Review Article

PD-L1, Inflammation and Glioblastoma

Robert Carlsson* and Shohreh Issazadeh-Navikas

Neuroinflammation Unit, Biotech research and innovation center (BRIC), Copenhagen University, Copenhagen, Denmark

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Abstract

Programmed death ligand 1 (PD-L1) is a transmembrane receptor that can contribute both to the termination of unwanted chronic inflammation, and to immune escape from desired immune responses by glioblastoma multiforme (GBM). Recent reports show that expression of the anti-inflammatory protein, PD-L1 on glioblastoma and other cancer cells, correlate with poor prognosis as a result of immune escape. On the contrary on stromal cells such as neurons, PD-L1 expression can correlate with a better prognosis, suggesting that the cell type where PD-L1 is expressed plays decisive role for the outcome of the disease. We discuss here the divergent functions of PD-L1 in the tumor microenvironment.

INTRODUCTION

Glioblastoma multiforme (GBM) is a grade IV WHO tumor with dismal patient prognosis and a median survival time of 12-14 months. Despite surgery and cytostatic treatment the tumor prevails. Better understanding of the initiation, maintenance, microenvironment and etiology of GBM is required for designing more successful treatments.

PD-L1 and GBM

Programmed death ligand 1 (PD-L1, also known as CD274 and B7H1) is a trans-membrane protein molecule with suggested proteoglycan moieties [1]. It is expressed predominantly in the heart and placenta at the mRNA level [2]. At the protein level PD-L1 expression can be found and induced on macrophages, T-lymphocytes and neurons [3].

The PD-L1 protein has an extracellular domain, a transmembrane domain and an intracellular domain. The extracellular domain (ECD) of the PD-L1 protein is comprised of IgC–like and IgV-like domains [2]. The ECD of PD-L1 interacts with the programmed death 1 (PD-1, also known as PDCD1) and B7.1 receptors. Blocking of the PD-L1 molecule with antibodies blocks cancer cells from killing cytotoxic T lymphocytes (CTLs), whereas siRNA knockdown of the PD-L1 molecule in glioblastoma makes the glioblastoma susceptible to CTL mediated killing [4,5]. Interaction with B7.1 results in the inhibition of T-lymphocyte responses *in vitro* and diabetogenic T-lymphocytes *in vivo* [6, 7].

PD-L1: PD-1 has an affinity of 0.77uM when glycosylated and in dimers and the monomeric non-glycosylated proteins has an affinity of 26.6uM. Dimeric PD-L1:B7.1 bind with an affinity of 1.99uM and the non-glycosylated monomeric proteins 80.7uM

[6,8]. This shows that important receptor ligand interactions can be made even though the apparent affinity is relatively low [8].

Of note, no functional properties are known to date for the intracellular domain of PD-L1, although some indications of an outside-in signaling exist upon B7.1 binding. CTLA4 and CD28 are the two other ligands for B7.1. When co-culturing CTLA4-/- CD28-/- T-lymphocytes with beads coupled with CD3 and B7.1 their proliferation is diminished, suggesting that B7.1 on the beads binds to PD-L1 on the T lymphocytes and down modulate anti-CD3 provoked proliferative capacity [6].

PD-L1 has been suggested to promote immune escape by tumors including glioblastomas [4,9,10]. Loss of phosphatase and tensin homolog (PTEN), a major GBM tumor suppressor, induces the expression of PD-L1 and possibly immune escape in GBM [5]. Impact of PTEN loss on up-regulation of PD-L1 in PTEN-GBMs is not the result of the gene regulation but it regulates PD-L1 post transcriptionally and correlates with the induction of PI3K. In melanoma the expression of PD-L1 is induced by infiltration of CD8+ T-lymphocytes [11], but a similar mechanism is not reported in GBM. Macrophages from GBM patients express higher levels of PD-L1 in the peripheral blood and in the tumor tissues [12]. Collectively these studies of immune evasion and the mechanism of escape would be for glioblastoma cells to express the PD-L1 protein that kills PD-1/B7.1 expressing T-lymphocytes invading the tumor. Except for immune evasion, it is yet unknown if PD-L1 expression by tumor cells serve additional biological advantages for malignant tumors to establish, grow and invade. Studies *in vitro* on co-cultures of cerebellar granular neurons (CGNs) and astrocytoma cells have shown that CGNs can inhibit the proliferation of the astrocytoma cells [13]. We have shown that killing of murine glioblastoma (GL261) cells is dependent on the

Carlsson et al. (2014)
Email: Robert.carlsson@bric.ku.dk

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expression and activity of PD-L1 receptor on neurons [3,14,15]. Furthermore, the killing of the gliomas was accompanied by upregulation of cleaved Caspase-3 and other hall markers of apoptosis [3]. This is the first finding describing the mechanism of action by which neurons kill glioblastoma cells.

