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The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy

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Abstract

Checkpoint inhibitor-based immunotherapies that target cytotoxic T lymphocyte antigen 4 (CTLA4) or the programmed cell death 1 (PD1) pathway have achieved impressive success in the treatment of different cancer types. Yet, only a subset of patients derive clinical benefit. It is thus critical to understand the determinants driving response, resistance and adverse effects. In this Review, we discuss recent work demonstrating that immune checkpoint inhibitor efficacy is affected by a combination of factors involving tumour genomics, host germline genetics, PD1 ligand 1 (PDL1) levels and other features of the tumour microenvironment, as well as the gut microbiome. We focus on recently identified molecular and cellular determinants of response. A better understanding of how these variables cooperate to affect tumour–host interactions is needed to optimize the implementation of precision immunotherapy.

Over the past several years, immune checkpoint inhibitors (ICIs), which target inhibitory receptors on T cells and reinvigorate antitumour immune responses (FIG. 1a), have begun to transform clinical cancer care. The humanized anti-cytotoxic T lymphocyte antigen 4 (CTLA4) antibody ipilimumab has doubled 10-year survival for metastatic melanoma compared with historical data[1–4] and was approved by the United States Food and Drug Administration (FDA) for clinical use in 2011. Blockade of another immune checkpoint molecule, programmed cell death 1 (PD1), or its ligand, PD1 ligand 1 (PDL1), was then

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Author contributions

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shown to provide a survival advantage in a number of different malignancies, with higher response rates and lower incidence of side effects than anti-CTLA4 (REFS[5–11]). Accordingly, antibodies targeting the PD1–PDL1 axis have been approved as second-line or first-line therapies for an ever-growing list of malignancies, including melanoma, lymphoma, lung cancers, renal cell cancer (RCC), head and neck squamous cell cancer (HNSCC), bladder cancer, liver cancer and gastro-oesophageal cancer[12]. However, despite these substantial advancements in clinical care, the majority of patients receiving ICIs do not derive benefit. Therefore, there exists intense interest in identifying and developing predictive biomarkers of ICI response, both to enable a precision medicine approach in cancer immunotherapy and to better understand and overcome mechanisms of resistance. Recent clinical trial results underscore the need for effective biomarker-based patient selection. For example, despite equally promising phase I/II trial results for the anti-PD1 antibodies pembrolizumab[13] and nivolumab[14], phase III results showed a statistically significant benefit only with pembrolizumab[15] and not nivolumab[16,17] as first-line therapy for non-small-cell lung cancer (NSCLC) using the predefined biomarkers in those trials. It is thought that differing assays, differences in patients accrued and differences in the criteria for assessing intratumoural PDL1 expression as a biomarker for patient selection may have contributed to this unexpected discrepancy[17,18]. In addition to guiding clinical trial design, precise and accurate predictive biomarkers will be critical for personalizing patient immunotherapy in the clinic.

Decisions regarding which immunotherapy to use or whether a combination approach is warranted should ideally be guided by rational mechanistic insight to maximize disease control, reduce side effects and minimize cost. Furthermore, although immunotherapies on a whole have fewer adverse effects than chemotherapy[10,15], it is crucial to identify patients most at risk of therapy-related toxicity so that they can be properly monitored and treated (BOX 1). This Review focuses on recent advances in the identification of predictive biomarkers for ICI response, toxicity and survival. We discuss mechanistic underpinnings of the interplay between tumour and host genomics, tumour microenvironment and immune function. There has been strikingly rapid progress in these areas. We recognize that we are unable to mention every study, as the literature is too vast to cover in complete detail. Therefore, we attempt to summarize only some of the more recent and salient observations in the field.

Tumour genomes and response

Tumour antigens and mutation burden.

In order to understand why some patients benefit from ICIs and others do not, it is important to first explore how the adaptive immune system detects and identifies tumours as foreign or non-self. Nearly all nucleated cells express major histocompatibility complex (MHC; encoded by the human leukocyte antigen (HLA) gene complex in humans) proteins on their cell surface. The MHC class I (MHC I) molecules bind short peptides (8–11 amino acids) derived from intracellular proteins for presentation on the surface of all nucleated cells to $CD8^+$ T cells[19–22] (FIG. 1b). Upon recognition of antigens (for example, pathogen-derived or tumour-derived epitopes), T cells are activated to proliferate and destroy cells presenting

the recognized antigen[23]. In general, tumour-associated antigens, that is, tumour proteins the immune system recognizes as non-self, can be classified into two broad categories: non-mutated self-antigens and MHC-I-restricted and MHC class II (MHC II)-restricted neoantigens, which arise as the result of non-synonymous somatic mutations[24–29].

Self-antigens comprise non-mutated proteins that are aberrantly expressed or overexpressed in tumour cells. For example, transcriptional or epigenetic reprogramming in tumour cells can lead to expression of proteins normally restricted to trophoblasts and male gametes, known as cancer–testis (CT) antigens, such as the melanoma-associated antigens (MAGEAs) and New York oesophageal squamous cell carcinoma 1 (NY-ESO-1). Because trophoblasts and male gametes generally lack expression of MHC I molecules, CT antigens are thought to be selectively presented to the immune system by tumour cells, suggesting that they may be effective targets for immunotherapy[30]. Lineage-specific differentiation markers, such as melan-A, PMEL and tyrosinase, represent another subclass of tumour self-antigens. These proteins exhibit tissue-specific expression and are often overexpressed in tumours[31].

While non-mutated tumour self-antigens can be targets for immunotherapy, the responses they elicit may be at least partially limited by central tolerance. Notably, few generalizable associations between tumour self-antigen expression and improved ICI response have been reported to date. However, a somewhat surprising relationship between increased expression of a particular subset of MAGEA CT antigens and poor ICI response has been reported[32]. Specifically, this eight-gene cluster, referred to as the ‘anti-CTLA4 resistance-associated MAGEA’ (CRMA) cluster, is associated with poor response to anti-CTLA4 but not anti-PD1 therapy. This result was observed in two cohorts of patients with melanoma who were treated with ipilimumab[32]. While the precise mechanism underlying this association is not known, it is thought that CRMA expression causes a decrease or defect in autophagy, which in turn may interfere with antigen-processing and presentation. Notably, CRMA expression was predictive of an overall survival (OS) disadvantage in an immunotherapy-treated population, but not in the immunotherapy-naive melanoma cohort in The Cancer Genome Atlas (TCGA), indicating that CRMA expression is a predictive biomarker specific to anti-CTLA4 therapy and not prognostic of disease outcome overall[32]. It will be important to determine the predictive value of this gene signature in the setting of anti-CTLA4 and anti-PD1 combination therapy. Treatment with combined ipilimumab and nivolumab improves response rates, but treatment-related toxicity is also increased significantly[7,33,34]. To minimize toxicity, it is crucial to determine which patients will not benefit from or do not require combination therapy, and the CRMA gene expression signature might be able to identify these patients.

Despite the contributions of self-antigens to tumour immunity, the primary targets of many mouse or human tumour immune responses are tumour-specific neoantigen peptides that arise from somatic mutations in cancer genomes[26,27,35–41] (FIG. 1b). Indeed, some of the best initial ICI response rates were observed in carcinogen-driven cancers such as melanoma[1] and NSCLC[5,6], which typically have high mutation burdens owing to the mutagenic effects of ultraviolet light and cigarette smoke, respectively[42].

It is reasonable to hypothesize that the number of non-synonymous single nucleotide variants (nsSNVs) in a tumour, referred to as the tumour mutation burden (TMB), may affect the odds of generating immunogenic peptides and thereby influence ICI response in patients. Indeed, in two melanoma cohorts, patients who experienced durable clinical benefit from ipilimumab (defined as stable or regressing disease for at least 6 months) had, on average, a higher pre-therapy TMB than those who did not benefit[40]. Furthermore, patients with a TMB greater than 100 mutations had a statistically significant OS advantage. A second study of patients with melanoma reaffirmed the association between high TMB and ipilimumab response[43]. Similar results linking TMB, response and progression-free survival (PFS) were observed in discovery and validation cohorts of patients with NSCLC treated with pembrolizumab; however, the optimal TMB cut-off for stratifying PFS in this study was higher[41].

Following the initial observations in melanoma and NSCLC, significant associations between high TMB and ICI response have been reported in a variety of tumour types[44] including urothelial carcinoma[11], small cell lung cancer[45], independent cohorts of NSCLC[46–48] and melanoma[49–51] and human papilloma virus (HPV)-negative HNSCC[52]. Furthermore, a meta-analysis across 27 tumour types showed a positive correlation between average response rate and log(TMB) by linear regression analysis[53]. Notable outliers in this analysis were Merkel cell carcinoma and RCC, both of which responded better than would be expected on the basis of TMB alone, and mismatch repair (MMR)-proficient colorectal carcinoma, which responded relatively worse than TMB alone would predict. These observations make clear that while the association between TMB and ICI response is largely robust, other factors are involved. For example, it is thought that proteins from oncogenic viruses such as Merkel cell polyomavirus and HPV may act as immunogenic neoantigens. Indeed, bioinformatic analysis of TCGA data sets revealed increased cytolytic activity in stomach, urothelial and HNSCC cancers when tumours were infected with oncogenic viruses, suggesting an association between virus-driven tumours and endogenous antitumour immune responses[54]. Moreover, different tumour types may have different operative immune checkpoints and thus differing responses to ICIs.

