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Targeting PD-1 in cancer: Biological insights with a focus on breast cancer

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ABSTRACT

Programmed cell death-1 (PD-1) receptor and its ligands physiologically regulate the activity of the adaptive immune system to limit excessive inflammatory processes, thus preventing normal tissue damage. Tumor cells escape from the host immune surveillance using this pathway, rendering it relevant therapeutic target. Despite the relevant clinical efficacy observed in patients with solid and hematological malignancies, the clinical benefit of these novel treatments is limited to a relatively restricted number of patients. A wide amount of genomic and immune related features is currently under investigation as potential predictive biomarkers for treatment selection. The results obtained so far are encouraging but still imperfect. Combination strategies using different immunotherapeutic agents or with other treatments (such as chemotherapy) are being investigated, showing promising but still not completely satisfactory results. This review aims to shed light on the main principles of targeting PD-1 in breast cancer, from biology through its functional and clinical implications.

1. Introduction

The immune system plays a fundamental role in active host defenses against foreign agents while its regulation is critical for eschewing autoimmunity, often triggered by persistent self-reactive T lymphocytes that survive central thymic selection and relocate to the periphery. Antigen (Ag) recognition alone (signal 1) is insufficient for T cell activation as a secondary activation signal, predominantly binding of the costimulatory receptor CD28 to the B7-1 (CD80) and/or B7-2 (CD86) ligands on Ag-presenting cells (APCs), is required (signal 2) (Mueller et al., 1989). Various inhibitory and costimulatory receptors are orchestrated to deliver negative or positive signals during the tight regulation controlling T cell-mediated immune responses (Zhu et al., 2011).

Inhibitory receptors, expressed on the surface of immune cells, are termed immune checkpoint molecules because once they interact with their ligand, cell-to-cell signals are generated to restrain T cell Ag-directed events (Bardhan et al., 2016). Cytotoxic T lymphocyte Ag 4 (CTLA-4) is an example of an important regulatory receptor that directly competes with CD28 for CD80/CD86 binding to deliver a negative stop signal to T cells (Chen, 2004). The function of these checkpoint receptors is to modulate the duration and amplitude of physiological immune responses, maintain self-tolerance and minimize peripheral tissue damage (Pardoll, 2012). This is an alternative mechanism of peripheral tolerance, working together with T cell anergy, deletion and regulatory T cell (Treg)-mediated suppression to control the breadth and intensity of immune responses. In recent years, immune checkpoint pathways have been extensively studied to understand the mechanisms of immune escape in cancer, with a particular focus on their ability to inhibit tumor Ag-specific T cells.

Many of these pathways can now be specifically blocked by therapeutic antibodies (Abs) or modulated by recombinant forms of ligands or receptors as a form of cancer immunotherapy referred to as immune checkpoint blockade (ICB) (Pardoll, 2012). Anti-CTLA-4, anti-programmed cell death-1 (PD-1) and anti-programmed cell death-ligand 1 (PD-L1) Abs were granted approval from the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of a broad spectrum of neoplastic diseases (Porcu et al.,

