Expert Opinion

Vaccines as early therapeutic interventions for cancer therapy: neutralising the immunosuppressive tumour environment and increasing T cell avidity may lead to improved responses

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Importance of the field: Considerable progress has been made in identifying the antigens recognised by the immune system. This has led to the success of monoclonal antibody therapy and the recent approval of prophylactic vaccines that give excellent protection against cervical cancer. Provenge will shortly be the first therapeutic vaccine to be approved.
Areas covered in this review: Our aim is to discuss the recent success with prophylactic cancer vaccines for prevention of cancer and the progress with therapeutic vaccines design to eradicate established tumours. Therapeutic vaccines need to stimulate high-avidity T cell responses that can recognise and kill tumours. How this can be achieved in cancer patients is discussed. The immunosuppressive tumour environment also needs to be modified to allow extravasation and efficacy of the vaccine induced T cells.
What the reader will gain: An insight into the limitations of present cancer vaccine approaches and how they can be manipulated to give more effective anti-tumour responses.
Take home message: A combination of more effective vaccines that stimulate high-avidity T cells, in combination with drugs or monoclonal antibodies that neutralize immunosuppressive factors within the tumour environment are needed to improve the efficacy of immunotherapy of cancer.

Keywords: cancer vaccines, immunotherapy, T cell avidity, tumour environment

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1. Introduction

Vaccination strategies are either 'prophylactic', aimed at preventing disease, or 'therapeutic', aimed at resolving an ongoing condition. Prophylactic vaccines to prevent virally induced cancer have been very successful but have a very limited application. In contrast it has been more difficult to develop therapeutic vaccines as they have to overcome the immunosuppressive tumour environment and they must stimulate potent T cell responses. T cell avidity, an assessment of potency, is measured by the amount of MHC–peptide required to elicit effector function. Thus high-avidity T cells can release cytokines or kill in response to low doses of MHC–peptide.

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Article highlights.

- Prophylactic vaccination prevents tumours induced by viral infection.
- The first therapeutic vaccine for the treatment of prostate cancer is about to be approved.
- Therapeutic cancer vaccines must stimulate high-avidity T cell responses.
- Vaccines targeting dendritic cells stimulate high-avidity T cell responses.
- Immunosuppressive factors within the tumour environment must be neutralised to allow T cells to unleash their full effector function.

This box summarises key points contained in the article.

Only high-avidity T cells are effective at targeting the low amounts of cognate MHC–peptide expressed by tumour cells and therefore new therapeutic vaccines should aim to stimulate high-frequency and high-avidity T cell responses.

2. Prophylactic vaccines

Most vaccines for infectious diseases are termed 'prophylactic' vaccines and are designed to induce an immune response to prevent the disease. These prophylactic vaccines usually induce neutralising antibodies, which bind to the pathogens and enhance their phagocytosis by monocytes. The pathogen is rapidly removed and the individual does not suffer the morbidity associated with the disease. Prophylactic vaccines for cancer are more difficult, as cancer is a multi-stage disease driven by genetic instability. This results in heterogeneous cell phenotypes which are impossible to predict. The only exceptions to this rule are the virally induced cancers and in particular hepatocellular carcinoma and cervical cancer. The incidence of the former has decreased with hepatitis B virus (HBV) vaccination and the later shows a strong association with human papillomavirus (HPV) infection. The huge recent success with the prophylactic vaccines, Gardasil (Merck and Co) and Cervarix (GlaxoSmithKline) both of which are now being used to immunise female teenagers and women in the USA and UK/Europe, will have a dramatic effect on the incidence of cervical cancer [1,2]. These prophylactic vaccines induce neutralising antibodies to the common HPV subtypes. Vaccination of young people prior to exposure will therefore prevent viral infection and the subsequent development of the cancer. They have achieved excellent protection against the HPV subtypes included in the vaccine (16 and 18 for Gardasill; 6, 11, 16 and 18 for Cervarix) which includes the major oncogenic subtypes, but do not confer protection against the more rare types. Although, a major breakthrough for protection from cervical cancer, these vaccines may not prevent all cervical cancer as they do not cover all HPV subtypes. Both limiting exposure to the virus and screening will still have a role to play in controlling this disease.

3. Therapeutic vaccines

The problem with cancer is that it is a heterogeneous disease. With the exception of a few genetic predispositions it is impossible to predict who will get which type of cancer and indeed if they will get cancer at all. Even if prophylactic cancer vaccines where developed for the five most common cancers, and there were shared antigens with an available T cell repertoire, high-avidity T cells recognising self antigens will induce autoimmunity (vitiligo is frequently associated with melanoma regression). Whilst this is acceptable in patients with cancer it would not be acceptable in healthy adults, especially as they may never get cancer. The alternative is to immunise with tumour-specific epitopes but as these are uncommon they would only protect a minority of individuals. It is therefore preferable to commence vaccination once the cancer has developed. It is then possible to obtain a tumour sample to identify the cell-associated proteins that may be targets for vaccination. These 'therapeutic vaccines', designed to target established tumours have two major problems. The first is the tumour environment which has evolved to avoid the immune response. This may be further complicated in metastatic patients as the entire host can be immune-suppressed and may be an obstacle to effective priming of an effective immune response. Thus treating patients with early stage disease or patients with minimal residual disease would be the favoured option. The second problem is generating T cells with high enough avidity or potency to recognise and kill tumour cells expressing the cognate MHC–peptide. We are now beginning to understand the mechanisms required to stimulate these responses and overcome the tumour environment to make a significant impact on cancer.

