


REVIEW

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Biomarkers in immune checkpoint inhibition therapy for cancer patients: what is the role of lymphocyte subsets and PD1/PD-L1?

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Abstract

Although durable clinical responses are achieved in a significant number of patients given Immune checkpoint inhibitors (ICI), like anti-CTLA-4 and anti-PD-1 inhibitors, some of the cancers have shown little or no response to ICI therapy. Even within the known responsive cancers, there is often a subset of non-responsive patients. Due to the accelerated FDA approval of these immunotherapies, the biomarker development has not been able to keep pace. Appropriate predictive, prognostic and surrogate biomarkers are needed to maximally exploit the benefits from ICI therapy for correct and timely stratification of patients to treatment, for monitoring treatment effect, and for avoiding costs and unwanted toxicities when therapy is likely to be ineffective. As the number of clinical trials exploring the utility of these treatments, both as stand-alone and as combination therapy for several cancers is escalating dramatically, the need for appropriate biomarkers is further amplified.

This review discusses the potential biomarkers being investigated in ICI therapies, focusing mainly on immunohistochemical expression of PDL-1 and the immune correlates. Various immune components discussed here include the cells of innate (natural killer or NK cells) and adaptive (CD4+ and CD8+ cells) immunity, regulatory and inhibitory immune cells (regulatory T cells or Tregs and myeloid derived suppressor cells or MDSCs), as well as cytokines. Immune checkpoint molecule, programmed death receptor ligand-1 (PD-L1) and various molecules and pathways influencing its expression are also discussed.

Keywords: CTLA-4, PD-L1, PD-1, Immune checkpoint inhibitor therapies, Immunotherapies, Biomarkers, Neoantigens

Introduction

Recent advances in immunotherapy have revolutionized the treatment of cancer, adding a fourth pillar to the historic three pillars of surgery, chemotherapy, and radiotherapy. Approval of Interleukin-2 (IL2) for the treatment of metastatic renal cell carcinoma (RCC) in 1992, and for metastatic melanoma (MM) in 1998, by Food and Drug Administration (FDA), were important breakthroughs in immune therapy [1, 2], providing the

first 'proof-of-principle' that solely targeting the immune system could lead to durable control of metastatic cancer. The major milestones in the development of immunotherapies are summarized in Fig. 1.

Progress in cancer immunotherapy has been remarkable ever since, especially with the clinical development of Immune checkpoint inhibitors (ICI), like anti-CTLA-4 and anti-PD-1 antibodies inhibitors [3–5]. Durable clinical responses were achieved in a significant number of patients that were given immunotherapies. The cancers with the highest median mutational loads, like melanoma, non-small cell lung cancer (NSCLC), squamous cell cancer of head and neck (SCCHN), bladder cancer and gastric cancers, have demonstrated greater than 15% response rates to anti-PD-1 or anti-PD-L1 therapy [6–8].

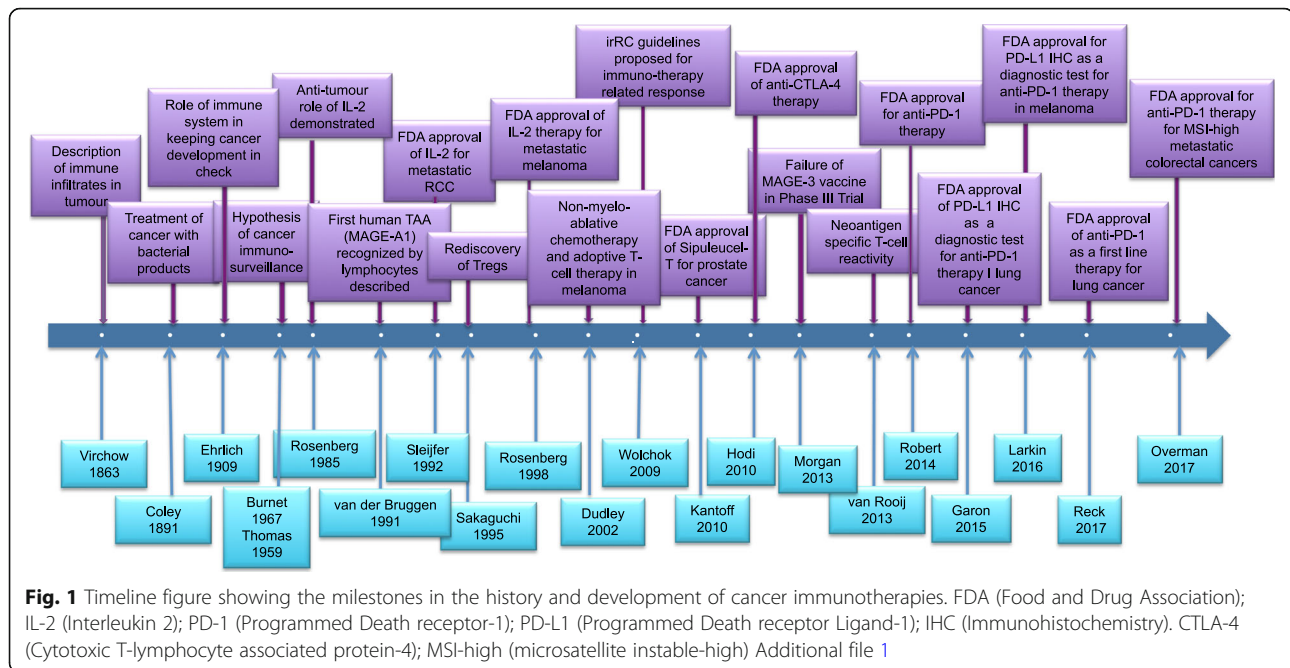
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Melanoma, a cancer with one of the highest mutational loads among human tumors, showed a particularly high response rate to anti-PD-1 therapy (30–40%) [9]. ICI therapy has also shown promising results in platinum refractory germ cell testicular cancer. [10]. With such promising results, immunotherapy drugs are positioned to become a backbone therapy for most tumor types. However, cancers with relatively low median mutational loads, such as pancreatic and prostate cancer, have shown little response to PD-1 pathway-blocking antibodies [7, 11]. In addition, immunotherapy is not without its toxicities, sometimes resulting in patient death [12].

