

PD-L1: PD-1 Interaction Contributes to the Functional Suppression of T-Cell Responses to Human Uveal Melanoma Cells In Vitro

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PURPOSE. To assess the expression of PD-L1 on human uveal melanomas and its potential to suppress T-cell function.

METHODS. A panel of primary and metastatic uveal melanoma cell lines was evaluated for PD-L1 expression by RT-PCR and flow cytometric analysis. Uveal melanoma-containing eyes were examined for PD-L1 expression by immunohistochemistry. PD-L1 function was tested by coculturing IFN- γ -pretreated uveal melanoma cells with activated Jurkat T cells for 48 hours and assessing T-cell production of IL-2 by ELISA.

RESULTS. Five of the nine primary and one of the five metastatic uveal melanoma cell lines tested constitutively expressed PD-L1 protein at various levels. However, all primary and metastatic uveal melanoma cell lines upregulated PD-L1 expression after stimulation with IFN- γ . Immunohistochemistry demonstrated that PD-L1 was not expressed by primary uveal melanomas in situ. IL-2 production by activated Jurkat T cells was decreased significantly when the cells were cocultured with IFN- γ -pretreated uveal melanoma cells. More than 70% of IL-2 production was restored by addition of either anti-PD-L1 or anti-PD-1 antibody to the coculture assays ($P < 0.01$).

CONCLUSIONS. Expression of PD-L1 by uveal melanoma cells regulates T-cell function by suppressing IL-2 production. The results imply that the presence of IFN- γ in the tumor local microenvironment promotes upregulation of PD-L1 expression by uveal melanoma, which may, in part, promote immune escape by impairing T-cell function. The selective blockade of PD-L1 is a potential strategy in T-cell-based immunotherapy for uveal melanoma. (*Invest Ophthalmol Vis Sci.* 2008;49:2518–2525) DOI:10.1167/iovs.07-1606

Uveal melanoma is the most common intraocular tumor in adults. It occurs with a frequency of six to seven cases per 1 million adults.¹ Approximately 1500 new cases are diagnosed in the United States annually.² Although the incidence is less than 1% of the annual cancer registrations, the prognosis of uveal melanoma is poor. Sixty-two percent of patients die within 5 years from the time of diagnosis, and 90% die within 15 years.³ Liver metastases are the main cause of death. Up to 95% of the patients who die of uveal melanoma have liver metastases.^{4–7} Uveal melanoma metastases are difficult to treat, because they are resistant to most conventional therapies,

including chemotherapy and antiangiogenic agents.³ Although many therapies have been developed, the 5-year survival rate of patients with uveal melanoma has not improved in more than 25 years.^{1,4,8}

Immunotherapy is a novel approach to the treatment of metastatic uveal melanoma. Much effort has focused on active immunization strategies that are designed to promote expansion and differentiation of tumor antigen-specific T cells in vivo. Although tumor vaccines against uveal melanoma have achieved this goal, elevated numbers of tumor-specific T cells rarely control tumor regression.^{9,10} Adoptive transfer of in vitro expanded tumor-antigen-specific T cells is an alternative approach that results in the presence of an even greater number of activated T cells that can produce proinflammatory cytokines and kill tumor cells directly.^{11–13} However, CD8⁺ T-cell responses are infrequent and, when present, decline rapidly, which suggests that the tumor microenvironment can suppress the function of activated T cells, resulting in tumor escape from immune-mediated destruction. Our laboratory has been keenly interested in the evasive mechanisms that uveal melanomas use to escape immune surveillance.

Several factors have been implicated as tumor escape mechanisms, including both soluble and membrane-bound molecules, such as transforming growth factor (TGF)- β , interleukin-10, FasL, complement regulatory proteins, and tumor necrosis factor-related apoptosis inducing ligand (TRAIL).^{14–16} Recently, the cell membrane-bound molecule, PD-L1, has been added to the list of molecules that may contribute to the tumor's capacity to evade immune elimination. PD-L1 is a type 1 transmembrane glycoprotein belonging to the B7 family.^{17–20} Its receptor, PD-1, is a transmembrane protein belonging to the Ig superfamily that lacks the relevant motif for binding to B7-1 and B7-2.²¹ PD-1 is expressed on thymocytes,^{22,23} on mature T and B cells after activation,^{24–26} and on myeloid cells.²² Interaction of PD-1 with PD-L1 downregulates T-cell proliferation and cytokine production and induces T-cell apoptosis.^{27–29}

The purpose of our study was to assess the expression of PD-L1 on human uveal melanoma and its potential to promote tumor immune escape by modulating T-cell function.