While the aggregate data show a significant pan-cancer association between TMB and ICI response by tumour type[53], clear associations on a case-by-case basis within certain tumour types and situations are not observed. For example, clinical studies of patients with RCC[55,56], patients with HPV-positive HNSCC[52] and patients with melanoma pretreated with ipilimumab (who subsequently received anti-PD1)[50] show no significant pre-therapy association between TMB and response from inhibition of the PD1 pathway. Additionally, a large-scale bioinformatic analysis of 19 cancer types concluded that immune infiltration was associated with mutation or neoantigen burden in cancers driven by recurrent mutations but not in those driven by copy number alterations, such as breast and pancreatic cancer[57]. Interestingly, this study again identified RCC as a distinct outlier; it had the highest immune infiltration score of all cancers studied yet harboured a low mutation burden[57]. Furthermore, even when a statistically significant association exists, TMB alone does not clearly discriminate all responders from non-responders — there are some patients with high TMB who do not respond and vice versa. It has been suggested that owing to the complexity of

tumour–immune interactions, static biomarkers may not be sufficient to accurately predict response and, as such, dynamic biomarkers may be required[58].

A recent comprehensive genomic analysis of matched pre-therapy and on-therapy melanoma tumours has begun to test the utility of dynamic biomarkers in immunotherapy. Patients in this study received anti-PD1 therapy and either had previously progressed on anti-CTLA4 or were immunotherapy-naive. Notably, pre-therapy TMB associated with OS only in the immunotherapy-naive group and not in the aggregate cohort. However, early (4-week) on-therapy change in TMB (ΔTMB) was strongly associated with anti-PD1 response and OS in the entire cohort[50]. Although ΔTMB appears to be a robust indicator of anti-PD1 efficacy, its clinical utility may be challenging to implement. Because determination of ΔTMB requires an on-therapy biopsy, this metric cannot be used as a guide for initial treatment decisions; however, it may be helpful in making an early assessment of whether patients are responding to anti-PD1 therapy. Sequencing of circulating cell-free DNA (cfDNA), which is discussed below, is being studied as a way to monitor ΔTMB in a non-invasive manner. Other assays for assessing TMB are discussed in BOX 2. On its own, pre-therapy TMB can help inform treatment decisions but does not currently provide unambiguous sensitivity or specificity. However, a deeper look at the mechanisms underlying tumour–immune interactions reveals that TMB is likely just the ‘tip of the iceberg’ for tumour immunogenomics.

Neoantigen-based prediction: quantity, heterogeneity and quality.

It has been hypothesized that elevated TMB increases the chances of generating immunogenic neoantigens[59]. It follows that true neoantigen burden, that is, the number of mutations actually targeted by T cells, may have a stronger relationship with ICI response than does TMB. This raises the important question of how best to identify immunogenic neoantigens from genomic data. Traditionally, computational neoantigen predictions have focused on peptide binding to MHCs on the basis of anchor residue identities (typically positions 2 and 9 of a nonamer MHC-I-restricted peptide)[27,39–41,60–65]. However, neoantigen burden determined by this method generally performs no better than overall TMB in predicting ICI response or survival, and the positive predictive value of these predictions in functional assays is quite low[40,41,43,46,62]. Therefore, it is likely that features beyond MHC binding affinity define functionally important neoantigens. The development of tools and models to predict neoantigens has been reviewed in detail elsewhere[65–68]. Here, we discuss some recent studies relating neoantigen features to clinical outcomes with ICIs.

One critical feature of a neoantigen may simply be whether it is present in the majority or minority of cells within a tumour (FIG. 1c). In most cases, tumours evolve in a stepwise fashion, thereby creating a clonal hierarchy. Owing to the exquisite specificity of the adaptive immune system, an immunogenic neoantigen must be present within a cell for that cell to be targeted and eliminated by a T cell response. Therefore, it was hypothesized that neoantigens derived from clonal mutations (or homogeneous tumours) may elicit more effective tumour immune responses than neoantigens derived from subclonal mutations (or heterogeneous tumours) and that intratumoural heterogeneity (ITH) may therefore influence response to ICIs[69]. Indeed, by using a combination of predicted neoantigen load and ITH cut-offs to stratify patients, TMB-based survival predictions were improved across three

independent ICI-treated cohorts[69]. Similar results were observed in a fourth cohort of patients with melanoma treated with anti-PD1 in a separate study[50]. Notably, in this study, clonal neoantigen burden was predictive of survival only in patients who had not received prior ipilimumab therapy, even though anti-PD1 response was statistically indistinguishable regardless of prior ICI exposure[50]. This suggests that therapeutic history may influence the predictive power of immunotherapy biomarkers but not the efficacy of the drugs themselves (see also BOX 3). Importantly, it should be noted that high ITH has previously been associated with poor clinical outcome outside the immunotherapy setting[70,71]. Here, a key question is whether subclonal neoantigens are an indicator of the evolvability of tumours; if true, this would suggest that a reason for poor response to ICIs in tumours with high ITH is that they potentially harbour multiple mechanisms of immune evasion.

Another neoantigen property that may influence ICI response predictions is the potential for a peptide–MHC complex to be recognized and bound by a T cell receptor (TCR). Indeed, there are at least two biophysical binding events that influence peptide immunogenicity: peptide–MHC binding and TCR recognition of the peptide–MHC complex (FIG. 2). Current prediction algorithms do not take TCR recognition potential into account and therefore likely overestimate true neoantigen burden.

Owing to the vast intra-individual and inter-individual diversity of TCR repertoires, prediction of TCR binding to peptide–MHC complexes is notoriously difficult. The challenge can be conceptualized via one question: what qualities constitute ‘non-selfness’ of mutant peptides? One approach to this question has been to consider the relative immunogenic impact of point mutations that occur outside versus at the MHC-binding anchor positions. Mutations that generate novel MHC-binding sites may be more immunogenic on average because they enable MHC presentation of self proteome regions previously ‘invisible’ to the immune system. In other words, non-anchor position residues in these peptides may appear foreign to T cells, whereas mutations that occur outside of anchor positions create only a single residue of foreign sequence within peptides for which the immune system has likely generated tolerance (FIG. 2). In practice, such neoantigens can be distinguished by assessing predicted MHC binding affinity (half-maximal inhibitory concentration (IC_{50})) of the wild-type peptide relative to that of the mutant peptide, a metric termed the differential agretopicity index (DAI)[72]. High DAI values indicate that a mutation significantly increases peptide–MHC binding compared with the wild-type sequence, while a low DAI indicates unchanged or decreased MHC binding affinity. High DAI values correspond with a high rate of experimental neoantigen validation in a murine model of methylcholanthrene-induced fibrosarcoma[72]. Additionally, two studies found that DAI outperforms TMB and traditionally defined neoantigen burden for survival prediction in two of three previously published ICI-treated cohorts[73,74]. It should be noted that both studies[73,74] applied TMB cut-off values that differed from those used in the original reports[41,43]. Nonetheless, multi-variate Cox regression demonstrated that mean DAI is predictive of OS in these two cohorts independent of overall TMB, age and gender[73].

Another reported method for assessing neoantigen foreignness is based on sequence homology with experimentally validated immunogenic microbial epitopes catalogued in the Immune Epitope Database (IEDB)[75]. Considering the possible selective pressure of host–

pathogen competition on TCR genetic loci[76] and the observation that TCRs can tolerate considerable amino acid substitution in their cognate epitopes[77,78], it was postulated that peptides resembling microbial sequences might have a higher likelihood of being detected as non-self by the evolutionarily sculpted TCR repertoire[75,79]. The authors developed a neoantigen fitness model incorporating three elements — tumour clonality, DAI and microbial epitope homology, which was quantified as a nonlinear function of alignment scores[75]. Performance of this model was compared with standard TMB in three independent cohorts of ICI-treated patients using each of the three components either alone or in combination. Importantly, the fitness model incorporating all three elements successfully predicted survival in all three cohorts[75]. As a control, the authors applied the same fitness model but did so on the basis of homology with IEDB epitopes that did not elicit T cell responses in vitro. As expected, predictive power was lost for the melanoma cohorts; however, this had no effect on survival prediction in the lung cancer cohort. This may highlight a limitation of using IEDB as a reference for immunologically active versus inert peptides, as IEDB does not account for all possible HLA contexts. Another caveat of this model is that the function relating alignment score to predicted TCR binding requires optimization of two parameters, which can vary between cancer types[75,79]. Thus, determination of unique parameters for each cancer and/or therapeutic agent may be required before the model can be applied more generally[80]. In a separate study, a machine learning approach incorporating nine immunogenicity features and integration of expression levels of gene mutations was used to develop the Neopepsee algorithm[81]. Application of Neopepsee to independent cohorts of patients with melanoma and leukaemia improved neoantigen prediction sensitivity and specificity compared with traditional classification methods. Similar to the neoantigen fitness model, one of the strongest features driving classification was neoepitope similarity to known pathogenic epitopes[81].

Mutation signatures and microsatellite instability.

A mutation can be classified according to the specific base change that occurs and its surrounding sequence context. Certain mutation processes or mutagens, for example, MMR deficiency (MMRd) or ultraviolet light, produce specific mutational signatures[42]. Interestingly, the cigarette smoke-associated mutation signature, but not self-reported smoking history itself, is strongly associated with increased therapeutic response and extended PFS in patients with NSCLC treated with anti-PD1 therapy[41]. It was noted in this same study that mutations of genes involved in the DNA MMR pathway, as well as in other DNA damage repair pathways, were enriched in patients who derived durable clinical benefit. Subsequently, it was found that tumours with MMRd exhibiting micro-satellite instability (MSI) are highly sensitive to ICI therapy regardless of the tissue of origin[82,83]. Further supporting the relationship between TMB and high sensitivity to ICI is the observation that tumours with high MSI generate numerous neopeptides owing to the hypermutated phenotype[82–84]. Mechanistically, MSI-positive tumours are a specific type of high TMB tumour, with MMRd generating a high mutational load. Notably, MMRd generates many insertion and deletion (indel) mutations[82]. Some of these indels result in frameshifts that produce neoantigens that may be more immunogenic on average owing to their greater sequence divergence from self-peptides (FIG. 2). In one sense, it is not surprising that MMRd is associated with improved ICI response owing to increased TMB;

however, it is worth noting that MMRd-induced mutations tend to be predominantly subclonal, leading to highly heterogeneous tumours[42]. As discussed above, subclonal neoepitopes tend to be less effective in driving tumour clearance[69]. It is possible that the sheer volume of subclonal neoepitopes in MMRd tumours ensures that every cell possesses at least one effective immune determinant; however, it will be important to explore whether MMRd may also stimulate immune responses through antigen-independent mechanisms.

