



PERSPECTIVES

The foundations of immune checkpoint blockade and the ipilimumab approval decennial

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Abstract | Cancer immunity, and the potential for cancer immunotherapy, have been topics of scientific discussion and experimentation for over a hundred years. Several successful cancer immunotherapies — such as IL-2 and interferon- α (IFN α) — have appeared over the past 30 years. However, it is only in the past decade that immunotherapy has made a broad impact on patient survival in multiple high-incidence cancer indications. The emergence of immunotherapy as a new pillar of cancer treatment (adding to surgery, radiation, chemotherapy and targeted therapies) is due to the success of immune checkpoint blockade (ICB) drugs, the first of which — ipilimumab — was approved in 2011. ICB drugs block receptors and ligands involved in pathways that attenuate T cell activation — such as cytotoxic T lymphocyte antigen 4 (CTLA4), programmed cell death 1 (PD1) and its ligand, PDL1 — and prevent, or reverse, acquired peripheral tolerance to tumour antigens. In this Review we mark the tenth anniversary of the approval of ipilimumab and discuss the foundational scientific history of ICB, together with the history of the discovery, development and elucidation of the mechanism of action of the first generation of drugs targeting the CTLA4 and PD1 pathways.

In 1968, Ingegerd Hellström, Karl Hellström and colleagues observed that lymphocytes isolated from the blood of cancer patients could react against cancer cells isolated from the same patients¹. They noted that it “may seem paradoxical that lymphocytes from patients destroy [...] autochthonous neoplastic cells in vitro.” The authors speculated that certain counteracting factors might block the destructive effect of immune lymphocytes in vivo; the identity of these factors was a mystery. We now possess a more sophisticated understanding of immune responses to cancer cells and can appreciate these phenomena in the larger context of immune homeostasis and self-tolerance. Activation and attenuation of immune responses is controlled by multiple — and often functionally redundant — pathways. In 1995, Goodnow and colleagues synthesized the emerging complexity of these pathways and proposed that the existence of “multiple and distinct checkpoints presumably minimizes the risk that one or two inherited or

somatically acquired mutations leads to uncontrolled autoantibody production, analogous to the role of checkpoints in the cell cycle that minimize neoplasia”². Pardoll then used the term “immunologic checkpoint” in the context of cancer therapy³, and Korman, Peggs and Allison coined the term “checkpoint blockade”⁴ to describe cancer therapies targeting immune attenuation pathways. Goodnow’s original concept of immune checkpoints was quite broad, encompassing both deletional and non-deletional mechanisms of self-tolerance; however, over time, the phrase checkpoint blockade has been more narrowly applied to drugs that specifically block activation-attenuating receptors on T cells, and that is how we use the term in this Perspective.

The first T cell-attenuating receptor identified was cytotoxic T lymphocyte antigen 4 (CTLA4), and in 1996, Leach, Krummel and Allison⁵ showed that an antibody to mouse CTLA4 could induce immune rejection of a transplanted

syngeneic tumour. It was not by chance that Allison was the first to extend the discovery of immune checkpoint receptors to cancer immunotherapy, given that his research had intersected cancer immunotherapy for over two decades. In a 1975 paper⁶ focused on adjuvants for improving immune responses to tumour antigens, Allison and colleagues wrote that “stimulating an immune response with modified cells from the tumour to be treated is a promising approach to cancer immunotherapy”. In 2011, ipilimumab, an anti-CTLA4 antibody, became the first successful immune checkpoint blockade (ICB) therapy for cancer (FIG. 1). In the decade following this approval, multiple drugs that block a second immunological checkpoint pathway, the one mediated by programmed cell death 1 (PD1) and its ligand, PDL1, have been approved^{7,8}. ICB is now the standard of care for multiple indications and is the first class of immunotherapy to have a broad impact on survival for cancer patients across a wide variety of tumour histologies and treatment settings⁹. The tenth anniversary of the approval of ipilimumab provides an opportunity to review the impact of ICB as well as the new understanding of the mechanism of action of these drugs provided by the flood of preclinical and clinical research prompted by this impact.

Roots of checkpoint blockade

The concept of ICB presupposes that cancer cells can be recognized by the immune system, and that acquired peripheral immune tolerance inhibits the resulting immune-mediated destruction of cancer cells without completely deleting all potentially cancer-reactive lymphocytes. It also presupposes that peripheral tolerance is mediated by druggable immune checkpoints, and that it can be reversed by blockade of those checkpoints. For this reason, the discoveries, beginning in the 1940s, of cancer cell antigens, peripheral tolerance and the pathways and molecules that mediate peripheral tolerance represent the scientific foundation of ICB.

Cancer cell antigens

The modern era of tumour immunology arguably began with the development of inbred strains of mice as a tool for studying

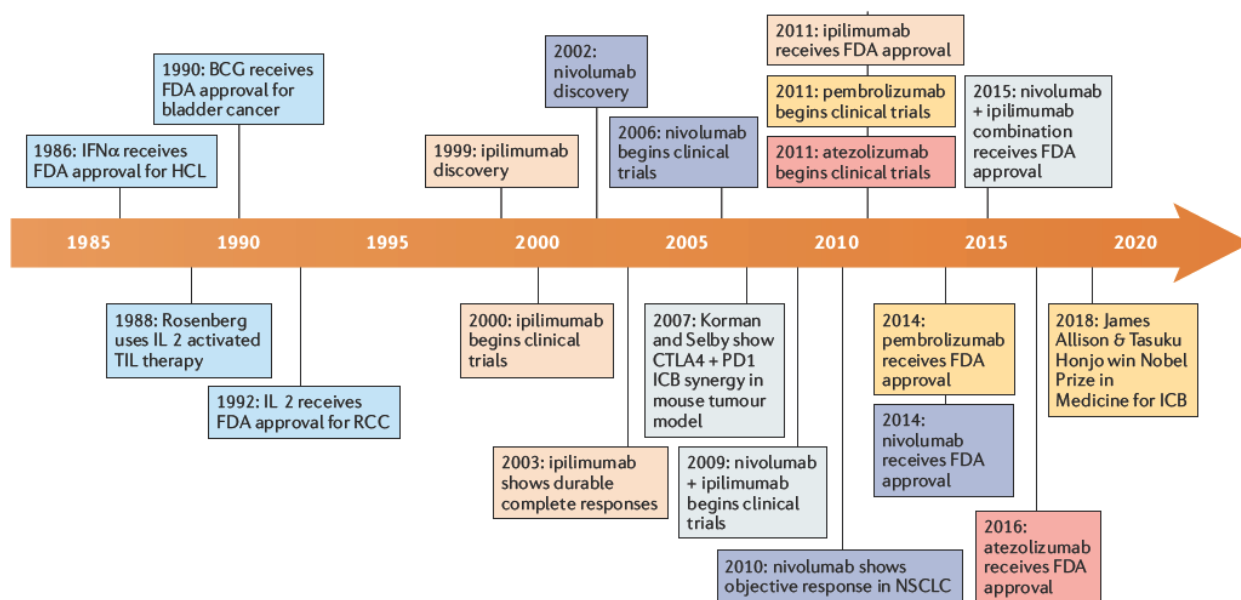


Fig. 1 | Milestones in discovery and development of immunotherapeutics. Key milestones for US regulatory approvals of pre-immune checkpoint blockade (ICB) immunotherapeutics: interferon α (IFN α), bacillus Calmette-Guérin (BCG) and IL-2 and for demonstration of ex vivo activated tumour-infiltrating lymphocyte therapy⁴⁷ are shown in blue. Key milestones in the development of the first four FDA-approved ICB drugs^{7,9}: ipilimumab, nivolumab, the combination of nivolumab and ipilimumab²³,

pembrolizumab and atezolizumab are shown in orange, purple, grey, yellow and red, respectively. The 2018 Nobel Prize in Physiology or Medicine was awarded to James Allison and Tasuku Honjo for the discovery of cancer therapy by inhibition of negative immune regulation²⁸. CTLA4, cytotoxic lymphocyte antigen 4; HCL, hairy cell leukaemia; NSCLC, non-small cell lung cancer; PD1, programmed cell death 1; RCC, renal cell carcinoma; TIL, tumour-infiltrating lymphocyte.

resistance to transplanted tumours^{10,11} (BOX 1). In 1943, Ludwick Gross showed that a methylcholanthrene-induced tumour derived from a C3H strain mouse could be used to immunize other C3H mice against the same tumour, demonstrating that cancer cells harbour immunologically recognized antigens that distinguish them from normal self¹². This, and later work by Prehn and Main in 1957 (REF.¹³), established the concept that cancer cells are the products of somatic mutations that lead to the expression of tumour-specific transplantation antigens, which are recognized by the host immune system as foreign¹⁴. Tumour-specific transplantation antigens could be direct products of somatic mutations, which induced the expression of gene products that are not expressed in normal adult cells (neoantigens), or — in the case of virally induced tumours — viral antigens.

Boon and colleagues cloned the first tumour-specific transplantation antigen, a neoantigen produced through chemical mutagenesis of a mouse tumour cell line¹⁵, and 2 years later, in 1991, the first human tumour-specific transplantation antigen, melanoma-associated antigen 1 (MAGE1), was identified, which is recognized by melanoma tumour-infiltrating cytotoxic

T lymphocytes (CTLs)¹⁶. MAGE1 falls into the second category of tumour-specific transplantation antigens: an unmutated gene product with restricted expression in normal cells, which allows for escape from self-tolerance.

Non-deletional T cell tolerance

The mouse tumour transplantation studies carried out in the 1950s established that chemically induced mouse tumours could be recognized by the immune system. However, the relevance of these experimental systems to human tumour immunology was undermined by the fact that patients present with cancer. If the immune system can recognize a cancer antigen, why does cancer exist? Burnet proposed in 1957 that cancers are, in fact, frequently cleared by the immune system before they are even detected, and that in patients who present with cancer, either the immune system has acquired tolerance to the cancer, or the malignant cells have lost antigens through somatic mutation and selection^{17,18}. In addition to proposing a theory of 'immune surveillance' of cancer, Burnet's 1957 paper¹⁸ also included a conceptual outline of a cancer immunotherapeutic as having "the converse of the effect of cortisone in damping down immunological reactivity".

In 1962 Brent and Gowland reported induction of tolerance to mismatched skin grafts in adolescent mice¹⁹, and in 1964 Mitchison reported the induction of tolerance to a specific soluble antigen in adult mice; a phenomenon that he called "immune paralysis"²⁰. In a 1968 review, he considered both deletional and non-deletional hypotheses to explain immune paralysis, and concluded that the evidence favoured immune cell deletion²¹.

In the 1970s, in vivo experiments provided conflicting results for the fate of B lymphocytes in models of immune tolerance. In 1970, Ada²² used radioactively labelled antigen to follow antigen-binding B cells and found that tolerance to model antigens was not associated with depletion of the reactive lymphocytes. However, in 1973 Weigle and colleagues used a similar system to follow antigen-reactive B cells, and found that tolerance was accompanied by the disappearance of reactive cells²³. In 1980, Nossal and Pike observed antigen-binding, but functionally unresponsive, B cells from mice that had been previously exposed to tolerogenic doses of fluorescein-coupled γ -globulin, either in utero or perinatally²⁴. They used the term 'clonal anergy' to describe this process, and to distinguish it from their earlier, deletional, model for

B cell tolerance, which they had termed 'clonal abortion'²⁵.

Non-deletional explanations for T cell tolerance did not gain traction until the demonstration in 1987 by Jenkins and Schwartz that antigen presentation by chemically inactivated antigen-presenting cells (APCs) rendered T cells non-responsive²⁶. The authors called this dysfunctional, non-responsive state 'anergy', a term that had been used for decades to describe the loss of response to a tuberculin delayed type hypersensitivity (DTH) test, and did not have a mechanistic connotation²⁷. Jenkins and Schwartz proposed that T cell activation required a first signal provided by antigen recognition together with a second signal provided by an activated APC (consistent with Bretscher and Cohn's two-signal hypothesis for explaining activation or the induction of peripheral B cell tolerance²⁸). A year earlier, Martin and colleagues had reported that T cells could be activated *in vitro* in the absence of APCs by simultaneous antibody-mediated signalling through the T cell receptor (TCR) complex and CD28 (REF.²⁹). CD28 would later be found to mediate the second signal elicited by activated APCs via engagement of CD80 (also known as B7-1) and CD86 (also known as B7-2), which are induced on activated APCs.

In 1988, Jamieson and Ahmed demonstrated non-deletional T cell tolerance *in vivo*, finding that exposure to lymphocytic choriomeningitis virus (LCMV) *in utero* does not cause a permanent deletion of specific T cells³⁰. They also noted, ironically, that this was an experimental system³¹ that Burnet had cited in his original formulation of clonal deletion as an explanation for self-tolerance^{32,33}.