For many years it was known that malignant cells could induce an immune response, which in some cases, destroyed the tumor; however the complexity of the immune system and a lack of technological solutions prevented in-depth study. There are a large number of possible immune responses, but T cells, especially CD8⁺ T cells, mediate the most critical part of the control of malignant cells. CD8⁺T cells are activated by antigen presented by Class I MHC molecules. Mutations in beta 2 microglobulin (B2M), one of the components of Class I MHC molecules have been associated with the response to immunotherapies [13]. CD8⁺cells are cytotoxic T cells, but continued stimulation by antigen renders exhaustion or induces expression of molecules similar to those expressed by CD4⁺CD25⁺ regulatory T cells or Treg cells, including cytotoxic T- lymphocyte associated protein-4 (CTLA-4), programmed death receptor-1 (PD-1), programmed death receptor ligand-1 (PD-L1), T-cell immuno-receptor with Immunoglobulin and ITIM domains (TIGIT), lymphocyte activation gene-3 (LAG-3), and T-cell immunoglobulin

and mucin domain-3 (TIM-3). The CD8⁺ effector T cells bind to the respective ligands expressed by tumor cells and inhibit the immune response, or causing T cell death, depending on downstream events. For instance, CTLA-4 also binds to and blocks CD80 and CD86, preventing further T cell activation through CD28, whereas TIM-3 binds to galectin, resulting in death of the cell expressing TIM-3. The best studied target, the PD-L1 ligand, and its receptor, PD-1, have lead to a rich new generation of therapeutic antibodies recently approved by the FDA, or are under clinical development. However, despite intense efforts, no universal biomarker predicting responses or toxicity has been developed so far. To date, the best-studied biomarkers include tumour mutational load, expression of PD-L1 on tumour cells, or PD-1 receptor on infiltrating lymphocytes.

Current biomarker search is directed at identifying which patients will respond to ICI therapy and which patients will develop side effects. In this review, we discuss immune checkpoint molecule (PD-L1), different molecules/pathways influencing its expression; components of the immune system including cytokines, the cells of innate (natural killer or NK cells) and adaptive immunity (CD4⁺ and CD8⁺ cells), regulatory immune cells (Tregs and myeloid derived suppressor cells or MDSCs). Table 1 summarizes the current status of these immune biomarkers in immune checkpoint inhibition therapy.

Immune checkpoint inhibitor therapies

T-cell recognition of tumor antigens drives the process of elimination of cancer cells via immunological mechanisms. Immune checkpoints minimize collateral tissue

Table 1 Summary of biomarkers currently under investigation for Immune Checkpoint Inhibition (ICI) therapies

Biomarker	Type	Pros	Cons	References
PD-L1	Predictive Therapeutic	<ul style="list-style-type: none"> • First FDA approved diagnostic for anti-PD1 therapy in NSCLC an melanoma • Direct target of anti-PD1/PD-L1 therapy 	<ul style="list-style-type: none"> • Does not correlate well in all the cancer types • Quite a few technical and biological variabilities from cancer to cancer and patient to patient • Not 100% correlation between its expression and anti-PD1 treatment response 	<p>Garon, et al. 2015 [19] Borghaei, et al. 2015 [21] Brahmer, et al. 2015 [20] Larkin, et al. 2015a [22] McDermott, et al. 2016 [31]</p>
Molecules influencing the expression of PD-L1	Predictive	<ul style="list-style-type: none"> • Very standard markers and therefore easy to access 	<ul style="list-style-type: none"> • Not too many • Indirect • Controversial reports on their correlation to ICI therapies 	<p>Parsa, et al. 2007 [34] Song, et al. 2013 [35] Hellmann 2015 [36] Larkin, et al. 2015c [38]</p>
Cytokines	Predictive Surrogate	<ul style="list-style-type: none"> • Gives an idea about the activation status of other immune correlates • Could be used in conjunction with immune cell data to give a complete picture of the immune system • Uses less invasive method since could be assessed directly in the blood 	<ul style="list-style-type: none"> • Different studies have reported changes in different types of cytokines • Larger studies are needed • Also need to check the tumors for the defects in cytokine signaling 	<p>Chang et al., 2013 [40] Selby et al., 2017 Yamazaki et al., 2017 [43] Zaretsky et al., 2016 [45] Gao et al., 2016 [46]</p>
NK cells	Predictive Surrogate	<ul style="list-style-type: none"> • Important as they offer the first line of defense • Involved in the production of important cytokines, brings about the activation/ maturation of immune cells 	<ul style="list-style-type: none"> • Controversial data from different studies on the changes in the number of NK subpopulations for anti-PD-1 treatment • Larger studies, and homogenization of the methods of detection are needed 	<p>Tietze et al., 2017 [63] Talerico et al., 2015 [64] Talerico et al., 2016 Liu et al., 2017 [66]</p>
CD8 + T cells	Predictive Surrogate Therapeutic	<ul style="list-style-type: none"> • High pre-treatment numbers of CD8 + T cells significantly correlate with better treatment outcomes for ICI therapies • Increased numbers are also predictive of irAE, allowing for close monitoring of the patient for early intervention • Tumor specific CD8 + T cells have a distinct profile which may allow for more accurate monitoring of treatment response • Uses less invasive method since could be assessed directly in the blood 		<p>Gros, et al. 2016 [74] Daud, et al. 2016 [72] Ngiow, et al. 2015 [73] Larkin, et al. 2015b [75]</p>
CD4 + T cells	Surrogate Therapeutic	<ul style="list-style-type: none"> • One of the very few markers for anti-CTLA-4 therapy. • CD4+ ICOS+ T-cells increases in a dose-dependent manner, highlighting their potential as a surrogate marker for pharmacodynamic monitoring of treatment response in anti-CTLA-4 therapy 	<ul style="list-style-type: none"> • It's role in combating cancer was recently unraveled and therefore it is relatively underexplored 	<p>Tran, et al. 2014 [77] Ng Tang, et al. 2013 [79]</p>
Regulatory T-cells T _{regs}	Predictive Surrogate Therapeutic	<ul style="list-style-type: none"> • High pre-treatment T_{regs} number in general is predictive of negative 	<ul style="list-style-type: none"> • A few controversial reports on the correlation between T_{reg} number and 	<p>Hodi, et al. 2008 [93] Lowther, et al. 2016 [92] Romano, et al. 2015 [95]</p>

