Cancer Biol Med 2016. doi: 10.20892/j.issn.2095-3941.2016.0015

REVIEW

Immuno-oncology combinations: raising the tail of the survival curve

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ABSTRACT There have been exponential gains in immuno-oncology in recent times through the development of immune checkpoint inhibitors. Already approved by the U.S. Food and Drug Administration for advanced melanoma and non-small cell lung cancer, immune checkpoint inhibitors also appears to have significant antitumor activity in multiple other tumor types. An exciting component of immunotherapy is the durability of antitumor responses observed, with some patients achieving disease control for many years. Nevertheless, not all patients benefit, and efforts should thus now focus on improving the efficacy of immunotherapy through the use of combination approaches and predictive biomarkers of response and resistance. There are multiple potential rational combinations using an immunotherapy backbone, including existing treatments such as radiotherapy, chemotherapy or molecularly targeted agents, as well as other immunotherapeutics. The aim of such antitumor strategies will be to raise the tail on the survival curve by increasing the number of long term survivors, while managing any additive or synergistic toxicities that may arise with immunotherapy combinations. Rational trial designs based on a clear understanding of tumor biology and drug pharmacology remain paramount. This article reviews the biology underpinning immuno-oncology, discusses existing and novel immunotherapeutic combinations currently in development, the challenges of predictive biomarkers of response and resistance and the impact of immuno-oncology on early phase clinical trial design.

KEYWORDS Combination drug therapy; oncology; clinical trials; PD-1; PD-L1; CTLA4; biomarkers; immunotherapy

Introduction

Immunotherapy has been utilized as a strategy for treating cancer for over 100 years since the use of Coley's toxins for the treatment of sarcoma[1]. For many years, the focus of immunotherapy has been activation of the immune response either through antitumor vaccines or direct stimulation with interleukin-2 (IL-2) or interferon. These approaches provided incontrovertible evidence that the immune system could be harnessed to fight cancer, however responses have been few and far between, although often durable when they did occur. It has only been through further study of the mechanistic basis of the immune system that recent breakthroughs have come, most notably through checkpoint inhibition[2]. By providing durable responses, partly due to memory immune responses, immunotherapy has shifted the focus of new treatments from the slope of the survival curve [e.g. median overall survival (OS) and progression free survival (PFS)] to the tail of the survival curve (e.g. 2- or 5-year survival rates)[3,4].

There remains however, significant scope for increasing the effectiveness of immunotherapy and in doing so raising the tail of the curve. For example, combination strategies with immunotherapy have great potential to be able to do this by combining different but synergistic immunotherapies or the pairing of immunotherapy with other modalities of antitumor treatments, such as chemotherapies, molecularly targeted therapies and radiotherapy. Early phase trials, carefully designed with a thorough understanding of the underlying mechanisms involved in both therapeutics and cancer biology, are critical for the safe and rational development of combination regimens involving immunotherapeutics. In this review, we will discuss the biology underpinning immunotherapy and detail key clinical immuno-oncology advances. We will also focus on strategies combining existing immunotherapy with chemotherapy, radiotherapy, molecularly targeted agents and novel immunotherapeutics, as well as the challenges and implications for early phase trial design.

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Received February 2, 2016; accepted March 11, 2016.
Available at www.cancerbiomed.org

Immune surveillance, editing and suppression

The underlying theory behind immune surveillance is that many tumors are eliminated by the immune system, while some cancers develop ways and means to escape the immune response[5]. A clinically apparent tumor is one that, even under the pressure of the immune system, has escaped from immune recognition[6,7].

The two main pathways in which tumors escape the immune response include 'immuno-editing' whereby tumor variants resistant to immune effectors are selected, with the progressive formation of an immune suppressive environment within the tumor[8-11]. During cancer immuno-editing, the host immune system shapes tumor fate in three phases through the activation of innate and adaptive immune mechanisms, namely elimination, equilibrium and escape[9,10]. Genetic mouse models have shown that T cell recognition of tumor antigens drives the adaptive immunological elimination, or 'sculpting' of a developing cancer[12,13]. It has become recognized that interferons play an important role in coordinating tumor-innate immune system interactions[14,15].

Much work has focussed on the development of the tumor immunosuppressive microenvironment, and the emergence of T cell tolerance (**Figures 1 and 2**)[16]. The local interaction between the immunologic milieu of dendritic cells (DCs), myeloid derived suppressor cells (MDSCs), regulatory T (Treg) cells, stromal cells and T cells is crucial to the induction of tolerance. Often it is the development of inhibitory processes, and in particular expression of inhibitory pathways via co-inhibitory pathways or immune checkpoints that inhibit T cell responses. This ultimately leads to T cell exhaustion, which is a state of T cell dysfunction that arises in many cancers[17,18]. It is defined by poor effector function, sustained expression of inhibitory receptors and a transcriptional state distinct from that of functional effector or memory T cells[19]. Importantly, T cell exhaustion prevents the optimal control of tumors.

Key negative regulatory pathways or 'checkpoints' control auto-reactivity and the improved understanding of their involvement in cancer has revolutionized tumor immunotherapy[20]. Cytotoxic T lymphocyte antigen 4 (CTLA4) is expressed mainly on the surface of activated $CD4^+$ T cells and Treg cells, and plays a central role in maintaining immune tolerance by inhibiting T cell co-stimulation[21]. Programmed cell death 1 (PD-1) is mainly expressed on activated T cells upon T cell receptor (TCR) engagement and on Treg cells, and also identifies exhausted T cells[22]. When bound to its ligand, programmed cell death 1 ligand (PD-L1) expressed on tumor cells, PD-1 downregulates T cell activity and leads to T cell exhaustion. Thus, the rationale for targeting this checkpoint in cancer therapy is to release the 'brakes' on pre-existing tumor-reactive T cells and to generate new T cell responses[23]. These novel agents have revolutionized the treatment of cancer and have led to impressive clinical benefits across a number of different tumor types. However, the majority of patients do not respond to these agents, and indeed some tumor types appear particularly resistant.

Key questions still remain as to why some tumors escape immune control while others do not. For example, what determines why some tumor cells are eliminated, while other tumor clones progress further into equilibrium and escape? In addition, what has occurred in the large proportion of patients who have no obvious or apparent immune reaction with their cancer, and did they ever develop one? If not, can one be engineered? Can the immune system be stimulated into developing a robust antitumor immune response? Will a personalized cancer immunotherapeutic strategy ever be designed specifically for an individual patient and their individual tumors in the future?

Current immunotherapeutic strategies

The CTLA4 monoclonal antibody ipilimumab was the first checkpoint inhibitor to be approved by the U.S. Food and Drug Administration (FDA). CTLA4 is expressed on T cells and when bound to its ligand B7 on DCs, prevents the required co-stimulatory signal from activating the T cell. Ipilimumab through CTLA4 blockade releases this checkpoint causing T cell activation. Additionally, ipilimumab causes antibody-dependent cellular cytotoxicity dependent depletion of Treg cells at the site of the tumor[24] Ipilimumab, with or without a gp100 peptide vaccine, was the first systemic agent to improve OS in patients with metastatic melanoma, as compared with gp100 alone. Although objective antitumor response rates were low (~10%), approximately 20% of patients had a durable response out to 10 years. Immunological memory, one of the cardinal features of the adaptive immune system may be responsible for this prolonged response. Specifically this is the ability of the adaptive immune system to provide long lived and maintained responses to specific epitopes or antigens[25]. This sustained benefit 10 years after treatment highlights the potential of immunotherapy and the role of immunotherapy in raising the tail of the survival curve. Treatment was not without toxicity however, with 10%-15%

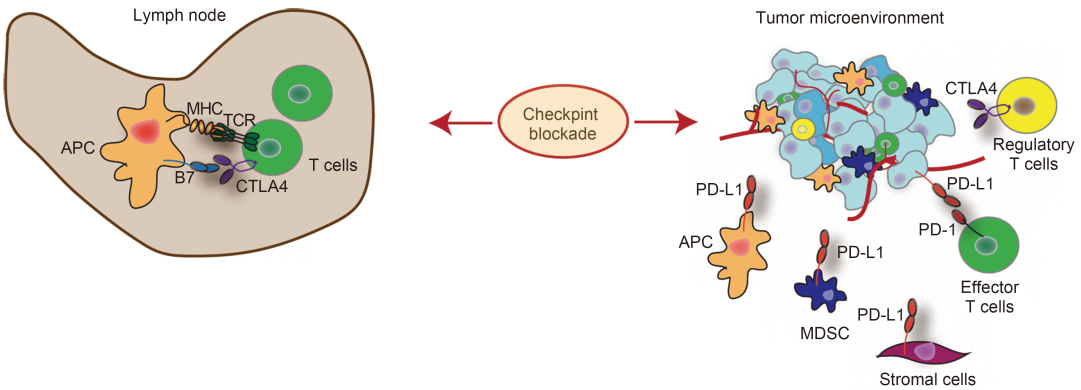

Figure 1 Current immunotherapeutic strategies with checkpoint inhibition.

