Novel tumour antigens and the development of optimal vaccine design

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Abstract: The interplay between tumours and the immune system has long been known to involve complex interactions between tumour cells, immune cells and the tumour microenvironment. The progress of checkpoint inhibitors in the clinic in the last decade has highlighted again the role of the immune system in the fight against cancer. Numerous efforts have been undertaken to develop ways of stimulating the cellular immune response to eradicate tumours. These interventions include the identification of appropriate tumour antigens as targets for therapy. In this review, we summarize progress in selection of target tumour antigen. Targeting self antigens has the problem of thymic deletion of high-affinity T-cell responses leaving a diminished repertoire of low-affinity T cells that fail to kill tumour cells. Thymic regulation appears to be less stringent for differentiation of cancer–testis antigens, as many tumour rejection antigens fall into this category. More recently, targeting neo-epitopes or post-translational modifications such as a phosphorylation or stress-induced citrullination has shown great promise in preclinical studies. Of particular interest is that the responses can be mediated by both CD4 and CD8 T cells. Previous vaccines have targeted CD8 T-cell responses but more recently, the central role of CD4 T cells in orchestrating inflammation within tumours and also differentiating into potent killer cells has been recognized. The design of vaccines to induce such immune responses is discussed herein. Liposomally encoded ribonucleic acid (RNA), targeted deoxyribonucleic acid (DNA) or long peptides linked to toll-like receptor (TLR) adjuvants are the most promising new vaccine approaches. These exciting new approaches suggest that the 'Holy Grail' of a simple nontoxic cancer vaccine may be on the horizon. A major hurdle in tumour therapy is also to overcome the suppressive tumour environment. We address current progress in combination therapies and suggest that these are likely to show the most promise for the future.

Keywords: checkpoint inhibitors, cytotoxic CD4 T cells, neo-epitopes, stress-induced post-translational modifications, T-cell avidity

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Introduction

Prophylactic vaccines, stimulating protective antibody responses, have been very effective at preventing infectious disease and cancers induced by the human papilloma virus (HPV) and associated with the hepatitis B virus (HBV). In contrast, vaccines administered after disease onset to induce a therapeutic cellular immune response have yet to show significant efficacy. This is in part due to the disease process that induces an immunosuppressive environment. Induction of cellular immunity requires uptake of antigens by antigen-presenting cells (APCs) such as dendritic cells (DCs). These cells migrate to the local draining lymph node, process antigens and present peptides on major histocompatibility complex-1 (MHC-I) and MHC-II molecules. Typically, peptides presented on MHC-II stimulate the T-cell receptors (TCRs) of CD4 T cells that secrete a variety of cytokines to amplify the immune response. CD4 T cells are the first cells to migrate to the site of infection, or tumour site. They release cytokines such as interferon gamma

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(IFNγ) and tumour necrosis factor alpha (TNFα) which can directly induce apoptosis of target cells and also upregulate MHC to enhance T-cell killing. Furthermore, they induce an inflammatory cascade to promote the extravasation of other immune effector cells such as CD8 T cells whose TCRs recognize peptide presented on MHC-I. Both CD4 and CD8 T cells not only respond to TCR stimulation and are influenced by cytokines, but also a wide variety of costimulatory molecules that either amplify or repress TCR signalling. These include toll-like receptor (TLR) ligands (reviewed in Sasai and Yamamoto[1]) and costimulatory molecules such as OX40, ICOS and CD28, as well as negative regulatory mechanisms acting through CTLA4 and PD-1 pathways (summarized in Table 1).[2]

In the early stages of tumourigenesis, tumours express a range of stress-related molecules that activate DCs and the immune system. This process is termed 'immunosurveillance' and can result in tumour rejection. Tumours that rapidly acquire mutations are very heterogeneous, which allows selection of clonotypes that are resistant to immune attack. This process, known as 'immune editing', results in increasingly immunosuppressive tumour environments and rapid tumour growth. It also results in tumours with a wide spectrum of tumour-infiltrating lymphocytes from very low levels known as 'cold' or 'immune desert' tumours to very high levels termed 'hot' or 'inflamed' tumours.[3] This is particularly evident in colorectal tumours where the density and type of immune infiltrate (immunoscore) is a better prognostic indicator than tumour, node and metastasis staging.[4] Some tumours, particularly pancreatic tumours have a third phenotype known as 'immune excluded', where a physical barrier prevents infiltration.[3]

The clinical benefit of first-generation cancer vaccines has been limited by the choice of antigen, suboptimal design of the vaccines and by the immunosuppressive tumour environment. Second-generation vaccines and combinatorial studies may overcome some of these limitations.

Tumour antigen

Early studies concentrated on stimulating T-cell responses targeting overexpressed self antigens. The TCR repertoire is both positively and negatively selected on self antigens in the thymus therfore high-affinity T cells to strongly binding self peptides are deleted, and low-affinity T cells to weakly expressed self antigens are not positively selected. Most self epitopes will stimulate moderate avidity T-cell responses that are only triggered by high levels of expression of the cognate MHC:peptide on the tumour. As there are about one million MHC molecules on any one tumour cell, but a billion possible T-cell epitopes, any one peptide MHC complex is only presented at low density. High-avidity T cells are stimulated by low levels of cognate MHC:peptide and rapidly kill tumour cells. In contrast, moderate-avidity T cells require much higher levels of signalling to reach their activation threshold and this is rarely achieved by tumour cells. Many early vaccines targeting self antigens triggered moderate-avidity T cells, which failed to result in tumour regression. Indeed, this could be one reason for the recent failure of the PROSTVAC cancer vaccine as it targeted the self-antigen prostate-specific antigen (PSA).

