

## REVIEW ARTICLE

# Predictive biomarkers for immune checkpoint blockade and opportunities for combination therapies

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Received 7 May 2019; received in revised form 7 June 2019; accepted 16 June 2019  
Available online 3 July 2019

## KEYWORDS

IFN- $\gamma$ ;  
Immune checkpoint;  
Microbiota;  
Microsatellite  
instability;  
Neoantigen;  
PD-L1;  
Radiotherapy

**Abstract** Immune checkpoint blockade therapies (ICBs) are a prominent breakthrough in cancer immunotherapy in recent years (named the 2013 “Breakthrough of the Year” by the Science magazine). Thus far, FDA-approved ICBs primarily target immune checkpoints CTLA-4, PD-1, and PD-L1. Notwithstanding their impressive long-term therapeutic benefits, their efficacy is limited to a small subset of cancer patients. In addition, ICBs induce inadvertent immune-related adverse events (irAEs) and can be costly for long-term use. To overcome these limitations, two strategies are actively being pursued: identification of predictive biomarkers for clinical response to ICBs and multi-pronged combination therapies. Biomarkers will allow clinicians to practice a precision medicine approach in ICBs (biomarker-based patient selection) such as treating triple-negative breast cancer patients that exhibit PD-L1 staining of tumor-infiltrating immune cells in  $\geq 1\%$  of the tumor area with nanoparticle albumin-bound

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Peer review under responsibility of Chongqing Medical University.

(nab)—paclitaxel plus anti-PD-L1 and treating patients of MSI-H or MMR deficient unresectable or metastatic solid tumors with pembrolizumab (anti-PD-1). Importantly, the insights gained from these biomarker studies can guide rational combinatorial strategies such as CDK4/6 inhibitor/fractionated radiotherapy/HDACi in conjunction with ICBs to maximize therapeutic benefits. Further, with the rapid technological advances (e.g., ATCT-Seq), we predict more reliable biomarkers will be identified, which in turn will inspire more promising combination therapies.

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## Introduction

Harnessing the patients' own immune system to attack tumor cells (cancer immunotherapy) is not recent. In 1891, William B. Coley injected live streptococcal organisms into a patient with inoperable cancer to stimulate the patient's own immune system and successfully cured the patient, which was the first documented case of cancer immunotherapy.<sup>1</sup> However, due to the lack of understanding of the immune system back then and the rapid development of chemotherapy and radiotherapy in 1950s, immunotherapy has not become a mainstay in cancer care until recently. With the increased understanding of the cancer-immunity cycle,<sup>2</sup> it has become evident that immune cells play a key role in constraining malignant cell growth. This dynamic "seek-and-hide game" between immune cells and cancer cells was best described by Schreiber and colleagues in 2002,<sup>3</sup> termed "the cancer immuno-editing", consisting of three stages: elimination, equilibrium, and escape. While detailed mechanisms underlying each stage are not fully understood, a clear realization is that elegantly-coordinated cooperation between the innate and adaptive immunity is indispensable for successful tumor eradication.<sup>4</sup> Dysregulation of this process results in tumor escape from immunosurveillance. Among the multiple mechanisms proposed for tumor immune evasion, hijacking the "immune checkpoints" by tumor cells has taken the central stage in recent years, owing to the impressive clinical responses of therapeutic agents targeting "immune checkpoints". In 2013, the Breakthrough Prize of the Year in Life Sciences was awarded to Jim Allison, in recognition of his pioneering work in this field.

Immune checkpoints are a group of inhibitory molecules that are induced during an active immune response, serving as a negative feedback mechanism to limit collateral tissue damage. CTLA-4 and PD-1 were the first characterized immune checkpoints.<sup>5</sup> As aforementioned, tumor cells can coopt CTLA-4 and PD-1 negative signals to evade immunosurveillance. Thus, blocking CTLA-4 and PD-1 with antibodies (collectively known as ICBs) significantly suppresses tumor growth and promotes long-term survival of tumor-bearing mice,<sup>6,7</sup> associated with rejuvenated effector functions of tumor-infiltrating T cells (TILs) including IFN- $\gamma$  signaling, as demonstrated by us and others.<sup>8,9</sup> In 2011, based on substantially-improved survival of patients in phase III clinical trials,<sup>10,11</sup> the FDA approved the very first ICB, ipilimumab, which is an anti-human CTLA-4

monoclonal antibody, to treat patients with advanced melanoma. This opened a new era in cancer immunotherapy, and since then, anti-PD-1 (pembrolizumab and nivolumab) and anti-PD-L1 (atezolizumab, avelumab, and durvalumab) immunotherapies have also gained FDA approvals to treat patients with various types of cancer (<https://www.fda.gov/Drugs>), culminating in the awarding of the 2018 Nobel Prize to Jim Allison and Tasaku Honjo. This ever-growing list of ICB approvals is depicted in Fig. 1 with their respective FDA approval times shown.

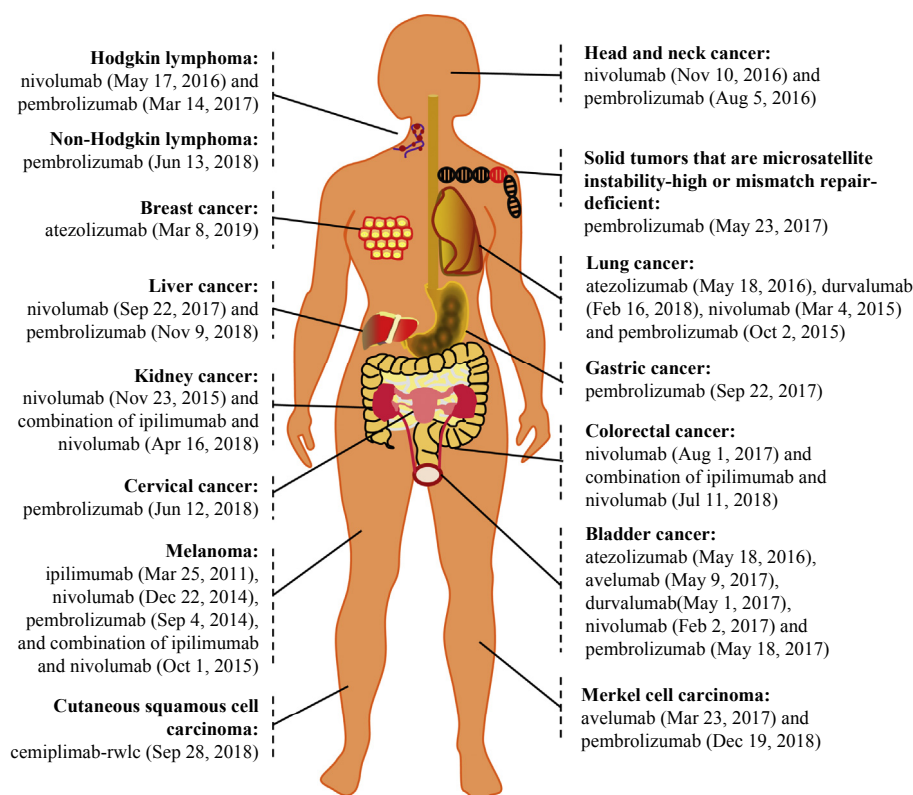
In spite of the impressive clinical response of ICBs, their therapeutic efficacy is generally limited to a minority of cancer patients (~20–40%),<sup>12</sup> except for the Hodgkin's lymphoma that shows ~80% response rate (discussed below). In addition, ICBs induce inadvertent life-threatening irAEs in some patients and the cost for long-term treatment is high. Therefore, intensive research efforts have been focused on identifying predictive biomarkers for clinical response to ICBs and designing rational combination therapies of ICBs with other therapeutic agents that can synergize with ICBs to augment overall therapeutic efficacy. Here, we provide a focused review on the most-investigated biomarkers (Fig. 2) and discuss combination strategies that are underway or under development.