MSI-positive colorectal cancers (CRCs) are highly $CD8^+$ T cell infiltrated compared with microsatellite stable (MSS) colon cancers[85]. Counterbalancing this active immune phenotype, MSI-positive colon tumours also express high levels of multiple immune checkpoint molecules including PD1, PDL1, CTLA4, lymphocyte activating 3 (LAG3) and the interferon-γ (IFNγ)-inducible immune inhibitory metabolic enzyme indoleamine 2,3-dioxygenase 1 (IDO1)[86]. These findings suggest that both the genomics of MSI-positive tumours and their respective microenvironment may contribute to the high objective response rates commonly observed in these types of tumours. With the recent approval of nivolumab and pembrolizumab for the treatment of MSI-positive cancers of any histology in 2017, PD1 blocking antibodies have become the first drug to be granted FDA approval on the basis of a specific tumour genetic characteristic agnostic of tumour histology[87].

There are many types of mutations that cause genomic hypermutation. These include mutations in *POLE* and *POLD*, which encode DNA polymerases, and genes in the homologous recombination DNA repair pathway. Mutations in these pathways may theoretically increase neoantigen load and be associated with better response to ICI treatment. Ongoing work is attempting to determine whether these mutations predict ICI efficacy, in a manner similar to MMR mutations.

Insertion and deletion mutations.

The main types of mutations considered in most current analyses of TMB and ICI response are nsSNVs. However, indel mutations may also be a rich source of immunogenic neoantigens[88–90] and may help explain some of the apparent anomalies in the relationship between TMB and ICI response. For example, as mentioned above, in MSI-positive tumours, indel-based neoantigens may be targeted. Interestingly, RCC has a good rate of response to ICIs (approximately 25%) yet only a modest TMB compared with other cancer types[53]. RCC had the highest indel mutation burden of the 19 cancer types assessed in a pan-cancer TCGA analysis, and frameshift indel mutations were found to generate three times more candidate neoantigens per mutation than nsSNVs[91]. Despite this, no association between mutation burden of any kind (nsSNVs or indels) and response was found in patients with RCC who were treated with anti-PD1 or anti-PDL1 in separate studies[55,56]. Conversely, indel burden was positively associated with response in patients with melanoma treated with anti-CTLA4 or anti-PD1; however, high indel load was not associated with a survival advantage[91]. This finding underscores the fact that initial tumour response does not always correlate with survival advantage and highlights the need to deepen our understanding of the effects of different types of mutations for predicting ICI benefit.

Somatic copy number alterations.

In addition to nsSNVs and indels, somatic copy number alterations (SCNAs) are another feature of the tumour genomic landscape that may impact ICI response. Another pan-cancer TCGA analysis found that arm and whole chromosome-level but not focal SCNAs are negatively associated with immune infiltration in 10 out of the 12 cancer types tested[92]. This finding was subsequently replicated in a larger study of TCGA patients[93]. Interestingly, a combined SCNA and TMB score was a better predictor of response and OS than either feature alone in two independent cohorts of patients with melanoma treated with anti-CTLA4[92]. Notably, one of these cohorts contained a unique subgroup of patients who showed no evidence of tumour response yet experienced a long-term survival advantage. The SCNA score was significantly lower in this subgroup than in patients with no survival advantage. TMB did not differ between these groups, suggesting that the SCNA score provides additional discriminatory power. Indeed, it was shown that SCNA score is a significant predictor of survival independent of TMB in the setting of anti-CTLA4 melanoma therapy[92]. Similar results have been found in other cohorts of patients with melanoma treated with anti-CTLA4 (REF.[94]) or anti-PD1 but only when the anti-PD1-treated patients had not progressed on prior anti-CTLA4 therapy[50]. While it has been speculated that SCNAs may interfere with neoantigen loading onto MHCs[92], it is difficult at this time to conclude whether SCNAs play a direct mechanistic role in ICI resistance, as SCNAs are known to be a negative prognostic indicator for cancer outcomes in general[95]. Alternatively, it may be that SCNAs result in loss of genes needed for immune activity, such as the HLA genes, which reside on the commonly deleted chromosome 6. Nonetheless, it appears likely that SCNA burden will provide useful information in predictive models for ICI response.

Specific mutated genes as predictive factors.

Mutational analyses often enable subclassification of traditional histopathological tumour types into molecularly defined subtypes[96–99]. Somatic mutation of specific genes may influence the ability of tumour cells to succumb to or evade immune surveillance. In one study of patients with melanoma treated with either anti-PD1 or sequential anti-CTLA4 and anti-PD1 therapy, no association was detected between melanoma molecular subtype and ICI response[50]. However, in a separate study of patients with melanoma, anti-CTLA4 and anti-PD1 combination therapy provided a survival advantage over anti-PD1 monotherapy in patients with mutant, but not wild-type, *BRAF*[7]. Furthermore, significantly increased anti-PDL1 response was observed in the luminal cluster II subtype of urothelial carcinoma[11], and the mutant *KRAS* and *LKB1* (also known as *STK11*) subtype of NSCLC was associated with decreased response and survival in three independent cohorts of anti-PD1-treated patients[100]. Notably, there was no significant difference in TMB between this subtype and NSCLC with mutant *KRAS* alone or mutant *KRAS* and *TP53*, suggesting that *LKB1* deficiency is an independent indicator of poor anti-PD1 response in *KRAS*-mutant NSCLC[100]. Additionally, inactivation of *PTEN* has been reported to be associated with resistance to ICI treatment in melanoma preclinical models and patients[101] and in patients with uterine leiomyosarcoma[102]. In a study of 35 patients with RCC, it was found that loss-of-function mutations in the *PBRM1* gene, which encodes a component of the SWI/SNF chromatin-remodelling complex, were enriched in patients who responded to anti-PDL1

therapy[55]. In the same study, this finding was corroborated in an independent cohort of 63 patients treated with a variety of ICI single or combination therapies[55]. However, a larger study of 305 patients found that *PBRM1* mutations were associated with response to the vascular endothelial growth factor receptor (VEGFR) inhibitor sunitinib but not with anti-PDL1 therapy[56]. The reasons for this discrepancy are not immediately apparent but may be due to confounding factors that were not accounted for in the first, smaller study. Moreover, *PBRM1* mutations may be prognostic of RCC outcomes in general[99,103–105].

There have been several reports of non-subtype-defining genetic mutations that are associated with ICI resistance or response. Mutations or deletions of genes involved in the IFNγ signalling pathway and antigen presentation (the so-called deletion signature[106]) have been reported in patients with melanoma and MMRd CRC with primary[106,107] or acquired[108] resistance to ICI therapy; however, the number of patients analysed in these studies was small. In larger cohorts of patients with melanoma and NSCLC, alterations in these genes were rare and were frequently detected in responders[46,47,50]. These discrepancies could be due to differences in the functional impact of the mutations detected. The predictive value of these mutations will need to be examined further in larger data sets.

In an examination of 19 genes recurrently mutated in melanoma[98], it was discovered that mutations in the genes *SERPINB3* and *SERPINB4*, which encode related protease inhibitors, were enriched in tumours of patients with melanoma who responded to anti-CTLA4. *SERPINB3* and *SERPINB4* mutations were associated with a significant survival advantage that was independent of TMB, tumour stage and patient age in two independent cohorts[109]. Notably, *SERPINB3* and *SERPINB4* mutations are not associated with survival in the non-ICI-treated TCGA melanoma cohort, indicating that these mutations may be predictive of ICI response but not prognostic of disease outcome outside the setting of immunotherapy[109].

Another tumour-specific genetic or epigenetic alteration that may affect ICI response is the aberrant expression of endogenous retroviruses (ERVs) found throughout the human genome. Indeed, pan-cancer analyses identified associations, both positive and negative, of ERV RNA expression with T cell activity[54] and patient prognosis[110] in various cancer types. Furthermore, therapies that induce expression of ERVs, such as DNA methyltransferase inhibitors and cyclin-dependent kinase 4 (CDK4) and CDK6 inhibitors, enhance the efficacy of ICIs in mouse models[111,112]. In the TCGA cohort of patients with RCC, ERV signatures that associated positively or negatively with prognosis were identified[110]. Furthermore, increased transcript expression of specific ERVs was associated with response[110] and improved survival[113] in patients with RCC treated with ICIs.

High-throughput CRISPR screening has identified a number of genes that may be associated with ICI response, such as *PTPN2* (REF.[114]), *APLNR*[115] and SWI/SNF complex genes[116]. Furthermore, there is increasing evidence that several autoimmune diseases, including rheumatoid arthritis, autoimmune glomerulonephritis, systemic lupus erythematosus and multiple sclerosis, are influenced by epistatic interactions[117]. Thus, it will be of great importance to study how epistasis influences antitumour immunity and response to ICI therapy.

Patient germline genetics

Tumour mutational landscape meets host genetics: the role of HLA.

