The expression profiles and regulation of PD-L1 in tumor-induced myeloid-derived suppressor cells

Chunwan Lu, Priscilla S. Redd, Jeffrey R. Lee, Natasha Savage, and Kebin Liu

Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA, USA; Charlie Norwood VA Medical Center, Augusta, GA, USA; Georgia Cancer Center, Augusta University, Augusta, GA, USA; Pathology, Medical College of Georgia, Augusta, GA, USA

ABSTRACT

Programmed death-ligand 1 (PD-L1) is an inhibitory ligand that binds to PD-1 to suppress T cell activation. PD-L1 is constitutively expressed and inducible in tumor cells, but the expression profiles and regulatory mechanism of PD-L1 in myeloid-derived suppressor cells (MDSCs) are largely unknown. We report that PD-L1 is abundantly expressed in tumor-infiltrating leukocytes in human patients with both microsatellite unstable and microsatellite stable colon cancer. About 60% CD11b<sup>+</sup>CD33<sup>+</sup>HLA-DR<sup>+</sup> MDSCs from peripheral blood of human colon cancer patients are PD-L1<sup>+</sup>. PD-L1<sup>+</sup> MDSCs are also significantly higher in tumor-bearing mice than in tumor-free mice. Interestingly, the highest PD-L1<sup>+</sup> MDSCs were observed in the tumor microenvironment in which 56–71% tumor-infiltrating MDSCs are PD-L1<sup>+</sup> in vivo. In contrast, PD-L1<sup>+</sup> MDSCs are significantly less in secondary lymphoid organs and peripheral blood as compared to the tumor tissues, whereas bone marrow MDSCs are essentially PD-L1<sup>−</sup> in tumor-bearing mice. IFNγ is highly expressed in cells of the tumor tissues and IFNγ neutralization significantly decreased PD-L1<sup>+</sup> MDSCs in the tumor microenvironment in vivo. However, IFNγ-activated pSTAT1 does not bind to the cd274 promoter in MDSCs. Instead, pSTAT1 activates expression of IRF1, IRF5, IRF7 and IRF8 in MDSCs, and only pSTAT1-activated IRF1 binds to a unique IRF-binding sequence element in vitro and chromatin in vivo in the cd274 promoter to activate PD-L1 transcription. Our data determine that PD-L1 is highly expressed in tumor-infiltrating MDSCs and in a lesser degree in lymphoid organs, and the pSTAT1-IRF1 axis regulates PD-L1 expression in MDSCs.

Introduction

Programmed death-ligand 1 (PD-L1, also known as CD274 or B7-H1) is the ligand for T cell repressive receptor PD-1 that functions to activate the T cell co-inhibitory pathways to maintain self-tolerance, and therefore act as an immune checkpoint under physiological conditions. However, under pathological conditions such as cancer, PD-L1 is abundantly expressed in tumor cells and binds to PD-1 to suppress T cell activation to promote tumor cell immune escape and progression. Consequently, anti-PD-L1/PD-1 immune check point immunotherapy has shown durable efficacy in various types of human cancer.

However, colorectal cancer, except for the microsatellite instability (MSI) subtype of colorectal cancer which only represents less than 4% of all colorectal cancer, stands out as one of the few cancer types that does not respond to immune check point immunotherapy. What distinct colorectal cancer from other human cancers in terms of response to anti-PD-L1/PD-1 immunotherapy immunologically is currently unknown. It has been observed that the expression level of PD-L1 is a response predictor to PD-1 pathway blocking antibody immunotherapy, suggesting that the expression level of PD-L1 is a determining factor of the PD-1 pathway-mediated cancer immune resistance against elimination by endogenous tumor-specific T cells. Therefore, PD-L1 expression level and types of cells that express PD-L1 might be an underlying mechanism of colorectal cancer resistance to immune check point immunotherapy.

The expression and regulation of PD-L1 in tumor cells have been extensively studied. It has been shown that PD-L1 is constitutively expressed in various types of tumor cells and regulated by oncogenes. Furthermore, PD-L1 expression can be upregulated by inflammatory cytokines such as IFNγ. In addition to expression in tumor cells, PD-L1 is also expressed on subsets of T cells, NK cells, macrophages, myeloid DCs, B cells, epithelial cells and vascular endothelial cells. PD-L1 is notably expressed on mature macrophages, particularly in activated macrophages. It has been shown that classically activated macrophages upregulate PD-L1, whereas alternative macrophages activated by IL-4 also upregulate PD-L1, albeit at a lesser degree. In T, B, myeloid and dendritic cells, PD-L1 expression is also upregulated upon cellular activation. Therefore, immune cells are another subset of major cell types that express PD-L1.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that exhibit potent suppressive activity against T cell activation and function. MDSCs suppress autoimmunity and regulate tissue repair under physiological conditions, but rapidly expand and accumulate under pathological conditions.
conditions, including inflammation, infection and cancer. The phenotypes of both human and mouse MDSCs are still not well defined. In humans, MDSCs are generally defined as CD11b^+ CD33^+ HLA-DR^+ with additional markers such as lineage-specific antigens, CD14, and CD15. In mice, CD11b^+ Gr1^+ are commonly used to define the MDSC phenotype and Ly6G and Ly6C are used to further define granulocytic and monocytic subsets of MDSCs. In human cancer patients and tumor-bearing mice, MDSCs massively accumulate to suppress tumor-reactive T cells to promote tumor progression. MDSCs thus are key targets in cancer immunotherapy. PD-L1 expression has been observed in MDSCspromote tumor progression. MDSCs thus are key targets in cancer conditions, including inflammation, infection and cancer. The phenotypes of both human and mouse MDSCs are still not well defined. In humans, MDSCs are generally defined as CD11b^+ CD33^+ HLA-DR^+ with additional markers such as lineage-specific antigens, CD14, and CD15. In mice, CD11b^+ Gr1^+ are commonly used to define the MDSC phenotype and Ly6G and Ly6C are used to further define granulocytic and monocytic subsets of MDSCs.