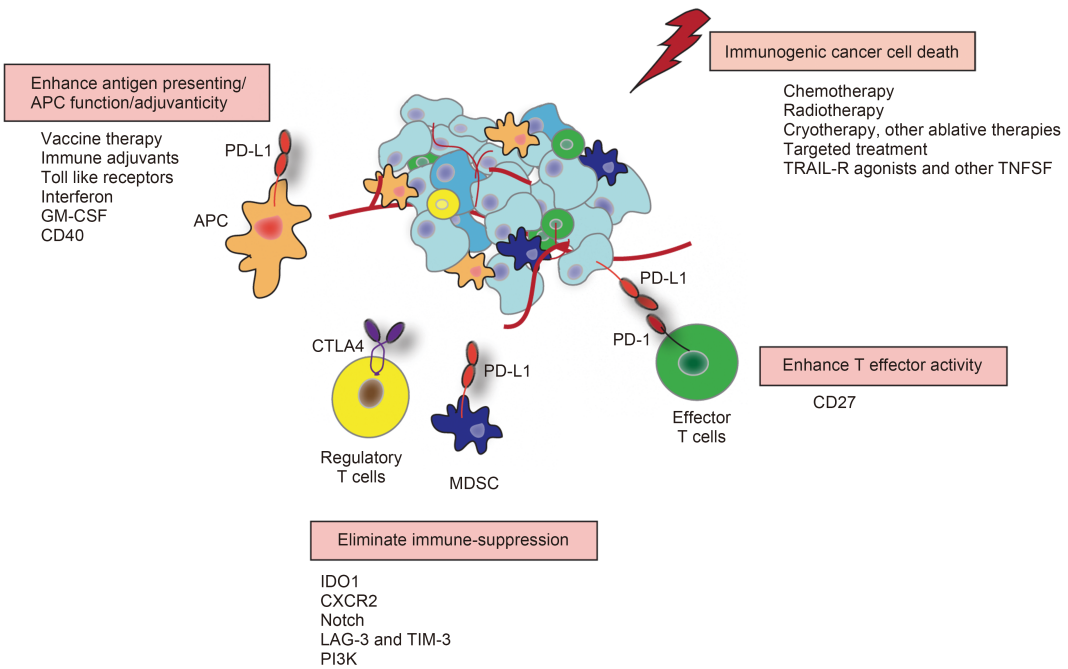

Figure 2 Future combination strategies with checkpoint inhibition. Multiple strategies can be used in combination with checkpoint inhibition to enhance the antitumor function of the immune system as shown. Immunogenic cell death can be promoted by chemotherapy, radiotherapy, targeted therapies as well as novel agents. Antigen presentation can be enhanced using vaccine therapies, immune adjuvants and other novel agents. Immune-suppression can be reduced or eliminated by attenuating suppressor cells and the microenvironment milieu and effector T cell function can be enhanced. TRAIL-R, TRAIL-receptor; TNF-SF, tumor necrosis factor super family; IDO1, indoleamine 2,3-dioxygenase 1; CXCR2, chemokine (C-X-C Motif) receptor 2; LAG-3, lymphocyte-activation gene 3; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; GM-CSF, granulocyte-macrophage colony-stimulating factor

grade 3-4 immune related adverse events (irAE) and overall 45% grade 3-4 adverse events (AE). There were also 14 (2.1%) treatment related deaths[26,27].

Tremelimumab is another anti-CTLA4 antibody that when tested in a phase 3 trial against chemotherapy, failed to provide a significant median OS advantage. However, the 3-year survival was 20% in the tremelimumab arm, similar to that seen with ipilimumab. Furthermore, a recent analysis of clinical data from four phase 1 and 2 tremelimumab trials has revealed long term responses with 20% 5-year survival and 15% 12-year survival[28,29].

Subsequent to anti-CTLA4 antibodies was the

development of antibodies that target the PD-1/PD-L1 axis. PD-1 and its ligand are expressed on T cells and tumor, respectively. PD-1, when activated by PD-L1 has a direct inhibitory effect on the T cell, preventing activation. The expression of PD-L1 by tumor is one of the key components leading to immune exhaustion and subsequently immune escape as described above. Multiple agents targeting both PD-1 and PD-L1 are in development across a multitude of tumor types[16].

Nivolumab, a monoclonal antibody against PD-1, has been shown to improve OS in patients with metastatic melanoma[30]. It has also, when compared to docetaxel shown superior OS as second line treatment in both squamous and non-squamous non-small cell lung carcinoma (NSCLC)[31,32]. Pembrolizumab, a monoclonal antibody also targeting PD-1, has likewise demonstrated survival benefits in melanoma and NSCLC[33,34]. Both nivolumab and pembrolizumab have been compared to ipilimumab in patients with metastatic melanoma and were found to have superior response rates (30%-40%) and improved OS. Importantly because of the selective nature of the immune activation, toxicity was much lower with both drugs compared to ipilimumab[34,35]. Multiple tumor types have been shown in early phase trials to benefit from anti-PD-1 treatment with activity seen in renal cell carcinoma (RCC), glioblastoma, small cell lung cancer (SCLC) , head and neck squamous cell cancer (HNSCC), bladder cancer, gastro-esophageal cancer, and hepatocellular cancer (HCC). Atezolizumab, a monoclonal antibody targeting PD-L1, is earlier in development but has shown clinically significant activity in melanoma, RCC, mismatch repair (MMR) deficient colorectal carcinoma (CRC), bladder cancer, and triple negative breast cancer (TNBC) amongst other tumors. A number of other PD-1/PD-L1 antibodies are also currently in development[36].

Although the full potential of checkpoint inhibitors as single agent therapy has not yet been fully elucidated, with many early and late phase clinical trials still ongoing, what is apparent is that not all patients will benefit from this novel class of drugs. Certain tumors such as CRC appear to be less immunogenic and have been associated with low response rates to checkpoint inhibition[37]. Furthermore, even in so called immunogenic tumors such as melanoma, a large proportion of patients do not respond to single agent checkpoint inhibition[34,35]. Validated predictive biomarkers of response and resistance have thus far been challenging to develop for these novel inhibitors[38]. An alternative strategy is to use immunotherapies in novel combination regimens, which holds great promise in broadening the applicability and increasing the effectiveness of immunotherapy.

A better understanding of the underlying mechanisms that underpin the immune response to tumors has identified a number of potential targets that have the ability to augment the response to checkpoint inhibition when used in combination therapy[36]. Robust preclinical and early clinical evidence point to antitumor synergies in combining immunotherapies by improving antitumor responses and durability through the stimulation of the memory immune response. The basic biological rationale for combining such therapies is to increase the immunogenicity of the tumor, thereby leading to improved antitumor effects[39]. There are multiple different approaches that can be taken in order to do this: enhancing antigen presentation and major histocompatibility complex (MHC) class I expression, tumor microenvironment changes that make immune response more favorable such as downregulation of Treg cells or enhancing T cell infiltration, and targeting the effector T cell with stimulatory measures or blockade of inhibition[40].

Multiple different therapies, both approved and in development, have been identified as showing promise in combination with immunotherapy (**Table 1**). Chemotherapy, radiotherapy, targeted therapies (small molecule and antibody), vaccine therapy and immunotherapies have all shown promise, although it is only through thorough understanding of the underlying biology that rational combinations and schedules can be designed[36,39,40].

Combinations with chemotherapy

Emerging evidence indicates that one of the mechanisms of actions of chemotherapy is via activation of the immune system through multiple pathways[41]. Cytotoxic cell death and subsequent antigen release is also thought to provide immune stimulation, common to many chemotherapies. Moreover, some chemotherapies, such as cyclophosphamide have been shown to reduce the number of circulating Treg cells, which are a key component in immunosuppression[42]. A number of different chemotherapies, including gemcitabine have also been shown to reduce MDSCs and hence interferon-gamma, which have inhibitory roles in the immune response[43]. The dual role of cytotoxicity and immune activation from chemotherapy has provided the biological rationale for the development of combinations with immunotherapy.