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PEMBROLIZUMAB (ANTI-PD-1)		
SINGLE AGENT		
TUMOR TYPE	**CRITERIA**	**OUTCOME**
MELANOMA	•Unresectable or metastatic disease •Stage III with LN involvement who underwent complete resection	•Ipilimumab naïve: improved OS (HR 0.69 (0.52, 0.90)) and PFS (0.58 (0.47, 0.72)) *vs ipilimumab*; ORR 33% *vs* 12% (**KEYNOTE 006**) •Ipilimumab refractory: OS (HR 0.86 (0.67, 1.10)), improved PFS (0.57 (0.45, 0.73)) *vs chemotherapy*; ORR 21% *vs* 4% (**KEYNOTE 002**) •RFS (HR 0.57 (0.46, 0.70)) *vs placebo* (**KEYNOTE 054**)
NSCLC	•≥ 1% TPS (PD-L1); no *EGFR* and no *ALK* mutations; 1st line; stage III not candidates for surgery or chemoradiation; metastatic •≥ 1% TPS (PD-L1); at least one prior chemotherapy regimen for metastatic disease (progression on or after platinum based chemotherapy); if *EGFR* or *ALK* positive mutations previous approved targeted therapy	•≥ 1% TPS: improved OS (HR 0.81 (0.71, 0.93)); PFS: no difference; ORR: no difference *vs chemotherapy* (**KEYNOTE 042**) •≥ 50% TPS: OS HR 0.69 (0.56, 0.85); PFS HR 0.81 (0.67, 0.99); ORR: no differences *vs chemotherapy* (**KEYNOTE 042**); improved OS (HR 0.60 (0.41, 0.89) and PFS (HR 0.50 (0.37, 0.68)) and ORR 45% *vs* 28% *vs chemotherapy* (**KEYNOTE 024**) •Improved OS (HR 0.71 (0.58, 0.88)); PFS (HR 0.88 (0.73, 1.04)); ORR: 18% *vs* 9% *chemotherapy* (**KEYNOTE 010**)
SCLC	•Metastatic disease; progression on or after platinum based chemotherapy; at least one previous line of treatment	•ORR: 19% (**KEYNOTE 158**)
HNSCC	•≥ 1% CPS (PD-L1); 1st line; metastatic, unresectable or recurrent •Recurrent or metastatic disease progressing on or after platinum	•Improved OS (HR 0.78 (0.64, 0.96)); PFS: no differences; ORR: 19% *vs* 35% *vs chemotherapy* (**KEYNOTE 048**) •ORR: 16% (**KEYNOTE 012**). •OS 0.82 (0.67, 1.01) *vs chemotherapy* (**KEYNOTE 040**)
UC	•≥ 10 CPS (PD-L1); not eligible for platinum; locally advanced or metastatic •Progression during or following platinum therapy or within 12 months of neoadjuvant or adjuvant treatment with platinum containing chemotherapy; locally advanced or metastatic	•ORR: 47% (**KEYNOTE 052**) •Improved OS (HR 0.73 (0.59, 0.91)); PFS: no difference; ORR: 21% *vs* 11% (p=0.002) *vs chemotherapy* (**KEYNOTE 045**)
MSI-H CANCER	•Unresectable or metastatic; prior treatments; no satisfactory alternative options •Colorectal: progression after fluoropyrimidine, oxaliplatin, and irinotecan	•ORR: 39.6% (**KEYNOTE 016, 164, 012, 028, 158**)
GC OR GEJ (ADENOCARCINOMA)	•≥ 1% CPS (PD-L1); recurrent locally advanced or metastatic; progression on or after two or more prior lines of therapy including fluoropyrimidine and platinum and if appropriate, HER2/neu-targeted therapy	•ORR: 13% (**KEYNOTE 059**)
CERVICAL CANCER	•≥ 1% CPS (PD-L1); recurrent or metastatic; progression on or after chemotherapy	•ORR: 14.3% (**KEYNOTE 158**)
HCC	•Previous treatment with sorafenib	•ORR: 17% (**KEYNOTE 224**). •OS and PFS no difference *vs placebo* (**KEYNOTE 240**)
. MCC	•Recurrent locally advanced or metastatic disease	•ORR : 56% (**KEYNOTE 017**)
IN COMBINATION WITH CHEMOTHERAPY or TARGETED THERAPY		
NSCLC	•*pemetrexed* and *platinum*: metastatic non squamous; no *EGFR* and no *ALK* mutations; 1st line; •*carboplatin* and either *paclitaxel* or *nabpaclitaxel*; metastatic squamous; 1st line	•Improved OS (HR 0.49 (0.38, 0.64)), PFS (HR 0.52 (0.43, 0.64)), ORR 48% *vs* 19% (p< 0.0001) *vs pemetrexed platinum chemotherapy* (**KEYNOTE-189**) •Improved OS (HR 0.64 (0.49, 0.85)), PFS (HR 0.56 (0.45, 0.70)), ORR 58% *vs* 35% (p 0.0008) *vs carboplatin* and either *paclitaxel* or *nabpaclitaxel* (**KEYNOTE-407**)
HNSCC	•*platinum* and *5-FU*: metastatic, unresectable or recurrent; 1st line	•Improved OS (HR 0.77 (0.63, 0.93)); PFS and ORR: no differences; *vs chemotherapy* (**KEYNOTE 048**)
RCC	•*axitinib*: advanced; 1st line	•Improved OS (HR 0.53 (0.38, 0.74); PFS (HR 0.69 (0.57, 0.84)); ORR (59% *vs* 36%, p<0.0001) *vs sunitinib* (**KEYNOTE 426**)

Fig. 1. Anti-PD-1 and anti-PD-L1 agents approved by the United States Food and Drug Administration (FDA) for the treatment of solid tumors. Figures summarize the main studies that investigated the efficacy of the anti-PD-1 and anti-PD-L1 agents approved in the various solid tumor types and patient subsets (latest update: 23/06/2019): Fig. 1**A** (pembrolizumab); Fig. 1**B** (nivolumab); Fig. 1**C** (atezolizumab); Fig. 1**D** (avelumab, durvalumab and cemiplimab).
Legend: *ALK*: anaplastic lymphoma kinase; CPS: combined proportion score; CRC: colorectal cancer; CSCC: cutaneous squamous cell carcinoma; *EGFR*: epidermal growth factor receptor; 5FU: 5-fluorouracil; GC: gastric cancer; GEJ: gastro esophageal junction; HCC: hepatocellular carcinoma; HNSCC: head and neck squamous cell carcinoma; HR: hazard ratio; LN: lymph node; MCC: Merkel cell carcinoma; MSI-H: microsatellite instability high; NSCLC: non-small cell lung cancer; ORR: objective response rate; OS: overall survival; PD-L1: programmed cell death-ligand 1; PFS: progression free survival; RCC: renal cell carcinoma; RFS: relapse free survival; SCLC: small cell lung cancer; TNBC: triple negative breast cancer; TPS: tumor proportion score; UC: urothelial carcinoma.

2019), in early and advanced settings, because they were shown to elicit durable clinical responses. To date, greater clinical benefit from these treatments has been observed in subsets of patients, including: advanced non-small cell lung cancer (NSCLC) with high PD-L1 expression (defined as positivity in ≥1% of tumor cells), particularly those with a tumor proportion score (TPS) ≥50% and microsatellite instability high (MSI-H) colorectal cancer (CRC) (Le et al., 2015). In less immunogenic tumors, such as breast cancer (BC), ICB has also been tested as monotherapy or in combination with other treatments (hormone therapy, radiotherapy, chemotherapy, targeted therapy and dual ICB blockade) in numerous clinical trials (Solinas et al., 2017a) with limited responding patients. Recently the anti-PD-L1 agent atezolizumab in association with nab-paclitaxel chemotherapy has been approved for the first line treatment of metastatic triple-negative breast cancer in patients with PD-L1 positive tumors (Schmid et al., 2018). Thus, there is a growing need to identify biomarkers that can reliably identify potential responders specifically selecting patients for treatment with these expensive and potentially toxic drugs. A summary of the benefit from PD-1/PD-L1 ICB drugs approved by FDA in the various tumor types and subgroups of patients is provided in Fig. 1 (last update: 23/06/2019). The aim of this review is to summarize the main principles of targeting PD-1 in cancer, with a focus on BC, from the biology to the functional and clinical implications.