The invention of major histocompatibility complex (MHC) tetramers by Davis and colleagues in 1996 (REF.³⁴) provided a tool for tracking the fate of T cells that have defined specificities during the course of an immune response. In 1998, using MHC tetramers to track virus-specific T cells in the mouse LCMV model, Zinkernagel and colleagues³⁵, and Ahmed and colleagues³⁶, found that virus-specific, but functionally unresponsive, CD8 cells persisted during chronic infection. The Zinkernagel group used the term 'anergy' to describe the functional state of these cells, and speculated that this was an intermediate state in a programme of 'exhaustion', a term that had been used to describe the process of clonal deletion³⁷. The Ahmed group specifically distinguished the process leading to the formation of these persistent but unresponsive cells from the

process of deletion (which they equated with the term 'exhaustion'). However, in a later paper³⁸, Ahmed, Wherry and colleagues coined the phrase 'functionally exhausted' to distinguish the progressive and hierarchical loss of function of effector T cells (T_{eff} cells) responding to a chronic infection or a persistent tumour, from the dysfunctional state imprinted on naive cells by exposure to antigen in the absence of signal 2 (such as CD28 co-stimulation) or signal 3 (such as IL-2) that Jenkins and Schwartz had termed 'anergy'. The term 'exhaustion' is now widely used; however, other terms, including

'stunned', have been used to describe dysfunctional T cells^{39–41}. Attaching labels to specific dysfunctional T cell states is now further complicated by the multiplicity of different dysfunctional subtypes that have been identified⁴².

Discovery of the T cell growth factor, IL-2, provided a means of reactivating some quiescent T cells⁴³. In 1981, Rosenberg and colleagues⁴⁴ and Vose and Moore⁴⁵ reported that lymphocytes from cancer patients could kill autologous tumour cells after *ex vivo* culture in the presence of IL-2. In a subsequent paper, Rosenberg et al. isolated

Box 1 | Ehrlich and Coley: early pioneers of cancer immunotherapy

Prior to the development of inbred strains of mice and the subsequent elaboration of modern transplantation immunology, there was a great deal of interest in tumour immunology, but very little actual scientific progress. In a 1929 review of the literature on immunity to transplantable tumours, Woglom³⁰⁹ expressed his frustration that only a small number of the 600 cited papers were of permanent value, while "far too large a proportion are merely ridiculous", demonstrating that "not everyone with an inoculating needle and a dozen white mice can plunge in and make a discovery". Nevertheless, the early literature on cancer immunity is abundant, making it easy to pick and choose to identify precursors to modern immunotherapy. For example, Paul Ehrlich and William Coley, neither of whom appear in Woglom's review, are frequently cited as pioneers of tumour immunology^{310,311}. Burnet's 1957 papers, in which he first outlined his theory of immune surveillance of cancer, also made no mention of either Ehrlich or Coley^{317,18}. Ehrlich's writings were not considered to have provided support for cancer immunotherapy until 1977, when Klein and Klein wrote a paper that criticized Burnet's theory³¹². They credited Ehrlich with articulating the original theory of immune surveillance, citing a 1909 paper that was Ehrlich's only published foray into cancer biology³¹³. This might have been intended as gentle ribbing directed at Burnet, because the conclusion of Ehrlich's paper was that natural rejection of cancer is not related to mechanisms associated with acquired immunity to pathogens and haptens. Instead, Ehrlich proposed a theory of 'athrepsis', or nutritional starvation, to explain the rejection of transplanted tumours in outbred strains of mice and rats. In 1926, Murphy dismissed Ehrlich's athrepsis theory on the basis of his observation that peripheral lymphocyte activation and lymphocytic infiltrates correlated with rejection of transplanted mouse tumours^{314,315}. Murphy found translational support for his mouse experiments in the work of MacCarty, a surgeon at the Mayo Clinic, who identified lymphoid infiltration as a positive prognostic marker for post-surgery survival in cancer patients³¹⁶. McCarty's papers also contributed to Burnet's theory of immune surveillance. The scientific historian, Arthur Silverstein, lamented that if Ehrlich had not been wedded to his theory of athrepsis, "the history of transplantation immunology would have developed far differently"³¹⁷. To be fair to Ehrlich, he was a careful experimentalist, and because inbred mouse strains had not yet been developed as a tool for the study of transplantation immunology, it was impossible for him to interpret tumour transplantation data correctly. Coley, also cited as a pioneer of tumour immunology, documented 140 patients with sarcoma treated with local injections of a bacterial extract in 1898 (REF.³¹⁸). Coley adopted this therapeutic strategy from earlier reports of streptococcal infection-induced tumour shrinkage³¹⁹. He believed that sarcomas were of "micro-parasitic or infectious origin", and that the toxins acted directly on the tumour cells, and not as a "result of inflammation". However, Coley's mechanistic hypotheses were not based on experimental data, and his therapy was very controversial during his lifetime. In 1894 the *Journal of the American Medical Association* published an editorial stating that "so far not a single well-authenticated case of recovery has been reported"³²⁰. Coley's toxins were still used sporadically, and in 1965 the American Cancer Society felt compelled to issue a statement that they had "found no evidence that treatment with Coley's mixed toxins results in any objective benefit in the treatment of cancer in human beings"³²¹. Coley's work does not seem to have had a substantial impact on the advancement of cancer immunotherapy except for the development of intravesical live bacillus Calmette-Guérin (the BCG vaccine, a live attenuated strain of *Mycobacterium bovis*) as a treatment for bladder cancer, which can be traced back to the 1959 discovery by Old, Clark and Benacerraf that BCG infection delayed the growth of transplanted tumours in mice³²². The authors did not at the time connect this observation with Coley's toxins; however, Old made that connection in later writings, also noting that part of the reason Coley's treatment "fell into disuse and [...] even into disrepute" was that they could not be explained mechanistically³²³. The Cancer Research Institute, which was founded by Coley's daughter, supported the clinical development of BCG^{324,325}, which was approved for treatment of non-muscle-invasive bladder cancer in 1990, and remains in use to this day.

tumour infiltrating lymphocytes (TILs) from six patients with melanoma and found that, after reactivation and expansion with recombinant IL-2, the lymphocytes from three of the patients were highly cytotoxic against autologous tumour cells but did not react against autologous normal cells or allogeneic tumour targets⁴⁶. In 1988, the Rosenberg group translated this observation into the clinic. They used ex vivo IL-2 reactivation and re-infusion of autologous TILs as a therapy in patients with metastatic melanoma⁴⁷. Eleven of the 20 patients treated experienced an objective response.

In addition to IL-2-mediated ex vivo reversal of T cell dysfunction, Rosenberg and colleagues used systemic administration of high-dose IL-2 to activate antitumour T cell responses in vivo, leading to FDA approval of recombinant IL-2 (aldesleukin) for the treatment of metastatic renal cell carcinoma (RCC) and melanoma⁴⁸. However, high-dose IL-2 therapy is also associated with considerable toxicity, and although responses are durable, they are also of low frequency, and efficacy has never been tested in a randomized clinical trial. The observed toxicity and heterogeneous antitumour activity of IL-2 therapy highlights the difficulty of identifying pathways and drugs that can activate an immune response within a tumour without also activating deleterious immune-related effects outside the tumour. This difficulty is compounded by the phenomenon of organ-specific peripheral tolerance. The homeostatic mechanisms that control immune activation do not necessarily act globally and can be restricted to particular organs or even within organs. Vitiligo provides an illustrative example, in which a T cell immune response to melanocytes can be observed at one location and be absent a millimetre away⁴⁹. Another example is provided by transgenic mouse models for organ-specific tolerance and organ-specific reversal of tolerance; Hammerling and colleagues describe double-transgenic mice expressing a liver-specific antigen and another transgene encoding a TCR that recognized such an antigen. In these mice, T cells were unresponsive to implanted liver-antigen-expressing tumours, and there was tumour growth with no signs of autoimmune hepatitis; however, if the tumour cells were also engineered to express IL-2, antitumour tolerance was broken and the tumours did not grow, whereas liver-specific tolerance was maintained⁵⁰. Breaking liver-specific tolerance in this model could only be accomplished by infection with *Listeria* (presumably via

activation of an innate immune response to the bacterial infection). These experiments highlight the challenge for successful ICB cancer therapy: reversing peripheral tolerance in the tumour microenvironment, without lowering the bar for T cell activation to the point where autoimmunity is unleashed against normal tissue.

Pathways in peripheral tolerance

By the mid-1990s, much of the conceptual framework for ICB was firmly established: cancer cells comprise neoantigens that are recognized by the immune system, but peripheral tolerance often leads to non-deletional inactivation of the resulting adaptive immune response. However, little was understood about the molecular interactions controlling immune attenuation. The discovery that CTLA4 and PD1 were T cell checkpoint receptors provided druggable targets and enabled ICB therapy.

CTLA4

The T cell transmembrane protein CTLA4 (REF⁵¹) was first characterized as a co-stimulatory receptor owing to its amino acid similarity and synteny with CD28 (REF⁵²). However, in 1994 and 1995, Bluestone, Allison, Sharpe, Mak and their colleagues independently demonstrated that CTLA4 is, in fact, an attenuator of T cell activation^{53–57}. CTLA4 and the activating receptor CD28 both recognize the co-stimulatory ligands T lymphocyte activation antigen B7-1 and B7-2 expressed on APCs. CTLA4 expression is tightly regulated and, prior to T cell activation, is largely sequestered in intracellular vesicles, which are rapidly shuttled between endocytic compartments and the plasma membrane via clathrin- and dynamin-mediated pathways⁵⁸.

Both B7-1 and B7-2 are expressed on professional APCs including macrophages, dendritic cells and B cells. Basal expression of B7-2 is typically higher than that of B7-1, although expression of both ligands is induced after cytokine stimulation (through interferon γ (IFN γ), tumour necrosis factor (TNF), and so on), activation by bacterial endotoxins (lipopolysaccharides) or antigen-dependent activation (Toll-like receptors (TLRs), MHC-II and so on). Interestingly, both the stimuli-specific dependence and kinetics of expression differ between the two ligands, with peak B7-1 expression occurring after that of B7-2 (REFS^{59,60}). B7-2 expression is largely absent from non-haematopoietic cells. However, B7-1 expression is induced more

widely; for instance, induction of B7-1 was observed in IFN γ -stimulated fibroblasts⁶¹. Therefore, although the ligands share similarities regarding their recognition by, and activation of, CD28 and CTLA4, unique biochemical characteristics and differences in their temporal and spatial expression result in distinct functional contributions.

The varied roles of CTLA4 and how it contributes to T cell inhibition.

CTLA4 functions within the immune system to suppress T cell activation via a remarkable number of mechanisms: first, direct competition with CD28 for B7 ligands, second, inhibition of TCR signalling via phosphatase recruitment to its cytoplasmic domain, third, alteration of CD28 localization within the immunological synapse, fourth, sequestration of B7 ligands from APCs by transendocytosis, and fifth, increasing T cell motility and reversing the CD28-induced stop signal (required to form a stable conjugate between T cells and APCs), which reduces the dwell time between APCs and naive T cells and results in decreased proliferation^{62,63}. Much work has sought to define the precise contribution of each proposed mechanism on the hyper-lymphoproliferative phenotype observed in CTLA4^{-/-} mice. The results overwhelmingly paint a complex picture in which context-dependent combinations of multiple mechanisms yield productive CTLA4-dependent inhibitory signals. For example, in several studies of CTLA4 in immunosuppressive regulatory T cells (T_{reg} cells), the CTLA4 cytoplasmic domain was required for attenuation of TCR signalling, but T_{reg} cell-induced immune suppression and anergy required only the extracellular ligand-binding domain of CTLA4 (REFS^{64–66}). This is consistent with early work by Thompson and colleagues showing that in CTLA4^{-/-} mice, expression of a transgene comprising only the ligand-binding extracellular domain blocked multiorgan lymphocytic infiltration; however, the cytoplasmic domain was required for complete rescue of the autoimmune phenotype⁶⁷. The contribution of both ligand binding and signalling domains is also demonstrated by other models of transgenic rescue of the CTLA4^{-/-} phenotype. Hyperproliferation can be delayed by expression of just the CTLA4 cytoplasmic domain; however, the onset of hyperproliferation is not fully reversed by expression of ligand-binding deficient CTLA4 alone. Multiple mechanisms, both ligand-dependent and -independent, seem to be critical for full CTLA4 functionality⁶⁸.

The multiple mechanisms by which CTLA4 elicits its inhibitory effects and in which immunological contexts these mechanisms are operative remains to be fully defined.

T_{reg} cells and the role of Fcγ receptors in anti-CTLA4 function. T_{reg} cells are an immunosuppressive subpopulation of CD4⁺ CD25⁺ cells characterized by the expression of the forkhead box P3 (FOXP3) transcription factor. In healthy patients, these cells have a role in self-tolerance and immune maintenance, preventing uncontrolled immune activation. T_{reg} cells use several mechanisms to confer immunosuppression including: IL-2 sequestration, which limits local T_{eff} cell proliferation and activation, secretion of inhibitory cytokines (such as IL-10), reduction of B7 ligands on APCs by transendocytosis via CTLA4 engagement, and direct killing by secretion of perforin and granzyme^{65,69–72}.

