Immune checkpoint blockade immunotherapy to activate anti-tumour T-cell immunity

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Summary

The tumour microenvironment plays a dual role in cancer: it can promote tumour progression by establishing pro-tumour survival conditions but can also suppress tumour progression by killing cancer cells or inhibiting their outgrowth. These dynamically interconnected processes are under intense investigation to better understand cancer pathophysiology and allow identification of new therapeutic approaches. The ability of cancer cells to evade anti-tumour T-cell activity in the microenvironment has recently been accepted as a hallmark of cancer progression. This review will highlight the most promising therapeutic approach aimed at activating anti-tumour T-cell immunity in the cancer microenvironment: blocking inhibitory immune regulatory proteins (immune checkpoint ligands and receptors). There is emerging evidence that haematological tumours co-opt immune checkpoints as a major immune resistance mechanism. Preclinical findings indicate that targeted therapies and blockade of immune checkpoints could be combined to promote therapeutic synergy and long-term anti-tumour immunity to improve clinical outcomes for cancer patients.

Keywords: cancer, PD-L1, PD-1, B7 family, lenalidomide.

Nonspecific cytotoxic agents remain the backbone of current treatments due to their ability to kill dividing abnormal cancer cells and their long clinical history, but are limited by their narrow therapeutic index, significant toxicities and acquired resistance (Riches et al, 2011). Improved understanding of cancer pathogenesis has identified new-targeted agents (biologics or small-molecule inhibitors) that aim to inhibit molecular pathways that are crucial for cancer cell growth and maintenance (Cortes et al, 2012; Brown, 2013). These drugs can induce striking tumour regressions in patients and have opened up a new paradigm for cancer treatment. The importance of the tumour microenvironment for the survival and progression of cancer cells has led to the concept that specifically targeting kinases in non-malignant microenvironment cells may also constitute an alternative to cytotoxic therapies (Lutzny et al, 2013).

However, similar to imatinib in chronic myeloid leukaemia (CML), tumour regressions effectuated by targeted drugs risk being followed by the development of progressive disease owing to the emergence or presence of drug-resistant cancer cell variants (Haber et al, 2011; Vanneman & Dranoff, 2012; Wilson et al, 2012) and/or cancer stem cells (Corbin et al, 2011; Chen et al, 2012a). Resistance is likely to involve secondary mutations within the protein target or compensatory changes within the target pathway that allows highly adaptable cancer clones to by-pass drug-mediated inhibition. Moreover, advanced and aggressive cancers have often lost their dependence on the therapeutic target and/or the tumour microenvironment pro-survival signals (van de Donk et al, 2012; Kridel et al, 2012). This has led cancer biologists to consider alternative therapy that has the potential to effectuate long-lived tumour control and match the cancer’s capability to evolve. It is now widely believed that to win the fight against cancer, it will be necessary to develop new strategies to kill the maximum number of cancer cells efficiently using the correct combination of targeted (or chemotherapeutic) agents but also to stimulate the immune response to harness the exquisite specificity and cytotoxic potency of CD8+ cytolytic T-lymphocytes (CTLs) against residual tumour and cancer stem cells.

Cancer immunotherapy and clinical success

The many genetic and epigenetic abnormalities that are characteristic of cancer cells provide a diverse set of antigens that the immune system can use to distinguish tumour cells from healthy cells. Importantly, cancer immunologists continue to identify a large array of immunogenic tumour-associated antigens that include unaltered tissue-differentiation antigens, mutated genes or neo-antigens, viral antigens and epigenetically-regulated antigens (Schreiber et al, 2011; Restifo et al, 2012). T-cell recognition of antigen through the T-cell receptor (TCR) is regulated by a balance between co-stimulatory and inhibitory signals (members of the B7/CD28 family (Paulos & June, 2010). These co-signalling molecules are
important regulators of the T-cell immune synapse receptor layer (Dustin & Depoil, 2011). Immune synapses control the assembly of signalling complexes and are the master regulators of T-cell activation and effector function in response to antigen-presenting cells (APCs) and target cells (e.g. tumour cells; Ramsay et al, 2008). The B7 (and tumour necrosis factor superfamily, TNF-family) members consist of membrane ligands that positively and negatively regulate antigen-dependent T-cell responses by engaging their activating or inhibitory co-receptors (Fig 1). Inhibitory ligands and receptors (immune checkpoints) have a central physiological role in tightly controlling the amplitude of T-cell activation in lymph nodes or effector responses in peripheral tissue (maintaining self-tolerance and limiting immune-mediated collateral tissue damage). The ability of tumours to co-opt these inhibitory pathways represents an emerging and critical T-cell resistance mechanism within the microenvironment. In addition, the loss of immunogenic antigen including loss or down-regulation of major histocompatibility complex (MHC) class I molecules and release of immunosuppressive cytokines in the tumour microenvironment are well-characterized cancer immune evasion mechanisms (Schreiber et al, 2011). It is believed that the ability of tumours to actively suppress CTL activity by utilizing immunosuppressive mechanisms and pathways has limited the clinical success of previous immunotherapy including therapeutic cancer vaccines (Ramsay et al, 2008, 2009; Le Dieu et al, 2009).