To gain immune recognition, the tumour must present an epitope that has avoided thymic regulation and binds with higher affinity than the self epitopes. This could be a neo-epitope which is formed by a mutation that occurs during oncogenesis, cancer-associated viral epitopes or post-translationally modified epitopes. More occasionally, there are epitopes from self antigens that have avoided thymic deletion.

Neo-epitopes

Tumour neo-antigens are the consequences of the genetic alteration accumulated by cancer cells during the tumourigenesis process. These can include single nucleotide variants, frameshifts and insertion and deletion (INDELs) mutations. The advent of next-generation sequence has now allowed rapid identification of these mutations. Most of these mutations are specific to each individual tumour, allowing the design of personalized vaccines. Somatic nonsynonymous mutations that accumulate in cancer should avoid thymic selection and stimulate high-avidity T cells. The higher the mutation rate within the tumour, the more possibilities of mutations that are potential targets for T cells. Unfortunately, most mutations do not increase MHC binding or TCR recognition and therefore do not stimulate strong T-cell responses. Tumours such as lung, melanoma, bladder and renal cancers that have high association with environmental carcinogens have the highest mutation rates[5] and are most likely to

Table 1. Overview of costimulatory and inhibitory receptors.

Coreceptor	Activatory/ inhibitory	Expressed on	Ligand	Ligand expression
CD4	Both	T cells, macrophages, monocytes	MHC-II	DC, some T cells, B cells, macrophages, monocytes
CD8	Both	T cells, DC subset	MHC-I	Most cells
CD244 (2B4)	Both	Macrophages, monocytes, basophils, NK cells, some T cells	CD48	Majority of leukocytes
CD28	Activatory	T cells	CD80 (B7-1), CD86 (B7-2)	DC, activated T cells, B cells, macrophages, monocytes
CD27	Activatory	T cells, B-cell subset and NK-cell subset	CD70	Activated DC, B cells and T cells
ICOS (CD278)	Activatory	Activated T cells, Th2 cells, NKT cells	ICOS-L (CD275/B7-H2)	DC, B cells, endothelial cells, macrophages, monocytes
CD30	Activatory	Activated T cells, B cells and NK cells	CD153	B cells, activated T cells
OX40 (CD134)	Activatory (inhibitory for Tregs)	Activated DC, B cells, T cells and monocytes and macrophages	OX40 ligand (CD252)	Activated T cells, Treg, NK cells, NKT cells and neutrophils
4-1BB (CD137)	Activatory	Macrophage, monocytes, DCs, NK cells and activated T cells	4-1BBL (CD137 ligand)	DC, B cells, macrophages, monocytes
CD40L (CD154)	Activatory	Platelets, activated T and B cells	CD40	DC, B cells, macrophages, monocytes, endothelial cells
CD270	Activatory	DC, B cells, T cells, NK cells, macrophages, monocytes	CD258, CD272, CD160	DC, activated T and B cells, NK cells, monocytes, macrophages
GITR (CD357)	Activatory	NK cells, activated T cells, Tregs	GITR ligand	DC, B cells, macrophages, monocytes, endothelial cells
CTLA4 (CD152)	Inhibitory	B cells, activated T cells	CD80 (B7-1), CD86 (B7-2)	DC, activated T cells, B cells, macrophages, monocytes
CD160	Inhibitory	T cells, NK cells, NKT cells	CD270, MHC-I	DC, T cells, B cells, NK cells, macrophages, monocytes
CD223 (LAG3)	Inhibitory	B cells, NK cells, activated T cells	MHC-II	DC, T cells, B cells, macrophages, monocytes
CD272 (BTLA)	Inhibitory	Activated T cells, B cells, DCs, Th1 cells	CD270	DC, T cells, B cells, NK cells, macrophages, monocytes
CD274 (PD-L1/ B7-H1)	Inhibitory	DC, T cells, B cells, NK cells, macrophages, monocytes	CD80 (B7-1)	DC, activated T cells, B cells, macrophages, monocytes
PD-1 (CD279)	Inhibitory	Activated B cells, DCs and T cells, monocytes, macrophages	CD274 (PD-L2/B7-H1) CD273 (PD-L2/B7-H2)	DC, T cells, B cells, NK cells, macrophages, monocytes
TIM3	Inhibitory	DC, B cells, T cells, monocytes, macrophages, NK cells	Galectin 9	T cells, Tregs

DC, dendritic cell; GITR, glucocorticoid-induced tumour necrosis factor receptor; MHC, major histocompatibility complex; NK, natural killer (cell); NKT, natural killer T (cell); Th, T-helper cell; Treg, regulatory T cell.

benefit from immunotherapy approaches.[6] The key to the success of these neo-epitope vaccines is the identification of immunogenic epitopes. Many algorithms have been designed to predict binding to common MHC-I alleles but programmes predicting MHC-II binding or TCR recognition are very limited. New algorithms rank neo-epitopes based upon their differential immunogenicity, thereby allowing rational neo-antigen selection for clinical immunotherapies.[7]