## Predictive biomarkers

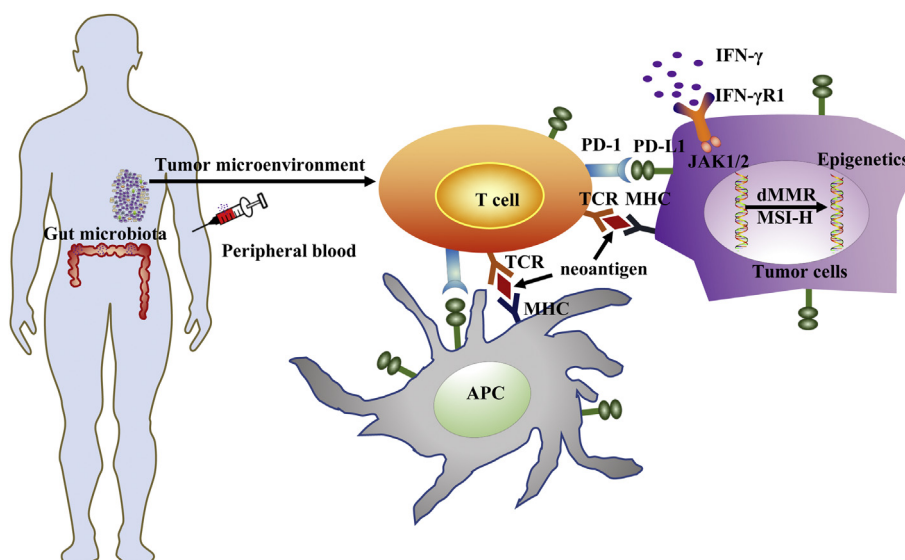
### PD-L1 expression

The PD-1/PD-L1 signaling pathway is an integral component of peripheral tolerance, serving as a rheostat in maintaining immune homeostasis. PD-L1 was successfully cloned in 1996.<sup>13</sup> Ligation of PD-L1 to PD-1 inhibits T cell receptor-mediated T cell proliferation and cytokine production<sup>14</sup> and prevents exuberant immune responses. PD-L1 is abundantly expressed on tumor cells as well as tumor-infiltrating immune cells (TILs) including dendritic cells, macrophage and T cells. Since anti-PD-1/L1 therapies primarily counteract PD-L1-mediated negative signals, understandably, PD-L1 expression in the tumor microenvironment (TME) has been the most-studied biomarkers, particularly for anti-PD-1/L1, which are summarized in Table 1.

There is a vast body of work assessing PD-L1 expression in tumor cells as a predictive biomarker to ICBs. However, the results have been splitting with both positive and



**Figure 1** The FDA approvals of ICBs for cancer treatment (as of 5/6/2019).



**Figure 2** The most-investigated predictive biomarkers for clinical response of immune checkpoint blockade therapies (ICBs): 1. PD-L1 expression; 2. Tumor-infiltrating immune cells (TILs); 3. IFN-γ signaling; 4. Neoantigens and tumor mutational burden; 5. Microsatellite instability-high (MSI-H) or mismatch repair (MMR) deficiency; 6. Epigenetics; 7. Peripheral blood biomarkers; 8. Microbiota.

negative correlations reported. In an initial clinical trial with limited number of patients, Topalian, et al found that while 9 out of 25 patients with PD-L1<sup>+</sup> advanced melanoma (36%) showed objective response to anti-PD-1 treatment, none of 17 patients with PD-L1<sup>-</sup> tumors exhibited objective response, suggesting that PD-L1 expression on tumor cells is

a promising predictive biomarker for clinical responses to anti-PD-1 therapy.<sup>15</sup> In support of this, it was demonstrated preclinically that PD-L1 expression on tumor cells indicates an immune-active TME, a prerequisite for effective anti-tumor responses induced by anti-PD-1 treatment.<sup>16</sup> Subsequently, favorable clinical response to ICBs was reported in

**Table 1** Clinical trials assessing PD-L1 expression as a predictive biomarker for clinical response to ICBs.

| Therapies                           | Tumor type                                      | Target cells | Detection Ab | Cut-off         | PD-L1 <sup>+</sup>                         | PD-L1 <sup>-</sup>                          | P value                                | Reference     |
|-------------------------------------|-------------------------------------------------|--------------|--------------|-----------------|--------------------------------------------|---------------------------------------------|----------------------------------------|---------------|
| Nivolumab                           | Melanoma, NSCLC, CRC, RCC, prostate cancer      | Tumor        | 5H1          | 5%              | 36% (9/25)                                 | 0% (0/17)                                   | P = 0.006                              | <sup>15</sup> |
| Ipilimumab + Nivolumab (concurrent) | Melanoma                                        | Tumor        | 28–8         | 5%              | 46% (6/13)                                 | 41% (9/22)                                  | P > 0.99                               | <sup>39</sup> |
| Ipilimumab + Nivolumab (sequenced)  |                                                 |              |              |                 | 50% (4/8)                                  | 8% (1/13)                                   | Unknown                                |               |
| Atezolizumab                        | NSCLC                                           | TILs         | SP142        | 1%<br>5%<br>10% | 31% (8/26)<br>40% (6/13)<br>83% (5/6)      | 20% (4/20)<br>18% (6/33)<br>18% (7/40)      | P = 0.015                              | <sup>34</sup> |
|                                     | All tumors studied (melanoma, NSCLC and others) | TILs         |              | 1%              | 29% (26/90)                                | 13% (8/60)                                  | P = 0.007                              |               |
|                                     |                                                 |              |              | 5%<br>10%       | 34% (19/56)<br>46% (15/33)                 | 16% (15/94)<br>16% (19/117)                 |                                        |               |
|                                     | NSCLC                                           | Tumor        |              | 1%<br>5%<br>10% | 25% (3/12)<br>33% (3/9)<br>38% (3/8)       | 26% (9/34)<br>24% (9/37)<br>24% (9/38)      | P = 0.920                              |               |
|                                     | All tumors studied (melanoma, NSCLC and others) | Tumor        |              | 1%              | 31% (9/29)                                 | 18% (22/121)                                | P = 0.079                              |               |
|                                     |                                                 |              |              | 5%<br>10%       | 39% (7/18)<br>47% (7/15)                   | 18% (24/132)<br>18% (24/135)                |                                        |               |
| Nivolumab                           | Nonsquamous NSCLC                               | Tumor        | 28–8         | 1%<br>5%<br>10% | 31% (28/123)<br>36% (34/95)<br>37% (32/86) | 9% (10/108)<br>10% (14/136)<br>11% (16/145) | P = 0.002<br>P = 0.002<br>P = 0.002    | <sup>21</sup> |
| Nivolumab                           | Squamous NSCLC                                  | Tumor        | 28–8         | 1%<br>5%<br>10% | 17% (11/63)<br>21% (9/42)<br>19% (7/36)    | 17% (9/54)<br>15% (11/75)<br>16% (13/81)    | P = 0.9364<br>P = 0.2908<br>P = 0.6411 | <sup>22</sup> |
| Pembrolizumab                       | NSCLC                                           | Tumor        | 22C3         | 50%<br>1%       | 45% (33/73)<br>28% (50/176)                | 15% (20/131)<br>11% (3/28)                  | p < 0.01                               | <sup>18</sup> |
| Ipilimumab + Nivolumab              | Melanoma                                        | Tumor        | 28–8         | 5%              | 72% (49/68)                                | 55% (115/210)                               | Unknown                                | <sup>12</sup> |
| Nivolumab                           | Melanoma                                        |              |              |                 | 58% (46/80)                                | 41% (86/208)                                |                                        |               |
| Ipilimumab                          | Melanoma                                        |              |              |                 | 21% (16/75)                                | 18% (36/202)                                |                                        |               |
| Ipilimumab + Nivolumab              | Melanoma                                        | Tumor        | SP142        | 5%              | 58% (14/24)                                | 55% (31/56)                                 | Unknown                                | <sup>25</sup> |
| Ipilimumab                          | Melanoma                                        |              |              |                 | 18% (2/11)                                 | 4% (1/27)                                   |                                        |               |
| Nivolumab                           | Untreated metastatic melanoma                   | Tumor        | 28–8         | 5%              | 53% (39/74)                                | 33% (45/136)                                | Unknown                                | <sup>19</sup> |
| Pembrolizumab                       | NSCLC                                           | Tumor        |              | 50%             | 45% (69/154)                               | Unknown                                     | Unknown                                | <sup>24</sup> |
| Atezolizumab                        | Urothelial carcinoma                            | TILs         | SP142        | 1%<br>5%        | 22% (45/207)<br>27% (27/100)               | 13% (13/103)<br>15% (31/210)                | Unknown                                | <sup>35</sup> |
| Nivolumab                           | NSCLC                                           | Tumor        | 28–8         | 5%              | 26 (55/211)                                | Unknown                                     | Unknown                                | <sup>26</sup> |
| Atezolizumab + Paclitaxel           | TNBC                                            | TILs         |              | 1%              | 59 (109/185)                               | 54 (143/265)                                |                                        | <sup>38</sup> |