In human cancer patients and tumor-bearing mice, MDSCs massively accumulate to suppress tumor-reactive T cells to promote tumor progression. MDSCs thus are key targets in cancer immunotherapy. PD-L1 expression has been observed in MDSCs in tumor-bearing mice and its expression has been linked to hypoxia and HIF-

However, the expression profiles of PD-L1 in MDSCs in both human cancer patients and tumor-bearing mice, as well as the molecular mechanisms underlying PD-L1 expression in MDSCs remain to be further determined. We made use of a recently developed and FDA-approved highly sensitive and specific anti-PD-L1 mAb, and examined PD-L1 expression profiles in leukocytes in human colon carcinoma specimens. We observed that in addition to tumor cells, PD-L1 is highly expressed in tumor-infiltrating leukocytes in all stages of colon cancer. MDSCs accumulate massively in the peripheral blood in human colon cancer patients and a large portion of these human MDSCs are PD-L1^+. Using mouse tumor models, we observed that the highest PD-L1-expressing MDSCs are in the tumor microenvironment. The second lymphoid organs such as the spleen contain less than 30% PD-L1^+ MDSCs, whereas MDSCs in BM are essentially PD-L1^- PD-L1 expression is apparently regulated by IFNγ since IFNγ neutralization diminished PD-L1^+ MDSCs in the tumor microenvironment. At the molecular level, we determined that IFNγ activates the expression of IRF1, IRF5, IRF7 and IRF8 in MDSCs, but only IRF1 binds to a unique IRF-binding consensus sequence element of the cd274 promoter chromatin to directly activate PD-L1 expression in MDSCs.

**Results**

**PD-L1 is abundantly expressed in human colon carcinoma and tumor-infiltrating immune cells.**

Various PD-L1 protein patterns have been observed in human colorectal carcinoma tissues. A highly specific and sensitive anti-PD-L1 mAb (Clone 28–8) has recently been developed and approved by FDA for detecting PD-L1 protein in human cancer patient tumor specimens. We made use of this human PD-L1-specific mAb and analyzed PD-L1 protein level in various stages of human colorectal carcinoma tissues. Abundant CD45^+ leukocytes are present in all 14 adenoma specimens analyzed (Fig. 1A.A1a and b). Thirteen of the 14 adenoma tissues exhibit PD-L1 protein in tumor cells, and the majority of tumor cells are PD-L1^+ (Fig. 1A and B1a and b). PD-L1^+ tumor-infiltrating leukocytes are present in all 14 specimens (Fig. 1B). All 14 carcinoma specimens also exhibit abundant CD45^+ leukocyte infiltration in the tumor (Fig. 1A.A2a and b) and have detectable PD-L1 protein in the tumor tissues (Fig. 1A and B2a and b). More than 50% of tumor-infiltrating CD45^+ cells are PD-L1^+ (Fig. 1B). CD45^+ leukocyte infiltration was also observed in both LN (Fig. 1A.A3a and b) and liver (Fig. 1A.A4 a and b) metastases. PD-L1 protein was detected in the metastatic colon cancer cells in the lymph nodes (Fig. 1A and B3a and b) and the liver (Fig. 1A and B4a and b). However, fewer PD-L1^+ leukocytes are present in liver metastases than in primary tumors and LN metastases (Fig. 1B).

To validate the specificity, human tonsil and adrenal tumor tissues were stained with this anti-PD-L1 antibody. As expected, membrane PD-L1 staining in epithelial cells surrounding crypts in the tonsil (Fig. 1A.C1a–c) and primarily membrane PD-L1 staining in adrenal tumor cells (Fig. 1A.D) were observed.

**Leukocytes in both MSI and MSS colon carcinoma tissues express PD-L1**

Human colorectal cancer, especially for the microsatellite unstable (MSI) colorectal cancer which accounts for approximately 4% human colorectal cancer, does not respond to anti-PD-L1/ PD-1 immunotherapy. Recent studies have shown that high level of PD-L1^+ myeloid cell infiltration in the tumor invasive front is a characteristic of MSI human colon carcinoma and PD-L1 expression in tumor cells is inversely correlated with MSI-high status in human colorectal cancer. We examined leukocyte infiltration profiles and PD-L1 expression level in MSI and microsatellite stable (MSS) colorectal carcinomas. Five of the seven MSI colon carcinomas exhibit high level of CD45^+ leukocyte infiltration throughout all tumor areas (Fig. 2A.I1 and Table S3). One carcinoma has high-level CD45^+ leukocyte infiltration in approximately 30% of the tumor area (Fig. 2A.I2 and Table S3). Another MSI colon carcinoma has low level of CD45^+ leukocytes in the tumor area (Fig. 2A.I3 and Table S3). For MSS colon carcinomas, four of the nine colon carcinomas exhibit high level of CD45^+ leukocyte infiltration in all tumor areas (Fig. 2A.S1 and Table S3). One carcinoma has high-level CD45^+ leukocyte infiltration in about 50% tumor areas (Fig. 2A.S2 and Table S3), and two MSS colon carcinoma has leukocyte infiltration in less than 20% tumor areas (Fig. 2A.S3 and Table S3).

Comparison of PD-L1 and CD45 staining in these MSI and MSS colon carcinoma revealed that majority of tumor-infiltrating CD45^+ leukocytes are PD-L1^+ (Fig. 2B.a1 and 2, Fig. 2B.b1 and 2). Quantification of PD-L1^+ cells in the CD45^+ cell population indicates that there is no significant difference in percentage of PD-L1^+CD45^+ cells between MSI and MSS colon carcinomas (Fig. 2C).

**PD-L1 is expressed in subsets of human leukocytes and highly expressed in tumor-induced human MDSCs**

To determine what types of tumor-infiltrating immune cells express PD-L1 as observed in the human colon tumor specimens, we collected peripheral blood from healthy normal donors and colon cancer patients. White blood cells were isolated and stained with CD11b-, CD33-, HLA-DR-, CD11c-, CD3-, CD19, CD337- and PD-L1-specific mAbs. CD11b^+CD33^+ HLA-DR^+ human MDSCs are significantly higher in blood of human colon cancer patients than in normal donors. Furthermore, the percentage of PD-L1^+ MDSCs reach about 60% of total MDSCs in the cancer patients and is significantly higher in blood from human colon cancer patients than that from normal donors (Fig. 3A). CD11c^+ dendritic cells
constitute a small fraction of human white blood cells and its level is not different between colon cancer patients and normal donors, but the percentage of PD-L1^CD11c^ cells is significantly higher in colon cancer patients than that in normal donors (Fig. 3B). T cell and B cell levels are remarkably lower in colon cancer patients as compared to normal donors, but the percentage of PD-L1 is detectable in a small fraction of T cells and B cells. In addition, the percentages of PD-L1^CD337^NK cells are dramatically higher in the blood from colon cancer patients than those from normal donors (Figs. 3C and D). Approximately 5% of human white blood cells are CD337^NK cells, and the percentage of NK cells increased to about 10% in human colon cancer patients. Interestingly, the percentage of PD-L1^NK cells is notably higher in human colon cancer patients than that in normal donors (Fig. 3E).