Despite these limitations, several groups have now successfully treated patients with neo-epitope vaccines. Carreno and colleagues, using a neo-epitope peptide-pulsed DC vaccine enhanced both pre-existing responses and induced responses that were undetectable prior to vaccination.[8] Sahin and colleagues treated 13 melanoma patients with an ribonucleic acid (RNA) polyepitope vaccine by intranodal delivery.[9] All patients developed T-cell responses against multiple neo-epitopes; the majority were CD4 responses. The cumulative rate of metastatic events was significantly reduced after vaccination, resulting in sustained progression-free survival. Two out of five patients with metastatic disease experienced objective responses but one had a late relapse due to outgrowth of a β2-microglobulin-negative tumour, and a third patient had a complete response when combined with anti-programmed cell death 1 (anti-PD-1) blockade therapy. Unexpectedly, high immunogenicity and antitumour responses of individual mutations identified their preferred recognition by CD4 T cells.[10] Ott and colleagues treated 6 melanoma patients with up to 20 epitopes expressed as long peptides admixed with the poly ICLC (stabilization of Polyinosinic:polycytidylic acid (poly I:C) with poly-L-lysine double-stranded RNA) agonist Hiltonol. The vaccine induced polyfunctional CD4 and CD8 responses targeting 58 (60%) and 15 (16%) of the 97 unique neoantigens respectively. Four out of six patients had no recurrences at 25 months after vaccination, while two with recurrent disease were subsequently treated with anti-PD-1 blockade and experienced complete regression with expansion of the neo-epitope-specific T cells.[11] Both these trials provide strong rationale for further development of this approach in patients whose tumours have a high mutation frequency, particularly in combination with checkpoint blockade.

The disadvantage of targeting somatic mutations is their great variability both within and between tumours. The former can lead to selection of tumours that no longer express the mutation[12] and the latter requires the design of a new vaccine for each patient, which is both expensive and time consuming. An alternative is to search for 'driver' mutations which are intimately involved in tumourigenesis and are expressed by all cells within the tumour. One such driver mutation is BRAFV600E which can also be targeted by small-molecule inhibitors[13] but has yet to be shown as a target of cell-mediated immune responses. However, inhibition of the mutant BRAF has been shown to enhance T-cell responses.[14] It remains to be seen if other common mutations can be identified and if they have a clinical utility, as they will only bind to selective human leukocyte antigen (HLA) molecules. A better alternative may be investigation of frameshift mutations, splice variants and INDELs.[15]

Although neo-epitope vaccines look promising for tumours with high mutational burden, many tumours have a low mutation frequency, meaning identification of neo-epitopes may be difficult and a different vaccine for these patients remains a high priority.

Post-translational modifications

Another attractive category of 'foreign' antigens, not subject to thymic deletion, derives from post-translational modifications in cancer-associated proteins. In many instances, these modifications are the result of the abnormal metabolism of cancer cells, such as abnormal phosphorylation or glycosylation. Cancer cells can present phosphorylated peptides in complex with MHC-I or MHC-II. Phosphorylation cascades are often altered in tumour cells, resulting in generation of novel phosphopeptides or an increase of phosphorylated proteins. Tumour-specific phosphopeptides have been shown to stimulate CD8 and CD4 responses and repertoires of T cells recognizing these tumour antigens have been detected in human subjects.[16–18] Results of a phase I trial incorporating two such peptides are awaited.

Aberrant N-acetylglucosamine (O-GlcNAc) can correlate with augmented cancer cell proliferation, survival, invasion and metastases. Elution of MHC-I peptides from cancer cells have identified numerous O-GlcNAc-modified epitopes. These peptides can stimulate multifunctional T-cell responses to the glycosylated but not the unglycosylated peptide and may form the basis for future cancer vaccines.[19]

In other instances, post-translational changes are the result of the stresses to which cancer cells are often subjected. A case in point is stress-induced autophagy leading to the post-translational conversion of arginine to citrulline by peptidylarginine deiminase (PAD) enzymes. These modified epitopes are presented on MHC-II and stimulate CD4 T-cell responses.[20] To this end, we have demonstrated that stress-induced citrullinated peptide epitopes induce potent, cytotoxic CD4 T-cell-mediated, antitumour responses that could constitute a completely new class of cancer treatment.[21]

Nutrient and oxygen deprivation, endoplasmic reticulum (ER) stress, as well as accumulated deoxyribonucleic acid (DNA) damage by rapidly dividing cancer cells creates a 'stressed' environment. The ability of established tumours to grow and survive depends largely on autophagic flux. However, the majority of tumours do not express MHC-II, as the immunosuppressive tumour environment inhibits inflammation. Vaccinating with citrullinated peptides induces pro-inflammatory, CD4 Th1 cells that are initially reactivated within the tumour environment by APCs, constitutively expressing MHC-II and autophagy. These reactivated CD4 T cells release IFNγ, which upregulates class MHC-II on the tumours, allowing direct recognition by the cytotoxic CD4 T cells. We have shown that citrullinated peptides stimulate CD4 T cells, which do not require CD8 responses for antitumour immunity,[21,22] and leave a memory response to prevent recurrence. Although our first vaccine targets are vimentin and enolase which are expressed by many cancers, other commonly expressed cytoskeletal, glycolytic, regulatory and chaperone proteins are also citrullinated under conditions of cellular stress.[23–25] In theory, combinations of a select few citrullinated peptides could be used to target all major solid cancers resulting in a step change in the approach to treating tumours. In addition to citrullination, other stress-induced post-translational modifications (siPTM) have been observed within MHC-bound peptides.[26,27] Our overarching hypothesis is that these vaccines based upon siPTM of proteins will elicit new or boost pre-existing immunity that will eliminate tumours with a low T-cell infiltrate.

