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Review

Enhancing cancer vaccines with immunomodulators

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Abstract

Harnessing the immune system to control cancer has been a challenge for cancer immunotherapists for many years. However, while specific immune responses to tumour-associated antigenic targets have been successfully induced in some patients, these responses have not always been sufficient to reproducibly and consistently mediate useful anti-tumour clinical activity. Many checks and balances have been incorporated into the immune response by nature to prevent or reduce the likelihood of autoimmunity or exaggerated protective inflammatory responses. Tolerance to self-antigens expressed on tumours is a major limitation in generating functional anti-tumour responses. In recent years, many of the pathways that mediate this tolerance have been identified, and reagents that can be used to manipulate these pathways have been clinically evaluated. These include: (a) pathways to activate professional antigen presenting cells, such as through Toll-like receptors, growth factors, such as GM-CSF, and the CD40 pathway; (b) use of cytokines, such as IL-2, IL-12, and Interferon α to enhance immune activation; and (c) pathways that inhibit T cell inhibitory signals, or Tregs. This article reviews clinical trials that have evaluated these approaches, and highlights promising combination vaccine/immunomodulator combination treatments based upon published clinical trial results.

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1. Introduction

There has been considerable interest in developing therapeutic vaccines for cancer. Vaccines have the advantage of being highly specific and relatively non-toxic, and hold the promise of delaying or preventing cancer recurrence, particularly in early-stage patients who may be at risk for recurrence after initial treatment. From an immunologic perspective, many cancer vaccines have been shown to induce significant specific humoral or T cell responses to the tumour antigens being targeted, and some have induced levels of CD8 T cells approaching those seen in response to foreign pathogenic viruses. Unfortunately, randomized phase III clinical trials employing a variety of vaccine technologies targeting different tumour antigens have not always shown consistent evidence of anti-tumour responses, whether the clinical endpoint has been objective response, time to progression, disease-free survival, or overall survival [1].

There are a number of possible explanations for these results. Many of the vaccines being studied may not optimally activate antigen presenting cells (APCs), and as a result, T cells may not be receiving the right intercellular signals needed to fully activate them or the required array of cytokines needed to efficiently polarize them. They may in fact be exposed to immunosuppressive cytokines, such as IL-10 or TGF-β. The vaccines themselves may not be eliminating the regulatory T cell compartment whose responsibility it is to "turn off" physiologic immune responses or to prevent the activation of auto-reactive T cell responses; in fact, they may even be activating it. Finally, subject selection in many initial phase I and II trials may result in a patient population with extensive disease in which many of these "suppressive" factors may be most prominent.

It is becoming apparent that strategies for developing more functionally active responses are essential if cancer vaccines are to succeed. To this end, researchers have been studying a number of immunomodulatory pathways that have the ability to enhance the functional response and in turn the clinical activity of cancer vaccines, and have been evaluating potentially powerful reagents to modulate these pathways. This review discusses these pathways and the clinically available reagents.

2. The response of the immune system to cancer: checks and balances

In order for an immune response to a cancer to be initiated, antigens from the tumour cells must be processed and presented by the innate arm of the immune system. To generate effective effector and memory T cells, antigen presenting cells must undergo activation and maturation. APCs are activated by recognition of various "danger signals" [2]. APCs are able to detect a limited set of conserved molecular patterns that are unique to the microbial world and have been termed pathogen-associated molecular patterns (PAMPs). These PAMPs can be recognized by a family or receptors expressed on APCs and some other cells called Toll-like receptors (TLRs) [3]. In addition, several other stimuli through other receptors expressed on APCs can contribute to APC maturation or activation. These include the CD40 receptor [4], and receptors to various cytokines, such as GM-CSF [5]. Without appropriate stimulation through these receptors, APCs may not become fully activated.

T cells recognize tumour antigens that are processed and presented by APCs. This presentation may be direct or through a process of cross-priming where apoptotic tumour, APC or other cells are phagocytosed by live APCs [6]. In order for both T helper cells and cytotoxic T cells to be effectively activated, they must receive second or co-stimulatory signals through members of the B7 family (CD80 and CD86) in addition to a signal through the T cell receptor (for a recent review, see [7]). Signals received through the CD28 receptor on T cells will activate the T cell, whereas signals through CTLA-4 will have an inhibitory effect. Other co-stimulatory signals from members of the TNF family (4-1BB Ligand or OX40) specifically for T helper cells may also play a role in this activation pathway. The activation process and the polarization of the response towards Th1 or Th2 cytokine profiles may also be influenced by the local cytokine milieu. In addition, the cytokine environment may play a role in stimulating the proliferation – or conversely, the induction of apoptosis – of the T cell.

T cells that are chronically exposed to antigen may become "exhausted" and are not unable to become effectively activated (for review, see [8]). This process is mediated by signalling through the Programmed death-1 (PD-1) receptor, which is part of the B7-CD28 family and is induced on exhausted T cells. Blockade of this receptor can re-invigorate and enhance activation of exhausted T cells stimulated through their antigen receptor.

APCs and APC subsets may also activate regulatory T cells (Tregs). Tregs may play a role in suppressing the T cell response so as to eventually terminate the immune response and prevent the development of autoimmunity. These cells may secrete inhibitory cytokines, such as IL-10 or TGF-β. They rely on cytokines such as IL-2 for activation and proliferation, and also may be regulated to some extent by tryptophan metabolism. Recently, increased numbers of Tregs have been found in the peripheral blood of patients with cancers such as ovarian or lung cancer [9,10]. Tumour cells themselves may secrete factors that may inhibit the activation of T cells (for review, see [11]). These factors may inhibit the expression of components of the T cell receptor signalling complex or downstream members of the signalling pathway.

As T cells, although not the only players, are thought to be important mediators of the immune response to cancer [12], this review will focus on strategies to enhance the functional activation of tumour-specific T cells that may be primed by various cancer vaccines. Understanding these processes of T cell activation may enable researchers to enhance the functional effector activity of cancer vaccines against tumour

antigens expressed on cancers. The ability to direct specific small molecule reagents or recombinant protein/monoclonal antibody reagents to these pathways may allow the enhancement of positive signals or the inhibition of negative signals. Such reagents are now being evaluated alone or in combination with various cancer vaccines. This review will focus on those currently being studied in the clinic.

3. Reagents available to enhance immune activation

3.1. Enhancing APC activity

3.1.1. Combination vaccine treatment with GM-CSF

Probably the most studied activator of dendritic cells has been granulocyte-macrophage colony stimulating factor (GM-CSF), which mediates a number of important immunologic activities. Recombinant GM-CSF or plasmid-encoded GM-CSF increases the numbers of immature dendritic cells (DCs) at vaccine sites [13], enhances DC1 maturation and migration, and may also enhance the immunogenicity of tumour cell vaccines [14].

A number of clinical trials have utilized GM-CSF as an adjuvant for peptide-, protein-, or viral-based vaccines. While these trials have been valuable in establishing the safety and feasibility of combination treatments, most of them have not been randomized, and it is difficult to dissect out the role of GM-CSF in whatever immunogenicity is seen. Since little can be learned about the contribution of the GM-CSF from many of these trials, they are not discussed in this review.

However, several trials using GM-CSF combination treatment were designed specifically to address the impact of GM-CSF in combination with cancer vaccines (Table 1).

von Mehren et al. evaluated the influence of GM-CSF on the immunologic response of the ALVAC-CEA/B7.1 viral vaccine [15]. Two groups of 30 patients each (non-randomized) were treated with 4.5×10^8 pfu ALVAC CEA/B7.1 given intradermally every 2 weeks for four injections, with or without 250 μg of GM-CSF starting 2 days before the vaccination and continuing for a total of 5 days with each vaccination. Biopsy of the vaccination sites revealed a significantly greater infiltration score for patients who received GM-CSF. In patients who received the vaccine alone, the infiltrations were mostly lymphocytic, whereas in those who received both vaccine and GM-CSF, they were more of a mixed inflammatory nature. The number of patients in the GM-CSF group who showed increases in T cell precursor frequency to CEA was not greater than the number in the other group (5/9 versus 7/11), but more patients in this group (11/25 versus 6/22) had stabilization of their disease. However, this difference was not statistically significant. The authors concluded that with the dose and schedule used in this trial, GM-CSF did not enhance the immunogenicity of the vaccine.