3.1 Tumour environment

There is an ongoing battle between the immune system and the tumour in a process known as 'Cancer immunoediting'. It comprises three phases: elimination, equilibrium and escape. During the elimination phase, mechanisms of innate and adaptive immunity serve to patrol peripheral tissues, singling out and eradicating abnormal or transformed cells. Seminal experiments by Shankaran *et al.* demonstrated that cells of the lymphoid system are essential for this process of immunosurveillance. Mice deficient in the RAG-2 gene product are unable to generate $\alpha\beta$T cells, B cells, NKT cells and $\gamma\delta$T cells. Following subcutaneous injection of a carcinogen, these mice developed tumours at the injection site faster and with more consistency than strain-matched wild-type controls [3]. Other studies involving depletion of NK and NKT cells have revealed a crucial role for this cell population in controlling tumour growth. Taken together, these experiments confirm a role for both innate and adaptive immune mechanisms in eliminating transformed cells [4]. There is less evidence in humans for elimination as these tumours would remain undetected. However, there is accumulating

evidence that cellular transformation is associated with upregulation of stress molecules that are recognised by the adaptive immune system [5,6]. During the equilibrium phase the immune system may control the growth of tumours. This competition between tumour growth and the immune system can persist for a number of years and can continue to restrict tumour growth even after the primary tumour has spread to secondary sites. Indeed in a number of tumours, T cell infiltration correlates with favourable prognosis [7-10]. In colorectal cancer T cell infiltration has recently been shown to be a better prognostic marker than tumour stage [11]. Similarly the stress related molecule, MHC class I chain-related molecule A (MICA), has been shown to be an independent predictor of good prognosis in colorectal and cervical cancer [12,13].

As tumours become genetically unstable they give rise to numerous new variants, thus increasing the likelihood of producing clones that are resistant to immune attack. Furthermore, by destroying susceptible cells, the immune system actively selects for these resistant tumour cell variants. This ultimately leads to the escape phase whereby the immunologically sculpted tumour expands in an uncontrolled manner [4,14]. There are many ways genetic instability can allow selection of transformed cells with the ability to avoid immune recognition. Tumours can produce immunomodulatory cytokines such as IL-10 [15] and TGF-β [16] which have a wide range of suppressive effects on the adaptive immune system. IL-10 inhibits dendritic cell (DC) maturation, promoting the induction of tolerance rather than immunity [17]. The presence of IL-10 in murine tumours has also been reported to promote tumour progression by driving the differentiation of regulatory CD4 cells [18]. TGF-β is another pleiotropic suppressive cytokine that acts on multiple cell types to promote tumour progression. It has long been known that TGF-β can potently inhibit the activation, proliferation and activity of T cells *in vivo* [19]. The inhibitory activity of this cytokine on CD8 T cells is a particular detriment of tumour immunity and was demonstrated by the adoptive transfer of TGFβ-receptor-deficient CD8 T cells which efficiently mediated rejection of established tumours in a murine model [20]. In addition to the effects on CD8 cells, TGF-β has also been reported to promote the generation of regulatory T cells (Tregs) *in vivo* [21]. Even if the activation of antigen-specific T cells is overcome by vaccination at distal sites, the induced T cells may still have a problem finding their target. If the tumour is not stimulating release of pro-inflammatory cytokines and chemokines the T cells will not get the directional cues to enter the tumour site. This anti-inflammatory environment can be overcome by activation of high-avidity tumour-specific CD4 T cells. These cells can outcompete the Tregs and secrete pro-inflammatory cytokines such as IFN-γ and TNF-α promoting further extravasation of both CD4 and CD8 effector cells [22]. However if the antigen-presenting cells within the tumour environment are not activated they will not present the cognate MHC–peptide in the context of costimulation and IL-12 to stimulate CD4 T cells to proliferate and release their vital cytokines. A further complication is that tumours can release high levels of chemokines. These abnormally high levels of chemokines can inhibit recruitment of effector T cells, in a process termed fugetaxis (chemorepulsion) but may still allow recruitment of Tregs [23,24].

Tregs are a small population of lymphocytes (1% – 3%) specialised in controlling responses to self antigens and therefore play a significant role in inhibiting anti-tumour immune responses. Their depletion has been shown to enhance vaccine approaches in patients and promote spontaneous tumour regression in animal models [25-27]. There are two main subsets of Tregs with distinct origins and mechanisms of action [28]. Natural regulatory T cells (nTregs: CD4 $CD25^{hi}$ $FoxP3^{+}$) are derived in the thymus and work in a contact-dependent manner through uncharacterised mechanisms [29,30]. Inducible Tregs (iTregs) are generated in the periphery, driven by interactions with DC and other antigen-presenting cells (APC) [31-33] and work in a cytokine-dependent/contact-dependent manner. These include T_H3 (TGF-b^{hi}) [34,35] and Tr1 (IL-10^{hi} IL-4^{-} IFNγ^{lo}) [34] cells. There are a growing number of reports implicating antigen-specific Tr1s in cancer, including responses to L antigen family member 1 (LAGE1) [36,37], antigen recognized by Treg cells 1 (ARTC1) [38] New York esophageal squamous cell carcinoma 1 (NY-ESO-1), survivin, transformation-related protein 1 (Trp1) and gp100 [39]. These tumour-antigen-specific cells secrete large amounts of IL-10 but also inhibit in a contact-dependent manner. Their induction in cancer has been linked to 'tumour programming' of DC [40]. *In vitro* multiple components of the tumour environment are capable of influencing the production of Tr1 cells by immature DC (iDC) [40,41]. These include stimulating iDC with a range of tumour-associated factors, for eample IL-10 [42,43], VEGF, vasoactive intestinal polypeptide (VIP) and prostaglandin E2 (PGE2) [44,45]. Each of these components are produced by various cancers and are therapeutic targets, for example blockade of PGE2 with COX-2 inhibitors and neutralisation of VEGF [46,47]. These tumour-conditioned iDC stimulate Tr1s, which preferentially home back to the tumour site in both colorectal and ovarian carcinoma [48,49]. Here they inhibit effector T cells and antigen-presenting cells, thus exacerbating the inhibitory environment of the tumour. In one study the mechanisms of DC inhibition by tumours was examined and was shown to be related to inhibition of MAP/ERK kinase kinase (MEKK) signalling which downregulates IL-12 secretion and the ability of the DC to stimulate a T_H1 response [50].

