaneous B16 tumors, we found strong, constitutive expression of both TNF-α and PD-L1 throughout tumor tissues **(Figure 4.6A)**. Co-localization studies revealed the source of TNF-α production was primarily F4/80^{+} macrophages, thus confirming *in vivo* the feedback loop described by our *in vitro* studies **(Figure 4.6B)**. We also conducted studies using TNFR$^{-/-}$ mice to assess the role of TNF-α in regulating tumor growth in the B16 model. We found B16 tumors grown in TNFR$^{-/-}$ mice were significantly smaller than those grown in WT mice **(Figure 4.7)**. Furthermore, there were significantly decreased numbers of PD-L1^{+} tumor-associated macrophages and dendritic cells but not tumor cells from TNFR$^{-/-}$ mice **(Figure 4.6C)**, supporting the results observed with *in vitro* studies.

Discussion.

Factors that regulate PD-L1 expression on TAM and monocytes have not been previously identified, which provided the impetus for the current study. In our investigations, we found that monocytes progressively upregulated PD-L1 expression as they matured and entered tumor tissues. In addition, we also identified a feed-forward loop regulating PD-L1 expression on TAM, wherein tumor-secreted versican triggered TNF-α secretion by macrophages. This in turn stimulated local upregulation of PD-L1 expression by the same macrophages. These findings suggest that PD-L1 expression by macrophages and monocytes in tumor tissues is regulated

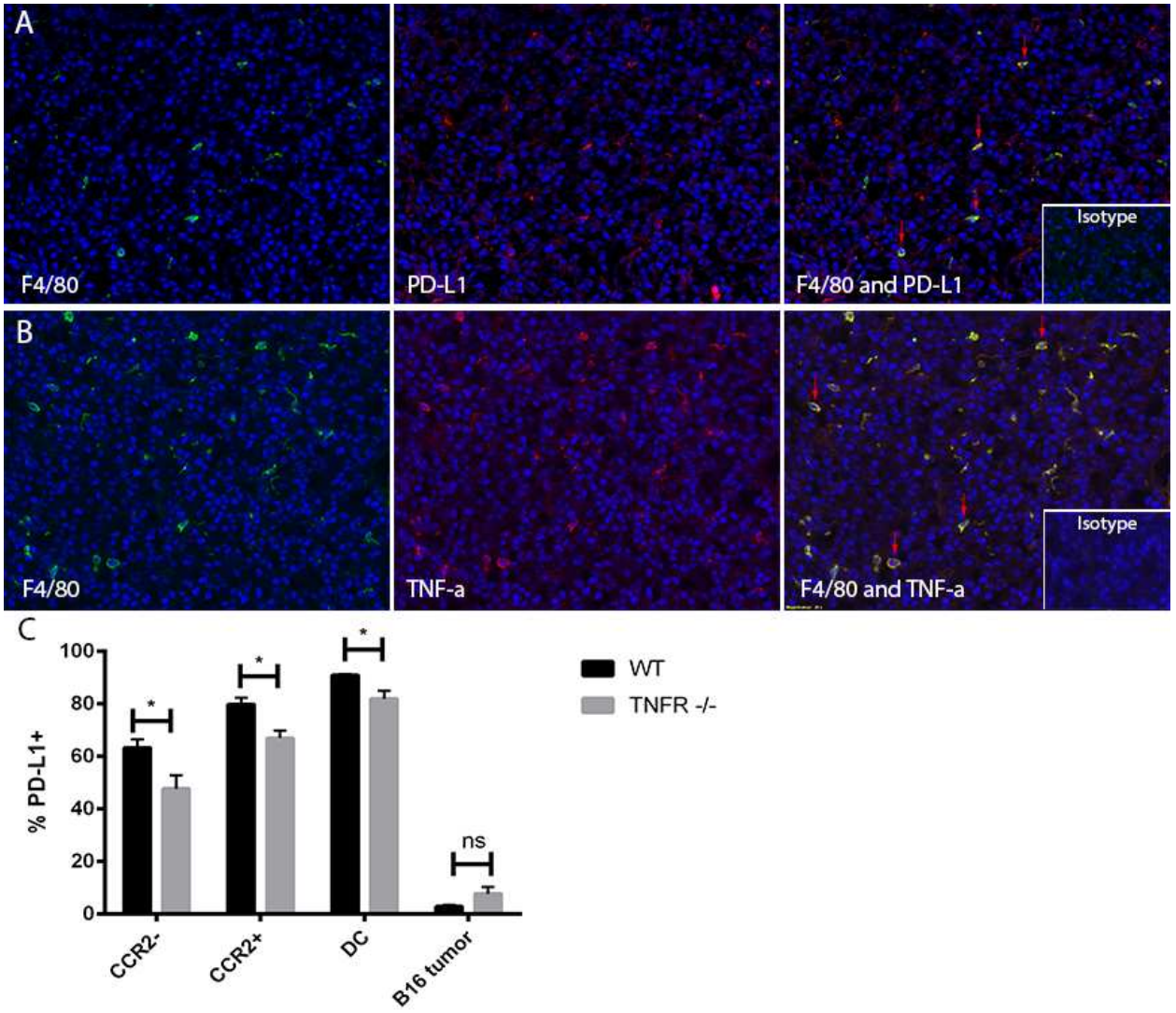

Figure 4.6 *In vivo* TNF-a production within tumor tissues and effects on tumor-associated macrophage PD-L1 expression. (A) B16 tumors were collected from the s.c. tissues of mice, cryosectioned, and immunostained for detection of F4/80 (green) and PD-L1 expression (red and counterstained with DAPI (blue). **(B)** Tumor tissues were immunostained for co-localization of F4/80 (green) and

TNF-α (red), and counterstained with DAPI (blue) for nuclear detection as noted in Methods. Depicted are representative images obtained from 6 separate tumors processed and immunostained for PD-L1, TNF-α, and F4/80. **(C)** Tumor tissues from WT and $TNFR^{-/-}$ mice were processed into single cell suspension for flow cytometric analysis of PD-L1 expression by macrophages ($CD45^{+}/CD11b^{+}/Ly6G^{-}/F4\text{-}80^{+}$) and dendritic cells ($CD11b^{+}/CD11c^{+}$). Mean percentages of $PD\text{-}L1^{+}$ cells were compared using non-parametric t-tests, with * = $p < 0.05$.

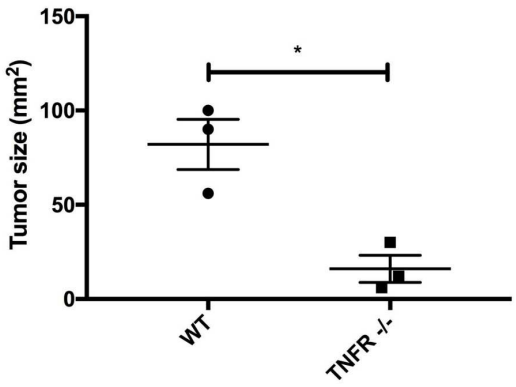

Figure 4.7 Effects of TNF-α on tumor size. B16 tumors were grown in WT and TNFR$^{-/-}$ mice and measured when the mice were euthanized. Tumor area was calculated as length x width in mm.

primarily by maturation and by locally produced TNF-α. Macrophage PD-L1 expression was largely independent of local IFN-γ production, in contrast to tumor cells, which were found to be much more dependent on IFN-γ for upregulated PD-L1 expression. Therefore, these results provide a clearer understanding of the regulation of PD-L1 in the TME.

Consistent with previous reports, we found that tumor cell PD-L1 expression was primarily regulated by endogenous production of IFN-γ[61,62], inasmuch as PD-L1 expression was significantly reduced in IFN-γ$^{-/-}$ mice. However, expression of PD-L1 by TAMs in IFN-γ$^{-/-}$ mice was unchanged compared to WT animals. These findings suggested alternative, IFN-γ-independent mechanisms for regulation of PD-L1 expression on TAM and monocytes.

One mechanism regulating PD-L1 expression by macrophages identified in this study was cellular maturation. For example, we observed that the level of PD-L1 expression (both in terms of the total percentage of PD-L1^{+} cells and the overall level of PD-L1 expressed by each cell) underwent significant upregulation as cells matured from bone marrow monocytes, to circulating monocytes, to macrophages in tumor and spleen tissues. Thus, monocytes likely

become more effective at downregulating T cell responses as they mature and enter tissues, which is consistent with the normal immune homeostatic role of the PD-1/PD-L1 axis[21,22].

Our investigations also revealed a previously unrecognized role for TNF-α in regulating PD-L1 expression by TAM. We found that TNF-α produced by monocytes themselves, in response to tumor-secreted versican, was the key cytokine responsible for upregulated PD-L1 expression on monocytes. The tumor-secreted versican triggered TNF-α production in a TLR2-dependent manner, consistent with the previously reported pro-inflammatory effects of tumor extracellular matrix proteins[184]. Versican is also involved in extracellular matrix formation during inflammatory reactions to provide a scaffold for the adhesion of recruited immune cells such as monocytes, and even enhances cellular adhesion to the scaffolds[56]. Furthermore, we determined that less mature monocytes (pro-inflammatory $CCR2^{+}$ monocytes) were more responsive to TNF-α than non-inflammatory, $CCR2^{-}$ monocytes. This finding suggests that within the TME, newly recruited inflammatory monocytes are likely to be a major source of high level PD-L1 myeloid cells, which would have the greatest negative impact on tumor-infiltrating T cells. These pro-inflammatory, $PD\text{-}L1^{hi}$ monocytes are also the most likely to be recruited in response to T cell-generated inflammation and CCL2 production, serving to counter-balance T cell inflammatory stimuli with anti-inflammatory immune regulatory responses[172,173].

Previous studies have reported that monocyte-derived IL-10 was an important stimulus for PD-L1 expression upregulation by human monocytes, and that TNF-α exerted only a marginal effect on PD-L1 expression[58]. In contrast, we found that IL-10 had little effect on monocyte PD-L1 expression, and that TNF-α was a very potent signal for PD-L1 upregulation. These differing findings may be explained by use of different monocyte populations (ie bone

marrow vs blood) obtained using different culture techniques. In addition, it is also possible that the murine and human monocytes respond differently to IL-10 and TNF-α.

In our studies, we found that tumor cells regulated monocyte PD-L1 expression via a contact-independent mechanism. TNF-α was produced by monocytes when cultured with tumor CM, and neutralization assays revealed that TNF-α was the principle cytokine responsible for this effect. We did not use mature macrophages for these assays because they have such high levels of PD-L1 expression that a treatment effect cannot be discerned (data not shown). Furthermore, monocytes derived from $TLR2^{-/-}$ mice showed decreased sensitivity to B16 CM, suggesting a TLR2-dependent signaling mechanism for regulating TNF-α secretion (and ultimately PD-L1 upregulation) by TAM. We found that the removal of versican from B16 CM significantly suppressed upregulation of TNF-α production and PD-L1 expression by monocytes cultured with B16 CM, indicating that versican produced by tumor cells plays a central role in regulating monocytes.

Interestingly, monocytes from $TLR2^{-/-}$ mice exhibited no decrease in TNF-α secretion compared to WT monocytes following exposure to 4T1 CM. This finding suggests an alternative mechanism by which CM from 4T1 cells induced TNF-α secretion. One possibility is production of MMP-3 by 4T1 cells, as they have been reported previously to produce MMP-3 in culture, which in turn was shown to induce macrophages to produce TNF-α[186,187].

Furthermore, we observed large numbers of macrophages expressing TNF-α and PD-L1 within tumor tissues, thus providing *in vivo* validation of the *in vitro* observations. We also observed that growth of B16 tumors was significantly suppressed in $TNFR^{-/-}$ mice with decreased expression of PD-L1 by TAM and dendritic cells but not on tumor cells, indicating the overall importance of PD-L1 expression by TAM in the regulation of tumor growth. The

reduction in PD-L1 expression by TAM from tumors in $TNFR^{-/-}$ mice would also be expected to enhance anti-tumor immunity, which could account for the reduced tumor growth. These data provide additional *in vivo* support for the existence of a TNF-α – PD-L1 immune regulatory pathway in tumors.

