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Evolving Dynamic Biomarkers for Prediction of Immune Responses to Checkpoint Inhibitors in Cancer

Afsheen Raza, Maysaloun Merhi, Allan Relecom, Queenie Fernandes, Varghese Inchakalody, Abdul Rahman Zar Gul, Shahab Uddin, Mohammed Ussama Al Homsy and Said Dermime

Abstract

Immune checkpoint inhibitors (ICIs) have been approved as first or second line therapy in a large group of cancers. However, the observation of potentially long-lasting responses was restricted to limited subset of patients. Efforts have been made to identify predictive factors of response to ICIs in order to select eligible patients and to avoid exposing non-responding patients to treatment side effects. Although several biomarkers have been identified, their predictive potential remains unsatisfactory. One promising emerging approach is to focus on dynamic biomarkers to directly characterize the response and, more importantly, to identify those patients presenting an immune response failure. Several studies have shown a strong correlation between specific circulating immune cell subsets and tumor immune infiltrates. Moreover, liquid biomarkers including soluble immune checkpoint molecules have potential in predicting the modulation of the immune response under immune checkpoint blockade. In this chapter, we will discuss current advances in the study of circulatory and intra-tumoral dynamic biomarkers as predictors of responses to ICIs therapy in cancer.

Keywords: dynamic biomarkers, serum soluble biomarkers, cellular immune response, immune checkpoint inhibitors, CTLA-4, PD-1, tumor control

1. Introduction

The immune checkpoint cell surface proteins like programmed death-ligand 1 (PD-L1), programmed death 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) represent important pathways of cancer immune evasion. Immune checkpoint inhibitors (ICIs) are identified as potent key players in providing therapeutic benefit in a range of solid cancers as well as in a subgroup of hematological malignancies. However, the response rates to these immune-modulatory molecules are sub-optimal and predictive biomarkers allowing to select for responsive cancer patients are lacking. The study of the dynamics of the immune system and of the tumors under immune checkpoint blockade has greatly improved knowledge on the

mechanisms of action of ICIs, allowing the identification of a number of novel candidate dynamic biomarkers predictive of ICI treatment response meriting further exploration in validation trials.

Tumor biopsy of tissue from primary or metastatic site is a major mainstay of treatment decisions as the molecular features and histology can reveal the complex cancer landscape. However, tissue biopsy has various limitations such as high heterogeneity, invasive nature of tissue sampling and skilled expertise/techniques required for analyzing and reporting making it a difficult specimen especially for treatment monitoring purpose [1]. Therefore, emphasis on utility of liquid biopsies as prognostic and predictive soluble biomarkers especially in cancer immunotherapy is gaining a lot of attention. The main advantages of blood-based specimens are that these are easy to extract and analyze with limited skilled expertise or techniques. Furthermore, biomarkers in the blood can represent dynamic alterations of the evolving cancer in response to treatment and can help in longitudinal monitoring. In addition to this, these can also be utilized for risk prediction of immune-related adverse events (irAE) which is an important and critical monitoring parameter [2].

In this chapter, we attempt to discuss in relevant details the purpose and role of immune modulatory molecules and of the different serum soluble biomarkers in various human and animal models with an aim to show insight on to their mechanisms of action and resistance, thus conveying information predictive of therapeutic response.

2. Effect of immune checkpoint blockade on effector T cells

2.1 Effect of PD-1 blockade on effector T cells

PD-1 is an important immune checkpoint expressed on activated T cells and known to regulate their functional activity. By binding to its ligands, PD-L1 or PD-L2, which are expressed on tumor cells and a variety of immune cells [3], PD-1 is able to inhibit downstream signaling of the T cells receptor (TCR) [4]. Hence, ICIs targeting the PD-1/PD-L1 axis are developed to modulate this negative feedback loop and restore T cells activity. Indeed, response to anti PD-1 drugs is characterized by the upregulation of genes associated with activation of effector T cells [5–7]. Moreover, the blocking of PD-1 has been linked to an expansion of CD8⁺ effector T cells within the tumor. Interestingly, this CD8⁺ T cells expansion was found to follow a specific gradient that decreases from the margins of the tumor into its center [5, 8, 9]. In addition, the noted expansion in CD8⁺ T cells was also found to coincide with an increase in CD8⁺ T cell clonality in the tumor microenvironment (TME) [8]. This is clearly indicative of the fact that CD8⁺ T cells expanded in the tumor upon blocking PD-1 are indeed tumor-reactive and stand as a consistent correlate of treatment benefit.

Another interesting aspect is the heterogeneity noted in the tumor infiltrating CD8⁺ T cells depending on their different phenotypes and functional states. One such subset is known to express high levels of PD-1 and co-express the following immune modulating proteins: the T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), the lymphocyte-activation gene-3 (LAG-3), the T cell immunoreceptor with Ig and ITIM domains (TIGIT) and CD39, which are all linked to T cells exhaustion [Tex] [10, 11]. Exhausted CD8⁺ T cells represent a dysfunctional cell state that develops due to chronic antigen exposition. However, exhausted CD8⁺ T cells in the TME have a higher potential for tumor antigens recognition and a higher clonal distribution than any other CD8⁺ T cells subset in the TME [12, 13].

Further, the Tex phenotype also defines an impaired capacity of producing effector cytokines such as IL-2, TNF- α and IFN- γ [12]. The inhibition of the PD-1/PD-L1 axis was initially suggested to reinvigorate the anti-tumoral immune response by reversing the state of these terminally exhausted tumor-reactive T cells. However, this function is challenged by the fact that this state of terminal exhaustion is characterized by a distinct epigenetic profile which limits its reversibility [14–16]. In support of this, recent evidence has shown that anti-PD-1 therapy can engage progenitor Tex subsets that co-express PD-1 and the lineage-determining transcription factor 1 (tcf-1), instead of terminally exhausted subsets [17, 18]. Similarly, certain studies on animal models also show that blocking PD-1 leads to the engaging of PD-1⁺/tcf-1⁺/CD8⁺ T cells inducing their self-regeneration or differentiation into effector tcf-1⁻ cells that eventually develop functional states of exhaustion [19, 20]. Interestingly, it has been shown that Tex subsets might promote the recruitment of other immune cells into the TME and consequently support the anti-tumoral immune response [12]. In addition, anti-PD-1 treatment was shown to engage memory-precursor like CD8⁺ T cell subsets, leading to their accumulation in the TME [21] and to a subsequent enhanced cytotoxicity towards cancer cells [22]. The on-treatment increase in memory CD8⁺ T cells was suggested to correlate with response to anti-PD-1 therapy in both preclinical models and clinical studies [9, 23].