One of the earliest combinations tested was ipilimumab and dacarbazine in a large phase 3 trial comparing dacarbazine alone to dacarbazine with ipilimumab. OS was significantly improved in the combination arm versus dacarbazine alone [hazard ratio (HR) 0.72, P=0.001, median

Table 1 Selected trials involving combination with immunotherapy

Agents tested	Study details	Main outcomes	Adverse events	Target	Author, year
Chemotherapy combinations with immunotherapy					
Dacarbazine (D) *vs.* ipilumamab + dacarbazine (I+D)	Phase 3 randomised 480 pts metastatic melanoma	Median OS I+D 11.2 months *vs.* D 9.1 months (HR=0.72 *P*=0.001); I+D 3 y survival 20.8% *vs.* D 12.2%	G3/4 AE I+D 56%, D 40%; G3 irAE I+D 41%, D 6%	CTLA4	Robert, 2011
Carboplatin + paclitaxel (CP) *vs.* CP + ipilimumab concurrent (CPIcon) *vs.* CP+ipilumumab phased (CPIph)	Phase 2 randomised 204 pts metastatic NSCLC	Phased ipilimumab irPFS 5.7 m *vs.* CP 4.6 m (HR=0.72, *P*=0.05); ORR CPIph 32%, CPIcon 21%, CP 14%	G3/4 irAE CP 6%, CPIcon 20%, CPIph 15%	CTLA4	Lynch, 2012
Carboplatin + paclitaxel (CP) *vs.* CP + ipilimumab concurrent (CPIcon) *vs.* CP+ipilumumab phased (CPIph)	Phase 2 randomised 130 pts extensive small cell lung cancer	Phased ipilimumab irPFS 6.4 m *vs.* CP 5.3 m (HR = 0.64, *P*=0.03) irORR CPIph 71%, CPIcon 49%, CP 53%	G3/4 irAE CP 9%, CPIcon 21%, CPIph 17%	CTLA4	Reck, 2012
Nivolumab + cisplatin/gemcitabine or cisplatin/pemetrexed or carboplatin/paclitaxel	Phase 1, 56 pts metastatic 1st line NSCLC	ORR 43%, 1 y OS 59%-87%	G3/4 AE 47%	PD-1	Antonia, 2014
Pembrolizumab + carboplatin/paclitaxel (CP) or carboplatin/ pemetrexed (CPem)	Phase 1, 44 pts metastatic NSCLC	Pembro + CP ORR 30% Pembro + CPem ORR 58%	G3/4 AE Pembro +CP 15%; Pembro + CPem 38%	PD-1	Papadimitra kopoulou, 2015
Atezolizumab + nab-paclitaxel	Phase 1, 32 pts metastatic TNBC	ORR 70.8%, SD 20.8%	G3/4 AE 56% (41% neutropenia)	PD-L1	Adams, 2015
Targeted therapy combinations with immunotherapy					
Ipilimumab + vemurafenib	Phase 1, 10 pts *BRAF* mutant metastatic melanoma		7/10 G2-3 hepatotoxcity	CTLA4 BRAF	Ribas, 2013
Durvalumab (Dur) + trametinib (T) + dabrafenib (Da) durvalumab + trametinib	Phase 1, 41 pts metastatic melanoma BRAF Mut Dur+T+Da BRAF WT Dur+T	ORR Dur+T+Da 16/21 (76%), Dur+T 6/20 (30%)	G3/4 AE Dur+T+Da 17 40%, Dur+T 17 40%	PD-L1 BRAF/MEK	Ribas, 2015
Tremelimumab + sunitinib	Phase 1, 21 pts metastatic RCC	PR 9/21 pts 43%	9/29 DLT 31% (3 acute renal failure)	CTLA4 VEGF	Rini, 2011
Nivolumab (N) + sunitinib (S) or pazopanib (P)	Phase 1, 37 pts metastatic RCC	ORR N+S 17/33 (52%) N+P 9/20 (45%)	G3/4 AE N+S 24/33 (73%), N+P 12/20 (60%)	PD-1 VEGF	Amin, 2014
Ipilimumab + bevacizumab	Phase 1, 46 pts metastatic melanoma	ORR 17%, clinical benefit rate 64%	G3/4 AE 13/46	CTLA4 VEGF	Hodi, 2014
Vaccine therapy combinations					
GVAX + CRS-207 *vs.* GVAX	Phase 2 randomized 90 pts metastatic pancreatic carcinoma	Median OS GVAX+CRS 6.1 months *vs.* 3.9 months m GVAX (HR=0.59; *P*=0.02)	Gvax+CRS 4/61 G3 transaminitis; 5/61 G3/4 lymphopenia	Vaccine	Le, 2015

Table 1 (*continued*)

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Agents tested	Study details	Main outcomes	Adverse events	Target	Author, year
T-VEC+ipilimumab	Phase 1, 19 pts metastatic melanoma	ORR 41%	G3/4 AE 32%	CTLA4	Puzanov, 2014
T-VEC+pembrolizumab	Phase 1, 21 pts	Not reported	G3/4 AE 29%	PD-1	Long, 2015
Immunotherapy combinations					
Nivolumab (N) + ipilimumab (I) *vs.* nivolumab (N) *vs.* ipilimumab (I)	Phase 3 randomized 945 pts metasatic melanoma	Median PFS N+I 11.5 months, N 6.9 months, I 2.9 months (HR N+I *vs.* I 0.57; 99.5% CI, 0.43 to 0.76; $P<0.001$; ORR N+I 57.6%, N 43.7%, I 19%	G3/4 AE N+I 55%, N 16.3%, 27% I	CTLA4 PD-1	Larkin, 2015
Pembrolizumab (P) + ipilimumab (I)	Phase 1, 17 pts metastatic NSCLC	ORR 54%	G3/4 AE 2/17 (6%) pts	CTLA4 PD-1	Patnaik, 2015
Pembrolizumab (P) + ipilimumab (I)	Phase 1, RCC, melanoma	ORR 6/17 pts (35%)	G3 AE 6/19 (31%) pts	CTLA4 PD1	Atkins, 2015
Durvalumab (Du) + tremelimumab (T)	Phase 1, 61 pts metastatic NSCLC	ORR 26%, SD 35%	G3/4 AE 31%	CTLA4 PD-L1	Antonia, 2015
Ipilimumab + epacadostat	Phase 1, 40 pts melanoma	ORR 30%, SD 30% DCR 30% pts with previous immunotherapy	G3 AE 23%	CTLA4 IDO1	Gibney, 2015
Ipilimumab + indoximid	Phase 1, 9 pts melanoma	Not reported	No DLT, 1/7pts colitis	CTLA4 IDO1	Zakharia, 2015
Pembrolizumab + epacadostat	Phase 1, 54 pts advanced solid tumors	ORR 10/19 (53%)	G3/4 irAE 8%	PD-1 IDO1	Gangadhar 2015

patients (pts); non small cell lung carcinoma (NSCLC); small cell lung cancer (SCLC); objective response rate (ORR); partial response (PR); stable disease (SD); disease control rate (DCR); adverse events (AE); immune related adverse events (irAE); dose limiting toxicity (DLT); progression free survival (PFS); overall survival (OS); immune related objective response rate (irORR); programmed cell death 1 (PD-1); programmed cell death 1 ligand (PD-L1); cytotoxic T lymphocyte antigen 4 (CTLA4); indoleamine 2,3-dioxygenase 1 (IDO1); vascular endothelial growth factor receptor (VEGFR).

OS 11.2 months *vs*. 9.1 months, 3-year survival 20.8% *vs*. 12.2%][27]. This study, in conjunction with a separate phase 3 study led to the approval of ipilimumab as a single agent for the treatment of metastatic melanoma. It is unclear, however, if the combination is required or only ipilimumab as there was no ipilimumab alone arm in this trial. However, an earlier phase 2 study comparing dacarbazine and ipilimumab *vs*. ipilimumab alone demonstrated improved response rates of 15% *vs*. 5% and improved survival of 14.3 months (95% CI, 10.2-18.8) *vs*. 11.4 months (95% CI, 6.1-15.6)[44]. Despite this, the approval and widespread use of ipilimumab is as a single agent.

Ipilimumab has also been combined with carboplatin and paclitaxel in two separate studies in NSCLC and SCLC. These studies also evaluated the timing of ipilimumab in relationship to chemotherapy. It was demonstrated that phased treatment with ipilimumab, starting after 2-4 cycles of chemotherapy, provoked superior immune responses compared to concurrent treatment. This suggests that 'induction chemotherapy' has a role in causing cell death and antigen release, as well as stimulating an immune response that is then potentiated by the CTLA4 blockade[45,46].

Treatment-related toxicity is increased when CTLA4 and chemotherapies are combined[44]. In the phase 2 trial comparing ipilimumab *vs*. ipilimumab/dacarbazine, the rate of grade 3 toxicity was 17.1% *vs*. 7.7% for the combination and single agent, respectively[44]. Likewise, in the phase 3 trial of ipilimumab/dacarbazine *vs*. dacarbazine, the rate of grade 3-4 immune-related adverse events was approximately 40%. Interestingly, 20%-30% of patients experienced grade 3-4 liver toxicity[27]. This compares to approximately 4% hepatotoxicity in other trials with ipilimumab monotherapy[47]. One possible explanation for this is that dacarbazine, which can result in hepatotoxicity, causes hepatic cell death and the release of hepatic specific neo-antigens. When this is combined with T cell activation from

ipilimumab, hepatotoxicity may subsequently ensue. With regards to hepatic toxicity, there were conflicting results between NSCLC and SCLC studies, with high rates in patients with SCLC and low observed rates in the NSCLC trial, suggesting that other patient-derived factors also interplay with toxicity[45,46].

There are currently limited data for PD-1/PD-L1 combinations with chemotherapy, although multiple ongoing studies are currently looking at various combinations. A phase 1 study of nivolumab with three different chemotherapy regimens: cisplatin/gemcitabine, cisplatin/pemetrexed, or carboplatin/paclitaxel in first line NSCLC patients showed overall response rates of 43%, with grade 3-4 adverse events in 47%. Notably, eleven patients withdrew from treatment, because of treatment-related toxicities, 10 during nivolumab monotherapy, a rate that is higher than usually seen with nivolumab when given as a single agent[48]. Pembrolizumab has also been combined with doublet chemotherapy in patients with metastatic NSCLC. Forty-four patients were treated with pembrolizumab 2 or 10 mg/kg and either carboplatin AUC6+paclitaxel 200mg/m^2 (cohort A) or carboplatin AUC 5 + pemetrexed 500 mg/m^2 (cohort C). Grade 3-4 toxicities were seen in 15% and 38% of cohort A and C, respectively. Common toxicities were transaminitis, colitis and rash. Preliminary objective response rate (ORR) was observed in 30% and 58%, respectively[49].

