Editorial

PD-L1 is a diverse molecule regulating both tumor-intrinsic signaling and adaptive immunosuppression

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Introduction

Programmed death-ligand 1 (PD-L1) is a 33.28 kDa protein on the surface of many immune and non-immune cells (1-3). Its primary function as a co-stimulatory molecule is well documented (4). PD-L1 serves as an 'immune checkpoint' and it binds to receptor programmed death-1 (PD-1) to regulate immune responses among antigen presenting cells and T-cells. Unfortunately, this exact mechanism is exploited by tumor cells where increased expression of PD-L1 results in immunosuppression of the adaptive tumor response by inhibiting T-cell proliferation, reducing T-cell survival, inhibiting cytokine release, and promoting T-cell apoptosis (5,6). This leads to T-cell exhaustion and immunosuppression in the tumor microenvironment (7).

PD-L1 expression can be constitutive and inducible. Constitutive expression is dependent upon cell genomics. Deleterious gene mutations influence cell signaling with downstream effects on PD-L1 expression levels. Inducible PD-L1 expression is dependent upon exposure of cells to IFNγ, TNFα, IL-1α, and IL-1β via TLRs or IFN receptors in the ERK and IFNγ signaling networks (1,4).

Inhibition of PD-1 and PD-L1 interactions at the cell surface have become a powerful strategy to reverse tumor-induced immunosuppression. Immunotherapy treatment decreases tumor growth, increases tumor-infiltrating T-cells, and decreases regulatory T cells. As a result, PD-L1 has emerged as a prominent biomarker. A number of commercially produced monoclonal antibody immunotherapies are now being clinically tested in a number of cancers and PD-L1 reactivity is being used to predict clinical treatment outcomes. Although antibodies to PD-L1 (αPD-L1) and PD-1 (αPD-1) have been used for the treatment of cancer and show promising outcomes, only a proportion of patients respond to the treatments (4), which indicates that there are other factors in play.

PD-L1 has tumor-intrinsic functions

Although tumor-expressed PD-L1 induces immunosuppression of the adaptive tumor response, Clark *et al*, in their recent study in *Cancer Research*, demonstrated that PD-L1 and PD-1 also have tumor-intrinsic functions (8). They showed that PD-L1 impacted tumor cell biology via autophagy and mTOR and suggested that there are non-immune, broader uses for PD-L1 as a biomarker for assessing cancer therapeutic responses. This study fills an important gap in our current understanding of the role of PD-L1 in tumor pathogenesis.

PD-L1 expression in cell lines and clones

Clark *et al*. developed a number of unique cell lines and clones with constitutive and inducible PD-L1 expression. A murine ID8 ovarian cancer cell line was used, an aggressive ID8 cell line was created (ID8agg), and a PD-L1 knockdown clone of ID8agg was created (ID8agg PD-L1^{lo}). Murine B16 melanoma cells were used that expressed basal PD-L1 that is further upregulated by IFNγ. A PD-L1 overexpressing clone of B16 was created (B16 PD-L1^{hi}) and

a PD-L1 knockdown clone of B16 was created (B16 PD-L1^{lo}). Both ID8 and B16 cell lines also expressed PD-1.

Wild-type (WT) mice, severely immune-deficient (NSG) mice, PD-L1 knockout (KO) mice, and T cell-deficient βδ (TCR KO) mice were used.

Effect of PD-L1 on proliferation

Clark *et al*. initially established that tumor intrinsic PD-L1 promoted cell proliferation in two distinct models (melanoma and ovarian cancer), that form tumors in two distinct anatomic compartments (skin and peritoneum) in an immune-independent fashion. Proliferation of cell lines and clones were related to PD-L1 expression. In proliferation assays, B16 PD-L1^{lo} and ID8agg PD-L1^{lo} cells had slower rates of proliferation than B16 cells, B16 PD-L1^{hi} cells, and ID8agg cells. In WT and NSG mouse challenge models, tumor volumes were also related to the PD-L1 expression levels of cell lines and clones.

