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Review

New Anticancer Immunotherapies

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Abstract. *The quest for immunotherapies against cancer has been ongoing for many years, and a greater understanding of the normal mechanisms involved in developing immune responses has now led to clinically effective therapies. With the licensing of Ipilimumab and Sipuleucel-T, immunotherapeutic strategies are taking their place alongside conventional treatments for cancer. This review will consider the different modalities of immunotherapy, highlighting clinical benefits observed and considering the immunological basis and evidence of their efficacy. Dendritic cell therapy, targeting activation and regulation of T cells, oncolytic virus vaccines and adoptive T cell therapies will all be considered, regarding the current situation and avenues for future exploration.*

The quest to find effective immunotherapies against cancer has been ongoing for over a century and Interferon and Interleukin-2 have been used for many years for selected indications. However, the licensing of Ipilimumab and Sipuleucel-T heralds a new era with a resurgence of interest in cancer immunotherapy. Definitive evidence for an anti-tumour immune response came some 50 years ago from experiments using transplantable tumours in inbred mice and further refined in immunodeficient transgenic mice using adoptive T-cell transfer experiments (85). A greater understanding of the complexities of the immune system and its interactions with tumours has led to immunotherapeutic strategies with measureable clinical benefit. This review will consider the current landscape of cancer immunotherapy, discussing newly licensed treatments along with selected therapies in development.

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The development of the immune response to cancer represents a complex interaction between the host and the cancer, involving the tumour itself, the stroma, antigen presenting cells (APC) including dendritic cells (DC) and macrophages, T and B lymphocytes, NK cells and others. A simplified model of how the immune response is generated is shown in Figure 1. APC such as DC take up tumour-associated antigen and with appropriate activating signals, traffic to the draining lymph node where they present antigen to naïve T cells (signal 1). The resultant T cell response at this stage is governed by co-stimulatory signals (signal 2) and cytokines secreted by APC (signal 3). The nature of the signal from DC to T cells determines the nature of the CD4 T-cell phenotype in terms of T helper (Th) 1, Th2, Th17, regulatory T cell (Treg) and other T cell subsets. Efficient activation of DC leads to robust co-stimulation and induction of Th1 and cytotoxic T cell (Tc) responses. An appropriate Th1 response and DC activation of CD8 T-cells generates antigen specific cytotoxic T-lymphocytes which subsequently home back to the tumour, where they induce T-cell killing of tumours. However, a multitude of mechanisms can lead to insufficient immune responses, including suppression of DC function leading to inefficient or inappropriate T cell activation. Antigen specific T-cells can also be down-regulated centrally or peripherally at the tumour site. Impaired activation of DC or suppressive signals from a tumour lead to anergic or regulatory T cell responses. If the T cell response generated is of a regulatory nature, this instead contributes to tumour-mediated immune suppression. Immunotherapeutic strategies target different steps in the induction of these responses in order to stimulate stronger, more effective immune reactions against cancer. Vaccination with antigen loaded, activated DC bypasses the need for effective activation within the often immune suppressive environment of the tumour. Oncolytic virus (OV) therapy has the potential to target tumours by two mechanisms; direct killing and induction of an immune response due to release of tumour antigen upon cell death coupled with effective viral danger signals which stimulate tumour-resident APC. Strategies to target T cell co-stimulation and regulation aim to enhance and prolong

effective activation of T cells and overcome suppressive signals from tumours. Protein antigen vaccination strategies aim to induce favourable immune responses *in vivo* to specific antigens. Adoptive T cell therapies circumvent the necessity to induce a T cell response *in vivo* and directly supply the effector arm of the immune system to the cancer patient.

Targeting T cell Activation and Regulation

The demonstration in phase 2 and phase 3 studies of the clinical benefit of Ipilimumab in advanced melanoma has highlighted the potential of therapeutic manipulation of immune co-stimulation and regulatory pathways in cancer therapy. The immune system has coordinated co-stimulatory and regulatory pathways for T cell activation, including up-regulation of CTLA-4 and PD-1 on T cells following T cell activation by DC to ensure appropriate termination of immune responses. Blocking of these so called 'immune checkpoints' can alleviate tumour-mediated immune suppression and allow responses to develop to poorly immunogenic antigens (Figure 2). CTLA-4 and PD-1 receptors on T cells are the current targets in clinical practice for such pathways, though more are in development. Expression of the CTLA-4 receptor is increased on T cells upon activation (14), being moved from endosomal stores to the cell surface. Here it competes with CD28 for binding of CD80 and CD86 on APC, with a higher affinity for these receptors (29, 99). The exact mechanisms by which CTLA-4 regulates T cell activation are still being elicited but appear to be numerous, including blocking CD28-induced up-regulation of T cell activating genes (74) and glucose uptake (69), attenuating the 'stop' signal induced by TCR ligation to maintain T cell-APC crosstalk (81) and causing internalisation of CD80 and CD86 on APC (72). Loss of CTLA-4 in murine models leads to development of lymphoproliferative disorders and severe autoimmune disease, demonstrating the crucial regulatory role of this receptor (96, 104). The PD-1 receptor is involved in T cell regulation in the periphery, binding to B7-H1 (PD-L1) or B7-DC (PD-L2) on peripheral tissues and APC. Like CTLA-4, PD-1 is up-regulated on T cells upon activation (2) but has to be produced by new protein expression rather than being held in intracellular stores. B7-H1 (PD-L1) is not expressed on normal tissues but is on many tumour cell lines and on freshly isolated human tumours (18). There is increased expression of PD-1 on tumour-infiltrating T cells with associated impairment of inflammatory cytokine production (3, 84). Thus, suppression of T cells *via* the PD-1 receptor is induced at the site of effector immune responses rather than being induced as an early regulatory mechanism in the manner of CTLA-4. The phenotype of PD-1 knockout mice is less severe than that of CTLA-4 knockout mice (64, 65) but it remains to be conclusively seen whether this translates into less toxicity with PD-1 targeted therapy.