MATERIALS AND METHODS

Cell Lines

All cell lines used in the study are shown in Table 1. Nine primary uveal melanoma cell lines designated OCM1, OCM3, OCM8, MEL202, MEL270, MEL285, MEL290, 92.1, and OM431, and five cell lines derived from metastases in human uveal melanoma patients, designated OMM1, OMM2.2, OMM2.3, OMM2.5, and OMM2.6 were used. OCM1, OCM3, and OCM8 were kindly provided by June Kan-Mitchell (University of California, San Diego, CA). MEL202, MEL270, MEL285, MEL290, OMM2.2, OMM2.3, OMM2.5, and OMM2.6 were kindly provided by Bruce Ksander (Schepens Eye Research Institute, Boston, MA). The primary uveal melanoma cell line OM431 was graciously given by Daniel Albert (University of Wisconsin, Madison, WI). The 92.1 cells were kindly provided by Martine Jager (University Hospital Leiden, Leiden, The Netherlands). OMM1 cells were isolated from a skin

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TABLE 1. Uveal Melanoma Cell Lines Used in This Study

Cell Line	Origin of Tumor or Cell Type	Source
OCM1	Primary uveal melanoma	June Kan-Mitchell
OCM3	Primary uveal melanoma	June Kan-Mitchell
OCM8	Primary uveal melanoma	June Kan-Mitchell
MEL202	Primary uveal melanoma	Bruce Ksander
MEL270	Primary uveal melanoma	Bruce Ksander
MEL285	Primary uveal melanoma	Bruce Ksander
MEL290	Primary uveal melanoma	Bruce Ksander
92.1	Primary uveal melanoma	Martine Jager
OM431	Primary uveal melanoma	Dan Albert
OMM1	Uveal melanoma metastasis	Gregorius Luyten
OMM2.2	Uveal melanoma metastasis	Bruce Ksander
OMM2.3	Uveal melanoma metastasis	Bruce Ksander
OMM2.5	Uveal melanoma metastasis	Bruce Ksander
OMM2.6	Uveal melanoma metastasis	Bruce Ksander
Jurkat	T lymphocyte	ATCC
K562	Chronic myelogenous leukemia	ATCC

metastasis developed from a uveal melanoma patient and were the generous gift of Gregorius Luyten (Erasmus University Rotterdam, Rotterdam, Netherlands). Human Jurkat T cells and chronic myelogenous leukemia K562 cells were obtained from the American Type Culture Collection (Manassas, VA).

Eye, liver metastases, and SC uveal melanoma cell lines were derived by injecting OCM1, OCM3, or OCM8 cells into the anterior chamber, spleen capsule, or subcutaneous tissues of BALB/c nude mice (National Cancer Institute, Bethesda, MD). Intrasplenic tumor cell injection is an effective method for producing liver metastases, as it facilitates tumor cell dissemination to the liver via the splenic-portal circulatory route.^{30–32} Tumors from each of these injection sites were isolated and cultured *in vitro*, as previously described.³³ Human corneal endothelial cells were established in our laboratory as described.³⁴

All cell lines were maintained in complete RPMI 1640 media (Bio-Whittaker, Walkersville, MD), containing 10% fetal bovine serum (HyClone, Logan, UT). The establishment of the human uveal melanoma cell lines and all research performed in this study adhered to the tenets of the Declaration of Helsinki. The animal experiments in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cytokines, Reagents, and Antibodies

Human recombinant interferon (IFN)- γ , phorbol 12-myristate 13-acetate (PMA), and phytohemagglutinin (PHA) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Functional grade purified mouse anti-human PD-L1 antibody (Clone: MIH1), anti-human PD-L2 antibody, mouse IgG1 isotype, mouse IgG2b isotype, and human IL-2 ELISA kits (Ready-SET-Go) were purchased from eBioscience (San Diego, CA). Anti-human PD-1 (Clone: 192106) antibody was purchased from R&D Systems, Inc. (Minneapolis, MN).

Cell Stimulation

Melanoma cells were grown to 80% confluence and stimulated with 500U/mL recombinant human IFN- γ in complete RPMI 1640 for 48 hours. The cells were then tested for PD-L1 mRNA and protein expression by RT-PCR and flow cytometry, respectively.

Reverse-Transcription-PCR

Total cellular RNA was prepared from lysed tumor cells (RNAqueous RNA isolation kit; Ambion, Austin, TX). The first-strand of cDNA was synthesized (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA). The resultant cDNA (0.5 μ L) was used in a 50- μ L reaction containing 0.1 μ M of each primer, 200 μ M of dNTP, 1.5 mM MgCl₂, 1 $\times$ reaction buffer, and 1 unit of *Taq* polymerase (Invitrogen, Carlsbad, CA). The primer sequences for human PD-L1 were as follows: forward, 5'-TTG

GGA AAT GGA GGA TAA GA-3'; reverse, 5'-GGA TGT GCC AGA GGT AGT TCT-3' (IDT, Coralville, IA). Human GAPDH was used as an internal control, and the primer sequences were as follows: forward, 5'-ACC ACA GTC CAT GCC ATC AC-3'; reverse, 5'-TCC ACC ACC CTG TTC CTG TA-3'.

