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Critical Reviews in Oncology/Hematology 89 (2014) 140–165

CRITICAL REVIEWS IN
Oncology
Hematology
Incorporating Geriatric Oncology

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Targeting the PD1/PD-L1 axis in melanoma: Biological rationale, clinical challenges and opportunities

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Accepted 15 August 2013

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Abstract

A dynamic interplay exists between host and tumor, and the ability of the tumor to evade immune recognition often determines the clinical course of the disease. Significant enthusiasm currently exists for a new immunotherapeutic strategy: the use of immunomodulatory monoclonal antibodies that directly enhance the function of components of the anti-tumor immune response such as T cells, or block immunologic checkpoints that would otherwise restrain effective anti-tumor immunity. This strategy is based on the evidence that development of cancer is facilitated by the dis-regulation and exploitation of otherwise physiological pathways that, under normal circumstances, down-regulate immune activation and maintain tolerance to self. Among these pathways an important role is covered by the Programmed death-1 (PD-1)/PD-Ligand (L) 1 axis. An emerging concept in cancer immunology is that inhibitory ligands such as PD-L1 are induced in response to immune attack, a mechanism termed "adaptive resistance". This potential mechanism of immune resistance by tumors suggests that therapy directed at blocking the interaction between PD-1 and PD-L1 might synergize with other treatments that enhance endogenous antitumor immunity. The anti-PD-1 strategy can be effective in several solid tumors such as renal cell carcinoma (RCC) or non-small cell lung cancer (NSCLC), however in this review we summarize the biological role of PD-1/PD-L1 on cancer by focusing our attention in the biological rationale, clinical challenges and opportunities to target the PD-1/PD-L1 axis in melanoma.
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Keywords: PD-1; PD-L1; Melanoma

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http://dx.doi.org/10.1016/j.critrevonc.2013.08.002

1. Introduction

1.1. Immune evasion as hallmark in cancer

Cellular transformation and tumor development result from an accumulation of mutational and epigenetic changes that alter normal cell growth and survival pathways [1]. In 2000, Hanahan and Weinberg reported six biological capabilities (hallmarks) acquired during the multistep development of human tumors: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [2]. In the last two decades, based on the emergence of new experimental data, two further hallmarks have been added to this list: reprogramming of energy metabolism and evading immune destruction [3].

This Copernican revolution had its foundation on the awareness that complete knowledge of cancer development cannot be achieved without recognizing the importance of the tumor microenvironment, a very important part of which is played by the immune system. A dynamic interplay exists between host and tumor, and the ability of the tumor to evade immune recognition (immune surveillance) often determines the clinical course of the disease [4].

The concept of cancer immune surveillance is based on the hypothesis that the immune system can suppress the development or progression of spontaneous malignancies [5]. Several data, first from animal models and later from studies in cancer patients, confirmed this original concept that the immune system can recognize and reject tumors, supporting the hypothesis that immune evasion by cancer cells plays an important role in the development and progression of tumors [6].

Based on this biological background, cancer immunotherapy focuses on the development of agents that can activate the immune system to recognize and kill tumor cells. However, until recently, all the efforts to therapeutically modulate the immune system were tempered by disappointing results from clinical trials with cancer vaccines and biochemotherapy regimens, as well as by the low response rates and high toxic effect associated with these two strategies [7–14]. A third approach known as adoptive cell therapy has shown clinical benefit for some patients although technical aspects and the complexity of the procedures have limited more widespread use [15]. Despite the initial lack of clinical success, extensive research over the past twenty years yielded the identification of innovative ways to manipulate the immune response to cancer.

The regulation of the immune system depends on a fine control system in which a key role is played by cellular receptors that ensure the activation or inhibition of cells involved in the control of infections and tumors and development of autoimmunity. Some of these mechanisms are activating and dictate whether the response arises, while others play the role of powerful repressors. Antagonist antibodies acting on such repressors result in enhanced immune responses, a goal that may also be achieved with agonist antibodies acting on the activating receptors.

More recently, significant enthusiasm arose for a fourth immunotherapeutic strategy: the use of immunomodulatory monoclonal antibodies that directly enhance the function of components of the anti-tumor immune response such as T cells or block immunologic checkpoints that would otherwise restrain effective anti-tumor immunity. This strategy is based on the evidence that development of cancer is enabled by the dis-regulation and exploitation of otherwise physiological pathways that, under normal circumstances, down-modulate immune activation and maintain tolerance to self. A series of therapeutic agents are under clinical development, and one of them, which is directed at the CTL-associated antigen 4 (CTLA-4) inhibitory receptor (Ipilimumab, Yervoy®), has been approved for the treatment of metastatic melanoma. The list of antagonist agents acting on repressors under development includes anti-CTLA-4, anti-Programmed death-1 (PD-1), anti-PD-Ligand 1 (L1) or (B7-H1), anti-KIR (killer cell Ig-like receptor), and anti-TGF-β. Agonist antibodies currently being investigated in clinical trials target CD40, CD137 (4-1BB), CD134 (OX40), and glucocorticoid-induced TNF receptor (GITR) (Fig. 1). Among these pathways an important role is covered by two immunologic checkpoints: the CTLA-4 and the PD-1/PD-L1 axis.

An emerging concept in cancer immunology is that inhibitory ligands such as PD-L1 are induced in response to immune attack, a mechanism termed adaptive resistance. This potential mechanism of immune resistance by tumors suggests that therapy directed at blocking interaction between PD-1 and PD-L1 might synergize with other treatments that enhance endogenous antitumor immunity.

In this review we summarize the biological role of PD-1/PD-L1 in cancer by focusing our attention on the biological rational, clinical challenges and opportunities to target the PD-1/PD-L1 axis in melanoma.

2. T cell recognition and immune checkpoints

Induction of immune response requires T cells to receive two sets of signals from antigen-presenting cells: the T cell receptor must recognize complexes of MHC with the antigen on the surface of an antigen-presenting cell (APC). T cells and the T cell receptor complex do not respond to antigen in solution, but even for the specific antigen they only respond to antigen-MHC complexes on the cell surface. This interaction is necessary, but not sufficient, for T cell activation.

T cell activation also requires a co-stimulatory signal involving interaction of CD28 on the T cell with CD80 or CD86 (B7 family genes) on the APC, which promotes T cell clonal expansion and cytokine secretion [16]. CD28 activates a signal transduction pathway acting through Phosphatidylinositide 3-kinases (PI3K), lymphocyte-specific protein tyrosine kinase (Lck) and adaptor proteins GRB2

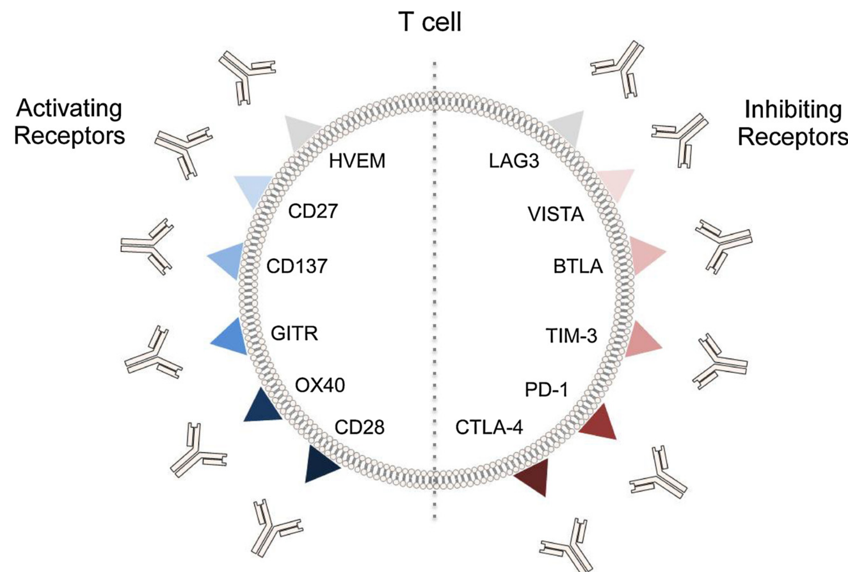

Fig. 1. Agonist antibodies (on the left) and antagonist antibodies (on the right). Antagonist agents under development acting on repressors include anti-CTLA-4 and anti-Programmed death-1 (PD-1).

and GRB2-related adaptor protein (Grb-2/ITK) to provide its co-stimulatory signal for T cell activation.

T cell-mediated immunity is regulated by balancing stimulatory and inhibitory signals that regulate the response [17]. In the absence of co-stimulatory molecules, the T cells enter an unresponsive state known as clonal anergy in which the T cells are incapable of providing antigen-specific immune responses [18].