It is not only T cells but myeloid cells that can also develop into suppressor cells. Myeloid-derived suppressor cells (MDSC) bring about antigen-specific T cell tolerance by various methods but one mechanism is the increased metabolism of L-arginine [51] These cells can be inhbited in mice with inhibitors of phosphodiesterase-5 [52]. Many human tumours

produce the immunosuppressive enzymes indolamine-2,3-dioygenase (IDO) which prevents the activation of T cells [53]. Stereoisomers of 1-methyl-tryptophan can inhibit these enzymes and may have a role to play in treatment of patients with cancer [54].

Tumour cells can also develop resistance to immune-cell-mediated death by becoming insensitive to apoptotic signals. Downregulation of the death receptor, Fas, has been reported in a number of human tumours. Engagement of Fas by its ligand, FasL, normally induces cellular apoptosis. Missense mutations resulting in disrupted Fas signaling have been reported in myeloma [55], non-Hodgkin's lymphoma [56] and melanoma [57]. In contrast, a variety of tumours have been reported to express FasL, which induces apoptosis in Fas susceptible target cells, such as activated T cells. This has been proposed as a mechanism by which tumours may induce activation-induced cell death in infiltrating T cells [58]. Finally, tumour cells have also been reported to block CD8-mediated cytotoxicity through the overexpression of a serine protease inhibitor that inactivates granzyme B [59]. In a last attempt to prevent T cell recognition tumour can lose expression of MHC class I. However, as complete loss of MHC class I can be recognised by NK cells, most tumours selectively downregulate only a single MHC allele that is the target for the cancer-induced T cell response [13,60]. Vaccine stimulation of naïve T cells to give a new response to an allele which has not been lost, can effectively overcome this block.

Thus although the tumour environment provides a formidable block to tumour immunity, the fact that the immune systems is still a strong prognostic factor in many tumours [7-10] suggests that it could be externally manipulated to provide an effective therapeutic approach. The key seems to be the stimulation of new high-avidity CD4 and CD8 anti-tumour T cell responses, perhaps in combination with depletion of Tregs or neutralisation of immunosuppressive cytokines/high-dose chemokines.

3.2 T cell avidity

As most solid tumours are not induced by viruses the target antigens are normally overexpressed or selectively expressed self antigens. High-affinity T cells recognising epitopes from self antigens have often been selectively deleted/anergised/regulated during mechanisms of central and peripheral tolerance. The remaining repertoire of T cells requires sufficient and appropriate inflammatory stimuli to promote their expansion and subsequent targeting of the tumour. While tumour-antigen-specific T cells can be identified in cancer patients they are often ineffective, having received neither sufficient nor appropriate activation to be effective. Analysis of these T cells reveals that they are of low functional avidity and are less efficient at recognising the naturally processed epitopes presented on MHC at the surface of tumour cells *in situ*. Even if the cancer is associated with viral infection such as Epstein–Barr virus (EBV) induction of nasopharyngeal cancer or hepatitis C induction of hepatocellular carcinoma, the viruses have evolved multiple means of immune suppression. These include induction of fatigued T cells, which only produce IFN-γ but not TNF-α or IL-2, do not lyse virally infected targets and express high levels of the death receptor PD-1 [61]. Recent studies in cancer patients have identified similar cells within the tumour environment [62]. The challenge is therefore to develop therapeutic vaccine approaches that re-educate the immune response to stimulate high-avidity T cell responses.

Antigen specific cytotoxic T lymphocytes (CD8 T cells) play a pivotal role in anti-viral and anti-tumour immunity. In both viral infection and tumour models, only high-avidity and not low-avidity CD8 T cells mediate viral clearance and tumour eradication [63-67]. The assignment of avidity (**Figure 1**) as it relates to antigen dose is determined functionally by the amount of peptide required for activation of effector function [63,68,69] and is therefore a measure of the overall strength of the interaction between a CD8 T cells and a target cell [63,70-72]. The difference between high and low avidity T cells that recognise the same MHC–peptide combination, is the dose of peptide that is required to elicit effector function. Thus high avidity T cells are more sensitive to lower thresholds of peptide.

Recent trials with cancer vaccines have stimulated measurable T cell responses but these have failed to control tumour growth. One explanation is that these T cells did not have high enough functional avidity to recognise the low levels of MHC–peptide presented on the surface of the cancer cell. This may not be the only explanation as a recent review of MAGE-A3 vaccines trials revealed that there was no difference in avidity of T cells cloned from regressing or progressing patients [73]. However this study is complicated by the very low frequency of the induced response and that the T cells were cloned in high dose peptide which has been shown to select for lower avidity responses [63,68,69]. This is also a concern for T cell recruitment into tissues, as the recent 'shop window model' of Marelli-Berg and Jarmin has shown that recognition of MHC–peptide on endothelial cells is a prerequisite for extravasation [74]. This may only allow extravasation of high-avidity T cells as the amount of cognate MHC–peptide on endothelial cells will be very low.