Weber et al. randomized 48 patients with resected stage IIA or IIB melanoma to vaccination with two melanoma class I binding peptides in incomplete Freund's adjuvant (IFA), with or without 5 days of GM-CSF at a 250 μg/dose given subcutaneously for 5 days between the two peptide vaccination sites [13]. Immune responses were seen in the majority of patients post-vaccination, as measured by interferon release

Table 1
Randomized clinical trials of GM-CSF and cancer vaccine combination treatments

Trial design	Patients	Immunological results	Clinical results	Ref.
ALVAC CEA/B7.1 ± GM-CSF 250 μg × 5 days (not randomized)	Sixty patients with advanced or metastatic CEA-expressing adenocarcinoma	No difference in frequency of immune responders, increased inflammatory infiltrate in GM-CSF vaccine sites	Increase disease stabilization with GM-CSF (11/25 versus 6/22)	[15]
Class I binding peptides + IFA ± 250 μg GM-CSF × 5 days (randomized)	Forty-eight patients with resected stage IIA or IIB melanoma	Increased cytokine producing and tetramer-reactive CD8+ T cells in GM-CSF group (not statistically significant)	Disease not measurable	[13]
Sequential vaccinations of recombinant fowlpox and vaccinia with CEA and TRICOM with or without 100 μg of GMCSF on Days 1–4	Fifty-eight patients with advanced CEA-expressing cancers of whom 25 patients in cohorts 7 and 8 received the vaccine or split dose of the vaccine with GM-CSF	No clear enhancement of CEA specific T cell precursors were documented in the groups	Patients who received GM-CSF with the vaccine had a longer progression-free survival versus those who did not receive the GM-CSF	[16]
3 HLA A2 binding peptides and Montanide ISA-51 alone, with GM-CSF 10 μg, with GM-CSF 50 μg (randomized)	Twenty-five patients with metastatic melanoma	Immune response in 9/25 patients—no increase with the two low doses of GM-CSF	–	[17]
Vaccine consisting of three allogeneic cell lines (VACCIMEL) with BCG with increasing doses of GM-CSF-placebo, 150, 300, 400, and 600 μg	Twenty melanoma patients in stages IIB, III and IV who were disease- free or had minimal disease	The addition of GM-CSF to VACCIMEL induced statistically significant increased delayed-type hypersensitivity	No conclusions were drawn concerning the clinical status of patients in relation to dose of GM-CSF	[18]

using ELISA assays or multiple cytokines using Luminex technology, or induction of tetramer-reactive T cells. There was a trend for GM-CSF to modestly increase the levels of immune activation as measured by all these assays.

Marshall et al. accrued 58 patients with metastatic CEA-expressing cancers to eight different treatment cohorts involving recombinant (r)vaccinia – CEA 6D TRICOM, and fowlpox CEA 6D TRICOM with or without GM-CSF [16]. Although no clear enhancement of CEA-specific T cell precursors were documented in the groups receiving GM-CSF, there was a trend towards improved survival in the groups receiving GM-CSF.

In another phase II clinical trial, 25 patients with melanoma were immunized with three melanoma HLA-A2-binding peptides [17]. Patients were randomized into three groups: (i) peptides with Montanide ISA-51; (ii) peptides + Montanide + GM-CSF 10 μg; or (iii) peptides + Montanide + GM-CSF 50 μg. Nine patients showed a successful immune response, as measured by delayed-type hypersensitivity testing to gp100 peptides. There did not seem to be any evidence of an enhanced immune response by combining the vaccine with either of the low-dose GM-CSF regimens used. The authors concluded that higher doses of GM-CSF may be needed.

Barrio et al. assessed the role of GM-CSF in a randomized phase I clinical trial of 20 staged IIB, III or IV melanoma patients [18]. The vaccine VACCIMEL consisted of three irradiated allogeneic melanoma cell lines with BCG as an adjuvant. In addition, patients received local injections of placebo, GM-CSF 150, 300, 400 or 600 μg split into four doses. The addition of GM-CSF to VACCIMEL was well tolerated and induced statistically significant increased delayed-type hypersensitivity (DTH) reactions, with the maximal effect occurring in the 400 μg total dose group.

3.1.2. Combination vaccine treatment with Toll-like receptor agonists

Toll-like receptor (TLR) agonists are another important group of molecularly defined activators of APC activity. Different APC subsets express unique profiles of TLRs that play critical roles in activation of the APCs (for review, see [3]), and these influence the activation, maturation, and features of the resulting immune response. TLR agonists are beginning to be evaluated in the clinic. Results from a clinical trial where CpG 7909 (Coley Pharmaceutical Inc.) was combined with the Engerix-B hepatitis vaccine demonstrated that antibody responses to Hepatitis B surface antigen appeared sooner and had higher peaks when the vaccine was administered with the CpG 7909 TLR [19].

TLRs are only beginning to be studied clinically in oncology. Perhaps the most studied are the TLR 9 agonists, or CpGs. Speiser et al. [20] evaluated eight HLA-A2-positive melanoma patients treated with a peptide vaccine containing the Melan-A 26–35 A2 binding analogue peptide mixed with incomplete Freund's adjuvant and CpG 7909. The vaccine was administered monthly, for four subcutaneous injections. Rapid and strong T cell responses occurred in all patients, and relatively high levels of circulating T cells (as high as 3% in some patients) were documented by direct ex vivo flow cytometry. These T cells had an effector memory phenotype (CD45-, CCR7-); they secreted Interferon γ; they expressed granzyme and perforin; and they were able to lyse syngeneic melanoma cells in an antigen-specific manner. When these responses were compared to those of an historical control group of patients treated with the peptide vaccine and IFA without the CpG, the increase in T cell responses was shown to be statistically improved in the group where the CpG was added.

Weihrauch et al. conducted a randomized phase II trial to evaluate several different vaccine combinations with CpG after chemotherapy with irinotecan, 5-FU, and leucovorin in 17 patients with newly diagnosed metastatic colorectal carcinoma [21]. Patients were all HLA-A2-positive, and the vaccine antigen was the CAP-1 CEA peptide. The first vaccination consisted of either (i) 1×10^7 CAP-1 pulsed dendritic cells, (ii) CAP-1 together with the double stem-loop immunomodulators (dSLIM-30L1), (iii) CAP-1 together with 50 μg GM-CSF, or (iv) 50 μg CAP-1 peptide alone. Double stem-loop immunomodulators (dSLIM) are covalently closed dumbbell-shaped DNA molecules containing unmethylated CpG motifs. Subsequent vaccinations involved either 50 μg CAP-1 with IL-2 and one of the following adjuvants: dSLIM, GM-CSF, or placebo. Toxicities were acceptable. Four patients had tetramer-documented increases in T cell precursor frequencies after direct ex vivo evaluation, and eight patients had increases after a 7-day in vitro culture. The highest frequency of tetramer-reactive T cells seen was 0.31%, in one patient. Responders came from groups receiving either a dendritic cell or non-dendritic cell initial vaccination and from the groups receiving CAP-1/dSLIM/IL-2, CAP-1/GM-CSF/ IL-2, or CAP-1 and IL-2. No striking immunologic differences between the adjuvants were detected.

Other CpG or TLR reagents are beginning to be evaluated in the clinic as monotherapies [22–25]. Most advanced amongst these are studies with TLR 7 or Imiquimod®, which is being tested as a topical adjuvant with various cancer vaccines. Published results of Imiquimod® or other TLR agonists in combination with cancer vaccines are not yet available.