In physiological conditions, immune checkpoints are crucial for maintaining self-tolerance and for protecting tissues from the damage of the immune response to pathogenic infection.

There is evidence that tumors resist immune attack by inducing tolerance toward tumor-specific T cells and by expressing ligands that bind inhibitory receptors such as immune checkpoints. Tumors can result in deregulation of the immune-checkpoint proteins and develop a mechanism for immune resistance, especially against T cells that are specific for tumor antigens [19].

Preliminary clinical data show that antibody blockage of immune checkpoints can substantially enhance therapeutic antitumor immunity [19,20]. Unlike conventional antibodies used for the treatment of tumors, antibodies that block immune checkpoints do not bind directly to the tumor cells, but target lymphocyte receptors or their ligands, in order to modulate their antitumor activity.

Several immunologic treatment options, such as the induction of an immune response or the administration of antibodies, have been investigated in melanoma and have shown interesting results [21]. CTLA-4 and PD-1 are the two most investigated immune checkpoint receptors in melanoma and cancer immunotherapy (Table 1) [22]. CTLA-4 is an inhibitory membrane receptor expressed exclusively on T cells, where it primarily regulates the amplitude of the early stages of T cell activation, counteracting the activity of the T cell co-stimulatory receptor, CD28.

As reported above, the engagement of the T cell antigen receptor by itself is not sufficient for full T cells activation; a second co-stimulatory signal is required. This co-stimulation is mediated by engagement of CD28 on the T cell surface by members of the B7 family on APC [23].

These co-stimulatory molecules are integral membrane proteins expressed on several cells with APC function. These molecules, including B7.1 (CD80) and B7.2 (CD86), bind to other ligands on T cells and provide the second signal for T-cells activation. Limited expression of B7 on APCs is a mechanism for maintenance of peripheral T cells tolerance, ensuring that T cells activation can only be stimulated by appropriate cells [24].

Interestingly, tumor cells do not express B7, and this contributes to their poor capacity to induce immune responses [25,26]. After activation, T cells express CTLA-4, a close homologue to CD28. CTLA-4 binds members of the B7 family with a much higher affinity than CD28 [27]. Accordingly, CTLA-4 expression on the surface of T cells decreases the activation of T cells by competing with CD28 for binding with CD80 and CD86. CTLA-4 exerts distinct effects on the two major subsets of CD4+ T cells: down-modulation of helper T cell activity and enhancement of regulatory T (T_{Reg}) cell immunosuppressive activity.

The specific signaling pathways by which CTLA-4 blocks T cell activation are still under investigation, although a number of studies suggest that activation of the protein phosphatases, SHP2 (also known as PTPN11) and PP2A, is important in counteracting kinase signals that are induced by TCR and CD28.

The central role of CTLA-4 for keeping T cell activation in check is dramatically demonstrated by the lethal

Table 1
Similarities and differences between CTLA-4 and PD-1.

	PD-1	CTLA-4
Biological function	Inhibitory receptor	Inhibitory receptor
Expression on	Activated T cells, activated B cells, activated NK cells, TILs in different tumor types	T cells at the time of their initial response to antigen (activated CD8+ effector T cells)
Major role	• Limitation of T cells activity in peripheral tissues following inflammatory responses • Limitation of autoimmunity	Regulation of the early stage of T cells activation
Ligands	• PD-L1 (B7-H1/CD274) • PD-L2 (B7-CD/CD273)	• CD80 (B7.1) • CD86 (B7.2)
Mechanism of action	• PD-1 binds to the ligand ↓ • Recruitment of phosphatase SHP-2 • Decreased expression of the cell survival protein Bcl-xL ↓ • PD-1 inhibits kinases (PI3K/AKT) that are involved in T cells activation	• CTLA-4 interacts with the ligand ↓ • Binding with PI3K, phosphatases SHP-2 and PP2A • Blockade of lipid-raft expression • Blockade of microcluster formation

NK cells, natural kill cells; CTLA-4, cytotoxic T-lymphocyte antigen 4; PD-1, Programmed death-1; PD-L1, Programmed cell death 1 ligand 1; PI3K, Phosphatidylinositide 3-kinase.

systemic immune hyperactivation phenotype of CTLA-4-knockout mice.

In experimental models, mice rapidly develop lymphoproliferative disease with multiorgan lymphocytic infiltration and tissue destruction, including particularly severe myocarditis and pancreatitis, and die at 3–4 weeks of age [28]. The severe phenotype of mice lacking CTLA-4 implies a critical role for CTLA-4 in down-regulating T cell activation and maintaining immunologic homeostasis. In the absence of CTLA-4, T cells are activated, can spontaneously proliferate, and may mediate lethal tissue injury.

3. PD-1/PD-L1 expression and biological function

The PD-1 receptor is a 50–55 kDa type I transmembrane glycoprotein of the Ig superfamily, with an extracellular domain showing 21–33% sequence identity with CTLA-4, CD28 and the inducible co-stimulatory (ICOS) molecule, but distinct function and ligand specificity [29,30]. PD-1 shows an extracellular IgV region, a transmembrane domain, and an intracellular tail. The cytoplasmic domain presents two tyrosine residues: one represents an immunoreceptor tyrosine-based inhibitory motif (ITIM), and the other an immunoreceptor tyrosine-based switch motif (ITSM) that may recruit phosphatases, similar to other negative regulators [31,32].

PD-1 expression is mostly detected at the cell surface (membrane) level, where it exerts its function of inhibitory receptor, however, PD-1 expression has also been observed in the cell cytoplasm [33]. It is as yet unclear whether cytoplasmic PD-1 exerts biological functions or merely serves as a repository of the protein, allowing an immediate response as soon as its expression at the membrane is requested.

PD-1 is expressed on CD4−/CD8− thymocytes in transition to CD4+/CD8+ stage and on mature T- and B cells upon activation, while it is not detectable on resting T cells. Thus, in comparison with CTLA-4, PD-1 has a slightly broader expression profile, also present on activated myeloid lineage cells such as monocytes, dendritic cells and NK cells [34,35]. PD-1 is quickly up-regulated on T lymphocytes after exposure to cognate antigen and its expression is controlled by several cytokines including IFN-γ, IL-2, IL-7, IL-15, and IL-21 [36]. Upon antigen clearance, PD-1 expression wanes accordingly. In normal tissues, PD-1 signaling in T cells regulates immune responses to diminish damage, and counteracts the development of autoimmunity by promoting tolerance to self-antigens.

Most biological functions of PD-1 have been elucidated by generating PD-1-deficient mice with gene knockout technology. PD-1-knockout mice spontaneously develop phenotypes of lymphoproliferative autoimmune diseases, accompanied by a marked accumulation of inflammatory cells in affected organs, including CD4+ and CD8+ T cell subsets [37]. Autoimmune manifestations in PD-1 mice are different from those observed in CTLA-4 −/− mice, which die at 3–4 weeks of age from massive lymphocytic infiltration and tissue destruction in multiple organs [38]. The PD-1 related autoimmune phenomena are overall milder and less frequent than those observed following anti-CTLA-4 therapy. Table 1 summarizes the main differences between CTLA-4 and PD-1.

Two ligands for PD-1, designated PD-1 ligand 1 (PD-L1; B7-H1/CD274) and PD-1 ligand 2 (PD-L2; B7-DC/CD273), have been identified based on the similarity to other B7

superfamilies. PD-L1 and PD-L2 are type I transmembrane glycoproteins composed of IgC- and IgV-type extracellular domains that present 40% amino acid identity sequence [34,39–41]. In contrast to the limited expression of PD-L2 on activated macrophages [42], PD-L1 is more broadly expressed on immune and non-hematopoietic cells. Specifically, PD-L1 is constitutively expressed on T- and B cells, macrophages, and dendritic cells, and is upregulated upon stimulation by proinflammatory cytokines including IFN.

Despite the fact that PD-L1 mRNA has been reported in a wide range of human healthy tissues and cell types (with high expression in placenta, heart, lung and liver, low expression in spleen, lymph nodes and thymus, and no expression in the central nervous system), constitutive PD-L1 protein is much less ubiquitous in normal tissues. PD-L1 protein has also been reported to be expressed on parenchymal cells such as pancreatic islet cells, endothelial cells, muscle cells and trophoblasts. Discrepancies between mRNA and protein expression are most likely attributable to a series of post-transcriptional controls that have not yet been completely clarified [43].