The differential effect of expression of PD-L1 on neurons and on glioblastoma raises a question; is the PD-L1 on neurons different from the PD-L1 expressed on the glioblastoma? Given that the PD-L1 protein can be glycosylated differentially, and/or bound to differential binding partners depending on the cellular context, one could speculate that there are either differences in the PD-L1 molecule itself, or among proteins it interacts with, that makes the difference between the neurons and the glioblastoma. An additional explanation might be that the glioblastomas expressing the PD-L1 molecule have disarmed it, by either e.g. differential glycosylation or proteins interaction, so that it is not conferring killing of nearby glioblastoma cells.

Glioblastoma subtypes

GBM cells within a tumor are morphologically and transcriptionally diverse. Investigation of the impact of silencing the oncosuppressors neurofibromatosis type I (NF1) and protein 53 (P53) for transformation of primary cells suggests that GBM can arise from many different types of cells, ranging from neural stem cells to astrocytes. In fact adult neurons were elegantly shown to be the origin of tumors for some of the evoked GBMs [16].

Despite the heterogeneity, molecular subtypes have been characterized; poor prognosis, as determined by genetic profiling, includes mesenchymal GBMs and tumors expressing neural stem cell markers, while tumors tumors expressing a proneural profile correlated with better prognosis [17]. Interestingly, only special culture conditions *in vitro*, i.e. addition of Epidermal growth factor (EGF) and Fibroblast growth factor (FGF) and no serum, maintain the *in vivo* gene expression profiles [18]. Classic glioblastomas often have amplifications or activating mutations in the Epidermal growth factor receptor (EGFR) gene resulting in overexpression and phosphorylation of the EGFR protein, whereas the oncosuppressor P53 rarely is mutated [19,20].

EGFR and Glioblastoma

EGFR mutation is one of the main driver mutations in GBM. EGFR is a transmembrane receptor tyrosine kinase (RTK) oncogene. Upon binding to EGF, the signaling pathway is activated and thereby it induces cell proliferation and/or differentiation. Alteration of EGFR activity can be triggered by mutations, fusion to nearby genes, inversions or amplification of the *egfr* gene [19]. These alterations of the *egfr* gene are present in more than 40% of GBMs. Normally the activation of EGFR requires EGF ligand binding, but in some occasions EGFR is mutated to escape requirement of EGF. EGFRvIII is a truncated version of the EGFR receptor that signals constitutively without the need for EGF. The frequency of the EGFRvIII mutation in GBM is debated but recent technology advances have shown it to be highly expressed in 11% GBM [19]. Recently the PD-1 pathway and EGFR were correlated and shown to be simultaneously expressed in a lung cancer model [9]. EGFR activity enhances the expression levels of the PD-L1 protein in vitro, as judged by the loss of cell surface staining of PD-L1 by lung cancer cells after treatment with an EGFR inhibitor, Gefitinib. Transformation of the bronchial epithelial cell line with a EGFR mutant enhances the expression of PD-L1 both at the mRNA level and as a protein on the cell surface. This study points out immune escape and a reduction in immune responses as the reason for the coexpression of EGFR and PD-L1 on the cancer cells [9]. Collectively these data suggest that the GBMs over expressing the EGFR molecule might also overexpress the PD-L1 molecule. In a mouse model of GBM it was shown that the EGFR tumorigenic activity is enhanced by deletion of PTEN. Loss of PTEN is inducing the expression of PD-L1 suggesting a link between over expression of EGFR, loss of PTEN and subsequent gain of PD-L1 expression [21]. Among the EGFR and PD-L1 molecules it is rather PD-L1 that seems to correlate with severity in GBM, as it is highly expressed in mesenchymal GBMs, suggesting the importance for PD-L1, the immune system and inflammation in GBM disease [22].

Inflammation and GBM

Inflammation can be both friend and foe in cancer. For certain cancer subtypes such as colon cancer, inflammation is suggested to be an inducer of mutations driving cancer progression together with the gut microbiota [23]. It is important to differ between chronic inflammation (CI) and a desired inflammation (DI), which is a physiological immune response fighting the tumor. For DI it has been suggested that PD-L1 is upregulated on tumors as a response to the immune attack and PD-L1 expression confers a better cancer prognosis in patients [24]. Conversely, PD-L1 expression on GBM has been shown to correlate with a bad prognosis [3]. Those *in vivo* findings are in contrast to each other and suggest an opposing outcome of PD-L1 expression on cancer cells depending on the cancer type.