In summary, our studies provide evidence for a new immune pathway for regulation of PD-L1 expression by monocytes and macrophages in tumor tissues, and potentially in other organs undergoing inflammatory insults. This pathway requires the release of pro-inflammatory mediators (such as versican) from tumors, plus the presence of inflammatory monocytes and macrophages that respond to the inflammation-associated signals by releasing TNF-α in a TLR2-dependent mechanism. The local release of TNF-α in turn leads to upregulation of PD-L1 expression, thereby triggering local immune suppression. Interruption of this pathway could have important therapeutic implications as a means of enhancing anti-tumor immunity. Thus, improved understanding of this and other pathways for regulation of PD-L1 expression by inflammatory monocytes TAM in tumor tissues will be important to the design of newer approaches to tumor immunotherapy[3,80,94].

CHAPTER 5

PD-L1 Signaling Regulates Proliferation and Activation of Tumor-associated Macrophages in Mice

Summary.

Tumor-associated macrophages (TAM) express high levels of PD-L1 and contribute to the immune-suppressive tumor microenvironment. While the role of PD-L1 signaling to PD-1 to induce T cell exhaustion is well established, much less is known about whether PD-L1 signals directly to macrophages and how these signals affect TAM function. Therefore, we used *in vitro* and *in vivo* models to investigate PD-L1 signaling in macrophages and the effects of sCD80, sPD-1, and PD-L1 blocking antibody on TAM phenotype. We observed that incubation of macrophages with PD-L1 antibody induced proliferation and spreading, along with upregulation of co-stimulatory molecules and spontaneous inflammatory cytokine production. Similar changes were observed in macrophages incubated with sCD80, in PD-L1$^{-/-}$ macrophages, and to a lesser extent in macrophages incubated with sPD-1. Furthermore, treatment with PD-L1 antibody upregulated mTOR pathway activity and RNAseq revealed an inflammatory macrophage phenotype. *In vivo*, significant tumor infiltration with macrophages was observed following treatment with PD-L1 antibody, along with evidence of macrophage activation. Studies conducted in Rag$^{-/-}$ mice showed upregulated co-stimulatory molecule expression by TAMs and reduced tumor growth in mice treated with PD-L1 antibody. Moreover, combined PD-1/ PD-L1 antibody treatment of mice with established melanomas elicited complete tumor elimination in half of the animals. These findings suggest that PD-L1 delivers a constitutive signal in macrophages to suppress mTOR pathway activity, leading to an immune-suppressive phenotype.

These results also suggest that PD-L1 antibodies induce qualitatively different anti-tumor effects than PD-1 antibody treatment, owing in part to the stimulatory effects of PD-L1 antibody on TAMs.

Background.

PD-L1 is often overexpressed by tumor tissues, and the level of expression by tumors is frequently associated with prognosis and response to treatment with PD-1 therapeutic antibodies[188-190]. Notably, a number of studies agree that the density of PD-L1-expressing macrophages in tumors is predictive of the efficacy of both PD-1 and PD-L1 antibody therapy[99,102,103]. For example, in some cases, response rates as high as 80% have been observed in patients where macrophages were positive for PD-L1 expression[99]. PD-L1 is a 290-amino acid transmembrane glycoprotein with a short cytoplasmic tail and has historically been viewed simply as a ligand for PD-1[191,192]. However, there has been emerging evidence suggesting that direct signaling by PD-L1 may play a role in regulating tumor growth and biology.

For example, PD-L1 signaling has been shown to promote epithelial-to-mesenchymal transition in several tumor types[193-195]. Conversely, downregulation of PD-L1 expression was associated with decreased tumor metastasis[193,194]. Other groups have reported that PD-L1 acts as an anti-apoptotic receptor in response to Fas ligation, and that PD-L1 was also associated with cancer stem cell proliferation[196,197]. In addition, upregulation of PD-L1 expression on tumor cells resulted in increased resistance to apoptosis upon treatment with chemotherapy[198].

PD-L1 signaling also regulates cellular functions. Tumor cell metabolism was shown to be regulated by PD-L1 through Akt/mTOR phosphorylation and signaling, and PD-L1 blockade resulted in decreased levels of glucose uptake and glycolysis[199]. This study also found that tumor

cells showed decreased glycolysis upon knockdown of PD-L1, suggesting that PD-L1 signals constitutively and that this activity may be blocked by PD-L1 antibodies. Furthermore, studies have reported an increase in ERK- and mTOR-mediated proliferation and survival of tumor cells following ligation of PD-L1 with human PD-1 or in culture with PD-L1-expressing T cells[200,201]. Ligation of PD-L1 on dendritic cells using soluble PD-1 led to a downregulation of maturation-associated markers, and a murine macrophage cell line showed a more regulatory, immune-suppressive phenotype when treated with a PD-L1 antibody[202,203]. Little is known, however, regarding the effects of PD-L1 signaling in macrophages in the tumor microenvironment (TME).

The goal of the present study was to investigate the role that PD-L1 expressed by tumor-associated macrophages (TAM) plays in regulating the biological functions of macrophages in the TME. TAM often express high levels of PD-L1, and we recently reported that PD-L1 expression by TAM was regulated primarily by locally produced TNF-α[204]. In a screen of several different tumors in humans, PD-L1-expressing macrophages were found to be more abundant than PD-L1-expressing tumor cells[99]. Other studies found that treatment with PD-L1 antibody induced antitumor activity even in models where PD-L1 was not expressed by the tumor cells themselves, suggesting that PD-L1 expression by macrophages may be more important to the overall effect of PD-L1 antibody treatment[102,103]. We hypothesized that this anti-tumor activity is mediated by changes in tumor macrophages following treatment with PD-L1 antibody. Therefore, we conducted *in vitro* studies to assess the impact of PD-L1 antibody, soluble PD-1, and soluble CD80 (another ligand of PD-L1[21]) on macrophage survival and activation. We also conducted global gene expression profiling of PD-L1 antibody-treated macrophages. In addition, the effects of PD-L1 antibody treatment on TAM phenotype were assessed in mouse tumor models, as well as the effects of combined PD-1 and PD-L1 antibody treatment.

Materials & Methods.

Mice. Wild-type C57Bl/6 mice and Rag1^{tm1Mom}/J mice were purchased from Jackson Laboratories and cared for in accordance with institutional and NIH guidelines. Mice were inoculated subcutaneously in the flank with 1 x 10^5 B16.F10 melanoma cells or PyMT breast carcinoma cells. On Day 7, B16-inoculated mice were randomly placed into 3 treatment groups and tumor take between the groups was verified to be statistically similar. They received intraperitoneal injections of PBS, 250 ug isotype (BioXcell, West Lebanon, NH) or PD-L1 antibody (BioXcell, clone 10F.9G2) in a volume of 100 ul every 3 days for 9 days. Treatment for PyMT-inoculated Rag$^{-/-}$ mice started on Day 18, and mice received injections of PBS, 100 ug isotype or PD-L1 antibody every 3 days for 9 days. Mice were humanely euthanized when the first tumors reached a size of 15 mm in diameter, according to Institutional Animal Care and Use Committee protocol. For combination blockade of PD-1 and PD-L1, mice were inoculated similarly and randomized on Day 7. They received intraperitoneal injections of 100 ug PD-1 antibody (BioXcell, clone RPM1-14), 100 ug PD-L1 antibody, or 100 ug PD-1 + 100 ug PD-L1 antibodies every 3 days for 9 days then once a week for a total of 68 days. Mice were euthanized individually as the tumors reached 15 mm in diameter for a survival study, and tumor area was calculated as length x width. Remaining mice were euthanized at 68 days and verified to be tumor-free.

Monocyte isolation and macrophage culture. Tibias and femurs were collected from healthy mice and bone marrow cells were harvested as described previously[204]. Tissues from PD-L1$^{-/-}$ C57Bl/6 mice were a kind gift from Raphael Nemenoff and Howard Li (University of Colorado, Denver). Bone marrow cells were cultured in PermaLife bags (OriGen Biomedical, Austin, TX)

with 10 ng/ml rM-CSF (R&D Systems, Minneapolis, MN) for one week. 50,000 M-CSF-cultured macrophages were washed with medium to remove M-CSF and plated in 96-well polystyrene cell culture plates (Corning, New York, NY) for 48 hours with or without treatment before use in experiments. Adherent bone marrow-derived macrophages were then harvested by pipetting with ice-cold 2mM EDTA (ThermoFisher Scientific, Waltham, MA) in PBS.

For enrichment of human macrophages, monocytes were selected from peripheral blood mononuclear cells by adherence overnight in cell culture plates and cultured for one week in 10 ng/ml rhM-CSF (Peprotech, Rocky Hill, NJ).

Reagents for treatment of bone marrow-derived macrophages. PD-L1 antibody (BioXcell, clone 10F.9G2) and irrelevant isotype-matched control antibody (BioXcell) were used at 100 ug/ml, which represents the reported minimum desired plasma antibody concentration for patients treated with PD-L1 antibody[99]. These antibodies were verified by the manufacturer to contain less than 0.002 EU/ug of endotoxin. A rat anti-mouse CD11b antibody (eBioscience, Waltham, MA, clone M1/70) was used as a macrophage specific antibody, but with a function unrelated to that of PD-L1, for *in vitro* antibody studies. For human macrophages, PD-L1 antibody (Biolegend, clone 29E.2A3) and irrelevant isotype-matched control antibody (Biolegend, San Diego, CA) were used at 200 ug/ml.

Recombinant mouse soluble PD-1 chimera protein with a human IgG1 Fc portion was obtained from R&D systems and used at 100 ug/ml to match the PD-L1 antibody concentration. Recombinant mouse soluble CD80 chimera protein with a human Fc portion was obtained from Biolegend and used at 100 ug/ml. Donkey anti-human IgG Fc from Jackson Immunoresearch (West Grove, PA) was used at 10 ug/ml to crosslink sPD-1 and sCD80, according to the

manufacturer's suggestions. CD16/CD32 Fc blocking antibody (eBioscience) was used at 100 ug/ml to match the PD-L1 antibody concentration.

Rapamycin (Sigma-Aldrich, St. Louis, MO) was used at 10 ug/ml as an indirect inhibitor of mTOR signaling and Torin2 (Sigma-Aldrich) was used at 0.15 uM as a direct inhibitor of mTOR[205,206]. IFN-γ and TNF-α from Peprotech were used to stimulate macrophages at 10 ng/ml.

Tumor cell lines. B16.F10 melanoma cells and PyMT breast carcinoma cells were obtained from ATCC and screened by PCR to ascertain that they were of murine origin, and free of mycoplasma contamination.

Tissue preparation for flow cytometry. Tumor tissues processed to a single cell suspension, as described previously[204]. Cells were immunostained using the following antibodies: Invitrogen: CD45 Pacific Orange (MCD4530), eBioscience: PD-L1 PE (clone MlH5), CD11b Pacific Blue (clone M1/70), CD86 PE (clone GL1), and MHCII APC (clone M5/114.15.2), Abd Serotec: F4-80 APC (clone Cl:A3-1), BD Pharmingen: Ly6C biotin (clone AL-21) and Ly6G FITC (clone 1A8). Cells were also stained with appropriately-matched isotype antibodies to assure specificity of immunostaining.

To quantitate intracellular cytokine production, macrophages were treated with protein secretion inhibitor Brefeldin (Biolegend) for 4 hours prior to staining. The cells were fixed with 4% PFA (Affymetrix, Santa Clara, CA) and permeabilized with 0.25% Saponin in FACS buffer (1% BSA in PBS with 0.05% sodium azide) prior to immunostaining for IL-12 PE (BD Pharmingen, San Jose, CA) and TNF-α PE (eBioscience, clone MP6-XT22). To measure mTOR pathway activity, polyclonal antibodies from Cell Signaling Technologies (Danvers, MA) were

used to stain for intracellular levels of mTOR and p-mTOR, and secondary donkey anti-rabbit IgG FITC (Jackson Immunoresearch) was used to detect primary antibody binding.

Human macrophages were immunostained with CD14 APC (Bio-Rad, Hercules, CA, clone TUK4), CD40 PE (eBioscience, clone 5C3) and CD86 FITC (BD Pharmingen, clone FUN-1). Appropriately-matched isotype control antibodies were also used to assure specificity of immunostaining.

Immunofluorescent tissue staining and imaging. Tumor tissues were prepared for immunofluorescent staining as described previously[204]. Primary antibodies for F4-80 (AbD Serotec, Raleigh, NC clone Cl:A3-1) and MHC II (eBioscience, clone M5/114.15.2) were used. Controls included immunostaining with appropriate concentrations of irrelevant isotype-matched antibodies.

Proliferation assay. A Click-iT assay kit was used to measure proliferation of macrophages (ThermoFisher Scientific). EdU was added the same day as PD-L1 antibody treatment to be incorporated into proliferating cells for 48 hours prior to analysis. Cells were then detached, stained, and proliferation was evaluated by flow cytometry or immunofluorescent imaging as described above.

