Immune regulation of canine tumour and macrophage PD-L1 expression


Department of Clinical Sciences, Flint Animal Cancer Center, Colorado State University, Ft. Collins, CO, USA

Abstract
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 immune regulation of PD-L1 expression by tumor cells and tumor-infiltrating macrophages in dogs. Therefore, 14 canine tumor cell lines, as well as primary cultures of canine monocytes and macrophages, were evaluated for constitutive PD-L1 expression and for responsiveness to immune stimuli. We found that PD-L1 was expressed constitutively on all canine tumor cell lines evaluated, although the levels of basal expression were very variable. Significant upregulation of PD-L1 expression by all tumor cell lines was observed following IFN-γ exposure and by exposure to a TLR3 ligand. Canine monocytes and monocyte-derived macrophages did not express PD-L1 constitutively, but did significantly upregulate expression following treatment with 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 the upregulation of PD-L1 expression by tumor cells and by tumor-infiltrating macrophages 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.

Introduction
The adaptive immune system depends on a network of numerous signals, both stimulatory and inhibitory. Balance in these signals is crucial to maintain self-tolerance while still protecting the host from pathogens and diseases like cancer. Under normal conditions, immune checkpoint molecules prevent autoimmunity by downregulating the tempo of T-cell responses. Dysregulation of immune checkpoint molecule expression is, however, a mechanism by which many cancers evade elimination by the host. The PD-1/PD-L1 axis is an important immune checkpoint pathway that plays a critical role in maintaining peripheral tolerance by downregulating T-cell activation and proliferation.1

Programmed death protein 1 (PD-1) is a cell surface protein molecule commonly upregulated on tumour-infiltrating lymphocytes,2 and its primary role is to limit the activity of T-cells during inflammatory responses.3 It is induced on T-cells upon activation as a mode of self-regulation and is highly expressed on regulatory T-cells, which is thought to enhance their proliferation to provide physiological homeostasis.4 Expression of the transmembrane-protein programmed death-1 ligand 1 (PD-L1) is increased on many tumours,5 and PD-L1 is also expressed on tumour-infiltrating lymphocytes.6 Tumour-associated macrophages express PD-L1 as well and this is thought to be one of the mechanisms by which these macrophages immunosuppressive effects in the tumour environment.7–9 Chronic exposure to antigen and to PD-L1 leads to T-cell exhaustion, which is characterized by poor effector function, low proliferative activity and the inability to persist as memory T-cells.10

PD-L1 expression on human tumour 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 oesophageal cancers. In mice, PD-L1 has been found to be expressed by ovarian, myeloma, lung, melanoma and mammary cancers, where it is involved with the escape of the tumour cells from the immune system. A recent study utilizing bovine lymphocytes reported that crosslinking of PD-L1 by PD-1-Ig increased cell death and decreased cytokine production in PD-L1 expressing cells, suggesting a role for PD-L1 in inducing immunosuppression. The upregulation of PD-L1 in inflamed tumours has been found to be mediated primarily by IFN-γ produced by tumour infiltrating T-cells in humans and mice.

The innate immune response has also been found to play a role in the regulation of PD-L1 expression in tumours. Toll-like receptors (TLRs) can recognize a wide range of pathogen-associated molecular patterns (PAMPs), which may also include certain tumour antigens. TLR expression is upregulated on many human tumours including colon, gastric, prostate, breast, ovary and brain tumours, where signalling by the TLRs has been implicated in augmenting the secretion of immune-modulatory cytokines. For example, TLR2 and TLR4 ligands have been shown to protect leukaemia blast cells found in acute myeloid leukaemia from T-cell killing through the induced expression of PD-L1.