PD-L1-deficient mice do not develop spontaneous autoimmune diseases, mild-to-moderate levels of lymphocyte accumulation are evident in the kidneys, liver, and lung, with a predominant CD3+/CD8+ component exhibiting significantly decreased apoptosis [44]. Although the mechanism underlying selective accumulation of CD8+ T cells in PD-L1−/− mice remains to be clarified, these findings implicate a role for PD-L1 in the maintenance of T and cell homeostasis in peripheral organs. Overall, these data support the hypothesis that the expression of this ligand in non-lymphoid tissue cells can prevent immune-mediated tissue damage [45].

3.1. Functional implications of PD1/PD-L1 interaction

PD-1/PD-L1 interaction inhibits T lymphocyte proliferation, survival, and effector functions, induces apoptosis of antigen-specific T cells, and promotes the differentiation of CD4+ T cells into Foxp3+ regulatory T cells. The mechanism by which PD-1 exerts the inhibitory effect has been partially explained. As soon as PD-L1 interacts with PD-1, there is a recruitment of Src homology region 2 domain-containing phosphatase-1 (SHP-1) and SHP-2, which dephosphorylate multiple members of the TCR signaling pathway. This abrogates downstream effects of T-cell activation, including cytokine production, cell-cycle progression, and the expression of survival proteins. Furthermore, the inhibition of RAS and PI3K/AK3 pathways cooperates to inhibit lymphocyte' function and survival (Fig. 2).

Considering that (1) PD-L1 is upregulated in hematopoietic and reticuloendothelial cells, in response to proinflammatory cytokines such as IFN-γ, and (2) PD-1 is expressed to various degrees on activated T cells, it is reasonable to speculate that the co-expression of ligand and receptor in inflamed tissues mitigates against collateral tissue destruction by T cells at these sites. The PD-1/PD-L1 axis would therefore be important in mitigating the inflammatory response and in turn potential autoimmune foci resulting from the dysregulation of the effector phase of the immune response. This mechanism is important in various physiological and pathological processes including feto-maternal tolerance, graft-versus-host disease, and various autoimmune diseases.

Another piece of the puzzle for understanding the central role of the PD-1/PD-L1 axis in the immune response derives from studies evaluating the developing of T cell exhaustion. While PD-1 is expressed on activated lymphocytes during an acute inflammatory process to limit tissue damage and, as soon as the exogenous stimulus is cleared, the PD-1 wanes accordingly, the persistent antigen exposure may prevent the down-regulation of PD-1. Since the immune response plays an important role in staving off cancer, mechanisms of immunosuppression hinder productive anti-tumor immunity. T cell exhaustion is one such mechanism. PD-1 has been identified as a marker of exhausted T cells in chronic disease states, including cancer, and blockade of PD-1–PD-L1 interactions has been shown to partially restore T cell function. The development of T cell exhaustion translates into a major immune resistance and promotes the immune evasion.

4. PD-1/PD-L1 in malignant diseases

Via immunohistochemistry, PD-L1 is constitutively expressed in many human cancers [46]. The finding that PD-L1 is commonly upregulated on many different tumor types including melanoma [47], ovarian cancer [48], lung cancer [49], clear cell renal carcinoma [50], urothelial carcinoma [51], squamous cell carcinomas of the head and neck [52,53], esophageal cancer [54], cervical cancer [55], breast carcinoma [56], pancreatic cancer [57], gastric cancer [58], Wilms tumor [59] and glioblastoma [60], and that PD-1 is expressed in TILs, has created an important rationale for mAb blockade of this pathway for cancer immunotherapy.

In addition to expression on tumor cells, PD-L1 can be detected on cells located in the tumor microenvironment, and high levels of PD-L1 expression have been reported in TILs and tumor-associated macrophages [49,61,62]. However, the ultimate meaning of the PD-L1 expression on tumor cells and other cells in the microenvironment remains to be fully determined.

PD-L1 expression on cancer cells may be an adaptive response to immune attack and induced by cytokines, or constitutive as a result of oncogenic processes, as occurs in *BRAFV600* mutated melanoma. The role of oncogenes to drive immunosuppression has been suggested but not as yet clarified so far.

Interestingly, in melanoma models there is evidence that *BRAFV600* mutation, along with the STAT3 signal, is essential for immune evasion by human melanomas. Recently, Khalili et al. demonstrated that the activation of the MAPK signaling pathway in melanoma cells by oncogenic *BRAF*^{V600E} leads to the production of interleukin 1 (IL-1)α/β

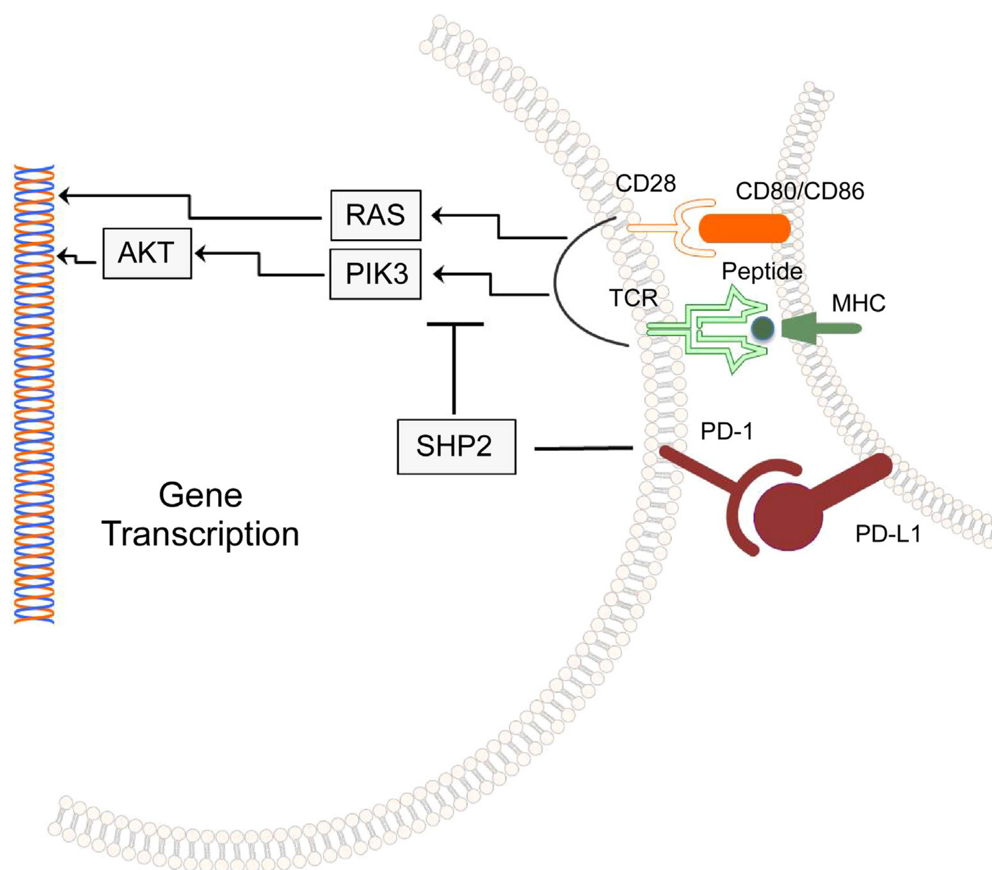

Fig. 2. Upon interaction of PD-L1 with PD-1, there is a recruitment of Src homology region 2 domain-containing phosphatase-1 (SHP-1) and SHP-2, which dephosphorylate multiple members of the TCR signaling pathway. This abrogates downstream effects of T-cell activation, including cytokine production, cell-cycle progression, and the expression of survival proteins. The inhibition of RAS and PI3K/AK3 pathways cooperates to inhibit lymphocytes' function and survival.

[63]. Tumor-associated fibroblasts (TAFs) respond to IL-1 by upregulating an immunomodulatory transcriptional program resulting in the production of COX-2, PD-1 ligands, and chemokines, as well as in the amplification of IL-1 signaling (Fig. 3). Collectively, these factors and signaling circuitries suppress cytotoxic T lymphocyte functions and ultimately promote tumor growth. These data highlight the hypothesis that both mechanisms (constitutive and induced) may concomitantly contribute to the PD-L1 expression on tumor cells and in other cells in the microenvironment, potentially fueling an immune tolerance state.

However, most of the data so far reported in literature are heterogeneous in terms of PD-L1 expression, variability in the assays and cell immunolocalization, as well as cut-off values for positive versus negative PD-L1 immunohistochemical expression. Furthermore, PD-L1 expression has been investigated in primary tumors and metastatic tissue samples, but little conflicting data is available with regard to the concordance/discordance of PD-L1 and PD-1 immunohistochemical expression in paired human tissues from primary melanomas and respective metastases.