2. PD-1: biology

The inhibitory receptor PD-1 (CD279) was discovered in 1992 in the laboratory of Tasuku Honjo, one of the 2018 Nobel prize winners in Physiology and Medicine (Ishida et al., 1992). The primary role of the PD-1 pathway is to regulate the effector phase of T cell-mediated inflammatory responses in peripheral tissues. PD-1′s interaction with its ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), induces and maintains T cell peripheral tolerance. In tumors, the PD-1:PD-L1/-L2 pathway has been shown to mediate potent inhibitory signals that effectively diminish T cell proliferation and effector functions, and thereby negatively impacting anti-tumor immunity. The success of therapeutically targeting this pathway is based on rescuing T cell activity directed against tumor cells (Bardhan et al., 2016).

Activated T cells upregulate PD-1 and continue to express this receptor in the periphery. In BC, flow cytometric analysis distinguishes three subsets of PD-1 positive T cells, ranging from low/negative (PD-1^{lo}) to intermediate (PD-1^{int}) and high (PD-1^{hi}) (Gu-Trantien et al., 2013; Solinas et al., 2017b; Myklebust et al., 2013). In the presence of chronic Ag exposure (signal 1) and chronic stimulation by cytokines (signal 3), the persistent induction of PD-1 on activated T cells can induce an exhausted, dysfunctional or anergic state, with these PD-1^{hi} cells co-expressing other inhibitory receptors such as T cell immunoglobulin and mucin domain-3 (TIM3) (Pardoll, 2012). Likewise,

NIVOLUMAB (ANTI-PD-1)		
SINGLE AGENT		
TUMOR TYPE	**CRITERIA**	**OUTCOME**
MELANOMA	•Advanced, unresectable or metastatic disease •Stage III with LN involvement or metastatic disease who underwent complete resection	•Previously treated metastatic melanoma: ORR 32% (**CHECKMATE 037**) •Previously untreated metastatic melanoma: improved OS (HR 0.42 (0.30, 0.60)); PFS (HR 0.43 (0.34, 0.56) and ORR (34% *vs* 9%) *vs dacarbazine* (**CHECKMATE 066**) •Improved RFS (HR 0.65 (0.53, 0.80)) *vs ipilimumab* (**CHECKMATE 238**)
NSCLC	•Locally advanced or metastatic disease after prior platinum; if *EGFR* mutated or *ALK* mutated, after targeted therapies for these mutations	•Second line of metastatic squamous disease: improved OS (HR 0.59 (0.44, 0.79)); PFS (HR 0.62 (0.47, 0.81); ORR (20% *vs* 9%, p=0.0083) *vs docetaxel* (**CHECKMATE 017**) •Second line of metastatic non-squamous disease: improved OS (HR 0.73 (0.60, 0.89)) and ORR (56% *vs* 36%, p=0.02) *vs docetaxel* (**CHECKMATE 057**)
SCLC	•Metastatic disease progressing after platinum therapy and at least one other line of therapy	•ORR: 12% (**CHECKMATE 032**)
RCC	•Advanced disease after prior (antiangiogenic) therapy	•Improved OS (HR 0.73 (0.60, 0.89)) and ORR (21.5% *vs* 3.9%) *vs everolimus* (**CHECKMATE 025**)
HNSCC	•Recurrent or metastatic disease progressing on or after platinum therapy	•Improved OS (HR 0.70 (0.53, 0.92)) *vs cetuximab* or *methotrexate* or *docetaxel* (**CHECKMATE 141**)
UC	•Locally advanced unresectable or metastatic disease progressing on or after platinum therapy or progressing within 12 months of neoadjuvant or adjuvant treatment with platinum	•ORR: 19.6% (**CHECKMATE 275**)
MSI-H CRC	•Metastatic disease progressed following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan	•ORR: 28% (**CHECKMATE 142**)
HCC	•Previous treatment with sorafenib	•ORR: 14.3% (**CHECKMATE 040**)
IN COMBINATION WITH IPILIMUMAB		
MELANOMA	•Advanced, unresectable or metastatic disease	•Improved OS (HR 0.55 (0.44, 0.69)); PFS (HR 0.42 (0.34, 0.51); ORR (50% *vs* 40%) *vs nivolumab* single agent (**CHECKMATE 067**)
RCC	•Advanced disease, previously untreated; intermediate or poor risk	•Improved OS (HR 0.63 (0.44, 0.89) and ORR: 41.6% *vs* 26.5% (p<0.0001) *vs sunitinib* (**CHECKMATE 214**)
MSI-H CRC	•Metastatic disease progressed following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan	•ORR: 46% (**CHECKMATE 142**)

Fig. 1. (*continued*)

ATEZOLIZUMAB (ANTI-PD-L1)		
SINGLE AGENT		
TUMOR TYPE	**CRITERIA**	**OUTCOME**
UC	•≥5% IC (PD-L1); locally advanced or metastatic disease not eligible for cisplatinum •Locally advanced or metastatic disease not eligible for any platinum, regardless of PD-L1 status •Locally advanced or metastatic disease progressing after or during platinum, or progressing within 12 months from neoadjuvant therapy or adjuvant therapy	•ORR: 28.1% (**IMvigor 210**) •ORR: 23.5% (**IMvigor 210**) •ORR: 14.8% (**IMvigor 210**)
NSCLC	•Locally advanced or metastatic disease after prior (platinum-based) chemotherapy; if *EGFR* mutated or *ALK* mutated, after targeted therapies for these mutations	•Improved OS (HR 0.79 (0.69, 0.91)) *vs docetaxel* (**OAK**)
IN COMBINATION WITH CHEMOTHERAPY AND TARGETED THERAPY		
NSCLC	•*bevacizumab* and *paclitaxel* and *carboplatin*; metastatic; non squamous; no *EGFR* or *ALK* mutations; 1st line	•Improved OS (HR 0.78 (0.64, 0.96)) with atezolizumab+*bevacizumab*+*paclitaxel*+*carboplatin*; PFS (HR 0.71 (0.59, 0.85)) with atezolizumab+*bevacizumab*+*paclitaxel*+*carboplatin*; and ORR (55% with atezolizumab+*bevacizumab*+*paclitaxel*+*carboplatin* *vs* 43% with atezolizumab+*paclitaxel*+*carboplatin* *vs* 42% with *bevacizumab*+*paclitaxel*+*carboplatin*) (**IMpower 150**)
TNBC	•*nab-paclitaxel*; ≥1% IC (PD-L1); locally advanced or metastatic disease	•Improved PFS (HR 0.60 (0.48, 0.77)); ORR (53% *vs* 33%) *vs placebo+nab-paclitaxel* (**IMpassion 130**)
SCLC	•*carboplatin* and *etoposide*; extensive stage; 1st line	•Improved OS (HR 0.70 (0.54, 0.91)); PFS (HR 0.77 (0.62, 0.96) *vs placebo+carboplatin+etoposide* (**IMpower 133**)