Pathogens are thought to be one of the strongest selective forces in human evolution, and the continuous interactions between humans and microorganisms have likely contributed to the tremendous immunologically related genetic variation found in human populations[118–120]. HLA genes are the most polymorphic genes in the human genome (FIG. 2), and they encode a critical component for immunosurveillance[19,121,122].

HLA class I (HLA-I) diversity is characterized by a remarkable sequence variation in the peptide-binding region (that is, the pocket where antigens are bound)[123,124]. The human genome contains up to six different primary HLA-I alleles, encoded by three genes (*HLAA*, *HLAB* and *HLAC*) located on chromosome 6 (REF.[19]). Each of these HLA-I variants presents a distinct, though often overlapping, repertoire of peptides termed the human immunopeptidome.

Our recent analysis of more than 1,535 patients with cancer treated with ICIs found that the presence of a more diverse array of HLA-I molecules (HLA-I heterozygosity) in a given individual is associated with increased survival, possibly owing to the ability to present a broader range of tumour antigens to T cells[125,126]. Interestingly, this effect of HLA-I heterozygosity on increased survival after ICI seemed mostly associated with the *HLAB* and *HLAC* loci, presumably because the MHC encoded by *HLAB* binds to a greater diversity of peptides and *HLAC* is generally expressed at higher levels in antigen-presenting cells than in other cell types[127,128]. Further, the association of HLA-I heterozygosity with extended survival was enhanced when also considering the TMB[125].

Notably, patients treated with anti-PD1 therapy who were heterozygous at all HLA-I loci had higher on-therapy clonality of TCRs than homozygous patients[125]. In other words, heterozygous patients were able to undergo better clonal expansion of their TCR repertoires. Given that only a small fraction of somatic mutations are usually immunogenic in a tumour[26,39,62,129–131], these findings indicate that somewhat small differences in the number of available HLA-I molecules may influence the strength of antitumour T cell responses after immunotherapy.

Additionally, specific HLA-I supertypes[132,133] are associated with survival after ICI therapy. For example, some *HLAB44* supertype alleles were associated with improved survival in patients with melanoma receiving ICIs[125]. The majority of alleles in the *HLAB44* supertype share a preference for glutamic acid (E) at anchor position 2 and polar and hydrophobic residues at the carboxyl terminus[134]. Interestingly, one of the most enriched amino acid mutations in melanomas is G>E, which indicates that there might be an enrichment of presentation of HLA-B44-restricted tumour-derived mutated epitopes in melanomas[125]. These observations highlight the need to understand how both the mutational signatures across cancer types and the HLA-I genotype of patients interact to impact the repertoire of neoepitopes presented in tumour cells. In addition to a possible enrichment of neoepitopes presented by *HLAB44* alleles, previously identified tumour-specific antigens commonly

expressed in melanomas are HLA-B44-restricted, including melanoma-associated antigen 3 (MAGEA3), which can be presented by *HLAB*44:03* and *HLAB*18:01* (REF.[135]).

During tumour progression, neoplastic cells must avoid immune destruction[136–138]. One mechanism that facilitates immune evasion is the disruption of the antigen presentation pathway. For example, tumours downregulate HLA-I expression[139–142], acquire damaging mutations in HLA-I genes[143], disrupt the function of the stabilizing β_2-microglobulin (β_2M) molecule[108,144–148] or harbour loss of heterozygosity (LOH) of HLA-I genes, wherein the maternal or paternal HLA-I haplotype is somatically lost[125,149–152]. Perhaps not surprisingly, some patients with germline heterozygous HLA-I loci can harbour somatic LOH at the HLA-I in their tumours, which has been associated with reduced response to ICI therapy[125]. A recent report showed that loss of HLA expression may affect ICI response[153]. Moreover, as mentioned above, the relevant role of the antigen presentation machinery in antitumour immune response has been further confirmed in CRISPR–Cas9 screens in preclinical models[115].

Other host immune-related genetic polymorphisms influencing response to immune checkpoint inhibitors.

People vary in their response to infectious diseases and can also exhibit different susceptibility levels to autoimmune and chronic inflammatory diseases, which is due in part to genetic variation in immune response genes[118,154–157]. Thus, it is likely that other host immune genetic polymorphisms have the potential to shape each individual cancer and contribute to the effectiveness of ICIs. Preclinical data indicate that anti-CTLA4 therapy can induce crystallizable fragment (Fc)γ receptor (FcγR)-dependent cytotoxicity in vitro and deplete intratumoural regulatory T (T_{reg}) cells in vivo[158]. This resulted in an increased ratio of intratumoural T effector cells to T_{reg} cells and enhanced antitumour immunity[158]. It was further shown that in patients with advanced melanoma, a single nucleotide polymorphism in the gene encoding the FcγR(*CD16A*^{V158F}) that resulted in an increased affinity for immunoglobulin G (IgG) was associated with better response to anti-CTLA4 in inflamed tumours[158]. It is possible that polymorphisms in other immune-related loci (for example, HLA class II genes, non-classical HLA-I genes and the genes that encode antigen peptide transporter 1 (TAP1) and TAP2, tumour necrosis factor (TNF), nuclear factor-κB (NF-κB) and Janus kinase (JAK)–signal transducer and activator of transcription (STAT) family members) may also be associated with tumour response to ICIs. Further work investigating how overall host genetic variation affects response to ICIs is warranted.

The immune microenvironment

PDL1 expression.

It is expected that PDL1 should be expressed for anti-PDL1 or anti-PD1 therapy to have an effect. Accordingly, immunohistochemistry (IHC) assays for PDL1 protein expression have been developed for clinical use. Currently, the FDA has approved PDL1 IHC as a companion diagnostic for anti-PD1 therapy for patients with NSCLC[159,160]. For example, pembrolizumab is approved for patients with NSCLC who are PDL1$^+$ (defined as PDL1 on at least 50% of tumour cells for first-line use and at least 1% for second-line use.) Despite

this, PDL1 expression remains an imperfect predictor of ICI response. Multiple studies in a variety of tumour types have found a positive correlation between PDL1 expression and ICI response or OS[6,11,13,15,46,161–164], while others have detected no association[16,52,165,166]. The details and implications of these findings have recently been reviewed extensively[159,160,167–169]. Here, we highlight a brief overview of some outstanding issues. Potential reasons for the contradictory results of PDL1 biomarker studies include the use of different detection assays, temporal and ITH of PDL1 expression and non-standardized criteria and cut-offs for assessing positivity, for example, percent $PDL1^+$ versus staining intensity. Furthermore, even when PDL1 expression is correlated with response, there are many patients with low to no detectable PDL1 expression who experience durable clinical benefit[169]. Therefore, PDL1 status is likely not a sufficiently comprehensive standalone biomarker for therapeutic decisions in the clinic. Importantly, a study of patients with NSCLC treated with combination anti-CTLA4 and anti-PD1 found that PDL1 staining and TMB are independent predictors of response and that a multivariable model including both factors improves the sensitivity and specificity of predictions[46]. Recently, in the Keynote 189 phase III trial, it was reported that PDL1 levels may be predictive of response to pembrolizumab plus chemotherapy in the setting of first-line treatment for patients with NSCLC[170]. The investigators observed that PDL1 expression enriched for responders to the immunotherapy plus chemotherapy regimen at all levels of PDL1 expression; however, the predictive effect of PDL1 positivity decreased markedly as the PDL1 threshold was relaxed. As another important caveat, some studies have found that the cell type on which PDL1 is expressed should also be considered[171]. In independent cohorts of patients with melanoma[172] and patients with urothelial carcinoma[11,173], it was found that PDL1 expression on tumour-infiltrating immune cells, but not on tumour cells themselves, was associated with response to anti-PD1 or anti-PDL1, respectively.

Tumour-infiltrating lymphocytes: density, phenotype and diversity.

It is thought that ICI therapy, particularly anti-PD1 and anti-PDL1, acts in part by reinvigorating a pre-existing tumour immune response[50,174,175]. Therefore, another potential predictor of ICI response is the density of tumour-infiltrating lymphocytes (TILs) within a tumour (see also BOX 4). In fact, TIL density is a strong positive prognostic indicator for some tumour types regardless of ICI therapy[176]. For example, a metric known as the Immunoscore, which involves quantification of $CD8^+$ T cells at the centre and periphery of a tumour, is a strong predictor of OS that can complement traditional tumour–node–metastasis (TNM) staging or MSI status in CRC[176–179]. Notably, single cell sequencing has shown that $CD4^+$ memory T cells are also enriched in human melanomas that respond to ICI[180]. In the context of anti-PD1 therapy, it has been reported that TIL density as measured by IHC at the invasive margin of a tumour, as opposed to central infiltration, is most strongly associated with anti-PD1 response[174]. This approach may be promising, but standardization has been difficult, and additional data on the generalizability of this assay are needed.

Immune-inflamed

The immune-inflamed profile is characterized by the presence of both $CD8^+$ and $CD4^+$ T cells in the parenchyma of the tumour; these T cells are spatially positioned in the proximity of tumour cells[250]. The inflamed tumour microenvironment is usually accompanied by the

expression of programmed cell death 1 ligand 1 (PDL1) on infiltrating immune cells and tumour cells[172,250,251] and the expression of other immune checkpoint molecules[86], which suggests that these type of tumours have pre-existing antitumour immune responses. These tumours have been associated with better response to ICI therapy[172,174].