3.1.3. Combination vaccine treatment and manipulating the CD40 pathway

Another approach to activate the innate immune system involves stimulation of dendritic cells through the CD40 pathway. CD40 is a member of the tumour-necrosis factor family, and is expressed by dendritic cells, B cells, monocytes, and some other normal cells, as well as by a variety of hematologic and solid malignancies. The natural ligand for CD40 is CD40 ligand, or CD154, which is expressed primarily on the surface of activated T cells, but agonist CD40 monoclonal antibodies may substitute for these cells in the activation of dendritic cells. Not all antibodies to CD40 have agonist

activity. Vonderheide et al. treated 29 patients with several different cancers with escalating doses of the agonist anti-CD40 monoclonal CP-870,893 (Pfizer) [26]. The maximum tolerated dose (MTD) was found to be 0.2 mg/kg. Symptoms that were attributable to cytokine release syndrome occurred, although the dose-limiting toxicities were grade 3 headaches and deep venous thrombo-embolism. A transient depletion of CD19 + B cells was seen in the peripheral blood, but a dose-related up-regulation of co-stimulatory molecules was seen in the remaining B cells after treatment. Four objective partial responses were seen in patients with melanoma, and were felt to be most likely related to immune activation, as expression of CD40 melanoma is generally low. No trials of this monoclonal antibody in combination with a cancer vaccine have yet been published.

There are thus some encouraging early results suggesting that GM-CSF [13] and CpG 7909 [20] given in combination with peptide vaccines may enhance the immunogenicity of these vaccines. Although with GM-CSF the trial results are mixed, there are suggestive clinical and/or immunological data to support a daily dose of 100 μg of GM-CSF for 4 days from several other trials with both viral and cellular vaccines [15,18]. Lower doses may not be useful. Further optimizing of vaccine combinations with these immuno-adjuvants seems warranted. The early results with the agonist anti-CD40 monoclonal antibody [26] suggest that manipulation of this pathway induces a functional immune response that can mediate clinical anti-tumour activity. Further evaluation of whether the immunogenicity of cancer vaccines may be enhanced by combination manipulation of this pathway is warranted.

3.2. Use of cytokines to enhance immune activation

3.2.1. IL-2

Interleukin-2 (IL-2) is an important T cell growth factor that has been studied extensively as a cancer therapy. High doses of IL-2, either alone or with lymphokine activated killer (LAK) cells or tumour-infiltrating lymphocyte (TIL) cells, can mediate anti-tumour activity. However, its therapeutic benefit is limited by its toxicity.

Many trials that have combined various cancer vaccines and IL-2 have been published (Table 2). In an early trial, Rosenberg et al. showed that IL-2 could enhance the anti-tumour activity of the HLA-A0201-restricted modified gp100 209 2M peptide vaccine [27]. Whereas no objective cancer regressions were seen in any of the 11 patients who received the peptide in incomplete Freund's adjuvant, objective remissions were seen in 8 of 19 patients who received the peptide plus a short course of high-dose IL-2 (720,000 IU/kg i.v.) every 8 h to tolerance. Interestingly, increases in specific T cell precursors in the peripheral blood were seen in 3 of 19 patients who received both peptide and IL-2 compared with 10 of 11 patients who received the vaccine without IL-2, leading the authors to postulate that the IL-2 may be promoting tumour cell homing and infiltration.

Slingluff et al. randomized 40 patients with resected stages IIB–IV melanoma to vaccination with four gp100 or tyrosinase peptides, a tetanus peptide, or low-dose IL-2 administered beginning on either Day 7 or Day 28 [28]. The peptides were administered in Montanide ISA-51 and 225 μg of GM-CSF. The vaccines were given on weeks 0, 1, 2, 4, 5, and 6, both subcutaneously and intradermally. T cell responses were assessed both from peripheral blood lymphocytes (PBL) and from a draining sentinel lymph node (SIN). T cell responses were observed from 37% of PBL and 38% of SIN from the patients receiving the IL-2 beginning on Day 7, and from 53% and 83%, respectively, from the group receiving the delayed IL-2. Because immune responses were higher in the group receiving the delayed IL-2 and occurred before the IL-2 was administered, it was concluded that the low-dose IL-2 regimen used in this study diminished the magnitude and frequency of CTL responses induced by these peptides. In addition, evaluation of immune responses in SIN was felt to be more sensitive than that done from PBL.

IL-2 was evaluated in combination with a dendritic cell vaccine pulsed with allogeneic melanoma cell lysates in patients with stage III or IV melanoma [29]. Thirteen patients received the vaccine alone, while seven received it together with low doses of subcutaneous IL-2. The vaccine was administered with Keyhole Limpet Hemocyanin (KLH) intradermally every 10 days for four injections, and the IL-2 dose was 2.4×10^6 IU/m^2 of rhIL-2 injected subcutaneously (s.c.) on Days 2, 3, and 4 of the second, third, and fourth vaccinations. There were no differences in the frequency of enzyme-linked immunospot (ELISPOT) or delayed-type hypersensitivity (DTH) responders in the two groups. For the overall group, there was an association between post-vaccination survival and disease stability and the induction of DTH responses. The authors concluded that although the DC vaccine was partially effective at triggering effective immunity, combining it with IL-2 in the fashion utilized in this trial did not enhance its activity.

Roberts et al. vaccinated 26 patients with advanced melanoma with g209-2M peptide once every 3 weeks with low-dose IL-2 (5 mIU/m^2 daily for 5 days during the 1st and 2nd weeks) [30]. No significant increases in tetramer or ELISPOT reactive T cells were seen, and there were no partial or complete responses.

Lindsey et al. evaluated the addition of IL-2 at various doses or schedules to a prime/boost vaccination approach with recombinant vaccinia–tyrosinase and recombinant fowlpox–tyrosinase in 64 patients with metastatic melanoma [31]. Vaccines were given intramuscularly at doses of 1×10^9 pfu every 4 weeks. Of these, 47 patients were enrolled in a trial where they were randomized to vaccine alone, vaccine followed immediately by low-dose s.c. IL-2, or vaccine followed immediately by high-dose intravenous (i.v.) IL-2. In a subsequent phase II trial, the entire vaccine course was given initially followed by treatment with high doses of i.v. IL-2. Evidence of a cellular response to tyrosinase occurred in a minority of patients, and this response was

Table 2
Clinical trials of Interleukin-2 and cancer vaccine combination treatments