Anti-Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4, also known as CD152) and, most recently, anti-programmed cell death 1 (PD-1, also known as PDCD1, CD279) and PD-1 ligand 1 (PD-L1, also known as CD274) fully humanized neutralizing monoclonal antibody (mAb) monotherapy have cleared the clinical acceptance bar with durable tumour responses and activation of long-term immunological memory detected in advanced solid tumours where conventional therapies have failed (Hodi et al, 2010; Brahmer et al, 2012; Topalian et al, 2012). The success of these clinical trials validates that activation of endogenous

**Fig 1.** Multiple co-signaling [co-stimulatory (+) and inhibitory molecules (−)] interactions regulate T-cell responses. The various interactions between T-cells (yellow cell) and antigen-presenting cells (APCs, blue cell) are shown that regulate the T cell response to antigen (peptide–major histocompatibility complex (MHC) molecule complexes that are recognized by the T-cell receptor (TCR) − indicated using a black arrow). These interactions occur at the initiation of T-cell activation in lymph nodes (APCs are dendritic cells, DCs) or in peripheral tissues or tumours (where effector responses are regulated). Many ligands can bind to multiple co-stimulatory and inhibitory receptors. One important family of membrane-bound ligands that bind both co-stimulatory and inhibitory receptors is the B7 family (immunoglobulin superfamily). Many of the receptors for more recently identified B7 family members have not yet been identified. Tumour necrosis factor (TNF) family members that bind to cognate TNF receptor family molecules represent a second family of regulatory ligand–receptor pairs (these receptors predominantly deliver co-stimulatory signals). Inhibitory ligands and receptors (immune checkpoints, indicated using red arrows) have a central role in tightly controlling the amplitude of T-cell activation and maintaining self-tolerance and limiting immune-mediated collateral tissue damage. In contrast to healthy cells, tumour cells exploit their function to induce impaired T-cell synapse formation and function. Co-stimulatory pathways and receptors (indicated using green arrows) that enhance the TCR signal can be activated using agonist antibodies that are in development or being tested in the clinic including anti-CD137 (also known as TNFRSF9) and anti-CD40. The B7 family is part of a complex signalling network that comprises other immunoglobulin superfamily members, the tumour necrosis factor (TNFRSF), chemokines, cytokines and adhesion molecules. A2aR (also known as ADORA2A), adenosine A2a receptor; B7RP1, B7-related protein 1; BTLA, B and T lymphocyte attenuator; GAL9 (also known as LGALS9), galectin 9; HVEM (also known as TNFRSF14), herpesvirus entry mediator; ICOS, inducible T-cell co-stimulator; KIR, killer cell immunoglobulin-like receptor; LG3, lymphocyte activation gene 3; PD-1 (also known as PDCD1), programmed cell death protein 1; PD-L1 (also known as CD274), PD-1 ligand; PD-L2 (also known as PDCD1LG2), PD-2 ligand; TIM-3 (also known as HAVCR2), T-cell membrane protein 3).
anti-tumour T-cells in cancer patients provides significant therapeutic effect (Pardoll, 2012a). The solid cancer field has led this translational research demonstrating that ‘active immunotherapy’ is a major modality for cancer therapy (Pardoll, 2012a). However, immune checkpoints are widely expressed by both haematological tumour cells and their microenvironment and are now starting to gain attention as new targets for novel immunotherapeutic intervention strategies (Paulos & June, 2010; Greaves & Gribben, 2012; Norde et al, 2012; Wilcox et al, 2012). This review will focus on the activation of anti-tumour conventional T-cell immunity in cancer. However, it is worth noting that anti-tumour immunotherapy using Gamma delta T-cells is also an active translational research approach and was the subject of a recent review (Braza & Klein, 2013).

**CTLA4 – a regulator of early stage T-cell activation in response to antigen**

CTLA4 (CD152) was the first co-inhibitory molecule identified (Brunet et al, 1998) and the first immune checkpoint receptor to be clinically targeted [US Food and Drug Administration (FDA) approval for metastatic melanoma, (Hodi et al, 2010)]. It is expressed exclusively on T-cells and regulates the amplitude of the early stages of T-cell activation (conventional naive and memory T-cells). CTLA4 counteracts the activity of the T-cell co-stimulatory receptor CD28 once antigen recognition occurs (Rudd et al, 2009). The stronger the CD28 signal, the greater the amount of CTLA4 that is trafficked to the T-cell surface. CD28 and CTLA4 share identical ligands: CD80 (B7.1) and CD86 (B7.2). CTLA4 expression on the surface of T-cells dampens the activation of T cells by outcompeting CD28 in binding to CD80 and CD86 and delivering inhibitory signals [activation of the protein phosphatase, SHP2 (also known as PTPN11) and PPA2], CTLA4 also mediates signalling-independent T-cell inhibition through the sequestration of CD80 and CD86 from CD28 ligation and by trans-endocytosis of CD80 and CD86 from APCs (Qureshi et al, 2011).

The influence of this B7/CD28 signalling in haematological malignancy has been illustrated clearly by a study showing that expression of CD86 on murine acute myeloid leukaemia (AML) tumour cells resulted in tumour rejection, whereas CD80⁺ AML tumours progressed in mice studies. Treatment with anti-CTLA4 mAb led to clearance of the CD80⁺ AML tumours. Thus, the tumour immunogenicity and the dominance of inhibitory versus stimulatory B7-ligand expression profiles in haematological malignancy during disease progression are likely to influence host T-cell immunity. CTLA4: CD80/CD86 interactions are relevant in multiple myeloma (MM) as targeting CTLA4 on T-cells from MM patients has been shown to promote the re-education of tumour-specific T-cells (Brown et al, 1998).

CTLA4 appears to exert its major immune regulatory activity on the intrinsic down-modulation of CD4⁺ T-cell activity and paradoxically on the extrinsic enhancement of regulatory T-cell (T_{reg}) activity (Peggs et al, 2009). CTLA4 is constitutively expressed by T_{reg}. Of note, there is an emerging role of CTLA4 as a cell extrinsic regulator of both conventional and regulatory T-cell responses (Wang et al, 2012). T_{reg} suppress anti-tumour T-cell immunosurveillance and their distribution and activity is linked to active cancer immune evasion (Pardoll, 2012b). Thus, CTLA4 blockade in the clinic is likely to boost effector T-cell helper activity and humoral immunity, while inhibiting the activity of T_{reg}.