A similar but very different approach is to create a novel multivalent vaccine by disrupting the degradation of intracellular proteins by the ubiquitin proteasome system. This also involves autophagosome products and is branded as the DRibbles vaccine, containing DRiPs (defective ribosomal products) and SLiPs (short-lived proteins), including tumour-associated antigens (TAAs). This involves blocking and stabilizing these agents and harvesting autophagosomes by membrane disruption and fractionalization to create an 'autologous vaccine'. Preclinical work showed that the DRibbles approach was much better than a whole-cell vaccine at enhancing IFNγ expression.[28] Combining this approach with anti-OX40 antibodies to provide costimulation enhanced protection in mice.[29]

Viral epitopes

Cancer caused by viruses and other infectious agents such as *Helicobacter pylori* constitute approximately 20% of cancer worldwide. In theory, none of the T cells recognizing these viruses should be subjected to thymic tolerance. Indeed, vaccines for 'HBV' and HPV are very effective at inducing protective antibody responses to reduce the cancer risk, if they are administered before exposure to the virus.[30] Despite the fact that many of the viral vaccines are directed against viral proteins involved in malignant transformation they are less effective once the virus has established a tumour. Antigen vaccination is preferable to vaccination with the whole virus as the latter exploits immune evasion and immune suppressive mechanisms that they have developed in the course of evolution. Indeed, the expression of E6 and E7 oncogenes by HPV can drive persistence and increase chance of cancer. Expression of these oncoproteins also influences innate immunity and promotes a suppressive tumour environment (reviewed in Smola et al.[31]). The best responses to date have been observed in patients with premalignant diseases such as cervical intraepithelial neoplasia or vulvar intraepithelial neoplasia, with up to 50% of patients achieving a partial or complete response after vaccination targeting HPV oncoproteins.[32–36] Yet this vaccine was of limited value in established cancers[37] due to the same peripheral tolerance/exhaustion mechanisms which operate in persistent infections and nonviral cancers.[31] Combination studies described in the checkpoint inhibitor section below section show good synergies and are resulting in more encouraging immune responses. Similar findings have been observed with a DC-based vaccine targeting the latent membrane protein 1 (LMP1) and 2 proteins from Epstein-Barr virus (EBV) in nasopharangeal carcinoma.[38] Other EBV-targeted vaccines have reached phase clinical trials but

have yet to prove efficacy in phase II studies.[39,40] Chronic infection with HBV and hepatitis C virus (HCV) can cause hepatocellular cancer (HCC). Therapeutic vaccines targeting HBV or HCV are more complex than other viral-induced cancers, as they do not contain oncogenic proteins but induce cancer due to inflammatory events. Most vaccines try to remove the infection prior to carcinogenesis but no good candidate has as yet been identified.[41]

Cancer–testis antigens and differentiation antigens

As previously discussed, high-avidity T cells recognizing self antigens are frequently deleted in the thymus leaving an attenuated low-avidity repertoire. However, thymic tolerance is not always complete, as has been elegantly shown by cloning T cells from regressing cancer patients. These CD8 T cells recognize differentiation antigens such as tyrosinase-related protein 2 (TRP-2)[42,43] or cancer–testis antigens.[44] Therapeutic vaccination of metastatic melanoma patients with peptides encoding these antigens was followed by tumour regression in a minority of the patients. In patients who do respond to the vaccine, the anti-vaccine T cells probably succeed in focally reversing this tumour-mediated immunosuppression and trigger a broad activation of other antitumour T cells, which proceed to destroy the tumour.[45]

In order to selectively stimulate high-avidity T cells that are capable of killing tumour cells, it is necessary to stimulate with low-dose antigen presented on activated DCs.[46,47] A DNA vaccine, SCIB1, incorporating HLA-A*0201 restricted epitopes from differentiation antigens glycoprotein 100 (gp100) and TRP-2 plus HLA-DR*0401 and HLA-DR7/DQ6/DR53 restricted epitopes from gp100 into the Complementarity-determining regions (CDR) regions of a human immunoglobulin G1 (IgG1) monoclonal antibody (mab) was administered to 35 melanoma patients. SCIB1 induced dose-dependent T-cell responses in 88% of patients with no serious adverse effects or dose-limiting toxicities. An 8 mg dose stimulated the strongest immune responses, and these were stronger in disease-free patients than in patients with detectable tumour. A total of 2/15 patients with measurable disease showed objective reductions in tumour burden, and 7/15 showed stable disease. All 20 fully resected patients showed a T-cell response and remained alive with a median observation time of 37 months. Clinical response was related to MHC-I/MHC-II expression on tumours prior to therapy ($p = 0.007$) whilst tumour recurrence was related to loss of MHC-I, loss of target antigens, a lack of a CD4 T-cell infiltrate and expression of programmed cell death ligand 1 (PD-L1).[48]

A similar approach was used with RNA vaccines encoding the cancer–testis antigen, NY-ESO-1 or tyrosinase complexed with liposomes and delivered intranodally, ensuring delivery to DCs. T-cell responses were induced to these antigens, although they were at lower frequency than those induced to neo-epitope vaccines administered to the same patients.[9]

A multipeptide melanoma vaccine containing six peptides encoding CD4 epitopes from melanocytic proteins induced high Th1 CD4 responses, as well as durable clinical responses and durable stable disease up to 7 years later in 7–12% of patients. Overall, survival for patients who received the vaccine was significant when compared with matched paired controls (5-year survival was 57% *versus* 16%, respectively ($p < 0.001$). Epitope spreading to CD8 T cells was induced in 45% of patients.[49–52] Ongoing trials are testing this vaccine with checkpoint blockade and BRAF/Mitogen-activated protein kinase kinase (MEK) inhibition.