Western blotting. A standard western blotting protocol from Bio-Rad was followed. Briefly, macrophages were treated with medium, irrelevant isotype antibody, or PD-L1 antibody for 5 hours. Samples were prepared under non-reducing, denaturing conditions and 6 ug total protein was loaded into a gel (BioRad). Polyclonal antibodies from Cell Signaling Technologies for Akt

and pAkt and were used to probe for their respectable proteins, followed by peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch). An antibody for β-actin (Sigma-Aldrich, clone AC-15) was used as a loading control, followed by donkey anti-mouse secondary antibody (Jackson Immunoresearch). Densitometric analysis to quantify band area was completed using ImageJ software and values were normalized to β-actin.

RNAseq. Bone marrow was collected from 9 mice and cultured as described above. After one week, macrophages were treated with medium only, irrelevant isotype or PD-L1 antibody antibodies for 24 hours. Total RNA was extracted using RNeasy micro Kit (Qiagen, Hilden, Germany) and RNA was submitted to Novogene (Sacramento, CA) for RNAseq. Samples were tested for quality control by Agilent 2100 Bioanalyzer system and by agarose gel electrophoresis. Sample RNA integrity number ranged from 7 to 8.9. After the QC procedures, mRNA was enriched using oligo(dT) beads and fragmented randomly, then cDNA synthesized by using mRNA template and random hexamers primer, after which a custom second-strand synthesis buffer (Illumina, San Diego, CA), dNTPs, RNase H and DNA polymerase I were added to initiate the second-strand synthesis. Second, after a series of terminal repair, a ligation and sequencing adaptor ligation, the double-stranded cDNA library was completed through size selection and PCR enrichment. Library was qualified by Agilent 2100 to test the insert size, then Q-PCR was used to quantify the library effective concentration. Quantified libraries were run on Illumina PE150 (HiSeq) with 250~300bp insert cDNA library for 20M raw reads/sample. Raw data was filtered by removing reads containing adapters and reads containing N > 10% by Novogene, Phred score >30.

The filtered reads obtained by RNAseq from Novogene were analyzed using Partek® Flow® software, version 6.0. Filtered reads were aligned with STAR pipeline aligned against RefSeq Transcripts 83 - 2017-11-01 mouse whole genome. Counts were calculated using Partek E/M. Transcript counts were normalized using Partek total count method per sample and then an offset of 0.0001 was added to avoid zero counts. Normalized transcript counts were used to generate PCA plot. To detect differentially expressed genes, ANOVA was performed on the normalized transcript counts. Differentially expressed genes were filtered using p-value with false discovery rate ≤ 0.05 and fold change ≤ -2 or ≥ 2. Further biological interpretations including gene ontology enrichment and pathway analysis was performed. Functional analysis was generated using Ingenuity pathway analysis (IPA, Qiagen Inc.) Version 01-12. IPA analysis settings included stringent filters for molecules and relationships in mouse, experimentally observed confidence, and filtered for tissue and primary cells.

Statistical analysis. Statistical comparisons between those data sets with two treatment groups were done using nonparametric t-tests (Mann-Whitney test). Comparisons between 3 or more groups were done using ANOVA, followed by Tukey multiple means post-test. Analyses were done using Prism7 software (GraphPad, La Jolla, CA) and statistical significance was determined for $p < 0.05$. Statistical comparisons for survival analysis were done with Kaplan Meier and log rank (Mantel Cox) tests, with statistical significance determined as $p < 0.01$.

Results.

Macrophage proliferation and size increase with PD-L1 antibody treatment. To first examine the effects of PD-L1 antibody on macrophage phenotype and function, we used *in vitro* generated

macrophages since they express high levels of PD-L1 **(Figure 5.1A)**. The most striking early observation was that macrophages cultured in the presence of PD-L1 antibody, were markedly more numerous and larger than control macrophages **(Figure 5.2A)**. The increased density of macrophages cultured with PD-L1 antibody could represent the effects of increased proliferation, survival, activation and spreading, or a combination of all 3.

To determine the cause of the increased macrophage density, we first assessed proliferation of macrophages incubated with PD-L1 antibody or control antibodies **(Figure 5.2B)**. We found that macrophages taken out of M-CSF continued to proliferate when treated with PD-L1 antibody compared to both untreated and irrelevant antibody-treated cells **(Figure 5.2C and 5.2D)**. Furthermore, live cell measurements of macrophages cultured with PD-L1 antibody revealed that macrophage size and numbers increased significantly over time (**Figure 5.2E and 5.2F)**. In addition, increased macrophage survival was demonstrated using the MTT assay **(Figure 5.2G)**. Hence, macrophages treated with PD-L1 antibody demonstrated changes in proliferation, survival and size.

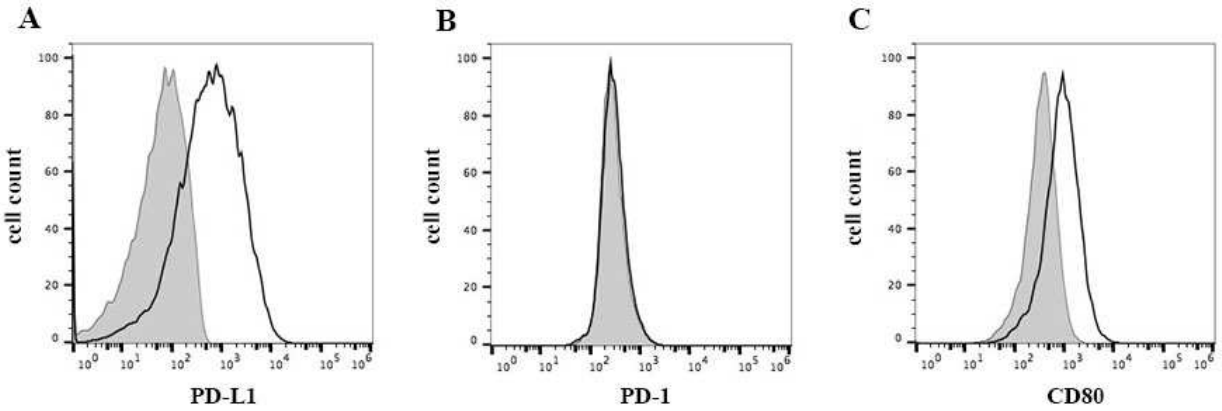

Figure 5.1 PD-1 and PD-L1 expression by bone marrow-derived macrophages. Bone marrow monocytes were cultured with M-CSF for one week for the enrichment of macrophages and plated for adherent culture overnight. They were then detached and processed for flow cytometric analysis of PD-L1 (A), PD-1 (B), and CD80 (C) expression. Histograms are shown in where isotype stain = gray filled and solid line = cultured macrophages.

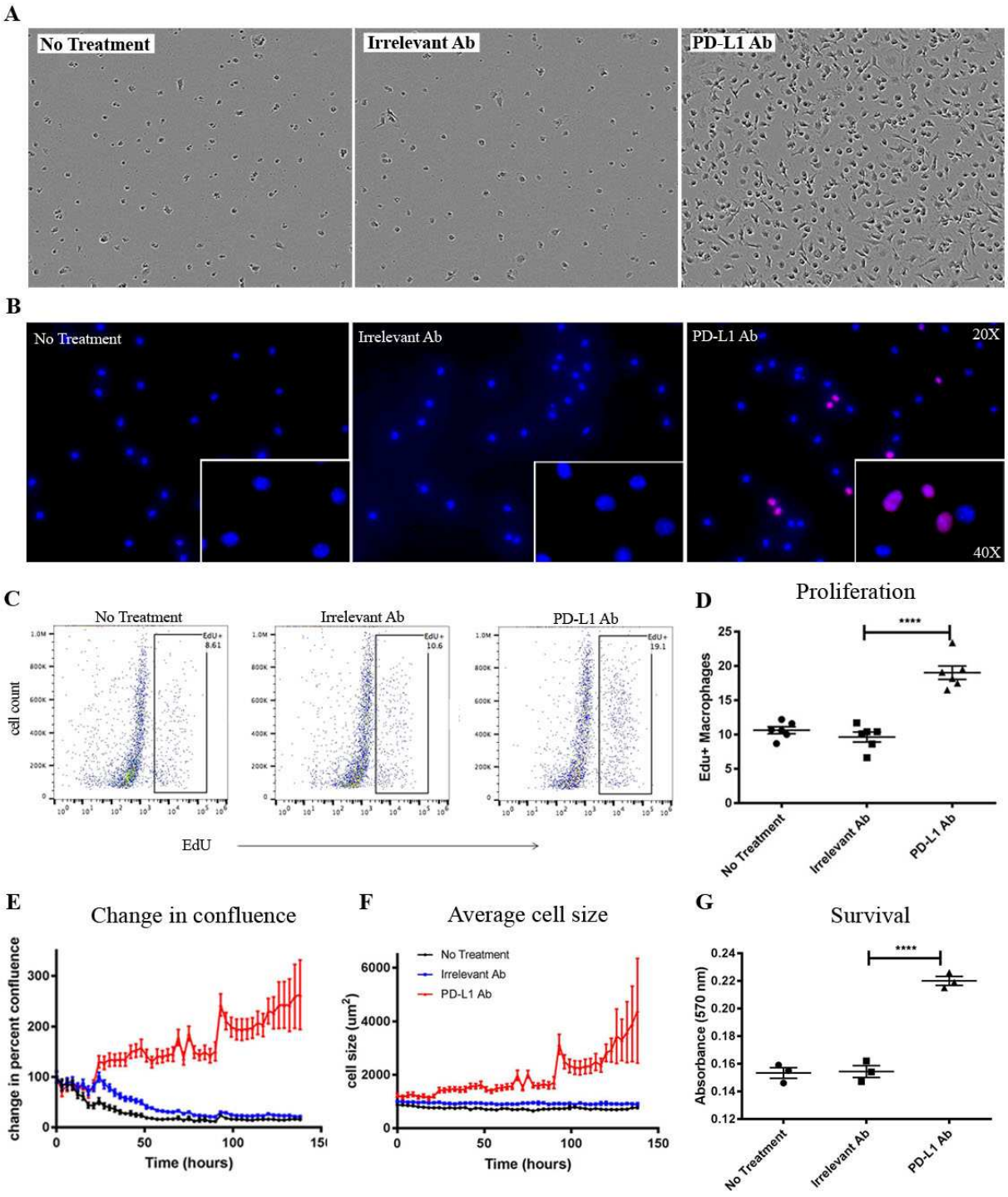

Figure 5.2 Macrophage proliferation and size increase with PD-L1 antibody treatment. Macrophages were treated with medium only, irrelevant isotype or PD-L1 antibody. After 6 days, images of the macrophages were taken (A). Proliferation was measured using EdU incorporation (B) and quantified using flow cytometric analysis (C) to be statistically compared using one-way ANOVA and Prism7 software (D). Changes in confluence (E) and average cell sizes (F) were measured every 3 hours for the 6 days with an IncuCyte ZOOM live cell imaging system. Lastly, an MTT was performed to measure survival of the cells and mean absorbances of

MTT substrate were compared statistically with one-way ANOVA and Prism7 software (G). Statistically significant differences were denoted as P-value **** < 0.0001. Similar results were obtained in 3 independent experiments.

PD-L1 antibody treatment induces macrophage activation. Studies were conducted next to determine whether PD-L1 antibody treatment stimulated macrophage activation, given the appearance of these macrophages in Figure 1. Compared to isotype antibody-treated macrophages, PD-L1 antibody treatment upregulated expression of co-stimulatory molecules CD86 and MHC II, consistent with macrophage activation **(Figure 5.3A)**. This effect was found to be titratable with increasing concentrations of PD-L1 antibody **(Figure 5.3B)** and the macrophage changes were first apparent after 48 hours of treatment **(Figure 5.3C)**. PD-L1 antibody-treated macrophages also spontaneously increased production of TNF-α and IL-12 **(Figure 5.3D)**, which suggested that PD-L1 antibody treatment skewed the macrophages towards a more inflammatory phenotype.