**Blockade of CTLA4 in the clinic and relevance to haematological malignancies**

In a phase III randomized clinical trial in 676 patients with metastatic melanoma, treatment with an antagonistic CTLA4-targeted antibody (ipilimumab) improved the median overall survival (OS) by 3-7 months. The median OS was 10.1 months for patients who were treated with ipilimumab alone and 10 months for patients treated with both ipilimumab and a peptide gp100 vaccine. In comparison, patients treated with gp100 vaccine alone that was equivalent to placebo had a median overall survival of 6-4 months (Hodi et al, 2010). The lack of impact of a single vaccine antigen suggests that multiple neo-antigens may need to be presented to generate effective *de novo* anti-tumour T-cell activity. Treatment with ipilimumab alone also led to a 36% reduction in the risk of progression compared to treatment with gp100 alone and a significant difference in the overall response rate (10.9%) and the disease control rate (28.5%) compared to treatment with gp100 alone (1.5% and 11.0% respectively). Forty-five percent of ipilimumab-treated patients were alive after 1 year, 24% of patients were alive after 2 years, and some patients had a durable clinical benefit that lasted for the 4–5 years follow-up. This was a dramatic improvement in the survival of patients with metastatic melanoma compared to a previously published meta-analysis that indicated that only 25% of patients with metastatic melanoma lived for 1 year (Korn et al, 2008). Ipilimumab has been the first treatment in history that has shown a survival benefit in advanced melanoma. A second randomized phase III trial confirmed the findings of the first trial by showing an OS of 11.2 months for patients who were treated with a combination of ipilimumab and standard dacarbazine chemotherapy, versus 9.1 months for patients who received dacarbazine alone (Robert et al, 2011).

Anti-CTLA4 (ipilimumab) monotherapy in a phase 1 study (O’Mahony et al, 2007) showed that two of four advanced non-Hodgkin lymphoma (NHL) patients developed a clinical response (one partial response for 14-months duration) and correlative science studies showed decreased T_{reg} levels. A larger trial showed ipilimumab to be well-tolerated and exhibit anti-tumour activity in patients with relapsed and refractory non-NHL including follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL; durable...
responses were also noted in a few patients; Ansell et al., 2009). However, correlative studies demonstrated infrequent activation of memory T-cells to recall antigens. This might reflect the fact that optimal T-cell activation and enhancement of antigen-specific effector T-cells in NHL may require additional blockade of immune-checkpoint receptor PD-1 (discussed later in this review; Myklebust et al., 2013). It is worth noting that not all cancer patients gain clinical benefit with anti-CTLA4 and there is no current reliably biomarker to predict response. Immune-related toxicity does remain a problem targeting this checkpoint receptor in cancer patients, likely due to its critical role as a regulator of T-cell activation. CTLA4-knock-out mice exhibit a dramatic immunological phenotype that induces death from destructive lymphoid infiltration into multiple organs (Tivol et al., 1995). However, careful dosing, schedules, improved clinical management of immune toxicities and revised response criteria (Hodi et al., 2010; Pardoll, 2012b) should allow effective use of anti-CTLA4, particularly in combination therapies. Clinical experience to date suggests impressive long-term survival benefit with anti-CTLA4 – supporting the concept that enhancing autologous tumour-reactive T-cell activity allows immunological control of tumour progression. This may represent the equilibrium phase of cancer immunodetection whereby the immune system keeps the tumour in check even in the absence of measurable tumour shrinkage (Schreiber et al., 2011).

PD-1 – a key regulator of antigen-specific effector T-cells and a new target for immune checkpoint immunotherapy

The immune-checkpoint receptor, PD-1 (PDCD1, CD279), has emerged as a very promising target for cancer immunotherapy (Pardoll, 2012a; Pardoll & Drake, 2012). In contrast to CTLA4, the major role of PD-1 is to limit the activity of effector T-cells in peripheral organs and tissue during inflammatory responses and to prevent autoimmunity. The expression of PD-1 is induced on activated T-cells when engaged by one of its ligands PD-L1 or PD-L2, (also known as PDCDC1LG2 or CD273). These ligands cross-compete and have different affinity for PD-1 binding (including PD-L1 conformational status) that is likely to have relevance in cancer (Ghiotto et al., 2010). PD-L1 is also able to bind to T-cell (and possibly APC)-expressed CD80 that mediates inhibitory signalling. This surprising result illustrates again the complex binding interactions and functions of B7/CD28 co-signalling molecules that are likely to differ in both health and disease (similar to the competing CD80/86 and CD28/CTLA4 interactions on naïve and activated T-cells described above). The PD-1 immunosuppressive signal inhibits kinases that are involved in T-cell activation through the phosphatase SHP2 (PD-1 forms negative co-inhibitory microclusters with the TCR; Yokosuka et al., 2012). Thus, PD-1 engagement inhibits the TCR ‘stop signal’ and prevents formation of signalling immune synapses (Ramsay et al., 2012). PD-1 is also expressed on activated NK-cells and B-cells (inhibitory function) and clinical blockade should enhance anti-tumour lytic activity and antibody production in cancer patients. Paradoxically, expression of inhibitory checkpoint receptors on Tregs [PD-1, CTLA4, (Francisco et al., 2009)] and myeloid-derived suppressor cells [PD-L1, PD-1, CTLA4, (Liu et al., 2009, 2008)] positively regulates their immunosuppressive function and therapeutic blockade is likely to diminish their activity in the tumour microenvironment and promote T-cell cancer immunosurveillance.