In contrast to conventional T_{eff} cells, T_{reg} cells constitutively express high levels of CTLA4, although the protein is still largely shuttled between membrane-proximal vesicular reserves and the cell surface^{65,73} (FIG. 2). CTLA4 activation may also cause positive feedback that supports the T_{reg} phenotype by increasing transforming growth factor β (TGFβ) secretion and FOXP3 expression^{64,66}. Similar to CTLA4 knockout mice, cell-specific knockout of CTLA4 in T_{reg} cells also results in lethality, albeit with delayed kinetics, emphasizing a role for CTLA4 in T_{reg} cell activity⁷². A key vesicular trafficking protein, lipopolysaccharide-responsive and beige-like anchor protein (LRBA) is particularly essential for maintaining CTLA4 levels on T_{reg} cells. Patients with autosomal recessive mutations in the *LRBA* gene develop immunodeficiency and autoimmune disorders similar to those observed in patients with CTLA4 heterozygous loss-of-function mutations, as well as those seen in a subset of patients treated with anti-CTLA4 therapy⁷⁴. Regardless of cause, functional CTLA4 loss diminishes the ability of T_{reg} cells to regulate the B7 ligand, resulting in the characteristic hyperproliferative phenotype associated with CD28 overstimulation.

After the approval of ipilimumab, several laboratories began to investigate the role of Fc and Fcγ receptors (FcγRs) in the activity of different anti-CTLA4 antibodies in mouse tumour models^{75–77}. FcγRs define the effector function of immunoglobulins; they display varying degrees of specificity for immunoglobulin isotypes and consist

of activating and inhibitory receptors that differ in their cellular expression⁷⁸. The most effective anti-CTLA4 antibodies bind to activating FcγR receptors and deplete intratumoural T_{reg} cells while sparing T_{reg} cells in the periphery, which results in an improved T_{eff}/T_{reg} cell ratio within the tumour. This specificity was a result of increased expression of CTLA4 in activated intratumoural T_{reg} cells along with antibody Fc binding to activated intratumoural myeloid cells that expressed increased levels of FcγRIV (an FcγR type found in mouse and not human) compared with myeloid cells in the periphery. Interestingly, anti-CTLA4 antibodies unable to bind activating FcγRs did not elicit antitumour activity in the tumour models tested⁷⁵. Because all the anti-CTLA4 antibodies tested were ligand blocking, as shown by the expansion and activation of peripheral T_{reg} cells, the role of ligand blockade could not be separated from the effects of T_{reg} cell depletion. Agents that selectively deplete T_{reg} cells can have potent antitumour activity in mouse subcutaneous tumour models^{79,80}, suggesting that CTLA4 blockade in T_{reg} cells does not alter their activity sufficiently to promote antitumour activity. Together, these data demonstrate that CTLA4 blockade alone, without FcγR engagement, has limited antitumour effects in mouse models.

In humans, peripheral T_{reg} cells were shown to expand in response to ipilimumab⁸¹, consistent with CTLA4-mediated inhibition of proliferation.

However, in vitro, in the presence of FcγRIII-bearing monocytic cells, ipilimumab could deplete T_{reg} cells⁸². Since ipilimumab (IgG1) and tremelimumab (IgG2) differ in their Fc regions and ability to engage FcγRs, these differences could result in differences in clinical activity related to their effects on T_{reg} cells. Testing the role of human Fc variants in vivo in mouse models with transgenic human FcγRs suggested that both isotypes could result in antitumour activity⁸³. Despite the failure of tremelimumab in a pivotal phase III trial⁸⁴, durable long-term responses have been observed with this antibody⁸⁵. Unfortunately, immune monitoring in human clinical trials has been limited by small sample numbers and longitudinal samples, with no clear evidence of effects on T_{reg} cell numbers^{82,86}.

Nevertheless, FcγRs may have a part in the intrinsic activity of anti-CTLA4 antibodies in T_{eff} cells, because an antibody against CTLA4 with improved binding to CD16 (FcγRIII, encoded by *FCGR3*) promoted greater T cell activation in the response to the superantigen staphylococcal enterotoxin A (SEA) in peripheral blood mononuclear cells⁸⁷. Specifically, CD16⁺ dendritic cells were implicated and the activity was independent of T_{reg} depletion (FIG. 2). Augmented proliferation of peripheral T cells by an afucosylated derivative of ipilimumab, which enhances binding to FcγRIII, was observed in cynomolgus macaques⁸⁸. Study of FcγR polymorphisms could provide support

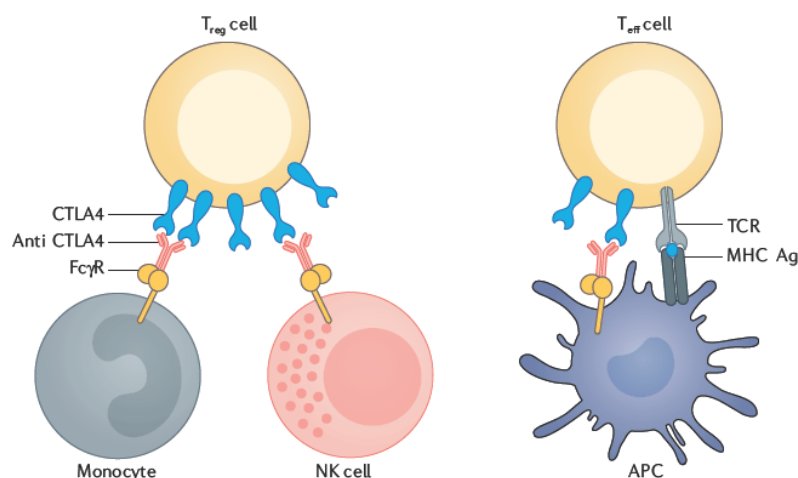


Fig. 2 | Roles of Fcγ receptors in anti-CTLA4 function. Upon binding of ipilimumab, activation of Fcγ receptors, probably CD16, on monocytes (or other myeloid cell types) or natural killer (NK) cells results in depletion of regulatory T cells (T_{reg} cells) (left), which express increased levels of CTLA4 compared with T_{eff} cells. In T_{eff} cells, recognition of major histocompatibility complex–antigen peptide complexes (MHC–Ag) by the T cell receptor (TCR) in the presence of an anti-CTLA4 antibody that had co engaged with FcγR on antigen-presenting cells (APCs) results in enhanced T cell activity (right).

for a role for Fc receptors in anti-CTLA4 activity; however, an analysis of FcγR polymorphisms in the pivotal phase III trial of ipilimumab did not reveal differences in response or survival for *FCGR3* and *FCGR2A* polymorphisms; this result was confirmed in analysis of other trials of ipilimumab in melanoma (unpublished data). Improved survival in melanoma seemed to be correlated with *FCGR3* polymorphisms in patients with high tumour mutational burden⁸³, but only a limited number of patients were analysed in this study.

Next generation of anti-CTLA4 antibodies. Given the observations regarding the role of the FcγRs and questions about whether CTLA4 blockade may occur in the tumour and/or the lymph node, various attempts have been initiated to improve the activity of anti-CTLA4. Analogous to observations in the mouse, augmenting the ability of Fc to bind to FcγRIII — either through an afucosylated derivative of ipilimumab (BMS-986218)⁸⁹ or an engineered Fc variant of an anti-CTLA4 antibody (AGEN-1181 or its mouse surrogate)⁹⁰ — improved the ability of anti-CTLA4 to deplete T_{reg} cells at the tumour site in mouse models. Interestingly, afucosylated ipilimumab uniquely induces lethal toxicity in cynomolgus macaques at high doses⁹¹. These toxicities seem to be due to increased T cell infiltration into tissues, predominantly in the gastrointestinal tract and kidney, and not through peripheral T_{reg} cell depletion.

Additional approaches to improving anti-CTLA4 therapy consists of limiting CTLA4 blockade to the tumour, with the goal of minimizing peripheral CTLA4 blockade to moderate the adverse event profile of anti-CTLA4. The simplest form of this approach is direct intratumoural injection of anti-CTLA4. Although this approach may limit adverse events⁹², its success requires that the kinetics of redistribution of the locally administered drug are slow enough to provide some degree of compartmentalization between tumour and normal tissue, and that locally activated T cells migrate to, and are effective at, other metastatic sites. An alternative ‘systemic’ approach to this problem relies upon a ‘proform’ of ipilimumab (BMS-986249)^{93,94}. Analogous to a prodrug requiring chemical transformation for the generation of an active molecule, this proform is a peptide sequence selected to specifically occlude the CTLA4 binding site of ipilimumab and is appended to the light chain of ipilimumab. The linker

sequence connecting the blocking peptide to ipilimumab contains a cleavage site sensitive to proteolytic enzymes that are prevalent and preferentially activated in the tumour microenvironment, thus affording site-selective CTLA4 blockade.

PD1

The second immune checkpoint receptor to be targeted for ICB therapy was PD1. Honjo and colleagues originally cloned the PD1 complementary (c)DNA (*PDCD1*) by subtractive hybridization in a search for lymphocyte transcripts induced by programmed cell death⁹⁵. In mice, PD1 expression was highest in the thymus and increased by in vivo administration of an anti-CD3 antibody. In 1996, the Honjo group reported the isolation of an antibody to PD1 and showed that the protein was induced on T and B lymphocytes by antigen receptor stimulation⁹⁶. In 1998, they reported that the phenotype of *Pdcd1*^{-/-} mice included moderate splenomegaly and augmented B cell responses⁹⁷. Further characterization of these PD1-deficient mice revealed a late onset of lupus-like autoimmune disease and augmented proliferation of T cells on in vitro stimulation with allogeneic cells⁹⁸. The authors proposed “that PD-1 is involved in the maintenance of peripheral tolerance by serving as a negative regulator of immune responses”. Cardiomyopathy in PD1-deficient Balb/c mice mediated by cardiac troponin 1 autoantibody was later described^{99,100}.

Discovery of PDL1 and PDL2, two inhibitory ligands for PD1. PDL1, a B7 family homologue previously reported by Chen and colleagues to be a T cell co-stimulatory ligand¹⁰¹, was identified by Freeman, Wood, Honjo and colleagues in 2000 as the principal ligand for PD1, and an inhibitor of T cell activation¹⁰⁰. In 2002, Chen and colleagues reported that PDL1 is expressed in a variety of human cancer tissues, and that in a mouse syngeneic tumour model, PDL1 expression on tumour cells counteracted the immune co-stimulatory effect of transfected B7-1 (REF¹⁰²). They also showed that tumour PDL1 expression allowed for increased tumour cell proliferation or survival, and increased T cell apoptosis, which was abrogated by an anti-PDL1 antibody. These results were consistent with the observation by Honjo and colleagues that *PDCD1* deletion augmented proliferation of T cells on stimulation with allogeneic cells in vitro⁹⁸, and the observation by Freeman and colleagues that PDL1 expression inhibited T cell activity¹⁰⁰.

In a later 2002 paper, Honjo and colleagues reported the inhibition of syngeneic tumour growth in PD1-deficient mice and in immunocompetent wild-type mice treated with anti-PDL1 antibodies¹⁰³. The authors noted the similarity of their results to those reported for anti-CTLA4 by Allison and colleagues in 1996 (REF⁵), and they proposed PD1 blockade and combination PD1–CTLA4 blockade as possible cancer therapies. PDL2, a second ligand with higher affinity for PD1, has also been identified^{104,105}. Whereas PDL2 may have a role in negative regulation, solely blocking PDL2 has not demonstrated any antitumour effects.

PDL1 and PDL2 expression mechanisms and key differences. PDL1 and PDL2 expression can be upregulated via both extrinsic and intrinsic mechanisms. The most common intrinsic mechanisms driving alterations in expression involve direct dysregulation of signalling cascades resulting from specific oncogenic driver mutations (such as EGFR, PTEN and so on)^{106,107}. However, increases in *CD274* (encoding PDL1) and *PDCD1LG2* (encoding PDL2) gene copy number and chromosomal polysomy are characteristics of advanced-stage Hodgkin lymphoma¹⁰⁸. Additionally, significant disruptions to the 3'UTR of *CD274*, which causes overexpression of PDL1, were also observed in about a third of patients with adult T cell leukaemias and less frequently in a variety of other solid tumours¹⁰⁹. Extrinsic upregulation occurs primarily in response to inflammation via IFNγ-dependent signalling, which is secreted by both tumour cells and resident antitumour immune effector cells within the tumour microenvironment^{110–112}.

PDL2 was identified as a second ligand for PD1 that competes directly with PDL1 for binding to PD1, although its expression is observably lower on tumour cells compared with PDL1^{104,105,113,114}. PDL2 is more specifically expressed on activated professional APC cells such as macrophages and dendritic cells than PDL1, which is ubiquitously expressed in both haematopoietic and non-haematopoietic cell lineages. Although PDL1 expression is highly predictive of patient responsiveness to PD1 targeted therapy, PDL2 expression often correlates with PDL1 expression in solid tumours, and patients with PDL1-negative tumours can still respond favourably to anti-PD1 treatment, which suggests that PDL2 also has a tumour-suppressive role^{114,115}. PDL2 expression is most frequently observed on activated APCs, and expression of both

ligands could be induced on endothelial cells, causing suppression of resident CD8⁺ T cell activation¹¹⁶. Additionally, endothelial cell expression of PDL1 and PDL2 in co-culture experiments demonstrated the capacity of these cells to enhance the immunosuppressive capacity of T_{reg} cells^{117,118}. Therefore, given the broad and inducible expression patterns of PD1 and its ligands, there are many different contexts in which the role of receptor–ligand engagement and the impact of PD1 blockade need to be established.