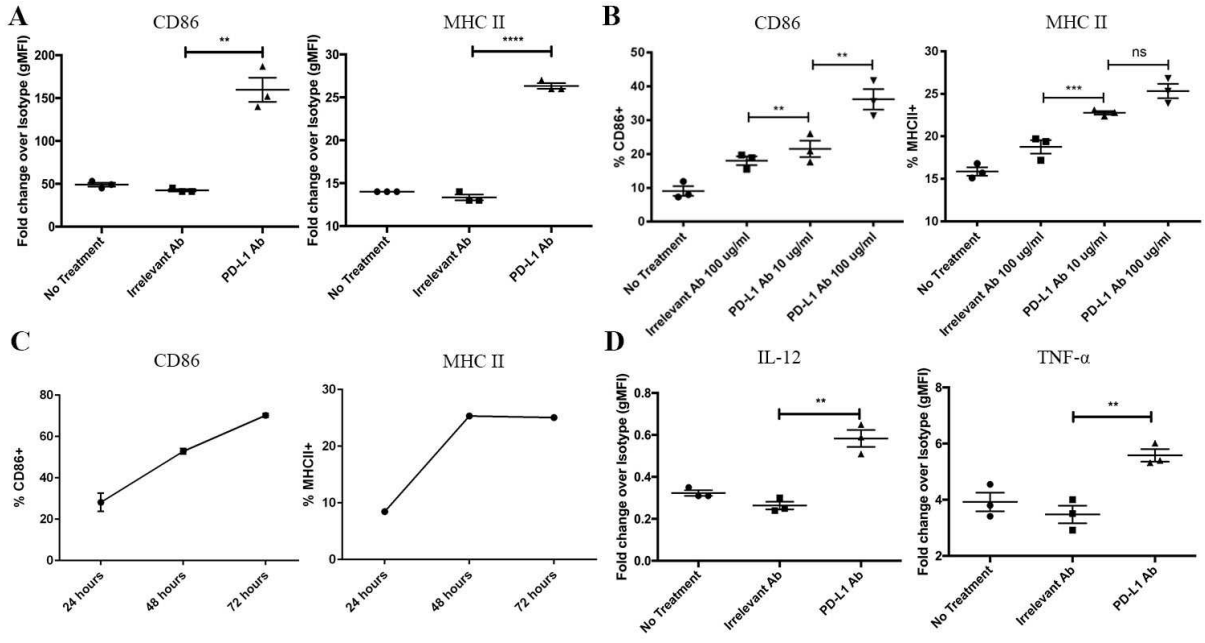

Figure 5.3 PD-L1 antibody treatment induces macrophage activation. Macrophages were treated with medium only, irrelevant isotype or PD-L1 antibody for 48 hours. They were stained for co-stimulatory molecule expression (CD86, MHCII) by flow cytometry and the geometric

mean fluorescence intensity (gMFI) is shown as fold increase over an irrelevant isotype stain (A). PD-L1 antibody was titrated (B) and macrophages were harvested at different time points following treatment with 100 ug/ml in (C). Finally, macrophages were permeabilized for staining of intracellular cytokines (IL-12 and TNF-α) by flow cytometry in (D). Fold changes were compared using one-way ANOVA and Prism7 software. Statistically significant differences were denoted as P-value ** < 0.005, *** < 0.0005, and **** < 0.0001. These data are representative of 3 experiments with similar results.

Similar changes were observed with human macrophages. Specifically, we found changes in morphology of macrophages following PD-L1 antibody treatment **(Figure 5.4A)** as well as upregulated expression of CD40 and CD86 **(Figure 5.4B)**.

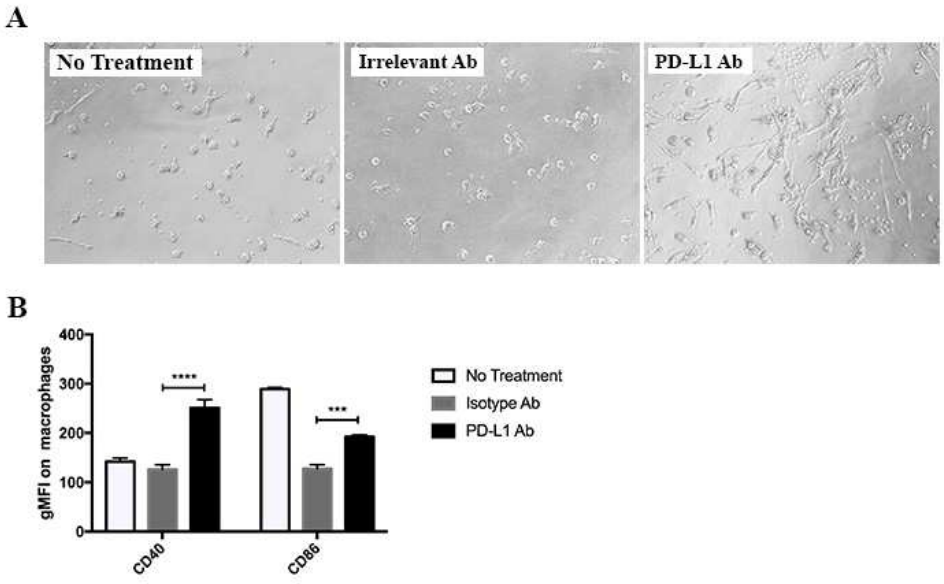

Figure 5.4 PD-L1 antibody treatment of human macrophages results in phenotypic changes consistent with those seen in murine macrophages. Macrophages were treated with medium only, an irrelevant isotype antibody, or PD-L1 antibody for 48 hours, and images were taken of the cells to assess changes in morphology (A). Surface expression of co-stimulatory molecules (CD40 and CD86) was compared by flow cytometry with two-way ANOVA and Prism7 software. Statistically significant differences were denoted as P-value *** < 0.0005 and **** < 0.0001, with similar results obtained in 2 independent experiments.

Binding of CD80 alters macrophage phenotype and activation more strongly than PD-1. We next wanted to see if the ligands for PD-L1 could induce the same effect as PD-L1 antibody treatment. To this end, we treated macrophages with crosslinked soluble PD-1 or CD80 to mimic

the effects of interaction with T cells expressing PD-1 or other macrophages expressing CD80. Treatment with both sPD-1 and sCD80 induced a change in macrophage morphology **(Figure 5.5A)** and also produced larger cells, consistent with the changes induced by incubation with PD-L1 antibody (**Figure 5.5B)**. The sPD-1 treated macrophages increased expression of the co-stimulatory molecule CD86, but not MHCII, suggesting partial macrophage activation. However, treatment with sCD80 greatly increased expression of CD86, MHC II, and TNF-α **(Figure 5.5C)**. Of note, we found that the macrophages do not express PD-1 either in our culture system **(Figure 5.1B)**, or directly taken from bone marrow, lungs, or spleens of healthy mice (data not shown). This is consistent with a recent paper that shows induction of PD-1 expression only upon migration into the tumor environment[207]. Furthermore, we observed that the macrophages expressed membrane-bound CD80 **(Figure 5.1C)** but not soluble CD80 in our culture system (data not shown). These data suggest that CD80 may be the strongest ligand for PD-L1, and that treatment with PD-L1-specific antibodies mimics this ligand-receptor interaction to activate macrophages.

Fc receptor engagement and treatment with a macrophage integrin antibody fail to activate macrophages. To assess the specificity of the observed changes mediated by PD-L1 antibody, macrophages were treated with a macrophage-specific antibody (CD11b) or with CD16/32 Fc receptor antibody. Macrophage morphological changes were not observed following treatment with CD11b antibody or Fc-receptor antibodies **(Figure 5.6)**. Changes in macrophage proliferation or expression of co-stimulatory molecules were also not observed **(Figure 5.7)**. Furthermore, pre-treatment of the macrophages with anti-Fc receptor antibody prior to incubation with PD-L1 antibody did not abrogate the changes mediated by PD-L1 antibody alone

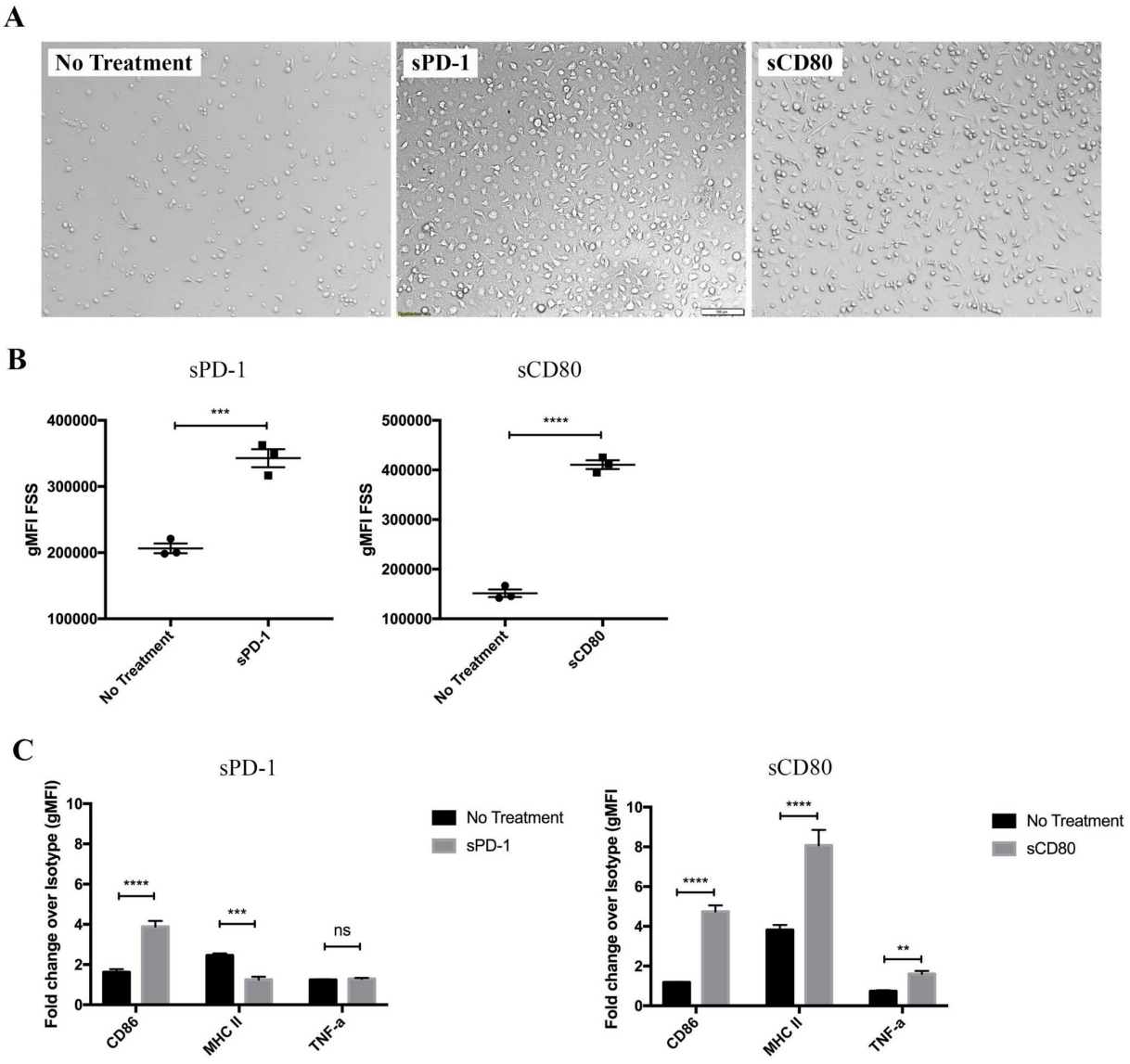

Figure 5.5 Treatment of macrophages with sCD80 alters macrophage phenotype and activation more strongly than crosslinked sPD-1. Macrophages were treated with crosslinked sPD-1 or sCD80 for 48 hours, and images were taken of the cells to assess changes in morphology (A). Size of the cells was quantified by flow cytometry in (B) and compared using nonparametric t-test and Prism7 software. In (C), co-stimulatory molecule expression (CD86 and MHC II) and intracellular TNF-α production were measured by flow cytometry. Geometric mean fluorescence intensity (gMFI) is shown as fold increase over an irrelevant isotype stain, and fold changes were compared using two-way ANOVA and Prism7 software. Statistically significant differences were denoted as P-value ** < 0.005, *** < 0.0005, and **** < 0.0001. Similar results were obtained in 2 repeated experiments.