Expression of the PD-L1/PD-1 pathway in cancer

It is becoming apparent that over-expression of immune checkpoint inhibitory ligands (e.g. PD-L1, PD-L2; Zou & Chen, 2008) is a major mechanism of cancer immune resistance, particularly in response to anti-tumour T-cell responses and pro-inflammatory cytokines [e.g. γ interferon (IFN-γ, IFNG; Pardoll & Drake, 2012)]. Chronic lymphocytic leukaemia (PLL) tumour cells show increased PD-L1 and marked up-regulation of this inhibitory ligand following immunostimulatory treatment (or exposure to reactive T-cells) compared to non-malignant B-cell control experiments (Grzywnowicz et al., 2012; Ramsay et al., 2012; Brusa et al., 2013). In keeping with this immune evasion mechanism, tumour-reactive T-cells from cancer patients (solid and haematological malignancies) express high levels of PD-1 receptor which induces a state of ‘exhaustion’ or ‘tolerance’ (Pardoll, 2012b). T-cell exhaustion is linked to chronic antigen exposure (persistent viral infection and cancer) that can be reversed with PD-1 blockade (Barber et al., 2006; Wherry, 2011). The CD8+ T-cell population in PLL patients has been shown to express multiple inhibitory receptors, including PD-1, that are features of T-cell exhaustion (Nunes et al., 2012; Ramsay et al., 2012; Riches et al., 2012). These dysfunctional effector T-cells are likely to arise through direct-contact interactions with PD-L1+ tumour cells expressing antigen in the immunosuppressive tumour microenvironment (cancer-induced ‘pseudo-exhaustion’; Ramsay et al., 2012). Tolerized PD1+ effector memory T-cells are also found in FL tumours but the identification of PD-L1 on ex vivo B-cell tumours has been more elusive (Myklebust et al., 2013). This is likely to reflect sensitivity of PD-L1 expression to the presence of an active cancer microenvironment (tumour cells up-regulate PD-L1 in response to cytokines) and possibly differences in immunohistochemistry (IHC) methodology (Zou & Chen, 2008; Andorsky et al., 2011; Brusa et al., 2013). IHC on a comprehensive FL tissue microarray has demonstrated increased expression of PD-L1 and other immune checkpoint ligands [CD200 and B7-H3 (also known as CD276)] on tumour cells compared to reactive lymph node tissue, as well as increased PD-1 on tumour-infiltrated T-cells (TILs) – with higher expression of these
immune checkpoints associated with poor prognosis patients. Herpesvirus entry mediator, HVEM (also known as TNFRSF14 or CD270) expression was associated with transformation to DLBCL (Ramsay et al., 2012). Functional assays using sorted primary FL tumour cells in co-culture with reactive T-cells identified that tumour-expressed immune checkpoints including PD-L1 deliver immunosuppressive signals towards T-cells. However, PD-L1 expressed on other immune microenvironment cells including tumour-associated macrophages (TAMs) and histocytes in FL are also likely to contribute to an immunosuppressive microenvironment (Myklebust et al., 2013). PD-L1 is expressed on adult T-cell leukaemia and lymphoma (ATLL) cells that may foster immune evasion of emerging tumour clones (Kozako et al., 2009) and also on T-cell-derived tumour cells (NHL) including tumour-infiltrated monocytes and dendritic cells (DCs) that inhibit T-cell activity and promote induction of T<sub>reg</sub> (Wilcox et al., 2009). Hodgkin lymphoma (HL) tumour cells have been shown to express both PD-L1 and PD-L2 that induces high PD-1 expression on infiltrating T-cells (Yamamoto et al., 2008). PD-L1 has been shown to enhance MM cell invasiveness and mediate the ability of tumour cells to evade T-cell and NK-cell recognition (Iwai et al., 2002; Benson et al., 2010). Notably, the immunomodulatory drug (IMiD) lenalidomide down-regulated MM-expressed PD-L1 and PD-1 expression on patient T-cells (Benson et al., 2010; Luptakova et al., 2013). The ability of this IMiD to down-regulate the PD-L1/PD-1 axis and repair lytic synapse activity (the assembly of key signalling molecules at the F-actin synapse) and motility of autologous tumour-reactive T-cells in cancer patients represents a powerful drug for cancer immunotherapy (Ramsay et al., 2008, 2013; Shanafelt et al., 2013). Of interest, there is growing evidence that reverse signalling of PD-L1 in cancer cells can render resistance to apoptosis (Azuma et al., 2008) and B7-H3 and B7-H4 (VTCN1) can stimulate growth and survival of malignant cells (Wilcox et al., 2012). This emerging data indicates a variety of pro-tumour roles for B7-related ligands.

Up-regulated immune checkpoint ligand expression and activity can be driven by constitutive oncogenic signalling known as ‘innate immune resistance’ and/or co-opted by tumour cells to suppress endogenous anti-tumour T-cell responses known as ‘adaptive immune resistance’ (not to be confused with innate or adaptive immunity; Dong et al., 2002; Parsa et al., 2007; Pardoll, 2012b; Taube et al., 2012; Fig 2). There is strong evidence that oncogenic signalling can increase PD-L1 expression on haematological tumour cells, independent of tumour microenvironment inflammatory signals. High-resolution copy number data and transcriptional analysis showed that PD-L1 and PD-L2 are key targets of the recurrent 9p24.1 amplification in HL and the related lymphoid malignancy primary mediastinal B-cell lymphoma (PMBCl; Green et al., 2010). In these tumours, the extended 9p24.1 region of amplification also includes the Janus kinase 2 (JAK2) locus. This JAK2 amplification increased JAK2 expression and augmented JAK/STAT signalling and associated CD274 (PD-L1) promoter activity and transcription. These studies show that gene amplification increased CD274 and PDCD1LG2 (PD-L2) expression and enhanced JAK2-mediated innate immune resistance (induction of immune checkpoint expression levels). Constitutive activating protein-1 (AP-1) activity and Epstein-Barr virus (EBV) infection have also shown to contribute to PD-L1 induction on HL cells (Green et al., 2012). Another innate immune resistance mechanism that induces up-regulation of PD-L1 and PD-L2 in PMBCl is recurrent genomic breaks of the MHC class II transactivator CIITA (Steidl et al., 2011). CIITA was shown to be a promiscuous partner with various in-frame gene fusions that impact on patient survival. Functional consequences of CIITA gene fusions were down-regulation of surface human leucocyte antigen (HLA) class-II expression and over-expression of PD-L1 and PD-L2. This study highlights multiple T-cell evasion mechanisms in operation, including reduced presentation of tumour antigen and protection from T-cell activity via an immune checkpoint ‘molecular shield’ (Zou & Chen, 2008; Steidl et al., 2011). Thus, genetically unstable tumour cells appear to co-opt immune regulatory proteins to evade T-cell recognition (Hanahan & Weinberg, 2011). The Phosphatidylinositol 3-kinase/Protein Kinase B (PI3K-AKT) pathway, commonly activated in many malignancies, was first shown to induce PD-L1 expression in glioblastoma (Parsa et al., 2007). Also, constitutive anaplastic lymphoma kinase (ALK) signalling in certain lymphomas (T-cell lymphoma and lung cancer) has been reported to drive PD-L1 expression through signal transducer and activator of transcription (STAT3) signalling (Marzec et al., 2008). It will be important for future research to understand how aberrant expression and activity of immune checkpoint ligands is driven by innate immune resistance oncogenic signalling (e.g. JAK-STAT signalling; Chen et al., 2012b).