Another role in this complicated puzzle is played by PD-1. Similar to exhausted virus antigen-specific T cells, which have been reported in chronic viral infections, the majority of TILs in melanoma express high levels of PD-1 compared with T cells from normal tissues and peripheral blood.

Previous studies on several human cancer types elegantly showed that overexpression of PD-L1 in tumor cells may allow immune evasion [46]. When PD-L1 binds with PD-1 receptor on T-cells, T-cell function is compromised through induction of apoptosis, suppression of proliferation, and inhibition of T -cell cytokines release, such as IFN-γ, IL-4 and IL-2. Furthermore, the PD-1/PD-L1 interaction inhibits T lymphocyte proliferation, survival and cytokines secretion, promotes the differentiation of CD4-positive/CD25-negative/Foxp3-negative T cells into Foxp3+ regulatory T cells (Treg), and induces apoptosis of tumor-specific T cells. Treg cells allow tumor cells to grow locally, and PD1/PD-L1 pathway on tumor cells causes immunosuppression by PD-L1-induced Treg cells.

One study showed that the majority of CD8+ TILs specific for the melanoma antigen MART-1 expresses significant levels of PD-1 compared with lower expression on MART-specific T cells from the peripheral blood of the same patients [64]. PD-1 expression by TILs in melanoma correlated with an exhausted T cell phenotype and impaired

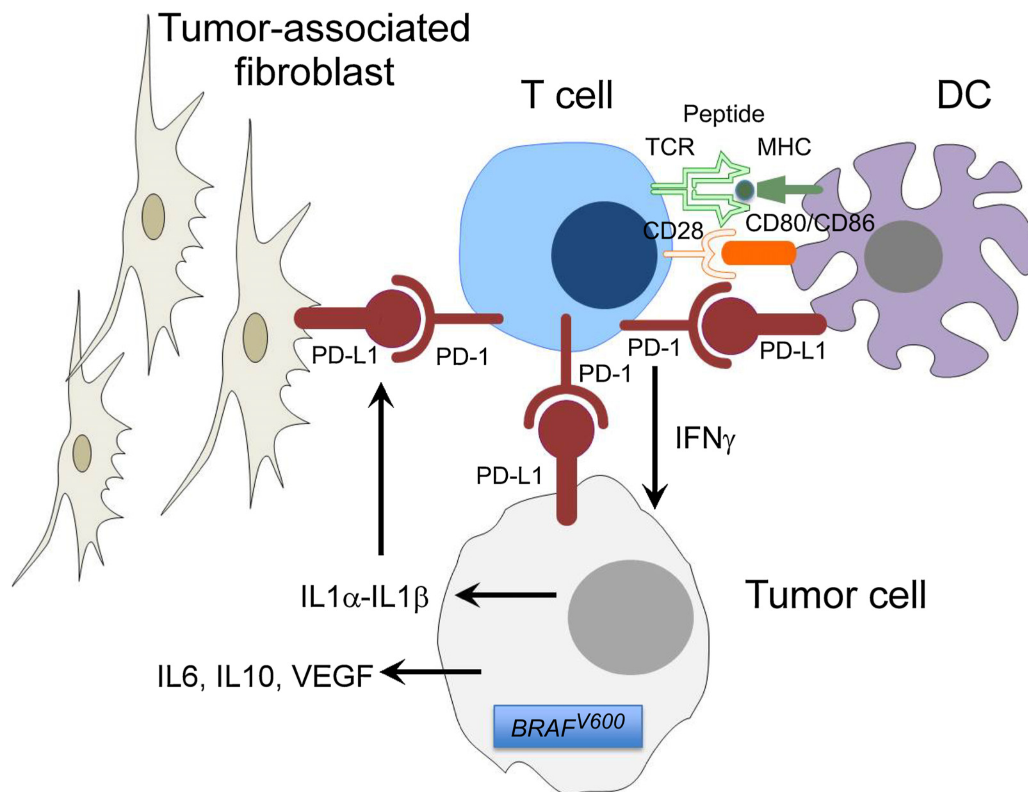

Fig. 3. The activation of the MAPK signaling pathway in melanoma cells by oncogenic *BRAF*^{V600E} leads to the production of interleukin 1 (IL-1)α/β. Tumor-associated fibroblasts (TAFs) respond to IL-1 by upregulating an immunomodulatory transcriptional program resulting in the production of COX-2, PD-1 ligands and chemokines as well as in the amplification of IL-1 signaling. Collectively, these factors and signaling circuitries suppress cytotoxic T lymphocyte (CTL) functions and ultimately promote tumor growth.

effector function. These data underscore once again that the assessment of circulating lymphocytes does not necessarily reflect what happens in the tumor microenvironment, at the biological interface between immune system and cancer. Therefore, the evaluation of the immunological profile in the circulation may be misleading in the assessment of the interaction between lymphocytes, APC, other cells in the microenvironment, and tumor cells.

In conclusion, all the above reported data suggest that the constitutively or the cytokine-induced PD-L1 expression would provide a selection advantage of cancer cells by inhibiting tumor-specific recognition and elimination by T cells. PD-L1/PD-1 axis is a potential point of contact between target molecules (BRAFV600), immune evasion and tumor growth. Therefore, therapeutic antibodies that block PD-1 in order prevent the inhibitory interaction between PD-1 and PD-L1 may partially circuit the cancer immune evasion and elicit the immune system to recognize and kill tumor cells.

5. Prognostic role of PD-L1 and PD-1 in malignancies

Tables 2 and 3 summarize studies on the prognostic role of PD-L1 and PD-1 in different tumor types. Overall, the results published so far are heterogeneous in terms of patient' selection criteria and methodology used for immunohistochemical analysis. Specifically, difficulties in comparing results arise from the following considerations: (i) available data are retrospective; (ii) patients with different tumor stages have been included; (iii) primary or metastatic sites have been included; (iv) different tumor histotypes have been compared within the same studies; (v) different types of tissues have been evaluated (frozen versus paraffin-embedded samples); (vi) monoclonal and/or polyclonal antibodies have been used; (vii) both cytoplasmic and/or membranous immunostaining have been evaluated to define positivity; and finally (viii) different scores of PD-L1 and/or PD-1 positivity have been reported. Thus, the prognostic role of PD-L1 and PD-1 in the context of different tumor types requires further investigation and only future studies will establish whether PD-L1 is an immune correlate and predictive marker for response to anti PD-1 antibody.

According to the aim of the present review we will focus on melanoma. At the time of the present manuscript, three studies reported the role of PD-L1 in melanoma patients.

Hino et al. evaluated the intensity of PD-L1 expression in melanoma specimens (59 primary tumors, 16 lymph nodes, and 4 in-transit metastases). By multivariate analysis, immunohistochemical PD-L1 expression correlated with Breslow thickness, disease free, and overall survival [47].

Table 2
PD-L1 expression and prognosis in malignancies.

Author	N^a	Tumor	Tissues/Detection Method	Immunostaining	Antibody	Prognostic role
Thompson [79]	306	Renal cell carcinoma	FFPE tissues/IHC	Cell-surface membrane ($\geq$5% tumor staining = PD-L1 positivity)	Anti-PD-L1 MoAb (5H1)	Yes: Association PD-L1 positivity tumor/ - Death (RR, 3.92; $P < 0.001$) - Overall mortality (RR, 2.37; $P < 0.001$) PD-L1 independent prognostic factor of cancer-specific death
Thompson [50]	429	Renal cell carcinoma	Fresh-Frozen tissues/IHC	Cell-surface membrane ($\geq$10% tumor staining = PD-L1 positivity)	Anti-PD-L1 MoAb (5H1)	Yes: Association PD-L1 positivity tumor/ death (RR, 4.53; $P < 0.001$) PD-L1 independent prognostic factor of cancer-specific death
Taube [66]	150	Melanoma	FFPE tissues/IHC	Cell-surface membrane PD-L1 expression ($\geq$5% tumor staining = PD-L1 positivity)	Anti-PD-L1 MoAb (5H1) Anti-PD-L1 polyclonal Ab (4059)	Yes: Association PD-L1 positivity tumor/ OS ($P = 0.032$)
Gadiot [65]	63	Melanoma	FFPE tissues/IHC	N/A ($\geq$1% tumor staining = PD-L1 positivity)	Several Anti-PD-L1 MoAbs Anti-PD-L1 polyclonal Ab (4059)	No