A direct link between immune suppression, CI and the severity of disease was recently shown in a cancer model *in vivo*. Depletion of suppressive T regulatory cells reduces tumor burden and CI in a breast cancer animal model [25]. For GBMs similar role for inflammation has been suggested. Immune surveillance through DI might be important to constrain the growth of the tumor cells but CI itself is most likely a negative factor. Cancer cells could also contribute to induction and/or maintenance of CI which in turn nourishes tumor development. In a murine GL261-induced glioma model, it is shown that gliomas can produce granulocyte macrophage colony-stimulating factor (GM-CSF) and stimulate infiltrating macrophages and microglia, suggesting that the malignant cells help maintain a CI state of the tumor [26].

Cytomegalovirus (CMV) DNA and/or protein products are often expressed in GBM. Valganciclovir (an antiviral drug) was used to treat the GBM in combination with surgery. Patients treated with the antiviral drug had an increased survival time (50%) compared to patients treated with surgery only [27], suggesting that a chronic CMV infection could possibly contribute to CI and thereby to GBM progression. Further more, nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) has been shown to induce proneural to mesenchymal transition of GBMs and correlated with CD44 expression [28]. Xenografts of mesechymal GBMs into immune compromised mice converted the GBMs from the mesenchymal subtype to the proneural, suggesting that an inflammatory condition is necessary to

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maintain the mesenchymal GBM phenotype [28]. Tumor necrosis factor alpha (TNF-α) could transform proneural to mesenchymal GBMs via the activity of NFkB. In addition the activity of NFkB regulated the expression of signal transducer and activator of transcription 3 (STAT3), CCAAT/enhancer-binding protein alpha (C/EBP-α) and transcriptional coactivator with PDZ-binding motif (TAZ) known to induce the mesenchymal GBM subtype [28]. Mesenchymal GBMs are among the most aggressive of GBMs expressing high amounts of PD-L1 [22]. In fact, NFkB has been shown to bind and up regulate the GM-CSF promoter in endothelial cells [29], and the PD-L1 promoter in macrophages in inflammatory conditions [30]. In addition, PD-L1 blockade has been shown to inhibit inflammation in a model of CI, suggesting a role for PD-L1 in maintaining the CI state [31]. Although a direct link needs to be established, NFkB induced expression of PD-L1 might be one of the inducers of the CI observed in GBM.

The molecular profile with low expression of PTEN and over expression of PD-L1, NFkB, GM-CSF and EGFR suggests that high grade GBMs, in addition to upregulating molecules that convey proliferative signals, express molecules that induces and maintains CI in the microenvironment of the tumor. In Figure 1 we schematize concerning the issues around PD-L1 expression in CI (A) and on neurons (B) as an example of the diverse affects PD-L1 expression can have within the tumor microenvironment.

PD-L1 directed immunotherapy in cancer

Two monoclonal antibodies are now available for treatment of the PD-1/PD-L1 axis. Both antibodies have shown to be efficacious in some solid tumor cancers [32,33].

For the PD-1 antibody therapy was efficient in 36% of the patients when PD-L1 was expressed on the tumor [33]. PD-L1 blockage by antibody induced tumor regression with an objective response rate of 6-17 % and prolonged stabilization of disease (12-41%) at 24 weeks [32]. Though the mechanism of action of these antibodies is debated, it is suggested by some that they act through the inhibition of immune escape [32, 33], but there is a possibility that the antibodies increase systemic levels of T-lymphocytes or both inhibit immune escape and increase levels of T-lymphocytes. Activation of anti-tumor T-lymphocytes is desirable to fight cancer progression. Therefore, it is of note to mention that activated T-lymphocytes are reported to express PD-L1 on their surface [34]. To our knowledge the direct importance of PD-L1 expressing T-lymphocytes for their anti-tumor activity has not been addressed. What an eventual anti-PD-L1 immunotherapy could impact suppressing desirable activated T-lymphocytes is also a relevant issue to consider.