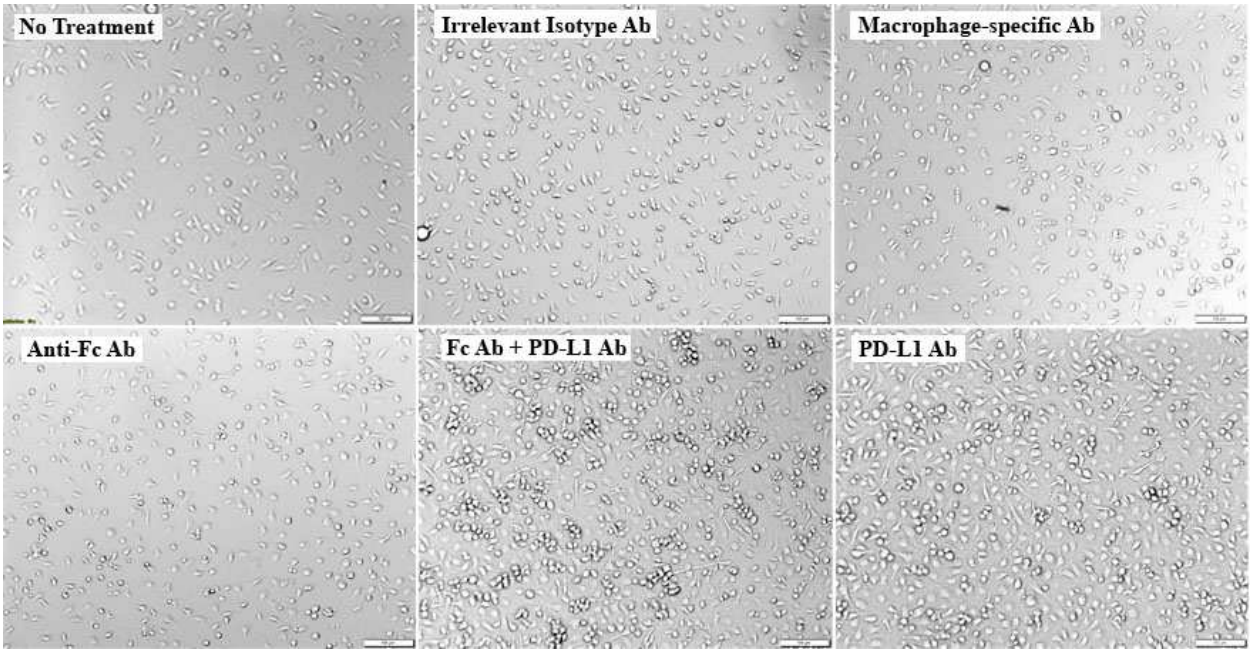

Figure 5.6 Morphology of macrophages treated with control antibodies. Macrophages were incubated with medium only, irrelevant isotype antibody, a macrophage-specific antibody (CD11b), Fc-blocking antibody, or pre-treated with Fc-blocking antibody for 1 hour before treatment with PD-L1 antibody. Photographs of the macrophages were taken after 48 hours with a confocal microscope.

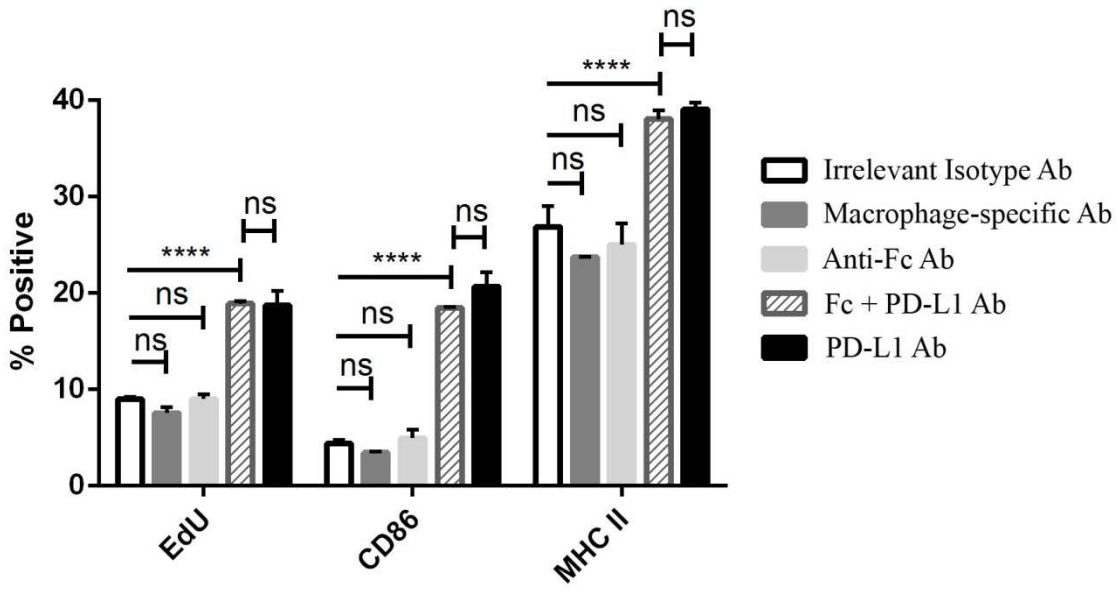

Figure 5.7 Control antibody treatments for macrophage proliferation and co-stimulatory molecule expression. Macrophages were incubated with irrelevant isotype antibody, macrophage-specific antibody (CD11b), Fc-blocking antibody, or pre-treated with Fc-blocking antibody before treatment with PD-L1 antibody. After 48 hours, proliferation measured by EdU incorporation and surface expression of co-stimulatory molecules (CD86 and MHC II) was compared by flow cytometry with two-way ANOVA and Prism7 software. Statistically significant differences were denoted as P-value **** < 0.0001, and similar results were obtained in 2 independent experiments.

(Figures 5.6 and 5.7). As a positive control for these studies, macrophages were stimulated with IFN-γ and TNF-α overnight (data not shown).

PD-L1 signals constitutively in macrophages to inhibit mTOR pathway signaling. We next sought to determine whether PD-L1 signals constitutively in macrophages or if PD-L1 signaling first requires ligation. To address this question, bone marrow derived macrophages were generated from PD-L1$^{-/-}$ mice. Macrophages from PD-L1$^{-/-}$ mice exhibited increased baseline proliferation **(Figure 5.8A)** and upregulated MHC II expression compared to wild-type macrophages **(Figure 5.8B).** These results are consistent with the concept that the PD-L1 molecule signals constitutively and negatively in macrophages, and that ligation with soluble CD80 or with certain PD-L1 antibodies inhibits this negative signaling, resulting in macrophage proliferation, survival and spontaneous activation.

Previous studies have reported involvement of the mTOR pathway in PD-L1 signaling in tumor cells[199-201], and mTOR signaling has been found to regulate metabolic programming of antigen presenting cells in normal tissues[208]. Therefore, we investigated the effects of mTOR inhibitors on macrophage activation following treatment with PD-L1 antibody. Treatment of PD-L1 antibody-cultured macrophages with either rapamycin (non-specific mTOR pathway inhibitor) or torin2 (targeted mTOR inhibitor)[205,206] dampened the changes induced by PD-L1 antibody treatment. Specifically, treatment with rapamycin and torin2 significantly blocked PD-L1 antibody-induced proliferation, TNF-α production, and expression of MHC II compared to macrophages treated with PD-L1 antibody alone (**Figure 5.8C**). We next assessed Akt and mTOR phosphorylation, since it was previously reported that PD-L1 signaling maintains mTOR pathway signaling in tumor cells[199]. We found increased p-Akt **(Figure 5.8D)** and p-mTOR

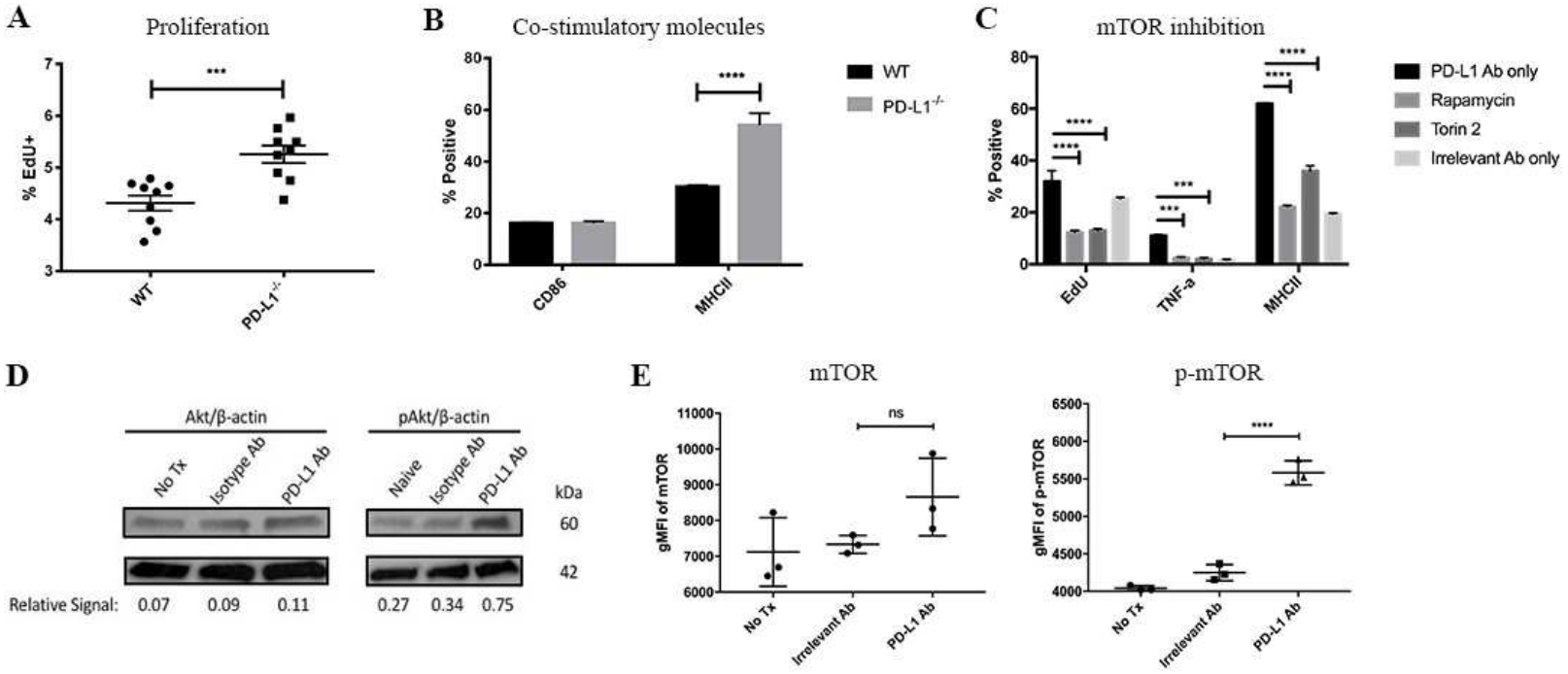

Figure 5.8 PD-L1 signals constitutively in macrophages to inhibit mTOR pathway signaling. Macrophages from wild-type and PD-L1$^{-/-}$ mice were cultured for one week as described in Methods. Proliferation was measured by EdU incorporation (A) and co-stimulatory molecule expression (CD86 and MHC II) was measured by flow cytometry (B). In (C), wild-type macrophages were treated with PD-L1 antibody in combination with mTOR inhibitors rapamycin and torin2 for 48 hours prior to phenotyping by flow cytometry. In (D), lysates from wild-type macrophages treated with medium, irrelevant isotype or PD-L1 antibody were used for assessment of Akt phosphorylation by western blot. Lastly, wild-type macrophages treated with medium, irrelevant isotype or PD-L1 antibody were also used for intracellular staining of mTOR and p-mTOR levels by flow cytometry (E). Statistical comparison for (A) was completed using nonparametric t-test, for (B) and (C) using two-way ANOVA, and for (E) using one-way ANOVA. All statistical comparisons were completed using Prism7 software. Statistically significant differences were denoted as P-value *** < 0.0005 and **** < 0.0001, and similar results were obtained in 2 additional, independent experiments.

(Figure 5.8E) in macrophages treated with PD-L1 antibody. Together, these results suggest that PD-L1 constitutively signals to block mTOR pathway signaling. Genetic elimination of PD-L1 expression or blockade of signaling by PD-L1 antibody appears to remove inhibition of this signaling pathway, resulting in the aforementioned macrophage changes.