blocking of PD-1 is known to cause an increase in the proliferation of CD8⁺ T cells very early during the course of treatment [24, 25]. This proliferative response can be observed in the periphery, where it peaks as early as 7 days following the introduction of anti-PD-1 therapy [24, 26]. In one of our studies on a gastric cancer patient undergoing anti-PD-1 therapy, we observed that the frequency of peripheral cytotoxic T cells CD8⁺/CD107⁺ specific for the NY-ESO-1 cancer testis antigen closely correlated with the patient clinical outcome [27]. Another study has reported that the proliferative response of peripheral CD8⁺/PD-1⁺ T cells could be predictive of durable clinical benefit in patients with solid tumors receiving anti-PD-1 therapy [26]. Thus, these studies support the fact that the systemic CD8⁺ effector T cells response plays a key role in tumor management under anti-PD-1 treatment and suggest their importance as a predictive biomarker of response to PD-1 blockade. In **Figure 1** we illustrate the dynamics of intra-tumoral effector T cells that are shown to correlate with anti-PD-1 treatment outcome.

2.2 Effect of CTLA-4 blockade on effector T cells

When T cells are engaged in an active immune response, the expression of the surface protein CTLA-4, a homolog of CD28 with high affinity to B7-1 and 2 ligands, will be upregulated. CTLA-4: B7-1/2 binding acts as a co-inhibitor of the TCR signal [28]. Thus, the primary role of the CTLA-4 checkpoint is to negatively regulate T cell activation especially during the priming phase upon binding to the B7 ligands expressed by antigen presenting cells (APC). In accordance with this, a major aspect of anti-CTLA-4 therapy is its ability to reinvigorate T cell proliferation and activation. Indeed, the effects of anti-CTLA-4 therapy are evident in several studies on mouse models. For example, CTLA-4 deficient mice were found to display rapidly lethal lymphoproliferation [29]. On the other hand, anti-CTLA-4 therapy led to the expansion of both CD4⁺ and CD8⁺ effector T cells in the tumor [30]. Although both T cells subsets are necessary in mediating tumor immune control [31], the increase in CD4⁺ T cells appeared to be of a greater importance than that of CD8⁺ T cells [30].

In few studies, CD8⁺ T cells expansion has been shown to translate into an effective response to immune checkpoint blockade therapy [7, 32]. However, a study on advanced melanoma patients undergoing treatment with the anti-CTLA-4 antibody tremelimumab, reports no association between the expansion of CD8⁺ effector T

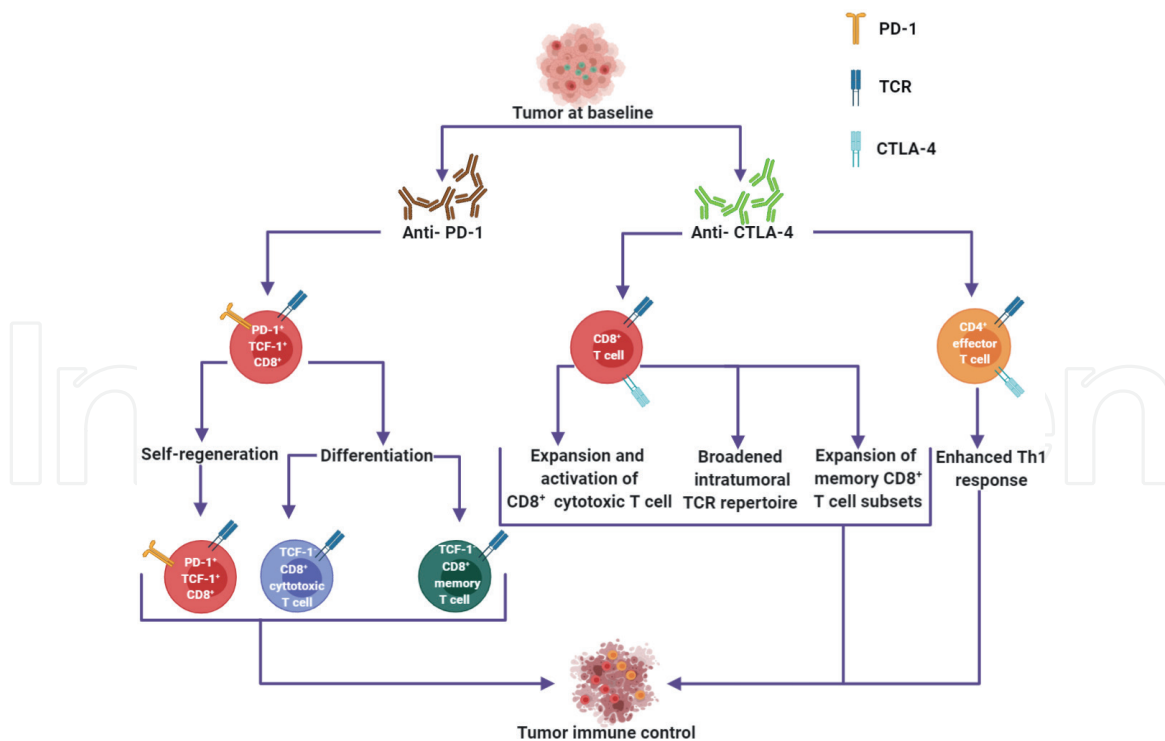


Figure 1.
Effect of immune checkpoint inhibition on effector T cells.

cells and successful anti-tumor response [33] despite similar CD8⁺ T cells activation profiles observed in lesions that responded to anti-CTLA-4 therapy and those that did not respond to this therapy [33]. These findings, possibly explained by the action of immunosuppressive elements in the TME, highlight that adequate CD8⁺ effector T cells function is necessary but not sufficient for complete suppression of tumor growth under immunotherapy [34]. Another interesting effect noted upon CTLA-4 blockade is the enhanced expansion of memory CD8⁺ T cell [35] which will help to promote long-term tumor control post-therapy. Moreover, this expansion of memory CD8⁺ T cells is considered as an indicator of treatment benefit in a few clinical studies [36, 37].