Note: Ipilimumab: anti-CTLA-4; Nivolumab, Pembrolizumab: anti-PD-1; Atezolizumab: anti-PD-L1; NSCLC: non-small cell lung cancer; CRC: colorectal cancer; RCC: renal cell carcinoma; TNBC: triple-negative breast cancer; TILs: tumor-infiltration T cells; TILCs: tumor-infiltrating immune cells; percentages (%) indicate rates of objective response.

patients with PD-L1<sup>+</sup> Hodgkin's lymphoma (reaching 87%),<sup>17</sup> non-small-cell lung cancer (NSCLC),<sup>18</sup> and melanoma,<sup>12,19</sup> which was further confirmed in expanded large scale analyses of patients with NSCLC.<sup>20–23</sup> Not only were PD-L1<sup>+</sup> NSCLCs more responsive to ICBs, more importantly, milder side effects were observed, leading to the approval of

pembrolizumab (anti-PD-1) to treat patients with metastatic PD-L1-expressing NSCLC by the FDA.<sup>24</sup> It is noteworthy to mention that in most of these studies PD-L1<sup>-</sup> tumors still responded to ICBs, albeit at lower rate, suggesting that PD-L1 expression in tumor cells is not a definitive biomarker. To this end, another study reported that

patients with previously-untreated PD-L1<sup>+</sup> melanoma did not show better clinical responses to combined anti-CTLA-4 and anti-PD-1 therapy than those with PD-L1<sup>-</sup> tumors.<sup>25</sup> This was corroborated by a more recent clinical trial of patients with previously-untreated stage IV or recurrent NSCLC after chemotherapy where distinguishable clinical responses of anti-PD-1 in PD-L1<sup>+</sup> vs PD-L1<sup>-</sup> tumors were not observed.<sup>26</sup> To reconcile these discrepant results, four factors can be considered: 1. The heterogeneity of PD-L1 expression in the tumor (in clusters) imposing a challenge to obtain a “representative” biopsy that accurately reflects the overall expression of PD-L1.<sup>27,28</sup> 2. Detection methods of PD-L1 expression: currently, PD-L1 expression is primarily evaluated by immunohistochemistry (IHC) of formalin-fixed paraffin-embedded (FFPE) tissues, which is highly dependent on the reliability and sensitivity of the detection antibodies<sup>29</sup> (Table 1). 3. Different cut-offs of PD-L1 expression (spanning from 1% to 50%) were used; additionally, the intensity of PD-L1 expression per cell need to be assessed, given the important role of tumor cell-intrinsic PD-L1 signaling in tumor cell proliferation<sup>30</sup> and metabolism.<sup>31</sup> 4. Different mechanisms for PD-L1 upregulation: intrinsically, PD-L1 expression can be modulated by key transcriptional factors such as Myc and HIF1 $\alpha$  and by epigenetic factors such as EZH2 and DNMT1<sup>32</sup>; extrinsically, IFN- $\gamma$  induces PD-L1 expression on the tumor cells.<sup>13</sup> Considering all these contributing factors and the inconsistent results, we argue that PD-L1 expression on tumor cells is more of a dynamic and inducible biomarker, reflecting a greater likelihood of response rather than a definitive predictor of clinical response to ICBs.

TILs are essential cellular components of the TME. Many of these cells express PD-L1 and govern anti-tumor response elicited by ICBs.<sup>33</sup> It is therefore pertinent to assess if PD-L1 expression on these cells predicts clinical response to ICBs with greater certainty than that of tumor cells. In support of this notion, PD-L1 expression on TILs, but not tumor cells, was associated with better response to anti-PD-L1 therapy across multiple cancer types.<sup>34</sup> This was further confirmed in a separate clinical trial in patients with metastatic urothelial carcinoma treated with anti-PD-L1.<sup>35</sup> PD-L1 expression on peripheral T cells was also positively correlated with progression-free and overall survival of patients with metastatic melanoma treated with anti-CTLA-4.<sup>36</sup> Importantly, PD-L1 expression in TILs of any intensity covering  $\geq 1\%$  of the tumor area, detected by the designated staining assay (the VENTANA PD-L1 (SP142) Assay),<sup>37</sup> was included in a recent approval by the FDA to treat TNBC patients with nanoparticle albumin-bound (nab)—paclitaxel plus PD-L1 blocking antibody.<sup>38</sup> Taken together, these results support that PD-L1 expression on TILs represents a more predictive biomarker to clinical benefit of ICBs compared to that of tumor cells.

### Tumor-infiltrating immune cells (TILs)

Responsive “hot” tumors to ICBs (e.g., melanoma and NSCLC) contain more TILs than non-responsive “cold” tumors (e.g., prostate and pancreatic cancer), underscoring the importance of TILs in predicting the efficaciousness of ICBs. However, absolute lymphocyte count alone was not

correlative with clinical responses in cohorts of melanoma patients treated with either concurrent regimen or sequenced regimen of anti-CTLA-4 and anti-PD-1<sup>39</sup>; rather, the profile of TILs appeared to be a strong predictor of patient survival in an early study using large cohorts of human colorectal cancer (CRC).<sup>40</sup> In this regard, RNA expression of genes associated with T cell activation and function in the TME positively correlated with clinical response to anti-CTLA-4 in patients with metastatic melanoma<sup>41</sup> and to anti-PD-L1 in patients with bladder tumors<sup>42</sup> and other tumors.<sup>34</sup> Besides T cell activation makers, the location of pre-existing CD8<sup>+</sup> cells (that is, at the invasive tumor margin) directly correlated with clinical response to anti-PD-1, which was further validated in a separate cohort of 15 patients with metastatic melanoma in the same study.<sup>43</sup> This result was confirmed from a different perspective in another study wherein constitutive activation of the WNT/ $\beta$ -catenin signaling in tumor cells mediates therapeutic resistance to ICBs by precluding T-cell infiltration into the tumor bed.<sup>44</sup> Collectively, these studies indicate that the immune contexture including functional state and localization of TILs may predict clinical response of ICBs.<sup>45</sup>