What is classified as high and low avidity? Functionally high avidity T cells efficiently kill virally infected or tumour targets whereas low avidity cells do not. An elegant study by Dutoit *et al.*, using CD8 cells cloned from melanoma patients, showed that an avidity of over 10^{-9} M was required for tumour recognition and CD8 clones from spontaneously regressing cancer patients had avidities in excess of 10^{-12} M [75]. This is similar to the avidity of CD8 T cell efficiently clearing viral infections. In contrast avidities < 10^{-8} M have rarely resulted in target recognition although they can recognise targets pulsed with high-dose peptide *in vitro*.

Interestingly although there has been much less reported on the potency of CD4 cells they appear to be of lower avidity than CD8 cells, with the strongest reported avidities being

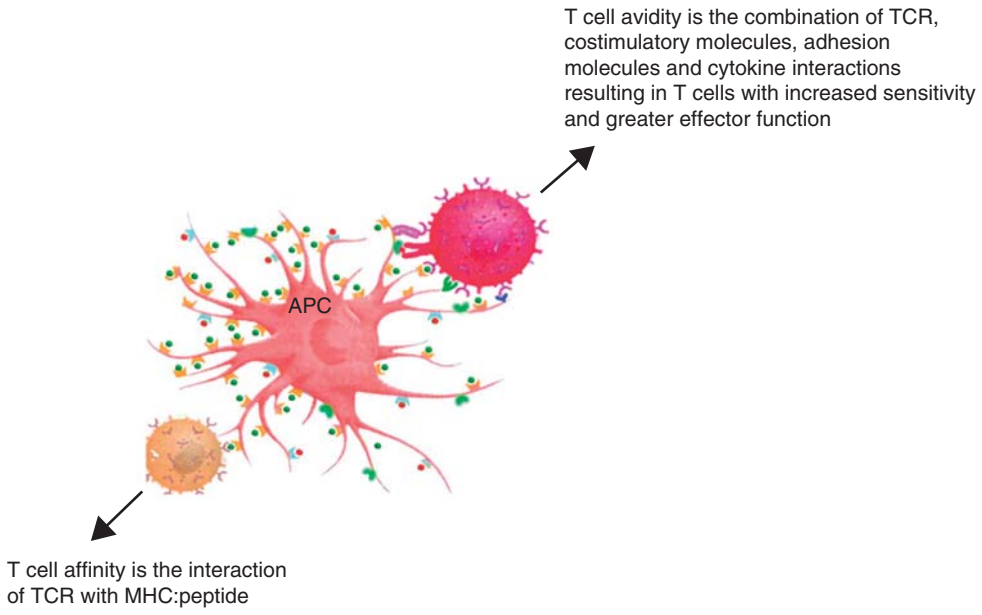

Figure 1. TCR affinity and T cell avidity.

in the nanomolar rather than picomolar range. The lower avidity of CD4 T cells may generally reflect the affinity of binding of the longer peptides to the open grooves of MHC class II antigens with subsequent reduction in TCR/peptide–MHC affinity, a key component of T cell avidity. The lower avidity of CD4 T cells may also be an advantage for immune regulation as CD4 Tregs may compete more easily with these lower-avidity effector CD4 cells. A recent study has shown that stimulation of high avidity CD4 cells ($> 10^{-8}$ M) can overcome T cell regulation of an anti-tumour response [22].

High-avidity T cells recognise targets expressing low numbers of MHC–peptide whereas low-avidity T cells require a much larger signal to be activated (**Figure 2**). As the T cell receptor (TCR) cannot undergo somatic hypermutation, how do T cells modulate their avidity? This appears to be a multifactoral process that has been reviewed by several groups [76-78]. How can this be exploited to produce more efficient cancer vaccines?

3.2.1 T cell repertoire and T cell receptor affinity

One contribution to T cell avidity is the affinity of recognition by the TCR of its cognate MHC–peptide. The TCR repertoire available for MHC peptide recognition is selected in the thymus on self antigen/MHC. Both lack of interaction, allowing death from neglect, and too strong an interaction, stimulating apoptosis, leads to a restricted repertoire of self-reactive T cells. However, this assumes all antigens are presented equally in the thymus. The recent identification of the autoimmune regulator (AIRE) transcription factor [79], which switches on a variety of differentiation antigens within the thymus, allows for selection and deletion of T cells recognising a wide variety of antigens. AIRE is expressed primarily by medullary thymic epithelial cells and functions to induce the ectopic expression of a large set of proteins that are considered to be restricted to peripheral tissues, such as preproinsulin II that is normally restricted to the pancreatic islet beta cells [80]. Deletion of high-affinity T cells can have an effect on the generation of high-avidity T cell responses as high-avidity CD8 responses could be generated in $p53^{-/-}$ but not in wild type mice [81]. This implicates TCR affinity as a key component of T cell avidity. In contrast, high-affinity T cell responses to Her2neu were not deleted but regulated in the periphery [82]. Although many tissue-specific antigens are ectopically expressed in the thymus, under the control of AIRE, and can impose negative selection of self-reactive T cells, not all self-antigens are expressed at levels sufficient to eliminate all autoreactive cells. In addition, many innocuous antigens are encountered through diet and environment and could potentially elicit pathogenic immune responses if they were not limited in the periphery [83]. Mechanisms of peripheral tolerance are therefore required to limit the activity of the self-reactive repertoire that escapes central tolerance. Clonal deletion of self-reactive T cells is not limited to the thymus and has been reported to occur in the peripheral lymphoid organs [84,85]. More recently, presentation of a peptide derived from tyrosinase in the lymph node, but not thymus, was found to result in rapid peripheral T cell deletion of TCR transgenic cells specific for that epitope. These observations suggest that direct antigen presentation by a population of LN-resident cells is responsible for the clonal deletion of self-reactive T cells in this system [86].