PD1 expression and its roles in various cell types. Induction of PD1 expression on T cells occurs during two different cellular processes: during differentiation of immature precursor T cells into effector and memory T cell populations, and during activation and/or reactivation of those circulating or resident effector and memory T cell subsets. After maturation in the thymus, naive precursor T cells, defined by expression of the surface marker CD45RA, express non-detectable levels of PD1. Differentiation is initiated by direct antigen recognition by the TCR, which causes rapid activation and translocation of nuclear factor of activated T cells (NFAT), a major driver of PD1 expression^{119–121}. Activation also signals the rapid demethylation of essential CpG islands in the *PDCD1* promoter, priming *PDCD1* for rapid transcription, which is subsequently reversed after antigen removal or during effector or memory T cell phenotypic switching¹²⁰. Antigen recognition leads to progressive changes in which T cells lose ‘stemness’ and proliferative capacity as they transition towards terminal cytotoxic and helper effector functions¹²². This process is driven by specific transcription factors, primarily TCF1 (encoded by *TCF7*) and T-bet, the expression of which inversely correlate with one another and which function to promote either a memory (TCF1 high, T-bet low) or effector (TCF1 low, T-bet high) phenotype^{123–126}. Notably, PD1 expression was found to increase rapidly during early differentiation and helps to define an initial population of stem-like memory cells that are primed for further differentiation into memory or effector subpopulations^{127–129}, expression tapers off after the early/intermediate phase of differentiation and is markedly lower on central memory and effector memory cell populations, although elevated in subsets of terminal cytotoxic and T_H1/2 effector cell populations^{130,131}. However, high PD1 expression is also a key marker of exhausted T cell populations that arise from exposure

to chronic infection or cancer^{132,133}. It is well established that blockade of PD1 restores antitumour CD8⁺ T cell cytotoxic activity by minimizing the tumour-suppressive effect of PDL1 and PDL2 expressed by tumour cells. Additionally, patients treated with anti-PD1 therapeutics who have circulating CD8⁺ T_{eff} cells with high PD1 expression have better overall responsiveness to anti-PD1 treatment and better outcomes¹³⁴.

However, the ‘exhausted’ phenotype has been further dissected into two specific states, a progenitor ‘stem-like’ state and a ‘terminal’ state. In mouse models of chronic LCMV infection and melanoma, PD1 is moderately expressed on progenitor exhausted CD8⁺ T cells, also defined by expression of CXCR5, and these cells were shown to expand with PD1 blockade, leading to better viral clearance and tumour suppression^{135–141}. These studies further demonstrate that terminally exhausted T cell populations, marked by high expression of PD1 and other inhibitory receptors, did not proliferate in response to anti-PD1 therapy. The implication from this work is that increased proliferation of the precursor population results in differentiation of fresh memory and effector populations that lead to stronger viral clearance and antitumour responses. This observation is in agreement with a study that found increased CD8⁺ T cell memory subset populations in patients with favourable anti-PD1 outcomes¹⁴². This suggests that PD1 blockade may primarily restore effector function by shifting this early activation path rather than reversing exhausted T cell phenotypes. It remains a possibility that treatment induces more subtle functional effects on terminally exhausted populations that could still be beneficial overall. Analysis of TCR clonotypes before and after PD1 blockade reveals that clonotypes not present in the tumour are expanded, consistent with new recruitment of antitumour T cells and not with reinvigoration of tumour-resident T cells¹⁴³.

Although there are substantial data regarding the ability of anti-PD1 therapy to rescue CD8⁺ T_{eff} cell function, there are fewer data regarding the rescue of exhausted tumour-infiltrating CD4⁺ T cells. In healthy patients, exhausted CD4⁺ T_{eff} cells with high PD1 expression are defective, producing cytokines while remaining non-proliferative, a phenotype that cannot be restored by blocking PD1 in vitro. Patients with glioblastoma showed enrichment of these defective CD4⁺ T_{eff} cells, which, as expected, were refractory to rescue with PD1 blockade; instead,

restoration of active PD1[−] CD4⁺ T_{eff} cell population was shown to be critical¹⁴⁴. Similarly, patients with non-small-cell lung cancer (NSCLC) with a high proportion of highly differentiated memory CD4⁺ T cells showed responsiveness to PD1 blockade and improved outcomes. However, patients with dysfunctional CD4⁺ T_{eff} cell responses, characterized by high PD1 and lymphocyte activation gene 3 protein (LAG3) expression, showed suppressed antitumour activity and poor responsiveness to PD1 treatment^{145,146}. These data further support the idea that anti-PD1 therapy is most beneficial when it can initiate proliferation and functional rescue of progenitor populations that are not terminally exhausted.

T_{reg} cells also express PD1, but its function in these cells is less clear^{62,147,148}. In mice, T_{reg} cell-specific deletion of *Pdcd1* resulted in T_{reg} cells that were more activated and showed higher immunosuppressive capacity, reversing experimental autoimmune encephalomyelitis and diabetes outcomes¹⁴⁹. This is in line with reports of anti-PD1 therapy causing an increase in tumour-infiltrating T_{reg} cell populations with corresponding increases in immunosuppressive phenotypes and increased CTLA4 expression^{150,151}. There is also growing evidence that one of the major contributors to poor responsiveness to anti-PD1 therapy is the overall ratio of PD1^{high} CD8⁺ T cells to CD4⁺ T_{reg} cells, with patients who have PD1^{low} CD4⁺ T_{reg} cells responding better to therapy than patients with PD1^{high} CD4⁺ T_{reg} cells^{152,153}. For these patients, blocking PD1 could initiate unwanted reactivation of tumour-resident T_{reg} cells propagating immunosuppressive activity via re-engagement of TCR–CD28. PDL1 can upregulate the T_{reg} cell phenotype both in vitro and in tumours, which was also linked to NOTCH activation^{69,154,155}. Conversely, in a mouse model of osteosarcoma, anti-PD1 therapy decreased the intratumoural T_{reg} cell population in addition to reducing tumour size¹⁵⁶. The mechanism driving T_{reg} cell reduction was not established, although antibody-dependent cellular cytotoxicity (ADCC) activation was discussed as one possibility¹⁵⁶. However, this seems unlikely owing to the reduced ADCC activity observed in vitro for IgG4 isotype antibodies, combined with the fact that no ADCC was observed with nivolumab treatment^{157–159}. Instead, these seemingly contradictory effects of anti-PD1 therapy on T_{reg} cell activity could be the result of the heterogeneous nature of the T_{reg} cell populations and what their phenotypes were at the start of treatment.

PD1 can also be expressed on macrophages and dendritic cells. In infection models, TLR activation triggered PD1 expression in macrophages, which suppressed the overall innate immune response to sepsis and *Mycobacterium tuberculosis*^{160,161}. The role of tumour-infiltrating macrophages in supporting tumour growth, promoting angiogenesis and stimulating metastasis is well characterized in many solid tumours. Tumour-infiltrating macrophages express PDL1 and PDL2 but there is evidence that they also express PD1 with expression levels that correlate with tumour progression in mice and humans^{162,163}. Furthermore, blockade of PD1 in animals showed increased macrophage phagocytosis, decreased tumour growth and improved survival, suggesting that anti-PD1 therapy efficacy may be in part the result of enhanced tumour-infiltrating macrophage activity^{162,163}. This is supported by the observation that elimination of PD1 from myeloid cell lineages resulted in antitumour activity superior to that in mice selectively lacking PD1 in T cells¹⁶⁴.

PD1 signalling and mechanisms for T cell inhibition. The cytoplasmic domain of PD1 contains two regulatory domains, an immunoreactive tyrosine-based inhibitory motif (ITIM) and an immunoreactive tyrosine-based switch motif (ITSM). Ligand engagement of PD1 induces proximity-based changes (increased receptor avidity, TCR clustering and so on), which results in the phosphorylation of Y248 in the ITSM domain. While phosphorylation of Y248 in the ITSM domain is required for SHP phosphatase recruitment, the direct role of the ITIM domain is unknown, although it is required for inhibitory function. Recruitment of SHP1 (also known as PTPN6) and/or SHP2 (also known as PTPN11) dephosphorylates TCR kinase ZAP70 and CD36. Canonically, this results in downregulation of PI3K, AKT and ultimately BCL-x_L, which reduces cell proliferation and increases apoptosis^{165,166}. It was reported independently by two groups that the target of PD1 signalling, contrary to the TCR model, is actually CD28. First, using a reductionist cell-free approach, Hui et al. established that PD1 activates SHP2, which subsequently preferred CD28 as a substrate over other substrates tested (TCR ζ , inducible T cell co-stimulator (ICOS) and so on)¹⁶⁷. This resulted in decreased CD28 phosphorylation and subsequent p85 recruitment. Additionally, Kamphorst et al. showed that blockade of PD1 required CD28

to reactivate exhausted T cells induced either by chronic infection or in a colon cancer model, demonstrating that blockade of PD1 resulted in increased CD28 phosphorylation and enhanced activation¹⁶⁸. Furthermore, blocking CD28 activity in addition to anti-PD1 therapy resulted in no reduction in tumour size or survival, which suggests that CD28 activity is required for anti-PD1 efficacy in reactivating 'exhausted' T cells. However, recent work using CD8⁺ TILs isolated from human NSCLC biopsy samples concluded that although CD28⁺ CD8⁺ TILs responded better to anti-PD1 treatment (higher reactivation), unlike previous observations in mice, the response in human cells was not dependent on B7/CD28 activity. Therefore, the authors conclude that CD28 is a marker for preferable anti-PD1 response but is not required¹⁶⁹. Additionally, in an ex vivo culture system looking at cytokine production as a readout of activation, Mizuno et al. found that, in fact, PD1 inhibited activation irrespective of CD28 or ICOS co-stimulation, which suggests that PD1 is primarily affecting the primary TCR signal¹⁷⁰. These experiments rely on the ability to selectively modulate one activity (such as loss of B7-1 binding) while not interfering with other activities, to see what the specific effect is. This is extremely difficult using blocking antibodies as described. Therefore, it remains a possibility that PD1 proximity to CD28 (or other co-stimulatory receptors) regulates activity in specific contexts and probably warrants further investigation. Interestingly, an antibody to PD1 that does not block interactions between PD1 and its ligand has been described that has functional activity in vitro and in vivo¹⁷¹. This antibody may define a novel conserved site that interacts with CD28 either directly or indirectly. The recently described regulation of CD28, CTLA4 and PD1 activity by *cis*-bound PDL1:B7-1 is another example of the complexities involved in modulating T cell activation, emphasizing the interrelatedness of these pathways^{172,173} (FIG. 3).

Exosomal and/or secreted PDL1. PDL1 can be secreted from cells via direct shedding or cleavage from the cell surface as well as via expression of specific messenger (m)RNA splice variants. PSL1 can also be secreted from the endocytic compartment on the surface on exosomes. Cell-surface-expressed PDL1 has been intensely studied as a potential indicator for likelihood of patient responsiveness to anti-PD1 therapy. In pembrolizumab treatment, a threshold of >50% PDL1-positive NSCLC cells was

set, on the basis of studies showing a tight correlation between high PDL1 expression and positive response¹⁷⁴. However, most other solid tumours fail to meet this threshold for PDL1 expression. PDL1 expression on exosomes (exoPDL1) has been detected in the serum of patients with a variety of cancers. High levels of exosomes were predictive of patients with metastatic melanoma that would benefit from anti-PD1 treatment¹⁷⁵. Levels of exoPDL1 also increased after treatment, with positive responders having the highest levels, suggesting that exoPDL1 might provide a means of clustering patients into responder/non-responder categories after treatment^{176,177}. By contrast, high circulating levels of soluble PDL1 were found to correlate with poor response to PD1 blockade. However, analysis of whole plasma includes both secreted and exosomal PDL1 fractions, which makes it impossible to ascertain the precise contribution of each form¹⁷⁸. A high level of soluble PD1 has also been identified as a biomarker for poor prognosis in both NSCLC and other tumours¹⁷⁹.

ICB: a new class of immunotherapy

Beginning with the approval of IFN α as a therapy for hairy cell leukaemia in 1986 (REF.¹⁸⁰), followed by the bacillus Calmette-Guérin (BCG) vaccine for bladder cancer and IL-2 for RCC and melanoma (FIG. 1), immunostimulatory drugs became part of the standard of care for certain cancers. However, immunotherapy was limited to a relatively small set of cancers, and the arrival of the first targeted cancer therapies, such as monoclonal antibodies (mAbs) (rituximab, trastuzumab, cetuximab and bevacizumab) and tyrosine-kinase inhibitors (such as imatinib, gefitinib, erlotinib, sunitinib and dasatinib) heralded the next generation of cancer therapy. Against this background, the discovery and early development of ICB drugs by our (A.J.K. and N.L.) research teams at Medarex and by teams at Pfizer, and later at Genentech, Merck and AstraZeneca, did not generate wide enthusiasm except within the specialized immunotherapy community. Ipilimumab and nivolumab were, respectively, the first CTLA4- and PD1-targeting drugs to enter human clinical trials.