Trial design	Patients	Immunological results	Clinical results	Reference
gp100 209-2M peptide with or without systemic high-dose IL-2	*Thirty patients of whom 11 received the peptide in IFA and 19 received the peptide in IFA plus systemic IL-2*	*Specific T cell precursors seen in 3 of 19 patients receiving peptide and IL-2 compared to 10 of 11 patients who received the vaccine without IL-2*	*8 of 19 patients (42%) receiving the vaccine with IL-2 had objective clinical regressions whereas none of the 11 receiving the peptide alone had an objective cancer regression*	[27]
B7-1 gene modified autologous tumour cell vaccine in combination with systemic IL-2	Fifteen patients with metastatic renal cell carcinoma	T cell infiltrates at DTH skin sites in three of four responding patients	Two patients with partial response and two patients with stable disease	[60]
Dendritic cell loaded with autologous tumour lysate in combination with IL-2	Twelve patients with metastatic renal cancer	Absence of cellular or humoral response	No objective response but extended stable disease	[61]
Vaccination of monocytes and dendritic cells pulsed with peptide with continuous infusion of IL-2	Sixteen patients with recurrent Ewing sarcoma	One patient with immunological response	All patients showed progressive disease	[62]
Prime boost vaccinia-CEA followed by avipox CEA or in the reverse order with GM-CSF followed by optional IL-2 treatment	Eighteen patients with advanced tumours expressing CEA. Seven of these patients were given vaccine followed by IL-2	T cell precursor frequencies continued to increase after IL-2 was added to vaccinations in HLA-A2 positive patients	No objective anti-tumour responses in any patients treated	[63]
PSA DNA vaccine with GM-CSF and IL-2 as adjuvants	Six hormone refractory prostate cancer patients	All three patients receiving the highest dose of vaccine showed an increase of T cell responses by ELISPOT	Decrease in serum PSA in one patient	[64]
Melanoma peptides (gp100 and Mar-1) in IFA. A second protocol included a second gp100 peptide. For patients who progressed given choice of high-dose IL-2	A total of 41 patients in the two protocols. A total of 22 patients received IL-2	No immunology noted after IL-2 treatment	Two objective responses that are comparable responses to IL-2 alone	[65]
DNA vaccination with plasmid expressing prostate-antigen with GM-CSF and IL-2	Nine patients with advanced hormone-refractory prostate cancer	PSA specific cellular immune response and an antibody response was detected in two of three patients in highest dose cohort	Four patients with metastases had stable disease, three patients had a decrease in slope of PSA levels	[66]
DNP-modified autologous vaccine and IL-2	Thirty-four metastatic melanoma patients of whom 24 received IL-2	11 out of 12 responding patients had strong skin reactivity to autologous cells	Response in 12 out of 34 patients (35%); 10 (2 complete response and 8 partial response) of these patients were treated with combination with IL-2	[67]
Four gp100 and tyrosinase-derived peptides, tetanus helper peptide and GM-CSF were followed by low-dose IL-2 administered beginning Day 7 (group 1) or Day 28 (group 2)	*Forty patients with resected stage IIB-IV melanoma*	*Magnitude of T cell responses to melanoma peptides was higher in group 2 at 53% of PBL and 83% of SINs (samples taken before IL-2 treatment) vs. 37 and 38%, respectively, in group 1. Concluded that low-dose IL-2 diminished T cell responses*	*Trend to better overall and disease-free survival in group 2. DFS at 2 years were 39 and 50% for groups 1 and 2, respectively*	[28]
Four melanoma peptides and tetanus helper peptide with GM-CSF and Montanide ISA-51 or pulsed on dendritic cell. Low-dose IL-2 was given to both groups (randomized)	Twenty-six advanced melanoma patients	GM-CSF arm: T cell response in 42% of PBLs and 80% of SINs. In DC arm, response is 11 and 13%, respectively	Objective clinical response in two patients in the peptide/GM-CSF arm and one patient in DC arm	[68]

Table 2 (*Continued*)

Trial design	Patients	Immunological results	Clinical results	Reference
Recombinant fowlpox encoding three forms of gp100. With progressive disease, patients were eligible for crossover to IL-2 treatment	Forty-six metastatic melanoma patients	ELISPOT reactivity was seen in one of seven patients receiving fowlpox native gp100; in 10 of 14 patients receiving fowlpox/modified gp100 and 12 of 16 patients receiving fowlpox/minigene gp100. Immune responses not noted after IL-2	Six out of 12 patients receiving fowlpox containing minigene construct of gp100 showed objective cancer regressions including 3 patients with complete regression. No responses in other two groups	[69]
Newcastle disease virus-modified with autologous melanoma cell lysate with IL-2	A total of 29 Melanoma patients with resectable stage III disease were treated with 8 patients receiving placebo	No immunology	No differences between vaccine and control groups	[70]
Dendritic cell pulsed with tumour cell lysates alone or in combination with low-dose IL-2	*Twenty patients with stage III or IV metastatic melanoma of whom 7 received combined therapy*	*Fifty percent (7 of 13) of the patients tested in vaccine group had ELISPOT response and 44% (3 of 3) in vaccine plus IL-2*	*Stable disease in 11 of 20 patients in trial and 4 of 7 patients who received combined therapy. No significant difference in clinical responses between two groups*	[29]
Dendritomas (purified hybrids from fusion of dendritic and tumour cells) combined with low-dose IL-2	Ten metastatic melanoma patients	Eight out of nine evaluable patients with T cell response	One patient with complete response (9 months after treatment) and two patients with stable disease for 9 and 4 months	[71]
G209-2M melanoma peptide vaccine followed by low-dose IL-2 on Days 1–5 and 8–13	*Twenty-six patients with advanced melanoma*	*Lack of evidence of induction of T cells by ELISPOT or tetramer*	*No objective responses*	[30]
Prime boost recombinant poxvirus vaccine encoding tyrosinase alone or concurrently with low s.c. or high i.v. dose IL-2	*Sixty-four refractory metastatic melanoma patients*	*Enhanced immunity in 6% of patients tested serologically and 25% tested for tyrosinase specific T cell responses for full length tyrosinase protein using RT-PCR*	*Prime boost in combination with IL-2 did not improve clinical responses (8 partial responses/12.5% of patients) over IL-2 alone*	[31]

Italic font: Discussed in text.

not enhanced in the groups of patients receiving IL-2. Objective clinical responses occurred at the same frequencies that were expected in patients receiving IL-2 alone.

Except for one report by Rosenberg et al. of an enhanced anti-tumour response when a peptide vaccine was combined with high doses of IL-2 [27], there are no convincing data to support combining various vaccines with lower doses of IL-2. In fact, the trial by Slingluff et al. suggested that low doses of IL-2 may have a detrimental effect on the immune response generated by a peptide vaccine [28].

3.2.2. IL-12

IL-12 is a heterodimeric glycoprotein produced by dendritic cells that is important for the generation of interferon γ-expressing cytotoxic T cells. It also plays a role in the generation of Th1 cells from progenitor cells. Spontaneous rejection of immunogenic tumours is reduced by elimination of IL-12.

Gajewski et al. evaluated whether IL-12 would enhance the immunization with autologous peripheral blood mononuclear cells pulsed with HLA-A02 epitopes from either MAGE-3 or Melan-A [32] (Table 3). Fifteen HLA-A02 patients were treated with 10^8 PBL pulsed with either of the above peptides every 21 days. Doses of either 0, 30, 100, or 300 ng/kg of IL-12 were injected s.c. adjacent to the vaccination sites on Days 1, 3, and 5 of each vaccination cycle. T cell responses, as assessed by direct ex vivo ELISPOT, occurred in 0/3, 3/3, 3/3, and 1/3 patients at 0, 30, 100, and 300 ng/kg/dose of IL-12. Of eight patients with immune responses, two had classical clinical responses, and four had minor or mixed responses. The authors concluded that the addition of low-dose IL-12 enhanced the immunogenicity and anti-tumour activity of the cellular vaccine.

Lee et al. evaluated 48 patients with high-risk stage III or IV melanoma treated with tyrosinase and gp100 peptides with or without IL-12 at 30 ng/kg administered intradermally at each vaccine/Incomplete Freund's Adjuvant injection site [33]. There were statistically significant increases in DTH and ELISPOT responses in the group that received IL-12. There was no difference detected in clinical response.

In a subsequent trial, Peterson et al. treated 20 HLA-A2 patients with metastatic melanoma with peripheral blood lymphocytes loaded with Melan-A HLA A2 peptides [34]. A dose of 4 μg of rh IL-12 was administered subcutaneously

Table 3
Clinical trials of Interleukin 12 and cancer vaccine combination treatments