Fig. 1. (*continued*)

during chronic viral infections, T cell proliferation and function in virus clearance is prevented by PD-1 upregulation on exhausted or dysfunctional virus-specific T cells, attenuating the $CD8^+$ T cell-mediated cytotoxicity at the effector phase (Watanabe et al., 2010; Day et al., 2006). Apart from its expression by activated and memory $CD4^+$ (Gu-Trantien et al., 2017) and $CD8^+$ T cells, PD-1 can be found on monocytes, natural killer (NK) cells and dendritic cells (DCs) (Probst et al., 2005). Its expression can also be induced on APCs and myeloid $CD11c^+$ DCs (Bardhan et al., 2016). B cells infiltrating BC were shown to be PD-1 negative, whereas they expressed its ligands PD-L1 and/or PD-L2, although by low proportions of cells (Solinas et al., 2017b).

Within the $CD4^+$ T cell subpopulation, which includes effector and regulatory cells (Tregs), PD-1 is differentially expressed. Intermediate levels of PD-1 are frequently detected on activated Tregs and other effector subpopulations (Gu-Trantien et al., 2017; Solinas et al., 2017b). $PD\text{-}1^{hi}$ is characteristic of follicular helper T cells (Tfh), a specialized population that provide help to B cells for maturation and are characteristically located in the germinal centers (GC) of secondary lymphoid organs (SLOs), like inflamed tonsils and lymph nodes (LNs). Tertiary lymphoid structures (TLS) arising at sites of chronic inflammation are structurally similar to SLOs. These organized lymphoid aggregates are commonly composed of a B cell follicle surrounded by a T cell zone (Iwai et al., 2002a; Wong et al., 2007; Nam-Cha et al., 2008; Roncador et al., 2007). TLS are functionally important temporary sites of adaptive immune responses generated in non-lymphoid tissues in response to persistent stimulation (Fig. 2). The generation of a TLS requires the action of chemokines, such as chemokine (C-C motif) ligand 9 (CCL19) and CCL21, necessary for the recruitment and disposition of T cells and DCs, and chemokine (C-X-C motif) ligand 13 (CXCL13), which plays a similar function for the recruitment of B cells (Hiraoka et al., 2016). In human tumors a positive association between TLS presence and improved prognosis has been demonstrated (Sautès-Fridman et al., 2016).

In the $CD8^+$ cytotoxic T lymphocyte (CTL) compartment, PD-1 is preferentially expressed on exhausted and Ag-specific populations during chronic viral infections (Golden-Mason et al., 2007; Zhang et al., 2007; Urbani et al., 2006; Petrovas et al., 2006; Conrad et al., 2011). Interestingly $CD8^+$ $PD\text{-}1^{hi}$ T cells were shown to maintain their

DURVALUMAB (ANTI-PD-L1)		
SINGLE AGENT		
TUMOR TYPE	CRITERIA	OUTCOME
UC	•Locally advanced or metastatic disease progressing after or during platinum, or progressing within 12 months from neoadjuvant therapy or adjuvant therapy with platinum	•ORR: 17% (**Study 1108**) •ORR in PD-L1 high (IC involve ≥1% of tumor area: TC ≥25% or IC ≥25%; if IC involve <1% of tumor area: TC ≥25% or IC=100%): 26% (**Study 1108**)
NSCLC	•Unresectable stage III disease, not progressed after platinum based chemoradiation therapy	•Improved PFS (HR: 0.52 (0.42, 0.65)) and ORR: 26% *vs* 14% *placebo* (**PACIFIC**)

AVELUMAB (ANTI-PD-L1)		
SINGLE AGENT		
TUMOR TYPE	CRITERIA	OUTCOME
MCC	•Metastatic disease	•ORR: 33% (**JAVELIN Merkel 200**)
UC	•Locally advanced or metastatic disease progressing after or during platinum, or progressing within 12 months from neoadjuvant therapy or adjuvant therapy with platinum containing therapy	•ORR: 16% (**JAVELIN Solid Tumor**)
IN COMBINATION WITH TARGETED THERAPY		
RCC	•*Axitinib*: advanced disease; 1st line	•Improved PFS (HR 0.69 (0.56, 0.84)) and ORR (51.4% *vs* 25.7%) *vs sunitinib* (**JAVELIN Renal 101 Trial**)

CEMIPLIMAB-rwlc (ANTI-PD-1)		
SINGLE AGENT		
TUMOR TYPE	CRITERIA	OUTCOME
CSCC	•Metastatic (nodal or distant) or locally advanced	•ORR 47.2% (**Study 1423** and **Study 1540**)

Fig. 1. (*continued*)

