Review

Second- and third-generation drugs for immuno-oncology treatment—The more the better?

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Abstract Recent success in cancer immunotherapy (anti-CTLA-4, anti-PD1/PD-L1) has confirmed the hypothesis that the immune system can control many cancers across various histologies, in some cases producing durable responses in a way not seen with many small-molecule drugs. However, only less than 25% of all patients do respond to immuno-oncology drugs and several resistance mechanisms have been identified (e.g. T-cell exhaustion, overexpression of caspase-8 and β-catenin, PD-1/PD-L1 gene amplification, MHC-I/II mutations). To improve response rates and to overcome resistance, novel second- and third-generation immuno-oncology drugs are currently evaluated in ongoing phase I/II trials (either alone or in combination) including novel inhibitory compounds (e.g. TIM-3, VISTA, LAG-3, IDO, KIR) and newly developed co-stimulatory antibodies (e.g. CD40, GITR, OX40, CD137, ICOS). It is important to note that co-stimulatory agents strikingly differ in their proposed mechanism of action compared with monoclonal antibodies that accomplish immune activation by blocking negative checkpoint molecules such as CTLA-4 or PD-1/PD-L1 or others. Indeed, the prospect of combining agonistic with antagonistic agents is enticing and represents a real immunologic opportunity to ‘step on the gas’ while ‘cutting the brakes’, although this strategy as a novel cancer therapy has not been universally endorsed so far. Concerns include the prospect of triggering cytokine-release syndromes, autoimmune reactions and hyper immune stimulation leading to activation-induced cell death or tolerance, however, toxicity.

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has not been a major issue in the clinical trials reported so far. Although initial phase I/II clinical trials of agonistic and novel antagonistic drugs have shown highly promising results in the absence of disabling toxicity, both in single-agent studies and in combination with chemotherapy or other immune system targeting drugs; however, numerous questions remain about dose, schedule, route of administration and formulation as well as identifying the appropriate patient populations. In our view, with such a wealth of potential mechanisms of action and with the ability to fine-tune monoclonal antibody structure and function to suit particular requirements, the second and third wave of immuno-oncology drugs are likely to provide rapid advances with new combinations of novel immunotherapy (especially co-stimulatory antibodies). Here, we will review the mechanisms of action and the clinical data of these new antibodies and discuss the major issues facing this rapidly evolving field.

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1. Introduction

In recent decades, systemic treatment options for patients with different types of cancers have evolved from chemotherapy through targeted-therapies to the more recent immuno-oncology agents, and emerging evidence on the role of the anti-tumour activity of the immune system has generated great interest in immunotherapy even for tumours that were historically considered as non-immunogenic [1].

Immuno-oncology is a novel therapeutic strategy currently being evaluated for many malignancies. This approach differs from traditional modalities, which target the tumour directly or aim to disrupt the tumour blood supply, as it is designed to potentiate the patient’s immune response to tumour cells. Immunotherapy is now emerging as a major modality in cancer treatment focussing on development of inhibitors or co-stimulatory agents of the cellular mediators of cancer-induced immunosuppression (immune checkpoints) to boost anti-tumour immune responses. Different immunologic approaches targeting immune checkpoint pathways are showing promise in development, and preclinical and clinical evidence provides the rationale for investigating the combination of co-stimulatory and inhibitory monoclonal antibodies to establish a novel or re-instating a pre-existing anti-tumour immune response.

The immune system is capable of identifying tumour-associated antigens (so-called neo-antigens) and eliminating the tumour cells expressing them. Expression of these neo-antigens (new epitopes) is regarded to be a consequence of new mutations (e.g. EGFR and/or DNA damage) [1]. Immune checkpoints refer to multiple inhibitory and co-stimulatory pathways that counteract certain crucial steps of T-cell-mediated immunity to maintain self-tolerance and modulate the duration and amplitude of immune responses.

Recently, the understanding of several checkpoints that shut down the immune system as an immunosuppressive mechanism in tumours has evolved a paradigm shift in cancer treatment [2]. Immune checkpoints are initiated primarily through T cell inhibiting and stimulating receptors and their ligands, including cytotoxic T lymphocyte-associated protein 4 (CTLA-4, CD152), PD-1 (programmed cell death-1, CD279) and PD-L1 (CD274) or PD-L2 (CD273; programmed cell death ligand-1, -2), among many others (reviewed by Refs. [3,4]).

Understanding how the immune system affects cancer development and progression has been one of the most challenging questions in immunology. It is now generally accepted that the immune system plays a dual role in cancer: it cannot only suppress tumour growth by destroying cancer cells or inhibiting their out-growth but also promote tumour progression either by selecting for tumour cells that are more fit to survive in an immunocompetent patient or by establishing conditions within the tumour microenvironment that facilitate tumour out-growth (‘cancer immune-editing’) [5].

To improve response rates following immune therapy and to overcome resistance, novel second- and third-generation immuno-oncology drugs are currently evaluated in ongoing phase I–III trials (either alone or in combination) including novel inhibitory compounds (e.g. TIM-3, VISTA, LAG-3, IDO, KIR) and newly developed co-stimulatory antibodies (e.g. CD40, GITR, OX40, CD137, ICOS). Here, we will review the mechanisms of action and the clinical data of these new molecules and discuss the major issues facing this rapidly evolving field.

2. Immune cells in defence against tumours

The most essential role of the immune system in humans is to eradicate invading pathogens by inducing a protective immunity and not to jeopardise the host by inducing tolerance to self-tissues. This is achieved through a fine tuning of antigen-presenting cells, T cell, B cells and NK cell activities (in concert with the B7 protein family) in initiation, differentiation, the effector phase and termination of the immune response [6].
2.1. T-cell activation and T helper cells

T-cell response is initiated by specific recognition of cognate peptides presented by MHC proteins on antigen-presenting cells (APCs; monocytes, macrophages, dendritic cells, B cells) through T-cell receptors (TCRs), which are referred to as the ‘first signal’ for T-cell activation. Although other CD4+ T cell subpopulations may exist, naive T helper populations can be differentiated into Th1, Th2, Th17 (against extracellular pathogenic bacteria) and regulatory T cells (Treg). Among them, Th1 and Treg appear to be the most important players in tumour defence. The differentiation of T helper cells is dependent on three signals. First, the interaction of TCR and MHC-II-peptides complexes, second, the signal via CD28 after binding to co-stimulating molecules CD80/CD86 (B7.1, B7.2) on APC, and finally, the expression of cytokines resulting in the differentiation to a specific T helper cell type. To stop an immune reaction, T cells express CTLA-4 instead of CD28 which leads to the induction of inhibitory signals. The activation of macrophages by Th1 cells is mediated by CD40L-CD40 interactions and interferon-γ. However, the ultimate magnitude and quality of T-cell response is determined by a balance between co-stimulatory and inhibitory signals that are transduced into T cells, which is referred to as the ‘second signal’ [1,7] (Fig. 1).

Following TCR engagement by cognate peptide-MHC complexes, co-stimulating and -signalling receptors are often mobilised and co-localised with TCRs, forming the immunological synapse between APCs and T cells. This synaptic interface is the place where the crosstalk between co-signalling ligands and receptors synergise or antagonise with TCR signalling, rendering T cells activated or inhibited [8] (for details see Fig. 1).

The importance of CD4+ helper T cells as part of the T-cell response in tumour immunity is not yet fully understood. CD4+ T cells play a role in anti-tumour immune responses by providing cytokines for differentiation of native CD8+ T cells into effector and memory cytotoxic T cells. In addition, Th cells specific for tumour antigens can secrete cytokines (e.g. TNF-α, interferon-γ) that can increase MHC-I expression on tumour cells [9].

2.2. Cytotoxic T cells

The principal mechanism of the adaptive immune protection against tumours is mediated via CD8+ T cells. The TCR of CD8+ T cells binds intracellular altered self-peptides from tumour cells presented on MHC-I molecules and kill the tumour cells. As CD4+ T cells, CD8+ (cytotoxic) T cells are generated in the thymus. However, rather than the CD4 molecule, cytotoxic T cells express a dimeric co-receptor, CD8, usually composed of one CD8α and one CD8β chain. CD8+ T cells recognise peptides (8-13mer) presented by MHC-I molecules, found on all nucleated cells. The CD8 heterodimer then binds to a conserved portion (the α3 region) of MHC-I during T-cell/antigen-presenting cell interactions [9].

