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Mechanisms of checkpoint inhibition-induced adverse events

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Summary

Immune checkpoint inhibition has revolutionized the treatment of several solid cancers, most notably melanoma and non-small-cell lung cancer (NSCLC). Drugs targeting cytotoxic T lymphocyte antigen (CTLA)-4 and programmed cell death 1 (PD-1) have made their way into routine clinical use; however, this has not been without difficulties. Stimulation of the immune system to target cancer has been found to result in a reduction of self-tolerance, leading to the development of adverse effects that resemble autoimmunity. These adverse effects are erratic in their onset and severity and can theoretically affect any organ type. Several mechanisms for immune-related toxicity have been investigated over recent years; however, no consensus on the cause or prediction of toxicity has been reached. This review seeks to examine reported evidence for possible mechanisms of toxicity, methods for prediction of those at risk and a discussion of future prospects within the field.

Keywords: cancer, checkpoint, CTLA-4, immunotherapy, PD-1

Introduction

A relationship between immune cells and cancer was hypothesized in the 19th century [1]. A series of groundbreaking discoveries in the latter half of the 20th century led to an understanding of the importance of tumour-promoting inflammation and avoidance of immune destruction in tumorigenesis [2,3]. Ultimately, this has led to the development of paradigm-changing immunotherapies as a novel class of cancer therapeutics.

Cytotoxic T lymphocyte-associated protein-4 (CTLA-4/CD152), a member of the immunoglobulin superfamily mainly expressed on activated lymphocytes, was shown to play an inhibitory role in T cell responses [4]. Then, in 1996, it was demonstrated that CTLA-4 inhibition could enhance the anti-tumour response in mice [5]. During this time, further immune checkpoints such as programmed cell death protein 1 (PD-1/CD279) were described [6]. Since the mid-1990s our understanding of the role of inhibitory checkpoints in cancer immune evasion has grown, and with it the range of potential targets for checkpoint inhibitor (CPI) therapy. Several immune checkpoints [including V-domain immunoglobulin (Ig) suppressor of T cell activation (VISTA), lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and ITIM domain (TIGIT), T cell immunoglobulin-3 (TIM-3) and CD134 (OX40)] and their inhibitors, are under investigation in both

preclinical and clinical trials [7]. The potential underlying successful strategies for recruiting an immune response against cancer was acknowledged by *Science* magazine in 2013 in naming cancer immunotherapy the 'breakthrough of the year' [8]. Ultimately, the advances in checkpoint inhibition led to a Nobel prize for James P. Allison and Tasuku Honjo in 2018 for the discovery of cancer therapy by inhibition of negative immune regulation.

Clinical development of CPIs began with ipilimumab (a fully human, IgG1κ monoclonal, anti-CTLA-4 IgG1 antibody), closely followed by the PD-1 targeting antibodies pembrolizumab (a humanized, engineered, monoclonal, anti-PD-1 IgG4 antibody) and nivolumab (a fully human, monoclonal, anti-PD-1 IgG4 antibody). Antibodies to the PD-1 ligand (PD-L1) followed, and collectively these antibodies are licensed alone and in combination for a growing number of cancer indications. Early human studies indicated that up-regulation of the immune response through CPI led to specific immunomodulation-related adverse effects known as immune-related adverse effects (irAEs), and increasing clinical use of these agents has shown that these effects pose a significant health challenge [9].

The CTLA-4 pathway

CTLA-4 is expressed on naive T cells after stimulation [10] and is constitutively expressed on forkhead box protein 3 $(FoxP3)^+$ regulatory T cells [11]. It regulates T cells in the early immune response, predominantly in lymph nodes, and acts as a competitive CD28 homologue. It has a greater affinity for B7-1 (CD80), and to a lesser degree for B7-2 (CD86), than does CD28 for these ligands (Fig. 1) [12]. T cell receptor signalling up-regulates CTLA-4 expression on the cell surface, reaching maximal expression 48–72 h post-stimulation [12,13]. CTLA-4 ligation triggers an inhibitory feedback loop within the cell, mediated through the tyrosine phosphatase Src homology region 2-containing protein tyrosine phosphatase 2 (SHP-2) and the serine/threonine phosphatase PP2A, which dephosphorylate downstream signalling kinases (Fig. 2). CTLA-4 also acts extracellularly, and has been shown to transendocytose CD80/CD86 [14], resulting in degradation of these ligands and impaired co-stimulation via CD28. As such, studies have shown that CTLA-4 downmodulates helper T cell activity and enhances immunosuppression mediated by regulatory T cells [15].