Table 2 (*Continued*)

Author	*N*[a]	Tumor	Tissues/Detection Method	Immunostaining	Antibody	Prognostic role
Hino [47]	59	Melanoma	FFPE tissues/IHC	Cytoplasm area	Anti-PD-L1 MoAb (27A2)	Yes: Association PD-L1 positivity tumor/ - OS ($P = 0.0402$) - PFS ($P = 0.0522$) PD-L1 independent prognostic factor of OS and PFS
Ohigashi [54]	41	Esophageal cancer	Frozen tissues/IHC; mRNA analysis (Real-time quantitative PCR)	Cell-surface membrane or Cytoplasm area (≥10% tumor staining = PD-L1 positivity)	Anti-PD-L1 MoAb (MIH1) Anti-PD-L2 MoAb (MIH18)	Yes: Association - PD-L1 mRNA expression positivity/ OS ($P = 0.025$) - PD-L2 mRNA expression positivity/ OS ($P = 0.003$) - PD-L1 positivity and PD-L2 positivity status/ OS ($P = 0.0008$) PD-L1 and PD-L2 = independent Prognostic factors of OS
Wu [58]	102	Gastric cancer	FFPE tissues/IHC	Cytoplasm area (some nuclear membrane localization)	Anti PD-L1 MoAb (2H11)	Yes: Association PD-L1 positivity tumor/ OS ($P < 0.01$) PD-L1= independent prognostic factor of OS (RR = 2.803; $P = 0.040$)

Konishi [49]	52	Non-small cell lung carcinoma	Frozen tissues/IHC	Cell-surface membrane and/or Cytoplasm area (focal or scattered pattern)	Anti-PD-L1 MoAb (MIH1) Anti-PD-L2 MoAb (MIH14)	No
Mu [80]	109	Non-small cell lung carcinoma	FFPE tissues/IHC	Cell-surface membrane and/or Cytoplasm area (focal or scattered pattern)	Anti-PD-L1 (clone not specified)	Yes: Association PD-L1 positivity tumor/ 3 years OS ($P = 0.034$)
Gao [81]	240	Hepatocellular carcinoma	FFPE tissues/IHC	Cell-surface membrane and/or Cytoplasm area (focal or scattered pattern)	Anti-PD-L1 MoAb Anti-PD-L2 MoAb	Yes: Association PD-L1 positivity tumor/ - DFS ($P = 0.047$) - OS ($P = 0.029$) PD-L1 independent prognostic factor of DFS ($P = 0.015$)
Cariani [82]	42	Hepatocellular carcinoma	Frozen tissues/mRNA analysis (Real Time quantitative PCR) FFPE tissues/IHC (on 15 HCC samples)	Cytoplasm area	Anti-PD-L1 polyclonal Ab	Yes: Association PD-L1 mRNA expression positivity/ OS ($P < 0.05$)

Table 2 (*Continued*)

Author	N^{a}	Tumor	Tissues/Detection Method	Immunostaining	Antibody	Prognostic role
Zeng [83]	109	HBV-related Hepatocellular carcinoma	FFPE tissues/IHC	NA (≥50% tumor staining = PD-L1 positivity)	Anti-PD-L1 MoAb	Yes: Association PD-L1 positivity tumor/ - TFS ($P \leq 0.001$) - OS ($P \leq 0.001$) PD-L1 independent prognostic factor of TFS ($P \leq 0.001$) and OS ($P = 0.007$)
Karim [55]	115	Cervical cancer	FFPE tissues/IHC	Cell-surface membrane (PD-L1/PD-L2)	Anti-PD-L1 MoAb (5H1) Anti-PD-L2 MoAb Anti-PD1 MoAb	No
Nomi [57]	51	Pancreatic cancer	Frozen tissues/IHC mRNA analysis (Real-time quantitative PCR)	Cell-surface membrane and Cytoplasm area (≥10% tumor staining = PD-L1 positivity)	Anti-PD-L1 MoAb (MIH1) Anti-PD-L2 MoAb (MIH18)	Yes: Association PD-L1 positivity tumor/ OS ($P = 0.016$) PD-L1 independent prognostic factor of OS ($P = 0.022$)

Nakanishi [51]	65	Urothelial cancer	Frozen tissues/IHC	Cell-surface membrane and/or Cytoplasm area (focal pattern)	Anti-PD-L1 MoAb (MIH1)	Yes: Association: PD-L1 positivity tumor/ - OS ($P = 0.021$) - Post-resection recurrence ($P = 0.026$)
Cho [62]	45	Oral squamous cell carcinoma	FFPE tissues/IHC	Cell-surface membrane and/or Cytoplasm area	Anti-PD-L1 polyclonal Ab (ab82059)	No
Hsu [84]	74	Nasopharyngeal carcinoma	FFPE tissues/IHC (46 samples) Snap-frozen tissue (28 samples)	NA	Anti-human PD-1 polyclonal Ab Anti-PD-L1 MoAb (MIH1) Anti-PD-L2 MoAb (MIH18)	No
Droeser [85]	1420	Colorectal cancer	FFPE tissues-TMA/IHC	NA (Score 2–3 tumor staining = PD-L1 positivity; *Intensity of PDL1 Staining*= Score 0: negative Score 1: very weak expression Score 2: moderate expression but weaker than placenta) Score 3: equivalent to or stronger expression than placenta)	Anti-PD-L1 MoAb (27A2) Anti-PD-L1 Polyclonal Ab (ab82059)	Yes: Association PD-L1 positivity tumor (MoAb)/ OS ($P = 0.0001$) PD-L1 positivity tumor (polyclonal Ab)/ OS ($P = 0.008$) PD-L1 trend for independent prognostic factor ($P = 0.052$)

Table 2 (*Continued*)

Author	N[a]	Tumor	Tissues/Detection Method	Immunostaining	Antibody	Prognostic role
Hamanishi [48]	70	Ovarian cancer	FFPE tissues/IHC	NA (Score 2–3 tumor staining = PD-L1/ PD-L2 positivity; *Intensity of PD-L1 and PDL2 staining* = -Score 0: negative -Score 1: very weak expression -Score 2: moderate expression but weaker than placenta (PD-L1) and tonsil (PD-L2) -Score 3: equivalent to or stronger expression than placenta or tonsil)	Anti-PD-L1 MoAb (27A2)	Yes: PD-L1 independent prognostic factor of: - OS (RR, 4.26; 95% CI, 1.39–12.94; $P = 0.011$) - PFS (RR, 2.57; 95% CI, 1.11–5.93; $P = 0.027$)
Topalian [67]	42	18 Melanoma 10 Non-small cell lung carcinoma 7 Colorectal cancer 5 Renal cell carcinoma 2 Prostate cancer	FFPE tissues/IHC	NA (≥5% tumor staining = PD-L1 positivity)	Anti-PD-L1 MoAb (5H1)	Yes: Association Pretreatment PD-L1 positivity tumor/ Objective Response after treatment with anti-PD-1 antibody

IHC, immunohistochemistry; FFPE, formalin-fixed and paraffin-embedded; OS, overall survival; DFS, disease free survival; PFS, progression free survival; RR, risk ratio; Ab, antibody; MoAb, monoclonal antibody; TMA, tissue microarray; TFS, treatment free survival; NA, not applicable; PCR, polymerase chain reaction.

[a] Number.

Table 3
PD-1 and prognosis in malignancies.