Developing ipilimumab and nivolumab

The first antibodies to CTLA4, which were used for in vitro functional testing and in vivo modelling, consisted of hamster anti-mouse CTLA4 (REFS^{54,56}) and mouse

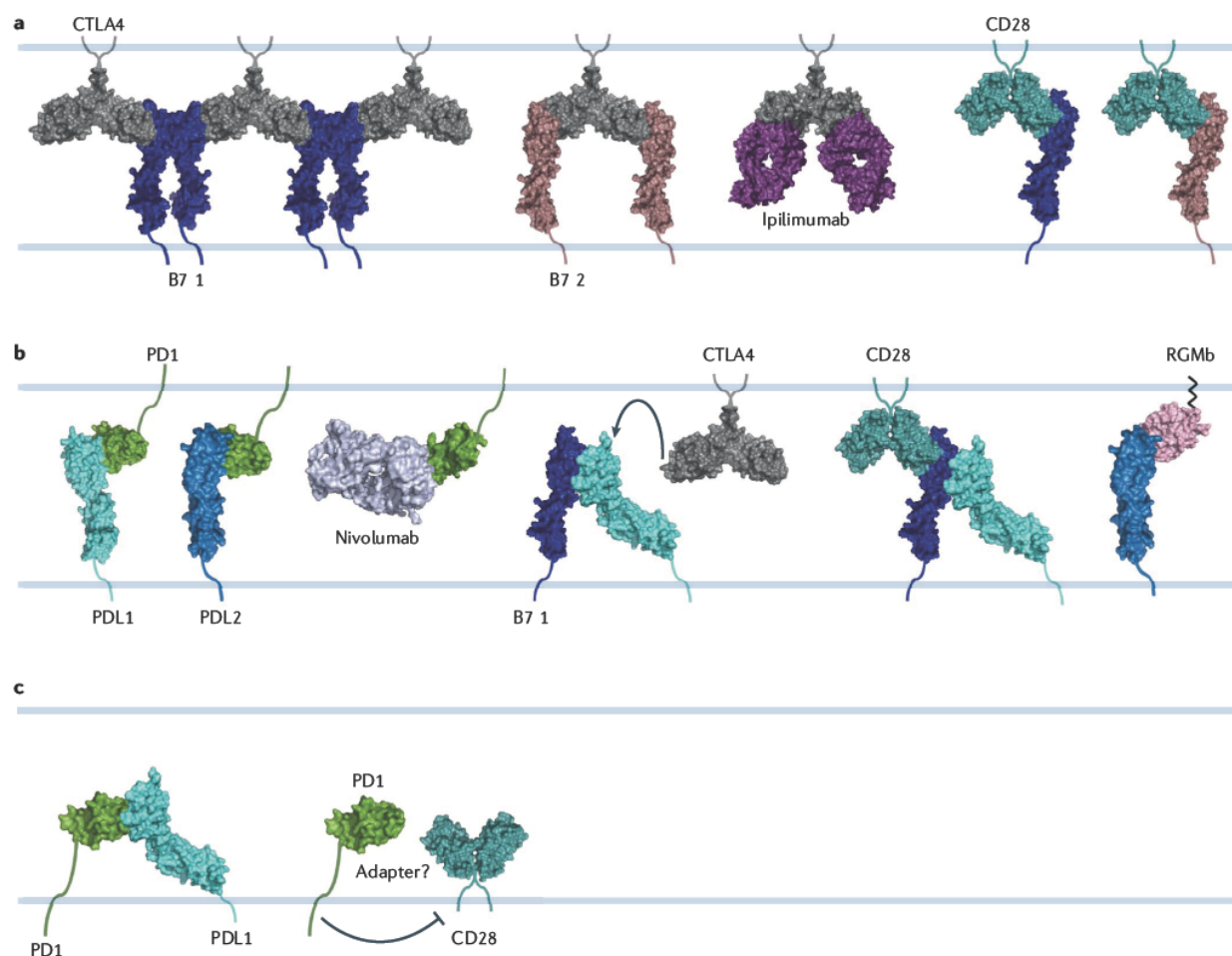


Fig. 3 | Interactions of CTLA4 and PD1 with their ligands and antibodies. TCR peptide-MHC engagement triggers cytoskeletal rearrangements that form specific contact zones within the immunological synapse, termed the centre, peripheral or distal supramolecular activation clusters (cSMAC, pSMAC and dSMAC). Concentrated signalling occurs in the cSMAC, where selective enrichment of CD28, CTLA4, PD1 and other co-stimulatory and co-inhibitory molecules tune the strength of human immune responses. Therefore, characterization of these interactions is critical in establishing the chemical and physical determinants driving immunological synapse formation, stability and regulation. **a** | Structure and function of CTLA4 and its ligands B7 1 and B7 2. CTLA4 (PDB ID: 3OSK) and CD28 (PDB ID: 1YJD) recognize the ligands via a conserved MYPPPY motif, although CTLA4 binds to both B7 1 (dissociation constant $K_d = 0.38 \mu\text{M}$) and B7 2 ($K_d = 2.6 \mu\text{M}$) with higher affinity than CD28 ($K_d = 4.2 \mu\text{M}$ and $19.1 \mu\text{M}$ for B7 1 and B7 2, respectively)^{285,286}. B7 1 (PDB ID: 1DR9) and B7 2 (modelled on the basis of PDB IDs: 1NCN and 1DR9) ligands form a concave hydrophobic patch that contacts the MYPPPY loop^{287–290}. The dimeric structure of CTLA4 combined with the higher tendency of B7 1 to homodimerize may support multivalent ‘zippering’ of these molecules at the immunological synapse (PDB ID: 1I8L), a process that is less likely for B7 2 owing to its reduced dimerization^{140,287–289}. Depictions of CD28 are based on biochemical data and the CD28:Fab structure (PDB ID: 1YJD) because the structures of CD28 bound to B7 1/B7 2 have not been determined. Ipilimumab blocks CTLA4 binding to B7 ligands. The crystal structure of ipilimumab in complex with CTLA4 (PDB ID: 5TRU) confirmed the occlusion of the B7 ligand binding site and highlighted key features of ipilimumab selectivity for CTLA4 over CD28 (REF.¹⁸²). **b** | Structure and function of PD1 and its ligands PDL1 and PDL2. PD1 (PDB ID: 3RRQ) recognizes PDL1 (PDB IDs: 4Z18 and 3SBW) and PDL2 (PDB ID: 3RNQ) and exhibits selectivity

for its ligand with higher affinity for PDL2 than PDL1 ($K_d \approx 2.0 \mu\text{M}$ and $8.0 \mu\text{M}$, respectively)^{141,291–295}. PD ligand-dependent enrichment of PD1 within the cSMAC results in recruitment of SHP2 and subsequent TCR downregulation^{296,297}. By contrast, RGMb (PDB ID: 4BQ6, with complex inferred) is a newly described ligand for PDL2 that competes directly with PD1 and supports T cell activation²⁹⁸. Similarly, PDL1 binds B7 1, and recent reports demonstrate that B7 1 primarily engages PDL1 in cis, resulting in disruption of the B7 1 dimer^{172,299–302}. Formation of the B7 1-PDL1 complex restricts access of PD1 to PDL1, and CTLA4 but not CD28 inhibits cis complex formation, probably via receptor-driven reorganization of B7 1 such that B7 1 dimer formation is favoured over the B7 1-PDL1 cis complex^{172,302}. Crystal structures of PD1 bound to monoclonal antibodies, including the FDA-approved nivolumab (PDB ID: 5GGR) and pembrolizumab (PDB: 5JXE), provide insight into their function-blocking activities, demonstrating that their binding epitopes directly overlap portions of the PD ligand recognition surface^{303,304}. **c** | Proposed alternative interactions for PD1. PD1 is co-expressed with PDL1 on a subset of tumour cells from patients with NSCLC. Additionally, PD1 may interact with PDL1 in cis, competing with inhibitory PD1-PDL1 trans interactions¹⁷³. Although the role of the cis PD1-PDL1 interaction within the tumour microenvironment remains unclear, this provocative work warrants future investigation. PD1-dependent recruitment of SHP2 directly inhibits its CD28 activation via dephosphorylation^{167,168} and therefore PD1 blockade may lead to reactivation of exhausted T cells, contrary to conventional models in which PD1 orchestrates TCR dephosphorylation and downregulation. Direct binding between the extracellular domains of PD1 and CD28 has not been described; however, non-ligand-blocking anti-PD1 monoclonal antibodies disrupted this PD1-dependent inhibition of CD28, suggesting that an additional uncharacterized surface on PD1 contributes to this activity¹⁷¹.

anti-human CTLA4 (REF.⁵²). The first two CTLA4-directed antibodies to enter clinical testing were both human-sequence anti-human CTLA4, developed using transgenic mice comprising germline configuration human immunoglobulin genes that rearrange to direct a repertoire of B cells expressing human antibodies. Ipilimumab (also known as MDX-010 and BMS-734016) and tremelimumab (CP 675,206) differed in the choice of Fc regions (IgG1 for ipilimumab, which binds to multiple activating FcγRs and is proficient for ADCC, and IgG2 for tremelimumab, which is largely limited in FcγR binding except for FcγRIIA H131)⁷⁸. Whereas the role of the Fc isotypes would become a central question regarding the mechanism of action of CTLA4 many years after their introduction into clinical development, ipilimumab was shown to mediate little ADCC activity towards activated T cells using NK cells *in vitro*¹⁸¹. An IgG1 format was originally selected for ipilimumab to facilitate rapid entry into the clinic (the antibody was derived from a transgenic mouse comprising human Fv region sequences and a human IgG1 constant region, and the initial clinical material was manufactured directly from fermentation of the original hybridoma cell line). To date, checkpoint blockade of CTLA4 has relied on antibodies for blocking the interaction of CTLA4 with its ligands. This inhibition must not interfere with the CD28–B7 ligand interactions required for co-stimulation of primary T cell responses. Notably, although ipilimumab contacts the conserved motif (MYPPPY) required for ligand binding by both CTLA4 and CD28, specificity for CTLA4 is afforded by a number of primary sequence and structural features unique to CD28, which preclude ipilimumab binding¹⁸² (FIG. 3). We discovered ipilimumab in 1999, and the first patients were dosed in June 2000 (REF.⁷). Tremelimumab began clinical testing in 2002 (REF.¹⁸³).

The first two PD1-directed antibodies to enter the clinic, nivolumab and pembrolizumab, both use human IgG4 Fc regions comprising stabilizing hinge region mutations. We discovered nivolumab in 2002 using human immunoglobulin transgenic mice¹⁵⁸, whereas pembrolizumab is the product of grafting the complementarity determining regions of a mouse mAb onto the homologous human antibody variable region to further reduce the immunogenicity of the mouse variable regions⁹. Atezolizumab, the first PDL1-directed antibody to enter the clinic, was also created by complementarity

determining region grafting⁹. It uses a human IgG1 Fc that is mutated to eliminate FcγR binding. Nivolumab entered the clinic in 2006, and pembrolizumab and atezolizumab both entered the clinic in 2011 (REF.⁹). Antibodies to PD1 differ from anti-PDL1 antibodies with respect to the molecular interactions they affect; anti-PD1 antibodies block PD1 interactions with both PDL1 and PDL2, whereas anti-PDL1 antibodies block interactions with PD1 as well as the interactions of PDL1 with B7-1. Given these differences, these antibodies may be expected to differ in their functional consequences and, moreover, work in concert; however, no formal head-to-head trial comparing anti-PD1 with anti-PDL1 has yet been performed.

Initial assessment of ipilimumab

The first clinical trials for ipilimumab (MDX-010) used single-dose, 3 mg/kg, infusions in patients with melanoma, ovarian cancer^{184,185} and prostate cancer¹⁸⁶. Intriguing anecdotal evidence of tumour responses was reported in patients with melanoma and ovarian cancer^{184,185}. Significant, and durable, tumour responses were then observed in a subsequent multi-dose study combining 3 mg/kg ipilimumab every 3 weeks with two peptide vaccines for the melanocyte differentiation antigen, gp100, in HLA-A2*02:01 patients with melanoma¹⁸⁷. Two complete responses and a partial response (each of >11 months duration) were documented, but safety signals of serious concern were noted (see below). These initial antitumour responses spurred a series of trials in melanoma that included combinations with a variety of approved therapeutics, such as IL-2 (REF.¹⁸⁸) and dacarbazine¹⁸⁹.