Table 1 Summary of biomarkers currently under investigation for Immune Checkpoint Inhibition (ICI) therapies (*Continued*)

Biomarker	Type	Pros	Cons	References
		treatment outcome to ICI therapies • Being a direct target for anti-CTLA4 therapy, holds potential as a surrogate marker for monitoring treatment response in this specific type of ICI therapy	treatment outcome for ICI therapies • Many studies have not considered all the different subtypes of Tregs	Li, et al. 2016 [96]
Myeloid derived suppressor cells (MDSCs)	Predictive Surrogate Therapeutic	• High pretreatment MDSC numbers are predictive of negative ICI treatment outcome • Can be utilized as a marker for pharmacodynamic monitoring of treatment response • Targeting MDSCs restores sensitivity to ICI treatments, and therefore this approach is being considered for ICI combination therapies		Tarhini, et al. 2014 [100] Bjoern, et al. 2016 [101] Meyer, et al. 2014 [102] De Henau, et al. 2016 [103]

damage that may result from uncontrolled immune activation under normal circumstances. In cancer cells however, bolstering of these pathways becomes a major mechanism of immune resistance, resulting in blockade of T effector cell function specific for tumour antigens. As immune checkpoints are initiated by ligand–receptor interactions, aberrant stimulation can be blocked by antibodies or modulated by recombinant forms of ligands or receptors. These blocking molecules form the basis for ICI therapies [14, 15]. Figure 2a and b depict the activation of CTLA-4 and PD-1/PD-L1 axis. The most commonly targeted immune checkpoint molecules are anti-CTLA-4 and anti-PD1 (and anti-PD-L1) antibodies, which bind to their receptors, physically blocking specific ligand interactions, and releasing the T-cells from their immunosuppressive signals (Fig. 2c). Other checkpoint blockade targets include lymphocyte-activation gene 3 (LAG-3), Tumor Necrosis Factor (Ligand) Superfamily, Member 4 (NFRSF4) or OX40 and T-cell immunoglobulin and mucin protein 3 (TIM-3), which are all commonly co-expressed on exhausted PD-1+ T-cells. Targeting of these receptors has demonstrated encouraging results in mouse models, and is also being investigated in clinical trials (NCT02817633) [16–18].

Biomarkers for immune-checkpoint therapy

Although treatments have focussed on immune checkpoint targets to date, the search for biomarkers have considered a broader view of the cancer-immune system milieu, and many authors believe that it is the interplay between the tumor landscape of individuals and their immune response to it, that determines the response to

immune therapies. Here, in addition to discussing the only FDA approved test, immunohistochemistry (IHC) for PD-L1 expression, we discuss the molecules/pathways influencing the expression of PD-L1 entering into a detailed discussion of different components of the immune system that are being explored as potential predictive and/or surrogate biomarkers for ICI therapies.

Immune checkpoint molecules

Given that immune checkpoint molecules are the direct targets of ICI therapies, it is not surprising their evaluation was the first attempt at defining possible predictive biomarkers; this has been moderately successful, as immunohistochemical expression of PD-L1 was the first FDA approved biomarker for the treatment response to anti-PD-1 therapies. However, the methodologies and appropriate cut-off values of PD-L1 expression are still a matter of debate.

Programmed death receptor ligand – 1 (PD-L1) expression

In October 2015, the FDA approved a PD-L1 immunohistochemistry (IHC) test (PD-L1 IHC 22C3 pharmDx) as a companion diagnostic for pembrolizumab in treating advanced NSCLC [19]. At that time, the PD-L1 IHC 28–8 pharmDx assay was also approved as a complementary, but not required, diagnostic test for nivolumab in lung cancer [20, 21]. The PD-L1 IHC 28–8 pharmDx assay was subsequently also approved in January 2016 as a complementary test for nivolumab treatment in melanoma [22]. These approvals were based on a number of clinical studies, including a nivolumab study where patients with PD-L1 positive tumors (defined as > 5% cells