CD4⁺ T cells expanding in the tumors of mouse models under anti-CTLA-4 treatment are found to exhibit a Th1-like effector phenotype with a noticeable expression of ICOS, a known marker of follicular T helper cells [30]. Interestingly, this phenotype of CD4⁺ T cell was observed in mice after genetic knock-down of negative co-inhibitory molecules such as CTLA-4 [38]. Moreover, ICOS-deficient mice showed an impaired anti-tumor T cell response to anti-CTLA-4 therapy [39]. In addition, a tumor microenvironment that is rich in CD4⁺ Th1 effector T cells infiltration was shown to be critical to develop response to anti-CTLA-4 therapy in castration resistant prostate cancer [40]. Furthermore, a higher expression of Th1 associated genes was observed in melanoma tumors of patients responding to ipilimumab compared to non-responders, supporting the functional relevance of a Th1- response in CTLA-4 inhibitor treatment benefit [41]. Interestingly, while the presence of CD4⁺ Th1 cells is predictive of response to anti-CTLA-4, a peripheral blood profile rich in Th17 cells was reported to be rather predictive of autoimmune toxicity under anti-CTLA-4 therapy [42]. Moreover, several clinical studies have reported an increase of ICOS⁺/CD4⁺ T cells in the tumor and peripheral blood of patients treated with anti-CTLA-4 [43–49]. Additionally, an increased proliferation of both peripheral CD4⁺ and CD8⁺ T cells is observed as early as 3 weeks after the first dose of anti-CTLA-4 treatment [50–52]. Such a response may partly be due to the bulk expansion in the periphery of specific T cells against known tumor antigens in anti-CTLA-4 treated patients [53–55]. Of note, an increase in absolute

lymphocyte count (ALC) was shown to correlate with enhanced overall survival and response to ipilimumab in several studies [56–59].

The distribution of the TCR repertoire may be described by different metrics. For example, TCR richness invoke the number of unique T cell clones while its evenness refers to the frequency of their distribution. Some studies reported an increase in the richness of the TCR repertoire under anti-CTLA-4 therapy [60, 61]. On the contrary, the evenness of the TCR repertoire under anti-CTLA-4 therapy is comparatively less impacted [60, 62, 63]. This increase in richness of the TCR repertoire under the effect of anti-CTLA-4 therapy is indicative of unleashed T-cell priming possibly allowing for enhanced tumor immune control through the promotion of new anti-tumor T cells responses [64]. However, in a study on metastatic melanoma and prostate cancer, it has been shown that enhanced clinical outcomes under CTLA-4 blockade are associated with less clonotype loss and on-treatment stability of existing high-frequency TCR clonotypes [61]. These findings suggest that response to anti-CTLA-4 treatment occurs despite the remodeling of the peripheral TCR repertoire rather than as a result of it. In **Figure 1** we illustrate the dynamics of intra-tumoral effector T cells that are shown to correlate with anti-CTLA-4 treatment outcome.

3. Effect of immune checkpoint blockade on immune suppressive T cells

3.1 Effect of PD-1 blockade on immune suppressive T cells

Regulatory T cells (Tregs), an immunosuppressive subset of T cells, are known to be closely involved in the regulation of the immune responses to cancer [65–67]. The tumor-infiltrating subsets of Tregs are characterized by their high surface expression of PD-1 [68–70] and the PD-1/PD-L1 axis is known to modulate Tregs function via cell-intrinsic pathways. For example, the blocking of PD-1 in animal models reduced the immunosuppressive function of Tregs and declined their expression in the TME [71, 72]. Moreover, studies on murine models have shown that PD-1/PD-L1 pathway mediates the conversion of CD4⁺ Th1 effector T cells into induced Foxp3⁺ Tregs (iTregs) [73, 74]. Conversely, certain preclinical studies show that anti-PD-1 therapy is associated with an increase rather than a decrease in Tregs infiltration in the TME [24, 75]. The proliferation of Tregs under anti-PD-1 therapy could be explained by a treatment-induced reversal of the exhausted state of PD-1^{Hi} in the TME [76, 77]. The expansion of Tregs upon blockade of PD-1/PD-L1 axis has been observed at the tumor level as well as in the peripheral blood. One particular study showed that patients with gastric adenocarcinoma responding to anti-PD-1 displayed an on-treatment decrease in intra-tumoral Tregs, whereas non-responders had post-treatment tumor biopsies exhibiting an infiltration of highly proliferative effector Tregs (Foxp-3^{hi}/CD45⁻ CD4⁺) [78]. This suggests that on-treatment changes in intra-tumoral T reg infiltration could be a relevant dynamic parameter to account for in predicting anti-PD-1/PD-L1 treatment response. The predictive insight provided by the on-treatment dynamics of circulating Tregs under PD-1/PD-L1 blockade has also been investigated. In a study conducted on melanoma patients exposed to nivolumab, an expansion of circulating Tregs under therapy was shown to positively correlate with treatment benefit [79]. These observations suggest that Tregs dynamics under anti-Pd-1 therapy may contribute predictive insight into treatment benefit when monitored both in the TME and in the periphery.

Interestingly, another immunosuppressive CD4⁺ T cell subset found to be regulated by anti-PD-1 therapy has been recently identified. These cells known as 4PD-1^{Hi} express high levels of PD-1 while lacking Foxp-3 expression [80]. Also, these 4PD-1^{Hi} cells were shown to accumulate in the tumor and to inhibit T cells effector

function. Therefore, 4PD1^{Hi} cells are considered as a marker of tumor progression. Anti-PD-1 treatment was shown to reduce the proliferation of 4PD-1^{Hi} cells, whereas anti-CTLA-4 treatment showed an exactly opposite effect on these cells. In line with this interesting observation, the downregulation of 4PD-1^{Hi} cells under anti-PD-1 treatment was further documented as a biomarker of treatment response under anti-PD-1 pembrolizumab antibody in a melanoma patient cohort. In addition, some preclinical studies support the fact that anti-PD-1 therapy can induce the expansion of specific CD8⁺ T cells immunosuppressive subsets [30]. Indeed, a recent study showed that PD-1 blockade in sub-optimally primed T cell conditions supported the proliferation of dysfunctional immunosuppressive CD8⁺ T cells expressing PD-1 and high levels of CD38 and this effect was associated with treatment failure and tumor resistance in cancer patients [81]. The dynamics of these T cell subsets under treatment may therefore provide valuable predictive information, pending the validation of these candidate biomarkers in larger scale studies. The proposed mechanisms of anti-PD-1 action on Tregs subsets are summarized in **Figure 2**.