Trial design	Patients	Immunological results	Clinical results	Reference
gp100 peptide alone or plus A: IL-12 i.v. (250 ng/kg) B: GM-CSF s.c. (100 or 500 μg) C: IL-2 i.v. alone	Fifty-four patients with metastatic melanoma	Decrease in circulating precursers with IL-2, GM-CSF or IL-12	Six out of 16 patients (38%) that received peptide plus IL-2 had objective cancer regressions. No clinical responses for the vaccine alone, IL-12 or GM-CSF arms	[72]
MAGE-3 or Melan-A peptide-pulsed dendritic cells with IL-12 (0, 30, 100, 300 ng/kg/dose)	*Fifteen patients with metastatic melanoma*	*Patients receiving low to moderate doses of IL-12 developed specific T cell responses*	*Of the eight patients showing increased immunity, six had evidence of clinical activity with 1 Complete Response, 1 Partial Response, 1 minor and 3 mixed responses*	[32]
gp100 and tyrosinase peptides with IFA with or without IL-12 at 30 ng/kg	*Forty-eight patients with high-risk resected stage III or IV melanoma*	*Immune response: by ELISA in 33 out of 38 patients by Tetramer in 37 of 42 patients Significant increase in DTH with IL-12. Significant increased ELISPOT to gp100 and tyrosinase with IL-12*	*Median follow up of 24 months with 24 patients relapsed and 10 died. The time-to-relapse curve was not enhanced in the group receiving IL-12.*	[33]
Treatment with Melan-A peptide-pulsed PMBC with IFA and with IL-12 (4 μg)	*Twenty patients with metatastatic melanoma*	*ELISPOT: increase of T cells against Melan-A and a correlation between Melan-A responses and clinical responses*	*Two patients with Complete Response, five patients had a minor or Mixed Response and four patients with Stable Disease.*	[34]
Melan-A and influenza peptides with recombinant human IL-12 (0, 10, 30 and 100 ng/kg) given either i.v. or s.c.	*Twenty-eight patients of which 23 patients had visceral metastases*	*T cell responses in 3 of the 12 i.v. patients and none of the s.c. patients. No dose-related relationship could be identified between clinical and immunological responses*	*IL-12 (iv): 1 Complete Response, 1 Stable Disease IL-12 (sc): 1 Partial Response, 5 Stable Disease*	[35]
Melanoma peptides (gp100, MART-1 and tyrosinase) with Montanide given with A: IL-12 (30 ng/kg) and Alum B: IL-12 (100 ng/kg) and Alum C: IL-12 (30ng/kg) + GM- CSF (250 μg) (randomized)	*Sixty patients with high risk resected melanoma*	*Higher post-vaccine immune response to IL-12/Alum in combination with GM-CSF. ELISPOT response in 100 ng/kg dose IL-12 were higher than other two arms*	*Risk of recurrence was lowest for group B, intermediate for group A and highest for C Significant association of immune response to MART-1 to relapse-free survival*	[36]

Italic font: Discussed in text.

on Days 1, 3, and 5 of 21-day vaccination cycles. The therapy was well tolerated, with no grade 3 or 4 toxicities documented. Specific immune responses to Melan-A, as documented by direct ex vivo ELISPOT, occurred in the majority of patients after vaccination. Two of 20 patients had complete responses, five patients had minor or mixed responses, and four patients had stable disease.

Cebon et al. evaluated 28 patients with metastatic melanoma with increasing doses of either intravenous or subcutaneous IL-12 (0, 10, 30, or 100 ng/kg) in combination with Melan-A peptides combined with HLA-A2 influenza peptides [35]. Toxicities were greater in patients treated with the i.v. IL-12, with grade 3 toxicities documented. Clinical and immunologic responses were seen with both routes, and were not clearly dose related. Overall, however, increases in specific T cell precursors were seen in only 3 of the 12 patients who received the IL-12 by the i.v. route, and in none of the patients who received it s.c. Nine of the patients had an immunologic response to influenza protein. Two patients had objective clinical responses, including a complete response of cutaneous disease and a partial response in a patient with hepatic metastases. There were six patients with stable disease. There were no clear relationships between the clinical and immunologic responses, and the clinical responses were not clearly dose- or route-dependant.

Most recently, Hamid et al. randomized 60 patients with high-risk resected melanoma to vaccination with gp100, mart and tyrosinase peptides with either (i) IL-12 30 ng/kg with Alum, (ii) IL-12 100 ng/kg with Alum, or (iii) IL-12 30 ng/kg and GM-CSF 250 μg [36]. Significantly higher responses were seen in the group receiving the Alum and high-dose IL-12. This group was also found to have the highest relapse-free survival.

The results evaluating IL-12 in combination with several different vaccine technologies are mixed. While Gajewski et al. showed that immune responses were greatest when their PBL-peptide vaccine [32] was combined with increasing doses of IL-12, and that the combination of IL-12 with peptide-pulsed PBMC could even yield some objective responses in the work of Peterson et al. [34], there was no clear enhancement in the immunogenicity of a peptide vaccine by IL-12 in the trial by Cebon et al. [35]. Lee et al. and Hamid et al. however, both showed improved immunologic responses in groups receiving IL-12, with the Hamid et al. study indicating a relationship to clinical response [33,36].

3.2.3. *Interferon α*

Interferon α, one of the Type 1 interferons, is probably the most studied of the cytokines in the clinic, and has a number of immunologic effects. It binds to a heterodimeric receptor, and initiates a signalling pathway that activates gene expression of a number of interferon-sensitive genes. It up-regulates many immunologically important genes, such as MHC, co-stimulatory molecules, and many potential tumour-associated antigens. It may promote the activation and function of APCs, the function of CTLs, and the generation of memory T cells, as well as the activation and proliferation of natural killer (NK) cells. Interferon has been shown to be active in many pre-clinical tumour models, and has been approved by the FDA for the adjuvant therapy of patients with stage 3 melanoma.

Mitchell et al. treated 18 patients with metastatic melanoma after they had failed to respond to vaccine therapy with the allogeneic cell lysate vaccine Melacine (and Detox adjuvant) [37] (Table 4). Patients received 5–6 million units of Interferon α three times a week subcutaneously for at least 2 months. Eight of the patients (44.4%) had a major objective clinical response, including five complete responses. The median duration of responses was 11 months, and the median survival duration of the responders exceeded 32 months. Specific cytolytic T cell responses, presumably induced by the previous vaccine therapy, were documented in all five of the complete responders.

Kirkwood et al. reported the results from a randomized phase II trial of 107 patients with stages IIB, III, or IV melanoma treated with the GM2-KLH vaccine in combination with the adjuvant QS 21, either alone, concurrently or followed by the high-dose Interferon α regimen [38]. Only 64 of the patients completed the entire 1-year course of treatment. More patients discontinued therapy in groups 1 and 2 because of the interferon toxicity, and more patients in group 3 discontinued because of progressive disease. The induction of antibody response to GM2 was comparable in all three groups. The relapse-free survival for patients in groups 1 and 2 who received interferon was greater than for group 3, although the result did not reach statistical significance. There were no clear differences in outcome or immunology between the two different interferon regimens (concomitant versus post-vaccination).

Vaishampayan et al. expanded their experience with this approach in a phase II trial that combined Melacine with pre-treatment cyclophosphamide and post-treatment Interferon α [39]. Cyclophosphamide was given at a dose of 300 mg/m^2 i.v. 3 days before the first dose of Melacine, and interferon was given at 5 million units/m^2 s.c. three times a week starting immediately after the fourth vaccination and given until progression. Melacine was given with Detox adjuvant at a dose of 10^7 cell equivalents administered s.c. weekly for 4 weeks and then at week 6. Forty-seven patients with metastatic melanoma were treated. The toxicity was well tolerated. In 39 evaluable patients, the overall response rate was 10.2%, and 64% of patients had disease stabilization of at least 16 weeks. The median time to disease progression in the evaluable patients was 8 months, and the median survival time for the entire group was 12.5 months. No immunologic responses were reported. The authors note that the response rates seen in the earlier trial was not reproduced, although they were encouraged by the degree of disease stabilization observed. They noted that this trial differed from the earlier one in that the interferon was started earlier, during vaccine therapy. (The administration of cyclophosphamide was also an addition, but

Table 4
Clinical trials of Interferon α and cancer vaccine combination treatments