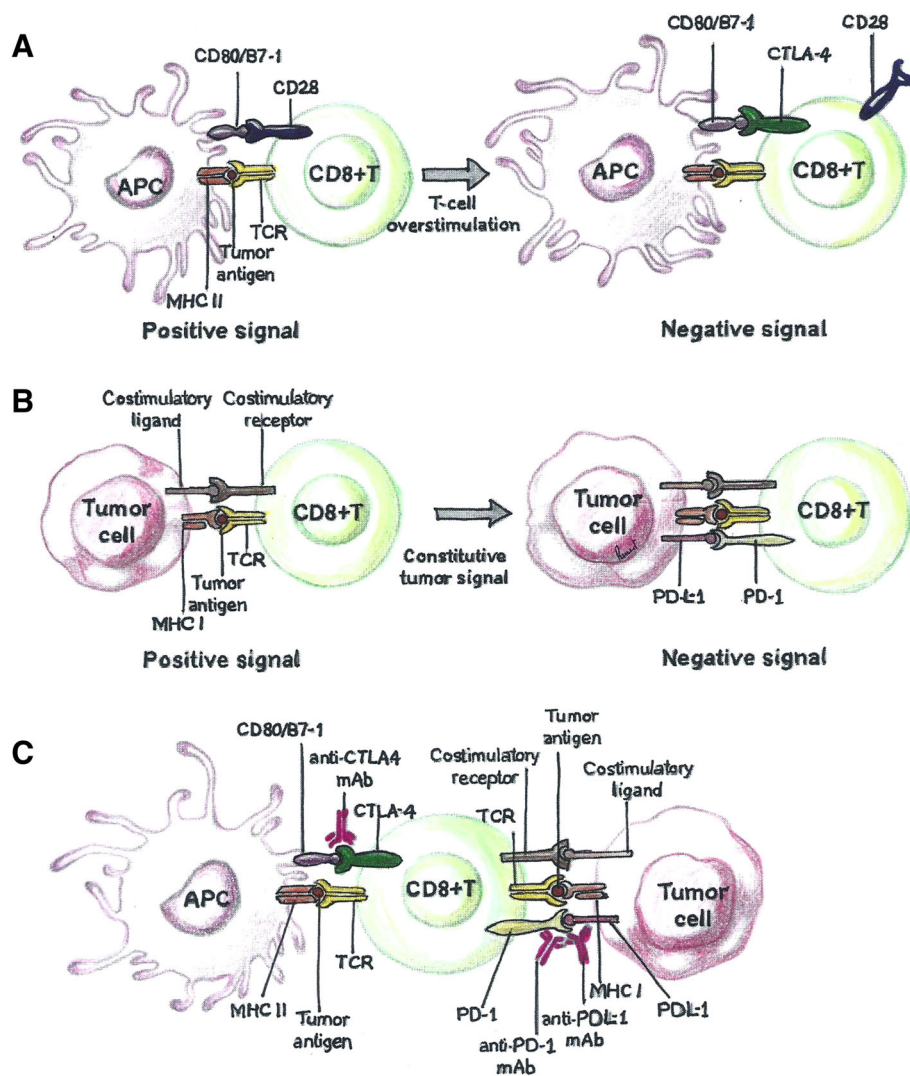


Fig. 2 Immune checkpoint inhibition pathways in tumors **(a)** Activation of CTLA-4 pathway: Naive and memory T cells express high levels of cell surface CD28 but do not express CTLA-4 on their surface. Instead, CTLA-4 is sequestered in intracellular vesicles. CTLA-4 is a CD28 homologue with higher affinity for the ligands CD80 (B7-1) or CD86 (B7-2) that it shares with CD28. After the T-cell receptor (TCR) is triggered by an antigen encounter, CTLA-4 is transported to the cell surface. The stronger the stimulation through the TCR and CD28, the greater the amount of CTLA-4 that is expressed on the T-cell surface, resulting in a net negative signal transduced by the CTLA-4: CD80/86 interaction. This mechanism results in CD8⁺T-cell inhibition, despite the presence of widely varying concentrations and affinities of ligand for the TCR. The negative T-cell inhibition or immunosuppressive signal is characterized by decrease in cytokine secretion, CD8⁺T-cell inactivation and loss of its cytotoxic function **(b)** Activation of PD-1/PD L-1 pathway: PD-1, a member of the B7/CD28 family of co-stimulatory receptors, regulates T-cell activation through binding to its ligands, programmed death ligand 1 (PDL-1) and programmed death ligand 2 (PDL-2). During inflammatory responses in tissues or in the setting of chronic antigen exposure (including tumor antigens), activated T-cells up-regulate PD-1 and continue to express it in tissues. In case of tumors, constitutive tumor signal (tumor antigen and PDL-1 expression) results in overstimulation of CD8⁺T cells and inhibits them by inducing a state of energy or exhaustion. **c** During Immune Checkpoint Inhibition therapy, anti CTLA-4, anti-PD-L1 or anti-PD-1 antibodies bind to their corresponding target molecules preventing the receptor-ligand interactions of these immune checkpoint inhibitors. This releases CD8⁺T-cells from immunosuppression imposed on them either by tumor cells via PD-1/PDL-1 pathway or by CTLA-4 pathway, which then resume their anti-oncogenic role

expressing PD-L1), were found twice as likely to respond to treatment as compared to the overall study population [6, 7], as well as other studies involving non-small cell lung cancer (NSCLC), melanoma, squamous cell carcinoma of head and neck (SCCHN), renal cell cancer (RCC) and bladder cancer [19–21, 23, 24]. These

studies used different PD-L1 IHC assays and different proposed cut-off values for PD-L1 expression, but in general, conclusions suggested that baseline PD-L1 expression in tumor specimens predicted a greater likelihood of response to anti-PD-1 and anti-PD-L1 therapies.

Nevertheless, it has become apparent that correct assessment of PD-L1 protein expression is impeded by both biological and technical shortcomings. There is wide variability in the expression of PD-L1 across different tumor types, and also within the same tumor type [8, 25–27]. In a study on a collection of commercially available archival formalin fixed paraffin embedded (FFPE) samples including 30 tumors with multiple samples per case, and 20 pairs of matched primary versus metastatic tissues, discordant results were noted in 6% of the multi-sampled cases and of 30% of the matched primary versus metastatic cases. [28]. Further, proprietary IHC tests currently in use or in development use different monoclonal antibodies (mAbs) for PD-L1 detection, and these have been developed in isolation without cross-comparisons. Not all anti-PD-L1 mAbs produce similar staining results as different clones target different epitopes in the PD-L1 protein. The IHC technique itself may have many variables, including antigen retrieval conditions and temperatures, mAb concentrations and incubation times, and detection systems. The conditions under which the specimens are collected and the time of collection with respect to the initiation of treatment might also affect conclusions. Finally, the method of tissue collection also matters; surgical resection compared to needle biopsy could result in a false-negative PD-L1 evaluation [29]. No doubt, PD-L1 IHC as a diagnostic test will continue to evolve as more information regarding clinical correlations from randomized trials becomes available.

With regard to clinical significance, the correlation between PD-L1 expression and long-term outcomes from anti-PD-1 therapy in terms of PFS and OS is yet to be firmly established. Presumably, tumor heterogeneity, as well as differences in population sampling explains why some trials in RCC find a positive correlation of intratumoral PD-L1 expression with response rates and PFS and OS, whereas others do not [30, 31]. Collectively these observations indicate that tumor PD-L1 IHC expression cannot be the sole determinant by which responsiveness to PD-1/PD-L1 targeting is assessed. To overcome some of these issues, PD-L1 expression on circulating tumor cells (CTCs) with respect to ICI therapy is also being evaluated [32, 33]. Validation studies of this approach can lead to a non-invasive, liquid biopsy method of accessing PD-L1 expression to monitor disease.