Atezolizumab has been combined with the nanoparticle albumin-bound paclitaxel (nab-paclitaxel) in a phase 1 trial in patients with metastatic TNBC. Objective response rates of 70.8% and stable disease in 20.8% were observed. Grade 3 and 4 adverse events occurred in 56% of patients with 41% experiencing grade 3 or 4 neutropenia[50].

Combinations with radiotherapy

Radiotherapy has been reported to stimulate immune responses. Indeed, the well recognized albeit rare abscopal response appears to be immune-mediated[51,52]. Direct cytotoxicity by ionizing radiation leads to increased neo-antigen expression and the induction of inflammatory cytokines that attract T cells and DCs, as well as enhance antigen uptake and cross presentation by DCs[53]. Animal models have shown promising synergy between anti-CTLA4 and anti-PD-L1 antibodies in inducing immune mediated response[54,55]. It has also been demonstrated that hypofractionated radiotherapy is superior to solitary doses in inducing immune responses[56]. Illustrating the potential benefit of radiotherapy is a case report of a patient who received palliative radiotherapy to a bone metastasis many months after the development of disease progression following ipilimumab therapy. Despite the length of time after receiving ipilimumab, there was significant regression of multiple metastatic lesions distant to the radiotherapy site[57]. A number of trials assessing the combination of radiotherapy and novel immunotherapies are currently underway[58].

Cryotherapy and other ablative therapies may also induce immune responses via antigen release and damage-associated molecular patterns that activate immune responses[59]. Preclinical models have shown synergy between radiotherapy and CTLA4 blockade. A pilot study of 20 patients has looked at combining ablative therapies [transarterial chemo-embolization (TACE) or radiofrequency ablation (RFA)] with tremelimumab in patients with HCC. The combination was well tolerated with no dose limiting toxicities (DLFs) observed. Four of 12 (33%) patients with evaluable disease outside the field of TACE/RFA had objective responses[60].

Combinations with molecularly targeted agents

Molecularly targeted therapies also show promise in combination with immunotherapy. There is increasing evidence that one of the mechanisms of certain targeted agents is through immune modulation[61,62]. Indeed, although drug resistance is a frequent occurrence with targeted inhibitors, such as imatinib in gastrointestinal stromal tumors (GIST) and *BRAF*/*MEK* inhibitors in melanoma, there appears to be a subset of patients who are long term responders[63,64]. Furthermore, there is also some evidence, in patients with GIST treated with imatinib, that induction of an immune response, as measured by interferon-gamma levels, is predictive of improved survival and long term responses[63]. This is suggestive that it may be through underlying immune mechanisms that some targeted therapies may deliver long term responses.

BRAF inhibitors have substantial activity in *BRAF* V600E mutant melanomas and other *BRAF* mutant tumors[65]. They however appear to cause a paradoxical activation of the *RAF* pathway in *RAF* wildtype cells. It has been shown that this activation also occurs in T cells and results in T cell activation and proliferation, which could be potentiated further via checkpoint blockade[62]. Tumor biopsy studies have demonstrated increased tumor infiltrating lymphocytes and neoantigen expression in patients treated with dabrafenib and trametinib, compared to baseline. This preclinical work provided the rationale for undertaking combination studies of immune checkpoint and *BRAF*/*MEK* inhibitors. It was predicted that the combination of these two different

modalities of therapies may potentially synergize the high response rates of *BRAF* directed therapy with the durable responses of checkpoint inhibition[66]. Unfortunately, a phase 1 trial of the *BRAF* inhibitor vemurafenib and ipilimumab in advanced melanoma was closed early after considerable toxicity was observed. Starting at the recommended dose of each drug (960 mg vemurafenib and 4 mg/kg ipilimumab), four of six patients in the first cohort developed dose limiting grade 3 elevations in liver function tests. In a second cohort assessing 720 mg of vemurafenib with 4 mg/kg of ipilimumab, three of four advanced melanoma patients experienced grade 2-3 liver transaminitis. Although all adverse events were asymptomatic and reversible with glucocorticoids, the combination was deemed too toxic and the study was closed to further accrual[67].

Despite this setback, great interest remains with the combination strategy of targeting the immune checkpoint and *BRAF*/*MEK* signalling pathway. In view of the favorable toxicity profile and selective T cell activation of PD-1/PD-L1 antibodies compared to CTLA4 inhibitors, a phase 1 trial is currently investigating the combination of the anti-PD-L1 agent durvalumab with the *MEK* inhibitor trametinib or a triple regimen with the *BRAF* inhibitor dabrafenib and trametinib[66]. In *BRAF* mutant patients, the full doses of dabrafenib and trametinib were combined with 3 mg/kg and 10 mg/kg of durvalumab. *BRAF* wildtype patients were treated with trametinib and durvalumab both in combination and sequentially with induction trametinib. Treatment appeared tolerable, with no differences in toxicities observed across the patient groups, with mostly *BRAF*/*MEK* inhibitor toxicities observed and low incidences of grade 3 autoimmune toxicities. Antitumor efficacy appears promising, but longer follow-up is required to provide more data on efficacy[66].

Combining vascular endothelial growth factor receptor (VEGFR) inhibitors with immunotherapeutic agents has demonstrated mixed results. A phase 1 trial of tremelimumab with sunitinib was discontinued early due to treatment-related toxicities with high rates of rapid onset renal failure[68]. In contrast, the combination of nivolumab with either sunitinib or pazopanib appeared well tolerated with durable antitumor responses reported[69]. The choice of the immunotherapy backbone in this situation is likely key to the difference in toxicity seen. CTLA4 blockade causes non-specific and widespread T cell activation, whereas blocking the PD-1/PD-L1 axis increases T cell responses only in tissues expressing PD-L1, providing a much more selective and specific response on tumor-directed T cells[36]. A similar parallel is seen with the development of hepatotoxicity with the combination of vemufarenib and CTLA4 inhibitors, in contrast to the seemingly more tolerable combination of dabrafenib/trametinib and durvalumab.

The cancer vasculature has multiple roles in the modulation of tumor immune responses[70]. In particular, vascular endothelial growth factor (VEGF) is known to suppresses DC maturation and can modulate lymphocyte endothelial trafficking[71]. Sunitinib has been shown to reduce MDSCs in the tumor microenvironment[72]. As such, considerable potential exists for the combination of immune checkpoint therapy and VEGF targeted therapy.

Bevacizumab has been combined with ipilimumab in a phase 1 trial of 46 patients with metastatic melanoma. Treatment-related toxicities were manageable, although interestingly there was one episode of immune mediated arteritis. Antitumor activity was higher than would have been expected for either agent alone with 17% ORR and 64% clinical benefit rate[73]. Bevacizumab has also been combined with nivolumab as part of a maintenance strategy in NSCLC after platinum-based chemotherapy with significant increase in toxicity above nivolumab alone[74]. Atezolizumab has also been tested in combination with bevacizumab with or without FOLFOX chemotherapy in a phase 1 trial with no increase in toxicity observed, although antitumor activity data are awaited[75].

The antibody drug conjugate brentuximab vedotin, which is highly active in Hodgkin's lymphoma, is also being looked at in combination with immunotherapy. Increased antigen release caused by brentuximab has the potential to synergize with checkpoint blockade[76]. A number of clinical trials combining brentuximab vedotin with nivolumab and/or ipilimumab are underway[77].

Combinations with vaccine therapies

Vaccine therapy has a long history as single agent immunotherapy with generally disappointing results. Although vaccines are able to provoke immune responses, translating this observation into clinical benefit has been challenging[78]. Armed with a better understanding of the immune system, new vaccine models have now been developed and are being used in combination with increasing success. A number of different strategies have been used in combination with checkpoint inhibition to increase antigen presentation via a range of vaccination approaches, ranging from simple peptide to more complex engineered cellular vaccines, DC vaccines, virus vectored vaccines and oncolytic viral vaccines[78].

Single peptide vaccines in combination with CTLA4

blockade have been shown to be able to induce peptide specific immune responses. However, a large phase 3 study combining gp100 peptide with ipilimumab did not provide any additional clinical benefit above and beyond ipilimumab[26].

Sipuleucel-T is an autologous dendritic cell vaccine that targets prostatic acid phosphatase and has been shown to improve OS in patients with metastatic castration-resistant prostate cancer (CRPC). It does so however without impacting on PFS or affecting prostate specific antigen (PSA) levels[79]. Sipuleucel-T has now been combined with the next generation androgen receptor inhibitor enzalutamide in patients with CRPC. Early results of a phase 2 trial involving this combination have shown that that this regimen is tolerable, and results in objective radiological and PSA tumor marker responses[80]. Interestingly, the sequencing of both therapies did not affect patient outcomes with similar results seen with concurrent or sequential treatment[80].