In these early clinical studies of ipilimumab, investigators noted that the appearance of new lesions did not always indicate treatment failure, and that some of these new lesions shrank or disappeared with continued treatment¹⁹⁰. Wolchok et al.¹⁹¹ devised new criteria for evaluation of efficacy to better capture the clinical activity of ipilimumab and potentially other immunotherapies, and to provide guidance for treatment discontinuation. These new guidelines assisted clinical development by allowing patients that were benefiting to remain on treatment¹⁹². However, development was still hampered by the absence of methods for measuring target engagement, receptor occupancy or biomarkers for patient selection. In addition, although these newly defined ‘immune-related response criteria’ were

demonstrated to correlate with survival, they remained experimental and did not provide accepted regulatory surrogate end points. The initial plan for seeking regulatory approval was based instead on conventional response criteria that had been used for non-immune-based cancer therapies. That plan, which was prepared in consultation with the FDA, involved three different phase II trials. The results of one of these trials were revealed in December of 2007. That study was an open label monotherapy trial in stage III and IV melanoma, and it did not meet the previously defined success criteria of 10% or greater WHO-defined objective responses. This result, together with data from a second phase II trial that was reported at ASCO a few months later¹⁹³, prompted a revision of the plan for seeking regulatory approval. That second trial randomly assigned patients with stage III and IV melanoma into three different dose cohorts receiving 0.3, 3 or 10 mg/kg (REF.¹⁹⁴). The overall survival curves from that trial demonstrated dose-dependent activity, but showed a late separation compared with what would be expected from conventional cancer therapies in which objective response rates or progression-free survival (PFS) can be used as meaningful surrogates for survival. The revised regulatory plan scrapped surrogate end points and focused instead on two randomized phase III trials in which the end points were changed to overall survival¹⁹⁵.

The first of these trials was a pivotal study in which patients were assigned in 3:1:1 ratio to three arms: ipilimumab, with and without gp100 peptides, and gp100 peptides alone for four doses every 3 weeks¹⁹⁶. The trial demonstrated an increase in median overall survival of 10.1 and 10.0 months for the ipilimumab with gp100 peptides and the ipilimumab-alone groups, respectively, compared with 6.4 months for the gp100-peptide-vaccine-only arm. More importantly, a significant percentage of patients were alive at 24 months (21.6% and 23.5% for the ipilimumab groups versus 13.6% for the control vaccine group). We note that long-term survival at 5 years was achieved by nearly 20% of the patients. Adverse events were observed in 10–15% of the patients in the ipilimumab groups versus 3% of the patients in the peptide group. These results led to the approval of ipilimumab for the treatment of metastatic melanoma in March 2011, 11 years after the initiation of clinical trials and 15 years after the initiation of the preclinical programme. An important phase IV follow-on trial compared 3 mg/kg versus 10 mg/kg of

ipilimumab monotherapy in metastatic melanoma and demonstrated that a dose of 10 mg/kg was superior to 3 mg/kg (31% versus 23% overall survival at 36 months) but with increased adverse events (30% grade 3/4 with 24% discontinuation at 10 mg/kg versus 14% grade 3/4 with 10% discontinuation at 3 mg/kg)¹⁹⁷. Despite the improved survival with the higher dose, 3 mg/kg every 3 weeks for four cycles (Q3W × 4) remained the dose for ipilimumab in metastatic melanoma.

Along with the unprecedented antitumour activity of ipilimumab, the clinical development of ipilimumab also uncovered a new class of adverse events that were considered to be 'autoimmune'¹⁹⁸ (BOX 2). These events, which came to be known as immune-related adverse events (IRAEs), were found to be most prevalent in certain organs such as the skin, colon and endocrine systems, but with increasing numbers of patients treated, effects on additional organ systems were identified¹⁹⁹. IRAEs can be of a serious nature, with colitis appearing as the most common grade 3 or 4 toxicity. The treatment of serious IRAEs typically involves cessation of ICB therapy and treatment with systemic glucocorticoids. Other immunosuppressive drugs, such as TNF blockers, are also used when steroids are insufficient. Retrospective analysis of clinical trial data did not reveal correlations between survival and either toxicity or steroid use²⁰⁰. With the development of algorithms for treatment of IRAEs, better management of these toxicities has allowed for broader use²⁰¹. Nivolumab and other PD1/PDL1 pathway inhibitors are also associated with IRAEs; however, their frequency and severity are lower than those observed with ipilimumab²⁰². In particular, serious gastrointestinal toxicities and hypophysitis are not frequent in patients treated with anti-PD1/PDL1 antibodies. However, pneumonitis, a potentially serious IRAE, is more frequently observed with PD1 pathway blockade than with CTLA4 blockade²⁰³. The combination of ipilimumab and nivolumab increases the frequency and severity of adverse events (BOX 2).

Initial assessment of nivolumab

Although ipilimumab was initially dosed at the potentially efficacious dose of 3 mg/kg, in March 2006, the severe toxicities associated with TGN1412, a CD28 agonist antibody²⁰⁴, mandated a more cautious single-dose escalation trial for nivolumab in patients with refractory solid tumours. The application for first-in-human clinical testing of nivolumab may have been the

Box 2 | Novel adverse events uncovered by checkpoint blockade

Toxicities as a consequence of immune checkpoint blockade (ICB) were expected given the phenotype of CTLA4^{-/-} mice, in which lymphocytic infiltration into essential organs results in lethality at 4–6 weeks of age. However, ant body treatment of mice or cynomolgus macaques did not reveal, to any significant extent, the variety of adverse events that were observed in clinical trials. Whether immune-related adverse events (IRAEs) from ICB are due to polyclonal T cell activation or activation of self-reactive antigen-specific T cells or loss of regulatory T (T_{reg}) cell function remains to be elucidated. In studies of CTLA4^{-/-} mice in which the T cell receptor β chain was fixed, T lymphocytic infiltrates were shown to be tissue specific³²⁶. Developing mouse models that replicate adverse events upon therapeutic treatment is an important area of study. Single cell transcriptomic analysis of intestinal T cells in patients receiving ICB revealed that colitis was associated with the activation of IFN γ producing T effector (T_{eff}) cells without alteration in the levels of T_{reg} cells³²⁷.

One of the more unusual IRAEs observed with ipilimumab is hypophysitis³²⁸. The mechanism of this specific toxicity may be distinct from toxicities caused by generalized immune activation and could instead be linked to surface expression of CTLA4 in the pituitary. It has been hypothesized that ipilimumab binding to CTLA4⁺ pituitary cells could lead to direct, Fc dependent, drug mediated cytotoxicity perhaps via complement^{329,330}.

The observation that a fraction of ICB treated patients experience tumour hyperprogression has generated concern that it may be drug related in some cases³³¹. Proposed mechanistic hypotheses for this phenomenon include activation of PD1⁺ T_{reg} cells³³¹ and reprogramming of tumour associated macrophages to an immunosuppressive phenotype mediated by Fc γ receptor binding to anti PD1 antibodies³³².

first application post-TGN1412 considered by the FDA for a T cell activating drug and, after considerable discussion, a protocol was approved in which the initial cohort of 39 patients was dosed at 0.3, 1, 3 and 10 mg/kg, and an expansion cohort at 10 mg/kg, with some patients receiving additional doses if tumour responses were observed¹⁵⁷. The first patient in the study was treated just 7 months after the TGN1412 trial failure. Significant antitumour responses were observed with nivolumab: a complete response in patients with colorectal cancer (CRC) with high microsatellite instability (MSI-H), partial responses in patients with melanoma and RCC, and two mixed responses (in patients with NSCLC and melanoma). As PD1 can be detected on circulating central memory T cells, receptor occupancy could be measured. Nivolumab was detected on peripheral T cells for several months after a single dose of 0.3 mg/kg (REF¹⁵⁷).

These results motivated a multidose trial in a broad assortment of indications including NSCLC, RCC and melanoma²⁰⁵. A total of 296 patients were treated every 2 weeks with doses ranging from 0.1 mg/kg to 10 mg/kg for up to 2 years. No maximum tolerated dose was observed; however, 14% of the patients experienced serious drug-related adverse events, and three deaths were associated with pneumonitis (see below), an inflammatory lung toxicity that is rarely observed with ipilimumab¹⁹⁴. Complete or partial objective responses were observed in patients with NSCLC (18%), melanoma (28%), and RCC (27%). Phase III trials were then initiated in each of these

indications^{206–209}, and nivolumab gained FDA approval for refractory melanoma in December of 2014, 8 years after entering the clinic. FDA approval for its use in refractory NSCLC and RCC was granted the following year. Dose–response data were obtained for patients with melanoma, RCC or NSCLC, and 3 mg/kg Q2W was the selected dosage regimen for all tumour types²¹⁰.

Impact of ICB on cancer therapy

Since the approval of ipilimumab in 2011, seven additional ICB therapeutic mAbs have been approved by the FDA for a broad range of different cancer indications (FIG. 4). The PD1/PDL1 pathway is now recognized as a critical checkpoint for immune responses in many different cancers, and PD1/PDL1-blocking agents as monotherapies or in combination with CTLA4 blockade or combined with chemotherapies or targeted therapies have become the standard of care in front-line metastatic melanoma, lung, kidney, liver, head and neck, triple-negative breast cancer (TNBC) and other cancers.

Clinical exploration of ICB continues in a number of settings, including adjuvant and neoadjuvant therapies. The activity of ipilimumab in metastatic melanoma led to its assessment and eventual approval in the adjuvant setting²¹¹. Ipilimumab treatment (Q3W × 4 followed by monthly dosing at 10 mg/kg) after surgical tumour resection resulted in a 10.5% increase in recurrence-free survival in favour of ipilimumab over placebo after a median follow-up of 5.3 years. However, adjuvant use of nivolumab or pembrolizumab has supplanted the use of ipilimumab, owing to

its superior benefit and reduced incidence of adverse events (70.5% and 75.4% 12 months recurrence-free survival for nivolumab and pembrolizumab, respectively)^{212,213}.

Neoadjuvant therapy using anti-PD1 and combination of anti-PD1 with anti-CTLA4 have also demonstrated a high level of pathological responses. In this setting, the antibody is administered several weeks prior to surgery and response is measured by pathological analysis of the resected tumour as well as survival. A pilot study of nivolumab in the neoadjuvant setting in NSCLC resulted in major pathological responses in 9 of 20 patients²¹⁴ along with the expansion of specific T cells in 8 of the 9 patients assessed. In a study of neoadjuvant ipilimumab plus nivolumab in melanoma,

the 2-year recurrence-free survival was 84%²¹⁵. High tumour mutational burden and high IFN γ -related gene signatures were associated with improved responses. Many neoadjuvant trials that include ICB are in progress in most solid tumour histologies²¹⁶.

Biomarkers

Although most of the approved uses of ICB therapeutics do not require diagnostic tests, these are required for some indications such as NSCLC, TNBC and MSI-H and mismatch repair-deficient (dMMR) cancers (FIG. 4). The expression of PDL1 on malignant cells, was identified as a potential biomarker for response to PD1 blockade in the phase I trial of nivolumab¹⁵⁷, and confirmed by analyses of larger subsets of patients from

the same trial^{205,217}. PDL1 expression makes intuitive sense as a biomarker given that it is induced by common γ -chain cytokines²¹⁸, and could therefore indicate the presence of a pre-existing, but quiescent, tumour immune response, with the potential for ICB-mediated reactivation. The role of tumour PDL1 expression in blocking immune clearance of cancers has been termed 'adaptive resistance'¹¹²; however, the phrase 'reactive resistance' might be more descriptive given that the biological implication of the word 'adaptive' implies a selection process. Establishment of a threshold for determining PDL1 positivity is one of the challenges for using this biomarker as a diagnostic test, and different indications use different thresholds ranging