An initial PCR denaturation step was performed at 94°C for 4 minutes. The general cycling parameters for PCR were as follows: denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 45 seconds for 35 cycles, with a final extension step at 72°C for 10 minutes. PCR amplification products were run on 1.5% agarose gels (Bio-Rad), prestained with 1 $\times$ nucleic acid stain (GelStar; Cambrex Bioscience Rockland Inc., Rockland, ME), and visualized (Typhoon 9410 imager; GE Healthcare, Piscataway, NJ).

Flow Cytometric Analysis

Expression of human PD-L1 and PD-L2 protein was assessed by flow cytometry. In brief, melanoma cell suspensions were prepared and washed in fluorescence-activated cell sorter buffer consisting of phosphate-buffered saline (PBS; pH 7.2) containing 2% fetal bovine serum. Cells were incubated with anti-PD-L1 antibody (2 μ g/mL), anti-PD-L2 antibody (2 μ g/mL), or mouse IgG1 isotype control (2 μ g/mL) for 60 minutes at 4°C, washed three times, and incubated with FITC-labeled secondary antibody (BD PharMingen, San Jose, CA) for 30 minutes at 4°C. The cells were then washed three additional times in PBS, fixed in 2% formalin, and assessed for fluorescence (FACScalibur flow cytometer; BD Bioscience, San Diego, CA).

Immunohistochemistry on Primary Human Uveal Melanoma-Containing Eyes

Cell membrane expression of PD-L1 in primary uveal melanoma-containing eyes was determined by immunohistostaining with anti-human PD-L1 monoclonal antibody (clone: MIH1; eBioscience). Tumor-bearing eyes from patients with uveal melanoma were embedded in paraffin and cut into 4- μ m sections. Tissue sections were incubated with 1 μ g/mL anti-PD-L1 or mouse IgG1 isotype control, followed by staining with the ABC system (Vectastain Elite; Vector Laboratories, Burlingame, CA), and counterstaining with methyl green as described elsewhere.³³

Effect of PD-L1 on Human T-Cell IL-2 Production

Melanoma cells were pretreated with recombinant IFN- γ (500 U/mL) and grown to 80% confluence, harvested, and cocultured with Jurkat T cells in the presence of 1 μ g/mL PHA and 50 ng/mL PMA at different effector-to-target (E-T) ratios for 48 hours. Jurkat T cells were added either directly to the melanoma cells or placed in the top chamber of a cell migration system (Transwell; Corning, Inc., Corning, NY), which separates the two chambers by a semipermeable membrane with 0.4- μ m pore size that prevents cells from entering the other chamber. In parallel experiments, IFN- γ -stimulated melanoma cells were preincubated with 10 μ g/mL anti-PD-L1 antibody or mouse IgG isotype control at 4°C for 1 hour before coculturing with Jurkat T cells in the presence of 20 μ g/mL anti-PD-L1 antibody or mouse IgG1 isotype control for 48 hours. In other experiments, IFN- γ -stimulated melanoma cells were cocultured with Jurkat T cells in the presence of 10 μ g/mL anti-PD-1 antibody or mouse IgG2b isotype control. Supernatants were harvested 48 hours later and assessed for IL-2 by ELISA.

Statistical Analysis

Results were expressed as the mean $\pm$ SD of at least triplicate samples. Data were analyzed by using Student's *t*-test. *P* < 0.05 was considered statistically significant. Each assay was performed at least twice.

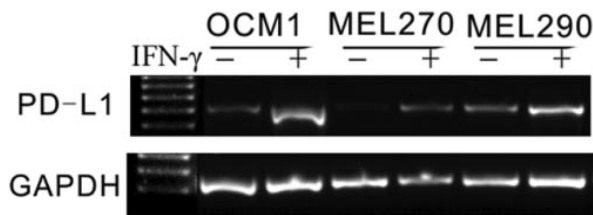


FIGURE 1. mRNA expression of PD-L1 on uveal melanoma cell lines as determined by RT-PCR. PD-L1 mRNA expression on OCM1, MEL270, and MEL290 uveal melanoma cells, with or without IFN- γ stimulation, was detected by RT-PCR. GAPDH expression was used as an internal control. -, without IFN- γ stimulation; +, 48 hours after 500 U/mL IFN- γ stimulation.

RESULTS

Expression of PD-L1 by Primary and Metastatic Uveal Melanoma Cells

First, we evaluated PD-L1 mRNA expression on eight human uveal melanoma cell lines and two metastatic cell lines by RT-PCR. PD-L1 primer specificity was confirmed by amplification of a 534-bp band from human corneal endothelial cell cDNA, which served as the positive control. Examples of undetectable, intermediate, or high levels of PD-L1 mRNA expression in uveal melanoma cells are depicted in Figure 1. Six of eight primary uveal melanoma cell lines and two metastatic cell lines expressed PD-L1 mRNA at various levels (Table 2). Human chronic myelogenous leukemia K562 cells served as the negative control.²⁷

Next, we determined whether PD-L1 gene transcription by primary and metastatic uveal melanomas coincided with PD-L1 protein expression. We detected the expression of PD-L1 protein on nine primary uveal melanoma cell lines and five metastatic cell lines by flow cytometric analysis. Examples of PD-L1 protein expression are depicted in Figure 2A. PD-L1 protein was constitutively expressed at various levels on five of nine primary uveal melanoma cells lines (OCM3, OCM8, MEL285, MEL290, and OM431) and one of five metastatic cell lines (OMM1; Fig. 2B). Although OCM1, OMM2.3, and OMM2.6 cells expressed PD-L1 mRNA, they did not constitutively express PD-L1 protein on their cell membranes, which indicates a posttranscriptional control of PD-L1 surface expression. Jurkat T cells, which do not express PD-L1 protein on their surface,²⁷ served as the negative control.