3.2 Effect of CTLA-4 blockade on regulatory T cells

Several observations suggest the action of anti-CTLA-4 on Tregs to be key in mediating treatment effects on the tumor. Indeed, some studies involving murine tumor models reported anti-CTLA-4 treatment to simultaneously induce an increased expansion of peripheral Tregs and a decreased expansion of intra-tumoral Tregs [82–84]. This dual action of anti-CTLA-4 on Tregs proliferation could be due to the higher expression of CTLA-4 on exhausted tumor-infiltrating Tregs, where their decline under treatment is suggested to be mediated by a distinct Fc-gamma receptor dependent mechanism of anti-body-mediated cell-mediated cytotoxicity (ADCC)

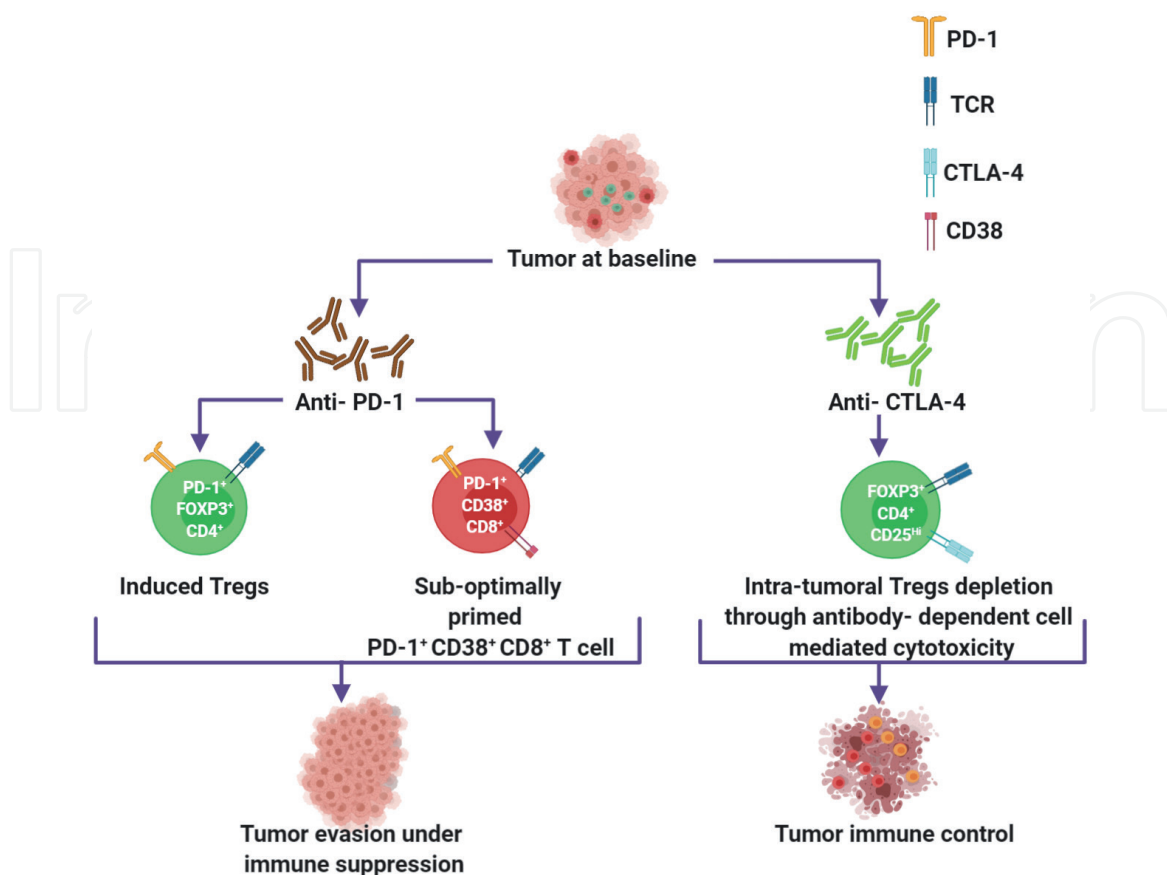


Figure 2.
Effect of immune checkpoint inhibition on immune suppressive T cells.

[85]. This feature was suggested to play a key role in tumor control under CTLA-4 blockade since it has been demonstrated that an on-treatment increase in intra-tumoral Tregs stands as the correlate of an optimal treatment response [82, 83, 86, 87]. Moreover, in another study in different murine models, the therapeutic activity of ipilimumab was found to essentially rely on this Fc-dependent Tregs depletion and not on the checkpoint inhibitor action of the drug [88]. However, there is noticeable inconsistencies in observed Tregs dynamics under CTLA-4 blockade in humans. Indeed, certain studies document a remarkable expansion of Tregs in the peripheral blood of patients treated with anti-CTLA-4 [51, 89–91], while other studies report a declined or unchanging frequency of peripheral Tregs during this therapy [37, 49, 92]. In addition, the ability of the peripheral Tregs dynamics to predict a treatment response to anti-CTLA-4 treatment is also quite unclear. For example, their change in frequency is found to correlate both negatively [93] and positively [90] with anti-CTLA-4 treatment benefit. Yet some other studies show no correlation at all between the change in Tregs frequency and a treatment response to anti-CTLA-4 therapy [56, 94, 95]. Observations of the intra-tumoral Tregs dynamics under anti-CTLA-4 is also inconclusive. A contradictory effect is put forth by a cohort on regionally advanced melanoma patients treated with 2 neoadjuvant doses of ipilimumab. This study reported a reversed association between the change in intra-tumoral Tregs frequency and treatment benefit [90]. Similarly, another study reported a marked decline in intra-tumoral Tregs levels in melanoma patients responding to ipilimumab compared to non-responding ones [96]. On the contrary, two other studies report increasing frequencies of Tregs in biopsies of patients undergoing anti-CTLA-4 therapy [13, 32]. It is interesting to note that the accumulation of Tregs within the tumors upon CTLA-4 blockade may be induced by a feedback loop triggered by a successful cytotoxic T cell response [97]. This may account for the positive correlation between the intra-tumoral levels of Tregs and patient long-term survival as reported by some studies involving solid tumors [98, 99]. These observations nonetheless suggest that Tregs dynamics under CTLA-4 treatment, in the TME and possibly in the periphery, should be accounted for when monitoring for treatment effects. The depletion of intra-tumoral Tregs under CTLA-4 blockade is illustrated in **Figure 2**.