**Figure 1.** PD-L1 protein level in human colon carcinoma tissues. (A) Human colon carcinoma tissues were stained with anti-human CD45 (A1a–A4a and A1b–A4b) and anti-human PD-L1 (B1a–B4a and B1b–B4b) monoclonal antibodies, respectively. Brown color indicates CD45 and PD-L1 protein levels, with counterstaining by hematoxylin in blue. Shown are representative images; A1 & B1: colon adenoma; A2 & B2: colon adenocarcinoma; A3 & B3: Lymph node metastases; A4 & B4: Liver metastases. a: images of whole tissue discs. b: amplified area as shown in a. Yellow arrows indicate CD45-positive cells and red arrows point PD-L1-positive cells. Human tonsil (C1a & C1b) and adrenal tumor (D) tissue were used as positive controls of PD-L1 protein. G: Germinal center. Black arrow indicates lymphoid cells. (B) Quantification of PD-L1^CD45^ cells in human colon carcinoma. PD-L1^+^ cells (B1a–B4a & B1b–B4b) of the CD45^+^ cell (A1a–A4a and A1b–A4b) in adenoma (n = 13), adenocarcinoma (n = 15), LN metastases (n = 6) and liver metastases (n = 7) were counted and expressed as % PD-L1^+^ cells/CD45^+^ cells per tumor tissue.

**MDSCs from spontaneous mouse colon cancer express PD-L1**

To study PD-L1 expression regulation in vivo, we made use of the AOM-DSS-induced spontaneous colon cancer mouse model. AOM-DSS induces tumor formation in the colon
(Fig. 4Aa). Analysis of the tumor-bearing colon tissues revealed that most of the colon tissues still have the normal colon structures (Fig. 4Ab). The tumor tissues represent various stages of the colon cancer including adenoma (Fig. 4Ac) and colon adenocarcinoma (Fig. 4Ad). Analysis of spleens from these tumor-bearing mice revealed that, as expected, the percentage of CD11b\(^+\)Gr1\(^+\) mouse MDSCs is significantly higher in tumor-bearing mice than that in tumor-free mice and the percentage

---

**Figure 2.** Leukocyte infiltration patterns in human MSI and MSS colon carcinoma tissues. (A) MSI and MSS human colon carcinoma tissues were stained with anti-human CD45 antibody. Brown color indicates CD45 staining, with nuclei counterstaining by hematoxylin in blue. I1-3: tumor tissues from three patients with MSI colon cancer. S1-3: tumor tissues from three patients with MSS colon cancer. Shown are representative images in low-power magnification. (B) High-power magnification of images showing CD45 (a1 and b1) and PD-L1 (a2 and b2) staining levels in MSI (a) and MSS (b) colon cancer tissues, respectively. (C) Quantification of PD-L1\(^+\)CD45\(^+\) cells in MSI (n = 7) and MSS (n = 9) colon carcinoma tissues. PD-L1\(^+\) cells of the CD45\(^+\) cells as shown in (A) were counted and expressed as percentage of PD-L1\(^+\) cells/CD45\(^+\) cells.

**Figure 3.** PD-L1 expression in MDSCs of human colon cancer patients. (A) Peripheral blood specimens were obtained from consented healthy donors (n = 10) and colon cancer patients (n = 10). White blood cells were isolated and stained with anti-human CD11b, anti-human CD33, anti-human HLA-DR and anti-human PD-L1 mAbs. HLA-DR\(^-\) cells were gated out and analyzed for CD11b\(^+\)CD33\(^+\) cells (top panel). HLA-DR\(^-\)CD11b\(^+\)CD33\(^+\) cells were then gated and analyzed for PD-L1\(^+\) cells (bottom panel). (B–E) White bloods cells from healthy donors (n = 10) and colon cancer patients (n = 10) as shown in (A) were stained with anti-human CD11c and anti-human PD-L1 (B), anti-human CD3 and anti-human PD-L1 (C), anti-human CD19 and anti-human PD-L1 (D), and anti-human CD33 and anti-human PD-L1 mAbs and analyzed by flow cytometry. % PD-L1\(^+\) cells in the indicated subsets of immune cells were quantified. Significance was determined by two-sided Student’s t-test.
of PD-L1+ MDSCs is dramatically higher in tumor-bearing mice than that in tumor-free mice (Fig. 4B). Further analysis of CD11b+Ly6G+ granulocytic MDSCs (gMDSCs) and CD11b+Ly6C+ monocytic MDSCs (mMDSCs) indicate that, as expected, both gMDSCs and mMDSCs are remarkably higher in tumor-bearing mice than in tumor-free mice. The percentages of PD-L1+ gMDSCs and mMDSCs are also noticeably higher in tumor-bearing mice than those in tumor-free mice with the highest PD-L1+ MDSCs in the mMDSC population (Fig. 4B). We also analyzed PD-L1 expression levels in CD11c+ dendritic cells (C), T and B cells (D) and NK cells (E) and observed no significant differences in the percentages of PD-L1+ cells in these subsets of leukocytes between tumor-bearing mice and tumor-free mice (Figs. 4C–E).
PD-L1 expression in MDSCs depends on MDSC anatomic locations in vivo

The above AOM-DSS-induced spontaneous colon cancer mouse model mimics human spontaneous colon cancer. However, the MDSC level in the AOM-DSS colon cancer-bearing mice (Fig. 4) is much lower than that in human patients (Fig. 3). We then established two orthotopic colon cancer mouse models and one orthotopic pancreatic cancer mouse models (Fig. 5A). Tumors were collected from these three types of tumor-bearing mice and analyzed for PD-L1 level in tumor-infiltrating MDSCs. Spleens and bone marrows were also collected and analyzed for PD-L1 levels in the MDSCs. As high as 65%, 56% and 71% PD-L1-expressing MDSCs were observed in tumor-infiltrating MDSCs in the CT26, MC32a and PANC02-H7 tumors (Fig. 5B). PD-L1-expressing MDSC levels are significantly lower in spleen and blood in all three orthotopic mouse models. BM MDSCs are essentially PD-L1-negative (Fig. 5B).