Immune-excluded

The immune-excluded phenotype is characterized by the presence of different immune cell types that cannot penetrate the parenchyma of the tumours but instead are contained in the stroma that surrounds the cancer cells[172,250,252,253]. The presence of tumour-derived WNT–β-catenin signalling can result in T cell exclusion and resistance to ICI therapy[254]. more recently, increased transforming growth factor-β (TGFβ) signalling was shown to promote exclusion of $CD8^+$ T cells from the tumour parenchyma[173,255]. These studies found that blockade of TGFβ signalling in preclinical models that allows for lymphocyte infiltration into the tumour converts the tumour microenvironment to a more inflamed state and makes it more susceptible to programmed cell death 1 (PD1)–PDL1 checkpoint blockade.

Immune-desert

The immune-desert phenotype is characterized by the absence of abundant T cells in either the parenchyma or the stroma of the tumour[172,250,256]. Not surprisingly, these tumours do not respond well to ICI therapy[172].

Increasing evidence suggests it is not only the location and density of TILs but also their phenotype that impacts ICI outcomes. For example, although TIL density did not significantly change with anti-PD1 therapy in patients with melanoma who had previously progressed on anti-CTLA4, response was associated with increased levels of a T effector cell activity metric known as the cytolytic score (CYT)[50] (calculated as the geometric mean of perforin 1 (PRF1) and granzyme transcript levels[54]). Notably, CYT is significantly associated with TMB on a case-by-case basis regardless of tumour type, supporting the notion that immune control of cancer involves T cell-mediated cytolytic responses against tumour neoantigens derived from nsSNVs[54].

In addition to T effector activity status, other T cell phenotypic markers are associated with ICI response. For example, the level of PD1 expressed on the surface of TILs may be related to the efficacy of an anti-tumour immune response. Specifically, an analysis of TILs from patients with NSCLC revealed three distinct populations of $CD8^+$ T cells on the basis of PD1 expression[181]. The intratumoural population with the highest PD1 expression was termed $PD1^{Tumour}$ ($PD1^T$) because they were transcriptionally distinct from the more terminally exhausted $PD1^{hi}$ cells typically associated with chronic viral infections. Of the three isolated $CD8^+$ T cell populations, only the $PD1^T$ cells were capable of mounting a cytokine response against autologous tumour cells in vitro, suggesting that this cell population was enriched in tumour antigen-specific T cells. Importantly, pre-therapy levels of $PD1^T$ cells were positively associated with anti-PD1 response[181]; however, these results remain to be tested in a validation cohort. By contrast, studies in mice indicate that T cells with high PD1 expression may exhibit an irreversibly exhausted phenotype as a result of T_{reg} cell interactions[182], chromatin remodelling[183,184] or transcriptional reprogramming[185–187]

in response to chronic antigen exposure. Additionally, a study of patients with HNSCC found that the percentage of intratumoural T cells with high PD1 expression was directly associated with poor PFS[188] following resection; however, these patients were not treated with ICI, and patient HPV status may be a confounding variable in HNSCC. Another study of patients with NSCLC treated with anti-PD1 identified a unique population of $CD4^{+}FOXP3^{-}PD1^{hi}$ T cells ($4PD1^{hi}$) that accumulated in tumours and peripheral blood as a function of tumour burden[189]. Importantly, an on-therapy decrease in peripheral blood $4PD1^{hi}$ cell count was significantly associated with improved OS in this cohort[189].

A unique feature of the NSCLC-associated $PD1^{T}$ T cells is their expression of the chemokine CXCL13, which specifically recruits B and T follicular helper (T_{FH}) cells[181]. Interestingly, $4PD1^{hi}$ cells had a T_{FH} cell-like phenotype[189]. Whether $PD1^{T}$ and $4PD1^{hi}$ T cells are mechanistically linked in the tumour microenvironment is currently unknown; however, an intratumoural CXCL13–T_{FH} cell–B cell axis was previously shown to be associated with prolonged survival in patients with non-ICI-treated CRC[190]. Although it is generally thought that B and T_{FH} cells are not primary components of antitumour immune responses, these findings suggest that this compartment of the tumour microenvironment may warrant further investigation in the context of ICI response.

The application of novel single cell RNA sequencing (scRNA-seq) platforms allows high-resolution phenotypic characterization that is not possible with bulk sequencing methods. Unsupervised clustering of single cell transcriptome data from 32 patients with metastatic melanoma treated with ICIs identified two major intratumoural $CD8^{+}$ T cell phenotypes: memory-like and exhausted[180]. The ratio of memory-like to exhausted TILs was strongly associated with response to ICIs. Furthermore, it was found that the transcription factor TCF7 is selectively expressed in T cells with a memory-like phenotype. As such, the ratio of $CD8^{+}TCF7^{+}$ to $CD8^{+}TCF7^{-}$ TILs was strongly associated with response and improved survival in an independent cohort of patients with melanoma treated with anti-PD1, even when no significant differences in total T cell infiltration were detected[180].

An effective T cell response involves activation and expansion of specific antigen-reactive T cell clones. Therefore, clonal architecture of the intratumoural or peripheral T cell repertoire may be another indicator of tumour immunogenicity related to ICI response. T cell repertoire diversity can be quantified using two complementary metrics: richness, that is, the number of unique TCR sequences, and clonality, that is, the equality of sequence distribution (where low clonality indicates equal distribution of all clones and high clonality indicates a skewed or oligoclonal population, with a few clones predominating)[50]. The relationship between intratumoural or peripheral T cell repertoire diversity and ICI response appears to be complex, as some studies find that pre-therapy[94,174] or post-therapy[191] TIL clonality levels are positively correlated with response, while others show that only on-therapy increases of clonality are associated with anti-PD1 response[50,192]. Another study of 30 patients with melanoma found no association of TIL diversity with anti-PD1 response or survival but did not test for on-therapy changes[193]. Interestingly, a study of 29 patients with urothelial cancer treated with anti-PDL1 found that pre-therapy peripheral T cell clonality was negatively associated with PFS and OS, while intratumoural T cell clonality had no association with survival[194]. Similarly, a study of 25 patients with metastatic pancreatic

cancer found that peripheral T cell clonality was negatively associated with survival in patients treated with anti-CTLA4 but not anti-PD1 (REF.[195]). To further complicate matters, prior immunotherapy exposure may influence T cell repertoire dynamics during subsequent anti-PD1 administration. Specifically, an on-therapy increase of intratumoural T cell richness was associated with anti-PD1 response in patients with melanoma who had progressed on anti-CTLA4 therapy, whereas an increase in T cell clonality was associated with anti-PD1 response in immunotherapy-naive patients with melanoma[50]. These results suggest that more work will be required to clarify the utility of intratumoural and peripheral T cell diversity as indicators of ICI response.

Tumour immune microenvironment: beyond T cells.

Many other types of immune cells may affect ICI efficacy. Although none are currently being measured by FDA-approved assays for prediction of ICI efficacy, work is being done to decipher the effects of these cell types on response rates. In order to gain a more comprehensive view of the tumour immune microenvironment, methods for extracting immune cell phenotype and abundance data from RNA sequencing results have been developed[190,196,197]. Such computational immune deconvolution was applied to pre-therapy and on-therapy tumour samples from patients with melanoma and revealed that anti-PD1 response was associated with an increase in $CD8^+$ T cells and natural killer cells and a decrease in macrophages[50]. This and additional data hint at a potential inhibitory role of myeloid cells in ICI response[198–200]. A similar correlation between tumour-associated macrophages (TAMs) and poor anti-PD1 response was observed in an independent cohort of patients with melanoma[201]. Mechanistically, TAMs may sequester therapeutic anti-PD1 antibodies from T cells[202].

Furthermore, peripheral blood levels of a poorly differentiated population of myeloid cells, known as myeloid-derived suppressor cells (MDSCs), correlate with poor anti-CTLA4 response in patients with melanoma[203–206]. Indeed, in a study that used immune deconvolution and machine learning to identify a set of parameters predictive of CYT across all TCGA tumour samples, one of the most critical features identified was a lack of MDSCs[197]. The other primary features of this model, known as the immunophenoscore, were enrichment of $CD8^+$ T cells, γδ T cells and natural killer cells, along with depletion of T_{reg} cells[197]. Notably, the immunophenoscore demonstrated high sensitivity and specificity in stratifying responders and non-responders in two independent cohorts of ICI-treated patients[197]. In an independent study of patients with RCC, a high intratumoural myeloid signature was associated with a nearly sixfold decrease in median PFS in patients receiving anti-PDL1, again highlighting the inhibitory impact of myeloid cells on ICI response[56]. Notably, coadministration of a VEGF inhibitor improved median PFS more than eightfold in patients with tumours with a high myeloid content[56], suggesting that the myeloid signature may be a useful biomarker in selecting combination therapies for patients with RCC.

Specific subsets of myeloid cells may be important in regulating T cell exclusion. It was reported that exclusion of T cells from pancreatic carcinomas may be regulated by $LY6C^{low}F4/80^+$ macrophages. These macrophages, which reside outside the tumour microenvironment, orchestrate sites of acquired T cell immune privilege, and eliminating

them promotes the capacity of immunotherapies to induce T cell-dependent tumour killing[207]. In summary, examination of the tumour microenvironment shows substantial promise as a predictive biomarker for ICI efficacy.

Transcriptomic and epigenetic signatures.