The PD-1/PD-L1 pathway

Lymphoid and myeloid cells widely express PD-1 [16]. PD-1 ligation suppresses T cells in peripheral tissues, and the PD-1/PD-L1 pathway has an important role in the maintenance of self-tolerance. The binding of PD-1 to its ligands, PD-L1 and PD-L2, inhibits T cell proliferation and the production of proinflammatory cytokines [17] (Fig. 3). This inhibitory function is mediated through the tyrosine phosphatase SHP-2, resulting in the dephosphorylation of proximal signalling kinases [18] (Fig. 2). While PD-L2 expression is more limited, PD-L1 is expressed

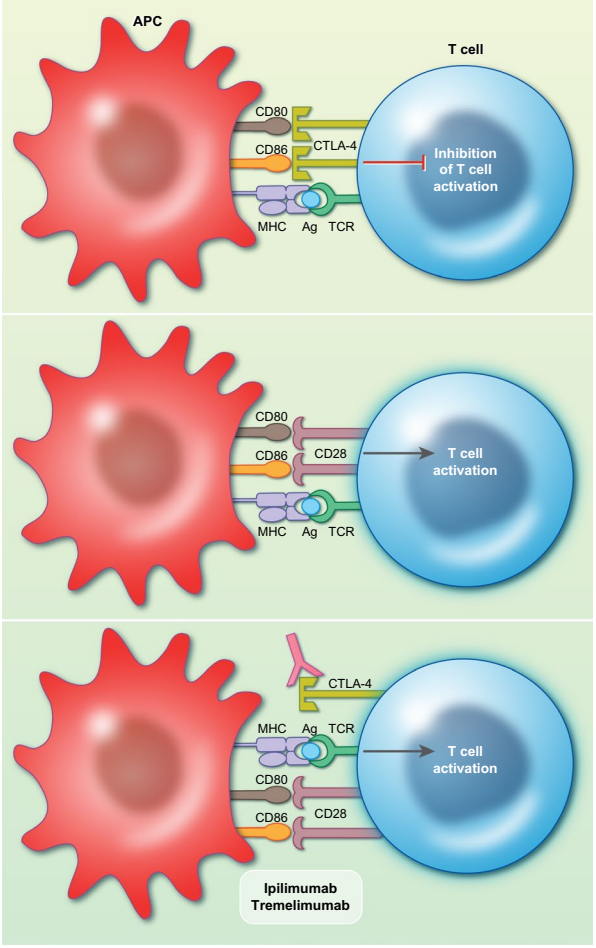

Fig. 1. The cytotoxic T lymphocyte antigen 4 (CTLA-4) pathway is a target of immune checkpoint inhibitors. The CTLA-4 pathway negatively regulates T cells in the early immune response. For initial T cell activation, two signals are required. Upon the delivery of signal 1 via T cell receptor–major histocompatibility complex (TCR–MHC) interaction, CTLA-4 is up-regulated on the cell surface, with peak expression at 48–72 h post-TCR stimulation. Signal 2 of T cell activation is attained via interaction of CD28 with the co-stimulatory molecules CD80 and CD86. As a CD28 homologue, CTLA-4 also binds CD80 and CD86, but with a greater affinity than CD28. CTLA-4 ligation with CD80/CD86 results in reduced CD28 binding, as well as negative downstream signalling through CTLA-4, both of which result in inhibition T cell activation. This pathway has become a target of novel anti-cancer therapies known as checkpoint inhibitors. Ipilimumab and tremelimumab are the two current CTLA-4-targeting monoclonal antibodies.

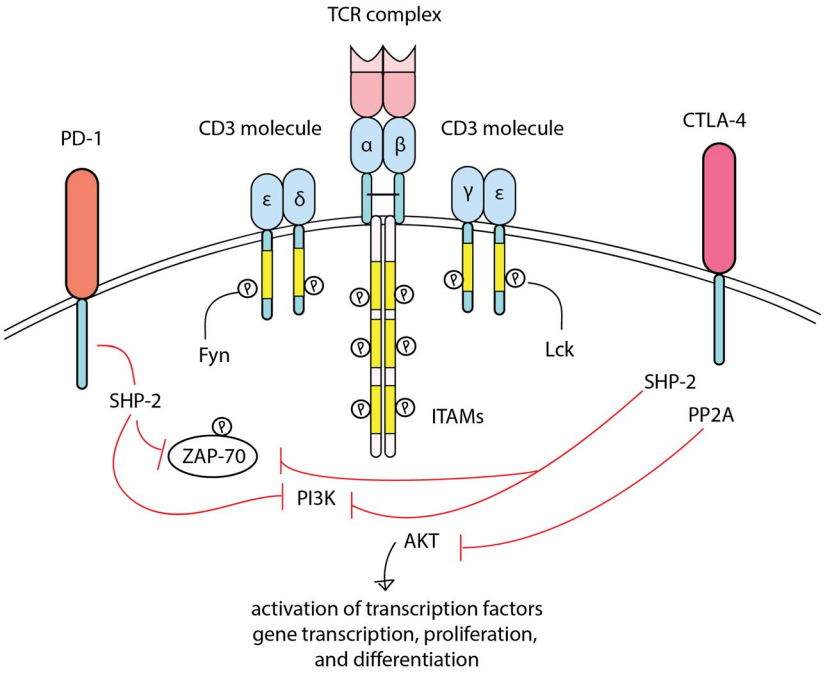

Fig. 2. Downstream signalling of programmed cell death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) is mediated by signalling phosphatases. Engagement of the T cell receptor (TCR) with major histocompatibility complex (MHC) begins a cascade of intracellular signalling resulting in T cell activation. The TCR cannot signal intracellularly itself; this is accomplished instead by the adjacent CD3 chains containing immunoreceptor tyrosine-based activation motifs (ITAMs). Following TCR engagement, the ITAM motifs are phosphorylated by Fyn and Lck kinases, resulting in ZAP-70 recruitment. ZAP-70 is then phosphorylated by Fyn and Lck, activating it, and allowing the continuation of the downstream signalling. PD-1/programmed cell death ligand 1 (PD-L1) binding suppresses this pathway through the actions of the phosphatase Src homology region 2-containing protein tyrosine phosphatase 2 (SHP-2), which dephosphorylates ZAP-70 and PI3K, inhibiting the signalling cascade. CTLA-4 exerts its actions similarly through SHP-2, but also through PP2A, which dephosphorylates AKT, further inhibiting the pathway.

more broadly on leucocytes, non-haematopoetic cells and non-lymphoid tissues [19,20].