The first of this class of therapies to be licensed is Ipilimumab (Yervoy, Bristol-Myers Squibb), an IgG1κ monoclonal antibody against CTLA-4. In phase 1/2 trials, clinical activity was seen in castration-resistant prostate cancer, metastatic melanoma and ovarian cancer (36, 90). Ipilimumab has gone on to show benefit in phase 3 trials in metastatic melanoma in both previously treated and untreated patients. In previously treated patients, Ipilimumab 3 mg/kg 3-weekly for 4 treatments (plus maintenance if appropriate) was compared with Ipilimumab plus gp100 peptide vaccine or gp100 peptide vaccine alone (37). Median overall survival (OS) in Ipilimumab-containing arms was 3.6 months longer than in the gp100-alone arm (Median OS 10.0 months *vs*. 6.4 months, HR for death 0.68, $p<0.001$). Response rates by conventional RECIST criteria were low at 5.7% (complete response + partial response) for Ipilimumab + gp100 and 10.9% for Ipilimumab alone, compared to 1.5% for gp100 alone. Disease control rates (complete response + partial response + stable disease) were 20.1% for Ipilimumab + gp100, 28.5% for Ipilimumab alone and 11.0% for gp100 alone. In 13/38 objective responders in the two Ipilimumab-containing arms, the duration of the response was greater than 2 years. In addition, late responses after completion of Ipilimumab treatment were seen in patients with previous progressive disease and further improvements were seen in patients with stable disease or partial responses. Toxicities in this trial were predominantly immune-mediated adverse events (IRAE), with 60% of patients experiencing some grade of IRAE. 10-15% of patients receiving Ipilimumab experienced grade 3-4 side effects, most commonly diarrhoea, colitis and rashes. Toxicities lasted 5-6 weeks and required corticosteroid treatment when grade 2+, resolving in a median of 2-3 weeks. 4 patients were treated with Infliximab for severe colitis and 8 required permanent hormone replacement due to autoimmune destruction of the pituitary gland. In treatment-naive patients with metastatic/unresectable stage III melanoma, Ipilimumab 10 mg/kg 3-weekly for 4 treatments (with the option of maintenance treatment) in combination with dacarbazine was compared to dacarbazine alone (77). Median OS was 2.1 months longer in patients receiving Ipilimumab (11.2 months *vs*. 9.1 months, HR for death 0.72, $p<0.001$). Despite demonstrating a difference in overall survival, disease control (complete response, partial response or stable disease) was not significantly different between the two groups at 33.2% and 30.2% ($p=0.41$) respectively for combination *vs*. dacarbazine alone. In keeping with the findings from the earlier study, some patients continued to improve from partial to complete response 6 months after treatment. Grade 3-4 adverse events occurred in 56% of patients receiving Ipilimumab compared to 26% for dacarbazine alone but in contrast to the second-line study, there were no drug-related deaths. The commonest grade 3-4 toxicity in the combination arm was deranged liver function tests in 17-20% of patients, and less frequent severe colitis was seen than in the second-line study.

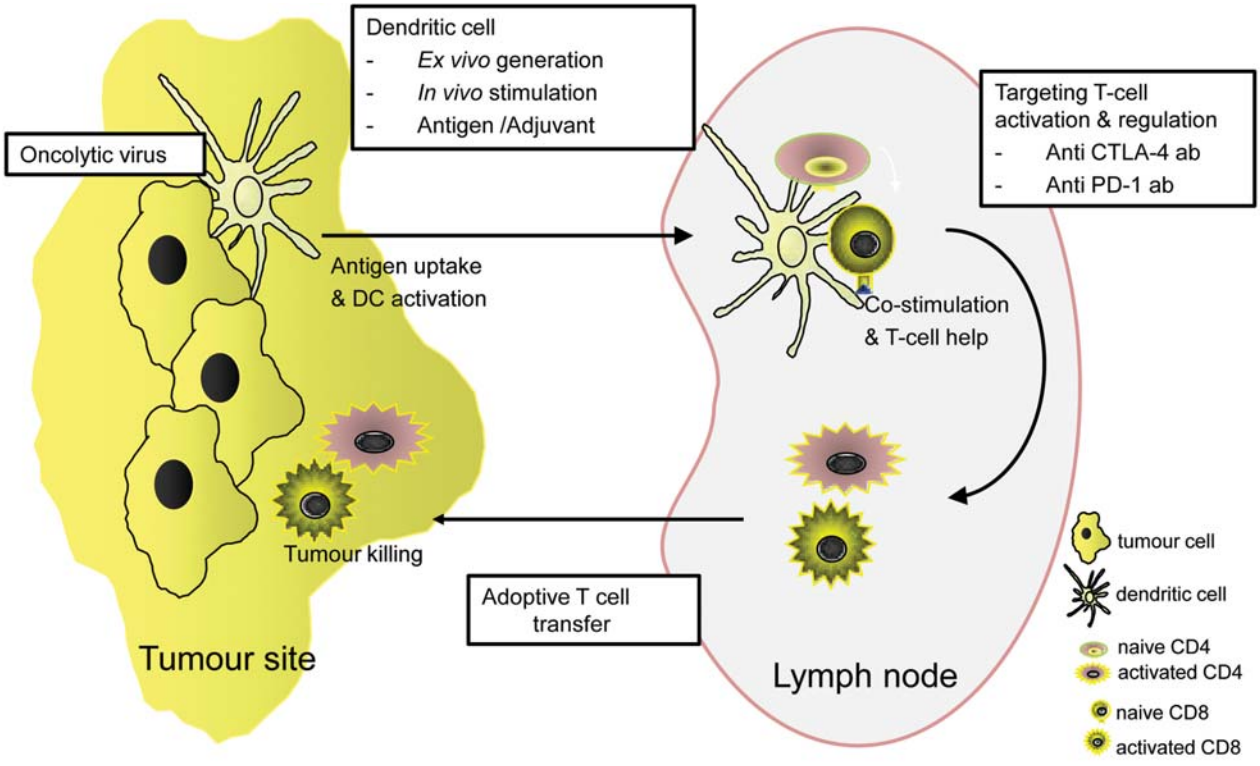
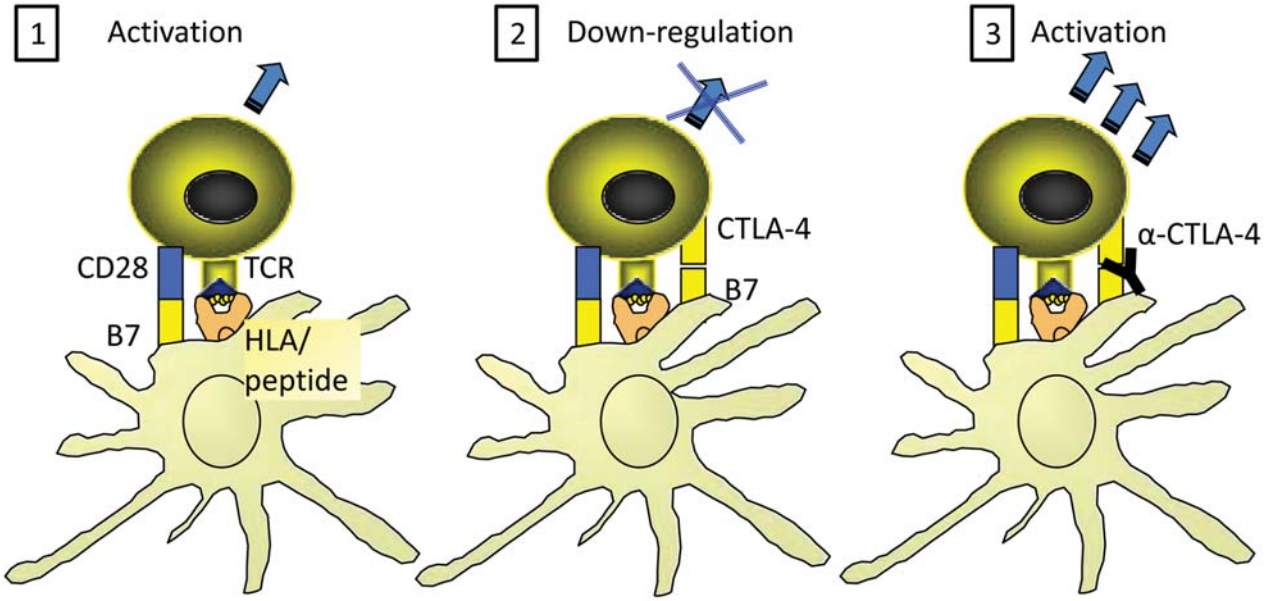