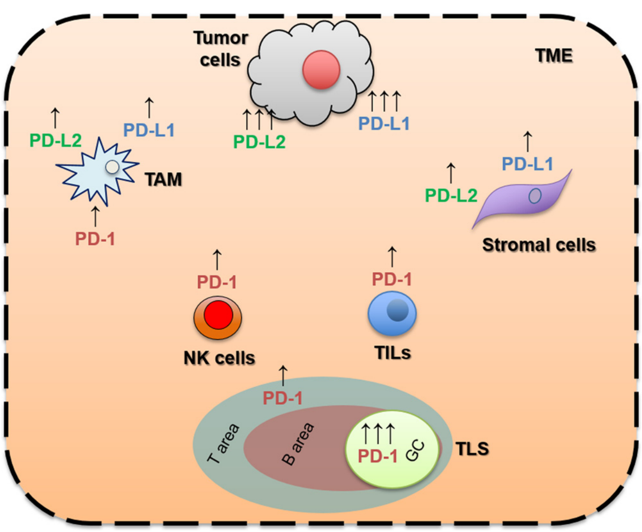

Fig. 2. PD-1 and its ligands' expression in the breast cancer (BC) tumor microenvironment (TME). PD-1 is highly expressed in cells of tertiary lymphoid structure-germinal centers (TLS-GC), whereas lower levels of expression are present in the TLS T cell areas, in TIL localized in the stroma, in natural killer (NK) cells and in tumor associated macrophages (TAM). PD-L1/L2 are expressed by tumor cells, TAM, stromal cells and rarely by TIL.

cytotoxic activity, although having defects in pathways associated with metabolic and mitochondrial functions (Trautmann et al., 2012). Furthermore, PD-1 surface expression by T cells is maintained by continued Ag stimulation, as observed during lymphocytic choriomeningitis virus (LCMV) infections where PD-1 is downregulated after viral epitope escape (Blattman et al., 2009). In cancer PD-1 expression identifies exhausted *versus* dysfunctional CTL TILs (Wherry, 2011).

PD-1 expression on T cells can be induced by several cytokines (*i.e.*: interleukin (IL)-2, IL-7, IL-10, IL-15, IL-21, as well as transforming growth factor-beta (TGF-β)) (Kinter et al., 2008; Francisco et al., 2010). In contrast, ligands for Toll-like receptor (TLR)1/2, TLR7, and TLR9 reduced the expression of PD-1 on Ag-activated $CD8^+$ T cells and these effects were mediated by IL-12 released by professional APCs, suggesting a role of the innate immune activation during effector T-cell development (Zahm et al., 2018). Interestingly, not the same effects were seen for the expression of other inhibitory immune checkpoint molecules, such as CTLA-4, lymphocyte activation gene 3 (LAG3) and TIM3.

In macrophages, constitutive and IFN-alpha (α) mediated PD-1 expression are regulated by interferon (IFN)-sensitive responsive element (ISRE) and signal transducer and activator of transcription (STAT)1/2 (Cho et al., 2008). In the tumor microenvironment (TME), the expression of PD-1 by tumor-associated macrophages (TAM) is associated with an inhibition of phagocytosis and of anti-tumor immunity (Gordon et al., 2017).

PD-1 can also be selectively induced on myeloid DCs by *Listeria monocytogenes* infection or by TLR2, TLR3, TLR4, or nucleotide-binding oligomerization domain (NOD) ligation, whereas it is inhibited by IL-4 and TLR9 (Yao et al., 2009).

3. PD-1 and T cell exhaustion in infections

$CD8^+$ effector T cells behave differently in acute and chronic viral infections and PD-1 plays important regulatory roles in these settings.

During acute infection, naïve Ag-specific $CD8^+$ T cells are activated, proliferate, and differentiate into effector $CD8^+$ T cells, being able to clear the virus. Most of these virus-specific effector $CD8^+$ T cells become apoptotic and a very small number (5–10%) of long-lived memory cell population arises, protecting against secondary infections (Wherry et al., 2004).

During chronic viral infections, persistent antigenic (signal 1) and cytokine stimulation (signal 3) lead to the loss of effector T cells and to their failure to develop into memory $CD8^+$ T cells. Consequently T cells become unresponsive to Ags and persist in a dysfunctional, exhausted or anergic state, in which they are unable to efficiently clear the virus (Shin and Wherry, 2007).

In the process of exhaustion, a hierarchical loss of effector functions takes place. The first functions to be lost are IL-2 production, high proliferative capacity and *ex vivo* killing functions. Afterwards, at more intermediate stages of dysfunction, cells lose the ability to produce tumor necrosis factor (TNF) and their proliferative potential. The latest exhaustion steps ultimately lead to virus-specific cells that partially or, in some cases, completely lack the ability to produce large amounts of IFN-γ or beta (β) or to degranulate. The final stage of exhaustion is represented by the physical deletion of virus-specific T cells (Wherry, 2011).

However, dysfunctional or exhausted T cells are not functionally inert. Indeed, in the simian immunodeficiency virus (SIV) infection, depletion of $CD8^+$ T cells (including exhausted T cells) generated a rapid increase in viral titers and the progression to acquired immune deficiency syndrome (AIDS) (Jin et al., 1999; Schmitz et al., 1999). This suggests an important role for the residual function of SIV-specific effector T cells in maintaining *equilibrium* between host and pathogen and for constraining the chronic infection (Jin et al., 1999). Further, dysfunctional or exhausted T cells produce low levels of IFN-γ and/or β and express high levels of Granzyme B (GZMB). Interestingly one subset of these cells has been shown to have some residual cytotoxicity, despite their dysfunctional or exhausted state (Wherry, 2011; Blackburn et al., 2009; Paley et al., 2012; Herbst et al., 2014).