There are a lot of important studies investigating their function in helping CD8+ T cells, interaction with APC or being directly cytotoxic to tumours, as well as their possible usage as therapeutic cells being induced by specific ectopic or neo-antigenic-based vaccines.

In an early study of Oseendorp and co-workers [10], a specific viral T helper epitope was used for vaccination resulting in protection against MHC-II negative virus-induced tumour cells and other virus-transformed tumour cells. CD8+ cytotoxic T cells were identified as main effector cells for killing the tumours, but Th1 cells were also induced. Although these Th1 cells did not directly recognise tumour cells, the requirement of cross-priming of tumour antigens through antigen-presenting cells and subsequent induction of Th1 cells was suggested to mediate full protection against MHC-II negative tumour cells [10].

In addition, signal transduction via CD40 without CD4+ T cells can also prime CD8+ T cells, indicating that CD4+ T cell help for CTL by CD40-CD40 ligand (L) interactions is an additional step in the activation of CD8+ T cells [11]. Interestingly, transfer of few naive tumour-reactive CD4+ T cells into lymphopenic recipients using an advanced melanoma mouse model led to T cell proliferation and regression of tumours. These CD4+ T cells had developed cytotoxic activity based on MHC-II-restricted tumour cell recognition [12].

Friedman et al. [13] investigated CD4+ tumour-infiltrating T lymphocytes (TLs) in patients with metastatic melanoma. They detected tumour-specific CD4+ lymphocytes in 20% of the patients indicating a possible effective function of CD4+ T cells in adoptive cell therapy. In this context, Linnemann et al. [14] found tumour neo-antigen-reactive CD4+ T cells in four out of five melanoma patients suggesting a high occurrence of CD4+ T-cell reactivity in melanoma. Although therapeutic-induced CD4+ responses would be attractive, each melanoma patient had individual mutations leading to neo-antigens which means that personalised immunotherapies have to be developed [15].

Activation of naive CD8+ T cells requires the interaction with professional APCs, a process where CD8+ T cells check the presented MHC-I peptide complexes via TCR. This is the first signal; for clonal expansion, the cytotoxic T cells have to be activated by co-stimulating molecules such as CD80/CD86. To generate long-lasting memory T cells and to allow repetitive stimulation of cytotoxic T cells, dendritic cells have to interact with both, activated CD4+ helper T cells and CD8+ T cells. During this process, CD4+ Th1 cells ‘license’ the dendritic cells to give a potent activating signal to the naive CD8+ T cells. Cytotoxic T
cells are very important for tumour surveillance [15]. When CD8+ T cells recognise specific altered self-peptides on MHC-I molecules and become activated, there are three major mechanisms to kill malignant cells. The first is secretion of cytokines, primarily TNF-α and interferon-γ (IFN-γ), which have anti-tumour and anti-viral microbial effects. The second major function is the production and release of cytotoxic granules. These granules, also found in NK cells, contain two families of proteins, perforin and granzymes. Perforin forms a pore in the membrane of the target cell, similar to the membrane attacking complex of complement. This pore allows the granzymes also contained in the cytotoxic granules to penetrate the malignant cell. Granzymes are serine proteases which cleave the proteins inside the cell ultimately resulting in apoptosis of the target cell. The third major function of CD8+ T-cell destruction of tumour cells is via Fas/FasL interactions. Activated
CD8+ T cells produce FasL (CD178), which binds to its receptor, Fas (CD95), expressed on the surface of the target cell. This binding causes the Fas molecules on the surface of the target cell to trimerise, which pulls together signalling molecules. These signalling molecules result in the activation of the caspase cascade, which also results in apoptosis of the target cell [16].

2.3. Regulatory T cells

Regulatory T (T\textsubscript{reg}) cells play an important role in preventing immune responses to self-antigens (peripheral tolerance), but increased numbers of T\textsubscript{reg} cells in peripheral blood and in the tumour microenvironment are commonly seen in patients with invasive and metastatic cancers [17]. T\textsubscript{reg} cells are generally identified as T cells that express CD4, CD25 proteins on their surfaces and the intracellular transcription factor FoxP3 as a specific marker and are found in blood as well as in lymphoid tissues; however, they are preferentially recruited to tumours (presumably by binding to CCR4, which is also expressed on T\textsubscript{reg}) and are a significant predictor of poor patient prognosis [18]. T\textsubscript{reg} cells also block production of interferon-\gamma and suppress Th1 cells (and also Th2, Th17) cell activation by antigen-presenting dendritic cells [18] through secretion of immunosuppressive cytokines IL-10 and transforming growth factor \( \beta \), and thereby terminating the immune response. The own expression of IL-10 and transforming growth factor \( \beta \) is one strategy of tumour cells to induce an immunological tolerance.

2.4. B cells

B cells are important producers of specific anti-tumour antibodies and can also function as APCs. CD4+ helper T cells (e.g. T\textsubscript{h}2 cells) are also mandatorily required to provide co-stimulatory signals for B cell activation (‘receiving help’ to produce the correct immunoglobulins). Protein antigens that are recognised by specific B-cell receptors, which are membrane-bound immunoglobulins, are endocytosed and processed to generate peptides that bind to MHC-II molecules and are presented to T\textsubscript{h}2 cells. Notably, the antigen that is presented to B cells is generally in its intact, native conformation and is not processed by APCs. On activation, T\textsubscript{h}2 cells express CD40L which binds to CD40 on activated B cells and induces B cell proliferation and differentiation (Fig. 2 for details). B cells then participate in a two-step differentiation process that yields both short-lived plasmablasts for immediate protection and long-lived plasma cells and memory B cells for persistent protection. Antigens that activate B cells with the help of T\textsubscript{h}2 cells are known as T-cell-dependent antigens and include foreign proteins [19].

As outlined, the second co-stimulatory signal for B cells stems from T\textsubscript{h}2 cells (‘thymus-dependent’). In contrast, the second co-stimulatory signal can also be provided by PAMPs (pathogen-associated molecular patterns; e.g. molecular structures that are produced by microbial pathogens). In addition, B cells also recognise endogenous molecules that are produced by or released from damaged and dying cells (damage-associated molecular patterns [DAMPs]). Cellular receptors for PAMPs and DAMPs are called pattern recognition receptors (PRRs; e.g. toll-like receptors 1-10, NOD-like receptors). Binding of PAMPs and DAMPs to PRRs will then activate signal transduction pathways that promote the antimicrobial and pro-inflammatory activity of the cells (‘thymus-independent’). However, B-cell activation via PRRs will neither result in an immunoglobulin isotype switching (mainly IgM is produced) nor in a somatic hypermutation. In addition, no memory cells are generated because these features are largely dependent on helper T cells, which are not activated by non-protein antigens [20].

2.5. Natural killer cells

Natural killer cells (NK cells) are able to kill many types of tumour cells, especially cells that have a reduced MHC-I expression and express ligands for NK cell-activating receptors. In particular, NK cells respond to the absence of MHC-I molecules on the cell membrane because the recognition of MHC-I molecules provides inhibitory signals to NK cells. The loss of expression of MHC-I molecules observed in many tumours makes tumour cells a good target for NK cells. In addition, some tumours also express MIC and ULB proteins which are ligands for the NKG2D-activating receptor on NK cells (Fig. 3). Moreover, NK cells can also be activated by binding of the Fc fragment of certain anti-tumouricidal antibodies to Fc receptor III (CD16). Notably, some tumours can prevent NK cell-induced lysis by secreting ‘decoy’ ligands that block the NKG2D receptor on NK cells [21,22] (Fig. 3).