Canine PD-1 and PD-L1 genes are conserved among canine breeds, and cross-reactive antibodies that also recognize canine PD-L1 have been identified. However, the expression of PD-L1 by a broad panel of different canine tumour types has not been previously investigated, nor has the immune regulation of canine PD-L1 expression on tumour cells been studied. Therefore, the purpose of the current study was to investigate PD-L1 expression by a series of canine tumour 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 expressed by tumour-infiltrating macrophages in humans, we investigated the expression of PD-L1 by canine macrophages generated from blood monocytes, and the effects of cytokines on macrophage PD-L1 expression. A recently developed canine PD-L1 antibody was used in these studies, and expression of PD-L1 by canine tumour cell lines and canine monocytes and macrophages was assessed by flow cytometry and by immunocytology. The tumour PD-L1 response to treatment with recombinant canine IFN-γ and TLR ligands was also assessed, as was the PD-L1 response to cytokine-enriched conditioned medium derived from activated canine T-cells.

We found that all canine tumour cell lines screened expressed low basal levels of PD-L1, though there was a high degree of variability between tumour cell lines, especially amongst haematopoietic-origin tumours. In all tumour lines evaluated, PD-L1 expression could be strongly upregulated by IFN-γ and certain TLR ligands. In contrast, canine monocytes and monocyte-derived macrophages did not constitutively express PD-L1 though canine monocyte-derived macrophages were responsive to treatment with IFN-γ and exhibited significant PD-L1 upregulation. These findings indicate that canine tumours may broadly express the checkpoint molecule ligand PD-L1, and that upregulation of PD-L1 expression on tumour cells and tumour infiltrating macrophages by T-cell cytokines such as IFN-γ may be an important determinant of overall tumour immunity in dogs.

Materials and methods

Cell lines

Fourteen different canine tumour cell lines were evaluated in this study. All cell lines were validated and screened to be genetically unique. 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 tumour (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).
Tumour cell culture

Tumour cells were grown in MEM medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO, USA) and 5% CTM [10 000 μg/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, USA) and strongly adherent cells were harvested by treatment with 0.25% Trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) (Gibco) followed by Trypsin/EDTA inactivation with media. Viability of cells was determined using 0.4% trypan blue stain (Gibco) for live and dead cell discrimination.

After tumour cells were harvested from culture, 1.0 × 10^5 viable cells were plated in triplicate wells of 24-well polystyrene cell culture plates (Falcon, Durham, NC, USA). The cells were then treated with cytokines, supernatants from Concanavalin A (ConA) activated peripheral blood mononuclear cells (PBMCs), or TLR ligands for 24 h at 37 °C. The supernatants were generated by incubating primary canine PBMCs in 10 μg/mL conA (Sigma-Aldrich, St. Louis, MO, USA) 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 lymphocyte separation medium (LSM; MP Biomedicals, Solon, OH, USA) 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 h in culture and adherent monocytes were cultured for seven days in 10 ng/mL human M-CSF (Peprotech, Rocky Hill, NJ, USA) 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 tumour culture medium. Tumour cells were cultured in the presence of IFN-γ in complete medium for 24 h 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, (pI:C) was purchased from InVivogen (San Diego, CA, USA) and was used at 10 μg/mL. Plasmid DNA (pDNA) was produced by Juvaris Biotherapeutics, Inc (Pleasanton, CA, USA) and used at 10 μg/mL. Lipopolysaccharide (LPS, E. coli serotype 0111:B4) was obtained from Sigma-Aldrich and used at 1 μg/mL. R848 from InVivogen was used at 10 μg/mL. These concentrations were chosen based on the working concentration ranges established by in vitro titration studies and by ranges suggested by manufacturers. The activity of TLR agonists in stimulating canine TLRs was assessed by stimulation of canine monocyte-derived macrophages and measurement of IL-6 secretion (data not shown).