### IFN- $\gamma$ signaling

One of the essential effector molecules in antitumor immunity is IFN- $\gamma$ . Pioneering work from the Schreiber group using either IFN- $\gamma$  blocking antibody<sup>46</sup> or mouse models lacking essential IFN- $\gamma$  signaling molecules<sup>47</sup> convincingly demonstrated a pivotal role of IFN- $\gamma$  signaling in immunosurveillance. Binding of IFN- $\gamma$  to its receptors (IFNGR1 and IFNGR2) recruits and activates the Janus kinases, JAK1 and JAK2, leading to subsequent phosphorylation, dimerization, and activation of STAT1 (signal transducer and activator of transcription 1). Dimerized STAT1 translocates to the nucleus and modulates transcription of IFN- $\gamma$ -regulated genes,<sup>48</sup> resulting in immune cell activation. As such, we contemplated that ICBs may act through this pathway to exert their therapeutic benefits. Using IFNGR1<sup>-/-</sup> mice and adoptive T-cell therapy (ACT) approach wherein T cells harvested from the IFNGR1<sup>-/-</sup> mice were injected into recipient mice bearing palpable tumors, we found that loss of IFNGR1 in T cells completely abrogates the efficaciousness of combined anti-CTLA-4 and anti-PD-1 therapy.<sup>9</sup> Furthermore, melanoma patients that were not responsive to anti-CTLA-4 therapy harbor more copy number loss of IFN- $\gamma$  signaling genes. Consistent with this, knock-down of IFNGR1 in B16 melanoma tumor cells attenuates therapeutic effects of anti-CTLA-4.<sup>49</sup> In a separate study, Zaretsky, et al reported that loss-of-function mutations in JAK1 and JAK2 in melanoma patients causes therapeutic resistance to anti-PD-1.<sup>50</sup> These results together point to an indispensable role of IFN- $\gamma$  signaling in determining therapeutic efficacy of ICBs, suggesting that activation of IFN- $\gamma$  signaling may serve as a predictive biomarker for clinical response to ICBs. To this end, a recent clinical trial of patients with melanoma, head and neck squamous carcinoma, and gastric cancer showed that gene expression profiles (GEPs) including IFN- $\gamma$  signature genes successfully separate responders from



non-responders to anti-PD-1 therapy.<sup>51</sup> More recently, another study revealed that *Ifng* expression correlates with longer progression-free survival of patients with melanoma and NSCLC in response to anti-PD-1.<sup>52</sup> Paradoxically, a separate study reported that chronic IFN- $\gamma$  signaling in the tumor cells induced acquired resistance to ICBs via STAT1-related epigenomic changes.<sup>53</sup> In sum, expression of IFN- $\gamma$  signature genes may predict positive clinical response to ICBs in the early stage, but once resistance is developed as in recurrent tumors persistent IFN- $\gamma$  signaling may attenuate therapeutic efficacy of ICBs.

### Neoantigens and tumor mutational burden (TMB)

Tumor progression is accompanied with acquisition and accumulation of mutations. These mutations can lead to aberrant expression of self-antigens, known as tumor-associated antigens <TAAs>, or expression of sequence-modified proteins, known as tumor-specific neoantigens. Both TAAs and neoantigens can be recognized by the immune system, initiating anti-cancer immune responses. T cells recognizing TAAs and neoantigens have been utilized in ACT, a therapeutic modality utilizing patients' own T cells that are genetically or pharmacologically manipulated, expanded *ex vivo*, and reinfused into the patients to cure cancer. While ACT of T cells against TAAs has thus far only generated modest clinical effects,<sup>54</sup> ACT of T cells recognizing mutant neoantigens such as *ERBB2IP* in metastatic cholangiocarcinoma<sup>55</sup> and *PPP1R3B* in advanced melanoma<sup>56</sup> has exerted potent tumor suppression, indicating a predominant role of immune responses against neoantigens in tumor control. Interestingly, clinical activity of ICBs also relies on immune responses against neoantigens.<sup>57</sup> Combining genomics and bioinformatics approaches, Gubin, et al showed that anti-CTLA-4 and anti-PD-1 improve both the quality and magnitude of neoantigen-specific intratumoral T cell responses, which orchestrate tumor rejection.<sup>58</sup> This was also observed in a patient with stage IV melanoma whose T cell response against *ATR* (ataxia telangiectasia and Rad3 related) mutation was greatly expanded by anti-CTLA-4, resulting in objective clinical response.<sup>59</sup> Further, patients with advanced NSCLC and melanoma who have enriched clonal neoantigens are more sensitive to anti-PD-1 and anti-CTLA-4 therapies.<sup>60</sup>

In keeping with an important role of neoantigen in ICBs, tumor mutational burden (TMB) also exhibits positive correlation with clinical responses to ICBs. Melanoma patients with higher TMB showed improved long-term benefits after anti-CTLA-4 therapy.<sup>61</sup> Likewise, NSCLC patients with higher TMB were more responsive to anti-PD-1 therapy and had enhanced neoantigen-specific CD8<sup>+</sup> T cell reactivity.<sup>62</sup> In an expanded analysis of accumulative data from 1638 patients with different types of tumor, high TMB was found to be an independent predictor of clinical response to anti-PD-1/PD-L1 immunotherapy; response rates of cancer patients with high and low TMB were 58% and 20%, respectively; and PFS of high vs low TMB was 12.8 months vs 3.3 months, respectively.<sup>63</sup> To pinpoint the role of specific mutations, exploratory efforts revealed that cancer

patients with concurrent mutations of TP53 and KRAS<sup>64</sup> or BRCA-2 mutation<sup>65</sup> had greater clinical benefit to anti-PD-1.

Nevertheless, not all mutations and neoantigens correlate positively with therapeutic benefits of ICBs. Some mutations are not translated into neoantigen; rather, they mediate acquired resistance to ICBs.<sup>50</sup> For instances, loss-of-function mutation of PTEN<sup>66</sup> and activation of PI3K-AKT<sup>67</sup> render tumors resistant to ICBs. Given the essential role of T cells in governing therapeutic effects of ICBs, Heemskerk, et al proposed the concept of cancer antigenome, which states that only those mutations capable of producing T cell-recognizable neoantigens without inducing therapeutic resistance may predict clinical response.<sup>68</sup> Using whole-exome cDNA sequencing, Robbins et al successfully identified mutated antigens that can be recognized by adoptively transferred tumor-reactive T cells.<sup>69</sup> Subsequently, various other "readable" neoantigens have been reported and summarized as in Table 2.<sup>55,56,59,61,69–75</sup> To name a few, neoantigen IDH1 (R132H) can be presented by MHC class II and subsequently activates CD4<sup>+</sup> T<sub>H</sub>1 response, which has the potential to predict response to immunotherapy.<sup>74</sup> Hydroxysteroid dehydrogenase-like protein 1 (*HSDL1L25V*) mutation can be recognized by CD8<sup>+</sup> T cell initially, but this CD8<sup>+</sup> T cell response was then suppressed by immunosuppressive TME,<sup>75</sup> suggesting treatment with ICBs may revamp this CD8<sup>+</sup> T cell response and eradicate tumors. There are other neoantigens ALMS1, C6ORF89 and FNDC3B identified in two chronic lymphocytic leukemia patients, but their T cell-reactivity awaits to be evaluated.<sup>72</sup> Using a neoantigen fitness model that integrates antigen presentation by MHC and effector T cell recognition, Luksha, et al successfully predicted the survival of patients with melanoma treated with anti-CTLA-4 and patients with NSCLC treated with anti-PD-1.<sup>76</sup> Moreover, newly-developed deep-sequencing technologies will enable systematic analysis of T cell reactivity in an individual tumor to provide a profile of patient-specific neoantigens. The personalized neoantigen profile may be THE predictive biomarker for ICBs. Given that not all mutations will be translated to neoantigen, TMB can be of some reference value, but will remain as an imperfect biomarker to clinical responses to ICBs.