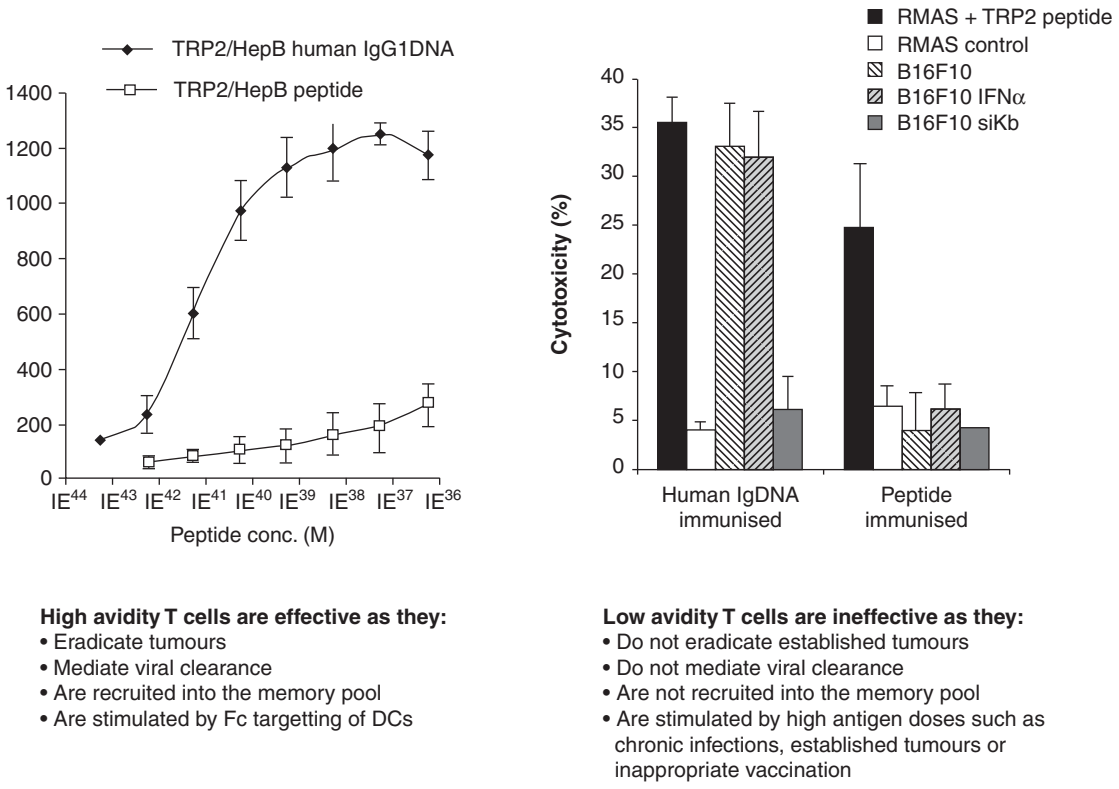

Figure 2. Why is T cell avidity important? Epitopes encoded with human IgG molecules, which allow Fc targeting of DCs stimulate high-avidity T cell responses that recognised low doses of cognate peptide–MHC. In contrast peptides stimulate low-avidity T cell responses which only recognise very high doses of MHC–peptide. The hIgG-induced high-avidity T cells kill peptide-pulsed targets but more importantly kill B16F10 tumour cells expressing low levels of MHC but not B16F10 in which MHC has been completely downregulated by siRNA. In contrast the peptide-stimulated low avidity T cells only recognised targets pulsed with high-dose peptide, they fail to kill B16 F10 even if MHC has been upregulated by IFNα.

Studies in TCR transgenic mice have shown that it is possible to generate high- and low-avidity responses from a single TCR [69]. This suggests that TCR engagement is a component of T cell avidity but that it may be influenced by the conditions under which T cells are stimulated. It also suggests that thymic selection should not restrict the generation of high-avidity T cells when they are stimulated under the appropriate conditions. One method for enhancing MHC peptide recognition to self antigens is to make conservative changes within the peptide to either allow enhanced MHC or TCR binding [87-90]. Indeed some of these heteroclitic epitopes have been used in patients [91] and Dr Schwartzentruber and colleagues [91] reported at the American Association of Clinical Oncology meeting in 2009 the results of a Phase III randomized trial comparing heteroclitic peptide plus IL-2 with IL-2 alone. They found that response rate and progression free survival was improved in the vaccine plus IL-2 arm compared with the IL-2 arm alone. Although the heteroclitic epitopes stimulated high avidity response to themselves, recognition of the native epitopes was usually 10 – 100-fold less efficient [92]. One recent exception is a heteroclitic CD4 epitope which was identified from *Mycoplasma penetrans* HF-2 permease (MPHF2) protein due to its homology to a pan HLA-DR MAGE-A6 epitope. This epitope has a conservative amino acid change but stimulates a higher avidity response to MAGE-A6 than the native peptide [93]. Several studies have shown that high-avidity T cells stain can stain intensely with tetramers and this can be used *in vitro* to select high- and low-avidity CD8 cells [66]. Using an extension of this approach Casian Yee has shown that *ex vivo* selection, expansion and re-infusion of high avidity CD4 [94] and CD8 cells [95] can lead to tumour regression even in patients with extensive disease. Similarly, expansion of tumour-infiltrating lymphocytes in high doses of IL-2 and re-infusion into patients [96] has lead to cure in some patients although further studies are required. These studies although expensive and patient-specific have shown that if sufficient numbers of cells with sufficient avidity to lyse tumour cells can be induced it is possible to cure cancer patients even with extensive disease.