Transcriptome profiling of PD-L1 antibody-treated macrophages showed inflammatory phenotype, increased survival and proliferation, and decreased apoptosis. To gain a comprehensive understanding of the changes to macrophages mediated by PD-L1 antibody treatment, RNAseq was conducted. We found that the gene expression profiles of macrophages treated with PD-L1 antibody were quite distinct from macrophages treated with isotype antibody or untreated macrophages **(Figure 5.9A)**. We identified 1823 differentially expressed genes following PD-L1 antibody treatment, with 643 genes upregulated and 1180 genes downregulated compared to isotype antibody treatment **(Figure 5.9B)**.

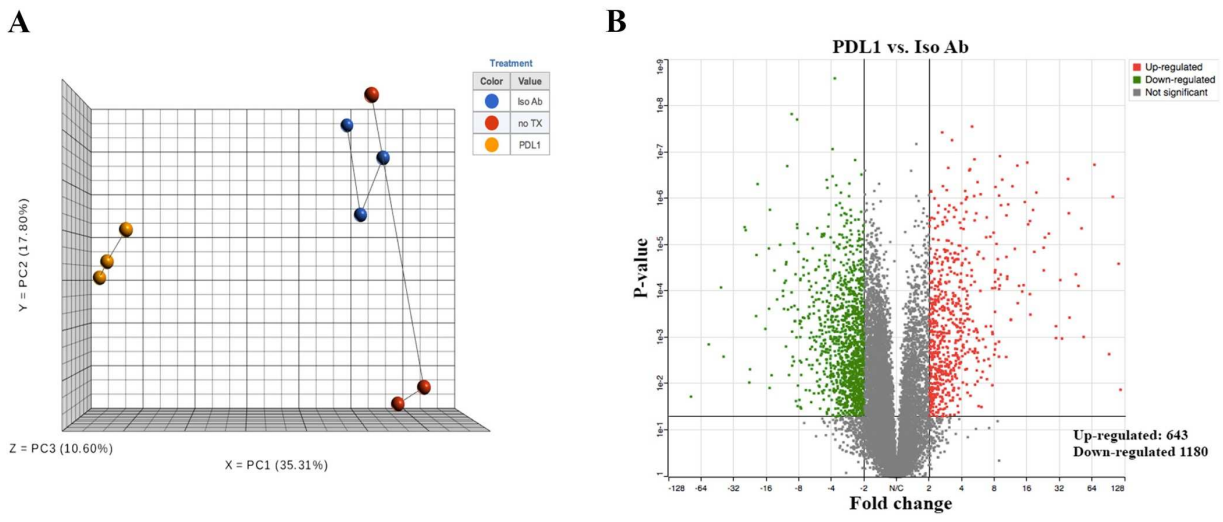

Figure 5.9 Transcriptome profiling of PD-L1 antibody-treated macrophages showed inflammatory phenotype, increased survival and proliferation, and decreased apoptosis. Macrophages were treated with medium only, irrelevant isotype antibody, or PD-L1 antibody for 24 hours prior to RNA extraction for RNA sequencing analysis. The PCA plot (A) depicts the

relationship and grouping of the samples based on global gene expression with medium only in red, isotype antibody in blue, and PD-L1 antibody in yellow. The volcano plot (B) shows the number of genes up- and down-regulated on PD-L1 antibody-treated macrophages compared to isotype antibody-treated macrophages for P value with FDR ≤ 0.05 and fold change ≤ -2 (left, green) or ≥ 2 (right, red). Gene Ontology enrichment analysis was used to classify all significantly upregulated (C) and downregulated (D) genes into biological processes, with the enrichment score on the x-axis. These data were generated using macrophages from 9 mice, with 3 mice in each treatment group.

To investigate the likely biological functions regulated by PD-L1, we used Ingenuity Pathway Analysis (IPA) software. We first identified the top differentially expressed genes by PD-L1 antibody-treated macrophages compared to those treated with isotype antibody. **Figure 5.10A** first depicts the 25 most upregulated genes by PD-L1 antibody-treated macrophages, which were increased up to 115-fold over isotype antibody-treated macrophages. The most highly upregulated gene was found to be *Serpinb2*, one of the most highly induced macrophage genes following LPS stimulus where it prevents macrophage death[209]. Serpinb2-deficient mice show impaired macrophage infiltration and an alternative/anti-inflammatory macrophage phenotype[210], suggesting an important role for this protease in promoting inflammatory macrophage function. The second most upregulated gene was *Saa3* (serum amyloid A3), which increases in response to acute inflammation and drives pro-inflammatory macrophage differentiation[211,212]. Not surprisingly, classical pro-inflammatory macrophage markers *Cd38*, *Il1a*, *Nos2*, and *Il6* were also upregulated. Other upregulated genes include *Slfn4* and *Slc7a2*, which are found to be upregulated only during macrophage activation[213,214], and upregulated chemotaxis genes *Ccl7* (MCP3), *Cxcl3* (MIP-2β), *Ccl2* (MCP-1), and *Fpr2*[215]. Interestingly, upregulated *Fpr1* is reported to only increase inflammatory macrophage chemotaxis while anti-inflammatory macrophages are unresponsive to FPR1[216]. Finally, upregulated *Dll4* and *Tarm1* promote pro-inflammatory activation and cytokine secretion by macrophages[217,218].

Figure 5.10A next depicts the top 25 downregulated genes in PD-L1 antibody-treated macrophages decreased by over 50-fold compared to isotype antibody-treated macrophages. Of these, a gene characteristic of anti-inflammatory macrophages (*Slco2b1*) was strongly downregulated[219]. We also found downregulated expression of *Angptl4*, which codes for a protein that is decreased in pro-inflammatory macrophages[220]. Finally, downregulated *Pparg*, *Tle1,* and *Clec10a* further show a suppressed anti-inflammatory phenotype of these macrophages and suggest a push towards an inflammatory state mediated by PD-L1 antibody treatment[221-223].

IPA analysis revealed that the top 10 signaling pathways altered by PD-L1 antibody treatment included upregulated inflammatory pathways, a downregulated anti-inflammatory pathway, an upregulated survival and proliferation pathway, and downregulated apoptosis pathways **(Figure 5.10B)**. The top pathway with the strongest statistical significance was the "Type I Diabetes Mellitus", which involves increased key components of the macrophage inflammatory response TNF-α, iNOS, and IL-1β **(Figure 5.10B)** The second pathway was "LXR/RXR activation", which also involves increased TNF-α as well as strongly upregulated inflammatory mediators iNOS, IL-1, IL-1β and IL-6 **(Figure 5.10B)** "PPAR signaling" was downregulated with decreased PDGF, which promotes an immune-suppressed environment[224], and decreased PPARγ, which skews macrophages towards an anti-inflammatory phenotype[221,225] **(Figure 5.10B)**. "TNFR2 signaling" was upregulated, showing increased TNFR2 and A20 that lead to increased proliferation and survival[226,227] **(Figure 5.10B)**. Also downregulated were two apoptosis pathways that emerged with decreased calpain and SERCA levels[228,229] **(Figure 5.10B)**.

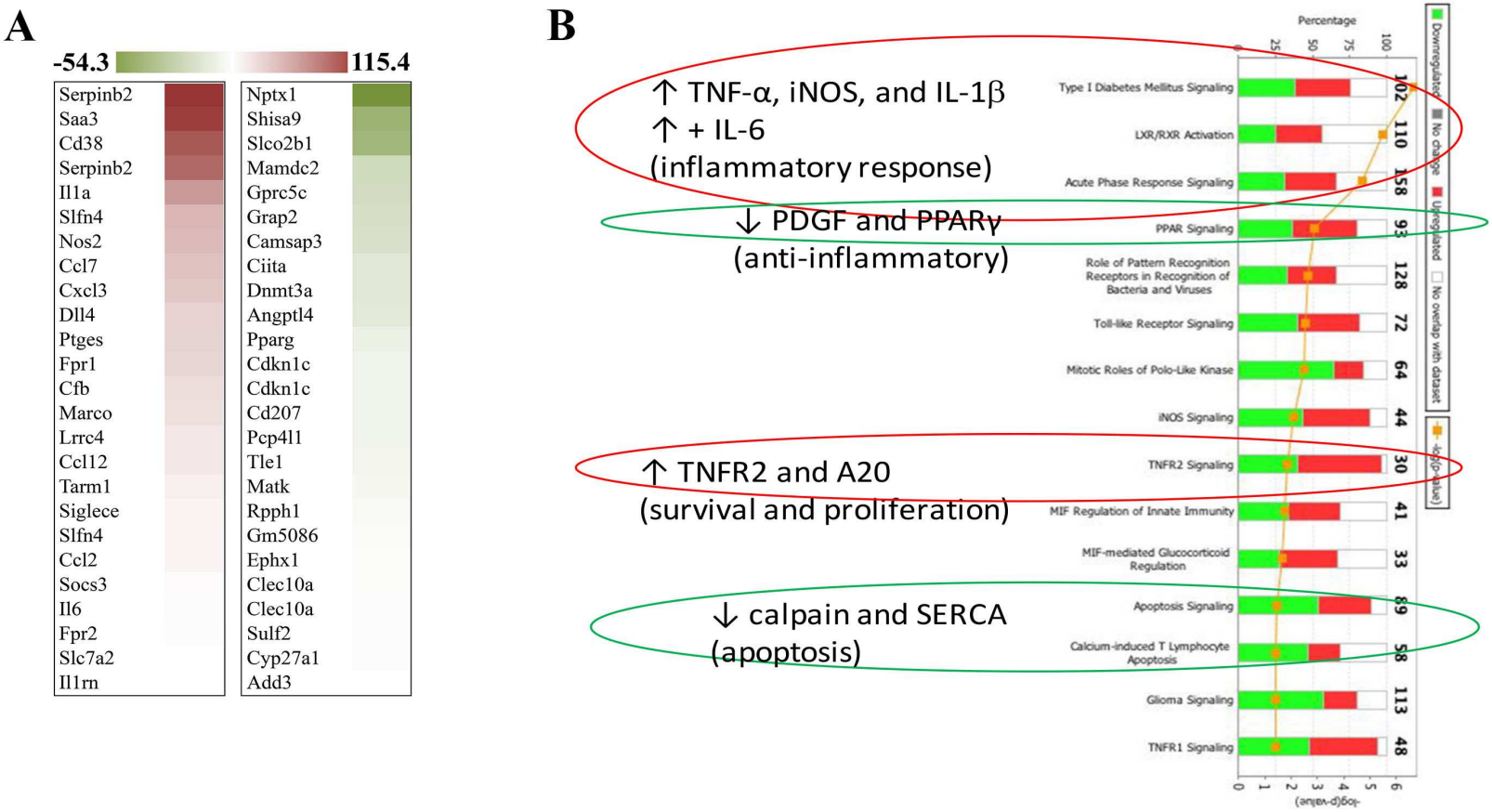

Figure 5.10 Ingenuity Pathway Analysis of PD-L1 antibody-treated macrophages showed inflammatory phenotype, increased survival and proliferation, and decreased apoptosis. Ingenuity Pathway Analysis of significantly up- and down-regulated genes was next used to identify altered signaling pathways. (A) depicts the top 25 upregulated (left, red) and downregulated (right, green) genes and the genes were classified into signaling pathways in (B), with the yellow line depicting statistical significance. Upregulated values are shown in red and downregulated values in green. These data were generated using macrophages from 9 mice, with 3 mice in each treatment group.

Accumulation of activated macrophages in tumors following PD-L1 antibody treatment and slowed tumor growth in RAG$^{-/-}$ mice. The *in vivo* effects of PD-L1 antibody treatment on TAM were investigated in mice with established B16 tumors. This tumor model was chosen due to recent studies conducted in our lab which showed high expression of PD-L1 on macrophages in B16 tumor tissues[204]. Treatment of mice with PD-L1 antibody triggered a significant increase in numbers of TAM, while the numbers of macrophages in the spleen and lymph nodes of PD-L1 antibody-treated mice were not altered **(Figure 5.11A)**. The numbers of CD4$^+$ and CD8$^+$ T cells in these tumors were also increased **(Figure 5.11B)**. In agreement with our *in vitro* data, TAM from PD-L1 antibody-treated mice had significantly increased MHC II expression **(Figure 5.11C)**. There was also a large overall increase in F4-80$^+$ macrophages in tumor tissues, including MHC II$^+$ macrophages **(Figure 5.11D)**.