3.3 Effect of PD-1 and CTLA-4 blockade on myeloid cell compartment

Monocytes, macrophages and dendritic cells are involved in antigen presentation and T cells priming and thereby serve as a bridge between the innate and adaptive immune response. However, chronic inflammation arising due to cancer disturbs the myeloid cell line maturation process, leading to the generation of myeloid derived suppressor cells [MDSCs] and tumor-associated macrophages [TAMs], that are both suppressors of the anti-tumor immune response [100]. These tumor associated monocytes and macrophages are known to display a wide variety of phenotypes with both pro-inflammatory [M1] and immunosuppressive [M2] functions [101]. Likewise, several studies on animal models have found that treatment with ICIs has the ability of bringing about a spectacular transformation of the intra-tumoral myeloid cell compartment from an immunosuppressive configuration to a more pro-inflammatory one [102, 103]. It has been suggested that the increased INF- γ secretion by renewed T cells would possibly indirectly mediate this myeloid cell reprogramming in the TME under immune checkpoint therapy [102]. Also, it was observed that dual PD-1 and CTLA-4 blockade induces an increase in intra-tumoral pro-inflammatory macrophages, as shown in animal models [104]. In addition, potential direct mechanisms of regulation of MDSCs by anti-PD-1 or anti-CTLA-4 treatment were also identified. As an example, an induced expression of CTLA-4 on monocyte-derived dendritic cells [mDCs] acts as a negative

regulator of mDCs-associated cytokine secretion and antigen-specific CD4⁺ T cell proliferation [105]. Moreover, subsets of intra-tumoral MDSCs that express PD-1 and CTLA-4 are found to display decreased arginase 1 expression and activity upon anti-CTLA-4 or anti-PD-1 treatment in mice [106]. In murine models, it has been shown that arginase 1 impairs T cells functions and contributes to immune evasion [107]. Furthermore, it has been recently reported that anti-PD-1 therapy was able to prevent the block in myeloid cell lineage maturation, thereby allowing the myeloid precursors to mature into effector macrophages and dendritic cells contributing favorably to the anti-tumoral immune response [108].

A decline in circulating MDSCs under anti-CTLA-4 is found to correlate with patient outcome in several studies [37, 90, 109, 110], although this association is not universally reported [111]. Moreover, these studies showed discordant observations regarding the dynamics and predictive value of the major MDSCs subsets (monocytic MDSCs (mo-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs) subsets) [112]. In addition, several studies showed that anti-PD-1 treatment had no effect on the level of circulating mo-MDSC and PMN-MDSC subsets [9, 113, 114]. Yet a particular study revealed a prominent restructuring of the myeloid compartment after initiation of anti-PD-1 therapy in metastatic melanoma patients when studied

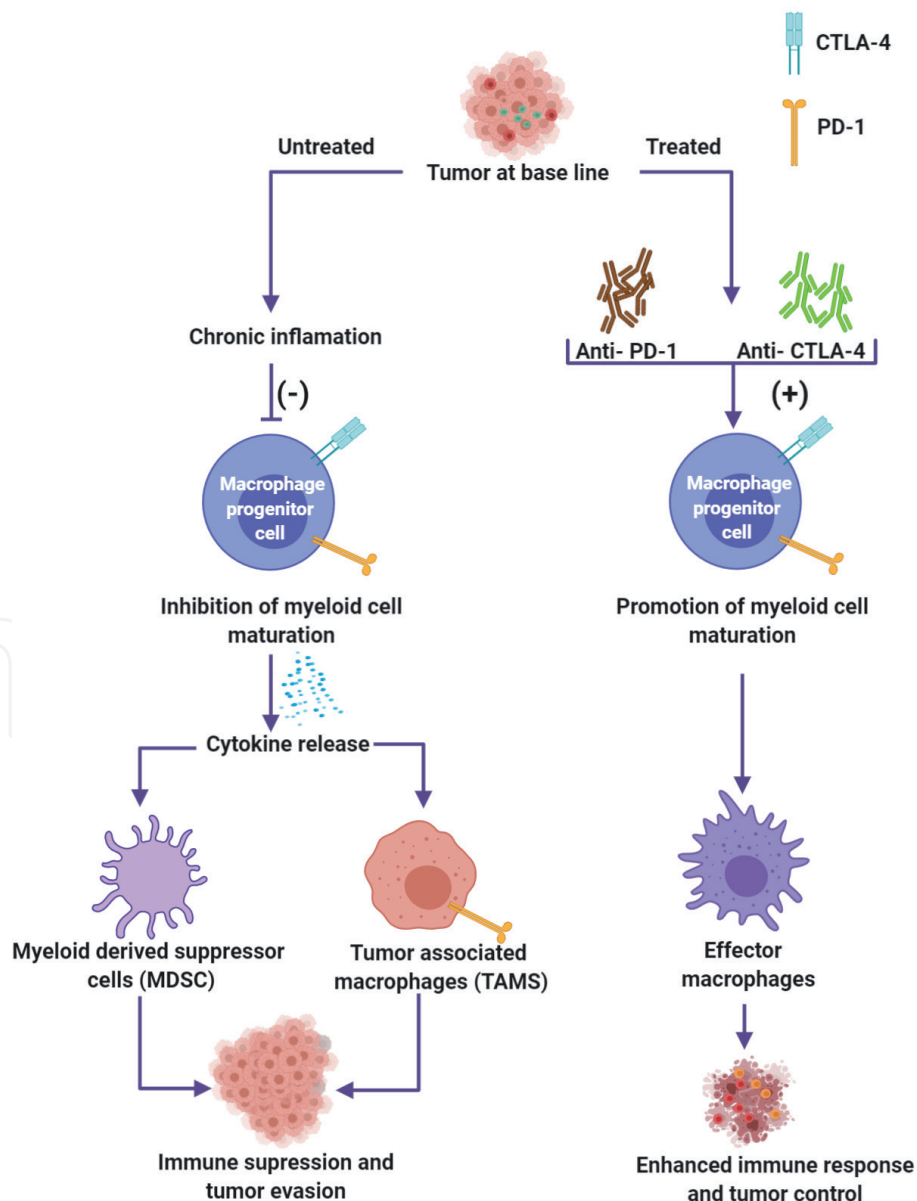


Figure 3.
Effect of PD-1 and CTLA-4 blockade on myeloid cells.

under the lens of high dimensional single cell analysis platforms [113]. Therefore, the ability to monitor the evolution of myeloid cells under immune checkpoint blockade appears to be of great predictive value, considering the important role that this cell compartment possibly plays in the modulation of the anti-tumoral immune response by either promoting or preventing the effector T cells response observed upon these therapies. One example of the described mechanisms of anti-PD-1 and anti-CTLA-4 effect on myeloid cells compartment is illustrated in **Figure 3**.

4. Dynamic predictive and prognostic soluble biomarkers in cancer immunotherapy

In the subsections below, we will focus on blood-based candidate biomarkers that can be utilized as predictive or prognostic markers in cancer immunotherapy.