An alternate DC vaccine has been tested in combination with docetaxel/prednisolone in patients with CRPC. Importantly, the addition of immunotherapy in the form of DC therapy did not seem to increase toxicity. All of the toxicities observed were grade 1-2 and were within the recognized range of adverse events for docetaxel. Although this was a single arm study with no comparator arm, efficacy was potentially enhanced with a median PFS of 19 months observed compared with an expected PFS of 13 months based on the Memorial Sloan Kettering Cancer Centre (MSKCC) nomogram. Furthermore, the administration of chemotherapy and corticosteroids did not prevent the induction of antitumor T cells, but did lead to a decline in immune-inhibitory Treg cells[81].

Autologous tumor vaccines also show promise as combination immunotherapy. GVAX is a vaccine with allogeneic tumor cells that have been transfected with granulocyte and macrophage colony stimulating factor (GM-CSF). A recent phase 2 study in patients with metastatic pancreatic carcinoma of GVAX with or without CRS-207, live-attenuated Listeria monocytogenes-expressing mesothelin, led to significant improvements in median OS. Median OS was 9.7 *vs.* 4.6 months (arm A *vs.* B; HR: 0.53; P=0.02)[82]. GVAX has previously been combined with ipilimumab in prostate cancer and pancreatic cancer trials and found to be a safe combination with some evidence of clinical benefit, although further research is required[83,84]. Current studies are underway combining GVAX/CRS207 with nivolumab[85].

Oncolytic viruses are treatments that may be wildtype or modified live viruses that provide a similar role to vaccination by replicating in tumor cells and releasing antigenic proteins. The most advanced oncolytic virus is talimogene laherparepvec (T-VEC), which is a herpes simplex virus that has been genetically modified via deletion of viral genes to reduce pathogenicity and insertion of the GM-CSF gene. The expression of GM-CSF in the virus promotes local GM-CSF production that aids in the recruitment and activation of antigen presenting cells (APCs) and then T cell responses. In a phase 3 study with intraregional injection of T-VEC compared to GM-CSF alone, there was an increase in median OS from 18.9 to 23.3 months with a P-value very close to significance (P=0.051). The ORR was 26.4% in the T-VEC group, with 16% durable responses. A different oncolytic virus coxsackie virus has similar responses in patients with metastatic melanoma[86]. On the basis of this trial, T-VEC has recently gained FDA approval for the treatment of metastatic melanoma.

There is also robust preclinical rationale for the combination of T-VEC with anti-CTLA4 or anti-PD-1/PD-L1 inhibitors. A phase 1 trial in patients with advanced melanoma combining T-VEC with ipilimumab led to response rates of 41%. Adverse events were manageable, with 32% grade 3-4 adverse events reported[87,88]. T-VEC in combination with pembrolizumab has been evaluated in a phase 1b/3 trial in patients with metastatic melanoma, with safety data for the phase 1 component recently reported. Combination therapy at full doses of each individual component was tolerable, with no DLTs observed and 29% grade 3-4 treatment-related adverse events reported, most commonly rash, fatigue and pyrexia. This trial has now commenced the phase 3 component[87].

Combinations with immunotherapies

Combination of anti-CTLA4 and anti-PD-1 has been shown to improve outcomes at the risk of increased toxicity. Nivolumab and ipilimumab given in combination was compared to single agent nivolumab and single agent ipilimumab for untreated metastatic melanoma. Response rates were 19%, 43.7%, and 57.6% in the ipilimumab, nivolumab and combination arms, respectively. OS has not yet been published but PFS was also significantly improved. Median PFS was 2.9 months, 6.9 months, and 11.5 months, respectively for the three arms. Although the trial was powered only to compare nivolumab *vs.* ipilimumab and combination *vs.* ipilimumab, even taking this into account the HR for PFS between combination and nivolumab was 0.74 (95% CI 0.6-0.092) in an exploratory analysis. This

conclusively showed that the combination of CTLA4 and treatment was superior to either as single agent[35].

Toxicity, however, was higher in the combination arm, with 27%, 16% and 55% grade 3 or 4 adverse events for the ipilimumab, nivolumab and combination arms, respectively. Despite the high level of adverse events, it is worth noting that patients who were required to come off treatment due to irAE had an overall higher response rate of 67%, with many of these after cessation of treatment[35]. More recent studies have demonstrated that the need to treat irAE with corticosteroids does not impact on outcomes[89]. Ongoing studies are looking at the combination in other tumor histologies.

In a pre-planned subgroup analysis, the benefit for the combination group was seen predominantly in patients whose tumors were negative for PD-L1 on immunohistochemistry at study entry. This suggests, that patients who are PD-L1+ have an immune response that has been inactivated through the PD-1/PD-L1 checkpoint. As such they did not benefit from CTLA4 blockade that causes non-specific T cell activation. In contrast the patients whose tumors were PD-L1 negative, needed the CTLA4 blockade to activate T cells and move them into tumors, and subsequent further PD-1 blockade maintains the immune response in the tumors[35].

Pembrolizumab has been combined with ipilumumab in a phase 1 trial for patients with previously treated advanced NSCLC, the results of which have been presented at American Society of Clinical Oncology (ASCO) annual meeting 2015. The toxicity profile was manageable with only 2/17 grade 3 events, and no DLT. Efficacy results revealed 54% complete response (CR) and partial response (PR) rates across all dosing cohorts. Three separate dose combinations were investigated, pembrolizumab 10 mg/kg + ipilumumab 3 mg/kg, 10 + 1 and 2 + 1. Despite small numbers of patients, the lower dose combinations did not appear to compromise efficacy[90]. This same combination with pembrolizumab 2mg/kg and ipilimumab 1 mg/kg every three weeks has also been tested in a phase 1 study in patients with metastatic melanoma or metastatic RCC. In a preliminary report, 6/9 patients experienced DLTs- grade 3 irAE, which met the prespecified definition of tolerability. PRs were seen in 6/17 (35%) evaluable patients[91].

Durvalumab and tremelimumab were studied in a combination phase 1 dose escalation trial in patients with NSCLC. Increasing doses of durvalumab were tolerable up to 20 mg/kg every 2 or 4 weeks with a constant tremelimumab dose of 1 mg/kg, however attempts to increase tremelimumab resulted in increasing irAE, most frequently colitis. About 18% of patients required discontinuation. The maximal tolerated dose (MTD) has not yet been defined. ORR was 26% and SD 35% across all cohorts. Importantly, 3 of 10 patients with PD-L1-negative tumors had partial responses, underlining the ability of the combination treatment to overcome negative PD-L1[92].

An alternative approach has been combination with agents that act to enhance the innate immune response. Agents such as toll-like receptors agonists have shown significant efficacy in tumor models and have been shown to enhance the activity of anti-CTLA and PD-1 antibodies. PF-3512676 and antibody to toll-like receptor 9 (TLR9) was explored in a combination with tremelimumab in a phase 1 study in patients with solid tumors and melanoma. Two of 17 patients had partial responses. At full doses of tremelimumab and the TLR9 agonist however significant toxicity was seen with 2 DLT of grade 3 diarrhea that was manageable with corticosteroids. Furthermore, there was an increase in late immune mediated toxicity occurring outside the three week DLT period[93].

Pegylated-interferon is another such agent that helps to activate the innate immune response that has shown early evidence of efficacy in combination with checkpoint blockade. Two small trials in combination with ipilimumab and tremelimumab reported 30% and 24% response rates in patients with melanoma, higher than would generally be expected for these agents. These combinations however require further validation in larger trials[94,95].

Combinations with other checkpoint inhibitors

Similar to how PD-1 is unregulated by cancer cells in order to produce T cell exhaustion, tumors may also upregulate other proteins that act to dampen T cell activity. T cell immunoglobulin domain and mucin domain-3 (TIM3) and lymphocyte activating gene 3 (LAG3) are two such checkpoint molecules, with novel antibody therapeutics targeting these key targets already in preclinical development[96].

LAG3 is expressed on CD4+ cells and binds to MHC class 2 molecules and appears to have a key role in Treg function. It is also overexpressed on CD8+ T cells in models of tumor-induced tolerance. Blockade of LAG3 in preclinical models does not completely restore T cell function, but there does appear to be potent synergy with anti-PD-1 treatments in animal models, which are able to reject even poorly immunogenic tumor[97] This therefore raises the prospect of clinical synergy when given with anti-PD-1 therapy. Multiple

agents are currently in clinical development, including the novel anti LAG3 antibody BMS-986016, which is currently in phase 1 testing in combination with nivolumab[36].

TIM3 is a glycoprotein receptor that is expressed on T cells, which has been shown to be present on PD-1+ CD8+ exhausted tumor infiltrating lymphocytes. Animals that lack TIM3 do not develop overt autoimmunity, suggesting a more subtle role in immunosuppression compared to PD-1. However, in animal models pre-disposed to autoimmunity, blockade of TIM3 can accelerate the development of autoimmunity[98]. Synergy with PD-1 blockade has been observed in animal models, with some evidence that triple therapy with TIM3/PD-1/4-1BB may provide further advantages[98,99]. Monoclonal antibodies targeting TIM3 are currently in preclinical development[36].