Effect of IFN- γ Stimulation on PD-L1 Expression by Uveal Melanoma Cells

IFN- γ , a key proinflammatory molecule released by activated T cells, is known to upregulate PD-L1 expression on a variety of cell types.²⁷⁻²⁹ To determine whether PD-L1 gene expression could be upregulated with IFN- γ stimulation, MEL270, OCM1, and MEL290 cells, which expressed undetectable, intermediate, or high levels of PD-L1 message, were cultured for 48 hours in the presence of 500 U/mL of

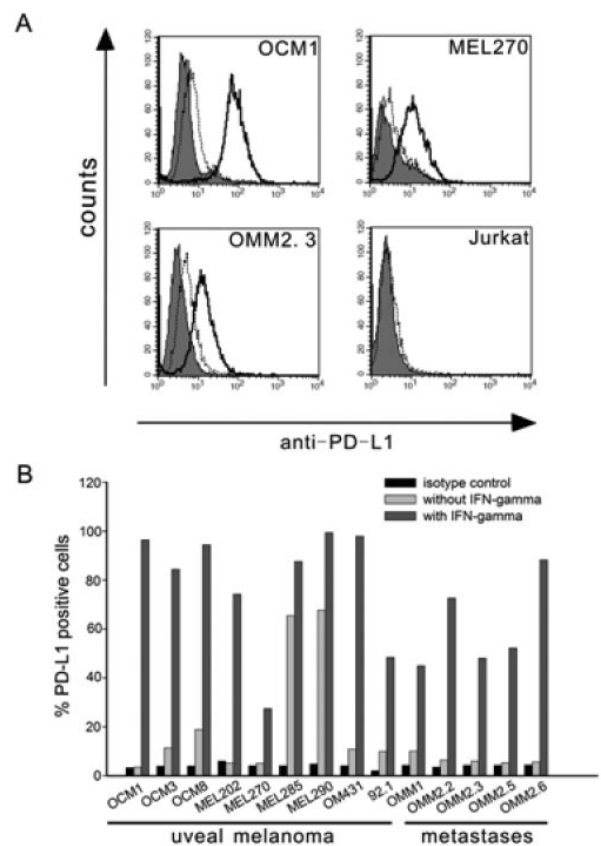


FIGURE 2. Expression of PD-L1 protein on primary and metastatic uveal melanoma cells. Melanoma cells, with or without IFN- γ stimulation, were incubated with either anti-human PD-L1 antibody or isotype control antibody, washed, incubated with FITC-labeled secondary antibody, and examined by flow cytometry. (A) Flow cytometric profiles of two uveal melanoma cell lines (OCM1, MEL270) and a metastatic human uveal melanoma cell line (OMM2.3). Human Jurkat T cells were used as the negative control. Shaded areas depict profiles of cells incubated with isotype control IgG. *Dashed traces*: profiles of melanoma cells not stimulated with IFN- γ , but incubated with anti-PD-L1 antibody. *Bold traces*: profiles of IFN- γ -pretreated cells incubated with anti-PD-L1. (B) Percentage of melanoma cells expressing PD-L1 protein. Data represent the average of results of two independent experiments performed in triplicate.