Antibodies

A murine anti-canine PD-L1 monoclonal antibody (clone 4F9) was used in these studies to detect PD-L1 expression. This antibody was developed by Merck Research Laboratories (Kenilworth, NJ, USA) and was used for flow cytometry, immunofluorescence staining and Western blotting studies. An isotype matched, irrelevant antibody was used at the same concentration (eBioscience, San Diego, CA, USA). Binding of mouse mAbs was detected using a labelled donkey anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Mouse anti-human CD11b (clone Bear1) was obtained from Immunotech-Beckman Coulter (Marseilles, France) and used for flow cytometric evaluation of primary canine macrophages. A polyclonal goat anti-canine IFN-γ antibody from Novus Biologicals (Littleton, CO, USA) was...
PD-L1 expression by canine tumour cell lines. Canine tumour cells were cultured in 24-well plates overnight in complete medium and stained the following day for detection of PD-L1 expression by flow cytometry. The cells were incubated with the appropriately matched concentration of irrelevant isotype (grey filled line) or PD-L1 antibody (bold white line) and the data were analysed using FlowJo Software. PD-L1 expression was determined with respect to the geometric mean fluorescence intensity of the isotype. This experiment was repeated four times with similar results each time.

Western blot

A standard Western blotting protocol from (Bio-Rad, Hercules, CA, USA) was followed. Briefly, the tumour cells were harvested by scraping, then lysed in the presence of protease inhibitors (Thermo Fisher Scientific, Waltham, MA, USA) 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 μg total protein was loaded into a 4–15% gradient mini PROTEAN TGX BioRad gel. Non-fat dry milk (5%) 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.
Regulation of canine PD-L1 expression

Figure 2. PD-L1 expression by canine tumour 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 (red) or irrelevant isotype antibody and with DAPI nuclear stain (blue). The coverslips were mounted on glass slides and the images were obtained using confocal microscopy, with instrument setting held constant between slides in order to compare the relative intensity of PD-L1 staining between tumour cell lines.

Flow cytometry

Tumour cells were harvested from culture using 0.25% trypsin/1 mM EDTA (Gibco), washed with PBS and incubated in 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, USA) were added to tumour cells for 20 min 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, USA). 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 analysed using FlowJo Software (Ashland, OR, USA).

Monocyte-derived macrophages were detached from tissue culture plastic with 2 mM 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 for tumour cells.

Immunofluorescence imaging

Tumour 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 min, and rehydrated in 1X PBS prior to staining. After blocking with 5% donkey serum and Streptavidin block (Vector Laboratories, Burlingame, CA, USA), 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
Figure 3. Effect of IFN-γ stimulation on PD-L1 expression by canine tumour cells. (A) Effects of tumour incubation with titrated concentrations of recombinant canine IFN-γ. Canine melanoma, osteosarcoma, transitional cell carcinoma and histiocytic sarcoma cells were cultured with 0 (grey line), 1 (dotted line), 10 (dashed line) or 100 ng/mL rcIFN-γ (bold line) in complete medium overnight. The cells were then immunostained for detection of PD-L1 expression, and flow cytometry was used to compare the mean fluorescence intensity of each treatment group. This titration experiment was conducted four times, with similar results each time. (B) Treatment of all canine tumour cell lines with 10 ng/mL IFN-γ. Tumour cell lines were treated with 10 ng/mL rcIFN-γ overnight and stained with an irrelevant isotype antibody (grey filled line) or PD-L1 antibody (unstimulated is the dotted line and IFN-γ treated is the bold line) and analysed by flow cytometry for PD-L1 expression. These data are representative of six different independent experiments.

Probes, Eugene, OR, USA) and mounted onto Superfrost slides (VWR, Radnor, PA, USA) with Fluoromount Aqueous Mounting Medium (Sigma-Aldrich).

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

RT-PCR

Total RNA was isolated from canine tumour cell lines with and without IFN-γ treatment using an RNeasy Mini Kit (Qiagen, Frederick, MD, USA), 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 $= 2^{-\Delta\Delta Ct}$ 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 tumour cell lines, using flow cytometric analysis. We found all 14 tumour cell lines expressed PD-L1 under basal conditions, with the two histiocytic sarcoma cell lines expressing the highest levels (Fig. 1). The cell lines with the lowest levels of surface PD-L1 expression were the lymphoma cell line and the two hemangiosarcoma cell lines. We also assessed the intracellular expression of PD-L1 by fixing and permeabilizing tumour cells on coverslips and assessing PD-L1 expression microscopically (Fig. 2). We found that all four tumour cell lines screened expressed PD-L1, when staining intensity was compared with that of the isotype control antibody.