Author	N°	Tumor	Tissues/detection method	Cell immunolocalization/cut-off for positivity	Antibody	Prognostic role
Hsu [84]	74	Nasopharyngeal carcinoma	FFPE tissues/IHC (46 samples) Snap-frozen tissue (28 samples)	NA	Goat polyclonal antibody anti-human PD-1 (R&D Systems, Minneapolis)	YES → Correlation: PD-1 + -CD8 expression/OS ($P = 0.05$). PD-1 + -CD8 expression/DFS ($P = 0.007$). PD-1 + CD8 expression/locoregional recurrence free Survival ($P = 0.004$). PD-1 + -CD8 expression/locoregional recurrence-free survival (four groups) ($P = 0.004$). High PD-1 + -CD8 group had a 6.5 times higher risk of locoregional recurrence ($P = 0.005$), a 6.5 times higher risk of treatment failure ($P = 0.013$), and a 9.5 times higher risk of death ($P = 0.015$); PD-1 expression in intratumoral CD8 cells: independent effect on the post-treatment outcome ---------------- NO → No Correlation: PD1 + -CD8 expression/distant metastasis-free survival ($P = 0.31$). PD-1 + -CD4 expression/ OS ($P = 0.56$). PD-1 + -CD4 expression/ DFS ($P = 0.90$)

Table 3 (*Continued*)

Author	N°	Tumor	Tissues/detection method	Cell immunolocalization/cut-off for positivity	Antibody	Prognostic role
Wahlin [86]	70	Follicular lymphoma	FFPE tissues/IHC Tissue microarray (TMA)	Non-nuclear Quantification: cell count = PD-1 positive cells/sum of total cellular area Compartment: total/follicular/interfollicular	Anti-PD-1 Ab non-specified	YES → Correlation Follicular PD1 positivity/ favorable prognosis (RR = 0.34, $P = 0.01$) along with interfollicular CD8+ cells (RR = 0.86, $P = 0.014$) independent of FLIPI
Takahashi [87]	82 (patients treated by standard R-CHOP as initial therapy)	Follicular lymphoma	NA	NA PD-1 positivity staining: > 14.4%	Anti-PD1 MoAb (Abcam 52587)	NO → No correlation CR rate/PD-1 positivity patients No correlation PD-1 positive cells (≥7.5%)/PFS ($P = 0.20$) No correlation PD-1 positive cells (≥7.5%)/OS ($P = 0.60$) PD-1 is not a Independent prognostic factor Among male patients ($n = 43$): correlation PD-1 positive cells/worse PFS ($P = 0.03$)
Richendollar [88]	91	Follicular lymphoma	Tissue microarray (TMA)/IHC	NA PD-1 positivity: >35.6 PD-1 positivity cells/HPF	Anti-PD1 Polyclonal (Abcam)	YES Association PD1 positivity T cells/decreased OSl (HR = 1.64, $P = 0.10$) PD1: independent prognostic factor for decreased OS
Muenst [89]	189	Hodgkin lymphoma	Tissue microarray (TMA)/IHC	NA PD-1 positivity: >23 PD-1 positivity cells/mm2	Anti-PD-1 Goat antihuman MoAb (AF1086 R&D Systems)	YES → PD1 positivity T cells/worse OS ($P = 0.005$) NO → PD1 is not a Independent prognostic factor ($P = 0.022$)

Shi [90]	56	Hepatocellular carcinoma	FFPE tissues/IHC, FACS	Both cell-surface membrane and cytoplasm area	Anti-PD1 Mouse antihuman MoAb (R&D Systems) Anti-PD-L1 Mouse antihuman MoAb (Biolegend), FACS with PE-conjugated PD-1 and PD-L1 (eBiosciences)	YES → Correlation High levels of intratumoral PD1 + CD8+ T cells/ shorter DFS ($P < 0.001$)
Zeng [83]	109 (cryoablation) 23 (IHC)	HBV-related Hepatocellular carcinoma	FFPE tissues/IHC	NA	NA	YES Correlation PD-1/TFS ($P \leq 0.001$) PD-1/OS ($P = 0.001$) Circulating PD-1 expression = independent poor prognostic factor for TFS ($P = 0.046$)

N°, number of patients; FLIP, Follicular Lymphoma International Prognostic Index; R-CHOP, treatment with Rituximab, Cyclophasfamide, Doxorubicin, Vincristine and Prednisolone; FFPE, formalin-fixed and paraffin-embedded; CR-rate, complete response rate; OS, overall survival; PFS, progression free survival; TFS, treatment free survival; FACS, flow cytometry analysis; RR, risk ratio; MoAb, monoclonal antibody.

Gadiot and colleagues evaluated paraffin-embedded tissues of 63 patients with stages III-IV melanoma diagnosed by the Netherlands Cancer Institute between 2000 and 2004. PD-L1 was analyzed in benign nevi ($n = 10$), primary melanomas ($n = 43$), satellite metastases ($n = 8$), in transit-metastases ($n = 21$), and distant organ metastases ($n = 22$). Interestingly, the authors showed that 20% of the benign nevi samples were PD-L1-positive, while only 5% of primary melanomas were PD-L1 positive. Satellite metastases, in-transit metastases, lymph nodes and distant metastases were PD-L1 positive in 25%, 40%, 14%, and 18% of cases, respectively. PD-L1 was more expressed in satellite and in-transit metastases compared with lymph node, and distant metastases. In this study, PD-L1 did not correlate with disease free or overall survival [65].

Finally, Taube et al. demonstrated a correlation between PD-L1 and the presence of TILs in both nevi and malignant melanoma: 98% of PD-L1 positive melanomas were associated with TILs compared with only 28% of PD-L1 negative melanomas. Furthermore, PD-L1 positive melanocytes were almost always localized adjacent to TILs. Interestingly, a positive correlation between surface PD-L1 expression by tumor cells (≥5%) and overall survival in metastatic disease in 56 patients was reported. On the other hand, there was no significant difference in survival in relation to PD-L1 expression in primary melanoma [66].

There are important differences in the first two above referenced studies [47,65]. PD-L1 was evaluated with different immunostaining protocols. In Hino's study, it is unclear whether the authors tested their antibodies against isotype controls and PD-L1Fc protein blockade to ensure lack of background or unspecific signals.

Another likely explanation for the conflicting results could be that small series of fewer than 100 melanoma patients from diverse origin were analyzed. The most noticeable difference between the Asian melanoma patient population [47] and the cohort population evaluated by Gadiot is the tumor type. In the latter, superficial spreading and nodular melanomas were predominantly diagnosed, whereas in the cohort of Hino et al., mainly patients with acral lentiginous melanomas were included. The latter have been shown to preferentially express c-kit amplifications or mutations, whereas the melanomas from the European cohort are more likely to express BRAF mutations. Finally, more women were included in Hino's study. Women are known to have a better prognosis in melanoma and may have influenced the prognostic analysis of PD-L1.

Finally, Taube et al. suggest that patients with both PD-L1 expression and TILs in melanoma may have improved prognosis compared with the group with PD-L1 expression without TILs [66]. This study seems to suggest that the evaluation of PD-L1 expression is important but not sufficient to understand the intricate interplay between melanoma and the immune system. PD-L1 overexpression in melanoma with TILS could be suggestive of an active and efficient immune response, and therefore may correlate with a better prognosis.

6. Clinical trials and future developments

We performed an extensive "Medline" and Cancerlit literature review (1995–2013). Various combinations of search terms were used depending on the requirements of the database being searched. These terms included "PD-1" or "PD-L1" or "B7-H1", in combination with "cancer patients" or "melanoma" or "tumor" or "tumour" or "activity" or "safety" or "phase 1" or "immunotherapy" or "immune checkpoints" or "lymphocytes" or "randomized" or "prospective" or "clinical" or "early phase" or "nivolumab" or "lambrolizumab". In addition, relevant references in each article were scanned, and we did manual searches of abstracts from the annual meetings of the American Society of Hematology (1993–2012), American Society for Clinical Oncology (1993–2013), European Haematology Association (1993–2012), and European Society for Medical Oncology (1998–2013).

Table 4 summarizes the main prospective phase 1 studies evaluating anti PD-1/PD-L1 antibodies in cancer patients. Topalian and coll. enrolled 296 patients with advanced melanoma, non-small-cell lung cancer, castration-resistant prostate cancer, or renal-cell or colorectal cancer to receive BMS-936558 (anti-PD-1 antibody) at a dose of 0.1–10.0 mg/kg of body weight every 2 weeks for 8 week-treatment cycles for 12 cycles until progression or complete response [67]. Common treatment-related adverse events included fatigue, rash, diarrhea, pruritus, decreased appetite, and nausea. Grade 3 or 4 drug-related toxicities occurred in 14% of patients; moreover, there were three deaths from pulmonary toxicity. Drug-related serious adverse events occurred in 32 of 296 patients (11%). The spectrum, frequency, and severity of treatment related adverse events were generally similar across the dose levels tested. Drug-related adverse events of special interest (e.g., those with potential immune-related causes) included pneumonitis, vitiligo, colitis, hepatitis, hypophysitis, and thyroiditis. In this trial, objective responses were observed in 28% of melanoma patients. Interestingly, partial sustained, long term responses were observed at each cohort level (0.3–10 mg/kg). At a dose of 3.0 mg/kg, objective responses were noted in 7 of 17 patients (41%). As measured by standard RECIST criteria, objective responses were long lasting, with response durations of 1 year or more in 13 out of 26 patients who had a response with 1 year or more of follow-up.

In the same study immunohistochemical analysis was performed on pretreatment tumor specimens obtained from 42 patients. Objective response was reported in a subgroup of patients (9/25 patients: 36%) with PD-L1-positive tumors. These preliminary data would suggest a relationship between PD-L1 expression on tumor cells and objective response.