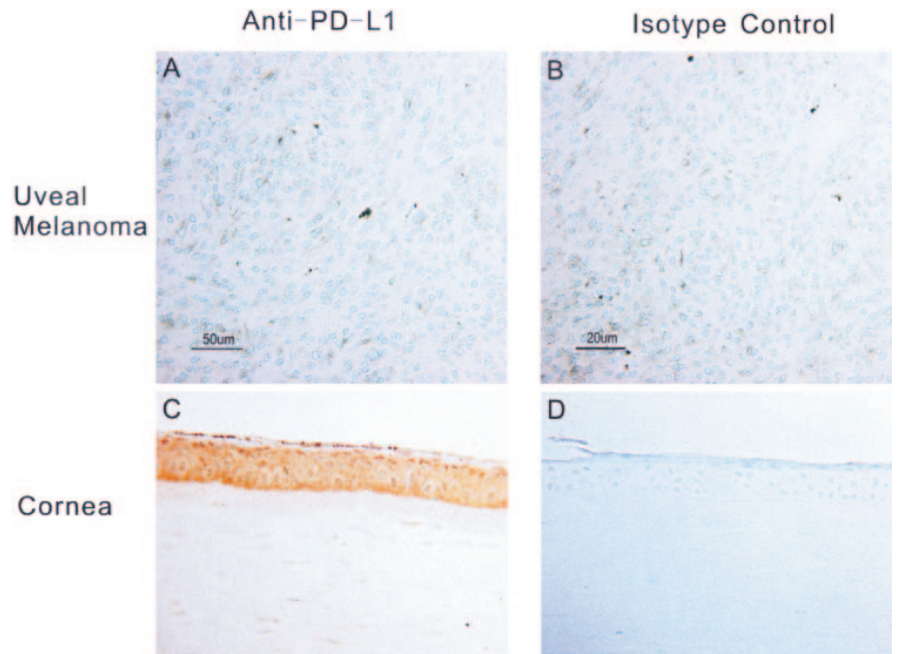
human recombinant IFN- γ before RT-PCR analysis. The results demonstrated that IFN- γ induced upregulation of PD-L1 message in all three cell lines, including MEL270 uveal melanoma cells in which it was previously undetectable (Fig. 1). Moreover, as shown in Figure 2, all the primary and metastatic uveal melanoma cell lines upregulated PD-L1 protein expression significantly after IFN- γ stimulation ($P < 0.01$).

TABLE 2. mRNA Expression of PD-L1 mRNA on Primary and Metastatic Uveal Melanoma Cell Lines

Cell Line	Pos. Control HCN	Primary Uveal Melanoma								Metastases		Neg. Control K562
		OCM1	OCM3	OCM8	OM431	MEL202	MEL270	MEL290	MEL285	OMM2.3	OMM 2.6	
PD-L1	+	+	++	+	++	-	±	+++	+++	+	+	-

+++ , strong expression; ++ , moderate expression; + , positive expression; - , no expression.

FIGURE 3. PD-L1 expression on primary uveal melanoma in situ by immunohistochemistry. Paraffin-embedded sections of uveal melanoma-containing eyes were incubated with anti-PD-L1 or mouse IgG1 isotype control antibody and counterstained with methyl green. Immunohistochemical staining with (A) anti-PD-L1 or (B) mouse IgG1 isotype control on primary uveal melanoma. (C) Human corneal epithelium served as the internal positive control for PD-L1 staining. (D) Mouse IgG1 isotype control staining of human corneal epithelium.



Expression of PD-L1 in Primary Uveal Melanomas In Situ

Because in vitro culture of uveal melanoma cell lines may not reflect PD-L1 expression in situ, we used immunohistochemical staining to examine eye sections from five patients with uveal melanoma for PD-L1 expression. None of the five primary uveal melanomas stained positively for PD-L1. An example of PD-L1 staining in a uveal melanoma-containing eye is shown in Figure 3. Human corneal epithelium, which expresses PD-L1, served as the positive control.

Influence of the Local Microenvironment on PD-L1 Expression by Uveal Melanoma Cells

It is known that the immune-privileged ocular microenvironment contains factors that regulate gene expression.³⁵ When uveal melanomas metastasize to the liver or skin, they encounter a non-immune-privileged microenvironment, which may promote differential expression of molecules that influence immune evasion and survival of melanoma cells. To test whether the microenvironment of the eye, liver, or skin influences PD-L1 expression on uveal melanoma cells, we established eye, liver, and skin metastases cultures of OCM1, OCM3, and OCM8 uveal melanoma cell lines, and evaluated PD-L1 protein expression by flow cytometry.

The results showed that, with the exception of OCM1 liver metastases, each of the eye, liver, or skin metastasis-derived uveal melanoma cells constitutively expressed PD-L1 protein on their cell membranes (Fig. 4). Moreover, each of the cell cultures upregulated PD-L1 expression significantly after in vitro treatment with IFN- γ .

Correlation of PD-L1 Expression on Uveal Melanoma Cells with Suppressed IL-2 Production by T Cells

Cytokine production is an important criterion for evaluating T-cell function. To test whether uveal melanoma expression of PD-L1 affects T-cell function, we cocultured Jurkat T cells with IFN- γ -pretreated OCM1 cells at different E:T ratios for 48 hours

and assessed by ELISA their production of IL-2. As shown previously, more than 90% of OCM1 cells expressed PD-L1 after stimulation with IFN- γ for 48 hours. Jurkat T cells do not express PD-1, the receptor of PD-L1, unless they are activated.²⁹ Accordingly, Jurkat T cells were stimulated with 1 μ g/mL PHA and 50 ng/mL PMA during the various in vitro assays. After 48 hours of stimulation, approximately 70% of Jurkat T cells expressed PD-1 according to flow cytometry data (data not shown).

Coculturing Jurkat T cells with OCM1 cells at an E:T ratio of 4:1 resulted in a significant reduction in IL-2 production by the Jurkat T cells (Fig. 5A; $P < 0.05$). Inhibition of IL-2 production was dose dependent, with maximum inhibition occurring at an 8:1 E:T ratio.