4.1 Blood cell counts/ratios, C-reactive protein and lactate dehydrogenase

Changes in the blood cell counts and their ratios including neutrophils, lymphocytes, neutrophil to lymphocyte ratio as well as C-Reactive Protein (CRP) and Lactate Dehydrogenase (LDH) have been reported as prognostic/predictive outcome markers for immunotherapy [2]. Several studies have shown that low neutrophils and high lymphocytes are associated with overall survival (OS) in cancer patients [58, 115, 116]. For example, melanoma patients on nivolumab treatment having absolute lymphocyte counts of $>1000/\mu\text{L}$ and absolute neutrophil count of $<4000/\mu\text{L}$ were observed to have better overall survival [115]. On the other hand, pre-treatment neutrophil-to-lymphocyte ratio (NLR) and derived NLR (dNLR) can also serve as an index of the systemic inflammatory response and therefore considered as useful indicators of response in immunotherapy. Pre-treatment NLR/dNLR levels and survival association studies in advanced cancers including melanoma, non-small-cell lung cancer (NSCLC) and genitourinary have reported that high pre-treatment NLR and dNLR levels are associated with poor progression free survival (PFS)/OS with increased risks of death in immunotherapy treated patients indicating their usefulness as predictive and prognostic biomarkers [117–120].

CRP is an inflammatory marker that induces the expression of acute-phase proteins such as neutrophils and has been correlated with poor prognosis in several cancers [121, 122]. With regards to immunotherapy, post treatment increased CRP levels have been associated with inflammation, disease progression and in some cases immune-related adverse events. On the other hand, low CRP levels post immunotherapies have been associated with better antitumor response/survival [93, 123].

LDH is a final enzyme in the glycolysis pathway that catalyzes the interconversion of pyruvate and lactate. In cancers, high levels of LDH leads to increased utilization of glycolysis as their energy requirement in the microenvironment [124]. Studies have confirmed that LDH is a significant negative prognostic factor for immunotherapy treated stage 4 melanoma patients [125]. Elevated baseline LDH in melanoma and lung cancer patients treated with pembrolizumab/nivolumab is associated with poor OS and higher risk of death [126–128]. Similar results have been reported for advanced esophageal squamous cell carcinoma patients treated with the anti-PD-1 immune checkpoint inhibitor camrelizumab where elevated LDH levels were found to correlate with poor OS [129].

The fact that blood cell counts/ratios, CRP and LDH tests are performed as part of a routine diagnosis and also are highly assessable/measurable at various treatment timelines in patients making them attractive dynamic biomarkers.

4.2 Soluble immune checkpoint inhibitors

Soluble forms of immune checkpoints (sICs) are shed in the plasma/serum and have been associated with modulation of the immune system by affecting the binding capacity of immunotherapeutic drugs and thus influencing the efficiency of immune system. Studies have demonstrated that sICs can serve as markers for prognosis, response to treatment and overall response rate (ORR) in immunotherapy treated patients [130]. In addition to this, these markers can also be important for prediction of immune related adverse events which is an area poorly explored with respect to these biomarkers. Here, in this sub-section, we will discuss sICs evidenced in literature as prognostic and predictive markers in ICIs treatment.

4.2.1 Soluble immune inhibitory markers

4.2.1.1 Soluble PD-1, PDL-1 and PDL-2

sPD-1 has been documented to inhibit all three PD-L1/PD-1 interactions: PD-L1/CD80, PD-L1/PD-1, and PDL2/PD-1 [131]. Researchers have demonstrated that expressed sPD-1 blocks PD-L1/PD-1 interactions that can lead to inhibition of tumor growth via various mechanisms including blockade of PD-L1 on tumor cells, upregulation of CD8⁺ T cells, reduction in the expression of IL-10, increased production of inducible nitric oxide synthase, TNF- α and IFN- γ and enhancement of the immune response through interaction with immune cells [132–134]. sPD-1 has been reported as a modulator of immune response during ICIs treatment in serum of cancer patients. A study on 22 NSCLC patients observed that sPD-1 decreased significantly in clinically responding patients during Nivolumab treatment. In addition to this, patients with performance status of 0 had a decreased sPD-1 during treatment and these patients were found to have better immune fitness with low levels of immunosuppression [135]. Similarly, a study on 177 unresectable metastatic melanoma patients treated with anti-PD-1 showed interesting results. High pre-treatment serum concentrations of PD-1 and PD-L1 were correlated with poor prognosis and survival. The authors postulated that circulating serum PD-1 molecules might be directly targeted by therapeutic anti-PD-1 antibodies and this interaction might impair the effectiveness of anti-PD-1 therapy via neutralization. It is possible that this is a tumor escape mechanism that facilitates poor outcome. Thus, quantification of circulating PD-1 and PD-L1 molecules can translate into prognostic and predictive factors in immunotherapy treated patients [136].

sPD-L1 is produced by tumor cells/activated mature DCs and is known to have structural similarities to mPD-L1 [137]. It has been postulated that sPD-L1 has the capability to exert a competing effect against anti-PD-L1 drugs. A study by *Gong et al.* reported that NSCLC patients who were refractory to ICIs treatment secreted a sPD-L1 variant (without the transmembrane domain) in serum and this variant competed, bound and then inhibited the activity of immunotherapeutic drug in such patients [138]. This is a critical finding as it gives evidence on the dynamic role of sPD-L1 and its utility as a biomarker of response in immunotherapy. Furthermore, studies on various cancers have also reported on this aspect. For example, a study on ipilimumab treated melanoma patients showed that patients with high pre-treatment sPD-L1 levels showed poor prognosis and disease progression [139]. This is an interesting observation and sheds light on the dynamic nature of this biomarker. Similarly, two studies on NSCLC have documented that high levels of sPD-L1 correlated with poor prognosis, OS, and abdominal metastasis [140, 141]. However, to date no correlation of sPD-L1 with tissue PD-L1 expression has been reported indicating the dynamic nature of secreted PD-L1 that is distinct from tissue PD-L1.

Several published studies evidence consistent data on its utility as a monitoring tool to test the efficacy of ICIs as a prognostic biomarker. Further studies with systematic uniform methodologies may allow better understanding of sPD-L1 as an effective tool for patient stratification with regards to anti-PD-1 therapy benefit.

sPD-L2 is a splice variant protein product that lacks transmembrane domain and is secreted into the blood. Distinct expression pattern of PD-L2 variants in leukocytes of distinct cellular status have been observed suggesting that modulation of sPD-L2 expression may have an influence on the outcome of the immune response [142]. However, limited data on sPD-L2 as an immune related biomarker is available. A study by *Zizari et al.*, on NSCLC patients treated with Nivolumab showed that sPD-L2 was dynamically modulated during ICIs treatment. The concentrations of sPD-L2 were found to be significantly lower in responding patients. In addition to this, soluble mediators including low PD-L1, CD137, Tim-3 and BTLA-4 in combination with low sPD-L2 are associated with favorable clinical response indicating that sPD-L2 works in synergy with other molecules to modulate the immune response. This allows understanding on the dynamic interaction of soluble immune modulators as useful biomarkers of response [135]. In addition to response prediction, a study on NSCLC patients treated with Nivolumab reported interesting results with respect to immune related adverse events. Low sPD-L2 concentration at diagnosis as well as in pre-treatment samples was found to be associated with occurrence of immune grade 3–4 toxicity indicating that sPD-L2 can also serve as potential predictive biomarker for immune related adverse events in ICIs treated patients [143].