### Microsatellite instability-high (MSI-H) or mismatch repair (MMR) deficiency

Normal cells rely on the DNA mismatch repair (MMR) system to rectify errors in association with DNA replication. In contrast, many types of cancer cells are defective in MMR, causing accumulation of erroneous genetic sequences that are typically repeated, known as microsatellites, presenting a microsatellite instability-high (MSI-H) phenotype. Mechanistically, epigenetic hyper-methylation of the promoter of MLH1 DNA mismatch repair gene can silence the *MLH1* gene, significantly increasing microsatellite instability.<sup>77</sup> Le, et al demonstrated for the first time that patients' MMR status faithfully predicts clinical response to anti-PD-1 immunotherapy; the objective response rates of patients with MMR-deficient vs MMR-proficient colorectal cancer (CRC) were 40% (4 of 10 patients) vs 0% (0 of 18 patients). The objective response rate was even higher in patients with other types of MMR-deficient cancer, reaching

78% (5 out of 7 patients).<sup>78</sup> Subsequently, the same group conducted an extended clinical trial where they prospectively evaluated patients with different subtypes of MMR-deficient cancers treated with anti-PD-1.<sup>79</sup> Remarkably, among these 12 different tumor types, they observed an overall objective radiographic response in 53% of patients, among whom 21% had complete responses. These responses were durable and median progression-free survival and overall survival had not yet been reached by the time that the study was published. Analysis of the peripheral blood T cells from a responding patient (subject 19) revealed a rapid *in vivo* expansion of neoantigen-specific T cell clones reactive to mutant neopeptides derived from the tumor.<sup>79</sup> These data corroborated an early study showing that MSI-H colon cancer was infiltrated with activated CD8<sup>+</sup> CTLs and T<sub>H</sub>1, but these cells were suppressed by multiple immune checkpoints including PD-1, PD-L1, CTLA-4, LAG-3 and IDO, which may explain why ICBs are highly efficient in MSI-H CRC.<sup>80</sup> On the basis of these results, FDA approved pembrolizumab (anti-PD-1) to treat patients with unresectable or metastatic solid tumors that are either MSI-H or MMR-deficient, regardless of the tumor type. In addition, another anti-PD-1 antibody (nivolumab) and anti-CTLA-4 antibody (ipilimumab) were approved to treat patients with MSI-H or MMR-deficient CRC. Thus, MSI-H and MMR deficiency are arguably the most successful predictive biomarkers for clinical response to ICBs.

## Epigenetic modifications

In addition to genetic alterations (mutations, MMR deficiency, loss of IFN- $\gamma$  signaling genes, etc.) correlative with tumor sensitivity to ICBs, epigenetic modulations (mainly, DNA methylation and post-translational histone

modifications) could also contribute to therapeutic effects of ICBs.<sup>66,81,82</sup> In support, it was stated that ICB-induced functional rejuvenation of T cells largely depends on the DNA methylation states of exhausted T cells.<sup>83</sup> Using assay for transposase-accessible chromatin with sequencing (ATAC-seq), Philip, et al found that tumor-infiltrating antigen-specific CD8<sup>+</sup> T cells undergo two discrete chromatin states: a plastic dysfunctional state when loss of T cell functionality can be rejuvenated and a fixed dysfunctional state when T cell dysfunction cannot be rescued.<sup>84</sup> Chromatin states are delicately controlled by “writers”, enzymes that introduce epigenetic changes such as DNA methyltransferases (DNMTs) and histone methyltransferase (EZH2), and “erasers”, enzymes that eliminate epigenetic alterations such as histone deacetylase (HDACs). In general, DNA methylation of the cytosine residues in the CpG islands is associated with the closed heterochromatin state and transcriptional repression/silencing; histone acetylation of lysine residues (H3K9, H3K14, H4K5, and H4K16) is associated with open euchromatin state and active gene transcription; with regard to histone methylation, the closed or open chromatin states depend on the extent of methylation, that is, mono-, di-, or tri-methylation: typically, monomethylation of H3K9, H3K2, and H3K79 histone proteins leads to active transcription, whereas trimethylation results in transcriptional repression.

Accordingly, DNMT inhibitors (DNMTi) demethylate the promoters of genes encoding cancer-testis antigens (CTAs), leading to increased expression of CTAs and immunogenicity.<sup>85</sup> DNMTi can also induce interferon-stimulated genes (ISGs) and immune signaling in tumor cells by activating the transcription factor IRF7 and augmenting cytosolic dsRNAs derived from endogenous retroviral sequences (ERVs), a phenomenon known as “viral mimicry”.<sup>82,86</sup> Hence, DNMTi

**Table 2** Neoantigens in anti-tumor immune responses.

| Neoantigens                     | Functions                                                              | Tumor                           | Recognized by                         | Reference     |
|---------------------------------|------------------------------------------------------------------------|---------------------------------|---------------------------------------|---------------|
| PPP1R3B                         | Regulate glycogen synthesis in liver                                   | Melanoma                        | CD4 <sup>+</sup> and CD8 <sup>+</sup> | <sup>56</sup> |
| PPP1R3B and PLEKHM2             | PLEKHM2 related to abnormal localization of lysosomes                  | Melanoma                        | CD4 <sup>+</sup> and CD8 <sup>+</sup> | <sup>69</sup> |
| ATR                             | DNA damage sensor                                                      | Melanoma                        | CD4 <sup>+</sup> and CD8 <sup>+</sup> | <sup>59</sup> |
| FND3CB, ALMS1, and C6ORF89      |                                                                        | Chronic lymphocytic leukemia    | CD8 <sup>+</sup>                      | <sup>72</sup> |
| IDH1 (R132H)                    | Catalyze the oxidative decarboxylation of isocitrate to 2-oxoglutarate | Gliomas                         | CD4 <sup>+</sup> T <sub>H</sub> 1     | <sup>74</sup> |
| Candidates for each patients    | Unknown                                                                | Melanoma                        | CD8 <sup>+</sup>                      | <sup>61</sup> |
| ERBB2IP                         | Regulate ERBB2 function and localization                               | Metastatic cholangiocarcinoma   | CD4 <sup>+</sup> T <sub>H</sub> 1     | <sup>55</sup> |
| HSDL1 (L25V)                    | Unknown                                                                | Ovarian Cancer                  | CD8 <sup>+</sup>                      | <sup>75</sup> |
| B2M, HLA-A, -B and -C and CASP8 | B2M associated with MHC I heavy chain                                  | CRC and others                  | CD8 <sup>+</sup>                      | <sup>73</sup> |
| MUC16                           | Form a protective mucous barrier                                       | Pancreatic cancer               | CD8 <sup>+</sup>                      | <sup>70</sup> |
| PBRM1                           | Related to transcriptional activation of nuclear hormone receptors     | Clear cell renal cell carcinoma | Unknown                               | <sup>71</sup> |

Note: T<sub>H</sub>1: IFN- $\gamma$ -producing CD4<sup>+</sup> T cells.