In mouse models therapeutically targeting the PD-1 pathway through ICB induced reinvigoration of a significant fraction of cells, characterized by a T-bet^{hi} Eomes^{lo} PD-1^{int} profile, which mirrors a "partially" exhausted phenotype that can be rescued with immunotherapy, since these cells were able to proliferate after PD-1 ICB. In contrast, the Eomes^{hi} PD-1^{hi} population had an exhausted phenotype with their functions unable to be rescued after PD-1 ICB blockade. This happens most probably because these cells express other alternative receptors, mediating non-redundant inhibitory pathways (Paley et al., 2012). Similar subsets of effector T cells defined by reciprocal patterns of T-bet, Eomes and/or PD-1 expression have been found in human patients with hepatitis C virus or HIV infections (Buggert et al., 2014; Urbani et al., 2006), where PD-1 ICB resulted in an increase of pathogen-specific T cells and in the decrease of viral load (Watanabe et al., 2010; Day et al., 2006).

4. PD-1 expression in cancer

PD-1 protein expression has also been described in tumor cells as observed in hepatocellular carcinoma (HCC), NSCLC, carcinoids, renal, urothelial and testis cancers, and melanoma (Yao et al., 2018; Kleffel et al., 2015; Du et al., 2018a; Li et al., 2017). Despite documented efficacy (*i.e.*, durable responses) of anti-PD-1 or anti-PD-L1 drugs in NSCLC (Postow et al., 2015), (particularly in tumors with positive PD-L1 expression on tumor cells), the expression of tumor-intrinsic PD-1 signaled rapid tumor progression in a NSCLC patient treated with anti-PD-1 (Du et al., 2018a).

A variety of studies further investigated the clinical significance of PD-1 expression by immune cells in cancer patients. We will list some of the reported investigations, since this narrative review does not aim to provide an exhaustive overview of the knowledge in this topic.

A high degree of PD-1 expression (*i.e.*, PD-1^{hi}) was found in was found in C-X-C motif chemokine receptor 5 $(CXCR5)^+$ $CD8^+$ T cells isolated from tumor invaded lymph nodes from patients with anaplastic thyroid cancer. These cells expressed lower levels of other immune checkpoint molecules, such as CTLA-4 and TIM3 with respect to $CXCR5^-$ $CD8^+$ T cells. Functional studies revealed that, upon stimulation, PD-1^{hi} $CXCR5^+$ $CD8^+$ T cells presented stronger downregulation of CD27, higher expression of IL-2, IFN-γ, and TNF-α, higher proliferation capacity, higher expression of cytotoxic molecules GZMA, GZMB, and perforin, resulting in more potent functionality compared to their $CXCR5^-CD8^+$ T cell counterpart (Zhou et al., 2018a).

In primary BC, an upregulation of PD-1 was suggested by a significant hypomethylation of CpG islands in the promoter regions of PD-1 compared with normal tissues (Sasidharan Nair et al., 2018). Notably, previous studies linked PD-1 gene expression with better prognosis in HER2-positive BC patients (Gu-Trantien et al., 2013; Solinas et al., 2017b) (not treated with the anti-HER2 agent trastuzumab, since it was not the standard of care at that time). In a study performed in the neoadjuvant setting, triple negative BC (TNBC) patients with high PD-1 (and PD-L1) expression experienced significantly higher rates of non-pathologic complete response (pCR). This finding suggests a link between the expression of PD-1 and PD-L1 and the relative low response to standard treatments. Further, univariate analysis showed that PD-1 expression was also significantly associated with shorter disease-free survival (DFS) in TNBC (Asano et al., 2018). However, this result needs to be interpreted with caution because it could have been influenced by the laboratory techniques used for the detection of either PD-1 or PD-L1.

Protein expression of PD-1 on TIL by IHC was found in 20% (Barrett et al., 2018) - 40% of TNBC (Buisseret et al., 2017). It was associated with some clinicopathological factors (among them: Ki67 and the histological grade) and with either poor (Zhou et al., 2018b) and better prognosis (Brockhoff et al., 2018). PD-1 was also detected in the GC of tumor draining lymph nodes (Ye et al., 2018) and of TLS in BC(Solinas et al., 2017b; Buisseret et al., 2017; Gu-Trantien et al., 2013), probably signaling the presence of activated Tfh cells, previously linked to a better prognosis particularly in the HER2-positive subtype (Ishida et al., 1992).

In peripheral blood, high PD-1^+CD4^+ and PD-1^+CD8^+T lymphocyte levels were independent risk factors for predicting worse survival in patients with metastatic gastric cancer, suggesting that high PD-1 expression on circulating T cell subsets may represent a future novel prognostic biomarker (Shi et al., 2018). However, these findings need further confirmation.

5. PD- 1/PD-Ls pathway and anti-tumor immunity

The hypothesis that blockade of the PD-1/PD-Ls inhibitory pathway can promote anti-tumor immunity raised after the observations that: 1) exposure to IFN-γ (signifying a spontaneous response to the tumor from the immune system) can induce the expression of inhibitory ligands (PD-L1 and PD-L2) on APCs, regulating their activity and 2) tumor cell lines and primary tumor cells can express PD-L1 (Latchman et al., 2001; Dong et al., 2002), as potential mechanisms of immune escape. Further it was observed that overexpression of PD-L1 on a mouse mastocytoma cell line inhibited the cytolytic activity of $CD8^+$ T cells through PD-1 ligation, promoting tumor growth and invasiveness (Iwai et al., 2002b).

Further, in the tumor microenvironment (TME), an active PD-1/PD-L1 pathway promotes the survival of tumor cells *via* PD-L1 mediated anti-apoptotic signals (Dong et al., 2002) and inhibits the activation of signaling pathways, which are critical for the survival, expansion and differentiation of T cells recognizing tumor Ags. The imbalanced activation of signaling events in T cells results in tumor tolerance by inhibiting T effector and memory cell generation and by promoting the differentiation of dysfunctional *versus* exhausted T and Treg cells. Importantly, a high level of PD-1 expression, correlating with an exhausted phenotype and an impaired effector function, has been detected on tumor-infiltrating T cells with respect to T cells in normal tissues and peripheral blood from matched patients and from healthy donors (Ahmadzadeh et al., 2009).