4.2.1.2 Soluble CTLA-4, TIM-3 and LAG-3

The major source of sCTLA-4 is Tregs, monocytes and immature DCs [144]. sCTLA-4 has been reported in several studies as a plausible marker for response in ICIs treatment. For example, in melanoma patients treated with ipilimumab, high pre-treatment expression of sCTLA-4 was associated with response to treatment and longer OS [145]. Another study on metastatic melanoma patients treated with ipilimumab showed similar results with high levels of sCTLA-4 at baseline associated with disease responsiveness and survival. Interestingly, the study observed that in responding patients, sCTLA-4 concentration increased with subsequent treatment cycles while in progressing patients, sCTLA-4 decreased subsequently indicating that sCTLA-4 can serve as a valuable dynamic marker for treatment monitoring. In addition to this, it was also observed that patients with high pre-treatment sCTLA-4 were at a higher risk of developing immune related adverse events providing further insight into its utility as a biomarker of response/adverse event monitoring [146]. However, in different cancers, its utility may be distinct based on its interaction with other molecules and subsequent immune modulation. For example, a recent study on NSCLC patients treated with nivolumab showed that lower concentration of sCTLA-4 at 3 months of clinical evaluation was associated with response. In addition to this, patients with performance status of 0 consistently maintained a lower expression of sCTLA-4 from the time of treatment initiation until 3 months of clinical evaluation indicating that sCTLA-4 can be an indicator of immune fitness in ICIs treated patients [135]. It is postulated that during ICIs treatment, sCTLA-4 might be involved in enhancing the ability of host cytotoxic T cells to attack tumor cells and thereby enhancing the antitumor effect of immunotherapy.

sTIM-3 secreted in blood lacks mucin and transmembrane domains. It is postulated that sTIM-3 is shed from the cell surface due to metalloproteinase-dependent cleavage and may serve as a decoy receptor for TIM-3 ligands thereby interfering with the inhibitory function of TIM-3 [147]. However, the exact function of sTIM-3 is still unknown. A study on plasma levels of sTIM-3 in ICIs treated patients observed that NSCLC

patients treated with nivolumab had a lower level of sTIM-3 at three months of clinical evaluation and this correlated with the response and longer survival of the patient [135]. Though, the study does give an indication of sTIM-3 as biomarker of response, further studies are needed to understand its dynamic nature in ICIs treatment.

sLAG-3 plays a role in immune pathways and has been associated as a Th1 activity marker in serum. sLAG-3 has been reported to bind to MHC class II and induce maturation of dendritic cells thereby facilitating attack on tumor cells [148, 149]. This makes sLAG-3 an attractive biomarker in ICIs treatment. Though several studies on LAG-3 and cellular response has been documented, there is paucity of data on the utility of sLAG-3 in serum of ICIs treated patients. A study on nivolumab treated NSCLC patients reported that sLAG-3 was significantly increased during treatment and this increase was retained in non-responding patients. In addition to sLAG-3, other soluble mediators including sPD-1 and sPDL-2 were also increased in these patients indicating the dynamic interactive nature of LAG-3 and its role as predictive marker [135].

4.2.1.3 Soluble IDO, CD163 and NKG2DL

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that acts as an immune checkpoint and inhibits T-cell proliferation by starving the cells from tryptophan (trp) in order to sensitize them to apoptosis [150]. IDO facilitates tumor immune escape and several preclinical studies have associated IDO activity with immunotherapeutic resistance [151]. A study on nivolumab treated NSCLC patients found that lower baseline level of IDO activity in serum was significantly associated with better PFS/OS while higher levels were associated with early progression indicating that high serum levels can serve as early marker of response/indicator of resistance to anti-PD-1 treatment [152]. On the other hand, a larger study on 27 NSCLC patients treated with nivolumab indicated the dynamic nature of sIDO at baseline, 2 months and at disease progression. The study aimed to evaluate the dynamic nature of IDO as a predictor of response. Interestingly, the authors observed that at baseline, IDO activity was higher in responding patients than non-responding patients. However, in patients who did not benefit from immunotherapy, a statistically significant increase was observed between baseline sample and sample taken at the time to disease progression indicating the potential utility of IDO as therapeutic resistance marker to anti-PD-1 treatment [153].

CD163 is the hemoglobin/haptoglobin complex scavenger receptor expressed exclusively on circulating monocytes/tissue macrophages. It is involved in anti-inflammatory functions associated with macrophages and therefore plays an important role in suppressing anti-tumor immune responses [154]. sCD163 is secreted in plasma via proteolytic shedding and is considered a specific marker for TAM. To demonstrate the utility of sCD163 as a marker of response in immunotherapy, *Fujimura et al.* conducted a study on 59 cases of advanced cutaneous melanoma and 16 cases of advanced mucosal melanoma treated with nivolumab. It was observed that in advanced cutaneous melanoma group, sCD163 was significantly increased at 6 weeks of treatment in the response group as compared to non-response group indicating that sCD163 is an early marker of response in nivolumab treated patients [155]. On the other hand, another study by the same group determined the utility of sCD163 as predictor of immune related adverse events (irAE) in nivolumab treated advanced melanoma patients. It was observed that at day 42 of treatment, the absolute value of sCD163 significantly increased in patients with adverse nivolumab-induced, immune-related events indicating that sCD163 can also serve as a valuable predictor of irAEs in immunotherapy [155]. However, due to limited published data, further studies will provide a better understanding on this marker.

NKG2D is an activating immunoreceptor of cytotoxic lymphocytes and is expressed on T, NK, and NKT cells. NKG2D has eight ligands including MIC (MICA

and MICB) or ULBP (ULBP1, ULBP2, ULBP3, ULBP4, RAET1G, and RAET1L) family. These NKG2D ligands are absent on normal cells but are usually overexpressed on tumor cells. Soluble NKG2D ligands (sNKG2DLs) are generated by proteolytic shedding of tumor cells which boosts tumor immune escape by binding and subsequent endocytosis/degradation of NKG2D receptor on NK/T cells thus suppressing antitumor immune responses [156, 157]. Various sNKG2DLs have been studied as predictive biomarkers of response in immunotherapy. A study in melanoma patients by *Maccalli et al.*, showed that absence of soluble sMICB, sULBP-1 and sULBP-2 in baseline serum of anti-PD-1 treated patients correlated with improved survival while detectable levels of these molecules was correlated with poor survival [158]. Similarly, another study by the same group on melanoma patients showed that elevated sULBP2 in early-stage patients on ICIs treatment was a strong indicator of poor prognosis indicating the clinical usefulness of sULBP2 as a distinguishing marker for classifying prognosis in early- and late-stage melanoma patients on treatment [156].