Trial design	Patients	Immunological results	Clinical Results	Reference
Patients who were previously treated with Melacine vaccine and who had failed to respond were then treated with 5 or 6 MU/m^2 of IFN-α s.c. three times a week for at least 2 months	Eighteen patients with disseminated melanoma	Cytolytic T cell precursors have been increased in five of five HLA-A2+ responding patients and in five of eight non-responders	Eight of 18 (44.4%) patients had a major objective clinic response induced by IFN-α including site-specific complete remission in 5 patients. Medium survival of responders was 36 months and of the non-responders was 7.3 months. The median duration of response was 11 months	[37]
Combination of GMK (GM2-KLH/QS-21) and IFN-α Arm A: vaccination with GMK plus induction therapy of IFN-α on Day 1 Arm B: vaccination with GMK plus induction therapy of IFN-α on Day 28 Arm C: vaccine	One hundred seven patients with stages IIB, III or IV melanoma	No differences in the percentage of antibody responders or titer threshold during the trial for the three arms of the trial. INF α does not affect the anti-GM2 antibody response	Median follow-up time was 23.9 months with median RFS time for Arm C of 14.9 months, arm B of 30.7 months and was not reached for arm A. Results did not reach statistical significance	[38]
Melanoma vaccine consisting of two allogeneic cell lines and Detox-PC adjuvant (Melacine) with Cyclophosphamide and IFN-α (given after fourth dose of Melacine at 5 MU/m2 three times a week)	Forty-seven patients with metastatic melanoma	N/A	Of 39 evaluable patients, 2 patients had minimal response; another 25 patients (64%) had stabilization of disease. Overall response rate of 10.2%	[39]
Viral vector vaccine expressing gp100 followed by high-dose IFN-α	Seven patients with metastatic melanoma or high risk of developing metastases	High doses of IFN-α recalled gp100 reactive T cells. No recalls in T cell responses for patients who did not respond initially to vaccine treatment	Tumour regression was observed in two of the three patients with clinically evident metastatic disease. Both of these patients were immunological responders	[40]
Tumour-derived heat shock protein peptide complexes Gp-96 (HSPPC-96) in combination with GM-CSF and IFN-α (3 MU s.c. twice weekly, 1 and 3 days after the last administration of GM-CSF)	Thirty-eight pretreated metastatic melanoma patients	Increase in class I HLA-restricted T and NK cell-mediated post-vaccination response in 5 out of 17 and 12 out of 18 patients tested. Four out of the five patients with T cell responses also had stable disease	There were 20 patients who received at least one cycle of HSPPC-96. Eleven patients had stable disease and one patient remained disease free after one cycle	[42]
Melanoma peptides (gp100, Melan-A) plus IFN-α (3MU s.c. on Days −1, 0, +1 with respect to peptides)	Ten pretreated patients with metastatic melanoma	Five of the seven evaluable patients, a consistent enhancement of CD8+ T cells recognizing Melan-A and gp100 was observed	Of the seven patients who completed at least the first vaccination cycle, two patients had stabilized disease for +24 and +13 months; prolonged disease-free interval of +11 months for one patient	[41]

this was not thought to be responsible for the lower response rate.)

Astsaturov et al. treated seven patients with a course of high-dose Interferon α (20 million units/m^2 i.v. × 20 doses) from 1.5 to 17 months post-vaccination with the canary pox vaccine ALVAC-gp100 (209-2M, 280-9V) [40]. All patients required dose reductions of interferon because of toxicities. Four of the patients who had had T cell responses to the earlier vaccine monotherapy had a recall in the T cell precursor frequency to gp100 with the interferon therapy. There were no recalls in the other three patients who had not responded initially to the vaccine therapy. The T cells recalled by the interferon had greater functionality, as measured in a cytotoxic T lymphocyte chromium release assay. Clinical responses were seen in the only two patients of the four immunologic responders who had measurable disease.

Di Pucchio et al. performed a pilot phase I/II clinical trial to determine the effects of Interferon α administered as an adjuvant to a small group of patients with metastatic melanoma treated with Melan-A 26–35 (27L) and gp100 209–217 (210 M) peptides [41]. The peptides were administered intradermally every 2 weeks for four injections, and the interferon was administered at a dose of 3 million units s.c. on Days −1, 0, and +1 with respect to the peptides. The peptides and interferon were injected in close proximity to each other, adjacent to draining lymph node regions. There were no objective clinical responses, but two patients had prolonged periods of stable disease (+24 and +13 months). Five of the seven patients showed a progressive increase in the frequency of T cells producing Interferon γ to the Melan-A and to a lesser extent the gp100 peptides. In three of the five patients, the increase in T cells was confirmed by an increase in tetramer-reactive T cells. All tested patients showed an increase in terminally differentiated effector cells of the CD45RA + CCR7-phenotype. Three of seven patients showed an increase of effector memory T cells of the CD45-CCR7-phenotype. An increase in the percentage of CD14 + CD2 + monocytes was seen after both the first and fourth interferon/peptide treatment with respect to pretreatment values. There was also an increase in the allo-proliferation (of allogeneic T cells) induced by post-vaccination monocytes of three non-progressor patients. The authors concluded that interferon may play a role in expanding effector memory T cell pool that could eventually differentiate into effector T cells. They concluded that further clinical trials would be required to confirm and understand the adjuvant activity of Interferon α.

In contrast to the above results, Pilla et al. evaluated the effects of vaccination on 38 patients who had undergone surgery for metastatic melanoma. The vaccine consisted of autologous tumour-derived heat shock protein gp96-peptide complex [42], administered subcutaneously every week for four injections. GM-CSF was given for 3 days before, on the same day, and the day after the vaccination, s.c. at the same site at a dose of 75 μg/injection. Interferon α was given at 3 million units s.c. twice weekly, 1 and 3 days after the last administration of GM-CSF during the first cycle, and at a site different from the GM-CSF. Patients with stable disease received ongoing vaccination. The treatment was well tolerated, and 20 patients received at least four injections. Eleven of 18 patients with measurable disease showed stable disease. There was evidence of increased T cell precursor frequencies post-vaccination in 5 of 17 patients. Four of the five patients with ELISPOT responses had stable disease clinically. The authors concluded that the immunologic and clinical results were not better than those seen previously in historically treated patients without GM-CSF or interferon.

There are several positive and encouraging results from studies employing combinations of several different vaccine technologies and different doses and schedules of Interferon α. In two trials, objective clinical responses were seen by the combination with either a cellular vaccine [37] or the ALVAC viral vaccine [40]. In addition, the trial by Di Pucchio et al. showed a temporal relationship for increases in peptide-specific effector and effector memory T cells subsets after interferon therapy [41]. In this trial, the interferon was administered locally and close to the vaccination site; the other trials used different doses and schedules. Further studies to optimize how to administer Interferon α with various vaccine technologies are warranted, to build upon these results.

3.3. Reagents that inhibit T cell inhibitory signals

3.3.1. Anti-CTLA-4

The CTLA-4 receptor is a member of the immunoglobulin supergene family. It is an inducible receptor found on activated CD4 and CD8 T lymphocytes, and it binds to CD80 and CD86 with up to 2500 greater avidity than does CD28 [43]. Engagement of this receptor inhibits activation, and in fact promotes cell cycle arrest and decreased cytokine production. As this receptor is up-regulated on T cells after activation, the physiologic role of signals through this receptor may be to dampen T cell responses and prevent excessive or autoimmune responses. Homozygote knock-outs of CTLA-4 result in early lethality from excessive polyclonal proliferation. CD4+, CD25+ Tregs constitutively express CTLA-4.

A blocking humanized monoclonal antibody (Ipilimumab) of the IgG1/κ isotype has been generated that binds to CTLA-4 and blocks the binding of CD80 or CD86 to CTLA-4. As a monotherapy, this monoclonal antibody can cause regression or prevent the outgrowth of various established murine tumours in syngeneic mice. The degree of anti-tumour activity may be related to the relative immunogenicity of the tumour. Dramatic synergy was seen with combination with the murine GVAX melanoma vaccine and anti-CTLA-4, with complete prevention of tumour outgrowth. This protection was associated with development of vitiligo and increased infiltrations of CD4 and CD8 T

lymphocytes at the tumour sites [44]. These and other data provided the rationale for clinical evaluation of anti-CTLA-4 as a monotherapy or in combination with cancer vaccines, such as GVAX.