Bioinformatic analysis of RNA sequencing data has been used to uncover global tumour and microenvironment features associated with ICI response or resistance. One study identified a pre-therapy signature comprising 26 transcriptomic sub-signatures that associated with anti-PD1 resistance in a cohort of 28 patients with melanoma[51]. However, this signature was not associated with anti-CTLA4 or anti-PD1 response in two other independent melanoma cohorts[50,51], suggesting that it may reflect features unique to the original cohort analysed. Another study of patients with melanoma treated with anti-PD1 identified a pre-therapy RNA expression signature associated with on-therapy changes of tumour clonal architecture[50]. It was hypothesized that because changes of tumour clonal structure were associated with response and OS, the pre-therapy RNA signature may also be predictive of therapeutic outcomes. Indeed, this RNA signature associated with improved survival in two independent melanoma cohorts, one treated only with anti-PD1 and another treated with either anti-CTLA4 or anti-PD1 (REF.[50]). An RNA signature that has been validated in multiple clinical trials is the IFNγ-related inflammatory NanoString profile for pembrolizumab[208]. Preliminary data suggest that this inflammation gene signature has predictive value in a number of cancer types[28,209]. Recent work has harnessed the exquisite cellular resolution of single cell transcriptome profiling to identify a tumour cell transcriptional programme associated with T cell exclusion[210]. This signature was predictive of poor ICI response and reduced survival in multiple independent cohorts of patients with melanoma. Notably, in vitro experiments indicated that this T cell exclusion programme can be reversed by CDK4 and CDK6 inhibition, thus suggesting a potential combination strategy for patients with tumours expressing this transcriptional programme[210].

Beyond genomic and transcriptomic signatures, epigenetic alterations may also be associated with response to ICIs. Using DNA methylation microarrays, an epigenetic signature that associates with ICI response was identified and validated in independent cohorts of patients with NSCLC[211]. Furthermore, the methylation status of a single gene, *FOXP3*, was also found to be predictive of ICI response[211].

Systemic markers

Ideally, routine clinical biomarkers should be assessed in a minimally invasive manner. Therefore, there is great interest in developing whole blood-derived or serum-derived predictive biomarkers of ICI response. Importantly, peripheral blood neutrophil to lymphocyte ratio values greater than five were associated with decreased PFS and OS in multiple studies of anti-CTLA4 and anti-PD1 across a wide range of cancer types[212–216]. Several other features of peripheral blood composition have been associated with ICI response, including total lymphocyte count, T cell clonality, monocyte count, circulating T_{reg} cell levels, cytokine levels (for example, IL-6, IL-8 and IL-10), relative eosinophil count, circulating monocytes or MDSCs and lactate dehydrogenase (LDH) activity. These

and other systemic factors have recently been reviewed comprehensively[217–219]. Notably, although typically a negative prognostic indicator in melanoma, pre-therapy levels of circulating VEGFC were associated with improved PFS in a cohort of 76 patients with melanoma treated with anti-CTLA4 and anti-PD1 combination therapy[220]. Additionally, tumour cells can release extracellular exosomes containing PDL1 on their surface[221,222]. An on-therapy increase in circulating exosomal PDL1 was associated with response in a cohort of 39 patients with melanoma treated with anti-PD1 (REF.[221]), while increased expression of PD1 and CD28 on exosomes was associated with improved PFS in a cohort of 59 patients treated with anti-CTLA4 (REF.[223]). Although promising in terms of feasibility, clinical implementation of these blood-based biomarkers will require large-scale prospective validation.

It is also possible that tumour genomic factors associated with ICI response may be determined from blood-derived cfDNA, an approach known as a liquid biopsy[218]. For example, it may be possible to determine relative TMB through sequencing analysis of cfDNA. Indeed, the number of mutations detected in cfDNA was positively associated with ICI response and OS in a trial of 69 patients representing 23 different cancer types[224]. Furthermore, retrospective analysis of cfDNA from two large-scale randomized trials (n = 211 and 583) demonstrated that cfDNA-derived TMB is robustly associated with improved survival in patients with NSCLC treated with anti-PDL1[225]. It will be important to determine whether this non-invasive approach can also be used to measure relative ΔTMB at an early on-therapy time point (4 weeks)[50] to potentially improve sensitivity and specificity of response predictions.

Commensal microbiota

Commensal microorganisms, collectively known as the microbiota, influence human immune responses in health and disease[226,227]. Importantly, it appears that the diversity and composition of the gut but not the oral microbiota can influence ICI response in mice[228,229] and humans[230–234]. Four independent studies in which baseline faecal samples were analysed have found an association between specific gut bacteria and ICI response in melanoma[230,231,233], NSCLC, RCC and urothelial cancer[232]. Although there is some overlap between the four reports, each study identified different strains of bacteria that associate with response or resistance. The reasons for these variable results are not immediately apparent but may include differences in microbial sequencing and analysis techniques as well as geographic variations in the distribution of gut flora. Nonetheless, in each case, adoptive transfer of response-associated bacteria to germ-free or antibiotic-treated mice was able to confer ICI sensitivity, suggesting that the identified bacteria were sufficient to promote ICI response. Furthermore, increased microbiota diversity, irrespective of species identity, was associated with improved ICI response in humans[232,233]. Notably, one study found that the ratio of response-associated (‘good’) to resistance-associated (‘bad’) bacteria was able to clearly stratify responders from non-responders[231]; however, it remains to be determined whether this metric is generally applicable in independent cohorts. An important implication of these findings is that factors affecting the gut microbiota will likely affect ICI therapy response. Indeed, patients treated with antibiotics, but not proton-pump inhibitors, during the course of ICI therapy had decreased antitumour response[232]. It is not entirely

clear whether this decreased tumour response is due to depletion of the gut microbiota or simply a result of the infections that necessitated antibiotic use. Nonetheless, these results are tantalizing and implicate the gut microbiota as an influential factor in antitumour immunity and ICI response.

Conclusions

How close are we to a unified predictive model for ICI efficacy? In short, we are not there yet. The current understanding of the clinical response to ICI therapy unequivocally indicates that there cannot be a single biomarker to identify patients who will likely benefit from this immunotherapy. Therefore, the development of a predictive model that takes into account the different components that affect tumour–host interactions is needed (TABLE 1). Importantly, this type of quantitative model will provide a unique opportunity to evaluate the individual contribution of each of these elements for response to ICIs and to assess the presence of confounding factors. Predictive models will require a combination of different types of data for training and evaluation. These variables may include, for example, DNA sequencing data of the tumour to estimate TMB, detect presence or absence of specific genetic alterations, assess ITH and estimate the fraction of SCNAs; RNA sequencing data to evaluate whether the immune phenotype will favour sensitivity to ICIs; germline DNA sequencing data to detect patient genetic polymorphisms (for example, HLA diversity); and IHC for PDL1 expression and expression of other checkpoint molecules and features of the tumour microenvironment. Furthermore, these predictive models will require a continuous process of model update and re-evaluation as more knowledge on the molecular determinants of response to ICIs is unravelled. Such quantitative models for response to ICIs will have profound implications in the area of precision immuno-oncology. Ultimately, clinical use will be governed not just by the science but also by feasibility and reproducibility in the ‘real world’ clinical setting, cost and investment to establish prospective validation. The ongoing intensive work to establish and understand biomarkers for ICI response prediction holds great promise for maximizing patient benefit from these transformative therapies.

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Competing interests

T.A.C. is a co-founder of Gritstone Oncology and holds equity. T.A.C. holds equity in An2H. T.A.C. acknowledges grant funding from An2H, AstraZeneca, Bristol-Myers Squibb, Eisai, Illumina and Pfizer. T.A.C. has served as an adviser for An2H, Bristol-Myers Squibb, Eisai, Illumina and MedImmune. T.A.C. holds ownership of intellectual property on using tumour mutation burden to predict immunotherapy response, with a pending patent, which has been licensed to Personal Genome Diagnostics. J.J.H.’s spouse is a full-time employee of Regeneron Pharmaceuticals.

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Box 1 |

Biomarkers of toxicity and understanding how toxicity relates to response

Although immune checkpoint inhibitors (ICIs) generally induce fewer toxic side effects than standard chemotherapy, it is still important to anticipate which patients may experience severe immune-related adverse events (irAEs). Low baseline interleukin 6 (IL-6) levels and female sex are independent risk factors for development of irAEs, and low IL-6 levels were also associated with increased overall survival (OS) in 140 patients with melanoma treated with anti-cytotoxic T lymphocyte antigen 4 (CTLA4)[235]. Another study used a proteome array to identify baseline serum antibody reactivity in 78 patients treated with ICIs. machine learning identified baseline antibody signatures associated with irAEs. These signatures achieved greater than 90% sensitivity and specificity[236]. Additionally, the constitution of the gut microbiota may also be associated with ICI-induced colitis. Baseline representation of species from the Bacteroidetes phylum was associated with decreased risk of ICI-induced colitis in a prospective study of 34 patients with melanoma treated with anti-CTLA4 (REF.[237]). While these are promising results, all these factors must be tested in larger independent cohorts to determine their general applicability as predictors of ICI-induced irAEs. Importantly, there have been conflicting reports of whether irAEs are associated with ICI response. A number of studies have reported a positive association between the on-therapy incidence of irAEs and response or OS for patients treated with anti-CTLA4 (REF.[238]) or anti-programmed cell death 1 (PD1)[239–241], while others found no association with OS[242,243]. Whether or not irAEs are associated with ICI response, it is important to note that many patients with irAEs experience clinical benefit from ICIs, even if ICI infusions are discontinued owing to toxicity.

Box 2 |

Assessing tumour mutation burden in the clinic

Tumour mutation burden (TMB) can be assessed using a number of next-generation sequencing (NGS) platforms, including whole-exome sequencing, whole-genome sequencing or targeted panel sequencing. Whole-exome sequencing of tumour and normal paired samples enables the most accurate, unbiased identification of somatic mutation burden. NGS using panels that cover predefined cancer genes can also be used to estimate TMB[11,244]. Currently, US Food and Drug Administration (FDA)-approved NGS panels that can be used to estimate TMB include the MSK-IMPACT panel and the FoundationOne CDx panel, with other solutions being developed. Because TMB is a continuous variable, establishing cut-offs for use for immunotherapy response prediction will be important. These cut-offs are likely to be different for different tumour types. ongoing work is progressing on this front to address this issue. Harmonization of reporting methods also needs to be achieved. Notably, a multi-institutional effort to tackle this issue for TMB is currently being conducted by the Friends of Cancer Research Consortium.