The functional mechanism that leads to the induction of higher-avidity T cells rather than low-avidity ones is thought to involve the level of costimulation at the time of induction. While MHC-peptide affinity plays a role in avidity, the

costimulatory signals coming from counter-receptors, adhesion molecules and cytokines play a significant role in promoting higher avidity T cells.

3.2.2 Costimulation, adhesion and cytokines

In an elegant study Hodge *et al.* showed incremental increases in T cell avidity by adding B7.1, Intracellular adhesion molecule-1 (ICAM-1), lymphocyte function-associated antigen 3 (LFA3), anti-CTLA-4 and GM-CSF to poxvirus vectored vaccines encoding either a viral or a tumour antigen (carcinoembryonic antigen (CEA)) [97]. Using all these factors they generated T cells with avidity greater than 10^{-11} M which efficiently lysed tumour cells. However, they could generate avidities of > 10^{-12} M to the viral antigen suggesting some repertoire deletion to the tumour antigen CEA. This is in line with previous studies, which have shown higher avidity responses to CEA in knockout mice compared with wild type mice [98]. A role for CD40 in stimulating high-avidity responses has also been demonstrated, as DC generated rapidly (48 h) with IFN-γ, TNF-α and CD40 L stimulating high-avidity T cells. This was related to IL-12 secretion, as when it was neutralised, T cell avidity was reduced [99]. Survival of $CD8^+$ T cells during homeostasis is also dependent on the pro-survival cytokine IL-7 which acts on T cells to stimulate expression of anti-apoptotic genes [100]. Recently, IL-7 signalling in CD8 T cells has been reported to modulate expression of the CD8 co-receptor [101]. A role for IL-15 has also been demonstrated with this cytokine not only selecting for high-avidity T cells by homeostatic proliferation but also increasing expression of CD8αβ heterodimer and secretion of IFN-γ [102]. The former has also been shown by several groups to be a key factor in differentiating, high- from low-avidity T cells.

3.2.3 CD8αβ heterodimer

The co-receptors of the TCR play an important role in T cell avidity. Renard *et al.* showed that T cell hybridomas lacking CD8 had lower avidity recognition of target [103]. If CD8α was transfected into these cells they stimulated higher-avidity responses but if both CD8α and CD8β where co-transfected into these cells this had the most profound influence on T cell avidity [104]. Similarly studies with transgenic TCR clones showed that the most obvious difference between high- and low-avidity clones was expression of the CD8αβ heterodimer [105]. This is thought to be related to the palmitylation of the CD8 β subunit that allows association with p53lck and assembly within lipid rafts [104,106]. This allows more efficient signalling per TCR/MHC–peptide recognition. These results are further supported by showing that high-avidity clones have greater TCR capping, suggesting a more potent immunological synapse, in response to MHC–peptide than low-avidity clones. Capping was inhibited in high-avidity T cells by depleting cells of cholesterol using the raft inhibit methyl β-cyclodextrin [105].

3.2.4 CD4 help

The presence of CD4 T cell helper responses may also be important for the generation of high-avidity CD8 T cell responses. An increase in avidity is characteristic of CD8 memory responses. This enables CD8 T cells to respond faster and with greater cytotoxicity upon re-encounter with cognate antigen than lower-avidity primary CD8 responses stimulated for the first time through the TCR. The role of CD4 help in the generation of CD8 T cell responses is controversial and has been reported to depend upon the source of the antigen [107] or the presence of IL-21 [108]. For example, similar levels of anti-virus CD8 T cells responses that are capable of controlling viral infection have been observed in mice that are deficient in CD4 cells [109]. More recently, several studies have dissected the importance of CD4 T cell help during CD8 priming and recall responses. The consensus seems to be that although CD4 T cell responses are not essential for the generation of primary CD8 responses, help during the priming stage is absolutely required for the generation of higher-avidity CD8 T cell memory [110-112]. CD4 cells provide significant help to T cells by provision of cytokine-mediated costimulation. In support of these observations, vaccine studies have demonstrated an important role for CD4 help in eliciting high avidity CD8 T cell responses. Altering the affinity of a CD4 epitope derived from the human immunodeficiency virus (HIV) so as to enhance CD4 T cell responses was found to significantly enhance both the frequency and avidity of the CD8 T cell response induced by immunisation with a CD4–CD8 linked peptide [113]. Although this study did not titrate the peptide concentration required to elicit the CD8 T cells response, CD8 cells from mice immunised with the modified helper epitope displayed 50% maximal lysis at an effector:target ratio of 6:1 compared with 25:1 from mice immunised with the unmodified helper epitope, suggesting more potent T cells. More recently, co-immunisation with a CD8 epitope and a CD4 helper epitope was found to generate higher avidity CD8 T cell responses capable of delaying tumour growth *in vivo*, compared with mice that were immunised with the CD8 epitope alone [114]. Collectively, these studies highlight an important role of CD4 help for the generation of high-avidity memory CD8 T cells responses. How then can high-avidity CD4 and CD8 T cells be stimulated *in vivo*?