IFN-γ has been reported to upregulate PD-L1 expression in human and mouse systems. Therefore, the effects of canine rIFN-γ on PD-L1
expression on canine tumour cell lines was assessed. We found that there was a titratable PD-L1 upregulation effect from exposure of tumour cells to increasing concentrations of rIFN-γ (Fig. 3A). A mid-range concentration of IFN-γ (10 ng/mL) was thereafter selected for the remaining studies. This concentration of IFN-γ was found to upregulate PD-L1 expression by all the tumour cell lines evaluated except the lymphoma cell lines, though to differing degrees depending on the specific cell line (Fig. 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 five 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 directly correspond to the amount of PD-L1 protein expressed by the cells when compared between tumour cell lines (Fig. S1, Supporting Information). These results were also demonstrated using immunocytology, where PD-L1 expression was increased after treatment with IFN-γ (Fig. 4). Finally, the 4F9 canine PD-L1-specific antibody was shown to bind a protein of the correct predicted molecular weight of glycosylated PD-L1, 50 kDa. In addition, expression of PD-L1 was increased after treatment of histiocytic sarcoma cells with IFN-γ (Fig. 5).

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 (e.g., IL-4, GM-CSF, TGF-β) have been shown to upregulate PD-L1 expression on different murine cell types, we were interested to determine the net effect of all cytokines produced by activated T-cells on canine tumour PD-L1 expression. A diverse
Regulation of canine PD-L1 expression

Figure 5. 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. Cell lysates were prepared 24 h later and immunoblotted, as described in Methods. Lane 1 = MW standard, Lane 2 = HS-1 lysates, unstimulated, Lane 3 = HS-1 lysates, IFN-γ-stimulated.

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. The T-cell cytokine mixture was then evaluated for upregulation of tumour PD-L1 expression. We found that the T-cell cytokine mixture strongly upregulated PD-L1 expression (Fig. 6A), above PD-L1 expression levels observed following incubation with equivalent amounts of 8 ng/mL IFN-γ alone (Fig. 6B). These findings suggested that IFN-γ in combination with other T-cell cytokines can stimulate even greater upregulation of tumour 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 (Fig. 7). These findings suggest that IFN-γ is the primary cytokine produced by activated canine T-cells responsible for upregulating PD-L1 expression on canine tumour cells and macrophages, though other T-cell derived cytokines appeared to play a role as well in amplifying the overall PD-L1 stimulatory effect of IFN-γ.

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 tumour cells. Therefore, adherent canine monocytes were evaluated for PD-L1 expression after overnight culture (Fig. 9A) and again after 7 days in culture in human M-CSF (Fig. 9B). Freshly isolated monocytes expressed low to negative levels of PD-L1 following brief overnight...
Figure 6. Effect of cytokines produced by concanavalin A activated canine T-cells on tumour PD-L1 expression and comparison with IFN-γ. (A) PD-L1 upregulation following exposure to serial dilutions of supernatants from ConA-stimulated PBMCs. Canine melanoma, osteosarcoma, transitional cell carcinoma and histiocytic sarcoma cells were exposed to four dilutions of supernatants from ConA-stimulated PBMC overnight. Groups include medium only (light grey filled line), 1:100 dilution of ConA supernatants (dotted line), 1:10 dilution of Con A supernatant (dashed line) and 1:1 dilution of ConA supernatant with medium (bold line). The cells were then harvested and stained with PD-L1 for flow cytometric analysis as previously described. This experiment was repeated four times. (B) Comparison of PD-L1 upregulation by supernatants from ConA-stimulated PBMCs and by 4 ng/mL rIFN-γ. The same tumour cell lines tested in (A) were incubated overnight with medium only (grey filled line), 4 ng/mL rIFN-γ (dotted line), or a 1:1 dilution of PBMC ConA supernatants (bold line) and the cells were immunostained for PD-L1 expression 24 h later. These data are representative of three independent experiments.
Regulation of canine PD-L1 expression

Figure 7. IFN-γ neutralization in conditioned medium from conA-stimulated PBMCs. Canine histiocytic sarcoma cells were cultured overnight in regular media, conditioned medium from conA-stimulated PBMCs and conditioned medium with an IFN-γ neutralizing antibody, or conditioned medium with control antibody. The next day, the cells were then harvested and stained for PD-L1 upregulation by flow cytometric analysis, as described in Methods.