In conditions of antigen persistence, such as chronic infection or cancer, repetitive T cell stimulation leads to a state of exhaustion and poor response. In a model of chronic viral exposure exhausted $CD8^+$ T cells express multiple inhibitory receptors, including PD-1, with PD-1 inhibition enhancing the T cell response [21]. PD-1 blockade may also enhance natural killer (NK) cell activity and antibody production [16]. Notably, while both CTLA-4 and PD-1 are induced following T cell receptor (TCR) signalling, CTLA-4 is rapidly and continuously internalized from the cell surface while, in the context of chronic stimulation, PD-1 expression is maintained [22,23].

Regulatory approvals for ipilimumab, nivolumab, pembrolizumab and other PD-1/PD-L1-directed antibodies have been awarded alone and in combination among multiple cancer indications. Detailed reviews on discovery, proposed mechanisms and implications of blockade can be found elsewhere [16,18,24,25]. This review focuses on current state-of-the-art research on mechanisms and effects of irAEs and potential biomarkers that could help to predict their development.

Toxicity of immune checkpoint inhibitors

Immune modulation resulting from checkpoint inhibition can alter normal self-tolerance and result in immune-related adverse effects. In preclinical cynomolgus monkey models the irAEs seen in humans were not observed [9]. As immunotherapy has made its way into regular clinical use, prediction of immune toxicity both in preclinical study and in clinical practice remains a major challenge. The advantages and disadvantages of different preclinical models have recently been reviewed by Ochoa de Olza *et al.* [9].

IrAEs are diverse, affecting almost every organ system by mechanisms that are not yet entirely understood. They can appear early, presenting within the first few days of treatment or much later, even after discontinuation of treatment [26]. Commonly affected systems include cutaneous, gastrointestinal, hepatic, respiratory and endocrine systems. A variety of possible reasons for some of the observed organ-specific irAEs have been postulated [26]. This includes the expression of CTLA-4 on normal pituitary cells [27,28], the enhancement of pre-existing anti-thyroid antibodies [29] and shared, potentially cross-reactive,

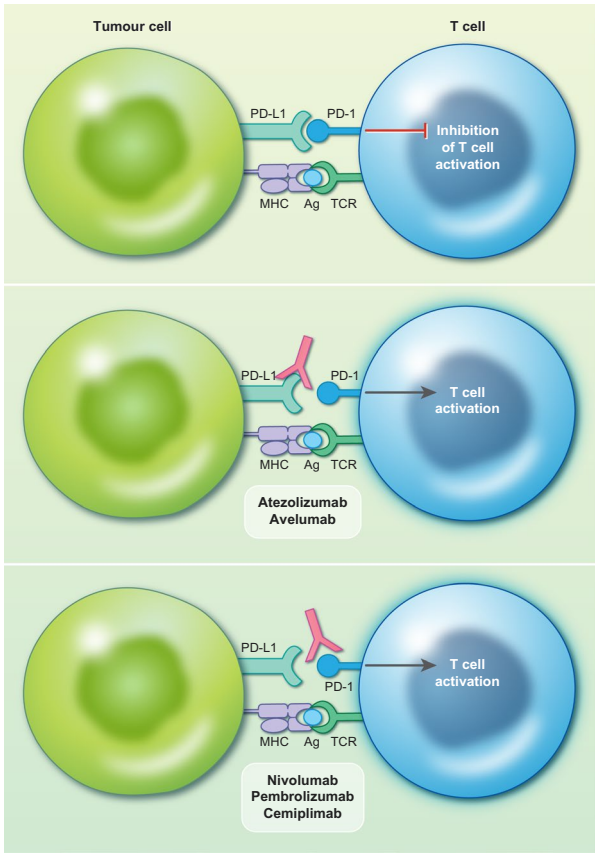

Fig. 3. The programmed cell death 1/programmed cell death ligand 1 (PD-1/PD-L1) pathway is utilized by tumours as immunosurveillance evasion strategy and is a target of immune checkpoint inhibitors. The PD-1/PD-L1 pathway supresses T cells in peripheral tissues and is involved at a later stage in the immune response than cytotoxic T lymphocyte antigen 4 (CTLA-4). For initial T cell activation, two signals are required. Signal 1 is delivered in the form of T cell receptor–major histocompatibility complex (TCR–MHC) binding, while signal 2 is achieved through the engagement of co-stimulatory molecules by CD28. After a T cell has been activated, the engagement of PD-1 on the T cell with PD-L1 on a tumour cell leads to T cell suppression, allowing tumours to avoid elimination by T cells. This pathway has become a target of novel anti-cancer therapies known as checkpoint inhibitors. Nivolumab, pembrolizumab and cemiplimab are the three current PD-1-targeting monoclonal antibodies, while atezolizumab and avelumab are the two current PD-L1-targeting antibodies.

antigens between tumour and tissue (for example, similar T cell clones in myocardium and tumour found in a patient with myocarditis [30], and vitiligo seen in melanoma patients treated with checkpoint inhibitors [31]).