Hodi et al. reported the activity and safety of BMS-936558 in patients with previously treated advanced melanoma [68]. BMS-936558 was administered every two weeks at doses of 0.1–10 mg/kg during dose-escalation and/or cohort expansion. Patients received up to 12 cycles (4 doses/cycle) of

Table 4
Main prospective phase 1 studies evaluating anti PD-1/PD-L1 antibodies in cancer patients.

Author	Tumors	Drug	Dose/schedule (i.v.)	Pts (*n*)	OR (%)	PFS	OS
Anti PD-1 antibody							
Topalian [67]	Melanoma	BMS936558	0.1 mg/kg	296	–	*At 24 weeks*	NR
	NSCLC		0.3 mg/kg		6%		
	CRPC		1.0 mg/kg		32%	47%	
	RCC		3 mg/kg		18%	67%	
	CRC		10 mg/kg			56%	
			Every 2 weeks				
Brahmer [91]	Melanoma	MDX1106	0.3 mg/kg	39	0	NR	
	NSCLC		1.0 mg/kg		0		
	CRPC		3 mg/kg		17%		
	RCC		10 mg/kg		10%		
	CRC						
Berger [92]	Advanced	CT-011	0.2 mg/kg	17*		NR	At 21 days
	Hematologic		0.6 mg/kg				
	malignancies		1.5 mg/kg		6%		76%
			3 mg/kg				
			6 mg/kg				
McDermott [93]	RCC	BMS936558		34		*At 24 weeks*	NR
			1.0 mg/kg		28%	50%	
			10 mg/kg		31%	67%	
			Every 2 weeks				
Patnaik [94]	Melanoma	MK3475		9			
	NSCLC		1.0 mg/kg				
	RC		3 mg/kg		NR	NR	NR
	Sarcoma		10 mg/kg				
	Carcinoid		Every 2 weeks				
Kudchadkar [95]	Melanoma	BMS936558	1.0 mg/kg	30			
		Plus vaccines#	3 mg/kg		NR	NR	NR
			10 mg/kg				
			Every 2 weeks				
Hodi [68]	Melanoma	BMS936558	0.1 mg/kg	95	20%		
			0.3 mg/kg		20%		
			1.0 mg/kg		29%	NR	NR
			3 mg/kg		41%		
			10 mg/kg		20%		
			Every 2 weeks				
Brahmer [96]	NSCLC	BMS936558	1.0 mg/kg	75	6%	NR	NR
			3 mg/kg		28%		
			10 mg/kg		19%		
			Every 2 weeks				
Anti PD-L1 antibody							
Brahmer [70]	Melanoma			160		*At 24 weeks*	
	NSCLC		0.3 mg/kg		0	*42%°*	
	CRPC	BMS936559	1.0 mg/kg		6%	*31%•*	NR
	RCC		3 mg/kg		29%	*22%∞*	
	CRC		10 mg/kg		19%		
	OC					*NR*	
	PC						
Tykodi [97]	Melanoma	BMS936559		162			
	NSCLC		0.3 mg/kg				
	OC		1.0 mg/kg		10%	NR	NR
	PC		3 mg/kg				
	RCC		10 mg/kg				
	CRC		Every 2 weeks				

NSCLC, non-small cell lung cancer; CPRC, castration-resistant prostate cancer; RCC, renal cell carcinoma; CRC, colorectal cancer; OC, ovarian cancer; PC, pancreatic cancer; RC: rectal cancer.
MART-1/gp100/NY-ESO-1 peptides with adjuvant Montanide ISA 51.
* 8 out of 17 patients: acute leukemia.
°: Melanoma patients.
•: NSCLC.
∞: ovarian cancer.
NR: not reported.
N: number.

Table 5
Ongoing clinical trials evaluating anti PD-L1 in cancer patients.

Drug	Phase	Trial	Disease	Primary outcome measures
Monotherapy Non-randomized				
MPDL3280A	1	NCT01375842	Solid tumors or hematologic malignancies	- DLTs
BMS-936559	1	NCT01452334	Non-Hodgkin's lymphoma Hodgkin lymphoma Multiple myeloma chronic Myelogenous leukemia	- Safety - Tolerability
MPDL3280A	2	NCT01846416	PD-L1-positive NSCLC	- OR
BMS-936559	1	NCT00729664	Solid tumors	- Safety - MTD - DLT
BMS-936559	1	NCT01455103	Melanoma	- Immunomodulatory effects
Combination therapy Non-randomized				
- MPDL3280A - Vemurafenib	1	NCT01656642	Melanoma	- Incidence of DLTs - Nature of DLTs - Incidence, nature or severity of adverse events and laboratory abnormalities
- MPDL3280A - Bevacizumab - Chemotherapy	1	NCT01633970	Advanced solid tumors	- Safety - DLTs/MTD

DLT, dose limiting toxicity; NSCLC, non-small cell lung cancer; OR, objective response; MTD, maximum tolerated dose.

treatment or until PD or CR. Ninety-five melanoma patients with Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) $\leq$ 2 were treated with BMS-936558 at 0.1 (n = 13), 0.3 (n = 17), 1 (n = 28), 3 (n = 17), or 10 mg/kg (n = 20). The majority of patients (60/95) had received interferon-alpha or IL-2 (prior anti-CTLA-4 excluded). Seven out of 95 patients previously received B-raf inhibitor therapy. Sixty out of 95 patients had previously received more than 2 lines of treatment. The incidence of grade 3–4 related adverse events (Aes) was 19% and included gastrointestinal (4%), endocrine (2%), and hepatobiliary disorders (1%). There were no drug-related deaths. Clinical activity was observed at all dose levels, including patients with visceral or bone metastases. Of 20 patients with objective response, 12 had object response duration $\geq$1 year, and 6 patients were on study with objective response duration between 1.9 and 11.3 months. Several patients had prolonged stable disease.

Recently Hamid et al. evaluated the anti-PD-1 antibody lambrolizumab at a dose of 10 mg/kg of body weight every 2 or 3 weeks or 2 mg/kg every 3 weeks in 135 patients with advanced melanoma [69].

The confirmed response rate across all dose cohorts was 38%, with the highest confirmed response rate, 52%, observed in the cohort that received 10 mg/kg every 2 weeks. The response rate did not differ significantly between patients pretreated or not with ipilimumab. At a median follow-up of 11 months, among patients achieving a response to treatment, long term responses were reported in 81% of patients. The overall median progression-free survival among the 135 patients was longer than 7 months. Common adverse events treatment-related were fatigue, rash, pruritus, and diarrhea; most of the adverse events were low grade.

Finally, Brahmer et al. evaluated 207 patients (75 with non-small cell lung cancer, 55 with melanoma, 18 with colorectal cancer, 7 with gastric cancer, and 4 with breast cancer), using anti-PD-L1 antibody (BMS-936559) in a multicenter Phase I trial at multiple escalating dose (from 0.3 to 10 mg/kg) [70]. Anti-PD-L1 antibody was administered every 14 days in 6-week cycles, for up to 16 cycles or until the patient had a complete response or confirmed disease progression. Grades 3–4 immune-related toxic effects occurred in 9% of patients. An objective response, complete or partial, was observed in 9/52 patients with melanoma (29% response rates at 3 mg/kg), 2/17 with renal cell cancer, 5/49 with non-small cell lung cancer (mostly non-squamous subgroup) and 1/17 with ovarian cancer. Prolonged stabilization of disease was observed for 12–41% lasting at 24 weeks [70].

Overall, with anti PD-1 or anti PD-L1 antibodies, the incidence of serious (grade 3–4) adverse effects was at a range similar to that with CTLA-4-blocking antibodies, but most were less clinically significant with the exception of pneumonitis, which led to death as a result of toxicity. Blocking the ligand PD-L1 with the fully human IgG4 antibody BMS-936559 resulted in a slightly lower frequency of objective tumor responses and also fewer adverse effects in phase I testing. These initial experiences, together with the current initial

Table 6
Ongoing clinical trials with anti-pd-1 antibodies.