To demonstrate the macrophage response to PD-L1 antibody treatment in both a different tumor model and in the absence of T cells, PyMT tumors were implanted into Rag$^{-/-}$ mice. There was no difference in the number of TAM in this tumor model **(Figure 5.11E)**. However, increased numbers of TAM from mice that were treated with PD-L1 antibody expressed co-stimulatory molecules CD86 and MHC II suggesting activation of these cells **(Figure 5.11F)**. Furthermore, PD-L1 antibody treatment slowed tumor growth in mice compared to irrelevant antibody controls **(Figure 5.11G)**. These results suggest a significant protective effect mediated by TAM that are activated by PD-L1 antibody treatment.

Combined therapy with PD-1/PD-L1 antibodies induces early tumor regression and tumor-free survival. Because PD-L1 antibody treatment appeared to exert other effects on TAM in addition to simply blocking the PD-1 interaction on T cells, we predicted that combined treatment with

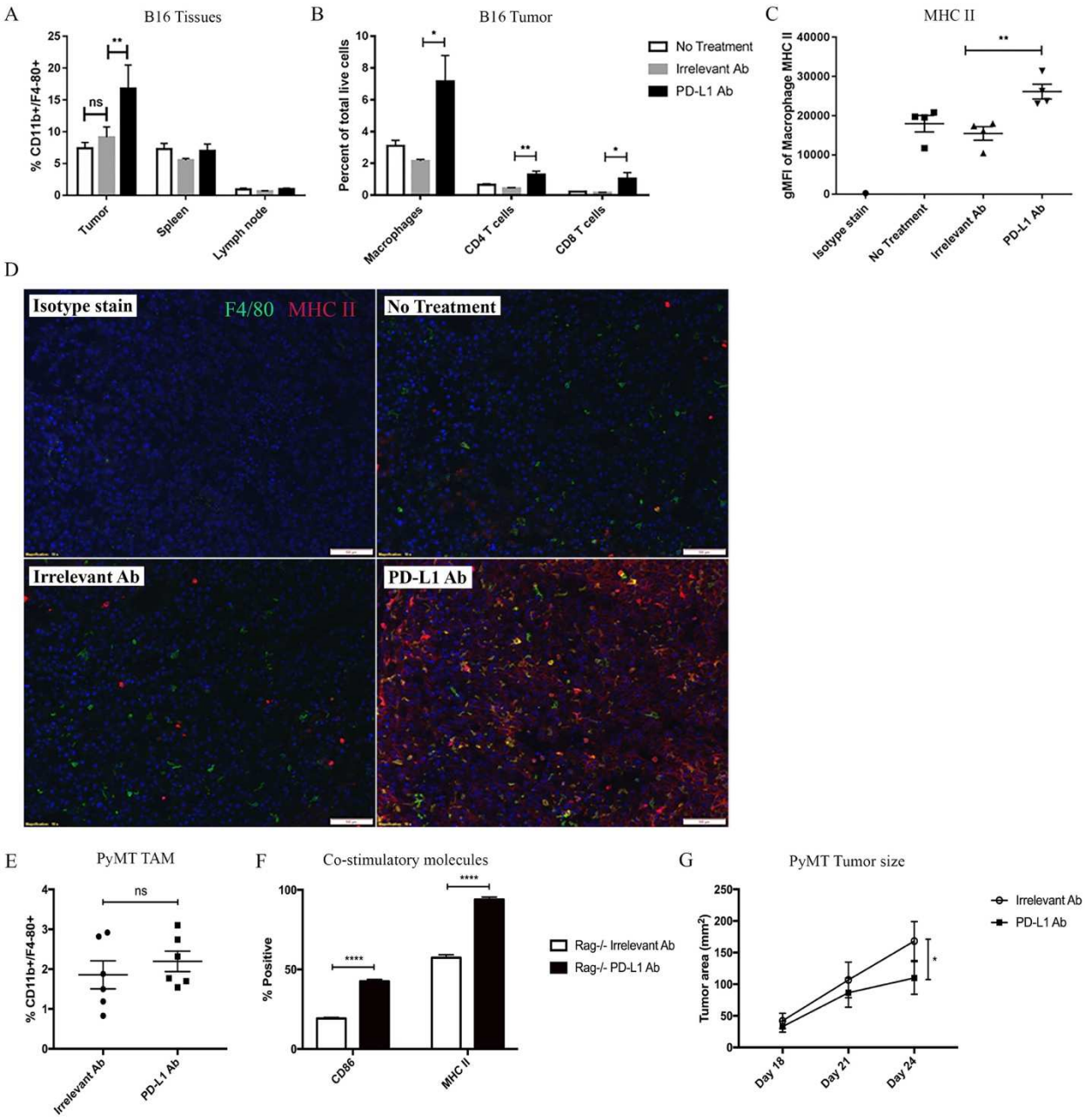

Figure 5.11 Accumulation of activated macrophages in tumors following PD-L1 antibody treatment and inhibited tumor growth in Rag$^{-/-}$ mice treated with PD-L1 antibody. B16 melanoma cells were injected into C57Bl/6 mice and the mice were treated with PBS, irrelevant isotype or PD-L1 antibody. Tumor, spleen, and lymph node tissue was harvested for flow cytometric analysis of macrophage populations (A) and percentages of tumor macrophages and T cells are shown in (B). We also measured surface expression of tumor macrophage MHC II (C) by flow cytometry, and tumor tissues were stained for F4-80 (green) and MHC II (red) expression before counterstaining with DAPI (blue) for imaging at 10X magnification (D). Statistical comparison of cell numbers and expression of surface markers were compared by two-way ANOVA using Prism7 software, and these data are representative of 2 repeated experiments with 4 mice in each group. Next, PyMT breast carcinoma cells were injected into Rag$^{-/-}$ mice and

the mice were treated with irrelevant isotype or PD-L1 antibody. Tumor tissue was harvested for flow cytometric analysis of macrophage percentages (E) and surface expression of costimulatory molecules (F). Tumor growth was also compared (G). Statistical comparison of cell numbers was completed using nonparametric t-test and both expression of surface markers and tumor sizes were compared by two-way ANOVA using Prism7 software. Statistically significant differences were denoted P-value * < 0.05, ** < 0.005, *** < 0.0005, and **** < 0.0001, and these are pooled data from 2 experiments for a total of 6 mice in each group.

PD-1/PD-L1 antibodies would induce significantly greater antitumor activity than treatment with either antibody alone. To address this question, B16 melanoma tumor-bearing mice with palpable cutaneous tumors were treated with PD-1 antibody, PD-L1 antibody, or a combination of PD-1 and PD-L1 antibodies. We observed that treatment with PD-1 and PD-L1 antibodies alone resulted in delayed tumor growth and slightly improved survival compared to sham-treated mice **(Figure 5.12A and 5.12B)**. Remarkably, combination PD-1/PD-L1 antibody therapy induced early tumor regression and, eventually, complete tumor rejection. Furthermore, survival was significantly improved, with 50% of animals being tumor-free out to 68 days post treatment.

Discussion.

PD-L1 is a co-inhibitory checkpoint molecule known for its role in dampening T cell responses. However, studies have found that PD-L1 blockade in patients whose tumor cells do not express PD-L1 is still effective in inducing tumor regression[102,103]. A recent study also showed that treatment of tumor-bearing mice with PD-L1 antibody decreased tumor growth in mice deficient in T cells, which points to a myeloid cell-dependent mechanism of action[230]. TAM significantly contribute to overall tumor immune-suppression, and TAM express high levels of PD-L1 in many tumor types[99,204]. Several reports have suggested additional roles for PD-L1 in regulating tumor cell and dendritic cell activity[193,194,196,197,199-203], but little is known concerning its role in regulating macrophage functions.

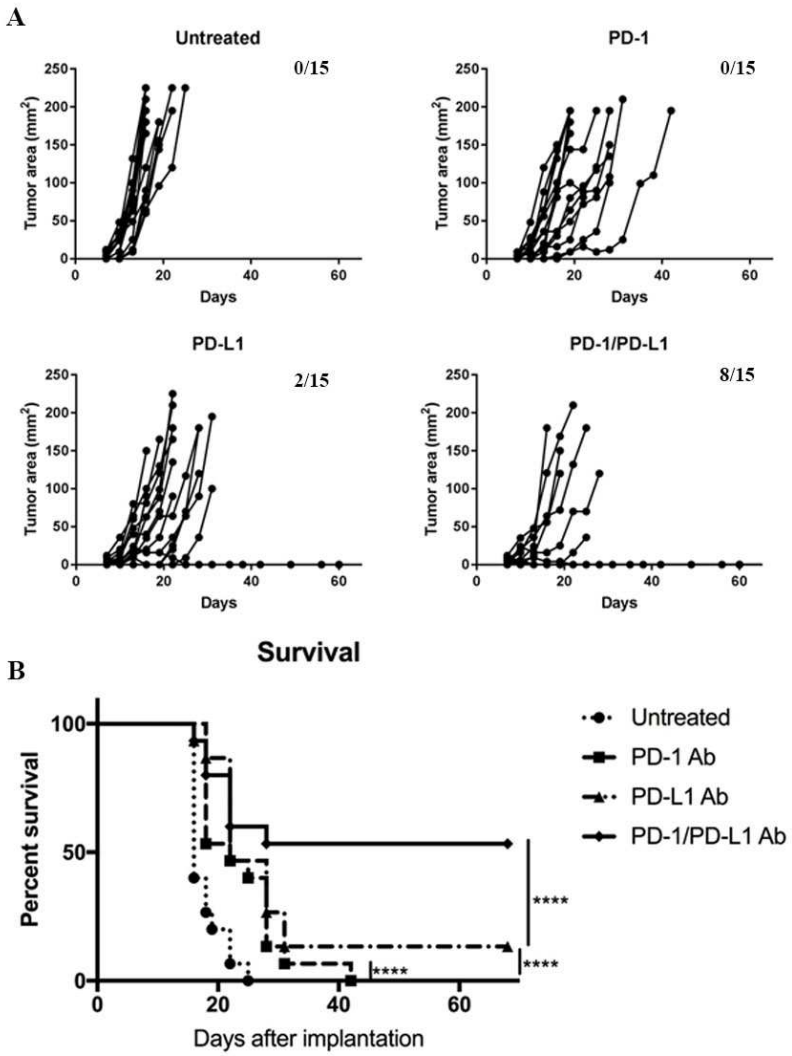

Figure 5.12 Combined therapy with PD-1/PD-L1 antibodies induces early tumor regression and tumor-free survival. B16 were implanted into C57Bl/6 mice and mice were treated with PD-1 antibody, PD-L1 antibody, or a combination of PD-1 and PD-L1 antibodies. Tumor growth was measured every 3 days and the number of tumor-free mice at the end of the study is noted for each group (A). Survival curves are shown in (B). Plots represent pooled data from 2 individual experiments for a total of 15 mice per group. Survival curves were compared by Kaplan Meier and log rank (Mantel Cox) test using Prism7 and statistically significant differences were denoted P-value **** < 0.0001.

Key findings from our studies were that PD-L1 antibody-treated macrophages exhibited significantly greater proliferation, enlargement, and survival, compared to irrelevant antibody

treated macrophages. The PD-L1 antibody-treated macrophages were also activated, as revealed by upregulation of co-stimulatory molecules and spontaneous pro-inflammatory cytokine production. Furthermore, we confirmed changes in macrophage morphology and increased co-stimulatory markers on human macrophages treated with PD-L1 antibody. These findings are particularly remarkable, as M-CSF-cultured macrophages have been shown to have weak ability to produce pro-inflammatory cytokines, even with LPS stimulation[231]. Overall, the picture that emerges is that of the PD-L1 antibody-treated macrophage as an activated, proliferating macrophage with a pro-inflammatory phenotype.

Other studies confirmed the apparent negative signaling properties of PD-L1 expressed by macrophages. For example, treatment with soluble CD80 completely recapitulated the phenotype of PD-L1 antibody-treated macrophages. In addition, treatment with soluble PD-1 and untreated bone marrow derived macrophages generated from PD-L1$^{-/-}$ mice also exhibited a partially activated phenotype. These data suggest that CD80 acts as a ligand for PD-L1 to activate macrophages, and that treatment with PD-L1 antibody mimics this interaction. These findings expand on the known co-stimulatory role of CD80 in T cell activation to also implicate CD80 in stimulating macrophage activation. On the other hand, PD-1 is known to restrain T cell activation and these data suggest that it partially activates macrophages. These seemingly conflicting roles may be part of a system of maintaining homeostasis, by maintaining some level of immune activation to prevent an overly immune-suppressed environment.