IFNγ is a master inflammatory cytokine that has been shown to upregulate PD-L1 expression in tumor cells.21,23 We next sought to determine whether IFNγ also regulates PD-L1 expression in MDSCs. We first analyzed IFNγ expression level in the tumor microenvironment. RNA was isolated from normal colon tissues, normal pancreas, the orthotopic CT26 tumor tissues and the orthotopic PANC02-H7 tumor tissues. Real-time PCR analysis revealed that IFNγ mRNA level is dramatically higher in both the CT26 tumor and the PANC02-H7 tumor as compared to that in normal colon and pancreas, respectively (Fig. 5C). These observations suggest that IFNγ is highly expressed in the tumor microenvironment. To functionally determine that IFNγ upregulates PD-L1 expression in the
tumor-infiltrating MDSCs, we treated tumor-bearing mice with IFNγ neutralization antibody. IgG was used as isotype control for the in vivo treatment. Analysis of tumor tissues indicated that IFNγ neutralization mAb significantly decreases PD-L1-expressing MDSCs in the tumor microenvironment in both the CT26 and PANC02-H7 tumor-bearing mice (Fig. 5D).

Establishment of J774M cells as a MDSC-like cell line

The above observation demonstrated that the vast majority of tumor-infiltrating MDSCs are PD-L1+, and PD-L1 expression in MDSCs are regulated at least in part by IFNγ in tumor-bearing mice. To validate this finding in a defined MDSC system, we sorted CD11b+Gr1+ cells from myeloid cell line J774 and established a stable cell line termed J774M. J774M is CD11b+Gr1+ and thus phenotypically resembles tumor-induced MDSCs (Fig. 6A). Interestingly, almost all J774M cells are PD-L1+ (Fig. 6A). Treatment of J774M cells with IFNγ in vitro significantly increased PD-L1 protein level on J774M cell surface (Fig. 6B) and PD-L1 mRNA level in J774M cells (Fig. 6C), suggesting that IFNγ regulates PD-L1 expression in the transcription level in myeloid cells. To determine whether J774M cells are immunosuppressive as tumor-induced MDSCs, CD3+ T cells were purified from BALB/c mouse spleens and cultured in anti-CD3 and anti-CD28-coated plates. J774M cells to the T cell cultures at various ratios. Analysis of T cell proliferation revealed that J774M cells exhibit potent inhibitory activity against T cell activation and proliferation at a dose-dependent manner (Figs. 6D and E). Taken together, our data determined that J774M cells phenotypically and functionally mimic tumor-induced MDSCs, and IFNγ upregulates PD-L1 expression in MDSCs.

IFNγ-activated pSTAT1 does not directly regulate PD-L1 expression

We next used J774M cells to elucidate the molecular mechanisms underlying IFNγ transcriptional activation of PD-L1 in MDSCs. IFNγ treatment of J774M cells rapidly induced STAT1 phosphorylation (Fig. S1A). However, our ChIP analysis determined that pSTAT1 does not bind to the cd274 promoter region in J774M cells (Fig. S1B). It is therefore apparent that pSTAT1 regulates PD-L1 expression indirectly. Although IFNγ-activated pSTAT1 can act directly to regulate gene expression, it is well demonstrated that IFNγ can regulate IRF expression. IRF proteins function as master transcription factors to regulate gene expression in various cell types, particularly in myeloid cells.19,49,50 Analysis of the nine known IRF family members indicated that treatment of J774M cells with IFNγ upregulated expression of IRF1, IRF5, IRF7 and IRF8 (Figs. S1C and D). Therefore, IRF1, IRF5, IRF7 and IRF8 are potential regulators of PD-L1 expression in MDSCs.

Mouse cd274 promoter contains three potential IRF-binding consensus sequence elements

The human CD274 promoter regulatory region contains two IRF-binding consensus sequence elements that are responsible for IFNγ-activated PD-L1 transcription activation.21 However, analysis of the mouse cd274 promoter regulatory region revealed that there is only 41% sequence similarity in this region between human and mouse genomes (Fig. S2). Furthermore, the two human IRF-binding consensus sequence elements are not present in the mouse cd274 promoter region (Fig. S2). However, we identified three potential IRF-binding consensus sequence elements in mouse cd274 promoter region (Figs. S2 and 7A).

IFNγ-activated IRF1 binds to the cd274 promoter to activate cd274 transcription

To determine whether and which of the four IRFs binds to these IRF elements in the cd274 promoter region, we designed three DNA probes containing these three IRF-binding consensus sequence elements, respectively (Fig. 7A). Nuclear extracts were prepared from IFNγ-treated J774M cells and analyzed for protein–DNA interactions using these three DNA probes by EMSA. IRF1-, IRF5, IRF7- and IRF8-specific antibodies were used to identify specific IRF binding to the DNA probes. We observed no IRF5, IRF7 or IRF8 binding to these three DNA sequences (Fig. S3). However, specific IRF1 binding to one of the three potential IRF-binding consensus sequence elements was detected (Fig. 7B). To validate this finding, we performed ChIP with IRF1-specific antibodies in J774M cells after IFNγ treatment. Five PCR primer pairs were designed to span the cd274 promoter region from −4,000 to +1,000 (Fig. 7C). PCR analysis of immunoprecipitated chromatin showed that IRF1 binds to the cd274 promoter region near the transcription start site. These data thus indicate that IFNγ may activate STAT1 that regulates IRF1 expression and IRF1 then binds to the cd274 promoter region to upregulate cd274 transcription. Indeed, there exist two potential pSTAT1-binding consensus sequence elements in the mouse irf1 promoter region (Fig. S4A), and EMSA analysis showed that pSTAT1 binds to one of these two GAS elements in the irf1 promoter region (Fig. S4B).