In addition, the prostate-specific antigen (PSA) has been used as a target antigen in the PROSTVAC vaccine. This has limited normal tissue expression and may induce less immune tolerance. This showed promise in preclinical testing and early clinical trial but has had limited efficacy in a recent phase III clinical study.[53,54] One of the limitations of the use of whole antigen is the competition in MHC binding from all possible peptide epitopes within the same APC. Thus, leading to the preferential binding of the highest-affinity peptides, which are most likely to be those subject to immune tolerance. The delivery of this vaccine as a recombinant viral vector vaccine may also have contributed to reduced efficacy, since immune responses may be subverted away from the target antigen and induced against the viral vector.

Optimal vaccine design

Stimulating any T-cell response requires uptake, processing and presentation on activated DCs. The DCs are usually activated by adjuvants, classically targeting TLRs. The dose of vaccine is also

crucial, as low-but-sufficient dose is required to select the highest-affinity TCR to give the highest-avidity CD8 T-cell response.[47] In contrast, CD4 T cells require a higher dose, as binding to MHC-II is lower affinity. This is difficult to achieve with a complex vaccine that presents multiple epitopes, such as cell-based,[55] protein[56] or oncolytic viral vaccines.[57] Low doses fail to stimulate a response, and high doses present the highest-binding epitopes to which the T cells have been deleted in the thymus. If the tumour has a high mutation frequency that generates strong MHC-binding epitopes that outcompete the self epitopes, they may stimulate a *de novo* T-cell response. The advantage of oncolytic viruses is that they kill a low number of tumour cells *in vivo*, allowing efficient presentation of low-dose antigen to activated DCs *in vivo*. The disadvantage is that low doses may fail to stimulate efficient CD4 responses required to reverse tumour immunosuppression and perhaps explains the much lower response rate in non-injected lesions.

One solution is to use epitope-based vaccines that focus the immune response on epitopes, such as neo-epitopes and post-translational modifications that have avoided thymic deletion. Their disadvantage is that they are HLA restricted. For personalized neo-epitope vaccines, this is less of a problem, as the vaccine is designed to match a patient's HLA type. For broader-based vaccine, selection of common HLA types such as HLA-A2, HLA-B7, B35, B44 and HLA-DP4, are desirable. The epitopes can be encoded within nucleic acid, peptide or DC vaccines. Encoded nucleic-acid vaccines are poorly transfected and translated and thereby deliver a low dose that is ideal for CD8 responses but less efficient for CD4 responses. Combining nucleic acid vaccines with electroporation (EP) or nanoparticle/liposomal delivery is more effective. In contrast, high doses of peptide are required to elicit any immune response, which is ideal for CD4 responses but induces a high-frequency, low-avidity CD8 response. New approaches with long peptides, amphiphilic peptides and linking adjuvants to peptides are showing encouraging responses. The complexity of the DC network is just beginning to be uncovered and choosing the correct subset for *ex vivo* loading of tumour epitopes is being explored.

Nucleic acid vaccines

The idea of using DNA vaccines to stimulate immune responses in humans was very appealing due to their simplicity and elegance. Early studies in the 1990s showed induction of strong immunity against viral and tumour antigens in small animals.[58] DNA has a number of advantages as a vaccine platform. It is the only recombinant vaccine that does not induce vector immunity, allowing repeated administration. Additionally, it is safe, rapid, simple and cost effective to manufacture. It is also relatively stable at room temperature, making cold-chain transport less critical compared with other vaccine platforms. Its flexibility is enormous, allowing combinations of multiple plasmids encoding a variety of antigens.

Plasmid DNA vaccines have intrinsic adjuvant activity, resulting in recruitment of large numbers of inflammatory cells to the site of immunization. The mechanisms underlying DNA vaccine-induced immunity are complex and have yet to be fully elucidated but are thought to involve promiscuous and discriminative DNA sensors expressed by APCs. CpG oligodeoxynucleotide motifs signal through TLR9 to promote the activation and maturation of DCs.[59] Interestingly, DNA vaccine activity was still observed in TLR9 knockout mice, implicating additional endosomal and cytosolic DNA sensors that mediate adjuvant activity.[60] DNA sensors such as TANK Binding Kinase 1 (TBK-1) and Stimulator of interferon genes (STING) activate TLR-independent pathways and induce type I IFN.[61] More recently, the helicase DDX41 was identified as a new DNA sensor in myeloid DCs.[62] In addition, Retinoic acid-induced gene I (RIG-1) can also stimulate type I IFNs by sensing cytosolic DNA in association with RNA polymerase III.[63] A DNA-dependent activator of IFN regulatory factors (DAI/DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immunity.[64]