Box 3 |

Effects of prior cytotoxic therapies

Because immune checkpoint inhibitor (ICI) response is associated with increased tumour mutation burden (TMB), it is tempting to speculate that prior therapy with DNA-damaging agents may improve response to ICIs by increasing TmB; however, sufficient data are not currently available to support this notion. Prior treatment with chemotherapy was associated with increased overall survival relative to patients with prior surgery or radiation therapy in an observational study of patients with head and neck squamous cell carcinoma treated with anti-programmed cell death 1 ligand 1 (anti-PDL1)[52]; however, larger trials of anti-programmed cell death 1 (anti-PD1) in the neoadjuvant or first-line settings for melanoma or non-small-cell lung cancer, respectively, have shown response and survival values similar to or better than those observed in heavily pretreated patients[15,47]. Importantly, prior treatment with chemotherapy may increase the prevalence of subclonal mutations and promote intratumoural heterogeneity[69,84]. As discussed in the main text, both of these factors are inversely associated with ICI response. Furthermore, analysis of neoantigens observed in ovarian tumours that relapsed following primary chemotherapy treatment showed that less than 5% of novel neoantigens were attributable to chemotherapy-associated signatures. Rather, the vast majority of mutations acquired upon relapse had arisen from mutational processes inherent to the tumours before chemotherapy exposure[245]. Together, these results suggest that chemotherapy exposure is not a predominant factor influencing neoantigen generation.

Box 4 |

Tumour–immune phenotypes influence response to immune checkpoint inhibitors

In 1998, it was found that the exclusion of $CD8^+$ T cells from the vicinity of tumour cells correlated with poor long-term clinical outcome in human colorectal carcinoma[246]. This result was validated in subsequent studies[177,247–249]. Similar characteristics of the tumour microenvironment can affect the efficacy of immune checkpoint inhibitors (ICIs)[172,174]. The overall immune infiltration of tumours can be broadly classified into three patterns: immune-inflamed, immune-excluded and immune-desert[250].

Predictive biomarkers

Measurable biological entities or phenotypes that are associated with response to a specific therapy.

MHC class I

(MHC I). A group of cell surface molecules (major histocompatibility complex (MHC) molecules) present on essentially all nucleated cells that bind and present peptides of approximately 8–10 amino acids in length to $CD8^+$ T cells.

MHC class II

(MHC II). A group of cell surface molecules (major histocompatibility complex (MHC) molecules) expressed primarily on professional antigen-presenting cells such as dendritic cells and macrophages that bind and present peptides of approximately 13–25 amino acids in length to $CD4^+$ T cells.

Central tolerance

The elimination of developing self-reactive T cell clones in order to prevent autoimmunity.

Static biomarkers

Biomarkers determined at a single point in time.

Dynamic biomarkers Biomarkers

determined by the relative change in a measurement over time.

Sensitivity

A measure of the proportion of patients who respond to therapy who were predicted to respond according to a biomarker, for example, biomarker-positive patients who actually respond divided by all patients who actually respond.

Specificity

A measure of the proportion of patients who do not respond to therapy who were predicted not to respond according to a biomarker, for example, biomarker-negative patients who do not respond divided by all patients who do not respond.

Positive predictive value

The probability that a biomarker-positive patient will benefit from a therapy.

Clonal mutations

Mutations acquired early in tumorigenesis that are present in all or most clones. These are also sometimes referred to as truncal mutations.

Subclonal mutations

Mutations acquired later in tumorigenesis that are present in a much smaller percentage of clones relative to clonal mutations.

Somatic copy number alterations

(sCNAs). Focal or chromosome-level deletions and amplifications that arise in tumours.

Epistatic interactions

Interactions of multiple genes that influence a phenotype.

HLA-I supertypes

Individual human leukocyte antigen class I (HLA-I) alleles can be classified into discrete HLA-I supertypes on the basis of similar peptide anchor binding specificities.

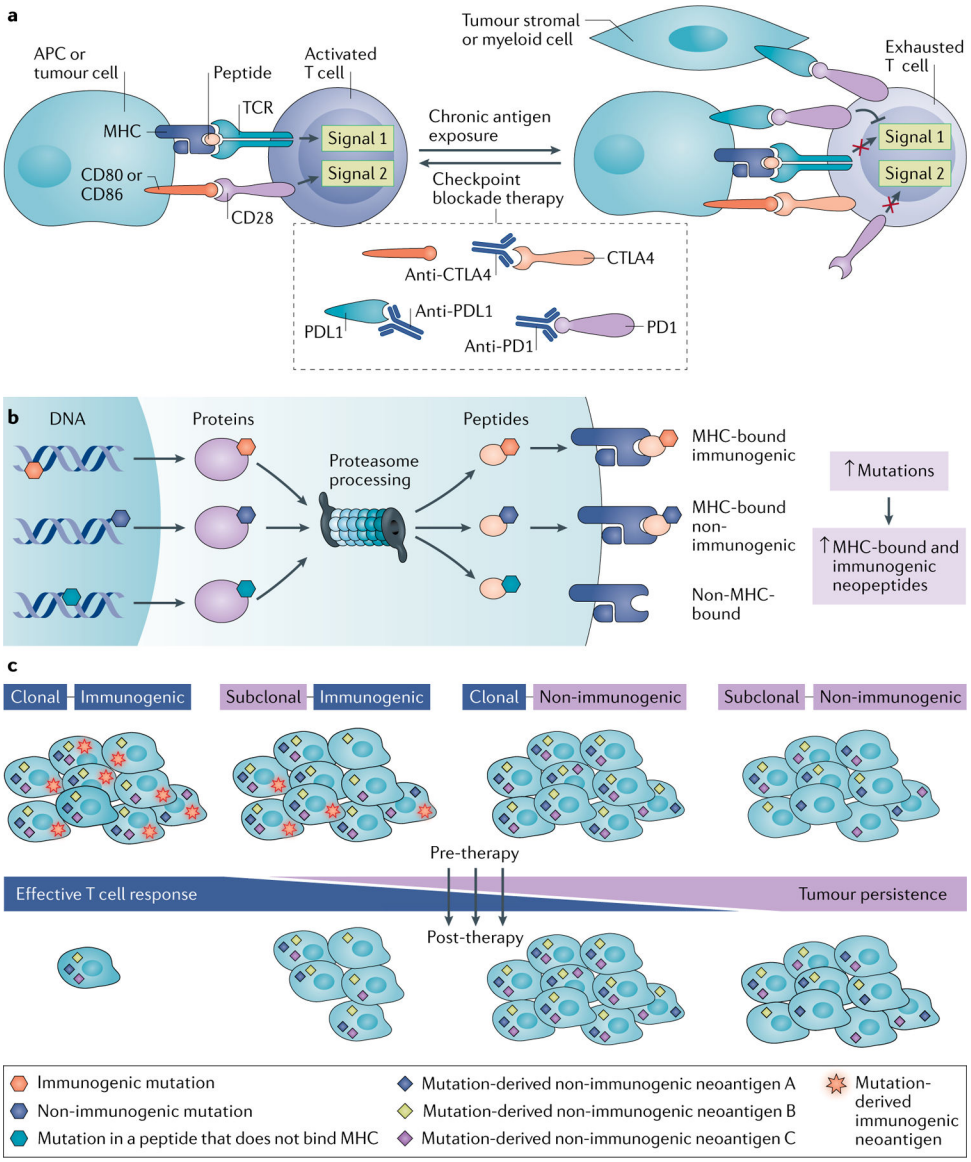

Fig. 1 |. Immune checkpoint blockade and somatic mutations.

a | T cells recognize and become activated against peptide antigens through ligation of T cell surface receptors. Two signals are required for T cell activation. Signal 1 is generated by the binding of major histocompatibility complex (MHC)-presented immunogenic peptide antigen to the heterodimeric T cell receptor (TCR). Signal 2, also referred to as co-stimulation, is transduced via ligation of the T cell co-stimulatory surface receptor CD28 to its ligand CD80 (also known as B7–1) or CD86 (also known as B7–2) on the surface of professional antigen-presenting cells (APCs). Once activated, T cells begin to express co-inhibitory cell surface receptors, such as cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death 1 (PD1). Like CD28, CTLA4 binds CD80 and CD86, but with significantly higher affinity. CTLA4 ligation with CD80 or CD86 blocks co-stimulation (signal 2) and prevents continued T cell activation. Blockade of the CTLA4–CD80 or CTLA4–CD86 interaction therefore promotes activation of T cells in secondary lymphoid

organs. Binding of PD1 to its ligand, PD1 ligand 1 (PDL1), inhibits signalling downstream of the TCR, thereby blocking signal 1. PDL1 is frequently expressed on tumours or in the tumour microenvironment. Therefore, PD1-targeted or PDL1-targeted antibody therapeutics can reinvigorate exhausted T cells at the tumour site. **b** | In tumours, mutated or aberrantly expressed proteins are processed via the immunoproteasome into peptides. These peptides can be loaded onto MHC class I (MHC I) molecules depending on the identity of their anchor residues (often positions 2 and 9). MHC-I-presented mutant peptides may or may not elicit a $CD8^+$ T cell response depending on a number of factors including peptide sequence, TCR sequences and immune infiltration. A high mutation burden increases the chances of generating MHC-presented immunogenic neoepitopes. **c** | Both peptide immunogenicity and intratumoural clonal heterogeneity influence tumour immune responses.