2.6. B7 proteins

The B7 family (see Table 1 for details) is a group of surface glycoproteins that share structural features with immunoglobulins (Ig), whose extracellular domains bear homology to IgV and IgC domains of Ig [23]. A hallmark of the B7 family molecules is their capability to co-stimulate or co-inhibit T-cell responses in the presence of peptide/MHC complex-mediated TCR signalling [24]. B7 family members primarily bind to the members of CD28 family including CD28, CTLA-4, PD-1, ICOS, and BTLA that transmit co-signals into the T cells. In addition, they are also involved in the activation of NK cells (binding the NKp30 receptor, Fig. 3).

The B7 co-stimulatory ligands are important for full activation of naïve T cells in the lymphoid organs, in which APCs, particularly dendritic cells (DCs), are the primary cellular source providing the ligands including...
CD80/CD86. In contrast, B7 co-inhibitory ligands are crucial for the termination of over-activated T cell response, maintenance of self-tolerance and protection of tissues from damage induced by invading pathogens. In addition, co-inhibitory ligands expressed on tumour microenvironment actively inhibit the effector functions of TILs or induce the generation of Treg cells [24,25] thus playing a role as important immune checkpoint proteins that are also involved in the immune resistance of cancer cells.

3. Clinical data
To date, mono-immunotherapy using CTLA-4 or PD-1/PD-L1 antagonistic antibodies in humans has shown promising therapeutic outcomes, proving that immunotherapy with T-cell checkpoint inhibitors is one of the most promising new therapeutic approaches. With these new therapies, long-term stabilisation of disease and overall survival (OS) of patients have been achieved [26].

Although anti-CTLA4, anti-PD-1 and anti-PD-L1 therapies have shown encouraging activity with good tolerability in patients eligible for clinical trials, caution is required when treating patients in clinical practice. The potential for severe immune-mediated toxicity (e.g. liver, gastrointestinal, lung) needs to be recognised, with clear algorithms in place to assist in rapid identification, evaluation and treatment of such toxicities. Considering only roughly 20% of unselected patients treated with anti-PD-1/PD-L1 antibodies will have a meaningful response to therapy [27], other therapies will need to be pursued in most patients, and efforts to
identify predictive biomarkers of response to this class of drugs remain of importance.

In parallel, additional identification and characterisation of multiple T-cell checkpoints will drive the further clinical development of other checkpoint inhibitors, such as TIM3, LAG-3, KIR, VISTA and others.

Another attractive alternative is to combine checkpoint inhibitors with agonistic antibodies that activate immune cells. The reversal of anergic/exhausted T cells by checkpoint blockade may allow these cells to be more potently activated and to develop full anti-tumour activity. Possible approaches for activating immune cells include the use of antibodies that target co-stimulatory receptors (e.g. CD137, OX40, CD40 and GITR).

Antibodies that target stimulatory molecules have been tested in early-phase clinical trials, and they all have their own spectrum of side-effects to be considered. It will now be critical to extensively test the anti-tumour efficacy and safety of combining immune-modulatory therapies (e.g. T-cell checkpoint inhibitors and T-cell activation agonists) pre-clinically using better animal models of cancer that are more clinically relevant.

4. Co-stimulatory targets

4.1. 4-1BB (CD137)

The 4-1BB protein receptor (CD137) CD137 (4-1BB) is a surface glycoprotein that belongs to the tumour necrosis factor receptor superfamily [28]. CD137 is an inducible co-stimulatory molecule expressed on a variety of immune cells, including activated CD4+ and CD8+ T cells, NK cells, monocytes and dendritic cells [28,29].

On T cells specifically, CD137 functions as a co-stimulatory receptor induced on TCR stimulation. In this context, ligation of CD137 leads to increased T-cell proliferation, cytokine production, functional maturation and prolonged CD8+ T-cell survival [30]. Consistent with the co-stimulatory function of CD137 on T cells, agonistic monoclonal antibodies against this receptor have been shown to provoke powerful tumour-
specific T-cell responses capable of eradicating tumour cells in a variety of murine tumour models [31].

Lin et al. [32] could show that CD137 was upregulated on human NK cells after Fc-receptor triggering. In addition, CD137 stimulation was also found to enhance NK-cell function in mice [33]. These findings led to the hypothesis that since Fc-receptor triggering results in the upregulation of CD137 expression on NK cells, stimulation via CD137 could enhance NK cell killing by ADCC and thereby augment the efficacy of rituximab. This concept was then confirmed by Kohrt et al. [34]. They could demonstrate that in a syngeneic murine lymphoma model and in a xeno-transplanted human lymphoma model, sequential administration of rituximab followed by anti-CD137 monoclonal antibody (BMS-663513) had potent anti-lymphoma activity in vivo [34]. These results established a proof of concept for the enhancement of rituximab through immunomodulation of effector cells and support the testing of anti-CD137 agonists in combination with rituximab in clinical trials for patients with lymphoma.

Utomilumab (PF-05082566; Pfizer, New York, USA) is a fully humanised monoclonal agonistic antibody that stimulates CD137 signalling (co-stimulatory checkpoint molecule) [35]. Preclinical studies have suggested that combining utomilumab with checkpoint inhibitors (e.g. PD-1, PD-L1, CTLA-4 antagonists) results in an enhanced immune response in tumour models [36]. Preclinical data [34] and data from a phase I trial that evaluated utomilumab in combination with rituximab in

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**Table 1**

Function and therapeutic implications of B7 protein co-signalling family members.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Modulation</th>
<th>Drug</th>
</tr>
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<tbody>
<tr>
<td>B7.1</td>
<td>CD80; ‘second’ co-stimulatory signal for B and T cells by binding to CD28, increases IL-2 production and T cell proliferation</td>
<td>Co-stimulation</td>
<td>Ipilimumab; tremelimumab, TGN1412&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B7.2</td>
<td>CD86; ‘second’ co-stimulatory signal for B and T cells by binding to CD28, increases IL-2 production and T-cell proliferation</td>
<td>Co-stimulation</td>
<td>Ipilimumab, tremelimumab, TGN1412&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B7-DC</td>
<td>CD273 (PD-L2); mainly expressed on APCs</td>
<td>Inhibition</td>
<td>rHlgM12B7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>B7-H1</td>
<td>CD274 (PD-L1); expressed on APCs and other cells (e.g. tumour cells)</td>
<td>Inhibition</td>
<td>Avelumab, durvalumab, atezolizumab, CA-170</td>
</tr>
<tr>
<td>B7-H2</td>
<td>CD275; ligand for ICOS, secreted by APCs and dendritic cells</td>
<td>Co-stimulation</td>
<td>GSX3359609, JTX-2011</td>
</tr>
<tr>
<td>B7-H3</td>
<td>CD276; inhibits APCs and stimulates T&lt;sub&gt;reg&lt;/sub&gt; cells (results in IL-2 suppression). Receptor is unknown (TLT-2?)</td>
<td>Inhibition</td>
<td>8H9&lt;sup&gt;c&lt;/sup&gt;, Enoblituzumab</td>
</tr>
<tr>
<td>B7-H4</td>
<td>Receptor not known (BTLA?), suppresses T-cell expansion and cytokine production, stimulation of T&lt;sub&gt;reg&lt;/sub&gt; (via BTLA?)</td>
<td>Inhibition</td>
<td>AMP-110&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>B7-H5</td>
<td>VISTA; mainly expressed by T&lt;sub&gt;reg&lt;/sub&gt; and tumours, depletes cytokine synthesis (e.g. IL-2, TNF-α, IFN-γ) and T cell function, induces FoxP3 synthesis</td>
<td>Inhibition</td>
<td>JNJ-61610588, CA-170</td>
</tr>
<tr>
<td>B7-H6</td>
<td>CD337; binds to the NKp30 receptor that delivers an activating signal into NK cells (leads to increased production of IFN-γ and IL-2 and enhances ADCC of NK cells). BAT3: second ligand for NKp30 (secretion following DNA damage)</td>
<td>Co-stimulatory</td>
<td>None</td>
</tr>
<tr>
<td>B7-H7</td>
<td>Ligand: CD28H; strongly promotes CD4&lt;sup&gt;+&lt;/sup&gt; T-cell proliferation and cytokine production (e.g. IL-2, IL-10, IFN-γ, TNF-α) via an AKT-dependent signalling cascade</td>
<td>Co-stimulatory</td>
<td>None</td>
</tr>
</tbody>
</table>

Abbreviations: IL, interleukin; IFN, interferon; BTLA, B and T lymphocyte attenuator; APC, antigen-presenting cell; NKp30, natural killer cell protein 30; ADCC, antibody-dependent cellular cytotoxicity; TNF, tumour necrosis factor; VISTA, v-domain Ig suppressor of T-cell activation; TLT-2, TREM-like transcript-2; BAT3, HLA-B-associated transcript 3; FoxP3, forkhead box protein 3.