Accumulating evidence suggests that individual checkpoint inhibitors, although sharing commonalities, favour specific organ trophic toxicities (Fig. 4). Anti-CTLA-4-associated irAEs are generally more severe, and trend towards (entero-) colitis and hypophysitis. Hyperthyroidism and pneumonitis are more common with anti-PD-1/PD-L1 targeting [32], with pembrolizumab having been reported to frequently cause vitiligo, implying tolerance break and autoimmunity [33–42]. Interestingly, the incidence of irAEs appears to increase with the administered dose of ipilimumab, but not anti-PD-1 agents [39,43]. Combining anti-CTLA-4 and anti-PD-1 targeting increases efficacy, but also toxicity [44,45].

Management of immune checkpoint inhibitor-related toxicity

To date there are no prospective trials to guide clinical management of irAEs. Multi-disciplinary consensus has been published through European Society for Medical Oncology and American Society of Clinical Oncology guidelines [46,47]. Grading of severity of toxicity is based on the Common Terminology Criteria for Adverse Events (CTCAE) system used to grade symptoms and side effects of drugs in clinical trials. For most toxicities, recommendations are to temporarily cease the CPA if grade 2 toxicity occurs, to cease and start high-dose systemic corticosteroids (with slow subsequent tapering) upon 3 toxicity, and to permanently discontinue the drug if grade 4 toxicity occurs. Endocrinopathies, controlled by hormone replacement therapy, and skin rashes managed with topical steroids are notable exceptions [46,47]. Anti-tumour necrosis factor (TNF)-α biologicals or other immunosuppressive agents are considered in steroid-unresponsive cases [48]. Treatment of irAEs with systemic steroids does not appear to worsen overall cancer outcomes [49]. Retreatment with CPIs following irAEs is controversial; a retrospective analysis by Alaiwi and colleagues found that in 339 metastatic renal cell carcinoma patients treated with CPI, 16% had their treatment interrupted owing to irAEs. Of those who received retreatment, the median break before recommencement was 1·2 months, and in the 41% who developed irAEs following retreatment the median time to recurrence of irAEs was 2·1 months [50]. This highlights the need for methods to differentiate irAEs to enable identification of toxicities likely to reoccur on reintroduction of CPIs.

Immune checkpoint inhibitor-related toxicity and response rates

The relationship between irAEs and response remains controversial [49,51]. A growing number of studies support the theory that discontinuation of treatment due to irAEs does not affect response rates [52]. Higher response rates in patients with toxicity have been described in melanoma patients [53,54]. There is evidence that the development of vitiligo in melanoma patients is associated with improved progression free-survival (PFS) and overall survival (OS). A systematic review of 27 studies reported a cumulative incidence of vitiligo of 3·4% and association with PFS (hazard ratio = 0·51) and OS (hazard ratio = 0·25) [55]. In addition, a prospective study of 67 melanoma patients receiving pembrolizumab reported that

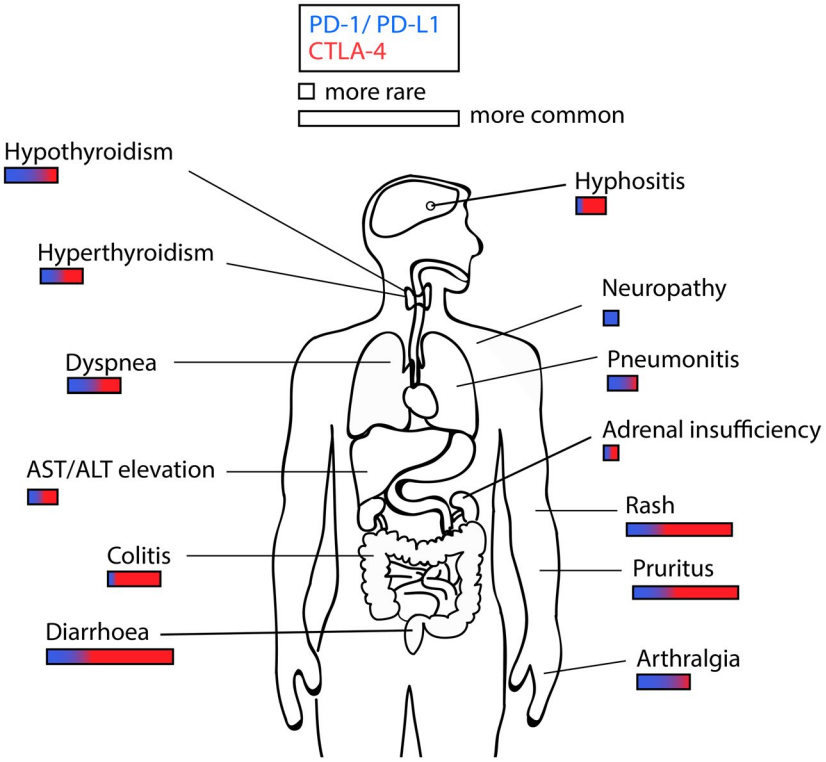

Fig. 4. Immune checkpoint inhibitors have unique risk profiles. Drugs targeting different immune checkpoints have been found to trend towards specific immune-related adverse effects (irAEs). The length of the bar corresponds to the relative incidence of these irAEs, while the colour is indicative of the pathway targeted. Increased proportions of red within the bar signifies toxicity more commonly associated with cytotoxic T lymphocyte antigen 4 (CTLA-4) inhibitors, while blue represents toxicity more commonly associated with programmed cell death 1/programmed cell death ligand 1 (PD-1/PD-L1) inhibitors.