Figure 1. *Anti cancer immune response*.



1. T-cell activation following binding of T cell receptor (TCR) to HLA-peptide complex on DC (signal 1) and CD28 and B7.1 on DC (signal 2).
2. CTLA-4 is up-regulated on activated T-cells, binds to B7.1 displacing CD28 binding and down-regulating T-cell activity
3. Blocking of CTLA-4/B7.1 binding with anti-CTLA-4 antibody (α-CTLA-4) stops this negative signal

Figure 2. *Mode of action of anti CTLA-4 antibodies*.

Ipilimumab is currently being investigated in a range of cancers including prostate cancer, renal cancer, lung and breast cancer and haematological malignancies. Its role in the adjuvant setting is being tested and a large phase 3 EORTC trial in high risk melanoma has been completed and the results are awaited.

Despite growing understanding of the role of CTLA-4 in regulation of T cell responses, the exact mechanism by which Ipilimumab produces clinical benefit is still under investigation. In addition, identifying predictors of and barriers to response are important to aid selection of patients and identify future targets for novel immunotherapies. To date there are no reliable biomarkers that predict response to Ipilimumab. Laboratory studies carried out along with clinical trials suggest that ICOS expression on circulating $CD4^+$ T cells is increased after treatment with Ipilimumab and that this increase is not due to induction of ICOS-expressing Tregs (54). The ability of $CD4^+$ T cells to produce IFNγ in response to CD3 stimulation was also increased after treatment and Ipilimumab could lead to development of NY-ESO-responsive $CD4^+ICOS^{hi}$ T cells (54). This study also examined bladder tumour tissue from patients treated with Ipilimumab and found higher ICOS and lower FoxP3 expression in $CD4^+$ cells in tumour tissue compared to tumour from untreated bladder cancer patients. Gene expression profiling of pre- and post-treatment tumour biopsies from melanoma patients treated with Ipilimumab compared patients who responded to treatment with those who did not (42). Many of the genes with higher expression in pre-treatment samples from responders compared to non-responders were T cell and other immune markers, suggesting that these tumours had a more permissive baseline immune profile enabling response to Ipilimumab. Unsurprisingly, genes up-regulated following treatment were most frequently immune-related genes and the up-regulation was stronger in responders than in non-responders. Biopsies of melanoma lesions following Ipilimumab treatment showed a correlation between the degree of necrosis and the ratio of infiltrating $CD8^+$ T cells to $FoxP3^+$ T cells (35). NY-ESO antibody and $CD4^+$ and $CD8^+$ responses were studied in melanoma patients treated with Ipilimumab (106), with a statistically significant difference in the rate of NY-ESO antibody positivity at baseline between those experiencing clinical benefit (classified as complete response, partial response or stable disease) and non-responders (55 *vs.* 31%, $p=0.0481$). Due to the fact that patients with NY-ESO antibodies (at baseline or induced by Ipilimumab treatment) did not all respond, analysis of T cell responses to NY-ESO was also carried out. The rate of $CD8^+$ NY-ESO responses was higher in the antibody seropositive patients who responded compared to antibody seropositive patients who did not (6/9 *vs.* 1/8, $p=0.0498$). Overall, seropositivity to NY-ESO and presence of $CD8^+$ T cells recognising NY-ESO were both associated with response to Ipilimumab.

In contrast to the improvement in survival seen with Ipilimumab, a phase 3 study of Tremelimumab(an IgG2 anti-CTLA-4 antibody, Pfizer) failed to demonstrate a survival benefit against single agent dacarbazine or temozolamide in first line treatment of metastatic melanoma (73) despite promising earlier phase studies. Possible reasons for this failure include early analysis of the data, difficulties with the now well documented phenomenon of apparent progression before tumour response and the fact that many patients went on to receive Ipilimumab, potentially masking late benefits in favour of Tremelimumab.

Like CTLA-4, PD-1 is also up-regulated on T cells upon activation. Ligands for PD-1 are expressed not only on APC but also on tumour cells in the periphery. Antibodies targeting the PD-1/PD1-L axis have shown significant promise in phase 1 and recently reported phase 2 studies in several tumour groups. Blocking antibodies to both PD-1 and PD-L1 have been developed and several of these are engineered IgG4 antibodies, removing the risk of antigen-dependent cellular cytotoxicity and complement-mediated cytotoxicity. BMS-936558 (Bristol-Myers Squib) is one such IgG4 anti-PD-1 antibody which has completed a phase 1/2 study in advanced melanoma, non-small cell lung cancer, renal and colorectal cancer and castration-resistant prostate cancer (97). 296 patients were treated in dose-escalation and expansion cohorts at 0.1, 1, 3 and 10 mg/kg biweekly for up to 24 months depending on response. Responses were seen at all doses and there was no clear dose-response relationship. Objective responses were seen in 14/76 (18%) evaluable lung cancer patients, 26/94 (28%) melanoma patients and 9/33 (27%) renal cancer patients. Responses were often durable, for example out of the 18 responding melanoma patients who had reached at least 1 year of follow-up, 13 continued to respond at this time point. In a subset of patients, tumour PD-L1 expression was examined and it was found that 9/25 (36%) patients with tumours positive for PD-L1 responded to anti-PD-1 therapy, whilst none of the 17 patients with tumours negative for PD-L1 responded. The potential of tumour PD-L1 expression as a biomarker for response to anti-PD-1 therapy will be explored further in later phase trials. Median PD-1 receptor occupancy on circulating T cells after 8 weeks of treatment was 64-70%, with no significant dose-response relationship. Severe drug-related adverse events in this study occurred in 11% of patients and included 3 patients (1%) with grade 3-4 pneumonitis, 3 patients (1%) with grade 3-4 diarrhoea, 1 patient with each of grade 3-4 pruritis, rash, hypothyroidism, hyperthyroidism and infusion related reaction. 4 patients had grade 3-4 deranged liver function tests. Gastrointestinal and hepatic toxicity of all grades and grade 1-2 pneumonitis responded to dose interruption +/– steroid treatment. Grade 3-4 pneumonitis was treated with Infliximab and/or mycophenolate but resulted in 3 treatment-related deaths. Responses with other anti-PD-1 antibodies have also been