can expose hidden immunogenic signals in the tumor cells, which in turn enhance immune signaling in tumor cells. EZH2 is another epigenetic regulator that has well-known immunoregulatory functions in sustaining  $T_{reg}$  stability,<sup>87</sup> inhibiting  $T_H1$  cytokine production,<sup>66</sup> driving T cell exhaustion,<sup>88</sup> and suppressing NK cell activity.<sup>89</sup> Expression of EZH2 in ovarian cancer cells epigenetically repressed production of chemokines CXCL9 and CXCL10 by mediating H3K27me3 (trimethylation of histone H3 lysine 27), which blocked T cell infiltration into the tumor bed.<sup>90</sup> Similar effects were observed with expressing polycomb repressive complex 2 (PRC2) (EZH2 is the catalytic subunit of PRC2) in colon cancer.<sup>91</sup> In  $T_{reg}$  cells, Ezh2 sustains their immunosuppressive activity and prevents tumor-infiltration by  $CD4^+$  and  $CD8^+$  T cells.<sup>92</sup> As such, genetic depletion of EZH2 in  $T_{reg}$  led to robust antitumor immunity; pharmacological inhibition of EZH2 in human T cells elicited phenotypic and functional alterations of  $T_{reg}$  cells and enhanced cytotoxic activity of  $T_{eff}$  cells; and, more importantly, EZH2 inhibition worked in synergy with anti-CTLA-4 therapy to boost overall therapeutic efficacy.<sup>93</sup> These findings corroborated a previous study where synergistic effects between EZH2 inhibition and anti-CTLA-4 and IL-2 immunotherapy in suppressing melanoma growth were described.<sup>94</sup> The third group of epigenetic regulators are HDACs. Utilization of HDAC inhibitors has led to some clinical successes in the treatment of cutaneous T cell lymphomas.<sup>95</sup> A recent study showed that a class I/IV HDAC inhibitor (mocetinostat) upregulated PD-L1 and class I/II human leukocyte antigen (HLA) antigen-presentation genes both *in vitro* and *in vivo*. In a syngeneic mouse tumor model, mocetinostat decreased intratumoral  $T_{reg}$  cells and MDSCs and concurrently increased intratumoral  $CD8^+$  T cells. In *ex vivo* assays, patient-derived, mocetinostat-treated  $T_{reg}$  showed significant downregulation of Foxp3. The combination of mocetinostat and anti-PD-L1 increased anti-tumor activity, as compared to monotherapies in two syngeneic HNSCC models.<sup>96</sup> Similarly, a pan-HDAC inhibitor increased acetylation of the PD-L1 gene promoter and upregulated PD-L1 expression in both human and murine melanoma cells lines, and when combined with anti-PD-1, led to more pronounced tumor regression using the B16F10 syngeneic murine melanoma model.<sup>97</sup> Given these immunomodulatory effects, epigenetic modulations (DNA methylation, histone methylation and acetylation) may represent putative biomarkers for ICB responsiveness. Targeting epigenetic alterations with inhibitors (DNMTi, EZH2i, and HDACi) may serve as an effective means to overcoming therapeutic resistance to ICBs, as they open up otherwise closed chromatin structures associated with therapeutic resistance.

### Peripheral blood biomarkers

While tumor biopsies may directly reflect the TME, they are difficult to obtain sometimes, especially for biopsies post-ICBs. Given this, some studies exploited peripheral blood samples for biomarker identification. Peripheral blood contains DNAs, RNAs and proteins released from tumor tissues, which to some extent reflect the dynamic changes in the TME. For example, the level of circulating tumor DNAs (ctDNAs) in the blood during the early stage of

treatment revealed invaluable hints on therapeutic efficacy.<sup>98</sup> Detection of hypermutated ctDNAs, as an indicator of the tumor mutational burden (TMB), positively correlated with better clinical responses to ICBs across various cancer types including melanoma, lung cancer, and head and neck cancer.<sup>99</sup> Using blood-based cell-free DNA (cfDNA) assay to measure TMB in plasma, another group successfully identified patients with NSCLC that had significant improvement of PFS upon anti-PD-L1 treatment.<sup>100</sup> Furthermore, Wang, et al constructed a cancer gene panel comprised of 150 genes (NCC-GP150), based on the plasma levels of ctDNAs. Their results indicated that NCC-GP150 matched well with tissue TMB measured by whole-exome sequencing. More importantly, patients whose NCC-GP150 detected 6 or more blood-based-TMB exhibited superior response rates to anti-PD-1/L1 therapies.<sup>101</sup> With respect to potential use of blood RNAs as predictive biomarkers, a previous report concluded that a four-gene signature including cathepsin D (CTSD), phospholipase A2 group VII (PLA2G7), thioredoxin reductase 1 (TXNRD1), and interleukin 1 receptor-associated kinase 3 (IRAK3) in the blood could predict survival of patients with melanoma treated with anti-CTLA-4 antibody.<sup>102</sup> Serum level of protein VEGF was also found to be associated with clinical responses in melanoma patients treated with anti-CTLA.<sup>103</sup>

Blood cellular components including  $CD4^+$  and  $CD8^+$  T cells and myeloid cells (monocytes, neutrophils, etc.) have been explored as predictive biomarkers for clinical response to ICBs. Two groups found that increases of central memory  $CD4^+$  T cells ( $CD27^+$ ,  $FAS^+$ ,  $CD45RA^+$ , and  $CCR7^+$ )<sup>104</sup> and IL-9-producing  $T_H9$  cells<sup>105</sup> correlated with clinical responses of melanoma patients treated with anti-PD-1. Another report detected  $PD-1^+$  tumor antigen-specific  $CD8^+$  T cells in the peripheral blood of melanoma patients,<sup>106</sup> which also existed in peripheral blood of patients responsive to anti-CTLA-4 and anti-PD-1.<sup>60</sup> Proliferating  $Ki67^+CD8^+$  effector-like T cells in the blood were another marker associated with better clinical outcomes of NSCLC patients treated with anti-PD-1-therapy.<sup>107</sup> In addition, blood myeloid cells may predict clinical response to ICBs. In patients treated with anti-PD-1 antibody, lower baseline levels of MDSCs were seen in non-relapsing melanoma patients treated with anti-PD-1<sup>108</sup>; conversely, high numbers of MDSCs were associated with poor survival of melanoma patients treated with anti-PD-1.<sup>109</sup> To track down the specific subtypes of MDSCs associated with clinical response of ICBs, Krieg, et al demonstrated that the frequency of  $CD14^+CD16^-HLA^-DR^{Hi}$  monocytes in the pre-treatment blood samples is a strong predictor for progression-free survival and overall survival of patients with stage IV melanoma treated with anti-PD-1 immunotherapy.<sup>110</sup> With this key finding, blood  $CD14^+CD16^-HLA^-DR^{Hi}$  monocytes as a predictive biomarker should be prospectively evaluated in clinical trials. Although peripheral blood analysis of ctDNAs, RNAs, and proteins can provide valuable insights into the clinical responses of cancer patients to ICBs, more investigations are needed before these biomarkers can be applied in the clinic, given the abundance of DNAs and proteins in peripheral blood is usually much lower than that in the TME. More sensitive methods need to be developed for accurate detection of ctDNAs, RNAs, and proteins.



## Microbiota

An emerging concept in immunology is that commensal bacteria are not just a mere symbiotic partner living inside our body, but actively shape immune responses, contributing to antitumor immunosurveillance. Accumulating evidence indicates that microbiota affect DC function, T cell trafficking and infiltration into the tumor site, and T<sub>reg</sub> cell stability.<sup>111</sup> The interconnection between microbiota and ICBs was initially suggested in a study where PD-1 deficiency can perturb microbial communities in the gut and mucosal immunity by acting through the axis of follicular helper T cells (T<sub>FH</sub>)-plasma cells-IgA secretion in the germinal center of Peyer's patches.<sup>112</sup> Direct evidence on the importance of microbiota in therapeutic effects of ICBs came from a study showing that fecal transfer of commensal *Bifidobacterium* bacteria boosted therapeutic effects of anti-PD-L1 by enhancing DC function and subsequent CD8<sup>+</sup> T cell priming and accumulation in the TME.<sup>113</sup> Interestingly, unlike anti-PD-L1, anti-CTLA-4 required *Bacteroides* species, but not *Bifidobacterium* bacteria for its therapeutic efficacy; *B. thetaiotaomicron* or *B. fragilis*-specific T cell responses were associated with anti-CTLA-4 efficacy; adoptive transfer of *B. fragilis*-specific T cells and fecal microbial transplantation from humans to mice confirmed that treatment of melanoma patients with anti-CTLA-4 favored the outgrowth of *B. fragilis* with anticancer properties.<sup>114</sup> Consistent with these preclinical results, the reliance of therapeutic effects of ICBs on specific commensal bacteria was also observed in cancer patients. In a clinical trial of melanoma patients treated with anti-PD-1, oral and fecal microbiome samples were prospectively collected for taxonomic profiling using 16S ribosomal RNA (rRNA) gene sequencing and metagenomic whole-genome shotgun (WGS) sequencing. Significantly higher alpha diversity and greater abundance of the *Ruminococcaceae* and *Faecalibacterium* were observed in responders; in contrast, low alpha diversity and high relative abundance of *Bacteroidales* were seen in non-responders.<sup>115</sup> Perplexingly, a separate study revealed a different spectrum of commensal bacteria (*Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium*) abundantly present in the fecal samples of melanoma patients responding to anti-PD-1 therapy; and reconstitution of germ-free mice with fecal material from responding patients led to improved tumor control, augmented T cell responses, and greater efficacy of anti-PD-L1 therapy.<sup>116</sup> The underlying reasons for this discrepancy are currently unknown, which may be due to the different methods of sampling and sequencing, different experimental designs, and different patient populations/diets in these clinical trials. In addition to melanoma, correlation of microbiome with clinical response to anti-PD-1 was observed in patients with advanced NSCLC, RCC, or urothelial carcinoma treated with anti-PD-1/PD-L1 antibodies<sup>117</sup>; metagenomic analyses of patient stool samples at diagnosis revealed correlations between clinical response to ICBs and the relative abundance of *Akkermansia muciniphila*. Consistent with previous reports, fecal microbiota transplantation (FMT) from responders into germ-free or antibiotic-treated mice restored the