Combinations with immune pathway agonists

Also in development are antibodies that target agonists of T cell co-stimulation and as such stimulate the immune response. These stimulatory receptors belong to the tumor necrosis factor receptor super family (TNFRSF). Tumor necrosis factor (TNF) is an important immune stimulant, with TNF having long been used with marginal efficacy in melanoma and sarcoma[100]. The TNFRSF includes a number of different receptors some of which are in development currently and hold promise in providing synergistic activity with checkpoint blockade[36].

4-1BB is a TNF receptor that is found on both T cells and natural killer (NK) cells with a co-stimulatory role. It has also been found on neutrophils, myeloid lineage and some DCs. Activation of 4-1BB has been shown to activate T cells and increase survival and effector functions. It plays important roles in immune homeostasis through Treg function as well as developing antitumor immune memory. As such the results of activation can be immune activation or restriction, and is most likely context development[101]. Preclinical data suggests likely synergistic activity with checkpoint inhibitors, but also with antibodies that cause antibody-dependent cell-mediated cytotoxicity (ADCC) as this process upregulates 4-1BB[102,103]. Two molecules, urelumab and PF-05082566 are in development in both single agent phase 1 and combination studies. A single agent phase 2 with urelumab had high rates of hepatotoxicity and was closed early, leading to efforts to better define a safe dose in currently ongoing studies[104]. Urelumab in combination with elotuzumab (NK cell activator and enhancing ADCC) is currently being tested in patients with multiple myeloma[105]. Other trials combining urelumab with rituximab, cetuximab and pembrolizumab amongst others are underway[106]. PF-050825266 was shown to be tolerable, with activity seen in 9 of 24 patients in a phase 1 study[107]. Combination therapy with rituximab has been reported in a phase 1 study and found to safe and efficacious in non-Hodgkin's lymphoma (NHL)[108]. It is also undergoing testing in combination with pembrolizumab[36].

OX40 or tumor necrosis factor receptor superfamily, member 4 (TNFRSF4), also known as CD134 is found on multiple T cell subsets, neutrophils and NK cells, and is believed to have a role in the survival of activated T cells and the establishment of T cell memory. It is expressed transiently after T cell activation[109]. There is preclinical evidence for its use in combination with 4-IBB agonists, as well as anti-PD-1, CTLA4 and TIM3 antibodies[99,110,111]. 9B12, a mouse IgG1 anti-human OX40 monoclonal antibody has been tested in prostate cancer in combination with standard treatments and shown to enhance immune activation via increased CD4+, CD8+ and NK cells, although no antitumor responses were seen. Given the need for T cells to become activated first to express OX40 it may be that combination with an agent aimed at increasing T cell activation is the best use of OX40 agonists rather than as monotherapy[109]. Further fully humanized monoclonal antibodies are currently in development, in particular MEDI6469, an OX40 specific antibody, which is being tested in a number of settings including single agent and in combination with tremelimumab and durvalumab[112].

CD40 is expressed on APCs and B-cells among other cell types rather than T cells. Its ligand CD40L is expressed on T cells amongst multiple other cell types. Activation of CD40 induces APC maturation and expression of co-stimulatory molecules that promotes T cell activation[109]. CP-870893, the agonistic antibody to CD40 has been tested in combination with tremelimumab in the phase 1 clinical trial setting[113]. An objective response rate of 27% was observed, however significant toxicity was also reported, with colitis and uveitis found to be dose limiting. CD40 has been shown in models to increase expression of PD-L1 on tumor infiltrating macrophages and monocytes, and as such, there is good rationale for combining CD40 targeted agents with anti-PD-1 inhibitors, which may be associated with relative fewer toxicities than tremelimumab[114].

There are multiple other members of the TNFRSF that are in preclinical development and have promising potential. Glucocorticoid-induced TNFR-related protein is expressed on Treg cells and may be upregulated in CD4 and CD8 T cells. It has shown synergy in combination with anti-PD-1 antibodies in preclinical models[115]. CD27 is a co-stimulatory

molecule expressed on effector T cells and memory B cells, as well as some NK cells. CD27 appears to have an important role in sustained T cell function as well as development of T cell memory and IgG antibody production in germinal centers. An agonistic antibody CDX-1127 has been tested in a phase 1 study in NHL and solid tumors, and was found to be tolerable with evidence of antitumor activity. Combination studies are currently being planned[116]. DR3 (TNFRSF25) and its ligand TNF-like 1A are involved in immune homeostasis, T cell accumulation and cytokine production. Agonists to DR3 have been shown to increase T effector populations and may thus be another potential immunotherapy combination partner[117].

Finally, herpes virus entry mediator (HVEM), which is expressed on APC, endothelium and lymphocytes, is a receptor to multiple ligands including TNF family proteins LIGHT and B and T cell attenuator (BTLA) and CD160. Despite a member of the TNF super family, HVEM is inhibitory in its actions on the immune system. However depending on the context of its activation can also be stimulatory. BTLA binding to HVEM is an important suppressor of immune activation, and is known to be unregulated in multiple tumor types. As such, it has great potential as a target, however detailed understanding of its mechanism of action will be required in order to target it effectively given the potential to either stimulate or inhibit immune responses[118].

Targeting the tumor microenvironment

The tumor microenvironment (TME) through multiple complex mechanisms is involved in the development of immune escape and immune-editing. As such, multiple targets in the TME exist that show promise in increasing tumor immunogenicity. One of the most developed targets in this area is indoleamine 2, 3-dioxygenase 1 (IDO1), although multiple other targets including MDSCs, chemokines, and Treg cells are still in pre-clinical development[40].

IDO1 is a cytosolic enzyme that is required for the catabolism of tryptophan, an essential amino acid. IDO1 is produced by tumor cells and MDSCs in response to inflammatory signals, such as interferon-gamma. Increased IDO1 levels lead to an increased breakdown of tryptophan into its metabolites which are known to supress T cell activity[119]. Cancer cell production of IDO1 is thought to be a key mechanism of TME immunosuppression. A number of IDO1 inhibitors are in clinical development. For example, GDC-0919, a small molecule potent inhibitor of IDO1 is currently undergoing phase 1 clinical trial testing. Preliminary results suggest some evidence of activity with 44% patients having a best response of stable disease for 4 months. Toxicity was manageable with one DLT of gastrointestinal haemorrhage, and although 58% patients developed grade 3-4 adverse events, many of these were disease rather than treatment-related[120].

Epacadostat (INCB024360) has been tested in single agent phase 1 trials, as well as in combination with ipilimumab. The combination was tested in patients with metastatic melanoma, with three different dose levels of epacadostat. The intermediate dose level of 50mg BD was well tolerated. Grade 3 adverse events occurred in 23% of patients. Antitumor activity appeared promising, with a disease control rate (DCR) of 60% (30% overall response rate) in immunotherapy-naive patients and 30% DCR in patients who had received prior immunotherapy treatments. About 50% of patients who had either CR/PR or SD had durable benefit beyond 6 months. The activity in patients with previous immunotherapy was particularly promising as this potentially suggests a role in overcoming resistance[121]. Combination with pembrolizumab in a phase 1 trial of patients with advanced solid tumors also showed promising activity, with tumor size reductions in 15 of 19 evaluable patients and an objective response in 10 of 19 (53%) patients. There were low rates of irAE (8%)[122]. A third IDO1 inhibitor in development is indoximod, which is a tryptophan analogue. A combination study with ipilimumab in patients with advanced melanoma has recently reported safety results, with the combination found to be tolerable up to 1,200 mg BID, which is the maximum biologically achievable dose of indoximod. No DLTs were observed, and a phase 2 trial is now planned[123].

Tumor growth factor Beta (TGFβ) is a cytokine with important roles in the TME including angiogenesis and immunosuppression. Overexpression is associated with poor outcomes in multiple different tumor types[124]. LY2157299 (galunisertib) is a small molecule kinase inhibitor that blocks TGFβ. Preclinical models have shown synergy with chemotherapy and with anti-CTLA4[125]. A combination study with nivolumab is currently being planned.

Antibodies targeting colony stimulating factor 1 receptor (CSF-1R) have been shown in both preclinical and early phase clinical trials to increase the CD8/CD4 ratio by depleting Tregs and inducing depletion of immunosuppressive macrophages in tumors. These effects raise the potential for their combination with immunotherapy, and future trials are planned[126].

CXCR4 is a receptor for the chemokine CXCL12 and

through tumor hypoxia, has been shown to augment an immunosuppressive tumor microenvironment through multiple pathways including Treg localization[127]. Preclinical studies have shown antitumor synergy with anti-PD-1 therapies, raising the potential for further combination studies[128]. A number of CXCR4 inhibitors are in development, the most advanced being ulocuplumab, which is currently being tested in combination with nivolumab[129].