EP, concomitant with injection of DNA vaccines has been explored as an approach to resolve the issue of poor transfection after injection with plasmid DNA alone. Brief electric pulses induce a transient increase in the permeability of cell membranes, resulting in enhanced plasmid DNA uptake and protein expression levels.[65] In addition, EP is largely thought to provide an adjuvant effect with local tissue damage and subsequent expression of pro-inflammatory cytokines.[66] Use of the EP device to administer a DNA vaccine caused transient pain and, on occasion, injection-site haematoma, but was successfully given on 218 occasions, including five patients who have now each received 15–17 immunizations over a period of up to 42 months. Discomfort from the

EP procedure only limited treatment to three doses in a single patient.[48]

An alternative method of DNA delivery is the use of cationic lipids, such as polyethylenimine (PEI) or polylysine.[67–72] Polyplexes and lipoplexes consisting of highly ordered structures of DNA molecules trapped within positively charged supramolecular assemblies have clearly demonstrated their transfection efficiency *in vitro*.[72] However, aggregation in tissue fluids, toxicity and low *in vivo* efficiency has thus far hampered their clinical use. More recently, cationic vectors have been combined with nonionic amphiphilic polymers. Poloxamine block copolymers have a tetrafunctional structure consisting of four polyethylene oxide/polypropylene oxide (PEO/PPO) blocks centred on an ethylenediamine moiety. Block copolymers are adsorbed on nanoparticles through binding of their hydrophobic PPO segments while their hydrophilic PEO segments extend outwardly. DNA molecules collapsed into oligomolecular complexes exhibit new structural characteristics. These negatively charged nanospheres are able to deliver reporter and therapeutic genes to skeletal and heart muscle cells *in vivo*.[73] Clinical trials are pending.

It has been known for some time that one of the components vital for induction of efficient cell-mediated immunity is the inclusion of T-cell help. Stevenson and colleagues have developed an elegant method of providing T-cell help within DNA vaccines by fusing their CD8 epitope to a CD4 epitope from tetanus toxin (FrC-DOM).[74] Upon translating this into the clinic, it was found in a prostate cancer study that 29/30 patients receiving DNA with EP exhibited enhanced antibody responses and CD4 T-cell responses to DOM and 55% demonstrated detectable CD8 responses to prostate-specific membrane antigen (PSMA).[75] Anti-PSMA responses were associated with an increase in PSA doubling time.[75] In a second study targeting the carcinoembryonic antigen (CEA), DOM-specific immune responses demonstrated successful vaccine delivery. A total of 50% of patients without measurable disease expanded anti-CAP-1 CD8+ T cells [specific for HLA-A*0201 restricted CAP-1 peptide from CEA (aa605-613)] compared with only 20% with advanced disease. The gastrointestinal adverse event of diarrhoea was reported by 48% of patients and linked to more frequent decreases in CEA ($p < 0.001$) and improved immunologic responses compared with patients without diarrhoea. In advanced-disease patients, decreases in CEA were associated with better overall survival (OS) [hazard ratio (HR) = 0.14, $p = 0.017$]. CAP-1 peptide was detectable on MHC-I of normal bowel mucosa and primary colorectal cancer tissue by mass spectrometry, offering a mechanistic explanation for diarrhoea through CD8+ T-cell attack. It also highlights the need to be aware of autoimmune responses when targeting self antigens.[76]

A novel DNA vaccine approach encodes the specific T-cell epitope within the CDR region of an antibody. The constant fragment crystallizable (Fc) region of the antibody targets the high-affinity Fc receptor CD64, which is only found on activated and not immature APCs.[47,77] The DNA is administered with EP which ensures good transfection, but inclusion of T-cell epitopes within the CDRs disrupts the antibody folding and leads to production of low levels of antibody. This results in higher-affinity CD8 responses than whole DNA vaccines, peptides or peptide-pulsed DC vaccines.[47] In line with the CEA-DOM study, the DNA vaccine encoding epitopes within the CDR regions of a human IgG antibody stimulated T-cell responses that were stronger in patients without tumour present at screening than in patients with detectable tumour, suggesting that tumour load may attenuate the response.[48] It also suggests that previous vaccine studies in patients with tumour load may have underestimated the measurable effects due to systemic or local immune suppression. DNA vaccination may therefore be particularly effective in early-stage patients with a low tumour burden.

Several groups have investigated the use of RNA for vaccination. Due to the instability of RNA, stabilized antigen encoding RNA formulations have been used and have shown antigen specific T-cell responses albeit with low antitumour activity.[78,79] This could have been related to the choice of encoding whole self antigens rather than neo-epitopes, as recent studies with these RNA vaccines delivered into the lymph node stimulated potent antitumour responses.[9] Similar to DNA vaccines, the efficiency of delivery of RNA is essential to the success of the vaccine. Recent studies using RNA lipoplexes have allowed systemic delivery that targets lymph nodes *in vivo*, where it is efficiently taken up by DCs and self adjuvants, inducing the release of type I IFN.[80] Lipid nanoparticles can protect the RNA from degradation and can be

engineered to express specific ligands, enabling targeting to specific cell types (reviewed in Reichmuth et al.[81]).