To test whether the inhibition of Jurkat T cell IL-2 production was cell-contact dependent, a coculture system (Trans-

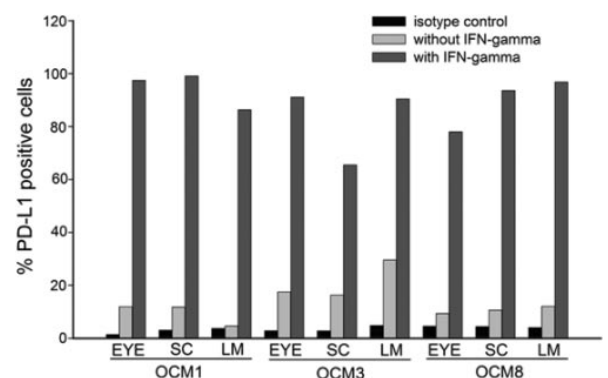


FIGURE 4. PD-L1 expression on uveal melanoma derived metastases. Eye, skin (SC), or liver metastases (LM) derived from OCM1, OCM3, and OCM8 cells were incubated with anti-PD-L1 or mouse IgG1 isotype control, followed by incubation with FITC-conjugated secondary antibody. The results showed the percentage of metastatic uveal melanoma cells expressing PD-L1. Data represent the average of results of two independent experiments performed in triplicate.

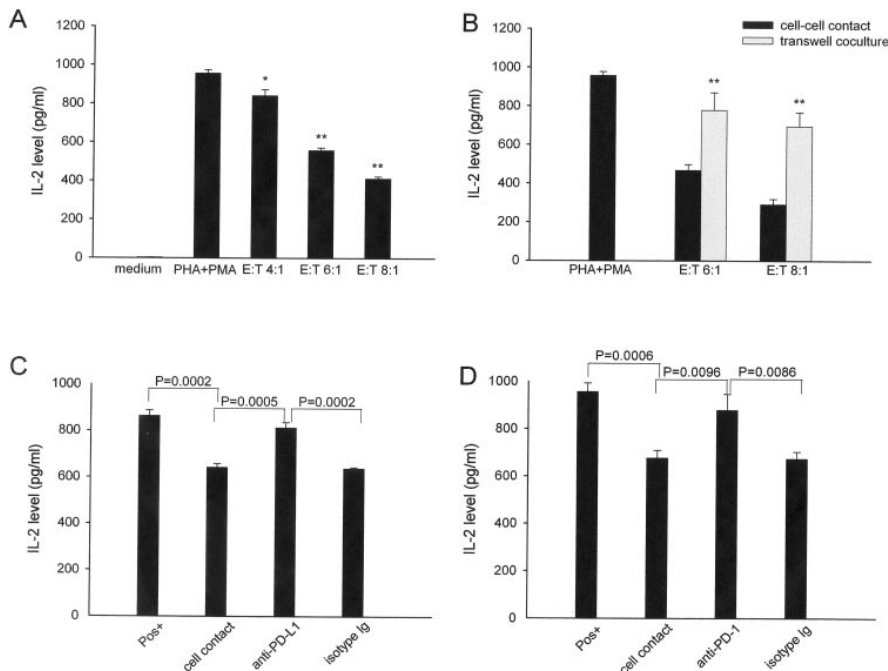


FIGURE 5. Uveal melanoma-expressed PD-L1 inhibits IL-2 production by human Jurkat T cells. OCM1 cells were pretreated with 500 U/mL IFN- γ for 48 hours, washed and cocultured with human Jurkat T cells by cell-cell contact or transwell coculture in the presence of PHA and PMA for 48 hours. IL-2 production was assessed by ELISA. (A) Inhibition of IL-2 production of Jurkat T cells by coculture with OCM1 cells at different E:T ratios. (B) Enhanced IL-2 production in transwell cocultures. (C) Restoration of IL-2 production by addition of anti-PD-L1 antibody to cell-contact cocultures. Pos+, positive control. IL-2 levels in the transwell cocultures were used as a positive control to discount the suppression of IL-2 production by soluble factors. (D) Restoration of IL-2 production by addition of anti-PD-1 antibody to cell-contact cocultures. IL-2 levels in the transwell cocultures were used as a positive control. The results shown are typical of those in three independent experiments. * $P < 0.05$; ** $P < 0.01$.

well; Corning, Inc.) was used in which a semipermeable 0.4- μ m pore size membrane separated Jurkat T cells from OCM1 melanoma cells. Figure 5B demonstrates that the uveal melanoma-associated inhibition of IL-2 production was cell-contact dependent, as IL-2 levels were significantly suppressed in Jurkat T cell and OCM1 melanoma cell cocultures that allowed cell contact ($P < 0.01$) when compared with IL-2 levels in separated OCM1 melanoma and Jurkat T cell cocultures. Of interest, the transwell cocultures of OCM1 melanoma and Jurkat T cells also suppressed some of the IL-2 production, which indicated that soluble factors produced by uveal melanomas may also influence T-cell function.