In a number of solid tumors (urothelial, ovarian cancer, BC, cervical, CRC, pancreatic, gastric, melanoma, glioblastoma, NSCLC, and hematological malignancies), PD-L1 and to a lesser extent PD-L2 expression has been detected, correlating with adverse prognosis (Thompson et al., 2004; Ohigashi et al., 2005; Wu et al., 2006; Hamanishi et al., 2007; Nakanishi et al., 2007; Nomi et al., 2007; Marzec et al., 2008). PD-L1 expression on tumor cells is mediated by cell intrinsic mechanisms, activated by oncogenic mutations (Marzec et al., 2008). Other cellular components of the TME, including macrophages (mostly pro-tumor M2), myeloid DCs, myeloid derived suppressor cells (MDSCs), stromal fibroblasts and endothelial cells were found to express PD-L1 and PD-L2 (Garon et al., 2015; Topalian et al., 2012; Robert et al., 2015) (Fig. 2). Induction of PD-L1 expression on both tumor cells and tumor-infiltrating immune cells by local inflammation (*i.e.*, type I/II IFN-γ released by activated T cells) is a condition defined as “adaptive immune resistance” (Taube et al., 2012).

In some tumor types, the expression of PD-L1 within the TME was associated with clinical responses to PD-1/PD-L1 ICB (Garon et al., 2015; Topalian et al., 2012; Robert et al., 2015). The absence of PD-L1 upregulation on tumor cells or tumor-infiltrating immune cells was associated with the lack of therapeutic responses and with disease progression (Herbst et al., 2014). It should be noted that in the reported studies investigating the predictive role of PD-L1 in clinical trials, different cut-offs for positivity of PD-L1 and heterogeneous approaches regarding the evaluation of PD-L1 expression only on tumor cells or also on tumor-infiltrating immune cells were used (Garon et al., 2015; Topalian et al., 2012; Robert et al., 2015), rendering harder the interpretation of these observations and suggesting the need for an improved standardization for PD-L1 assessment in cancer. Another important (dichotomic) biomarker that is now being used for the selection of patients to anti-PD-1 ICB is the MSI-H status, identifying subsets of patients with advanced CRC and endometrial cancer that can benefit from this treatment (Dudley et al., 2016).

Other immune factors that were associated with the efficacy of PD-1/PD-L1 ICB were: 1) the presence of $CD4^+$ T cells specific to tumor-derived mutant peptides (Sun et al., 2017; Urban and Schreiber, 1992; Schumacher et al., 2014; Ott et al., 2017; Flament et al., 2015); 2) higher expressions of PD-1 on $CD4^+$ T lymphocytes and NK cells in the peripheral blood of patients treated with anti-PD-1 (nivolumab or pembrolizumab with or without chemotherapy) after the first cycle of immunotherapy (Du et al., 2018b). Interestingly, in the non-responder group lower expression of CTLA-4, GITR, and OX40 in peripheral blood was observed after the second cycle of immunotherapy (Du et al., 2018b).

6. Discussion and conclusions

Clinical data from different tumor types have showed that immunotherapies targeting immune checkpoint pathways generate relevant clinical benefits, including durable responses and prolonged survival in cancer patients. Indeed, the efficacy of single agent PD-1/PD-L1 mAbs has been proven in many trials, representing in a short time a new opportunity to treat cancer. Despite these impressive results obtained across different solid and hematological malignancies, this novel (immune)therapeutic strategy seems to be really effective only in a subset of patients that we are not yet able to precisely select before starting immunotherapy.

As already stated the predictive role of PD-L1 expression is controversial, considering that its absence or low presence is not an absolute indicator of immunotherapy’s lack of activity. On the contrary, a strong PD-L1 positivity does not give the certainty of a response (Herbst et al., 2016; Reck et al., 2016; Rittmeyer et al., 2017; Borghaei et al., 2015). All these heterogeneous findings highlight that PD-L1 is an imperfect, fragile and unreliable marker, particularly if used alone. Indeed other biological and molecular features involved in the complex, dynamic and multiple mechanisms of response to ICB, could be exploited as additional and hopefully more reliable biomarkers to allow an optimal patient selection.

An emerging role in predicting the clinical efficacy of ICB has been described for tumor infiltrating immune cells, including TIL (Loi et al., 2017; Emens et al., 2019), from the TME (Quiroga et al., 2016; Suyama et al., 2018; Hellmann et al., 2018). The main limit of this evaluation is the difficulty to obtain a quantitative and repeatable analysis on routine cytological or histological specimens, compromising the possibility of a robust and correct assessment between different trials and the use in clinical practice. In addition, mismatch-repair (MMR) deficient tumors, the microsatellite instability (MSI) high tumors have been shown to be very sensible to ICB in terms of activity and efficacy in several adult and pediatric malignancies. Remarkably for the first time in the history of cancer therapy, FDA approved a treatment based on a common biomarker rather than considering the histotype or the anatomical site of origin of the tumor (Administration, U. S. F. D., 2017).

Unfortunately, the use of IHC or polymerase chain reaction (PCR) assays allows detecting a MMR deficiency only in a little portion of screened subjects, while a larger genome analysis may be able to identify tumors with a higher number of somatic mutations, usually associated with an elevated neo-Ag load (Schumacher and Schreiber, 2015) and higher likelihood of benefit from ICB. In this scenario, the tumor mutational burden (TMB) has been recently explored as a predictive biomarker of response/clinical benefit from ICB (Hellmann et al., 2018), showing promising findings. Noteworthy the main limits with this biomarker are: 1) the lack of standardization of TMB assessment, 2) the disagreement about positivity thresholds, 3) the high costs and 4) the difficult reproducibility.