Effects of antibodies

αPD-L1 was reported to alter PD-L1-mediated cell-intrinsic growth signals. Proliferation of B16 cells but not B16 PD-L1^{lo} cells and proliferation of ID8agg cells but not ID8agg PD-L1^{lo} cells were reduced in cell culture. Clark *et al*. also noted that the effects of αPD-L1 were directed to tumor cells and not other PD-L1-expressing cells. In WT mice, αPD-L1 arrested tumor growth of B16 cells, but not B16 PD-L1^{lo}cells. In PD-L1 KO mice, αPD-L1 arrested tumor growth of B16 cells (and was fully protective), but not B16 PD-L1^{lo}cells. In NSG mice incapable of mediating tumor-specific immunity or antibody-dependent cellular cytotoxicity, αPD-L1 slowed tumor growth of B16 cells. In WT and NSG mice, αPD-L1 did not arrest tumor growth of ID8agg cells or improve survival, despite reducing the proliferation ID8agg cells in culture.

Role of autophagy

Clark *et al*. also observed that tumor PD-L1 suppressed tumor autophagy in their melanoma and ovarian cancer models, suggesting that autophagy disruption could be a relatively general tumor PD-L1 effect. A total of 1,269 differentially expressed basal genes were found in PD-L1 altered tumor-intrinsic signaling of RNA-seq data between ID8agg cells and ID8agg PD-L1^{lo} cells. The set of genes were found to impact (I) cell viability after specific insults, (II) differences in survival in serum starvation, and (III) differences in autophagy inhibitors and TNFα.

There were differences in cell viability. PD-L1^{hi} B16 cell viability was lower under serum starvation conditions than B16 cells or B16 PD-L1^{lo} cells.

There were differences in survival under serum starvation conditions. The autophagic flux, defined as the conversion of LC3-I to LC3-II (e.g., LC3-II/LC3-I ratio) was higher in ID8agg PD-L1^{lo} cells versus ID8agg cells and serum starvation did not increase LC3-II further in ID8agg PD-L1^{lo} cells suggesting that tumor PD-L1 regulated basal and starvation-induced autophagy in ID8agg. Flux was also blunted in B16 cells and autophagy was induced during serum starvation in B16 PD-L1^{lo} cells. LC3 foci formation was also higher in B16 PD-L1^{lo} cells than in B16 cells under basal conditions and serum starvation was indicative of higher autophagosome formation. This was consistent with tumor cell PD-L1-mediated suppression of autophagy in B16.

Furthermore, there were differences in the effects of autophagy inhibitors and TNFα. B16 cells and ID8agg cancer cells expressing PD-L1 had low basal autophagy and were disproportionately sensitive to autophagy inhibitor-mediated growth reduction compared to clones with lower PD-L1 expression. The results were verified in cells and mice with the autophagy inhibitor chloroquine. B16 PD-L1^{lo} cells were more resistant to chloroquine than B16 PD-L1^{hi} cells. Similarly, ID8agg PD-L1^{lo} cells were more resistant to chloroquine than ID8agg cells. Work in WT, NSG, and TCR KO mice confirmed the observation that tumor PD-L1 sensitized B16 cells but not ID8agg cells to autophagy inhibitors *in vivo*. Chloroquine improved survival against ID8agg cell or ID8agg PD-L1^{lo} cell challenge in WT mice. Thus this outcome was independent of T cell immunity and supported the concept that tumor PD-L1 expression predicted autophagy-dependent growth.

mTOR

ID8agg cell RNA-seq data also identified potential PD-L1 regulated mTOR (mammalian target of rapamycin) signaling. mTOR is a network that regulates cell metabolism, growth, proliferation, and longevity that regulates cellular processes including autophagy through the multi-protein complexes mTORC1 and mTORC2. Clark *et al.* reported that tumor PD-L1 regulated mTOR and promoted basal mTORC1 signaling as assessed by P70S6KT389 phosphorylation during serum starvation and

other treatments. PD-L1^{lo} cells proliferated more slowly than control lines, suggesting that lower mTORC1 activity in PD-L1^{lo} cells compromises their growth. Rapamycin is a specific mTOR inhibitor that complexes with FKBP-12 and binds to mTOR inhibiting it's activity. Rapamycin suppressed mTORC1 and abolished P70S6KT389 phosphorylation in B16 cells, ID8agg cells, and B16 PD-L1^{lo} cells, suggesting that PD-L1 regulation of metabolic activity is mTORC1-dependent. However, other mTORC1 substrates, mTORC2, or other rapamycin effects could also mediate specific PD-L1 dependent effects.