Peptide vaccines

Therapeutic vaccines require targeting and activation of DCs to stimulate both CD4 and CD8 T-cell responses. Initial trials concentrated on short peptides encoding CD8 T-cell epitopes that without CD4 help resulted in short-lived responses. Short peptides (<15 amino acids) do not require processing by APCs and can bind directly to HLA class I molecules of all nucleated cells. Thus, most of these peptides bind to non-APCs that have no costimulatory molecules and thereby cause tolerization.[82–84] If the short peptide is combined with a depot, T cells elicited by the vaccine migrate to the vaccination site rather than the tumour and appear to die there.[85] In contrast, immunization with synthetic long peptides (SLP) did not tolerize but stimulated a robust CD8 T-cell response due to efficient processing within APCs.[82,86–88] SLPs can also encompass CD4 epitopes and it has been shown they are even more efficient at activating CD4 responses than processing from intact proteins.[89,90] Furthermore, physical linkage of peptides to TLR agonists enhances the immune responses observed in three ways: (a) efficient targeting to DCs; (b) maturation of DCs to express costimulatory molecules, and secrete cytokines and chemokines; and (c) formation of an antigen depot within DCs to allow prolonged presentation of the peptide.[91–94]

An alternative solution is to use amphiphilic peptides that self assemble into nanostructures. In combination with poly I:C, a TLR3 agonist, they generate larger numbers of CD8 and CD4 T cells that result in reductions in the tumour growth rate in animal models.[95,96]

Dendritic-cell vaccines

The only approved therapeutic autologous cell-based vaccine is sipuleucel-T (Provenge®) where peripheral blood mononuclear cells are pulsed with prostate antigens. It has shown a modest survival benefit of 3 months, but the cost and time of production have severely limited its use. Dendritic cells are the major professional APC of the immune system and have been used for many years in development of vaccine therapies. Autologous DCs made *in vitro* by cytokine polarization are the usual candidates for this therapeutic approach. The goals for design of DC vaccines rely upon using an efficient strategy to load tumour antigen onto the DC and optimize the best way to deliver the vaccine efficiently so it can target the lymph nodes. A plethora of studies are published in which DCs derived from blood monocytes *in vitro* have been loaded with peptides, antigens and tumour lysates and have shown some promise in preclinical studies (reviewed in Shang et al.[97] and Markov at al.[98]). This involves the culture of the immature DCs that are able to catch antigen by mechanisms such as macropinocytosis, phagocytosis and receptor-mediated endocytosis with the soluble antigen. Nucleic acids have also been used as a source of antigen for DCs; however, these are not able to be directly taken up by DCs. They therefore require the combination with mechanisms to internalize the nucleic acid such as electroporation, liposome formulations and viral-vector delivery. Despite efficacy in preclinical studies, these approaches have shown similar limited benefit in clinical studies.[98] It is possible that this is due to a number of different issues. The activation state of the DC will determine the immune response generated, as DCs can tolerize responses, as well as activate them. It is well known that immature DCs can lead to the induction of immune tolerance.[99] The extensive culture required for generation of monocyte-derived DCs may also impact their immunogenicity. Recently, work has shifted to the use of different DC subsets derived directly from blood with minimal *in vitro* manipulation and the incorporation of both CD4 and CD8 epitopes, with an improvement in clinical responses.[100,101]

The low efficacy of DC based and other vaccine therapies in clinical studies to date will suggest that generation of an immune response does not necessarily guarantee clinical outcome. This can be in part put down to the negative effect of the tumour on the immune system.

Overcoming the inhibitory tumour environment

Vaccines are likely to be more effective against immune 'hot' tumours which permit high immune infiltrates. 'Cold' or 'immune desert' tumours that are low or deficient in immune infiltrates often have developed suppressive mechanisms to exclude immune cells which may not be reversed by vaccines alone. These include checkpoint blockade of T cells,[102–108] myeloid suppressor cells,[109,110] immunosuppressive cytokines such as

transforming growth factor-β and interleukin-10,[111–115] and metabolic enzymes such as indoleamine 2,3 dioxygenase.[116,117] To overcome these suppressive mechanisms generated by the tumour, therapies will ultimately need to combine vaccination to stimulate the immune response with other therapies that help reverse the suppressive tumour environment.