The alternative mechanism for PD-L1 up-regulation on tumours is their adaptation (or defence) to endogenous tumour-specific immune responses (this is termed adaptive immune resistance). In adaptive immune resistance, the tumour uses the natural physiology of PD-L1 (protection of peripheral tissue from inflammation-induced T-cell mediated damage) in order to suppress the cytolytic activity of CTLs. PD-L1 is induced on tumour cells in response to interferons — predominantly IFN-γ (also known as IFNG) produced by Th1 CD4<sup>+</sup> and CD8<sup>+</sup> CTLs and NK-cells. PD-L1 is post-transcriptionally regulated by type I and II interferons (Keir et al., 2008). This mechanism of adaptive immune resistance supports the concept that T-cell immunosurveillance exists in cancer (Schreiber et al., 2011). However, the tumour ultimately resists the activity of effector T-cells by up-regulating its expression of immune checkpoint ligands (e.g. PD-L1) that engage inhibitory receptors (e.g. PD-1) expressed on the infiltrated effector T-cells and prevent anti-tumour immunity. In addition, the highly immunosuppressive microenvironment cells (TAMs and T<sub>reg</sub>) that are hallmarks of
haematological malignancy also express these inhibitory checkpoints that prevent T-cells making contact with target antigen-expressing tumour cells (Dave et al., 2004; Zou & Chen, 2008; Francisco et al., 2009; Ramsay et al., 2013). Functional studies to model tumour microenvironment cell interactions have shown that direct-contact co-culture of CLL or lymphoma cells with allogeneic T-cells or autologous tumour-associated T-cells induces a negative feedback loop whereby inhibitory ligands and receptors are significantly up-regulated and these immune checkpoint pathways consequently inhibit T-cell immune synapse signalling (Ramsay et al., 2012). In contrast, functional studies using non-malignant cell counterparts had no impact on T-cell function (Ramsay et al., 2012). Another study using functional assays showed that PD-L1 blockade on NHL tumour cells (anaplastic large cell lymphoma and DLBCL) enhances cytokine production of autologous tumour-reactive T-cells (Andorsky et al., 2011). This functional data using primary haematological tumour cells provides evidence that the ability of tumours to utilize immune checkpoint axes represents an important escape phase of human cancer immunoediting (Schreiber et al., 2011; Ramsay et al., 2012). CML tumour cells express PD-L1 (up-regulated further on aggressive blast crisis tumour cells) that is associated with CML-specific T-cells expressing high levels of PD-1 receptor. Fifty to ninety percent of total CD8+ T-cells from CML patients were

Fig 2. The two major mechanism of immune checkpoint expression on tumour cells. (A) Innate immune resistance. Constitutive oncogenic signalling can up-regulate immune checkpoints (e.g. PD-L1) expression on tumour cells, independent of tumour microenvironment inflammatory signals. Activation of AKT and signal transducer and activator of transcription 3 (STAT3) pathways have been shown to drive PD-L1 expression. This mechanism is likely to regulate multiple immune-checkpoint ligands co-opted by cancer cells (B) Adaptive immune resistance. In some tumours, PD-L1 is not constitutively expressed (or expressed at very low levels) and its expression is induced in response to inflammatory signals (e.g. IFNγ) that are produced by an active anti-tumour T-cell response (or tumour microenvironment inflammatory signals). Adaptive induction has been shown to be a common mechanism for the expression of multiple immune checkpoint molecules in haematological tumours. IFNγ (also known as IFNG), interferon-γ; MHC, major histocompatibility complex; TCR, T cell receptor.
found to be PD-1+ indicating that it may not only be the CML-specific CTLs that are suppressed in cancer by aberrant inhibitory immune checkpoint signalling (Mumprecht et al., 2009). CLL patients also exhibit tumour-induced global T-cell defects that are probably induced by the large circulating tumour load inherent to chronic leukaemias and direct-contact immunosuppressive interactions with immune checkpoint-expressing tumour cells in the tumour microenvironment (Ramsay et al., 2008, 2012). Importantly, blocking adaptive immune resistance (PD-L1/PD-1 axis) in blast crisis CML experiments prolonged survival in pre-clinical mouse studies (Mumprecht et al., 2009). Thus, it is gaining acceptance that revisiting immunotherapy (e.g. interferon therapy) in CML may have a beneficial role in targeting tyrosine kinase inhibitor-resistant tumour clones and the highly evasive CML stem cell (Corbin et al., 2011; Perl & Carroll, 2011; Hamilton et al., 2012). An important recent study has demonstrated clearly that CML stem cells are indeed immunogenic and can be targeted by specific CTLs, provided that the target antigen is leukaemia-specific and that the immunotherapy is applied in a situation with a low leukaemia load, probably after cytoreduction (Schurch et al., 2013). Targeted immune checkpoint blockade is likely to maximally activate CTL lytic activity against CML blasts and may also have a role in allogeneic haematopoietic stem cell transplant immunotherapy (alloSCT; see later section in this review covering this immunotherapy) that is currently the only curative treatment for CML. PD-L1 has been shown to mediate resistance of acute myeloid leukaemia (AML) blasts including dormant leukaemia cells (that up-regulate PD-L1 and CD80) against CTL activity in mouse models of AML (Saudemont & Quesnel, 2004; Zhang et al., 2009). Another pre-clinical study showed that PD-L1/PD-L1 blockade coupled with PD-1+ Treg depletion improved the therapeutic efficacy of adoptive AML-reactive CTLs in advanced AML disease (Zhou et al., 2010).