seen in advanced haematological malignancies(5, 6). Further phase 2 studies with BMS-936558 (NCT01354431 and NCT01358721) are underway and phase 3 trials are planned. Other ongoing or planned phase 1 trials of anti-PD-1 antibodies include combinations with peptide vaccination (NCT01176474 and NCT01176461), DC-tumour cell fusion vaccine (NCT01441765, NCT01096602 and NCT01067287) and conventional chemotherapy (NCT01454102).

The PD-L1 antibody which has been most extensively studied thus far is BMS-936559 (Bristol-Myers Squib), also an engineered fully human IgG4 antibody. In a phase 1/2 dose escalation and expansion cohort study, patients with advanced melanoma, non-small cell lung cancer, renal, colorectal and ovarian cancer were initially treated at 0.3, 1, 3 and 10 mg/kg biweekly for up to 96 weeks (8). Expansion cohorts were then treated at 10 mg/kg for all five initial cancer types and additionally at 1 and 3 mg/kg for melanoma and non-small cell lung cancer due to clinical activity observed at these doses in the initial treatment cohorts. Pancreatic, breast and gastric cancer patients were also treated at 10 mg/kg in the expansion phase of the trial. In patients evaluable for response, 9/52 melanoma patients had an objective response (1 at 1 mg/kg, 5 at 3 mg/kg (2 complete responses) and 3 at 10 mg/kg (1 complete response)). In non-small cell lung cancer, there were 5 partial responses out of 49 patients, with 1 at 3 mg/kg and 4 at 10 mg/kg. In ovarian and renal cancer there were 1/17 and 2/17 partial responses respectively, all at the 10 mg/kg dose. Duration of responses was again prolonged as with the anti-PD-1 antibody, with 5/9 responding melanoma patients continuing to respond at 1 year of follow-up and 3/5 responding non-small cell lung cancer patients continuing to respond at 24 weeks. Stable disease lasting more than 24 weeks was seen in 14/52 melanoma patients, 6/49 non-small cell lung cancer patients, 3/17 ovarian cancer patients and 7/17 renal cancer patients. Grade 3-4 treatment-related adverse events were seen in 9% of patients but drug-related grade 3 adverse events were rare, consisting of 1 patient experiencing each of sarcoidosis, endophthalmitis, diabetes, myasthenia gravis and adrenal insufficiency, and 1 case of a grade 3 infusion reaction. Treatment of these adverse events was with treatment interruption +/– steroids and there were no treatment-related deaths. Infusion reactions were more common than with the anti-PD-1 antibody, occurring in 10% of patients, mainly those treated at 10 mg/kg. All of these reactions were manageable with antihistamines, antipyretics +/– steroids. It remains to be seen whether tumour PD-L1 expression is a predictor of response to anti-PD-L1 treatment as appears possible with anti-PD-1 therapy.

Dendritic Cell Vaccines

DC are key APC, activating and directing the adaptive immune system and their potential as vaccines for cancer therapy has long been recognised (4). DC vaccines have been refined as understanding of DC has progressed, and Sipuleucel-T (Provenge, Dendreon) is the first licensed DC based therapy. Sipuleucel-T is an autologous active cellular immunotherapy consisting of peripheral blood mononuclear cells (PBMC) activated with a recombinant fusion protein, PA2024 (prostatic acid phosphatase, a prostate specific tumour antigen, fused to granulocyte-macrophage colony stimulating factor (GM-CSF)). Vaccine manufacture consists of 3 biweekly leucopharesis procedures and a 3 day *ex vivo* manufacture prior to reinfusion. At a central facility, the leucopharesis product undergoes PBMC isolation, monocyte depletion and incubation with PA2024 for 36-44 hrs. The resultant cell mixture contains DC precursors, T cells, B cells and macrophages in variable proportions (Table I). Early phase studies demonstrated that Sipuleucel-T induced T cell proliferative responses to PA2024 in all patients and that clinical efficacy in term of time to progression was related to DC number and T or B cell responses (88). In these studies, time to progression was longer in patients who received more than 10^8 DC per infusion compared to less than 10^8 per infusion (31.7 weeks *vs*. 12.1 weeks, $p=0.013$) and in those who developed a detectable T or B cell response to seminal fluid derived PAP compared to those who did not (34 *vs*. 13 weeks, $p<0.027$). T cell proliferative responses to the fusion protein (PA2024) were undetectable at baseline but could be detected in 100% of patients after Sipuleucel-T infusion. Cytokine analysis determined that the T cell response generated was predominantly Th1, secreting IFNγ but not IL-4. Infused $CD54^+$ dendritic cells also expressed CD40, CD86 and high levels of HLADR, confirming their mature phenotype. Thus it appears likely that the DC portion of the therapy and induction of a detectable immune response to naturally occurring PAP are both key to clinical benefit. Whether the other cellular constituents also play a role in maturation of DC and induction of adaptive responses, or whether pure circulating DC alone could be an even more potent therapy is not yet clear.

In two initial phase 3 studies, Sipuleucel-T failed to achieve its primary endpoint of improvement in time to progression but did show a significant reduction in risk of death and a trend towards increased survival respectively (34,

Table I. *Cellular constituents of Sipuleucel-T.*

Cellular constituent of Sipuleucel-T	Burch *et al*. (15) Mean % (SD)	Small *et al*. (88) Mean % (SD)
DC ($CD54^{bright}$) (87)	18.6 (9.4)	11.2 (11.5)
T cells ($CD3^+$)	65.1 (12.0)	62.3 (16.4)
Monocytes ($CD14^+$)	16.6 (7.8)	14.4 (7.1)
B cells ($CD19^+$)	5.0 (2.4)	7.2 (4.2)
NK cells ($CD56^+$)	ND	13.8 (30.3)

89). In a definitive phase 3 trial of 512 patients with metastatic castration-resistant prostate cancer, Sipuleucel-T prolonged median OS (the primary endpoint) by 4.1 months compared to placebo of PBMC cultured without PA2024 (25.8 *vs*. 21.7 months, HR for death 0.78, p=0.03)(45). This difference was maintained at 3 years, with survival of 31.7% compared to 23.0% for placebo. Mild acute side-effects from Sipuleucel-T were common (chills, fever, fatigue, nausea and headache) but more severe side effects of grade 3+ only occurred in 6.8% (chills and fatigue most commonly) and just 0.9% of patients were unable to complete 3 infusions.