Discussion

In this study we observed that all the canine tumour cell lines evaluated expressed detectable levels of PD-L1 under basal (i.e., unstimulated) conditions (Figs. 1 and 2). Moreover, all the canine tumour cell lines were highly responsive to treatment with IFN-γ (Figs. 3 and 4). The histiocytic sarcoma lines were the most responsive to IFN-γ. In addition, the histiocytic sarcoma cell lines were also the most responsive to stimulation with several different TLR agonists (Fig. 8). 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 responsive to activation by TLR ligands.35,36

IFN-γ is recognized as the principle cytokine that upregulates PD-L1 expression on many tumour types in human and mouse models.25,26 Recently, it was reported that IFN-γ also upregulates PD-L1 expression on certain canine tumour lines when used at a high concentration.30 We observed that all of the canine tumour cell lines evaluated in the present study 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 suggested that the presence of other immune-modulating cytokines may further upregulate PD-L1 expression by canine tumour cells, or that other cytokines may increase the activity of IFN-γ in inducing PD-L1 upregulation. For example, it has been reported that IL-4 and TNF-α together can synergistically induce PD-L1 expression by human renal cell carcinoma cells.32 Thus, cytokines such as IL-4 and TNF-α from T-cells may potentially be active in upregulating PD-L1 expression on canine tumour cells.

Human tumour cells have been found to express various TLRs and to upregulate PD-L1 following activation by ligands for TLR2, TLR3 and TLR4.29,37 We found that the TLR3 agonist p: C induced upregulation of PD-L1 on melanoma, osteosarcoma, transitional cell carcinoma, and histiocytic sarcoma. Moreover, monocyte-derived macrophages also did not express PD-L1 even after 1 week in culture with human M-CSF. However, canine monocyte-derived macrophages were responsive to IFN-γ stimulation, as their PD-L1 expression was significantly increased (P-value = 0.001) after overnight treatment with canine rIFN-γ.

PD-L1 expression on primary tumours

Expression of PD-L1 by a canine histiocytic sarcoma tumour biopsy was assessed to compare the staining intensity to in vitro histiocytic sarcoma cell lines. Fresh frozen tumour samples were immunostained for PD-L1 expression. PD-L1 was found to be highly expressed in the histiocytic sarcoma tumour tissues, with expression located on both the cell membrane and within the cytoplasm (Fig. 10). The levels of in vivo expression by histiocytic sarcoma cells were similar to those expressed by the HS-1 cell line in vitro, suggesting that canine histiocytic sarcomas may be somewhat unique with respect to high levels of PD-L1 expression. A larger study is currently in progress to assess PD-L1 expression by canine tumours, using a panel of different canine tumour tissue biopsies (Faulhaber, et al., manuscript in preparation).
Figure 8. PD-L1 expression response to tumour cell activation with TLR ligands. Canine melanoma, osteosarcoma, transitional cell carcinoma and histiocytic sarcoma cells were treated with media only (grey filled line), pDNA (long dashed line), LPS (dotted line), pI:C (bold line) or R848 (dashed line) overnight before analysis for PD-L1 expression by flow cytometry. This experiment was repeated six times.

sarcoma cells, while all four TLR agonists evaluated (i.e., agonists for TLR3, TLR4, TLR7/8 and TLR9) triggered upregulated PD-L1 expression on canine histiocytic sarcoma cells. These data suggest that innate immune responses may also play a role in regulating canine tumour PD-L1 expression.