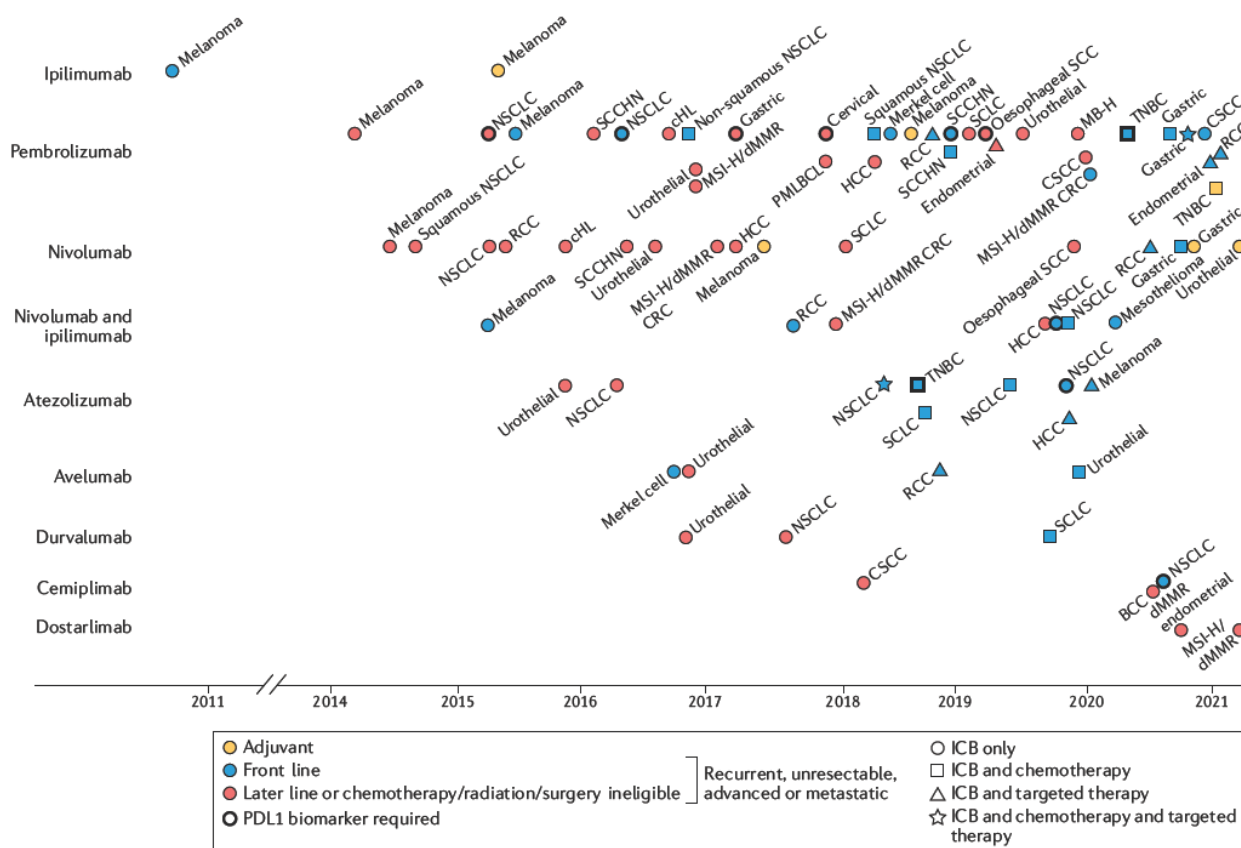


Fig. 4 | FDA approvals of ICB therapeutics. US regulatory approvals for immune checkpoint blockade (ICB) drugs ipilimumab (anti CTLA4 antibody), pembrolizumab (anti PD1 antibody), nivolumab (anti PD1 antibody), atezolizumab (anti PDL1 antibody), avelumab (anti PDL1 antibody), durvalumab (anti PDL1 antibody), cemiplimab (anti PD1 antibody) and dostarlimab (anti PD1 antibody) are listed. These approvals include several accelerated approvals that require additional confirmatory trials for full approval³⁰⁵. For some indications, ICB drugs are used in combination with conventional chemotherapy, targeted agents (atezolizumab with bevacizumab for non small cell lung cancer (NSCLC) or hepatocellular carcinoma (HCC), pembrolizumab or avelumab with axitinib for renal cell

carcinoma (RCC), pembrolizumab with lenvatinib for endometrial cancer and RCC, atezolizumab with cobimetinib and vemurafenib for melanoma, nivolumab with cabozantinib for RCC, pembrolizumab with trastuzumab for gastric cancer), or in combination with another ICB drug, cHL, classical Hodgkin lymphoma; CRC, colorectal cancer; CSCC, cutaneous squamous cell carcinoma; MB H, high mutational burden cancers; dMMR, mismatch repair deficient cancers; MSI H, high microsatellite instability cancers; PMLBCL, primary mediastinal large B cell lymphoma; SCCHN, squamous cell cancer of the head and neck; SCLC, small cell lung cancer; TNBC, triple negative breast cancer. The designation 'Gastric' includes oesophageal, oesophageal junction and gastric cancers.

from 1% to 50% positivity to determine treatment eligibility. In addition to malignant cells, PDL1 is also expressed on tumour-infiltrating immune cells¹¹² and correlates with response^{219,220}. A diagnostic test based on this biomarker is used for treatment decisions for atezolizumab in urothelial cancer, TNBC and NSCLC.

Cancer cell somatic mutation frequency, or tumour mutational burden, is another biomarker that has been explored for ICB therapy. Somatic mutations are a hallmark of cancer and are likely to be the major source of neoantigens recognized by the immune system^{221,222}. Positive correlations between tumour mutational burden and clinical benefit from ICB therapy were first reported for CTLA4 blockade in patients with melanoma^{223,224}, and subsequently for PCD1 blockade and PD1 and CTLA4 combination blockade therapies in NSCLC^{225,226}. Pembrolizumab was approved as a treatment option for all patients with metastatic solid tumours harbouring ≥ 10 mutations per megabase, who had progressed on prior therapy and had no satisfactory alternative treatment options²²⁷, but tumour mutational burden and PDL1 expression seem to be independent predictors of ICB clinical benefit²²⁸. Interestingly, tumour mutational burden is not a predictive biomarker for anti-PD1 and anti-CTLA4 combination therapy²²⁹. The presence of dMMR, which may be a surrogate for somatic mutation and neoantigen expression, has also emerged as a biomarker for ICB^{230–232}.

Ipilimumab and nivolumab dual ICB

In 2005, we performed the first mouse tumour experiments combining anti-CTLA4 and anti-PD1^{233,234}. These experiments revealed that efficient tumour rejection could be achieved in a variety of tumour models when the two antibodies were administered concurrently. In addition, they also showed that the dosing of one antibody relative to the other could be altered, foreseeing potential dose de-escalation. Combination of checkpoint inhibitors together with a cellular vaccine were also studied in the nonimmunogenic B16 melanoma model²³⁵. In vitro studies revealed that in the mixed lymphocyte reaction, in which purified T cells respond to allogeneic dendritic cells, the addition of ipilimumab to nivolumab resulted in a dose-dependent increase in IFN γ over that observed for nivolumab alone²³⁶. We note that ipilimumab alone was not effective in augmenting the mixed lymphocyte reaction. We first observed this in vitro synergy in early 2004, and it was one factor in our

decision later that year to take nivolumab into the clinic despite the existing resource constraints imposed on a small biotech by our ipilimumab development activities. In retrospect, the role of both PD1 and CTLA4 in CD28 co-stimulation provides a simple mechanistic explanation for this synergy. PD1 can be thought of as an on/off switch for CD28 signalling mediated by SHP2, whereas CTLA4 acts as a rheostat by titrating B7 ligation. Activation of the superantigen response in peripheral blood mononuclear cells was also enhanced in the combination, as shown for the combination of another CTLA4 antibody and nivolumab²³⁷. Cynomolgus macaques were used to assess the safety of the combination and although some observations (such as increase in spleen cellularity) were unique to the combination, serious adverse events were not observed, consistent with the inability of the cynomolgus macaques to foreshadow the profile in humans²³⁶. However, another study in cynomolgus macaques using higher dosage of ipilimumab and lower dosage of nivolumab showed induction of inflammatory infiltrates, including myocarditis²³⁸.

In 2009, the first patients were treated in a dose-escalation study of the combination. From the earliest patients treated, it became clear that frequent and deep responses occurred²³⁹. These results led to a phase III study in which combined ipilimumab and nivolumab (ipilimumab at 3 mg/kg Q3W $\times$ 4 and nivolumab at 3 mg/kg Q3W) was compared with ipilimumab or nivolumab regimens alone. The combination, in a 5-year follow-up, was observed to give 52% long-term survival compared with 44% for nivolumab and 26% for ipilimumab alone²⁴⁰. Further follow-up at 6.5 years maintained the observed effects on overall survival (49%, 42% and 23%), including a prolonged treatment-free interval following discontinuation in the combination arm compared with the monotherapy arms²⁴¹. The study was not powered to have a statistical comparison with nivolumab alone. Importantly, grade 3–4 adverse events were reported in 55% of patients in the combination arm versus 16.3% for nivolumab and 27.3% for ipilimumab at 3 years. Discontinuation rates, another metric for assessing tolerability of the therapies, were 29.4% for the combination therapy and 5.1% for nivolumab and 13.2% for ipilimumab. The first approval of the combination regimen was for melanoma in 2015.

Although the regimen for melanoma used the approved dosage of each of the

therapies, subsequent trials in several other tumour types used reduced levels of ipilimumab, such as 1 mg/kg at an extended dose interval, now typically every 6 weeks, for a limited number of doses while maintaining nivolumab therapy. In patients with NSCLC, decreasing the frequency of ipilimumab dosing seems to reduce toxicity while still providing efficacy²⁴². Notwithstanding the improvement in response rates and survival, concerns about the adverse events remain. We note that, in addition to the well-described toxicities, several lethal cases of myocarditis were observed^{243,244}. Myocarditis is relatively rare in ICB but is enhanced in combination therapy. Because of its potential mortality it also tends to be reported (reviewed in REF.²⁴⁵). However, despite these concerns, exploration of combination therapy has continued. The prediction of which patients will experience adverse events remains an important unmet area of study.

The combination of ipilimumab and nivolumab has now been approved in six different indications in a variety of dosing regimens (FIG. 5). The combination was approved in RCC, in which patients with intermediate or poor risk showed improved survival compared with chemotherapy (60% versus 47% at 30 months in intermediate- or poor-risk patients and 64% versus 56% in the intent-to-treat population)^{209,246}. Additional approvals were obtained in NSCLC, MSI-H/dMMR CRC, hepatocellular carcinoma and pleural mesothelioma. In NSCLC, the ipilimumab and nivolumab combination is approved for patients with squamous and non-squamous pathologies with PDL1 expression of $>1\%$. This offers the first chemotherapy-free regimen for lung cancer. However, given the importance of chemotherapy in lung cancer, a short course of chemotherapy (two cycles of platinum doublet chemotherapy) in addition to ipilimumab–nivolumab has been tested and is approved for NSCLC independently of PDL1 status²⁴⁷. We note that tests of ipilimumab in patients with NSCLC using either phased or concurrent dosing with chemotherapy failed to show significant clinical benefit^{248,249}.

Is there a molecular basis for the effectiveness of the combination of anti-CTLA4 and anti-PD1 blockade? Mechanistic studies in mouse tumour models, although more tractable, are biased by the role of anti-CTLA4 in T_H1 cell depletion, an activity that remains unclear in human studies. Anti-CTLA4 and anti-PD1 may provide enhanced activity by activating separate, non-redundant, pathways acting

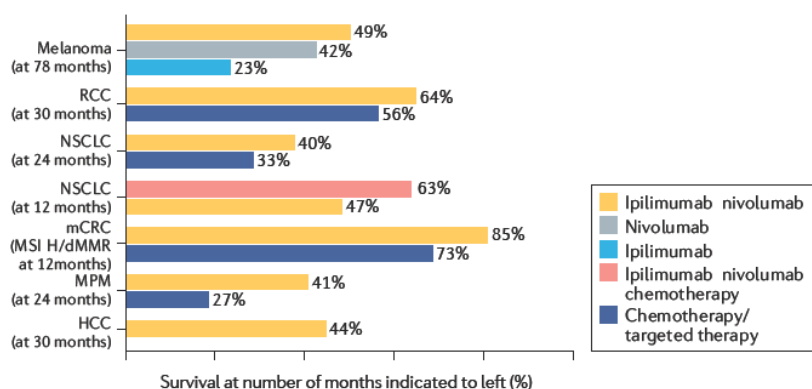


Fig. 5 | Combination therapy with CTLA4 and PD1 inhibitors. The figure shows those approved indications (see FIG. 4) where ipilimumab and nivolumab demonstrate enhanced activity: melanoma²⁴¹ (3 mg/kg ipilimumab every 3 weeks for four cycles (Q3W × 4) together with 1 mg/kg nivolumab Q3W × 4 followed by 3 mg/kg nivolumab Q3W); renal cell carcinoma (RCC)²⁴⁶ (3 mg/kg nivolumab and 1 mg/kg ipilimumab Q3W × 4, followed by 3 mg/kg nivolumab Q2W for 2 years or until progression); non small cell lung cancer (NSCLC) at 12 months³⁰⁶ (1 mg/kg ipilimumab Q6W × 4 and 3 mg/kg nivolumab Q2W × 4, followed by 3 mg/kg nivolumab) and NSCLC²⁴⁷ at 24 months (360 mg nivolumab Q3W and 1 mg/kg ipilimumab Q6W plus two cycles of chemotherapy); microsatellite high/deficient mismatch repair colorectal cancer (mCRC MSI H/dMMR)²³⁸ (3 mg/kg nivolumab and 1 mg/kg ipilimumab Q3W × 4 followed by 3 mg/kg nivolumab Q2W); malignant pleural mesothelioma (MPM)³⁰⁷ (nivolumab 3 mg/kg Q3W and ipilimumab 1 mg/kg Q6W followed by 3 mg/kg nivolumab); HCC³⁰⁸ (nivolumab 1 mg/kg and ipilimumab 3 mg/kg).

on different cell types. For example, CTLA4 inhibition can elicit and/or expand the repertoire of tumour-reactive T cells (as assessed by TCR sequencing²⁵⁰) by lowering the threshold of T cell activation, and anti-PD1, principally, can reactivate clonally expanded tumour-reactive T cells.

An alternative explanation is that CTLA4 and PD1 contribute to the exhausted phenotype acting within the same effector cell^{251,252}. The added effects of co-blockade were first observed in anti-viral effector T cells in LCMV for PD1 and LAG3 (REF. 253). Interestingly, co-blockade of CTLA4 and PD1 was ineffective in LCMV²⁵⁴. The presence of CTLA4^{high}/PD1^{high} double-positive T cells in human tumours may be a significant component of the efficacy of combination therapy. CTLA4^{high}/PD1^{high} CD8⁺ T cells have been observed in human melanoma tumours by flow cytometry²⁵⁵ as well as in both CD4⁺ and CD8⁺ T cells in multiple tumour histologies by flow cytometry and immunohistochemistry²⁵⁶. In melanoma²⁵⁵, double-positive T cells were partially exhausted and their abundance correlated with the response to anti-PD1. CTLA4^{high}/PD1^{high} T cells could be activated by concurrent blockade and may be more specifically targeted by bispecific dual PD1/CTLA4 blockers²⁵⁶. CTLA4 blockade-mediated enhancement of an in vitro mixed lymphocyte reaction (discussed above²³⁶) depends on PD1

blockade, which is also consistent with a cell-intrinsic mechanism of synergy.