DC vaccination approaches have primarily involved generation of DC *ex vivo*, most commonly from monocytes by culture with GM-CSF and IL-4 with cytokine cocktails for DC maturation. To date, despite some initial clinical responses, these studies have been largely disappointing. More recently, with our increased understanding of DC biology, attempts have been made to generate more immunogenic DC capable of inducing better adaptive immune responses. A formulation of monocyte-derived DC (moDC) (αDC1) has been generated which produces high levels of IL-12p70, the key cytokine for inducing Th1 responses (59, 103). Clinical efficacy (stable disease, partial or complete response) has been seen in recurrent glioblastoma multiforme (6/13 patients) and anaplastic glioma (6/9 patients) after intra- or peri-nodal injection of αDC1 loaded with multiple glioma-associated antigen epitopes and intramuscular poly-IC:LC(66). Intra-tumoural injection of adenoviral-transduced DC with inducible IL-12p70(82), fusion of tumour cells with DC(80) and enhanced activation of DC by electroporation with mRNA expressing antigen along with CD40 ligand, a constitutively active TLR4 and CD70 (TriMixDC)(63) have also shown activity in early phase trials.

To overcome time-consuming and expensive *ex vivo* generation protocols, *in vivo* targeting of DC is also being considered. A soluble LAG3-Ig fusion protein (IMP321) which targets $MHC2^+$ cells (DC and monocytes) has been studied in phase 1 trials in advanced renal cancer(11) and with paclitaxel chemotherapy as first line treatment for metastatic breast cancer(13). LAG-3 binds to the MHC2 receptor, leading to phenotypic maturation, inflammatory cytokine and chemokine production(12) and enhanced ability to activate Tc cells. The trial in previously treated metastatic renal cancer patients demonstrated that IMP321 was safe, and at higher doses enhanced T cell activation and an increase in effector memory CD8 cells was seen along with more frequent disease stabilisation(11). In the breast cancer study, IMP321 was given by subcutaneous injection every 2 weeks for up to 24 weeks on day 2 and day 16 of 28-day cycles of paclitaxel chemotherapy (given day 1, 8 and 15). The timing of IMP321 injections was to take advantage of tumour antigen released due to cell death induced by chemotherapy. No dose limiting toxicities secondary to IMP321 were seen and there were no detectable anti-IMP321 antibodies. Immunomonitoring was done 13 days after each IMP321 injection to detect sustained alterations in immune subsets/activation. In the majority of patients, significant increases in absolute monocyte counts, NK cells, activated CD8 T cells and proportions within PBMC of plasmacytoid and myeloid DC and effector memory T cells were seen. There was also an increase in maturation/activation markers on monocytes in patients receiving the highest dose of IMP321. Whilst the increase in each individual subset or marker was mostly of a modest magnitude, the combined net effect on the immune response is likely to be clinically meaningful. 10 patients had stable disease at 6 months and 15 had a partial response, exceeding the expected response from paclitaxel alone from a historical control group. The kinetics of disease response also suggested that there was an immune component, since there was a dose-response relationship between IMP321 and ongoing tumour shrinkage during chemotherapy maintenance after completion of IMP321 treatment. Larger studies will be required to ascertain the clinical utility of this approach.

Oncolytic Virus Therapy

The aim of therapy with OV is to induce preferential killing of tumour cells in conjunction with immune stimulation to induce systemic anti-tumour immunity. OV show preferential killing of tumour over normal cells due to a combination of overexpression of viral receptors, impaired mechanisms to prevent viral replication and impaired anti-viral interferon responses (19, 50, 58). Some are derived from non-pathogenic viruses such as reovirus and Newcastle disease virus which, in a non-modified form, are harmless to normal cells but cytotoxic to tumour cells. Other OV therapies are modified forms of pathogenic viruses such as herpes simplex virus (HSV), adenovirus and measles virus. Tumour cell death and the resulting antigen release, combined with virally mediated activation of local APC can induce systemic anti-tumour immunity. In some OV, modifications have been made, *e.g.* incorporation of the GM-CSF gene to enhance activation of APC.

Several OV are approaching or are currently in phase 3 testing based on promising responses in phase 2 trials. $Oncovex^{GM\text{-}CSF}$ (Biovex) is a modified GMCSF-expressing herpes simplex virus type 1 (HSV-1) which has deletions of the neurovirulence factor ICP34.5 that blocks cellular anti-viral responses to minimise viral infection of normal cells and deletion of ICP47 which blocks antigen presentation on infected cells(55). Deletion of ICP47 also brings US11 under the control of a more potent promoter, and increased expression of this protein leads to enhanced viral replication and anti-tumour cytotoxic effect. Intra-tumoural injection produced objective responses in 13/50 melanoma patients with unresectable stage IIIc or IV disease (26%, 8 complete

response, 5 partial response)(83) with stable disease in a further 10 patients for more than 3 months. 92% of those responding had responses which lasted 7-13+ months, and shrinkage was seen in non-injected lesions including visceral disease, demonstrating that systemic immunity was induced. In keeping with other immunotherapy trials, responses were often preceded by apparent progression with this occurring in 4 patients who subsequently had a complete response and 2 who proceeded to partial response. Laboratory analyses showed that there was a local reduction in $CD4^+CD25^{hi}FoxP3^+$ Tregs, $CD8^+FoxP3^+$ suppressor T cells and myeloid-derived suppressor cells in Biovex injected lesions and melanoma-specific $IFN\gamma^+$ elispot responses were detectable after in PMBCs and lymphocytes from both injected lesions and non-injected lesions(47). A phase 3 trial in unresectable stage IIIb/c or IV melanoma with a control arm of intra-tumoural GM-CSF injections (NCT00769704) has completed recruitment and is due to report on the primary outcome measure of improvement in durable response rate in summer 2012.