For the canine tumour cell lines evaluated in this study, PD-L1 expression was observed to be located on both the cell membrane and within the cytoplasm of the same cell. This is different than some studies of PD-L1 expression on human tumour cells, where PD-L1 has been reported to be expressed only on the plasma membrane or in the cytoplasmic compartment of tumour cells, even within the same tumour sample. Other studies of human tumour cells have demonstrated both cytoplasmic and surface staining of PD-L1 in the same cells. Our findings also add additional tumour cell lines and mechanistic insights to a previous study that evaluated PD-L1 expression by canine tumour cell lines.

Tumour-associated macrophages (TAM) comprise a large population of tumour stromal cells. We did not observe constitutive PD-L1 expression following short-term cultures of canine monocytes, which is in contrast to findings from a prior study in mice. We also have not observed expression of PD-L1 on circulating canine monocytes (Coy et al.; manuscript in preparation). However, we did find that treatment with IFN-γ
Figure 9. PD-L1 expression and regulation by IFN-γ in 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) Monocytes incubated with huM-CSF. Primary canine monocytes were cultured in huM-CSF for 1 week to drive macrophage differentiation (isotype is light grey filled and PD-L1 is bold). (C) Monocyte-derived macrophages stimulated with IFN-γ. Monocyte-derived macrophages cultured in M-CSF were treated overnight with rIFN-γ (isotype is light grey filled, baseline PD-L1 is dotted and IFN-γ treated is bold). These data are representative of results from three separate studies.

Figure 10. PD-L1 expression by canine histiocytic sarcoma tissue biopsy. Tumour biopsy was obtained from a dog with malignant histiosarcoma and imbedded in OCT and cryosectioned. Tumour tissues were immunostained as described in Methods and imaged by confocal microscopy. Intense expression of PD-L1 (red) was observed throughout the tumour section. Cell nuclei were stained with DAPI (blue). Immunostaining with an isotype control antibody is displayed in the inset.

significantly upregulated PD-L1 expression by cultured canine macrophages, which is in agreement with previous rodent studies. It should also be noted that the source of macrophages was different in our study (blood) as compared to 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, IFN-γ has also been shown to play a role in inducing the production of anti-inflammatory cytokines such as IL-1Ra and IL-18BP. These counter-regulatory, immune-suppressive activities of IFN-γ allow IFN-γ to also protect the host from tissue damage caused by uncontrolled inflammation. Therefore,
the induction of PD-L1 expression may be another means by which IFN-γ acts to dampen the duration and intensity of host inflammatory responses.

Upregulated expression of PD-L1 by tumour cells is an example of a phenomenon known as adaptive immune resistance. PD-L1 expression by tumour cells may therefore be useful as a marker of the level of adaptive immune resistance exhibited by tumours, as well as a predictor of the effectiveness of PD-1/PD-L1 blockade for cancer immunotherapy. It has recently been shown that the use of PD-L1 blocking antibodies enhances cytokine secretion by peripheral blood mononuclear cells and tumour-infiltrating cells in vitro in dogs, suggesting that PD-L1 blockade may be an effectiveness means of immunotherapy for certain canine tumours. Studies are currently underway in our laboratory assessing the patterns of PD-L1 expression by canine tumour cells in vivo (using tumour biopsies) to determine the degree of PD-L1 expression heterogeneity and tumour-type association (Faulhaber; manuscript in preparation).

The tremendous potential for checkpoint molecule blockade, based on the results of recent human clinical trials, suggests that such an approach also has considerable merit for treatment of canine cancer.

Acknowledgements

These studies were supported by Merck Research Laboratories and by the Shipley Foundation.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. qRT-PCR of canine tumour cell lines with and without IFN-γ treatment. Five canine tumour 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 $2^{-\Delta C_t}$.

References


32. Quandt D, Jasinski-Bergner S, Mueller U, Schulze B and Seliger B. Synergistic effects of IL-4 and TGFα on the induction of B7-H1 in renal cell carcinoma.