3.3 Stimulation of high avidity CD4 and CD8 responses in vivo

3.3.1 Antigen dose

Early *in vitro* studies showed a role for antigen dose in avidity selection of CD8 and CD4 T cells. Interestingly low-dose peptide selected high-avidity T cells whereas high-dose stimulated low-avidity responses [63,115]. This also seemed to be true *in vivo* with high-dose peptide stimulating a larger frequency of low-avidity T cells whereas low dose stimulated a smaller but higher avidity response. However, upon re-challenge the

high-avidity responses expanded more rapidly than the lower-avidity responses suggesting that low-dose is a more effective vaccination [116].

A major goal for cancer vaccine is the generation of high-avidity memory responses that regress established tumour and prevent regrowth. Several studies have shown that high-avidity T cells are preferentially selected into memory but the conditions under which these cells were primed were crucial. If the prime does not stimulate high-avidity T cells then an effective memory response is not generated upon subsequent challenge. There is a further complication for vaccination studies as high-avidity T cells can also be deleted *in vitro* by high-dose antigens [63]. This is induced by TNF-α binding to TNFRII and downregulation of the anti-apoptotic protein Bcl2 [117]. Thus the failure of many vaccines may be due to either too high a dose, which fails to stimulate a high avidity response, or if the prime was efficient, boosting with too high a dose may result in deletion of the high-avidity T cells. Deletion of high-avidity T cells may have been one of the factors in the failure of the recent Phase III clinical trials with tumour cell vaccines or peptide-pulsed DC vaccines [118]. High-dose-antigen-induced death of T cells is also operative *in vitro* and is crucial in monitoring responses to vaccination in animals or patients. Despite the elegant studies of Alexander-Miller *et al.* [63,117], clearly showing that high-avidity T cells are selectively killed by 1 – 10μg/ml of peptide leaving an unrepresentative low avidity T cell population, most studies still use this dose for *in vitro* expansion and analysis of T cell responses. This at best underestimates the efficacy of the *in vivo* T cells and at worst perpetuates or selects for low-avidity responses that only respond to artificially high levels of MHC–peptide. Dose may be less of an issue if it is presented on mature DC as these cells only stimulate high-avidity responses [119]. However this is a major limitation with vaccines that do not target activated DC such as peptide, protein and cellular vaccines and may explain the poor objective clinical response rate of less than 10% [120].

3.3.2 Role of DC

As previously mentioned mature DC always stimulate high-avidity responses, and hence vaccines targeting these cells should be effective. Targeting mature DC has been tried both *ex vivo* and *in vitro*. *ex vivo* DC have been expanded from cancer patients and pulsed with a variety of antigens including peptides, proteins or tumour lysates [119]. Vence *et al.* [39] showed some clinical response to DC pulsed with melanoma lysate which correlated with T cell proliferation and secretion of type I cytokines using a technology called EPIMAX. Unfortunately this technology does not measure T cell avidity or tumour lysis. Disappointingly a recent Phase III clinical trial in melanoma failed to show a significant survival advantage for patients immunised with peptide-pulsed DC [121]. As T cell avidity was not measured it is difficult to assess whether the vaccine failed due to stimulation of ineffective low-avidity, T cell responses or if high-avidity T cells were stimulated but failed to survive in the tumour environment. A more promising approach is to use DC stimulated for 48 h with IFN-γ, TNF-α and CD40 ligand as these DC have been shown to secrete larger amounts of IL-12 and stimulate high-avidity T cells. This approach is currently being tested in breast cancer patients [122].

Clinical trials employing PBMC pulsed with vaccine for the treatment of prostate cancer have had more promising results. A recent placebo-controlled Phase III trial in patients with metastatic asymptomatic hormone-refractory prostate cancer reported on the benefits of immunisation with autologous PBMC loaded with prostatic acid phosphatase (PAP) antigen in a fusion protein with GM-CSF. Although no improvement in time to disease progression was observed, immunisation with the PAP-loaded PBMC vaccine demonstrated a statistically significant 4.5 month improvement in overall survival. This was associated with an eightfold improvement in the induction of antigen-specific T cell responses following immunisation with the PAP-loaded PBMC vaccine compared with the placebo control [123]. This vaccine, known as Provenge, has now been filed for approval by the FDA.

A major hindrance to this immunisation strategy is the requirement for MHC-matched PBMC in order to induce MHC-restricted T cell responses. This requires the generation of large numbers of clinical-grade autologous PBMC for patient-specific therapy – a process that can be protracted and costly [124]. Targeting DC *in vivo* may prove to be a more viable approach.

3.3.3 Targeting DC in vivo

The ability to target vaccine antigens to DC *in vivo* offers an attractive alternative to the use of autologous DC vaccines, which are patient-specific, expensive and difficult to manufacture. Several groups have attempted to achieve this through targeting vaccine modalities to specific receptors on the surface of DC that facilitate antigen processing and presentation.

DEC-205 represents one such receptor that facilitates enhanced antigen uptake and presentation in DC. Hawiger *et al.* observed that when antigens were targeted to DEC-205 in this manner, an initial burst of T cell proliferation was induced but this was not prolonged. Furthermore, a normal T effector cytokine profile was not induced and the T cells became anergic to antigenic restimulation. In contrast, when anti-DEC-205 targeting was combined with an agonistic anti-CD40 antibody [125] or in conjunction with CpG oligonucleotides the growth of established melanomas in a murine model was inhibited [126].