Reolysin (Oncolytics Biotech) is a type III reovirus which is selectively cytotoxic to tumour cells (32) and is in phase 3 trials in head and neck cancer. Infection of normal cells leads to PKR phosphorylation which halts reovirus replication. In contrast, tumour cells with activating mutations in the Ras signalling pathway (either Ras itself or upstream proteins such as EGFR or PDGFR) cannot activate PKR upon viral infection and reovirus continues to replicate leading to cell death (16, 91, 92). Intra-tumoural Reolysin injection has been tested alone (93), in combination with chemotherapy (33) and in combination with radiotherapy (31), but current development is with intravenous delivery. Phase I studies with intravenous Reolysin alone (27, 102) or in combination with chemotherapy (17) proved safe, with no virus-associated dose limiting toxicity and mild-moderate flu-like symptoms as the main side effects. Objective responses were seen when combined with chemotherapy and in one patient with a tumour known to have an activating Ras mutation treated with virus alone, and stabilisation of disease with reduction in tumour markers was also seen after virus monotherapy. These factors combined with detection of viral genome in post-treatment tumour biopsies suggested that the virus was able to target tumour and produce anti-tumour activity (17, 27, 102). *In vitro* infection of DC with reovirus led to phenotypic maturation, inflammatory cytokine production and increased ability to activate T cells (23). DC maturation was retained in the presence of tumour cells, indicating that DC could still be activated by reovirus inside tumours (41). A further phase 1/2 study combined Reolysin with carboplatin and paclitaxel chemotherapy in unselected (phase 1) and head and neck cancers (46). Treatment was again well tolerated and out of 26 patients there was 1 complete response, 6 partial responses and 9 with stable disease by RECIST along with 2 further clinical responses in patients non-evaluable by RECIST criteria. The randomised double blind phase 3 trial currently recruiting (NCT01166542) is comparing addition of intravenous Reolysin or placebo to paclitaxel/carboplatin chemotherapy in metastatic/recurrent SCC of the head and neck resistant to prior platinum-based therapy.

Jennerex (JX-594, Jennerex Biotherapeutics) is a thymidine kinase inactivated vaccinia virus expressing GM-CSF that selectively replicates in EGFR-Ras pathway overexpressing tumours(94). In phase 1 studies JX-594 was safe, though dose-limiting toxicity was seen at the highest doses (grade 3 hyperbilirubinaemia when injected into liver metastases) (67). At the selected maximum tolerated dose (MTD) or lower, there was limited but manageable grade 1-3 toxicities. Following intra-tumoural virus administration, GM-CSF was detectable in serum samples for more than 48 hours in 3 patients treated with the MTD. Several days later there was a second peak of viral genome expression in 12/14 patients implying virus replication *in vivo*. The viral genome could also be detected in non-injected sites including ascites, pleural effusions and other solid lesions. Importantly for safety, there was no viral shedding in urine or on throat swabs. Of 10 evaluable patients for response, there were 3 partial responses, 6 with stable disease and 1 progressive disease. In a further phase 1 study of intravenous delivery of JX-594, higher doses of virus resulted in detectable viral replication in tumour tissue but not normal tissue with sustained viral replication(9). Responses included 1 partial response and 12 with stable disease out of 23 patients. In 3 further patients with hepatitis-B-associated hepatocellular carcinoma treated with intra-tumoural injections responses were also seen (56).

CG0070 (Cell Genesys), is a replication-competent adenovirus developed for intra-vesical treatment of bladder cancer which expresses GM-CSF under the control of the E2F-1 promoter. The E2F-1 transcription factor is regulated by the retinoblastoma (Rb) tumour suppressor protein, which is inactivated in more than half of bladder cancers. This leads to E2F-1 overexpression which will increase GM-CSF expression in tumour cells infected with CG0070. In a phase 1/2 trial in patients who had relapsed after intra-vesical BCG treatment(26), CG0070 was well tolerated with only one dose limiting toxicity of grade 3 lymphopaenia. Out of 13 patients known to have inactivation of Rb in their tumours, 9 had a complete response to treatment. In all patients, remissions lasted 3.0-38.2+ months with 6/35 still responding at the time of reporting. The phase 3 trial comparing intra-vesical CG0070 to standard chemotherapy in patients who have failed intra-vesical BCG therapy (NCT01438112) is due to start recruiting in September 2012 (Clinicaltrials.gov, accessed 20-4-12).

Adoptive transfer of T Cells

Adoptive transfer of tumour-infiltrating lymphocytes (TIL) has a long history but to date has only been possible in