Molecules/pathways influencing the expression of PD-L1

Increased PD-L1 expression by tumor cells in the tumour microenvironment (TME) is generally associated with a better response to anti-PD-1 therapy. In glioblastoma, constitutive oncogenic signalling in tumor cells drives PD-L1 expression. Deletion or silencing of *PTEN*, resulted in very high expression of PD-L1, implicating the role of PI3K–AKT pathway in PD-L1 upregulation,

resulting in immunoresistance in gliomas [34]. *PTEN* loss has also been associated with upregulation of PD-L1 in colorectal cancers [35]. In lung cancer, the anti-PD-1 response was lower in EGFR - mutant adenocarcinomas and this may be due to increased AKT signalling facilitated by EGFR [36]. Notably, mutant EGFR NSCLC is associated with never-smoker status, which correlates with a considerably lower response rate to anti-PD-1, likely due to lower mutation rates, than smoking-associated lung cancer. The presence of the common oncogenic BRAF-V600E mutation in melanoma did not correlate with PD-L1 expression [37]. In keeping with this, there was no difference in response to anti-PD1 therapy in melanoma in BRAF-V600E mutated versus BRAF wild-type tumors [38].

Components of immune system

ICI acts by manipulating the immune system, therefore, both cytokines as well as the immune cells hold the potential to be the ultimate predictive as well as surrogate biomarkers for these treatments.

Cytokines

Cytokines are small non-structural proteins that include interferons, interleukins, the chemokine family, mesenchymal growth factors, the tumor necrosis factor family and adipokines [39]. They are involved in the activation, differentiation, proliferation and chemotaxis of immune cells in immune-related inflammatory response and could be proinflammatory or anti-inflammatory. Their expression profile changes in the presence of tumors. Peripheral blood mononuclear cells (PBMCs) from stage I lung cancer patients were reported to possess distinct cytokine expression patterns compared to both non-cancer patients, and lung cancer patients following tumor removal [40]. Plasma from these patients also showed five altered cytokines (CCL3, IL8, IL1 β , CXCL10, sIL2R α). qPCR of PBMCs demonstrated a higher expression of CCL3, IL8 and IL1 β in lung cancer patients compared to the same patients at each of four sequential timepoints after removal of their tumors, whereas CXCL10 and IL2R α levels were unchanged. In the same study, similar changes in cytokine and gene expression were observed in in-vitro co-culture systems, when PBMCs from healthy donors were exposed to lung cancer cell line. Certain cytokines, especially interferon (IFN), can be produced by, and act on, both tumor cells and immune cells [41].

The role of cytokines as a biomarker in checkpoint inhibitor therapies is being explored. In vitro studies with combined ipilimumab and nivolumab showed enhanced cytokine secretion in superantigen stimulation of human peripheral blood lymphocytes and in mixed lymphocyte response assays [42]. A single arm, Phase II study (JAPIC-CTI #11681) evaluating the antitumor activities of nivolumab in advanced melanoma patients conducted

in Japan found that the pre-treatment serum levels of interferon- γ , and interleukin-6 and -10 were significantly higher in the patients with objective tumor responses than in those with tumor progression [43]. In a study presented at the European Lung Cancer Conference (ELCC) 2016, the authors reported the results from two of the Clinical trials, CheckMate 063 = NCT01721759; CheckMate 017 = NCT01642004 [44]. In CheckMate 063, patients ($N = 117$) received nivolumab 3 mg/kg Q2W until progressive disease (PD)/unacceptable toxicity. Checkmate 017 had two arms and the patients were randomized 1:1 to receive nivolumab 3 mg/kg Q2W ($n = 135$) or docetaxel (doc) 75 mg/m² Q3W ($n = 137$) until PD or discontinuation due to toxicity/other reasons. They reported that a select group of serum cytokines at baseline may be associated with OS benefit in patients with advanced metastatic squamous NSCLC and suggested that prospective validation of these preliminary findings was needed. Acquired resistance to PD-1 blockade immunotherapy in patients with melanoma was associated with defects in the pathways involved in interferon-receptor signalling in addition to antigen presentation [45]. Similarly, Gao et al. found that the melanoma tumors that were resistant to ipilimumab therapy had defects in IFN- γ pathway genes [46]. Further studies in this direction are warranted.

Immune cells

Cells that potentially predict treatment response include innate immune cells (NK cells), adaptive immune cells (CD4⁺ and CD8⁺) and inhibitory or regulatory immune cells (MDSCs or Tregs). In addition to their role as predictive and surrogate biomarkers in ICI treatment, immune cells were recently implicated in the prediction of immune response adverse events (irAEs) [22]. Changes in total lymphocyte counts, ratios of different cell populations as well as changes in various subpopulations have demonstrated a correlation with ICI treatment response. In addition, monitoring of different T-cell subpopulations like CD4⁺T, CD8⁺T cells, NK cells and Tregs, and also analysing the percentage proliferation in each subpopulation using the Ki67 marker, have been proposed as a potential surrogate markers to assess the treatment response for ICI therapies [47–51]. These are considered in more detail below.

Relative counts of immune cell subpopulations

Lymphocytic profiles have been explored as biomarkers in ICI therapies. On treatment with ipilimumab, metastatic melanoma (MM) patients with absolute lymphocyte counts (ALCs) more than 1000 lymphocytes per cubic millimetres of blood, particularly after the first or second course of ipilimumab (7 weeks), showed significantly improved survival [52]. The baseline expression of

immune-related tumor biomarkers and a post-treatment increase (at 3 weeks) in TILs was seen to be significantly associated with ipilimumab clinical activity [53]. In another similar study, in MM patients treated with ipilimumab, patients with baseline neutrophil to lymphocyte ratio (NLR) of ≥ 5 were associated with significantly increased PFS and OS, compared to those with an NLR < 5 [54, 55]. Another study described the cut-off ratio as ≥ 4 (Zaragoza, et al. 2016). Correlation between absolute eosinophil count (AEC) and response to checkpoint blockade was also demonstrated [56]. An association between absolute lymphocyte count (ALC) and OS in patients with MM treated with ipilimumab was also seen [56]. However, ALC did not significantly correlate with response to either PD-1 inhibitor monotherapy or combination therapy with nivolumab and ipilimumab [57, 58], even though it has been hypothesized that anti-CTLA-4 therapy drives T cell infiltration into tumors, creating a more optimal environment for anti-PD-1 therapy to work with [59, 60].