<sup>a</sup> In 2006, a London (UK)-based phase I clinical trial with TGN1412 (a CD28 superagonist monoclonal antibody) resulted in a catastrophe. Six healthy volunteers recruited to the trial developed a cytokine-release syndrome (CRS) due to the ‘superactivation’ of T cells by TGN1412. This led to a systemic burst of pro-inflammatory cytokines with the individuals requiring life support [82]. These unexpected clinical data demonstrated that an immune-mediated cytokine storm can lead to multi-organ failure even in the absence of infection, contamination of endotoxins, or underlying disease. It is currently unclear whether the severe effects of this type of CRS in humans is caused by the direct ligation of CD28 on T cells or by ligation and activation of other cell types and require further investigation.

<sup>b</sup> A clinical phase I trial for stage IV melanoma patients is ongoing (NCT00658892).

<sup>c</sup> The potential of this drug as a radio-immunotherapy (124I-labelled) is evaluated in an ongoing phase I trial (NCT01502917).

<sup>d</sup> AMP-110 is currently evaluated in a phase I trial for rheumatoid arthritis (NCT02277574).
patients with relapsed or refractory CD20-positive lymphomas demonstrated a significant anti-tumour efficacy of utomilumab/rituximab and no dose-limiting toxicities were observed. In addition, no patients discontinued treatment due to treatment-related adverse events [37].

The drug is currently evaluated in more phase I trials in combination with rituximab (NCT01307267), mogamulizumab (NCT02444793), an experimental OX40 agonist (NCT02315066), and avelumab (NCT02554812). The combination of utomilumab and pembrolizumab (NCT02179918) was evaluated in a small number of patients with advanced cancers, including pancreatic, colorectal, kidney, thyroid and lung cancer. Among the 23 patients, 6 patients had complete or partial responses. CRs occurred in one patient with small cell lung cancer (CR now for nearly a year) and one with kidney cancer (CR for more than a year) [38]. No safety issues were detailed.

4.2. ICOS

ICOS (inducible T cell co-stimulator, CD278) is expressed on activated T cells, whereas its ligand (B7-H2) is expressed mainly on B cells and DCs. The co-stimulatory B7-H2/ICOS pathway augments the T-cell effector function which leads to an enhanced cytokine production of T_{hl} and T_{h2} cells. In addition, ICOS stimulates IL-10 production suggesting that this pathway is also involved in the regulation of T_{reg} cell function [24].

The ICOS agonist GS3K359609 (GlaxoSmithKline, London, UK) is currently evaluated in a phase I trials (INDUCE-1, NCT02723955), whereas another compound (JTX-2011, Jounce Therapeutics, Cambridge, USA) is in phase II/III evaluation (alone or in combination with nivolumab; ICONIC trial, NCT02904226).

Currently, targeting of the B7-H2/ICOS pathway does not appear to be aggressively pursued for cancer treatment. However, this pathway may be of interest to be considered for the treatment with an anti-CTLA4 antibody (e.g. ipilimumab, tremelimumab) since mice studies suggest that the protective anti-tumour T-cell responses formed during this treatment are dependent on this pathway [39].

4.3. GITR

The glucocorticoid-induced TNF-R-related protein (GITR, CD357, a member of the TNF receptor superfamily) and its ligand (GITRL) can be detected in steady-state on T_{reg} cells with a further increasing expression on stimulation [40]. In addition, effector CD4+ and CD8+ T cells express GITR constitutively at low levels, but rapidly upregulate GITR expression on activation. GITR expression in humans has also been described in macrophages and NK cells [41]. GITR/GITRL molecules have been shown to be involved in inhibiting T_{reg} cells (e.g. in the tumour environment) and subsequent studies revealed GITR to also be upregulated on any activated T cells in humans, thus undermining its utility as a regulatory T-cell marker. From these preclinical findings, it was concluded that therapeutic GITR stimulation may activate anti-tumour immunity in humans and agonistic GITR antibodies are warrant to be tested in clinical trials [42].

To date, the most advanced monoclonal antibody targeting GITR, TRX518-001 (a humanised, Fc-disabled antibody, Leap Therapeutics, Cambridge, USA) is currently evaluated in two phase I trials (NCT01239134: melanoma and other solid tumours; NCT02628574: dose-escalation study in advanced tumours). First-in-human results (NCT01239134) have been presented most recently [43]. TRX518-001 was found to be well tolerated (up to 8 mg/kg) with no dose-limiting toxicities, related serious adverse events or related treatment emergent adverse events. Limited efficacy data were available (28/40 patients); 4 patients achieved a best response of stable disease. From this study, it was concluded that TRX518-001 treatment was safe and well tolerated and further investigation is warranted.

MK-4166 and MK-4126 (Merck & Co., Kenilworth, USA) are other agonistic monoclonal antibodies in advanced development which are currently tested in phase I clinical trials (combination of MK-4126 and pembrolizumab); both trials are currently recruiting patients (NCT02132754 and NCT02553499). In addition, other molecules targeting GITR are also in early clinical development (e.g. AMG228, BMS-986156, MEDI1873, GWN323, INCAGN01876; reviewed by Ref. [42] in this journal).

4.4. CD27-CD70

CD27 supports antigen-specific expansion of naïve T cells (differentiation of CD8+ T cells into effector cytotoxic T cells) and is vital for the generation of T-cell memory. It is also a memory marker of B cells. The ligand, the CD70 protein, is expressed on highly activated lymphocytes and plays in important role in boosting B cell activation (stimulation of immunoglobulin production). T cells are typically stimulated to divide and mediate anti-tumour effects through two signal mechanisms that involve the TCR stimulation (when engaged with an APC) and a co-stimulatory signal. CD27 can provide a potent co-stimulatory signal when engaged by its ligand CD70 (Fig. 2). Current preclinical data suggested that agonistic antibodies targeting the CD27−CD70 system would increase the capability of immune cells to kill malignant tumours [28,44].

Several molecules targeting the CD27−CD70 system are currently in early-phase clinical development.
ARGX-110 (Argenex, Breda, the Netherlands) is a newly developed monoclonal agonistic antibody targeting CD70. It has brought to the clinic and is currently being tested in two ongoing phase I trials (NCT01813539: advanced malignancies; NCT02759250: nasopharyngeal carcinoma). Recently, data from a phase I trial with MDX-1203 (Bristol-Myers Squibb, New York, USA; agonistic monoclonal antibody targeting CD70) were reported [45]. The most commonly reported adverse events included fatigue (85%), nausea (54%), decreased appetite (39%), anaemia, dyspnoea (35% each), constipation, peripheral oedema (31% each), vomiting (27%), cough, skin hyperpigmentation, thrombocytopenia (23% each). Exploratory efficacy analysis showed a best response of stable disease in 18 of 26 patients (69%) [45].

An agonistic CD27 fully humanised monoclonal antibody (varlilumab, Celldex Therapeutics, Hampton, USA) has now entered the clinic, both in haematologic malignancies and solid tumours (NCT01460134). Importantly, when T cells are activated through TCR stimulation and varlilumab, they undergo multiple cell divisions, secrete cytokines with a dominant pro-inflammatory signature (IFN-γ, IL-2 and TNF-α) and express activation markers consistent with an activated phenotype [9].