Since our research focused on PD-L1, a cell membrane-bound molecule that acts in a cell contact-dependent manner, the IL-2 levels in the transwell cocultures were used as the positive control for maximum IL-2 production in the following experiments to discount the suppression by the soluble factors. To verify that the cell-contact-dependent inhibition of IL-2 production was due to uveal melanoma-derived PD-L1, we set up a coculture in the presence of blocking antibody to PD-L1 or mouse IgG1 isotype control. Figure 5C demonstrates that addition of anti-PD-L1 blocking antibody restored 77% of Jurkat T-cell production of IL-2. By contrast, addition of the mouse IgG1 isotype control had no discernible effect on IL-2 production by Jurkat T cells.

To test whether the PD-L1-derived inhibition of IL-2 production was due to PD-L1: PD-1 interaction, anti-PD-1 blocking antibody or mouse IgG2b isotype control was added to the coculture systems. Addition of anti-PD-1 blocking antibody restored more than 70% of the IL-2 produced by Jurkat T cells (Fig. 5D).

These results suggest that uveal melanoma cell-derived PD-L1 is functional and inhibits cytokine production by activated T cells via interaction with PD-1. It has been reported that PD-L2, another ligand of PD-1, also affects T-cell cytokine production.³⁶ Experiments were performed to determine whether PD-L2 was also expressed on OCM1 uveal melanoma cells and contributed to the inhibition of IL-2 production by Jurkat T cells. Flow cytometry was used to examine OCM1

melanoma cells for PD-L2 expression. The results of a typical assay are shown in Figure 6A, which demonstrated that OCM1 melanoma cells did not express PD-L2, even after IFN- γ stimulation for 48 hours. Although the OCM1-mediated inhibition of Jurkat T cell production of IL-2 was PD-1-dependent, it was not PD-L2-mediated. This further supports the role of PD-L1 in the inhibition of IL-2 production by activated Jurkat T cells. Human retinal pigment epithelial ARPE-19 cells, which express PD-L2,³⁷ served as the positive control (Fig. 6B).

DISCUSSION

Our study demonstrates that PD-L1 is expressed constitutively in five of nine primary human uveal melanoma cell lines and

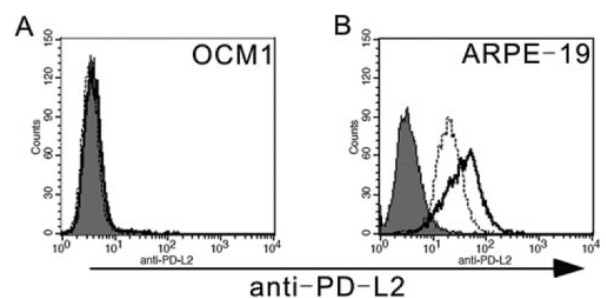


FIGURE 6. Detection of PD-L2 expression on uveal melanoma cells OCM1 by flow cytometry. OCM1 melanoma cells were cultured either in the presence (bold lines) or absence (dashed lines) of IFN- γ for 48 hours. The cells were then incubated with either anti-human PD-L2 antibody or isotype control antibody, washed, incubated with FITC-labeled secondary antibody, and examined by flow cytometry. Flow cytometry profiles of (A) OCM1 or (B) human retinal pigment epithelial ARPE-19 cells, which served as the positive control for PD-L2 expression. Shaded areas: profiles of cells incubated with isotype control IgG. The results shown are typical of those in three independent experiments.

one of five cell lines from uveal melanoma metastases, and its expression is markedly upregulated after stimulation by IFN- γ . However, primary uveal melanomas do not express PD-L1 in situ. The different microenvironments of the eye, skin, and liver do not appear to influence PD-L1 expression on human uveal melanoma cells. The results also demonstrate that the PD-L1 expressed on uveal melanoma cells after IFN- γ stimulation is capable of suppressing T-cell function, such as IL-2 production.

Uveal melanoma progression has been attributed to a variety of immune evasion strategies. These include secretion of immunosuppressive factors such as transforming growth factor- β ,^{38,39} expression of enzymes such as indoleamine 2,3-dioxygenase (IDO) to establish an immunosuppressive tumor microenvironment,⁴⁰ and expression of cell-membrane-bound immunosuppressive factors such as complement regulatory proteins^{16,41} and FasL.⁴² Recently, newly recognized cell-membrane-bound molecules, such as PD-L1 (B7-H1),^{21,26–28} PD-L2 (B7-DC),^{18,20,24} and B7-H4 (B7S1),⁴³ have been found to contribute to tumor evasion of immune suppression. Among these, PD-L1 is thought predominately to mediate inhibitory signals toward T cells. There is evidence that PD-L1 also contributes to immune privilege by inhibiting T-cell proliferation, inducing apoptosis of immune cells, and suppressing cytokine secretion by T cells.^{21,26–28} Our study results support the hypothesis that PD-L1 promotes uveal melanoma's escape from T-cell-mediated immune surveillance. This mechanism should be considered when designing T-cell-based immunotherapy for metastatic uveal melanoma.