Two human anti-CTLA-4 monoclonal antibodies are being studied in the clinic: MDX-010 and CP-675,206. Only phases I and II trials have been reported. There have been several general findings. First, in heavily pretreated patients, the anti-CTLA-4 monoclonals can induce objective responses as monotherapy in 7–15% of patients. Responses can be seen in various visceral sites, including lung and brain. Often there is evidence of immunologic infiltrates and necrosis in tumour sites, even at sites where regression is not seen. While various doses and schedules have been studied, the optimal administration remains to be defined. Treatment with anti-CTLA-4 is associated with many immune-related adverse events, including erythematous rash, immune infiltrates into the gastrointestinal tract causing colitis, hypophysitis, uveitis, nephritis, and hepatitis [45,46]. Interestingly, serious adverse events (i.e., grade 3 or 4) have been correlated with anti-tumour responses [47].

The humanized monoclonal 10D1 to CTLA-4, which cross-reacts to cynomologous monkey CTLA-4, was studied along with a hepatitis and melanoma vaccine in a toxicology experiment [48]. The humoral responses to an HBsAg vaccine were significantly enhanced by a single injection of the monoclonal 1 day before priming and boosting with the vaccine. There was an increase in antigen-specific CD8+ and CD8- T cell responses in one of four animals. There was no evidence of lymphocytic infiltrations in sections from the intestines or colon [48].

The CTLA-4 monoclonal was tested in combination with a cellular vaccine consisting of the human melanoma cell line (SKmel-GM) transfected with the GM-CSF gene. The vaccine was given monthly for six injections preceded by a single dose of the anti-CTLA-4 monoclonal at 10 mg/kg. Dramatic increases in the humoral response, as determined by a flow cytometry binding assay to the vaccine cells, were demonstrated. Greater antibody-dependant cellular cytotoxicity was seen from the sera of the anti-CTLA-4-treated monkeys. A T cell proliferative response to the tumour target was enhanced in one of the six animals. Specific ELISPOT assays could not be performed, as there was no specific antigen defined.

Hodi et al. infused CTLA-4 into nine cancer patients who had been previously vaccinated with several different cancer vaccines. Seven patients had metastatic melanoma, and two had ovarian carcinoma [49]. Three of the melanoma patients and both ovarian cancer patients had previously received irradiated autologous tumour cells engineered to secrete GM-CSF; three of the melanoma patients had been immunized with autologous dendritic cells engineered to express gp100 and MART-1 by adenoviral gene transfer; and one melanoma patient had received the gp100 peptide and high-dose IL-2. Anti-CTLA-4 was administered as a single dose at 3 mg/kg. The anti-CTLA-4 treatment was well tolerated. Evidence of autoimmunity with auto-antibodies occurred in four patients. The melanoma patients developed skin rashes, with evidence of dying melanocytes and T cell infiltrates on biopsy, and extensive tumour necrosis occurred in three of them. Reduction or stabilization of CA-125 occurred in both of the ovarian cancer patients. The authors conclude that specific characteristics of the pre-existing tumour immunity may influence the responses to CTLA-4 blockade.

Phan et al. treated 14 patients with metastatic melanoma with serial i.v. administration of anti-CTLA-4 (MDX-010) in conjunction with s.c. vaccination with two HLA-A2-01 peptides from gp100 (209–217 (210M) and 280–288 (288V)) [47]. All patients were HLA-A2-01-positive. Patients received infusions of the anti-CTLA-4 monoclonal every 3 weeks, followed by subcutaneous immunization with the two peptides in incomplete Freund's adjuvant in two separate arms. Patients received from one to six cycles. Six patients had grade 3/4 autoimmune toxicities, including dermatitis, enterocolits, hepatitis, and hypophysitis. Tumour regressions occurred in three patients, including two complete and one partial response. Regressions of pulmonary, subcutaneous, and brain metastases were seen. Using ELISPOT assays, no increases in T cell precursor frequency to the gp100 epitopes were seen, and thus the authors concluded that the anti-CTLA-4 did not appear to enhance the documented immunologic responses expected from the peptides. Changes in the phenotype of CD3+, CD4+ T cell subsets were seen.

Sanderson et al. vaccinated 19 patients with high-risk resected stages III and IV melanoma with three peptide epitopes from gp100, MART-1, and tyrosinase [50]. Anti-CTLA-4 was administered intravenously every 4 weeks at escalating doses from 0.3 to 1 and 3 mg/kg, and the vaccine peptides were administered in incomplete Freund's adjuvant subcutaneously immediately after each infusion. Patients received 6 months of treatment, followed by two treatments 3 months apart. The maximal tolerated dose was found to be 1 mg/kg of anti-CTLA-4, based upon the occurrence of grade 3 toxicity in three patients at 3 mg/kg of anti-CTLA-4 treatment. Disease relapse occurred in only three of eight patients with evidence of autoimmunity, compared to nine of 11 patients without autoimmunity. Immune responses to gp100 and MART-1 were measured by tetramer and ELISPOT assays, with 43% of patients having an increase in ELISPOT reactivity to either the gp100 or MART-1 peptides. Homing receptor expression for GI mucosa (CCR9) increased by 41% on CD4+ T cells, and 8 of 13 patients had significant increases in staining of their CD4 T cells for CCR9 after treatment with MDX010. The authors concluded that the level of immunity seen seemed greater than would be expected for treatment with the peptides alone, and noted that the immunologic effects (such as those seen by Phan et al.) may be greater on the CD4+ subsets.

Attia et al. extended their experience to 56 patients with metastatic melanoma treated with peptides and anti-CTLA-4 [51]. Patients were treated with doses of either 1 mg/kg or

3 mg/kg of anti-CTLA-4 every 3 weeks, and received concomitant vaccinations with two modified HLA-A201 gp100 peptides after each infusion. Peptides were administered s.c. in incomplete Freund's adjuvant. All patients were HLA-A201 positive. Two patients achieved a complete response, and five had partial responses. Tumour regressions were seen in visceral sites including lung, liver, brain, and lymph nodes, as well as in subcutaneous sites. Objective responses were seen in five of 14 patients with grade 3 or 4 autoimmune toxicities compared to 2 of 42 patients with no autoimmune toxicities. There was no relationship of toxicity or objective responses to the doses of the anti-CTLA-4. Results from immunologic assessments using in vitro sensitization tests suggested that treatment with anti-CTLA-4 did not increase the frequency or the magnitude of the immune response. The authors concluded that breaking tolerance to self-antigens may be a prerequisite for breaking tolerance to tumour-associated antigens, in the pursuit of achieving clinical responses in cancer.

Anti-CTLA-4 monoclonal antibody therapy is clearly a very active biologic agent, and can non-specifically reduce immune tolerance. As of yet, results from trials where anti-CTLA-4 has been combined with various cancer vaccines have not clearly shown enhancement of the magnitude or functionality of specific immune responses. Further trials are needed to identify cancer vaccines that might synergize with anti-CTLA-4 and to define optimal doses, schedules, and timing of anti-CTLA-4/cancer vaccine combination therapies.

3.3.2. *Specific elimination of Tregs*

The existence of both naturally occurring and adaptive Tregs has been considered for many years. More recently, there has been clear experimental evidence in many murine models that T cells of the CD4+ phenotype that are derived from the thymus can prevent or reduce autoimmune disease [52]. For example, adult thymectomy and subsequent sublethal irradiation produced Type 1 diabetes and thyroiditis in selected strains of rats. It has also been shown that Tregs can be generated outside the thymus in certain conditions. These can be generated using a variety of approaches [52], usually involving T cell activation in the presence of immunomodulating cytokines, such as IL-10 or repetitive stimulation from non-professional APCs. These cells have a CD4+, CD25+ phenotype and express high levels of IL-10, TGFβ, and IL-5. They also express CTLA-4 and Fox P3. In several animal models, depletion of Tregs leads to enhanced anti-tumour immunity [53,54].