Varlilumab is currently in phase I/II development for the treatment of colorectal cancer, NSCLC, metastatic melanoma, ovarian cancer, renal cell carcinoma and head-and-neck squamous cell carcinoma. In addition, combinations with nivolumab are also in clinical development (NCT01460134). Preliminary results presented most recently showed that the combination is well tolerated [46], data on the clinical efficacy were not detailed.

Varlilumab is currently undergoing an extensive development programme. In an ongoing phase, I/II trial varlilumab is evaluated in combination with atezolizumab (anti-PD-L1 antibody) in patients with advanced cancers (NCT02543645). Other ongoing combination phase I/II studies with varlilumab include ipilimumab (metastatic melanoma: NCT02413827) and sunitinib (renal cell carcinoma: NCT02386111). Results have not been detailed.

4.5. OX40

OX40 (CD134) and its ligand OX40L (secreted by APCs and DCs) are essential for enhancing the activation of CD8+ T cells (co-stimulatory second signal). The expression of OX40 following antigen encounter is largely transient for both CD4+ and CD8+ T cells (24–72 h), with the duration of OX40 expression by CD8+ T cells reported to be shorter than for CD4+ T cells stimulation of OX40 results in increased secretion of IFN-γ and subsequently PD-L1 overexpression [47,48]. OX40 agonists have the ability to directly regulate T<sub>reg</sub> cells and some preclinical studies have provided evidence that OX40 agonists can push T<sub>reg</sub> cells in both directions, depending on the context of stimulation and the cytokine milieu [49]. Whether OX40 functions via T<sub>reg</sub> cell suppression, deletion or both, treatment with these agonists should diminish the inhibitory effects mediated by T<sub>reg</sub> cells and thereby promote anti-tumour CD8+ T-cell responses necessary to maintain long-term anti-tumour immune responses [50].

Preclinical studies have demonstrated that treatment of tumour-bearing mice with OX40 agonistic antibodies resulted in tumour regression which formed the basis to further evaluate this strategy in clinical trials [50]. Recently, monotherapy with an OX40 agonistic antibody (9B12, a murine IgG antibody; Providence Health, Portland, USA) was tested in a phase I trial in patients with solid tumours (NCT01644968) with promising results [51]. Twelve out of 30 patients receiving 9B12 had regression of at least one metastatic lesion with only one cycle of treatment, and no significant adverse events were reported. However, in the meantime, the development of murine antibodies has been precluded continued treatment [51].

Currently agonistic OX40 monoclonal antibodies (e.g. MOX0916, PF-04518600, MEDI0562, MEDI6469, MEDI6383) are evaluated in several phase I/II clinical trials either as monotherapy or in combination with other immune-modulating agents (durvalumab [NCT02221960], tremelimumab/rituximab [NCT02205333], utomilumab [NCT02315066], atezolizumab/bevacizumab [NCT02410512]; extensively reviewed in this journal by Ref. [52]).

Indeed, the clinical evaluation of OX40 agonists represents an exciting new chapter in cancer immunotherapy and further studies and patients’ immune response monitoring will provide more insight how to best combine the novel strategy with existing therapies since it will be unlikely that targeting OX40 alone will be sufficient to cure all patients across various histologies. However, there is a great promise that combination of immunotherapies incorporating both OX40 agonists and checkpoint inhibitors may add additional benefit to cancer patients. In this regard, trials incorporating multiple complementary interventions are under development [50]. One particularly intriguing concept is a triple combination of an OX40 agonist with concomitant PD-1 and CTLA-4 blockade (‘triple threat’). This type of immunotherapy approach may help augment the efficacy of dual PD-1/CTLA-4 blockade by enhancing the expansion, survival and cytolytic activity of CD8+ T cells. Conducting of this study is ongoing.

4.6. CD40

CD40 is a TNF receptor superfamily member expressed on APCs, B cells (key receptor for B-cell activation via T<sub>h2</sub> cells) and monocytes as well as many non-immune cells and a wide range of tumours [53]. Interaction with its
trimeric ligand (CD40L: CD154) on activated T helper cells results in APC activation, required for the induction of adaptive immunity (e.g. activation of macrophages by T_{H1} cells) and resulting in the enhanced secretion of pro-inflammatory cytokines. The consequences of CD40 signalling are clearly multi-faced and depend on the type of cells expressing CD40 and the microenvironment in which the CD40 signal is provided [54].

In several preclinical models, CD40 agonistic antibodies have shown remarkable therapeutic activity in the treatment of CD40-positive B-cell lymphomas with 80–100% of mice cured [55]. In addition, CD40 agonists were also able to clear bulk tumours from mice with near terminal disease [55]. To date, 6 monoclonal antibodies targeting CD40 have entered clinical trials: CP-870893 (Pfizer, New York, USA, now RG-7876, Roche, Basel, Switzerland), APX005M (Apelesh, San Carlos, USA), ADC-1013 (Alligator Bioscience, Lund, Sweden), lucatumumab (Novartis, Basel, Switzerland), Chi Lob 7/4 (University of Southampton, UK) and dacetuzumab (Seattle Genetics, Bothell, USA; reviewed by Ref. [53]). Notably, these compounds show diverse activities ranging from strong agonism (CP-870873) to antagonism (lucatumumab), and currently there is no satisfactory explanation for this heterogeneity [56].

The effort over the last 10 years to develop CD40 agonists as a new class of drug for cancer patients has been overwhelming and has been recently summarised in two reviews [57,58]. Briefly, in the first clinical trial, CD40 agonists demonstrated clinical activity and led to long-lasting CR in a patient with advanced head-and-neck tumour [53]. Similarly, testing a single dose of CP-870893 resulted in 4/29 responses in patients with advanced cancer [56,57] with one of these patients (who received 9 subsequent doses) still remaining in CR more than 5 years later.

Currently, several clinical trials are ongoing with the above-mentioned CD40 agonists (see Refs. [57,58] for review). Although agonistic CD40 antibodies have been found to trigger elevations in liver enzymes, decreases in platelet numbers and infusion-related reactions in these trials, overall toxicity has not been a major issue with CD40 agonists in clinical settings. However, to improve the clinical effectiveness of these drugs, several major challenges including determining the optimal dose and schedule, identifying appropriate biomarkers, and establishing the optimal combination therapy remain to be addressed.

Agonistic CD40 monoclonal antibodies represent a promising strategy for cancer patients and the initial clinical trials reported so far have shown clinical activity in the absence of disabling toxicity. Moreover, some responses have been dramatic and very durable, but response rates still remain 20% in the monotherapy setting. Therefore, it is conceivable (at least for solid tumours) that CD40 agonists might be most effectively used in combinations with other modalities (e.g. chemotherapy, radiation and so forth). In addition, the prospect of trials combining agonistic CD40 antibodies with other immune checkpoint modulators targeting CTLA-4 (NCT01103635), or PD-1/PD-L1 (NCT02304393, NCT02410512) is enticing and represents a novel immunologic treatment opportunity for cancer patients.

5. Inhibitory targets

5.1. VISTA (B7-H5)

VISTA (v-domain Ig suppressor of T cell activation, also known as B7-H5; Table 1) has been identified as an inhibitory ligand (homology to PD-L1). VISTA is preferentially expressed on mature myeloid APCs and to a lesser extent on T cells and activated T_{reg} cells (FoxP3 expression is stimulated in T_{reg}). VISTA functions through an unknown receptor inhibiting T cell proliferation and cytokine production and by arresting cell cycle [59]. In addition, B7-H5 expressed on tumour cells can enhance tumour-invasive growth by augmenting type 1 matrix metalloproteinase [60]. Preclinical studies with VISTA blockade have shown promising improvement in anti-tumour T-cell responses, leading to impeded tumour growth and improved survival [61].

Antibodies targeting VISTA for cancer immunotherapy are already under development. JNJ-61610588 (Johnson & Johnson, Princeton, USA), a fully human IgG1 kappa anti-VISTA monoclonal antibody, is the first antibody being in evaluated in clinical trials. Currently, a phase I study of safety, pharmacokinetics, and pharmacodynamics of JNJ-61610588 in patients with advanced cancers is ongoing (NCT02671955). In addition, combination studies with PD-1/PD-L1 antagonist are in preparation.