Targeting Tregs as a means of augmenting immunotherapy also holds great promise. Tregs are known to downregulate immune responses, and at least part of the activity of ipilimumab is through the depletion of Tregs, as well as the induction of effector T cell responses. A number of antitumor agents are currently in development aimed at reducing Tregs. For example, mogamulizumab is a monoclonal antibody that targets CCR4, which is highly expressed on Tregs. Treatment with mogamulizimuab has been shown to reduce Treg counts and induce immune responses, and also has single agent activity in T-cell leukemia[130,131]. Trials combining mogamulizumab and either tremilimumab, durvalumab and nivolumab are in planning[132,133]. Focal adhesion kinase inhibitors (FAKi) have also been shown to reduce Tregs and increase the CD8/CD4 ratios[134]. The FAK inhibitor VS-6063 is currently being combined with pembrolizumab in a phase 1 trial[135].

Trial designs for immunotherapy combination strategies

What is important in the development of these combinations is intelligent trial design based on a thorough understanding of the underlying immunomodulatory mechanisms. Both the scheduling and sequencing of drugs is important and needs to be carefully considered. For example, unlike conventional anticancer treatments, immunotherapy has the potential to result in long lasting antitumor effects that may still be active when subsequent treatments are delivered. A poignant example is IL-2 and ipilimumab. When administered after IL-2 therapy, ipilimumab appears to be effective, but the observed toxicity is significantly higher than would be expected from either drug alone[136]. In the case of ipilimumab and PD-1 therapy, important data are drawn from the phase 1 combination study in which a treatment arm of patients with advanced melanoma with prior ipilimumab exposure received nivolumab. Importantly, toxicity did not appear higher in this group than would be expected for single agent nivolumab[137].

The scheduling of different antitumor agents in combination may also be important. For example, the use of induction chemotherapy prior to starting ipilimumab appears to be beneficial, likewise the use of hypofractionated radiotherapy is superior to other schedules[46,56]. Furthermore, with novel combinations of immunotherapies, timing in the schedule is likely to be important, especially for targets that are induced or appear transiently such as OX40.

Other important considerations to make when designing immunotherapy combination early phase trials include the incorporation of biomarker studies, monitoring of toxicities and the determination of antitumor efficacy.

Incorporating biomarker studies into immuno-oncology trials

As discussed in this review, anti-CTLA and anti-PD-L1/PD-1 antibody treatments have revolutionised the treatment of some tumor types and have the potential to reform cancer treatment as a whole. These treatments are not without patient risk however and come at high financial costs, therefore identifying biomarkers predictive of response and/or resistance is essential. Furthermore, incorporating biomarker enrichment strategies into early phase trials and beyond can be beneficial as it allows for continuous reassessment and refinement of the biomarkers and technologies involved[138]. Various immunologic correlates with clinical benefit to anti-CTLA4/PD-1/PD-L1 have been demonstrated in the literature, however a robust predictive biomarker has yet to emerge[139].

To date, the failure to relate immune response activation with clinical benefit may in part be due to current assay limitations, rather than a true lack of biological correlation. For example, T cell immune response assays that measure the activation of the immune response exist, however results of such assays are notoriously inconsistent between laboratories[140]. Harmonization of assay protocols and reporting through the use of standard operating procedures have the potential to reduce inter-laboratory variability (http://miataproject.org/) and increase the odds of identifying previously unidentified predictors of response.

There have been conflicting studies on the usefulness of PD-L1 expression in predicting responses to anti-PD-1/PD-L1 antibody treatments, in part perhaps due to the use of different anti-PD-L1 antibodies and cut-offs to determine positivity[141]. In a phase 1 study of nivolumab in patients with advanced melanoma, there were no responses in 17 patients with PD-L1 negative tumors in comparison to 9 responses out of 25 (36%) with PD-L1 positive tumors (P=0.006)[142]. Subsequent studies in melanoma patients have however been variable, with some showing a positive correlation with

tumor PD-L1 expression and others not[137,143,144]. For NSCLC, PD-L1 expression appears to correlate more consistently with response to anti-PD-1/PD-L1 antibody treatments across studies[33,145], however, in all tumor types, a healthy response rate to anti-PD-1/PD-L1 therapies is still seen in PD-L1 negative tumors. Such an observation is probably not unexpected since there is growing evidence that PD-L1 expression changes over time and with treatment[146,147]. It is thus possible that single measurements of PD-L1 expression from archival tumor biopsies may not be sufficiently representative of metastatic disease. Interestingly, as mentioned earlier in this review, in a recent phase 3 study in previously untreated patients with metastatic melanoma, tumors negative for PD-L1 expression had a PFS benefit from the combination of anti-CTLA4 and anti-PD-1 treatment compared to either treatment alone[35]. By contrast, the combination of nivolumab and ipilimumab showed no PFS benefit compared to nivolumab alone in patients with PD-L1 positive tumors suggesting that only those tumors lacking PD-L1 expression might benefit from immunotherapy combination approaches. Currently therefore, PD-L1 expression in archival tumor samples should not be used to exclude patients from anti-PD-L1 treatments and further work is required to fully understand the usefulness of PD-L1 expression as a biomarker predicting clinical response to anti-PD-1/PD-L1 treatments[148,149].

The presence of melanoma antigen-directed activated T cells is prognostic for the improved survival of melanoma patients and upregulation of such tumor-targeting T cell subsets correlates with clinical benefit to anti-CTLA4 antibody treatment[150,151]. Despite this however, attempts to focus the immune system towards specific antitumor antigens using vaccines in combination with ipilimumab have failed to show any additional benefit over ipilimumab alone, questioning the robustness of these approaches as predictive biomarkers of response[139,152]. Nevertheless, it is now recognized that tumors with a high mutational load generate increased levels of neo-antigens, and specific neo-antigen signatures have been shown to correlate with antitumor responses to anti-CTLA4 treatments[153].

Similarly, a higher non-synonymous mutation burden in NSCLC tumors was associated with improved objective response, durable clinical benefit, and PFS after treatment with pembrolizumab[154]. In keeping with this, CRCs with MMR deficiencies demonstrated significant antitumor responses and survival benefits following pembrolizumab treatment compared to MMR proficient tumors[37]. Combining cytotoxic chemotherapy with immunotherapy aims to increase tumor antigen presentation and it will be interesting to see if measuring levels of activated T cell subsets or neo-antigens will predict for clinical benefit in this setting.

Another approach will be to characterize the composition of the immune infiltrate in the tumor before and after treatment. In mice, tumor depletion of regulatory T cells following antibody treatment against OX40 correlates with tumor regression, therefore Treg depletion or reduction of Treg dependent cytokines such as IL-10, are both potential biomarker options for predicting clinical benefit[155,156]. A recent study has suggested that pre-existing CD8 expression at the invasive margin of tumors may be a biomarker for tumors with adaptive immune resistance and might also therefore predict response to PD-1 inhibitors[157]. Fresh invasive tumor biopsies are unlikely to be practical outside a research setting, however recent advances in the ability to non-invasively image inflammatory responses using positron emission tomography (PET) may enable the dynamic monitoring of immune reactivation in tumors following treatment[158].

Monitoring of toxicities in early phase clinical trials

Close monitoring and early recognition of immunotherapy toxicities as well as effective management with established protocols and standard operating procedures are critical when testing new immunotherapy combinations. For example, toxicities associated with immune checkpoint inhibitors are widely different in their underlying mechanisms involved, clinical features and necessary treatments, in contrast to conventional cytotoxic chemotherapies. In addition, as new combination regimens are developed, it is entirely possible that new or unexpected toxicities will arise.

As we continue to build exponentially on the use of different immunotherapies, it is likely that our experience in recognizing and managing immune-related toxicities will no doubt improve. It is important to recognize that such immune-related adverse events range from mild to life threatening, with inflammatory colitis being responsible for the majority of immunotherapy-related deaths. To illustrate this point, irAEs may occur in up to 62% of patients treated with ipilimumab and involve the skin (rash, pruritus), gastrointestinal tract (diarrhea, colitis), liver (hepatitis) and endocrine system (hypothyroidism, hypopituitarism, adrenal insufficiency, hypophysitis), with grade 3 or 4 adverse events occurring in up to 20% of patients[26,34]. Rarely, neurological toxicity can also occur and cases of transverse myelitis and

Guillain-Barre syndrome have both been described[159]. The spectrum of toxicities associated with anti-PD-1/PD-L1 antibodies is similar to anti-CTLA treatments, although toxicity generally occurs less frequently and is often less severe, with grade 3-4 AEs occurring in approximately 10% of patients[34]. Treatment-related pneumonitis is perhaps the exception, which has been described more commonly with anti-PD-L1/PD-1 therapies compared to anti-CTLA antibodies and is a potential concern with anti-PD-1/PD-L1 combination studies.

Whilst concurrent targeting of CTLA and PD-1/PD-L1 significantly improves response rates in the melanoma clinic, the incidence of grade 3-4 toxicities are more than doubles (55% for combination *vs.* 27% for ipilimumab alone) and 36%-45% of patients discontinue combination treatment due to toxicity, most commonly diarrhea or colitis[35,144]. Up to 90% of patients receiving nivolumab in combination with ipilimumab require some form of immunosuppressive treatment, although in >80% patients, grade 3-4 immune-related adverse events do resolve completely[35,144]. The combination of ipilimumab with other anti-PD-1/PD-L1 antibodies also appears to result in similar toxicity profiles[90]. Combination studies of anti-PD-1/PD-L1 antibodies with cytotoxic chemotherapy have yielded impressive response rates, with associated high rates of adverse events[27,48]. Pneumonitis in particular appears to occur more frequently when anti-PD-1/PD-L1 antibodies are used in combination with either chemotherapies or molecularly targeted agents[48].