Apart from the well-known predictive factors that have been studied in cancer immunotherapy, the heterogeneous biological role(s) played by PD-1 in the diverse cells of the TME further add to the complexity of this scenario. The principal aim of this review was to summarize the most recent knowledge about PD-1 biology in cancer, together with its functional and clinical implications.

Interesting data on PD-1 expression on T cells, *i.e.*, its low, intermediate or high degrees seem relevant and promising when referring to responses to ICB. Indeed some $PD\text{-}1^{hi}$ cells (either $CD4^+$ and $CD8^+$ T cells) can express other inhibitory receptors such as TIM3 and LAG3 (Pardoll, 2012). One could speculate that this dysfunctional *versus* exhausted condition probably render patients not-responders to single agent ICB, whereas these may be the appropriate patients for a combination therapy with new inhibitory checkpoint molecule modulators (such as anti-LAG3 or anti-TIM3 agents) (Puhr and Ilhan-Mutlu, 2019).

Furthermore, a role that still needs to be clarified is the presence of TLS (Sautès-Fridman et al., 2016), as well as that of $CD4^+$ (either T helper and Tregs) and $CD8^+$ T cells expressing PD-1 at various degrees ($PD\text{-}1^{int}$ or $PD\text{-}1^{hi}$), whose putative predictive role in these structures needs to be investigated in trials of immunotherapy. Remarkably, $CXCL13^+$ $PD\text{-}1^{hi}$ $CD8^+$ TIL are highly predictive for response to PD-1 blockade in NSCLC(Thommen et al., 2018).

Noteworthy, in addition to its role in the most studied $CD4^+$ and $CD8^+$ T cells, it would be interesting to investigate the functional effect (s) of anti-PD-1 ICB on other immune cells such as monocytes, NK cells, APCs, myeloid DCs and TAM. This will allow us to detect new possible mechanisms of efficacy or resistance to ICB (Bardhan et al., 2016; Solinas et al., 2017b; Cho et al., 2008; Gordon et al., 2017).

Lastly, one of the most important topics on which research efforts are currently concentrated is the recent finding of PD-1 intrinsic expression on tumor cells. Past studies on PD-1 expression were focused only on immune cells, making its potential role and functions in tumor cells largely unexplored and consequently unclear. Intrinsic PD-1 protein expression has been primarily observed in melanoma, HCC and NSCLC, but while the biologic role of PD-1 in T cells has been widely studied, the meaning of high levels of the PD-1 expression on the surface of tumor cells should necessarily be further investigated, also because it was considered a risk factor for predicting worse survival (Du et al., 2018a; Yao et al., 2018; Kleffel et al., 2015; Li et al., 2017). Recent evidences highlighted that overexpression of PD-1 on melanoma and HCC tumor cells promotes tumor growth through phosphorylation of the effector proteins of the mammalian target of rapamycin (mTOR) signaling. These findings suggest that anti-PD-1 ICB could exert its anti-tumor activity by both restoring anti-tumor immunity and blocking the pro-tumorigenic drivers generated by cancer-intrinsic PD-1. These evidences could support the use of combination therapies with mTOR inhibitors and PD-1 ICB, previously unexplored in these settings (Li et al., 2017; Kleffel et al., 2015).

To increase the number of patients who would primarily benefit from ICB, different strategies have been studied, including combining immunotherapies with each other or pairing them with other types of cancer treatments such as chemotherapy or radiotherapy, to exploit their potential synergistic effect (Galluzzi et al., 2018). The best results were obtained when combining chemotherapies with anti PD-1/PD-L1 agents in the first line setting of metastatic NSCLC and metastatic TNBC or using the anti-PD-1 nivolumab *plus* the anti-CTLA-4 ipilimumab in previously untreated advanced melanoma patients, with no evident relationships between PD-L1 expression and clinical benefit in all of these studies (Gandhi et al., 2018; Socinski et al., 2018; Larkin et al., 2015; Schmid et al., 2018). However, as expected, these combinations of immunotherapeutic agents are more effective but unfortunately more toxic than either single agents are in both melanoma and NSCLC. Thus, a major challenge for these new strategies is how to maximize benefits and minimize toxicities, as well as identifying patterns of toxicities (Porcu et al., 2019; Solinas et al., 2018), assessing responses at imaging (Solinas et al., 2017c; Porcu et al., 2018), recognition of abscopal responses (Hlavata et al., 2018).

Combination of approved ICB together or with other anti-cancer treatments are being tested also in other tumor types, such as bladder and renal cancers, head and neck tumors, HCC, *etc*. Moreover, a broad range of new inhibitory and stimulatory checkpoint modulators are under rapid development as monotherapies or, most frequently, in combination with approved ICB, with the aim to improve anti-tumor immunity and rescue the activity of exhausted T cells (Popovic et al., 2018).

Furthermore pre-clinical data suggest that other strategies involving ICB with diverse treatments, such as cytokines, adoptive T cell therapy, oncolytic viruses, vaccines and small molecules are able to unleash optimal, robust and durable anti-tumor immune responses in immunologically cold tumors (*i.e.*, characterized by a low or absent spontaneous immune infiltration) (Marshall and Djamgoz, 2018). However, more efforts are needed for a rapid translation of these promising results in everyday clinical practice.

Strong evidences support the effectiveness and efficacy of ICB in oncology and a considerable inter-disciplinary effort involving clinical, translational and basic researchers needs to be done in order to extend these results to a wider number of patients, taking into account all the potential limitations, such as safety profiles, efficacy and patient selection.

In this scenario, understanding the biology and therapeutic role of PD-1 could represent an interesting challenge to use these research-based evaluations for elaborating a new generation of immunotherapeutic drugs, aiming to definitely cure and possibly prevent cancer.

Declaration of Competing Interest

Authors declare they have no conflicts of interest.

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