Checkpoint inhibition

Although cognate recognition of MHC:peptide by the TCR is the primary signal for T-cell activation, other costimulatory molecules can fine tune this response either positively or negatively. Some costimulatory molecules (CD28, CD27) are present on naïve cells and are essential in T-cell activation, whereas others (4-1BB, OX40 and CD40L) are expressed during T-cell activation and modulate polarization and differentiation into memory. To regulate these responses, inhibitory receptors (cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), PD-1, LAG-3, Tim-3) are induced to return the T cells to a resting state. In cancer, due to the inability of the T cells to clear the disease, persistent antigen results in chronic activation or 'exhaustion' of T cells. Blocking the inhibitory receptors has been shown to reverse the poor functionality of these T cells and re-establishes tumour control.[118] This approach was first proven in patients with advanced melanoma, based on the improved OS of patients treated with the anti-CTLA-4 directed mab, ipilimumab.[103,119] Blocking of CTLA-4 has also shown some success with long-term clinical benefit for a proportion of refractory malignant mesothelioma patients treated with the mab, tremelimumab.[120] Anti-CTLA-4 antibodies have been shown to deplete regulatory T cells (Tregs) *via* antibody-mediated cellular cytotoxicity in murine models[121,122] and it has been claimed that the clinical activity of these antibodies is mediated at least in part by this mechanism. As Tregs can inhibit both the priming and the function of vaccine-induced T-cell responses, depletion of these cells could increase vaccine efficacy. Initially, the antitumour activity of an anti-PD-1 antibody was shown in melanoma, renal cell carcinoma and lung cancer.[106] These studies have now been extended to a wide range of cancers including Hodgkin's lymphoma, head and neck cancer, urothelial tumours and microsatellite unstable tumours using antibodies that block either PD-1 or PD-L1. Nevertheless, the clinical efficacy of PD-1 pathway inhibition as monotherapy has been limited to subsets of patients within most tumour types studied to date, with response rates of 20% or less in many cancers, including common types, such as breast, colon, and prostate cancer. There is a correlation between the number of mutations within a tumour and its ability to stimulate a T-cell response, as most mutations are not immunogenic.[102] A vaccine may increase the repertoire and number of responding T cells, allowing rapid tumour infiltration. In response to this brisk immune infiltrate and especially if IFNγ is released, the tumour will upregulate PD-L1 in a process known as 'acquired resistance'.[123] This will limit the efficacy of the vaccine, but synergy with checkpoint inhibition could enable eradication of tumours. In animal models, combined therapies of vaccines and checkpoint inhibition resulted in synergistic antitumour responses.[124] Indeed, combination of CTLA-4 and PD-1 blockade is more effective than either modality alone in promoting the rejection of various murine tumours secreting granulocyte–macrophage colony-stimulating factor[125] or McDonough Sarcoma fms-like tyrosine kinase 3 (Flt-3)[126] or other vaccines.[127]

In animal models, SCIB1 DNA vaccination was associated with increased infiltration of CD4 and CD8 T cells within the tumour, in addition to being associated with upregulation of PD-L1 within the tumour environment. PD-1 blockade also resulted in increased CD8 T-cell infiltration and an antitumour response with 50% of mice showing long-term survival. In line with the hypothesis that PD-1/PD-L1 signalling results in inhibition of proliferation of high-avidity T cells at the tumour site, the combination of PD-1 blockade with vaccination enhanced the number and proliferation of the CD8 T-cell infiltrate.[128] This resulted in a potent antitumour response with 80% survival in an aggressive mouse melanoma model. When another DNA vaccine, SCIB2, was given in combination with Treg depletion, CTLA-4 blockade or PD-1 blockade, long-term survival from established tumours was significantly enhanced to 56%, 67% and 100%, respectively.[129]

Clinical trials have begun to validate this concept. The response rate of melanoma patients receiving the oncolytic virus talimogene laherparepvec in combination with ipilimumab (30%) was compared with patients receiving ipilimumab alone (18%).[130] Patients treated with neo-epitope vaccines that recurred showed complete responses when they were subsequently treated with

checkpoint blockade. This suggests that priming a T-cell response prepares patients to respond well to checkpoint inhibition.[9] In a phase II study of autologous monocyte-derived mRNA electroporated DCs plus anti-CTLA-4 in melanoma patients, the 6-month disease-control rate was 51%, which included eight complete and seven partial responses.[131] In a small trial of 22 patients with incurable HPV16+ oropharyngeal cancer, the overall response rate to combined anti-HPV SLP vaccination and anti-PD-1 checkpoint blocker (nivolumab) treatment was 36% *versus* 16% in published monotherapy with nivolumab.[132] Many of the responses were deep (two complete responses, six partial responses) and durable, warranting a randomized phase II trial.

Chemotherapy

Preclinical evidence shows that certain chemotherapeutics can synergize very effectively with therapeutic cancer vaccines. These principles have recently been carried into the clinic. For example, patients with HPV16+ recurrent or metastatic cervical cancer lived significantly longer if they had robust T-cell responses to an SLP vaccine against the HPV16 E6/E7 antigens. Vaccination was started 2 weeks after the 2-day cycle of carboplatin and paclitaxel chemotherapy, when increased immunosuppressive myeloid cell numbers had declined to the level of healthy donors. The longer survival was not due to generally better immunocompetence because high and low immune responders to vaccination had equal T-cell competence as measured by their recall responses to unrelated common microbial antigens.[133]

Using a spontaneous mouse model of gastrointestinal stromal tumour, oncogenic *Kit* tyrosine-kinase blockade augmented endogenous T-cell responses by inhibiting tumour cell production of the immunosuppressive enzyme indoleamine 2,3-dioxygenase. It was further shown that the combination of kinase inhibition and T-cell immunotherapy synergized to enhance antitumour efficacy. These findings established a critical link between cell-autonomous oncogenic signalling and cell-nonautonomous immunosuppression. It introduced the paradigm that the antitumour effects of kinase inhibition are partially T-cell dependent, and was the proof of concept for combining targeted therapy with immunotherapy.[134,135]

The development of new vaccine designs and identification of novel targets for vaccination is increasing rapidly and showing promising results in many preclinical studies. A few of these are also beginning to show promise in the clinical setting. Combinations with checkpoint blockade therapies and chemotherapy will ultimately lead to enhanced effects, provided adverse side effects can be minimized. Overall, vaccine design and development has progressed rapidly and shows considerable promise for the future.

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Conflict of interest statement

Lindy G Durrant is a director of Scancell Ltd. Victoria A Brentville and Katherine Cook are employees of Scancell Ltd.

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