25% developed vitiligo, and complete or partial response to treatment was associated with higher rates of vitiligo [12 of 17 treatment responders (71%) *versus* 14 of 50 non-responders (28%)] [56]. Enterocolitis [57], hypophysitis [58] and thyroid dysfunction [29] have reportedly been associated with anti-tumour response or improved outcome. Beyond melanoma, a study of 559 patients with NSCLC reported an increased overall response rate (ORR), PFS and OS in patients with any-grade irAEs compared to those without at multivariate analysis [59].

Prediction of immune checkpoint inhibitor-related toxicity

The ability to predict irAEs will allow clinicians to risk-assess patients prior to starting treatment. Numerous clinical and molecular biomarkers have been investigated aiming to characterize patients at higher risk of irAEs.

Clinical factors to predict checkpoint inhibitor-related toxicity

The simplest method to identify patients at risk utilizes clinical features. Groups historically considered high risk, and therefore excluded from many clinical trials, include those with autoimmune disease, stem cell transplants, solid organ transplants and chronic viral infections.

A retrospective analysis of 30 advanced melanoma patients with pre-existing autoimmune disease found that 27% experienced a flare of their autoimmune condition after receiving ipilimumab [60]. With regard to transplantation, cancer has been reported as the second leading cause of death in solid organ transplant recipients, making this subset of patients highly relevant for potential CPI use [61]. However, a systemic analysis reports that, of 39 patients with allograft transplantation, 41% experienced allograft rejection post-CPI, with similar rates for both CTLA-4 and PD-1 targeting agents. High mortality rates were also reported, with death occurring in 46% of these patients and graft loss in 81% [62].

Despite initial safety concerns, a recent review reports that of 73 HIV patients treated with CPIs, only 8·6% developed grade 3 or above irAEs. Furthermore, objective response rates were 30% for NSCLC, 27% for melanoma and 63% for Kaposi sarcoma [63]. While these results are limited in terms of patient numbers, clinical trials investigating CPI use in HIV-infected individuals will

shed further light on the matter. CPI efficacy in virus-associated cancers has been reviewed recently [64]. Notably, the results from two clinical trials, Keynote-012 and NCT02207530, suggest that human papillomavirus (HPV)-positive HNSCC patients respond better to CPIs than those with HPV-negative status. Grade 3 or above irAEs occurred in 13 and 8% of patients, respectively [65,66].

Various clinical parameters have been considered to potentially predict CPI toxicity. Age in NSCLC does not appear to predict for increased risk of toxicity; however, older patients were more likely to require intervention to manage irAEs [67]. A study investigating the impact of body composition parameters on ipilimumab toxicity found sarcopenia and low muscle attenuation to be significantly associated with high-grade adverse events on multivariate analysis [odds ratio (OR) = 5·34 and 5·23, respectively] [68]. A prospective study of 67 patients with advanced NSCLC treated with nivolumab showed a significant association between grade 3 or worse treatment-related adverse events with symptomatic central nervous system (CNS) metastases, performance status (PS) 2 and serum albumin < 30 g/l at the time of the first treatment cycle. However, in a multivariate analysis only symptomatic CNS metastases were significantly associated with worse toxicity [69]. A study of 140 melanoma patients treated with ipilimumab were analysed if irAEs were associated with anthropometric features (age and sex), tumour burden, surrogate markers [S-100 and LDH (lactate dehydrogenase)] and a panel of potential blood biomarkers [C-reactive protein (CRP), beta-2 microglobulin, vascular endothelial growth factor (VEGF), interleukin (IL)-2, IL-6, granulocyte and lymphocyte subpopulations]. Only female sex (OR = 1·5) and low IL-6 level (OR = 2·84) were significantly and independently associated with irAEs [70]. While there may be clinical variables that suggest risk of irAE, there is as yet no consensus suitable to implementation in clinical practice.

Biomarkers of checkpoint inhibitor toxicity

Full blood count. Simple changes in full blood count parameters may predict toxicity risk. Jaber *et al*. reported a rise in eosinophils compared with baseline in eight stage IV melanoma patients with treatment-related skin reactions [71]. A retrospective, multi-institution review of 156 patients treated with ipilimumab found an increase in absolute eosinophilia count from baseline to week 4 and from baseline to week 7 to be associated with development of irAEs [72]. A study of 29 melanoma, lung and renal cell carcinoma patients who developed irAEs while on CPIs found that 70% of patients had lymphopenia immediately prior to the development of their toxicity [73]. While none of these studies reported associations between differences in full blood count prior to treatment and irAEs, the ability to follow eosinophils or absolute lymphocytes during the course of treatment could identify at-risk patients early.