Natural killer (NK) cells

NK cells are involved in the innate immunity and are the first line of defense of immune system, guarding against the tumors and fighting the infections. Natural killer T-cells (NKT cells) are CD3⁺, whereas CD56^{dim} NK, CD56^{bright} NK are both CD3⁻. Majority of NK cells in the peripheral blood are CD56^{dim} and are relatively more mature and have higher cytotoxic activity than CD56^{bright} cells, which are mainly involved in cytokine secretion [61, 62]. Low baseline levels of NK cells and CD56^{dim} NK cells as well as normal levels of CD56^{bright} NK cells correlated significantly with a positive response to ipilimumab [63]. High levels of CD56^{bright} NK cells predicted a negative outcome. NK cells and CD56^{dim} and CD56^{bright} NK cell subpopulations in patients with a normal baseline did not change with treatment. NK cells and CD56^{dim} NK cells showed a significant increase with treatment, whereas CD56^{bright} NK cells were unaffected by treatment. The baseline levels of NK cells also correlated with the number of metastatic organs. In the same study, no such correlation was found in patients treated with pembrolizumab. One previous study also reported the activation and proliferation of CD56^{dim} NK cells in response to ipilimumab treatment [64]. Here authors also observed an increase in cytotoxic CD8⁺ T cells, and proposed that ipilimumab treatment induces NK cell maturation, which might in turn drive activation of CD8⁺ T cells. In melanoma patients being treated with anti-CTLA4 therapy, the survival correlated with low expression of the inhibitory receptor TIM-3 on circulating T and NK cells at baseline and through the treatment, and with increased frequency of mature circulating CD56^{dim} NK cells during treatment [65]. Survival also correlated with low levels of IL-15 in the serum. Authors

also unravelled the contribution of IL-15 in the expression of PD-1 and TIM-3 on both T-cells and NK cells in *in vitro* experiments. The increased PD-1 expression on NK cells correlated with poorer survival in oesophageal and liver cancers [66]. *In vitro* experiments demonstrated that anti-PD-1 antibody markedly enhanced cytokine production and degranulation in NK cells and suppressed apoptosis in them. In addition, anti-PD1 treatment of mouse xenografts showed a significant suppression of growth, which was completely abrogated by NK depletion. NK cells, in addition to being exploited in adoptive NK cell therapy [67], are also emerging as potential biomarkers in ICI therapies and therefore, need to be explored further.

CD4+ and CD8+ T-cells

CD4+ and CD8+ T cells are cells of adaptive immunity. Similar to chronic infections, T cells in the tumor micro-environment (TME) also exhibit an exhausted phenotype and function. Exhausted T cells in cancer express high levels of inhibitory receptors, including PD-1, CTLA-4, TIM-3, LAG-3, BTLA and TIGIT, as well as showing impaired effector cytokine production, such as IL-2, TNF- α , IFN- γ and granzyme B [68]. ICI therapies with anti-CTLA-4 and/or anti-PD-1 monoclonal antibodies can reinvigorate tumor-infiltrating T lymphocytes resulting in clinical benefits to a large number of patients with advanced cancer. In ICI therapy, CD8+ Cytotoxic T lymphocytes (CTLs) are believed to drive tumor shrinkage, as they can recognize and target cancer cells that present either tumor-specific antigens such as cancer testis antigens or somatic neoantigens [69].

Neoantigen specific T-cells were found to be present in cancers and were amplified as a result of checkpoint blockade therapy [70]. For melanoma, CD8+ T cell density at the invasive tumor edge was correlated with response to anti-PD-1 treatment [71]. Tumeh et al. created a predictive model for the patients treated with pembrolizumab for advanced melanoma. Responding patients had higher pre-treatment numbers of CD8+, PD-1, and PD-L1 expressing cells, both at the tumor margins and in the inside of the tumors. The pre-existing density of CD8+T cells was more closely correlated with response to anti-PD-1 therapy than was PD-L1 expression [71].

In a study on freshly isolated metastatic melanoma samples from 2 cohorts of 20 patients each, the data suggested that the relative abundance of partially exhausted tumor-infiltrating CD8+T cells predicts response to anti-PD-1 therapy [72]. Even the expression of PD-1 on CD8+T cells dictates the response to anti-PD-1. It was shown that PD-1 expression level on CD8+T cells must be below a certain threshold level to make the tumors sensitive to anti-PD-1 therapy. The same study, using mouse xenograft models, demonstrated that Tregs depletion made

tumors more sensitive to anti-PD1 therapy tumors [73]. Another study demonstrated that PD-1 expressing CD8+T cells are neoantigen specific and could be utilized in isolating and expanding this specifically reactive T-cell population [74]. They also demonstrated that the tumor antigen-specificity of PD-1 expressing T cells in the blood cell compartment very much resembles that of tumor-resident PD-1-expressing T cells. The PD-1 expressing population of circulating T lymphocytes may provide a valuable window into the tumor-resident antitumor lymphocytes (TILs) by a non-invasive, simplified method. CD8+ T-cell clonal expansion could be used as a biomarker that can be used to monitor severe irAEs for early intervention.

Immune checkpoint inhibitors in combination, significantly increase the rate of therapeutic benefit, however, as described previously, the proportion of patients experiencing grade 3–4 irAEs virtually doubles when ipilimumab is combined with nivolumab [75]. In one study the authors found that CD8+T -cell clonal expansion was significantly greater in patients developing grade 2–3 irAEs vs. grade 0–1 irAEs, and occurred even before the development of the grade 2–3 immune-related toxicities, the median time between the blood draw and the onset of toxicity being 13 days [76]. A few recent studies have detected the presence of neoantigen specific CD4+T amplification as well [77]. ICOS (inducible co-stimulator) is expressed on the cell surface of activated T cells and plays a role in T cell expansion and survival. As reviewed by Yuan et al., ICOS expression on CD4+ T cells was shown to increase in a dose-dependent manner in patients with bladder cancer, breast cancer and mesothelioma after treatment with either ipilimumab or tremelimumab [49]. A sustained increase in CD4+ICOS+ T cells was observed over 12 weeks after CTLA-4 blockade therapy and correlated with improved survival in four independent studies [78]. Therefore, CD4+ ICOS+ T cells may have a potential to be reproducible pharmacodynamic biomarker to indicate biological activity for CTLA-4 blockade therapy [79].