specialist units and for only a small population of cancer patients. Initial studies demonstrated the feasibility of this approach using unselected TIL from subcutaneous and lymph node tumours with response rates of ~30% (78, 79). Subsequently, impressive response rates of up to 60-70% in those receiving TIL treatment were seen in metastatic melanoma patients prepared with non-myeloablative chemotherapy with cyclophosphamide and fludarabine +/– total body irradiation (TBI) and stem cell rescue with high dose bolus IL-2 following TIL infusion (21, 22). These protocols were labour-intensive, requiring multiple separate cultures and screening of T cells for IFNγ response against autologous tumour cell lines or HLA-matched allogeneic lines. Due to the screening requirement and the prolonged time taken to generate sufficient T cells for therapy, up to 60% of patients enrolled were unable to proceed to TIL treatment. The challenge was therefore to develop a quicker, simplified protocol of TIL generation. It was noted that in fact, IFNγ secretion levels at screening of TIL cultures did not correlate with subsequent clinical response, but that telomere length (22, 40, 108), high CD27 expression (39, 40) and shorter time in culture (79) did, suggesting that early-effector T cells are more effective (70). In addition, telomere length seemed to correlate with shorter culture (108). It was also demonstrated that persistence of infused TIL correlated with tumour response and in those responding, higher CD27 and CD28 expression was observed in persisting compared to non-persisting clones (40, 70, 75, 107). These observations led to the development of 'Young-TIL' protocols, using unselected or $CD8^+$ selected TIL produced in one bulk culture with no IFNγ screening process required. This change improved the proportion of patients progressing to the treatment phase by reducing failures due to inability to establish an autologous tumour line, lack of HLA-matching to allogeneic lines or failure at the IFNγ screening stage. In addition, the move to a single bulk culture makes this protocol more feasible for more centres as the requirement for extensive tissue culture expertise and time is reduced dramatically. The Rosenberg group tested a protocol of $CD8^+$ enrichment prior to rapid bulk expansion without IFNγ screening. In a non-randomised phase 2 study 56 patients were treated with TIL + bolus high-dose IL-2 following non-myeloablative chemotherapy with cyclophosphamide and fludarabine +/– TBI of 3x6Gy and autologous stem cell rescue(20). 53/122 patients who had tumour removed for TIL preparation were subsequently treated, a significant improvement on previous protocols. 19/33 patients treated without TBI responded (16 partial responses, 3 complete responses) and 11/23 treated with TBI responded (9 partial responses, 2 complete responses). It was noted that successful TIL generation was correlated with a high percentage of cells in initial suspensions being lymphocytes, with the mean lymphocyte percentage being 52% in patients for whom TIL cultures could be generated compared to only 8% in patients for whom TIL culture failed ($p<0.0001$). Toxicities were as expected for IL-2 and in addition, 28/56 experienced at least 1 episode of febrile neutropaenia and there were 2 treatment-related deaths. 11 of the 30 responders did not have evidence of tumour recognition by their TIL which would have been required on standard protocols. There is now a randomised phase 2 study being carried out comparing $CD8^+$ enriched short term cultured TIL plus high-dose bolus IL-2 after non-myeloablative chemotherapy with fludarabine and cyclophosphamide against high-dose IL-2 alone in metastatic melanoma. At the time of writing, recruitment for this study has been completed and results are awaited. The Schachter group used a similar protocol without $CD8^+$ selection or TBI in two phase 2 studies. In the first (7), 20/27 metastatic melanoma patients received treatment with TIL (average 68 days after resection), of whom 10 had a response (2 complete responses, 8 partial responses), 4 had stable disease and 6 had progressive disease. Median OS in the non-responders (stable disease + progressive disease) was 5.7 months, and had not been reached at a median follow-up of 9.3 months in the responding group. The 2 patients with a complete response were disease free at 4 months and 20 months. In this study, the age of TIL in responders was significantly less than in non-responders and the expansion rate was significantly higher, leading to a higher number of cells being infused. There was some but not complete correlation between age of TIL infused and rate of expansion, suggesting that both factors were important in inducing response. Total $CD8^+$ count infused in the responding group was higher than in the non-responders (4.1×10^8 *vs.* 2.2×10^8, $p=0.047$) but in contrast to previous studies, overall CD27 and CD28 expression did not differ. This study confirmed that selection of TIL on the basis of IFNγ secretion in response to autologous cell lines or HLA-matched cell lines was not a requirement for clinical response, since four of the responding patients were not assessable (no autologous cell line established and incompatible HLA type for use of other cell lines) and the TIL of one patient with a complete response would have 'failed' on standard IFNγ secretion assays. In the second study (86), TIL could not be generated from 5/65 patients and a further 10 patients deteriorated too quickly, resulting in 50/65 patients receiving treatment. Of those evaluable for response at the time of reporting, 16/38 patients had responded (5 complete responses, 11 partial responses) with 11 patients experiencing stable disease. The toxicity of this regime was as expected but considerable, with 92% experiencing neutropaenic sepsis, 28% pulmonary congestion grade 3-4 and 9% grade 3-4 hypotension, with a mean hospital stay of 20 days for treatment. Response was again associated with higher numbers of $CD8^+$ T cells infused (4.1×10^{10} *vs.* 2.2×10^{10}, responders *vs.* non-responders, $p=0.009$) and shorter time to generate TIL (14 days *vs.* 18 days, responders *vs.* non-responders, $p=0.005$).

The search for more predictable and potent tumour-reactive T cells whilst circumventing the time-consuming and laborious TIL generation procedures has led to attempts to genetically modify peripheral blood leucocytes (PBL) to recognise tumour cells. This involves either transfer of genes encoding MHC-restricted tumour-specific T cell receptors (TCR) or transfection with chimeric antigen receptors (CAR). CAR consist of non-MHC restricted antigen recognition using monoclonal antibody variable regions fused with T cell activating moieties/TCR constant regions (CD3ζ +/– co-stimulatory signals such as CD28) in one molecule(24). Additional advantages of these approaches include application of T cell immunotherapy to cancers which do not typically have a large infiltrating lymphocyte population, and the lack of the requirement for MHC-associated antigen presentation by tumours in the case of CAR.

Due to the fact that most tumour antigens are not *de novo* antigens and are expressed at low levels in normal tissues, there is an inherent risk that introduction of very high affinity receptors to these antigens (which would normally have been thymically deleted due to recognition of self-antigens) will lead to significant toxicity. Indeed, there have been several case reports from early phase trials of severe adverse reactions including death. In a study using scFv(G250)-transduced PBL directed at CAIX on renal carcinoma, considerable liver toxicity was seen. After biopsies demonstrated CAIX expression on biliary epithelial cells, this was presumed to be due to targeting of normal tissues by genetically modified T cells (53). In another study in chronic lymphocytic leukaemia where a CAR targeting CD19 expression was used (10), one subject developed pyrexia, hypotension, renal failure and subsequently died following T cell infusion. Post-mortem did not support a diagnosis of tumour lysis syndrome, and serum cytokine levels measured during treatment showed an elevation in inflammatory cytokines after cyclophosphamide treatment but before infusion of T cells. Ultimately, a likely diagnosis of a septic episode was reached and the modified T cells were not felt to be responsible. In a study using a CAR based on the mAB Trastuzumab (targeting HER2) along with CD28, 4-1BB and CD3ζ moieties, a patient with colon cancer metastatic to the lungs and liver developed respiratory distress and pulmonary infiltrate on chest x-ray 15 minutes after T cell infusion(61). Serum cytokine analysis was consistent with a cytokine storm and despite intubation and treatment in intensive care the patient died. The same group described transient severe colitis when treating bowel cancer patients with T cells expressing a CAR targeting CEA (68). All 3 patients treated experienced a subsequent fall in CEA levels, one patient experienced partial response on RECIST criteria and another had 17% tumour regression. Both those who had tumour shrinkage however progressed by 6 months.

Most early studies with genetically modified T cells however generally proved safe but had variable efficacy. A study in ovarian cancer did not demonstrate objective clinical benefit or persistence of infused T cell clones or tracking to tumour (48) but another in lymphoma did show persistence of modified T cells for 4-9 weeks when infusion was followed by IL-2 treatment and one patient had a partial response(95). Another study used PBL transduced with a retrovirus encoding a low-affinity MART-1-recognising TCR identified from a melanoma patient treated with TIL. Melanoma patients were treated using the same non-myeloablative chemotherapy and bolus IL-2 as standard TIL protocols (60). 2/17 patients had a partial response and had detectable engineered T cells 1 year later, other patients had no clinical response but had detectable engineered T cells for at least 2 months. The same group carried out a similar trial using a high-affinity MART-1 TCR or murine gp100 TCR and saw persistence of T cells, elevation of serum IFNγ after treatment and more toxicity in terms of normal melanocyte destruction (uveitis, hearing loss and vitiligo requiring steroid treatment) (44). Response rates were higher with responses in 6/20 MART-1 treated patients and 3/16 gp100 treated patients. An interesting observation in this trial was the finding that although infused tetramer positive cells were 3.5% $CD45RA^+$ and 94% $CD45RO^+$, when tetramer positive cells from peripheral blood were analysed a month after infusion, CD45RA expression had increased to 27% and CD45RO expression had decreased to 66%, suggesting that either the $CD45RA^+$ population had proliferated preferentially or that the $CD45RO^+$ cells had reverted to RA^+. A further study used a TCR against NY-ESO, an attractive target due to its restricted expression on tumours but not normal adult tissue (76). 11 patients with metastatic melanoma and 6 with synovial cell sarcoma were treated, all with tumours expressing NY-ESO. Responses in the melanoma patients included a complete response in 2 (ongoing at 22 and 20 months), and partial responses in 3 (one ongoing at 9 months). 4 of the synovial cell sarcoma patients experienced a partial response with one continuing to 18 months. Another option being explored is the use of virus specific T cells, with the hypothesis that these cells will receive better co-stimulation, enhancing anti-tumour activity and persistence *in vivo*. A trial in neuroblastoma patients used selected EBV-specific T cells for transduction with a GD-2-specific CAR and demonstrated that these cells were safe, and that EBV-specific cells persisted longer than non-virus-specific activated T cells transduced with the same CAR. When PBMC were isolated up to 24 weeks after infusion of modified T cells, the EBV-specific modified cells could still be detected and could respond to EBV-positive stimuli. Tumour necrosis or regression was seen in 4/8 evaluable patients, with one complete response and one partial response (71).