### Blockade of the PD-L1/PD-1 pathway in the clinic and relevance in haematological malignancies

Two large clinical trials of anti–PD-1 antibody (BMS-936558, termed nivolumab; Topalian et al., 2012) and anti–PD-L1 antibody (BMS-936559, inhibits binding to both PD-1 and CD80; Brahmer et al., 2012) showed that neutralizing antibodies targeting these immunoregulatory proteins induced durable tumour response rates of 10% to 15% in advanced solid cancer patients. This represents the highest rate of anti-tumour activity of the many immunotherapy approaches tested in the clinic over the past 30 years (Mellman et al., 2011). Objective and durable tumour responses were seen in patients with lung cancer, which has been notoriously resistant to immunotherapy. As predicted by the distinct phenotypes of Pdcd1 (also known as Pd-1)-knockout mice (Nishimura et al., 1999) versus Ctila4-knockout mice (Tivol et al., 1995), the frequency of immune-related toxicities from anti-PD-1 therapy appears to be less than anti-CTLA4 treatment. These initial observations in solid cancer suggest that antibodies blocking PD-1 or PD-L1 are likely to provide a new benchmark for repairing anti-tumour activity with immunotherapy. Following the path of ipilimumab, anti-PD-1 nivolumab is predicted to reach regulatory approval based on the phase I results and on-going phase III trials. Haematological malignancies are responsive to immunotherapy with the demonstrated curative potential of alloSCT (graft-versus-leukaemia, GVL effect) and use of rituximab (anti-CD20) mAb therapy (Riches et al., 2010). The activity of PD-L1/PD-1 antibodies in haematological malignancies are currently being explored and are showing promise in early-phase clinical trials.

A phase I study of anti-PD-1 (CT-O11) mAb in diverse haematological tumours has shown that the immunotherapy was well tolerated and had a 33% overall response rate with one complete remission (Berger et al., 2008). These results are encouraging but there is emerging consensus in the cancer immunology field that additional inhibitory immune checkpoint pathways will remain active in the tumour microenvironment with monotherapy alone and combinatorial checkpoint blockade will be required to maximize clinical responses (Pardoll & Drake, 2012; Ramsay et al., 2012). Antibodies and small molecule inhibitors (drugs) targeting numerous immune checkpoint molecules are now entering the clinic including the inhibitory ligands B7-H3, B7-H4 (also known as VTCN1) and T-cell immunoglobulin and mucin-domain-containing molecule 3 (TIM-3, also known as HAVCR2; Pardoll, 2012b; Pardoll & Drake, 2012).

### Multiple immune checkpoint targets in cancer

Eight B7 family ligand members have been identified to date including CD80, CD86, PD-L1, PD-L2, ICOS-L (also known as ICOSLG), B7-H3, B7-H4 and B7-H6 (also known as NCRLG1) and have been reviewed in Greaves and Gribben (2012). Many tumour cells express multiple inhibitory ligands and TILs express multiple inhibitory receptors. Therefore, there are many opportunities to enhance anti-tumour immunity through multiple blockade of immune checkpoints. Some B7 family inhibitory ligands including B7-H3 and B7-H4 do not yet have defined receptors, but mouse knock-out experiments support an immune inhibitory role for these ligands (Yi & Chen, 2009). B7-H3 and B7-H4 are up-regulated on tumour cells and tumour-infiltrating microenvironment cells (endothelial cells and TAMs). Preclinical mouse models of cancer have shown that blockade of individual immune-checkpoint ligands or receptors can enhance anti-tumour immunity and dual blockade of coordinately expressed receptors can produce additive or synergistic anti-tumour activities. PD-1 and lymphocyte-activation gene 3 (Lag3) are co-expressed on tumour-reactive CD8+ and CD8+ T-cells in many tumour types. In contrast to Lag3+/Pdcd1+/− mice that developed lethal autoimmune
organ failure, dual anti-LAG3/anti-PD-1 antibody treatment cured most mice of established tumours that were largely resistant to single antibody treatment (without autoimmune toxicity; Woo et al, 2012). Moreover, Lag3−/−/Pdcd1−/− mice showed marked survival and clearance of multiple transplantable tumours. LAG3 is known to enhance the function of Treg and its only known ligand to date is MHC class II molecules. Co-expression of TIM-3 and PD-1 has been shown to identify a CD8+ T-cell exhaustion phenotype in mice with advanced AML (Zhou et al, 2011). The only confirmed ligand of TIM-3 to date is galectin-9 (also known as LGALS9, a galectin that is up-regulated in various types of cancer that inhibits T effector cell responses). Blocking the PD-L1/ PD-1 axis or Galectin-9/TIM-3 axis alone was insufficient to rescue mice from AML lethality, but an additive effect was identified in reducing tumour burden and lethality when both immune checkpoint pathways were blocked. Further evidence highlighting the importance of multiple immune checkpoint pathways in cancer was provided in a recent study reporting that simultaneous blockade of CTLA4, PD-1 and LAG-3 yielded effective adoptive cell transfer (ACT – discussed later in review) in a mouse model of leukaemia (Berrien-Elliott et al, 2013). Targeting B and T-cell lymphocyte attenuator (BTLA) along with PD-1 and TIM-3 enhances the expansion, proliferation and cytokine production of tumour-specific CD8+ T-cells in melanoma patients. BTLA was identified as an inhibitory receptor on T-cells following demonstration of enhanced T-cell responses in Btla-knockout mice (Watanabe et al, 2003). HVEM was shown to be the BTLA ligand (Sedy et al, 2005). This is an interesting example in which a TNF family member interacts with an immunoglobulin supergene family member. BTLA has been shown to be a relevant inhibitory receptor for T-cells in the melanoma tumour microenvironment (Derre et al, 2010).