Cytokines. A study of bladder cancer patients reported elevated levels of the proinflammatory cytokine interferon (IFN)-γ following CTLA-4 blockade; however, this was not associated with an increased risk of irAEs. A study performed in 52 melanoma patients undergoing ipilimumab treatment reported significantly higher serum levels of the proinflammatory cytokine IL-17 levels (at weeks 7 and 12) in patients who went on to develop colitis compared to patients with no irAEs [74]. Tarhini *et al*. reported elevated baseline IL-17 associated with severe diarrhoea or colitis in melanoma patients treated with neoadjuvant ipilimumab [75].

Two further studies have investigated large panels of cytokines in the prediction of irAEs. Sixty-five cytokines were analysed in 98 melanoma patients treated with CPIs. Eleven cytokines [granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage CSF (GM-CSF), fractalkine, fibroblast growth factor (FGF)-2, interferon (IFN)-α2, IL-12p70, IL-1α, IL-1β, IL-1RA, IL-2 and IL-13] were associated with severe irAEs and were combined to form a 'CYTOX' score. The area under the curve (AUC) of this score to discriminate severe toxicity was 0·68 at baseline for a validation cohort [76].

In a similar study, a 40-cytokine panel was applied pre- and post-treatment with CPI [the majority (53 of 65) of these patients had lung cancer treated with anti-PD-1 therapy (n = 49)] [77]. They found that reduced baseline levels of several cytokines, in particular C-X-C motif chemokine ligand (CXCL)9, CXCL10, CXCL11 and CXCL13, were significantly associated with the development of irAEs. CXCL9, CXCL10 and CXCL11 bind to C-X-C motif chemokine receptor (CXCR3) are chemotactic for activated T cells and have previously been implicated in a number of autoimmune conditions. This axis has also been shown to regulate the differentiation of naive T cells to T helper 1 cells and leads to migration of immune cells to tumour sites. Notably, increased CXCR3 and CXCL9 and CXCL11 expression has been linked to increased accumulation of tumour-infiltrating lymphocytes and positive clinical outcome. The observation that prior to CPI exposure these inflammation-associated chemokines are in fact lower than average in patients that go on to develop more severe irAEs may suggest that a 'baseline' inflammatory state in an organ at risk is not the explanation for irAE evolution. This requires confirmation in larger patient cohorts followed by in-depth study of possible associated mechanisms.

These studies used different assays, in different patient groups, treated with different checkpoint inhibitors. Together, these differences outline the challenges for

implementing these findings into clinical use. Further large data sets are required to drive our understanding of irAEs.

Gene expression. Longitudinal, whole-blood gene expression profiling in a cohort of 162 melanoma patients, 49 of whom developed grade 2 or above gastrointestinal (GI) irAEs, revealed 27 probe sets with differential mean expression between GI irAEs and no irAEs. Differential gene expression within these probe sets related to three groups: the immune system, the cell cycle and cellular trafficking. Notably, increases in CD177 and CEA cell adhesion molecule 1 (CEACAM1), two neutrophil activation markers, were closely associated with GI irAEs; however, the sensitivity of these to predict GI irAEs was low [78]. Patients who developed GI irAEs were found to have a higher baseline expression of IL-32, a cytokine associated with secretion of chemotactic factors by cells of the innate immune system and the production of proinflammatory cytokines IL-2 and IL-8. IL-32 is implicated in the pathobiology of inflammatory bowel disease and rheumatoid arthritis. In addition to these makers of inflammation, a number of immunoglobulin-related genes were significantly increased at 11 weeks in the GI irAE group. This reflects previous reports of the inhibitory function of CTLA-4 on immunoglobulin and cytokine production by plasma cells [79]. In the absence of an external pathogen the authors propose that this could be due to the generation of antibodies to self-antigens, or those expressed by the intestinal flora.

Whole exome sequencing (WES) of DNA extracted from 87 tumours from 49 patients with metastatic melanoma treated with CPIs demonstrated 14 mutated genes to be enriched in tumour samples from 10 patients with colitis compared to those free of colitis. In patients with any irAE, seven mutated genes were enriched [80]. These findings suggest that specific mutations within the tumour may have an association with toxicity risk. In 89 patients treated with PD-1/PD-L1 CPIs, 150 validated germline miRNA-based biomarkers were tested. Using χ^2 analysis, 12 biomarkers were found to be associated with response and 12 associated with toxicity, including germline mutations in 3′ untranslated regions as well as miRNA promoter regions. Specificity was reported to be 89% for response and 76% for toxicity [81]. These findings further support the idea that mutations within the tumour, but also the host immune system, may impact the development of toxicity.

Antibodies. A Phase I study of patients with stage III/IV metastatic melanoma assessed the benefit of combining anti-CTLA-4 treatment and intralesional bacillus Calmette–Guérin (BCG) injections. The trial was stopped, with only five patients enrolled, after two patients developed high-grade irAEs. Although no conclusions can be drawn from this small study, interestingly a protein array of plasma from both patients showed an increase in the repertoire of antibodies against self- and cancer-antigens [82].