To functionally validate that IRF1 regulates PD-L1 expression in MDSCs, J774M cells were transfected with scramble or IRF1-specific siRNA, respectively. Real-time PCR analysis indicates that IFNγ upregulates both IRF1 and PD-L1 expression, and silencing IRF1 expression diminished PD-L1 increase in J774 M cells (Fig. 7D). We therefore conclude that IFNγ induces rapid phosphorylation of STAT1 that binds to the GAS element in the irf1 promoter region to activate irf1 transcription. The upregulated IRF1 then binds to the IRF-binding consensus DNA sequence in the cd274 promoter to activate PD-L1 expression in MDSCs.

Discussion

The PD-L1 expression profiles in human colorectal carcinoma have been extensively studied. Overall, the results vary greatly from study to study. It was reported that PD-L1 protein is present in 37% of mismatch repair (MMR)-proficient and 29% of MMR-deficient human colon carcinoma.6 In other studies, PD-L1 protein was observed in 22–38.9%,43 48.21%,44 54.5%45 and 89%6 of tumor cells in human colorectal cancer tissues. A more recent study has detected PD-L1 expression in 89% human colorectal cancer cases.6 Furthermore, analysis of PD-
Figure 6. Establishment of a stable MDSC-like cell lines. (A) CD11b<sup>+</sup>Gr1<sup>+</sup> cells were sorted from J774 myeloid cell line and establish a CD11b<sup>+</sup>Gr1<sup>+</sup> stable MDSC-like cells lines termed J774M. (B) J774M cells were treated with IFNγ for approximately 20 h. Cells were then stained with PD-L1-specific mAb and analyzed by flow cytometry. The MFI of PD-L1 was quantified and presented at the right panel. Column: mean; Bar: SD. (C) J774M cells were treated with IFNγ as in B and analyzed by qPCR for PD-L1 mRNA level with β-actin as internal normalization control. (D) CD3<sup>+</sup> T cells were purified from BALB/c mouse spleens and labeled with CFSE. Cells were then seeded in anti-CD3 and anti-CD28-coated 96-well plate (1 × 10<sup>5</sup> cells/well). At the same time, J774M cells were added to the culture at the indicated cell density (top left corner) and the cell mixture were cultured for another 72 h. Cells were collected and stained with CD11b-specific mAb. CD11b<sup>+</sup> cells were gated and analyzed for CFSE intensity. (E) Quantification of CD3<sup>+</sup> T cell proliferation as determined by CFSE intensity as shown in (D). Column: mean; Bar: SD.
L1 expression level in various types of human cancers revealed that while PD-L1 is highly expressed in human melanoma, non-small cell lung carcinoma, and renal cell carcinoma, human colorectal carcinoma is essentially PD-L1–. This is possible that this discrepancy in PD-L1 expression patterns in human colorectal carcinoma between different studies is due to the subtypes of the colorectal cancers. It has recently been shown that while MSI colon cancer cells are PD-L1+, there was virtually no discernible PD-L1 expression on tumor cells of MSS colon cancer cells by IHC. However, it is also possible that discrepancies of PD-L1 expression patterns of human colorectal carcinoma in these various studies might be due to the various anti-PD-L1 antibodies used in these studies. A highly sensitive and specific anti-PD-L1 mAb (Clone 28–8) has recently developed and approved by FDA for detecting PD-L1 protein in human tumor tissues by IHC. Using this newly developed PD-L1-specific mAb, we detected PD-L1 protein in all human colon cancer tissues of various stages from adenoma to liver metastases. Based on these observations, we conclude that PD-L1 is expressed in all stages of human colon carcinoma.

The PD-L1 expression patterns of tumor-infiltrating leukocytes in human colorectal carcinoma tissues also vary greatly between different studies. It was reported that while colon carcinoma cells express high level of PD-L1, tumor-infiltrating immune cells are essentially PD-L1–. In another study, few tumor-infiltrating leukocytes were found to be PD-L1+. It was also reported that while tumor-infiltrating CD163+ myeloid cells expressed high level of PD-L1 in the MSI colon cancer, no clear PD-L1+ tumor-infiltrating CD163+ cells were detected in MSS colon cancer tissues. In this study, we observed that tumor-infiltrating leukocytes are abundantly present in human colon carcinoma tissues and the majority of these leukocytes are PD-L1+. We also shown here that MDSCs constitute a large fraction of human leukocytes in human cancer patients and these tumor-induced MDSCs express significantly higher level of PD-L1 than the MDSC-like cells from normal donors. This observation is validated by our analysis of blood sample from human colon cancer patients and mouse tumor models. MDSCs massively accumulate in the peripheral blood of human colon cancer patients and a large portion of these MDSCs are PD-L1+. Our finding is consistent with one report of human colorectal cancer MDSC PD-L1 expression. Only a fraction of human colon cancer, the MSI human colon cancer, responds to anti-PD-L1/PD-1 immunotherapy, and it has been shown that CD163+ myeloid cells heavily infiltrate at invasive tumor front in MSI colon carcinoma but not in MSS colon carcinoma. In this study, although we observed that tumor-infiltrating leukocytes are abundantly present in human colon carcinoma tissues and the majority of these leukocytes are PD-L1+, we also observed that MDSCs massively accumulate in the peripheral blood of human colon cancer patients and a large portion of these MDSCs are PD-L1+. This finding is consistent with one report of human colorectal cancer MDSC PD-L1 expression. Only a fraction of human colon cancer, the MSI human colon cancer, responds to anti-PD-L1/PD-1 immunotherapy, and it has been shown that CD163+ myeloid cells heavily infiltrate at invasive tumor fronts and heavily CD45+ leukocyte infiltration was also observed in the majority of MSS colon carcinoma analyzed. Further studies in a larger cohort of human MSI and MSS colon carcinomas are clearly required. Nevertheless, our observations clearly indicate that leukocytes also infiltrate MSS colon carcinomas and the majority of tumor-infiltrating leukocytes are PD-L1+ in both MSI and MSS colon carcinomas.

Interestingly, the highest percentages of PD-L1-expressing MDSCs are present in the tumor tissues. Considering the fact
that MDSCs massively accumulate in human cancers including colorectal cancer, our findings thus raise the notion that in addition to the tumor cells, the PDL1 MDSCs might be another major source of PD-L1 that inhibits tumor-infiltrating CTL activation and function in the tumor microenvironment. The relative role of these PDL1 MDSCs in colon cancer immune escape and resistance to immune checkpoint immunotherapy requires further studies.