4.2.2 Soluble immune stimulatory markers

4.2.2.1 Soluble CD27 and CD28

CD27 is expressed in lymphocytes and is activated by its ligand, CD70, which is a member of the tumor necrosis factor receptor superfamily. Upon binding of CD70 to CD27, soluble CD27 (sCD27) is cleaved off by metalloproteinases and is secreted in serum, plasma, and urine samples. Studies have suggested that changes in sCD27 levels reflect the activity of systemic immunity [159]. In various hematological malignancies increased levels of sCD27 have been reported to correlate with poor prognosis [160]. A study on 16 advanced lung cancer patients on anti-PD-1 treatment were tested for their pretreatment sCD27 levels and correlated with their response patterns. It was observed that a sCD27 level was higher in patients with longer survival and in such patients the duration of treatment was shorter. The authors suggested that sCD27 levels can serve as prognostic marker for predicting effectiveness of ICIs in advanced lung cancer [161].

CD28 is a second messenger of T cell activation and is a critical immune checkpoint for recognition of dendritic cells by T cells. Previous studies have suggested that PD-1 antibodies rely on the activation of the CD28/B7 pathway to rescue the depletion CD8⁺ T cells and then achieve anti-tumor effects [162]. Soluble sCD28 has been reported as a modulator of T cells for proliferation and is therefore considered an attractive biomarker of response to ICIs treatment [163]. Recently, a study on 44 patients with various cancers (lung, tongue, esophageal and nasopharyngeal, colorectal, cholangiocarcinoma, gastric, duodenal adenocarcinoma, renal cell carcinoma, hepatocellular carcinoma, and malignant melanoma) on anti-PD-1 treatment were tested for serum CD28 along with other soluble markers. It was observed that patients with higher baseline sCD28 expression had a longer PFS and responded better to treatment than non-responsive patients [164]. This dynamic change in sCD28 during treatment gives a credible index in terms of its predictive efficiency as a promising response marker. However, larger studies on this aspect are needed to understand the role of this marker.

5. Conclusion

Using ICIs have shown promising effect in treating cancers. However, only small group of patients are responsive to this treatment strategy. Tumor resistance to the immune response can be mediated by the involvement of several immunological pathways. In this chapter, we reviewed the different immunological pathways that

can be modulated by immune checkpoint blockade and more specifically PD-1 and CTLA-4 inhibitors. We have summarized all the findings obtained in pre-clinical and clinical trials reporting an impact of anti-PD-1 and anti-CTLA-4 on intra-tumoral and peripheral immune response. Interestingly, the study of the dynamics of the immune system under CTLA-4 and PD-1 inhibitors shows a noticeable distinction in their regulatory mode of action on the anti-tumoral and peripheral immune response. Moreover, the findings discussed in this chapter show that CTLA-4 and PD-1 inhibitors do not only restore intra-tumoral effector T cells activity upon exhaustion but are also able to induce a consequential remodeling of the tumor microenvironment as well as the systemic immune response. Indeed, the field of immunological liquid biomarkers is fast evolving with many novel predictive and prognostics markers gaining attention. Though, liquid biopsies have many advantages including minimal invasiveness, longitudinal monitoring and simultaneous parallel testing with highly sensitive/specific high throughput applications. Although several studies state the utility of soluble ICIs markers, it is observed that the characteristic feature of these markers is to modulate the immune response in synergy with each other. This makes them attractive candidates as up and down regulation of a combination of markers can allow better understanding of the immune modulatory and dynamic nature of soluble immune molecules involved in ICIs treatment. However, there are several limitations that need to be addressed for these markers. Mainly, standardization of sampling/measurement techniques as well as larger validation studies are required to verify the utility of these markers as promising tools to guide and monitor treatment decisions in ICIs treated patients. Finally, identification of dynamic biomarkers for prediction of ICIs tumor control and for monitoring of patient response under treatment is gaining considerable knowledge through recent technologies including proteomics and transcriptomics. Progress along this approach is critical to build reasoning for novel therapeutic combinations and to set forth a more personalized cancer immunotherapeutic strategy.

Acronyms and abbreviations

ICI	immune checkpoint inhibitors
PD-L1	programmed death-ligand 1
PD-1	programmed death- 1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
TCR	T cell receptor
TME	tumor microenvironment
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
LAG-3	lymphocyte-activation gene-3
TIGIT	T cell immunoreceptor with Ig and ITIM domains
Tex	exhausted T cells
Teff	effector T cells
ALC	absolute lymphocyte count
MDSCs	myeloid derived suppressor cells
TAM	tumor-associated macrophages
irAE	immune-related adverse events
CRP	C-reactive protein
LDH	lactate dehydrogenase
OS	overall survival
NLR	neutrophil-to-lymphocyte ratio
dNLR	derived Neutrophil-to-lymphocyte ratio
NSCLC	non-small-cell lung cancer
PFS	progression free survival

sICs	soluble forms of immune checkpoints
ICI	immune checkpoint inhibitors
ORR	overall response rate
sPD-1	soluble programmed cell death protein 1
sPDL-1	soluble programmed death-ligand 1
sPDL-2	soluble programmed cell death 1 ligand 2
sCTLA-4	soluble cytotoxic T-lymphocyte-associated protein 4
sTIM-3	soluble T-cell immunoglobulin and mucin domain-3
sLAG-3	soluble lymphocyte-activation gene 3
sIDO	soluble indoleamine 2,3-dioxygenase
sCD163	soluble cluster of differentiation 163
NKG2D	natural killer group 2D
sNKG2DL	soluble natural killer group 2D ligands
MICA	major histocompatibility complex class I-related chain A
MICB	major histocompatibility complex class I-related chain B
NK	natural killer
NKT	natural killer T cells
ULBP 1,2,3,4	UL-16-binding proteins 1,2,3,4
RAET1G	retinoic acid early transcript 1G
RAET1L	retinoic acid early transcript 1 L
sCD27	soluble cluster of differentiation 27
sCD28	soluble cluster of differentiation 28

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