Serum from 49 melanoma patients was analysed in a study addressing the role of IgG and its four subclasses in predicting response to CPI. While raised IgG2 at baseline was significantly associated with response to treatment, no association was found between any IgG class and adverse effects [83]. In a more extensive study, a human proteome microarray analysis of baseline serum interrogated pretreatment subsets of IgG antibodies associated with irAEs in a set of 78 stage IV melanoma patients treated with CPIs. Differentially expressed antibodies were categorized for each treatment cohort. From these, 914 antibodies were associated with severe toxicity in the anti-CTLA-4 cohort, 723 in the anti-PD-1 cohort and 1161 in the combination cohort. Interestingly, there was minimal overlap in these differentially expressed antibodies between each group, suggesting that discrete, treatment-specific antibody sets may be associated with toxicity. The antigen targets for these autoantibodies were significantly enriched for proteins in organs commonly affected by irAEs, such as skin and liver, or involved in pathways associated with immune pathology (such as apoptosis), indicating a potential causative role for these autoantibodies in toxicity. In addition, autoantibodies associated with several autoimmune conditions were found to be elevated in patients who developed toxicity; for example, anti-complement factor H (anti-CFH), which has been associated with age-related macular degeneration, haemolytic uraemic syndrome and membranoproliferative glomerulonephritis. These data support the theory that certain patients may possess an underlying subclinical autoimmune phenotype, rendering them at higher risk for toxicity [84].

Changes in adaptive immunity. A number of studies have looked into clonality in relation to toxicity. Wang *et al*. [85]used flow cytometry and microarray analysis of overall gene expression in $CD4^+$ and $CD8^+$ T cells before and after CTLA-4 blockade in melanoma. The genes most impacted by CTLA-4 blockade were immune-associated genes related to the cell cycle. Increased expression of Ki67, a cellular marker for proliferation and cell cycling, was found on both $CD4^+$ and $CD8^+$ T cells after treatment. The study reported that a decreased absolute percentage of $Ki67^+CD8^+$ T cells at 6 months was associated with the development of irAEs (P = 0·02, OR = 0·47), as well as a low baseline percentage of $Ki67^+$eomesodermin $(EOMES)^+CD4^+$ T cells (P = 0·008, OR = 8·0). EOMES is a transcription factor known to control the function of effector $CD8^+$ T cells, and a low baseline percentage of $Ki67^+EOMES^+CD8^+$ T cells and $EOMES^+CD8^+$ T cells was found to be significantly associated with relapse in this study. This supports the theory that certain patients may

have a baseline immune phenotype contributing not only to their risk of developing toxicity, but also the likelihood of response to treatment.

A further study evaluated the immunomodulatory effects of CTLA-4 blockade in peripheral mononuclear cells of 21 patients treated for metastatic melanoma within a Phase II clinical trial. The complementary determining region 3 (CDR3) from the TCR-β chain was analysed at baseline and 30–60 days after treatment. An increase in unique productive sequences was found in 19 of 21 patients, indicating an increased T cell diversity after treatment. This increase in diversity, however, was not associated with treatment response. In contrast, a significant difference in the total unique CDR3s between patients experiencing toxicity and those without was seen, suggesting mobilization of clonal autoreactive T cells in irAE aetiology [86].

In a Phase II trial, TCR sequencing was undertaken in patients with metastatic prostate cancer treated with anti-androgen and ipilimumab. Of these patients, > 40% experienced grade 3 toxicity, and baseline $CD4^+$ or $CD8^+$ T cell clonality was not associated with treatment benefit or toxicity [87]. However, they report a clonal expansion of $CD8^+$ T cells prior to the development of toxicity, with a median time between blood draw and toxicity of 13 days. The number of expanded $CD8^+$ T cell clones was strongly associated with risk of irAE, with a 2% increase in risk with each additional expanded clone. Expansion of 55 or more $CD8^+$ T cell clones gave a sensitivity of 100% in predicting patients who would go on to experience grades 2–3 irAEs. In a further prostate cancer trial, T cell repertoire after combination treatment with CTLA-4 blockade and GM-CSF was studied. Increased $Ki67^+$ lymphocytes were found following treatment, but there was no association with irAEs. Next-generation sequencing of the TCR from peripheral blood found an immediate decline in clonality following treatment, indicating a more diverse peripheral blood TCR repertoire. Again, greater diversification and a broad expansion of lower-frequency clonotypes was seen in patients with irAEs compared to patients without irAEs, and broadening in the repertoire was seen within the 2 weeks of treatment preceding irAE onset [88]. irAEs were associated with an increase in pre-existing TCR clones, as well as newly detected TCR clones. While the lack of clonality differences in pretreatment between patients with and without irAEs indicates that this tool is not suitable for risk stratification prior to treatment, it has potential for monitoring to allow earlier detection and intervention for irAEs.