It is well documented that PD-L1 is constitutively expressed and inducible in tumor cells. IFNγ is an essential component of the host cancer immune surveillance system. Loss of IFNγ expression and function lead to increased cancer incidence including augmented colon carcinoma growth in vivo. However, IFNγ is also a master inducer of PD-L1 in human tumor cells. The human CD274 gene promoter region has two IRF-binding consensus sequence elements and IFNγ-activated IRF1 binds to these two DNA elements to upregulate PD-L1 expression in human tumor cells. The regulation of PD-L1 in MDSCs is much less studied. It has been shown that PD-L1 expression in MDSCs is regulated by HIF-1α in the tumor microenvironment. Here, we show that IFNγ also regulates PD-L1 expression in mouse MDSCs. Surprisingly, the mouse cd274 gene promoter region DNA sequence only has 41% similarity to the human CD274 gene promoter. Furthermore, the mouse cd274 gene promoter contains three putative IRF-binding consensus sequence elements that are distinct from those in human CD274 promoter. However, IFNγ-activated IRF1 only binds to one of these three DNA elements to activate cd274 transcription in MDSCs. Therefore, our data determined that PD-L1 expression in MDSCs is also regulated by IFNγ that activates pSTAT1 to directly regulate IRF1 transcription, and IRF1 directly binds to an unique IRF-binding consensus element to upregulate PD-L1 expression in MDSCs. Although IFNγ is essential for host cancer immune surveillance against colon cancer development, chronic IFNγ signaling results in spontaneous colon cancer development. It is possible that the chronic IFNγ signaling might contribute to increased level of PD-L1 expression in MDSCs in the tumor microenvironment to promote colon cancer development. Therefore, targeting chronic IFNγ signaling in the tumor microenvironment to decrease PD-L1 expression level in MDSCs in the tumor microenvironment might be an effective approach to reverse immune suppression to inhibit colon cancer progression, which also requires further studies.

Materials and methods

Tumor cells and specimens

The mouse colon carcinoma cell line CT26 was obtained from American Type Culture Collection (ATCC) (Manassas, VA). ATCC has characterized these cells by morphology, immunology, DNA fingerprint and cytogenetics. The murine MC32a cell line were kindly provided by Dr Jeffrey Schlom (National Cancer Institute) and characterized as previously described. PANC02-H7 cells were kindly provided by Dr Min Li (University of Oklahoma Health Sciences Center) and characterized as previously described. Human colorectal carcinoma progression tissue microarrays (TMA) were provided by the Cooperative Human Tissue Network (CHTN) Atlantic Division (Charlottesville, VA). MSI and MSS human colon carcinoma specimens were characterized and provided by CHTN Southern Division (Birmingham, AL) (Table S3). Peripheral blood specimens were collected from consented healthy donors at the Shepherd Community Bank and from de-identified colon cancer patients at Augusta University Medical Center. All studies of human specimens were performed according to protocols approved by Augusta University Institutional Human Research Protection Committee.

Orthotopic mouse tumor models

C57BL/6 and BALB/c mice were obtained from the Jackson Laboratory. The spontaneous colon cancer mice were generated by treatment with azoxymethane (AOM) and dextran sodium sulfate (DSS) as previously described. For orthotopic mouse tumor models, mouse was continuously anesthetized under 2% isoflurane in oxygen flow. For CT26 orthotopic tumor model, a small abdominal incision was made at the right side of BALB/c mice and the cecal wall was identified. CT26 tumor cells (1 × 10^6 cells in 20 μL saline) were injected into the cecal wall of BALB/c mice using a sterile tuberculin syringe. For MC32a orthotopic tumor model, MC32a tumor cells (1 × 10^6 cells in 20 μL saline) were injected into the cecal wall of C57BL/6 mice using a sterile tuberculin syringe. For PANC02-H7 orthotopic tumor model, a small abdominal incision at the right side near the spleen of C57BL/6 mice and the pancreas were pulled out with a sterile forcep. Tumor cells (1 × 10^6 cells in 20 μL saline) were injected into the pancreas using a sterile tuberculin syringe. All mouse studies are performed according to protocols approved by Augusta University Institutional Animal Care and Use Committee.

J774M cell line

The myeloid cell line J774 was obtained from ATCC. J774 cells were stained with CD11b- and Gr1-specific antibodies and analyzed by flow cytometry. The CD11b^+Gr1^+ cells were sorted and cultured to establish a stable CD11b^+Gr1^+ cell line termed J774M.

Western blotting analysis

Western blotting analysis was performed as previously described. Antibodies are listed in Table S1.

Gene expression analysis

Cells were lysed in Trizol (Life Technologies) to isolate total RNA. cDNA was synthesized from total RNA and used for analysis of gene expression using gene-specific primers (Table S2) in the StepOne Plus Real-Time PCR System (Applied Biosystems).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out as previously described using the SimpleChIP Enzymatic Chromatin IP Kit (cell signaling) according to the manufacturer’s instructions as previously
described. The *pd-l1* promoter DNA was detected by real-time PCR using promoter DNA-specific primers as listed in Table S2.

**Immunohistochemistry**

Immunohistochemical staining was performed as previously described. TMA slides were deparaffinized and rehydrated, followed by treatment with the Universal HIER antigen retrieval reagent (Abcam, Cat# ab208572) according to the manufacturer’s instructions. The slides were then blocked with goat serum, rinsed and probed with anti-human PD-L1 Rab-MAb (Abcam, clone 28–8) and CD45 (Biolegend) antibodies. The tissues were probed with rabbit specific IHC polymer detection kit (Abcam, Cat# ab209101) according to the manufacturer’s instructions. The stained tissues were counterstained with hematoxylin (Richard-Allan Scientific, Kalamazoo, MI).

**Electrophoresis Mobility Shift Assay (EMSA) of protein–DNA interactions**

Sequences of DNA probes containing *irf1* and *cd274* promoter GAS and IRF1-binding consensus elements are listed in Table S2. EMSA was performed as previously described. Briefly, the end-labeled probes were incubated with nuclear extracts for 20 min at room temperature. Anti-pSTAT1 (Santa Cruz) and anti-IRF1, anti-IRF5, anti-IRF7 and anti-IRF8 antibodies (Santa Cruz) were included to identify specific protein–DNA complexes. DNA–protein complexes were separated by electrophoresis in 6% polyacrylamide gels and identified using a phosphoimage screen (Molecular Dynamics) and the images were acquired using a Personal Molecular Imager (BioRad).