Establishing the maximum tolerated dose (MTD) is a traditional phase 1 trial endpoint and is often used to determine the recommended phase 2 dose. Whilst the MTD is valid for cytotoxic treatments in view of their dose-dependent toxicities, its usefulness in immunotherapy trials remains controversial. Although the frequency and severity of immune-related toxicities appears to be dose-dependent for anti-CTLA4 antibodies such as ipilimumab, there does not appear to be such a correlation between dose and toxicity for anti-PD-1/PD-L1 antibodies such as pembrolizumab[47,160]. These differences may reflect the varied underlying mechanisms of action underpinning anti-CTLA4 versus anti-PD-1/PD-L1 antibodies, and also question the use of the MTD as a primary endpoint in early phase 1mmunotherapy monotherapy and combination trials. Novel, adaptive phase 1 trial designs that incorporate the establishment of an optimum biological dose (OBD) range have been implemented successfully, however an OBD range will ultimately be challenging to define with novel immunotherapies, unless a robust, biologically-relevant, pharmacodynamic (PD) biomarker exists[161].

Currently, the severity and timing of conventional definitions for DLTs are pre-defined in trial protocols. However, DLTs for immunotherapy trials clearly need to incorporate irAEs and differentiate between those that are quickly reversible or treatable (such as skin toxicity or single-organ endocrine disorders) versus those that are potentially life-threatening if treatment is not withdrawn (such as colitis). DLTs are traditionally defined as occurring within the first treatment cycle (often 3-4 weeks), however most irAEs are known to occur outside this window, peaking around 6-8 weeks and are often slow to resolve. Such factors need to be considered when defining immunotherapy trial-specific DLT periods and criteria[162]. The design of combination studies involving immunotherapies and conventional cytotoxic chemotherapy will be particularly challenging and may require different DLT windows depending on the nature of the AE.

Another important consideration is that for both anti-CTLA4 and PD-1/PD-L1 inhibitors, autoimmunity may correlate with clinical response. For example, two-thirds of patients who discontinued nivolumab/ipilimumab treatment due to toxicity went on achieve an objective response[144,163]. If the presence of irAEs correlates with response, trial protocols need to establish clear guidelines for the management of such toxicities, which should not necessarily result in patient withdrawal from study.

Finally, whilst OS is not usually a primary outcome measure for phase 1 studies, the growing trend to combine phase 1 and 2 studies during clinical drug development means statistical consideration of such outcomes is important early on. To date, OS benefits for immunotherapies have been demonstrated in a minority, but are generally substantial for those who do respond, as demonstrated by the 'long tail' on the Kaplan-Meier survival curve. This unique response pattern results in delayed separation of Kaplan-Meier survival curves, which if analyzed using standard statistical methods, may fail to demonstrate a statistically significant OS benefit[164,165]. Use of randomised phase 2 studies, helps to identify occasions when the above may occur and when utilized, will better guide the statistical design of subsequent phase 3 studies.

In the future, with increased experience and knowledge of the toxicity profiles associated with immunotherapy drugs, both as single agents and in combination with other therapeutics, we should be able to prospectively optimize and adapt combination trial designs. However, at the current time, for new immunotherapy combination trials being conducted, careful consideration and monitoring of dose, schedule, clinical outcomes and trial parameters will require

thoughtful flexibility and adaptation to ensure the best outcomes for both patients and the study compound.

Assessing antitumor responses in immunotherapy combination trials

Upon the observation of objective clinical and radiological responses with immunotherapies in early phase trials, it became clear that unique kinetics and patterns of disease response occurred in contrast to other anticancer treatments. It was recognized that tumor infiltration of immune cells could result in a period of apparent or "pseudo" tumor growth prior to delayed tumor shrinkage. In addition, the initial immune-cell infiltration into previously unidentified malignant lesions could be falsely interpreted as progressive disease using standard response evaluation criteria in solid tumors (RECIST). Consequently, immune-related response criteria were defined to address these unique clinical scenarios[166].

The immune-related RECIST criteria have been modified to be more in line with RECIST 1.1 and now incorporate the use of uni-dimensional measurements and a reduced number of target lesions[167,168]. Assessing disease response during immunotherapy combination studies will be particularly challenging as the phenotypes and kinetics of tumor responses may alter once again. This is a complex and dynamic area, and ultimately both the timing and criteria for response assessments will require thoughtful consideration so as to avoid both premature treatment cessation of effective combinations, as well as the persistence of treatment in patients deriving no clinical benefit. Such strategies may require the personalization of radiological assessments to each combination regimen and perhaps even the individualization of such criteria and imaging modality to each patient. For example, with immuno-oncology combinations, it will be important to establish a priori specific trial criteria for treatment discontinuation, which may incorporate standard or immune-related RECIST criteria, depending on the partner drugs involved.

Increased efforts should also focus on the development of novel functional and molecular imaging techniques to assess the micro-structural properties of tumors in order to provide an early surrogate biomarker of treatment-related changes, rather than size-based parameters. Examples of such modern imaging modalities include novel PET and functional magnetic resonance techniques.

Conclusions

Immunotherapy is a rapidly evolving and complex field that offers great potential to deliver substantial benefits to patients with a range of different cancers. A number of approved therapies already exist that offer impressive and long lasting responses in some patients. It is now clear however that a substantial proportion of patients do not respond to the immunotherapies currently available. Better patient selection through the use of more precise predictive biomarkers of response may potentially help; however, no clinically validated robust biomarkers currently exist. As such, it is highly likely that combination therapies will be required to increase responses to immunotherapy. Already, the combination of nivolumab and ipilimumab through the targeting of different but complimentary pathways has been shown to substantially increase response rates and PFS in metastatic melanoma. Long term data is awaited to confirm that responses are truly durable and that the tail of the survival curve has been raised as with ipilimumab. However this combination and many other combinations discussed herein hold much promise in delivering this outcome. The improvements in outcomes do however come at the cost of increased drug-related toxicities. As already discussed in this review, multiple other combination therapies hold great promise to increase the immunogenicity of tumors and thereby improve outcomes in patients who would not necessarily benefit from single agent treatment. Chemotherapy, radiotherapy and targeted therapies all hold genuine promise as potential partners with different immunotherapeutic agents. Caution of course still needs to be applied since combination therapies always bear the risk of leading to synergistic toxicities. The use of anti-PD-1/PD-L1 agents as the immunotherapy backbone rather than anti-CTLA4 antibodies may potentially mitigate combination toxicities to some degree.

It is critical for the design of immunotherapy-based combination clinical trials to be based on strong preclinical rationale coupled with a thorough understanding of established scientific principles. Although there is much hope for chemotherapy or molecularly targeted therapies to increase tumor immunogenicity, there is still great potential that exists for some treatments to be immunosuppressive and as such, negate the potential benefit of the immunotherapy involved. Many combinations are currently still in preclinical or early phase trial development. Most if not all of these combinations require meticulous clinical trial testing initially in phase 1 trials to confirm their safety profiles, but also to assess for preliminary signals of antitumor activity. Such trial

designs will need to incorporate any preclinical and clinical data on the unique toxicity profiles in question, so as to inform the starting doses of both drugs and the subsequent dosing and scheduling of the combination. Of course, significant chronic toxicities may develop beyond the first cycle of treatment, and such factors will need to be considered when evaluating the overall safety profile of the novel combination. But likewise, the potential for certain toxicities to predict antitumor response will need to taken into account, for example the higher rate of response in patients who experienced grade 3/4 irAE when treated with combination ipilimumab and nivolumab[35]. Trial designs will also need to consider different endpoints of drug effectiveness such as durable control rates. Although RECIST response rates will remain important, the meaningful benefit of immunotherapy has thus far been with the impressive durable survival rates, and the elevation of the tail of the survival curve. As such, capturing this metric and developing valid predictive biomarkers for this endpoint will be important in future trials. Phase 1 trials will also need to be designed to learn as much about the combinations being tested through detailed pharmacokinetic testing for drug-drug interactions and if possible, tumor biopsies to confirm putative mechanisms of action and to assess putative predictive biomarkers of response.

Much has now been established about the immune response to tumors and the associated mechanisms of immune escape. However, we are clearly still at the tip of the iceberg with regards to exploiting such targeted immunological strategies. Hypothesis-testing, biomarker-driven early phase clinical trials are ideally placed to help improve our burgeoning knowledge of the complex biology involved with such treatments through the testing of novel immunotherapy-based combination regimens in advanced cancer patients. It is only through such strategies that we will finally be able to fulfil the promise that such new treatments bring to the advancement of modern oncology.

Conflict of interest statement

No potential conflicts of interest are disclosed.

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Cite this article as: Harris SJ, Brown J, Lopez J, Yap TA. Immuno-oncology combinations: raising the tail of the survival curve. Cancer Biol Med. 2016; 13: 171-193. doi: 10.20892/j.issn.2095-3941.2016.0015