Molecular delineation of the dominant immune checkpoint pathways active in different cancer types and during disease progression will be essential to design effective immunotherapy strategies for patients. This research approach has been used to show that the co-ordinated activity of multiple inhibitory B7-related molecules (up-regulated CD200, PD-L1, B7-H3 and HVEM) on primary human leukaemia and lymphoma cells (CLL, FL, DLBCL, MM, and HL) induces adaptive immune resistance (tumour-induced T-cell dysfunction; Ramsay et al, 2012). Notably, CD200 is over-expressed on cancer cells including cancer stem cells and has been shown to attenuate T-cell and NK-cell anti-tumour immunity including the induction of Treg activity in both solid and haematological malignancies (Kawasaki & Farrar, 2008; Coles et al, 2011, 2012). Thus, CD200 may represent an attractive immune checkpoint target for leukaemia immunotherapy (Wong et al, 2010, 2012). Functional screening assays identified that CD200R (also known as CD200R1), PD-1 and BTLA are the T-cell inhibitory co-receptors that transmit the tumour immunosuppressive signalling (Ramsay et al, 2012). In contrast to healthy cells, tumour cells exploited the combined action of these multiple immune checkpoint signalling axes to induce T-cell immune synapse dysfunction and suppressed effector function in both previously healthy allogeneic and autologous tumour-reactive T-cell populations. Expression of immune checkpoints in the tumour microenvironment was associated with the success of neutralizing antibodies in the functional assays – suggesting the definition of potential biomarkers to determine the dominant immune checkpoint pathways in cancer. This study also identified a novel immunomodulatory mechanism of action of lenalidomide – blocking tumour-cell induced T-cell synapse dysfunction (Ramsay et al, 2012). Both ex vivo and in vivo lenalidomide treatment prevented induction of the T-cell defect and down-regulated immune checkpoints on tumour cells and their co-receptors on T-cells. This discovery, that lenalidomide can block adaptive immune resistance, provides important mechanism of action data for the observed clinical activity of this agent in on-going large clinical trials in leukaemia and lymphoma (Shanafelt et al, 2013). The ability of this drug to promote anti-tumour T-cell immune synapse activity by blocking immune checkpoint pathways (Ramsay et al, 2012) and repair cytoskeletal T-cell immune synapse signalling (Ramsay et al, 2008) in cancer patients represents an attractive immunotherapy. These findings suggest that lenalidomide should be considered in combination with immune checkpoint PD-L1 and PD-1 antagonist antibodies.

**AlloSCT and immune checkpoint therapy**

Tumour relapses remain a serious problem after allogeneic stem cell transplantation. Immune checkpoints have also been shown to contribute to T-cell dysfunction in patients who relapse following alloSCT (Norde et al, 2012). PD-L1 was highly up-regulated on immature human leukaemic progenitor cells, whereas costimulatory molecules, such as CD80 and CD86, were not expressed. Blocking PD-1 signalling led to elevated proliferation and IFN-γ production of minor histocompatibility antigen (MiHA)-specific T cells co-cultured with PD-L1-expressing leukaemia cells. Moreover, patients with relapsed leukaemia after initial MiHA-specific T-cell responses displayed high PD-L1 expression on CD34+ leukaemia cells and increased PD-1 levels on MiHA-specific CD8 + T cells. Importantly, blocking PD-1/PD-L1 interactions augmented proliferation of MiHA-specific CD8+ memory T-cells from relapsed patients. These results suggest that blocking the PD-1 immune checkpoint offers a promising immunotherapeutic strategy following alloSCT in patients with recurrent or relapsed disease. Anti-CTLA4 mAb has also been used successfully in a phase II trial to treat relapsed HL, myeloma and leukaemia after alloSCT (Norde et al, 2012) without inducing graft-versus-host disease. The beneficial role of NK-cells in anti-tumour immune responses has been clearly demonstrated with data showing enhanced GVL in alloSCT. This immune activity is mediated by mismatches

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between donor NK inhibitory receptors and recipient patient HLA alleles – this reduces NK inhibitory receptor activity. Future research will determine the translational relevance of therapeutic blockade of NK inhibitory receptors or the use of agonist antibodies specific for co-stimulatory receptors, such as the tumour-specific B7-H6/NKP30 (also known as NCR3) immunostimulatory axis (Brandt et al, 2009; Fig 1 includes co-stimulatory pathways and receptors that enhance T-cell activity and that can be activated with agonist antibodies in the clinic).