Regulatory T-cells (Tregs)

Under normal physiology, Tregs constrain immune responses and keep other types of T cells from mounting hyper-aggressive responses. They are recruited to the tumor sites by way of chemokines produced by tumor cells. The induction of forkhead box P3 (FoxP3) expression and conversion of CD4+ CD25⁻ T cells into Tregs is attributed to the factors produced by TME like hypoxic conditions, STAT3 and HIF-1 α [80–83].

Myeloid derived suppressor cells (MDSCs) may also have a role in the induction and/or expansion of Tregs [84]. Tregs contribute to immunosuppression by inhibiting both CD4+ and CD8+T cells, NK (natural killer) cells and dendritic cells (DC), using a variety of molecules

and pathways. Tregs were able to kill CD8⁺T cells through granzyme B and perforin, could impair CD4⁺T cell responses by pathways involving COX and PGE2, suppressed NK cells via TGF- β and DC by way of IL-10 and TGF- β [85–88]. In most studies, Treg cells were associated with immunosuppression and worst prognosis. However, some studies showed Tregs to be associated with better prognosis in some cancer types, which seems to be counter intuitive considering the suppressive role of Tregs. Infiltration of CD8 and FOXP3⁺ Tregs were independently associated with a better outcome in the breast cancer patients treated with neoadjuvant chemotherapy [89]. Improved survival was associated with high-density infiltration of FOXP3⁺ Tregs in colorectal cancers [90]. One explanation for this positive correlation, suggested by the authors who found that the presence of Tregs in stroma in gastric cancers were associated with improved outcome, was that Tregs most likely inhibit local inflammatory processes that would normally promote tumor carcinogenesis [91]. In addition, the changes in Treg phenotype might also have a bearing on its relationship to the prognosis of the disease. For example, one of the studies showed that dysfunctional, exhausted Tregs that highly express PD-1 and secrete IFN- γ might be a positive indicator of benefit following anti-PD-1 therapies [92].

The decreased numbers of Treg cells in the tumor tissue relative to CD8⁺ T effector cells, following Ipilimumab treatment have been strongly correlated with clinical benefit from early on [93, 94]. Patients with glioblastoma multiforme when treated with a PD-1 blocking antibody had a higher proportion of dysfunctional Tregs cells [92]. Melanoma patients treated with ipilimumab demonstrated antibody-dependent cell-mediated cytotoxicity and (ADCC)-mediated Tregs depletion. The prior presence of innate immune cells (macrophages) to mediate ADCC was important for ICI activity [95]. Further, PD-L1 and (forkhead box P3) FOXP3⁺ Tregs may work synergistically or participate in the same molecular pathway and their up-regulated expression may promote immune evasion in breast cancer [96]. These findings provide a theoretical basis for the development of immunotherapies targeting PD-L1 and FOXP3⁺ Tregs simultaneously in the treatment of breast cancer, and perhaps other cancers with similar TME.

Myeloid derived suppressor cells (MDSCs)

Similar to Tregs, tumor resident MDSCs play a suppressive role in tumor biology. Hatzioannou et al. reviewed the cytokines and molecules implicated in the proliferation of MDSCs and their suppressive characteristics, including IL-6, IL-1 β , TNF- α , IL-13/4, and IFN- γ , STAT3, NF- κ B and HIF-1 [97]. One of the most established mechanisms of MDSC suppression is that these molecules secrete the enzyme ARG-1 in the TME, causing

uptake of L-arginine by T-cells and subsequent reduction of the production of CD3- ζ chain, in turn resulting in the inhibition of T-cell proliferation [98]. Inhibition of the NK cell response is also mediated by MDSC through the production of TGF- β , thereby blocking IFN- γ production and inhibiting their cytotoxicity. MDSCs produce IL-10, which is one of the components of Type 2 immune response. Type 2 immune response favors tumor growth by promoting angiogenesis as well as by inhibiting cell-mediated immunity and subsequent tumor cell killing [99]. Therefore, through the production of IL-10, MDSCs also skew the phenotype of macrophages toward Type 2 immune responses that favors tumor growth [97]. They may also have a role in the induction and/or expansion of inhibitory Tregs [84].

Many pre-clinical and clinical studies have demonstrated the immunosuppressive role of MDSCs in various different types of cancers. The presence of MDSCs is therefore likely to modulate the effect of ICI therapies. Decrease in circulating MDSCs (myeloid derived suppressor cells) in patients being treated with ipilimumab was associated with improved PFS [100]. A high baseline frequency of MDSCs and high levels of IL-6 was associated with a reduced response to therapy [101]. Similar observations from a study by Meyer et al. suggest that the frequency of monocytic MDSCs may be used as predictive marker of response, as low frequencies identify patients more likely to benefit from ipilimumab treatment [102]. Targeting myeloid cells could possibly overcome resistance to ICI in patients with high levels of suppressive MDSC infiltration in tumours. Efforts have already begun in that direction with selective pharmacologic targeting of the gamma isoform of phosphoinositide 3-kinase (PI3K γ), highly expressed in myeloid cells, that results in restored sensitivity to ICI [103]. This inhibitor is currently being evaluated in a phase 1 clinical trial (NCT02637531).

Discussion

In 2013, immunotherapy was designated as the 'Breakthrough of the Year' by the Science Magazine [104]. Despite the effectiveness of this treatment in multiple cancer types in the advanced setting, only a relatively small subset of patients responds. Apart from immune-mediated treatment resistance to immune therapies, other mechanisms can either potentially enhance or contribute to treatment resistance. For instance, chemotherapeutic agents such as anthracyclines and oxaliplatin may eventually lead to secondary expansion of immunosuppressant cells in the host, exhaustion of immune effectors and the emergence of chemo-resistant tumor clones [105]. Timing of immune therapies may also play a role in improving outcomes. Earlier treatment in the disease course is an obvious possibility; the incidence of distant metastases after surgical

intervention may be reduced [106]. Combining immunotherapies with first line therapies may also result in better treatment outcomes [107]. For example, radiotherapy is believed to increase the efficacy of ICI by triggering the release of TAAs and /or damage-associated molecular pattern molecules (DAMPs), both of which may activate the innate and adaptive immune system, and enhance tumour-cell immunogenicity [105, 108, 109]. On the other hand, radiotherapy tends to induce clonal diversity in tumors through the induction of further mutations, which may lead to eventual resistance to immune therapies.