In 2015 data for CA-170 (Curis, Cambridge, USA), a first-in-class oral, small-molecule antagonist that selectively targets PD-L1 (EC_{50}: 17 nM) and VISTA (EC_{50}: 37 nM), both of which function as negative checkpoint regulators of immune activation, have been detailed [62]. Preclinical ex vivo data demonstrate that CA-170 can induce effective proliferation and IFN-γ production (produced by activated T cells and a marker of T cell activation) by T cells that are specifically suppressed by PD-L1 or VISTA. In addition, CA-170 also appears to have anti-tumour effects similar to anti-PD-1 or anti-VISTA antibodies in multiple in vivo tumour models. In preclinical toxicology studies, CA-170 appeared safe when administered at multiple dose levels using a once daily oral dosing schedule [62]. CA-170 is currently investigated in a phase I trial in patients with advanced solid tumours and lymphomas (NCT02812875).

For VISTA (B7-H5), many questions still remain, not least being the identity of its receptor. As with B7-H3 and B7-H4, answering this question may be challenging, but would facilitate therapeutic development.
5.2. Mogamulizumab

Mogamulizumab is a recombinant, humanised monoclonal antibody of the IgG subclass 1 kappa (IgG1κ) isotype that targets CCR4-expressing cells that is being developed by Kyowa Kirin Pharma (Tokyo, Japan) for the treatment of T cell malignancies including PTCL, CTCL, ATL and solid tumours [63]. Mogamulizumab targets CCR4 (cellular chemokine receptor), a chemo- kine receptor that is preferentially expressed by Th₂ and Treg cells. In response to its ligands, CCL17 (TARC) and CCL22 (MDC), CCR4 promotes T-cell migration to extranodal sites, including the skin. The antibody depletes CCR4-expressing cells by antibody-dependent, cell-mediated cytotoxicity (ADCC). Defucosylation of its Fc region culminates in enhanced Fc receptor binding, permitting ADCC at lower antigen densities and at lower ratios of effector cells to target cells. In addition, mogamulizumab depletes Treg cells, an important therapeutic target in many human cancers because of their role in suppressing host anti-tumour immunity. Therefore, in addition to directly targeting malignant T cells expressing CCR4, mogamulizumab may favourably influence the tumour microenvironment on Treg depletion without triggering clinically significant autoimmune complications [64].

This forms the rationale for current evaluation of mogamulizumab in combination with other immune checkpoint inhibitors including tremelimunab/durvalumab (CTLA-4 antagonist; PD-L1 antagonist, phase I study, advanced solid tumours, NCT02301130) and nimolumab (PD-1 antagonist, phase I and phase II studies, advanced solid tumours, NCT02476123, NCT02705105), utomilumab (CD137 agonist, phase I study, advanced solid tumours, NCT02444793). In addition, the IDO-1 inhibitor KHK2455 alone and in combination with mogamulizumab in patients with locally advanced or metastatic solid tumours is also investigated in an ongoing phase I trial (NCT02867007).

5.3. B7-H3

B7-H3 (CD276) is an inhibitory member of the B7 protein family (Table 1). It inhibits APCs and stimulates Treg cells which results in IL-2 suppression (termination of the immune response), however, the receptor is still unknown (TLT-2?) [24].

Recently, a humanised IgG1 monoclonal antibody (including Fc engineering) targeting B7-H3 has been developed and brought to the clinic (enoblituzumab, MacroGenics, Rockville, USA). Data from an ongoing phase I study showed that enoblituzumab was well tolerated at all dose levels (up to 15 mg/kg), with grade 3/4 drug-related adverse events seen in only 4% of the patients. In addition, no severe immune-related adverse events, and no drug-related treatment discontinuations were observed (NCT01391143). In this phase I study of enoblituzumab, initial monotherapy anti-tumour activity in this heavily pre-treated patient population was observed across several tumour types, including patients with prostate and bladder cancer as well as melanoma [65].

Currently, enoblituzumab is evaluated in combination with other checkpoint inhibitors, including ipilimumab (NCT02381314) and pembrolizumab (NCT02475213), in patients with B7-H3 positive melanoma, NSCLC and head-and-neck cancers (phase I trials). Results have not been reported yet.

5.4. TIM-3

TIM-3 (T cell immunoglobulin and mucin-domain containing 3) is a Th1 cell-specific surface protein which is involved in the suppression of macrophage activation (‘escape model’ following PD-1 inhibition), but it is mainly expressed on activated CD8+ T cells. TIM-3 stimulation (via its ligand galectin-9 secreted by Treg cells) results in a depletion of IFN-γ production by interrupting CD45 and Lck. In addition, Th1 activation is inhibited. Interestingly, although anti-TIM-3 therapy alone had only a modest effect in animal models, the combination of anti-TIM-3 and anti-PD-1/PD-L1 monoclonal antibodies significantly suppressed tumour growth and even resulted in cures in a small proportion of these treated tumour-bearing mice supporting the potential of blocking TIM-3 in combination with other immune checkpoint inhibitors for cancer treatment [44,66].

To date, only two TIM-3 antagonistic monoclonal antibodies are in early-phase clinical development (MBG453, TSR-022). TSG-022 (Tesaro, Waltham, USA) is currently evaluated as single agent in a phase I trial in patients with advanced solid tumours (NCT02817633). In addition, part 2 of the study will further explore the safety and clinical activity of TSR-022 as monotherapy and in combination with an anti-PD-1 antibody in patients with select tumour types. MGB453 (Novartis, Basel, Switzerland) is evaluated in a phase I-Ib/II open-label multicentre study as single agent (safety and efficacy) and in combination with PDR001 (novel anti-PD-1 antibody) in patients with advanced malignancies (NCT02608268). Both trials are currently recruiting patients.

5.5. LAG-3

LAG-3 (lymphocyte-activation gene 3, CD 223) binds MHC-II molecules and was found to play a more subtle role in modulating T-cell function than either CTLA-4 or PD-1. LAG-3 acts by regulating CD8+ T-cell expansion in immune reactions that have already been initiated (inhibitory signal) and by increasing the Treg cell activity. Of note, LAG-3 stimulation can also activate DCs. In mouse models, LAG-3 was found to be one of the key inhibitory receptors (along with PD-1) that contribute to T-cell exhaustion [67]. Moreover,
simultaneous blockade of LAG-3 and PD-1 synergistically enhanced T-cell activity and anti-tumour immunity in mice [68].

A monoclonal antibody directed against LAG-3 (BMS-986016, Bristol-Myers Squibb, New York, USA) has recently entered clinical trials, either alone or in combination with nivolumab (NCT01968109; N = 360, and NCT02061761). In addition, it is also evaluated in a phase I trial in combination with a CD137 agonistic antibody for patients with recurrent glioblastomas (NCT02658981). Furthermore, the LAG-3 inhibitory antibody LAG525 (Novartis, Basel, Switzerland) is undergoing phase I testing in combination with an anti-PD-1 antibody in patients with advanced cancers (NCT02460224).

In contrast, IMP321 (Prima Biomed, New South Wales, Australia) is the first-in-class co-stimulatory molecule targeting LAG-3 that activates DCs and thereby enhances the antigen presentation to cytotoxic CD8+ T cells. This leads to DC maturation, migration to the lymph nodes and enhanced cross-presentation of antigens to CD8+ T cells [69]. As a result, strong and sustained cytotoxic T-cell responses are obtained with repeated IMP321 injections in animal models. The compound is currently undergoing phase I testing in combination with pembrolizumab in patients with metastatic melanoma (TACTI-mel trial, NCT02676869).

5.6. KIR

The theme of early combination of checkpoint modulators is also being pursued in the case of monoclonal antibodies targeting the KIR receptor family (killer cell immunoglobulin-like receptor). NK cells use different innate receptors to sense their environment and respond to alterations induced by malignant cell transformation. In a process termed ‘licensing,’ NK cells use inhibitory KIRs for ‘self’-MHC-I molecules to maintain a state of responsiveness and to kill target cells that have lost MHC-I (e.g. tumour cells) [70]. In contrast, the recognition of missing or downregulated self-MHC-I molecules on tumour cells by licenced NK cells shifts, the NK cell receptor balance towards activation [71]. Taken together, the modulation of NK cell activity is therefore controlled by an array of germ line-encoded activating and inhibitory receptors, as well as modulating co-receptors (co-stimulatory KIRs; reviewed by Ref. [22]).