Despite the fact that the mechanism of action for PD-L1/PD-1 axis therapy in cancer is still discussed, it might be of importance to carefully consider when one should target the PD-L1/PD-1 axis (anti-PD-L1 in particular) in GBM, since there is a risk for sustained chronic inflammation and GBM progression upon treatment. This is concomitant with our results from studying
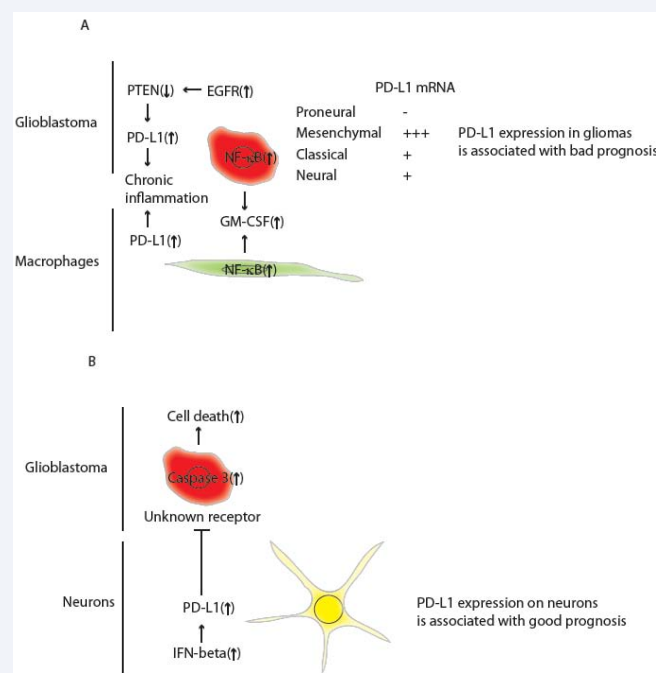

Figure 1 (A, B) The impact of programmed death ligand-1 (PD-L1) expression on prognosis of glioblastoma is dependent on the identity of the cells expressing PD-L1. A) In glioblastoma multiforme (GBM) PD-L1 expression on the mRNA level is associated with bad prognosis and the mesenchymal phenotype. PD-L1 protein is induced upon the loss of PTEN, which stabilizes the expression of PD-L1 post transcriptionally. Gain of epidermal growth factor receptor (EGFR) is also coupled to the loss of PTEN and the induction of an inflammatory milieu in the GBM and is associated with bad prognosis. On infiltrating macrophages, PD-L1 is induced by granulocyte macrophage colony-stimulating factor (GM-CSF) production of the gliomas, contributing to the pathology of the GBM. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) can induce both PD-L1 and GM-CSF, suggesting that it maintains chronic inflammation. B) Neurons nearby the tumor expressing PD-L1 are beneficial for the patient. PD-L1 is induced on the neurons by endogenous activity of interferon beta. PD-L1 on the neurons induces killing of the glioblastomas *in vitro* via direct cell-to-cell interaction through a postulated unknown receptor.

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GBM patients and its model, where high expression of PD-L1 on neurons adjacent to the gliomas correlated with better clinical prognosis likely because neuronal PD-L1 induces apoptotic cell death of gliomas [3]. Treatment with anti-PD-L1 in such setting could preclude neuronal killing activity and hence glioma progression. A similar scenario could be involved in other types of cancer where PD-L1 expression on stromal cells could contribute to prevention of tumor growth.

CONCLUDING REMARKS

Given the heterogeneity and complex etiology of the GBM and the origin of the tumor cells, it is not surprising that the environment that surrounds the tumors display wide varieties of responses. In particular, the immunosuppressive molecule PD-L1 has heterogenous functions depending on the context of expression. Hence, PD-L1 can convey immunosuppressive properties; 1. by inhibiting cytotoxic T-lymphocytes from destroying the tumor cells, 2. by contributing to sustained chronic inflammation as a result of modulating the immune cells so that the tumor microenvironment is activated by invasion of immune suppressive regulatory T cells, and/or by mediating the release of inflammatory mediators by immune cells with no anti-tumor activity. In some cancers, PD-L1 expression by tumor cells correlates with a good prognosis for the patient, whereas in glioblastoma many reports suggest that it is a bad prognostic marker. The interplay between the gliomas, the tumor microenvironment and infiltrating immune cells can hold key information to how to utilize PD-L1/PD-1 directed immunotherapies to successfully treat GBM patients. We suggest that the PD-L1 pathway can exert positive and negative effects on the GBMs (Figure 1). This divergent effect might be dependent on the glioma subclass, expression levels of PD-L1 and PD-L1 regulatory molecules, but most importantly on the cell type that express PD-L1 in the tumor microenvironment.

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