Several studies have demonstrated that targeting antigens to the mannose receptor (MR) can also improve the efficiency of immunisation protocols. A humanised antibody directed against human MR (B11) was generated and genetically fused to the whole protein pmel17, a splice variant of the melanocyte-specific protein gp100. In an autologous *in vitro* culture system, B11-pmel17 loaded DC were able to

stimulate CD4 proliferative responses, as well as directing the development of anti-melanoma CD8 cells [127]. More recently, the use of anti-MR conjugated antigen has been reported to target antigen to both the exogenous and cross-presentation pathways of antigen presentation in human-mannose-receptor-transgenic mice *in vivo* [128]. Moreover, when co-immunised with CpG-containing oligonucleotides to promote DC maturation, this vaccination strategy was able to induce protective immunity against challenge with a tumour cell line expressing the cognate antigen.

In addition to targeting DC-specific receptors, several groups have demonstrated enhanced immune responses through targeting of a range of vaccine modalities to the Fc receptors for IgG. Akiyama *et al.* showed that immunisation with DC pulsed with IgG-complexed-apoptotic tumour cells enhanced the *in vivo* generation of tumour-specific CD8 cells and tumour rejection, as compared with DC pulsed with apoptotic tumour cells alone [129]. Rafiq *et al.* showed that tumour immunity specific for ovalbumin (OVA)-expressing tumours could be provided by immunisation with OVA-I- pulsed wild type DC but not DC deficient in FcγR signaling, suggesting that the Fc might also provide FcγR-mediated maturation signals to DC in order to promote immunity rather than tolerance [130]. Fcγ receptor targeting has also been reported to improve the efficiency of DNA immunisation. Enhanced CD8 T cell, T_H1 and antibody responses were observed *in vivo* following immunisation with a DNA construct incorporating an IgG Fc fragment fused to a model hepatitis B antigen compared with a construct encoding antigen alone [131]. More recently, the enhanced immune responses observed following DNA immunisation with a construct encoding Fc was able to delay tumour growth and prolong survival in a murine model of prostate cancer [132]. A DNA vaccine that targets CD64, and encodes a human IgG1 antibody containing CD8 and CD4 epitopes within its CDR regions [133], has recently been shown to stimulate high-avidity T cells (> 10^{-12} M) that reject established tumours. The same CD8 epitope has also shown to be a rejection epitope in cancer patients and this vaccine will shortly enter clinical trials, with its major endpoint being to assess stimulation of high avidity T cells in cancer patients.

4. Expert opinion

Immunotherapy for cancer is fast becoming the fourth therapeutic modality alongside surgery, chemotherapy and radiotherapy. Six monoclonal antibodies and a prophylactic vaccine have been approved for cancer therapy. Provenge has been submitted for approval by the FDA as the first therapeutic vaccine for the treatment of established tumours. However recent clinical trials with other vaccines have demonstrated that the frequency of the T cell response alone is not a good indicator of tumour regression. This has lead to the suggestion that immune responses should not be measured and the efficacy of vaccines should be assessed in Phase III trials that only measure survival. Unfortunately this has lead to a number of large, expensive Phase III clinical trials that have failed to enhance survival. In retrospect it is impossible to distinguish vaccines that failed to stimulate an effective immune response (ineffective vaccines) from vaccines that stimulated a strong immune response (effective vaccines) but that failed to overcome the immunosuppressive tumour environment. Ineffective vaccines may stimulate low-avidity responses. Low-avidity CD8 T share some of the characteristics of virally fatigued T cells in that they can produce IFNγ in response to high dose peptide but do not lyse target cells and they express high levels of the death receptor PD-1. They are stimulated by high dose MHC–peptide in the absence of costimulation and are induced by chronic viral infection and may also be induced by tumours and inappropriate vaccination. In particular, great care is required that vaccination does not kill off any high-avidity T cell responses. These may have been induced by the tumour and have been controlling its growth (immune equilibrium). Vaccine-induced killing of these cells could result in vaccinated patients doing worse than those receiving placebo, as has recently been observed in several clinical trials [121,134].

Functional T cell avidity may be a better predictor of clinical response than T cell frequency and should be monitored in all new vaccine trails. If the vaccine targets a specific epitope the standardised method for measuring avidity is to titrate the epitope and measure responses either by ELISPOT or CTL lysis of peptide-pulsed antigen-negative target cells. It is more difficult to measure avidity if the vaccine targets unknown epitopes. This is best achieved by measuring CTL lysis of tumour cells. In our experience and those of many other groups there is a good correlation between T cell functional avidity and lysis of tumour cells. This should be the minimum assay for all cancer vaccines. It should preferably be lysis of autologous tumour but as it can be difficult to get tumour biopsies from all patients it should be against HLA-matched and -mismatched, antigen-negative and -positive target cell lines.

Adoptive cell transfer studies or T cells cloned from cancer patients show high-frequency, high-avidity CD4 and CD8 T cell responses that correlate with tumour regression. More recent vaccine studies have shown that epitope specific vaccines targeting activated DC stimulate high-avidity T cell responses that clear established tumours in animal models [135]. Effective vaccines that stimulate high-frequency, high-avidity T cell responses but fail to control tumour growth could be more effective in patients with minimal residual disease. Alternatively, effective vaccines could be combined with other approaches to encourage T cell recruitment within tumours, such as depletion of Tregs, anti-CTLA-4 therapy, neutralisation of immunosuppressive cytokines or high-dose chemokines.

We are at a point where we are beginning to understand how to stimulate effective anti-tumour T cell responses. While these may provide a large step forward in terms of immunotherapy it is also likely that they will provide a significant addition to combination therapies that will ultimately cure cancer patients.

Declaration of interest

LG Durrant is a shareholder and Director of Scancell Ltd V Pudney and RL Metheringham are employees of Scancell Ltd. The authors have been funded by the University of Nottingham and Scancell Ltd.

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