More recently, high-content flow cytometry has been used to identify subsets of T cells that respond to either PD1 blockade or to both PD1 and CTLA4 blockade, although similar analyses of ipilimumab monotherapy samples are unavailable. In these analyses, CD8⁺ T cells that are analogous to terminally differentiated exhausted-like T cells found in mouse tumours showed increased expansion in human tumours relative to peripheral blood. In addition, CD4⁺ T cells expressing ICOS that display an effector phenotype are also expanded, as has been observed for T_H1-like CD4⁺ T_{eff} cell subsets in mouse tumours that respond to anti-CTLA4 (REF. 257), consistent with the effects of anti-CTLA4 in CD4⁺ T cells.

ICB beyond CTLA4 and PD1

T cell function is controlled by the integration of positive and negative signals. T cell non-responsiveness is associated with the expression of multiple negative regulatory receptors, each of which may contribute to the exhausted phenotype^{250,252}. The inhibitory receptor LAG3 provides a useful illustration of these concepts. LAG3, discovered in 1990 as a CD4 homologue, was defined as a negative regulator of CD4⁺ T cell activation^{258,259}. LAG3 binds to MHC class II with higher affinity than CD4 but it can function on CD8⁺ cells too²⁶⁰. Fibrinogen-like protein 1 (FGL1), a soluble

protein expressed by tumour cells and hepatocytes, has recently been reported as negative regulatory ligand for LAG3 (REF. 261). LAG3 blockade alone has little effect in restoring exhausted LCMV-reactive T cells in vivo and, similarly, has little antitumour activity in mouse models, yet LAG3 blockade improves the effects of anti-PD1/PDL1 in both models^{253,262}. These results led to the testing of anti-LAG3 (BMS-986016 or relatlimab) in combination with anti-PD1 nivolumab in human malignancies, including a phase III in melanoma. This trial in melanoma demonstrated an improved PFS for the combination over that of nivolumab alone (PFS of 47.7% versus 36% at 12 months with a median PFS of 10.12 months versus 4.63 months) without a significant increase in adverse events (18.9% versus 8.7% grade 3/4 and 14.6% versus 6.7% discontinuations for all adverse events), thereby establishing the validity of dual ICB apart from anti-PD1/anti-CTLA4 (REF. 263).

Another pathway that has been actively studied and for which many antibodies are in clinical development²⁶⁴ is the nectin family, consisting of the activating receptor CD226 and its principal negative regulatory counterpart T cell immunoreceptor with Ig and ITIM domains (TIGIT)^{265,266}. Other negative regulatory receptors, such as PVRIG and CD96, may also affect this pathway. Ligands for the nectin family include CD155 (also known as poliovirus receptor) and PVRL2 (also known as Nectin-2). The functional relevance of isotype choice is also an issue in anti-TIGIT antibody development. As discussed for anti-CTLA4, simple functional blockade (use of non-FcγR binding antibodies) as opposed to antibodies that bind to activating FcγRs (with potential for depletion of activated TIGIT⁺ T_{reg} cells or CD8⁺ T cells) and improve TIGIT blockade⁸⁷ may have clinical implications.

Additional negative regulatory receptors and their ligands have also been studied preclinically and clinically in combination with anti-PD1/PDL1 inhibitors, including NKG2A/HLA-E, T cell immunoglobulin mucin receptor 3 (TIM3, also known as HAVCR2)/phosphatidylserine (PS), leukocyte immunoglobulin-like receptor subfamily B members 1 and 2 (ILT2, also known as LILRB1, and ILT4, also known as LILRB2)/HLA-G, P-selectin glycoprotein ligand 1 (PSGL1)/VISTA and killer cell immunoglobulin-like receptor 3DL3 (KIR3DL3)/HHLA2 (REFS 267–270). VISTA, described as a distant B7 family member with negative regulatory properties expressed by myeloid cells and neutrophils²⁷¹

within the tumour microenvironment²⁷², is a particularly compelling target. It is a ligand for the negative regulatory receptor PSGL1 (REFS^{273,274}), long known for its role in T cell migration via selectin binding. PSGL1 knockout mice can control LCMV infection and antibodies against VISTA can control tumour growth. We note that VISTA–PSGL1 interactions are governed by pH, thereby providing a way of targeting VISTA within the tumour microenvironments through an acidic pH-selective, PSGL1-blocking anti-VISTA antibody²⁷⁴.

Activating receptors on T cells have also been extensively studied as targets for immunotherapy, beginning with urelumab, an agonistic antibody to CD137. Urelumab showed monotherapy activity in clinical trials in melanoma; however, safety concerns related to liver toxicity impeded further exploration of that high-dose schedule^{275,276}. A tolerable lower-dose schedule was identified²⁷⁷ but a combination trial with nivolumab failed to demonstrate evidence of synergy²⁷⁸. Agonist antibodies against other co-stimulatory targets, such as TNF receptor superfamily member 4 (TNFRSF4, also known as OX40), GITR (also known as TNFRSF18), CD27 (also known as TNFRSF7), and ICOS have demonstrated excellent activity in mouse tumour models^{276,279}; however, agonists to the human orthologues have failed to show significant clinical activity. These antibodies were safe even at high doses and in combination with PD1/PDL1 inhibitors.

Reasons for the observed lack of efficacy of agonist antibodies to these targets include: insufficient agonist activity, poorly explored dosing and regimen required for activation, downregulation of the target receptor²⁸⁰, excessive activation that may result in T cell apoptosis²⁸¹, and lack of translatability of the mechanisms responsible for activity in mouse models to human tumours. For example, many of the agonist targets are expressed by both T_{eff} and T_{reg} cells. Optimal activity in mouse models is often observed with mouse IgG2a antibodies, which can exhibit potent antibody-dependent cellular phagocytosis (ADCP)/ADCC-mediated T_{reg} cell depletion but are possibly less effective as agonists. Translation from mouse models to human cancers is also complicated by the fact that human IgG1 antibodies (which is the common format for agonists) may not exhibit the same cell depletion activity in human solid tumours as that from mouse IgG2a antibodies in syngeneic mouse models. Differences in the number and function of intratumoural NK cells or other effector cells²⁸² as well as interspecies differences in FcγRs⁷⁸ may account for

these observations. In addition, antitumour activity in mouse models may be more dependent on T_{reg} cells than in established human tumours²⁸³.

Concluding remarks

ICB represents a distinct fifth pillar of cancer therapy beyond surgery, radiation, chemotherapy and targeted therapy. The discovery and development of ICB was

built on the foundation of the scientific understanding of tumour immunology. Current research efforts and clinical development in oncology are focused on how to enhance its therapeutic efficacy. At the centre of these efforts have been the insights into the molecules whose inhibition drives activation of T_{eff} cell responses, and attention is being directed towards immunosuppressive cells and pathways

Box 3 | Immune checkpoint blockade for infectious disease

The ability of checkpoint antibodies in restoring anti pathogen specific immune responses has led to their study in the therapy of infectious diseases. An early trial of nivolumab in hepatitis C virus (HCV) infection showed some benefit in reducing HCV RNA in blood using a single dose at 10mg/kg (REF³³³). In patients with hepatitis B virus (HBV) positive hepatocellular carcinoma (HCC), there were initial concerns that reactivation of HBV-reactive T cells might cause liver toxicity, but dose escalation studies of anti PD1 antibodies revealed no safety issues and some reductions of circulating levels of HBV surface antigen (HBsAg)³³⁴. PDL1 blockade in a woodchuck model of HBV infection augmented reductions in HBsAg levels³³⁵. These observations have prompted an interest in PD1 blockade in HBV cure strategies³³⁶. Another use for PD1 inhibitors was found in the treatment of patients with progressive multifocal leukoencephalopathy (PML), which is due to reactivation of John Cunningham (JC) virus in the nervous systems of patients undergoing treatment for autoimmune disorders. Nivolumab and pembrolizumab reduced JC viral load in the cerebrospinal fluid indicating that reactivation of PD1⁺ antigen-specific T cells occurred in these patients^{337,338}.

PD1 has also been studied in HIV infection, as a paradigm for chronic virus infection in humans. In people living with HIV, HIV specific CD8⁺ T cells have upregulated PD1 and reduced levels of cytokine expression compared with CMV-reactive T cells³³⁹. HIV specific CD4⁺ T cells are also functionally impaired with high expression of CTLA4^{340,341}. In ex vivo experiments, these cells can respond favourably to anti PDL1 ex vivo, leading to increased cytokine production³⁴². A trial of anti PDL1 antibody (BMS 936559) in people living with HIV on suppressive antiretroviral therapy (ART) showed a significant increase in antigen-specific T cells in two of six patients³⁴³.

Timing of the administration of anti PD1 in rhesus macaques infected with simian immunodeficiency virus (SIV) is important in demonstrating a functional effect. In viraemic rhesus macaques infected with SIV, anti-PD1 treatment augmented SIV-specific T cells and reduced plasma levels of SIV RNA³⁴⁴. By contrast, in SIV infected rhesus macaques on suppressive ART, anti PD1 had no effect on persistence of virus or on time to viral rebound after stopping ART³⁴⁵. However, administration of anti PD1 either during or immediately following ART discontinuation in SIV infected macaques resulted in a 2log reduction of peak virus load, suggesting that some antigen must be present to enhance the effects of ICB³⁴⁶. Finally, using anti PD1 antibodies at the time of initiation of ART resulted in an accelerated decline in viral load and enhanced HIV specific CD8⁺ T cell function³⁴⁷.

A major interest has emerged in the use of ICB to eliminate HIV latency. PD1⁺ T cells from people living with HIV on ART are enriched in latent virus³⁴⁸. Expression of PD1 and other immune checkpoint markers plays a part in establishing latent infection in resting CD4⁺ T cells³⁴⁹. Reversal of HIV latency could be observed in the presence of anti PD1 together with another activating stimulus such as bryostatins³⁵⁰. Recent trials of ICB in people living with HIV on ART with cancer have provided opportunities to study the effects of ICB on HIV latency. In a recent prospective observational study of pembrolizumab, anti PD1 treatment induced a significant increase in cell associated HIV RNA consistent with latency reversal³⁵¹. In another prospective study, treatment with nivolumab alone or with combined nivolumab and ipilimumab resulted in latency reversal in the combination arm and a decline in the reservoir in some participants³⁵².

ICB may also be relevant in the control of parasite and bacterial pathogens. *Plasmodium falciparum* infection in humans upregulates PD1 on CD4⁺ T cells. Treatment of mice with combined PD1 and LAG3 blockade results in reduced parasitaemia³⁵³. Increased levels of PDL2 expression in dendritic cells in individuals with non lethal malaria may limit PDL1 mediated cell exhaustion³⁵⁴. However, depending on the pathogen biology, ICB may result in poorer outcomes, such as when several cases of tuberculosis occurred after PD1 blockade in cancer patients³⁵⁵. These results were predicted by observations in mice with PD1^{-/-} CD4⁺ T cells, which had increased mortality and immune pathology after infection with *Mycobacterium tuberculosis*^{356,357}. The potential utility of PD1 inhibitors has also been evaluated in sepsis. Studies using the caecal ligation puncture model of sepsis revealed that PD1 knockout mice had improved survival³⁶⁰. PD1 expression may be relevant in the immunosuppressive phase of sepsis, although PD1/PDL1 blockade could exacerbate immune activation. An anti PDL1 antibody (BMS 936559) was tested in a single ascending dose study and shown to be safe³⁵⁸.

that limit the immune response to tumours. Other successful strategies, such as direct tumour targeting through antibodies or antibody–drug conjugates, T cell and NK cell redirected killing through bispecific antibodies, and cell therapy, will probably be used in concert with ICB in addition to the ‘traditional’ forms of cancer therapy such as surgery, radiation, chemotherapy and targeted therapies. In addition, ICB may find additional utility in the treatment of other diseases, particularly infectious diseases (BOX 3). Given the many potential targets in immuno-oncology, it is noteworthy that as of this writing only those antibodies that target CTLA4 and PD1 or PDL1 have been approved for clinical use. CTLA4 and PD1/PDL1 probably represent the most potent regulators of T cell function and are unlikely to be rivalled. However, other negative regulators, such as LAG3 and TIGIT, may contribute to antitumour activity in combination with CTLA4 and PD1/PDL1, increasing the number of responding patients in currently approved indications, expanding ICB to new indications and perhaps mediating response without an increase in adverse events. We are grateful to have had the opportunity to participate in this grand effort.

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

A.J.K. and N.L. are owners of BMS stock, the company that markets ipilimumab and nivolumab. S.C.G.-T. declares no competing interests.

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