**Cell surface protein analysis**

Cells were stained with fluorescent dye-conjugated antibodies that are specific for (1) human CD11b, CD33, HLA-DR, CD11c, CD3, CD19, CD337 and PD-L1; and (2) mouse CD11b, Gr1, Ly6G, Ly6C, CD11c, CD3, CD19, NK1.1 and PD-L1. Stained cells were analyzed by flow cytometry.

**In vitro T cell activation and co-culture with MDSCs**

Spleens were collected from BALB/c mice. Single-cell suspension was prepared and used to purify CD3+ T cells using the MojoSort mouse CD3 T cell isolation kit (Biolegend) according to the manufacturer’s instructions. For T cell activation, 96-well culture plate was coated with anti-mouse CD3 and anti-mouse CD28 MAb (0.25 μg/well in 150 μL PBS) overnight. The purified T cells were labeled with CFSE (Life Technologies) and then seeded in the coated plate at a density of 1.5 × 10^5 cells/well in RPMI medium plus 10% FBS. 774M cells were added to the culture at the same time and co-cultured for another 72 h. Cells were then analyzed by flow cytometry.

**Gene silencing**

Tumor cells were transiently transfected with scramble and IRF1-specific siRNAs (Santa Cruz, Cat# sc-35707) respectively and analyzed for IRF1 and PD-L1 expression by real-time PCR. IRF1 and PD-L1 specific PCR primers are listed in Table S2.

**Statistical analysis**

Statistical analysis was performed using ANOVA and paired Student’s t-test. A *p* < 0.05 was taken as statistically significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Dr Asha Nayak-Kapoor for collecting blood specimen from de-identified human colon cancer patients. We also thank Dr Kimya Jones in the Georgia Pathology Services in assistance for immunohistochemical staining of tumor specimens.

**Funding**

Kebin Liu was funded by National Cancer Institute CA182518 and VA Merit Review Award BX001962.

**Author contributions**

C.L., P.S.R.: performed experiments; C.L., P.S.R., J.R.L., N.S.: analyzed data; C.L., K.L.: designed studies and wrote manuscript.

**References**

1. Sun L, St Leger AJ, Yu CR, He C, Mahdi RM, Chan CC, Wang H, Morse HC, 3rd, Egyuagu CE. Interferon Regulator Factor 8 (IRF8) Limits Ocular Pathology during HSV-1 Infection by Restraining the Activation and Expansion of CD8+ T Cells. PLoS One 2016; 11:e0155420; PMID:27171004; http://dx.doi.org/10.1371/journal.pone.0155420
2. Simon S, Vignard V, Florenceau L, Dreno B, Khammari A, Lang F, Labarriere N. PD-1 expression conditions T cell avidity within an antigen-specific repertoire. Oncoimmunology 2016; 5:e1104448; PMID:26942093; http://dx.doi.org/10.1080/2162402X.2015.1104448
3. Lim TS, Chew V, Siew JL, Goh S, Yeong IP, Soon AL, Ricciardi-Castagnoli P. PD-1 expression on dendritic cells suppresses CD8 T cell function and antitumor immunity. Oncoimmunology 2016; 5:e1085146; PMID:27141339; http://dx.doi.org/10.1080/2162402X.2015.1085146
4. Rosenbaum MW, Bledsoe JR, Morales-Oyarvide V, Huynh TG, Minokenudson M. PD-L1 expression in colorectal cancer is associated with microsatellite instability, BRAF mutation, medullary morphology and cytotoxic tumor-infiltrating lymphocytes. Mod Pathol 2016; 29:1104-12; PMID:27198569; http://dx.doi.org/10.1038/modpathol
5. Dunne PD, McArt DG, O’Reilly PG, Coleman HG, Allen WL, Loughrey M, Van Schaeybroeck S, McDade S, Salto-Tellez M, Longley DB et al. Immune-derived PD-L1 gene expression defines a subgroup of stage II/III colorectal cancer patients with favorable prognosis that may be harmed by adjuvant chemotherapy. Cancer Immunol Res 2016; 4:582-91; PMID:27197082; http://dx.doi.org/10.1158/2326-6066.CIR-15-0302
6. Masugi Y, Nishihara R, Yang J, Mima K, da Silva A, Shi Y, Inamura K, Cao Y, Song M, Nowak JA et al. Tumour CD274 (PD-L1) expression and T cells in colorectal cancer. Gut 2016; PMID:27196573; http://dx.doi.org/10.1136/gutjnl-2016-311421
7. Dudley JC, Lin MT, Le DT, Eshleman JR. Microsatellite instability as a biomarker for PD-1 blockade. Clin Cancer Res 2016; 22:813-20; PMID:26880610; http://dx.doi.org/10.1158/1078-0432.CCR-15-1678
8. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D et al. PD-1 blockade in tumors...
with mismatch-repair deficiency. N Engl J Med 2015; 372:2309-20; PMID:26028255; http://dx.doi.org/10.1056/NEJMoa1500596
9. Kroemer G, Galluzzi L, Zitvogel L, Fridman WH. Colorectal cancer: the first neoplasia found to be under immunosurveillance and the last one to respond to immunotherapy? Oncoimmunology 2015; 4:e105897; PMID:26140250; http://dx.doi.org/10.1080/20012648.2015.1058597
10. Zitvogel L, Galluzzi L, Kroemer G, Targeting PD-1/PD-L1 interactions for cancer immunotherapy. Oncoimmunology 2012; 1:1223-5; PMID:23224358; http://dx.doi.org/10.4161/onci.21335
11. Chawla A, Philips AV, Alatrasch G, Mittendorf E. Immune check-points: A therapeutic target in triple negative breast cancer. Oncoimmunology 2014; 3:e283325; PMID:24848333; http://dx.doi.org/10.4161/onci.28325
12. Llosa NJ, Cruise M, Tam A, Wicks EC, Hechenbleikner EM, Taube JM, Blosser RL, Fan H, Wang H, Luber BS et al. The vigorous immune microenvironment of metastasable instable colon cancer is balanced by multiple counter-inhibitory checkpoints. Cancer Discov 2015; 5:43-51; PMID:25358689; http://dx.doi.org/10.1158/2159-8290.CD-14-0863
13. Brahmer JR, Tykodi SS, Chow LQ, Topalian SL, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsii K et al. Safety and activity of anti-PD-L1 antibody in patients with advanced NSCLC. N Engl J Med 2012; 366:2455-65; PMID:22658128; http://dx.doi.org/10.1056/NEJMoa1200694
14. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvalaj RD, Somasam JA, Atkins MB et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 2012; 366:2443-54; PMID:22265127; http://dx.doi.org/10.1056/NEJMoa1200690
15. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med 2002; 8:793-800; PMID:12091876; http://dx.doi.org/10.1038/nm0902-1039c
16. Spranger S, Spaapen RM, Zha Y, Williams J, Meng Y, Ha TT, Gajewski TF. Up-regulation of PD-1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. Sci Transl Med 2013; 5:200ra116; PMID:23986400; http://dx.doi.org/10.1126/scitranslmed.3005650
17. Song M, Chen D, Lu B, Wang C, Zhang J, Huang L, Wang X, Timmons CL, Hu J, Liu B et al. PTEN loss increases PD-L1 protein expression and affects the correlation between PD-L1 expression and clinical parameters in colorectal cancer. PLoS One 2013; 8:e65821; PMID:23785454; http://dx.doi.org/10.1371/journal.pone.0065821
18. Hong S, Chen N, Fang W, Zhan J, Liu Q, Kang S, He X, Liu L, Zhou T, Huang J et al. Upregulation of PD-1 by EML4-ALK fusion protein mediates the immune escape in ALK positive NSCLC: Implication for optional anti-PD-1/PD-L1 immune therapy for ALK-TKIs sensitive and resistant NSCLC patients. Oncoimmunology 2016; 5:e1094598; PMID:27141355; http://dx.doi.org/10.2147/onci.a109459
19. Tang Y, Fang W, Zhang Y, Hong S, Kang S, Yan Y, Chen N, Zhan J, He X, Qin T et al. The association between PD-L1 and EGFR status and the prognostic value of PD-L1 in advanced non-small cell lung cancer patients treated with EGFR-TKIs. Oncotarget 2015; 6:14209-19; PMID:25895031; http://dx.doi.org/10.18632/oncotarget.6394
20. Fang W, Zhang J, Hong S, Zhan J, Chen N, Qin T, Qin T, Tang Y, Zhang Y, Kang S et al. EBV-driven LMP1 and IFN-gamma up-regulate PD-L1 in nasopharyngeal carcinomas: Implications for oncotargeted therapy. Oncotarget 2014; 5:12189-202; PMID:25361008; http://dx.doi.org/10.18632/oncotarget.2608
21. Lee SJ, Jang BC, Lee SW, Yang Yi, Suh SI, Park YM, Oh S, Shin JG, Yoo S, Chen L et al. Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-gamma-induced upregulation of B7-H1 (CD274). FEBS Lett 2006; 580:755-62; PMID:16413358; http://dx.doi.org/10.1016/j.febslet.2005.12.093
22. Taube JM, Andrews RA, Young GD, Xu H, Sharma R, McMillan TL, Chen S, Klein AP, Pardoll DM, Topalian SL et al. Colocalization of inflammatory response with B7-H1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med 2012; 4:127ra37; PMID:22461641; http://dx.doi.org/10.1126/scitranslmed.3003689
53. Inamoto S, Itatani Y, Yamamoto T, Minamiguchi S, Hirai H, Zhao K. Circulating and tumor-infiltrating myeloid-derived suppressor cells in patients with colorectal carcinoma. PLoS One 2013; 8:e57114; PMID:23437326; http://dx.doi.org/10.1371/journal.pone.0057114