It is unclear why soluble PD-1, or genetic ablation of PD-L1 expression, did not fully recapitulate the phenotype of PD-L1 antibody-treated macrophages, but it is possible that the strength of the signal delivered by PD-L1 antibody and by soluble CD80 is much stronger than that delivered by soluble PD-1. In the case of genetic deletion of PD-L1 in PD-L1$^{-/-}$ mice,

compensatory pathways to regulate macrophage activation may have arisen during mouse development. These findings also raise the important point that not all PD-L1 antibodies are likely to be equivalent with agonistic or antagonistic properties, and that additional *in vitro* screening may be required to identify macrophage-activating clones. In support of this, we found that a different anti-murine PD-L1 antibody (clone MlH5) failed to induce the macrophage activating effects of the 10F.9G2 clone (data not shown).

We found that inhibiting the mTOR pathway could partially reverse the macrophage-activating effects of PD-L1 antibody treatment. Our studies also revealed upregulated mTOR pathway activity in macrophages following treatment with PD-L1 antibody. This is particularly significant because Akt/mTOR signaling regulates macrophage proliferation, activation, and metabolism[232,233]. Thus, PD-L1 may deliver a negative, constitutive signal to downregulate macrophage activation in the TME. Therefore, one of the major effects of *in vivo* treatment with PD-L1 antibodies may be to reverse the immune suppressive phenotype of TAM. It is also possible that treatment with PD-L1 antibody blocks its interaction with PD-1 on macrophages, which has been found to inhibit TAM phagocytosis and anti-tumor immunity[207].

Transcriptome profiling showed that PD-L1 antibody treatment changed the macrophages to a pro-inflammatory phenotype with increased survival and decreased apoptosis. Genes classically associated with pro-inflammatory macrophages were upregulated as well as chemoattractant and survival genes. Top downregulated genes included anti-inflammatory genes. Furthermore, IPA analysis yielded multiple upregulated inflammatory pathways, one downregulated anti-inflammatory pathway, one upregulated survival and proliferation pathway, and two downregulated apoptosis pathways. We did not find the mTOR pathway to be significantly altered at the level of gene expression. However, both our data as well as a previous

study[199] found that components of the mTOR pathway were changed at a phosphorylation level upon PD-L1 antibody treatment, versus at the level of transcription.

In the B16 tumor model, tumor-bearing mice treated with PD-L1 antibody exhibited increased numbers of TAM and these macrophages upregulated co-stimulatory molecule expression. As a result, it is likely that these macrophages were more inflammatory than TAM from sham-treated animals. Interestingly, some tumor cells themselves appeared to upregulate MHC II expression in animals treated with PD-L1 antibody, possibly mediated by IFN-γ released by increased numbers of tumor-infiltrating T cells. PyMT tumors established in mice lacking T cells ($Rag^{-/-}$) also showed increased inflammatory activation of TAM following PD-L1 antibody treatment. Furthermore, these animals had inhibited tumor growth compared to animals treated with irrelevant antibody. Recent reports demonstrated that reprogramming macrophages to a pro-inflammatory phenotype inhibited tumor progression and metastasis[234,235], which supports our findings that PD-L1 antibody-treated TAM had significant anti-tumor effects *in vivo*.

The use of PD-1- and PD-L1-blocking antibodies has shown great promise for tumor immunotherapy. However, checkpoint molecule blockade alone with a single antibody is not as effective as combined treatment, as revealed in patients treated with the combination of PD-1 and CTLA-4 antibodies[236]. Our findings suggest that the combination of PD-1 and PD-L1 antibodies, which heretofore would have been considered redundant, may in fact exhibit synergistic antitumor activity due to the additional activities of PD-L1 antibodies on macrophage function. For example, combined treatment with PD-1 and PD-L1 antibodies in mice with established B16 tumors resulted in complete tumor rejection in 50% of animals, compared with 0% of PD-1 antibody-treated animals and 10% of PD-L1 antibody-treated animals. The potency of combined PD-1/PD-L1 antibody therapy is likely due to the targeting of distinct, non-

overlapping cell populations in the tumor. Thus, it is reasonable to propose that combined therapy with PD-1 and PD-L1 antibodies be evaluated in clinical trials in suitable cancer patients, including those that may have failed treatment with PD-1 antibody alone.

CHAPTER 6

Final Conclusions and Future Directions

Immunotherapy has become one of the most promising modalities of cancer treatment, owing in part to the success of antibodies that bind immune checkpoint molecules. Among these checkpoints is the PD-1/PD-L1 signaling axis, which has been targeted to improve anti-tumor T cell responses in patients. These efforts have resulted in the FDA approval of several monoclonal antibodies for treatment of a variety of different cancers[85-87]. However, it has become apparent that only a small subpopulation of patients experience durable responses to immunotherapeutic agents, including those targeting PD-1 and PD-L1[95]. Thus, top priorities for the field include developing animal models that more readily translate to human medicine, and expanding our understanding of immune checkpoints to identify biomarkers that can predict patient response to immunotherapy.

The field of comparative oncology has recognized that spontaneously-occurring cancers in dogs is a precious resource for modeling human disease, and that progresses achieved in treating human disease can be translated to veterinary medicine. As a result, the success of human immunotherapy has prompted the development of canine-specific antibodies that are rapidly being tested for the treatment of canine cancers. We, and others, have shown that PD-1 and PD-L1 antibodies activate canine T cells, and the first use of a canine PD-L1 antibody in dogs with cancer showed promising anti-tumor activity[132,133]. However, little is known concerning the expression of immune checkpoints and their regulation in canine tumors. We therefore completed studies in Chapter 2 that revealed constitutive PD-L1 expression by all canine tumor cells lines screened, and inducible PD-L1 expression by primary canine

macrophages. We further showed that IFN-γ was primarily responsible for upregulation of PD-L1 expression by both cell types, though the tumor cells lines were responsive to stimulation by specific TLR ligands. These studies suggest that increased expression of PD-L1 is a mechanism of tumor-induced immune suppression in canine tumors, and that dogs with cancer may benefit from PD-1 and PD-L1 antibody blockade.

PD-1 antibody therapy is FDA approved for Hodgkin lymphoma and has been shown to be highly effective in the treatment of other human lymphomas[160-162]. Therefore, in the studies presented in Chapter 3, we conducted an in-depth profiling of PD-1 and PD-L1 expression by canine lymphoma. We found increased expression of PD-L1 by BCL compared to healthy B cells, and low to negative expression of both PD-1 and PD-L1 by all T cells analyzed. This suggests that BCL may be a promising disease for treatment with immunotherapy. However, TCL should not be discounted due to the lack of PD-1 or PD-L1 expression by malignant T cells, as studies have found PD-L1 antibody blockade to induce tumor regression even in cases where the tumor cells did not express PD-L1[102,103]. We believe this activity to be caused in part by the inhibition of PD-L1 signaling and concurrent anti-tumor activation of macrophages, as discussed in Chapter 5. This is supported by studies of human cancers that found increased PD-L1 expression by tumor-infiltrating immune cells to correlate with increased response to PD-L1 antibody therapy[99,157].

Most canine lymphoma patients develop drug-resistant disease within one year despite standard of care chemotherapy. The reasons for this are multifactorial, but emergence of drug-resistant cancer stem cell (CSC) populations is likely to play a dominant role. In Chapter 3, we show upregulated expression of both PD-1 and PD-L1 by canine lymphoma cell lines following selection by prolonged, high-dose chemotherapy treatment. Recent studies report a role for PD-

L1 in promoting epithelial-to-mesenchymal transition and tumor metastasis, and PD-L1 signaling has been found to increase CSC proliferation and resistance to apoptosis in response to chemotherapy[193-198]. Thus, studies should be conducted to determine the effect of PD-L1 signaling in canine lymphoma cells, and the effect of PD-L1 antibodies on CSC phenotype. It is plausible that increased PD-L1 expression in response to chemotherapy treatment not only shields CSC from immune-killing but also decreases their susceptibility to apoptosis and increases their ability to metastasize. If this is the case, dogs with lymphoma could benefit from combining chemotherapy to reduce the bulk of disease and PD-L1 antibody therapy to target CSC.

Interestingly, we identified a population of dual-positive PD-1^+/PD-$L1^+$ tumor-infiltrating T cells in dogs with BCL that was not present in healthy dog lymph nodes, and similar dual-positive cells have not been observed in the peripheral blood of dogs with cancer[132]. Future studies are warranted to elucidate the functional phenotype and the significance of these cells, as well as the factors that regulate their dual expression of PD-1 and PD-L1. PD-1 expression is historically known to be induced upon immune cell activation, and PD-L1 is primarily regulated by environmental cues. For example, in Chapter 4 we described a novel signaling loop in the tumor environment that parallels the regulation of PD-L1 on many cells by IFN-γ, in which a pro-inflammatory mediator (TNF-α) secreted by macrophages in response to a tumor cell-secreted protein (versican) stimulates the upregulation of an immune-suppressive molecule (PD-L1) in an autocrine fashion. It is likely that activation of tumor-infiltrating T cells in dogs with BCL increases their PD-1 expression, and secretion of inflammatory cytokines results in the induction of PD-L1 expression by the same cells. Similar to the case of PD-L1 expression by

canine lymphoma cells, the effect of PD-L1 signaling on tumor-infiltrating T cells should be investigated as well as the result of dual signals by PD-1 and PD-L1 in the same cell.

PD-L1 is primarily known as a ligand that binds PD-1 to deliver a negative signal to T cells, and the anti-tumor activity of PD-L1 antibodies has historically been attributed to the ability of the antibodies to stimulate an increase in the number and activation of T cells and to a decrease in the number and suppressive properties of Tregs[29,48,76-79]. However, the studies we described in Chapter 5 demonstrate a previously undescribed negative signaling role for PD-L1 on macrophages that results in significant anti-tumor activity when altered by PD-L1 antibody treatment. We found that PD-L1 constitutively signals in macrophages to induce or maintain an immune-suppressive phenotype, and that treatment with PD-L1 antibody or ligation with CD80 blocks this signaling to reprogram the macrophages to an inflammatory phenotype. These findings have important implications due to the increased expression of PD-L1 on TAM that we showed in Chapter 4. PD-L1 positivity in tumors, specifically by tumor macrophages, has been shown to be predictive of the efficacy of both PD-1 and PD-L1 antibody therapy[99,102,103]. This is indicative of the importance of these studies describing the previously unknown role of PD-L1 signaling in regulating macrophage activation, and also provides rationale for the use of PD-L1 expression by TAM as a biomarker. Therefore, expression of PD-L1 by tumor macrophages in patients should be taken into consideration for understanding how to optimize utilization of checkpoint molecule blockade with PD-L1 antibody, either in combination with PD-1 antibody or with other T cell expressed co-inhibitory checkpoint molecules. Another factor to be studied is the effect of PD-L1 signaling and PD-L1 antibody treatment on tumor cells, since it has been shown to alter tumor cell functions such as metabolism, survival, and proliferation[199-201]. This

may drastically change the desired effect of PD-L1 antibody therapy, since it will likely have off-target effects on tumor cells rather than simply activating T cells and macrophages.

Emerging evidence suggests that metabolic shifts are crucial in regulating macrophage functions[237]. However, metabolic processes that support changes in macrophage polarization is poorly understood[233]. Different macrophage activation states are hypothesized to be supported by distinct metabolic programs based on necessary functions. For example, inflammatory macrophages upregulate glycolysis to rapidly produce ATP that is needed during acute infections, and immune-suppressive macrophages utilize β-oxidation to more efficiently produce ATP for defending against slow-growing parasites[238]. It is likely that the phenotypic changes we observed in macrophages following PD-L1 antibody treatment are supported by metabolic shifts in these cells. The field of immunotherapy would greatly benefit from studies that focus on understanding the regulation of macrophages and other immune cells by immune checkpoint molecules at a metabolic level.

In summary, these studies describe the characterization of PD-L1 expression and regulation by canine tumors and macrophages as well as the discovery of a novel role for PD-L1 as a signaling molecule in macrophages. This work provides crucial knowledge for the development of checkpoint molecule inhibitors for canine immunotherapy, and further describes canine lymphoma as a potential target for PD-1 and PD-L1 blocking antibodies. It also shows a previously undescribed means of tumor-mediated immune-suppression via the upregulation of PD-L1 on macrophages. This high expression of PD-L1 suppresses not only T cell responses but also macrophage anti-tumor activity, and blocking PD-L1 with antibodies activates both cell populations. Finally, we demonstrate a novel mechanism for the effectiveness of PD-L1 antibody blockade and provide insight into the differences between PD-1 and PD-L1 antibody therapies.

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