Dannull et al. showed in preclinical experiments that human CD4+, CD25+ Tregs can be eliminated with a single short (6-h) exposure to DAB389-IL-2 without significant bystander toxicity [55]. DAB389-IL-2 (trade name Ontak®) is a recombinant conjugate of the active sequences of diphtheria toxin and IL-2, and was granted Orphan status by the FDA in 1999 for the treatment of cutaneous T cell lymphoma. They found that DAB389-IL-2 also abrogated DC-mediated activation of T cells in vitro, suggesting that the timing of DAB389-IL-2 treatment may be important and should be restricted to pre-vaccination. They treated seven renal cancer patients with a single dose of DAB389-IL-2 at 18 μg/kg 4 days prior to vaccination with RNA-loaded DCs (for gp100 and MART-1). Four control patients were treated with the vaccine alone. Significant reductions in Treg numbers were seen in the patients who received the DAB389-IL-2 infusion. This was associated with higher frequencies of tumour antigen-specific CD8 T cells when compared to patients receiving the vaccine alone. The duration of the Treg reduction was transient, and most Tregs reappeared by 2 months. Frequencies of tumour-specific CD8+ T cells reached as high as 0.9% after DAB389-IL-2 treatment.

Attia et al. treated 13 patients (12 with metastatic melanoma and one with metastatic renal cancer) with DAB389-IL-2 [56]. Ontak® was given for 5 consecutive days at doses of either 9 or 18 μg/kg, and was not combined with any vaccine. The treatment was repeated after 21 days. No significant impact of the Ontak® treatment upon immunologic parameters was seen. Specifically, there was no specific decrease in FoxP3 expression, although small decreases were seen in the patients who received the 18 μg/kg dose. There was no reduction in the suppressive effects of CD4+ CD25+ T cells. No clinical responses were documented.

In contrast, Mahnke et al. conducted a phase I trial to evaluate the toxicities, immune responses, and anti-tumour activity to vaccination of melanoma patients after depletion of Tregs [57]. Seven HLA-A201 patients with metastatic melanoma were treated with three daily infusions of Ontak® at one of two different doses (5 or 18 μg/kg). On the 4th day, they received vaccination with two HLA peptides (MART-1 and gp100) intradermally. This vaccination schedule was repeated a second time a month later. Prior to initiating the trial, Mahnke et al. showed that Ontak® could reduce Treg numbers in vitro, although depletion was not complete. In addition, the depletion was associated with decreased T cell suppressive activity from these cells. In the clinical trial, Ontak treatment was safe, and reductions of Tregs (CD4+, CD25+, FoxP3+) occurred in all patients from mean levels of approximately 5% pretreatment to about 1% post-treatment. This was also associated with decreased suppressive activity. In addition, there were enhanced contact dermatitis and proliferative responses to DCP post-Ontak® treatment. Finally, Ontak® treatment was associated with increases in tetramer and ELISPOT-reactive T cells to both gp100 and MART, as well as with increased specific cytotoxicity. No objective clinical responses were seen.

Two trials using Ontak® to target the high-affinity IL-2 receptor on Tregs have convincingly shown that Treg number and function can be reduced by Ontak® [55,57]. In one trial, increased numbers of antigen-specific CD8+ T cells were documented in patients treated with Ontak® and an RNA vaccine compared to those treated with the vaccine alone

[57]. These very preliminary results are encouraging, and further optimization with Ontak® or more specific reagents targeting Tregs holds promise.

4. Conclusions

Therapeutic cancer vaccines need help.

Although many of these vaccines have been shown to break tolerance to tumour-associated self-antigens and induce some level of humoral or T cell response in some cancer patients, for the most part this has not been sufficient to mediate significant anti-tumour clinical activity [58]. This is probably not surprising, given our expanding understanding of the complexity of the immune system and the many checks and balances that regulate it. It has become clear that the human immune system has evolved to ensure that immune reactions to foreign pathogens or self-antigens are tightly controlled to prevent excessive tissue damage or autoimmunity. Thus, whereas a cancer vaccine will perform an important function to focus a possible immune response, strategies to manipulate these checks and balances will likely need to be added in order to achieve a useful and functional clinically relevant response.

As reviewed in this paper, there are a number of different pathways that can be considered, and clinically applicable reagents to manipulate these pathways are starting to be studied. These reagents, already in the clinic, have been directed to a variety of different pathways, including activation of antigen presenting cells through different molecularly defined receptors (GM-CSF, CD40, TLRs); activation of cytotoxic T cells through type I cytokines (IL-2, Interferon α, and IL-12); and inhibition of some of the major inhibitory pathways, such as anti-CLTA-4 and Treg cells. In the very near future, there will be results from clinical trials of other reagents that are just beginning to be evaluated, such as antibodies or inhibitors to PD1, IL-10 and TGFβ, and a variety of other TLRs.

There have been many early phase clinical trials that have combined various cancer vaccines and immunomodulators. These trials have been valuable in demonstrating safety and to some extent providing information about doses and/or schedules that can be used or conversely that should not be used. However, with only a few exceptions, because of their design these trials have not demonstrated the immunologic or clinical value or enhancement of these combinations. Most of them have included small numbers of patients, who often have end-stage disease and have failed multiple previous treatments, all of which would likely have a negative impact on the intactness of their immune systems. Objective response endpoints may not be appropriate for immune therapies aimed at halting progression, and careful evaluation of disease stabilization, preferably as progression-free endpoint in a randomized setting, are not frequently evaluated [1]. Probably the biggest limitation of many of these trials is that combinations were not evaluated in a randomized fashion (e.g., with or without immunomodulator, or at various doses or schedules of the immune modulator). Their intent was to compare an immune or clinical result to that of historical controls, which of course is almost always difficult to interpret. The need for randomized comparisons of these vaccine/immunomodulator combinations in future trials cannot be overstated.

A number of the combination trials mentioned above are particularly noteworthy. In one of the few randomized clinical trials with GM-CSF, Weber et al. demonstrated that 5 days of 250 μg GM-CSF with peptide vaccines enhanced multiple immunologic parameters over that seen with the peptides alone [13]. Markovic et al. showed that lower doses of GM-CSF may not be effective [17]. The trial by Speiser et al., although not randomized, provided convincing evidence that CpG 7909 together with the Melan-A peptide vaccine could induce impressive effector memory T cell responses [20]. Randomized data from Slingluff et al. showed clearly that low doses of IL-2 were not effective in enhancing peptide vaccines, and in fact may diminish the responses [28]. Randomized data from Gajewski et al. provided some support that low doses of IL-12 may enhance peptide-pulsed cellular vaccines, although more definitive data are still required [32]. Probably the strongest data to support the value of adding a cytokine comes from Interferon α. In an early trial that combined Interferon α with the cellular vaccine Melacine, an unexpectedly high objective clinical response rate was seen (8 of 14 patients) [37]. More recently, Astsaturov et al. demonstrated objective clinical regressions in two patients treated with the viral vaccine ALVAC-gp100 followed by high doses of Interferon α [40]. Di Pucchio et al. have also showed that Interferon α in combination with a peptide vaccine generated convincing effector or effector memory T cell responses in all seven of seven treated patients [41]. The optimal dose and schedule of combination treatment with Interferon α still need to be defined. At present, although anti-CTLA-4 is a promising and active biologic agent, there is no clear evidence that specific immunogenicity induced by a cancer vaccine can be enhanced using the doses, schedules, and vaccines that have been studied to date [47,59]. Finally, the initial results from two trials with Ontak® show that Tregs in cancer patients can be reduced, and that T cell responses by some cancer vaccines can be enhanced [55,57].

These initial promising results provide leads for further exploration and optimization. In addition, many new immune modulators with improved functional profiles are being developed. These include novel TLR agonists, naturally occurring or molecularly modified cytokine variants, more specific manipulators of Tregs, and other agents to reduce or eliminate negative signals in T cells. There is reason to be optimistic that even more consistent functional and meaningful specific immune responses can be induced by combination therapies. As we move forward, it is important that we continue to build upon the promising early results and in addition, study new immunomodulator/vaccine combinations in well-designed clinical trials.

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