Work is ongoing to characterise the subsets of T cells which are most suitable for genetic modification, in terms of ability to induce a tumour-specific response, minimisation of toxicity and ability to engraft and persist *in vivo* (reviewed in (98)).

Considerations include use of virus specific T cells, naive or memory $CD8^+$ T cells, and within the memory population, central or effector memory $CD8^+$ T cells. In addition to the T cell population used and whether presence of $CD4^+$ T cells are also beneficial, other considerations include the constituents of engineered CARs in terms of co-stimulatory moieties and pre-infusion conditioning of patients(51).

Protein Vaccines

Vaccination with antigen, as protein, peptide or DNA is being investigated either alone or with accompanying adjuvant. The aim is to stimulate local APC at the vaccination site to generate an immune response. Use of cancer testis antigens is a popular choice due to their lack of expression on normal adult tissues. Many different adjuvant combinations have been tested and those which include toll-like receptor (TLR) agonists have shown the most promise. MAGE-A3 is a cancer testis antigen which is expressed on around 2/3 of malignant melanoma (43) and on several other tumour types. A recombinant MAGE-A3 vaccine was tested with two different adjuvant preparations in a randomised phase 2 trial in MAGE-A3 positive unresectable stage III or stage IV M1a (subcutaneous metastases only) melanoma (52). Adjuvant preparations were AS15 (a liposomal preparation of MPL, a TLR4 agonist + QS-21, saponin fractions + CpG, a TLR9 agonist) or AS02B (MPL + QS-21 in an oil-water emulsion). 36 patients were treated with each combination, and there were 3 complete responses (11, 28+ and 55+ months) and 1 partial response (6 months) in the AS15 arm and 1 partial response (7 months) in the AS02B arm. Grade 3 toxicity was low at 5.6% (AS15) and 2.8% (AS02B) with no grade 4 toxicity. The MAGE-A3/AS15 preparation is now being tested in a randomised placebo-controlled phase 3 adjuvant trial in fully resected lymph node positive melanoma (stage IIIB + C) (49). A randomised placebo controlled phase 2 trial of MAGE-A3 vaccine with adjuvant has also been carried out in completely resected stage IB or II non-small cell lung cancer (101). 182 patients were treated with MAGE-A3 vaccine or placebo, and non-significant improvements in disease free interval (HR 0.74), disease free survival (HR 0.73) and overall survival (HR 0.66) were seen in favour of MAGE-A3 treatment, suggesting potential benefit from adjuvant vaccination treatment, which will be evaluated in a phase 3 trial. Interestingly, translational research programmes in these phase 2 trials analysing RNA expression in tumours prior to therapy have identified a gene signature which associates with clinical benefit (57, 100). The phase 3 studies will validate this as a secondary endpoint.

Future

As well as further development of the individual therapies discussed above, combination strategies are likely to be employed with increasing frequency. Pure immunotherapy with complementary combinations such as DC vaccines with oncolytic virus (62) are already being investigated and would have the potential to synergise in their anti-tumour effect. Other rational combinations are dendritic cell vaccines or oncolytic viruses with anti-CTLA-4 or anti-PD-1/PD-L1 therapy. Combination of immunotherapy with drugs which have been noted to alter immune function is another area of interest, such as DC vaccination with Sunitinib treatment for metastatic renal carcinoma (25). Sunitinib has been observed to reduce Treg levels (1) and since these are likely to be one of the barriers to effective DC vaccine therapy, addition of Sunitinib may allow more effective induction of adaptive anti-tumour responses. It is now clear that some conventional chemotherapies induce more immunogenic cell death than

Table II. *Approaches to cancer immunotherapy.*

Targeting T cell activation and regulation
Anti-CTLA-4 antibodies: • Ipilimumab • Tremelimumab
Anti-PD-1 antibodies • BMS-936558 • MK3475 • CT-011 • AMP-224
Anti-PD-L1 antibodies • BMS-936559
Adoptive T cell therapy
Tumour infiltrating lymphocytes Genetically modified PBL • Chimeric antigen receptors • Engineered TCR
DC vaccines and APC targeting
Ex vivo generation • Sipuleucel-T • αDC1 *In vivo* targeting • IMP321
Oncolytic viruses
• $Oncovex^{GM\text{-}CSF}$ • Reolysin • Jennerex • CG0070
Protein vaccines
• MAGE-A3/AS15

others (28, 30) and selection of these chemotherapies for use with immunotherapy is likely to be more beneficial. In melanoma, the dramatic tumour responses seen with BRAF-targeted therapy such as Vemurafenib and Dabrafenib are associated with increased infiltration of tumours with $CD8^+$ and $CD4^+$ T cells (105) and no impairment in overall immune competency (38), suggesting that combining these drugs with blockade of CTLA-4, PD-1 or PD-L1 could allow a better immune response to develop. This combination is enticing, since targeted BRAF therapy can produce dramatic tumour shrinkage but with short-lived duration, whilst anti-CTLA-4 therapy is less effective against bulky disease and has much lower response rates, but responses are more often durable when they do occur.

In summary, immunotherapeutic techniques are being added to the panel of available treatments for cancer and the success of agents such as Ipilimumab and Sipuleucel-T have led to a renewed interest in cancer immunotherapy. The next few years are likely to bring many more immunotherapies into regular clinical use (Table II) and will hopefully provide more options for patients with tumours which respond poorly to conventional chemo and radiotherapy as well as providing the possibility of long term tumour control and even cure to others.

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