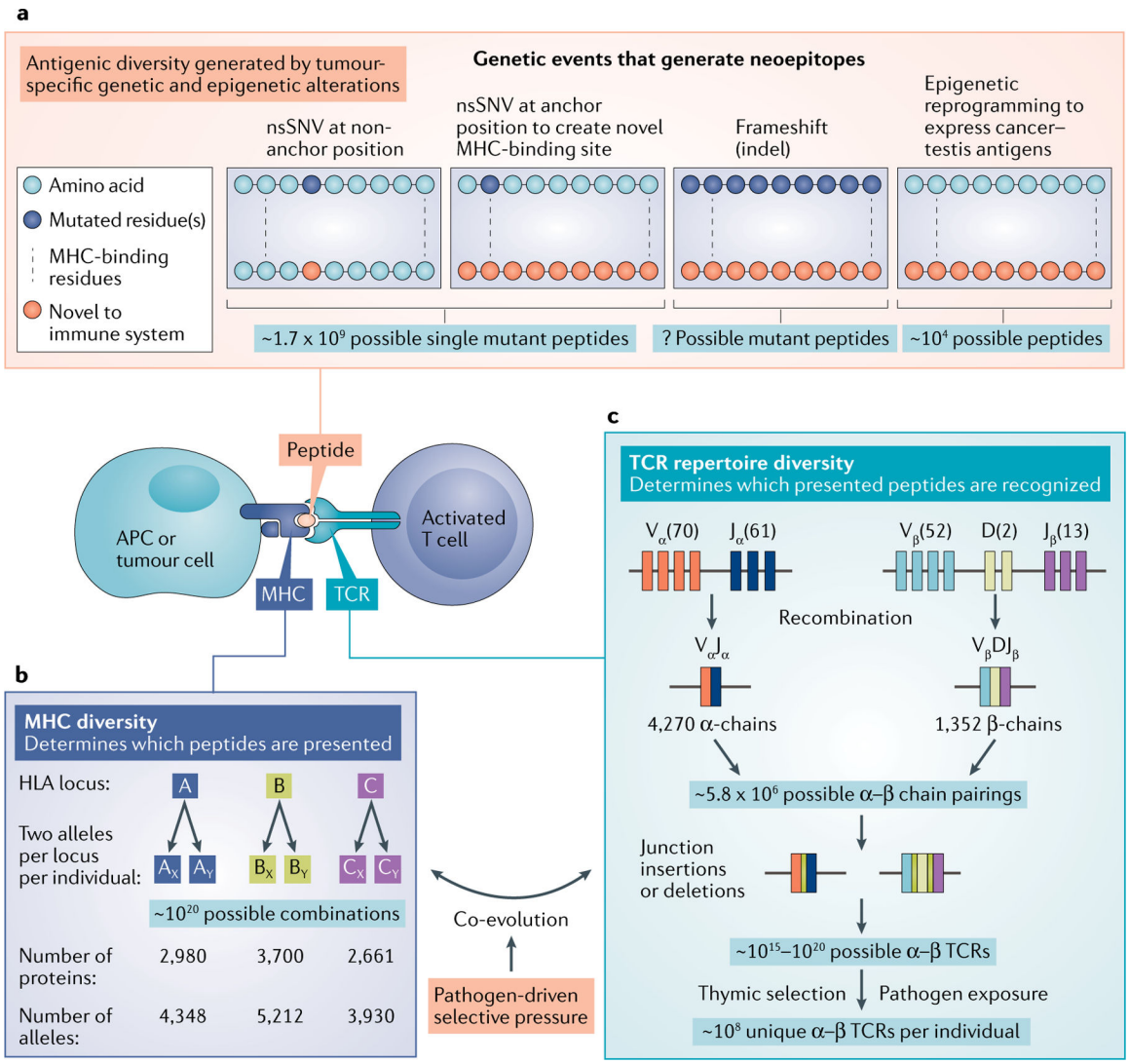

Fig. 2 |. Multiple sources of diversity converge to influence tumour immunity.

The immunological synapse, consisting of a major histocompatibility complex (MHC)-presented peptide and a cognate T cell receptor (TCR), is the lynchpin of adaptive tumour immunity. Accurate predictions regarding this critical ternary complex could translate into predictions of immune checkpoint inhibitor (ICI) efficacy; however, each contributing element is characterized by vast evolutionarily sculpted diversity, rendering predictions challenging. Many current predictive algorithms consider only peptide–MHC binding. Multivariable models incorporating peptide processing, human leukocyte antigen (HLA) genotype and TCR repertoire analysis will be critical for improving predictions of tumour immunity and ICI response. **a** | Immunogenic neoepitopes can arise from non-synonymous single nucleotide variants (nsSNVs), small insertion or deletion (indel) mutations resulting in frameshifts or epigenetic reprogramming that allows aberrant expression of genes normally restricted to trophoblasts or early phases of development. Each process generates peptides that are foreign to the immune system; however, the fraction of amino acids per peptide that appears foreign to the immune system will differ. An nsSNV at a non-anchor,

that is, non-MHC-binding, residue will generate a peptide that differs by only a single residue from a wild-type peptide previously presented to the immune system during thymic selection. As such, it is possible that an individual may have previously developed tolerance for neoepitopes generated in this manner. Alternatively, if an nsSNV generates a novel MHC-binding site, it is likely that the immune system has never been exposed to any part of the resultant MHC-presented peptide and that tolerance has not been developed. The same is true for neoepitopes generated via indel-induced frameshifts and epigenetic reprogramming. **b** | The HLA genes are among the most polymorphic in the human genome. There are thousands of known *HLAA*, *HLAB* and *HLAC* alleles. Each individual possesses two alleles of each HLA gene, resulting in vast inter-individual diversity in the ability to present tumour-derived neoepitopes. (Note that the HLA allelic combination estimate does not take into account lineage disequilibrium, which may reduce the total possible combinations empirically observed.) **c** | TCRs recognize and bind HLA-presented peptide epitopes. Every individual possesses a unique TCR repertoire generated via the semi-random recombination of variable (V), diversity (D) and joining (J) gene segments within every developing T cell of the body. TCR diversity is further multiplied by the deletion or insertion of nucleotides at gene segment junctions through the activity of terminal deoxynucleotide transferase. T cell clones subsequently undergo positive and negative thymic selection to enrich for T cells that bind self-MHC molecules but do not bind self-peptides. It is thought that both HLA and TCR genes have evolved to bind pathogen-derived sequences. This may influence which tumour-derived neoepitopes are most likely to elicit a productive T cell response. APC, antigen-presenting cell.

Table 1 |

Factors that predict response to immune checkpoint inhibitor therapy

Factor	Association with favourable clinical outcome	Validated in phase III clinical trial?	Predictive versus prognostic[a]	Cancer type	Tissue type for biomarker assessment[b]	Possible assay type for biomarker assessment
Tumour mutation burden	Positive	Yes	Predictive	Multiple cancer types	Blood or tumour tissue	NGS WES or targeted gene panel sequencing
PDL1 expression	Positive	Yes	Predictive	Multiple cancer types	Tumour tissue	Immunohistochemistry
Copy number variation	Negative	TBD	Prognostic, predictive or both	Multiple cancer types	Tumour tissue	NGS WES or targeted gene panel sequencing
HLA class I diversity	Positive	TBD	Predictive	Melanoma and NSCLC	Blood	NGS WES or PCR- based typing
LOH at HLA class I alleles	Negative	TBD	Predictive	Melanoma	Tumour tissue	TBD
T cell repertoire clonality change	Positive	TBD	Predictive	Melanoma	Tumour tissue or blood	TBD
T cell- inflamed microenvironment	Positive	TBD	Prognostic, predictive or both	Multiple cancer types	Tumour tissue	NGS RNA-seq or immunostaining
SERPINB3 or *SERPINB4* mutations	Positive	TBD	Predictive	Melanoma	Tumour tissue	NGS WES
Gut microbial diversity	Positive	TBD	Predictive	Melanoma	Oral or gut	PCR or NGS
Specific gut microbial species	Positive or negative	TBD	Predictive	Melanoma	Oral or gut	PCR or NGS
TGFβ expression	Negative	TBD	Predictive	Colon cancer and urothelial cancer	Tumour tissue	NGS RNA-seq or expression panel
Mutations in the β-catenin pathway	Negative	TBD	Predictive	Melanoma	Tumour tissue or blood	NGS WES, targeted gene panel sequencing or RNA-seq
JAK2 mutations (rare)[c]	Negative	TBD	Predictive	Melanoma	Tumour tissue or blood	NGS WES or targeted gene panel sequencing
B2M mutations (rare)[c]	Negative	TBD	Predictive	Melanoma	Tumour tissue or blood	NGS WES or targeted gene panel sequencing
STK11 mutations (common)	Negative	TBD	Predictive	NSCLC	Tumour tissue or blood	NGS WES or targeted gene panel sequencing

HLA, human leukocyte antigen; LOH, loss of heterozygosity; NSCLC, non-small-cell lung cancer; NGS, next-generation sequencing; PDL1, programmed cell death 1 ligand 1; RNA-seq, RNA sequencing; TBD, to be determined; TGFβ, transforming growth factor-β; WES, whole-exome sequencing.

[a] Predictive refers to a given biomarker that has an effect dependent on the immune checkpoint inhibitor therapy, and prognostic refers to a biomarker that has a specific effect independent of the therapy.

[b] Blood detection of mutations refers to cell-free DNA analysis.

[c] *JAK2* and *B2M* mutations are controversial. Responses have been seen in patients with these mutations. Intratumoural heterogeneity likely needs to be assessed along with these mutations.