Another interesting observation focused on PD-L1 as a pro-survival agent to the cytotoxic insults of chemotherapy agents and cytokines. B16 cells and ID8agg cells were resistant to cisplatin- and paclitaxel-mediated cytotoxicity and B16 cells, but not ID8agg cells, were resistant to TNFα-mediated cytotoxicity.

Finally, the results of this study carried over to human cell lines. Cell-intrinsic PD-L1 regulated proliferation, mTOR signaling, autophagic flux, and sensitivity to autophagy inhibitors in the human ES2 (hES2) ovarian cancer cell line. PD-L1 knock down eliminated hES2 mTORC2 activation (AktS473 phosphorylation) suggesting that PD-L1 regulation of cancer cell autophagy and autophagy dependence could be a common mechanism in PD-L1 expressing mouse and human cancer cells.

Implications

Overall, PD-L1 was reported to promote tumor cell proliferation and immune-independent growth in melanoma and ovarian cancer cell lines and clones in cell culture and metastatic melanoma spread in mice. αPD-L1 inhibited melanoma growth, but not ovarian cancer growth in an immune-independent fashion in mice. Tumor cell-intrinsic PD-L1 was found to alter autophagy inhibitor and mTOR inhibitor efficacies. Growth of melanoma cells but not ovarian cancer cells were slowed by autophagy inhibitors in mice. PD-L1 was found to promote basal mTORC1 activation in all cells tested and inhibited phosphorylation of the mTORC2 substrate Akt in mouse lines, but not a human cell line. These observations have important implications.

Clark *et al.* first suggested that tumor PD-L1 could be a biomarker for response to mTOR or autophagy inhibitors in selected cancers. PD-L1 expression could predict mTORC1 activity and rapamycin-sensitive tumor growth. They also suggested that autophagy reduction in cancer cells with elevated mTORC1 activity and low autophagic activity related to PD-L1 expression could be a catastrophic cell event, exploited in future combination therapy treatments. Their data suggested that autophagy inhibitors could boost αPD-L1 treatment of PD-L1^{+} cancers. αPD-L1 and/or αPD-1 immunotherapies could be combined with mTOR inhibitors in treating PD-1/PD-L1-replete tumors, or mTOR inhibitors could be used alone in treating PD-1^{lo}/PD-L1^{lo} tumors.

Remaining gaps and questions

While separating the intrinsic effects of PD-L1 within cells from the immune effects of PD-L1 among cells, Clark *et al.* left us with a number of new gaps in this concept. The first gap concerns the induction and maintenance of tumor PD-L1 reactivity. PD-L1 expression alone as a biomarker for immunotherapy selection is not sufficient and there is a need to consider input from tumor mutations, inflammatory cells, and other biomarkers (9-11). Most studies have focused on immune cell activation and de-activation only by predicting PD-L1, immune activation, and evasion. Prior studies have not focused on PD-L1 expression linked to proliferation, viability, and invasion as suggested by Clark *et al*. If their concept holds up, this work has many implications for the use of immuno-oncology drugs. And prior studies have not focused on non-immuno-oncology drug responses with or without PD-L1 inhibitors. A second gap is how to consider multi-gene tumor sequencing implications in this model. A third gap is how to predict responses to checkpoint inhibitors by looking beyond PD-L1 expression. Unfortunately, Clark *et al.* only presents trends that are difficult to derive from their data or quoted in their list of references. One question remains on how these receptors are signaling within the tumor cell and having an impact on autophagy and mTOR. Additional and further insights into the signaling pathways and or mechanisms involved are needed. Finally, the last gap concerns the differences among results. They are not consistent and varied between tumor types. There was not enough information to suggest whether these differences were related to either tumor-specific or compartment-specific events. This again indicates further complexities and nuances that may occur between differences in tumor types, microenvironments, and tumor genomics. Still, this article presented interesting, novel concepts and opened additional avenues for treating cancers utilizing therapeutics that have more than one cancer cell target.

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Conflicts of Interest: Kim A. Brogden has had a Cooperative Research and Development Agreement with Cellworks Group, Inc., San Jose, CA. Kim A. Brogden declares no competing financial interests in the findings of this study or with Cellworks Group, Inc., San Jose, CA. Taher Abbasi and Shireen Vali work for Cellworks Group, Inc., San Jose, CA, USA. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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