54. Zhang B, Wang Z, Wu L, Zhang M, Li W, Ding J, Zhu J, Wei H, Zhao K. Companion diagnostic assays for PD-1/PD-L1 checkpoint inhibitors in NSCLC. Expert Rev Mol Diagn 2016; 16:131-3; PMID:26559787; http://dx.doi.org/10.1586/14737519.2016.1177389

55. Jorgensen JT. Companion diagnostic assays for PD-1/PD-L1 checkpoint inhibitors in NSCLC. Expert Rev Mol Diagn 2016; 16:131-3; PMID:26559787; http://dx.doi.org/10.1586/14737519.2016.1177389

56. Ostrand-Rosenberg S. Tolerance and immune suppression in the tumor microenvironment. Cell Immunol 2016; 299:23-9; PMID:26353543; http://dx.doi.org/10.1016/j.clim.2015.09.011

57. Inamoto S, Itatani Y, Yamamoto T, Minamiguchi S, Hirai H, Iwamoto M, Hasegawa S, Takeko MM, Sakai Y, Kawada K. Loss of SMAD4 promotes colorectal cancer progression by accumulation of myeloid-derived suppressor cells through the CCL15-CCR1 chemokine axis. Clin Cancer Res 2016; 22:492-501; PMID:26341919; http://dx.doi.org/10.1158/1078-0432.CCR-15-0726

58. Chun E, Lavoie S, Michaud M, Gallini CA, Kim J, Soucy G, Odze D, Glickman JN, Garrett WS. CCL2 promotes colorectal carcinogenesis by enhancing polymorphonuclear myeloid-derived suppressor cell population and function. Cell Rep 2015; 12:244-57; PMID:26146082; http://dx.doi.org/10.1016/j.celrep.2015.06.024

59. Teng MW, Ngiew SF, Ribas A, Smyth MJ. Classifying cancers based on EGFR-driven lung tumors. Cancer Discov 2013; 3:355-63; PMID:24078774; http://dx.doi.org/10.1158/2159-8290.CD-13-0101

60. Bardhan K, Paschall AV, Yang D, Chen MR, Shankaran V, Ikeda H, Old LJ, Bedossa P, Hasegawa S, Taketo MM, Sakai Y, Kawada K. Loss of SMAD4 promotes colorectal cancer progression by accumulation of myeloid-derived suppressor cells through the CCL15-CCR1 chemokine axis. Clin Cancer Res 2016; 22:492-501; PMID:26341919; http://dx.doi.org/10.1158/1078-0432.CCR-15-0726