Combinatorial immune checkpoint blockade immunotherapy

The latest disease pathogenesis and immunology knowledge is driving our understanding of how cytotoxic therapies and targeted drugs in conjugation with immunotherapies may have complementary roles in cancer treatment (Vanneman & Dranoff, 2012). Surprisingly, although they were initially considered as immunosuppressive, emerging evidence exists that certain conventional cytotoxic chemotherapies, such as cyclophosphamide, can antagonize immunosuppression in the tumour microenvironment and promote anti-tumour immunity (Vanneman & Dranoff, 2012; Shanafelt et al, 2013). Moreover, targeted drugs have shown immunomodulatory activities including sensitizing tumours to CTL lytic activity and promoting endogenous in situ vaccination (DC priming). Haematological malignancies, such as CLL and lymphoma, have been at the forefront of chemoimmunotherapy (CIT) clinical use with the addition of rituximab mAb to intensive combination chemotherapies that include cyclophosphamide. Cyclophosphamide has also been used as the pre-conditioning regimen for the encouraging early phase CIT clinical use with the addition of rituximab mAb to intensive combination chemotherapies that include cyclophosphamide. Cyclophosphamide has also been used as the pre-conditioning regimen for the encouraging early phase clinical studies treating CLL patients with autologous T-cells genetically engineered to express chimeric antigen receptors (Kalos et al, 2011; Porter et al, 2011). However, CIT-resistant, toxicity and disease relapse remain major clinical problems in the treatment of cancer. A recent study has highlighted the potential for combinatorial immune checkpoint blockade in haematological malignancy (Ding et al, 2012). The study provided critical mechanistic insights into the dynamic interplay between tumours and CD4⁺ T-cells in the context of CIT. Cyclophosphamide was shown to have a potent immunostimulatory effect when combined with subsequent ACT of anti-tumour CD4⁺ T-cells. Therapeutic synergy via modulation of anti-tumour immunity was demonstrated using this CIT platform to treat a mouse model of aggressive lymphoma (transplanted A20HA tumours). Cyclophosphamide treatment was shown to prevent immune tolerance of transferred T-cells as well as induce direct tumour cytotoxicity. Critically, CIT promoted the expansion of poly-functional CD4⁺ effector T-cells that exhibited the ability to produce multiple Th1-type pro-inflammatory cytokines (IFN-γ, TNFα and IL2). Although CIT induced effective tumour regression in this lymphoma model, it was not curative as the majority of mice succumbed to late relapse. Serial analysis experiments showed that tumour relapse following CIT correlated with a decrease in CD4⁺ T-cell numbers and conversion from the polyfunctional phenotype to a PD-1high dysfunctional state. The authors then went to investigate antibody blockade of PD-1 on T-cells and PD-L1 on the cancer cells. Immune checkpoint blockade following CIT prevented tumour-induced PD-1-mediated CD4⁺ T-cell tolerization that allowed retention of the polyfunctional phenotype and maintenance of effective endogenous anti-tumour CTL activity. This treatment approach led to complete tumour regression and development of immunological memory. An important conceptual advance was the demonstration that in order to exploit the therapeutic opportunity created by CIT and prevent disease relapse, blockade of the negative immune regulatory PD-L1/PD-1 signalling axis was required. Future pre-clinical studies in haematological malignancy should strive to use genetic mouse models that spontaneously develop tumours (non-transplantation setting) with intact host immune systems (in order to accurately model cancer progression and immunoeediting (Schreiber et al, 2011). These studies should act as a prelude to developing novel combinatorial treatments for clinical trials. Indeed, clinical studies using ACT together with the administration of IL2 can lead to prolonged tumour eradication in human patients with metastatic melanoma (Rosenberg et al, 2011) or leukaemia (Kochenderfer et al, 2012) who have failed other treatment options. Melanoma represents the best example of translating cancer immunology research to clinical trials. Blocking immunosuppressive PD-L1/PD-1 signalling in melanoma has been shown to potentiate ACT in mice (Pilon-Thomas et al, 2010) and was shown to induce tumour regression in some patients (Brahmer et al, 2012; Topalian et al, 2012). It is important to note that ACT-based therapies are not yet FDA-approved and are only available in a limited number of locations worldwide at great expense.

Concluding remarks

In conclusion, the potential of drugs and antibodies that block immune checkpoints to complement existing therapies (CIT and alloSCT) and newer targeted agents (Brown, 2013) in haematological cancers merits investigation in an attempt to harness durable anti-tumour immunity in patients (Fig 3). Future challenges and next steps will be translating preclinical data to the clinic for these combination therapies. The development of knowledge-driven immune monitoring assays should aid design of clinical trials that take into consideration the immunological end points that can be used to determine whether agents have had an effect on their targets. Importantly, immunological correlative data should correlate with clinical outcomes. An example of such an immune-monitoring assay is the T-cell immune synapse bioassay, which has been used successfully to measure longitudinal T-cell function during a CIT induction and lenalidomide
consolidation phase II clinical trial in CLL (Shanafelt et al, 2013). This assay allowed simultaneous measurement of early T-cell activation signalling and effector cytolytic granzyme B expression at the T-cell: tumour cell immune synapse. Notably, this study showed that those patients who had a stronger response to therapy had a better recovery of T-cell immune synapse activity than those patients who only achieved partial remission or no response. Continued development of knowledge- and data-driven immune monitoring assays and biomarkers should be developed for each tumour type and immunotherapy approach. Another challenge for the field of cancer immunology is that clinical responses to immunotherapy can take months compared to more rapid responses with chemotherapy and can be associated with tumour enlargement prior to regression (Pardoll & Drake, 2012). The phase III clinical studies to date investigating ipilimumab and the sipuleucel-T vaccine in prostate cancer (Kantoff et al, 2010) indicate that immune-based mono- and combination therapies for metastatic cancers may be able to prolong patient survival without significant levels of tumour shrinkage and they may keep tumours in ‘check’ or a state of equilibrium following therapy. Taken together, these results indicate that criteria for response to cancer immunotherapy may need to be adjusted compared to those evaluating chemotherapy or oncogenic-targeted drugs, which tend to induce faster but shorter-lived responses. Greater knowledge on the effect of combinations of immune checkpoint blocking agents among themselves and together with conventional or newer targeted drugs should yield further clinical success in both solid and blood cancers. The recent clinical results showing durable tumour responses with anti-PD-L1/PD-1 immune checkpoint blockade immunotherapy in advanced solid cancers highlights an exciting opportunity to address unmet clinical needs in haematological cancers.

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Conflict of interest
There is no conflict of interest to disclose.

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