antitumor effects of PD-1 blockade. More importantly, supplementation of *A. muciniphila* to the FMT with non-responders' feces can restore the efficaciousness of PD-1 blockade in an IL-12-dependent manner by enhancing the recruitment of CCR9<sup>+</sup>CXCR3<sup>+</sup>CD4<sup>+</sup> T cells into the tumor bed.<sup>117</sup> Taken together, the baseline profiles of commensal bacteria may predict clinical response to ICBs: *Bacteroides thetaiotaomicron* or *B. fragilis* in anti-CTLA-4; *Bifidobacterium* bacteria in anti-PD-L1; and *Ruminococcaceae* + *Faecalibacterium*, or *Bifidobacterium longum* + *Collinsella aerofaciens* + *Enterococcus faecium*, or *Akkermansia muciniphila* in anti-PD-1. Given this key information, two pertinent questions to address are how to nutritionally cultivate "good" endogenous commensal bacteria and how to utilize exogenously-expanded microorganisms as an immune-adjuvant to boost therapeutic efficacy of ICBs.

## Combination strategies to boost therapeutic efficacy of ICBs

Although the "perfect" biomarkers await to be identified, the above-described seminal work provides promising leads for future explorations. Considering the complexity of the TME wherein the dynamic tug-of-war between host cells (including immune cells) and tumor cells takes place, we propose an interconnected network of multiple factors, rather than individual factors in isolation would be the eventual biomarker, following the philosophy "the whole is greater than the sum of its parts". As such, we contemplate that therapies imparting multiple biomarkers would work in stronger synergy with ICBs to boost the overall therapeutic efficacy. One good example is cyclophosphamide, a commonly-used platinum-based chemotherapy. When combined with anti-PD-1, cyclophosphamide promotes tumor antigen-specific immunity by inducing immunogenic cell death and release of tumor antigens and DAMPs (danger-associated molecular patterns), decreasing immunosuppressive immune cells (T<sub>reg</sub>), and increasing tumor-infiltration of CTLs and non-T<sub>reg</sub> CD4<sup>+</sup> T cells, leading to improved tumor-free survival of mice bearing cervical cancer.<sup>118</sup> Several other chemotherapeutic agents with multifaceted immunogenic effects also synergize effectively with ICBs to drive tumor eradication. Readers interested in the combinatorial therapies of chemotherapy and ICBs are encouraged to read excellent reviews on this.<sup>119,120</sup> Here, we discuss the most recent developments of combinatorial therapies, including small molecule inhibitors, metabolic inhibitors, and radiotherapies, in conjunction with ICBs.

In this era of precision medicine, small molecule inhibitors have been developed to target specific pathways that are mutated in tumor cells, including BRAFi/MEKi, imatinib (an antagonistic agent against BCR-ABL tyrosine kinase), and CDK4/6 inhibitor (abemaciclib). Interestingly, in addition to inducing tumor cell death, these inhibitors also exerted potent multifaceted immunomodulatory impacts on both innate and adaptive immune cells. For example, administration of abemaciclib to mice bearing CRC resulted in endogenous retroviral gene/dsRNA

response, anti-viral effector function, and significant downregulation of immunosuppressive mechanisms ( $T_{reg}$ , expression of PD-1, TIM-3, CTLA-4, and LAG-3, etc.).<sup>121</sup> Interestingly, it also increased PD-L1 expression, suggesting addition of anti-PD-2 to abemaciclib treatment would further enhance therapeutic efficacy. Indeed, combination therapy of abemaciclib and anti-PD-1 greatly enhanced tumor regression and improved OS rates in mice bearing breast cancers.<sup>122</sup> Similarly, greater therapeutic benefits have been reported for combination therapies of BRAFi/MEKi + ICBs in patients with BRAF<sup>V600E</sup> mutated melanoma,<sup>123,124</sup> and imatinib + anti-CTLA-4 in patients with advanced malignancies.<sup>125</sup>

One unusual metabolic aspect of tumor cells is that they rewire their metabolic machinery to engage glycolysis (a rather inefficient energy-producing pathway compared to oxidative phosphorylation), even in the presence of ample oxygen, the well-known Warburg effect.<sup>126</sup> This metabolic reprogramming of tumor cells produces substantial amount of immune-suppressive metabolites such as lactate, adenosine, and kynurenine that hinder immune cell infiltration into the tumor bed, interfering immune surveillance and leading to therapeutic resistance to ICBs. Multiple metabolic pathways have been shown to contribute to immunotherapeutic resistance, including PI3K-Akt-mTOR, CD73-CD39 adenosinergic pathway, IFN- $\gamma$ -JAK/STAT, and Wnt/ $\beta$ -catenin. To overcome the therapeutic resistance, inhibitors against these metabolic regulators have been actively tested in conjunction with ICBs. However, provided the important roles of these metabolic pathways in mediating immune cells functions,<sup>127,128</sup> special considerations should be given to when (relative to the onset of ICBs) and how (locoregional or systemic) these inhibitors should be administered. Among the four PI3K isoforms ( $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$ ), PI3K $\delta$  may represent an interesting target, in light of its indispensable role in  $T_{reg}$  survival and proliferation but dispensable in non- $T_{reg}$  cells. Coadministration of a PI3K $\delta$ -specific inhibitor (CAL101) with a tumor-specific vaccine to mice-bearing lung cancer significantly decreased  $T_{reg}$  and increased vaccine-induced CD8<sup>+</sup> T cells in the TME, inducing potent antitumor efficacy.<sup>129</sup> In preclinical MC38-OVA (colon) and RM-1 (prostate) subcutaneous tumor models, concomitant blockade of CD73 with ICBs has also shown potent synergistic effects.<sup>130</sup> As discussed above (the IFN- $\gamma$  signaling section), the IFN- $\gamma$ -JAK-STAT axis has dual functions in anti-tumor immunity: anti-tumorigenic during the early stage and pro-tumorigenic at the late stage when acquired resistance develops. Thus, prudent temporal administration of JAKi in relation to ICBs is of critical importance to achieve the optimal outcome.<sup>49,50,53</sup> WNT inhibitors (PKF115-58, IWP-L6, and XAV939) have yielded promising immunogenic effects in mouse models of melanoma and lymphoma, justifying their use in combination with ICBs in cancer therapy.<sup>44,131</sup> Clinical testing of some these combinatorial therapies is underway and results will be available in the future (<https://clinicaltrials.gov>). On a further note, with the availability of advanced sequencing technologies, personalized combination therapy based on specific mutations in the patient may become feasible in the near future.