Monotherapy
Non-randomized

Drug	Phase	Trial	Disease	Primary outcome measures
BMS-936558	1	NCT01621490	Melanoma	- Immunomodulatory effects
BMS-936558	1	NCT01592370	- Non-Hodgkin's lymphoma - Hodgkin lymphoma - Multiple myeloma - CML	- Safety and - Tolerability
BMS-936558	2	NCT01721759	NSCLC	- OR
BMS-936558	1	NCT01658878	HCC	- Safety
BMS-936558	1	NCT00836888	Solid tumors	- Safety - Pharmacokinetic
AMP-224	1	NCT01352884	Solid tumors	- Safety - Pharmacokinetic - Tolerability
Randomized				
BMS-936558 0.3 mg/kg versus BMS-936558 2 mg/kg versus BMS-936558 10 mg/kg (pretreated patients) versus BMS-936558 10 mg/kg (naïve patients)	1	NCT01358721	RCC	- Immunomodulatory activity
BMS-936558 versus Dacarbazine	3	NCT01721772	Melanoma	- OS
MK-3475 at different dose levels: Low (0.1 mg/kg) Intermediate (3 mg/kg) High (10 mg/kg) Patients with Melanoma and NSCLC will be randomized to: Low dose versus Intermediate dose versus High dose	1	NCT01295827	Any type of carcinoma or Melanoma or NSCLC Melanoma and NSCLC	- Safety - OR - Change from baseline in candidate biomarker expression levels in melanoma and NSCLC participants
- MK-3475 versus - Carboplatin *or* - Carboplatin + - Paclitaxel - Paclitaxel *or* - Dacarbazine *or* - Temozolomide	2	NCT01704287	Melanoma	- OR - PFS - OS
- BMS-936558 versus - Docetaxel	3	NCT01673867	NSCLC	- OS

Table 6 (*Continued*)

Drug	Phase	Trial	Disease	Primary outcome measures
Monotherapy Non-randomized				
- BMS-936558 versus - Docetaxel	3	NCT01642004	Squamous cell NSCLC	- OR - OS
- BMS-936558 versus - Dacarbazine *or* - Carboplatin *or* - Paclitaxel	3	NCT01721746	Melanoma	- OR - OS
- BMS-936558 versus - Dacarbazine	3	NCT01721772	Melanoma	- OS
- BMS-936558 versus - Everolimus	3	NCT01668784	RCC	- OS
Combination therapy Non-randomized				
- BMS-936558 - NY-ESO-1 (peptide vaccine) - gp100: 280–288 (peptide vaccine)	1	NCT01176474	Melanoma	- Time to relapse in patients with resected Stage IIIC/IV melanoma
- BMS-936558 - MART-1 (peptide vaccine) - NY-ESO-1 (peptide vaccine) - gp100: 209–217(210 M) (peptide vaccine) - gp100: 280–288(288V) (peptide vaccine)	1	NCT01176461	Melanoma	- OR
- BMS-936558 - BMS-986015 (ANTI-KIR):	1	NCT01714739	-NSCLC -Melanoma, -RCC -Colorectal cancer -Ovarian cancer	- Safety and - Tolerability
- BMS-936558 - Gemcitabine	2	NCT01313416	Pancreatic cancer	- Feasibility and - Safety
- BMS-982470 (Recombinant Interleukin-21, rIL-21) - BMS-936558	1	NCT01629758	Any type of carcinoma	- Safety
- BMS-936558 ± - DC RCC vaccine	2	NCT01441765	RCC	- Safety - OR
- BMS-936558 ± - DC AML vaccine	2	NCT01096602	AML	- Toxicity
- BMS-936558 - DC fusion vaccine	2	NCT01096602	Multiple myeloma	- Immunological response

Table 6 (*Continued*)

Monotherapy Non-randomized				
Drug	**Phase**	**Trial**	**Disease**	**Primary outcome measures**
- BMS-936558 - BMS-986015 (ANTI-KIR)	1	NCT01714739	Solid Tumors	- Safety - Tolerability
Randomized				
- Sipuleucel-T (vaccine) versus - Sipuleucel-T + - BMS-936558 versus - Cyclophosphamide + - Sipuleucel-T + BMS-936558	2	NCT01420965	Prostate cancer	- Feasibility - Immune efficacy
- BMS-936558 or - BMS-936558 Plus – Ipilimumab versus Ipilimumab alone	3	NCT01844505	Melanoma	- OS

NSCLC, non-small cell lung cancer; HCC, hepatocellular carcinoma; RCC, renal cell carcinoma; CML, chronic myelogenous leukemia; AML, acute myelogenous leukemia; DC, dendritic cell; KIR, killer-cell immunoglobulin-like receptor; RR, response rate; PFS, progression free survival; OS, overall survival; OR, overall response.

clinical testing of a series of other antibodies and blocking constructs to the PD-1/PD-L1 axis, provide an impetus for the continued clinical testing of these highly active immunotherapies for melanoma.

Tables 5 and 6 summarize ongoing clinical trials with anti PD-1 or anti PD-L1 antibodies.

Based on experimental data, blockade of B7-H1 or PD-1 is not expected to stimulate de novo immune responses but rather to enhance ongoing immune responses against tumor antigens, since this axis is implicated in the effective phase and not in the priming phase of the immune response. A combinational strategy to elicit the immune response could improve the objective response rate as well as the duration of response. In patients with advanced disease it is likely that the majority of TILs have an “exhausted” phenotype. The presence of inhibitory molecules would render these cells insensitive to the action of anti-PD-1. On the basis of these considerations, preliminary results in mice models suggest that combination therapies may be helpful in restoring an activating phenotype in the tumor microenvironment [71]. In experimental models combining B7-H1/PD-1 blockade with cancer vaccines [72–75], adoptive transfer of preactivated T cells, or T cell stimulation with anti-CD137 [76] often provides dramatic synergistic antitumor effects, in some cases eradicating well-established tumors.

Furthermore there is preclinical evidence that CTLA-4 and PD-1 could play complementary roles in regulating adaptive immunity. Curran et al. reported, in mice xenograft, that the combination of CTLA-4 and PD-1 blockade determines the accumulation of CTLA-4/PD-1 double-positive T effector cells within B16 melanoma cell lines [77]. These data suggest that T cells that would otherwise be functionally and proliferatively repressed are instead able to continue expanding and carrying out effector functions.

In the same model the combination of CTLA-4 and PD-1 blockade resulted more than twice as effective as either alone in promoting the rejection of B16 melanoma cell lines. Interestingly the further addition of PD-L1 antibody elevates the rate of tumor-free survival to 65% versus 10% with CTLA-4 blockade alone [77].

Recently, on the basis of these observations, a phase 1 study to investigate the safety and efficacy of combined CTLA-4 and PD-1 blockade [with the use of ipilimumab (anti-CTLA4 antibody) and nivolumab (anti-PD-1 antibody), respectively] in patients with advanced melanoma has been conducted [78]. In this study the objective-response rate for all patients in the concurrent-regimen group was 40%. At the maximum tolerated doses (nivolumab at a dose of 1 mg/kg of body weight and ipilimumab at a dose of 3 mg/kg), 53% of patients had an objective response, all with tumor reduction of 80% or more. Grade 3 or 4 toxicities occurred in 53% of patients and were generally reversible and qualitatively similar to those reported with monotherapy (ipilimumab or nivolumab alone).

7. Conclusion

The past decade has seen important strides in the field of melanoma research. Extensive research over the past twenty years yielded the identification of new and innovative ways to manipulate the immune response to cancer. Negative regulators of the immune system, called immunologic checkpoints, have been found to play important roles in restraining otherwise effective anti-tumor immunologic responses. Therapies that target the PD-1/PD-L1 axis have demonstrated promising clinical results. Treatment is generally well tolerated, but a novel spectrum of side effects, termed immune-related adverse events, has been experienced. Unfortunately, not all patients respond to these therapies, and evaluation of biomarkers predictive of response is ongoing.

Some open issues and scarce information may unfavorably impact the management of patients with advanced melanoma, therefore, priority actions are needed in this direction. In particular:

1. A standardized methodology to define the PD1/PD-L1 positive melanoma is strongly needed. Tumor heterogeneity in terms of PD-L1 expression, variability in the assays and cell immunolocalization as well as cut-off values for positive versus negative PDL-1 immunohistochemical expression are not uniformly defined and do not currently allow appropriate patient's classification.
2. The prognostic and predictive role of TILS (overall estimation and/or immunophenotyping) in addition to PD-1/PD-L1 remains to be determined in order to better stratify patients and to identify those who may benefit from therapies that target PD-1/PD-L1 axis.
3. The anti PD-1 and PD-L1 antibodies have demonstrated clinical activity in early clinical trials, but randomized studies with immune correlates are lacking so far.
4. Combinations of PD-L1/PD-1 blockade with cancer vaccines, adoptive transfer of pre-activated T cells, T cell stimulation with anti-CD137 or MAPK inhibitors should be investigated as they could provide synergistic antitumor effects.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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