Despite the wide distribution of PD-L1 mRNA in normal human tissues,^{18,22,44} constitutive surface expression of PD-L1 protein appears to be limited primarily to vascular endothelial cells and to the maternal-fetal interface,^{45,46} suggesting that there is a posttranscriptional control for expression of this inhibitory factor. Our data reflected posttranscriptional regulation of PD-L1 expression in uveal melanoma cells. OCM1, OMM2.3, and OMM2.6 cells constitutively express PD-L1 mRNA, but fail to express PD-L1 protein on their surface constitutively. The mechanisms regulating posttranscriptional PD-L1 expression are still unknown. We have been unable to obtain freshly enucleated uveal melanoma-containing eyes for in situ RT-PCR analysis, and thus, we can only speculate as to whether the PD-L1 message is present in the intact eye. However, we did not detect PD-L1 expression in primary uveal melanoma in situ by immunohistochemistry. It is conceivable that expression of PD-L1 is unnecessary for primary uveal melanomas, which reside in the immune-privileged environment of the eye and are thus shielded from many immune effector elements. However, uveal melanoma cells retain their capability to express PD-L1 under special circumstances. Our results show that all of the uveal melanoma cell lines examined were able to upregulate PD-L1 expression remarkably after IFN- γ stimulation. Once uveal melanoma cells metastasize to the liver, they lose the sanctuary provided by the eye and enter an organ that has access to T cells and possesses one of the highest concentrations of NKT cells of any organ the body.^{47,48} In the liver, uveal melanoma cells might encounter IFN- γ secreted by activated T cells in the tumor's local microenvironment and thus could respond by upregulating PD-L1 on their cell membrane and inhibit T-cell production of IL-2.

Previous work has shown that TGF- β , at concentrations found in the aqueous humor, significantly downregulates MHC class I antigen expression of uveal melanoma cells.³⁵ In addition, the ocular microenvironment promotes epigenetic changes in uveal melanoma gene expression.^{49,50} Therefore, to determine the effect of the local microenvironment on PD-L1 expression, we compared PD-L1 expression on uveal melanoma cells transplanted into the eye, liver, or subcutaneous

sites. The results showed that the microenvironment had little effect on PD-L1 expression, with the exception of one uveal melanoma liver metastasis (OCM1-LM cells), which did not constitutively express PD-L1 protein on the surface. All the uveal melanomas maintained their capacity to upregulate PD-L1 expression when stimulated with IFN- γ . Thus, the microenvironments of the eye, liver, and skin do not alter the capacity of uveal melanoma cells to upregulate PD-L1 expression when confronted with the proinflammatory T cell-derived cytokine, IFN- γ .

Adoptive cell transfer therapies using either melanoma-specific T-cell clones or tumor-infiltrating lymphocytes (TILs) seldom induce successful clinical responses. Although TILs display higher activation levels and a superior production of effector cytokines than do peripheral blood lymphocytes,^{51–54} they also express higher levels of PD-1.²⁸ Thus, despite their greater activation, TILs may be more susceptible to the immunoregulatory effects of PD-L1 displayed on uveal melanomas.

The present findings indicate that PD-L1 expressed on uveal melanoma cells is functional and suppresses T-cell IL-2 production, which is a key cytokine required for the proliferation and survival of activated T cells. Verbik et al.⁵⁵ have shown that uveal melanoma cells inhibit T-cell proliferation in mixed-lymphocyte cultures in a cell-to-cell contact-dependent manner. Although they did not identify the effector molecule expressed on the uveal melanoma cells, their findings show PD-L1 to be an attractive candidate that could explain this interesting phenomenon. PD-1 is not normally expressed on resting T cells.²⁶ However, PD-L1-mediated inhibition of IL-2 may play a role in activated effector T cells that infiltrate uveal melanomas in the eye or on uveal melanoma metastases in the liver. It is conceivable that IFN- γ released by tumor-infiltrating T cells induce the functional activation of PD-L1 on uveal melanoma cells. We observed that even though MEL290 cells constitutively expressed high levels of PD-L1 on their cell membranes, they did not suppress the production of IL-2 by Jurkat T cells. However, MEL290 cells pretreated with IFN- γ strongly suppressed IL-2 production by Jurkat T cells. We have also observed that normal human corneal epithelial cells constitutively expressed high levels of cell membrane PD-L1, yet do not inhibit IL-2 production unless they are pretreated with IFN- γ (Yang W, et al., unpublished findings, 2007). Thus, it appears that IFN- γ activates the PD-L1 molecule through a yet-to-be-determined mechanism. The identity of this mechanism is currently under investigation.

Our findings imply that the presence of IFN- γ elaborated by T cells that infiltrate liver metastases of uveal melanomas promotes upregulation of PD-L1 expression on uveal melanoma cells, which in turn, dampens T-cell proliferation by impairing the production of IL-2. Accordingly, the selective blockade of PD-L1 is a potential target for disrupting one of the immune escape mechanisms used by uveal melanomas.

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