A cardinal feature of the TME (especially in solid tumors) is hypoxia, which is primarily regulated by HIF1 $\alpha$  (hypoxia-

inducible factor-1 $\alpha$ ). Hypoxia induces therapeutic resistance through downregulation of MHC class-I molecules,<sup>132</sup> upregulation of immune checkpoints such as CTLA-4 and PD-L1 on tumor cells,<sup>133</sup> and impairment of DNA damage response proteins (leading to more aggressive phenotype).<sup>134,135</sup> Intriguingly, many of these immunosuppressive effects can be reversed by radiotherapy (RT), particularly fractionated RT (leading to reoxygenation). Beyond direct cytotoxic effects, RT may enhance anti-tumor immune responses via releasing tumor antigens and DAMPs, increasing MHC-I expression on cancer cells,<sup>136</sup> priming and mediating maturation of antigen-presenting cells,<sup>137</sup> driving T cell infiltration to irradiated tumors,<sup>138</sup> repolarizing tumor-associated macrophages to be inflammatory M1 macrophages,<sup>139</sup> and activating natural killer cells.<sup>140</sup> Of note, RT also leads to upregulation of PD-L1<sup>141</sup> and accumulation of  $T_{reg}$ <sup>142</sup> in irradiated tumors, justifying combination therapy of fractionated RT, anti-CTLA-4 (efficiently depleting intratumoral  $T_{reg}$ ) and anti-PD-1/L1 (blocking increased PD-L1 signaling) to treat hypoxic tumors.<sup>143</sup> This strategy was applied in a recent study with both mouse tumor models and patients with melanoma, resulting in optimal clinical response and immunity.<sup>144</sup> Additional supporting evidence came from clinical trials of combination of RT (palliative or ablative) with single ICBs (anti-CTLA-4, anti-PD-1, and anti-PD-L1), which showed prolonged progression-free survival rates and increased overall survival.<sup>145–150</sup> In spite of these promising clinical outcomes, details on timing, dosage, and fractionation of RT awaits further elaboration to achieve the optimal efficacy when used in combination with ICBs. Moreover, specific mechanisms underlying the abscopal effects of these combination strategies need to be identified, which may manifest novel therapeutic targets. Considering the largely undistinguished killing of tumor cells by RT, combined RT and ICBs may represent an effective approach to overcome therapeutic resistance of some tumors.<sup>49,50</sup>

Additionally, clinical successes with anti-CTLA-4 and anti-PD-1/L1 inspired research endeavors to characterize and target other co-inhibitory molecules including TIGIT (T cell immunoreceptor with Ig and ITIM domains),<sup>151</sup> LAG-3 (Lymphocyte-activation gene 3),<sup>152</sup> and TIM-3 (T-cell immunoglobulin and mucin domain-containing protein 3).<sup>153</sup> Combination therapies by blocking multiple immune checkpoints are being tested in the clinic with promising results available from some of these clinical trials. Thus far, the best outcome of the combination therapies of ICBs is from dual blockade of CTLA-4 and PD-1, which significantly improves the survival of patients with advanced melanoma.<sup>12,25,39</sup> One of the rationales to combine anti-CTLA-4 with anti-PD-1 is that anti-CTLA-4 upregulated PD-L1<sup>9</sup> and additional blockade with anti-PD-1 effectively annihilated this inhibitory signal, augmenting efficacy. HDACi or other epigenetic modulators can open closed chromatin structures and convert “cold” tumors to “hot” ones, which may also sensitize them to ICBs.<sup>154,155</sup> Inspired by the exciting findings on the role of microbiome in orchestrating therapeutic efficacy of ICBs, feces transplantation from responding patients and dietary supplementation boosting growth of beneficial bacterial genera may prove to be effective approaches to maximize clinical benefits of ICBs. With the identification of personalized neoantigen

becoming feasible, ACT with T cells against these neoantigens in combination with ICBs is another promising approach.<sup>156</sup> All in all, our improved understanding of how ICBs function and recent developments in precision medicine and RT open doors to new opportunities to develop combinatorial approaches with enhanced efficacy. Reliable biomarkers that responsively reflect the dynamics of the immune response, be it in isolation (for specific tumor types and specific therapies) or in combination, will provide guidance to select patients for those mechanistically-justified combination strategies.

## Concluding remarks

With the burgeoning applications of ICBs in cancer treatment, a clinical concern would be “leaving some patients who may respond to ICBs behind”. In the meantime, inappropriate or overtreatment of patients with ICBs should be avoided, considering their limited efficacy to a minor subset of patients with cancer, inadvertent toxicity (some can be life-threatening) and high financial cost. This conundrum can be solved with the identification of reliable predictive biomarkers of clinical responses to ICBs and rational combination therapies. As far as the biomarker development is concerned, concerted efforts from onco-immunologists have already resulted in successes, evidenced by the incorporation of some biomarkers in the clinical decision-making (as discussed: PD-L1 staining of TILs in  $\geq 1\%$  of the tumor area by the SP142 Assay as a pre-condition for anti-PD-1 therapy of TNBC patients<sup>37</sup> and MSI-H/MMR deficiency in solid tumors (regardless of tumor types) for anti-PD-1 therapy). However, much more work needs to be done to improve the predictability of biomarkers. With the rapid advances in both immunology (e.g., immunometabolism, microbiota, etc.) and biotechnology (deep-sequencing, ATAC-seq, imaging tracer, etc.), “better” and more reliable biomarkers will become available. For example, utilization of radiolabeled high-affinity PD-1 as a PET imaging tracer would provide an accurate evaluation of otherwise highly heterogeneous PD-L1 expression within the tumor. Further, this is a noninvasive method and easier for cancer patients to accept.<sup>157</sup> On the same token, with the neoantigen fitness model capable of predicting antigen presentation by MHC molecules and recognition by T cells and deep sequencing technology, “actual” immunogenic neoantigens can be discovered as more reliable biomarkers.<sup>76</sup> Furthermore, the majority of work so far has been done with analysis of samples collected at a single static time point (mostly, pre-treatment baseline), missing essential information on the dynamic responses to ICBs, which can be gained by implementing longitudinal tumor sample collection and analysis. Doing this can detect molecular determinants after the first round of ICB treatment that better reflect the clinical response and irAEs.<sup>158</sup> While it may be easier said than done, with the advancement of imaging technology using radio-labeled tracers, it is doable. In addition, most results have been generated through retrospective analyses and in some cases with limited number of patients, which should be confirmed prospectively in clinical trials with more patients.

With all the insights gained through the seminal work on predictive biomarkers, substantial efforts to combine agents that impact multiple biomarkers with ICBs are underway, in order to augment overall therapeutic efficacy. Of note, the majority of the therapeutic agents (small molecule inhibitors, metabolic inhibitors, radiotherapies, etc.) exerted therapeutic effects at the start, but eventually lost their efficaciousness. This may be ascribed to their immunostimulatory effects in the early stage, the “Yang” impact, and subsequent immunosuppressive effects when therapeutic resistance develops, the “Yin” counter-balance mechanism. Several stereotypical examples are MEK4/6 inhibitors, RT, and HDACi. Adding ICBs to these therapies will not only overcome the “Yin” counteractive responses (e.g. upregulation of PD-L1 and increase of  $T_{reg}$ ) but also further sustain and boost the short-lived “Yang” immunostimulatory effects, tipping the “Yin-Yang” balance to the benefit of tumor eradication. Deeper understanding of the underlying mechanisms of these different therapies will allow a more rational design of combination therapies. Considering the dynamic of induced immune responses, timing and dosing are the keys to achieving successful outcomes.

## Conflict of interest

A consensus has been reached among authors on the content of this review. All authors declare no conflict of interest.

## Acknowledgements

Work in our lab has been funded by the V Foundation Scholar Award (V2018-023), ACS-IRG (91-022-19), and R21 (1R21CA230475-01A1) to L.S. All authors contributed to writing. L.S. was responsible for the overall construction and final editing of this manuscript.

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