Conclusion

Prognostic and surrogate biomarkers are actively being investigated to maximally utilize the potential of ICI therapies. Given the modest success of potential biomarkers to date, it is unlikely that a single biomarker will suffice, but a better understanding of the interplay of multiple factors in an individual may lead to predictive profiles. Thus the genetic and immune profile of the cancer, or the ‘cancer-immune setpoint’, of an individual may help to predict treatment response for immunotherapies [110]. As discussed, PD-L1 expression by tumors, although FDA approved, is modestly effective, at best, for prediction of treatment response. The field is still evolving, however, and at present, it appears that a combination of absolute pre-treatment lymphocyte counts, ratios of different immune cell populations (e.g. T_{eff}/T_{reg}), or proportions of NK cells, CD4⁺T and CD8⁺T cells, Tregs, and MDSCs are the leading candidates. The products of these active immune cells may also be helpful in predicting treatment response, such as cytokine expression profiles [40]. These studies highlight the cross talk between the highly individualized tumor-immune landscapes of patients. Therefore, it makes sense that a multi-factorial, highly individualized biomarker profile for the most efficient delivery of ICI treatment is required.

Future directions

Based on the evidence from the studies discussed here and several other similar studies, it is clear that immunotherapy needs to be considered as a first or early line therapy, before the chemo- or radio-therapeutic agents would induce further mutations resulting in tumor heterogeneity, clonal diversity and in turn the resistant tumor clones. To make this possible, it is imperative that the ICI treatment responsive patients be identified and distinguished from non-responsive patients. For appropriate delivery of immunotherapies, use of these multiple biomarkers discussed here, combined with genetic profiling, would be very practical approach. This multifactorial biomarker approach may help screen as well as monitor the patients; better inform the design and help adjust the treatment regimes; save the crucial

time and exorbitant treatment costs by distinguishing responders from the non-responders in a timely manner; and facilitate the extension of ICI treatment to non-responders within the commonly treated immunogenic cancers as well as to other poorly immunogenic cancers currently non-responsive to ICI Additional file 1.

Additional file

Additional file 1: Refers to References for Figure 1 (in chronological order). (DOCX 130 kb)

Abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity; AEC: absolute eosinophil count; ALCS: absolute lymphocyte count; ARG-1: arginase-1; B2M: beta 2 microglobulin; BRAF: B-Raf proto-oncogene; BRAF-V600E: B-Raf proto-oncogene, valine to glutamic acid mutation; BTLA: B and T lymphocyte associated; CCL3: C-C motif chemokine ligand 3; CD28: cluster of differentiation-28 positive cells; CD3- ζ : CD3 antigen, epsilon polypeptide; CD4⁺ cells: cluster of differentiation-4 positive cells; CD4⁺CD25⁺: cluster of differentiation-4 positive cells and cluster of differentiation-25 positive cells; CD56^{bright} NK: cluster of differentiation-56 bright, natural killer; CD56^{dim} NK: cluster of differentiation-56 dim, natural killer; CD8⁺ cells: cluster of differentiation-8 positive cells; CD80: cluster of differentiation-80 dim, natural killer; CD86: cluster of differentiation-86 dim, natural killer; COX: cyclooxygenase; CTCs: circulating tumor cells; CTLA-4: cytotoxic T-lymphocyte associated protein-4; CTLs: cytotoxic T-lymphocytes; CXCL10: C-X-C motif chemokine ligand 10; DAMPs: damage-associated molecular pattern molecules; DC: dendritic cells; EGFR: epidermal growth factor receptor; ELCC: European Lung Cancer Conference; FDA: Food and Drug Administration; FFPE: formalin fixed paraffin embedded; FoxP3: forkhead box P3; HIF-1: hypoxia inducible factor- 1; HIF-1 α : hypoxia inducible factor- 1 α ; ICI: immune checkpoint therapy; ICOS: inducible co-stimulator; IFN: interferon; IFN- γ : interferon gamma; IHC: immuno histochemistry; IL-10: interleukin 10; IL-13/4: interleukin 13/4; IL-15: interleukin 15; IL1 β : interleukin 1 beta; IL2: interleukin 2; IL2R α : interleukin 6 R alpha; IL-6: interleukin 6; IL8: interleukin 8; irAEs: immune related adverse events; ITIM: immunoreceptor tyrosine-based inhibitory motif; JAPIC-CTI: Japan Pharmaceutical Information Centre- Clinical Trials Information; Ki67: name derived from the city of origin, ‘Kiel’, Germany; LAG-3: lymphocyte activation gene-3; mAbs: mono-clonal antibodies; MDSCs: Myeloid derived suppressor cells; MHC: major histocompatibility complex; MM: metastatic melanoma; NF- κ B: nuclear factor – kappa B; NFRSF4: Tumor Necrosis Factor (Ligand) Superfamily, Member 4; NK cells: natural killer cells; NLR: neutrophil to leucocyte ratio; NSCLC: non-small cell lung cancer; OS: overall survival; OX40: TNF receptor superfamily member 4; PBMCs: peripheral blood mononuclear cells; PD: progressive disease; PD-1: programmed death receptor- 1; PD-L1: programmed death receptor ligand-1; PFS: progression free survival; PGE2: prostaglandin E synthase 2; PI3K-AKT: phosphoinositide-3-kinase- serine –threonine kinase; PI3K γ : phosphoinositide-3-kinase gamma; *PTEV*: phosphatase and tensin homologue; qPCR: quantitative polymerase chain reaction; RCC: renal cell carcinoma; SCCHN: squamous cell cancer of head and neck; sIL2R α : soluble interleukin 2 receptor alpha; STAT3: signal transducer and activator of transcription; T cells: thymus lymphocyte cells; T_{eff}/T_{reg}: effector T cells / regulatory T cells; TGF- β : transforming growth factor- beta; TIGIT: T-cell immuno-receptor with Immunoglobulin and ITIM domains; TILs: tumor infiltrating lymphocytes; TIM-3: T-cell immunoglobulin and mucin domain-3; TME: tumor microenvironment; TNF- α : tumor necrosis factor - alpha; Tregs: regulatory T cells

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