Several monoclonal antibodies targeting KIRs are tested in preclinical models and in ongoing clinical trials (reviewed by Ref. [72]). Amongst these compounds, lirilumab (Bristol-Myers Squibb, New York, USA) is the most advanced compound and currently under extended clinical evaluation. Lirilumab (inhibitory monoclonal antibody targeting KIR2DL1-3) is undergoing phase I/II testing for solid tumours (lirilumab in combination with nivolumab for advanced solid tumours, NCT01714739) and mainly for haematological malignancies (lirilumab plus nivolumab and azacitidine in MDS patients [NCT02599649]; lirilumab plus azacitidine for AML [NCT02399917]; lirilumab in combination with rituximab for CLL patients [NCT02481297]). In addition, lirilumab was evaluated in a double-blind placebo-controlled randomised phase II study evaluating the efficacy of lirilumab as maintenance treatment administered in elderly patients with AML in first complete remission (EFFIKIR trial, N = 150, NCT01687387). The study is ongoing, but not recruiting patients. Results are expected early in 2017.

5.7. IDO

The immune-regulatory molecule IDO (indoleamine-pyrrole-2,3-dioxigenase-1,2) is a 45-kD haemoprotein essential for oxidative catabolism of tryptophan in the kynurenine pathway. IDO (two isoforms: IDO-1 and IDO-2) catalyses oxidative cleavage of the 2,3-double bond in the indole moiety of l-tryptophan, resulting in the production of the first kynurenine pathway metabolite, N-formyl kynurenine. This results in a decreased tryptophan level which then suppresses T cell proliferation, a mechanism being involved to terminate the immune reaction [73]. IDO expression has been correlated with decreased OS and PFS in several clinical studies and it has been suggested that high IDO-1,2 levels during treatment could be related to poor outcome to chemotherapy and/or radiotherapy and, at least in part, contribute to resistance to therapy [74]. In addition, IDOs are also important in developing resistance to immunotherapy, and it has been suggested that IDOs play a major role in resistance to ipilimumab (anti-CTLA-4 treatment results in IDO-1,2 overexpression and subsequently increased PD-1/PD-L1 stimulation). IDO-1,2 enzymes can be induced in APCs by inflammatory cytokines such as IFN-γ, IL-6 and TNF-α and then lead to Treg activation. In addition, most human tumours express IDO, which contributes to tumour-induced tolerance and suppression of the immune system [74]. Several preclinical studies have suggested that IDO inhibitors can overcome resistance to anti-neoplastic agents and X-irradiation and may stimulate the anti-tumour immune response [75].

Several IDO-1,2 inhibitors are currently undergoing an extensive development programme (extensively reviewed by Ref. [76]). Amongst them, indoximod (inhibits IDO-2, NewLink Genetics, Ames, USA) and the second-generation IDO-1 inhibitors such as the orally available agent epacadostat (formerly known as INCB024360; Incyte, Wilmington, USA) and GDC-0919 (NewLink Genetics, Ames, USA) have already entered phase II/III clinical development, whereas other molecules appear to be suboptimal for clinical development.

The first-in-man phase I clinical trial involving indoximod enrolled a total of 48 patients with refractory
solid malignancies (NCT00567931). In this dose-escalation study, oral indoximod was well tolerated up to a dose of 2000 mg twice a day. Moreover, of seven evaluable patients who received 200-mg indoximod per day, five patients experienced objective responses or disease stabilisation [77]. The results of another study investigating the clinical profile of indoximod have been published in part (NCT01191216) [78]. In this phase I clinical trial, indoximod was evaluated in combination with docetaxel in 27 patients with metastatic solid tumours to determine the MTD of indoximod given in combination with docetaxel. Patients were assigned to receive 300, 600, 1000, 1200 and 2000-mg indoximod p.o. twice a day, in combination with either 60 or 75 mg/m² docetaxel every 3 weeks. The most common side-effects were fatigue (58.6%), anaemia (51.7%), hyperglycaemia (48.3%), infections (44.8%), and nausea (41.4%). Out of 22 evaluable patients, four experienced partial responses and nine disease stabilisation. The authors recommended a dose of 1200-mg indoximod twice a day in combination with 75 mg/m² docetaxel every 3 weeks for evaluation in phase II studies. Most recently, encouraging interim results from a phase II trial have been reported (NCT02077881) [79]. In this trial, patients were treated with indoximod (1200-mg BID continuous dosing) in combination with gemcitabine (1000 mg/m², d1, 8, 15, qd29) and nab-paclitaxel (125 mg/m², d1, qd29). Eligible patients had treatment naïve metastatic pancreatic cancer or first-line salvage therapy after previous resection and adjuvant therapy. Treatment continues until disease progression or toxicity. Primary endpoint is OS. Secondary endpoints include response rate and OS (target enrolment: 98 patients). At this interim analysis, 30 patients to date had been enrolled and completed protocol treatment at least through imaging at the end of cycle 2. Of these, 11 (37%) have demonstrated an objective response by RECIST criteria including one patient with a confirmed complete response. One adverse event of immunological significance (colitis) was observed and required study withdrawal. Furthermore, indoximod is currently in phase II evaluation (N = 56) in combination with pembrolizumab for patients with metastatic melanoma (NCT02073123). In addition, indoximod is also studied in an ongoing randomised phase II trial (N = 154) for patients with metastatic breast cancer (NCT01792050: indoximod plus a taxane versus indoximod plus placebo). Both studies are ongoing, but currently not recruiting patients.

In an ongoing phase I/II randomised, blinded, placebo-controlled study, ipilimumab is evaluated in combination with placebo or epacadostat in patients with unresectable or metastatic melanoma (NCT01604889). In this setting, 7 patients were assigned to receive 300-mg epacadostat p.o. twice a day plus 3-mg/kg ipilimumab i.v. every 3 weeks; however, enrolment was stopped when 5 patients developed clinically significant elevations of circulating alanine transaminase. Six out of seven patients were evaluable at discontinuation and all exhibited disease stabilisation. A second cohort of eight patients receiving ipilimumab in combination with 25-mg epacadostat p.o. twice a day was enrolled. One of these subjects experienced dose-limiting hepatic toxicity (Grade 3 aspartate aminotransferase elevation), whereas immunological side-effects were manageable with temporary treatment discontinuation. At first evaluation, the disease control rate was 75%, 3 patients achieved radiologically confirmed partial responses, and 3 patient experienced disease stabilisation [80]. Furthermore, epacadostat is currently undergoing evaluation in several phase I–III trials including the combinations with pembrolizumab for metastatic melanoma (phase III, N = 600, primary endpoints OS and PFS; NCT02752074), with atezolizumab for metastatic NSCLC (phase I, N = 80, NCT02298153: ECHO-110 trial), with durvalumab for selected cancers (phase I/II, N = 185, NCT02318277: ECHO-203 trial), and with nivolumab for selected cancers (phase I, N = 291, NCT02327078: ECHO-204 trial). Results of these trials are awaited eagerly.

GDC-0919 (Genentech, San Francisco, USA) is evaluated in one ongoing phase I study as monotherapy in patients with advanced solid tumours (NCT02048709), results have not been reported so far. The discovery of the two different IDO isoforms has further complicated the clinical development of IDO inhibitors. As other IDO inhibitors are developed, the importance of IDO-1 and IDO-2 in mediating tumour immunosuppression will be better characterised. Equally important will be discovering the possible autoimmune toxicities and risk of latent infection reactivations with IDO-1-specific inhibitors. In addition, a better understanding of the various pathways affected by IDO-1,2 inhibition will help to identify suitable biomarkers for studying the effects of these agents.