Changes in lymphocyte biology during CPI therapy are not limited to T cells. One study found that circulating B cells decline, plasma blasts increase and $CD21^{low}$ B cells increase after combination therapy, but not monotherapy [89]. PD-1 expression on the $CD21^{low}$ B cells was higher than on other B cell subsets, and B cell receptor sequencing of these $CD21^{low}$ B cells showed increased clonality, both of which positively correlated with frequency of irAEs. Furthermore, patients with B cell changes were more likely to develop multi-organ immunotoxicity than those without, with these changes preceding toxicity by a median of 3 weeks. Early B cell changes correlated with grade 3 or higher irAEs. $CD21^{low}$ B cells have been associated with chronic immune stimulation including autoimmunity [90]. This study found no correlation between changes in circulating T, natural killer (NK) or myeloid cells and irAEs. It is likely that irAEs are not solely related to the T cell effects of CPIs.

As described, a number of studies have used a variety of methods to assess the variation in T cells, T cell receptor repertoire and B cells following CPI treatment. Evidence suggests a widening of the T cell repertoire and clonal expansion to be associated with development of toxicity. Most of these findings have been during the course of treatment as opposed to baseline, therefore while they help to direct ongoing research into pathogenesis of toxicity, clinical applicability is limited. In order to use the above findings to predict toxicity, patients would have to undergo T cell receptor repertoire analysis early in treatment, which may not be clinically feasible or affordable.

Microbiome. Increasing evidence demonstrates a close relationship between the microbiome and the immune system. Stool microbiome has been investigated for predictive markers of toxicity. A study of 34 patients with metastatic melanoma found that increased representation of *Bacteroidetes* phylum led to a reduced rate of ipilimumab-induced colitis, whereas a lack of bacterial modules involved in polyamine transport and B vitamin biosynthesis was associated with an increased risk of colitis [91]. Another study of 26 patients with metastatic melanoma found an enrichment of *Faecalibacterium* and other *Firmicutes* at baseline to be both beneficial for response to ipilimumab, but also associated with more frequent occurrence of colitis [92]. A description of two patients treated for refractory CPI-induced colitis with faecal microbiota transplant (FMT) reported a positive response, with improvement in symptoms in both patients. These patients were deficient in *Bacteroides* at the time of colitis manifestation, and were found to have an increase in *Bifidobacterium* following treatment [93]. In support of these observations, increased *Bifidobacterium* in a murine model has been associated with attenuation of CTLA-4-associated colitis [94].

While these studies point to an evident link between the microbiome and response/toxicity, as recently reviewed by Humphries and Daud [95], there is significant difficulty in interpreting studies related to the microbiome in checkpoint inhibition. Literature implicates a wide range of bacteria, highlighting the significant variation that can be found between microbiome studies. Furthermore, a large number

of these studies use murine models, and it has been shown that 85% of the mouse gut microbiome is not found in humans [96]. Further high-powered studies with consistent methods will be required to fully characterize the proposed associations before firm conclusions can be drawn.

Limitations

The findings discussed in this review carry a number of limitations that currently prevent translation to clinical use. Fundamentally, sample sizes are limited resulting in relatively low sensitivity and specificity. Studies aiming to investigate similar hypotheses, such as the case with Lim *et al.* [76] and Khan *et al.* [77], have used different methods, in different patient populations, once again making comparisons across studies challenging. Many of these studies describe changes in markers observed after, not before, exposure to checkpoint inhibitors. Understanding the variables that might change over the course of treatment could still be valuable, allowing clinicians to identify the possibility of irAEs early. However, how this could be used clinically remains to be determined. If the goal is prediction and prevention, then the gold standard should be a biomarker that could be measured before exposure to the drug, then combined with clinical characteristics to give a multivariate predictor of risk of toxicity.

Future directions

The described studies provide evidence to direct future research into the pathogenesis of toxicity following checkpoint inhibition; however, they may also facilitate a further understanding of the immune system itself. There have been reports of patients with no prior history of autoimmune disease that go on to develop seropositive RA post-checkpoint inhibition [97]. Although drug-induced, if the end-disease is indeed a true representation of RA, studying its development may offer valuable insight into the pathogenesis of this disease. This also applies other CPI-induced inflammatory syndromes that mimic their autoimmune counterparts. Although most studies focus on irAEs with regard to the adaptive immune system, it should be noted that PD-1 and its ligands are expressed on various myeloid cells, which may equally be affected by systemic treatment, contributing to the inflammatory conditions observed. As an example, a recent study showed marked changes in the myeloid compartment of murine tumours after successful checkpoint inhibition [98]. Extending our understanding to all compartments of the immune system is necessary to define toxicity. To achieve this, collaborative cohorts capable of recruiting vastly higher numbers of patients are essential to identify biomarkers of irAEs at cellular, proteomic and genetic levels. This will require national or international consortia approaches with standardization of sample collection to enable bulk analysis and validation cohorts.

Conclusion

Immune checkpoint inhibitors have undeniably revolutionized the treatment of many cancers; however, their capacity to cause a range of inflammatory syndromes has become clear. There is a pressing need to characterize the mechanisms by which these syndromes emerge and to predict the patients most at risk for their development. Increasing evidence suggests several possible mechanisms, and it is apparent that multiple factors are at play. A clearer understanding of irAE pathogenesis, including risk factors, will lead to improved methods of prevention, CPI stratification and patient management.

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Author contributions

I. E., P. U., M. B. and S. P. wrote and edited the paper. M. B. provided figures. E. P., W. W., S. R., L. T., N. P., S. K., A. C. and S. P. contributed to development of the questions, writing and editing of the final paper.

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