6. Conclusion

The use of immunotherapy to activate the immune system’s ability to eradicate cancer cells is an exciting new avenue for cancer treatment, but the exact clinical applications are still not clear. Co-signalling molecules comprise a complex molecular network that positively or negatively modulates T-cell responses. It is important to emphasise that agonistic molecules as immunostimulatory agents strikingly differ in their proposed mechanism of action compared with monoclonal antibodies that accomplish immune activation by blocking negative checkpoint molecules such as CTLA-4 or PD-1/PD-L1 (Table 2).

Indeed, the prospect of combining agonistic antibodies with CTLA-4, PD-1 or PD-L1 antagonistic antibodies is enticing and represents a real immunologic opportunity to ‘step on the gas’ while ‘cutting the brakes.’
Table 2
Summary of ongoing clinical trials with second- and third-generation immune-oncology drugs.

<table>
<thead>
<tr>
<th>Target (CDx)</th>
<th>Drug (company)</th>
<th>Clinical phase</th>
<th>Trial number</th>
</tr>
</thead>
</table>
| 4-1BB (CD137) | Utomilumab (Pfizer) | I | NCT010307267  
NCT02444793  
NCT02315066  
NCT02554812 |
| ICOS (CD278) | GSK3359609 (GlaxoSmithKline) | I | NCT0273955  
NCT02904226 |
| GITR (CD357) | TRX 518-001 (Jounce Therapeutics) | I | NCT01239134  
NCT02628574  
NCT02132754  
NCT02553494 |
| VISTA (B7-H5) | ARGX-110 (Argenix) | I | NCT01813539  
NCT02759250  
NCT01460134  
NCT0235918 |
| OX40 (CD134) | 9B12 (Providence Health) | I | NCT01649068  
NCT02410512  
NCT02219724  
NCT02315066  
NCT02221960  
NCT02205333  
NCT01103635  
NCT02304933  
NCT02410512 |
| CD70 | Varmilumab (Cellidex) | I/II | NCT02304393  
NCT02759250  
NCT0235918 |
| CD27 | CP870893 (Pfizer) | II | NCT02132754  
NCT02553494  
NCT02219724 |
| CD40 | CA-170 (Curis) | I | NCT02812875  
NCT02671955 |
| VISTA (B7-H5) | JNJ-61650588 (Johnson & Johnson) | I | NCT02205333  
NCT02304933  
NCT02410512  
NCT02132754 |
| Moganulizumab (CD194) | CCR4 (Kyowa Kirin) | I/II | NCT02301130  
NCT02476123  
NCT02750150  
NCT02444793  
NCT02867007 |
| B7-H3 (CD276) | Enoblituzumab (MacroGenics) | I | NCT02475123  
NCT02817633  
NCT02608268 |
| TIM-3 | TS-022 (Tesaro) | I | NCT01968109  
NCT02061761  
NCT02658981  
NCT02676869  
NCT02460224 |
| LAG-3 (CD223) | BMS-986016 (Bristol-Myers Squibb) | I | NCT01968109  
NCT02061761  
NCT02658981  
NCT02676869  
NCT02460224 |
| KIR (2DL1-3) | Lirilumab (Bristol-Myers Squibb) | I | NCT01714739  
NCT02599649  
NCT02399917  
NCT02481297  
NCT01687387 |
| IDO-1,2 | Indoximod (NewLink Genetics) | II | NCT00567931  
NCT01191216  
NCT02077881  
NCT02073123  
NCT01792050  
NCT02048709  
NCT01604889  
NCT02752074 |
| GDC-0919 | GSK3359609 (GlaxoSmithKline) | I | NCT02904226  
NCT01813539  
NCT02759250  
NCT01460134  
NCT0235918 |
| Lag525 (Novartis) | IMP321 (Prima Biomed) | I | NCT01604889  
NCT02752074  
NCT02298153  
NCT02311827  
NCT02327078 |

Promising clinical outcome stems from several clinical trials that have used novel immune checkpoint strategies for the treatment of cancers. It is quite clear that successful clinical trials with novel immune checkpoint modulators (either alone or in combination) must be preceded by understanding fundamental mechanisms of immune regulations led by co-signalling molecules in normal and pathological microenvironment.

In addition, ongoing trials are investigating the immuno-therapy combination of antagonists with additional co-stimulatory agents, and it is expected that the impact of treatment sequence of immuno-therapy (e.g. with chemotherapy or radiotherapy), and the possible role of maintenance therapy with immune checkpoint modulators should advance our knowledge and treatment options.

In addition, it will be mandatory to incorporate tissue analyses into future trials of second- and third-generation drugs for immune-oncology treatment to facilitate insight into predictive biomarkers of response and resistance. This might be most relevant in the first-line setting because such patients generally have a number of other therapeutic options (e.g. treatment with TKIs, chemotherapy, monoclonal antibodies).

For example, despite an emphasis on PD-L1 testing in ongoing trials, it is clear that the role of PD-L1 expression alone is far from being clear since there is still a number of pitfalls (e.g. heterogeneity of expression, threshold differences in PD-L1 assays, lack of a gold standard for PD-L1 positivity) [81]. In addition, it can be speculated that PD-L1 is not just ‘present’ (positive) or ‘absent’ (negative), since it is a biological continuum which might apply to other biomarkers as well.

Hence, the development of better predictive biomarkers may allow oncologists to identify particular patients’ subsets that are most likely to benefit from immune-oncology treatment in first-line, either alone or in combination with chemotherapy or other checkpoint inhibitors which are currently in clinical development.

Although patterns of checkpoint co-expression have only begun to be analysed in patients with cancer, the accumulating data could be extremely valuable in designing combination regimens; in fact it could very well turn out that a combination checkpoint modulation treatment strategy requires a personalised approach to achieve maximal efficacy.

Most importantly, combinations of several immune checkpoint molecules (antagonistic, agonistic) can result in increased clinical benefit, however, additional clinical work is required to understand the clinical potential for this strategy (e.g. safety, efficacy) to induce long-term clinical responses in patients with cancer.

Immuno-oncology drugs for cancer treatment—the more the better? Although the immune-oncology armamentarium will clearly increase in the near future,
there are still several challenges with single, dual or even triple combinations and the most appropriate way forward still remains to be identified. Apart from identifying suitable biomarkers, data from early clinical development provided evidence that the overall toxicity may not be a significant issue for the immune-oncology drugs; however, monitoring of new toxicity signals is mandatory in all ongoing clinical trials.

In addition, it still remains to be proven that modulation of the immune system can be optimally and reliably achieved in cancer patients treated at various doses, schedules, and combinations. However, checkpoint inhibition alone is not sufficient to promote tumour regression in a majority of patients (ORRs: 20% in the monotherapy setting) suggesting that the combination of checkpoint stimulating and inhibitory molecules appear to be an appropriate way to increase response rates. Generating a robust therapeutic immune response requires not only releasing the ‘brakes’ but also stepping on the ‘gas.’ Since anti-tumour immunity is directed by a dynamic constellation of signals, maximising the therapeutic benefit of lymphocyte agonists will likely depend on incorporating multiple complementary interventions, and there is great promise that combination immunotherapy incorporating both stimulating and inhibiting drugs may be able to do what single agents alone cannot—resulting in an amplification of cytolytic T cells.

In addition, synergy between stimulatory immune therapy and checkpoint blockade may yield ORRs at lower dosages than are required when each drug is used as a monotherapy which might further decrease toxicity. The most viable candidates for combinatorial therapies clearly are those that have already achieved FDA- and/or EMA-approval.

In summary, the challenge of increasing the curative immune response rates in a diverse population of patients will almost certainly require multiple complementary therapeutic modalities to overcome the immune-suppressive tumour microenvironment of established tumours. This then may provide a protective anti-tumour immune response. Therefore, the results of the ongoing combination studies with second- and third-generation immune-oncology drugs are eagerly awaited.

**Conflict of interest statement**

W. Dempke and S. Dale are the employees of Kyowa Kirin Pharmaceutical Development Ltd. (UK). K. Fenchel and P. Uciechowski declare not conflicts of interest.

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